Reduction of pulse "antinutritional" content by optimizing pulse canning process is insufficient to improve fat-soluble vitamin bioavailability

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17   **Abbreviated running title:** Pulse canning process and fat-soluble vitamin bioavailability

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20   Data described in the manuscript will be made available upon request pending application and

21   approval.

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23

24 **Abstract**

25

26 Some bioactive compounds found in pulses (phytates, saponins, tannins) display

27 antinutritional properties and interfere with fat-soluble vitamin bioavailability (i.e.,

28 bioaccessibility and intestinal uptake). As canned chickpeas are consumed widely, our aim

29 was to optimize the chickpea canning process and assess whether this optimization influences

30 fat-soluble vitamin bioavailability. Different conditions during soaking and blanching were

31 studied, as was a step involving prior germination. Proteins, lipids, fibers, vitamin E, lutein, 5-

32 methyl-tetrahydro-folate, magnesium, iron, phytates, saponins and tannins were quantified.

33 Bioaccessibility and intestinal uptake of vitamin D and K were assessed using in vitro

34 digestion and Caco-2 cells, respectively. Significant reductions of phytate, saponin and tannin

35 contents (-16 to -44%), but also of folate content (up to –97%) were observed under

36 optimized canning conditions compared with the control. However, bioaccessibility and

37 cellular uptake of vitamin D and K remained unaffected after in vitro digestion of test meals

38 containing control or optimized canned chickpeas.

39

40 **Keywords:** Chickpeas; germination; phytates; tannins; saponins; vitamin D; vitamin K;

41 bioaccessibility; Caco-2 cells.

1.   Introduction

Pulses display interesting nutritional properties, as they are good sources of proteins, fibers and micronutrients (Margier et al., 2018). Pulses are also a significant source of bioactive compounds such as phytates, saponins and tannins (Margier et al., 2018), the consumption of which demonstrates some interesting lipid-lowering properties. For instance, dietary fibers positively impact postprandial lipemia (Desmarchelier et al., 2019; Lee et al., 2020) and cholesterolemia (Schoeneck & Iggman, 2021). Phytates at high concentrations and tannins were shown to interfere with human lipase activity (Antoine et al., 2021). Finally, saponins have often been described as anti-obesogenic compounds (Marrelli et al., 2016), partly because they also interact with lipase activity (Antoine et al., 2021; Marrelli et al., 2016). However, these compounds can also be considered "antinutritional", as they can negatively interact with micronutrients such as minerals. In particular, phytates and tannins are divalent cation quenchers that can decrease iron and zinc bioavailability (Antoine et al., 2021; Lestienne et al., 2005). Furthermore, we recently showed that fibers, phytates, tannins and saponins from pulses can reduce fat-soluble vitamin bioavailability in vitro, and more precisely that they can modulate the bioavailability of vitamin K (Margier et al., 2019) and vitamin D (Antoine et al., 2021).

Different strategies can be set up to decrease pulses' "antinutritional" content, one of the most straightforward being the optimization of pulse cooking/transformation methods (Margier et al., 2018). As canned pulses are consumed widely, we decided to investigate the optimization of canning process. Chickpeas were selected as the pulse to be studied. The objective of this study was thus i) to identify and optimize the parameters of the canning process that could reduce chickpeas' levels of bioactive compounds displaying antinutritional properties (phytates, saponins and tannins); and ii) assess whether the decreases in phytates, saponins

67 and tannins resulting from canning process optimization were sufficient to improve the

68 bioavailability of vitamins D and K in test meals containing canned chickpeas.

## 2.	Material and methods

### 2.1.	Chemicals

Cholecalciferol (vitamin D_3), phylloquinone (vitamin K_1), γ-tocopherol, retinyl acetate (all >95% pure), phytic acid sodium salt hydrate, soyasaponin I, 4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), catechin 4-(dimethylamino)cinnamaldehyde (DMACA), 2-(N-cyclohexylamino) ethane sulfonic acid (CHES), 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), α-amylase from bacillus sp., protease from Streptomyces griseus, and 5-methyltetrahydrofolate (5-CH3-H4 folate calcium salt) were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Lutein (>95% pure) was from Extrasynthèse (Genay, France). Chicken pancreas conjugase was from R-Biopharm (Darmstadt, Germany). Isio4 oil was from Lesieur (Asnières-sur-Seine, France). Iron (III) chloride hexahydrate and vanillin (all 99% pure) were from Acros Organics (Noisy-le-Grand, France). Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose and trypsin-EDTA (500 and 200 mg/L, respectively), non-essential amino acids, penicillin/streptomycin and phosphate-buffered saline (PBS) were purchased from Life Technologies (Illkirch, France). Fetal bovine serum (FBS) came from PAA (Vélizy Villacoublay, France). Chickpeas (*Cicer arietinum* 'Eldorado'), were produced locally and provided by the GPS France cooperative (Manosque, France). All solvents were HPLC grade and obtained from Carlo Erba (Peypin, France).

### 2.2.	Chickpea preparation

2.2.1. Control canning process

The control canning protocol was devised by the Centre Technique de Conservation des Produits Agricoles (CTCPA, Avignon, France) and implemented in their pilot plant. In brief,

94 chickpeas were soaked in low-ionized water (hardness titration [HT]=30 °f) at 15 °C at a ratio

95 of 1:3 (Chickpeas:water; w/w) for 14h. Afterwards, immersion blanching was done at 90 °C

96 for 5 min. Chickpeas were then conditioned in cans (200 g), and hot brine (0.5% salt, 70°C) at

97 a ratio of 86:114 (w:w) was added. The cans were then sterilized at 127 °C for 16 min, and

98 subsequently cooled to 30 °C for 10 min.

99 *2.2.2. Preliminary tests*

100 Preliminary tests were performed to address the effect of modifying single parameters.

101 Soaking and blanching parameters were thus modified independently (Table 1). An

102 "antinutritional" compound analysis was conducted after each step, but not on the final

103 canned products.

104 • *Modification of the soaking step (Table 1)*

105 Hot water soaking (water at 80°C) was done in a Stephan® Combicut TC System (Stephan

106 Bvba, Nazareth, Belgium) for 2h. The "soft" water (HT=6 °f) was Volvic water (Société des

107 eaux de Volvic – Danone group, Volvic, France) and the "hard" water (HT=184 °f) was

108 Hépar water (Nestlé Waters Vosges, Vittel, France). Chickpeas were soaked at different

109 ratios: 1:3, or 2:3, or 1:10 (chickpeas:water; w:w). An agitation step could be added either

110 with a pump (Yamada, Arlington Heights, IL, USA) during soaking at 15 °C or with paddles

111 in a Stephan® Combicut TC during soaking at 80°C. Water could be replaced at the midpoint

112 of the soaking time. Citric acid (La Littorale - Erbslöh, Servian, France) and sodium

113 bicarbonate (Cérébos, Levallois-Perret, France) were used for a soaking at pH=5 and pH=9,

114 respectively. Finally, a test was done after the addition of a germination step performed before

115 soaking and blanching: chickpeas were immersed for 12 h in water (1:3, w:v), then kept

116 between thick layers of damp cotton cloth and allowed to germinate for 3 days at room

117 temperature (20-25°C).

118 • *Modification of the blanching step (Table 1)*

6

119   Tests on the blanching step were carried out on chickpeas previously soaked via the control

120   process. A longer blanching time was also tested (20 min). Moreover, the immersion

121   blanching was changed into either steaming or cascading. Steaming consisted of a 5-min

122   exposure to steam using a steam oven (Bourgeois, Faverge, France). Cascading was

123   performed using vertically flowing water in a steam-heated autoclave (Auriol, Marmande,

124   France).

125   *2.2.3. Optimization tests on the global process (Table 2)*

126   Once the preliminary tests were analyzed, different processes combining the most efficient

127   soaking and blanching parameters were tested. For reasons of technological practicality, hot

128   soaking was done with a high chickpea-to-water ratio (1:10) for a shorter time (2h).

129   Conditioning and sterilization parameters were not modified. Contrary to the preliminary

130   tests, bioactive compounds were only analyzed in the final products.

131

132   **2.3. Texture analysis**

133   The texture of the canned chickpeas was analyzed to check whether process modifications

134   induced changes in chickpea texture compared to chickpeas treated with the control process.

135   A multipurpose texture analyzer TA-plus (Lloyd Instruments, Farenham, UK) was used with

136   a method adapted from Chenoll and colleagues (Chenoll et al., 2009). The peak compression

137   force (N) was recorded as the hardness of the chickpeas.

138

139   **2.4. In vitro digestion experiments**

140   The test meals used for in vitro digestion experiments were made of 3.35 g of canned

141   chickpeas and 100 mg of Isio 4 oil. Isio 4 oil contributed minor amounts of fat-soluble

142   vitamins to the meal, without consequences to data interpretation, as oil was present in all

143   conditions. Each test meal was supplemented with 125 µM of fat-soluble vitamin D or K

144   dissolved in the oil. The in vitro digestion experiments were conducted as previously

145   described (Malapert et al., 2018). Aliquots of digesta and mixed micelles were taken and

146   stored at −80 °C under a nitrogen atmosphere until analysis. Bioaccessibility was calculated

147   as the percentage of vitamin recovered in mixed micelles divided by the amount of vitamins

148   recovered in the digesta at the end of the digestion.

149

150   **2.5.   Fat-soluble vitamin absorption by intestinal cells in culture**

151   *2.5.1.   Preparation of micelles from in vitro digestion*

152 Preliminary cytotoxicity tests were conducted. Briefly, Caco-2 cells were seeded and grown

153 for 3 days in 96-well plates, prior to a 1 h-incubation with diluted micelles obtained from in

154 vitro digestion. The cell culture medium was then removed, and a MTT solution (1 mg/mL)

155 was added for 2 h at 37 °C. The supernatant was then replaced by DMSO (100 μL per well)

156 and the absorbance was measured at 540 nm. In accordance with these tests, fat-soluble

157 vitamin micelles obtained from in vitro digestions were diluted in DMEM at a 1:6 ratio (v:v)

158 for further cell experiments (data not shown).

159   *2.5.2.   Cell experiments*

160 Caco-2 clone TC7 cells were cultured as previously described (Goncalves et al., 2016). For

161 each experiment, cells were seeded and grown for 2 weeks in 6-well plates (24 mm diameter,

162 1 μm-pore-size polycarbonate membrane; Becton Dickinson, Le Pont-de-Claix, France).

163 Twelve hours prior to each experiment, the complete medium (i.e., DMEM supplemented

164 with 16% FBS, 1% non-essential amino acids and 1% antibiotics) was replaced by serum-free

165 complete medium. At the beginning of the experiment, cell monolayers received diluted

166 micelles obtained from in vitro digestions. Cells were incubated for 1h at 37 °C with the

167 micelles. Next, cells were washed with 0.5 mL of ice-cold PBS to eliminated adsorbed fat-

168 soluble vitamins, then scraped and collected in 0.5 mL of ice-cold PBS. Vitamin absorption

169 percentage was calculated by dividing the amount of vitamin recovered in cells by the total

170 amount of vitamin delivered to the cells (i.e., vitamin found in the medium plus vitamin

171 remaining in the cells at the end of the experiment). All samples were stored at −80 °C until

172 analysis.

173

174 **2.6. Chickpea lyophilization**

175 The canned chickpeas were first lyophilized for 48h in a Cosmos freeze dryer (Cryotec, Saint-

176 Gély-du-Fesc, France) and then ground into powder using a Proline 700W blender (Proline,

177 Paris, France).

178

179 **2.7. Bioactive compound assays**

180 *2.7.1. Phytate assay*

181 Phytates from chickpea powder were analyzed using a spectrophotometric method derived

182 from that published by Dost & Tokul (Dost & Tokul, 2006). Briefly, phytates were extracted

183 from 200 mg of powder with 10 mL of chloridric acid (0.5 M, Fisher Scientific, Saint-

184 Herblain, France) for 1 h at room temperature (20-25°C). The extract was then centrifuged 5

185 min at 800×g and the supernatant was recovered. One hundred µL of supernatant was added

186 to 0.9 mL of water and 2 mL of iron (III) chloride hexahydrate. This mixture was mixed for

187 2h and 30 min at 40 °C and centrifuged 5min at 800×g. The absorbance of the supernatant

188 was measured at 480 nm against water using a Shimadzu UV 8000 spectrophotometer

189 (Shimadzu, Marne-la-Vallée, France). The results were expressed as phytic acid equivalent.

190 *2.7.2. Saponin assay*

191 Saponins were analyzed by a spectrophotometric method inspired by the method previously

192 published by Li and colleagues (Li et al., 2010). The first step was the extraction of saponins

193 from the chickpea powder. One gram of flour was added to 5 mL of methanol (80% in water),

194 mixed for 24 h at room temperature (20-25°C) and centrifuged 5 min at 800×g. This step was

195   repeated twice. Supernatants (0.2 mL) were completed with 0.3 mL of methanol (80% in

196   water), 0.5 mL of vanillin (8% in water), and 5 mL of sulfuric acid (72%). The mixtures were

197   incubated at 60°C for 10 min and placed into an ice bath. The absorbance of the mixtures was

198   measured at 544 nm against methanol (80% in water). The results were expressed as

199   soyasaponin I equivalent.

200   *2.7.3. Tannin assay*

201   Tannins were extracted from 0.12 g of chickpea powder with 12 mL of water. Two milliliters

202   of a water/methanol mixture (1:1; v:v), 4 mL of tannin extract, and 1 mL of DMACA solution

203   were mixed together. After 20 min, samples were centrifuged 5 min at 800×g and the

204   absorbance of the supernatants was measured at 640 nm against a water/methanol mixture

205   (1:1). The results were expressed as catechin equivalent.

206

207   **2.8. Lipid, protein, fiber and mineral assays**

208   Lipids were extracted from the chickpea powder. Briefly, 0.5 g of powder, 2 mL of distilled

209   water, and 5 mL of ethyl acetate were mixed for 6 min. The extract was centrifuged 15 min at

210   1100 × g and the supernatant was recovered. This procedure was repeated once with the

211   addition of ethyl acetate. To wash the extract, 3 mL of water and 0.5 mL of ethanol were

212   added to the supernatant, and the mixture was vortexed for 5 min and centrifuged 10 min at

213   1100×g. Pooled supernatants were evaporated to dryness under nitrogen. Total lipids were

214   measured by weighing the dry extracts.

215   The analysis of protein, fibers, magnesium and iron analysis was subcontracted to

216   Phytocontrol Agrifood (Nîmes, France), which is accredited to conduct these specific analyses

217   (Cofrac #1-1904).

218

219   **2.9. Vitamin assay**

220   *2.9.1. Vitamin E (γ-tocopherol) and lutein from chickpeas*

221 Vitamin E (γ-tocopherol) and lutein were extracted from 250 mg of chickpea powder using

222 the following method: 1 mL of distilled water was added to the sample. The internal standard

223 (retinyl acetate) was added to the sample in 1 mL of ethanol. The mixture was extracted twice

224 with 4 mL of hexane. After centrifugation (2500×g, 10 min at 4 ∘C), the hexane phase was

225 evaporated to dryness under nitrogen. The dried extract was dissolved in 200 µL of methanol–

226 dichloromethane (65:35, v:v). A final volume of 180 µL was used for HPLC analysis. Fat-

227 soluble vitamins and carotenoids were separated as previously described (Gleize et al., 2012).

228 Luteine and γ-tocopherol were detected at 445 and 298 nm, respectively, and identified by

229 retention time in comparison with pure standards. Quantification was performed using

230 Chromeleon 6.8 software (ThermoFisher Scientific, Villebon sur Yvette, France) to compare

231 the peak area with standard reference curves.

232   *2.9.2. Vitamin D and vitamin K assay from in vitro digestion and cell samples*

233 Vitamin D and vitamin K were extracted from 200 µL of digesta and mixed micelles, and

234 from 500 µL of apical medium and cells. The internal standard was retinyl acetate. Both

235 extraction and HPLC analysis were performed according to Antoine and al. (Antoine et al.,

236 2021) and Goncalves et al. (Goncalves et al., 2014), respectively. Vitamins were identified by

237 spectral analysis and/or retention time and co-injection in comparison with pure standards.

238 Quantification was performed using Chromeleon 6.8 software to compare the peak area with

239 standard reference curves.

240   *2.9.3. Folate assay*

241 Folates from chickpea powder were analyzed by a HPLC method derived from the method

242 published by Ruggeri and colleagues (Ruggeri et al., 1999). Powder (0.75g) was added to 10.5

243 mL of extraction buffer in ebullition (HEPES buffer, pH 7.85, containing 2% (w:v) sodium

244 ascorbate and 10 mM 2-mercaptoethanol). After flushing the samples with nitrogen, they

245 were heated for 10 min in a boiling water bath and homogenized with an Ultra Turrax device

246 for 30 s at a speed of 13500 rpm. Extraction buffer was then added (qsp 15 mL). The pH was

247 adjusted to 4.9 and 600 µL of chicken pancreas conjugase, 120 µL of rat serum and 600 µL of

248 α-amylase were added. Samples were incubated under agitation at 37°C for 3 h. The pH was

249 adjusted to 7.0, then 1.2 mL of protease were added, and samples were incubated for 1h as

250 previously described. Enzymes were then inactivated by boiling for 10 min. Samples were

251 centrifuged for 30 min at 2000×g. Supernatants were transferred to a strong anion exchanger

252 (SAX) column (ThermoFisher Scientific) to purify folates (solid phase extraction). A final

253 volume of 20 µL was used for HPLC analysis. The HPLC system comprised a Dionex

254 separation module (P680HPLC Pump and ASI-100 Automated Sample Injector, Dionex, Aix-

255 en-Provence, France) and a Jasco fluorimetric detector (Lisses, France). Analyses were

256 performed on a HypersilTM ODS C18 column (150×4.6 mm; 5µm) coupled with a 10×4 mm

257 C18-5µm HypersilTM ODS guard column (FisherScientific, Illkirch, France) kept at a

258 constant temperature (40 °C). The mobile phase consisted of a gradient of acetonitrile and

259 30mM phosphate potassium buffer at pH 2.6. The flow rate was 0.8 mL/min. The run time

260 was 35 min. The gradient profile of the mobile phase was set at 5-95% and changed linearly

261 to 17-83% from minute 9 to 30. The mobile phase then changed back to 5-95% from minute

262 30 to 35. 5-methyltetrahydrofolate, which is the main form of folate found in chickpeas

263 (Ruggeri et al., 1999), was detected at 356 nm after excitation at 290 nm, and identified by

264 retention time compared with a pure standard. Quantification was performed using

265 Chromeleon software to compare the peak area with standard reference curves.

266

267 **2.10. Statistics**

268 For all groups, data were expressed as mean ± SEM. Statistical analyses were performed

269 using GraphPad Prism software, version 8.4.3 (GraphPad Software, San Diego, California,

270 U.S.A.). Prior to ANOVA (fixed-effects models), data were tested for equality of variances.

271 The distribution of all dependent variables was graphically checked and was considered

12

272 normal. Tukey's test was used as a post-hoc test for pairwise comparisons. Values of $p \leq 0.05$

273 were considered significant.

3.   Results

3.1.   Chickpea contents in phytates, saponins and tannins can be decreased by modifying canning parameters

3.1.1. *Phytate and saponin contents*

Figures 1A and 1B show the results of preliminary tests. We observed a significant decrease of phytate content after hot soaking (PT1), mixed soaking (PT7) and acid soaking (PT9) (-36.0 ± 4.9%, -25.0 ± 7.0%, -25.0 ± 3.2%, $p \leq 0.05$, respectively), but also after soaking with low-ionized water (PT4), with a low chickpeas-to-water ratio (PT6) or after water replacement (PT8) (-19.0 ± 2.6%, -18.0 ± 6.3%, -17.0 ± 2.7%, $p \leq 0.05$, respectively) (Figure 1A). Moreover, Figure 1B shows that phytate content was significantly reduced when the chickpeas were blanched by cascading (PT14, -7.0 ± 1.7%, $p \leq 0.05$).

Concerning saponins, there was no significant diminution of their concentrations when soaking and blanching parameters were modified. Saponin content was slightly reduced when soaking was done with low-ionized water (PT4) or with a low chickpeas-to-water ratio (PT6) (-13.0 ± 3.1%, -10.1 ± 1.2%, respectively), but these values remained non-significantly different (Figure 1 A and 1B).

We then explored the phytate and saponin content in canned and sterilized chickpeas after these different parameters were combined. As shown in Figure 1C, phytate and saponin concentrations were both significantly decreased when chickpeas were canned after i) hot soaking with water replacement followed by immersion blanching (T3) (-26.0 ± 1.9%, -29.0 ± 1.7%, $p \leq 0.05$, respectively), ii) hot mixed soaking followed by cascading blanching (T8) (-37.0 ± 1.6%, -35.0 ± 2.2%, $p \leq 0.05$, respectively), and iii) hot soaking with water replacement followed by cascading blanching (T9) (-28.0 ± 0.7%, -39.0 ± 2.4%, $p \leq 0.05$, respectively). Moreover, we observed a significant reduction of saponin content after soaking at 15 °C with

299   i) low-ionized water and blanching by cascading (T10), ii) agitation blanching by immersion

300   (T5), iii) water replacement and cascading blanching (T12) and vi) germination (T13) before

301   soaking and blanching (-21.0 ± 2.1%, -17.0 ± 2.1%, -16.0 ± 1.7%, -29.0 ± 6.2%, p≤0.05,

302   respectively). These 7 conditions were chosen for further analysis.

303   *3.1.2. Tannin content*

304   Our results show that tannin content was reduced in 3 conditions among 7 (Figure 1D): i) hot

305   soaking with water replacement followed by immersion blanching (T3), ii) hot soaking with

306   water replacement followed by cascading blanching (T9), and iii) germinated chickpeas (T13)

307   (-44.1 ± 8.6%, -40.0 ± 7.6%, -43.0 ± 6.8%, p≤0.05, respectively). However, tannin content

308   increased significantly relative to the control when chickpeas were soaked in hot mixed water

309   and blanched by cascading (T8) (+74.0 ± 20.4%, p≤0.05).

310

311   **3.2.   The texture of optimized canned chickpeas is different from that of control**

312   **chickpeas**

313   The texture of the different batches of canned chickpeas was analyzed to check if process

314   modifications changed the chickpea texture compared to the control condition. Table 3 shows

315   that there was a significant increase in chickpea hardness after hot soaking followed by

316   immersion blanching: +43.9 ± 3.8% for hot soaking followed by immersion blanching (T1),

317   +48.4 ± 3.8% for mixed and hot soaking followed by immersion blanching (T2) and +41.9 ±

318   5.2% for hot soaking with water replacement followed by immersion blanching (T3).

319   Moreover, we observed a significant decrease in chickpea hardness after soaking at 15 °C and

320   blanching by cascading: -33.1 ± 3.1% after soaking at 15 °C with low-ionized water followed

321   by cascading blanching (T10) and -26.2 ± 4.1% after mixed soaking at 15 °C followed by

322   cascading blanching (T11).

323

3.3.   Protein, mineral and vitamin contents can be modified after optimized canning processes compared with control canning.

Table 4 shows the concentrations of proteins, fibers, iron and magnesium, lipids and vitamins in the 7 optimized canned chickpeas and in control chickpeas. There was a reduction in protein content after soaking at 15 °C with i) low-ionized water followed by cascading blanching (T10), ii) agitation followed by an immersion blanching (T5) and iii) a germination step (T13) (-35.8 ± 0.1%, -19.5 ± 0.1%, -23.6 ± 0.1%, p≤0.05, respectively). The magnesium content decreased similarly (-35.9 ± 0.04%, -21.5 ± 0.05%, -27.6 ± 0.06%, p≤0.05, respectively), but increased after hot soaking when water is replaced, followed by immersion blanching (T3, +12.8 ± 0.05%, p≤0.05). Moreover, our results show that relative to the control, iron content was increased after hot soaking with i) water replacement and immersion blanching (T3), ii) agitation and cascading blanching (T8) and iii) water replacement and cascading blanching (T9): +30.7 ± 1.1%, +24.5 ± 0.8%, +40.0 ± 1.6%, p≤0.05, respectively. We also found a higher iron content in chickpeas soaked in water at 15 °C with either i) low-ionized water (T10) or ii) water replacement (T12), both followed by a cascading blanching (+26.7 ± 0.8%, +67.3 ± 1.1%, p≤0.05, respectively).

Additionally, we noticed that germination induced a decrease in lipid content (-36.1 ± 4.6%, p≤0.05) (Table 4).

Vitamin E content significantly increased for 4 conditions relative to the control: +94.2 ± 16.7% for hot soaking with water replacement and cascading blanching (T9), +82.1 ± 17.3% for soaking at 15 °C with low-ionized water followed by cascading blanching (T10), +130.2 ± 14.3% for mixed soaking at 15 °C and immersion blanching (T5), and +102.4 ± 11.2% for soaking at 15 °C with water replacement followed by cascading blanching (T12). Conversely, a significant decrease in lutein content occurred in the germination condition relative to the control condition (-63.8 ± 3.8%, p≤0.05). Finally, 5-methyltetrahydrofolate content decreased

349   significantly for all conditions (from -12.1± 4.7 % to -97.1± 0.1 %), except for a mixed hot

350   soaking followed by a cascading blanching (T8).

351

352   **3.4.   Vitamin D and K bioavailability is not improved in meals containing optimized**

353   **canned chickpeas**

354   As shown in Figure 2A, there was no difference in vitamin D and K bioaccessibility between

355   meals containing control chickpea and meals containing optimized canned chickpeas. Similar

356   results were obtained regarding vitamin D and K uptake by differentiated Caco-2 cells (Figure

357   2B).

358

4. Discussion

Pulse components such as phytates, saponins and tannins have previously been shown to modulate fat-soluble vitamin bioavailability (Antoine et al., 2021; Margier et al., 2019). The objective of this study was to find technological solutions to address this negative effect. We thus modified several parameters of the canning process to decrease phytate, saponin and tannin content in chickpeas.

To this aim, we first modified some process parameters independently of each other. Our results indicate that phytate content can be significantly decreased by modifying soaking or blanching parameters. Under the conditions of our study, this is likely due to an improved release of these compounds, caused by the agitation of chickpeas during soaking, the increase of chickpea coat permeability at 80°C (Tabekhia & Luh, 1980), osmosis for a soaking with low-ionized water (Vijayakumari et al., 1996), a low chickpeas-to-water ratio or with water replacement/ cascading. Moreover, phytates can also be degraded by phytase during either hot or acid soaking, as phytase activity is increased by heat (Khokhar & Chauhan, 1986) and optimized at a pH of 4.6 (Wodzinski & Ullah, 1996).

According to our data, saponin content was not significantly reduced relative to the control condition when a single parameter was changed. A mild reduction in saponin content was observed after soaking with low-ionized water and with a low chickpeas-to-water ratio, reflecting previously published results (Shi et al., 2009), but these differences remained non-significant, likely because of the variability of the results. Yoshiki and colleagues showed that saponin content was decreased after blanching for 30 min (Yoshiki et al., 1998). Our blanching conditions (10 or 20 min) may thus not have been long enough.

After performing preliminary tests using single modifications, we then combined the most effective processes (13 conditions compared with the control process). The decreases in

384 phytate and saponin content were greater than those in preliminary tests because of the

385 combinations of different parameters, followed by the use of hot brine and sterilization, in

386 accordance with previous data (Jood et al., 1986; Tabekhia & Luh, 1980).

387 It is noteworthy that no difference in phytate and saponin contents were observed after

388 germination. According to previous results (Kyriakidis et al., 1998)(El-Adawy, 2002),

389 phytates were expected to decrease due to a rise in phytase activity during germination. We

390 hypothesize that we failed to observe a decrease in phytates due to the method used - as we

391 did not perform a purification step, we may also have detected the product of phytic acid

392 hydrolysis (phosphoric acid) together with the remaining phytic acid. Saponin assay may also

393 detect the hydrolysis product of saponins: sapogenin (Hiai et al., 1976; Price et al., 1986)

394 together with remaining saponins. In addition, Ruiz and colleagues showed a reduction in

395 saponin content 6 days after germination (Ruiz et al., 1996), while our germination period was

396 of only 3 days. Ayet and colleagues observed a decrease in saponin content 3 days after

397 germination, but it was followed by an increase after 6 days (Ayet et al., 1997). Different

398 germination times should thus be tested to fully comprehend these phenomena, and other

399 analytical methods such as mass spectrometry detection should be set up for further

400 investigations.

401 Following these intermediate results, 7 conditions inducing the greatest decreases in both

402 phytate and saponin content were ultimately selected to be fully characterized. Changes in

403 texture were observed between these conditions, but further investigations including a sensory

404 analysis are needed to confirm whether consumers would perceive these changes.

405 The first additional compound evaluated were tannins. A decrease in tannin content was

406 observed for the 2 conditions incorporating water replacement during the soaking step. As

407 tannins are water-soluble, they are likely better released by osmosis during soaking when the

408 water is replaced (Deshpande et al., 1986). A decrease in tannin content was also observed

19

409 after germination. This is consistent with previous works (Khandelwal et al., 2010)(Rao &

410 Deosthale, 1982): polyphenol oxidase activity increases during germination and induces

411 tannin hydrolysis. Conversely, an increase in tannin content occurred after hot (80 °C) mixed

412 soaking and cascading blanching. We suggest this is due to an inactivation of polyphenol

413 oxidase (Buckow et al., 2009).

414 We then checked whether compounds of interest remained unaltered after modifying the

415 canning process. Fiber content was not modified. Although the presence of fibers can

416 decrease vitamin bioavailability (Margier et al., 2019), its preservation is important because

417 most people lack fiber in their diets (Lairon et al., 2003). In accordance with previous data

418 (El-Adawy, 2002), lipid content decreased after germination only, as sprouting chickpeas use

419 lipids for growth. Lutein content also decreased during germination, presumably for the same

420 reason.

421 Water-soluble compounds such as minerals or proteins were expected to decrease when

422 soaking or blanching were optimized. Protein content decreased moderately under 3

423 conditions (immersion blanching, cascading blanching and germination) that likely increased

424 chickpea moisture and softness. These increases may facilitate the release of water-soluble

425 compounds. Our results differ from previous data (Chitra et al., 1996)(El-Adawy, 2002), but

426 authors compared raw chickpeas with germinated chickpeas.

427 Surprisingly, iron content significantly increased under 4 conditions (as did magnesium

428 content in 1 condition). We suggest that this effect may be due either to i/ lower phytate

429 content, leading to fewer quenched cations, or to ii/ greater disruption of the chickpea matrix,

430 which may favor mineral extraction prior analysis. Vitamin E content also increased under 4

431 conditions. Much as with iron, we suggest that greater softening of the vegetal walls would

432 make vitamin E more extractable, as has been observed in kidney beans (Margier et al., 2018).

433 Finally, 5-methyltetrahydrofolate contents were reduced after most of the optimized processes

434 relative to the control process. Although heat does not appear to be the main factor in

435 inducing folate loss (Delchier et al., 2013), folates are known to be highly susceptible to

436 leaching during food transformation processes (Scott et al., 2000).

437 In a second set of experiments, we assessed vitamin bioavailability after in vitro digestion of

438 test meals made with the different batches of canned chickpeas. We focused especially on

439 vitamin D_3 (cholecalciferol) and vitamin K_1 (phylloquinone), as these vitamins have very

440 different structures and thus may behave differently, as previously observed (Antoine et al.,

441 2021; Margier et al., 2019).

442 No difference was observed between the different conditions in term of bioaccessibility (i.e.,

443 the vitamin amount transferred from the solid phase of the meal to the aqueous phase of the

444 digesta during in vitro digestion). No difference was observed between the different

445 conditions in term of cellular uptake by Caco-2 cells in culture, either. This means that the

446 decreases in phytate, saponin and/or tannins contents in optimized canned chickpeas were not

447 important enough to modulate micelle formation, vitamin incorporation in mixed micelles or

448 vitamin membrane transporter functioning. In a previous study, we observed significant

449 differences in terms of vitamin K bioaccessibility and bioavailability when we compared

450 household cooked chickpeas vs canned chickpeas (Margier et al., 2019). The decreases in

451 phytate, saponin, tannin and fiber content were of -24%, -4%, -17,5% and -22%, respectively.

452 We suggest that the difference between the two studies may be due partly to a decrease in

453 fiber content not observed in the present study.

454 In conclusion, we identified different soaking and blanching parameters in the present study

455 that promoted decreases in phytate, tannin and saponin contents of canned chickpeas (-16 to -

456 44%). Further experiments are required to validate these data in other chickpea varieties and/

457 or in other pulse species. Additionally, when using these chickpeas in tests meals, we did not

458 observe any improvement in the bioaccessibility and the cellular uptake of vitamins D and K

459 as compared with the control conditions. Although further modification of the canning

460 process could lead to greater decreases in bioactive amounts, these modifications may be

461 more expensive and less environmentally friendly. We thus suggest that upstream solutions,

462 such as improved cultivar choice, should also be considered.

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613

614

Figure legends

616

617 **Figure 1: Bioactive compound contents in canned chickpeas.**

618 *A: Phytates and saponin content under different soaking conditions after preliminary tests. B:*

619 *Phytate and saponin content under different blanching conditions after preliminary tests.*

620 During preliminary tests (PT), canning process parameters were modified independently.

621 Phytate (white bars) and saponin (black bars) contents were analyzed in chickpea powder

622 after each step, but not in the final product. Soaking was done with hot (80°C), acidic (pH 5),

623 alkaline (pH 9), mixed, low- (HT = 6 °f) and high-ionized (HT = 184 °f) water, but also in

624 different chickpeas-to-water ratios (2:3 or 1:10), with water replacement and for a longer time

625 (24h). Blanching was tested for a longer time (20 min) and with different methods (cascading

626 or steaming). Data are expressed as mean ± SEM (n = 3).

627 *C: Phytate and saponin content in canned chickpeas.* Different processes combining the most

628 efficient soaking and blanching parameters were tested (T). Contrary to preliminary tests,

629 phytate (white bars) and saponin (black bars) contents were analyzed on the final product

630 powders, after conditioning and sterilization. Data are expressed as mean ± SEM (n = 9).

631 *D: Tannin content in canned chickpeas.* Once optimized conditions (low phytate and saponin

632 content) were chosen, tannins were analyzed in chickpea powders. Data are expressed as

633 mean ± SEM (n = 9).

634 A difference of $p \leq 0.05$ was considered significant. Cp: Control Process; Germ: Germination.

635 dm = dry matter.

636

637

638 **Figure 2. Vitamin D and K bioavailability in the presence of optimized chickpeas**

639   **A**: Vitamin D and K bioaccessibility. Test meals were made with the different canned

640   chickpeas and supplemented with vitamin D or vitamin K. Bioaccessibility was calculated as

641   the percentage of the vitamins recovered in mixed micelles divided by the vitamin amount

642   recovered from the digesta at the end of the digestion.

643   **B**: Vitamin D and K intestinal absorption. Mixed micelles obtained from in vitro digestions

644   were delivered to Caco2-TC7 cells after appropriate dilution. The apical side and the well

645   monolayers were collected after 1h of incubation. The vitamin absorbed was calculated as the

646   percentage of the vitamins recovered from the cells divided by the total vitamin amount

647   delivered to the cells .

648   Vitamin D: white bars; vitamin K: black bars; CP: Control Process; Germ: Germination. Data

649   are expressed as mean $\pm$ SEM (n = 6). A difference of $p \leq 0.05$ was considered significant.

650 **Tables**

651

652 **Table 1: Chickpea soaking and blanching parameters for preliminary experiments**

	SOAKING												BLANCHING				
Parameters	**CP**	**PT 1**	**PT 2**	**PT 3**	**PT 4**	**PT 5**	**PT 6**	**PT 7**	**PT 8**	**PT 9**	**PT 10**	**PT 11**	**Parameters**	**CP**	**PT 12**	**PT 13**	**PT 14**
Temperature (°C)	15	**80**	15	15	15	15	15	15	15	15	15	15	**Temperature (°C)**	90	90	90	90
Time (h)	14	14	**24**	14	14	14	14	14	14	14	14	14	**Time (min)**	5	**20**	5	5
Water hardness (°f)	30	30	30	**184**	**6**	30	30	30	30	30	30	30	**Mode**	Immersion		**Steaming**	**Cascading**
Chickpeas-to-water ratio	1:3	1:3	1:3	1:3	1:3	**2:3**	**1:10**	1:3	1:3	1:3	1:3	1:3	-	-	-	-	-
Agitation	-	-	-	-	-	-	-	Yes	-	-	-	-	-	-	-	-	-
Water replacement	-	-	-	-	-	-	-	-	Yes	-	-	-	-	-	-	-	-
Additive	-	-	-	-	-	-	-	-	-	Acid	Base	-	-	-	-	-	-
Germination	-	-	-	-	-	-	-	-	-	-	-	Yes	-	-	-	-	/

653

654 Different preliminary tests (PT) were done by modifying single soaking and blanching parameters of the control process (CP).

655

656

657   **Table 2: Optimization tests on global process**

		Control	T 1	T 2	T 3	T 4	T 5	T 6	T 7	T 8	T 9	T 10	T 11	T 12	T 13
Soaking	**Temperature (°C)**	15	**80**	**80**	**80**	15	15	15	**80**	**80**	**80**	15	15	15	15
	Time (h)	14	**2**	**2**	**2**	14	14	14	**2**	**2**	**2**	14	14	14	14
	Water hardness (°f)	30	30	30	30	**6**	30	30	30	30	30	**6**	30	30	30
	Chickpeas-to-water ratio	1/3	**1/10**	**1/10**	**1/10**	1/3	1/3	1/3	**1/10**	**1/10**	**1/10**	1/3	1/3	1/3	1/3
	Mixing	No	No	**Yes**	No	No	**Yes**	No	No	**Yes**	No	No	**Yes**	No	No
	Water replacement	No	No	No	**Yes**	No	No	**Yes**	No	No	**Yes**	No	No	**Yes**	No
	Additive	No	No	No	No	No	No	No	No	No	No	No	No	No	No
	Germination	No	No	No	No	No	No	No	No	No	No	No	No	No	**Yes**
Blanching	**Temperature (°C)**	90													
	Time (min)	5													
	Mode	Immersion							Cascading						Immersion

658

659   Different tests (T) based on the results of preliminary tests and combining the most efficient

660   soaking and blanching parameters were performed. Six soaking conditions were defined,

661   followed either by immersion blanching or cascading blanching. Conditioning and

662   sterilization parameters remained unchanged.

663

664   Table 3. Canned chickpea texture

665

	Texture Maximal applied force (N)
Control	9.58 ± 0.5
T1	13.77 ± 0.4 *
T2	14.02 ± 0.4*
T3	13.60 ± 0.5*
T4	8.33 ± 0.4
T5	8.60 ± 0.6
T6	12.10 ± 0.9
T7	10.14 ± 0.3
T8	10.00 ± 0.4
T9	9.50 ± 0.4
T10	6.53 ± 0.3*
T11	7.07 ± 0.4*
T12	8.50 ± 0.55

666

667   A compression force was applied on the pulses using an Uniaxial Microsystem TA XT texture

668   analyzer to determine the texture (compression forces are positively correlated with seed

669   hardness). Data are expressed as mean ± SEM (n = 30). A difference of $p \leq 0.05$ was

670   considered significant.

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672

673

Table 4. Protein, lipid, fiber, mineral, fat-soluble vitamin and folate content in canned
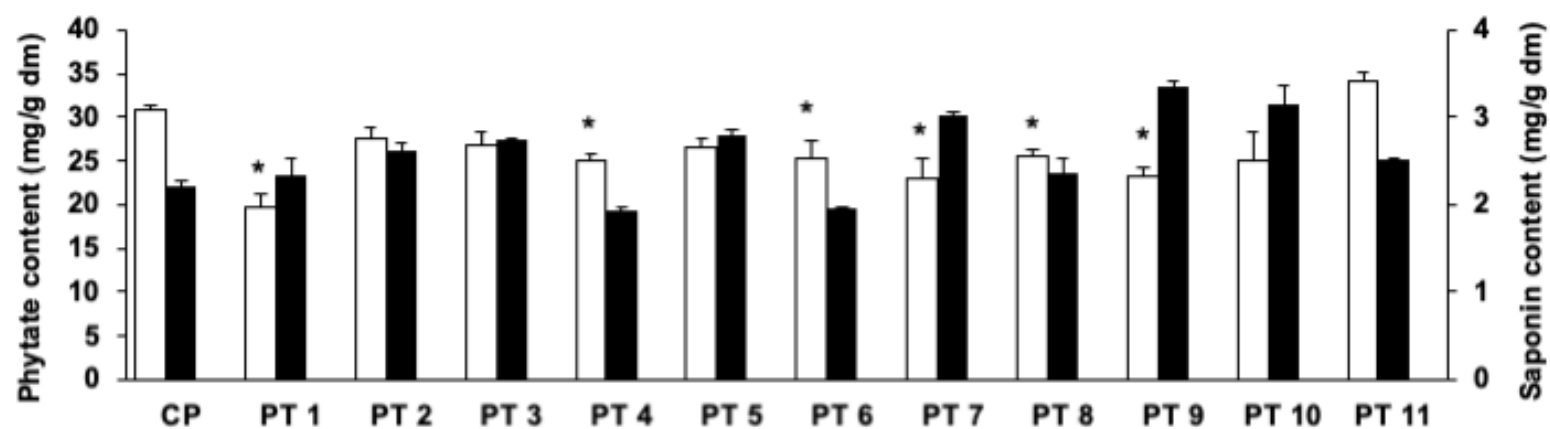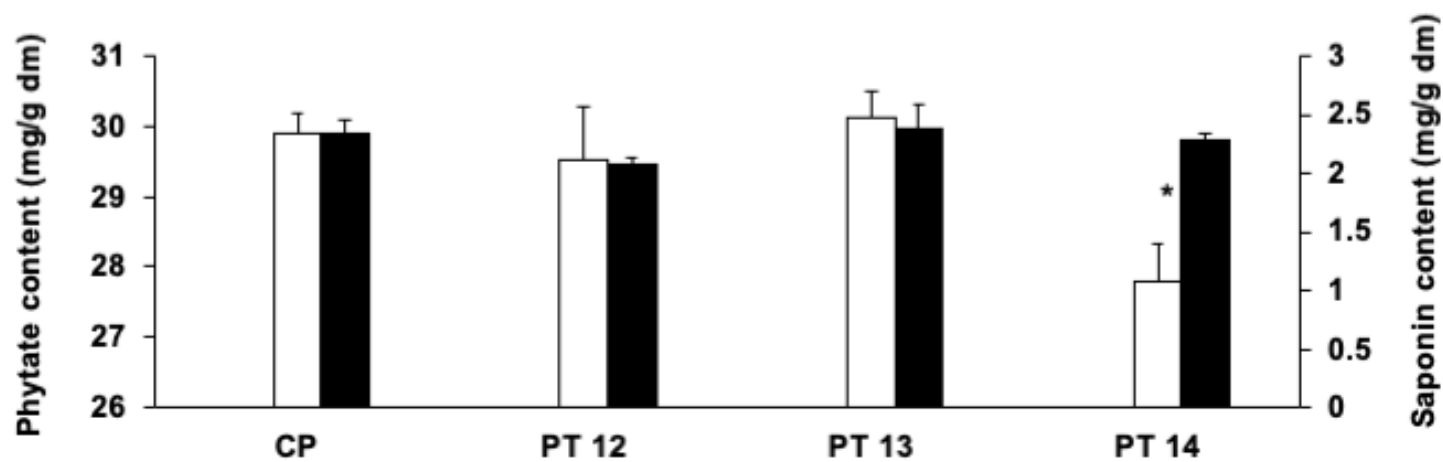
chickpeas

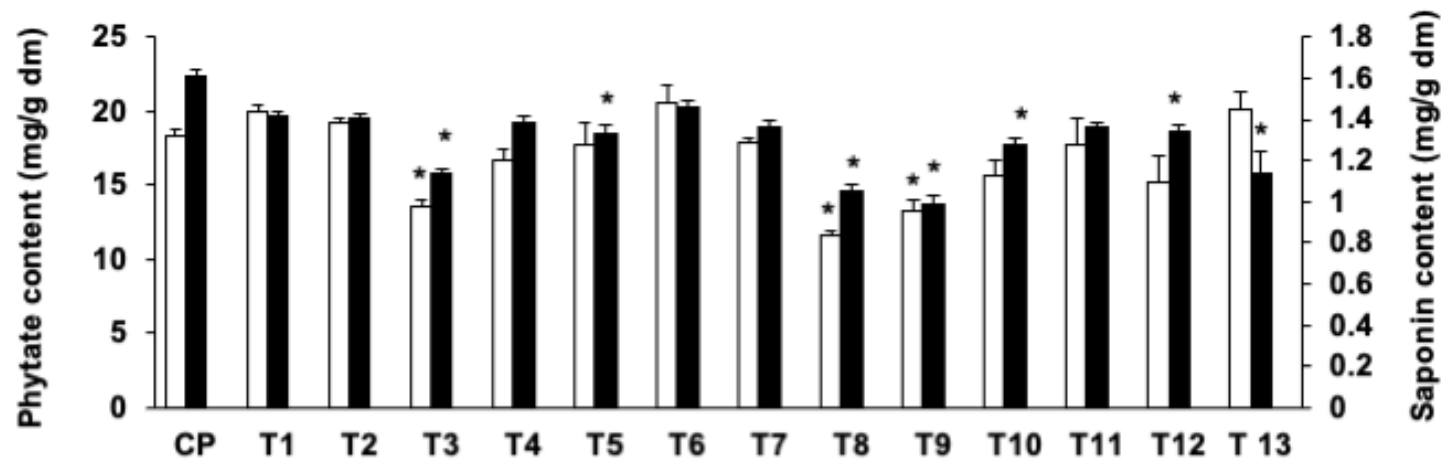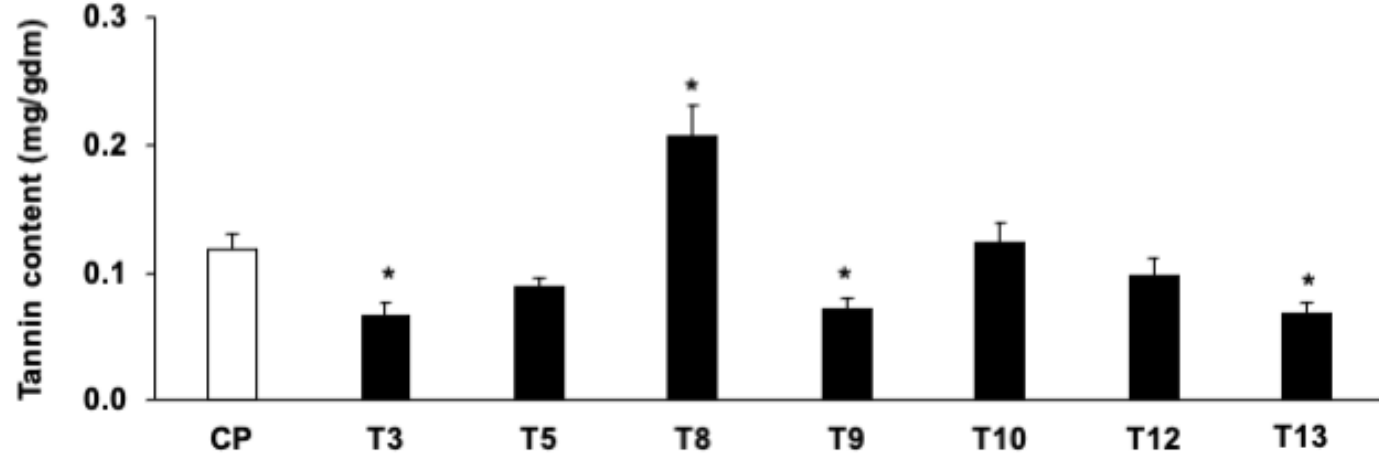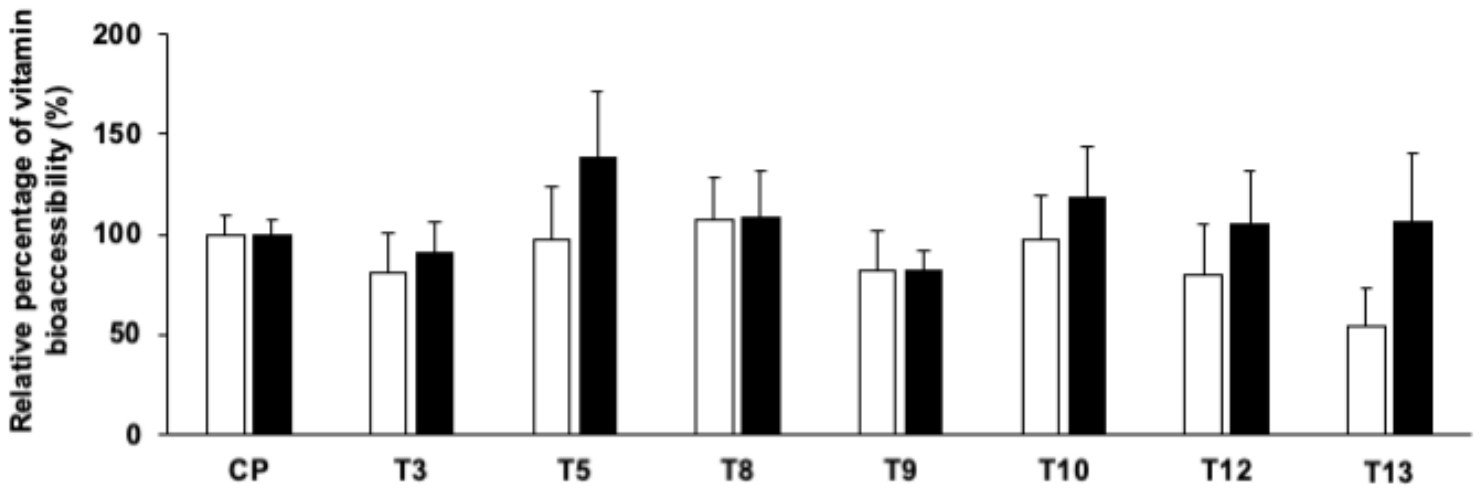|	Control process	T3	T5	T8	T9	T10	T12	T13
Proteins (g/100g dm)	23.1 ± 1.0	23.5 ± 1.1	18.6 ± 0.9*	21.4 ± 0.1	22.2 ± 1.0	14.8 ± 0.7*	22.1 ± 1.0	17.6 ± 0.8*
Total lipids (g/100g dm)	5.0 ± 0.1	4.8 ± 0.2	6.4 ± 1.6	6.0 ± 0.6	5.40 ± 0.05	5.1 ± 0.1	4.0 ± 0.3	3.2 ± 0.2*
Total fibers (g/100g dm)	14.9 ± 2.0	15.7 ± 1.9	14.8 ± 2.1	16.6 ± 2.0	14.1 ± 1.9	16.6 ± 2.0	14.9 ± 2.01	14.6 ± 1.9
Vitamin E (mg/100g dm)	2.2 ± 0.4	3.0 ± 0.3	5.2 ± 0.3*	3.1 ± 0.1	4.3 ± 0.4*	4.1 ± 0.4*	4.6 ± 0.3*	2.3 ± 0.3
Lutein (µg/100g dm)	226.7± 36.0	198.1 ± 20.8	321.4 ± 34,1	158.7 ± 5.1	140.7 ± 18.8	255.3 ± 38.5	220.0 ± 17.1	82.1 ± 8.6*
5-methyl-tetrahydro-folate (µg/100g dm)	32.1 ± 9.6	6.4 ± 1.4*	0.9 ± 0.1*	28.2 ± 1.5	1.8 ± 0.3*	3.5 ± 0.8*	1.8 ± 0.4*	4.9 ± 1.3*
Magnesium (mg/100g dm)	131.6 ± 9.5	148.5 ± 10.7*	103.3 ± 7.3*	122.9 ± 8.9	140.7 ± 10.1	84.4 ± 6.0*	116.6 ± 8.3	95.2 ± 6.7*
Iron (mg/100g dm)	32.2 ± 1.7	42.0 ± 1.6*	32.5 ± 1.7	40.0 ± 1.7*	45.0 ± 3.2*	40.7 ± 1.7*	53.8 ± 3.5*	27.2 ± 1.9
Dry matter (g/ 100g)	34.2	35.7	33.9	34.9	35.5	34.4	33.4	35.7

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The nutritional characterization of canned chickpeas was performed on chickpeas processed

according to either the control process (CP) or our 7 optimized conditions (See Table 2). dm =

dry matter. Data are expressed as mean ± SEM (n = 3). A difference of $p \leq 0.05$ was

considered significant.

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A

Relative percentage of vitamin bioaccessibility (%)

CP · T3 · T5 · T8 · T9 · T10 · T12 · T13

B

Relative percentage of vitamin absorption by Caco-2 cells (%)

CP · T3 · T5 · T8 · T9 · T10 · T12 · T13