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Running title: Comparing dual isotope and ileal balance methods

Abbreviations: AA: amino acid, AP: atom percent, APE: atom percent excess, Asx: asparagine + aspartate, C: carbon, DM: dry matter, GC-C-IRMS: gas chromatography combustion isotope ratio mass spectrometry, Glx: glutamine + glutamate, F: ileal flow rate, IAA: indispensable amino acid; PEG: polyethylene glycol, TF: transamination factor.

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Abstract

Background: The measurement of ileal amino acid (AA) digestibility is invasive and inappropriate when applied to vulnerable populations. The dual isotope method has been developed over the past 5 years as an alternative method.

Objective: The aim of this work was to compare the indispensable AA (IAA) digestibility values of two different proteins obtained using the dual isotope and the standard ileal balance methods in the same subjects.

Methods: Fifteen healthy adults completed the study. Over 4 h, they ingested nine successive portions of mashed potatoes containing the test protein (pea protein or casein) labeled intrinsically with ^{15}N and ^{2}H, and a ^{13}C-free AA mixture as a reference for the dual isotope method. Plasma was sampled regularly over the 8-hour postprandial period, while the ileal digesta was collected continuously via a naso-ileal tube. Isotopic enrichments (^{15}N, ^{13}C) were measured in the digesta for the direct determination of ileal IAA digestibility, while plasma enrichments (^{2}H, ^{13}C) were measured to determine IAA digestibility using the dual isotope method.

Results: The 4-h repeated meal procedure enabled the almost complete digestion of test proteins at 8 h and the attainment of a plasma isotopic plateau between 2.5 and 4 h. These conditions were necessary to perform the ileal balance and dual isotope methods simultaneously. For pea protein, the mean IAA digestibility was similar between the two methods, but significant differences (from 10 to 20%) were observed for individual IAA values. For casein, IAA digestibility was significantly lower with the dual isotope method for all the IAA analyzed.

Conclusion: Under our experimental conditions, the degree of agreement between the dual isotope and ileal balance methods varied among AAs and depended on the protein source. Further research is needed to validate the dual isotope method. This study was registered at clinicaltrials.gov as NCT04072770.

Key words: Dual isotope method, digestibility, amino acid, human, stable isotopes.

Introduction

Amino acid (AA) digestibility is defined as the proportion of dietary AA that is absorbed by the gut after ingestion. To estimate AA digestibility, the amount of residual dietary AA remaining in the digesta after absorption is determined (1, 2). Digestibility varies with the source (composition, structure), the food matrix (texture, technological process) and individual factors (physiological state, genetics) (3, 4) and is a major determinant of protein quality (5). In the search for new sources of sustainable, high quality protein, there has been a renewed interest of the scientific community in the evaluation of AA digestibility over the past 10 years (6). However, measuring protein digestibility in humans is methodologically challenging (7). The FAO recommends a separate evaluation of digestibility of each AA (5), which implies that digestive losses need to be measured at the ileal level rather than the fecal level in order to prevent misestimation due to the metabolic activity of the colonic microbiota (8).

The ileal digestibility of AA can be assessed in humans using different approaches. The direct method is based on determining the ileal AA balance by sampling the ileal contents following the ingestion of test proteins, often labeled with stable isotopes. The digesta can be collected from patients with an ileostomy (9, 10), but recruitment can be lengthy and the digestive process in such individuals may not be representative of healthy subjects (11). Another possibility is the installation of a naso-ileal tube in healthy volunteers that enables the continuous sampling of digesta and hence relevant measurements of ileal dietary AA losses (12-14). However, such a tube is invasive and this method may almost be impossible for vulnerable individuals (6, 15). Determining protein digestibility under different physiological conditions, such as in older adults, pregnant women or individuals with pathophysiological conditions, is necessary to ensure protein nutrition security for all. It is thus essential to develop minimally invasive approaches.

It has been suggested that the dual isotope method can produce approximate values for the AA digestibility of proteins (16). This indirect method is based on the simultaneous ingestion of a test protein and a reference source of AAs (a protein or mix of free AA) of known digestibility, followed by a comparison of their plasma AA enrichment patterns. Each protein is labeled with a stable isotope, ^{2}H being recommended for the test protein and ^{13}C for the reference. As splanchnic uptake is considered to be identical for the same AA from one protein to another, comparing the $^{2}H/^{13}C$ ratio in the meal and in the plasma for a given AA offers a good reflection of its digestibility in the test protein versus the reference. Several studies have been published

during the past five years that used the dual isotope approach to evaluate the digestibility of individual AA (17-23) and the results were encouraging, as the findings were similar to the expected values based on data in the literature (24). Nevertheless, there have been few direct comparisons between the data obtained with this indirect method and true ileal AA digestibility obtained using the ileal balance method. The development of a comparative protocol is challenging because both methods require specific procedures. For instance, a protocol involving 7-hour repeated meals is preferable for the dual isotope method in order to maintain a prolonged isotopic plasma plateau. By contrast, a bolus meal is preferred with the ileal sampling method in order to enable the complete collection of dietary AA ileal losses during the 8 hours that follow meal ingestion. A recent study compared the digestibility values of AA from ^{15}N-labeled sunflower proteins obtained with the ileal balance and dual isotope methods in the same subjects (25). The digestibilities of lysine and threonine (two indispensable AAs (IAAs) that do not undergo transamination) were evaluated, and lower values (10% in absolute terms) were obtained using the indirect method. ^{15}N-sunflower biscuit and a mix of free ^{13}C AA were given in the same meal but using different matrices (biscuit and apple puree or chocolate) and this may have induced a kinetic offset of plasma ^{13}C and ^{15}N AA enrichment, thus explaining these discrepancies.

The aim of the present work was to test the hypothesis that AA digestibility values obtained with the dual isotope method are similar to those obtained with the direct ileal balance method. Thus, we compared AA digestibility obtained with the dual isotope method and the direct ileal balance method in the same subjects using two different protein sources labeled with ^{2}H and ^{15}N. One plant and one animal source (pea protein isolate and milk casein) were selected and the reference was a mix of free AA. The ileal AA digestibility values of pea protein isolate and casein obtained with the ileal balance method have been described previously (26), but the values obtained from the two methods described in the present article were measured in the same subjects.

Methods

Test meals

We evaluated the digestibility of two purified test proteins that were doubly-labeled with ^{15}N and ^{2}H, as previously described (26). The labeling with ^{2}H makes it possible to determine AA digestibility values with the dual isotope method, and the labeling with ^{15}N has already been

used to determine AA digestibility values with the ileal balance method (26). For pea protein, the labeling protocol was implemented at the Unité Expérimentale du Domaine d'Epoisses, INRAE (Bretenière, France). Three micro-plots of yellow pea (*Pisum sativum*) were fertilized with two inputs of ^{15}N-labelled ammonium nitrate ($^{15}NH_4{}^{15}NO_3$) at the 4/6 leaves and floral bud stages. They were also watered with $^{2}H_2O$ for 5 consecutive days, starting from 50% bloom. The seeds were harvested at maturity and then air-dried and ground into flour. The protein fraction was extracted by acid precipitation, heat-treated and freeze-dried to obtain a protein isolate. For casein, a 5-day labeling protocol was carried out. A lactating cow at the AgroParisTech experimental (Thiverval-Grignon, France) was fed with ^{15}N-labelled ammonium sulfate ($(^{15}NH_4)_2SO_4$) for 4 days (days 2-5) and got administrated with oral doses of heavy water ($^{2}H_2O$) for 3 days (days 1-3). The labelled milk was collected on day 5 and then skimmed and pasteurized. The casein fraction was extracted by ultrafiltration followed by microfiltration, heat-treatment, and freeze-drying. Both the casein and pea protein isolates were tested for microbiological contamination. The nitrogen (N) contents were 12% and 12.5% in the pea protein isolate and casein, respectively. ^{15}N enrichment reached 0.71 atom percent (AP) for the pea protein isolate and 1.75 atom percent for casein. ^{2}H enrichment was 0.11 AP for the pea protein isolate and 0.14 AP for casein. The test meals were composed of mashed potatoes (Picard®, France) containing one of the test proteins (pea protein isolate or casein) and an algal ^{13}C-free AA mixture (97%, Eurisotop, Saint-Aubin, France). The composition of the ^{13}C-free AA mixture is shown in Supplemental Table 1. It did not contain any tryptophan, cysteine or glutamine. The food matrix was semi-liquid in order to minimize the phase shift between the test protein and the free AA during gastric emptying. A 4-h repeated feeding protocol was implemented to reach a transient isotopic enrichment plateau in the plasma; this was necessary to carry out the calculations for the dual tracer method and enable the maximal collection of dietary ileal losses during the 8 h following ingestion of the first meal (25). The meal was split into 12 portions and given in 9 mini-meals, the first being a loading dose of 4 portions. Each volunteer received a total of 45 g mashed potatoes (dry weight), 35 g pea protein isolate or 31 g casein isolate (corresponding to 4 g N from the test protein) and 400 mg of the ^{13}C-free AA mixture. The volunteers were given a glass of water every hour (with every other meal during the first 4 hours and alone thereafter) and were not allowed to ingest any other food during the 8 hours of the postprandial sampling period.

Study design

This study was approved by the Sud-Est III Ethics Committee and was registered at the www.clinicaltrials.gov database (NCT04072770), with nitrogen and AA true ileal digestibility of pea protein isolate and casein as the pre-declared primary outcomes and validation of the dual isotope method to determine AA digestibility as the pre-declared secondary outcome. The study was conducted at the Human Nutrition Research Center at Avicenne Hospital (AP-HP, Bobigny, France). The protocol was single-blinded using two-arm, parallel design, and the volunteers were allocated to one of the two groups (pea or casein) according to an alternating pattern in order to prevent any random seasonal effects. The subjects were blinded to the protocol, but the study personnel were not because they had to prepare the meals. The mashed potato containing pea and casein proteins was prepared in the morning before administration, and the meals looked similar.

Subjects

All subjects were certified as being in good health after blood tests (serologic tests for hepatitis B, C, and HIV) and a physical examination. The inclusion criteria were: BMI between 18 and 30 kg/m², age between 18 and 65 years, no allergies, negative serology for HIV and hepatitis B and C viruses, absence of pregnancy, no abusive drug or alcohol consumption, absence of severe disease, and <7 hours physical activity per week. All subjects received detailed information on the objectives and potential risks of the protocol from the doctor and nurse in charge of the study. After signing their written informed consent forms, 29 healthy volunteers were recruited between June 2019 and March 2020. The calculation of the size of each group (n = 7/group) was based on the primary outcome of the study and has been described previously (26). Several subjects did not complete the entire protocol or had to be excluded, and the final sample size was 15 (age, 36 ± 8.3 years old; BMI, 23.6 ± 2.4 kg/m^2). The principal causes of exclusion are described on the participant flowchart (Figure 1).

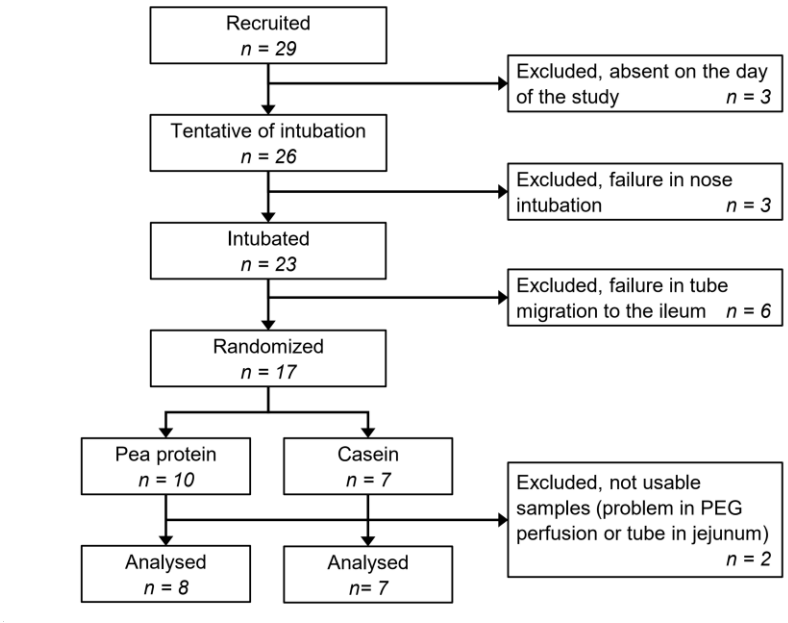

Figure 1. Participant flowchart, causes of failure and exclusion criteria.

Experimental protocol

The subjects were admitted to the hospital for 2 days (Figure 2). They arrived in a fasted state on the morning before the experiment, and a triple-lumen tube (polyvinyl chloride tubing, total length 2.5 m) was inserted into the stomach via the nose under local anesthesia. The tube was allowed to progress through the digestive tract for 24 hours, as previously described (26). In the early afternoon, the passage of the tube through the pylorus was checked by X-ray. The volunteers received an evening meal at 20:00 before fasting overnight. Early on day 2, migration of the tube to the terminal ileum was checked by X-ray and confirmed by measuring the pH of the intestinal effluent, which was collected by aspiration using a syringe through the second lumen of the tube. Polyethylene glycol 4000 (PEG-4000; 20 g/L) was used as a non-absorbable marker to calculate the intestinal flow rate and was perfused in the ileum at a rate of 1 mL/min, 20 cm above the digesta collection site. A catheter was inserted into a forearm vein of the volunteers to enable blood sampling. The 9 test meals were given every 30 minutes for 4 hours and were consumed within a limited period of time (10 minutes). The ileal content samples were collected continuously from 30 minutes before the first meal ingestion to the end of the procedure, 8 hours later. The digesta were pooled every 30 minutes, treated with a protease inhibitor (diisopropylfluorophosphate), frozen at -20°C and freeze-dried until analysis. Blood was sampled every 30 minutes for the first 4 hours, then hourly.

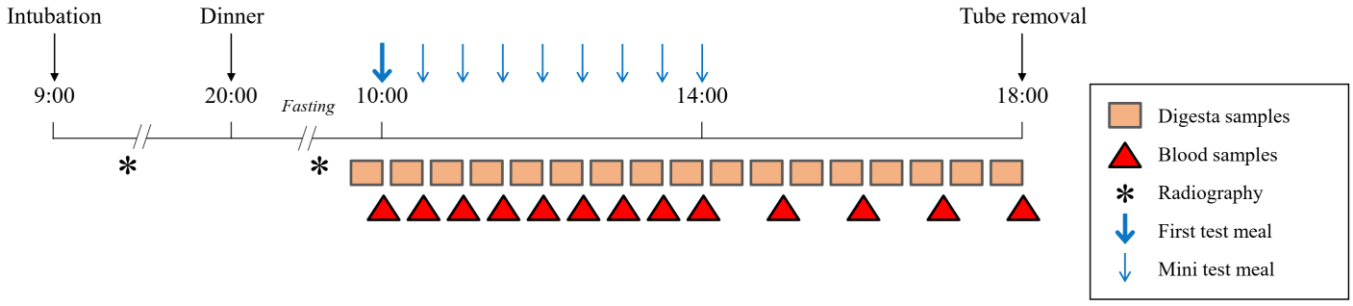

Figure 1. Experimental design of the study. The volunteers arrived in a fasted state on the morning before the experiment and a tube was inserted into the stomach via the nose. The tube was allowed to progress through the digestive tract for 24 h. The position of the tube was checked by X-ray in the afternoon of the first day and just before the experiment on the second day. On the day of the experiment, the 9 test meals were given every 30 min for 4 h. Samples of the ileal content was collected continuously from 30 min before the first meal ingestion to the end of the procedure, 8 h later, and pooled every 30 min. Blood was sampled every 30 min for the first 4 h, then hourly. The tube was removed at the end of the experiment.

Analytical procedures

Ileal AA digestibility evaluated using the ileal balance method was assessed from the AA content and ^{15}N enrichment of individual AA in digesta and test proteins. The analytical procedures and calculations have been described previously (26).

The PEG-4000 content of the digesta was measured using a turbidimetric method (27) to determine the ileal flow rate. Digesta samples were analyzed for AA content and ^{13}C enrichment in individual AAs, plasma samples were analyzed for ^{2}H and ^{13}C enrichment in individual AAs, and meals were analyzed for AA content and ^{13}C and ^{2}H enrichment in individual AAs. The ^{13}C enrichment values for the algal AA mixture used were those supplied in the technical data sheet. For the digesta and meals, 90 mg samples were hydrolyzed for 24 h with HCl 6N at 110°C. AA were then isolated using a hydrogen form resin (Dowex® 50WX8 hydrogen form 100-200 mesh, Sigma-Aldrich, Saint-Louis, USA) and derivatized with ethyl chloroformate, as previously described (26). For plasma, the isolation of free AA was performed on 1.5 mL samples. The ^{15}N, ^{2}H and ^{13}C enrichment of isolated AA was analyzed by gas chromatography (GC 6890N, Agilent Technologies, Les Ulis, France) coupled with an isotope ratio mass spectrometer (Isoprime, GV Instrument, Manchester, UK) via the GC5 Isoprime interface. The combustion furnace temperature was 950°C in N mode, 850°C in C mode, and 1100°C in H

mode (high temperature conversion). The temperature program for the GC column (RXI-17, 30 m long, 0.25 µm i.d., 0.5 µm film thickness; Restek, Lisses, France) started at 150°C, and was increased by 4°C per min up to 200°C and by 25°C per min up to 270°C, the final temperature being maintained for 10 min. The inlet temperature was set at 270°C. The injection volume was 1 µL. The injections were performed in splitless mode for N and H analyses, and in split mode (1:10) for C analyses. To measure the AA content in digesta and meals, 10 mg samples were hydrolyzed for 24 hours with HCl 6N at 110°C. Norvaline was added prior to hydrolysis as an internal standard. Calibration standards were composed of an AA mixture (Waters) with the addition of norvaline. Hydrolysates and standards were derivatized using the AccQTag Ultra Derivatization Kit (Waters) according to the manufacturer's protocol. AA quantification was performed on an Acquity HClass ultra-high performance liquid chromatography (UPLC) system with a photodiode array detector (PDA detector; Waters, Saint-Quentin-en-Yvelines, France). The AA were separated using an AccQ-Tag AA C18 column (2.1 × 100 mm; 1.7 µm bead size; Waters, France) and quantified as mmol/g of dry matter.

Calculations of the ileal digestibility of the reference ^{13}C-free AA mixture using the ileal balance method

The ileal flow rate (F) was evaluated every 30 minutes as follows:

$$F\ (mL/30min) = \frac{[PEG]_{solution}}{[PEG]_{digesta}} \times perfusion\ flow\ rate \times 30$$

Where $[PEG]_{solution}$ and $[PEG]_{digesta}$ were the concentrations of PEG-4000 in the perfused solution and in the digestive contents, respectively. The flow rate of the PEG perfusion was 1 mL/min.

The quantity of dietary ^{13}C-AA from the free AA mixture in digesta ($^{13}C\text{-}AA_{i\ diet}$) was calculated for each period of time (t):

$$^{13}C\text{-}AA_{i\ diet}\ (t)\ (mmol) = [AA_i]_{ileum}\ (t) \times DM\ (t) \times F(t) \times \frac{APE_{i\ ileum}\ (t)}{APE_{i\ meal}}$$

Where $[AA_i]_{ileum}$ was the concentration of AA_i in the digesta at t period (mmol/g), DM was the dry matter of digesta at t period (g/100 mL), F was the ileal flow rate at t period (mL/30 min) and APE was ^{13}C enrichment gained in AA_i in the digesta at t period as compared to the basal value (in AP). Basal enrichment in AA_i was defined for each volunteer independently as AA_i enrichment in the t = 0 digesta sample.

Real ileal ^{13}C-AA digestibility was calculated for each AA as follows:

$$\textit{Ileal } {}^{13}\textit{C-AA}_i \textit{ digestibility (\%)} = 1 - \frac{\sum_{t=0}^{t=8} 13CAA_{i\ diet}}{AA_{i\ ingested}} \times 100$$

Where $\sum_{t=0}^{t=8} 13CAA_{i\ diet}$ was the sum of dietary ^{13}C-AA$_i$ over 8 hours (mmol) and AA$_{i\ ingested}$ was the amount of AA$_{i\ ingested}$ by the volunteer (mmol). Ileal AA digestibility could not be calculated for tryptophan and cysteine because they were not present in the mixture.

Calculations of indispensable amino acid digestibility using the dual isotope method

The ratio of ^{2}H to ^{13}C enrichment (in APE) was measured in the meal for each IAA (i) as follows:

$$\textit{Meal ratio IAA}_i = \frac{{}^{2}H_{meal\ i}}{{}^{13}C_{meal\ i}}$$

The ratio of ^{2}H to ^{13}C enrichment (APE) in the plasma was calculated for each IAA at the isotopic plateau period or using the areas under the curve (AUC) of the enrichments over the 8 hours following the first meal intake. The plateau period was determined as consecutive, non-significantly different time points for ^{2}H and ^{13}C IAA enrichment in plasma (post-hoc Tukey test according to the mixed model with the group as the fixed factor and time as a repeated factor). The ratio of ^{2}H to ^{13}C enrichment (in APE) was thus measured in the plasma at the plateau period or with the AUC for each IAA (i) as follows:

$$\textit{Plasma ratio IAA}_i = \frac{{}^{2}H_{plasma\ i}}{{}^{13}C_{plasma\ i}}$$

The IAA digestibility for each IAA (i) was determined using the plasma and meal ratios as follows:

$$\textit{IAA}_i \textit{ digestibility (\%)} = \textit{ileal } {}^{13}\textit{C-IAAi digestibility} \times \frac{\textit{Plasma ratio IAA}_i}{\textit{Meal ratio IAA}_i} \times TF_{IAAi}$$

Where ileal ^{13}C-IAA$_i$ digestibility was determined using the ileal balance method in the present study (pooled individual ^{13}C-AA digestibility) and TF$_{IAA}$ was the transamination correction factor of AA$_i$ (1.070 for isoleucine, 1.081 for leucine, 1.002 for lysine, 1.053 for phenylalanine, 1.013 for proline and 1.048 for valine). The transamination factors were obtained from Devi *et al.* (19) and used to correct for the loss of ^{2}H atoms during transamination.

Statistical analysis

The values were expressed as mean ± standard deviation (SD). Differences between groups regarding ^{13}C-AA digestibility as well as ^{13}C- and ^{2}H-enrichment AUCs for each IAA were tested using an unpaired t-test. Differences between the digestibility methods (ileal balance, plateau dual isotope method and AUC dual isotope method) were estimated using a one-way ANOVA for each protein source. The IAA ^{13}C or ^{2}H plasma enrichment kinetics were analyzed using a mixed model with the group as a fixed factor and time as a repeated factor. Tukey post-hoc tests were performed to determine the concomitant plateau period for ^{13}C and ^{2}H. An overall significant difference was observed for $P < 0.05$. Statistical analyses were performed using Prism 6.04 (Graph Pad Software Inc.) and R version 4.0.3.

Results

Isotopic labeling of indispensable amino acids in pea protein and casein

The ^{15}N and ^{2}H-labelings of pea protein and casein IAAs are presented in Table 1. ^{15}N labeling was homogenous across IAAs for both pea protein and casein, with a lower enrichment of pea protein compared to casein (0.7 AP for pea and 1.6-1.9 AP for casein). IAAs from pea protein were also homogenously ^{2}H-labeled, with values ranging from 0.09 to 0.11 AP. By contrast, the ^{2}H-labeling of IAAs from casein was lower than pea, with more variable enrichment values ranging from 0.04 to 0.09.

Table 1. Indispensable amino acid composition and ^{15}N and ^{2}H enrichment of protein sources.

	Pea protein			Casein		
	Content (mg/g protein*)	^{15}N enrichment (AP)	^{2}H enrichment (AP)	Content (mg/g protein*)	^{15}N enrichment (AP)	^{2}H enrichment (AP)
Isoleucine	47.8	0.72	0.10	49.9	1.91	0.09
Leucine	79.5	0.72	0.10	92.5	1.60	0.07
Lysine	73.4	0.73	0.09	79.6	1.96	0.09
Phenylalanine	56.3	0.73	0.11	49.1	1.65	0.04
Threonine	35.9	0.71	0.10	41.5	1.79	0.06
Valine	49.1	0.73	0.11	61.3	1.74	0.07

Values are means of 3 replicates. * Protein content = N × 6.25. AP, atom percent.

Ileal AA digestibility of the reference ^{13}C-free AA mixture

The ileal AA digestibility values of the reference ^{13}C-free AA mixture were obtained from the collection of ileal digesta and are shown in Table 2. For each AA evaluated, there was no

difference between the ileal digestibility of the ^{13}C-free AA mixture when ingested in the pea protein or casein meal. We used the pooled digestibility values as a reference to calculate digestibility using the dual isotope method. The lowest ileal digestibility was observed for tyrosine (95.6 ± 2.5%) and the highest for phenylalanine (99.4 ± 0.3%). The mean AA digestibility of the ^{13}C-free AA reference mixture was slightly lower than 100%, reaching 98.2 ± 0.7%.

Table 2. True ileal AA digestibility of the reference ^{13}C-free AA mixture.

	With pea protein	With casein	*T-test*	Pooled
IAA digestibility (%)				
Isoleucine	98.7 ± 0.6	98.5 ± 1.1	*n.s.*	98.6 ± 0.9
Leucine	99.0 ± 0.4	98.8 ± 0.8	*n.s.*	98.9 ± 0.6
Lysine	98.2 ± 1.1	976 ± 1.5	*n.s.*	97.9 ± 1.3
Methionine	98.6 ± 0.7	98.8 ± 0.9	*n.s.*	98.7 ± 0.8
Phenylalanine	99.5 ± 0.3	99.3 ± 0.4	*n.s.*	99.4 ± 0.3
Threonine	97.5 ± 0.7	97.6 ± 1.0	*n.s.*	97.5 ± 0.8
Valine	98.6 ± 0.5	98.5 ± 0.8	*n.s.*	98.6 ± 0.6
Mean IAA	98.6 ± 0.6	98.5 ± 0.9	*n.s.*	98.5 ± 0.7
DAA digestibility (%)				
Alanine	99.3 ± 0.3	99.0 ± 0.7	*n.s.*	99,1 ± 0.5
Asx	97.0 ± 1.1	97.8 ± 0.8	*n.s.*	97.4 ± 1.0
Glx	97.4 ± 1.2	97.7 ± 1.2	*n.s.*	97.6 ± 1.2
Glycine	98.4 ± 0.6	98.0 ± 1.1	*n.s.*	98.2 ± 0.9
Proline	99.1 ± 0.2	99.3 ± 0.3	*n.s.*	99.2 ± 0.3
Serine	97.7 ± 0.9	96.0 ± 1.5	*n.s.*	96.8 ± 1.5
Tyrosine	95.0 ± 3.0	96.2 ± 1.6	*n.s.*	95.6 ± 2.5
Mean DAA	97.7 ± 0.9	97.9 ± 0.7	*n.s.*	97.8 ± 0.8
Mean all AA	98.2 ± 0.7	98.2 ± 0.8	*n.s.*	98.2 ± 0.7

Values are means ± SD. n = 7 for casein and n = 8 for pea protein for all AA, except serine (n = 4). Asx, asparagine + aspartate; DAA, dispensable amino acid; Glx, glutamine + glutamate; IAA, indispensable amino acid; n.s., not significant.

Meal and plasma isotopic enrichments in ^{2}H and ^{13}C

^{2}H and ^{13}C enrichment values in the pea protein and casein test meals are shown in Supplemental Table 2. For all the IAA analyzed, these values were higher in the pea protein meal than in the casein meal, except for ^{13}C phenylalanine. These differences were due to the higher ^{2}H enrichment in pea protein isolate than casein and to the higher IAA content of casein compared with pea protein and subsequent dilution of ^{13}C-AA enrichment in the meal.

The ^{2}H and ^{13}C plasma enrichment kinetics are presented in Figure 3. Three IAAs are missing from the results because their digestibility could not be evaluated using the ileal balance and/or

dual isotope methods. These absences could be explained by their low recovery or unsatisfactory isolation and/or derivation in the digesta hydrolysates (tryptophan, histidine; for ileal digestibility) or in plasma samples (for methionine; for digestibility determined with the dual isotope method). Plasma ^{2}H enrichments were more variable among IAAs for casein than for pea protein, ranging from very low enrichment (for phenylalanine) to high enrichment (for isoleucine). For both pea protein and casein, plasma ^{13}C enrichment was the highest for isoleucine and the lowest for lysine. The ^{13}C enrichment profile of plasmatic AA was quite similar between the pea protein and casein groups. A significant effect of time on plasma ^{13}C and ^{2}H enrichment was observed for all IAA with both pea protein and casein ($P < 0.0001$ for all, except for ^{2}H enrichment in threonine with casein where $P = 0.0263$). ^{2}H and ^{13}C-IAAs appeared in the plasma 30 min after the first ingestion of pea protein (Figure 3A and 3C) and casein (Figure 3B and 3D). For both pea protein and casein, a concomitant ^{13}C and ^{2}H isotopic enrichment plateau was reached between 2.5 h and 4 h for all IAA. After the plateau, enrichments declined gradually until the end of the protocol.

The AUCs were calculated from the plasma enrichment curves for ^{2}H and ^{13}C over the whole postprandial period (Supplemental Table 3). ^{2}H AUC values for isoleucine, leucine, phenylalanine, threonine and valine were higher with pea protein than with casein. For ^{13}C, AUC values did not differ between the two diet groups.

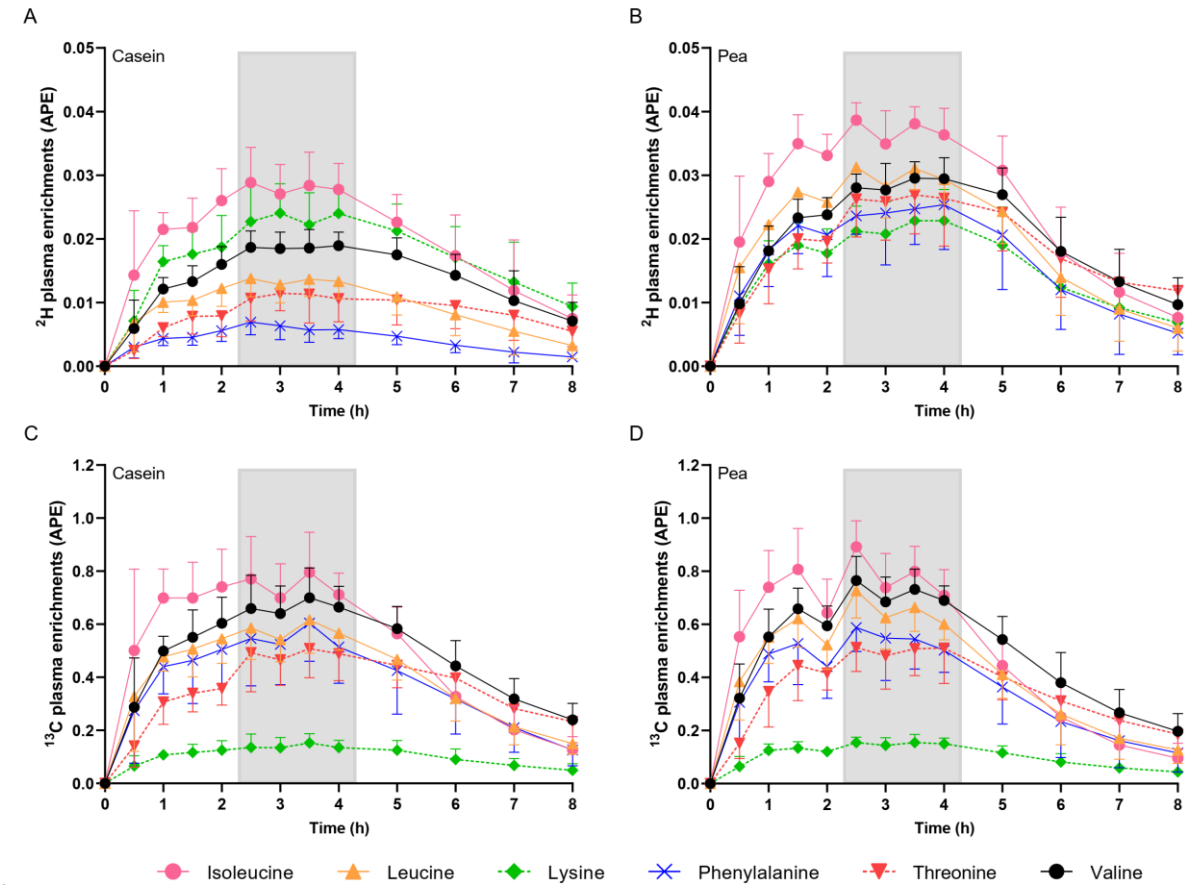

Figure 2. ^{2}H (A and B) and ^{13}C (C and D) enrichment of indispensable amino acids in plasma for the casein and pea protein groups. The grey area indicates the plateau level. n = 7 for casein and n = 8 for pea protein. Values are means ± SD. APE: atom percent excess.

Indispensable amino acid digestibility assessed using the ileal balance or dual isotope method

Table 3 summarizes the IAA ileal digestibility assessed using the ileal balance method and the digestibility values determined with the dual isotope method using plateau or AUC plasma ratios. The ileal AA digestibility of pea protein and casein was determined previously by means of ^{15}N labeling and is fully described elsewhere (26). Inter-individual variability was greater with the dual isotope method for both AUC and plateau calculations, especially for threonine in pea and lysine, and phenylalanine and threonine in casein.

The IAA digestibility values of pea protein determined using the dual tracer method ranged from 83.2 ± 7.6% (lysine) to 116.0 ± 21.2% (threonine) using the AUC calculation, and from 80.5 ± 7.7% (lysine) to 112.4 ± 13.7% (threonine) using the plateau calculation. No difference was observed between the AUC and plateau calculations with the dual isotope method, except for isoleucine. Pea AA digestibility was significantly higher with the dual isotope method

compared to the ileal balance values for threonine and isoleucine, whereas it was significantly lower for lysine and phenylalanine. No difference was observed between the two methods regarding leucine and valine.

The IAA digestibility values with casein determined using the dual tracer method ranged from 58.9 ± 5.7% (leucine) to 87.6 ± 9.5% (lysine) using the AUC calculation, and from 59.9 ± 7.3% (leucine) to 87.7 ± 5.3% (lysine) using the plateau calculation. No difference was observed between the AUC and plateau calculations using the dual isotope method for the pea isolate. Casein AA digestibility was consistently and significantly lower when evaluated with the dual isotope method for all AA, except for threonine where no difference was found due to high inter-individual variability. The difference between the methods ranged from 10% for isoleucine or lysine to 40% for leucine.

Table 3. Indispensable amino acid digestibility of pea protein and casein determined by the ileal balance method or dual isotope method, using either AUC or plateau values.

	Pea protein				Casein			
	Ileal balance	Dual isotope AUC	Dual isotope Plateau	*ANOVA*	Ileal balance	Dual isotope AUC	Dual isotope Plateau	*ANOVA*
Isoleucine	92.9 ± 3.8^{a}	109.2 ± 4.1^{b}	102.6 ± 4.4^{c}	*<0.0001*	94.4 ± 2.2^{a}	83.4 ± 4.5^{b}	84.2 ± 2.9^{b}	*0.0005*
Leucine	94.4 ± 2.8	96.4 ± 4.9	94.4 ± 5.8	*n.s.*	98.6 ± 0.4^{a}	58.9 ± 5.7^{b}	59.9 ± 7.3^{b}	*<0.0001*
Lysine	93.9 ± 2.6^{a}	83.2 ± 7.6^{b}	80.5 ± 7.7^{b}	*0.0002*	98.0 ± 0.5^{a}	87.6 ± 9.5^{b}	87.7 ± 5.3^{ab}	*0.0201*
Phenylalanine	94.6 ± 2.8^{a}	79.9 ± 6.4^{b}	82.4 ± 8.0^{b}	*0.0001*	99.2 ± 0.3^{a}	78.6 ± 13.4^{b}	82.6 ± 10.3^{b}	*0.0060*
Threonine	91.8 ± 4.0^{a}	116 ± 21.2^{b}	112.4 ± 13.7^{b}	*0.0074*	94.6 ± 1.3	82.6 ± 17.5	83.3 ± 17.4	*n.s.*
Valine	92.5 ± 3.8	93.1 ± 2.8	90.8 ± 3.2	*n.s.*	96.1 ± 1.3^{a}	82.9 ± 3.1^{b}	84.1 ± 2.8^{b}	*<0.0001*

Values are means ± SD. n = 8 for the pea protein group and n = 7 for the casein group. Mean values in a column within a protein source with unlike superscript letters were significantly different. AUC, area under the curve; IAA, indispensable amino acid; n.s., not significant.

Discussion

The aim of this study was to compare the digestibility values obtained using the minimally invasive dual isotope method and the standard direct ileal balance method, for two different protein sources, pea isolate and casein, doubly labeled with ^{15}N and ^{2}H. The 4-hour repeated meal procedure enabled the almost complete digestion of the test proteins at 8 hours and a plasma isotopic plateau between 2.5 and 4 hours was obtained. For the pea protein isolate, IAA digestibility values were either higher, similar or lower with the dual isotope method compared to the ileal balance method, depending on the IAA considered. For casein, systematically lower

digestibility values were obtained with the dual isotope method compared with the ileal balance method.

Methodological choices and isotopic plasma kinetics

Setting up a protocol to test the dual isotope method and the ileal balance method with the same volunteers was very challenging because the protocols are specific to each method. For the ileal balance method, a bolus meal is preferred to enable the complete collection of dietary AA ileal losses during the 8 hours that follow meal ingestion (12, 13, 28). By contrast, in most studies that use the dual isotope method, a plateau feeding protocol is implemented, with repeated meals being given to the volunteers over 7 to 8 hours (18-23). An alternative protocol proposed by the FAO is to give a bolus meal and calculate the ratio of AUC values for isotope enrichments rather than the ratio at the plateau (16), but this method has never been used to evaluate IAA digestibility. In order to apply the two methods to the same subject, we chose a hybrid feeding protocol, as we previously performed (25, 29). We split the test meal into 12 portions and administered it in 9 small meals, the first being a loading dose of 4 portions during the first 4 hours. The objective was to obtain a transient isotopic plateau while enabling almost complete digestion over 8 hours.

Furthermore, and contrary to the previously published study comparing the dual isotope and ileal balance methods (25), we used doubly labeled (^{15}N and ^{2}H) protein sources to prevent any confounding effect of transamination with ^{15}N labeling in the dual isotope method.

For the reference source of AAs in the dual isotope method, we chose to use a mixture of free ^{13}C AA rather than ^{13}C-spirulina as recommended by FAO (16), and that has been more widely employed (18, 19, 22, 23). However, the mean AA digestibility of ^{13}C-spirulina has been shown to be moderate (<90%) and has yielded variable outcomes among studies (19, 30). Moderate to low digestibility values are indeed usually associated with higher inter-individual variability (6), so using a unique value for spirulina digestibility in all volunteers is a source of uncertainty. By contrast, free AAs are supposed to be 100% digestible. The mean ileal AA digestibility we obtained in the present study for the free AA reference was 98.2%, with no difference between the pea isolate and casein test meals, and similar to previously observed levels (98.1%) (25). Digestibility was thus high, with low variability (< 1%) and good inter-study repeatability. We mixed the test protein and the ^{13}C-free AA reference in the same semi-liquid food matrix

(mashed potatoes) for the test meal in order to prevent the offsets observed previously between free AA and protein-bound AA kinetic absorption (25).

These methodologic choices could have had an impact on the concomitance of ^{13}C and ^{2}H-AA kinetics in the plasma. Indeed, a free AA mixture is known to be absorbed more rapidly than an intact protein of similar AA composition (31, 32). The repeated feeding pattern was implemented in order to spread over time the absorption of ^{13}C-AA, although it could not completely ensure the prevention of kinetic offsets between isotopes, as previously observed (25). However, the mix of free AAs and the test protein within the same matrix resulted in good concordance between ^{13}C and ^{2}H AA kinetics, regardless of the protein source. The feeding procedure made it possible to reach a transient plateau for both ^{2}H and ^{13}C isotopes, with a concurrent plateau period observed from 2.5 hours to 4 hours after consumption of the first meal. Because we collected blood every 30 minutes from 0 to 4 hours after the meal intake, the plateau period included four values. Further, almost complete digestion was achieved 8 hours after the first meal intake, despite the repeated meal protocol. Indeed, only 0.8 and 0.4 mmol of dietary nitrogen were found in the ileal digesta at 8 hours for casein and pea, respectively (26), which represented 0.3% and 0.1% of the nitrogen ingested and was in the same range as the findings of ileal balance studies with bolus feeding (12, 28, 33, 34). Thus, the feeding protocol we chose to compare the ileal balance and dual isotope methods enabled us to obtain a plasma isotopic plateau and complete digestion of the test meals simultaneously. Nevertheless, the ^{2}H and ^{13}C plasma enrichments had not returned to the basal level at 8 hours. It is therefore possible that the AUCs gave rise to minor errors in the estimation of IAA digestibility (0-1.5%).

Digestibility values obtained using the dual isotope method

The ileal digestibility results we obtained using the ileal balance method with ^{15}N-labeled casein and pea protein have been discussed previously (26). In the case of the dual isotope method, no differences were observed between the AUC and the plateau calculation method for either pea or casein. The dual isotope method yielded digestibility values that could be considered physiologically acceptable (< 100%) for leucine, lysine, phenylalanine and valine for pea, and for all the IAAs analyzed for casein. Isoleucine and threonine digestibility values reached > 100% for pea protein, which could be considered as being aberrant. For pea protein, the IAA digestibility values using the indirect method were either higher (isoleucine, threonine), similar (leucine, valine) or lower (lysine, phenylalanine) when compared to ileal digestibility. For casein, lower digestibility was always obtained with the dual isotope method for all IAAs.

Several factors could have contributed to these discrepancies. One of the important assumptions of the dual isotope method is that splanchnic removal is the same for AA derived from the test protein and reference source of AA (16). The percentage of dietary AA extracted by splanchnic tissues may vary as a function of different factors such as protein digestion and AA absorption (35, 36). A greater rise in plasma AAs and a higher AUC have been observed in healthy subjects following the bolus ingestion of free AA mixture compared with the ingestion of an equivalent amount of intact protein (32), but this does not account for the degree of splanchnic extraction. Further, no differences in splanchnic extraction have been observed between free and protein-bound glutamine (37), or between free and protein-bound leucine (38) in healthy humans. Hence, according to the literature, differences in splanchnic uptake between bound AAs from the test proteins and the reference free AAs seem unlikely. However, we cannot completely exclude this possibility and differential splanchnic extraction between the test proteins and the reference free AA mixture may have contributed to the inconsistency of our results with the dual isotope method. Furthermore, casein is also known to be a slowly digested protein and we found that casein nitrogen appeared 30 minutes later, and started to decrease in the ileal digesta 1 hour after dietary nitrogen from the pea protein isolate (26). These differences between pea protein isolate and casein digestion kinetics may have contributed to the differences in the deviation we observed from the ileal balance method.

In previously published studies using the dual isotope method, blood samples were collected from 5 to 8 hours after the first meal intake, during a prolonged plateau obtained from consumption of repeated meals over 7 to 8 hours (18-22). This prolonged plateau limits the risk of an offset of plasma kinetics due to differential intestinal absorption kinetics. In our study, the isotopic ratios were calculated with the AUC or during an early (from 2.5 to 4 h) transient plasma isotopic plateau. Using the AUC calculation, the underestimation of plasma isotopic enrichment for both ^{2}H and ^{13}C, due to the delayed return to baseline at 8 h, may have caused an underestimation of digestibility. As both ^{2}H and ^{13}C enrichment AUCs were underestimated and digestibility was calculated using their ratio, the errors on the final digestibility values were small, between 0 and 1.5% depending on the AAI. Using the plateau value, its duration might have been insufficient to completely buffer the differential absorption of AA even though isotopic plasma enrichment kinetics were comparable, and the plateaus for ^{2}H and ^{13}C enrichment were concurrent. The use of a protein as the reference source of AA may solve this issue, but a highly digestible, commercially affordable and highly intrinsically ^{13}C-labeled protein is not yet available.

The ^{2}H labeling of plant and animal proteins with deuterated water potentially could result in differential labeling (24). The administration of $^{2}H_2O$ to autotrophs such as plants gives rise to a random and homogenous distribution of ^{2}H labeling. By contrast, the administration of $^{2}H_2O$ to cows may result in a more heterogeneous distribution of ^{2}H labeling since it occurs through transamination exchanges at the level of the second carbon of AA or from *de novo* synthesis of AA from rumen bacteria. To account for possible losses of the α-carbon ^{2}H atom through transamination after the absorption of AA, we used the transamination factors determined by Devi *et al*. using LC-MS/MS (19), as usually done in studies using the dual isotope method (18, 22, 23). These transamination factors were estimated for a group of Indian subjects who had ingested ^{2}H-labeled legumes. However, it is questionable whether these factors are universally applicable to all protein sources and all populations. The use of an incorrect factor might have an important impact since a difference of 0.02 may result in an increase or decrease of nearly 2% of digestibility in highly digestible proteins. An assessment of transamination among protein sources and for different population groups is necessary in order to determine more specific corrective factors.

Conclusion

In conclusion, most of the AA digestibility values obtained using the dual isotope method were different from those obtained with the standard ileal balance method, and the discrepancies between methods differed depending on the protein sources. Using the dual isotope method, a prolonged isotopic plasma plateau, or the use of a protein as the reference, may be necessary to offset any differences in AA absorption between the test and reference proteins. The dual isotope method requires further research and validation but could enable major advances in the evaluation of protein quality for human nutrition, particularly in vulnerable populations.

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The data described in the manuscript will be made available upon request, pending application and approval.

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Supplemental tables

Supplemental Table 1. Amino acid composition and ^{13}C enrichment of algal free amino acids mixture, used as the reference for dual tracer method

	AA content (mmol/g)	^{13}C enrichment (AP)
Alanine	1.08	99
Arginine	0.21	98
Asparagine	0.39	99
Glutamine	0.71	99
Glycine	0.85	99
Histidine	0.06	98
Isoleucine	0.62	97.5
Leucine	0.91	99
Lysine	0.17	99
Methionine	0.10	99
Phenylalanine	0.48	99
Proline	0.29	99
Serine	0.25	95
Threonine	0.45	98
Tyrosine	0.07	97
Valine	0.81	99

AA, amino acid; AP, atom percent.

Supplemental Table 2. ^{2}H and ^{13}C enrichment (APE) of the test-meals used to calculate meal ratios in dual isotope method.

	^{2}H			^{13}C		
	Pea protein meal	Casein meal	*T-test*	Pea protein meal	Casein meal	*T-test*
Isoleucine	0.088 ± 0.001	0.080 ± 0.000	*<0.0001*	1.82 ± 0.01	1.64 ± 0.02	*<0.0001*
Leucine	0.081 ± 0.000	0.052 ± 0.000	*<0.0001*	1.52 ± 0.01	1.22 ± 0.01	*<0.0001*
Lysine	0.073 ± 0.000	0.071 ± 0.000	*<0.0001*	0.40 ± 0.00	0.39 ± 0.01	*0.0068*
Phenylalanine	0.094 ± 0.000	0.026 ± 0.001	*<0.0001*	1.67 ± 0.01	1.76 ± 0.02	*0.0011*
Threonine	0.076 ± 0.004	0.042 ± 0.004	*<0.0001*	1.71 ± 0.02	1.54 ± 0.03	*0.0004*
Valine	0.087 ± 0.001	0.054 ± 0.000	*<0.0001*	1.90 ± 0.01	1.53 ± 0.01	*<0.0001*

Values are means ± SD of 5 replicates. APE, atom percent excess.

Supplemental Table 3. Area under the curve (AUC, in APE/h) of the ^{2}H and ^{13}C plasma enrichment of indispensable amino acids.

	^{2}H			^{13}C		
	Pea protein	Casein	*T-test*	Pea protein	Casein	*T-test*
Isoleucine	0.21 ± 0.03	0.16 ± 0.02	*0.0058*	4.10 ± 0.49	4.15 ± 0.51	*n.s.*
Leucine	0.16 ± 0.03	0.08 ± 0.01	*< 0.0001*	3.47 ± 0.49	3.29 ± 0.46	*n.s.*
Lysine	0.12 ± 0.03	0.14 ± 0.03	*n.s.*	0.85 ± 0.16	0.82 ± 0.22	*n.s.*
Phenylalanine	0.13 ± 0.04	0.03 ± 0.01	*< 0.0001*	2.97 ± 0.75	3.04 ± 0.86	*n.s.*
Threonine	0.15 ± 0.03	0.07 ± 0.02	*0.0001*	2.91 ± 0.40	2.88 ± 0.50	*n.s.*
Valine	0.17 ± 0.03	0.11 ± 0.01	*0.0002*	4.03 ± 0.50	3.92 ± 0.54	*n.s.*

Values are means ± SD. n = 8 for pea protein group and n = 7 casein group. APE, atom percent excess; AUC, area under the curve; n.s., not significant.