Food and Agriculture Organization of the United Nations

Research approaches and methods for evaluating the protein quality of human foods

Report of a FAO Expert Working Group

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Abbreviations and Acronyms

AA	Amino acid
AAdiet	Dietary amino acid
AAtot	Total amino acid
APE	Nitrogen enrichment excess
AUC	Area under the curve
BV	Biological value
CODEX	Codex Alimentarius Commission
CP	Crude protein
DAA	Dispensable amino acid
DIAAS	Digestible indispensable amino acid score
DM	Dry matter
EAR	Estimated average requirement
EHC	Enzyme hydrolyzed casein
FAO	Food and Agriculture Organization of the United Nations
GC-MS	Gas chromatography-mass spectrometry
^{2}H	Deuterium
HP	High protein
IAA	Indispensable amino acid
IAAO	Indicator amino acid oxidation
IRMS	GC-pyrolysis-isotope ratio MS
iv	Intravenous
LP	Low protein
MS	Mass spectrometry
N	Nitrogen
NPPU	Net postprandial protein utilization
NPU	Net protein utilization

Ntot	Total nitrogen
PDCAAS	Protein digestibility corrected amino acid score
PPU	Postprandial protein utilisation
SD	Standard deviation
SID	Standardized ileal digestibility
SJRI	St. John's Research Institute
TD	True digestibility
TID	True ileal digestibility
TiO_2	Titanium dioxide
UNU	United Nations University
WHO	World Health Organisation

Section 1:
Background

The determination of protein requirements for human nutrition was reviewed by FAO for the first time in 1955 (FAO, 1957) and subsequently in 1963 and 1971 with the World Health Organization (WHO) (FAO, 1965 and FAO, 1973); 1981 with WHO and United Nations University (UNU) (WHO, 1985) and most recently in 2002 with WHO and UNU (WHO, 2007). Separate but related expert meetings on protein quality evaluation were held in 1989 with WHO (FAO, 1991) and by FAO in 2011 (FAO, 2013).

In regards to protein quality evaluation, the 1989 Expert Consultation focused its attention on the then newly developed PDCAAS (Protein Digestibility Corrected Amino Acid Score) method for evaluating protein, while the 2011 Expert Consultation reviewed extensively the more recent DIAAS (Digestible Indispensable Amino Acid Score) method and considered the advantages and disadvantages for advocating the replacement of PDCAAS with DIAAS. DIAAS measures the oro-ileal nitrogen balance by calculating the ileal digestibility of individual amino acids. In contrast, PDCAAS uses crude faecal digestibility values in measuring the oro-faecal nitrogen balance which includes contributions from intestinal secretions and colonic bacteria, thus underestimating the protein available for absorption.

While there was consensus at the 2011 consultation that ileal digestibility was superior conceptually to faecal digestibility, there was concern about whether there was sufficient published data available on true ileal amino acid digestibility on a wide range of human diets to allow the practical implementation of a system based on ileal digestibility. In addition, whereas substantial ileal amino acid digestibility data had been collected from pigs and rats using a standardised methodology for the sampling of ileal content, there was concern that the foods used were not representative of human diets including those in developing countries and that animal digestibility values were representative of those of humans. In order to address these concerns, the 2011 consultation established 2 subcommittees tasked to report after the formal consultation had finished but before the report was completed. The first subcommittee was tasked to collate all available human and animal ileal digestion data, while the second subcommittee was to evaluate this collated data to establish whether it was sufficient to warrant replacing PDCAAS with DIAAS as the preferred method at this time. The second subcommittee concluded that:

> "… currently available data were insufficient to support the application in practice….of true ileal amino acid digestibility in the calculation of DIAAS", and that "more data on the true ileal amino acid digestibility of human foods was urgently needed, determined in humans and animal models" (FAO, 2012a).

The overall outcome of the Expert Consultation was that "More inter-species (human, pig, rat) true ileal amino acid digestibility comparisons are urgently needed" (FAO, 2013) and it was recommended that FAO organize and convene an expert working group:

> "…to agree upon an experimental protocol to enable the development of a more robust data set of the true ileal amino acid digestibility of human foods..." and that "The protocol should include recommended best practice for a pig-based assay for true ileal amino acid digestibility determination" (FAO, 2013).

In addition, the second subcommittee recommended that:

> "If the data obtained from these studies (as specified under #3) convincingly support the move to ileal digestibility, assessment of the potential impact of this recommendation (to be used in the assessment of individual protein sources as well as mixed diets commonly consumed by humans) needs to be undertaken before the new evaluation model is implemented. This should include potential gains and/or losses to public health consequent upon the implementation of the new recommendations on the assessment of protein quality for humans" (FAO, 2012a).

Subsequently, recognizing the difficulties of obtaining human ileal digestibility values directly and in light of the increased application of stable isotopes in determining protein utilization, FAO expanded the working group's topic "to provide recommendations on the best methods to measure and predict digestion and efficiency of utilization of protein and amino acids in humans" (See Annex 6).

Planning for the working group began in mid-2013 soon after the Expert Consultation report was published. A first call for experts was announced in August 2013 but due to an insufficient number of responses from qualified scientists and the expanded topic, a second call was announced in December 2013. The 25 applications received were reviewed by two non-FAO scientists who were not directly involved in the working group planning. They each ranked the applicants according to the criteria based on the description in the call for experts. FAO invited the experts who received the highest rankings to attend this working group.

Each expert was requested to fill in a declaration of interests. Of the 10 experts, 6 gave information regarding advisory activities with a range of organizations that may have interests relevant to the working group topic. However, FAO did not find that the activities represented a potential competing interest that would warrant exclusion of any of the experts from the working group focusing on research methodologies.

FAO provided the complete funding for the expert working group meeting through a letter of agreement with the St. John's Research Institute in Bangalore, India, an integral part of the St. John's National Academy of Health Sciences, which hosted and organized the logistics for the meeting.

Section 2:
Introduction and Opening of the Working Group Meeting

The meeting was held at the St. John's Research Institute (SJRI), an IAEA Collaborating Centre, in Bangalore, India, from 2 to 5 March 2014. The opening welcome on behalf of the SJRI was given by Dr. Anura Kurpad, followed by a welcoming statement on behalf of FAO by Dr. Warren T K Lee. Subsequently, those in attendance introduced themselves and briefly described their research focus and its relationship to the objectives of the working group. Dr. Janice Albert of FAO introduced and explained the draft agenda and the plan for conducting the meeting (See Annexes 4 and 5). The introductory session was concluded by Dr. Robert Weisell with a presentation on the history of FAO's involvement with protein requirements and protein quality evaluation and a second presentation by Dr. D J Millward giving a perspective on methods for determining protein quality which was based largely on an article in the *British Journal of Nutrition* (Millward, 2012).

Section 3:
Rationale for the Working Group Meeting

Following the opening and introduction of the participants, the working group reviewed the aim of the meeting, which originated from the FAO Expert Consultation in 2011. Dr. Paul J Moughan, the chair of the 2011 FAO Expert Consultation on Dietary Protein Quality Evaluation in Human Nutrition provided an overview of salient points from the consultation for this working group's attention. These were:

1. Identify one or more experimental protocols to enable the development of a more robust data set of the true ileal AA digestibility of human foods; this would include more data on true ileal AA digestibility and more inter-species comparisons of true ileal AA digestibility.
2. The protocols should include recommended best practice for pig-based, rat-based and adult human-based assays of true ileal AA digestibility.
3. Direct measures of amino acid bioavailability and utilization often involving stable isotopes are being developed. These offer additional information about protein quality and should be considered for further development.

The participants discussed the review and sought clarification on some issues. One expert disagreed with the word 'true' digestibility and preferred the term 'standardised'. The group acknowledged there was a need to clarify the nomenclature (e.g. apparent, true, real, standardised) and suggested it as an item for future work.

Two experts who had also attended the 2011 FAO Expert Consultation drew attention to the report of the second subcommittee of the Expert Consultation, which was prepared following the Expert Consultation meeting in New Zealand (FAO, 2012a). This subcommittee assessed the advisability of using ileal digestibility over faecal digestibility and based largely on the report from the first subcommittee found that there was insufficient human ileal digestibility data to accommodate a change from true faecal crude protein digestibility (PDCAAS) to true ileal amino acid digestibility (DIAAS) at the time (FAO, 2012b). Further, the public health impact of this change in the way of evaluating protein had not been assessed. Attention was then given to the current working group task.

Section 4:
Protein Quality Evaluation and Public Health Considerations

The objective of the working group meeting was to bring together experts in the area of protein and amino acid nutrition to update our knowledge of the science related to protein and protein quality evaluation. However, the experts recognized the relevance of protein quality in addressing nutrition and public health and its effect on health and nutrition outcomes, a topic on which Dr. Ricardo Uauy made a presentation.

All aspects of protein's physiological role are of key importance in defining its impact on health and functional outcomes since the absolute amount of protein in itself does not reflect the true and total nutritional effects. Of primary importance is protein quality. Important body functions such as immunity and host defenses, linear growth and associated mental development are affected by protein quality. Recently, these topics were discussed in full at a workshop held in the spring of 2012 at Tufts University with the proceedings published in a special section of the *Food and Nutrition Bulletin* (FNB, 2013).

Recent literature indicates that protein quality and not only quantity is of importance to support linear growth and reduce stunting of children (Ghosh *et al*., 2012; Timby *et al*., 2014; Uauy, 2013) and mental development and performance of children (Steinberg *et al*., 1992; Yogman *et al*., 1982). Specific attention has been given to the effects on infant growth and development due to changes in infant feeding (Heine, 1999; Heine *et al*., 1996; Jackson *et al*., 2002; Lien, 2003; Lien *et al*., 2002; Markus *et al*., 2000; Markus *et al*., 2002) and the precursor roles of specific amino acid such as tryptophan in regards to serotonin (Borbély and Youmbi-Balderer, 1987; Fernstrom, 2012; Fernstrom, 2013; Fernstrom and Fernstrom, 1995; Fernstrom *et al*., 2013; Sharp *et al*., 1992; Yogman *et al*., 1982) and tyrosine in regards to adrenaline and noradrenaline. Moreover, the evidence produced over the past decade indicates that improved linear growth is associated with lower risk of death during childhood and to enhance educational performance and labour productivity later in life. A long-term follow up of 5 cohorts from developing countries that included as outcomes linear growth, educational performance and adult productivity revealed a significant effect of stunting on restricting educational attainment which was associated with lower adult income levels (Hoddinott *et al*., 2008). In one of the countries, Guatemala, a protein and energy supplement was compared to providing only energy; the results related to adult productivity indicated that the protein-energy supplemented group achieved a higher level of education and greater income in adult life. These follow up studies have indicated the need to improve the quality of foods considering linear growth and not weight as the relevant short term outcome that will impact adult productivity and long term health. The policy implications of these findings point to the first 1000 days (from conception to age 2 years) as the critical time for long term benefits for health, productivity and well-being of populations.

Section 5:
Relationship Between Animal and Human Studies and Bridging the Two

The working group was faced with the question "What would legitimize the use of animal experimental results for the application to humans?" It was agreed that there were limitations with both the animal and human methods. However, the goal was to address and accommodate these limitations.

While recognizing that human data are preferable, the 2011 Expert Consultation recommended that the initial routine evaluation of human foods would be more immediate by using the fully validated true ileal amino acid digestibility (TID) assay in the growing pig. The working group believed that the digestive and nutrient absorption systems of the pig is somewhat comparable to humans physiologically, although not totally. The pig models offered opportunities to develop relatively rapidly large data sets of the ileal digestibility of amino acids in a large number of human foods since the methods have been standardised worldwide. However, the working group stressed that validation studies further to those already published were needed to confirm the use of pig data sets to represent the digestibility of the same foods when consumed by humans. In addition, the generated data could be assumed to apply to healthy adult humans but not necessarily to human subjects with specific conditions (e.g. disease, age, under nutrition, tropical enteropathy/malabsorption, parasite burden, pregnancy and lactation).

Recently developed, direct *in vivo* isotopic methods for determining amino acid digestibility show great promise. In developing the isotopic method for determining amino acid digestibility in humans, direct comparison should be made with data from the pig-based true ileal amino acid digestibility assay. For example, with the Indicator Amino Acid Oxidation (IAAO) and the Net Postprandial Protein Utilization (NPPU) assays giving estimates of digestibility of the limiting amino acid in a dietary protein, it is likely that such a comparison can be undertaken in the near future. The experts recommended that research be conducted to compare the IAAO and the NPPU estimates of a limiting amino acid bioavailability in humans and pigs with the pig true ileal amino acid digestibility assay of the specific amino acid. There should also be bridging between other potentially more convenient, but at present theoretical isotope methods for determining amino acid digestibility that are being developed, by comparison with the true ileal amino acid digestibility assay in the growing pig.

Section 6:
Characteristics of an Ideal Method of Protein Quality Evaluation

Prior to the discussion of the different methods currently available and developing research protocols, one expert proposed a framework for the development of any new method for evaluation of protein quality. This was presented with a challenge as to what would be the characteristics of an 'ideal' method for determining the bioavailability of amino acids (protein quality) in humans.

As a starting point, the definition of bioavailability described in the report of the 2011 FAO Expert Consultation (FAO, 2013) was accepted for this purpose[1]. The goal was to establish a list of characteristics and parameters that could be used to guide the decision regarding the suitability and potential application of the available methods under consideration and methods that may be developed in the future.

The first and most important characteristic was that the ideal method would allow for direct measurement in humans. This was recognized, however, as potentially difficult to achieve due to the scarcity of non-invasive methods of measurement and ethical considerations. Thus, methods applicable to animals could be considered as long as sufficient cross-species comparison research was conducted to develop acceptably accurate predictions of amino acid bioavailability in humans.

The ideal method would also be directly applicable in all human life stages and conditions that could possibly affect the digestion, absorption, and metabolism of amino acids, and thus bioavailability. These human conditions would include: inter alia, age, health status, genetics, environment, prior nutritional status, parasitism and other conditions.

To be relevant to actual conditions, the method must be applicable to the widest possible range of human foods. From the perspective of public health, this would mean the inclusion of foods of low protein quality (e.g. pulses and grains), which represent common protein sources for the

[1] Bioavailability encompasses three properties of foods that can alter the proportion of an amino acid that can be utilized; these are:

1. Digestibility, which describes the net absorption of an amino acid.
2. Chemical integrity, which describes the proportion of the amino acid that, if absorbed, is in a utilizable form.
3. Freedom from interference in metabolism resulting from the presence in the food of substances that limit the utilization of the amino acid.

Of these, the greatest source of variation in bioavailability is, in most cases, digestibility.

poor. In addition, it must be able to capture the effects of a wide range of cooking and processing methods on protein bioavailability. Cooking and processing methods are known to produce anti-nutrient factors such as the Maillard-type reaction products, which reduce lysine bioavailability, and may also reduce bioavailability of other amino acids through a range of possible reactions with starch, sugars or mineral components of the diet.

Of critical importance is the ability to measure the bioavailability of all individual amino acids. However, not all methods may capture data on all amino acids. For example, methods that measure changes in postprandial net protein synthesis may work well for an individual limiting dietary indispensable amino acid (IAA), such as lysine in most cereals, the sulfur amino acids in legumes and tryptophan in maize, but would not be applicable to all IAAs or to dispensable amino acids (DAA). Since indispensable amino acids are of greatest importance, the first priority would be given to research on those indispensable amino acids that are most likely to be limiting for humans in that particular food, with firm recognition that the dispensable amino acids are also important in human nutrition and require consideration wherever possible.

Any method chosen must also be applicable to mixed human diets and be capable of measuring the effect of amino acid supplementation. Where the overall protein quality of a foodstuff, or a traditional mixed diet, is lowered because of the low bioavailability of a single amino acid, enhancement of the food with protein sources supplying the limiting amino acid or supplementation with the limiting amino acid can dramatically increase the capacity of the food or diet to meet protein and amino acid requirements for human growth, performance and health. Therefore, it would be desirable if the method could detect or measure or be used to calculate the effect of complementation or amino acid supplementation on overall quality of the diet.

Any method chosen must allow for wide implementation because this is necessary to create a large enough database of local foods and food processing/cooking methods to justify changing global recommendations. In this context, wide implementation means that the method must be applicable to a large range of potential users, i.e. research institutions and governments and industry with various levels of skill and background knowledge. This implies that the method should be understandable by these groups and the methodology clearly defined so that the introduction of errors is unlikely.

A significant consideration for wide implementation is that the cost must be reasonable; otherwise, the method is not feasible. In addition, if used directly in humans, the method should be minimally invasive; otherwise, recruitment of research subjects and approval by relevant human ethics committees becomes a potentially limiting factor.

A clear recognition of the 2011 Expert Consultation was that there is a very limited quantity of ileal amino acid digestibility data directly obtained in humans and therefore, any methods should be validated in animals as far as is possible. This means that the results obtained with the method must be comparable to very well described and widely accepted methods in amino acid ileal digestibility (True Ileal Digestibility, i.e. TID or Standardised Ileal Digestibility, i.e. SID) and bioavailability in animals and must give similar results to these methods.

Finally, it would be advantageous if the method could be potentially recognized and accepted, as a minimum, by the Codex Alimentarius Commission. This means that the method would gain wide acceptance and could be used potentially by food regulatory agencies in evaluating food health and nutrition claims by industry.

The Working Group concluded that the 'ideal' method does not currently exist in that no method meets all the criteria. Therefore, the Working Group proposed that a combination of the existing methods may be required, for example, the combination of pig TID measurement with IAAO. Different methods may also be required depending upon the specific research goals or foodstuffs. Ultimately, the goal is to accumulate sufficient data to enable the practical change from the PDCAAS to the DIAAS approach for assessing the protein quality of human foods and diets.

What would be the components of an "ideal" method for bioavailability of amino acids in humans?

- Direct measurement in humans. If not possible, need sufficient cross-species research to develop predictions from animal research;
- Directly applicable in all human life stages (i.e. age), health status, genetics, environment, nutritional status, other conditions;
- Applicable to wide-range of human foods and must capture effects of cooking and other technological processes applied to foods;
- Applicable to all individual amino acids. Give priority to indispensable amino acids (IAA) but desirable to include dispensable amino acids (DAA) or sum of DAA;
- Applicable to mixed human diets and supplementation;
- Must be capable of wide implementation because this is necessary to create a large enough database of local foods and food processing/cooking methods to justify moving worldwide recommendations.
  - Cost must therefore be reasonable if wide implementation is to be feasible;
  - Should be minimally invasive because this is desirable/necessary for wide implementation;
  - Validated in animals – values agree with well-described and accepted methods in amino acid digestibility (SID) and bioavailability.
- Potentially recognizable at least to the Codex Alimentarius Commission (CODEX).

The working group adopted this framework as a starting point for evaluating and developing several research protocols that could be used alone or in combination. In addition, the various methods could be part of a coordinated international effort to provide evidence in support of the effective future use of DIAAS.

Section 7:
Methods for Measuring Protein Digestibility in Human Foods

The Working Group considered five protocols that were either well-tested and used or showed considerable promise with future development. These are described in subsections 7.1 to 7.5 and include:

- True Ileal Amino Acid Digestibility
- Indicator Amino Acid Oxidation (IAAO)
- Postprandial Protein Utilization (PPU)
- Net Postprandial Protein Utilization (NPPU)
- A Dual Tracer Approach to Measuring DIAAS

Because these methods encompass a range in the state of development, varying levels of prescription and details can be provided. For the initial three methods, sufficient information is available for providing either extensive detail as to how to apply the method or caution and advice (See Annexes 1, 2 and 3).

7.1 TRUE ILEAL AMINO ACID DIGESTIBILITY

The 2011 FAO Expert Consultation concluded that presently it is difficult and expensive to determine true ileal amino acid digestibility directly in humans. Therefore, there was a need for an animal model for the more routine determination of amino acid digestibility.

It was agreed that the rat, being a nocturnal animal with a selective feeding habit and practicing coprophagy, is an inferior animal model to the pig, which is a meal-eating omnivore with a similar anatomy of the digestive tract (mouth to ileum) and similar digestive physiology to the adult human.

The similarities between the growing pig and adult human for protein and amino acid digestibility are well documented. Pig true ileal amino acid digestibility and ileal reactive lysine digestibility measures have been thoroughly validated in pig studies and have been shown to be accurate predictors of absorption in this animal. Published studies (based on four protein sources) show close inter-species agreement between growing pigs and the adult human for true ileal amino acid digestibility (Deglaire *et al.*, 2009a; Rowan *et al.*, 1994).

With rats, foods need to be finely ground before presentation to the animal to avoid particle selection by the animal, whereas the pig will generally consume foods eaten by humans and in

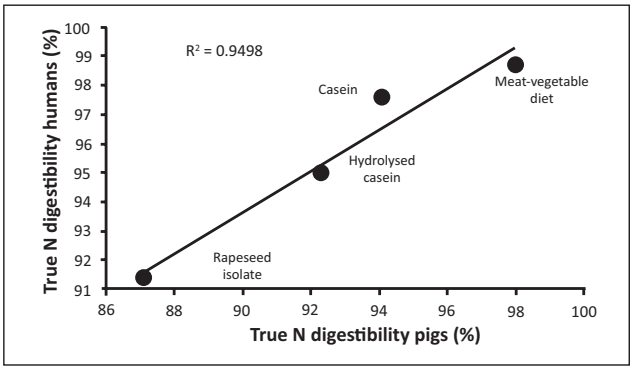
the form presented to humans for consumption. The fine grinding of foods for rats is likely to influence nutrient digestibility. Advantages of the pig as an animal model for the prediction of amino acid digestibility in humans were noted as:

- Excellent control and standardisation possibilities;
- Pig provides large samples of ileal digesta;
- All amino acids can be determined in a single assay;
- Pigs eat a wide variety of foods including virtually all foods consumed by humans;
- The availability of laboratories able to conduct pigs assays.
- Limitations of the pig model include its cost; possible future constraints of animal use due to ethical considerations.

Further, although the process of protein digestion in the pig is physiologically similar to that in humans, protein digestibility is unlikely to be identical. While not reflecting on the determination of protein quality in optimal conditions *per se*, there is the further public health consideration of determining the effect that different social and environmental conditions could have on the recommended protein quality of human diets. The human condition may be quite variable due to malnutrition, exposure to parasites and tropical enteropathy, etc. Consequently, the digestibility of amino acids in humans is likely to be variable and the determination of whether an additional allowance for protein quality is needed will require a measurement that can be used in humans in these conditions.

Consideration was given to developing robust statistical prediction equations relating protein/amino acid digestibility determined in the adult human with that determined in the growing pig. Such a relationship has been published (see Figure 1), but it was considered by the Working Group that this dataset should be expanded and extended by the addition of experimental results for additional protein sources, particularly covering protein sources with a low (<75%) and a medium (<85%) true ileal nitrogen and amino acid digestibility. Aspects of developing standardised protocols for the determination of true ileal amino acid digestibility (naso-ileal intubation for the adult human given liquids and powdered materials; adult human ileostomates for coarser materials, cannulated growing pig, laboratory rat) were discussed and subsequently developed (See Annex 1)

Figure 1: Human-growing pig comparison



Once the relationship for true ileal amino acid digestibility between pigs and humans has been developed over a wider range of digestibility values, application of the pig true ileal amino acid digestibility protocol should be encouraged to develop a complete dataset of the amino acid digestibility of human foods. The Working Group concluded that it would be valuable to strengthen the relationship of true ileal amino acid digestibility values between pigs and humans, based on a wide range of foods and diets, thus potentially broadening the application of the data base.

7.2 THE INDICATOR AMINO ACID OXIDATION (IAAO) METHOD

Background

The IAAO method was discussed at the 2011 Expert Consultation (FAO, 2013). Because it is one of only 2 published bioassay methods in humans that produce data on amino acid bioavailability in humans, it was considered in detail at the working group meeting in 2014.

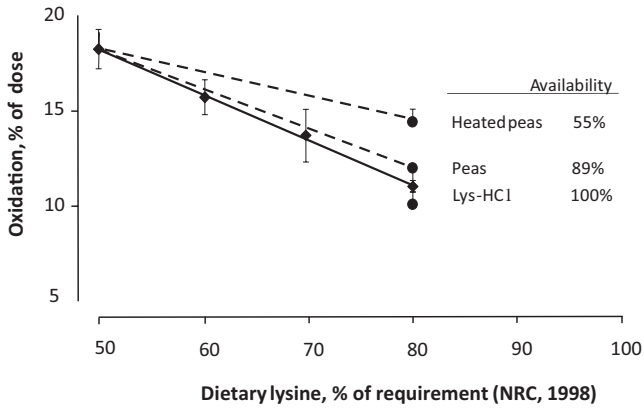
The IAAO method has been used extensively to measure the amino acid and protein requirements of pigs (e.g. Ball and Bayley, 1986; Moehn *et al.*, 2008) and humans (see reviews by Elango *et al.*, 2009, 2012a, 2012c; Pencharz and Ball, 2003). The most recent protein requirement report (WHO, 2007) noted the potential promise of using 24-hour IAAO balance instead of or in addition to 24-hour nitrogen balance for the determination of amino acid requirements of humans. A method, based upon the IAAO technique, was developed to measure the metabolic availability of the limiting amino acid in the diet of pigs (Levesque *et al.*, 2010; Moehn *et al.,* 2005, 2007) and subsequently adapted to humans (Humayun *et al.*, 2007, Prolla *et al.*, 2013). In the pig, the values obtained by IAAO are not different from those obtained by 'true' ileal digestibility. The term 'metabolic availability' was coined to describe the sum of the overall digestion, absorption and metabolic utilization for protein synthesis of amino acids from the dietary protein source. This is accepted as equivalent to the definition of bioavailability used in the report of the 2011 FAO Expert Consultation (FAO, 2013). The digestibility of the amino acid will have the largest influence on the IAAO response. However, because this method measures a relative change in net protein synthesis, any amino acid in the food that is unavailable, due to chemical complexes such as Maillard reactions or reduced utilization due to interference from other factors in the diet or creation of un-reactive amino acid by cooking methods (e.g. effect of acid treatment on methionine and tryptophan), will be accounted for.

The IAAO method is based on the fact that when any single amino acid is limiting for protein synthesis that all other amino acids are in excess and thus must be oxidized. The indicator amino acid is maintained at a constant intake; therefore, the decline in IAAO is linear with incremental addition of the limiting amino acid below the requirement intake. Therefore, this portion of the response can also be used to test the change in net protein synthesis with increasing intake of a food ingredient in which an amino acid is limiting.

Calculation of bioavailability in IAAO experiments

The slope of the oxidation line for increments of the food compared to the slope of the oxidation line for the same amino acid supplied as a 100% bioavailable synthetic amino acid allows a slope ratio calculation (Batterham, 1992; Littell *et al.*, 1997). The slope of the oxidation obtained with the crystalline form of the test amino acid represents the maximal unit increase in net protein synthesis and is equivalent to 100% bioavailability of the test amino acid. A shallower slope indicates that less amino acid per unit intake is available to support net protein synthesis. The ratio of the slopes therefore represents the bioavailability of the limiting amino acid in the food. Figure 2 demonstrates the typical response as reported by Moehn *et al.*, 2007.

Figure 2: Lysine availability in peas



The bioavailability of the amino acid is calculated by the slope ratio method as oxidation change per g protein bound amino acid divided by oxidation change per g free amino acid, using the following equation:

$$y = a + bSxS + bTxT$$

where xS and xT stand for the levels of intake for the standard and test sources respectively and bS and bT are the slopes for the standard and test sources respectively (Littell *et al.*, 1997). The bioavailability of the test source is then calculated as bS/bT.

There are several advantages to this slope ratio approach for this calculation. Because the measured response is a relative change, rather than an absolute value, this will reduce the effect of inter-laboratory variability. It also means that differences in rates of net protein synthesis among the subjects will have no influence because individuals are used as their own controls.

There are a number of key conditions that must be satisfied for correct use of IAAO and the slope ratio assay for amino acid bioavailability. A description of these may be found in Annex 2.

Choice of indicator Amino Acid

The choice of indicator amino acid and the essential and recommended characteristics of an indicator amino acid have been discussed frequently in publications (e.g. Elango *et al.*, 2012b; Levesque *et al.*, 2010; Zello *et al.*, 1993) and are critical to sensitively partition the response to graded test intakes between protein synthesis and oxidation. The criteria for selecting the indicator amino acid are: a) it must be an indispensable amino acid; b) it must have a carboxyl-labeled carbon that is irreversibly oxidized upon catabolism and is released to $^{13}CO_2$, which can be quantitatively measured in breath; c) it must have a small, well regulated pool within the body and d) it is not involved in quantitatively significant pathways other than incorporation into protein or oxidation to CO_2. Phenylalanine, in the presence of excess tyrosine, fits all four criteria. L-1-^{13}C-Phenylalanine has been successfully used to measure metabolic availability in pigs (Levesque *et al.*, 2011; Moehn *et al.*, 2005, 2007) and humans (Humayun *et al.*, 2007; Prolla *et al.*, 2013). It is possible that other amino acids, such as leucine, are usable as tracers; however, to date phenylalanine has been used in these protocols to determine dietary bioavailability of amino acids.

IAAO methodology for establishing the necessary linkage between the Pig TID database and Human Foods

To bridge the data from the human IAAO results on bioavailability to the pig TID database, corresponding IAAO experiments for bioavailability must be conducted in pigs as described by Moehn *et al.*, 2007. Initially, this must be conducted with a sufficiently large number of foods with a wide range in amino acid bioavailability so that robust prediction equations can be developed. Thereafter, this step need only be conducted for foods that have characteristics or bioavailability outside of the reference range of foods used in the first instance to develop corrections or adjustments to the pig TID database. If doubt exists about the accuracy of the correction factor used for any type of food studied by human IAAO bioavailability, then a pig IAAO experiment for bioavailability is required.

Critique of protocol and conclusions

The IAAO bioavailability method, which is based on minimal assumptions that have been validated in animals and humans, can be used to assess all protein sources and measures the bioavailability of individual amino acids. It is a relatively non-invasive method, requiring only the oral consumption of the food and stable isotope and collection of breath samples for analysis of $^{13}CO_2$; it is therefore readily adaptable to routine use for evaluation of amino acid bioavailability of proteins in human nutrition in a variety of human conditions in multiple countries. The method produces similar values for amino acid bioavailability in the pig for lysine, threonine and methionine as the more invasive true ileal digestibility (TID) pig method. Technically, the method is based on measures of overall amino acid utilization and therefore, meticulous control is necessary as any factor that differentially affects utilization of the amino acid from the test diet versus the reference diet will affect the outcome. This method has also been tentatively applied to humans but some additional method development work may be necessary in humans to ensure that very similar methods are

used in both pigs and humans (see "IAAO methodology - humans" section in Annex 2). Assuming such developments, this method will be able to provide the necessary correction or adjustment factors for bridging the TID pig results to the human model. To accomplish this, the identical IAAO metabolic availability experiments need to be conducted in both pigs and humans using a range of identical foods and compare these to the TID pig method. The drawback of the IAAO metabolic availability method is that it only measures availability of a single amino acid in the course of an experiment. Therefore, multiple experiments would be required for each food. The Working Group endorsed the IAAO method and encouraged further research in humans and the proposed experiments to conduct the necessary comparisons of pigs and human results described above. Of immediate importance would be increasing the number of data points representing various diets and foods of varying degrees of digestibility.

7.3 POSTPRANDIAL PROTEIN UTILIZATION (PPU)

Background

The PPU assay was developed to determine the overall efficiency of protein utilisation in the postprandial state (see Gibson *et al.*, 1996; Millward, 1998, 2003, 2012; Millward and Pacy, 1995; Millward *et al.*, 2000, 2002 for a detailed discussion of the limitations of the ^{13}C-1 leucine infusion approaches and of the metabolic model involved). The approach involves measurement of ^{13}C-1 leucine oxidation and balance in response to feeding during a constant infusion of trace amounts of ^{13}C-1 leucine. The change in leucine balance as a proportion of the change in leucine intake represents a value for the efficiency of post prandial protein utilisation (PPU) similar to net protein utilization (NPU) but will produce more realistic values than those from slope of nitrogen balance studies which have been argued to underestimate protein utilization (Millward, 2003; Millward, 2012).

The procedure included two protocols. In each one, leucine balance (leucine intake - leucine oxidation) is converted to nitrogen balance on the basis of leucine - nitrogen conversion factors for tissue protein and this is compared with the actual meal nitrogen intake.

The first protocol (Millward *et al.*, 2000) is a steady state, two intake slope assay, consisting of three consecutive 3-hour periods of fasting, low protein feeding and high protein feeding during a constant intravenous (IV) infusion of ^{13}C-1 leucine. Both the low protein (LP) and high protein (HP) diets are consumed in small meals at 30 minute intervals in an attempt to achieve a metabolic steady state. For such a protocol with both the LP and HP meals being isoenergetic, the PPU is influenced only by the protein intake (insulin levels are unchanged during the two feeding phases) and is calculated from the slope of the N balance curve between the LP and HP meal feeding:

$$PPU = NB/NI$$

The second protocol (Millward *et al.*, 2002) is a non-steady state, area under the curve (AUC) assay also involving a 9-hour iv ^{13}C-1 leucine infusion protocol. In the case of this protocol, a single meal is fed after the initial 3-hour infusion in the fasting state and the cumulative increase in

leucine oxidation above the basal post-absorptive state is measured over the six hours following the meal to give nitrogen utilisation, determined by N intake - cumulative excess N excretion. With this protocol, PPU is influenced by both the protein and energy component of the meal with the energy intake increasing insulin and suppressing proteolysis. PPU is calculated from total excess leucine oxidation, AUC leucine oxidation above fasting level:

PPU = N utilisation/NI

Both protocols produced similar PPU values for milk and wheat protein with the large meal values being slightly lower.

Adaptation of protocol to assess digestibility

The issues discussed at the working group meeting prompted the question of whether this protocol could be adapted to assess the two components of protein quality, i.e. digestibility and biological value.

In order to assess which of the two protocols is most sensitive to changes in digestibility, recalculation of the PPU value can be made using the observed leucine oxidation rates and varying values for digestibility. In the case of the two protocols described above, the digestibility of the wheat protein was assumed to be 93%. In Table 1 below, PPU values have been recalculated assuming digestibility levels of 100%, 93% (as in the published studies), 80% and 50%.

Table 1: PPU values for wheat evaluated by the two protocols assuming different values for the digestibility of the wheat protein used in the studies

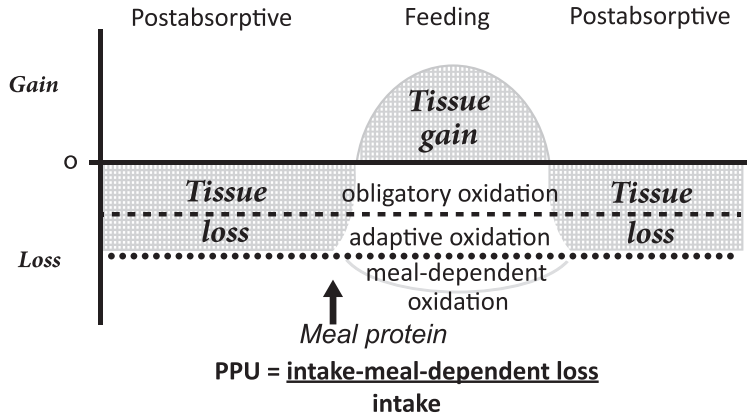
Assumed % Digestibility	PPU Small Meal	PPU Single Meal
100	*0.71*	*0.64*
93	*0.70*	*0.62*
80	*0.68*	*0.55*
50	*0.60*	*0.28*

The PPU calculation from the single meal protocol is much more sensitive to changes in digestibility. This difference between the two methods reflects the way that PPU is calculated in the two studies. Although in each case both the intake and balance (intake-loss) terms in the calculation are influenced by assumptions of digestibility of the wheat, the multiple small meal steady state slope calculation (change in balance as a fraction of the change in intake) is less sensitive than the single large meal AUC calculation of absolute balance/intake calculation. These results suggest that an assay based on the single meal AUC approach would be best suited to separately evaluate both the digestibility and biological value components of protein utilisation. Although the single meal protocol examines the sum of the effect of energy and protein on protein utilisation, it can be argued that this is more relevant in terms of physiology. Thus, it is proposed that the single meal protocol be adopted for examining the digestibility levels.

Suggested single meal assay of protein digestibility

The metabolic model as described by Millward, 2003 and Millward *et al.*, 2002 is shown in Figure 3.

Figure 3: Model assumed for determination of protein utilization during a single meal



PPU = intake-meal-dependent loss / intake

Dietary protein provides for both obligatory and adaptive metabolic demands that occur throughout the diurnal cycle. Leucine oxidation in the post absorptive state is assumed to trace overall amino acid oxidation and nitrogen excretion derived from tissue protein loss. Meal protein is utilized to provide for obligatory and adaptive demands and for tissue gain associated with repletion of post absorptive losses. Any increased leucine oxidation with feeding (meal-dependent oxidation measured as cumulative increased leucine oxidation) represents the inefficiency of meal protein utilization. Postprandial protein utilization (PPU) is calculated from cumulative increased oxidation and nitrogen intake after conversion of utilized leucine into nitrogen to account for differences in meal and tissue leucine-nitrogen ratios.

To examine the protein quality, one must determine the digestibility of the protein, food or diet. If the PPU of the protein or meal under test was compared with the PPU obtained with a meal of an amino acid mixture formulated to match the amino acid composition of the test protein, one can assume that any difference in PPU between the test protein and the amino acid mixture will be due to digestibility only. Thus, the PPU of the free amino acid meal will reflect biological value (amino acid score) whereas the PPU of the test meal will reflect digestibility x BV:

$$\frac{PPU_{\text{test protein}}}{PPU_{\text{amino acid mixture}}} = \frac{\text{Digestibility x } BV_{\text{test protein}}}{BV_{\text{amino acid mixture}}} = \text{Digestibility}_{\text{test protein}}$$

Such an assay allows a complete measure of the protein quality of the protein or mixed meal under test.

Critique of protocol and conclusions

As stated earlier, this method was developed for and to date has only been used to evaluate the biological value of the protein. However, the description above offers a possible adapted protocol, which should allow the digestibility of either a single source protein or a mixed meal to be assessed with a reasonable degree of confidence. It has the advantage of also allowing the biological value and the overall protein utilisation to be assessed and an estimate of the protein requirement of a population group although it does not allow the digestibility of individual amino acids to be assessed. It is stressed that the PPU value and digestibility measure obtained with the method is, like all the other proposed stable isotope methods including the IAAO and dual tracer methods, a measure of the whole body utilisation and actual digestibility in terms of entry of amino acids into the circulating metabolic amino acid pool rather than a measure of loss from the gut at any specific point (terminal ileum or anus). Thus, it is a different approach to and not strictly comparable with either ileal or faecal values as measured in the pig assay.

Its limitations are mainly: 1) the assumptions made in calculating leucine oxidation (see Gibson *et al.*, 1996) in that the route of isotope delivery is intravenous and thus bypassing the small intestine and 2) in converting leucine balance to N balance, i.e. the leucine N ratios of the dietary intakes (which can be measured) and of tissue proteins (which can only be estimated). However, the extent of errors introduced by incorrect conversions factors can be easily calculated. Clearly, because this assay is not firmly established investigators may wish to modify it to suit their own purposes and may require additional research and development for determining the bioavailability of protein bound amino acids. In addition although this approach as described involves developing an existing protocol so that it can measure both digestibility and biological value in human subjects, the same approach can in principle be applied to animal models where such studies were deemed appropriate.

7.4 NET POSTPRANDIAL PROTEIN UTILIZATION (NPPU)

Introduction

The nutritional efficiency of dietary protein utilization is based on the extent to which the dietary protein nitrogen is absorbed and retained by the organism while balancing the daily nitrogen losses. The net protein utilization is the percentage of ingested nitrogen that is retained in the body and is determined by measuring digestive, metabolic (urinary) and miscellaneous nitrogen losses. As the post-prandial phase is a critical period of dietary protein utilization, net postprandial protein utilization (NPPU) which is the immediate retention of dietary nitrogen following meal ingestion represents a reliable and sensitive approach for the assessment of dietary protein nutritional efficiency. NPPU is a measure of protein quality that takes into account true ileal protein nitrogen digestibility and the fraction of absorbed dietary nitrogen that is retained in the body during the post-prandial phase. NPPU is determined from the difference between total ingested dietary protein nitrogen corrected by true nitrogen ileal digestibility, and dietary protein nitrogen subjected to deamination and recovered in urea body pool and urinary nitrogen.

Description of method

Due to the presence of endogenous protein and amino acids, the NPPU approach uses a stable isotope-labelled dietary protein and AA to trace absorption and metabolic utilization of protein bound dietary amino acids (Fouillet *et al.*, 2002a; Fuller and Tomé, 2005) and to differentiate it from the endogenous protein, amino acids and derived metabolites (particularly ammonia and urea) already present in the intestinal lumen and in the blood.

There are a number of ways to intrinsically label proteins with stable isotopes, all of which are reported in the literature (for instance, Bos *et al.*, 2005, 2007; Fromentin *et al.*, 2012; Gausserès *et al.*, 1997; Mahé *et al.*, 1994a). Intrinsic and uniform labelling of dietary proteins with ^{15}N (e.g., milk, soya protein isolate and wheat) have been used in particular to measure the metabolic fate of dietary nitrogen after its consumption so as to allow the investigation of postprandial N transfers into different metabolic pools with Ileal digesta, faeces, blood and urine being sampled in human subjects. NPPU is calculated using true ileal digestibility and ^{15}N-labelled protein utilization parameters (Tomé and Bos, 2000).

In the NPPU method, human subjects are equipped with a blood catheter and a double-lumen intestinal tube introduced through the nose up to the terminal ileum. One lumen is used to perfuse a saline solution of phenol red as a non-absorbable marker of the flux of intestinal effluents, and the other is used to aspirate ileal effluent samples. Basal blood, urine and ileal effluent samples are collected. After 60 minutes of basal ileal sample collection, the subjects receive a single ^{15}N-labeled mixed meal (ingested ^{15}N). Then, during the 8 hours following meal ingestion, the ileal effluents and urine are collected continuously on ice and pooled at 1-hour intervals and blood samples are collected every hour.

Calculation of true ileal nitrogen and amino acid digestibility

Total nitrogen, dietary ^{15}N nitrogen, total amino acids, ^{15}amino acid (^{15}AA) and phenol red concentrations are measured in intestinal effluents. The total nitrogen (Ntot) and amino acid (AAtot) flow rates are derived from nitrogen and amino acid concentrations and ileal flow rates:

$$Ntot = F \times Ns/100 \quad \text{and} \quad AAtot = F \times AAs/100$$

where F is the flow rate of ileal effluents calculated from phenol red concentrations for each 1-hour period, Ns is the nitrogen content of the ileal sample and AAs is the content of each amino acid in the ileal sample.

The level of dietary nitrogen losses present in ileal samples (Ndiet) is determined from the dilution of the isotopic marker (^{15}N) in the samples and similarly, the amount of dietary amino acid losses (AAdiet) recovered in ileal samples was calculated for each amino acid:

$$Ndiet = Ntot \times (APEs/APEm) \quad \text{and} \quad AAdiet = AAtot \times (APEs/APEm)$$

where Ntot is the amount of total N in the sample, APEs and APEm are the ^{15}N enrichment excess (i.e. above the baseline value) of total nitrogen of the sample and the meal, respectively, AAtot

is the amount of individual amino acid (i.e. free amino acid peptides and protein-bound amino acids) in the sample and APEs and APEm are the ^{15}N enrichment excess of the individual sample and the meal, respectively.

Endogenous nitrogen and amino acid contributions were derived from the differences between total and dietary nitrogen and amino acids, respectively. Nitrogen and amino acid true digestibility (TD) in humans are calculated from the cumulated amounts of nitrogen and amino acids recovered at the ileal level and thus not absorbed in the small intestine, using the equation

$$TD = (\text{intake} - \text{ileal content}) \times 100/\text{intake}$$

where intake and ileal content are the cumulative amounts of nitrogen or amino acids ingested and recovered at the terminal ileum after 8 hours.

Calculation of NPPU

Dietary ^{15}N transferred to body urea (body ^{15}N-urea) and transferred to urine, mainly as urea, (urinary ^{15}N) are measured. NPPU is calculated as follows:

$$NPPU\ \% = 100 \times [\text{ingested } ^{15}N - (\text{ileal } ^{15}N + \text{body } ^{15}N\text{-urea} + \text{urinary } ^{15}N)]/\ \text{ingested } ^{15}N$$

Results for ileal digestibility and NPPU of protein sources

This NPPU method has been applied to a variety of different protein foods (Bos *et al.*, 1999, 2003, 2005, 2007; Gaudichon *et al.*, 1995; Gausserès *et al.*, 1997; Lacroix *et al.*, 2006, 2008; Morens, 2003; Mariotti *et al.*, 1999, 2000, 2002;). True ileal nitrogen digestibility measured for different protein sources were: milk protein, 95% (Bos *et al.*, 2003; Gaudichon *et al.*, 2002; Mahé *et al.*, 1994b); soya and pea protein, 91 % (Bos *et al.*, 2003; Gaudichon *et al.*, 2002; Gausserès *et al.*, 1997); wheat protein, 85-90% (Bos *et al.*, 2005; Juillet *et al.*, 2008); rapeseed protein, 84% (Bos *et al.*, 2007). In addition, True ileal digestibility values of dietary nitrogen and amino acids was also measured after the ingestion of milk or soya protein, with digestibility of amino acids ranging from 91% (glycine) to 99% (tyrosine) for milk protein, and from 89% (threonine) to 97% (tyrosine) for soya protein (Gaudichon *et al.*, 2002).

An average value of 70 % can be considered as the NPPU of dietary proteins (Bos *et al.*, 2005) when determined under optimized, controlled conditions in healthy adults. Comparison of the immediate post-prandial nitrogen utilization of different protein sources in adults adapted to an adequate level of protein intake shows values for post-prandial protein utilization ranging from 75-93% for high quality protein as milk protein to values of 63-66% for protein with lower quality such as wheat. Specific NPPU values determined for milk, soya protein isolate and wheat were 81%, 78% and 66%, respectively (Bos *et al.,* 1999; Bos *et al.,* 2005; Mariotti *et al.,* 1999; Tomé and Bos, 2000).

In addition, the kinetics of dietary N appearance in ileal effluent, plasma proteins, plasma free amino acids, body urea, urinary urea and urinary ammonia are calculated using a 13-compartment,

21 parameter model (Juillet *et al.,* 2006). The model calculations are fairly complex but allow one to predict the kinetics of dietary nitrogen in the body (Juillet *et al*., 2006). Both the amino acid profile of the protein and the kinetics of amino acid delivery to the blood can affect the postprandial splanchnic and peripheral uptake of amino acid in humans (Deglaire *et al*., 2009b; Fouillet *et al*., 2000, 2001, 2002b, 2003, 2009; Juillet *et al*., 2008). Increasing protein intake increases splanchnic catabolic use while splanchnic catabolic use and peripheral anabolic use are inversely affected (Fouillet *et al*., 2009). Interestingly, this approach shows a nutritional value of individual food proteins in the order animal protein (milk) > legume (soya, pea) protein > cereal protein (wheat).

Critique of method and conclusions

This method represents a major advance in the evaluation of dietary protein quality. The NPPU of Tomé *et al.* is very well developed in humans and with application to the pig model could very clearly be used to quantify the necessary correction or adjustment factors required to calculate human DIASS values from pig TID protocol studies. In those instances where the existing and well-described method in humans has been replicated in the pig, there has been excellent agreement of the values to the TID pig values (Deglaire *et al*., 2009a).

Like other protocols discussed, this research method needs to be extended over a wider range of human foods, especially those with amino acid digestibility in the range of 50-80%. This would create a viable bridge between the methods, as it will be possible to compare the results on the digestibility of the different proteins, including highly digestible and poorly digestible proteins, obtained by the different methods.

A drawback of the method is that it relies on the use of ^{15}N as a tracer and this stable isotope is currently expensive to produce. In addition, it is restricted only to foods that can be intrinsically labelled with ^{15}N. Another drawback of the current NPPU method is it being unacceptably invasive for widespread use in humans due to the use of the naso-intestinal intubation technique to collect the ileal digesta. Therefore, with the inclusion of this invasive procedure, this method is unlikely to be widely adopted for routine application. However, the Working Group was apprised that the method could be simplified and less-invasive and thus potentially more widely applicable. While the development of this simpler and less invasive method is still in progress, the Working Group was encouraged by this potential enhancement as it appears to have great promise. A significant advantage of the method is that within one experiment data on both total nitrogen and individual amino acid digestibility of the food are produced.

7.5 A DUAL TRACER APPROACH TO MEASURING DIAAS

Introduction

The measurement of the digestible indispensable amino acid score (DIAAS) (FAO, 2013) of proteins in humans is difficult because of the inaccessibility of the ileum. A dual stable isotope tracer approach, which is being developed, promises to provide a minimally invasive means to

estimate digestibility, without the requirement for intubation or fistulation of the gut. The method also minimizes subject burden by requiring the consumption of only a single test meal from which the appearance of plasma amino acids is measured. Furthermore, the method has the advantage in going beyond estimating the DIAAS, which largely describes the bioavailability of protein in foods or meals, to permitting the additional estimation of amino acid utilization, thus providing information on other aspects of protein quality.

The dual tracer method utilizes intrinsic labeling to allow the study of the digestion of a specific plant protein independent of other proteins in the test meal and independent of endogenous proteins in the body. However, appearance of amino acids in the accessible pool (blood) is not quantitative, due to uptake and metabolism of these amino acids in the splanchnic bed. By accompanying the test protein with a small quantity of a highly digestible protein labeled with a different isotope, and ingested in the same balanced meal, the effect of the splanchnic removal can be corrected. This principle presents a means to develop a non-invasive procedure that avoids intubation. The novelty of this new approach is the use of this dual labeling where measurement of amino acid appearance from a test protein is compared with a reference protein. The reference protein will be common to all measurements using this approach and originate from a commercial source of single-celled protein, highly enriched with a stable isotope. It is important that the reference protein is highly digestible and culturally acceptable; microalgal protein of certified quality meets this criterion. Its amino acid composition need not be exactly the same as accepted high quality proteins like egg protein (or human milk protein), as correction terms can be applied.

Intrinsic labeling

There is precedent for the intrinsic labeling of foods for human nutrition trials. For carbohydrate assimilation studies, cereal and legume starch have been labeled with ^{13}C (Edwards *et al.*, 2002; Priebe *et al.*, 2008; Verbeke *et al.*, 2010). For lipids and fat-soluble vitamins, ^{13}C and ^{2}H have been applied with equal success to label essential carbon skeletons (Bluck, 2009; Parker *et al.*, 1993; Tang *et al.*, 2005). For the intrinsic labeling of proteins, ^{15}N has commonly been applied in microbial, plant and animal proteins (Gaudichon *et al.*, 2002; Picou and Taylor-Roberts, 1969; Stack *et al.*, 1989). ^{15}N is unsuitable for tracing the carbon skeleton of individual indispensable amino acids, as transamination after digestion and absorption mixes the label through the alpha amino pool. However, plants proteins can also be labelled with either ^{13}C or ^{2}H.

Two isotopic forms of each amino acid would be exploited in this dual labeling approach. Deuterium (^{2}H) and ^{13}C can be used to trace the indispensable amino acid carbon skeleton equally well. However, tracer costs, together with the costs of intrinsic labeling of foodstuffs, argue that $^{2}H_2O$ rather than $^{13}CO_2$ should be used to label the test protein. Legumes are the protein sources currently under consideration, but developing intrinsically labeled proteins from other sources is feasible and possible. A field protocol is being developed to facilitate the efficient labeling of growing legumes by the application one or more pulses of $^{2}H_2O$ in a predetermined pattern after flowering.

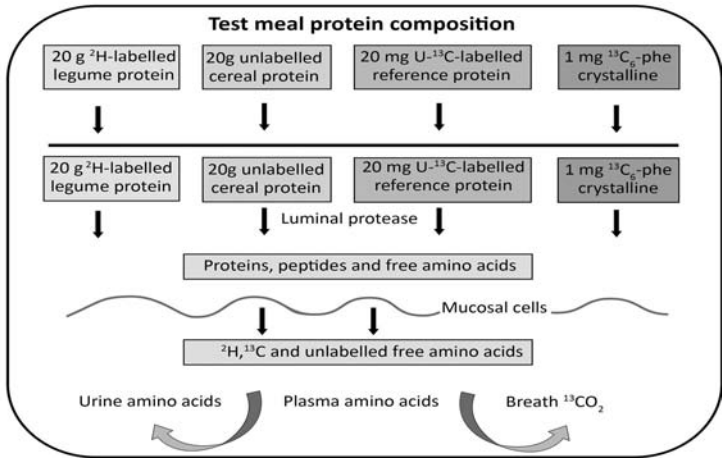
Test meals

Test meals comprising known quantities (about 20 g protein) of ^{2}H-labelled grain legumes will be processed and prepared into meals according to local cooking practices. Since the processing and preparation of food will impact the digestibility of protein, these variables will be carefully considered. A commercial source of highly enriched ^{13}C-enriched single cell protein (Spirulina; *Arthrospira sp.*) will be added at minimum quantity (20 mg protein) as the reference protein in each test meal.

Protocol

Adult subjects will undertake a controlled study where, following a test meal, blood and breath samples will be obtained over a time course of no more than 8 hours (Figure 4). Subjects will be asked to report to the nutrition facility after an overnight fast. In addition to anthropometric measurements, they will be asked to give basal breath and blood samples. Subjects will then consume a test meal containing 20 g of the ^{2}H-labeled grain legume protein under test, in a balanced meal including unlabeled cereals, vegetables and condiments, along with 20 mg of a ^{13}C-labelled reference protein and 1 mg of crystalline $^{13}C_6$-phenylalanine, obtained from a commercial source. Initially, for each labeled legume under study, blood and breath samples will be taken every 20 minutes for 4 hours and then hourly up to eight hours. Blood sampling frequency and duration will be reduced in subsequent measurements after the pilot results have been appraised.

Figure 4: Test meal protein composition



Analyses

Free amino acids will be isolated from blood plasma by cation exchange following plasma protein removal. Amino acids will also be prepared by acid hydrolysis from samples of each test and reference protein. Volatile derivatives (ethoxycarbonyl ethyl amino acid esters) will be prepared

from amino acids prior to analysis of multiple-atom ^{13}C enrichment by Gas Chromatography Mass Spectrometry (GC-MS) and of ^{2}H-enrichment by GC-pyrolysis-Isotope Ratio MS (IRMS), (MacDonald *et al.*, 2013). A single H atom is lost from most IAA during transamination, but this process can be predicted (Commerford *et al.*, 1983) and an equivalent post-digestive fate of test meal protein-derived IAA (labelled with ^{2}H) and reference protein-IAA (labeled with ^{13}C), can be assumed. The combination of analytical instruments chosen allows the use of the same volatile derivative for both analyses while using minimal sample for ^{13}C analysis with analysis of low-level ^{2}H analysis by IRMS. This strategy also permits analysis of different isotopologues by GC-MS so that a 1mg quantity of a crystalline amino acid with a unique signature (probably $^{13}C_6$-phenylalanine) can be added to the test meal to provide an absorption index.

Outputs and calculations

A variety of indexes of protein quality will be derived:

Digestibility: For each indispensable amino acid in the grain legume protein, digestibility will be calculated from the AUC of IAA from the test protein relative to IAA in the reference protein:

$$100 \times (\text{AUC } ^{2}H\text{-IAA}/\text{AUC } ^{13}C\text{-IAA}) \times [\text{test meal } ^{13}C\text{-IAA}/\text{test meal } ^{2}H\text{-IAA}]$$

Absorption: AUC of the appearance of plasma $^{13}C_6$-phe, normalized to tracer dose and body size, could be used as an index of absorption.

Validation studies

A number of validation studies will be undertaken. Firstly, if the test protein was the same protein as the reference protein but labeled with ^{2}H, then tracer equivalence in terms of splanchnic handling can be confirmed. Secondly, the availability of highly digestible animal proteins will also be studied. Feeding labeled grain legumes to a laying hen will produce eggs with protein labeled intrinsically with ^{2}H-IAA. A test meal based on intrinsically labeled egg protein will be used in a small number of subjects to demonstrate the protein quality outputs in an accepted highly digestible protein. It is also proposed that test meals containing ^{2}H-labelled milk protein will be assessed using this protocol. Thirdly, validation studies on intubated adult volunteers will be carried out. These invasive intubation studies will provide a bridge between bioavailability studies in the literature and the new non-invasive dual tracer method. Finally, as the largest database of protein bioavailability exists in animals (rats, pigs) it is proposed that the dual tracer protocol be translated to a center experienced in undertaking ileal digestion studies in pigs, in order to compare digestibility estimates with both methods. A typical protocol may involve a Latin square design where up to 6 test proteins were tested in 6 animals. The test proteins would be labeled and include those with a variety of digestibility, including common grain legumes.

Importantly, the studies described above will help to inform the design of a streamlined protocol suitable for use in larger studies and for use in children, with shorter duration and fewer blood samples. This will be dependent on the co-incident appearance in blood of the two tracers in each protein source, as evidence that the proteins in the test meal were emptied from the

stomach and subjected to small intestinal digestion in the same time frame. This will add strength to the proposed use of the appearance ratio of both tracers in individual IAA close to the time of maximal appearance. If tracer appearance is co-incident, then the ratio of tracer enrichment at time of maximum appearance can be used as a proxy of their area under the curve (AUC) ratio using a single blood sample, as opposed to a series of samples taken over an 8-hour period.

The dual tracer method for measuring DIAAS: larger studies and studies in children and infants

Larger studies and studies in children will benefit from a streamlined protocol using as few blood samples as feasible. Should it prove possible to prepare test meals so that tracers appear in coincidence, it is theoretically possible to derive parameters from a single blood sample taken close to the time of maximum tracer appearance. Following the validation phase, the protocol will be evaluated to judge if a shorter protocol, ideally using a single blood sample, can be adopted for use in children. For instance, Evenepoel and co-workers (2000) showed that a maximum of ^{13}C leucine appeared in blood occurred 2 hours after ingestion of a meal containing intrinsically labeled egg white protein.

Strengths and limitation of the dual tracer approach to measuring DIAAS

The dual tracer approach addresses the requirement to measure the bioavailability of individual indispensable amino acids. This approach is relatively non-invasive, requiring only blood sampling. The dual tracer approach has potential for use in children and infants, especially if tracers appear simultaneously and in co-incidence, facilitating use of a minimum number of blood samples. Studies will also be undertaken to determine if saliva or urine samples can be used to make these measurements more acceptable for use in infants. Multiple output terms are feasible in addition to digestibility. Additional parameters of protein quality, such as protein utilization (oxidation) can also be obtained if breath samples are collected in addition to blood samples. It is potentially possible to interpret an AUC analysis of unlabeled amino acids to derive an index of dispensable amino acid turnover. Finally, other measurements are accessible, such as analysis of the relative fractional synthetic rate of major plasma proteins.

The principal limitation of the dual tracer approach is the cost of procuring stable isotope labeled proteins together with the cost of analysis. Table 2 lists examples of the cost of representative tracers. It should be noted that these costs do not account for inefficiencies during intrinsic labeling of plants, which can be considerable. The cost of tracer procurement will be mitigated by use of state-of-the-art Mass Spectrometry (MS) so that minimal quantities of tracers can be used. It is not proposed to apply the method in very large groups, but in key groups with the intention in informing larger nutrition trials. Further, it should be noted that by minimizing the number of samples required to derive the necessary outputs, a single MS facility could support studies over a wide geographical area.

The need for extensive validation is another limitation. As a novel concept, validation studies are necessary for the approach to become widely accepted, but these can be accomplished under controlled conditions and in a small group of healthy adult volunteers. A final limitation is the assumption that in the dual tracer design, splanchnic removal is the same for amino acids derived from the test protein and reference protein.

Table 2: Example of current tracer costs (source: Cambridge Isotope Laboratories Inc., MA, USA, March 2014)

Plant Labeling Feedstock		**Test meal tracers**		
Material	Cost	Material	Cost	Cost /'dose'
$^{2}H_2O$	\$0.50 /g	Spirulina, whole cells	\$475 /g	\$16.5 /'dose'
$^{13}CO_2$	\$56.8 /g	$^{13}C_6$-phenylalanine	\$780 /0.25g	\$3 /'dose'
$(^{15}NH_4)_2SO_4$	\$48.0 /g			

Section 8:
Final considerations of the working group

Following the deliberations on research protocols and methods, the Working Group summarized their general recommendations and considerations for future work:

- The experts concluded that conceptually true ileal amino acid digestibility as expressed by DIAAS was preferable to the faecal crude protein digestibility as expressed by PDCAAS as the accepted protein and amino acid quality evaluation method. However, they also recognized that the complete value of DIAAS could not be realized until there are sufficient accumulated digestibility data for human foods as determined by competent national and/or international authorities. A meeting of experts and stakeholders may be required to assess the size and quality of the dataset and determine the timeline for full adoption and implementation of DIAAS.
- To allow an informed decision regarding DIAAS, there is the need to develop a fully accessible, robust database on amino acid digestibility of foods and diets from different regions of the world. In addition, there is a need to identify commonly consumed human foods focusing on the diets consumed by vulnerable populations of varying ages in low income countries with the need to conduct multiple studies using the same methodologies in different regions of the world.
- Before a final decision is made concerning the adoption of DIAAS, there will be a need to review the practical effects for public health policies and programmes in using true ileal amino acid digestibility (via DIAAS) instead of faecal crude protein digestibility (via PDCAAS). Preliminary work concerning the public health policies and programmes could be initiated in the near future.
- There is need for protocols to assess protein requirements in vulnerable groups with increased requirements due to physiological changes such as pregnancy, chronic inflammation and the effect of sanitation and hygiene. In addition, reference patterns for these vulnerable groups will need to be developed and applied to the amino acid requirements that will be used with the new DIAAS data.
- In discussing practical steps forward, there is the need to identify funds for research. The experts believed that public and private sector funding would be required to carry out this work and efforts should be made to encourage such funding. It would be most advantageous that there be coordination of the funding of the research and data collection.
- There is a need to foster collaboration with international and national agriculture research institutions for introducing stable isotopes into various plants. This would require training and funding for equipment to assist institutions. In addition, to ensure sustainability and relevance, the experts suggested that it would be advantageous for young researchers from developing countries to be given opportunities to participate in research on this topic.

- A long term recommendation is that there is a need to develop a method that can be used by government agencies to regulate specific foods in terms of protein quality.
- In addition, the potential for the additivity of DIAAS measurements in mixed meals and prepared food products that contain multiple ingredients must be assessed.
- There is a need to clarify the nomenclature related to digestibility (e.g. apparent, true, standardised).

Annex 1:
True ileal amino acid digestibility assay: a standard methodology

The 2011 FAO Expert Consultation on protein quality in human nutrition recommended the following:

> "It is recommended that the FAO convene a Working Group, as a matter of urgency, to agree upon an experimental protocol to enable the development of a more robust data set of the true ileal amino acid digestibility of human foods and agree upon a method for assessment of the potential impact of the use of true ileal amino acid digestibility data. The protocol should include recommended best practice for a pig-based assay for true ileal amino acid digestibility determination" (FAO, 2013).

The following protocols address this recommendation.

Human-based assay for true (corrected for basal endogenous amino acid losses) ileal amino acid digestibility

Background: The 2011 Expert Consultation stated that "digestibility should be based on the true ileal digestibility (i.e. determined at the end of the small intestine) of each amino acid preferably determined in humans ....." (FAO, 2013). When liquid or non-fibrous, powdered type foods (e.g., casein, gelatin, zein) are to be assessed, the naso/ileal intubation method as described by Gaudichon *et al.* (2002) is preferred. Where fibrous, coarse foods (e.g. cooked beans) are to be assessed whereby representative digesta sampling and tube blockages may be a problem, the protocol described by Moughan *et al.* (2008) involving adult ileostomates should be followed, with the protein source included in the subject's general diet for 7 days before digesta collection. Endogenous losses can be determined according to the method as described by Rowan *et al.* (1994) where the subject is its own control.

Pig-based assay for true (corrected for basal endogenous amino acid losses) ileal amino acid digestibility

Background: Determining true ileal amino acid digestibility in humans is difficult and expensive. Consequently, for the routine evaluation of foods, it is necessary to adopt an animal model. The Expert Consultation (FAO, 2013) recommended the pig as the best model for the adult human and where this model cannot be applied, the growing rat. True ileal amino acid (including reactive lysine) digestibility is the preferred method in pigs for predicting dietary amino acid uptake. The

ileal assay has been thoroughly validated for its application in the pig and is now used routinely to assess the protein quality of feedstuffs for pigs and for other simple-stomached farm animals. Research by Deglaire *et al.* (2009a) and Rowan *et al.* (1994) and has shown a high degree of agreement for true ileal amino acid digestibility between the adult human and the growing pig, but this is based on a limited number of foods. In light of this, the 2011 FAO Expert Consultation recommended that more work be conducted to compare true ileal amino acid digestibility values between the human and growing pig using protein sources that are representative of those consumed by human populations, to further validate the pig as a model animal. In addition, if a world database on the true ileal digestibility of human foods is to be generated, a standard protocol would be of considerable benefit. The following describes a standard protocol, developed by a number of scientists with expertise in the field.

Protocol

Approval from an appropriate animal ethics committee must be obtained and animals must be housed and cared for in accordance with the requirements of the organisation where the studies are conducted.

Animals:

30-100 kg commercial-type pigs

T-cannula (placed proximal to the ileo-caecal valve)

Minimum of 5 animals per test protein source

Minimum 5-day recovery from surgery

Feeding protocol:

Animals adapted to an appropriate human-type diet

Feed at 8% of body weight$^{0.75}$/d as 2 meals at ≥9 h interval between meals

All nutrient requirements, except protein/amino acids, to meet those prescribed by the NRC (2012)

Minimum of 5 days adaptation to the diet and environment

Water *ad libitum*

Test diet composition[1,2]:

Test protein source included at a CP[3] concentration of 100 g/kg diet DM

Mixture of purified sucrose, corn oil and cellulose (micro-crystalline) in a ratio of 10:5:3 (180 g/kg DM)

[1] In order to maintain a dietary CP concentration of 100 g/kg, for low protein foods where CP is <150 g/kg DM, the test ingredient can be diluted with a mixture of cellulose and corn oil (1:1). For foods where the CP concentration is <10 g/kg DM CP, the test diet should consist of the test ingredient, vitamin/mineral mixture and TiO_2.

[2] The composition of diets for comparing true ileal amino acid digestibility in pigs and to humans may differ from that shown above.

[3] Crude protein (CP) is estimated based on the determined N content of the protein source and using an N to protein conversion factor of 6.25.

TiO_2 (3 g/kg DM)

Vitamin/mineral premix at prescribed inclusion rate

The diets are made up to their total weight with purified corn starch

Test protein source should be:

a. fed in a similar form as consumed by humans (appropriately ground if required)
b. homogeneously distributed through the rest of the diet

Housing conditions:

Individually housed in pens without bedding materials able to be consumed in such a manner as to ensure that coprophagy does not occur

Thermoneutral zone

12 hour light/dark cycle

Digesta collection:

Collected over two periods of ≥9 hours, starting directly after a meal and separated by at least 2 meals

Collection bag to contain antimicrobial compounds

Collected digesta frozen as soon as possible

Digesta collected to be bulked over days and hours of collection per animal

Endogenous losses determination:

Feeding a protein-free diet

Each animal is its own control

Feeding at 8% of body weight$^{0.75}$/d fed as 2 meals at ≥9 h interval between meals

Chemical analyses of diet and digesta:

TiO2, amino acids[4], dry matter

Rat based assay for true (corrected for basal endogenous amino acid losses) ileal amino acid digestibility

Background: The Expert Consultation stated that "digestibility should be based on the true ileal digestibility (i.e. determined at the end of the small intestine) of each amino acid preferably determined in humans ....., but if this is not possible, in the growing pig ....., or in the growing rat, in that order (FAO, 2013). The following describes a standard protocol developed by a number of scientists with expertise in the field.

Protocol

Approval from an appropriate animal ethics committee must be obtained and animals must be housed and cared for in accordance with the requirement of the organisation where the studies are conducted.

[4] Amino acid analyses should be conducted according to the standard protocol as described by Rutherfurd and Gilani (2009).

Animals:

200 - 250 g male laboratory (e.g. Sprague-Dawley) rats

Minimum of 5 animals per test protein source

Feeding protocol:

Animals adapted to environment, diet and feeding regimen and fed an appropriate human-type diet for 9 days

Nine hourly feeds from 8.30-16.30 with feed freely available for 10 min each hour

All nutrient requirements, except protein/amino acids, to meet those prescribed by the NRC (1995)

Minimum of 5 days adaptation to the test diet and environment

Water *ad libitum*

Test diet composition[5]:

Test protein source included at a level of 100 g/kg CP[6] (% DM of the diet)

Mixture of purified sucrose, corn oil and cellulose (micro-crystalline) in a ratio of 10:5:3 (180 g/kg DM)

TiO_2 (3 g/kg DM)

Vitamin/mineral premix

The diets are then made up to their total weight with purified corn starch.

Test protein source should be finely ground and homogeneously distributed in the diet such that selective consumption of dietary components is not possible

Housing conditions:

Individually in wire-bottom cages to prevent coprophagy

Thermoneutral zone

12h light/dark cycle

Digesta collection:

Asphyxiation with CO_2 and decapitation or cervical dislocation

Dissection of the ileum section 20 cm anterior to ileal-caecal valve

Flushing of digesta using distilled deionised water

Collected digesta for each animal frozen as soon as possible

Stomach content checked for coprophagy

[5] In order to maintain a dietary CP concentration of 100 g/kg for low protein foods where CP is <150 g/kg DM, the test ingredient can be diluted with a mixture of cellulose and corn oil (1:0.6). For foods where the CP concentration is <10 g/kg DM CP, the test diet should consist of the test ingredient, vitamin/mineral mixture and TiO_2.

[6] Crude protein (CP) is estimated based on the determined N content of the protein source and using an N to protein conversion factor of 6.25.

Endogenous losses determination:
Feeding a protein-free diet
As with the rats fed the test diet, use the same housing and procedures.
Chemical analyses of diet and digesta:
Ti, amino acids[7], dry matter

[7] Amino acid analyses should be conducted according to the standard protocol as described by Rutherfurd and Gilani (2009).

Annex 2:
Detailed Protocol of the Indicator Amino Acid Oxidation (IAAO)

IAAO Methodology - Pigs:

The details of the protocol for pigs have been described in the experiments published by Levesque *et al.* (2010) and Moehn *et al.* (2007). The details of the protocol for humans have been described by Prolla *et al.* (2013). Additional comments and clarifications are provided below.

Reference diet

The basal diet should consist of highly digestible ingredient such as whey or casein to meet approximately 30% of the dietary requirement of the test amino acid. The same highly digestible protein must be used as the reference protein in the basal diet that will be used in the pig TID and human IAAO experiments for bioavailability, and comparability between the pig TID and the human experiments is required to calculate human DIAAS from the extended pig TID database.

The remaining amino acids should be supplemented as free amino acids. During the adaptation period to the test food, this will be in the pattern of the test food up to at least 120 % of the requirement of the pig (NRC, 2012) according to the rate of food intake. During the experimental period, when oxidation is being conducted, all of the free test amino acid must be removed from the diet and replaced, either with increments of the test amino acid to create the reference slope, or with increments of the test foods. All other amino acids must be provided in the diet to provide at least 120% of requirement (see Sub-section 7.2).

First limiting Amino Acid

The test AA must be first limiting in the diet to ensure that the intake of the test AA drives the change in indicator oxidation. This is achieved by providing all other amino acids, in the same pattern as the test protein, at approximately 120% of dietary EAR. This is approximately 2 SD above the mean requirement and thus can be assumed to exceed the demands of the majority of the population. Intakes higher than 120% may be chosen if there is uncertainty about the requirements for the amino acids or their availability in the test protein is expected to be very low or is unknown.

Linearity of oxidation response

The change in indicator oxidation to incremental changes in test AA must be linear to allow calculation of availability. This is accomplished by restricting the maximum intake of the test amino acid to no more than 60 to 80% of the EAR. This intake is necessary because intake must

be at least 2 SD below the requirement to ensure that none of the subjects in the population sample will be meeting their demand for the limiting amino acid. If an individual's intake were to exceed the requirement, the slope would not be linear because oxidation of the indicator amino acid would be in the plateau phase. Linear regression is used to determine the slope of the best-fit line.

Tests for linearity must always be conducted for the test amino acid. The researcher has a choice of measuring linearity of response to the test food. If the food is within the reference range of foods used to develop the correction factors for pig TID then this may not be necessary and only the intake containing 60-80% of the EAR will be required. However, if the test food is potentially outside of the reference range of foods used to develop corrections or adjustments to the pig TID database then it is recommended that linearity of response be conducted for the test food also.

Lack of interactions

The observed response must not be influenced by dietary nutrients other than the foodstuff being tested (Batterham, 1992; Littell *et al.*, 1995). Of course, if the goal of the experiment is to test interactions between foods or to study the influence of anti-nutrients, then these criteria can be waived after properly characterizing bioavailability of amino acids in the test foodstuff.

Adaptation period

The minimum adaptation period for pig IAAO bioavailability should be the same as the adaptation period used in the pig TID experiments (see Annex 1) to ensure comparability of experiments and transferability to the pig TID database. If the test food is highly fibrous, is suspected to be very poorly digested or contains anti-nutrients that require longer adaptation, then information on the minimum adaptation period should be obtained from the literature or the oxidation experiment can be repeated at intervals to determine the adaptation period.

Isotopic steady state

An isotopic steady state in oxidation of the indicator amino acid must be achieved during each individual test of oxidation. The percent (%) of the dose oxidized per unit time must only be calculated after the oxidation reaches a plateau otherwise the measurement will be unduly influenced by the rate of absorption and transport of all the amino acids rather than bioavailability of the test AA.

Subjects as own control

Each subject must be used as their own control in a repeated measurements design. Each study subject receives each of the series of 3 or 4 reference diets supplying free test AA (crystalline form) at incremental increases to a maximum of 80% of the EAR, as described above. Each subject also receives each of the test diets containing the protein-bound AA. The use of repeated

measurements within the same subject reduces the effect of individual variability, and increases the sensitivity of the estimate by accounting for between-subject variation, which is recognized as at least 20 % in humans (WHO, 2007) and can be up to 30% (Zello *et al.*, 1993).

Conditions for the indicator Amino Acid

There are a number of conditions regarding intake of the indicator amino acid that are critical for the successful application to assessment of protein quality and amino acid bioavailability by the IAAO method.

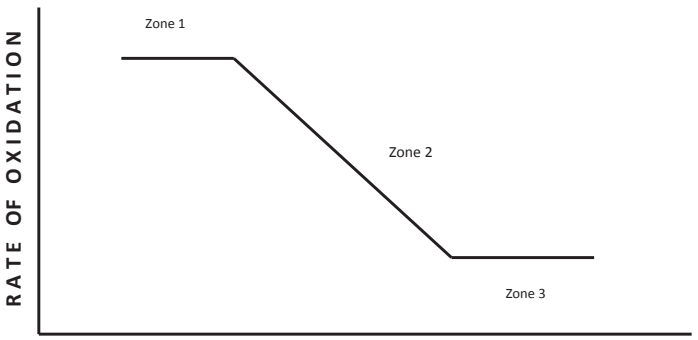
The indicator amino acid increases in excess as against its dietary requirement for protein synthesis, as intake of the test protein or amino acid decreases. To ensure the constant intake of indicator amino acid, on a bioavailable basis, requires knowledge of both the concentration of the indicator in the test foods and of its bioavailability.

Concentration of the indicator amino acid may be determined by direct amino acid analyses or nutrient composition tables. Bioavailability in humans will not be known unless it has been measured, which is rare. The Expert Consultation (FAO, 2013) and the Working Group concluded that TID amino acid values from the pig were highly correlated to the few measured values in humans. Therefore, bioavailability values for the indicator amino acid in the test protein may be taken from published TID values in the pig or the values predicted for humans from the pig data, when these become available. If these are not available for the test protein, then the preferred approach will be to measure pig TID of the indicator amino acid prior to the IAAO experiments. If this is not possible then an estimate may be made, with the proviso that the experiment may need to be repeated if a plateau response is observed at either lower or upper intakes of the test protein (See below), indicating that the estimated bioavailability value or the indicator amino acid was either too low or too high.

In addition to being constant, intake of the indicator amino acid, on a bioavailable basis, must not become either deficient or excessive (see Figure 5). The indicator amino acid increases in excess as against its dietary requirement for protein synthesis, as intake of the test protein or amino acid decreases. If intake of the indicator amino acid is in too great of an excess (zone 1 Figure 5), then the slope of the line at the lower intakes of the test protein or amino acid may plateau because at some point the intake may exceed the oxidative capacity of the body for the indicator amino acid. If the line is at plateau, it will no longer be in linear and decreasing slope (zone 2 Figure 5), and therefore the slope ratio analyses will be incorrect. Alternatively, if intake of the indicator amino acid were to be deficient (below dietary requirement on a bioavailable basis) then at upper levels of intake of the test protein or amino acid, the slope of the line will also plateau (zone 3 Figure 5) because protein synthesis will be limited by intake of the indicator amino acid, rather than intake of the test protein or amino acid. Again, the slope ratio analyses will be incorrect. The response shown in zone 3 will also occur if the intake of the test protein or amino acid exceeds its dietary requirement, which is what happens during IAAO requirement experiments. To ensure intake of the indicator amino acid is neither deficient nor excessive the dietary requirement of the test population must be known. For information on requirements,

the reader is referred to the many publications on amino acid requirements (e.g. WHO, 2007), particularly those that used amino acid oxidation; because if those experiments used diets based on free amino acids, the requirements represent the bioavailable amino acid requirement.

Figure 5: Response will be non-linear if the intake of the indicator amino acid is either in excess (Zone 1) or deficient (Zone 3)



IAAO Methodology - Humans:

The IAAO method to measure metabolic availability has been described by Prolla *et al.*, 2013. The following is a brief description and explanation of key issues and differences between the method of Prolla *et al.*, 2013 and the protocol recommended in the current report.

Isotope protocol

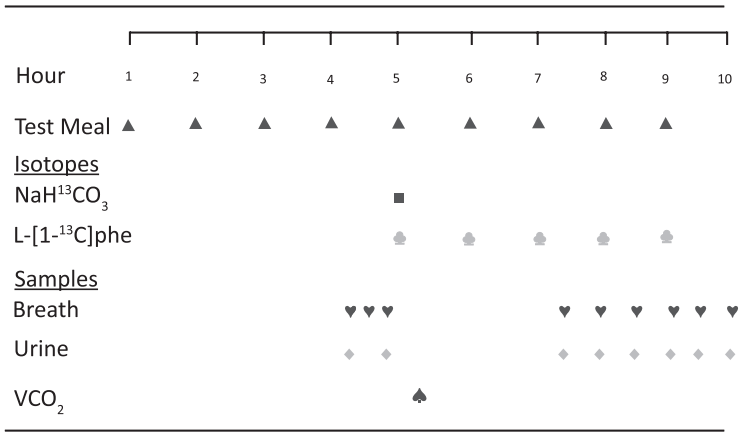
Test diets must have the appropriate amounts of phenylalanine + tyrosine, to ensure sensitive partitioning of response between protein synthesis or oxidation. The amount of L-[1-^{13}C] phenylalanine given during the study day must be subtracted from the dietary provision of phenylalanine such that the total intake of phenylalanine is 30 mg/kg/d with a tyrosine intake of 40 mg/kg/d (to ensure an excess of tyrosine). Previous human studies have used priming doses of $NaH^{13}CO_3$ (2.07 µmol/kg) and L-[1-^{13}C] phenylalanine (99 atom % excess, 40 µmol/kg). The continuous dose of L-[1-^{13}C] phenylalanine will be 15 µmol/kg/h, given hourly until the 9th meal (see Figure 6). If other isotopic choices are made, then additional studies need to be conducted, especially linearity of response to both the test amino acid and protein bound amino acid, to ensure adequate responses in indicator amino acid oxidation to measure amino acid availability.

Dietary composition

Diets should be designed to provide a protein intake of 1.0 g/kg/d. Diets should achieve an energy intake of 1.5 X Resting Metabolic Rate with approximately 52%, 36% and 12% of energy from carbohydrates, fat and protein, respectively. For the duration of all experiments, subjects should consume a daily multivitamin supplement and 500mg of choline to ensure adequate vitamin intake.

The study day diet should be consumed as 9 isonitrogenous and isocaloric hourly meals, each meal representing one twelfth of the subject's total daily protein and energy requirement. Each study day will be 10 hours long (see Figure 6).

Figure 6: Human IAAO study day protocol (Annex 2)



Test for linearity of free Amino Acid intakes

The first objective should be to ensure that metabolic availability is being measured during a predictable response to graded intakes of test amino acids in each individual subject. For example, recommended intakes to test for linearity of response to lysine could be in the range of: 10, 15, 20, 25 and 28 mg Lysine/kg/d which would meet 28.5, 43, 57, 71 and 80 %, respectively of the mean lysine requirement in adult men.

Basal and test protein diets

The basal diet should consist of highly digestible protein that attains a maximum of 30% of the dietary requirement of the test amino acid. As described in the pig IAAO protocol (see above) use of the same reference diet in both humans and pigs will allow comparisons across all experiments and species. The remaining amino acids should be supplemented as free amino acids. During the adaptation period to the test food, free amino acids will be provided in the pattern of the test food to a minimum of 120% of the requirement of the human EAR (WHO, 2007) for the most limiting amino acid. During the experimental period, when oxidation is being conducted, all of the free test amino acid must be removed from the diet and replaced, either with increments of the test amino acid to create the reference slope, or with increments of the test foods. All other amino acids must be provided in the diet to provide a minimum of 120% of mean requirement of the most limiting amino acid.

A highly digestible ingredient must be used as the reference protein and foundation of the basal diet. Whey is recommended because it has a relatively low phenylalanine content

and therefore a wide range of foods with high phenylalanine concentrations can be studied. Bioavailability of amino acids in milk proteins has been tested in humans (Bos *et al.*, 1999) and may be adopted for use in the model.

The test protein should be included in the diet to provide a maximum of 40% of the total test amino acid because approximately 40% will be provided by EHC in basal diet and the remainder as free amino acid. With this protocol, diets with amino acid bioavailability as low as 60% can be tested. The experimental subjects may not be able to consume 40% of their protein requirement from the test protein for several reasons. For example, they may not be physically able or willing to consume that quantity of the test protein; this may occur if either the protein concentration or the amino acid bioavailability is low. Alternatively, there may be anti-nutrients in the food which restrict its intake. Intake may need to be adjusted accordingly.

If the protein concentration of the food is low (e.g. <12%) or its expected amino acid bioavailability is less than 60%, then two approaches are possible; either the intake from free amino acids must be more than 120%, or the inclusion of the test protein must provide less than 40% of the total; otherwise the subjects will have a deficient intake of the test amino acid. Humans have the ability to oxidize large quantities of excess amino acids (Elango *et al.*, 2012b) therefore intake of any amino acids in excess of 120% is not considered to be an issue and therefore this approach is acceptable. Alternatively, inclusion of the test protein can be reduced to less than 40%, and the free amino acids supplemented to 120% or greater to ensure that the intake is adequate for even the least bioavailable amino acid.

Adaptation in response to test protein

Further research in the human protocol may be required to establish length of adaptation time for less digestible intact proteins. The adaptation times should be published and made available as soon as the research is complete. The minimum adaptation should be initially based on the pig TID protocol, i.e. 5 days. This period may vary with the test proteins, as more fibrous foods may require additional length of time to adapt and adaptation may need to be established for some specific foods. In addition, the Working Group considered that there may be situations and foods for which it may be valuable to study both adapted and non-adapted conditions.

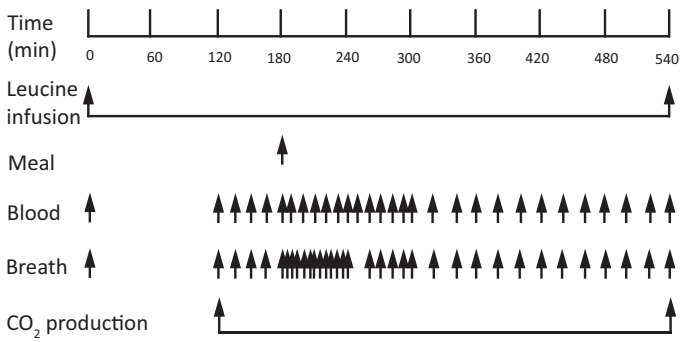
Effect of cooking/heat processing

Cooking of human diets may result in chemical complexes or loss of amino acids, which makes them unavailable for protein synthesis (e.g. Prolla *et al.,* 2013). Wherever possible, the test food must be prepared with the typical cooking method. If a variety of cooking methods are used for a specific test food then these should also be tested for the effect on individual amino acid availability. In addition, some foods are always cooked and prepared in mixtures and are not consumed separately, therefore, it is advisable that the food be tested separately, if at all possible, so that it can be used on an additive basis, but also tested in the mixture as cooked.

Annex 3:
The PPU Protocol

The experimental protocol is shown in Figure 7. The ^{13}C-1 leucine infusion lasts 9 hours and starts after an overnight fast. The single meal is given after 3 hours and the infusion is continued for a further 6 hours. Sampling times are shown in Figure 7 within each period for blood, breath, and carbon dioxide production. It is discussed in detail in Millward *et al*. (2002).

Figure 7: Experimental protocol



Experimental design factors

Subjects

The principle of the PPU measurement is that protein utilisation is measured during consumption of a meal representing 50% of the subject's usual daily intake. This means that the protein intake is not in excess of the capacity for net protein deposition which might limit utilisation. Whilst it is not necessary to exactly match the intake to individual habitual intakes it is important to ensure the meal will reasonably meet 50% of the subject population group's habitual intakes. If this is not known then intakes should be assessed by a 4 day food diary prior to the study. Before the studies begin, no restrictions on activities of daily living are needed although subjects should be encouraged to regulate their eating patterns to a diurnal cycle of 12 hour of feeding and 12 hour of fasting beginning at the expected time of the meal during the study day. It is also important that the baseline leucine oxidation rate, measured between 120 and 180 minutes of the infusion, is a true post-absorptive measurement after an overnight fast with the last meal 10 hours before the start of the infusion.

Meal composition

Test Meal: The composition of the meal should reflect the objective of the study: i.e. evaluation of a single source protein or of protein from a mixed meal. For a single source protein it is best that the P:E ratio and the carbohydrate-fat ratio are standardised although there may be constraints given the need to formulate a palatable meal which can be consumed within a short period of time (e.g.10-15 minutes). As examples, in a published study of wheat and milk utilisation (Millward *et al.*, 2002) the meals were designed to provide the protein at a relatively high protein-energy value: i.e. 0.5 g protein/kg and 30 kJ/kg, i.e. a protein-energy ratio of 30% and the fat content of the meal was kept as low as possible to maximize gastric emptying. Thus the milk-protein meal consisted of fresh skim milk and dissolved potato dextrose with low natural ^{13}C enrichment giving protein-carbohydrate-fat ratios of 32.3:65.7:2.0. The wheat-protein meal consisted of wheat gluten, plain flour, and low naturally ^{13}C-enriched potato dextrose. Wheat gluten was used to provide a high protein content in a small volume. It was fed as a dry-fried pancake made from dough prepared from an equal weight of gluten and plain flour with water and was served hot, accompanied by a drink containing dissolved potato starch with a small amount of sugar-free orange beverage. In this case the protein-carbohydrate-fat ratios were 36.7: 72.3: 1.0.

For mixed meals especially those based on plant protein sources it would be difficult to achieve 0.5g protein/kg from a single meal which could be consumed over a short time period because this would represent a major fraction of the daily intake. Thus for diets based on legumes and cereals, overall P:E ratios range from 9-11% (Millward & Jackson, 2004) so that at energy intakes of say 1.4 X BMR during the infusion, for a 70 kg adult this diet provides about 0.75g protein/kg/d and 126kJ/kg per day. Thus providing 30kJ/kg (25% of the day's intake) would provide only about 0.2g/kg/d. If the protein intake is too low then the error in measuring the AUC for the increase in leucine oxidation becomes large. A single meal based on 40% of the daily intake (50kJ/kg) would provide 0.3g protein/kg which is probably sufficient protein. Clearly it would be necessary to conduct meal size studies to assess the accuracy and practicality of the assay.

Free amino acid meal control: This meal serves to assess the biological value (free amino acid pattern relative to that of the metabolic demand) of the test meal. It must be formulated therefore with the same amino acid pattern and content as the test meal and with the same overall energy content.

Choice and quantity of tracer

In this assay where the actual rate of tracer oxidation is used in the calculation, the tracer oxidation rate must be measured as accurately as possible. This requires a measureable precursor in blood of the excreted $^{13}CO_2$. Thus ^{13}C-1 leucine is suitable since the ^{13}C enrichment of the transamination product of leucine, α-ketoisocaproate is easily measurable by standard GC-MS techniques. An infusion rate of 0.5mg/kg/h of L-[1-^{13}C] leucine (99% ^{13}C), has been shown to be sufficient with a priming dose of 0.5mg/kg. The bicarbonate pool is also primed with 0.2 mg/kg of $NaH^{13}CO_2$.

Annex 4:
FAO Working Group Meeting on Developing Protein Quality Evaluation Research Protocols 2-5 March

St John's Research Institute

Background

FAO convened an Expert Consultation on dietary protein quality evaluation in human nutrition in Auckland, New Zealand from 31 March to 2 April 2011. A primary objective was to assess the existing Protein Digestibility Corrected Amino Acid Score (PDCAAS) method for expressing protein quality and to examine the suitability and advisability of replacing it with the new measure "Digestible Indispensable Amino Acid Score" (DIAAS). Although the Expert Consultation recognized that the DIAAS was conceptually superior to the PDCAAS, it acknowledged that there was a need for more data, particularly related to humans. The Expert Consultation called upon FAO to organize a working group to develop research protocols that would allow the measurement of true ileal digestibility values across different animal models (pig, rat) and human studies using protein sources that are representative of those consumed by human populations.

Objective of the meeting

The objective of the 4-day working group meeting is to develop specific and viable research protocols related to assessing protein quality in human foods.

Expected outcomes

Immediate outputs:

The expected outcomes and outputs of the working group meeting are:

1) A short report describing the working group's deliberations and conclusions;
2) Specific and viable research protocols that can subsequently be submitted for funding;
3) Communication note reporting on the working group recommendations submitted for publication in one or more journals to notify the research community about the protocols.

Long term output:

Following the anticipated accumulation of data and information resulting from the research carried out, FAO would sponsor an expert meeting on protein quality and the implications for human nutrition.

Provisional Agenda

SUNDAY 2 March		
08:15	Pick up at Hotel	
08:30 – 09:00	Registration • Receive DSA • Wi-Fi access	Bangalore Meet Secretariat
09:00 – 10:30	- Administrative announcements & Welcome - Welcome - Overview of process - Self-introduction of participants with brief description of their work related to objectives of the meeting - Comments on the agenda & meeting facilitation - Brief explanation of FAO's interest and activity in protein requirements and quality assessment	A. Kurpad W. T. K. Lee J. Albert All participants J. Albert R. Weisell
10:30 - 11:00	Coffee break	
Session 1: Rationale for the Working Group - Facilitator: R. Ball		
11:00- 12:00	Brief review of 2011 Expert Consultation and salient points for the working group objectives	P. Moughan and R. Uauy (Chair & Vice-Chair of 2011 consultation)
12:00 – 12:30	Discussion	
12:30 – 13:30	Lunch	
Session 2: Context of protein quality evaluation – Facilitator: A. Kurpad		
13:30 – 14:00	Protein quality – implications for public health recommendations and Discussion	R. Uauy
14:00 – 14:30	A perspective on methods for determining protein quality and discussion	J. Millward
14:30 – 14:45	Coffee Break	
14:45 – 17:00	Discussion and identification of research gaps	All participants
	Evening is free	
MONDAY 3 March		
Session 3: Methods for measuring protein digestibility in human foods - Facilitator: D. Tomé		
08:30	Pickup at the hotel	
09:00 – 10:00	Current available data	W. Hendriks / P. Moughan
10:00 – 10:30	Discussion	
10:30 – 11:00	Coffee Break	
11:00 – 12:00	Determination of metabolic availability of protein bound amino acids using the "Indicator Amino Acid Oxidation Technique"	R. Ball / R. Elango
12:00 – 12:30	Discussion	
12:30 – 13:30	Lunch	

Session 4: Protein digestibility - continued. - Facilitator: R. Uauy		
13:30 – 14:30	Determination of metabolic availability of protein bound amino acids using stable isotope labelled protein	D. Tomé
14:30 – 15:00	Discussion	
15:00 – 15:15	Coffee Break	
15:15 - 16:15	Protein Quality: the promise of intrinsic labels with amino acid-specific analysis	T. Preston/ A. Kurpad
16:15 – 16:45	Discussion	
18:00	Pick up at Hotel for Dinner	
19:00	Dinner in a local restaurant	
TUESDAY 4 March		
Session 5 Limitations of current methods – animal and human - Facilitator: J Millward		
08:30	Pickup at the hotel	
09:00 – 10:30	Round table presentation and discussion – Limitations of animal models	W. Hendriks/ P. Moughan/R. Ball
10:30 – 11:00	Coffee Break	
11:00 – 12:30	Round table presentation and discussion – Limitations of current methods in human	D. Tomé/A. Kurpad/ R. Elango
12:30 – 13:30	Lunch	
13:30 – 14:15	Tour of the St. John's facility	Kurpad
Session 6: The way forward - Facilitator: T. Preston		
14:15 – 15:45	Technical discussion and preparation of protocols Assignment of protocol drafting groups	All
15:45 – 16:00	Coffee Break	
16:00 – 18:00	Preparation of protocols	Drafting groups
WEDNESDAY 5 March		
Session 7 Approval of the report Facilitators: R. Weisell and J. Albert		
08:30	Pickup at the hotel	
09:00 – 12:30	Presentation of protocols and funding opportunities	Drafting groups assign a presenter
10:30 – 11:00	Coffee Break	
11:00 – 12:30	Discussion continues	
12:30 – 13:30	Lunch	
13:30 – 15:00	Review of the draft report Preparation of communication for publication	All To be determined
15:00 – 15:15	Coffee break	
15:15 – 16:00	Approval of the report and annexes	All
16:00 – 16:15	Closing	T. Raj and W. T. K. Lee

Annex 5:
List of Participants at the FAO Working Group on Protein Quality

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Annex 6:
FAO Technical Workshop on Protein Quality Evaluation

Call For Experts

As follow up to the 2011 review of dietary protein quality evaluation in human nutrition*, FAO is in the process of identifying experts to participate in a working group to further consider a crucial issue from the 2011 review and to *provide recommendations on the best methods to measure and predict digestion and efficiency of utilization of protein and amino acids in humans.*

Experts would be expected to participate in a four day meeting at St. John's Research Institute, Bangalore, India in early March 2014 (dates to be determined).

Applicants should meet the following general criteria:

- Advanced University/College degree in nutrition science, food science, or related fields or a medical degree
- Experience with *in vitro/in vivo* models/assays for the digestion and efficiency of utilization of protein and amino acids
- Scientific publications in peer-reviewed journals, in particular, relevant publications within the most recent ten years
- Good knowledge of the English language, both written and oral.
- 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
- Experience with determination of requirements of protein and amino acids in humans.

Selection of experts

FAO places great value on the technical quality and independence of the participating experts as well as on the transparency of its selection process. FAO has a procedure for selecting experts that promotes the excellence and independence of opinions provided.

Applicants' curriculum vitae will be reviewed on the basis of the criteria listed above by a selection panel consisting of three or more individuals including at least two independent, internationally recognized external experts appointed by FAO. In selecting experts FAO will consider, in addition to scientific and technical excellence in the topic of the review, balanced geographic representation, including developing and developed countries, as well as gender. Selected experts may be requested to assist in the preparation of background papers.

Appointment of experts

The experts will be invited to contribute only in their individual scientific capacity. An expert will not represent the government of country of which he or she is a citizen, or the institution with which he or she is associated. The experts designated will not receive any remuneration; however, travel costs, subsistence allowance and other related expenses will be the exclusive responsibility of FAO.

Applications

Interested applicants should submit their curriculum vitae (CV) to the FAO Nutrition Division, at the address provided below.

Your CV should include a description of education and work experience and a list of peer- reviewed publications relevant to the factors indicated above (please do not include reprints in your submission unless specifically requested at a later date).

Before participating in any related activity, the selected experts will be required to declare all interests associated with the subjects that will be evaluated through completion of a standard form developed by FAO. You will be asked to indicate in writing any interest (financial and intellectual) on your part or that of your spouse that may affect your scientific independence as an expert, including one or more of the following conditions: employment (past or present) by any commercial enterprise or private or civil sector association; a recipient of research or other study grants from such enterprises or associations; or shareholdings in commercial enterprises active in fields related to nutrition. These declarations will be evaluated and retained by the FAO Secretariat. In addition, a confidentiality undertaking is also to be signed to ensure proper handling of dossiers and proprietary information.

Deadline

Applications should be sent to FAO by **17 January 2014**.

*Link to the Report of an FAO Expert Consultation on dietary protein quality evaluation in human nutrition and the two subcommittee reports): http://www.fao.org/ag/humannutrition/35978-02317b979a686a57aa4593304ffc17f06.pdf

http://www.fao.org/ag/humannutrition/nutrition/63158/en/

Send applications and queries to:

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IT IS PREFERABLE TO SEND THE APPLICATION BY E-MAIL IF POSSIBLE.
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December 2013

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Following the 2011 FAO Expert Consultation on dietary protein quality evaluation in human nutrition, a working group was convened in Bangalore, India from 2-5 March 2014 to explore and develop means for producing more data accessible worldwide of ileal amino acid digestibility of human foods, particularly for foods consumed in low income countries. The paucity of data, especially from human studies, remains an obstacle to the practical implementation of the DIAAS method for evaluating protein quality. The report considers protocols including recommended best practice for pig-based, rat-based and human based assays for true ileal amino acid digestibility determinations to support the generation of new data. The working group considered the development of protocols that would allow non-invasive measures of ileal amino acid digestibility in humans with primary reliance on novel approaches using minimally invasive stable isotopes tracers. Such an exercise would need to involve the determination of ileal protein and amino acid digestibility in both humans and animal models to allow the development of robust inter-species protein digestibility predictions.

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