The True Amino Acid Digestibility of 15N-Labelled Sunflower Biscuits Determined with Ileal Balance and Dual Isotope Methods in Healthy Humans

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Running title: Amino acid digestibility of sunflower biscuits

Abbreviations: AA: amino acid; Asx: aspartate and asparagine; AP: atom percent; APE: atom percent excess; AUC: area under the curve; BMI: body mass index; C: carbon; DAA: dispensable amino acid; Glx: glutamate and glutamine; IAA: indispensable amino acid; IRMS: isotope ratio mass spectrometer; N: nitrogen; PEG: polyethylene glycol; SD: standard deviation

Abstract

Background:

Sunflower is a promising protein source but data on amino acid (AA) digestibility are lacking in humans. Classically, the determination of AA digestibility requires ileal digesta sampling. The dual isotope method is minimally invasive but has not been compared to the conventional approach.

Objective:

This study aimed to determine the true ileal digestibility of sunflower AAs in healthy volunteers who ate biscuits containing ^{15}N protein isolate, in comparison with the dual isotope method.

Methods:

Twelve healthy volunteers (men and women, 40.4±10.5 years old, BMI 23.7±2.9 kg/m^2) were equipped with a naso-ileal tube. They consumed for 4h nine repeated meals comprising ^{15}N-sunflower protein biscuits together with ^{13}C-AAs, carried either in chocolate (SUN+C, n=7) or apple puree (SUN+P, n=5). Ileal digesta and blood were sampled throughout 8h after ingestion of the first meal. The ^{15}N and ^{13}C AA enrichments were measured in digesta to determine ileal digestibility directly, and in plasma to determine lysine and threonine digestibility using the dual isotope method. Differences between methods and between vector groups were analyzed using paired and unpaired *t*-tests, respectively.

Results:

Ileal digestibility of sunflower indispensable AAs (IAA) was 89±5.3%, threonine and lysine having the lowest digestibility. In the SUN+C meal, IAA digestibility was 3%

below that of SUN+P ($P < 0.05$). Mean free ^{13}C-AA ileal digestibility was 98.1±0.9%. No matter which matrix was used to carry ^{13}C-AAs, plasma ^{15}N and ^{13}C-AA kinetics displayed a 1h offset. Digestibility obtained with the dual isotope method (70.4±6.0% for threonine and 75.9±22.3% for lysine) was below the target values.

Conclusions:

The ileal digestibility of IAAs from a sunflower isolate incorporated in a biscuit was close to 90% in healthy adults. Under our experimental conditions, the dual isotope method provided lower values than the usual method. Further protocol developments are needed to validate the equivalence between both methods.

Clinical Trial Registry: The clinical trial was registered at www.clinicaltrials.gov database (NCT04024605).

Keywords: protein quality, amino acids, sunflower protein, ileal digestibility, dual isotope method

Introduction

Oilseeds like sunflower are good candidates to contribute to the increasing demand for plant protein sources for humans. As oil coproducts, sunflower cakes contain about 30% protein (1). The amino acid (AA) composition of sunflower is relatively well balanced, except for a moderate lysine deficiency (2). Besides AA composition, which is a key determinant of protein quality, AA digestibility also plays a role in the satisfaction of human AA requirements. Data on protein digestibility from sunflower cake have been collected in pigs, values varying from 72 to 82% (1,3–5). The AA digestibility of a sunflower isolate has recently been reported to be very high (95%) in rats (6). However, sunflower AA digestibility has never been assessed in humans.

To directly determine AA digestibility in humans, ileal samples can be collected either in ileostomates or in healthy volunteers. In the latter, digesta must be collected using a naso-ileal tube. This method allows investigation of protein digestion under many nutritional conditions. When coupled to the use of ^{15}N intrinsically labelled dietary protein, values of protein and AA ileal digestibility have been obtained for many protein sources (7–16). Whereas this method is the usual direct way to measure AA digestibility in healthy volunteers, its main drawback is its invasiveness.

The Food and Agriculture Organization (FAO) of the United Nations has proposed a minimally invasive method based on the measurement of isotope enrichments in plasma AAs after the ingestion of a test protein labelled with ^{2}H, together with a ^{13}C reference protein of known digestibility (17). ^{15}N was not proposed because of transaminations, but ^{15}N labeling is easier and less expensive for plant proteins, especially in field growing conditions. Interestingly, the dual isotope method was implemented earlier in cystic fibrosis patients, using ^{15}N-labelled spirulina as the test

protein and free ^{2}H phenylalanine as the reference amino acid of known digestibility, the $^{15}N/^{2}H$ ratio in phenylalanine being compared in plasma and meal to calculate spirulina phenylalanine digestibility (18). The method was further developed by another research group to determine AA digestibility in various ^{2}H-labelled protein sources, using ^{13}C-labelled spirulina as the reference protein, in Indian adults and children (19–24). This method is promising in the view of collecting data in various populations, including vulnerable people, but it needs to be validated by comparison with the usual direct determination of AA ileal digestibility. However, both methods present some specific constraints, especially in terms of feeding procedure, that may be challenging to address in one single protocol. In particular, the prolonged plateau variant of the dual tracer approach requires a fractioned feeding pattern for several hours while the direct ileal measurement requires that the food digestion is complete before the end of the experiment.

This study aimed to determine the true ileal amino acid digestibility of ^{15}N-labelled sunflower protein isolate incorporated in biscuits consumed by healthy volunteers. A second objective was to compare ileal digestibility values with those obtained with the dual isotope method within subjects.

Materials and methods

Subjects

The eligibility criteria were a BMI between 18 and 30 kg/m², an age between 18 and 65 years, a negative serology for HIV, hepatitis C virus antibodies, and hepatitis B virus surface antigens, and the absence of any dietary allergy and digestive disease.

The study PRODIGE was conducted in the Human Nutrition Research Center of Avicenne Hospital (APHP, Bobigny, France). It was registered at www.clinicaltrials.gov database (NCT04024605). All subjects provided a written informed consent for inclusion. Data were collected at the UMR PNCA (Paris, France). The study was approved by the Ethical Committee Sud Mediterranée IV (ref 180502) and authorized by the Health and Drug French Agency (ANSM, ref 2018062100214).

The number of volunteers was determined in accordance with previous studies on amino acid digestibility of protein sources (7–16), allowing for external comparison between sunflower isolate and other proteins assessed in the same conditions. It was also calculated to enable a comparison between the ileal balance and the dual isotope methods. The size group was n = 13 to reveal a difference of digestibility of 5 ± 5% in a within subject design (two-tails paired Student test), for a risk α=5% and a risk (1-β) = 90% (G*Power 3.1). Recruitment started in January 2019 and ended in April 2019. All volunteers signed their informed consent. Nineteen volunteers were recruited and the final sample size was n=12 (**Table 1**).

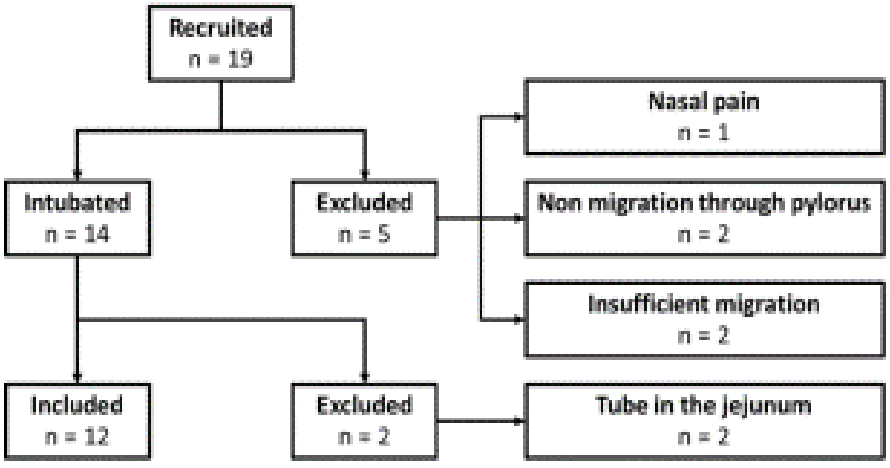
Table 1. Anthropometric characteristics of the subjects

	n = 12
Sex (F/M)	8/4
Age (years)	40.4 ± 10.5
BMI (kg/m²)	23.7 ± 2.9

Values are means ± SD. n = 12. BMI: body mass index.

Causes of failure were non-migration of the tube through the pylorus (n = 2), insufficient migration in the small intestine (n = 2) and nasal pain (n = 1). Two volunteers were excluded after the experiment because their tube was not positioned in the terminal ileum (**Figure 1**).

Figure 1. Flow chart of the study and exclusion criteria.



Test meals

Sunflower plants were labelled with two applications of ^{15}N-enriched fertilizer during growth (Terres Inovia, Pessac, France). Seeds were dehulled and de-oiled (Olead, Pessac, France), and proteins were extracted and isolated as described previously (6) (UMR LRGP, Nancy, France). The ^{15}N-labelled sunflower protein isolate was incorporated in chocolate chip biscuits (13 g) (Foodinnov, Rennes, France). Biscuits were composed of 17.2% (w/w) of ^{15}N-labelled sunflower isolate as the main protein source (**Supplementary Table 1**). They were cooked at 180°C for 13 min. The wheat flour used in the biscuit also had a small amount of gluten (2.8% of the biscuit). As the ^{13}C-labelled reference protein of known digestibility, algal free ^{13}C-AA mixture (97%) (Eurisotop, Saint-Aubin, France) was preferred to spirulina because of its high digestibility and subsequently low interindividual variability in contrast to spirulina (1). However, free ^{13}C-AAs were not incorporated in the biscuits because they could have been altered by the Maillard reaction during cooking. A separate matrix, a chocolate, was therefore initially chosen as the vector for ^{13}C-AAs to ensure a slow gastric emptying of the AAs in order to obtain a concomitant delivery of ^{15}N, provided by the biscuit, and ^{13}C. Due to the high additional energy and the high polyphenol content of chocolate (between 283 and 510 mg/100 g (25,26)), as well as the possible interaction with protein digestibility, we also decided to test apple puree, a vector that is less caloric and is lower in polyphenol contents. Finally, the chocolate was fed to seven subjects (SUN+C) and the puree to five subjects (SUN+P). Chocolate and puree compositions are given in **Table 2**.

Table 2. Composition of chocolate and puree

	Chocolate	Puree
	g/100 g	
Chocolate	88.3	/
Sugar	8.0	10.0
Fiber	/	2.0
Cocoa butter	2.0	/
Algal amino acid mixture	0.7	0.7
Energy (kcal/100 g)	391.7	44.0
Protein (g/100 g)[1]	7.3	1.0

[1] Chocolate and apple puree contain, respectively, 6.6 g and 0.3 g protein (N x 6.25)/100 g.

The test meal was split into nine portions. The first one was composed of four biscuits, and the other eight were comprised of one biscuit. In total, volunteers ingested 156 g of biscuits, including 26.8 g of ^{15}N-labelled sunflower isolate. Alongside biscuits, they also ingested chocolate or apple puree for a total amount of 400 mg of ^{13}C-AA mixture. Volunteers were allowed one glass of water hourly, or half a glass per meal portion. Sunflower isolate contained 14.2% nitrogen (N) and ^{15}N enrichment was 0.80 atom percent, two times the natural abundance. Biscuits contained 3.5% N, and the ^{15}N enrichment was 0.73 atom percent. Sunflower isolate was composed of 33.1% IAA and 66.9% dispensable amino acids (DAA) (**Table 3**). Amino acid composition of the algal mixture is presented in **Supplementary Table 2**.

Table 3. Amino acid composition of sunflower isolate in percentage of all amino acids

IAA		DAA	
Histidine	2.35	Alanine	3.91
Isoleucine	4.07	Arginine	9.14
Leucine	6.21	Asx	8.92
Lysine	4.31	Cysteine	3.31
Methionine	3.42	Glx	21.89
Phenylalanine	3.77	Glycine	7.36
Threonine	3.82	Proline	4.75
Valine	4.67	Serine	4.45
Tryptophan	1.09	Tyrosine	2.57

Asx: aspartate and asparagine. Glx: glutamate and glutamine.

Clinical protocol

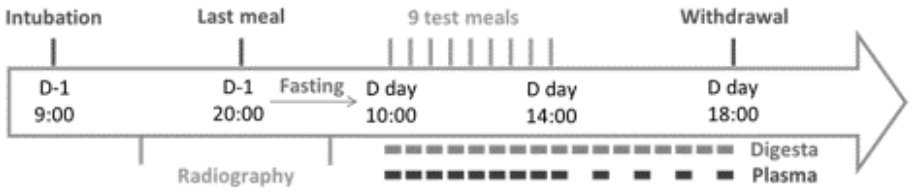
One week before the experiment, volunteers followed a standardized diet to achieve a mean protein intake of 1.3 g.kg of body weight^{-1}.day^{-1}. This quantity of protein corresponds to the mean consumption of protein in France (27). This diet standardization was performed to reduce the possible effects of the subjects' habitual diets.

The intestinal tube was composed of three lumens. One was used to inflate or deflate a balloon to help migration of the tube, another one allowed collection of ileal digestive contents, and the third to perfuse a non-absorbable marker of intestinal flow: polyethylene glycol (PEG 4000, 20 g/L, Biogaran, Colombes, France).

The day before the experiment (**Figure 2**), the tube was introduced through the subject's nose and was allowed to progress through the stomach and the digestive

tract for 24 h. The subject then fasted from 20:00. On the day of the experiment, the position of the tube was checked with radiography and by measuring the pH of the effluent (pH being 8.0 in the ileum). At 9:00 a catheter was inserted in the forearm vein of the subject. The intestinal perfusion of PEG 4000 was initiated at a rate of 1 mL/min. Basal collection of ileal effluent was performed for 30 min, as well as blood sampling. At 10:00 subjects began to eat the first meal (four portions of biscuits together with chocolate or apple puree), followed by one portion each half hour. The experiment lasted 8 h from the first meal to the removal of the tube. Digestive contents were collected continuously and pooled by half hour. The volume of digesta was measured and diisopropylfluorophosphate was added as anti-protease. Digestive contents were frozen at -20°C before being freeze-dried. Plasma was sampled every 30 minutes for four hours and every subsequent hour. After centrifugation, plasma supernatant was dispatched in aliquots and frozen at -20°C.

Figure 2. Experimental design



Analytical methods

The concentration of PEG 4000 in the digesta was assessed by the turbidimetric method (28) to determine the ileal flow rate.

^{15}N and ^{13}C enrichments in meal, digesta and plasma individual AAs were analyzed by gas chromatography (GC 6890N, Agilent Technologies, Les Ulis, France) coupled to an Isoprime isotope ratio mass spectrometer (Isoprime, GV Instrument, Manchester, UK) via the GC5 Isoprime interface (GC-C-IRMS). Analytical methods for ^{15}N enrichment determination were previously described (6). For ^{13}C enrichment, the combustion furnace temperature was 850°C. The GC column (RXI-17, 30 m long, 0.25 µm i.d., 0.5 µm film thickness; Restek) temperature program started at 150°C, rose to 200°C by 4°C/min and then to 270°C by 25°C/min; the final temperature was maintained for 10 min. The inlet temperature was set at 270°C. Plasma samples (2 µL) were injected in split mode (2:1) and digestive samples were injected with split 10:1.

Briefly, sunflower proteins and digestive contents were hydrolyzed for 24 h with hydrochloric acid 6N at 110°C. Amino acids for sunflower isolate, ileal contents and plasma were isolated using a hydrogen form ion exchange resin (Dowex® 50WX8 hydrogen form 100-200 mesh, Sigma-Aldrich, Saint-Louis, USA) and derivatized with ethyl chloroformate (29).

AA quantification in ileal digesta, meal and protein isolate were performed on an Acquity H-class UHPLC system with a PDA detector (Waters, Milford, USA) as previously described (30). For tryptophan, a basic hydrolysis was performed with barium hydroxide 2N. For sulfur AA, a performic acid oxidation was used before an acid hydrolysis. For the other amino acids, an acid hydrolysis was performed with hydrochloric acid 6N (31). The weight of each AA was calculated using free AA molecular weight (32).

Calculations

The ileal flow rate was evaluated for each period of 30 minutes (F, mL/30min) using the following formula:

$$F = \frac{[PEG]_{solution}}{[PEG]_{digesta}} \times \text{perfusion flow rate} \times 30$$

where [PEG] is the concentration of glycol in the perfused solution and in the digestive contents, and the perfusion flow rate of the PEG was set at 1 mL/min.

To determine AA ileal digestibility, it was necessary to determine the total amount of each AA ingested and the amount of exogenous AAs recovered in the ileal contents.

The total amount of AA ingested ($AA_{ingested\ i}$, mmol) was:

$$AA_{ingested\ i} = [AA]_{meal\ i} \times \text{protein ingested}$$

where $[AA]_{meal\ i}$ is the quantity of each AA "i" in the sunflower isolate (mmol/g), and "protein ingested" is the amount of isolate ingested by the subject (g).

The quantity of exogenous AA in digesta ($AA_{exo\ i}$, mmol) at every period was:

$$AA_{exo\ i}(t) = [AA]_{ileum\ i}(t) \times DM\ (t) \times F(t) \times \frac{APE_{ileum\ i}\ (t)}{APE_{isolate}}$$

where $[AA]_{ileum\ i}$ is the quantity of each AA "i" in the digestive contents at each period "t" (mmol/g), DM is the amount of dry matter in the digestive contents (g/100 mL), F is the ileal flow rate (mL/30 min), APE is the enrichment excess of each AA "i" in the digestive contents at each period "t" compared to the basal abundance (in atom percent) of ^{15}N and $APE_{isolate}$ that of sunflower isolate. Basal abundance is the abundance measured in the t = 0 sample of each volunteer.

The same calculation was used for the digestibility of ^{13}C-AAs, except that the APE of ^{13}C-AAs (i.e. 98.3 % as given by the supplier) was used instead of $APE_{isolate}$.

For each AA, the ileal digestibility (% of ingested AA) was:

$$\text{AA ileal digestibility}_{\text{i}} = 1 - \left(\frac{\sum AA_{exo\ i}}{\text{AA}_{\text{ingested i}}} \times 100 \right)$$

where $\Sigma AA_{exo\ i}$ for each AA "i" is the sum of exogenous AA over 8 h (mmol), and $AA_{ingested\ i}$ is the quantity of AA "i" from sunflower isolate for ^{15}N and the tracer dose for ^{13}C, respectively, that was ingested by the volunteer (mmol).

To calculate mean AA digestibility, the digestibility of each AA was weighted by the relative contribution of the AA in sunflower protein isolate.

The formula used to determine ileal digestibility of ^{13}C-free AAs was similar.

The ratio between the two isotopes in the meal was determined for each IAA (i):

$$\text{Meal ratio i} = \frac{^{15}\text{N}_{\text{meal i}}}{^{13}\text{C}_{\text{meal i}}}$$

The AUC (APE/h) for plasma enrichment in both isotopes was calculated for each IAA, and the ratio between the two isotope AUCs was determined for each IAA (i) as follows:

$$\text{Plasma AUC i} = \frac{\text{AUC}\,^{15}\text{N}_{\text{i}}}{\text{AUC}\,^{13}\text{C}_{\text{i}}}$$

Using the dual isotope method, IAA absorption ($IAA_{absorption\ plasma\ i}$) for every IAA (i) was determined using plasma AUC and meal ratios as follows:

$$\text{IAA}_{\text{absorption plasma i}} = {}^{13}\text{C AA digestibility}_{\text{i}} \times \frac{\text{Plasma AUC i}}{\text{Meal ratio i}}$$

where ^{13}C AA digestibility i is the ileal ^{13}C digestibility of the AA "i", determined by analysis of digestive content.

Statistical analysis

Data are expressed as means ± standard deviation (SD). The main outcome was the digestibility of individual AAs obtained by the two methods and their subsequent comparison. ^{13}C or ^{15}N enrichment kinetics data were analyzed in a mixed model with group as a fixed factor and time as a repeated factor. Differences between digestibility methods were estimated using a paired Student's *t*-test. Differences between IAA and DAA digestibility within groups as well as differences between vector groups (SUN+C or SUN+P) were estimated using an unpaired Student's *t*-test. All analyses were done using R (version 3.5.1, R project). Differences were considered statistically significant for a P_{value} inferior to 0.05.

Results

Ileal AA digestibility of ^{15}N-labelled sunflower

True amino acid digestibility of sunflower protein isolate was determined in the ileum (**Table 4**). In the SUN+C group, values were the lowest for glycine (~68 %) and the highest (~92%) for glutamine/glutamate (glx). Mean ileal IAA digestibility was 6 % higher ($P < 0.01$) than DAA digestibility and the variability among IAAs was lower. In the SUN+P group, values were also the lowest for glycine (~73%) but the highest methionine (~95%). For all AAs, there was no statistically significant difference between the two groups and no difference for mean digestibility of AAs ($P = 0.34$). Nevertheless, mean IAA digestibility was lower in SUN+C group than in SUN+P group ($P < 0.05$).

Table 4. Amino acid ileal digestibility of ^{15}N sunflower isolate

	SUN+CHOCO	SUN+PUREE	P_{value}	Pooled
		IAA digestibility (%)		
Isoleucine	87.7 ± 5.0	90.7 ± 6.0	0.36	89.0 ± 5.4
Leucine	89.9 ± 4.5	92.7 ± 5.2	0.35	91.1 ± 4.8
Lysine	86.4 ± 4.3	88.4 ± 4.8	0.48	87.2 ± 4.4
Methionine	91.1 ± 5.8	95.4 ± 2.6	0.16	92.9 ± 5.0
Phenylalanine	90.3 ± 4.9	92.6 ± 6.7	0.50	91.3 ± 5.5
Threonine	85.8 ± 5.1	89.3 ± 5.5	0.28	87.3 ± 5.3
Valine	88.3 ± 4.7	91.2 ± 6.1	0.38	89.5 ± 5.3
Mean IAA	**88.5 ± 5.0**	**91.5 ± 5.4**	**< 0.05**	**89.8 ± 5.3**
		DAA digestibility (%)		
Alanine	88.4 ± 4.5	91.0 ± 5.6	0.40	89.5 ± 5.0
Glycine	68.3 ± 9.5	73.4 ± 10.0	0.39	70.4 ± 9.6
Glx	92.2 ± 3.1	94.3 ± 3.8	0.33	93.1 ± 3.4
Proline	81.4 ± 6.4	85.9 ± 6.6	0.27	83.3 ± 6.6
Serine	82.1 ± 7.0	81.9 ± 7.1	0.98	82.0 ± 6.4
Mean DAA	**82.5 ± 10.6**	**85.4 ± 9.8**	**0.30**	**83.8 ± 10.3**
Mean (all AA)	**85.2 ± 4.7**	**88.2 ± 5.6**	**0.34**	**86.5 ± 5.1**

Values are means ± SD. N = 7 for all amino acids (AA) in the SUN+CHOCO group, except n=3 for serine. N=5 for the SUN+PUREE group, except n=4 for serine. Pooled (SUN+CHOCO + SUN+PUREE) values are presented. DAA: dispensable amino acid, Glx: glutamate and glutamine, IAA: indispensable amino acid.

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

For chocolate, ileal digestibility of ^{13}C AAs values varied from around 95 % for tyrosine to 99 % for alanine (**Table 5**). When incorporated in puree, ileal ^{13}C-AA

digestibility values ranged in the same range but phenylalanine was the most digestible. There was no statistical difference between IAA and DAA digestibility values (P = 0.83 for SUN+C and P = 0.98 for SUN+P). There was also no difference between chocolate and puree for mean (P = 0.87) and individual AA digestibility.

Table 5. Amino acid ileal digestibility of ^{13}C algal free amino acids.

	^{13}C algal AA digestibility			
	SUN+CHOCO	SUN+PUREE	P_{value}	Pooled
	IAA digestibility (%)			
Isoleucine	98.1 ± 1.1	97.8 ± 1.2	0.73	98.0 ± 1.1
Leucine	98.5 ± 0.9	98.3 ± 0.8	0.72	98.4 ± 0.8
Lysine	95.9 ± 2.3	95.6 ± 3.7	0.87	95.7 ± 2.8
Methionine	96.7 ± 1.7	97.8 ± 0.7	0.22	97.2 ± 1.4
Phenylalanine	98.8 ± 0.8	99.1 ± 0.7	0.54	98.9 ± 0.7
Threonine	97.4 ± 1.4	97.2 ± 1.3	0.81	97.3 ± 1.3
Valine	97.8 ± 1.2	97.6 ± 1.1	0.85	97.7 ± 1.1
Mean IAA	**97.6 ± 1.7**	**97.6 ± 1.8**	**0.95**	**97.6 ± 1.7**
	DAA digestibility (%)			
Alanine	98.9 ± 0.6	98.8 ± 0.9	0.88	98.8 ± 0.7
Glycine	97.9 ± 1.1	98.0 ± 1.1	0.89	97.9 ± 1.1
Glx	98.1 ± 1.1	98.0 ± 1.3	0.91	98.1 ± 1.1
Proline	98.6 ± 0.7	98.8 ± 0.5	0.58	98.7 ± 0.6
Serine	97.1 ± 1.6	97.0 ± 1.3	0.90	97.0 ± 1.3
Tyrosine	95.3 ± 2.7	95.1 ± 2.6	0.93	95.2 ± 2.5
Mean DAA	**97.7 ± 1.9**	**97.6 ± 1.8**	**0.87**	**97.7 ± 1.8**
Mean (all AA)	**98.1 ± 1.0**	**98.0 ± 0.9**	**0.87**	**98.1 ± 0.9**

Values are means ± SD. In the SUN+CHOCO group, n =3 for serine, n = 4 for threonine and n = 7 for other amino acids. In the SUN+PUREE group, n = 5 for all amino acids. Carbon

283 digestibility for each vector and pooled values are presented. DAA: dispensable amino acid,
284 Glx: glutamate and glutamine, IAA: indispensable amino acid.

285

286 **Plasma kinetics of ^{15}N and ^{13}C AA**

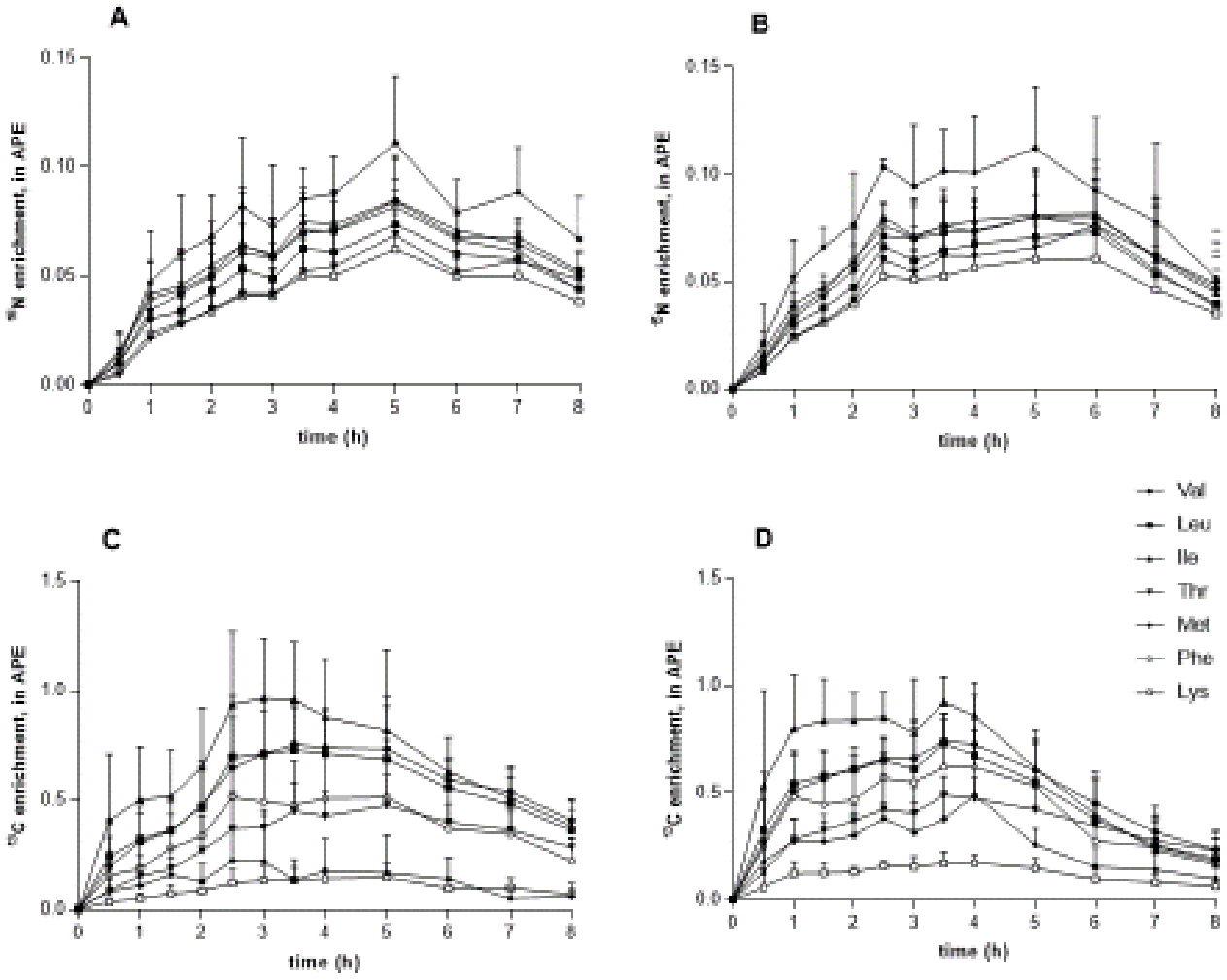
287 ^{15}N enrichment in the meal IAA ranged from 0.281 ± 0.07 APE for histidine and 0.379
288 ± 0.031 APE for lysine (**Supplementary figure 1**). For ^{13}C, it ranged from 0.211 ±
289 0.081 APE for histidine to 2.613 ± 0.470 APE for alanine.

290 With both chocolate and puree vectors, dietary ^{15}N IAAs appeared in plasma after 0.5
291 h (**Figure 3A** and **Figure 3B**), and we observed a plateau between 3 and 6 h after
292 ingestion of the first meal. Methionine was the most enriched IAA in blood, and
293 phenylalanine and lysine were the least enriched. ^{15}N enrichments in individual IAAs
294 did not differ between the puree and chocolate vectors, except for methionine, which
295 was more enriched in the SUN+P group (P = 0.02).

296

297

Figure 3. ^{15}N enrichment in plasma IAA for SUN+CHOCO group (A) and SUN+PUREE group (B) and ^{13}C enrichment in plasm
SUN+CHOCO group (C) and SUN+PUREE group (D).



Values are means ± SD. N = 7 for SUN+CHOCO and n = 6 for SUN+PUREE.

298

299 For dietary ^{13}C-IAAs (**Figure 3C** and **Figure 3D**), kinetics displayed different shapes
300 between groups but there was no statistical difference on average (group effect P =
301 0.08) between vectors. Free IAAs appeared and increased rapidly in the SUN+P
302 group with a plateau between 1 h and 4 h, while this occurred between 3 h and 6 h
303 for SUN+C group, as it did for ^{15}N in both groups. For both groups, isoleucine was
304 the most enriched IAA in ^{13}C, and lysine was the least enriched. There was a
305 difference in ^{13}C enrichment between SUN+C and SUN+P groups for methionine

306 only (P = 0.02). AUC was not different between groups for both isotopes (P = 0.28 for
307 ^{15}N and P = 0.90 for ^{13}C for mean AUC), except for ^{13}C methionine AUC, which was
308 double in the SUN+C than in SUN+P group (P = 0.03) (**Table 6**).

309

Table 6. AUC of isotopic IAA enrichment in plasma.

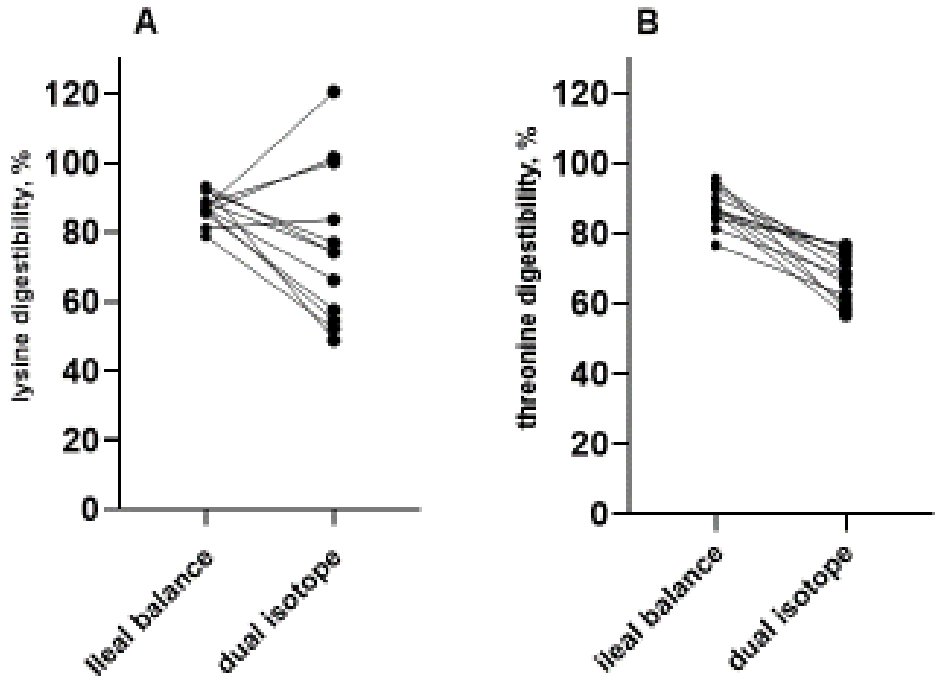
	AUC					
	^{15}N sunflower isolate			^{13}C algal AA		
	SUN+CHOCO	SUN+PUREE	P_{value}	SUN+CHOCO	SUN+PUREE	P_{value}
Valine	0.46 ± 0.09	0.48 ± 0.09	0.76	4.35 ± 0.65	4.04 ± 0.75	0.46
Leucine	0.40 ± 0.08	0.42 ± 0.08	0.60	4.20 ± 0.80	3.80 ± 0.87	0.43
Isoleucine	0.48 ± 0.08	0.50 ± 0.08	0.75	5.27 ± 0.94	4.75 ± 0.82	0.34
Threonine	0.37 ± 0.10	0.39 ± 0.09	0.73	2.64 ± 0.55	2.72 ± 0.51	0.81
Methionine	0.60 ± 0.11	0.69 ± 0.14	0.23	1.05 ± 0.61	2.01 ± 0.71	0.03
Phenylalanine	0.46 ± 0.09	0.47 ± 0.10	0.76	2.94 ± 0.84	3.29 ± 0.95	0.51
Lysine	0.33 ± 0.07	0.36 ± 0.07	0.55	0.80 ± 0.22	0.94 ± 0.23	0.30
Mean	**0.44 ± 0.11**	**0.47 ± 0.13**	**0.28**	**3.03 ± 1.71**	**3.08 ± 1.39**	**0.90**

310 Values are means ± SD. N = 7 for SUN+CHOCO group and n = 5 for SUN+PUREE group.
311 AA: amino acid, AUC: area under the curve.

312 **Digestibility of lysine and threonine determined with the dual isotope method**

313 The digestibility of sunflower lysine and threonine was calculated with the dual
314 isotope method and compared to conventional ileal digestibility. For lysine (**Figure**
315 **4A**), the value obtained with the dual isotope method was lower than ileal digestibility
316 in eight subjects, similar in one, and higher in three subjects, among which one value
317 was aberrant (far above 100 %). Mean lysine digestibility determined with the dual
318 isotope method was 75.9 ± 22.3%, and was not different (P = 0.66) from the value
319 obtained with the ileal balance method due to the high variability. The mean was 12%
320 lower than the ileal digestibility. For threonine **(Figure 4B**), there was a consistent
321 underestimation with the dual isotope method in all subjects, with a mean value of
322 67.7 ± 7.1%, i.e. 10% below the ileal digestibility (P < 0.0001).

Figure 4: Lysine (A) and threonine (B) digestibility obtained with the ileal balance and the dual isotope methods in each subject (n = 12).



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For other AAs that do transaminate, the same trend of an underestimation with the dual isotope method was observed, except for methionine, for which values were aberrant (**Supplementary Table 3**). The average digestibility of IAAs, excluding methionine, obtained with the dual isotope method around 73 %, i.e. 17% below the average ileal digestibility (P = 0.04).

Discussion

This study aimed to measure the true ileal AA digestibility of ^{15}N-labelled sunflower isolate incorporated in a biscuit, using a conventional method by determining the non-absorbed AAs in ileal digesta samples. We also aimed to test values obtained from the dual isotope method in the same protocol. Regarding the constraints of each method, the strategy chosen for the feeding procedure was a 4 h repeated meal, in the view of obtaining an isotopic plateau while allowing a nearly complete digestion over 8 h. ^{13}C-AAs were elected as the reference protein for their theoretical high digestibility. However, they had to be incorporated in separate uncooked matrixes to avoid any Maillard reactions and to limit the risk of kinetic offset. This study is the first to measure sunflower AA digestibility in humans and also pioneeringly addresses validation of the dual isotope method compared to the conventional method.

AA digestibility of sunflower isolate

The digestibility of AA from sunflower isolate, when incorporated into a cooked biscuit, was 86% on average, and the mean digestibility of IAA was close to 90%. Interestingly, the digestibility of IAAs was around 3% higher than that of DAAs glycine and, to a lower extent, proline and serine. This is consistent with data observed in pigs fed sunflower seeds and meals that showed a lower digestibility of glycine (5).

Among IAAs measured, lysine and threonine had the lowest digestibility, which is also in accordance with the lower lysine digestibility reported for canola and sunflower meals in pigs (5). In the present study, the AA digestibility of the protein isolate studied was lower by about 7-8% compared to what we have previously observed in rats with a similar isolate (6). In addition to the fact that protein digestibility is generally higher in rats than in humans (as observed for meat, for instance (13,33)), the nature of the test meal containing the protein isolate (i.e. cooked in biscuits including other ingredients like wheat flour, sugar and starch as well as chocolate chips) may also explain this substantial difference. Moreover, biscuits were ingested together with a vector containing polyphenols, the chocolate vector being of particular note. Dark chocolate contains between 280 and 840 mg/100 g of polyphenol, whereas apple puree contains 100 to 200 mg/100 g (25,26). Polyphenol is known to have a negative effect on protein digestion and digestibility (34,35), which is consistent with the -3 % digestibility we observed between the two groups.

Free ^{13}C-AAs as the reference protein for the dual isotope method

In previous studies (19–21), but not all (18,36,37), ^{13}C-labelled spirulina was used as the reference protein, according to FAO recommendation. Although the equivalence of dual isotope digestibility between ^{13}C-AAs and ^{13}C-spirulina has been demonstrated (22), the use of ^{13}C-labelled spirulina is a source of uncertainties due to its moderate digestibility. Digestibility of < 90% is generally associated to a higher variability among subjects (38). In rats, we found a mean AA digestibility of spirulina of 83% (39). The values ranged from 75 to 92%, illustrating that using a universal value among studies and individuals is a substantial source of error. To date, there is no data on spirulina AA digestibility obtained in humans at the ileal level. Studies

374 determining AA digestibility of spirulina with the dual isotope method have shown
375 interstudy variations with an overall difference of 2.7% among AAs, and even a
376 11.7% difference for lysine (19,22). It is usually assumed that free AAs have a 100%
377 digestibility, and our study provides a value of 98%, on average. This high
378 digestibility, associated to a low variability (1%), supports our hypothesis that using
379 free AAs may be a better option than ^{13}C-labelled spirulina because the values for
380 the ^{13}C reference AAs appear robust and reproducible. Accordingly, this strategy to
381 use 100% digestible, free AAs as the reference was chosen by another team (18,36)
382 to use in patients with pulmonary disorders with healthy subjects as controls. The
383 group from Bengalore also recently used a similar free ^{13}C-AA mixture to determined
384 milk digestibility in healthy Indian women (37).

385 **Influence of the feeding procedure**

386 However, this choice of reference protein had several drawbacks in our experimental
387 conditions because ^{13}C-AAs could not be incorporated directly in the sunflower
388 biscuits, owing to the Maillard reaction that occurs during cooking at 180°C, and to
389 which free AAs are especially sensitive. In other kinds of meals like mashed beans
390 (19) or protein drink (18), free AAs can be easily added to the final product. In our
391 case, a separate matrix was necessary to incorporate them to limit the risk of offset
392 between free ^{13}C and sunflower ^{15}N AA kinetic absorption. We selected two vectors:
393 chocolate because it could entrap the free AAs without any heat damage, and apple
394 puree for its ease of use.

395 The feeding procedures for the dual isotope method differs among studies: either
396 using a bolus (36) and calculating the ratio of AUC of isotope enrichments, as
397 proposed by FAO expert group (17); or repeated small meals over 7 to 8 h to obtain a
398 prolonged isotopic plateau, as developed by the team from Bangalore (19,20); or

399 alternatively, repeated meals during an intermediate time of 4 h to better correspond
400 to normal meal ingestion (40). In all cases, the feeding protocol and the choice of
401 meal tracers is a crucial point of the method.

402 We chose the intermediate feeding procedure to allow for an almost complete
403 digestion during the 8 h of ileal sample collections. The plasma appearance of ^{13}C-
404 AAs differed between the two vectors, being faster with puree than with chocolate.
405 This is not surprising, as we expected that AAs would be trapped in the chocolate
406 texture and thus be released more progressively than when added to the puree.
407 Plasma kinetics differed between the ^{13}C and ^{15}N labelling with a faster apparition of
408 ^{13}C-AAs, especially with the SUN+P group, and a more transient plateau for ^{15}N AAs.
409 This may be due to the fact that, although incorporated in a matrix, free AAs are more
410 rapidly absorbed than protein-bound AAs.

411 Using this protocol, we were able to calculate the digestibility of lysine and threonine
412 as they are not subjected to transamination processes in the body. For these two
413 AAs, as well as other IAAs that transaminate, we found at least a 10% lower
414 digestibility than with the conventional method. This means that under our
415 experimental conditions, plasma appearance of ^{15}N lysine and threonine was
416 underestimated relative to ^{13}C lysine and threonine. Two factors may explain this
417 difference. The first is the kinetic offset between ^{13}C and ^{15}N AAs. It is possible that
418 the AUC calculated for ^{15}N was more underestimated than for ^{13}C. Indeed, the return
419 to the basal level at 8 h seems to be more delayed for ^{15}N than for ^{13}C-AAs.
420 However, calculating the digestibility using the isotopic enrichments at the isotopic
421 plateau instead of AUC gave similar results (not shown). Because the plateau
422 obtained was transient and occurred at different times depending on the tracer and
423 the subjects, the AUC calculation was preferred.

The second factor may be that there was a differential splanchnic extraction of ^{15}N and ^{13}C-AAs. This is plausible, not only due to the kinetic offset, but also to the difference in the amount of AAs from the ^{13}C tracer dose (400 mg) and the sunflower isolate (20 g). It is likely that the low dose, appearing earlier, is more preserved by splanchnic tissues, as the liver is known to extract more AAs when there is a massive AA afflux (41,42).

This suggests that different protocols must be performed to test the adequacy between dual isotope and ileal balance digestibility. From these results, it seems preferable to not separate ^{13}C-AAs from the test meal, when possible, and to give either a bolus, which matches with the typical meal conditions but might result in kinetic offsets, or a prolonged plateau to limit the risk of differential splanchnic extraction, but which requires prolonging the digesta collection over 8 h. In our experimental conditions, the use of ^{13}C-labelled spirulina as the reference protein may have avoided the kinetic offset and resulted in a better adequacy between the two methods.

Additionally, the discrepancy between both methods might have been strengthened by an overestimation of ileal digestibility due to an incomplete recovery of digesta under the condition of 4h repeated meals. On the other hand, ^{15}N recycling in the intestine underestimates digestibility, what has been evaluated as ~ 1-1.5 % (43). Both pitfalls having compensatory effects, at least partially, the discrepancy we observed should mainly originate from the non-optimal dual tracer protocol we used. The obtention of such comparison data on a protein source that has already been evaluated with the dual isotope method and a prolonged plateau approach would have helped to better identify the reason for this difference.

To summarize, our study provides original data on AA sunflower isolate digestibility in humans, showing that IAAs are almost 90% digestible, but that the limiting AA, lysine, is slightly under this value. This study is also the first attempt to validate the dual isotope method against direct ileal digestibility determination. The use of ^{13}C free AAs as the reference protein appeared to be a convenient methodological choice, but caution must be exercised regarding the way they are delivered. As lysine and threonine digestibility was underestimated by about 10% with the dual isotope method, this shows that further methodological investigation, especially regarding the feeding protocol, is necessary to enable internal validation of this method in a single protocol.

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

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