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Influence of lupin and chickpea flours on acrylamide formation and quality characteristics of biscuits

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## Food Chemistry

# Influence of lupin and chickpea flours on acrylamide formation and quality characteristics of biscuits

--Manuscript Draft--

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| <b>Abstract:</b>             | <p>Asparagine and sugars are direct precursors of acrylamide; however, proteins and fibres can also influence it. In this study, biscuits prepared replacing wheat flour with increasing concentrations (20, 40, 60%) of lupin or chickpea flour were investigated. Asparagine concentration was equalized in all formulas to isolate the effect of other flour characteristics on the acrylamide formation during baking.</p> <p>The results showed that replacing wheat flour with lupin flour increased acrylamide from 583.9 up to 1443 µg/kg after 9 min of baking, while 20-40% chickpea flour reduced acrylamide to 354.4-312.6 µg/kg. The acrylamide reduction using chickpea was attributed to the lower interaction between precursors resulting from both the coarser particle size and the lower reactivity of carbohydrate in presence of chickpea proteins. Chickpea addition did not affect the colour and texture of biscuits, opening the possibility for large-scale implementation of this mitigation strategy in formulas with a similar initial asparagine content.</p> |
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**Highlights:**

- Asparagine concentration was equalized in all biscuit formulas.
- The type of flour can strongly affect acrylamide formation in biscuits.
- Lupin flour was not effective in reducing acrylamide content in biscuits.
- Chickpea flour did not influence the texture and colour of the final biscuits.
- The use of chickpea flour is a promising strategy for acrylamide control in biscuits.

1 **Influence of lupin and chickpea flours on acrylamide formation and quality**  
2 **characteristics of biscuits**

3  
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25 influence it. In this study, biscuits prepared replacing wheat flour with increasing concentrations (20,  
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27 formulas to isolate the effect of other flour characteristics on the acrylamide formation during baking.  
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32 presence of chickpea proteins. Chickpea addition did not affect the colour and texture of biscuits,  
33 opening the possibility for large-scale implementation of this mitigation strategy in formulas with a  
34 similar initial asparagine content.

35

36 **Keywords:**

37 Acrylamide; Biscuits; Legume flours; Asparagine; Bakery products.

38

39 **Chemical compounds studied in this article:**

40 Acrylamide (PubChem CID: 6579); Acrylamide-d<sub>3</sub> (PubChem CID: 12209671); Asparagine  
41 (PubChem CID: 6267); Sucrose (PubChem CID: 5988).

42

43 **1. Introduction**

44 Bakery products, including biscuits, are popular foods worldwide. However, these products, together  
45 with coffee and potato products, contribute to the dietary intake of acrylamide (AA), a toxic  
46 compound classified as “probably carcinogenic to humans” (group 2A) by the International Agency  
47 for Research on Cancer. The formation of AA in foods is due to the simultaneous presence of reducing  
48 sugars and asparagine combined with processing conditions (temperatures above 120 °C and low  
49 humidity) triggering the Maillard reaction (Mesías et al., 2016).

50 International regulations about the maximum tolerable levels of AA in foods became more restrictive  
51 over the years (European Commission, 2007; 2011; 2013; 2017; 2019), calling for the application of  
52 mitigation measures at the food industry level. Asparagine in flours is the main AA precursor,  
53 therefore, several studies have investigated the effect of different flour sources and mixtures on the  
54 AA formation in bakery products (Miśkiewicz et al., 2012; Sazesh & Goli, 2020; Žilić et al., 2020).  
55 In general, it has been proved that cereal or non-cereal varieties having higher amounts of free  
56 asparagine resulted in biscuits with higher concentrations of AA (Manolache et al., 2019; Mesías et

57 al., 2016; Miśkiewicz et al., 2012; Sazesh & Goli, 2020). In contrast, Žilić et al. (2020) observed that  
58 asparagine concentrations in different flours tested (i.e. wheat, oats, rye, barley, triticale, maize) did  
59 not significantly correlate with AA concentrations measured in biscuits prepared with the different  
60 formulations. No correlation between asparagine concentration in the starting ingredient and AA in  
61 the final product was found also by Capuano et al. (2009) who prepared bread crisp with wheat, rye  
62 and whole-wheat flours and toasted them at different time-temperature conditions. These  
63 observations indicated that other flour compounds and properties can influence the extent of Maillard  
64 reaction and, consequently, the AA formation.

65 Some proteins characteristic can influence the AA formation in different food products (Miśkiewicz  
66 et al., 2012, 2020; Rydberg et al., 2003; Tareke et al., 2002). Rydberg et al. (2003) studied the effect  
67 of protein-rich ingredients (i.e. cod meat) added to potato-based products observing a reduction in  
68 AA in the final products up to 70%. It has been suggested that this effect may result from a protective  
69 action of the proteins by scavenging the AA formed. In another study, chickpea proteins extract  
70 showed a mitigation effect of AA formation in a biscuit-like low-moisture model system (Miśkiewicz  
71 et al., 2020). It was suggested that the observed 40% reduction of AA formation was due to the  
72 increased thermal stability of the reducing sugars by the chickpea proteins extract. In the presence of  
73 chickpea proteins extract, the carbohydrates presented a higher ordering of their crystallographic  
74 structures and this reduced their availability to react with asparagine and lead to AA formation  
75 (Miśkiewicz et al., 2020). On the other hand, legume flours are usually higher in dietary fibre content  
76 than cereal ones (Rebello et al., 2014). High-fibre okara flour (a soya by-product) has been shown to  
77 promote the Maillard reaction and hence AA formation in biscuits by reducing the water activity of  
78 the dough and thus increasing the concentration of AA precursors (Palermo et al., 2012). From the  
79 published studies, it is not easy to understand whether the differences in the concentration of AA  
80 found in the final biscuits are indeed solely related to variations in the initial amount of asparagine in  
81 the flours or due to the effect of the dietary fibre and protein content in the flours.

82 The aim of the present study was to investigate the potential of biscuit formulations prepared with  
83 different types of flour and a standardised starting content of asparagine in terms of AA mitigation.  
84 Biscuits were formulated by replacing 20, 40 and 60% of wheat flour with protein-rich legume flours  
85 from lupins and chickpeas. Asparagine was added proportionally to all formulations to have the same  
86 concentration in all biscuits. In this way, we were confident to evaluate the possible role of other  
87 flours characteristics, such as proteins and dietary fibres addition described previously, on AA  
88 formation. In addition to the chemical composition, several structure-related effects on the formation  
89 of AA during baking were investigated, together with the impact on the colour and texture  
90 characteristics of the final products.

91

## 92 **2. Materials and methods**

93

### 94 **2.1. Biscuit ingredients and chemicals**

95 Wheat flour (Molen De Vlijt, Wageningen, The Netherlands), lupin flour (Frank Food Products,  
96 Twello, The Netherlands), chickpea flour (NutsinBulk, Dublin, Ireland) and other biscuit ingredients  
97 were purchased from local and online markets (Wageningen, The Netherlands).

98 Petroleum ether, formic acid, Carrez I and Carrez II solutions were purchased from Sigma-Aldrich  
99 (St. Louis, MO, USA). The HPLC gradient analytical standard as AA ( $C_3H_5NO$ , molecular weight  
100 71.08 g/mol, CAS No. 79-06-1), AA- $d_3$  solution (500 mg/L in acetonitrile, CAS No. 122775-19-3),  
101 L-asparagine ( $C_4H_8N_2O_3$ , molecular weight 132.12 g/mol, CAS No. 70-47-3), D-(-)-fructose  
102 ( $C_6H_{12}O_6$ , molecular weight 180.16 g/mol, CAS No. 57-48-7), D-(+)-glucose ( $C_6H_{12}O_6$ , molecular  
103 weight 180.16 g/mol, CAS No. 50-99-7) and sucrose ( $C_{12}H_{22}O_{11}$ , molecular weight 342.30 g/mol,  
104 CAS No. 57-50-1) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile and  
105 methanol were purchased from Actu-All Chemicals (Oss, The Netherlands). Ethanol was purchased  
106 from VWR Chemicals (Radnor, PA, USA) and the Oasis MCX cartridge from Waters (Milford, MA,  
107 USA). Milli-Q water was produced by Milli-Q PURELAB Ultra, ELGA LabWater (Lane End, UK)  
108 and the total dietary fibre assay kit was purchased from Megazyme (Illinois, Chicago, IL, USA).

109

### 110 **2.2 Preparation of biscuit samples**

111 The different biscuit doughs were formulated with 100% wheat flour and with wheat flour partially  
112 replaced by 20, 40 and 60% of lupin flour or chickpea flour. The sample codes according to their  
113 flour percentages were: W for 100% wheat flour (control); L20, L40, L60 for wheat flour replaced  
114 with 20%, 40%, 60% lupin flour and C20, C40, C60 for wheat flour replaced with 20%, 40%, 60%  
115 chickpea flour.

116 The biscuit doughs were prepared according to the basic recipe from the AACC method 10-54  
117 (AACC, 2009) and added with pure asparagine to reach the same asparagine concentration in all  
118 formulations. The proportion of baking ingredients was: total flour (250.0 g), sucrose (105.0 g),  
119 shortening (100.0 g), sodium chloride (3.13 g), sodium bicarbonate (2.5 g), ammonium bicarbonate  
120 (1.25 g), high-fructose corn syrup (3.75 g), non-fat dry milk (2.5 g), distilled water and asparagine.  
121 The amounts of distilled water and external asparagine added to reach the same concentration in the  
122 raw dough of approximately 17% and 65.5 mg/kg, respectively, were calculated from the moisture  
123 and asparagine contents determined in the different flours and considering their percentages in each  
124 biscuit formulations. In detail, the added amounts, accurately weighed with a microbalance (XP6,

125 Mettler Toledo, USA), of asparagine in samples W, C20, C40, C60, L20, L40 and L60 were 15.58,  
126 13.31, 11.04, 8.78, 10.38, 5.19 and 0 mg, respectively.

127 To ensure homogeneous distribution in the dough, asparagine, high-fructose corn syrup and sucrose  
128 were solubilized in water at room temperature for 1 min using Thermomix TM5 (Vorwerk,  
129 Wuppertal, Germany) by setting the speed control to position 2. Successively, the other dry  
130 ingredients and shortening were added and mixed thoroughly for 1 min by setting the speed regulator  
131 to position 5 and reversing the direction of rotation after 30 s. The dough was shortly kneaded by  
132 hand to compact it, wrapped in plastic foil and let to rest for 20 min in a refrigerator at 4 °C. For some  
133 subsequent analyses, parts of the raw dough samples were freeze-dried and finely grounded with a  
134 mortar.

135 The raw dough was rolled out to a thickness of about 3 mm by a pasta filler machine (Marcato,  
136 Campodarsego, Italy) and cut by using a stainless-steel circular cup pastry of 6 cm diameter. For each  
137 formulation and baking batch, 8 biscuits were baked in an electrical oven (OV185C, Inventum,  
138 Arnhem, The Netherlands) with convection mode at 175 °C for 5, 7 and 9 min. The different baking  
139 conditions were chosen in preliminary tests to obtain biscuits that were neither undercooked nor  
140 overcooked. The biscuits were placed in the middle position of a baking tray inside the oven; for each  
141 baking cycle, the air temperature inside the oven chamber was recorded every 20 s using a digital  
142 thermometer equipped with type K thermocouples (Pro 206-3722, RS Components, Corby, UK) to  
143 ensure equal temperature exposure between the baking cycles. After baking, biscuits were removed  
144 from the oven, placed on a grid and kept cooling at room temperature for about 1 h.

145 All biscuit formulations and baking times were performed in triplicate, resulting in 24 biscuits per  
146 sample at each baking time (24 × 7 samples × 3 baking times, a total of 504 biscuits).

147

## 148 **2.3 Characterization of flours and biscuits**

149

### 150 2.3.1 Proximal analysis

151 The wheat, lupin and chickpea flours were analysed for protein, fat, dietary fibre, ash and  
152 carbohydrate contents.

153 The total protein content (g/100 g) was determined by weighted 15 mg of each flour in steel crucibles  
154 using Dumas method with a protein analyser (Flash EA 1112, Thermo Fisher Scientific, Waltham,  
155 MA, USA). The conversion factor of 6.25 to determine crude protein content was used.

156 The fat content (g/100 g) was measured using the Soxhlet method (Gerhardt, Königswinter,  
157 Germany). Approximately 5 g of each flour was weighted in cellulose extraction thimbles and  
158 extracted continuously with 200 mL of petroleum ether at 60 °C for 3 h. After cooling down



159 overnight, the solvent was evaporated under vacuum in a rotavapor (R-200, Büchi, Flawil,  
160 Switzerland) at 60 °C and the fat content was determined gravimetrically.

161 The total dietary fibre content (g/100 g) was determined on 1 g of each flour by an enzymatic-  
162 gravimetric method using a total dietary fibre assay kit.

163 The ash content (g/100 g) was determined by weighing 1 g of each flour into ceramic crucibles and  
164 incinerating for 5 h at 525 °C in a muffle furnace (Gallenkamp and Co., London, UK). After  
165 combustion and cooling down the ash content was determined gravimetrically.

166 The carbohydrate content (g/100 g) was determined by subtracting the amounts of protein, fat, dietary  
167 fibre, ash and water (described in section 2.3.5) from 100 g of the flour sample. Using this method,  
168 the calculated carbohydrate value includes sugars, starch and may also contain small amounts of other  
169 minor compounds.

170 All analyses were performed in duplicate for each flour type.

171

### 172 2.3.2 Particle size

173 The particle size (Dv90 (µm)) of the flours was measured by a laser particle size analyser (Mastersizer  
174 3000, Malvern Panalytical, Malvern, UK). The obscuration in all measurements was 0.5-10%, air  
175 pressure was 2 bar and hopper height was 3 mm with a feed rate of 50%. The flours were analysed as  
176 opaque particles according to Fraunhofer approximation. The particle sizes were calculated by the  
177 supplier's software (version 3.62, Malvern Instruments, Malvern, UK) and the Dv90 (µm) value,  
178 representing the maximum particle diameter below which 90% of the sample falls, was evaluated.

179 The analysis was carried out in triplicate for each flour type.

180

### 181 2.3.3 Hydration properties

182 The water holding capacity (WHC) and the water binding capacity (WBC) of the flours were  
183 determined based on Sarangapani et al. (2016). Both WHC and WBC were evaluated in 1 g of flour  
184 mixed with 10 mL of distilled water. For WHC the mixture was kept for 24 h at room temperature,  
185 then the non-absorbed water was discarded, and the hydrated sample was weighted. For WBC, the  
186 mixture of sample and water was centrifuged for 3 min at  $1363 \times g$  and 20 °C (Heraeus Multifuge  
187 X3R, Thermo Fisher Scientific, Waltham, MA, USA). The non-absorbed water was removed, and  
188 the hydrated sample was weighted.

189 The results were expressed in g of water/g of solid; both measurements were done in triplicate for  
190 each flour type.

191

### 192 2.3.4 pH

193 The pH of flours and raw doughs was determined according to the method described by Mesías et al.  
194 (2015). 1 g of the ground sample was mixed with 100 mL of deionized water, vortexed for 3 min and  
195 kept at room temperature for 1 h. After centrifugation at  $4816 \times g$  and  $20\text{ }^{\circ}\text{C}$  for 10 min (Heraeus  
196 Multifuge X3R, Thermo Fisher Scientific, Waltham, MA, USA), the pH of the supernatant was  
197 measured using a pH-meter (1100L, VWR, Radnor, PA, USA).

198 The measurement was performed in duplicate for each flour and raw dough.

199

#### 200 2.3.5 Moisture content

201 The moisture content of flours (g/100 g), raw doughs (%) and baked biscuits (%) was determined by  
202 a gravimetric method. For each sample, about 3 g of ground product was exactly weighted and dried  
203 at  $105\text{ }^{\circ}\text{C}$  in an oven (Heraeus Series 6000, Thermo Scientific, Berlin, Germany) until constant  
204 weight.

205 The analysis was carried out in triplicate for each flour, dough and baking batch per sample.

206

#### 207 2.3.6 Water activity

208 The water activity ( $a_w$ ) of flours, raw doughs and biscuits was determined at  $25\text{ }^{\circ}\text{C}$  with an  $a_w$ -meter  
209 (LabMaster, Novasina AG, Lachen, Switzerland) setting both time and temperature factors stability  
210 at 2 min.

211 The measurement was performed in duplicate for each flour, dough and baking batch per sample.

212

#### 213 2.3.7 Weight loss

214 The weight loss (%) of 8 biscuits was calculated as the percentage change in weight before and after  
215 each baking cycle per sample.

216

#### 217 2.3.8 Colour

218 The colour of flours, whole surfaces of raw and baked biscuits was performed with an IRIS V400  
219 electronic visual analyser (Alpha MOS, Toulouse, France) equipped with a 25 mm lens, lower and  
220 upper illumination and using a black background with a size of  $210 \times 297$  mm. ImageJ software (NIH,  
221 USA) was used for processing and quantification of CIE  $L^*$  (lightness),  $a^*$  (redness) and  $b^*$   
222 (yellowness) parameters of RGB images. From the numerical values of the measured parameters, the  
223 browning index (BI) was calculated by the following equations (Sakin-Yilmazer et al., 2013):

224

$$225 \text{ BI} = \frac{[(X-0.31) \cdot 100]}{0.17}, \text{ where } X = \frac{a^* + 1.79 \cdot L^*}{5.645 \cdot L^* + a^* - 3.012 \cdot b^*}$$

226

227 The colour measurements of each flour were carried out in triplicate and on the two surfaces of 5  
228 biscuits for each baking batch per sample.

229

### 230 2.3.9 Texture

231 The texture analysis of biscuits was performed at room temperature with Texture analyser TA.XT2  
232 (Stable Micro Systems, Surrey, UK) equipped with a load cell of 50 kg and a three-point bending test  
233 holder and probe. The distance of two beams of sample holder was 20 mm and the other setting were:  
234 pre-test speed of 5.00 mm/s, test speed of 1.00 mm/s, post-test speed of 10.00 mm/s and distance of  
235 5 mm. The downward movement was advanced till the biscuit was broken. The texture was described  
236 by the hardness (N), determined by means of maximum force, fracturability (1/mm), expressed as  
237 one/breakpoint distance between the origin of curve till the point where the biscuit breaks, and  
238 crispness, evaluated by the linear distance between the first and the last peak registered (Romani et  
239 al., 2012).

240 Force vs distance curves were obtained from 8 biscuits for each baking batch per sample.

241

## 242 **2.4 Quantification of asparagine and acrylamide by LC-MS/MS**

243

### 244 2.4.1 Sample extraction

245 Flours, raw doughs and baked biscuits were analysed for asparagine and AA contents. Asparagine  
246 and AA were extracted according to Žilić et al. (2020) with minor modifications. Briefly, 1 g of  
247 grounded sample was triple extracted with 20 mL of 10 mM formic acid in Milli-Q water. Each time  
248 the extract was vortex for 1 min and centrifuged for 10 min at  $4816 \times g$  and  $20\text{ }^{\circ}\text{C}$  (Heraeus Multifuge  
249 X3R, Thermo Fisher Scientific, Waltham, MA, USA). The combined supernatant was collected and  
250 stored in a freezer at  $-20\text{ }^{\circ}\text{C}$  until analysis (maximum 2 weeks).

251 For asparagine determination, the formic acid extract (5 mL) was centrifuged for 10 min at  $20817 \times$   
252  $g$  and  $20\text{ }^{\circ}\text{C}$  (5430 R, Eppendorf AG, Hamburg, Germany). For better clarification, 4 mL of  
253 supernatant were centrifuged for 7 min at  $20817 \times g$  and  $20\text{ }^{\circ}\text{C}$ , then 1 mL of clear supernatant was  
254 mixed with 1 mL of acetonitrile and filtered with  $0.2\text{ }\mu\text{m}$  PTFE filters ( $\text{Ø}15\text{ mm}$ ) into an amber glass  
255 autosampler vial. For AA determination, the formic acid extract (4.75 mL) with AA- $\text{d}_3$  solution (100  
256  $\mu\text{L}$ ) were clarified with 0.125 mL of Carrez I and 0.125 mL of Carrez II. The mixture was vortexed  
257 and centrifuged for 3 min at  $10621 \times g$  and  $20\text{ }^{\circ}\text{C}$ . For better clarification, 2 mL of supernatant was  
258 collected and centrifuged for 10 min at  $20817 \times g$  and  $20\text{ }^{\circ}\text{C}$ . Solid phase extraction cleaning was  
259 carried out according to Mogol & Gökmen (2014) using the Oasis MCX cartridge and collecting the  
260 sample in an amber glass autosampler vial.

261

#### 262 2.4.2 LC-MS/MS methods

263 Samples analyses were carried out with a Nexera UPLC system (Shimadzu Corporation, Kyoto,  
264 Japan) coupled with an LCMS-8050 triple quadrupole mass spectrometer (Shimadzu Corporation,  
265 Kyoto, Japan). The UPLC unit consisted of a SIL-30AC autosampler, an LC-20ADXR solvent  
266 delivery module, a DGU-20ASR degassing unit, a CTO-20AC column oven and an FCV-20AH<sub>2</sub>  
267 valve unit.

268 The chromatographic separation of free asparagine was performed injecting 5  $\mu$ L of samples on a  
269 SeQuant® ZIC HILIC 3.5  $\mu$ m, 4.6  $\times$  150 mm (Merck KGaS, 64271, Darmstadt, Germany) attached  
270 to a SeQuant® ZIC HILIC PEEK coated guard column 20  $\times$  2.1 mm (Merck KGaS, 64271,  
271 Darmstadt, Germany). The flow rate was set at 0.7 mL/min and the column temperature at 40 °C. The  
272 mobile phases consisted of 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid  
273 (solvent B) with the following elution profile (min/%B): 0.0/90, 4.0/70, 10.0/20, 13.0/20, 15.0/90 and  
274 18.0/90.

275 The chromatographic separation of AA was performed on Acquity PREMIER BEH C18 column (1.7  
276  $\mu$ m, 2.1  $\times$  50 mm) connected to an Acquity UPLC BEH C18 VanGuard Pre-column, (130 Å, 1.7  $\mu$ m,  
277 2.1  $\times$  5 mm) (Waters Chromatography B.V, Etten-Leur, The Netherlands) with a flow rate of 0.2  
278 mL/min at 40 °C column temperature. A gradient mixture of mobile phases A (0.1% formic acid) and  
279 B (methanol with 0.1% formic acid) was used for elution following the elution profile (min/%B) of:  
280 0.0/5, 2.5/70, 5.0/90, 6.0/90, 7.0/5 and 11.0/5.

281 Positive ionisation mode was used for both MS analyses. The voltage of the turbo ion-spray ionization  
282 was 4.0 kV. The temperature of the electrospray ionization probe, desolvation line and heat block was  
283 set at 300 °C, 250 °C and 400 °C, respectively. The pressure of the collision-induced dissociation gas  
284 was 4 kPa whereas the flow rates of the drying gas, nebulizer gas and heating gas were set at  
285 10 mL/min, 3 mL/min and 10 mL/min, respectively. The electrode voltage of Q1 pre bias (collision  
286 cell energy entrance potential), collision cell Q2 (collision energy), Q3 pre bias (collision cell energy  
287 exit potential), parent and fragment ion m/z of the multiple reaction monitoring transitions were  
288 optimized using support software (Shimadzu Corporation, Kyoto, Japan). For single reaction  
289 monitoring (SRM), the dwell time was set at 4 or 42 msec, respectively for asparagine and AA, and  
290 the most abundant fragment ion was selected for quantitation. The second and third fragments in ion  
291 yield were selected as a structural confirmation based on the optimized SRM transition. The  
292 precursor/product ion transitions m/z 133.20 $\rightarrow$ 74.00, 133.20 $\rightarrow$ 87.05 and 133.20 $\rightarrow$ 28.15 were  
293 monitored for asparagine; 72.00 $\rightarrow$ 55.10, 72.00 $\rightarrow$ 27.10 and 72.00 $\rightarrow$ 44.00 were monitored for  
294 acrylamide; 75.25 $\rightarrow$ 58.05, 75.25 $\rightarrow$ 30.05 and 75.25 $\rightarrow$ 44.05 were monitored for acrylamide-d<sub>3</sub>. Data

295 were processed with LabSolutions (Shimadzu Corporation, Kyoto, Japan). The recovery and matrix  
296 effects were satisfactory since the average recovery for AA was  $93 \pm 7\%$  and average matrix effects  
297 were  $101 \pm 10\%$  and  $105 \pm 6\%$  for asparagine and AA, respectively.

298 The sample extraction was repeated twice for each flour and each batch per sample and the analytical  
299 measurements were replicated twice for each extract. The results were expressed as  $\mu\text{g}/\text{kg}$  for AA and  
300  $\text{mg}/\text{kg}$  for asparagine on dry matter basis.

301

## 302 **2.5 Glucose, fructose and sucrose contents**

303

### 304 2.5.1 Sample extraction

305 Raw doughs and biscuits were analysed for glucose, fructose and sucrose contents. The sample  
306 extraction process was based on Nguyen et al. (2016) with slight modifications. Grounded biscuit  
307 (2.5 g) or freeze-dried dough (2.5 g) was mixed with 1:1, v/v of Milli-Q water and ethanol mixture  
308 (25 mL). The samples were incubated for 1 h at  $50\text{ }^\circ\text{C}$  in a water bath and cooled down for 20 min at  
309 room temperature. Then the samples were centrifuged at  $1962 \times g$  and  $20\text{ }^\circ\text{C}$  for 10 min (Heraeus  
310 Multifuge X3R, Thermo Fisher Scientific, Waltham, MA, USA). The supernatant (1.5 mL) was  
311 centrifuged at  $20817 \times g$  and  $20\text{ }^\circ\text{C}$  (5430 R, Eppendorf AG, Hamburg, Germany) for 10 min and 1  
312 mL was collected into a glass tube. The water/ethanol solvent was evaporated with a sample  
313 concentrator (SBHCONC/1, Stuart, Staffordshire, UK) under nitrogen flush at  $50\text{ }^\circ\text{C}$  for 4.5 h. The  
314 sample was reconstituted with acetonitrile (20 mL) and Milli-Q water (20 mL) and stored in a freezer  
315 at  $-20\text{ }^\circ\text{C}$  until measurement (maximum 1 week). Before analysis 1.5 mL of sample was passed  
316 through CA ( $\text{O}28\text{ mm}$ ;  $0.2\text{ }\mu\text{m}$ ) filters and transferred into an autosampler vial.

317

### 318 2.5.2 UPLC-ELSD method

319 The samples were analysed according to the procedure provided by Waters' technical application  
320 notebook with an Acquity UPLC-H Class Plus System (Waters, Milford, MA, USA) equipped with  
321 an Acquity Evaporative Light Scattering (ELSD) detector, an Acquity UPLC BEH Amide column  
322 ( $1.7\text{ }\mu\text{m}$ ,  $2.1 \times 100\text{ mm}$ ) and an Acquity UPLC BEH Amide VanGuard pre-column ( $130\text{ \AA}$ ,  $1.7\text{ }\mu\text{m}$ ,  
323  $2.1\text{ mm} \times 5\text{ mm}$ ) (Waters, Milford, MA, USA). The mobile phase A consisted of Milli-Q water and  
324 acetonitrile mixture (8:2, v/v) with 0.2% triethylamine (TEA) while mobile phase B consisted of  
325 acetonitrile/water 3:7, v/v with 0.2% TEA. The flow rate was  $0.25\text{ mL}/\text{min}$ . The gradient changes  
326 with the following elution profile (min/%A): 0.00/100, 6.00/40, 6.01/100 and 18/100. Before the first  
327 injection, the column was equilibrated with 100% A,  $0.25\text{ mL}/\text{min}$  for 30 min. The injection volume  
328 was  $1.3\text{ }\mu\text{L}$  and the column temperature was  $35\text{ }^\circ\text{C}$ . The pressure of ELSD conditions was 40 psi with

329 a drift tube temperature of 40 °C and a data rate 10 pps. Operating the software was carried out using  
330 a Waters Acquity Control console and data processing was performed with Chromeleon  
331 Chromatography Data System (version 7.2.10, Thermo Scientific Corp, Waltham, MA, USA). The  
332 quantification was done by an external calibration curve ranging from 85-1360 mg/L (sucrose) and  
333 45-720 mg/L (glucose and fructose).

334 The sample extraction was repeated twice for each batch per sample and the analytical measurement  
335 was conducted twice for each extract. The results for sucrose content were expressed as g/kg on dry  
336 matter basis.

337

## 338 **2.6 Data analysis**

339 Data processing and statistical analyses were performed with Excel (Microsoft, Redmond, WA, USA)  
340 and STATISTICA 8.0 (StatSoft Inc., Tulsa, UK) software. The results were reported as mean  $\pm$   
341 standard deviation of replications. Parametric unidirectional analysis of variance (ANOVA), followed  
342 by Tukey's post-hoc comparison test, with a significance level of 95% ( $p < 0.05$ ), were used to  
343 determine significant differences between the samples. The relationships between AA level and  
344 quantity of legume flours and asparagine content of the biscuits prepared with different formulations  
345 were determined by linear correlation with  $r^2$  coefficient in the range between  $-1$  (negative  
346 relationship) and  $+1$  (positive relationship).

347

## 348 **3. Results and discussion**

349

### 350 **3.1 Characteristics of flours**

351 The free asparagine content and other tested characteristics of wheat, lupin and chickpea flours to be  
352 related to the AA content and quality characteristics of the final biscuits are reported in **Table 1**. All  
353 flours had protein, fat, dietary fibre and ash contents comparable to those provided by the respective  
354 flour manufacturers and findings in the literature (Cardoso et al., 2019; Hall et al., 2017; Torra et al.,  
355 2021; Žilić et al., 2020).

356 The free asparagine content, which is the main factor influencing AA concentration in bakery  
357 products (Žilić et al., 2020), was significantly higher in legume flours than in the wheat one in the  
358 percentages of 117.4% and 51.2% for lupin and chickpea flour, respectively. This is coherent with  
359 the significantly higher protein content of legume flours (32.2 and 17.1 g/100 g for lupin and chickpea  
360 respectively) compared to wheat flour (11.2 g/100 g). The amount of free asparagine determined in  
361 wheat flour was in line with the ones reported by Hamlet et al. (2008), Capuano et al. (2009) and Žilić  
362 et al. (2020). The asparagine concentrations found in lupin flour were significantly much higher than

363 the literature reports (about 28 mg/kg), while those identified in chickpea flour were significantly  
364 much lower (about 420 mg/kg) (Bartkiene et al., 2016; Barutcu et al., 2009). Free asparagine  
365 accumulation in crops can be very variable because it largely depends on growing conditions as well  
366 as the processing methods (Miśkiewicz et al., 2012; Žilić et al., 2020).

367 Besides composition, the flours used in this study also differed in their particle size, which could  
368 influence the rate of the Maillard reaction and the formation of AA modulating the interaction and  
369 reactivity of chemical constituents in complex food mixtures (Betoret & Rosell, 2020; Sun et al.,  
370 2019). Chickpea flour had the highest Dv90 value, indicating that 90% of the sample had a particle  
371 size of 410.0 µm or less, while wheat one had the lowest particle size, with a Dv90 of 156.3 µm.

372 Moisture and  $a_w$  levels also play a crucial role in the interaction of AA precursors as well as in the  
373 rate of the Maillard reaction (De Vleeschouwer et al., 2007). The moisture of the flours ranged  
374 between 6.2-12.8 g/100 g (lupin-wheat flour) and the  $a_w$  varied in the range 0.34-0.65 (lupin-wheat  
375 flour). The moisture and  $a_w$  discrepancies found in the studied flours are probably due to the different  
376 compositions and milling processing conditions.

377 Regarding the hydration properties, the lupin flour had the significant highest value of WHC and  
378 WBC, probably due to the highest dietary fibre content (40.8 g/100 g) compared to the other flours  
379 (11.8 and 2.8 g/100 g for chickpea and wheat, respectively). Wheat and chickpea flours had very  
380 similar WHC values, about 2 g water/g solid, and differed significantly only for WBC. In addition,  
381 these hydration properties may also result from the presence of various types of hydrophilic  
382 carbohydrates and the varying structure of proteins (Farooq & Boye, 2011).

383 The pH values of the water-soluble fraction of the flours were close to neutral. Compared to the pH  
384 of wheat flour (6.1), the pH of lupin flour equal to 6.0 was significantly lower, while that of chickpea  
385 flour equal to 6.7 was significantly higher.

386 Regarding colour, legume flours were both less light with lower  $a^*$  and higher  $b^*$  colour parameters  
387 due to their yellowish colour compared to wheat flour which remains lighter and whiter. The presence  
388 of a range of pigments in the cotyledons and seed coats of several legumes gives them a distinct  
389 colour (Tetrycz et al., 2020).

390

### 391 **3.2 Influence of legume flour on the contents of acrylamide and free asparagine of biscuits**

392 Besides the amount of free asparagine, other flour properties can lead to different rates of AA  
393 formation (Miśkiewicz et al., 2020). To assess the effect of other flours differences on AA formation  
394 independently from their asparagine content, the concentration of asparagine in all biscuit doughs  
395 was standardized. The highest asparagine concentration was recorded in the lupin flour; therefore, no  
396 additional asparagine was added to the L60 dough, achieving an asparagine value of  $65.5 \pm 7.0$  mg/kg

397 on dry matter basis. In the other type of biscuits samples, the amount of added free asparagine was  
398 adjusted according to the percentage of used flours and their asparagine concentration (**Table 1**). The  
399 asparagine values of the different dough samples after standardisation, together with AA and sucrose  
400 concentrations detected in all biscuit samples, are reported in **Table 2**.

401 Looking at the data it is clear that despite the same free asparagine content, the use of legume flours  
402 compared to wheat flour led to a different formation of AA during the baking of the biscuits. The  
403 wheat control sample showed an increase in AA during baking, reaching values above the reference  
404 level reported in Commission Regulation (EU) 2017/2158 (350 µg/kg) of 421.2, 481.9 and 583.9  
405 µg/kg (d.m.) after 5, 7 and 9 min, respectively. AA values in wheat biscuits were significantly higher  
406 compared to the ranges reported in previous studies, probably due to the addition of pure asparagine  
407 done to equalize the asparagine concentrations in all formulations (Manolache et al., 2019; Mesías et  
408 al., 2016; Sazesh & Goli, 2020; Žilić et al., 2020).

409 The use of different percentages of lupin and chickpea flour in the biscuit recipes resulted in different  
410 rates of AA formation compared to the wheat flour. When 40 and 60% of lupin flour was used, the  
411 AA content increased by 105.6 and 173.2%, 80.7 and 161.7%, 84.2 and 147.1% after 5, 7 and 9 min  
412 of baking, respectively, compared to wheat samples. The biscuits had a significantly higher AA  
413 content than in the wheat sample, proportionally to the amount of lupin flour used ( $r^2 = 0.99$ ,  $r^2 = 1.00$   
414 and  $r^2 = 0.99$ , respectively for 5, 7 and 9 min of baking). Similar results were obtained in studies by  
415 Bartkiene et al. (2013, 2016) who described higher AA levels of 43.3 and 78.5% by increasing lupin  
416 flour in bread and biscuit products, respectively, compared to control samples without added lupin  
417 flour. The resulting increase in AA proportional to the amount of lupin flour was attributed to a higher  
418 asparagine content in lupin flour compared to wheat flour (Bartkiene et al., 2016). In the present  
419 study, since the initial asparagine concentrations were standardized, the increase in AA in biscuits  
420 could be due to the higher dietary fibre content of lupin flour (40.8 g/100 g) compared to wheat one  
421 (2.8 g/100 g) (**Table 1**). The presence of a high percentage of dietary fibre contributed to reducing  
422 the  $a_w$  of the biscuits during baking as described in the next section 3.3 “Influence of legume flours  
423 on the main characteristics of biscuits” and this may have favoured the Maillard reaction and AA  
424 formation due to a higher concentration of reaction substrates (Palermo et al., 2012). However,  
425 conflicting results have been found in the literature, some studies stated that the Maillard rate is higher  
426 at high  $a_w$  values (0.6-0.8) where the mobility of the reactants is greater, whereas at very low  $a_w$  the  
427 reactants become too concentrated limiting their diffusion and interaction (van Boekel, 2001).

428 Low AA levels were obtained in biscuits with 20 and 40% of chickpea flour, leading to a reduction  
429 of about 50% in AA compared to the wheat control sample after each baking time (**Table 2**). This  
430 result can confirm a possible effect of chickpea proteins in the thermal stability of reducing sugars



431 previously described by Miśkiewicz et al. (2020). The authors, using a Differential Scanning  
432 Calorimetry (DSC) analysis, reported that the melting point of glucose and fructose with 1% of  
433 chickpea proteins extract (extract composition: protein=82.70 g/100 g, fructose=0.05 mg/g d.m.,  
434 glucose=0.02 mg/g d.m., sucrose=0.12 mg/g d.m. and maltose=1.49 mg/g d.m.) increased, due to a  
435 higher ordering of the crystallographic structures of the carbohydrates. This helped to reduce the  
436 reaction speed between reducing sugars and asparagine slowing down the formation of AA  
437 (Miśkiewicz et al., 2020). In addition, the lower interaction between AA precursors may also have  
438 resulted from the coarsest particle size of the chickpea flour. However, no such effect was noticed for  
439 lupin biscuit samples despite a larger flour particle size than wheat flour, indicating a greater effect  
440 of dietary fibre content by decreasing the  $a_w$  as previously explained. When wheat flour was  
441 substituted with 60% of chickpea flour, significant AA increases of 79.5, 45.7 and 7.8% were detected  
442 compared to wheat samples at 5, 7 and 9 min, respectively. Probably because at this chickpea flour  
443 percentage, the effect of its dietary fibre content (11.8 g/100 g) on moisture and  $a_w$  control prevails  
444 over the positive effect of chickpea proteins on AA formation described above (Miśkiewicz et al.,  
445 2020; Palermo et al., 2012). The percentages of chickpea flour in the biscuits had a non-significant  
446 correlation to the amount of AA determined after baking ( $r^2 = 0.71$ ,  $r^2 = 0.82$ ,  $r^2 = 0.64$ , respectively  
447 at 5, 7 and 9 min). Overall, the AA values in chickpea biscuits measured in this research activity are  
448 high compared to the result obtained by Miśkiewicz et al. (2012), probably in relation to differences  
449 in the biscuit's formulations, as well as in the baking process parameters.

450 Concerning the asparagine concentrations detected in biscuits (**Table 2**), in wheat samples the values  
451 were negatively correlated with AA levels ( $r^2 = -0.91$ ), confirming the dominant role of this amino  
452 acid in the formation of AA. A similar result was observed for lupin biscuits, with negative linear  
453 correlation coefficients  $r^2$  of  $-1.00$  for L20,  $-0.91$  for L40 and  $-0.96$  for L60. A relatively good  
454 negative linear correlation between AA and asparagine contents was also found for some chickpea  
455 samples with an  $r^2$  of  $-0.90$  for C20 and an  $r^2$  of  $-0.97$  for C40; no correlation was found for sample  
456 C60, where the AA concentrations did not vary significantly between 5 and 9 min of baking.  
457 However, the percentages of asparagine reduction from the dough (time 0) to biscuit baked for 9 min  
458 were much lower in chickpea samples than in wheat and lupin ones, especially at the flour percentages  
459 of 20 and 40. For wheat samples, a reduction in asparagine of 25.1% was measured, for samples L20  
460 and L40 a reduction of 26.4 and 40.6%, respectively, and for samples C20 and C40 only a reduction  
461 of 7.8 and 14.3%, respectively. This clearly confirmed the hypothesis made by examining the data on  
462 AA formation and confirmed the observation of Miśkiewicz et al. (2020): the use of chickpea flour  
463 at 20 and 40% reduced the reaction rate between free asparagine and reducing sugars.

464 No reducing sugars (i.e. glucose and fructose) could be detected in either the dough or the biscuits,  
465 this could be due to their participation in the Maillard reaction or other thermal reactions (e.g.  
466 caramelization, pyrolytic reactions). The initial contents of sucrose did not change significantly  
467 during baking (**Table 2**), demonstrating any hydrolysis of this sugar during baking has already been  
468 detected by some previous studies (Gökmen et al., 2007; Nguyen et al., 2016; Schouten et al., 2022).  
469 Indeed, the sucrose content in the samples did not significantly influence the Maillard reaction  
470 development.

471

### 472 **3.3 Influence of legume flours on some quality characteristics of biscuits**

473 The use of different flours in the biscuit formulation led to variations in moisture,  $a_w$  and weight loss  
474 of the biscuit samples at different baking times, as displayed in **Table 3**. The amount of water added  
475 in the dough recipes was standardised and calculated based on the moisture content of each flour to  
476 achieve similar moisture content (around 17%) and  $a_w$  (around 0.80) in all doughs. This was because,  
477 in addition to the chemical characteristics of the flour, moisture and  $a_w$  of biscuit doughs are  
478 parameters that can influence the formation of AA. As expected, both moisture and  $a_w$  values of all  
479 biscuit samples decreased with increasing baking time. For all baking times tested the moisture and  
480  $a_w$  of the wheat samples were significantly higher than of the lupin and chickpea biscuits, except for  
481 the C60 sample after 5 min of baking in which the values were significantly the same. The low  
482 moisture and  $a_w$  values of the lupin and chickpea samples could be related to differences in the  
483 macronutrient compositions (e.g. dietary fibre, carbohydrates) and particle size of the legume flours  
484 (**Table 1**). In lupin samples, both moisture and  $a_w$  decreased with the increase of the amount of lupin  
485 flour, while in chickpea samples these parameters tended to decrease without a clear trend related to  
486 the increased amounts of chickpea flour. As reported in previous studies, the weight loss was greatest  
487 in the first few minutes of baking (5 min); the formation of a dry surface layer caused a reduction in  
488 water vapour flow although the mass transfer continued until the end of baking (9 min) leading to an  
489 increase in weight loss percentage (Thorvaldsson & Skjöldebrand, 1998). The weight loss after  
490 baking of the lupin biscuits was significantly greater compared to wheat samples, while for chickpea  
491 ones were no significant differences (**Table 3**).

492 No significant differences ( $p < 0.05$ ) were found in the pH of the biscuit doughs, resulting in a range  
493 between 8 and 8.8, suggesting that the leavening agent and other common ingredients used in the  
494 recipe were able to compensate for the slight initial differences between the flours (**Table 1**).

495 The use of different flours in the formulation resulted in variations in the colour and texture properties  
496 of the baked biscuits as indicated in **Table 4**. The  $L^*$  (lightness) values of the dough decreased  
497 significantly with the increasing amount of legume flours that showed a more yellow and intense

498 colouring than the matt and greyish wheat flour (**Table 1**). Similar results were previously described  
499 by Bartkiene et al. (2016) analysing biscuits obtained with lupin flour. Moreover, for all types of  
500 biscuits, the upper L\* value significantly decreased with the increase of baking time. Compared to  
501 the wheat sample, after 5 min of baking, the L\* value of the upper surface of the biscuits decreased  
502 significantly for samples L20, L40, L60, C40 and C60, whereas after 7 and 9 min of baking L\*  
503 decreased significantly only for samples C40 and C60. When comparing samples prepared with lupin  
504 and chickpea flour after 7 and 9 min of baking, samples C40 and C60 showed a lower lightness than  
505 samples L40 and L60, indicating more intense colour changes. The BI (browning index) values of  
506 the different types of dough (**Table 4**) also increased over the percentage of legume flours. For all  
507 wheat, lupin and chickpea samples, the upper surface of the biscuits became more brown during the  
508 prolongation of the baking time due to Maillard and caramelisation reactions (Lara et al., 2011). At  
509 all baking times, a significantly higher upper BI was determined when the legume flours were used  
510 at 40 and 60%. Differences in L\* and BI results were also found in the biscuits lower surfaces that  
511 were less light and darker than the upper ones. The differences in surface colour of all biscuits were  
512 also noticeable from the visual appearance shown in **Figure 1**.

513 As reported in **Table 4** the different flours used for biscuit formulations caused differences also in  
514 terms of texture which is an important quality parameter in bakery products correlated with  
515 consumers' perception of the freshness (Zoulias et al., 2000). The assessed texture properties were  
516 hardness and fracturability, which indicate the firmness of the structure, and crispness, which is a  
517 measure of structure friability. Generally, hardness is often considered an undesirable characteristic  
518 of biscuit products, while fracturability is related to a pleasant sensory characteristic as long as it does  
519 not become excessive (Zoulias et al., 2000). For all formulations, biscuits increased in hardness,  
520 fracturability and crispness with increasing baking times as noted in previous research (Lara et al.,  
521 2011; Romani et al., 2012; Schouten et al., 2022). Compared to the wheat samples, hardness increased  
522 significantly when the amount of lupin flour was increased in the formula, due to the high dietary  
523 fibre content, while it decreased with more chickpea flour, probably attributed to its coarser particle  
524 size. However, no significant differences in hardness were found between the wheat and C20 samples  
525 at all tested baking times. As expected, the harder biscuits were also the less fracturable ones. The  
526 lupin biscuits presented lower fracturability values than the wheat and chickpea ones after all tested  
527 baking times, although not always significantly. The crispness was significantly different between  
528 wheat and lupin samples after 5 and 7 min of baking and for L40 and L60. On the other hand, wheat  
529 samples differed significantly, although slightly, compared to chickpea samples only when chickpea  
530 flour was used at 60%. The highest dietary fibre content, WH and WB capacities of lupin flour, as

531 well as the greater particle size of chickpea one (**Table 1**), may have contributed to the texture results  
532 of the biscuits made from them.

533

#### 534 **4. Conclusions**

535 The standardization of the initial asparagine concentration in the different formulations has been an  
536 effective approach to assess the effect of flours of different origins on AA formation in biscuits.

537 The use of lupin flour was not effective in the mitigation of AA levels in biscuits. Especially at high  
538 concentrations, lupin flour accelerated the AA formation reactions probably due to its higher dietary  
539 fibre content causing lower moisture content and  $a_w$  in biscuits.

540 Interestingly, chickpea flour showed good potentiality for controlling AA formation mainly when it  
541 was used at a concentration between 20 and 40%. The effect is likely due to the composition and  
542 particle size of the chickpea flours and to the effect on the thermodynamic properties of carbohydrate  
543 compounds by the addition of chickpea proteins. In biscuits formulated with chickpea flour,  
544 concentrations of AA were found to be lower than the reference value given in Commission  
545 Regulation (EU) 2017/2158 (350  $\mu\text{g}/\text{kg}$ ) despite the very high amount of asparagine added used in  
546 our biscuits.

547 Interestingly, the use of a limited concentration of chickpea flour did not substantially change some  
548 quality characteristics of the final biscuits such as colour and texture, while it improved protein and  
549 dietary fibre content. Further studies are needed to evaluate the sensory acceptability of chickpea  
550 addition to the biscuits; however, the use of chickpea flour instead of other flours with similar  
551 asparagine content can be a simple and effective solution to mitigate the AA formation in biscuits  
552 and other low moisture bakery products.

553

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559

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687 <https://doi.org/10.1080/10942910009524643>
- 688
- 689 **Figure 1.** Visual appearance of the raw biscuits (A) and of upper (B) and lower (C) surfaces of  
690 biscuits baked at 175 °C for different times formulated with different flours (W: only wheat flour;  
691 L20, L40, L60: 20%, 40%, 60% of lupin flour; C20, C40, C60: 20%, 40%, 60% of chickpea flour).



1 **Influence of lupin and chickpea flours on acrylamide formation and quality**  
2 **characteristics of biscuits**

3  
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23 **Abstract:**

24 Asparagine and sugars are direct precursors of acrylamide; however, proteins and fibres can also  
25 influence it. In this study, biscuits prepared replacing wheat flour with increasing concentrations (20,  
26 40, 60%) of lupin or chickpea flour were investigated. Asparagine concentration was equalized in all  
27 formulas to isolate the effect of other flour characteristics on the acrylamide formation during baking.  
28 The results showed that replacing wheat flour with lupin flour increased acrylamide **from 583.9 up to**  
29 **1443 µg/kg after 9 min of baking**, while 20-40% chickpea flour reduced acrylamide to 354.4-312.6  
30 µg/kg. The acrylamide reduction using chickpea was attributed to the lower interaction between  
31 precursors resulting from both the coarser particle size and the lower reactivity of carbohydrate in  
32 presence of chickpea proteins. Chickpea addition did not affect the colour and texture of biscuits,  
33 opening the possibility for large-scale implementation of this mitigation strategy in formulas with a  
34 similar initial asparagine content.

35

36 **Keywords:**

37 Acrylamide; Biscuits; Legume flours; Asparagine; Bakery products.

38

39 **Chemical compounds studied in this article:**

40 Acrylamide (PubChem CID: 6579); Acrylamide-d<sub>3</sub> (PubChem CID: 12209671); Asparagine  
41 (PubChem CID: 6267); Sucrose (PubChem CID: 5988).

42

43 **1. Introduction**

44 Bakery products, including biscuits, are popular foods worldwide. However, these products, together  
45 with coffee and potato products, contribute to the dietary intake of acrylamide (AA), a toxic  
46 compound classified as “probably carcinogenic to humans” (group 2A) by the International Agency  
47 for Research on Cancer. The formation of AA in foods is due to the simultaneous presence of reducing  
48 sugars and asparagine combined with processing conditions (temperatures above 120 °C and low  
49 humidity) triggering the Maillard reaction (Mesías et al., 2016).

50 International regulations about the maximum tolerable levels of AA in foods became more restrictive  
51 over the years (European Commission, 2007; 2011; 2013; 2017; 2019), calling for the application of  
52 mitigation measures at the food industry level. Asparagine in flours is the main AA precursor,  
53 therefore, several studies have investigated the effect of different flour sources and mixtures on the  
54 AA formation in bakery products (Miśkiewicz et al., 2012; Sazesh & Goli, 2020; Žilić et al., 2020).  
55 In general, it has been proved that cereal or non-cereal varieties having higher amounts of free  
56 asparagine resulted in biscuits with higher concentrations of AA (Manolache et al., 2019; Mesías et

57 al., 2016; Miśkiewicz et al., 2012; Sazesh & Goli, 2020). In contrast, Žilić et al. (2020) observed that  
58 asparagine concentrations in different flours tested (i.e. wheat, oats, rye, barley, triticale, maize) did  
59 not significantly correlate with AA concentrations measured in biscuits prepared with the different  
60 formulations. No correlation between asparagine concentration in the starting ingredient and AA in  
61 the final product was found also by Capuano et al. (2009) who prepared bread crisp with wheat, rye  
62 and whole-wheat flours and toasted them at different time-temperature conditions. These  
63 observations indicated that other flour compounds and properties can influence the extent of Maillard  
64 reaction and, consequently, the AA formation.

65 Some proteins characteristic can influence the AA formation in different food products (Miśkiewicz  
66 et al., 2012, 2020; Rydberg et al., 2003; Tareke et al., 2002). Rydberg et al. (2003) studied the effect  
67 of protein-rich ingredients (i.e. cod meat) added to potato-based products observing a reduction in  
68 AA in the final products up to 70%. It has been suggested that this effect may result from a protective  
69 action of the proteins by scavenging the AA formed. In another study, chickpea proteins extract  
70 showed a mitigation effect of AA formation in a biscuit-like low-moisture model system (Miśkiewicz  
71 et al., 2020). It was suggested that the observed 40% reduction of AA formation was due to the  
72 increased thermal stability of the reducing sugars by the chickpea proteins extract. In the presence of  
73 chickpea proteins extract, the carbohydrates presented a higher ordering of their crystallographic  
74 structures and this reduced their availability to react with asparagine and lead to AA formation  
75 (Miśkiewicz et al., 2020). On the other hand, legume flours are usually higher in dietary fibre content  
76 than cereal ones (Rebello et al., 2014). High-fibre okara flour (a soya by-product) has been shown to  
77 promote the Maillard reaction and hence AA formation in biscuits by reducing the water activity of  
78 the dough and thus increasing the concentration of AA precursors (Palermo et al., 2012). From the  
79 published studies, it is not easy to understand whether the differences in the concentration of AA  
80 found in the final biscuits are indeed solely related to variations in the initial amount of asparagine in  
81 the flours or due to the effect of the dietary fibre and protein content in the flours.

82 The aim of the present study was to investigate the potential of biscuit formulations prepared with  
83 different types of flour and a standardised starting content of asparagine in terms of AA mitigation.  
84 Biscuits were formulated by replacing 20, 40 and 60% of wheat flour with protein-rich legume flours  
85 from lupins and chickpeas. Asparagine was added proportionally to all formulations to have the same  
86 concentration in all biscuits. In this way, we were confident to evaluate the possible role of other  
87 flours characteristics, such as proteins and dietary fibres addition described previously, on AA  
88 formation. In addition to the chemical composition, several structure-related effects on the formation  
89 of AA during baking were investigated, together with the impact on the colour and texture  
90 characteristics of the final products.

91

## 92 **2. Materials and methods**

93

### 94 **2.1. Biscuit ingredients and chemicals**

95 Wheat flour (Molen De Vlijt, Wageningen, The Netherlands), lupin flour (Frank Food Products,  
96 Twello, The Netherlands), chickpea flour (NutsinBulk, Dublin, Ireland) and other biscuit ingredients  
97 were purchased from local and online markets (Wageningen, The Netherlands).

98 Petroleum ether, formic acid, Carrez I and Carrez II solutions were purchased from Sigma-Aldrich  
99 (St. Louis, MO, USA). The HPLC gradient analytical standard as AA ( $C_3H_5NO$ , molecular weight  
100 71.08 g/mol, CAS No. 79-06-1), AA- $d_3$  solution (500 mg/L in acetonitrile, CAS No. 122775-19-3),  
101 L-asparagine ( $C_4H_8N_2O_3$ , molecular weight 132.12 g/mol, CAS No. 70-47-3), D-(-)-fructose  
102 ( $C_6H_{12}O_6$ , molecular weight 180.16 g/mol, CAS No. 57-48-7), D-(+)-glucose ( $C_6H_{12}O_6$ , molecular  
103 weight 180.16 g/mol, CAS No. 50-99-7) and sucrose ( $C_{12}H_{22}O_{11}$ , molecular weight 342.30 g/mol,  
104 CAS No. 57-50-1) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile and  
105 methanol were purchased from Actu-All Chemicals (Oss, The Netherlands). Ethanol was purchased  
106 from VWR Chemicals (Radnor, PA, USA) and the Oasis MCX cartridge from Waters (Milford, MA,  
107 USA). Milli-Q water was produced by Milli-Q PURELAB Ultra, ELGA LabWater (Lane End, UK)  
108 and the total dietary fibre assay kit was purchased from Megazyme (Illinois, Chicago, IL, USA).

109

### 110 **2.2 Preparation of biscuit samples**

111 The different biscuit doughs were formulated with 100% wheat flour and with wheat flour partially  
112 replaced by 20, 40 and 60% of lupin flour or chickpea flour. The sample codes according to their  
113 flour percentages were: W for 100% wheat flour (control); L20, L40, L60 for wheat flour replaced  
114 with 20%, 40%, 60% lupin flour and C20, C40, C60 for wheat flour replaced with 20%, 40%, 60%  
115 chickpea flour.

116 The biscuit doughs were prepared according to the basic recipe from the AACC method 10-54  
117 (AACC, 2009) and added with pure asparagine to reach the same asparagine concentration in all  
118 formulations. The proportion of baking ingredients was: total flour (250.0 g), sucrose (105.0 g),  
119 shortening (100.0 g), sodium chloride (3.13 g), sodium bicarbonate (2.5 g), ammonium bicarbonate  
120 (1.25 g), high-fructose corn syrup (3.75 g), non-fat dry milk (2.5 g), distilled water and asparagine.  
121 The amounts of distilled water and external asparagine added to reach the same concentration in the  
122 raw dough of approximately 17% and 65.5 mg/kg, respectively, were calculated from the moisture  
123 and asparagine contents determined in the different flours and considering their percentages in each  
124 biscuit formulations. In detail, the added amounts, accurately weighed with a microbalance (XP6,

125 Mettler Toledo, USA), of asparagine in samples W, C20, C40, C60, L20, L40 and L60 were 15.58,  
126 13.31, 11.04, 8.78, 10.38, 5.19 and 0 mg, respectively.

127 To ensure homogeneous distribution in the dough, asparagine, high-fructose corn syrup and sucrose  
128 were solubilized in water at room temperature for 1 min using Thermomix TM5 (Vorwerk,  
129 Wuppertal, Germany) by setting the speed control to position 2. Successively, the other dry  
130 ingredients and shortening were added and mixed thoroughly for 1 min by setting the speed regulator  
131 to position 5 and reversing the direction of rotation after 30 s. The dough was shortly kneaded by  
132 hand to compact it, wrapped in plastic foil and let to rest for 20 min in a refrigerator at 4 °C. For some  
133 subsequent analyses, parts of the raw dough samples were freeze-dried and finely grounded with a  
134 mortar.

135 The raw dough was rolled out to a thickness of about 3 mm by a pasta filler machine (Marcato,  
136 Campodarsego, Italy) and cut by using a stainless-steel circular cup pastry of 6 cm diameter. For each  
137 formulation and baking batch, 8 biscuits were baked in an electrical oven (OV185C, Inventum,  
138 Arnhem, The Netherlands) with convection mode at 175 °C for 5, 7 and 9 min. The different baking  
139 conditions were chosen in preliminary tests to obtain biscuits that were neither undercooked nor  
140 overcooked. The biscuits were placed in the middle position of a baking tray inside the oven; for each  
141 baking cycle, the air temperature inside the oven chamber was recorded every 20 s using a digital  
142 thermometer equipped with type K thermocouples (Pro 206-3722, RS Components, Corby, UK) to  
143 ensure equal temperature exposure between the baking cycles. After baking, biscuits were removed  
144 from the oven, placed on a grid and kept cooling at room temperature for about 1 h.

145 All biscuit formulations and baking times were performed in triplicate, resulting in 24 biscuits per  
146 sample at each baking time (24 × 7 samples × 3 baking times, a total of 504 biscuits).

147

## 148 **2.3 Characterization of flours and biscuits**

149

### 150 2.3.1 Proximal analysis

151 The wheat, lupin and chickpea flours were analysed for protein, fat, dietary fibre, ash and  
152 carbohydrate contents.

153 The total protein content (g/100 g) was determined by weighted 15 mg of each flour in steel crucibles  
154 using Dumas method with a protein analyser (Flash EA 1112, Thermo Fisher Scientific, Waltham,  
155 MA, USA). The conversion factor of 6.25 to determine crude protein content was used.

156 The fat content (g/100 g) was measured using the Soxhlet method (Gerhardt, Königswinter,  
157 Germany). Approximately 5 g of each flour was weighted in cellulose extraction thimbles and  
158 extracted continuously with 200 mL of petroleum ether at 60 °C for 3 h. After cooling down

159 overnight, the solvent was evaporated under vacuum in a rotavapor (R-200, Büchi, Flawil,  
160 Switzerland) at 60 °C and the fat content was determined gravimetrically.

161 The total dietary fibre content (g/100 g) was determined on 1 g of each flour by an enzymatic-  
162 gravimetric method using a total dietary fibre assay kit.

163 The ash content (g/100 g) was determined by weighing 1 g of each flour into ceramic crucibles and  
164 incinerating for 5 h at 525 °C in a muffle furnace (Gallenkamp and Co., London, UK). After  
165 combustion and cooling down the ash content was determined gravimetrically.

166 The carbohydrate content (g/100 g) was determined by subtracting the amounts of protein, fat, dietary  
167 fibre, ash and water (described in section 2.3.5) from 100 g of the flour sample. Using this method,  
168 the calculated carbohydrate value includes sugars, starch and may also contain small amounts of other  
169 minor compounds.

170 All analyses were performed in duplicate for each flour type.

171

### 172 2.3.2 Particle size

173 The particle size (Dv90 (µm)) of the flours was measured by a laser particle size analyser (Mastersizer  
174 3000, Malvern Panalytical, Malvern, UK). The obscuration in all measurements was 0.5-10%, air  
175 pressure was 2 bar and hopper height was 3 mm with a feed rate of 50%. The flours were analysed as  
176 opaque particles according to Fraunhofer approximation. The particle sizes were calculated by the  
177 supplier's software (version 3.62, Malvern Instruments, Malvern, UK) and the Dv90 (µm) value,  
178 representing the maximum particle diameter below which 90% of the sample falls, was evaluated.

179 The analysis was carried out in triplicate for each flour type.

180

### 181 2.3.3 Hydration properties

182 The water holding capacity (WHC) and the water binding capacity (WBC) of the flours were  
183 determined based on Sarangapani et al. (2016). Both WHC and WBC were evaluated in 1 g of flour  
184 mixed with 10 mL of distilled water. For WHC the mixture was kept for 24 h at room temperature,  
185 then the non-absorbed water was discarded, and the hydrated sample was weighted. For WBC, the  
186 mixture of sample and water was centrifuged for 3 min at  $1363 \times g$  and 20 °C (Heraeus Multifuge  
187 X3R, Thermo Fisher Scientific, Waltham, MA, USA). The non-absorbed water was removed, and  
188 the hydrated sample was weighted.

189 The results were expressed in g of water/g of solid; both measurements were done in triplicate for  
190 each flour type.

191

### 192 2.3.4 pH

193 The pH of flours and raw doughs was determined according to the method described by Mesías et al.  
194 (2015). 1 g of the ground sample was mixed with 100 mL of deionized water, vortexed for 3 min and  
195 kept at room temperature for 1 h. After centrifugation at  $4816 \times g$  and  $20 \text{ }^\circ\text{C}$  for 10 min (Heraeus  
196 Multifuge X3R, Thermo Fisher Scientific, Waltham, MA, USA), the pH of the supernatant was  
197 measured using a pH-meter (1100L, VWR, Radnor, PA, USA).

198 The measurement was performed in duplicate for each flour and raw dough.

199

#### 200 2.3.5 Moisture content

201 The moisture content of flours (g/100 g), raw doughs (%) and baked biscuits (%) was determined by  
202 a gravimetric method. For each sample, about 3 g of ground product was exactly weighted and dried  
203 at  $105 \text{ }^\circ\text{C}$  in an oven (Heraeus Series 6000, Thermo Scientific, Berlin, Germany) until constant  
204 weight.

205 The analysis was carried out in triplicate for each flour, dough and baking batch per sample.

206

#### 207 2.3.6 Water activity

208 The water activity ( $a_w$ ) of flours, raw doughs and biscuits was determined at  $25 \text{ }^\circ\text{C}$  with an  $a_w$ -meter  
209 (LabMaster, Novasina AG, Lachen, Switzerland) setting both time and temperature factors stability  
210 at 2 min.

211 The measurement was performed in duplicate for each flour, dough and baking batch per sample.

212

#### 213 2.3.7 Weight loss

214 The weight loss (%) of 8 biscuits was calculated as the percentage change in weight before and after  
215 each baking cycle per sample.

216

#### 217 2.3.8 Colour

218 The colour of flours, whole surfaces of raw and baked biscuits was performed with an IRIS V400  
219 electronic visual analyser (Alpha MOS, Toulouse, France) equipped with a 25 mm lens, lower and  
220 upper illumination and using a black background with a size of  $210 \times 297 \text{ mm}$ . ImageJ software (NIH,  
221 USA) was used for processing and quantification of CIE  $L^*$  (lightness),  $a^*$  (redness) and  $b^*$   
222 (yellowness) parameters of RGB images. From the numerical values of the measured parameters, the  
223 browning index (BI) was calculated by the following equations (Sakin-Yilmazer et al., 2013):

224

$$225 \text{ BI} = \frac{[(X-0.31) \cdot 100]}{0.17}, \text{ where } X = \frac{a^* + 1.79 \cdot L^*}{5.645 \cdot L^* + a^* - 3.012 \cdot b^*}$$

226

227 The colour measurements of each flour were carried out in triplicate and on the two surfaces of 5  
228 biscuits for each baking batch per sample.

229

### 230 2.3.9 Texture

231 The texture analysis of biscuits was performed at room temperature with Texture analyser TA.XT2  
232 (Stable Micro Systems, Surrey, UK) equipped with a load cell of 50 kg and a three-point bending test  
233 holder and probe. The distance of two beams of sample holder was 20 mm and the other setting were:  
234 pre-test speed of 5.00 mm/s, test speed of 1.00 mm/s, post-test speed of 10.00 mm/s and distance of  
235 5 mm. The downward movement was advanced till the biscuit was broken. The texture was described  
236 by the hardness (N), determined by means of maximum force, fracturability (1/mm), expressed as  
237 one/breakpoint distance between the origin of curve till the point where the biscuit breaks, and  
238 crispness, evaluated by the linear distance between the first and the last peak registered (Romani et  
239 al., 2012).

240 Force vs distance curves were obtained from 8 biscuits for each baking batch per sample.

241

## 242 **2.4 Quantification of asparagine and acrylamide by LC-MS/MS**

243

### 244 2.4.1 Sample extraction

245 Flours, raw doughs and baked biscuits were analysed for asparagine and AA contents. Asparagine  
246 and AA were extracted according to Žilić et al. (2020) with minor modifications. Briefly, 1 g of  
247 grounded sample was triple extracted with 20 mL of 10 mM formic acid in Milli-Q water. Each time  
248 the extract was vortex for 1 min and centrifuged for 10 min at  $4816 \times g$  and  $20\text{ }^{\circ}\text{C}$  (Heraeus Multifuge  
249 X3R, Thermo Fisher Scientific, Waltham, MA, USA). The combined supernatant was collected and  
250 stored in a freezer at  $-20\text{ }^{\circ}\text{C}$  until analysis (maximum 2 weeks).

251 For asparagine determination, the formic acid extract (5 mL) was centrifuged for 10 min at  $20817 \times$   
252  $g$  and  $20\text{ }^{\circ}\text{C}$  (5430 R, Eppendorf AG, Hamburg, Germany). For better clarification, 4 mL of  
253 supernatant were centrifuged for 7 min at  $20817 \times g$  and  $20\text{ }^{\circ}\text{C}$ , then 1 mL of clear supernatant was  
254 mixed with 1 mL of acetonitrile and filtered with  $0.2\text{ }\mu\text{m}$  PTFE filters ( $\text{Ø}15\text{ mm}$ ) into an amber glass  
255 autosampler vial. For AA determination, the formic acid extract (4.75 mL) with AA- $\text{d}_3$  solution (100  
256  $\mu\text{L}$ ) were clarified with 0.125 mL of Carrez I and 0.125 mL of Carrez II. The mixture was vortexed  
257 and centrifuged for 3 min at  $10621 \times g$  and  $20\text{ }^{\circ}\text{C}$ . For better clarification, 2 mL of supernatant was  
258 collected and centrifuged for 10 min at  $20817 \times g$  and  $20\text{ }^{\circ}\text{C}$ . Solid phase extraction cleaning was  
259 carried out according to Mogol & Gökmen (2014) using the Oasis MCX cartridge and collecting the  
260 sample in an amber glass autosampler vial.



261

#### 262 2.4.2 LC-MS/MS methods

263 Samples analyses were carried out with a Nexera UPLC system (Shimadzu Corporation, Kyoto,  
264 Japan) coupled with an LCMS-8050 triple quadrupole mass spectrometer (Shimadzu Corporation,  
265 Kyoto, Japan). The UPLC unit consisted of a SIL-30AC autosampler, an LC-20ADXR solvent  
266 delivery module, a DGU-20ASR degassing unit, a CTO-20AC column oven and an FCV-20AH<sub>2</sub>  
267 valve unit.

268 The chromatographic separation of free asparagine was performed injecting 5  $\mu$ L of samples on a  
269 SeQuant® ZIC HILIC 3.5  $\mu$ m, 4.6  $\times$  150 mm (Merck KGaS, 64271, Darmstadt, Germany) attached  
270 to a SeQuant® ZIC HILIC PEEK coated guard column 20  $\times$  2.1 mm (Merck KGaS, 64271,  
271 Darmstadt, Germany). The flow rate was set at 0.7 mL/min and the column temperature at 40 °C. The  
272 mobile phases consisted of 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid  
273 (solvent B) with the following elution profile (min/%B): 0.0/90, 4.0/70, 10.0/20, 13.0/20, 15.0/90 and  
274 18.0/90.

275 The chromatographic separation of AA was performed on Acquity PREMIER BEH C18 column (1.7  
276  $\mu$ m, 2.1  $\times$  50 mm) connected to an Acquity UPLC BEH C18 VanGuard Pre-column, (130 Å, 1.7  $\mu$ m,  
277 2.1  $\times$  5 mm) (Waters Chromatography B.V, Etten-Leur, The Netherlands) with a flow rate of 0.2  
278 mL/min at 40 °C column temperature. A gradient mixture of mobile phases A (0.1% formic acid) and  
279 B (methanol with 0.1% formic acid) was used for elution following the elution profile (min/%B) of:  
280 0.0/5, 2.5/70, 5.0/90, 6.0/90, 7.0/5 and 11.0/5.

281 Positive ionisation mode was used for both MS analyses. The voltage of the turbo ion-spray ionization  
282 was 4.0 kV. The temperature of the electrospray ionization probe, desolvation line and heat block was  
283 set at 300 °C, 250 °C and 400 °C, respectively. The pressure of the collision-induced dissociation gas  
284 was 4 kPa whereas the flow rates of the drying gas, nebulizer gas and heating gas were set at  
285 10 mL/min, 3 mL/min and 10 mL/min, respectively. The electrode voltage of Q1 pre bias (collision  
286 cell energy entrance potential), collision cell Q2 (collision energy), Q3 pre bias (collision cell energy  
287 exit potential), parent and fragment ion m/z of the multiple reaction monitoring transitions were  
288 optimized using support software (Shimadzu Corporation, Kyoto, Japan). For single reaction  
289 monitoring (SRM), the dwell time was set at 4 or 42 msec, respectively for asparagine and AA, and  
290 the most abundant fragment ion was selected for quantitation. The second and third fragments in ion  
291 yield were selected as a structural confirmation based on the optimized SRM transition. The  
292 precursor/product ion transitions m/z 133.20 $\rightarrow$ 74.00, 133.20 $\rightarrow$ 87.05 and 133.20 $\rightarrow$ 28.15 were  
293 monitored for asparagine; 72.00 $\rightarrow$ 55.10, 72.00 $\rightarrow$ 27.10 and 72.00 $\rightarrow$ 44.00 were monitored for  
294 acrylamide; 75.25 $\rightarrow$ 58.05, 75.25 $\rightarrow$ 30.05 and 75.25 $\rightarrow$ 44.05 were monitored for acrylamide-d<sub>3</sub>. Data

295 were processed with LabSolutions (Shimadzu Corporation, Kyoto, Japan). The recovery and matrix  
296 effects were satisfactory since the average recovery for AA was  $93 \pm 7\%$  and average matrix effects  
297 were  $101 \pm 10\%$  and  $105 \pm 6\%$  for asparagine and AA, respectively.

298 The sample extraction was repeated twice for each flour and each batch per sample and the analytical  
299 measurements were replicated twice for each extract. The results were expressed as  $\mu\text{g}/\text{kg}$  for AA and  
300  $\text{mg}/\text{kg}$  for asparagine on dry matter basis.

301

## 302 **2.5 Glucose, fructose and sucrose contents**

303

### 304 2.5.1 Sample extraction

305 Raw doughs and biscuits were analysed for glucose, fructose and sucrose contents. The sample  
306 extraction process was based on Nguyen et al. (2016) with slight modifications. Grounded biscuit  
307 (2.5 g) or freeze-dried dough (2.5 g) was mixed with 1:1, v/v of Milli-Q water and ethanol mixture  
308 (25 mL). The samples were incubated for 1 h at  $50\text{ }^\circ\text{C}$  in a water bath and cooled down for 20 min at  
309 room temperature. Then the samples were centrifuged at  $1962 \times g$  and  $20\text{ }^\circ\text{C}$  for 10 min (Heraeus  
310 Multifuge X3R, Thermo Fisher Scientific, Waltham, MA, USA). The supernatant (1.5 mL) was  
311 centrifuged at  $20817 \times g$  and  $20\text{ }^\circ\text{C}$  (5430 R, Eppendorf AG, Hamburg, Germany) for 10 min and 1  
312 mL was collected into a glass tube. The water/ethanol solvent was evaporated with a sample  
313 concentrator (SBHCONC/1, Stuart, Staffordshire, UK) under nitrogen flush at  $50\text{ }^\circ\text{C}$  for 4.5 h. The  
314 sample was reconstituted with acetonitrile (20 mL) and Milli-Q water (20 mL) and stored in a freezer  
315 at  $-20\text{ }^\circ\text{C}$  until measurement (maximum 1 week). Before analysis 1.5 mL of sample was passed  
316 through CA ( $\text{Ø}28\text{ mm}$ ;  $0.2\text{ }\mu\text{m}$ ) filters and transferred into an autosampler vial.

317

### 318 2.5.2 UPLC-ELSD method

319 The samples were analysed according to the procedure provided by Waters' technical application  
320 notebook with an Acquity UPLC-H Class Plus System (Waters, Milford, MA, USA) equipped with  
321 an Acquity Evaporative Light Scattering (ELSD) detector, an Acquity UPLC BEH Amide column  
322 ( $1.7\text{ }\mu\text{m}$ ,  $2.1 \times 100\text{ mm}$ ) and an Acquity UPLC BEH Amide VanGuard pre-column ( $130\text{ \AA}$ ,  $1.7\text{ }\mu\text{m}$ ,  
323  $2.1\text{ mm} \times 5\text{ mm}$ ) (Waters, Milford, MA, USA). The mobile phase A consisted of Milli-Q water and  
324 acetonitrile mixture (8:2, v/v) with 0.2% triethylamine (TEA) while mobile phase B consisted of  
325 acetonitrile/water 3:7, v/v with 0.2% TEA. The flow rate was  $0.25\text{ mL}/\text{min}$ . The gradient changes  
326 with the following elution profile (min/%A): 0.00/100, 6.00/40, 6.01/100 and 18/100. Before the first  
327 injection, the column was equilibrated with 100% A,  $0.25\text{ mL}/\text{min}$  for 30 min. The injection volume  
328 was  $1.3\text{ }\mu\text{L}$  and the column temperature was  $35\text{ }^\circ\text{C}$ . The pressure of ELSD conditions was 40 psi with

329 a drift tube temperature of 40 °C and a data rate 10 pps. Operating the software was carried out using  
330 a Waters Acquity Control console and data processing was performed with Chromeleon  
331 Chromatography Data System (version 7.2.10, Thermo Scientific Corp, Waltham, MA, USA). The  
332 quantification was done by an external calibration curve ranging from 85-1360 mg/L (sucrose) and  
333 45-720 mg/L (glucose and fructose).

334 The sample extraction was repeated twice for each batch per sample and the analytical measurement  
335 was conducted twice for each extract. The results for sucrose content were expressed as g/kg on dry  
336 matter basis.

337

## 338 **2.6 Data analysis**

339 Data processing and statistical analyses were performed with Excel (Microsoft, Redmond, WA, USA)  
340 and STATISTICA 8.0 (StatSoft Inc., Tulsa, UK) software. The results were reported as mean  $\pm$   
341 standard deviation of replications. Parametric unidirectional analysis of variance (ANOVA), followed  
342 by Tukey's post-hoc comparison test, with a significance level of 95% ( $p < 0.05$ ), were used to  
343 determine significant differences between the samples. The relationships between AA level and  
344 quantity of legume flours and asparagine content of the biscuits prepared with different formulations  
345 were determined by linear correlation with  $r^2$  coefficient in the range between  $-1$  (negative  
346 relationship) and  $+1$  (positive relationship).

347

## 348 **3. Results and discussion**

349

### 350 **3.1 Characteristics of flours**

351 The free asparagine content and other tested characteristics of wheat, lupin and chickpea flours to be  
352 related to the AA content and quality characteristics of the final biscuits are reported in **Table 1**. All  
353 flours had protein, fat, dietary fibre and ash contents comparable to those provided by the respective  
354 flour manufacturers and findings in the literature (Cardoso et al., 2019; Hall et al., 2017; Torra et al.,  
355 2021; Žilić et al., 2020).

356 The free asparagine content, which is the main factor influencing AA concentration in bakery  
357 products (Žilić et al., 2020), was significantly higher in legume flours than in the wheat one in the  
358 percentages of 117.4% and 51.2% for lupin and chickpea flour, respectively. This is coherent with  
359 the significantly higher protein content of legume flours (32.2 and 17.1 g/100 g for lupin and chickpea  
360 respectively) compared to wheat flour (11.2 g/100 g). The amount of free asparagine determined in  
361 wheat flour was in line with the ones reported by Hamlet et al. (2008), Capuano et al. (2009) and Žilić  
362 et al. (2020). The asparagine concentrations found in lupin flour were significantly much higher than

363 the literature reports (about 28 mg/kg), while those identified in chickpea flour were significantly  
364 much lower (about 420 mg/kg) (Bartkiene et al., 2016; Barutcu et al., 2009). Free asparagine  
365 accumulation in crops can be very variable because it largely depends on growing conditions as well  
366 as the processing methods (Miśkiewicz et al., 2012; Žilić et al., 2020).

367 Besides composition, the flours used in this study also differed in their particle size, which could  
368 influence the rate of the Maillard reaction and the formation of AA modulating the interaction and  
369 reactivity of chemical constituents in complex food mixtures (Betoret & Rosell, 2020; Sun et al.,  
370 2019). Chickpea flour had the highest Dv90 value, indicating that 90% of the sample had a particle  
371 size of 410.0 µm or less, while wheat one had the lowest particle size, with a Dv90 of 156.3 µm.

372 Moisture and  $a_w$  levels also play a crucial role in the interaction of AA precursors as well as in the  
373 rate of the Maillard reaction (De Vleeschouwer et al., 2007). The moisture of the flours ranged  
374 between 6.2-12.8 g/100 g (lupin-wheat flour) and the  $a_w$  varied in the range 0.34-0.65 (lupin-wheat  
375 flour). The moisture and  $a_w$  discrepancies found in the studied flours are probably due to the different  
376 compositions and milling processing conditions.

377 Regarding the hydration properties, the lupin flour had the significant highest value of WHC and  
378 WBC, probably due to the highest dietary fibre content (40.8 g/100 g) compared to the other flours  
379 (11.8 and 2.8 g/100 g for chickpea and wheat, respectively). Wheat and chickpea flours had very  
380 similar WHC values, about 2 g water/g solid, and differed significantly only for WBC. In addition,  
381 these hydration properties may also result from the presence of various types of hydrophilic  
382 carbohydrates and the varying structure of proteins (Farooq & Boye, 2011).

383 The pH values of the water-soluble fraction of the flours were close to neutral. Compared to the pH  
384 of wheat flour (6.1), the pH of lupin flour equal to 6.0 was significantly lower, while that of chickpea  
385 flour equal to 6.7 was significantly higher.

386 Regarding colour, legume flours were both less light with lower  $a^*$  and higher  $b^*$  colour parameters  
387 due to their yellowish colour compared to wheat flour which remains lighter and whiter. The presence  
388 of a range of pigments in the cotyledons and seed coats of several legumes gives them a distinct  
389 colour (Tetrycz et al., 2020).

390

### 391 **3.2 Influence of legume flour on the contents of acrylamide and free asparagine of biscuits**

392 Besides the amount of free asparagine, other flour properties can lead to different rates of AA  
393 formation (Miśkiewicz et al., 2020). To assess the effect of other flours differences on AA formation  
394 independently from their asparagine content, the concentration of asparagine in all biscuit doughs  
395 was standardized. The highest asparagine concentration was recorded in the lupin flour; therefore, no  
396 additional asparagine was added to the L60 dough, achieving an asparagine value of  $65.5 \pm 7.0$  mg/kg

397 on dry matter basis. In the other type of biscuits samples, the amount of added free asparagine was  
398 adjusted according to the percentage of used flours and their asparagine concentration (**Table 1**). The  
399 asparagine values of the different dough samples after standardisation, together with AA and sucrose  
400 concentrations detected in all biscuit samples, are reported in **Table 2**.

401 Looking at the data it is clear that despite the same free asparagine content, the use of legume flours  
402 compared to wheat flour led to a different formation of AA during the baking of the biscuits. The  
403 wheat control sample showed an increase in AA during baking, reaching values above the reference  
404 level reported in Commission Regulation (EU) 2017/2158 (350 µg/kg) of 421.2, 481.9 and 583.9  
405 µg/kg (d.m.) after 5, 7 and 9 min, respectively. AA values in wheat biscuits were significantly higher  
406 compared to the ranges reported in previous studies, probably due to the addition of pure asparagine  
407 done to equalize the asparagine concentrations in all formulations (Manolache et al., 2019; Mesías et  
408 al., 2016; Sazesh & Goli, 2020; Žilić et al., 2020).

409 The use of different percentages of lupin and chickpea flour in the biscuit recipes resulted in different  
410 rates of AA formation compared to the wheat flour. When 40 and 60% of lupin flour was used, the  
411 AA content increased by 105.6 and 173.2%, 80.7 and 161.7%, 84.2 and 147.1% after 5, 7 and 9 min  
412 of baking, respectively, compared to wheat samples. The biscuits had a significantly higher AA  
413 content than in the wheat sample, proportionally to the amount of lupin flour used ( $r^2 = 0.99$ ,  $r^2 = 1.00$   
414 and  $r^2 = 0.99$ , respectively for 5, 7 and 9 min of baking). Similar results were obtained in studies by  
415 Bartkiene et al. (2013, 2016) who described higher AA levels of 43.3 and 78.5% by increasing lupin  
416 flour in bread and biscuit products, respectively, compared to control samples without added lupin  
417 flour. The resulting increase in AA proportional to the amount of lupin flour was attributed to a higher  
418 asparagine content in lupin flour compared to wheat flour (Bartkiene et al., 2016). In the present  
419 study, since the initial asparagine concentrations were standardized, the increase in AA in biscuits  
420 could be due to the higher dietary fibre content of lupin flour (40.8 g/100 g) compared to wheat one  
421 (2.8 g/100 g) (**Table 1**). The presence of a high percentage of dietary fibre contributed to reducing  
422 the  $a_w$  of the biscuits during baking as described in the next section 3.3 “Influence of legume flours  
423 on the main characteristics of biscuits” and this may have favoured the Maillard reaction and AA  
424 formation due to a higher concentration of reaction substrates (Palermo et al., 2012). However,  
425 conflicting results have been found in the literature, some studies stated that the Maillard rate is higher  
426 at high  $a_w$  values (0.6-0.8) where the mobility of the reactants is greater, whereas at very low  $a_w$  the  
427 reactants become too concentrated limiting their diffusion and interaction (van Boekel, 2001).

428 Low AA levels were obtained in biscuits with 20 and 40% of chickpea flour, leading to a reduction  
429 of about 50% in AA compared to the wheat control sample after each baking time (**Table 2**). This  
430 result can confirm a possible effect of chickpea proteins in the thermal stability of reducing sugars

431 previously described by Miśkiewicz et al. (2020). The authors, using a Differential Scanning  
432 Calorimetry (DSC) analysis, reported that the melting point of glucose and fructose with 1% of  
433 chickpea proteins extract (extract composition: protein=82.70 g/100 g, fructose=0.05 mg/g d.m.,  
434 glucose=0.02 mg/g d.m., sucrose=0.12 mg/g d.m. and maltose=1.49 mg/g d.m.) increased, due to a  
435 higher ordering of the crystallographic structures of the carbohydrates. This helped to reduce the  
436 reaction speed between reducing sugars and asparagine slowing down the formation of AA  
437 (Miśkiewicz et al., 2020). In addition, the lower interaction between AA precursors may also have  
438 resulted from the coarsest particle size of the chickpea flour. However, no such effect was noticed for  
439 lupin biscuit samples despite a larger flour particle size than wheat flour, indicating a greater effect  
440 of dietary fibre content by decreasing the  $a_w$  as previously explained. When wheat flour was  
441 substituted with 60% of chickpea flour, significant AA increases of 79.5, 45.7 and 7.8% were detected  
442 compared to wheat samples at 5, 7 and 9 min, respectively. Probably because at this chickpea flour  
443 percentage, the effect of its dietary fibre content (11.8 g/100 g) on moisture and  $a_w$  control prevails  
444 over the positive effect of chickpea proteins on AA formation described above (Miśkiewicz et al.,  
445 2020; Palermo et al., 2012). The percentages of chickpea flour in the biscuits had a non-significant  
446 correlation to the amount of AA determined after baking ( $r^2 = 0.71$ ,  $r^2 = 0.82$ ,  $r^2 = 0.64$ , respectively  
447 at 5, 7 and 9 min). Overall, the AA values in chickpea biscuits measured in this research activity are  
448 high compared to the result obtained by Miśkiewicz et al. (2012), probably in relation to differences  
449 in the biscuit's formulations, as well as in the baking process parameters.

450 Concerning the asparagine concentrations detected in biscuits (**Table 2**), in wheat samples the values  
451 were negatively correlated with AA levels ( $r^2 = -0.91$ ), confirming the dominant role of this amino  
452 acid in the formation of AA. A similar result was observed for lupin biscuits, with negative linear  
453 correlation coefficients  $r^2$  of  $-1.00$  for L20,  $-0.91$  for L40 and  $-0.96$  for L60. A relatively good  
454 negative linear correlation between AA and asparagine contents was also found for some chickpea  
455 samples with an  $r^2$  of  $-0.90$  for C20 and an  $r^2$  of  $-0.97$  for C40; no correlation was found for sample  
456 C60, where the AA concentrations did not vary significantly between 5 and 9 min of baking.  
457 However, the percentages of asparagine reduction from the dough (time 0) to biscuit baked for 9 min  
458 were much lower in chickpea samples than in wheat and lupin ones, especially at the flour percentages  
459 of 20 and 40. For wheat samples, a reduction in asparagine of 25.1% was measured, for samples L20  
460 and L40 a reduction of 26.4 and 40.6%, respectively, and for samples C20 and C40 only a reduction  
461 of 7.8 and 14.3%, respectively. This clearly confirmed the hypothesis made by examining the data on  
462 AA formation and confirmed the observation of Miśkiewicz et al. (2020): the use of chickpea flour  
463 at 20 and 40% reduced the reaction rate between free asparagine and reducing sugars.

464 No reducing sugars (i.e. glucose and fructose) could be detected in either the dough or the biscuits,  
465 this could be due to their participation in the Maillard reaction or other thermal reactions (e.g.  
466 caramelization, pyrolytic reactions). The initial contents of sucrose did not change significantly  
467 during baking (**Table 2**), demonstrating any hydrolysis of this sugar during baking has already been  
468 detected by some previous studies (Gökmen et al., 2007; Nguyen et al., 2016; Schouten et al., 2022).  
469 Indeed, the sucrose content in the samples did not significantly influence the Maillard reaction  
470 development.

471

### 472 **3.3 Influence of legume flours on some quality characteristics of biscuits**

473 The use of different flours in the biscuit formulation led to variations in moisture,  $a_w$  and weight loss  
474 of the biscuit samples at different baking times, as displayed in **Table 3**. The amount of water added  
475 in the dough recipes was standardised and calculated based on the moisture content of each flour to  
476 achieve similar moisture content (around 17%) and  $a_w$  (around 0.80) in all doughs. This was because,  
477 in addition to the chemical characteristics of the flour, moisture and  $a_w$  of biscuit doughs are  
478 parameters that can influence the formation of AA. As expected, both moisture and  $a_w$  values of all  
479 biscuit samples decreased with increasing baking time. For all baking times tested the moisture and  
480  $a_w$  of the wheat samples were significantly higher than of the lupin and chickpea biscuits, except for  
481 the C60 sample after 5 min of baking in which the values were significantly the same. The low  
482 moisture and  $a_w$  values of the lupin and chickpea samples could be related to differences in the  
483 macronutrient compositions (e.g. dietary fibre, carbohydrates) and particle size of the legume flours  
484 (**Table 1**). In lupin samples, both moisture and  $a_w$  decreased with the increase of the amount of lupin  
485 flour, while in chickpea samples these parameters tended to decrease without a clear trend related to  
486 the increased amounts of chickpea flour. As reported in previous studies, the weight loss was greatest  
487 in the first few minutes of baking (5 min); the formation of a dry surface layer caused a reduction in  
488 water vapour flow although the mass transfer continued until the end of baking (9 min) leading to an  
489 increase in weight loss percentage (Thorvaldsson & Skjöldebrand, 1998). The weight loss after  
490 baking of the lupin biscuits was significantly greater compared to wheat samples, while for chickpea  
491 ones were no significant differences (**Table 3**).

492 No significant differences ( $p < 0.05$ ) were found in the pH of the biscuit doughs, resulting in a range  
493 between 8 and 8.8, suggesting that the leavening agent and other common ingredients used in the  
494 recipe were able to compensate for the slight initial differences between the flours (**Table 1**).

495 The use of different flours in the formulation resulted in variations in the colour and texture properties  
496 of the baked biscuits as indicated in **Table 4**. The  $L^*$  (lightness) values of the dough decreased  
497 significantly with the increasing amount of legume flours that showed a more yellow and intense

498 colouring than the matt and greyish wheat flour (**Table 1**). Similar results were previously described  
499 by Bartkiene et al. (2016) analysing biscuits obtained with lupin flour. Moreover, for all types of  
500 biscuits, the upper L\* value significantly decreased with the increase of baking time. Compared to  
501 the wheat sample, after 5 min of baking, the L\* value of the upper surface of the biscuits decreased  
502 significantly for samples L20, L40, L60, C40 and C60, whereas after 7 and 9 min of baking L\*  
503 decreased significantly only for samples C40 and C60. When comparing samples prepared with lupin  
504 and chickpea flour after 7 and 9 min of baking, samples C40 and C60 showed a lower lightness than  
505 samples L40 and L60, indicating more intense colour changes. The BI (browning index) values of  
506 the different types of dough (**Table 4**) also increased over the percentage of legume flours. For all  
507 wheat, lupin and chickpea samples, the upper surface of the biscuits became more brown during the  
508 prolongation of the baking time due to Maillard and caramelisation reactions (Lara et al., 2011). At  
509 all baking times, a significantly higher upper BI was determined when the legume flours were used  
510 at 40 and 60%. Differences in L\* and BI results were also found in the biscuits lower surfaces that  
511 were less light and darker than the upper ones. The differences in surface colour of all biscuits were  
512 also noticeable from the visual appearance shown in **Figure 1**.

513 As reported in **Table 4** the different flours used for biscuit formulations caused differences also in  
514 terms of texture which is an important quality parameter in bakery products correlated with  
515 consumers' perception of the freshness (Zoulias et al., 2000). The assessed texture properties were  
516 hardness and fracturability, which indicate the firmness of the structure, and crispness, which is a  
517 measure of structure friability. Generally, hardness is often considered an undesirable characteristic  
518 of biscuit products, while fracturability is related to a pleasant sensory characteristic as long as it does  
519 not become excessive (Zoulias et al., 2000). For all formulations, biscuits increased in hardness,  
520 fracturability and crispness with increasing baking times as noted in previous research (Lara et al.,  
521 2011; Romani et al., 2012; Schouten et al., 2022). Compared to the wheat samples, hardness increased  
522 significantly when the amount of lupin flour was increased in the formula, due to the high dietary  
523 fibre content, while it decreased with more chickpea flour, probably attributed to its coarser particle  
524 size. However, no significant differences in hardness were found between the wheat and C20 samples  
525 at all tested baking times. As expected, the harder biscuits were also the less fracturable ones. The  
526 lupin biscuits presented lower fracturability values than the wheat and chickpea ones after all tested  
527 baking times, although not always significantly. The crispness was significantly different between  
528 wheat and lupin samples after 5 and 7 min of baking and for L40 and L60. On the other hand, wheat  
529 samples differed significantly, although slightly, compared to chickpea samples only when chickpea  
530 flour was used at 60%. The highest dietary fibre content, WH and WB capacities of lupin flour, as



531 well as the greater particle size of chickpea one (**Table 1**), may have contributed to the texture results  
532 of the biscuits made from them.

533

#### 534 **4. Conclusions**

535 The standardization of the initial asparagine concentration in the different formulations has been an  
536 effective approach to assess the effect of flours of different origins on AA formation in biscuits.

537 The use of lupin flour was not effective in the mitigation of AA levels in biscuits. Especially at high  
538 concentrations, lupin flour accelerated the AA formation reactions probably due to its higher dietary  
539 fibre content causing lower moisture content and  $a_w$  in biscuits.

540 Interestingly, chickpea flour showed good potentiality for controlling AA formation mainly when it  
541 was used at a concentration between 20 and 40%. The effect is likely due to the composition and  
542 particle size of the chickpea flours and to the effect on the thermodynamic properties of carbohydrate  
543 compounds by the addition of chickpea proteins. In biscuits formulated with chickpea flour,  
544 concentrations of AA were found to be lower than the reference value given in Commission  
545 Regulation (EU) 2017/2158 (350  $\mu\text{g}/\text{kg}$ ) despite the very high amount of asparagine added used in  
546 our biscuits.

547 Interestingly, the use of a limited concentration of chickpea flour did not substantially change some  
548 quality characteristics of the final biscuits such as colour and texture, while it improved protein and  
549 dietary fibre content. Further studies are needed to evaluate the sensory acceptability of chickpea  
550 addition to the biscuits; however, the use of chickpea flour instead of other flours with similar  
551 asparagine content can be a simple and effective solution to mitigate the AA formation in biscuits  
552 and other low moisture bakery products.

553

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559

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687 <https://doi.org/10.1080/10942910009524643>
- 688
- 689 **Figure 1.** Visual appearance of the raw biscuits (A) and of upper (B) and lower (C) surfaces of  
690 biscuits baked at 175 °C for different times formulated with different flours (W: only wheat flour;  
691 L20, L40, L60: 20%, 40%, 60% of lupin flour; C20, C40, C60: 20%, 40%, 60% of chickpea flour).

**Table 1.** Characterization of wheat, lupin and chickpea flours.

| <b>Propriety</b>                 | <b>Wheat flour</b>        | <b>Lupin flour</b>        | <b>Chickpea flour</b>     |
|----------------------------------|---------------------------|---------------------------|---------------------------|
| Moisture (g/100 g)               | 12.8 ± 0.1 <sup>a</sup>   | 6.2 ± 0.1 <sup>c</sup>    | 11.4 ± 0.1 <sup>b</sup>   |
| Water activity (a <sub>w</sub> ) | 0.65 ± 0.0 <sup>a</sup>   | 0.34 ± 0.01 <sup>c</sup>  | 0.63 ± 0.01 <sup>b</sup>  |
| Protein (g/100 g)                | 11.2 ± 0.0 <sup>c</sup>   | 32.2 ± 0.3 <sup>a</sup>   | 17.1 ± 0.2 <sup>b</sup>   |
| Fat (g/100 g)                    | 1.2 ± 0.0 <sup>b</sup>    | 6.1 ± 0.0 <sup>a</sup>    | 5.9 ± 1.0 <sup>a</sup>    |
| Dietary fiber (g/100 g)          | 2.8 ± 0.8 <sup>c</sup>    | 40.8 ± 1.2 <sup>a</sup>   | 11.8 ± 0.7 <sup>b</sup>   |
| Ash (g/100 g)                    | 0.6 ± 0.1 <sup>b</sup>    | 3.0 ± 0.2 <sup>a</sup>    | 3.1 ± 0.2 <sup>a</sup>    |
| Carbohydrates (g/100 g)          | 71.38 ± 0.66 <sup>a</sup> | 11.65 ± 0.90 <sup>c</sup> | 50.65 ± 1.06 <sup>b</sup> |
| Asparagine (mg/kg)               | 88.4 ± 7.4 <sup>c</sup>   | 192.2 ± 11.8 <sup>a</sup> | 133.7 ± 10.6 <sup>b</sup> |
| Particle size (Dv90 (µm))        | 156.3 ± 1.5 <sup>c</sup>  | 210.3 ± 4.5 <sup>b</sup>  | 410.0 ± 3.5 <sup>a</sup>  |
| WHC (g water/g solid)            | 2.0 ± 0.1 <sup>b</sup>    | 4.7 ± 0.1 <sup>a</sup>    | 1.8 ± 0.1 <sup>b</sup>    |
| WBC (g water/g solid)            | 0.7 ± 0.0 <sup>c</sup>    | 1.7 ± 0.0 <sup>a</sup>    | 0.9 ± 0.0 <sup>b</sup>    |
| pH                               | 6.1 ± 0.0 <sup>b</sup>    | 6.0 ± 0.0 <sup>c</sup>    | 6.7 ± 0.0 <sup>a</sup>    |
| Lightness (L*)                   | 82.1 ± 0.6 <sup>a</sup>   | 73.3 ± 0.9 <sup>b</sup>   | 74.8 ± 0.1 <sup>b</sup>   |
| Green-red parameter (a*)         | -0.5 ± 0.1 <sup>a</sup>   | -1.0 ± 0.2 <sup>b</sup>   | -1.2 ± 0.1 <sup>c</sup>   |
| Yellow-blue parameter (b*)       | 8.4 ± 0.2 <sup>c</sup>    | 22.9 ± 1.1 <sup>a</sup>   | 21.5 ± 0.0 <sup>b</sup>   |

Different letters in the same line indicate significant differences among samples ( $p < 0.05$ ).

**Table 2.** Asparagine, acrylamide and sucrose contents on dry matter (d.m.) basis in wheat, lupin, chickpea doughs (time 0) and biscuits baked at 175 °C for 5, 7, 9 minutes (W: only wheat flour; L20, L40, L60: 20%, 40%, 60% lupin flour; C20, C40, C60: 20%, 40%, 60% chickpea flour).

| Baking time (min)              | W                            | L20                          | L40                           | L60                            | C20                          | C40                           | C60                          |
|--------------------------------|------------------------------|------------------------------|-------------------------------|--------------------------------|------------------------------|-------------------------------|------------------------------|
| <b>Asparagine (mg/kg d.m.)</b> |                              |                              |                               |                                |                              |                               |                              |
| 0                              | 66.4 ± 5.9 <sup>a, A</sup>   | 71.6 ± 8.0 <sup>a, A</sup>   | 73.8 ± 12.3 <sup>a, A</sup>   | 65.5 ± 7.0 <sup>a, A</sup>     | 57.9 ± 6.5 <sup>b, A</sup>   | 68.7 ± 6.0 <sup>a, A</sup>    | 69.0 ± 6.8 <sup>a, A</sup>   |
| 5                              | 72.9 ± 7.8 <sup>a, A</sup>   | 61.4 ± 6.5 <sup>b, B</sup>   | 61.1 ± 12.4 <sup>b, B</sup>   | 55.4 ± 5.4 <sup>b, C</sup>     | 77.3 ± 2.3 <sup>a, A</sup>   | 62.7 ± 3.7 <sup>a, B</sup>    | 67.3 ± 8.0 <sup>a, B</sup>   |
| 7                              | 70.1 ± 8.0 <sup>a, A</sup>   | 59.3 ± 8.3 <sup>bc, B</sup>  | 55.5 ± 13.5 <sup>b, B</sup>   | 53.6 ± 6.5 <sup>b, B</sup>     | 70.3 ± 5.8 <sup>a, A</sup>   | 60.9 ± 4.0 <sup>b, B</sup>    | 56.8 ± 6.3 <sup>b, B</sup>   |
| 9                              | 49.7 ± 8.5 <sup>b, B</sup>   | 52.7 ± 5.2 <sup>c, AB</sup>  | 43.8 ± 14.5 <sup>c, C</sup>   | 46.6 ± 3.3 <sup>c, C</sup>     | 53.4 ± 2.4 <sup>c, A</sup>   | 58.9 ± 4.0 <sup>c, A</sup>    | 50.4 ± 6.9 <sup>b, B</sup>   |
| <b>Acrylamide (µg/kg d.m.)</b> |                              |                              |                               |                                |                              |                               |                              |
| 0                              | <LOD                         | <LOD                         | <LOD                          | <LOD                           | <LOD                         | <LOD                          | <LOD                         |
| 5                              | 421.2 ± 3.1 <sup>a, C</sup>  | 438.7 ± 23.0 <sup>a, C</sup> | 866.1 ± 39.6 <sup>a, B</sup>  | 1150.9 ± 13.2 <sup>a, A</sup>  | 232.9 ± 66.0 <sup>a, D</sup> | 203.9 ± 35.0 <sup>a, D</sup>  | 756.0 ± 53.4 <sup>a, B</sup> |
| 7                              | 481.9 ± 13.7 <sup>b, D</sup> | 497.9 ± 36.2 <sup>b, D</sup> | 871.0 ± 14.6 <sup>a, B</sup>  | 1261.2 ± 13.7 <sup>ab, A</sup> | 227.7 ± 19.6 <sup>a, E</sup> | 270.6 ± 36.4 <sup>ab, E</sup> | 702.3 ± 42.9 <sup>a, C</sup> |
| 9                              | 583.9 ± 21.1 <sup>c, C</sup> | 559.6 ± 42.1 <sup>c, C</sup> | 1075.6 ± 24.9 <sup>b, B</sup> | 1443.0 ± 15.8 <sup>b, A</sup>  | 354.4 ± 48.7 <sup>b, D</sup> | 312.6 ± 10.2 <sup>b, D</sup>  | 629.6 ± 24.6 <sup>a, C</sup> |
| <b>Sucrose (g/kg d.m.)</b>     |                              |                              |                               |                                |                              |                               |                              |

|   |                              |                              |                              |                              |                              |                              |                              |
|---|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|
| 0 | 184.0 ± 16.7 <sup>a, A</sup> | 187.1 ± 11.8 <sup>b, A</sup> | 185.5 ± 9.8 <sup>a, A</sup>  | 211.0 ± 18.8 <sup>a, A</sup> | 202.7 ± 13.9 <sup>a, A</sup> | 200.6 ± 14.8 <sup>a, A</sup> | 226.1 ± 8.4 <sup>a, A</sup>  |
| 5 | 192.8 ± 19.6 <sup>a, A</sup> | 230.1 ± 9.2 <sup>a, A</sup>  | 205.9 ± 11.8 <sup>a, A</sup> | 237.6 ± 17.9 <sup>a, A</sup> | 208.8 ± 10.0 <sup>a, A</sup> | 207.8 ± 19.9 <sup>a, A</sup> | 236.0 ± 9.8 <sup>a, A</sup>  |
| 7 | 195.1 ± 13.4 <sup>a, A</sup> | 231.1 ± 6.5 <sup>a, A</sup>  | 206.3 ± 8.1 <sup>a, A</sup>  | 236.2 ± 8.2 <sup>a, A</sup>  | 194.9 ± 20.8 <sup>a, A</sup> | 206.8 ± 11.6 <sup>a, A</sup> | 241.6 ± 13.9 <sup>a, A</sup> |
| 9 | 194.1 ± 7.0 <sup>a, B</sup>  | 244.0 ± 3.9 <sup>a, A</sup>  | 214.9 ± 8.8 <sup>a, A</sup>  | 226.7 ± 6.4 <sup>a, A</sup>  | 220.1 ± 13.2 <sup>a, A</sup> | 213.4 ± 30.3 <sup>a, A</sup> | 239.9 ± 10.3 <sup>a, A</sup> |

Different lowercase letters in the same column of each compound and different capital letters in the same line indicate significantly different differences among samples ( $p < 0.05$ ). LOD = Limit of detection.



**Table 2.** Asparagine, acrylamide and sucrose contents on dry matter (d.m.) basis in wheat, lupin, chickpea doughs (time 0) and biscuits baked at 175 °C for 5, 7, 9 minutes (W: only wheat flour; L20, L40, L60: 20%, 40%, 60% lupin flour; C20, C40, C60: 20%, 40%, 60% chickpea flour).

| Baking time (min)              | W                            | L20                          | L40                           | L60                            | C20                          | C40                           | C60                          |
|--------------------------------|------------------------------|------------------------------|-------------------------------|--------------------------------|------------------------------|-------------------------------|------------------------------|
| <b>Asparagine (mg/kg d.m.)</b> |                              |                              |                               |                                |                              |                               |                              |
| 0                              | 66.4 ± 5.9 <sup>a, A</sup>   | 71.6 ± 8.0 <sup>a, A</sup>   | 73.8 ± 12.3 <sup>a, A</sup>   | 65.5 ± 7.0 <sup>a, A</sup>     | 57.9 ± 6.5 <sup>b, A</sup>   | 68.7 ± 6.0 <sup>a, A</sup>    | 69.0 ± 6.8 <sup>a, A</sup>   |
| 5                              | 72.9 ± 7.8 <sup>a, A</sup>   | 61.4 ± 6.5 <sup>b, B</sup>   | 61.1 ± 12.4 <sup>b, B</sup>   | 55.4 ± 5.4 <sup>b, C</sup>     | 77.3 ± 2.3 <sup>a, A</sup>   | 62.7 ± 3.7 <sup>a, B</sup>    | 67.3 ± 8.0 <sup>a, B</sup>   |
| 7                              | 70.1 ± 8.0 <sup>a, A</sup>   | 59.3 ± 8.3 <sup>bc, B</sup>  | 55.5 ± 13.5 <sup>b, B</sup>   | 53.6 ± 6.5 <sup>b, B</sup>     | 70.3 ± 5.8 <sup>a, A</sup>   | 60.9 ± 4.0 <sup>b, B</sup>    | 56.8 ± 6.3 <sup>b, B</sup>   |
| 9                              | 49.7 ± 8.5 <sup>b, B</sup>   | 52.7 ± 5.2 <sup>c, AB</sup>  | 43.8 ± 14.5 <sup>c, C</sup>   | 46.6 ± 3.3 <sup>c, C</sup>     | 53.4 ± 2.4 <sup>c, A</sup>   | 58.9 ± 4.0 <sup>c, A</sup>    | 50.4 ± 6.9 <sup>b, B</sup>   |
| <b>Acrylamide (µg/kg d.m.)</b> |                              |                              |                               |                                |                              |                               |                              |
| 0                              | <LOD                         | <LOD                         | <LOD                          | <LOD                           | <LOD                         | <LOD                          | <LOD                         |
| 5                              | 421.2 ± 3.1 <sup>a, C</sup>  | 438.7 ± 23.0 <sup>a, C</sup> | 866.1 ± 39.6 <sup>a, B</sup>  | 1150.9 ± 13.2 <sup>a, A</sup>  | 232.9 ± 66.0 <sup>a, D</sup> | 203.9 ± 35.0 <sup>a, D</sup>  | 756.0 ± 53.4 <sup>a, B</sup> |
| 7                              | 481.9 ± 13.7 <sup>b, D</sup> | 497.9 ± 36.2 <sup>b, D</sup> | 871.0 ± 14.6 <sup>a, B</sup>  | 1261.2 ± 13.7 <sup>ab, A</sup> | 227.7 ± 19.6 <sup>a, E</sup> | 270.6 ± 36.4 <sup>ab, E</sup> | 702.3 ± 42.9 <sup>a, C</sup> |
| 9                              | 583.9 ± 21.1 <sup>c, C</sup> | 559.6 ± 42.1 <sup>c, C</sup> | 1075.6 ± 24.9 <sup>b, B</sup> | 1443.0 ± 15.8 <sup>b, A</sup>  | 354.4 ± 48.7 <sup>b, D</sup> | 312.6 ± 10.2 <sup>b, D</sup>  | 629.6 ± 24.6 <sup>a, C</sup> |
| <b>Sucrose (g/kg d.m.)</b>     |                              |                              |                               |                                |                              |                               |                              |

|   |                              |                              |                              |                              |                              |                              |                              |
|---|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|
| 0 | 184.0 ± 16.7 <sup>a, A</sup> | 187.1 ± 11.8 <sup>b, A</sup> | 185.5 ± 9.8 <sup>a, A</sup>  | 211.0 ± 18.8 <sup>a, A</sup> | 202.7 ± 13.9 <sup>a, A</sup> | 200.6 ± 14.8 <sup>a, A</sup> | 226.1 ± 8.4 <sup>a, A</sup>  |
| 5 | 192.8 ± 19.6 <sup>a, A</sup> | 230.1 ± 9.2 <sup>a, A</sup>  | 205.9 ± 11.8 <sup>a, A</sup> | 237.6 ± 17.9 <sup>a, A</sup> | 208.8 ± 10.0 <sup>a, A</sup> | 207.8 ± 19.9 <sup>a, A</sup> | 236.0 ± 9.8 <sup>a, A</sup>  |
| 7 | 195.1 ± 13.4 <sup>a, A</sup> | 231.1 ± 6.5 <sup>a, A</sup>  | 206.3 ± 8.1 <sup>a, A</sup>  | 236.2 ± 8.2 <sup>a, A</sup>  | 194.9 ± 20.8 <sup>a, A</sup> | 206.8 ± 11.6 <sup>a, A</sup> | 241.6 ± 13.9 <sup>a, A</sup> |
| 9 | 194.1 ± 7.0 <sup>a, B</sup>  | 244.0 ± 3.9 <sup>a, A</sup>  | 214.9 ± 8.8 <sup>a, A</sup>  | 226.7 ± 6.4 <sup>a, A</sup>  | 220.1 ± 13.2 <sup>a, A</sup> | 213.4 ± 30.3 <sup>a, A</sup> | 239.9 ± 10.3 <sup>a, A</sup> |

Different lowercase letters in the same column of each compound and different capital letters in the same line indicate significantly different differences among samples ( $p < 0.05$ ). **LOD = Limit of detection.**

Table 3

**Table 3.** Moisture (%), water activity ( $a_w$ ) and weight loss (%) values of wheat, lupin and chickpea doughs (time 0) and biscuits baked at 175 °C for 5, 7, 9 minutes (W: only wheat flour; L20, L40, L60: 20%, 40%, 60% lupin flour; C20, C40, C60: 20%, 40%, 60% chickpea flour).

| Baking time (min)                        | W                           | L20                          | L40                         | L60                         | C20                          | C40                          | C60                          |
|--|-----------------------------|------------------------------|-----------------------------|-----------------------------|------------------------------|------------------------------|------------------------------|
| <i>Moisture (%)</i>                      |                             |                              |                             |                             |                              |                              |                              |
| 0  | 17.0 ± 0.1 <sup>a, A</sup>  | 16.8 ± 0.4 <sup>a, A</sup>   | 17.1 ± 0.1 <sup>a, A</sup>  | 17.0 ± 0.2 <sup>a, A</sup>  | 16.5 ± 0.2 <sup>a, A</sup>   | 17.0 ± 0.1 <sup>a, A</sup>   | 17.0 ± 0.1 <sup>a, A</sup>   |
| 5  | 9.7 ± 0.3 <sup>b, A</sup>   | 9.0 ± 0.1 <sup>b, B</sup>    | 8.7 ± 0.5 <sup>b, B</sup>   | 8.4 ± 0.4 <sup>b, C</sup>   | 8.8 ± 0.4 <sup>b, B</sup>    | 8.9 ± 0.1 <sup>b, B</sup>    | 9.4 ± 0.2 <sup>b, AB</sup>   |
| 7  | 5.6 ± 1.1 <sup>c, A</sup>   | 5.2 ± 0.1 <sup>c, B</sup>    | 5.0 ± 0.4 <sup>c, BC</sup>  | 4.4 ± 0.2 <sup>c, C</sup>   | 5.3 ± 0.0 <sup>c, B</sup>    | 5.4 ± 0.1 <sup>c, B</sup>    | 5.3 ± 0.1 <sup>c, B</sup>    |
| 9  | 3.0 ± 0.2 <sup>d, A</sup>   | 2.5 ± 0.2 <sup>d, B</sup>    | 2.6 ± 0.3 <sup>d, B</sup>   | 2.2 ± 0.2 <sup>d, C</sup>   | 2.8 ± 0.1 <sup>d, B</sup>    | 2.8 ± 0.1 <sup>d, B</sup>    | 2.5 ± 0.1 <sup>d, B</sup>    |
| <i>Water activity (<math>a_w</math>)</i> |                             |                              |                             |                             |                              |                              |                              |
| 0  | 0.82 ± 0.01 <sup>a, A</sup> | 0.82 ± 0.00 <sup>a, A</sup>  | 0.81 ± 0.00 <sup>a, A</sup> | 0.81 ± 0.01 <sup>a, A</sup> | 0.81 ± 0.00 <sup>a, A</sup>  | 0.81 ± 0.01 <sup>a, A</sup>  | 0.81 ± 0.01 <sup>a, A</sup>  |
| 5  | 0.65 ± 0.01 <sup>b, A</sup> | 0.61 ± 0.00 <sup>b, B</sup>  | 0.59 ± 0.02 <sup>b, B</sup> | 0.58 ± 0.01 <sup>b, B</sup> | 0.62 ± 0.00 <sup>b, B</sup>  | 0.60 ± 0.00 <sup>b, B</sup>  | 0.62 ± 0.01 <sup>b, AB</sup> |
| 7  | 0.47 ± 0.01 <sup>c, A</sup> | 0.41 ± 0.01 <sup>c, BC</sup> | 0.39 ± 0.03 <sup>c, C</sup> | 0.37 ± 0.01 <sup>c, C</sup> | 0.42 ± 0.01 <sup>c, BC</sup> | 0.42 ± 0.01 <sup>c, BC</sup> | 0.42 ± 0.01 <sup>c, B</sup>  |
| 9  | 0.21 ± 0.01 <sup>d, A</sup> | 0.18 ± 0.01 <sup>d, B</sup>  | 0.19 ± 0.03 <sup>d, B</sup> | 0.19 ± 0.00 <sup>d, B</sup> | 0.19 ± 0.01 <sup>d, B</sup>  | 0.19 ± 0.00 <sup>d, B</sup>  | 0.20 ± 0.03 <sup>d, B</sup>  |
| <i>Weight loss (%)</i>                   |                             |                              |                             |                             |                              |                              |                              |
| 0  | -                           | -                            | -                           | -                           | -                            | -                            | -                            |
| 5  | 8.1 ± 0.4 <sup>c, A</sup>   | 8.3 ± 0.2 <sup>c, A</sup>    | 8.9 ± 0.6 <sup>c, A</sup>   | 8.9 ± 0.1 <sup>c, A</sup>   | 8.5 ± 0.4 <sup>c, A</sup>    | 8.7 ± 0.2 <sup>c, A</sup>    | 8.3 ± 0.2 <sup>c, A</sup>    |
| 7  | 11.4 ± 0.4 <sup>b, B</sup>  | 12.2 ± 0.1 <sup>b, AB</sup>  | 12.7 ± 0.4 <sup>b, A</sup>  | 13.0 ± 0.3 <sup>b, A</sup>  | 12.3 ± 0.2 <sup>b, AB</sup>  | 12.2 ± 0.1 <sup>b, AB</sup>  | 12.4 ± 0.3 <sup>b, AB</sup>  |
| 9  | 14.5 ± 0.4 <sup>a, B</sup>  | 14.8 ± 0.3 <sup>a, A</sup>   | 14.9 ± 0.3 <sup>a, A</sup>  | 15.3 ± 0.1 <sup>a, A</sup>  | 14.7 ± 0.2 <sup>a, AB</sup>  | 14.7 ± 0.2 <sup>a, AB</sup>  | 14.7 ± 0.7 <sup>a, AB</sup>  |

Different lowercase letters in the same column of each parameter and different uppercase letters in the same line indicate significant differences among samples ( $p < 0.05$ ).

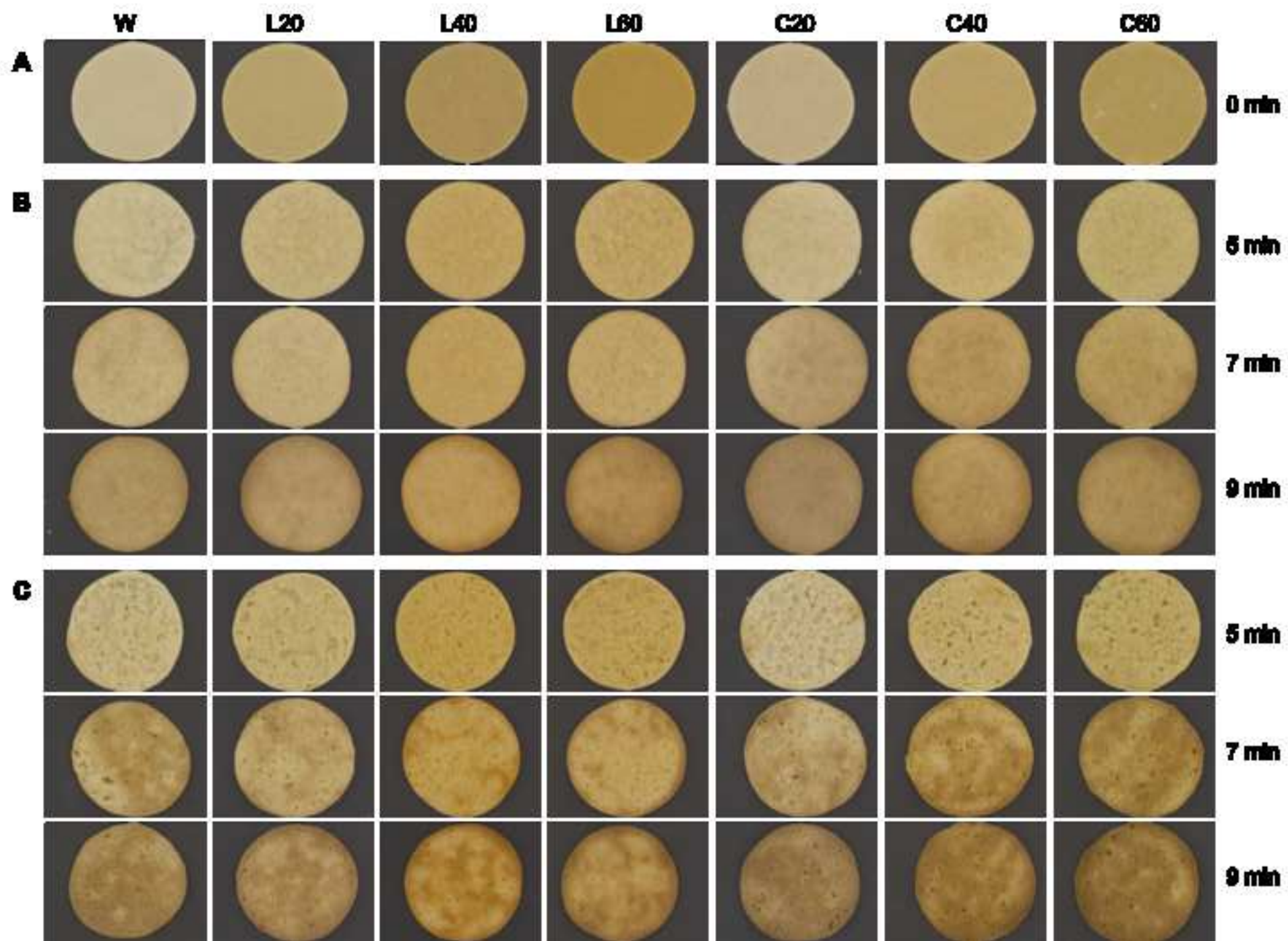
**Table 4.** Colour ( $L^*$ , BI) and texture (hardness, fracturability, crispness) parameters of wheat, lupin, chickpea doughs and biscuits baked at 175 °C for 5, 7, 9 minutes (W: only wheat flour; L20, L40, L60: 20%, 40%, 60% lupin flour; C20, C40, C60: 20%, 40%, 60% chickpea flour).

| Baking time (min)                      | W                           | L20                         | L40                         | L60                          | C20                         | C40                         | C60                          |
|--|-----------------------------|-----------------------------|-----------------------------|------------------------------|-----------------------------|-----------------------------|------------------------------|
| <i>Lightness (<math>L^*</math>)</i>    |                             |                             |                             |                              |                             |                             |                              |
| 0                                      | 72.9 ± 0.3 <sup>a, A</sup>  | 66.4 ± 0.0 <sup>b, B</sup>  | 63.5 ± 0.3 <sup>b, C</sup>  | 60.3 ± 0.2 <sup>c, D</sup>   | 70.1 ± 0.5 <sup>a, AB</sup> | 66.6 ± 0.42 <sup>b, B</sup> | 62.7 ± 0.3 <sup>b, C</sup>   |
| 5                                      | 73.1 ± 0.4 <sup>a, A</sup>  | 70.5 ± 0.5 <sup>a, B</sup>  | 68.0 ± 1.9 <sup>a, C</sup>  | 66.8 ± 0.6 <sup>a, D</sup>   | 72.3 ± 0.5 <sup>a, AB</sup> | 70.7 ± 0.6 <sup>a, B</sup>  | 68.1 ± 0.3 <sup>a, C</sup>   |
| 7                                      | 67.6 ± 1.8 <sup>b, A</sup>  | 68.8 ± 1.0 <sup>ab, A</sup> | 67.5 ± 1.9 <sup>a, A</sup>  | 66.5 ± 0.7 <sup>a, AB</sup>  | 66.3 ± 2.0 <sup>b, AB</sup> | 65.4 ± 2.0 <sup>b, BC</sup> | 63.3 ± 2.1 <sup>b, C</sup>   |
| 9                                      | 61.5 ± 1.9 <sup>c, A</sup>  | 62.6 ± 1.8 <sup>c, A</sup>  | 61.2 ± 1.6 <sup>b, A</sup>  | 61.3 ± 1.7 <sup>b, A</sup>   | 59.4 ± 2.1 <sup>c, AB</sup> | 58.4 ± 2.2 <sup>c, B</sup>  | 56.0 ± 1.9 <sup>c, C</sup>   |
| <i>Browning index (BI)</i>             |                             |                             |                             |                              |                             |                             |                              |
| 0                                      | 37.4 ± 0.2 <sup>d, E</sup>  | 63.9 ± 0.0 <sup>b, C</sup>  | 72.8 ± 0.0 <sup>b, B</sup>  | 82.7 ± 0.3 <sup>b, A</sup>   | 43.2 ± 0.7 <sup>c, D</sup>  | 72.9 ± 0.1 <sup>c, B</sup>  | 82.5 ± 0.8 <sup>c, A</sup>   |
| 5                                      | 44.0 ± 2.1 <sup>c, D</sup>  | 56.3 ± 1.5 <sup>b, C</sup>  | 73.9 ± 1.5 <sup>b, B</sup>  | 78.7 ± 3.1 <sup>b, AB</sup>  | 42.2 ± 1.2 <sup>c, D</sup>  | 66.4 ± 1.9 <sup>c, C</sup>  | 82.0 ± 7.4 <sup>c, A</sup>   |
| 7                                      | 60.0 ± 3.3 <sup>b, C</sup>  | 59.1 ± 2.5 <sup>b, C</sup>  | 73.5 ± 1.5 <sup>b, B</sup>  | 79.4 ± 1.5 <sup>b, AB</sup>  | 58.9 ± 3.6 <sup>b, C</sup>  | 80.4 ± 5.5 <sup>b, A</sup>  | 84.0 ± 4.6 <sup>b, A</sup>   |
| 9                                      | 68.7 ± 3.3 <sup>a, C</sup>  | 69.0 ± 3.2 <sup>a, C</sup>  | 78.8 ± 6.5 <sup>a, B</sup>  | 91.2 ± 3.7 <sup>a, A</sup>   | 69.6 ± 3.3 <sup>a, C</sup>  | 92.8 ± 4.6 <sup>a, A</sup>  | 92.7 ± 3.2 <sup>a, A</sup>   |
| <i>Lightness (<math>L^*</math>) **</i> |                             |                             |                             |                              |                             |                             |                              |
| 0                                      | 72.9 ± 0.3 <sup>a, A</sup>  | 66.4 ± 0.0 <sup>b, B</sup>  | 63.5 ± 0.3 <sup>b, C</sup>  | 60.3 ± 0.2 <sup>b, D</sup>   | 70.1 ± 0.6 <sup>a, AB</sup> | 66.6 ± 0.4 <sup>a, B</sup>  | 62.7 ± 0.3 <sup>a, C</sup>   |
| 5                                      | 70.8 ± 0.4 <sup>b, A</sup>  | 68.4 ± 0.3 <sup>a, B</sup>  | 67.3 ± 0.4 <sup>a, B</sup>  | 64.3 ± 0.8 <sup>a, C</sup>   | 69.6 ± 0.6 <sup>a, A</sup>  | 67.8 ± 0.6 <sup>a, B</sup>  | 53.0 ± 1.0 <sup>b, D</sup>   |
| 7                                      | 62.6 ± 1.9 <sup>c, AB</sup> | 64.5 ± 0.8 <sup>b, A</sup>  | 65.9 ± 0.6 <sup>b, A</sup>  | 64.3 ± 0.6 <sup>a, A</sup>   | 61.4 ± 1.5 <sup>b, B</sup>  | 59.9 ± 1.5 <sup>b, B</sup>  | 47.8 ± 1.5 <sup>c, C</sup>   |
| 9                                      | 55.8 ± 1.7 <sup>d, B</sup>  | 57.2 ± 1.7 <sup>c, B</sup>  | 60.4 ± 1.9 <sup>c, A</sup>  | 58.7 ± 1.8 <sup>c, A</sup>   | 53.6 ± 1.7 <sup>c, B</sup>  | 51.7 ± 1.1 <sup>c, C</sup>  | 41.9 ± 1.4 <sup>d, D</sup>   |
| <i>Browning index (BI) **</i>          |                             |                             |                             |                              |                             |                             |                              |
| 0                                      | 37.4 ± 0.2 <sup>d, E</sup>  | 63.9 ± 0.0 <sup>d, C</sup>  | 72.8 ± 0.0 <sup>c, B</sup>  | 82.7 ± 0.3 <sup>c, A</sup>   | 43.2 ± 0.7 <sup>d, D</sup>  | 72.9 ± 0.1 <sup>d, B</sup>  | 82.5 ± 0.8 <sup>d, A</sup>   |
| 5                                      | 52.4 ± 2.0 <sup>c, E</sup>  | 66.6 ± 1.2 <sup>c, D</sup>  | 78.4 ± 12.2 <sup>b, C</sup> | 102.5 ± 2.5 <sup>ab, B</sup> | 52.1 ± 2.6 <sup>c, E</sup>  | 78.3 ± 7.0 <sup>c, C</sup>  | 111.4 ± 4.3 <sup>c, A</sup>  |
| 7                                      | 83.0 ± 3.3 <sup>b, BC</sup> | 75.9 ± 2.2 <sup>b, C</sup>  | 78.5 ± 13.0 <sup>b, C</sup> | 98.7 ± 2.8 <sup>b, B</sup>   | 77.0 ± 2.7 <sup>b, C</sup>  | 106.2 ± 3.5 <sup>b, B</sup> | 158.7 ± 12.6 <sup>b, A</sup> |
| 9                                      | 93.7 ± 3.8 <sup>a, D</sup>  | 87.6 ± 3.6 <sup>a, D</sup>  | 88.1 ± 16.6 <sup>a, D</sup> | 109.4 ± 3.4 <sup>a, C</sup>  | 92.3 ± 2.8 <sup>a, D</sup>  | 127.8 ± 2.9 <sup>a, B</sup> | 191.3 ± 11.8 <sup>a, A</sup> |

| <i>Hardness (N)</i>                |                             |                            |                            |                              |                            |                            |                            |
|------------------------------------|-----------------------------|----------------------------|----------------------------|------------------------------|----------------------------|----------------------------|----------------------------|
| 0                                  | -                           | -                          | -                          | -                            | -                          | -                          | -                          |
| 5                                  | 6.5 ± 1.0 <sup>c, C</sup>   | 6.9 ± 1.3 <sup>c, C</sup>  | 9.5 ± 1.5 <sup>b, B</sup>  | 13.0 ± 1.7 <sup>c, A</sup>   | 6.3 ± 1.2 <sup>c, C</sup>  | 5.6 ± 0.8 <sup>c, D</sup>  | 4.1 ± 0.6 <sup>c, E</sup>  |
| 7                                  | 21.7 ± 3.8 <sup>b, D</sup>  | 24.4 ± 3.4 <sup>b, C</sup> | 27.1 ± 3.4 <sup>a, B</sup> | 33.6 ± 2.0 <sup>b, A</sup>   | 21.7 ± 2.0 <sup>b, D</sup> | 21.4 ± 2.3 <sup>b, D</sup> | 18.9 ± 2.1 <sup>b, E</sup> |
| 9                                  | 26.5 ± 2.2 <sup>a, C</sup>  | 27.5 ± 2.4 <sup>a, B</sup> | 27.9 ± 2.7 <sup>a, B</sup> | 31.2 ± 3.5 <sup>a, A</sup>   | 26.3 ± 3.3 <sup>a, C</sup> | 24.6 ± 2.8 <sup>a, D</sup> | 24.0 ± 2.5 <sup>a, D</sup> |
| <i>Fracturability (1/mm)</i>       |                             |                            |                            |                              |                            |                            |                            |
| 0                                  | -                           | -                          | -                          | -                            | -                          | -                          | -                          |
| 5                                  | 0.7 ± 0.2 <sup>c, AB</sup>  | 0.6 ± 0.1 <sup>c, B</sup>  | 0.6 ± 0.1 <sup>b, B</sup>  | 0.5 ± 0.1 <sup>c, C</sup>    | 0.7 ± 0.1 <sup>c, A</sup>  | 0.7 ± 0.1 <sup>c, A</sup>  | 0.7 ± 0.0 <sup>c, A</sup>  |
| 7                                  | 0.9 ± 0.2 <sup>b, A</sup>   | 0.8 ± 0.2 <sup>b, AB</sup> | 0.8 ± 0.2 <sup>a, AB</sup> | 0.7 ± 0.1 <sup>b, B</sup>    | 1.00 ± 0.2 <sup>b, A</sup> | 1.1 ± 0.1 <sup>b, A</sup>  | 1.1 ± 0.2 <sup>b, A</sup>  |
| 9                                  | 1.2 ± 0.1 <sup>a, A</sup>   | 0.9 ± 0.2 <sup>a, B</sup>  | 0.9 ± 0.2 <sup>a, B</sup>  | 1.0 ± 0.2 <sup>a, AB</sup>   | 1.2 ± 0.2 <sup>a, A</sup>  | 1.3 ± 0.2 <sup>a, A</sup>  | 1.3 ± 0.1 <sup>a, A</sup>  |
| <i>Crispness (linear distance)</i> |                             |                            |                            |                              |                            |                            |                            |
| 0                                  | -                           | -                          | -                          | -                            | -                          | -                          | -                          |
| 5                                  | 9.2 ± 1.4 <sup>c, B</sup>   | 8.9 ± 1.8 <sup>c, BC</sup> | 12.5 ± 1.9 <sup>c, A</sup> | 13.00 ± 2.8 <sup>c, A</sup>  | 8.9 ± 1.4 <sup>c, BC</sup> | 8.3 ± 1.0 <sup>c, C</sup>  | 6.8 ± 0.4 <sup>c, D</sup>  |
| 7                                  | 25.8 ± 3.8 <sup>b, CD</sup> | 27.6 ± 4.0 <sup>b, C</sup> | 32.3 ± 4.9 <sup>b, B</sup> | 37.89 ± 3.9 <sup>b, A</sup>  | 26.5 ± 3.4 <sup>b, C</sup> | 26.4 ± 4.0 <sup>b, C</sup> | 23.0 ± 2.0 <sup>b, D</sup> |
| 9                                  | 33.6 ± 4.3 <sup>a, B</sup>  | 34.6 ± 3.2 <sup>a, B</sup> | 34.6 ± 5.5 <sup>a, B</sup> | 42.36 ± 13.6 <sup>a, A</sup> | 33.0 ± 5.6 <sup>a, B</sup> | 33.2 ± 5.7 <sup>a, B</sup> | 32.3 ± 3.8 <sup>a, B</sup> |

Different lowercase letters in the same column of each parameter and different uppercase letters in the same line indicate significant differences among samples ( $p < 0.05$ ).

\*\* Lower surface of the biscuits.



# Graphical Abstract

AIM

Investigate the effect of various flour characteristics on acrylamide (AA) formation and certain quality properties of biscuits.

## BISCUIT SAMPLES

Formulations:

**W** (control)  
100%  
Wheat flour



W

vs

**L20, L40, L60**  
20%, 40%, 60%  
Lupin flour

**C20, C40, C60**  
20%, 40%, 60%  
Chickpea flour



L40

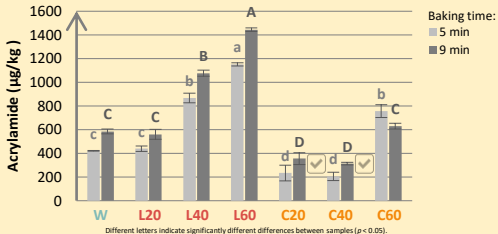


C40

- All flours had different composition and physical properties.
- The asparagine content has been standardised in the formulations.

Baking: electric oven (convection mode) at 175°C for 5, 7 and 9 min.

## ACRYLAMIDE RESULTS



- ✓ The use of **C flour** at 20 and 40% reduced the AA content compared to **W**.
- ✓ The results were attributed to a C flour coarser particle size and type of proteins.
- ✓ **C flour** addition did not affect the colour and texture of the final biscuits.

**Declaration of interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:



**Author statement**

Maria Alessia Schouten: Writing - Original Draft, Investigation, Formal analysis

Christos Frygas: Investigation, Methodology, Writing - Review & Editing

Silvia Tappi: Writing - Review & Editing

Santina Romani: Writing - Review & Editing, Supervision, Funding acquisition

Vincenzo Fogliano: Conceptualization, Writing - Review & Editing, Supervision, Funding acquisition