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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

1 **Abstract**

2 Background:

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

7 Objective:

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

11 Methods:

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

21 Results:

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

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

28 Conclusions:

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

33

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

36

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

39 Introduction

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

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

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

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

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

80 **Materials and methods**

81 **Subjects**

82 The eligibility criteria were a BMI between 18 and 30 kg/m², an age between 18 and
83 65 years, a negative serology for HIV, hepatitis C virus antibodies, and hepatitis B
84 virus surface antigens, and the absence of any dietary allergy and digestive disease.

85 The study PRODIGE was conducted in the Human Nutrition Research Center of
86 Avicenne Hospital (APHP, Bobigny, France). It was registered at
87 www.clinicaltrials.gov database (NCT04024605). All subjects provided a written
88 informed consent for inclusion. Data were collected at the UMR PNCA (Paris,
89 France). The study was approved by the Ethical Committee Sud Méditerranée IV (ref
90 180502) and authorized by the Health and Drug French Agency (ANSM, ref
91 2018062100214).

92 The number of volunteers was determined in accordance with previous studies on
93 amino acid digestibility of protein sources (7–16), allowing for external comparison
94 between sunflower isolate and other proteins assessed in the same conditions. It was
95 also calculated to enable a comparison between the ileal balance and the dual
96 isotope methods. The size group was $n = 13$ to reveal a difference of digestibility of 5
97 $\pm 5\%$ in a within subject design (two-tails paired Student test), for a risk $\alpha=5\%$ and a
98 risk $(1-\beta) = 90\%$ (G*Power 3.1). Recruitment started in January 2019 and ended in
99 April 2019. All volunteers signed their informed consent. Nineteen volunteers were
100 recruited and the final sample size was $n=12$ (**Table 1**).

101

102

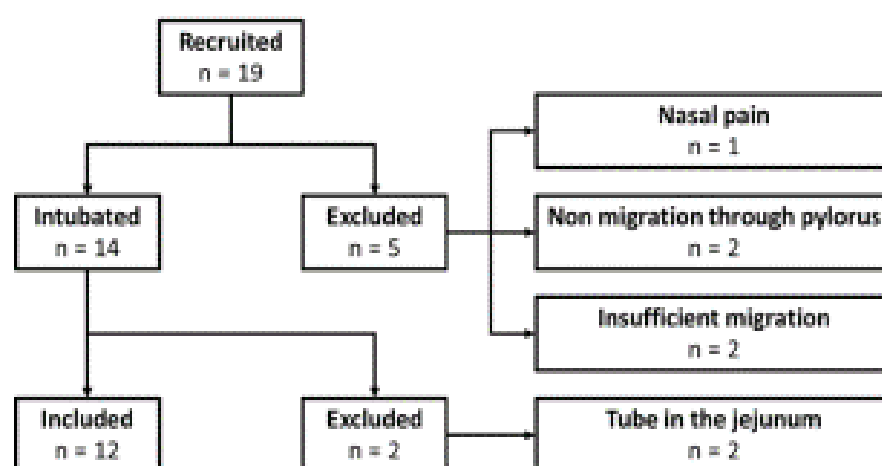
Table 1. Anthropometric characteristics of the subjects

	n = 12
Sex (F/M)	8/4
Age (years)	40.4 ± 10.5
BMI (kg/m ²)	23.7 ± 2.9

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

104

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

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

109

110

111 **Test meals**

112 Sunflower plants were labelled with two applications of ^{15}N -enriched fertilizer during
113 growth (Terres Inovia, Pessac, France). Seeds were dehulled and de-oiled (Olead,
114 Pessac, France), and proteins were extracted and isolated as described previously
115 (6) (UMR LRGP, Nancy, France). The ^{15}N -labelled sunflower protein isolate was
116 incorporated in chocolate chip biscuits (13 g) (Foodinnov, Rennes, France). Biscuits
117 were composed of 17.2% (w/w) of ^{15}N -labelled sunflower isolate as the main protein
118 source (**Supplementary Table 1**). They were cooked at 180°C for 13 min. The wheat
119 flour used in the biscuit also had a small amount of gluten (2.8% of the biscuit). As
120 the ^{13}C -labelled reference protein of known digestibility, algal free ^{13}C -AA mixture
121 (97%) (Eurisotop, Saint-Aubin, France) was preferred to spirulina because of its high
122 digestibility and subsequently low interindividual variability in contrast to spirulina (1).
123 However, free ^{13}C -AAs were not incorporated in the biscuits because they could have
124 been altered by the Maillard reaction during cooking. A separate matrix, a chocolate,
125 was therefore initially chosen as the vector for ^{13}C -AAs to ensure a slow gastric
126 emptying of the AAs in order to obtain a concomitant delivery of ^{15}N , provided by the
127 biscuit, and ^{13}C . Due to the high additional energy and the high polyphenol content of
128 chocolate (between 283 and 510 mg/100 g (25,26)), as well as the possible
129 interaction with protein digestibility, we also decided to test apple puree, a vector that
130 is less caloric and is lower in polyphenol contents. Finally, the chocolate was fed to
131 seven subjects (SUN+C) and the puree to five subjects (SUN+P). Chocolate and
132 puree compositions are given in **Table 2**.

133

134

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.

135

136 The test meal was split into nine portions. The first one was composed of four
137 biscuits, and the other eight were comprised of one biscuit. In total, volunteers
138 ingested 156 g of biscuits, including 26.8 g of ¹⁵N-labelled sunflower isolate.

139 Alongside biscuits, they also ingested chocolate or apple puree for a total amount of
140 400 mg of ¹³C-AA mixture. Volunteers were allowed one glass of water hourly, or half
141 a glass per meal portion. Sunflower isolate contained 14.2% nitrogen (N) and ¹⁵N
142 enrichment was 0.80 atom percent, two times the natural abundance. Biscuits
143 contained 3.5% N, and the ¹⁵N enrichment was 0.73 atom percent. Sunflower isolate
144 was composed of 33.1% IAA and 66.9% dispensable amino acids (DAA) (**Table 3**).

145 Amino acid composition of the algal mixture is presented in **Supplementary Table 2**.

146

147

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

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

149

150 **Clinical protocol**

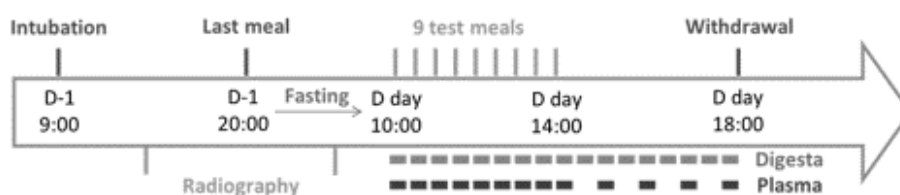
151 One week before the experiment, volunteers followed a standardized diet to achieve
 152 a mean protein intake of 1.3 g.kg of body weight⁻¹.day⁻¹. This quantity of protein
 153 corresponds to the mean consumption of protein in France (27). This diet
 154 standardization was performed to reduce the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

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

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

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

Figure 2. Experimental design



175

176 Analytical methods

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

179 ¹⁵N and ¹³C enrichments in meal, digesta and plasma individual AAs were analyzed
180 by gas chromatography (GC 6890N, Agilent Technologies, Les Ulis, France) coupled
181 to an Isoprime isotope ratio mass spectrometer (Isoprime, GV Instrument,
182 Manchester, UK) via the GC5 Isoprime interface (GC-C-IRMS). Analytical methods
183 for ¹⁵N enrichment determination were previously described (6). For ¹³C enrichment,
184 the combustion furnace temperature was 850°C. The GC column (RXI-17, 30 m long,
185 0.25 µm i.d., 0.5 µm film thickness; Restek) temperature program started at 150°C,
186 rose to 200°C by 4°C/min and then to 270°C by 25°C/min; the final temperature was
187 maintained for 10 min. The inlet temperature was set at 270°C. Plasma samples (2
188 µL) were injected in split mode (2:1) and digestive samples were injected with split
189 10:1.

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

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

202

203 Calculations

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

$$206 \quad F = \frac{[\text{PEG}]_{\text{solution}}}{[\text{PEG}]_{\text{digesta}}} \times \text{perfusion flow rate} \times 30$$

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

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

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

$$212 \quad AA_{\text{ingested } i} = [AA]_{\text{meal } i} \times \text{protein ingested}$$

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

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

$$216 \quad AA_{\text{exo } i}(t) = [AA]_{\text{ileum } i}(t) \times DM(t) \times F(t) \times \frac{APE_{\text{ileum } i}(t)}{APE_{\text{isolate}}}$$

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

223 The same calculation was used for the digestibility of ^{13}C -AAs, except that the APE of
224 ^{13}C -AAs (i.e. 98.3 % as given by the supplier) was used instead of APE_{isolate} .

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

$$226 \quad \text{AA ileal digestibility}_i = 1 - \left(\frac{\sum \text{AA}_{\text{exo } i}}{\text{AA}_{\text{ingested } i}} \times 100 \right)$$

227 where $\sum \text{AA}_{\text{exo } i}$ for each AA “i” is the sum of exogenous AA over 8 h (mmol), and
 228 $\text{AA}_{\text{ingested } i}$ is the quantity of AA “i” from sunflower isolate for ^{15}N and the tracer dose
 229 for ^{13}C , respectively, that was ingested by the volunteer (mmol).

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

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

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

$$234 \quad \text{Meal ratio } i = \frac{^{15}\text{N}_{\text{meal } i}}{^{13}\text{C}_{\text{meal } i}}$$

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

$$238 \quad \text{Plasma AUC } i = \frac{\text{AUC } ^{15}\text{N}_i}{\text{AUC } ^{13}\text{C}_i}$$

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

$$241 \quad \text{IAA}_{\text{absorption plasma } i} = ^{13}\text{C AA digestibility}_i \times \frac{\text{Plasma AUC } i}{\text{Meal ratio } i}$$

242 where $^{13}\text{C AA digestibility } i$ is the ileal ^{13}C digestibility of the AA “i”, determined by
 243 analysis of digestive content.

244 **Statistical analysis**

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

254

255 **Results**

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

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

266

267

Table 4. Amino acid ileal digestibility of ¹⁵N 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

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

272

273 Amino acid digestibility of the reference ¹³C-free AA mixture

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

276 digestibility values ranged in the same range but phenylalanine was the most
 277 digestible. There was no statistical difference between IAA and DAA digestibility
 278 values ($P = 0.83$ for SUN+C and $P = 0.98$ for SUN+P). There was also no difference
 279 between chocolate and puree for mean ($P = 0.87$) and individual AA digestibility.
 280

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

281 Values are means \pm SD. In the SUN+CHOCO group, $n = 3$ for serine, $n = 4$ for threonine and
 282 $n = 7$ 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 ¹⁵N and ¹³C AA**

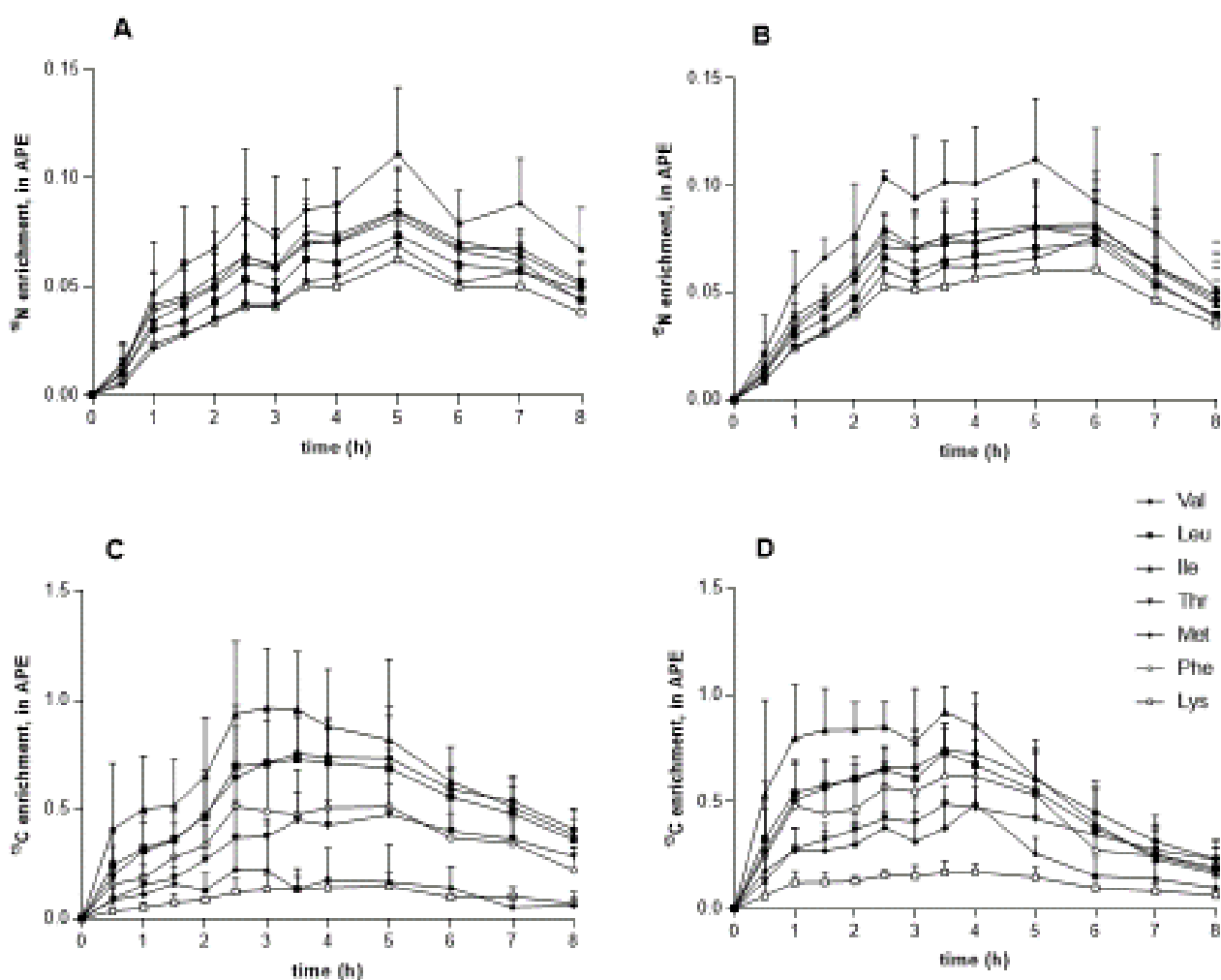
287 ¹⁵N enrichment in the meal IAA ranged from 0.281 ± 0.07 APE for histidine and 0.379
288 ± 0.031 APE for lysine (**Supplementary figure 1**). For ¹³C, it ranged from 0.211 ±
289 0.081 APE for histidine to 2.613 ± 0.470 APE for alanine.

290 With both chocolate and puree vectors, dietary ¹⁵N 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. ¹⁵N 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 plasma IAA for SUN+CHOCO group (C) and SUN+PUREE group (D).



Values are means \pm 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

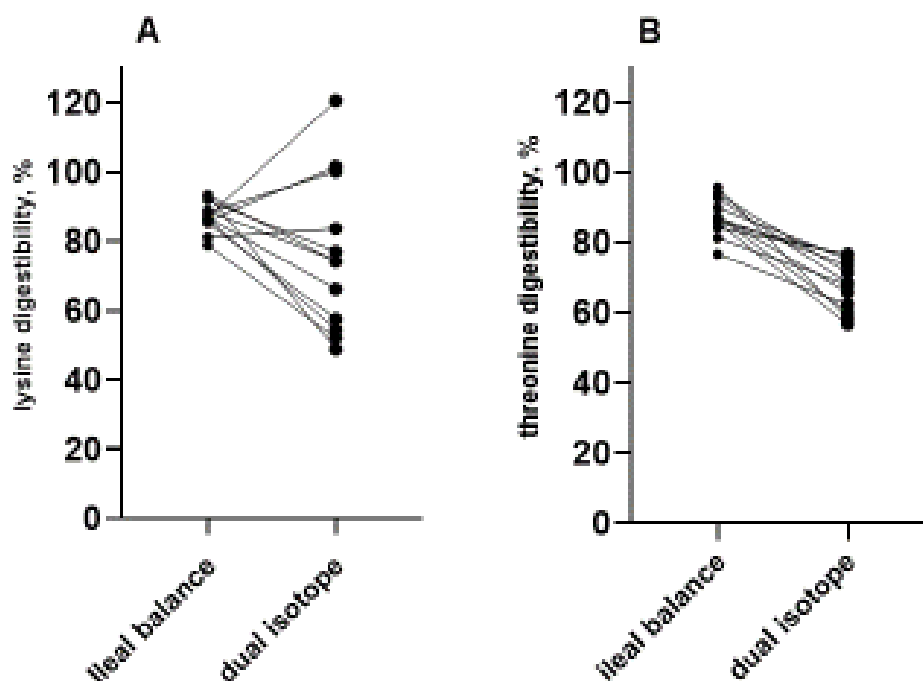
310 Values are means \pm SD. $N = 7$ for SUN+CHOCO group and $n = 5$ for SUN+PUREE group.

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 $75.9 \pm 22.3\%$, 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 $67.7 \pm 7.1\%$, 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).



323

324

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

330

331 **Discussion**

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

343 **AA digestibility of sunflower isolate**

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

349 Among IAAs measured, lysine and threonine had the lowest digestibility, which is
350 also in accordance with the lower lysine digestibility reported for canola and
351 sunflower meals in pigs (5). In the present study, the AA digestibility of the protein
352 isolate studied was lower by about 7-8% compared to what we have previously
353 observed in rats with a similar isolate (6). In addition to the fact that protein
354 digestibility is generally higher in rats than in humans (as observed for meat, for
355 instance (13,33)), the nature of the test meal containing the protein isolate (i.e.
356 cooked in biscuits including other ingredients like wheat flour, sugar and starch as
357 well as chocolate chips) may also explain this substantial difference. Moreover,
358 biscuits were ingested together with a vector containing polyphenols, the chocolate
359 vector being of particular note. Dark chocolate contains between 280 and 840
360 mg/100 g of polyphenol, whereas apple puree contains 100 to 200 mg/100 g (25,26).
361 Polyphenol is known to have a negative effect on protein digestion and digestibility
362 (34,35), which is consistent with the -3 % digestibility we observed between the two
363 groups.

364 **Free ¹³C-AAs as the reference protein for the dual isotope method**

365 In previous studies (19–21), but not all (18,36,37), ¹³C-labelled spirulina was used as
366 the reference protein, according to FAO recommendation. Although the equivalence
367 of dual isotope digestibility between ¹³C-AAs and ¹³C-spirulina has been
368 demonstrated (22), the use of ¹³C-labelled spirulina is a source of uncertainties due
369 to its moderate digestibility. Digestibility of < 90% is generally associated to a higher
370 variability among subjects (38). In rats, we found a mean AA digestibility of spirulina
371 of 83% (39). The values ranged from 75 to 92%, illustrating that using a universal
372 value among studies and individuals is a substantial source of error. To date, there is
373 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 Bangalore 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.

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

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

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

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

458

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463

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465 conducted the research; RK, AQ, OG and RB: provided essential materials; RT, JC
466 and CG: analyzed data; RT, JC and CG: wrote the paper; RT, CG: had primary
467 responsibility for final content; and all authors: read, modified and approved the final
468 manuscript.

469

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

Bibliography

1. Liu JD, Li QY, Zeng ZK, Li P, Xu X, Wang HL, Zhang S, Piao XS. Determination and Prediction of the Amino Acid Digestibility of Sunflower Seed Meals in Growing Pigs. *Asian-Australas J Anim Sci* 2014;28:86–94.
2. Janicki J, Sobkowska E, Warchalewski J, Nowakowska K, Chełkowski J, Stasińska B. Amino Acid Composition of Cereal and Oilseed. *Food Nahr* 1973;17:359–65.
3. Nørgaard JV, Fernández JA, Jørgensen H. Ileal digestibility of sunflower meal, pea, rapeseed cake, and lupine in pigs. *J Anim Sci* 2012;90:203–5.
4. Almeida FN, Htoo JK, Thomson J, Stein HH. Digestibility by growing pigs of amino acids in heat-damaged sunflower meal and cottonseed meal. *J Anim Sci* 2014;92:585–93.
5. González-Vega JC, Stein HH. Amino acid digestibility in canola, cottonseed, and sunflower products fed to finishing pigs. *J Anim Sci* 2012;90:4391–400.
6. Tessier R, Khodorova N, Calvez J, Kapel R, Quinsac A, Piedcoq J, Tomé D, Gaudichon C. ¹⁵N and ²H Intrinsic Labeling Demonstrate That Real Digestibility in Rats of Proteins and Amino Acids from Sunflower Protein Isolate Is Almost as High as That of Goat Whey. *J Nutr* 2020;150:450–7.
7. Bos C, Mahé S, Gaudichon C, Benamouzig R, Gausserès N, Luengo C, Ferrière F, Rautureau J, Tomé D. Assessment of net postprandial protein utilization of ¹⁵N-labelled milk nitrogen in human subjects. *Br J Nutr* 1999;81:221–6.
8. Gaudichon C, Mahé S, Benamouzig R, Luengo C, Fouillet H, Daré S, Van Oycke M, Ferrière F, Rautureau J, Tomé D. Net Postprandial Utilization of [¹⁵N]-Labeled Milk Protein Nitrogen Is Influenced by Diet Composition in Humans. *J Nutr* 1999;129:890–5.
9. Mariotti F, Mahé S, Benamouzig R, Luengo C, Daré S, Gaudichon C, Tomé D. Nutritional Value of [¹⁵N]-Soy Protein Isolate Assessed from Ileal Digestibility and Postprandial Protein Utilization in Humans. *J Nutr* 1999;129:1992–7.
10. Gaudichon C, Bos C, Morens C, Petzke KJ, Mariotti F, Everwand J, Benamouzig R, Daré S, Tomé D, Metges CC. Ileal losses of nitrogen and amino acids in humans and their importance to the assessment of amino acid requirements. *Gastroenterology* 2002;123:50–9.
11. Bos C, Juillet B, Fouillet H, Turlan L, Daré S, Luengo C, N'tounda R, Benamouzig R, Gausserès N, Tomé D, et al. Postprandial metabolic utilization of wheat protein in humans. *Am J Clin Nutr* 2005;81:87–94.
12. Bos C, Airinei G, Mariotti F, Benamouzig R, Bérot S, Evrard J, Fénart E, Tomé D, Gaudichon C. The Poor Digestibility of Rapeseed Protein Is Balanced by Its Very High Metabolic Utilization in Humans. *J Nutr* 2007;137:594–600.

13. Oberli M, Marsset-Baglieri A, Airinei G, Santé-Lhoutellier V, Khodorova N, Rémond D, Foucault-Simonin A, Piedcoq J, Tomé D, Fromentin G, et al. High True Ileal Digestibility but Not Postprandial Utilization of Nitrogen from Bovine Meat Protein in Humans Is Moderately Decreased by High-Temperature, Long-Duration Cooking. *J Nutr* 2015;145:2221–8.
14. Calvez J, Benoit S, Piedcoq J, Khodorova N, Azzout-Marniche D, Tomé D, Benamouzig R, Airinei G, Gaudichon C. Very low ileal nitrogen and amino acid digestibility of zein compared to whey protein isolate in healthy volunteers. *Am J Clin Nutr* 2021;113:70–82.
15. Mariotti F, Pueyo ME, Tomé D, Bérot S, Benamouzig R, Mahé S. The Influence of the Albumin Fraction on the Bioavailability and Postprandial Utilization of Pea Protein Given Selectively to Humans. *J Nutr* 2001;131:1706–13.
16. Mariotti F, Pueyo ME, Tomé D, Mahé S. The bioavailability and postprandial utilisation of sweet lupin (*Lupinus albus*)-flour protein is similar to that of purified soyabean protein in human subjects: a study using intrinsically N-labelled proteins. *Br J Nutr* 2002;87:315.
17. FAO Expert Working Group. Research approaches and methods for evaluating the protein quality of human foods [Internet]. 2014. Available from: <http://www.fao.org/3/a-i4325e.pdf>
18. Engelen MPKJ, Com G, Anderson PJ, Deutz NEP. New stable isotope method to measure protein digestibility and response to pancreatic enzyme intake in cystic fibrosis. *Clin Nutr* 2014;33:1024–32.
19. Devi S, Varkey A, Sheshshayee MS, Preston T, Kurpad AV. Measurement of protein digestibility in humans by a dual-tracer method. *Am J Clin Nutr* 2018;107:984–91.
20. Kashyap S, Shivakumar N, Varkey A, Duraisamy R, Thomas T, Preston T, Devi S, Kurpad AV. Ileal digestibility of intrinsically labeled hen's egg and meat protein determined with the dual stable isotope tracer method in Indian adults. *Am J Clin Nutr* 2018;108:980–7.
21. Kashyap S, Varkey A, Shivakumar N, Devi S, Reddy B H R, Thomas T, Preston T, Sreeman S, Kurpad AV. True ileal digestibility of legumes determined by dual-isotope tracer method in Indian adults. *Am J Clin Nutr* 2019;110:873–82.
22. Kashyap S, Shivakumar N, Varkey A, Preston T, Devi S, Kurpad AV. Co-ingestion of Black Tea Reduces the Indispensable Amino Acid Digestibility of Hens' Egg in Indian Adults. *J Nutr* 2019;149:1363–8.
23. Devi S, Varkey A, Dharmar M, Holt RR, Allen LH, Sheshshayee MS, Preston T, Keen CL, Kurpad AV. Amino Acid Digestibility of Extruded Chickpea and Yellow Pea Protein is High and Comparable in Moderately Stunted South Indian Children with Use of a Dual Stable Isotope Tracer Method. *J Nutr* 2020;150:1178–85.

24. Shivakumar N, Kashyap S, Kishore S, Thomas T, Varkey A, Devi S, Preston T, Jahoor F, Sheshshayee M, Kurpad AV. Protein-quality evaluation of complementary foods in Indian children. *Am J Clin Nutr* 2019;109:1319–27.
25. Ovaskainen M-L, Törrönen R, Koponen JM, Sinkko H, Hellström J, Reinivuo H, Mattila P. Dietary Intake and Major Food Sources of Polyphenols in Finnish Adults. *J Nutr* 2008;138:562–6.
26. Scalbert A, Williamson G. Dietary Intake and Bioavailability of Polyphenols. *J Nutr* 2000;130:2073S-2085S.
27. Afssa. Apport en protéines: consommation, qualité, besoins et recommandations [Internet]. Agence française de sécurité sanitaire des aliments; 2007 p. 461. Available from: <https://www.anses.fr/fr/system/files/NUT-Ra-Proteines.pdf>
28. Russell RW, Mc Gilliard AD, Berger PJ, Young JW. Evaluation of Turbidimetric Determination of Polyethylene Glycol. *J Dairy Sci* 1982;65:1798–803.
29. Fromentin C, Sanders P, Nau F, Anton M, Fromentin G, Tomé D, Thibault J-N, Gaudichon C. A pilot study for the intrinsic labeling of egg proteins with ¹⁵N and ¹³C. *Rapid Commun Mass Spectrom* 2012;26:43–8.
30. Guillin FM, Gaudichon C, Guérin-Deremaux L, Lefranc-Millot C, Airinei G, Khodorova N, Benamouzig R, Pomport P-H, Martin J, Calvez J. Real ileal amino acid digestibility of pea protein compared to casein in healthy humans, a randomized trial. *Am J Clin Nutr* 2021;
31. Rutherford SM, Gilani GS. Amino Acid Analysis. *Curr Protoc Protein Sci* John Wiley & Sons, Ltd; 2009;58:11.9.1-11.9.37.
32. Rutherford SM, Dunn BM. Quantitative Amino Acid Analysis. *Curr Protoc Protein Sci* John Wiley & Sons, Ltd; 2011;63:3.2.1-3.2.6.
33. Oberli M, Lan A, Khodorova N, Santé-Lhoutellier V, Walker F, Piedcoq J, Davila A-M, Blachier F, Tomé D, Fromentin G, et al. Compared with Raw Bovine Meat, Boiling but Not Grilling, Barbecuing, or Roasting Decreases Protein Digestibility without Any Major Consequences for Intestinal Mucosa in Rats, although the Daily Ingestion of Bovine Meat Induces Histologic Modifications in the Colon. *J Nutr* 2016;146:1506–13.
34. Gilani GS, Wu Xiao C, Cockell KA. Impact of Antinutritional Factors in Food Proteins on the Digestibility of Protein and the Bioavailability of Amino Acids and on Protein Quality. *Br J Nutr* 2012;108:S315–32.
35. Peace RW, Sarwar G, Touchburn SP, Botting HG. Effects of soybean trypsin inhibitors and dl-methionine on growth and serum parameters in young rats. *Nutr Res* 1991;11:1197–208.
36. Kirschner SK, Deutz NEP, Jonker R, Olde Damink SWM, Harrykissoon RI, Zachria AJ, Dasarathy S, Engelen MPKJ. Intestinal function is impaired in patients with Chronic Obstructive Pulmonary Disease. *Clin Nutr* 2021;40:2270–7.

37. Kashyap S, Shivakumar N, Sejian V, Deutz NE, Preston T, Sreeman S, Devi S, Kurpad AV. Goat milk protein digestibility in relation to intestinal function. *Am J Clin Nutr* 2021;113:845–53.
38. Gaudichon C, Calvez J. Determinants of amino acid bioavailability from ingested protein in relation to gut health. *Curr Opin Clin Nutr Metab Care* 2021;24:55–61.
39. Tessier R, Calvez J, Khodorova N, Gaudichon C. Protein and amino acid digestibility of ¹⁵N Spirulina in rats. *Eur J Nutr* 2021;60:2263–9.
40. van der Wielen N, Khodorova NV, Gerrits WJJ, Gaudichon C, Calvez J, Tomé D, Mensink M. Blood 15N:13C Enrichment Ratios Are Proportional to the Ingested Quantity of Protein with the Dual-Tracer Approach for Determining Amino Acid Bioavailability in Humans. *J Nutr* 2020;150:2346–52.
41. Morens C, Gaudichon C, Fromentin G, Marsset-Baglieri A, Bensaïd A, Larue-Achagiotis C, Luengo C, Tomé D. Daily delivery of dietary nitrogen to the periphery is stable in rats adapted to increased protein intake. *Am J Physiol-Endocrinol Metab* 2001;281:E826–36.
42. Gorissen SH, Horstman AM, Franssen R, Kouw IW, Wall BT, Burd NA, de Groot LC, van Loon LJ. Habituation to low or high protein intake does not modulate basal or postprandial muscle protein synthesis rates: a randomized trial. *Am J Clin Nutr* 2017;105:332–42.
43. Deglaire A, Moughan PJ, Airinei G, Benamouzig R, Tomé D. Intact and hydrolyzed casein lead to similar ileal endogenous protein and amino acid flows in adult humans. *Am J Clin Nutr* 2020;111:90–7.