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**The true amino acid digestibility of <sup>15</sup>N-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  $^{15}\text{N}$  protein isolate, in comparison with the dual  
10 isotope method.

### 11 Methods:

12 Twelve healthy volunteers (men and women,  $40.4 \pm 10.5$  years old, BMI  $23.7 \pm 2.9$   
13  $\text{kg/m}^2$ ) were equipped with a naso-ileal tube. They consumed for 4h nine repeated  
14 meals comprising  $^{15}\text{N}$ -sunflower protein biscuits together with  $^{13}\text{C}$ -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  $^{15}\text{N}$  and  $^{13}\text{C}$  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  $^{13}\text{C}$ -AA ileal digestibility was  $98.1 \pm 0.9\%$ .  
25 No matter which matrix was used to carry  $^{13}\text{C}$ -AAs, plasma  $^{15}\text{N}$  and  $^{13}\text{C}$ -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](http://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  $^{15}\text{N}$  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  $^2\text{H}$ , together with a  $^{13}\text{C}$   
59 reference protein of known digestibility (17).  $^{15}\text{N}$  was not proposed because of  
60 transaminations, but  $^{15}\text{N}$  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  $^{15}\text{N}$ -labelled spirulina as the test

63 protein and free  $^2\text{H}$  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  $^2\text{H}$ -labelled protein  
67 sources, using  $^{13}\text{C}$ -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  $^{15}\text{N}$ -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/m<sup>2</sup>, 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](http://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



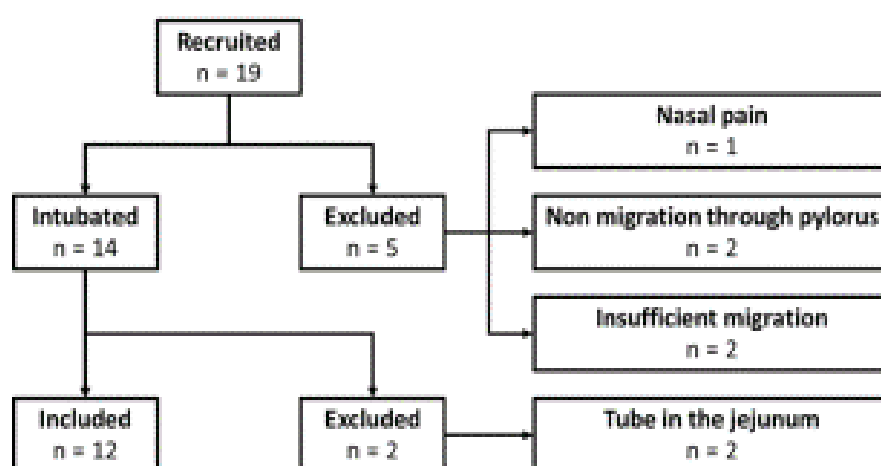
**Table 1.** Anthropometric characteristics of the subjects

	n = 12
Sex (F/M)	8/4
Age (years)	40.4 ± 10.5
BMI (kg/m <sup>2</sup> )	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  $^{15}\text{N}$ -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  $^{15}\text{N}$ -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  $^{15}\text{N}$ -labelled sunflower isolate as the main protein  
118 source (**Supplementary Table 1**). They were cooked at  $180^\circ\text{C}$  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  $^{13}\text{C}$ -labelled reference protein of known digestibility, algal free  $^{13}\text{C}$ -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  $^{13}\text{C}$ -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  $^{13}\text{C}$ -AAs to ensure a slow gastric  
126 emptying of the AAs in order to obtain a concomitant delivery of  $^{15}\text{N}$ , provided by the  
127 biscuit, and  $^{13}\text{C}$ . 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) <sup>1</sup>	7.3	1.0

<sup>1</sup> 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 <sup>15</sup>N-labelled sunflower isolate.

139 Alongside biscuits, they also ingested chocolate or apple puree for a total amount of  
140 400 mg of <sup>13</sup>C-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 <sup>15</sup>N  
142 enrichment was 0.80 atom percent, two times the natural abundance. Biscuits  
143 contained 3.5% N, and the <sup>15</sup>N 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**

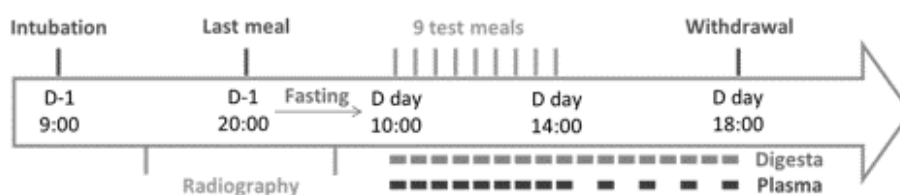
151 One week before the experiment, volunteers followed a standardized diet to achieve  
 152 a mean protein intake of 1.3 g.kg of body weight<sup>-1</sup>.day<sup>-1</sup>. This quantity of protein  
 153 corresponds to the mean consumption of protein in France (27). This diet  
 154 standardization 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  $^{15}\text{N}$  and  $^{13}\text{C}$  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  $^{15}\text{N}$  enrichment determination were previously described (6). For  $^{13}\text{C}$  enrichment,  
184 the combustion furnace temperature was  $850^{\circ}\text{C}$ . The GC column (RXI-17, 30 m long,  
185  $0.25\ \mu\text{m}$  i.d.,  $0.5\ \mu\text{m}$  film thickness; Restek) temperature program started at  $150^{\circ}\text{C}$ ,  
186 rose to  $200^{\circ}\text{C}$  by  $4^{\circ}\text{C}/\text{min}$  and then to  $270^{\circ}\text{C}$  by  $25^{\circ}\text{C}/\text{min}$ ; the final temperature was  
187 maintained for 10 min. The inlet temperature was set at  $270^{\circ}\text{C}$ . Plasma samples ( $2\ \mu\text{L}$ )  
188 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  $110^{\circ}\text{C}$ . 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  $^{15}\text{N}$  and  $APE_{\text{isolate}}$  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  $^{13}\text{C}$ -AAs, except that the APE of  
224  $^{13}\text{C}$ -AAs (i.e. 98.3 % as given by the supplier) was used instead of  $APE_{\text{isolate}}$ .

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  $^{15}\text{N}$  and the tracer dose  
 229 for  $^{13}\text{C}$ , 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  $^{13}\text{C}$ -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  $^{13}\text{C}$  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.  $^{13}\text{C}$  or  $^{15}\text{N}$  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  $P_{\text{value}}$  inferior to 0.05.

254

## 255 **Results**

### 256 **Ileal AA digestibility of $^{15}\text{N}$ -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 <sup>15</sup>N sunflower isolate

	SUN+CHOCO	SUN+PUREE	P <sub>value</sub>	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
<b>Mean IAA</b>	<b>88.5 ± 5.0</b>	<b>91.5 ± 5.4</b>	<b>&lt; 0.05</b>	<b>89.8 ± 5.3</b>
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
<b>Mean DAA</b>	<b>82.5 ± 10.6</b>	<b>85.4 ± 9.8</b>	<b>0.30</b>	<b>83.8 ± 10.3</b>
<b>Mean (all AA)</b>	<b>85.2 ± 4.7</b>	<b>88.2 ± 5.6</b>	<b>0.34</b>	<b>86.5 ± 5.1</b>

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 <sup>13</sup>C-free AA mixture

274 For chocolate, ileal digestibility of <sup>13</sup>C AAs values varied from around 95 % for  
 275 tyrosine to 99 % for alanine (**Table 5**). When incorporated in puree, ileal <sup>13</sup>C-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  $^{13}\text{C}$  algal free amino acids.

$^{13}\text{C}$ algal AA digestibility				
	SUN+CHOCO	SUN+PUREE	$P_{\text{value}}$	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
<b>Mean IAA</b>	<b>97.6 ± 1.7</b>	<b>97.6 ± 1.8</b>	<b>0.95</b>	<b>97.6 ± 1.7</b>
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
<b>Mean DAA</b>	<b>97.7 ± 1.9</b>	<b>97.6 ± 1.8</b>	<b>0.87</b>	<b>97.7 ± 1.8</b>
<b>Mean (all AA)</b>	<b>98.1 ± 1.0</b>	<b>98.0 ± 0.9</b>	<b>0.87</b>	<b>98.1 ± 0.9</b>

281 Values are means ± 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 <sup>15</sup>N and <sup>13</sup>C AA**

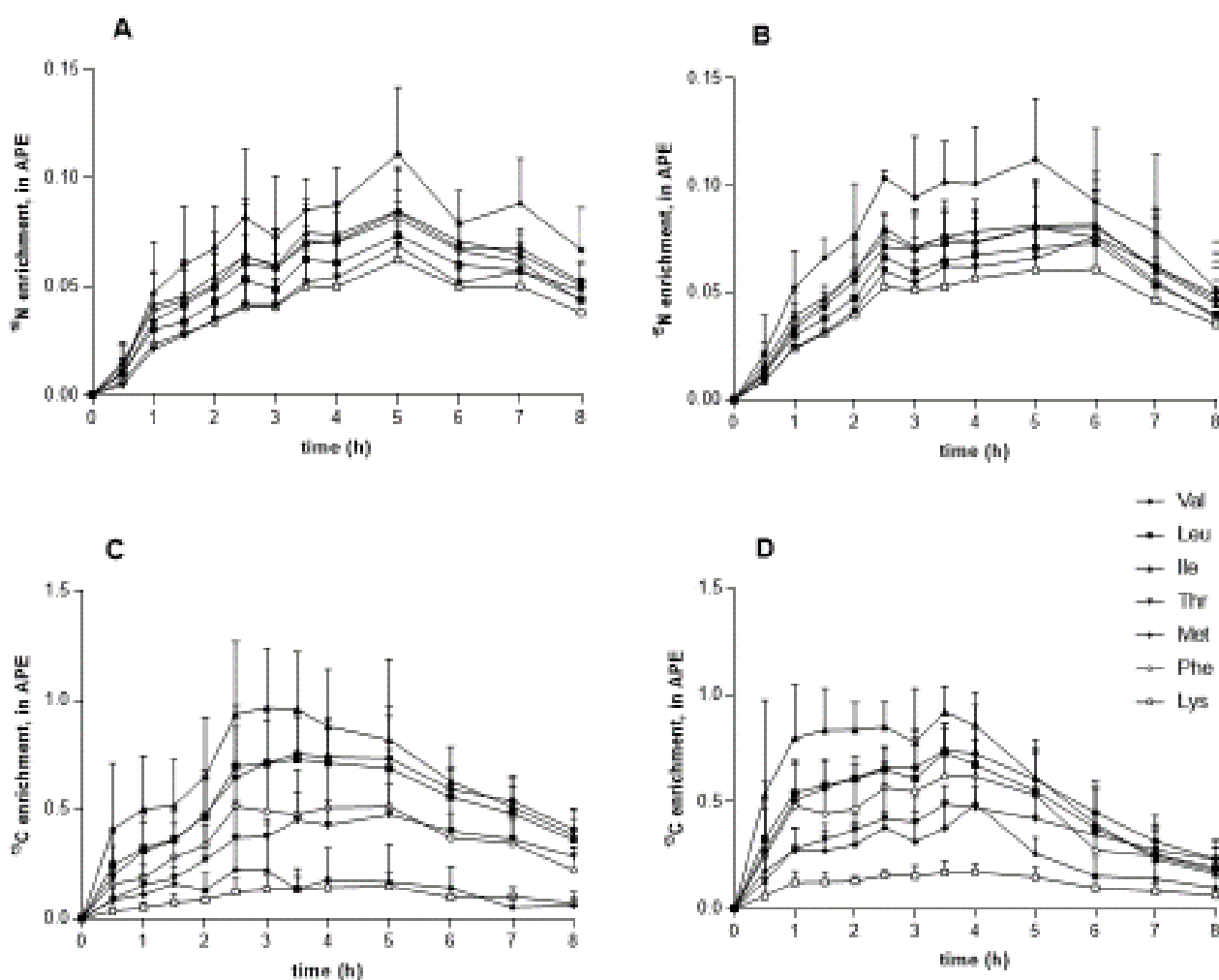
287 <sup>15</sup>N enrichment in the meal IAA ranged from 0.281 ± 0.07 APE for histidine and 0.379  
288 ± 0.031 APE for lysine (**Supplementary figure 1**). For <sup>13</sup>C, it ranged from 0.211 ±  
289 0.081 APE for histidine to 2.613 ± 0.470 APE for alanine.

290 With both chocolate and puree vectors, dietary <sup>15</sup>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. <sup>15</sup>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.  $^{15}\text{N}$  enrichment in plasma IAA for SUN+CHOCO group (A) and SUN+PUREE group (B) and  $^{13}\text{C}$  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  $^{13}\text{C}$ -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  $^{15}\text{N}$  in both groups. For both groups, isoleucine was

304 the most enriched IAA in  $^{13}\text{C}$ , and lysine was the least enriched. There was a

305 difference in  $^{13}\text{C}$  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  $^{15}\text{N}$  and  $P = 0.90$  for  $^{13}\text{C}$  for mean AUC), except for  $^{13}\text{C}$  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						
$^{15}\text{N}$ sunflower isolate				$^{13}\text{C}$ algal AA		
	SUN+CHOCO	SUN+PUREE	$P_{\text{value}}$	SUN+CHOCO	SUN+PUREE	$P_{\text{value}}$
Valine	$0.46 \pm 0.09$	$0.48 \pm 0.09$	0.76	$4.35 \pm 0.65$	$4.04 \pm 0.75$	0.46
Leucine	$0.40 \pm 0.08$	$0.42 \pm 0.08$	0.60	$4.20 \pm 0.80$	$3.80 \pm 0.87$	0.43
Isoleucine	$0.48 \pm 0.08$	$0.50 \pm 0.08$	0.75	$5.27 \pm 0.94$	$4.75 \pm 0.82$	0.34
Threonine	$0.37 \pm 0.10$	$0.39 \pm 0.09$	0.73	$2.64 \pm 0.55$	$2.72 \pm 0.51$	0.81
Methionine	$0.60 \pm 0.11$	$0.69 \pm 0.14$	0.23	$1.05 \pm 0.61$	$2.01 \pm 0.71$	0.03
Phenylalanine	$0.46 \pm 0.09$	$0.47 \pm 0.10$	0.76	$2.94 \pm 0.84$	$3.29 \pm 0.95$	0.51
Lysine	$0.33 \pm 0.07$	$0.36 \pm 0.07$	0.55	$0.80 \pm 0.22$	$0.94 \pm 0.23$	0.30
<b>Mean</b>	<b><math>0.44 \pm 0.11</math></b>	<b><math>0.47 \pm 0.13</math></b>	<b>0.28</b>	<b><math>3.03 \pm 1.71</math></b>	<b><math>3.08 \pm 1.39</math></b>	<b>0.90</b>

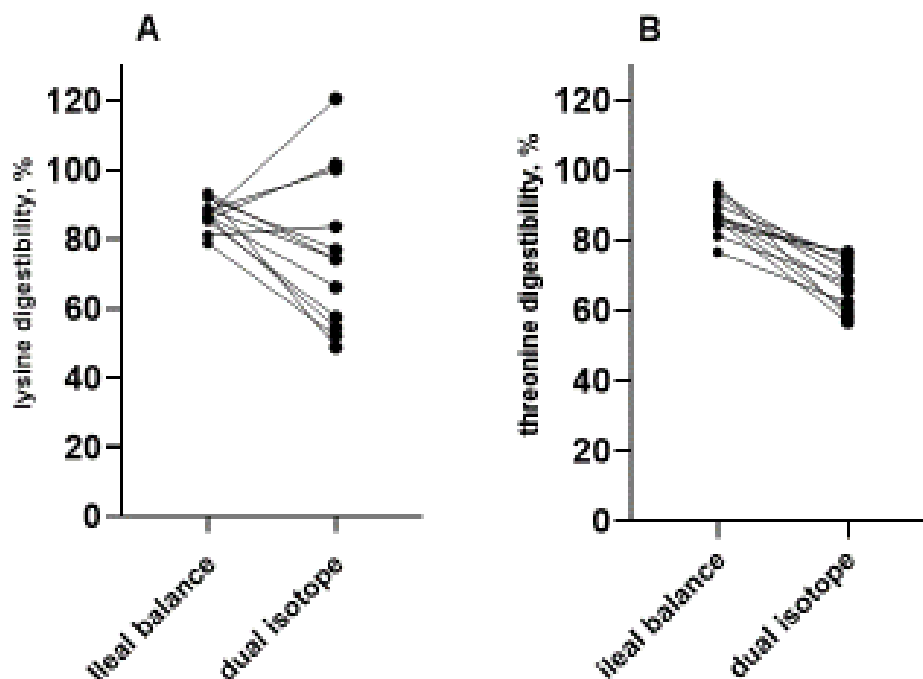
310 Values are means  $\pm$  SD.  $N = 7$  for SUN+CHOCO group and  $n = 5$  for SUN+PUREE group.

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  $^{15}\text{N}$ -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.  $^{13}\text{C}$ -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 <sup>13</sup>C-AAs as the reference protein for the dual isotope method**

365 In previous studies (19–21), but not all (18,36,37), <sup>13</sup>C-labelled spirulina was used as  
366 the reference protein, according to FAO recommendation. Although the equivalence  
367 of dual isotope digestibility between <sup>13</sup>C-AAs and <sup>13</sup>C-spirulina has been  
368 demonstrated (22), the use of <sup>13</sup>C-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  $^{13}\text{C}$ -labelled spirulina because the values for  
380 the  $^{13}\text{C}$  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  $^{13}\text{C}$ -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  $^{13}\text{C}$ -AAs could not be incorporated directly in the sunflower  
388 biscuits, owing to the Maillard reaction that occurs during cooking at  $180^{\circ}\text{C}$ , 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  $^{13}\text{C}$  and sunflower  $^{15}\text{N}$  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  $^{13}\text{C}$ -  
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  $^{13}\text{C}$  and  $^{15}\text{N}$  labelling with a faster apparition of  
408  $^{13}\text{C}$ -AAs, especially with the SUN+P group, and a more transient plateau for  $^{15}\text{N}$  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  $^{15}\text{N}$  lysine and threonine was  
416 underestimated relative to  $^{13}\text{C}$  lysine and threonine. Two factors may explain this  
417 difference. The first is the kinetic offset between  $^{13}\text{C}$  and  $^{15}\text{N}$  AAs. It is possible that  
418 the AUC calculated for  $^{15}\text{N}$  was more underestimated than for  $^{13}\text{C}$ . Indeed, the return  
419 to the basal level at 8 h seems to be more delayed for  $^{15}\text{N}$  than for  $^{13}\text{C}$ -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  $^{15}\text{N}$   
425 and  $^{13}\text{C}$ -AAs. This is plausible, not only due to the kinetic offset, but also to the  
426 difference in the amount of AAs from the  $^{13}\text{C}$  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  $^{13}\text{C}$ -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  $^{13}\text{C}$ -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,  $^{15}\text{N}$  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  $^{13}\text{C}$  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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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.

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