Influence of protein-phenolic complex on the antioxidant capacity of flaxseed (*Linum usitatissimum* L.) products

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Abstract

The impact of the naturally-present phenolic compounds and/or proteins on the antioxidant capacity of flaxseed products- phenolic fraction, protein concentrates and hydrolysates- before and after simulated gastrointestinal digestion was studied. For that, whole and phenolic reduced products were assessed. Four glycosylated phenolic compounds – secoisolariciresinol and ferulic, p-coumaric and caffeic acids – were identified in flaxseed products. Phenolic fraction exerts the highest antioxidant capacity that increased by alkaline hydrolysis and by simulated gastrointestinal digestion. The action of Alcalase® and digestive enzymes resulted in an increase of the antioxidant capacity of whole and phenolic reduced products. Principal component analysis showed that proteinaceous samples act as antioxidant is by H+ transfer, while those samples containing phenolic compounds exert their effects by both electron donation and H+ transfer mechanisms. Protein/peptide-phenolic complexation, confirmed by fluorescence spectra, exerted a positive effect on the antioxidant capacity, mainly in protein concentrates.

Keywords

Flaxseed, antioxidant capacity, protein, phenolic compounds, enzymatic hydrolysis, simulated gastrointestinal digestion

20 **Introduction**

21 Several studies have shown the antioxidant potential of peptides released from 22 enzymatic hydrolysis of different protein sources.[1-3] This source of antioxidants has attractive 23 characteristics for the food industry, once it is non-toxic and has recognized nutritional value.[1] 24 In food industry, peptides can be an alternative to the use of synthetic antioxidants, preventing 25 lipid peroxidation and maintaining the sensory characteristics of the products.[1] In the human 26 body, they may assist the antioxidant defense system in the prevention or deceleration of the 27 progression of oxidative stress-associated diseases.[4,5]

28 It has been reported that procedures used to obtain protein plant protein isolates can 29 also facilitate the extraction of polyphenols.[6,7] After enzymatic hydrolysis, both released 30 peptides and phenolic compounds might be responsible for the antioxidant activity of 31 hydrolysates.[1,8] In this respect, the antioxidant activity of plant-derived matrices has been 32 associated with protein components, phenolic compounds and their complexes, although few 33 studies on protein hydrolysates have considered the presence and contribution of phenolic 34 compounds.[9]

35 Flaxseed is an oilseed widely studied for its beneficial health effects. It is a source of 36 alpha linolenic fatty acids, phenolic compounds and soluble fiber, has anti-inflammatory and 37 antioxidant capacities, and has been related to reduce risk of chronic diseases such as cancer, 38 obesity, and diabetes.[10,11] Flaxseed is the richest source of plant lignans, due to its high 39 content of secoisolariciresinol diglucoside (SDG).[12] In this seed, SDG along with non-lignan 40 phenolic compounds, such as ferulic, p-coumaric and caffeic acids, are constituents of an 41 oligomeric structure called lignan macromolecule.[13,14] These phenolic compounds have 42 phytoestrogenic and antioxidant properties[12,14] and, therefore, they may exert potential 43 benefits on human health.[15]

44 A previous study carried out in our laboratory with flaxseed protein concentrates
45 containing phenolic compounds have shown that simulated gastrointestinal digestion was
46 equal or more effective than Alcalase® hydrolysis to obtain antioxidant hydrolysates.[16]
47 However, the specific contribution of phenolic compounds and peptides on the antioxidant
48 capacity of flaxseed protein hydrolysates, as well as on other plant-derived hydrolysates, is
49 not fully elucidated. Therefore, the aim of the present study was to evaluate the influence of
50 naturally-present phenolic compounds and their complexes with proteins on the antioxidant
51 potential of flaxseed products before and after simulated gastrointestinal digestion.

52

53 **MATERIALS AND METHODS**

54 **Reactives**

55 Partially defatted brown flaxseed meal (FM) was obtained from Cisbra Ltd. (Panambi,
56 RS, Brazil). Alcalase® 2.4 L, pepsin, pancreatin, bile salts, Folin-Ciocalteu reagent, gallic
57 acid, [(±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid] (Trolox), 2,4,6-tri (2-
58 pyridyl)-s-triazine (TPTZ), 2,2'-Azobis (2-methylpropionamidine) dihydrochloride (AAPH),
59 sodium fluorescein, secoisolariciresinol, caffeic, *p*-coumaric and ferulic acids were purchased
60 from Sigma (St. Louis, MO, USA). Trifluoroacetic acid (TFA) and acetonitrile were
61 purchased from Merck (Hohenbrunn, Germany). All other chemicals and reagents were of
62 analytical grade.

63

64 **Preparation of flaxseed defatted flour, protein concentrates and phenolic fractions**

65 A flow chart of the preparation of flaxseed protein products and phenolic fraction is
66 shown in Figure 1.

67 To obtain the defatted flaxseed meal (DFM), FM was defatted with hexane in a ratio
68 of 1:3 (w/v) for 24 h at room temperature. For polyphenols extraction, three consecutive steps

69 with 63% ethanol solution (v/v) were performed.[13] Two first steps were performed for 4 h
70 under stirring and room temperature, whereas the last one was made overnight. After each
71 extraction phase, DFM was centrifuged at 2500 x *g* for 30 min, and filtered obtaining the
72 phenolic-reduced DFM (phr-DFM) and the phenolic isolate (Phi).

73 Flaxseed protein concentrate (FPC) and phenolic-reduced FPC (phr-FPC) were
74 prepared from DFM and phr-DFM, respectively, following Dev & Quensel protocol.[17] The
75 corresponding source sample was dispersed in deionized water at a product:water ratio of 1:10
76 (w/w), and after adjusting its pH to 9.0 with 0.5 M NaOH, the solution was stirred at room
77 temperature for 30 min and centrifuged (2500 x g/30 min; 25 °C). The supernatant containing
78 protein was filtered and its pH adjusted to 4.2 with 0.5 M HCl. The precipitated protein was
79 then separated by centrifugation (2500 x g/30 min), washed three times with acidified water
80 (pH 4.2), and suspended in deionized water adjusting its pH to 6.0 with 0.5 M NaOH.
81 Flaxseed products were freeze-dried and stored at -20 °C until their use.

82

83 **Hydrolysis of flaxseed products**

84 The hydrolysis of FPC and phr-FPC was performed with Alcalase® under the
85 following conditions: protein concentration of 5% (w/v), 60 °C, pH 8.5, and enzyme substrate
86 (E:S) ratio 1:90 (w/w). The hydrolysis reaction was monitored using the pH-stat method using
87 an automatic titrator DL model Metler 21 (Schwerzenbach, Switzerland) with a stirring
88 system coupled to a thermostatic bath. After 180 min, the reaction was stopped by heating at
89 90 °C for 10 min. Then, the pH of the hydrolysates was adjusted to 6.0, and they were freeze-
90 dried, and stored at -20 °C. The degree of hydrolysis (% DH) of flaxseed protein hydrolysate
91 (FPH) and phenolic reduced flaxseed protein hydrolysate (phr-FPH) obtained from FPC and
92 phr-FPC, respectively, was calculated according to the equation described by Adler Nissen.[18]

To assess whether the conditions for obtaining the protein hydrolysate may change the phenolic compounds present in the FPC, Phi (5% w/v) was subjected to the same conditions of temperature and time of the hydrolysis reaction, but without addition of Alcalase® to obtain the phenolics hydrolysate (Phh) (Figure 1).

Chemical composition

The chemical composition of flaxseed products was determined according to AOAC procedures.[19] Protein and lipid contents were determined according to Kjeldahl (N x 6.25)[19], and Bligh & Dyer[20] methods, respectively.

Characterisation of flaxseed products by RP-HPLC

The chromatographic analysis of flaxseed products was carried out using a reverse-phase high-performance liquid chromatography (RP-HPLC) system with an automatic injector and a diode-array absorbance detector (Agilent, 1200 Series, Snoqualmie, WA, USA). Separation was carried out onto a Luna C18 column (250 mm x 4.6 mm, Phenomenex, Torrance, CA, USA) at a flow rate of 1 mL/min. The mobile phase was constituted by solvent A (0.04% TFA in water) and solvent B (0.03% TFA in acetonitrile). The gradient was from 0 to 80% of solvent B over 40 min. The absorbance was measured at 214 and 280 nm. The samples were filtered through a 45-µm membrane, and 50 µL were injected. Sample concentration of flaxseed flours, concentrates and hydrolysates was adjusted to 8 mg/mL. In the case of digested samples, concentration was adjusted to 4 mg/mL. The Star Chromatography Workstation software (Agilent) was used to record and process data.

Identification of phenolic compounds by UPLC-MS/MS

The identification of ferulic, *p*-coumaric and caffeic acids and SDG was carried out using a UPLC-Q-Tof system comprised of an AcquityTM UPLC system coupled to a

118 XevoTM G2-XS Q-Tof (Waters Corp., Milford, MA, USA), with an electrospray source
119 ionization (ESI) in negative mode. The instrument control and data processing were
120 performed by MassLynx software (Waters Corp.) version 4.1. Samples were analyzed in MSE
121 mode, in which precursor and fragment information are collected from the same analysis. For
122 the MS operating conditions the following parameters were set: capillary voltage 2.5 kV, cone
123 voltage 30 V, source temperature 150 °C, desolvation temperature 550 °C, cone gas flow 50
124 L/h and desolvation gas flow 900 L/h. The instrument was previously calibrated with a
125 sodium formate solution, and all runs were acquired with real time lockspray correction for
126 mass accuracy (deprotonated rutin ion, mass/charge (m/z) 609.1456). Spectra were acquired
127 every 0.1 s, on a *m/z* range of 100-1200. High energy spectra were acquired from *m/z* 50-1200
128 using a collision energy ramp from 20-30 eV.

129 The chromatographic separation was carried out on a BEH C18 column (50 mm × 2.1
130 mm × 1.7 µm) (Waters Corp.). Mobile phases consisted of 0.1% formic acid in water (A) and
131 0.1% formic acid in methanol (B). The gradient program was as follows: B was ramped from
132 5-95% in 4 min, followed by a 0.5 min lapse at 95% B. Then, mobile phase composition was
133 restored to initial conditions for 0.5 min. Flow rate was set to 0.6 mL/min, the injection
134 volume was 5 µL, and the column oven and sample manager were kept at 45 °C and 10 °C
135 respectively. Each standard or sample was properly diluted in ultrapure water, and filtered
136 through 0.45 µm polytetrafluoroethylene membrane before being injected onto the system.

137

138 **Analysis of protein–phenolic complex by fluorescence spectroscopy**

139 The intrinsic fluorescence analysis was performed to study the formation of the
140 protein-phenolic complex among proteins/peptides and polyphenols according to Kanakis, et
141 al. [21] with some modifications. FPC, phr-FPC, FPH and phr-FPH were dispersed in aqueous
142 solutions at a protein concentration of 4 mg/mL. The fluorescence spectra of the Phi and Phh

143 (4 mg sample/mL) were also recorded. All solutions were prepared at 24.0 ± 1 °C and kept in
144 the dark. Fluorescence spectra were recorded at λ_{exc} = 280 nm and λ_{emi} from 290 to 500 nm,
145 and data were acquired using an ISS PC1 Fluorimeter (Champaign, IL, USA).
146
147
148 **Simulated gastrointestinal digestion**
149 Simulated gastrointestinal digestion was performed as reported by Martos et al. [22] with
150 modifications. The samples were dispersed in gastric juice (35 mM NaCl), and the pH was
151 adjusted to 2.0 with 1 M HCl, thus obtaining a concentration of 5.9 mg protein/mL (DFM,
152 phr-DFM, FPC, phr-FPC, FPH, phr-FPH) and 1.2 mg phenolic compounds/mL (Phi and Phh).
153 Mixtures were left in a water bath at 3 °C for 15 min under constant stirring. Then, pepsin was
154 added (E:S 1:20, w/w), and the pH was again adjusted to 2.0. The mixture was left in a
155 thermostatic bath at 37 °C for 60 min under stirring. At the end of gastric phase, the pH of
156 samples was adjusted to 6.8 with 1 M $NaHCO_3$, 1 M $CaCl_2$, and 9 mg/mL bile salt and they
157 were maintained in a water bath at 37 °C for 15 min under stirring. Then, pancreatin was
158 added (E:S 1:10, w/w), the pH was adjusted to 6.8 with 1 M $NaHCO_3$, and the volume was
159 made up to 4 mL with deionized water. The intestinal digestion was carried out at 37 °C for
160 60 minutes under stirring. To stop the reaction, the digest was heated at 90 °C for 10 min
161 under stirring and centrifuged (11000 x g) for 15 min. The supernatants (digests) were frozen
162 and kept at -20 °C until further use.
163
164 **Antioxidant capacity**
165 The antioxidant capacity of the samples before and after simulated digestion was
166 measured in the aqueous extracts. To obtain the extracts, lyophilized non digested samples
167 (1% w/v) and digested samples (3% v/v) were suspended in deionized water, shaken for 30

168 min, centrifuged at 36000 x *g* for 30 min at 10°C, filtered through N°1 Whatman qualitative
169 filter paper, and stored at -20 °C in dark until use. All the antioxidant capacity assays were
170 carried out using a Synergy™ HT Multi-Mode Microplate Reader (Biotek®, Winooski, VT,
171 USA).

172 **Folin-Ciocalteau reagent reducing substances (FCRRS).** The procedure was carried
173 out according to Medina.[23] Briefly, 450 µL of deionized water and 50 µL of appropriately
174 diluted samples, gallic acid standard solutions (0, 50, 100, 200, 300, 400, 500, 600 µg/mL) or
175 deionized water for blank were added and mixed. The Folin-Ciocalteu reagent (50 µL) was
176 added, mixed, and allowed to react for 5 min. Then, 500 µL of 7% Na_2CO_3 and 200 µL of
177 deionized water were added and mixed. The mixture was left to react at room temperature in
178 the dark for 90 min. The absorbance was measured at 760 nm and the results were expressed
179 as mg gallic acid equivalent (GAE) per gram of sample (mg GAE/g sample).

180 **Ferric reducing antioxidant power (FRAP).** The FRAP assay was carried out
181 according to Benzie & Strain [24] with modifications. In the dark, 30 µL of sample extract,
182 standard or blank was mixed with 90 µL of water and 900 µL of the FRAP reagent (450 µL of
183 0.3 M acetate buffer, pH 3.6, 225 µL of 10 mmol TPTZ in 40 mmol HCl and 225 µL of 20
184 mmol $FeCl_3$). The mixture was incubated at 30 °C for 30 min. The absorbance was measured
185 at 595 nm and the results were expressed as µmol of Trolox equivalent (TE) per gram of
186 sample (µmol TE/g sample).

187 **Oxygen radical absorbance capacity (ORAC).** ORAC assay was performed
188 according to Davalos et al. [25] Briefly, 20 µL of sample extract and 120 µL of sodium
189 fluorescein in potassium phosphate buffer (pH 7.4) (final concentration 0.378 µg/mL) were
190 mixed in water with 60 µL of AAPH (final concentration 108 mg/mL). Potassium phosphate
191 buffer was used as a blank. Trolox solutions (25-500 mM) were used as standard.
192 Fluorescence was measured every minute for 80 min with an excitation wavelength of 485 nm

193 and an emission wavelength of 520 nm. The antioxidant capacity was expressed as μmol TE/g
194 sample, based on the area under the curve (AUC) for the decline in the fluorescence time.

195 **Metal chelation activity.** Fe^{2+}-chelating activity was determined by measuring the
196 formation of the Fe^{2+}-ferrozine complex according to Carter [26] with adaptations. Samples
197 were diluted (0.4-6.4 mg/mL) in 100 mM sodium acetate buffer (pH 4.9), stirred for 30 min
198 and centrifuged at 27821 x *g*. Sample solution (250 μL) was mixed with 30 μL $FeCl_2$ (50
199 μg/mL), and incubated for 30 min at room temperature. Then, ferrozine (12.5 μL, 40 mM)
200 was added. Ethylenediamine tetraacetic acid (EDTA) was used as a positive control at the
201 same concentration used for samples. The chromophore formed by binding of Fe^{2+} ions to
202 ferrozine was measured at 562 nm. Iron chelating activity was calculated using the equation 1:
203 $\% \text{ Chelating Activity} = [(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100$

204

205 **Statistical Analysis**

206 Data were presented as means and standard deviations. All antioxidant assays were
207 carried out in triplicate. A paired t-test was performed to determine the difference between the
208 FCRRS content and the antioxidant capacity before and after simulated gastrointestinal
209 digestion. The antioxidant capacity results were analyzed by ANOVA followed by the
210 Tukey´s test. The statistical analyses were carried out using the software SPSS 15.0 (SPSS
211 Inc, Chicago, IL, USA).

212 Principal component analysis (PCA) was performed to understand how the phenolic
213 compounds and the digestive process can influence the antioxidant behavior of the samples.
214 Data were autoscaled and analyzed using Pirouette Statistics, version 3.11.

215

216 **RESULTS AND DISCUSSION**

217 **Chemical characterisation of flaxseed products**

218 The chemical composition (proteins, moisture, ash, lipids, and dietary fiber) of
219 flaxseed products is shown in Table 1. The basis product of this study (FM) contained 29.3%
220 protein, 8.4% moisture, 4.2% ash, 14.9% lipids, and 35.1% dietary fiber. Defatting FM
221 resulted in a reduction of lipids content and a slight increase in both proteins and dietary fiber.
222 DFM was subjected to ethanol treatment in order to remove polyphenols. This process
223 slightly increased the protein content of phr-DFM (from 33.4 to 35.7%), as well as its
224 moisture and fiber levels (Table 1). The resulting product, Phi, containing extracted
225 polyphenols also contained a little amount of protein (5.21%), indicating that ethanol
226 extraction also extracted some flaxseed protein from defatted meal. Protein extraction allow to
227 obtain two products, FPC and phr-FPC which protein contents were 73.9 and 82.1%,
228 respectively, 2.2- and 2.3-times higher than those obtained for their source products, DFM
229 and phr-DFM. However, these values were lower than that reported in the literature.[27] This
230 fact could be due to the extraction method employed that did not include seed coat removal
231 previous to the defatting procedure. The levels of other components of FPC decreased after
232 protein extraction, and they were similar to those reported in our previous study.[16]
233 FPC and phr-FPC were hydrolysed by Alcalase® for 180 min. The DH for both
234 products was 18.3 ± 0.2 and 17.2 ± 0.7%, respectively, with no significant differences
235 between them (p <0.05). These results suggest that the phenolic fraction present in protein
236 concentrate did not interfere on Alcalase® activity.
237
238 **Chromatographic analysis of flaxseed products: effects of enzymatic hydrolysis**
239 The chromatographic profiles of flaxseed products before and after Alcalase®
240 hydrolysis are shown in Figure 2. The chromatograms were obtained at 214 and 280 nm
241 because these two wavelengths allow detecting both proteins/peptides and phenolic
242 compounds.[28,29] The 214 nm-chromatogram of FPC (Figure 2A) showed numerous peaks

243 eluting between 8 and 20 minutes which intensity was notably lower in phr-FPC and Phi
244 products (Figure 2B and 2C). The intensity of a peak eluted at 15 min and detected at both
245 214 and 280 nm was much higher in the Phi than in FPC chromatogram, suggesting that it
246 might correspond to a phenolic compound extracted with ethanol, as it was not observed in
247 phr-FPC sample. Similarly, other components of lignan macromolecule present in FPC and
248 Phi could elute as a wide peak with retention time between 20 and 23 min. This peak was also
249 visible in the chromatographic profile of DFM product (data not shown). A previous analysis
250 of flaxseed lignan macromolecule by size exclusion-HPLC with diode array detection, had
251 also described the elution of a wide peak at 280 nm.[14] The authors suggested that the
252 heterogeneity of lignan macromolecule could be responsible for this result, considering the
253 sample as a mixture of molecules with similar molecular weight but a slightly different
254 composition of individual phenolic compounds. According to Johnsson, et al. [30], and Struijs,
255 et al. [31] SDG ester linked to hydroxymethyl-glutaric acid forms the backbone of the lignan
256 macromolecule that is also comprised of the hydroxycinnamic acids, *p*-coumaric acid
257 glucoside, and ferulic acid glucoside.

258 After hydrolysis with Alcalase®, the chromatographic profiles of flaxseed products
259 drastically changed in both appearance and intensity of eluted peaks. This was due to the
260 release of peptides after the action of the microbial enzyme on flaxseed proteins. Profiles of
261 FPH and phr-FPH (Figure 2D and 2E) were similar, indicating that phenolic compounds
262 accompanying proteins in FPC did not affect Alcalase® activity, as it had been observed
263 measuring the DH. Notable changes were also observed for Phh compared with Phi that could
264 be due to modifications in phenolic compounds resulting from partial hydrolysis of lignan
265 macromolecule under conditions (pH 8.5 and 60 °C) used to simulate enzymatic hydrolysis.

266 In order to identify phenolic compounds, FPH, phr-FPH, Phi, and Phh products were
267 subjected to UPLC-MS/MS analysis, injecting standards under the same experimental

268 conditions. Figure 3A-3D shows the extracted ion chromatogram (EIC) of four phenolic
269 compounds identified, ferulic, *p*-coumaric and caffeic acids, and SDG. Presence of these four
270 compounds was confirmed in all samples except phr-FPH which is produced from a flaxseed
271 product free of phenolic compounds. In the case of ferulic and *p*-coumaric acids (Figure 3A
272 and 3B), more than one peak could be observed. Analysis of the mass spectra indicates that
273 these peaks corresponded to modified versions of the phenolic compounds, mainly
274 glycosylated forms, which fragment upon ionization conditions generated the same ions.
275 These results strongly suggest that modified phenolic compounds are belonging to lignan
276 macromolecule. According to Li, Yuan, Xu, Wang and Liu [15] phenolic compounds in lignan
277 macromolecule are firstly esterified by ethanol and subsequently hydrolysed by alkali to
278 produce SDG and other phenolic glycosides. These phenolic glycosides and SDG were more
279 clearly detected in Phh.

280

281 **Protein-peptide-phenolic compounds complex**

282 Once confirmed the presence of phenolic compounds in flaxseed products, potential
283 interactions and the subsequent formation of complexes between proteins/peptides and
284 polyphenols were investigated by fluorescence spectroscopy. Emission spectra from 290 to
285 500 nm of FPC, phr-FPC and Phi are shown in Figure 4A. Figure 4B shows spectra
286 corresponding to FPH, phr-FPH and Phh samples.

287 The fluorescence of folded proteins is a result of the fluorescence from individual
288 aromatic residues. Most of the intrinsic fluorescence emissions are due to excitation of Trp
289 residues, with some emissions due to Tyr and Phe. Trp has an emission peak ranging from
290 308 to 350 nm depending on the local environment and the degree of solvent exposure of the
291 chromophore.[32] In our study, the highest fluorescence intensity was measured for phr-FPC
292 and phr-FPH products which did not contain phenolic compounds. However, presence of

293 these components in FPCand FPH resulted in a notable reduction (~50%) of fluorescence
294 intensity as well as a shift in the maximum emission peak (from 348 nm in FPC to 356 nm in
295 phr-FPC, and from 358 nm in FPH to 360 nm in phr-FPH). This reduction could be due to
296 modifications in protein/peptide chains caused by the formation of protein/peptide complexes
297 with phenolic compounds in FPC and FPH products. Kanakis et al.[21] had reported that when
298 proteins interact with other molecules, Trp fluorescence changes depending on the impact of
299 the interaction on the spatial conformation of the protein structure. A negligible emission was
300 observed for Phi and Phh samples (Figure 4A and 4B), which chemical characterisation had
301 demonstrated very low protein content.

302

303 **Impact of simulated gastrointestinal digestion of peptide profile and antioxidant activity**

304 The chromatograms of flaxseed products after simulated digestion, obtained at 214
305 and 280 nm, are shown in Figure 5 (A-F). As it can be observed, the action of digestive
306 proteases on FPC and phr-FPC led to a high number of peptides that eluted between 8 and 20
307 min (Figure 5A and 5B). Both samples after digestion showed similar profiles, indicating that
308 phenolic compounds present in FPC did not affect the enzymatic action of pepsin and
309 pancreatin. Only the wide peak eluting at 20-23 min, corresponding to lignan macromolecule,
310 was still visible in the chromatogram of digested FPC (dFPC). The same behavior was
311 observed for FPH and phr-FPH, samples resulting from Alcalase® hydrolysis (Figures 5D and
312 5E). Moreover, these profiles were similar to those shown by their digested parent products
313 (dFPC and dphr-FPC), suggesting that peptides visible in the chromatograms were released by
314 the action of pepsin and pancreatin on flaxseed proteins that had been not previously degraded
315 by the microbial enzyme.

316 In the case of phenolic compounds fraction (Phi and Phh), different behavior was
317 observed after their simulated digestion. Small differences in the profile of digested Phi (dPhi,

318 Figure 5C) were detected comparing with non-digested sample (Phi, Figure 2C) that could be
319 due to phenolics modifications caused by pH and temperature changes. Analysis of Phh and
320 digested Phh (dPhh) showed similar chromatographic patterns (Figure 2F and 5F), thus
321 indicating that phenolic compounds modified by alkaline conditions were not further affected
322 by digestive conditions.

323 In order to evaluate the potential contribution of protein/peptide and phenolic
324 compounds on the antioxidant capacity of flaxseed, different products were subjected to
325 analysis using several measuring methods (ORAC, FRAP, FCRRS, and metal chelating
326 activity). The results would also allow evaluating the impact of Alcalase® hydrolysis and
327 gastrointestinal digestion on the antioxidant activity. Figure 6 shows the results obtained from
328 different assays before (Figure 6A, 6C, 6E, and 6G) and after simulated digestive process
329 (Figure 6B, 6D, and 6F).

330 Before simulated digestion, Phi and Phh products showed the highest ORAC and
331 FRAP values as well as the greatest chelating potential. This might be associated with the
332 higher concentration of nucleophilic centers comparing with other flaxseed products, as well
333 as with the possible synergisms among different phenolic compounds. The ability of the
334 phenolic compounds to act as multifunctional antioxidant, as chain-breaking or metal
335 chelating agent, can be explained by the nucleophilic character of the aromatic rings in its
336 structure.[33] The ORAC value of Phh was 1.5-times higher than that of Phi, which can be
337 related to the release of compounds of lower molecular weight and lower hydrophobicity
338 resulting from alkaline conditions, as it was observed by chromatographic analysis (Figure
339 2C, 2F). Among these compounds, glycosylated ferulic, caffeic, and *p*-coumaric acids could
340 be responsible for the increase in the ORAC value. These phenolic acids act as antioxidant
341 mainly through a hydrogen atom (H^+) transfer mechanism due to the reactivity of their phenol
342 moiety, although they also could act via electron donation.[34] The iron chelating ability of Phi

343 and Phh was similar ($p > 0.05$) (Figure 6G), and about 2-times higher than that of FPH and
344 phr-FPH. The potential of FPH and phr-FPH may be compromised by the dietary fiber
345 content (Table 1) because these high molecular weight polysaccharides could interfere with
346 the iron-peptides interaction, hindering the formation of the chelate.[35] No significant
347 differences were observed between both hydrolysates, suggesting that their potential is due to
348 peptides, especially those containing His, Glu, Asp, and Cys residues[36] rather to the presence
349 of polyhydroxylated rings in phenolic compounds. Some studies have shown that iron
350 chelating by peptides may facilitate absorption of this mineral by intestinal cells[37-39]
351 increasing its bioavailability, while some classes of phenolic compounds may exert an
352 opposite effect.[40] However, it has been demonstrated that iron chelating by peptides or
353 phenolic compounds may maintain the metal more stable and less prone to interactions,[41]
354 which prevents free iron to catalyze human body reactions involving ROS, leading to the
355 oxidation of unsaturated lipids and promoting oxidative damage in cells.[42] Thus, both peptides
356 as phenolic compounds can have a beneficial effect due to its ability to chelate iron.

357 Alcalase® hydrolysis was responsible for an increase in the antioxidant potential of
358 flaxseed products up to 6 and 4 times, as determined by ORAC and FRAP assays,
359 respectively, compared with non-hydrolysed products. Similar results have been previously
360 reported for other plant protein hydrolysates.[43] Although the absolute values of the antioxidant
361 capacity of samples containing protein and phenolic compounds were higher than those
362 measured for products only containing proteins, the relative increase on the antioxidant
363 capacity as a result of hydrolysis with Alcalase® was similar. Therefore, this increase might
364 be associated with the release of peptides during enzymatic hydrolysis rather than with
365 changes in phenolic compounds.[1] FPH showed the highest FCRRS content ($p < 0.05$) with a
366 value 2- and 1.4-times higher than that measured in phr-FPH and Phi products, respectively
367 (Figure 6E), indicating that exposure of both aromatic rings of the phenolic compounds and

368 aromatic residues of proteins during hydrolysis with Alcalase® was responsible for this
369 antioxidant mechanism of action.

370 As expected, the antioxidant capacity of flaxseed products was maintained or
371 increased after gastrointestinal digestion (Figure 6B, 6D, and 6F), indicating that the digestive
372 process might exert a beneficial effect on the release of bioactive compounds, regardless of
373 the mechanism evaluated. This effect was higher than that demonstrated for Alcalase®
374 hydrolysis. In the case of the effect of digestion on the antioxidant capacity of Phi, a
375 significant increase of FRAP value and FCRRS content was observed, while ORAC value did
376 not change after the action of digestive enzymes. However, three antioxidant values were
377 increased when simulated digestion was performed on Phh, indicating that alkaline conditions
378 favored the access of nucleophilic sites of phenolic compounds to radicals in spite of
379 chromatographic profiles of Phh and dPhh were similar. In literature, the effect of digestion
380 on the phenolic compounds antioxidant capacity is contradictory and dependent on the
381 digested product. Tarko et al. [44] showed that the antioxidant capacity of the phenolic
382 compounds from apple and plum increased, while those from pear and banana decreased after
383 simulated digestion. The antioxidant activity of FPC and phr-FPC, without previous
384 Alcalase® hydrolysis, significantly increased after being subjected to simulated
385 gastrointestinal digestion. Although the chromatographic profile of dFPC and dphr-FPC were
386 similar, the antioxidant behavior was different, with highest capacity shown by product
387 containing both proteins and phenolic compounds. This was also observed for products
388 previously hydrolysed by Alcalase® (dFPH and dphr-FPH).

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391

392 **Influence of phenolic compounds on antioxidant behavior and antioxidant capacity of**
393 **flaxseed products**

394 PCA was performed to understand how the presence of phenolic compounds
395 influenced the antioxidant potential of flaxseed products. PCA displays similarities and
396 differences among samples from their spatial distribution. ORAC, FRAP and FCRRS values
397 were considered as independent variables, while flaxseed deffated flours, protein concentrates
398 and hydrolysates, and their digests were the dependent variables. Samples were then
399 distributed according to different oxidation assays, allowing knowing the predominant
400 antioxidant mechanism for each sample (Figure 7).

401 Samples were centered on two main axes or principal components (PC), PC1 that
402 explained 89.3% of the variance and PC2 that explained 9.7%. Thus, both components
403 explained 99.0% of the antioxidant behavior of flaxseed products. As it can be observed in
404 Figure 7, FPC and phr-FPC are located in the same quadrant, indicating that there are no
405 differences between the antioxidant mechanisms of these samples. Same results were obtained
406 for source flours, DFM and phr-DFM. However, once subjected to Alcalase® hydrolysis, the
407 behavior of the hydrolysates changed, and FPH and phr-FPH were located in opposite
408 quadrants. Phr-FPH, only containing peptides, was located at the downleft quadrant,
409 indicating that its antioxidant activity was mainly mediated through protons transference and
410 peroxyl radicals chelation. However, FPH, containing both peptides and phenolic compounds,
411 was located at the upper right quadrant, suggesting that phenolic compounds were the main
412 responsible for reducing ferric to ferrous iron (FRAP assay). After simulated gastrointestinal
413 digestion, similar behavior was observed with peptides released from the action of digestive
414 enzymes responsible for ORAC and FCRRS values, and phenolic compounds contributing to
415 iron reduction.

416 The presence of polyphenols contributed positively, but in a variable way, on the
417 antioxidant capacity of the majority of protein samples. Comparing FPC and phr-FPC, the
418 presence of phenolic compounds led to an increase of about 80% on antioxidant capacity
419 measured by ORAC and the FRAP assays. For the hydrolysate, the presence of phenolic
420 compounds also led to an increase of nearly 80%, measured by FRAP, but only 15% as
421 measured by ORAC. After simulated digestion, the influence of the phenolic compounds on
422 the antioxidant capacity of the samples determined by the FRAP remained high, between 70
423 and 80%, while lower influence was observed when the antioxidant capacity was measured by
424 ORAC (Figure 6). This impact can be explained either by synergism between the antioxidant
425 compounds or formation of protein-phenolic complex. In proteinaceous samples, antioxidants
426 compounds are SDG, caffeic, ferulic and p-coumaric acids,[34] as well as flaxseed peptides, in
427 Alcalase® hydrolysates or in digested samples.[45] Together, such compounds may have their
428 antioxidant potential increased, since a non-oxidized compound is able to regenerate the other
429 which has been oxidized, in a similar way that synergism occurs between α-tocopherol and
430 flavonoids or α-tocopherol and ascorbate.[46]

431 In the protein concentrates, the positive influence of phenolic compounds on
432 antioxidant capacity can be either due to their high amount of phenolic hydroxyl groups
433 and/or by unfolding the protein structure due to protein-phenolic complex formation. In most
434 cases, formation of complexes increases the exposure of nucleophilic centers formed by
435 hydrophobic amino acid residues at the N-terminal portion, or the presence of His, Trp, Phe,
436 Tyr, Cys in the protein moieties.[1] Although the formation of complex promotes the
437 participation of protein as an antioxidant, it can, in turn, compromise the performance of
438 polyphenols in this process, masking their bioactivity.[47] Thus the resulting antioxidant activity
439 is due the increasing the antioxidant capacity of proteins and decreasing that of phenolic
440 compounds.

441 On the other hand, in the hydrolysates, the formation of complex is hindered due to the
442 small contact surface between peptides and phenolic compounds.[48] Thus protein hydrolysis
443 can weaken the protein-phenolic complexes, by reducing the interactions between these
444 compounds and increasing both the availability of the nucleophilic sites of peptides and
445 phenolic compounds. This greater exposure of regions capable of neutralizing reactive species
446 and the possible synergism between these two classes of compounds may also explain the
447 higher antioxidant capacity of the hydrolysates when compared to the concentrates.

448 In conclusion, the phenolic fraction showed the highest antioxidant capacity among
449 the flaxseed products studied, which was enhanced by both alkaline hydrolysis and simulated
450 gastrointestinal digestion, possibly by releasing SDG and *p*-coumaric, caffeic and ferulic
451 phenolic acids. The hydrolysis by both Alcalase® and digestive enzymes also resulted in an
452 increase of the antioxidant activity of protein concentrates with/without phenolic compounds.
453 Peptides released act through protons transference and peroxyl radicals chelation while
454 phenolic compounds were, furthermore, responsible for the iron reduction.

455 The formation of protein-phenolic complexes may have a positive effect on the
456 antioxidant capacity of plant protein concentrates. In our study, we have found that flaxseed
457 protein-phenolic complexes favored the exposure of protein moieties capable of acting as an
458 antioxidant, which would complement the antioxidant potential of phenolics, with a positive
459 relationship between these two classes of compounds. This would have a dual effect of
460 phenolic protection against oxidative degradation along the gastrointestinal tract and
461 establishment of a positive antioxidant environment. Animal models should be needed to
462 evaluate the bioavailability of peptides and phenolic compounds as well as to confirm the *in*
463 *vivo* antioxidant effects providing health benefits against oxidative stress-associated disorders.

464 **CONFLICT OF INTEREST**

465 The authors declare that there are no conflicts of interest.

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609

610 **FIGURE CAPTIONS**

611 **Figure 1**. Flow chart of the preparation of flaxseed products from flaxseed meal (FM)

612 **Figure 2**. Chromatogram profiles (RP-HPLC) recorded at 214 nm and 280 nm of flaxseed
613 products before and after Alcalase® hydrolysis. (A) flaxseed protein isolate (FPC), (B)
614 phenolic reduced flaxseed protein isolate (phr-FPC), (C) phenolic compounds isolate (Phi),
615 (D) flaxseed protein hydrolysate (FPH), (E) reduced phenolic flaxseed protein hydrolysate
616 (phr-FPH), and (F) phenolic compounds hydrolysate (Phh)

617 **Figure 3**. Extracted ion chromatogram (EIC) of (A) ferulic acid (*m/z* 193.1 ± 0.5), (B) p-
618 coumaric acid (*m/z* 163.0 ± 0.5), (C) caffeic acid (*m/z* 179.0 ± 0.5) and (D) secoisolariciresinol
619 diglucoside (SDG) (*m/z* 685.3 ± 0.5). EICs correspond (from bottom to top) to phenolic
620 reduced flaxseed protein (phr-FPH), flaxseed protein hydrolysate (FPH), phenolic compounds
621 hydrolysate (Phh) and phenolic compounds isolate (Phi)

622 **Figure 4**. Fluorescence emission spectra of (A) flaxseed protein isolate (FPC), phenolic
623 reduced flaxseed protein isolate (phr-FPC) and phenolic compounds isolate (Phi), and (B)
624 flaxseed protein hydrolysate (FPH), phenolic reduced flaxseed protein (phr-FPH) and phenolic
625 compounds hydrolysate (Phh).

626 **Figure 5**. Chromatogram profiles (RP-HPLC) recorded at 214 nm and 280 nm of flaxseed
627 products after simulated gastrointestinal digestion. (A) Digested flaxseed protein isolate
628 (dFPC), (B) Digested phenolic reduced flaxseed protein isolate (dphr-FPC), (C) digested
629 phenolic compounds isolate (dPhi), (D) digested flaxseed protein hydrolysate (dFPH), (E)
630 digested reduced phenolic flaxseed protein hydrolysate (dphr-FPH), and (F) digested phenolic
631 compounds hydrolysate (dPhh)

632 **Figure 6**. Antioxidant capacity of flaxseed protein products determined by ORAC (A and B),
633 FRAP (C and D), FCRRS levels (E and F) and quelating capacity (G), before (A, C, E, G) and
634 after simulated gastrointestinal digestion (B, D, F). Values expressed as mean of duplicates

635 (each in triplicate) ± standard deviation. Bars with different letters differ from each other by
636 ANOVA, post hoc Tukey test ($p < 0.05$). Bars of the same sample with phenolics and reduced
637 phenolics with '*' are different from each other by T test ($p < 0.05$).

638 **Figure 7**. Principal component analysis (PCA) on the measured parameters: ORAC, FRAP
639 and FCRRS before and after Alcalase hydrolysis and simulated gastrointestinal digestion. (▼)
640 Defatted flaxseed meal (DFM); (♦) Phenolic reduced defatted flaxseed meal (phr-DFM); (○)
641 Flaxseed protein isolate (FPC); (□) Phenolic reduced flaxseed protein isolate (phr-FPC); (Δ)
642 Flaxseed protein hydrolysate (FPH); (■) Phenolic reduced flaxseed protein hydrolysate (phr-
643 FPH); (◊) Digested DFM (dDFM); (*) Digested phr-DFM (dphr-DFM); (♣) Digested FPC
644 (dFPC); (♥) Digested phr-FPC (dphr-FPC); (+) Digested FPH (dFPH); (♠) Digested phr-FPH
645 (dphr-FPH).
646

647

648 **TABLES**

649 **Table 1.** Chemical composition (expressed as %) of flaxseed meal (FM), defatted flaxseed
650 meal (DFM), phenolic reduced defatted flaxseed meal (phr-DFM), flaxseed protein isolate
651 (FPC), phenolic reduced flaxseed protein isolate (phr-FPC), flaxseed protein hydrolysate
652 (FPH), phenolic reduced flaxseed protein hydrolysate (phr-FPH), phenolic isolate (Phi) and
653 phenolic hydrolysate (Phh). Results are the mean ± standard deviation

Sample	Proteins[a]	Moisture	Ash	Lipids	Dietary Fiber
FM	29.28 ± 0.38	8.42 ± 0.00	4.20 ± 0.00	14.86 ± 0.87	35.12 ± 0.31
DFM	33.36 ± 0.94	8.18 ± 0.16	4.48 ± 0.03	6.83 ± 0.17	38.84 ± 0.32
phr-DFM	35.68 ± 0.01	11.94 ± 0.31	4.44 ± 0.04	6.41 ± 0.06	40.73 ± 0.49
FPC	73.91 ± 1.00	3.82 ± 0.12	1.57 ± 0.05	4.73 ± 0.17	13.01 ± 0.29
phr-FPC	82.05 ± 1.04	3.07 ± 0.22	1.64 ± 0.08	1.11 ± 0.05	14.91 ± 0.11
FPH	67.85 ± 0.22	ND	ND	ND	11.94 ± 0.27
phr-FPH	75.18 ± 0.31	ND	ND	ND	13.67 ± 0.08
Phi	5.21 ± 0.17	ND	ND	ND	ND
Phh	4.77 ± 0.13	ND	ND	ND	ND

654 [a] N (%) x 6.25; ND: Not determined

655