

Nutritional and Health-Related Environmental Studies (NAHRES)

Bioavailability of proteins from plant based diets

Brief Summary

Protein quality has a great importance in meeting the nutritional needs of populations across the developing world throughout the life course, but in particular during pregnancy and early childhood. Protein quality is likely to have an impact on the WHO Global Nutrition Target 2025 of reducing the prevalence of stunting (low height-for-age) in children under 5 years by 40%. Stunting has been shown to reduce capacity for learning in children, and hence productivity in adults. Nutrition underpins sustainable development. Uncertainty over the quality of diet, specifically with reference to its protein quality potentially impacts a nation's health, economy, agriculture and nutrition security, and hence Sustainable Development Goals (SDGs). In particular SDG 1 – End Poverty in all its forms; SDG 2 – End Hunger, achieve food security, improved nutrition, and sustainable agriculture; SDG 3 – Ensure Healthy lives; SDG 4 – Ensure quality education and learning; SDG 5 – Ensure gender equality and empowerment. Estimates of amino acid digestibility based on analysis of faeces relative to foods ingested do not represent the fraction of amino acid absorbed. Ideally, amino acid bioavailability should be measured from oro-ileal balances (the difference between ingested amino acids and the amino acids leaving the ileum), since absorption occurs only in the small intestine and colonic bacteria can recycle nitrogen. However this is virtually impossible to measure non-invasively in healthy humans. The measurement of intrinsically stable isotope labelled plant protein offers a solution to this problem. Either $^{13}\text{CO}_2$ or $^2\text{H}_2\text{O}$ can be used. $^2\text{H}_2\text{O}$ is economically feasible and appropriate for plant proteins. Indispensable amino acids (IAA) become labelled within the food matrix and following preparation and ingestion of the food, their appearance in the blood gives a unique bioavailability measure. If a labelled test meal is accompanied by a trace quantity of a differently labelled reference protein, such as casein, or single cell proteins, comparative tracer appearance gives a direct measure of digestion/availability of the protein under test. This CRP aims to develop and validate novel, minimally invasive techniques to assess protein digestibility and utilization from plant based diets, as they are consumed by vulnerable populations, in regions habitually relying on plant based diets. A novel approach to assessing protein quality will be developed based on the use of ^{15}N , ^{13}C and/or ^2H labelled amino acids or intrinsically-labelled proteins. Intrinsically labelled local varieties of grain legumes will be grown in collaboration with local agriculture colleges/institutes.

Background

a) Rationale/definition of the problem

Protein quality has a great importance in meeting the nutritional needs of populations across the developing world throughout the life course, but in particular during pregnancy and early childhood. Protein quality is likely to have an impact on the WHO Global Nutrition Target 2025 of reducing the

prevalence of stunting (low height-for-age) in children under 5 years by 40%. Stunting has been shown to reduce capacity for learning in children, and hence productivity in adults. Nutrition underpins sustainable development. Uncertainty over the quality of diet, specifically with reference to its protein quality potentially impacts a nation's health, economy, agriculture and nutrition security, and hence Sustainable Development Goals (SDGs). In particular SDG 1 – End Poverty in all its forms; SDG 2 – End Hunger, achieve food security, improved nutrition, and sustainable agriculture; SDG 3 – Ensure Healthy lives; SDG 4 – Ensure quality education and learning; SDG 5 – Ensure gender equality and empowerment.

The last WHO/FAO/UNU Expert Consultation on the Protein and Amino Acid Requirements of Man in 2002 determined that the requirements of indispensable amino acids (IAA) were higher than previously thought¹. Conversely the new data on protein accretion based on ⁴⁰K counting (a measure of body cell mass), suggested that the total protein needs of children should be slightly lowered. Thus the evidence supports a slightly lower need to total protein but a greater need for some of the essential amino acids (notably isoleucine, leucine, lysine, valine, threonine, phenylalanine + tyrosine). Protein quality therefore becomes of greater relevance in order to meet the IAA needs of children and adults alike. More specifically, protein quality is likely to have an impact on the broader issues of reducing stunting and the successful development of human capital. A landmark early life intervention study from Guatemala, of energy alone or a balanced protein and energy supplement in the first 2-3 years of life, has shown that the group that received the protein supplement did better in terms of their height as well as their earning capacity as adults². Equally, these considerations need to be environmentally sensitive, and engage with agricultural science with an emphasis of the economical production of high quality plant sources of protein, such as legumes.

The immediate implication is that dietary protein quality matters to human health. Protein quality, in terms of IAA content has a greater importance in meeting the needs of populations across the developing world, as well as the nutritional quality of food commodities currently available to meet the human health needs through the human lifecycle. Uncertainty over the quality of diet, specifically with reference to its protein quality, does potentially impact a nation's health, economy, agriculture and nutrient security. International organizations have begun to address these issues. For example, the FAO released a report on Dietary Protein Quality Evaluation in Human Nutrition in 2013³, which was a prelude to the 2012 WHO technical note on foods for the management of moderate acute malnutrition in children⁴. The IAEA held a Consultants' Meeting in October 2013 to discuss stable isotopic methods to measure protein quality in humans, followed by a FAO Expert Working Group Meeting in March 2014 in Bangalore, to specifically define the most appropriate research methodologies to measure protein digestibility and utilization in humans⁵.

Poor dietary quality has a marked negative impact on the sensitive periods of pregnancy and the first two years of life (the first 1000 days). Dispensable and indispensable amino acids play key roles in the achieving healthy growth in early life. The faster the growth rate the greater the need for IAA, however, even non-essential amino acids play key roles in promoting lean body mass gain and linear growth in particular by modulating the secretion of hormones (i.e. arginine stimulates insulin secretion thus lowering serum glucose which in turn drives growth hormone and lean mass growth including height gain). These amino acids also are responsible for stimulating insulin like growth factors (IGFs) which promote length gain, specific proteins in milk and other animal foods are relevant in this process. Thus we need to characterize the best amino acid mix to promote healthy lean mass gain without inordinate fat mass gain. This is not predicted by the amino acid need for accretion, but is best explained by the role of amino acids as regulators of hormones and growth factors responsible for tissue growth.

Protein quality also impacts the quality of life at the later part of life. Women need to enter pregnancy in a well-nourished state, and eat appropriately during pregnancy. Foetal development, and child

development during early life, is critically dependent on the appropriate amount of dietary proteins, for both appropriate growth and body composition, that may determine their human capacity and potential at adulthood. Protein synthesis rates in early gestation are associated with birth length⁶. Low intake of high quality protein (providing branched chain amino acids) is associated with sarcopenia, which is an important component of strength, mobility and quality of life at older ages. Meeting population nutrient needs must also relate to the requirements of living under real life conditions such as repeated infections, poor environmental sanitation and psychosocial stress, all of which increase amino acid losses and prevent a strong anabolic response leading to recovery. Additionally, the effect of food processing and heating may alter absorption and utilization of some amino acids such as lysine, which may be already limiting protein quality of many predominantly cereal, based diets. In simple-stomached animals possessing a well-developed hind-gut (and this includes humans), a profuse and diverse microbiota acts on undigested material entering the large bowel, with a significant degree of metabolism of protein, peptides and amino acids. Ammonia, one of the products of the bacterial breakdown of protein and amino acids, is absorbed from the hindgut, but amino acids, as such, are not considered to be absorbed from the large intestine in nutritionally meaningful amounts^{7,8}.

Faecal protein is largely microbial protein, and compositionally bears no resemblance to the array of dietary amino acids remaining undigested at the end of the ileum. Given that the bacterial protein does not directly relate to undigested food amino acids and to the food protein ingested, it is illogical to determine amino acid digestibility at the faecal level. Estimates of amino acid digestibility based on analysis of faeces relative to foods ingested do not represent the fraction of amino acid absorbed. Accordingly, measurements of digestibility determined at the ileal level are critical for determining amino acid losses of both dietary and endogenous origin^{7,9}. Faecal-ileal digestibility differences can be substantial and both amino acid and protein ileo-faecal digestibility differences have been shown across a wide range of simple-stomached animal species. Thus there is no reason to assume that the human, with a well-developed colon, will be any different and the limited experimental evidence available from human studies supports this concept. Ideally, amino acid bioavailability should be measured from oro-ileal balances (the difference between ingested amino acids and the amino acids leaving the ileum), since absorption occurs only in the small intestine and colonic bacteria can recycle nitrogen. However this is virtually impossible to measure non-invasively in healthy humans.

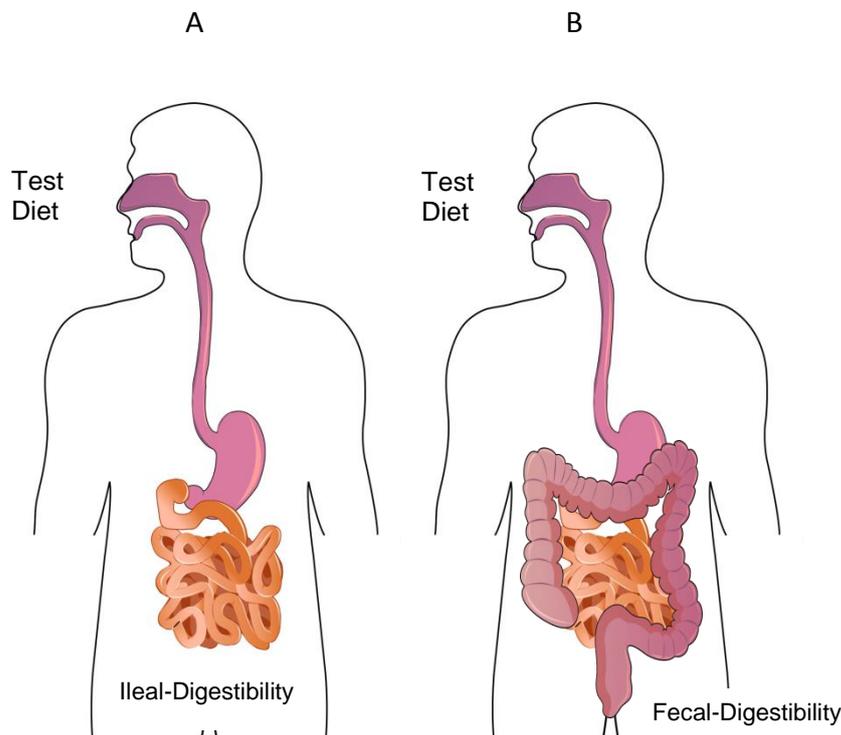


Figure 1

A depicts the traditional way of measuring protein digestibility – as the difference in nitrogen content of oral intake and faecal output. The oro-faecal nitrogen balance under-estimates digestibility since faecal nitrogen contains nitrogen fixed from the body ammonia pool by colonic bacterial, as well as the nitrogen in intestinal secretions and desquamations.

B depicts the correct way of measuring protein digestibility – as the difference in nitrogen content of oral intake and ileal output. The colon is not shown in Figure B, since colonic processes that affect nitrogen content of the colonic contents, are not considered in this oro-ileal balance. In addition, in this method, amino acid digestibility can also be measured for specific amino acids. One error that is not considered in this method is that intestinal secretions and desquamations are not accounted for. Therefore this oro-ileal balance does not measure ‘true’ ileal digestibility.

The measurement of intrinsically stable isotope labelled plant protein offers a solution to this dilemma. Either $^{13}\text{CO}_2$ or $^2\text{H}_2\text{O}$ can be used. $^2\text{H}_2\text{O}$ is economically feasible and appropriate for plant proteins. Indispensable amino acids (IAA) become labelled within the food matrix and following preparation and ingestion of the food, their appearance in the blood gives a unique bioavailability measure. If a labelled test meal is accompanied by a trace quantity of a differently labelled reference protein, such as casein, or single cell proteins, comparative tracer appearance gives a direct measure of digestion/availability of the protein under test. Mass spectrometry instrumentation using newer sensitive methods (GC-C-IRMS, LCMS), allows for minimally invasive sampling, such as the use of saliva.

b) New methods for protein quality measurement

The nutritional efficacy of a protein meal is related to the absorption of dietary AA through the intestine and their relative fate in anabolic and catabolic pathways. Protein digestibility predicts the

dietary intake which is made available as amino acids to the organism after digestion and absorption. It is measured as the proportion of ingested nitrogen or amino acids that is absorbed in the intestine following protein consumption. Protein digestion is however a complex process as there are continuous movements and exchange of protein, amino acids and nitrogen between the gut lumen and the systemic pools. Amino acids released from protein hydrolysis in the intestinal lumen are absorbed along the small intestine. *True or apparent digestibility* discriminates between exogenous nitrogen (food) and endogenous nitrogen losses (secretions, desquamations etc.). The fraction that enters the large intestine is degraded by the microbiota with the release of ammonia. *Faecal digestibility* of protein is the difference between nitrogen ingested and excreted in the faeces and does not take into account this colonic metabolism. *Ileal digestibility* measured at the terminal ileum is considered more accurate for dietary amino acid digestibility as a measure of dietary intake made available as amino acids to the organism. One reason for the increased accuracy is that ileal digestibility is measured at the level of individual amino acids.

c) Methodological considerations

This project aims at developing a novel approach based on the use of ^{15}N , ^{13}C and/or ^2H labelled amino acids or intrinsically-labelled proteins with two or three different tracers and evaluation of an isotopic signature of circulating absorbed dietary amino acid availability. As a reference method, in the Net postprandial protein utilization (NPPU), 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 $^{15}\text{N}/^{13}\text{C}/^2\text{H}$ -labeled mixed meal (ingested $^{15}\text{N}/^{13}\text{C}/^2\text{H}$). Then during the 8 h following meal ingestion the ileal effluents, blood and urine are collected. The method allows a direct determination of ileal digestibility by measurement of the quantity of dietary protein not absorbed in the small intestine and entering the large intestine. It also allows the determination of the retention of dietary amino acids in the body by subtracting ileal and urinary losses to the total ingested protein. This method is however invasive. It can be used as a reference direct method but not as a routine method.

Non-invasive methods based on meal intake and blood and urine sampling should be developed using either single labelled amino acid or intrinsically-labelled protein. ^{15}N , ^{13}C and/or ^2H stable isotope-labelled dietary protein and amino acids may be used to trace absorption and metabolic utilization of protein bound dietary amino acids. Single labelled amino acid oxidation methods use a comparison between an ingested amino acid mixture considered as 100% digestible and the tested protein to evaluate amino acids released in the blood; amino acid availability is indirectly measured by the oxidation of a tracer amino acid provided in the meal (Indispensable amino acid oxidation method) or infused in the blood (Post-prandial utilization method). In the uniformly labelled protein based methods the principle is to give a mix of a) one or two reference protein (such as algae protein hydrolysate) labelled with ^{15}N , ^{13}C and/or ^2H (100% or known digestibility); b) the tested protein of unknown digestibility labelled with ^{15}N or ^2H ; c) the unlabelled tested protein of unknown amino acid digestibility. The isotopic signature in the meal and in the plasma is determined from the $^2\text{H}/^{13}\text{C}$ and/or $^{15}\text{N}/^{13}\text{C}$ ratio of each indispensable amino acid. Differences in the signature between the meal and the plasma should allow digestibility (bioavailability) to be calculated at the level of individual amino acid. The signature of the dietary protein can potentially be extended to proteomic and metabolomics signature from plasma and urine.

Study design: Phase 1a) – validation of the experimental approach: reference proteins

In the first instance, small groups (~10) of healthy adults will be recruited for validation and familiarisation studies of a protocol designed to assess the bioavailability of plant proteins, namely grain legumes. The key grain legumes under study will be among the eight species regarded as global priorities by CGIAR¹⁰ (2012). The intention is to apply intrinsic labelling where the bioavailability of indispensable amino acids IAA will be measured in locally-produced and prepared grain legumes. In the first instance, the protocol will be introduced by establishing the methodology in several centres by measuring the bioavailability of a single-cell reference protein in comparison to whole milk protein. The reference protein will be the same in every group and will be acquired from a commercial source by the IAEA. This is likely to be a small quantity of highly ¹³C-enriched single cell protein that has been applied worldwide in human nutrition. The test protein in the first place will be whole milk protein, will be produced with a low level deuterium enrichment within the study. This will permit the ¹³C-single cell protein to be characterised against a well-studied animal protein known to possess high digestibility. Tracer AA appearance in the circulation (blood; saliva, urine and breath will also be sampled) and the relative appearance of test and reference protein will be taken as a measure of relative bioavailability which can be converted into an absolute measure once the reference protein has been characterised. The basic measurement will be of stable isotope enrichment in individual amino acids.

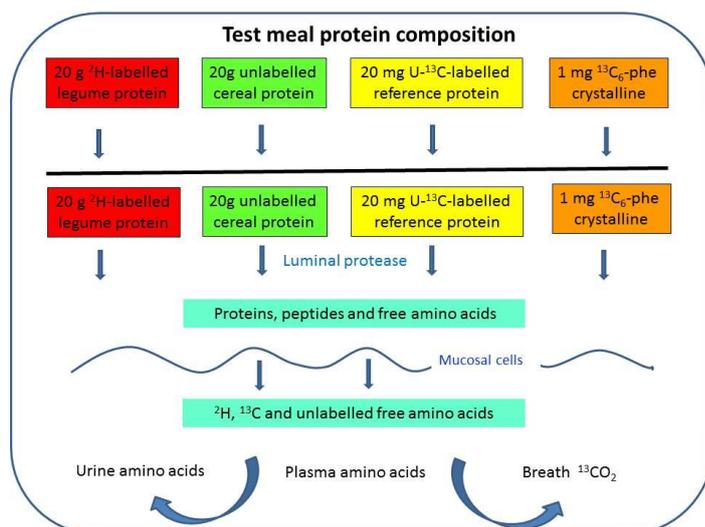


Figure 2 Schematic of provisional dual tracer protein bioavailability protocol (FAO, 2014)

Phase 1b) – production of intrinsically-labelled grain legumes

In collaboration with local agricultural experts, key grain legumes will be chosen and grown on a pilot scale (100-1000 g dry grain) in the first instance. Approximately 2 weeks after anthesis (flowering), the water supply will be amended with deuterium oxide so as to label the developing seed protein at low level. Seeds will be taken to maturity and harvested. Sub-samples of dried seed will be milled to fine flour and characterised for their isotopic, macronutrient and micronutrient composition. This will include total N and C analysis, protein hydrolysis and amino acid analysis using the same MS techniques as used to measure AA appearance in the circulation. Once the intrinsic labelling methodology has been established at pilot scale, production will be scaled up, so that labelled grain legumes can be made available on a larger scale (1-10 kg dry weight seed) and in the species/varieties identified during the first Research Coordination Meeting.

Phase 2) - Bioavailability of locally-produced grain legumes

In this phase, the bioavailability of intrinsically-labelled grain legumes will be estimated by combining these into test meals along with the reference single-cell protein. Initially, the test meal will be prepared according to local custom, probably in combination with a cereal. Eventually, processing and preparation variables will be studied along with grain legume varieties and differing protein/energy ratios. A group of healthy volunteers will be recruited who will each receive several meals (4 test grain legumes; milk protein) in a random fashion with at least two weeks wash-out period between treatments. The same approach as used in the pilot study of milk protein will be adopted to study the bioavailability of grain legumes through simultaneous comparison with a modest quantity ¹³C-enriched single cell protein combined in the same test meal.

One of the major objectives of the final research coordination meeting will be to review and examine the data set to determine if a minimally-invasive protocol can be applied in future studies (shorter duration; fewer blood samples supported by an optimal pattern of saliva and breath samples). Such future studies may extend to early life, to pregnancy and to those with suspected disease-induced maldigestion or malabsorption.

Inclusion and exclusion criteria

Inclusion

Healthy, non-pregnant adults 18-65 years of age
 Not taking any medication, including NSAIDs
 No taking of antibiotics within 4 weeks of study
 BMI within 18-35?

Exclusion

Children and elderly (over 65)
 Pregnancy
 Diagnosed with non-communicable or communicable disease
 Taking medication, including NSAIDs
 Antibiotic therapy within 4 weeks

Intrinsic labelling

Grain legumes will be grown and under local conditions by agriculture specialists collaborating with each human nutrition centre. As much as possible, local practice will be followed (soil; fertilizer; irrigation; pesticide application) in order to produce a grain quality similar to that in the local market. The proposal is to use the most economical non-radioactive isotope deuterium (²H) in the form of heavy water (deuterium oxide), which will be applied in a bolus at the optimal time, approximately two weeks after anthesis. The irrigation water supply will be amended by a modest quantity of deuterium oxide as acquired for the CRP by the IAEA. During the pilot phase, the exact labelling procedure will be adjusted at each participating site so as to be optimal for the local conditions. Each grain legume will be taken to maturity and dried pending use. A subsample will be milled and shared between each participating analytical laboratory for characterisation and for QC purposes.

d) Analytical techniques

In the human studies, breath will be analysed by continuous-flow isotope ratio MS (IRMS) to determine the enrichment and exhalation profile of CO₂ (the oxidation product of the reference protein). Urine samples will be analysed for total N (TUN) and ¹⁵N by Elemental Analyser-IRMS (EA-IRMS). Plasma and saliva samples for IAA analysis will be subjected to protein precipitation (acid

treatment or ultrafiltration) and cation exchange purification of free amino acids. AA will be eluted in strong alkali and dried pending derivatisation (samples could be transported to the analytical laboratory at this stage. Alternatively, they could be transported once volatile AA derivatives had been formed). Analysis of low level deuterium enrichment in AA will be undertaken by GC-pyrolysis-IRMS. Analysis of the ^{13}C enrichment in AA will be made on the same derivatised AA samples by organic MS (GCMS or LCMS).

Sub-samples of dried seed will be milled to fine flour and characterised for their isotopic, macronutrient and micronutrient composition. This will include gross N & C analysis (EA-IRMS), protein hydrolysis and amino acid analysis using the same MS techniques as used to measure AA appearance in the circulation. Analysis will also include assessment of freedom from pathogens and toxins. Samples of milk protein will be similarly characterised in each analytical centre by GC-pyrolysis-IRMS and EA-IRMS (total N, total C and ^{15}N). Finally, samples of the ^{13}C -enriched reference protein will be processed in each analytical laboratory and characterised by organic MS. Analysis of reference and milk proteins in all analytical facilities will help standardise and harmonise AA analysis.

e) Selected references

1. World Health Organization, Protein and Amino Acid Requirements in Human Nutrition. Report of a joint FAO/WHO/UNU Expert Consultation, WHO Technical Report Series 935, WHO, Geneva (2007).
2. Hoddinott, J., Maluccio, J., Behrman, J.R., Flores, R. & Martorell, R., The impact of nutrition during early childhood on income, hours worked, and wages of Guatemalan adults. *The Lancet*, 371 (February 2008) 411-416.
3. Food and Agriculture Organization, Dietary Protein Quality Evaluation in Human Nutrition.: Report of an FAO Expert Consultation. FAO Food and Nutrition Paper No. 92. FAO, Rome (2013).
4. World Health Organization, Technical Note: Supplementary foods for the management of moderate acute malnutrition in infants and children 6-59 months of age, WHO, Geneva (2012).
5. Food and Agriculture Organization, Research approaches and methods for evaluating the protein quality of human foods; Report of a FAO Expert Working Group, FAO, Rome (2014).
6. Duggleby, S.L., Jackson, A.A., Relationship of maternal protein turnover and lean mass during pregnancy and birth length, *Clin. Sci.* **101** (2001) 65-72.
7. Moughan, P.J. Amino acid availability: aspects of chemical analysis and bioassay methodology, *Nutr. Res. Rev.* **16** (2003) 127-141.
8. Miner-Williams, W.M., Stevens, B.R., Moughan, P.J., Are intact peptides absorbed from the healthy gut in the adult human? *Nutr. Res. Rev.* **27** (2014) 308-329.
9. Fuller, M.F., Tomé, D. In vivo determination of amino acid bioavailability in humans and model animals. *J. AOAC Int.* **88** (2005) 923-934.
10. CGIAR. 2012. CGIAR Research Program on Grain Legumes Leveraging legumes to combat poverty, hunger, malnutrition and environmental degradation. Consortium of International Agricultural Research Centers, Montpellier.

Overall Objective

To develop minimally invasive methods to establish quality protein as an important factor of sustainable diet, and nutrition for health along the life course.

Specific Research Objectives

1. To quantify dietary indispensable amino acids made available to the body for optimal growth, development and function along the life course.
2. To produce intrinsically stable isotope labelled protein foods to achieve objective 1, including locally available common foods e.g. grain legumes, cereal, milk, eggs, single cell proteins, and fish and flesh foods.
3. To develop and characterize a high quality protein that is representative of different diets around the world; to be used as an internal control in the test at all centres.
4. To develop a simple method to assess protein quality in vulnerable populations.
5. To contribute towards defining protein digestibility to inform the practical application of the proposed protein quality index called DIAAS (Digestible Indispensable Amino Acid Score).

Outcomes

Improved health and quality of life throughout the life course

Outputs

1. New data on indispensable amino acid bio-availability from local diets.
2. A supply of intrinsically stable isotope labelled grain legumes.
3. High quality protein representative of different diets around the world.
4. A new method to assess protein quality in vulnerable populations.
5. New data on protein digestibility.
6. Improved capacity to use nuclear techniques to assess protein quality of human diets.
7. Preparation of a Human Health Series publication on intrinsic labelling of foods.
8. Publications in the form of scientific reports and peer-reviewed papers and conference presentations.

Assumptions

Assumptions include that partnerships can be established between local agriculture institutes and nutrition researchers, sufficient high quality proposals are received, and that additional funds will be obtained to support field implementation and analysis costs.

Proposal submission forms

Research institutions in Member States interested in participating in this CRP are invited to submit proposals directly to the Research Contracts Administration Section (NACA) of the International Atomic Energy Agency: Official.Mail@iaea.org or to Ms Christine Slater: C.Slater@iaea.org. The forms can be downloaded from <http://cra.iaea.org/cra/forms.html>. For more information about research contracts and research agreements, please visit [our web-site](#)

Deadline for submission of proposal

Proposals must be received **no later than 31 July 2015**. Transmission via Email is acceptable if all required signatures are scanned.

For additional information, please contact: Christine Slater, Nutrition Specialist
Nutritional and Health-related Environmental Studies Section Division of Human Health International Atomic Energy Agency (IAEA) A-1400 Vienna, Austria Phone: + 43 1 2600 26059 Fax: + 43 1 2600-7
C.Slater@iaea.org