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The use of kidney bean flour with intact cell walls reduces the formation of acrylamide in biscuits

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# Journal Pre-proof

The use of kidney bean flour with intact cell walls reduces the formation of acrylamide in biscuits

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Journal Pre-proof

1 **The use of kidney bean flour with intact cell walls reduces the formation of acrylamide in**  
2 **biscuits**

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**Abstract:**

Acrylamide (AA) is formed by Maillard Reaction (MR), during high-temperature and low moisture processes in several food categories, including bakery products. AA is classified as a toxic and carcinogenic compound and therefore, mitigation strategies are necessary to control its concentration in foods. Selecting ingredients with a reduced amount of asparagine, the main AA precursor, is the most effective method of reducing the AA formation in bakery products.

In the present study, the hypothesis that cotyledon cell walls integrity could prevent the formation of AA by modulating the dehydration rate and reducing the availability of asparagine during the MR development during baking was investigated. Legumes have strong cotyledon cell walls that can be preserved during flour production: in this study two kidney bean flours with intact and broken cell walls were used. The integrity of the cotyledon cell walls was assessed by scanning electron microscopy while AA, its precursors, moisture and water activity were measured in bean flours, raw doughs and baked biscuits. The biscuits were also characterised for the main baking parameters such as weight loss, pH, colour and texture.

The results showed that biscuits formulated with bean flour with intact cell walls had a 15% less amount of AA than those made with the bean with damaged cell walls at the end of the cooking time. Furthermore, the use of the two different bean flours led to different quality characteristics of the biscuits in terms of hue angle and browning index, as colour parameters, and hardness, which is related to the texture properties. These differences were attributed to the different development of the MR and in the structure of the biscuit during baking. The use of intact plant tissue legume flours in alternative biscuits formulations is a feasible and low-cost approach that can contribute to the AA mitigation strategy.

**Keywords:**

Acrylamide; Biscuits; Legumes; Bakery products; Cell walls; Food structure.

**1. Introduction**

Acrylamide (AA) has been classified as a toxic and carcinogenic compound that is formed naturally during high-temperature processes  $> 120$  °C in several food categories (IARC, 1994; SNFA, 2002). This toxic compound is formed mainly in carbohydrate-rich foods, such as potato, coffee and bakery products, during the Maillard reaction (MR) initiated between reducing carbohydrates (glucose, fructose, etc.) and asparagine (Capuano & Fogliano, 2016; Mousavi Khaneghah et al., 2020; Sarion et al., 2021). Given the risks associated with this compound,

55 monitoring and reducing AA in foods has been an important issue for food legislation (European  
56 Commission, 2011, 2013, 2017, 2019; FoodDrinkEurope, 2019).

57 Several studies have recently investigated the effect of various flours on the formation of AA in  
58 bakery products. Flour is the main source of asparagine, which is the limiting factor for the  
59 formation of AA in bakery products (Krishnakumar & Visvanathan, 2014; Miśkiewicz et al., 2012;  
60 Negoită et al., 2017; Salazar et al., 2012; Sarion et al., 2021; Sazesh & Goli, 2020; Žilić et al.,  
61 2020). The origin, milling process and pre-treatments (e.g. fermentation) of the different flours  
62 used in the formulation of some bakery products could influence the final AA levels (Sarion et al.,  
63 2021). For example, replacing the commonly used wheat flour with flours from alternative sources,  
64 including pseudo-cereals (e.g. quinoa) and legumes (e.g. chickpeas), could lead to a reduction in  
65 AA formation thanks to low asparagine concentration and high protein content that could bind to  
66 reducing sugars limiting their availability for the MR, respectively (Miśkiewicz et al., 2020;  
67 Sazesh & Goli, 2020). The level of asparagine in flour could be controlled by carrying out specific  
68 fermentation treatments of flours to produce safe and nutritionally valuable bakery products low  
69 in AA (Bartkiene et al., 2016, 2017). In addition, it can be assumed that the flours microstructure  
70 could also influence the mechanistic pathway of AA formation by changing the availability and  
71 mobility of reaction precursors, including asparagine. However, the influence of cell walls  
72 integrity on AA formation was not investigated, thus far. In the case of legume flours, it is possible  
73 to hypothesise that the use of a legume ingredient characterised by a low cell walls integrity, could  
74 lead to increased availability of AA precursors during baking, on the contrary, legume ingredients  
75 characterised by intact cell walls could limit it, reducing AA formation. Legumes are mainly  
76 composed of cotyledon cells characterized by strong walls composed of a complex network of  
77 polysaccharides that can remain intact during some flour manufacturing (Huisman et al., 2003;  
78 Jones & Boulter, 1983; McNeil et al., 1984; Jenkins et al., 2002). Recent studies have provided  
79 evidence that the presence of intact cotyledon cell walls within plant tissues during digestion  
80 restricts the access of digestive enzymes and the hydrolysis of intracellular starch in navy beans  
81 and kidney beans (Berg et al., 2012; Rovalino-Córdova et al., 2018), lipid digestion in almond and  
82 hazelnut (Grundy et al., 2016; Capuano et al., 2018) as well as protein digestion in soybeans (Zahir  
83 et al., 2018). On the other hand, an increase in macronutrients digestions has been observed when  
84 the cotyledon cell walls structure is damaged by mechanical or enzymatic treatments either before  
85 or after cooking. Therefore, these findings can also be applied to the availability of AA precursors  
86 as most proteins and carbohydrates are primarily located in cotyledon cells of the legume seeds  
87 (Chigwedere et al., 2019; Glahn et al., 2016; Pallares Pallares et al., 2021; Rovalino-Córdova et  
88 al., 2019). In addition, the microstructure of legume flours may also play a role in the water

89 distribution inside the dough and in the dehydration rate during baking, influencing the kinetic of  
90 the MR (Sarion et al., 2021).

91 The purpose of the present study was to examine the effect of two legume flours having intact and  
92 damaged cell walls on AA formation in biscuits during baking. To test this, biscuit doughs were  
93 prepared, according to a standard recipe, by partially replacing wheat flour with kidney bean flour  
94 as a mash, obtained by boiling and mashing the beans or as a fine powder prepared by grinding  
95 the beans with a cryo-mill. The integrity or non-integrity of the cotyledon cell walls of the two  
96 obtained kidney bean flours were analysed by scanning electron microscopy (SEM). In addition,  
97 kidney bean flours, raw doughs and baked biscuits were evaluated for AA content, its precursors,  
98 moisture and water activity as well as for the main baking quality parameters.

99

## 100 **2. Materials and methods**

101

### 102 **2.1 Preparation of kidney bean flours**

103 Dry kidney beans were purchased from the local market (Windkorenmolen De Vlijt, Wageningen,  
104 The Netherlands) and stored in the dark at room temperature until use. The bean maceration was  
105 performed according to Rovalino-Córdova et al. (2018) with minor modifications. In brief, 100 g  
106 of dry beans were soaked in 200 mL of ice-chilled distilled water for 15 h and de-hulled by manual  
107 separation of the seed coat (moisture =  $49.4 \pm 1.0\%$  and  $a_w = 0.97 \pm 0.01$ ).

108 The bean flour characterised by damaged cotyledons cell walls, hereinafter Damaged Bean Flour  
109 - DBF was prepared by grinding two batches (60 g) of de-hulled beans into a fine powder using a  
110 cryogenic mill (mod. 6870D, SpexSamplePrep, Metuchen, NJ, USA) with liquid nitrogen and  
111 setting 3 steps of 5 min each at 24 back-and-forth cycles per second (cps).

112 The bean flour characterised by intact cotyledons cell walls, hereinafter Intact Bean Flour - IBF,  
113 was obtained by boiling de-hulled beans in distilled water (ratio 2:1, w/v) at about  $100 \pm 1$  °C for  
114 1 h under stirring to avoid burning of the material in the bottom of the beaker. The cooking  
115 temperatures were monitored using a digital thermometer equipped with type K thermocouples  
116 mod. RS Pro 206-3722 (RS Components, Corby, UK). The cooked beans, without discarding the  
117 water (in order not to remove any possible quantity of asparagine released into the water), were  
118 gently crushed with a mortar and pestle to obtain a uniform mash.

119

### 120 **2.2 Microscopy analysis of kidney bean flours**

121 The integrity of the cotyledon cell walls of IBF and DBF, even during the baking process, was  
122 investigated using scanning electron microscopy (SEM).

123 A portion (50 g) of IBF and DBF with a thickness of 1.5 cm were heated at 170 °C for 10 min in  
124 an electric oven (mod. OV185C, Inventum, Arnhem, The Netherlands) to test the stability of the  
125 integrity of the cotyledon cell walls at high temperature. This bean sample and a part of each flour  
126 were snap-frozen in liquid nitrogen and subsequently freeze-dried for SEM analysis.

127 The sample was attached on SEM sample holders using carbon adhesive tabs (EMS, Washington,  
128 WA, USA). The sample was then sputter-coated with 12 nm Tungsten (mod. EM SCD 500, Leica,  
129 Vienna, Austria) and analysed in a FEI Magellan 400 field emission scanning electron microscope  
130 (2 kV, 13 pA) (mod. Magellan 400, FEI, Eindhoven, The Netherlands). Size measurements were  
131 performed using the xT microscope control software (FEI, Eindhoven, The Netherlands).

132 Three independent preparations of DBF and IBF, before and after baking, were analysed by SEM.

133

### 134 **2.3 Preparation of biscuit samples**

135 The biscuits were formulated by mixing the refined wheat flour (Windkorenmolen De Vlijt,  
136 Wageningen, The Netherlands) with IBF or DBF (14% of beans on dry matter basis). In detail, the  
137 biscuit doughs were prepared with ingredients purchased from local and online markets  
138 (Wageningen, The Netherlands) following the standard AACC method 10-54 recipe (AACC,  
139 2009) with small modifications as reported in **Table 1**. The amounts of distilled water added in  
140 both formulations were standardised according to the different moisture contents of the added bean  
141 flours and were  $62.4 \pm 0.4\%$  and  $50.5 \pm 0.2\%$  for mash bean and flour bean, respectively.

142 High-fructose corn syrup and sucrose were solubilized in water at room temperature for 1 min  
143 using Thermomix TM5 (Vorwerk, Wuppertal, Germany) by setting the speed regulator to position  
144 2. Successively, the other dry ingredients and shortening were added and mixed thoroughly for 1  
145 min by setting the speed regulator to position 5 and reversing the direction of rotation after 30 s.  
146 The dough was shortly kneaded by hand to compact it, wrapped in plastic foil and let to rest at 4  
147 °C for 20 min. For some subsequent analyses, parts of the raw dough samples were freeze-dried  
148 and finely ground with a mortar.

149 The dough was rolled out to a thickness of about 3 mm by a pasta filler machine (Marcato,  
150 Campodarsego, Italy) and cut by using a stainless-steel circular cup pastry of 6 cm diameter. For  
151 each formulation and baking batch, 6 biscuits were baked in an electrical oven (mod. OV185C,  
152 Inventum, Arnhem, The Netherlands) with convection mode at 170 °C for 5, 7, 9 and 11 min. The  
153 baking temperature and times studied were selected after preliminary trials in order to obtain  
154 biscuit samples baked at different levels; after 5 min the biscuits were undercooked and after 11  
155 min the biscuits were overcooked. The optimal baking time was in the range of 7-9 min. The  
156 biscuits were placed on a baking tray in the middle position and for each baking cycle, the air



157 temperature inside the oven chamber was monitored every 20 s using a digital thermometer  
158 equipped with type K thermocouples (mod. RS Pro 206-3722, RS Components S.r.l., Corby, UK).  
159 After baking, biscuits were removed from the oven, placed on a grid and kept cooling at room  
160 temperature for about 1 h.

161 All biscuit formulation and baking time were performed in triplicate; a total of 72 baked biscuits  
162 plus the raw ones for each sample were prepared.

163

## 164 **2.4 Analysis of the main quality parameters**

### 165 2.4.1 Moisture

166 Moisture (%) of bean flours, raw doughs and baked biscuits was determined on ground products  
167 (3 g) by gravimetric method at 105 °C until constant weight (AOAC, 1980) using an oven (mod.  
168 Heraeus Series 6000, Thermo Scientific, Berlin, Germany).

169 Three replicates were performed for each sample and baking batch.

170

### 171 2.4.2 Water activity

172 Water activity ( $a_w$ ) of bean flours, raw doughs and baked biscuits was determined on ground  
173 samples using mod. LabMaster aw-meter (Novasina AG, Lachen, Switzerland) at 25 °C, setting  
174 both the time and temperature factors stability at 2 min, respectively.

175 Three replicates were performed for each sample and baking batch.

176

### 177 2.4.3 Weight loss

178 Weight loss (%) was determined as the percentage of weight variation of biscuits ( $n = 6$ ) before  
179 and after each baking cycle per sample.

180

### 181 2.4.4 pH

182 The pH of bean flours, raw doughs and baked biscuits was determined based on the method  
183 described by Mesías et al. (2015). The grounded sample (1 g) was mixed with distilled water (100  
184 mL), vortexed for 3 min and held for 1 h at room temperature. After centrifugation at 4816 rpm  
185 and 20 °C for 10 min (mod. Heraeus Multifuge X3R, Thermo Fisher Scientific, Waltham, MA,  
186 USA), pH of the supernatant was measured with mod. 1100L pH meter (VWR, Radnor, PA, USA).

187 Three replicates were performed for each sample and baking batch.

188

### 189 2.4.5 Colour

190 The colour of raw and baked biscuits was measured using an IRIS V400 electronic visual analyser  
191 (Alpha MOS, Toulouse, France) equipped with a 25 mm lens and lower and upper illumination.  
192 The processing of RGB images in the CIE L\*a\*b\* scale was performed with ImageJ analysis  
193 software (NIH, Bethesda, MD, USA). From numerical values of the measured parameters, hue  
194 angle (h°) and browning index (BI) were calculated by the following equations (McGuire, 1992;  
195 Sakin-Yilmazer et al., 2013):

$$196 \quad h^\circ = [(\tan^{-1}(b^*/a^*)/2\pi)] \cdot 360$$

$$197 \quad BI = [(x-0.31) \cdot 100]/0.17, \text{ where } x = (a^*+1.79 \cdot L^*)/(5.645 \cdot L^*+a^*-3.012 \cdot b^*)$$

198 The colour measurements were carried out on the two surfaces of 6 biscuits for each baking batch  
199 per sample.

200

## 201 2.4.6 Texture

202 The texture of baked biscuits was performed at room temperature with Texture analyser TA.XT2  
203 (Stable Micro Systems, Surrey, UK) equipped with a load cell of 50 kg and a three-point bending  
204 test holder and probe. The distance of two beams of sample holder was 20 mm and the other setting  
205 were: pre-test speed of 5.00 mm/s, test speed of 1.00 mm/s, post-test speed of 10.00 mm/s and  
206 distance of 5 mm. The downward movement was advanced till the biscuit was broken. The texture  
207 was described by the hardness (N), calculated by means of maximum force values; index of  
208 crispness, calculated by using the linear distance between the first and the last peaks registered  
209 (Tylewicz et al., 2019), and fracturability (1/mm), expressed as one/breakpoint distance between  
210 the origin of curve till the point where the biscuit breaks (Romani et al., 2012).

211 Force vs distance curves were obtained from 6 biscuits for each baking batch per sample.

212

## 213 2.5 Quantification of asparagine and acrylamide

### 214 2.5.1 Sample extraction

215 The sample extraction process for asparagine and AA determinations in bean flours, raw doughs  
216 and baked biscuits was performed according to the method described by Žilić et al. (2020) with  
217 minor modifications. Ground sample (1 g) was triple extracted with 10 mL, 5 mL and 5 mL of 10  
218 mM formic acid (Sigma-Aldrich, St. Louis, MO, USA) in Milli-Q water (Milli-Q PURELAB  
219 Ultra, ELGA LabWater, Lane End, UK). For each volume, the extract was vortexed for 1 min at  
220 maximum speed and centrifuged for 10 min at 4700 rpm and 20 °C (mod. Heraeus Multifuge X3R,  
221 Thermo Fisher Scientific, Waltham, MS, USA). The combined supernatant was collected in a 50  
222 mL tube and stored in a freezer at -20 °C until analysis (maximum 2 weeks).

223 For asparagine determination, 5 mL of formic acid extract was centrifuged for 10 min at 14000  
224 rpm and 20 °C (mod. 5430 R, Eppendorf AG, Hamburg, Germany). For better clarification, 4 mL  
225 of supernatant was centrifuged for 7 min at 14000 rpm and 20 °C. Then 1 mL of clear supernatant  
226 was mixed with 1 mL of acetonitrile (Actu-All Chemicals, Oss, The Netherlands) and filtered with  
227 0.2 µm and Ø15 mm PTFE filters (Phenomenex, Torrance, CA, USA) into an amber glass  
228 autosampler vial.

229 For AA quantification, 4.75 mL of the formic acid extract with 100 µL of 5000 µg/L AA-d<sub>3</sub>  
230 solution (CAS No 122775-19-3, Sigma-Aldrich, St. Louis, MO, USA) were clarified with 0.125  
231 mL of Carrez I and 0.125 mL of Carrez II solutions (Sigma-Aldrich, St. Louis, MO, USA). The  
232 mixture was vortexed and centrifuged for 3 min at 10000 rpm and 20 °C. For better clarification,  
233 2 mL of supernatant was collected and centrifuged for 10 min at 14000 rpm and 20 °C. For the  
234 solid phase extraction (SPE) clean-up, according to Mogol and Gökmen (2014), the Oasis MCX  
235 cartridge (Waters, Milford, MA, USA) was activated with 1 mL of methanol (Actu-All Chemicals,  
236 Oss, The Netherlands) and conditioned with 1 mL of Milli-Q water with a speed of 1 drop/second.  
237 Subsequently, 1 mL of clean extract was passed through to preconditioned cartridge (1  
238 drop/second) into an amber glass autosampler vial. The first 7-8 drops of the sample were carefully  
239 discarded to avoid any dilution. The recovery (RE%) of the SPE procedure was  $93.3 \pm 5.3\%$ ,  
240 determined by evaluating the analyte signal responses of 1 mL of standard solutions composed of  
241 980 µL of AA (250 µg/L) and 20 µL of AA-d<sub>3</sub> (5000 µg/L) before (SB) and after (SA) passing it  
242 through SPE ( $RE\% = (\text{area SA}/\text{area SB}) \times 100$ ).

243 The sample preparations were repeated twice for each sample and baking batch per sample.

244

#### 245 2.5.2 LC-MS/MS method

246 LC-MS/MS analyses were carried with a Nexera UPLC system coupled with an LCMS-8050 triple  
247 quadrupole mass spectrometer (Shimadzu Corporation, Kyoto, Japan). The UPLC unit consisted  
248 of a SIL-30AC autosampler, an LC-20ADXR solvent delivery module, a DGU-20ASR degassing  
249 unit, a CTO-20AC column oven and an FCV-20AH2 valve unit. The HPLC gradient analytical  
250 standards such as AA (C<sub>3</sub>H<sub>5</sub>NO, molecular weight 71.08 g/mol, CAS No 79-06-1) and L-  
251 asparagine (C<sub>4</sub>H<sub>8</sub>N<sub>2</sub>O<sub>3</sub>, molecular weight 132.12 g/mol, CAS No 70-47-3) were purchased from  
252 Sigma-Aldrich (St. Louis, MO, USA).

253 The chromatographic separation of free asparagine was performed by injecting 5 µL of samples  
254 on a SeQuant® ZIC HILIC (3.5 µm, 4.6 × 150 mm, Merck KGaA, Darmstadt, Germany) attached  
255 to a SeQuant® ZIC HILIC PEEK coated guard column (20 × 2.1 mm, Merck KGaA, Darmstadt,  
256 Germany). The flow rate was set at 0.7 mL/min and the column temperature at 40 °C. The mobile

257 phases consisted of 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent  
258 B). MS data were collected for 18 min with the following elution profile (min/B%): 0.0/90, 4.0/70,  
259 10.0/20, 13.0/20, 15.0/90 and 18.0/90. Asparagine quantification was performed with an external  
260 calibration curve ranging from 39 to 10000  $\mu\text{g/L}$ .

261 The chromatographic separation of AA was performed on a Thermo Scientific<sup>TM</sup> Hypercarb  
262 column (5  $\mu\text{m}$ , 2.1  $\times$  50 mm, Thermo Fisher Scientific, Waltham, MA, USA) connected to an  
263 OPTI-GUARD<sup>®</sup> 1 mm Guard C18 column (Sigma-Aldrich, St. Louis, MO, USA). The flow rate  
264 was set at 0.2 mL/min and the column temperature at 40 °C. The mobile phases consisted of 0.1%  
265 formic acid (solvent A), methanol with 0.1% formic acid (solvent B). MS data were collected for  
266 10 min with the following elution profile (min/B%): 0.0/5, 2.5/5, 5.0/50, 6.0/50, 7.0/5 and 10.0/5.  
267 A calibration curve ranging from 15 to 1000  $\mu\text{g/L}$  of AA was used for quantification. Each  
268 calibration solution was spiked with AA-d<sub>3</sub> at a concentration of 100  $\mu\text{g/L}$ .

269 Positive ionisation mode was used for the MS analysis. The voltage of the turbo ion-spray  
270 ionization was 4.0 kV. The temperatures of the electrospray ionization probe, desolvation line and  
271 heat block were set at 300 °C, 250 °C and 400 °C, respectively. The pressure of the collision-  
272 induced dissociation gas was 4 kPa whereas the flow rates of the drying gas, nebulizer gas and  
273 heating gas were set at 10 mL/min, 3 mL/min and 10 mL/min, respectively. The electrode voltage  
274 of Q1 pre bias (collision cell energy entrance potential), collision cell Q2 (collision energy), Q3  
275 pre bias (collision cell energy exit potential), parent and fragment ion m/z of the multiple reaction  
276 monitoring transitions were optimized using support software (Shimadzu Corporation, Kyoto,  
277 Japan). For single reaction monitoring (SRM), the dwell time was set at 4 or 42 ms, respectively  
278 for asparagine and AA, and the most abundant fragment ion was selected for quantitation (**Table**  
279 **2**). The second and third fragments in ion yield were selected as a structural confirmation based  
280 on the optimized SRM transition reported in **Table 2**.

281 The analytical measurements were repeated three times for each extract, reporting the results as  
282  $\mu\text{g/kg}$  for AA and mg/kg for asparagine on dry matter basis. Quantification was performed with a  
283 significant linear regression ( $R^2= 0.99$ ) of an external calibration curve in the range of 39.06 -  
284 10000  $\mu\text{g/L}$  for asparagine and of 15.63 - 1000  $\mu\text{g/L}$  for AA spiked with 100  $\mu\text{g/L}$  of AA-d<sub>3</sub>. The  
285 coefficients of variations (CV%) of calibration curves intra-day were in the range of 7.0-11.5%  
286 and of 8.5-12.9% for asparagine and AA, respectively. In addition, the analyte signal for AA of  
287 both calibration curve and samples was corrected with the internal standard (AA-d<sub>3</sub>). The corrected  
288 areas (AA/AA-d<sub>3</sub>) of the MS peak signals were plotted on the concentration.

289 The limit of quantification (LOQ) and of detection (LOD) were calculated from the standard  
290 deviation of the intercept (S) and the slope (s) of the calibration line ( $\text{LOQ} = 10 \times S/s$  and  $\text{LOD} =$

291 3.3×S/s). For asparagine, the LOQ was  $0.8 \pm 0.5 \mu\text{g/L}$  and the LOD was  $0.3 \pm 0.2 \mu\text{g/L}$ , while for  
292 AA, the LOQ was  $3.65 \pm 2.01 \mu\text{g/L}$  and the LOD was  $1.20 \pm 0.66 \mu\text{g/L}$  indicating a very sensitive  
293 method. The matrix effects ( $\text{ME}\% = (\text{B}/\text{A}) \times 100$ ), as an indicator of the ionization suppression or  
294 enhancement effects, were calculated by comparing the analytes signal responses of the analytes  
295 standard solution with a known concentration (A) to that of the extract sample spiked with the  
296 analyte at the same concentration (B). In detail, evaluating two distinct repetitions, the ME% were  
297  $95 \pm 5\%$  and  $110 \pm 5\%$  for asparagine and AA, respectively.

298

## 299 **2.6 Quantification of glucose, fructose and sucrose**

### 300 2.6.1 Sample extraction

301 The sample preparation for glucose, fructose and sucrose determinations in raw doughs and baked  
302 biscuits was performed based on the method described by Nguyen et al. (2016) with slight  
303 modifications. Ground biscuits (2.5 g) or freeze-dried doughs (2.5 g) were mixed with 25 mL of  
304 Milli-Q water and ethanol (VWR Chemicals, Radnor, PA, USA) mixture (1:1, v/v) and vortexed  
305 for 1 min. The samples were incubated for 1 h at  $50 \text{ }^\circ\text{C}$  in a water bath and vortexed again before  
306 being cooled down for 20 min at room temperature. Then the samples were centrifuged at 3000  
307 rpm and  $20 \text{ }^\circ\text{C}$  for 10 min and 1.5 mL of supernatant was collected. Then the supernatant was  
308 centrifuged at 14000 rpm and  $20 \text{ }^\circ\text{C}$  for 10 min and 1 mL was collected into a glass tube. The  
309 water/ethanol solvent was evaporated with a sample concentrator (mod. SBHCONC/1, Stuart,  
310 Staffordshire, UK) under nitrogen flush at  $50 \text{ }^\circ\text{C}$  for 4.5 h. After solvent evaporated, the sample  
311 was reconstituted with 20 mL of acetonitrile and 20 mL of Milli-Q water and vortexed for 1 min.  
312 Samples were stored in a freezer at  $-20 \text{ }^\circ\text{C}$  until measurement (maximum 1 week). Before analysis  
313 1.5 mL of sample was passed through  $\text{Ø}28 \text{ mm}$  and  $0.2 \mu\text{m}$  CA filters (Phenomenex, Torrance,  
314 CA, USA) and transferred into an autosampler amber glass vial.

315 The sample preparations were repeated twice for each sample and baking batch per sample.

316

### 317 2.6.2 UPLC-ELSD method

318 The samples were analysed with an Acquity UPLC-H Class Plus System (Waters, Milford, MA,  
319 USA) equipped with an Acquity Evaporative Light Scattering (ELSD) detector and following the  
320 procedure provided by Waters' technical application notebook (Waters Corporation, 2009). Sugars  
321 were separated on an Acquity UPLC BEH Amide column ( $1.7 \mu\text{m}$ ,  $2.1 \times 100 \text{ mm}$ ) with an Acquity  
322 UPLC BEH Amide VanGuard pre-column ( $130\text{Å}$ ,  $1.7 \mu\text{m}$ ,  $2.1 \text{ mm} \times 5 \text{ mm}$ ) (Waters, Milford,  
323 MA, USA). The mobile phase A consisted of Milli-Q water and acetonitrile mixture (8:2, v/v) with  
324 0.2% triethylamine (TEA, Sigma-Aldrich, St. Louis, MO, USA) while mobile phase B consisted

325 of acetonitrile/Milli-Q water (3:7, v/v) with 0.2% TEA. The flow rate was 0.25 mL/min and the  
326 gradient changes with the following elution profile (min/A%): 0.00/100, 6.00/40, 6.01/100 and  
327 18/100. Before the first injection, the column was equilibrated with 100% A, 0.25 mL/min for 30  
328 min. The injection volume was 1.3  $\mu$ L and the column temperature was 35 °C. Set up a seal wash  
329 with acetonitrile/water (1:1), strong needle wash and weak needle wash with acetonitrile/water  
330 (8:2) and acetonitrile/water (7:3) respectively. The pressure of ELSD conditions was 40 psi with  
331 a drift tube temperature of 40 °C and a data rate of 10 pps. Operating the software was carried out  
332 using a Waters Acquity Control console and data processing was performed with Chromeleon  
333 Chromatography Data System (version 7.2.10, Thermo Scientific Corp, Waltham, MA, USA). The  
334 quantification was done by an external calibration curve ranging from 85-1360 mg/L (sucrose) and  
335 45-720 mg/L (glucose and fructose). The HPLC gradient analytical standard such as sucrose  
336 ( $C_{12}H_{22}O_{11}$ , molecular weight 342.30 g/mol, CAS No 57-50-1), D-(+)-glucose ( $C_6H_{12}O_6$ ,  
337 molecular weight 180.16 g/mol, CAS No 50-99-7) and D(-)-fructose ( $C_6H_{12}O_6$ , molecular weight  
338 180.16 g/mol, CAS No 57-48-7) were purchased from Sigma-Aldrich (St. Louis, MO, USA).  
339 The analytical measurements were conducted three times for each extract. The results for sucrose  
340 content of doughs and baked biscuits were expressed as g/kg on dry matter basis. Quantification  
341 was performed with a significant linear regression ( $R^2 = 0.99$ ) of an external calibration curve in  
342 the range of 85-1360 mg/L (sucrose) and of 45-720 mg/L (glucose and fructose). The CV% of  
343 calibration curves intra-day were in the ranges of 3.0-8.7%, 6.4-12.7%, 9.8-14.7% for sucrose,  
344 glucose and fructose, respectively.  
345 The LOQ and LOD were calculated based on the standard deviation of the intercept and the slope  
346 of the calibration line. For sucrose, the LOQ was  $8.8 \pm 3.0$  mg/L and the LOD was  $2.9 \pm 1.0$  mg/L,  
347 for glucose, the LOQ was  $6.8 \pm 3.1$  mg/L and the LOD was  $2.2 \pm 1.1$  mg/L, while for fructose the  
348 LOQ was  $0.2 \pm 1.1$  mg/L and the LOD was  $0.05 \pm 0.1$  mg/L. The ME% was  $115 \pm 5\%$ ,  $105 \pm 5\%$   
349 and  $97 \pm 7\%$  for sucrose, glucose and fructose, respectively.

350

## 351 **2.7 Data analysis**

352 The results were reported as mean value  $\pm$  standard deviation of replications. The data processing  
353 and statistical analysis were performed in Excel (Microsoft, Redmond, USA) and STATISTICA  
354 8.0 software (StatSoft Inc., Tulsa, UK). Significant differences between data were calculated by  
355 unidirectional analysis of variance (ANOVA) followed by Tukey's post-hoc comparison test, with  
356 a significance level of  $p < 0.05$ . The relationship between the average values of AA, asparagine,  
357 sucrose and the physical characteristics (i.e. weight loss, moisture,  $a_w$ , pH, colour and texture  
358 parameters) of the biscuits prepared with different formulations were evaluated with Pearson

359 correlation coefficient ( $r$ ). An  $r$ -value between  $0.60 \leq r \leq 1.00$  indicates a positive linear  
360 correlation,  $-1.00 \leq r \leq -0.60$  indicates a negative linear correlation and  $-0.60 < r < 0.60$  indicates  
361 no correlation, with a significance level  $p < 0.05$ .

362

### 363 **3. Results and discussion**

364

#### 365 **3.1 Kidney bean flours characterization**

366 SEM analysis was used to compare the cell walls structure of the two kidney bean flours, namely  
367 DBF and IBF. In **Figure 1** the related scanning electron micrographs (SEM) are shown. SEM  
368 images A and B confirmed that the protocols employed effectively produced two kidney bean  
369 flours having a different amount of damaged cell walls. In the DBF (**Figure 1A**) the cotyledon cell  
370 material was completely damaged by milling, whereas in the IBF (**Figure 1B**) the intact cell walls  
371 were clearly visible and the cell surface was rough and homogeneous without cracks.

372 The two flour preparations also have significantly different moisture contents and  $a_w$  values,  $49.6$   
373  $\pm 1.2\%$  and  $0.97 \pm 0.00$  for DBF and  $63.4 \pm 1.1\%$  and  $0.96 \pm 0.01$  for IBF, respectively. Moreover,  
374 the two bean flours also differed in pH values,  $7.0 \pm 0.0$  and  $6.9 \pm 0.0$  for IBF and DBF,  
375 respectively. The slightly lower pH of the DBF can be attributed to the release of cellular contents  
376 due to the breakdown of the cotyledons cell walls.

377 In general, legume cell walls are assembled as a continuous network of cellulose-xyloglucan in  
378 combination with a pectin matrix that serves as filling for the spaces between network constituents,  
379 increases cell walls thickness and adds coherence (Cosgrove, 2000; Vincken et al., 2003). During  
380 the cooking process, two simultaneous processes occur inside and outside the cotyledon cells;  
381 gelatinisation of the intracellular starch and protein denaturation are accompanied by softening as  
382 a result of plasticisation or partial solubilisation of the middle lamella, which leads to separation  
383 of individual cotyledon cells (Klamczynska et al., 2001; Wang, 2008; Wang et al., 2003). Other  
384 studies showed that low-temperature boiling of whole legume beans does not lead to a breakdown  
385 of the cell walls, but only causes a deterioration of the external appearance and softens the texture  
386 (Leelayuthsoontorn & Thipayarat, 2006; Pieniazek & Messina, 2016; Rovalino-Córdova et al.,  
387 2018).

388 The resistance of the cotyledon cell walls of the IBF after heating at  $170^\circ\text{C}$  for 10 min was assessed  
389 by SEM (**Figure 1C**) and it was found that the organisation of the cell structure remained  
390 unchanged. In addition, there was no formation of porous cavities on the surface of the cell walls.  
391 A difference compared to the not baked IBF was noticeable, probably caused by dehydration and  
392 loss of water that occurred during the heating treatment.

393

### 394 **3.2 Influence of kidney bean flours on acrylamide content and main quality characteristics** 395 **of biscuits**

396 The two different types of kidney bean preparations led to a different formation rate of AA in  
397 biscuits during baking, the trends are presented in **Figure 2**. As expected, no AA was found in the  
398 raw dough samples and there was no significant difference in AA levels between samples DBF  
399 and IBF from 5 min of baking (with values of  $321.7 \pm 6.3 \mu\text{g/kg}$  and  $320.6 \pm 3.5 \mu\text{g/kg}$ ,  
400 respectively) until 7 min of baking (with values of  $379.3 \pm 17.5 \mu\text{g/kg}$  and  $353.9 \pm 29.1 \mu\text{g/kg}$ ,  
401 respectively). However, after 9 and 11 min of baking time, significantly lower AA values were  
402 obtained in the biscuits prepared with IBF compared to those of DBF. In detail, AA levels were  
403  $513.9 \pm 28.5 \mu\text{g/kg}$  and  $452.6 \pm 17.1 \mu\text{g/kg}$  after 9 min of baking and  $704.9 \pm 74.8 \mu\text{g/kg}$  and  $596.8$   
404  $\pm 57.3 \mu\text{g/kg}$  after 11 min of baking for DBF and IBF samples, respectively. As initially  
405 hypothesised, the difference in the rate of AA formation could be attributed to the different  
406 availability of AA precursors and the different moisture content of the DBF and IBF biscuit  
407 samples during baking (**Table 3**). As reported in the table, asparagine levels in the biscuits  
408 decreased during baking in both formulations, but to a greater extent for the DBF biscuit samples  
409 probably because the cell breakage released free asparagine in the dough favouring the MR with  
410 reducing sugars. No significant differences between DBF and IBF samples in terms of both  
411 asparagine and sucrose were found in the raw doughs (0 min). Nevertheless, after 11 min of baking,  
412 the asparagine content in DBF biscuits was reduced by 50%, whereas in IBF biscuits by only 40%  
413 indicating its less availability in flours containing intact cell walls. For both types of biscuits,  
414 asparagine content was significantly negatively correlated with AA levels with an  $r$ -value of  $-0.87$   
415 and  $-0.79$  for the DBF and IBF samples, respectively (**Figure 3**). No reducing sugars (i.e. glucose  
416 and fructose) could be detected in either the dough or the biscuit samples. The sucrose contents  
417 did not correlate with AA levels ( $r = -0.23$  for DBF and  $r = -0.66$  for IBF) and did not change  
418 significantly during the baking of the biscuits in accordance with previous studies (Graf et al.,  
419 2006; Gökmen et al., 2007; Nguyen et al., 2016; Schouten et al., 2021). This result confirms that  
420 the key factor responsible for the AA formation in bakery products is the presence of free  
421 asparagine (Krishnakumar & Visvanathan, 2014; Miśkiewicz et al., 2012; Negoită et al., 2017;  
422 Salazar et al., 2012; Sarion et al., 2021; Sazesh & Goli, 2020; Žilić et al., 2020).

423 Besides the presence of precursors, the main factor determining the amount of AA formed in  
424 biscuits is the presence of water. The two sets of samples started from similar initial moisture and  
425  $a_w$  (about 16 % and 0.80, respectively), both values progressively decreased in both biscuit samples  
426 during baking. The moisture content was significantly lower in DBF biscuits than in IBF biscuits



427 from 5 to 11 min of baking while the  $a_w$  results became significantly different between the samples  
428 only at 7 and 9 min. The faster water loss in the DBF biscuits can be probably attributed to cell  
429 walls damage of this sample that facilitated water migration and evaporation during biscuit-dough  
430 baking. In contrast, in the IBF sample, this phenomenon is slower, probably thanks to the higher  
431 water holding capacity exerted by the intact cell walls. The different kinetics of water loss, together  
432 with the low availability of asparagine, are the two main factors contributing to the higher levels  
433 of AA in the DBF sample in agreement with the results of several studies that have reported that  
434 low moisture and  $a_w$  values trigger the MR and thus the formation of AA (Bråthen et al., 2005;  
435 Esposito et al., 2020; Matthäus et al., 2004).

436 The two different types of kidney bean flour also led to a different development of the main baking  
437 parameters of biscuit samples as reported in **Table 4**. The weight loss determined during the baking  
438 of the biscuit followed a similar trend to the moisture content, indicating a greater weight loss in  
439 the DBF biscuit sample than in the IBF sample after 5, 7 and 9 min of baking. In general, the  
440 biscuits made with DBF had a slightly lower pH than those made with IBF, but this did not affect  
441 the AA levels ( $r = -0.46$  for DBF biscuits and  $r = -0.33$  for IBF biscuits), probably because both  
442 biscuit samples had significantly similar and basic pH values. The lowest pH values were reached  
443 after 11 min of baking,  $8.0 \pm 0.1$  and  $8.3 \pm 0.2$  for DBF and IBF samples, respectively. The different  
444 dough formulations also led to a different development of the biscuit surface colour during baking,  
445 as can be appreciated in the example pictures shown in **Figure 4**. Colour is influenced by dough  
446 composition, water content and process conditions such as temperature and duration of baking.  
447 For both DBF and IBF biscuits, during baking, the values of  $L^*$  (lightness) and  $h^\circ$  (hue angle) of  
448 upper and lower surfaces decreased indicating a concomitant reduction in lightness and change to  
449 a redder hue. This colour change was proportional to the AA content for both samples, showing a  
450 negative correlation with  $L^*$  ( $r = -0.87$  and  $-0.86$  for upper surface and  $r = -0.92$  and  $-0.90$  for  
451 the lower surface, for samples IBF and DBF respectively) and  $h^\circ$  ( $r = -0.76$  and  $-0.80$  for upper  
452 surface and  $r = -0.80$  and  $-0.79$  for lower surface, for samples IBF and BF respectively). In  
453 addition, together with an increased formation of AA, the biscuits became browner during baking  
454 as indicated by an increased BI (browning index) ( $r = 0.90$  and  $0.95$  for upper surface and  $r = 0.95$   
455 and  $0.95$  for lower surface, for samples IBF and DBF respectively). The upper surface of the DBF  
456 samples, with a higher AA, showed a less bright colour than the IBF samples for the 9 and 11 min  
457 baking times, and a more red hue for the baking time of 7 and 9 min. In addition, the upper surface  
458 of the DBF samples showed a general darker colour, demonstrated by higher BI values, compared  
459 to the IBF samples at the same baking times, although not significantly so for the shorter baking  
460 time of 5 min. The bottom surface of the biscuits was more similar between the samples in terms

461 of  $L^*$  and  $h^\circ$  values, probably because the heat transfer from the baking tray could impact the  
462 colour formation. On the other hand, the BI of the lower surface of the biscuit was significantly  
463 higher for the IBF samples than for the DBF samples for the baking times of 9 and 11 min (**Table**  
464 **4** and **Figure 4**). Concerning texture proprieties, together with the formation of AA hardness ( $r =$   
465 0.95 for DBF and 0.88 for IBF) and fracturability ( $r = 0.91$  for DBF and 0.92 for IBF), which  
466 indicate the firmness of the structure, and crispness ( $r = 0.98$  for DBF and IBF), which is a measure  
467 of the friability of the structure, increased with longer baking times in both DBF and IBF biscuits  
468 as noted in previous research (Lara et al., 2011; Romani et al., 2012; Schouten et al., 2022). These  
469 texture parameters are useful structural properties in assessing the quality of biscuits because of  
470 their close association with the perception of freshness (Lara et al., 2011). At the same baking  
471 time, IBF samples showed significantly higher hardness values than DBF samples after 9 and 11  
472 min of baking. In addition, as a consequence, these samples showed a lower value of fracturability  
473 and a higher value of crispness, although not significantly for each baking time. In general, a lower  
474 moisture content leads to a higher sample hardness (Aguilera, 2005; Martinez-Navarrete et al.,  
475 2004; Pereira et al., 2013; Romani et al., 2015), but in the present case, the opposite is true. The  
476 harder IBF samples also had a significantly higher moisture content than the DBF samples. These  
477 findings demonstrated that the relationship between water content and hardness in the biscuits  
478 matrix is not always straightforward, as the texture is also affected by other physico-chemical  
479 aspects such as product composition (e.g. interactions between ingredients, such as fat, sugar,  
480 starch) and its micro- and macro-structure characteristics (Aguilera, 2005; Martinez-Navarrete et  
481 al., 2004; Romani et al., 2015, 2016). Therefore, the use of different bean flour preparations in  
482 formulations leads to the specific structural characteristics of biscuit doughs, making further  
483 optimisation on a case-by-case basis for specific biscuit formulations necessary.

484

#### 485 **4. Conclusions**

486 The results obtained in this study suggested that the use of kidney bean flour with different  
487 cotyledon cell integrity as an ingredient in the biscuits has an influence on the formation of AA.  
488 SEM analysis showed that direct grinding of the beans promoted mechanical rupture of the  
489 cotyledon cell walls while boiling the beans, before reduction to a homogeneous mash, maintained  
490 their integrity.

491 As hypothesised, the integrity of the cotyledon cell walls leads to a decreased formation of AA.  
492 Biscuits made with intact bean flour developed a significantly lower level of AA than those made  
493 with damaged bean flour, probably because its precursor asparagine is less available and the rate

494 of water loss during baking is slower, reducing the extent of the MR. This result was also  
495 confirmed by different evolution of the main quality characteristics in the biscuit samples.

496 In the present experiments, the AA concentrations at the end of the baking time were above the  
497 reference values of 350 µg/kg specified in the EU Regulation 2017/2158 for the category “biscuits  
498 and wafers” (European Commission, 2017) for both biscuit formulations. This confirms the urgent  
499 need to implement an applicable mitigation strategy to reduce the consumers’ exposure to this  
500 contaminant.

501 The overall results indicated that an accurate design of the legume flour preparation (milling and  
502 cooking process) could be a useful strategy to modulate the AA formation kinetics in biscuit  
503 products.

504

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511

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678



679 **Figure captions:**

680 **Figure 1.** Scanning electron micrographs performed at 250, 1000 and 5000× times magnification  
681 of freeze-dried Damaged Bean Flour - DBF (A), Intact Bean Flour - IBF (B) and heated Intact  
682 Bean Flour - IBF (C) from kidney beans.

683  
684 **Figure 2.** Levels of acrylamide ( $\mu\text{g}/\text{kg}$ ) expressed on dry matter (d.m.) basis of biscuits formulated  
685 with Damaged Bean Flour (DBF) and Intact Bean Flour (IBF) during baking at 170 °C for 5, 7, 9  
686 and 11 min. Different letters indicate significant differences among samples ( $p < 0.05$ ).

687  
688 **Figure 3.** Linear correlation between asparagine concentrations ( $\text{mg}/\text{kg}$  d.m.) and acrylamide  
689 levels ( $\mu\text{g}/\text{kg}$  d.m.) found in biscuits formulated with Damaged Bean Flour (DBF) and Intact Bean  
690 Flour (IBF) after 5, 7, 9 and 11 min of baking at 170 °C.

691  
692 **Figure 4.** Visual appearance of the top and bottom surfaces of biscuit samples formulated with  
693 14% of Damaged Bean Flour - DBF (A) and Intact Bean Flour - IBF (B) baked at 170 °C for 5, 7,  
694 9 and 11 min.

**Table 1.** Recipes of biscuit samples used in the experiment.

<b>Ingredient</b>	<b>Damaged Bean Flour (DBF) biscuits</b>	<b>Intact Bean Flour (IBF) biscuits</b>
Wheat flour	172.0 g	172.0 g
Boiled bean	-	74.4 g
Milled bean	56.5 g	-
Fine sucrose	84.0 g	84.0 g
High fructose syrup	3.0 g	3.0 g
Shortening	80.0 g	80.0 g
Non-fat dry milk	2.0 g	2.0 g
Salt	2.5 g	2.5 g
Sodium bicarbonate	2.0 g	2.0 g
Ammonium bicarbonate	1.0 g	1.0 g
Distilled water	19.5 mL	1.54 mL

**Table 2.** HPLC-MS/MS acquisition parameters of the optimized SRM transition adopted for the quantification of acrylamide and asparagine.

<b>Compound</b>	<b>Precursor ion (m/z)</b>	<b>Product ion (m/z)</b>	<b>Dwell time (ms)</b>	<b>Q1 Pre Bias (V)</b>	<b>Q2 (V)</b>	<b>Q3 Pre Bias (V)</b>
Asparagine	133.20	74.00	4	-14.0	-15.0	-13.0
	133.20	87.05	4	-14.0	-12.0	-16.0
	133.20	28.15	4	-10.0	-25.0	-28.0
Acrylamide	72.00	55.10	42	-30.0	-15.0	-23.0
	72.00	27.10	42	-11.0	-23.0	-29.0
	72.00	44.00	42	-12.0	-24.0	-16.0
Acrylamide-d <sub>3</sub>	75.25	58.05	42	-30.0	-15.0	-23.0
	75.25	30.05	42	-11.0	-23.0	-29.0
	75.25	44.05	42	-12.0	-24.0	-16.0

**Table 3.** Levels of asparagine (mg/kg), sucrose (g/kg) expressed on dry matter (d.m.) basis, moisture content (%) and water activity ( $a_w$ ) of biscuits formulated with Damaged Bean Flour (DBF) and Intact Bean Flour (IBF) baked at 170 °C for different times.

<b>Baking time (min)</b>	<b>Asparagine (mg/kg d.m.)</b>	<b>Sucrose (g/kg d.m.)</b>	<b>Moisture (%)</b>	<b>Water activity (<math>a_w</math>)</b>
<i>DBF biscuits</i>				
0	161.1 ± 17.1 <sup>ab</sup>	199.4 ± 50.7 <sup>a</sup>	16.6 ± 0.4 <sup>a</sup>	0.81 ± 0.01 <sup>a</sup>
5	163.2 ± 15.7 <sup>ab</sup>	187.5 ± 24.0 <sup>a</sup>	9.6 ± 0.2 <sup>c</sup>	0.67 ± 0.01 <sup>b</sup>
7	147.9 ± 17.6 <sup>bc</sup>	180.8 ± 27.7 <sup>a</sup>	5.9 ± 0.2 <sup>e</sup>	0.45 ± 0.01 <sup>d</sup>
9	114.8 ± 12.5 <sup>d</sup>	204.2 ± 23.4 <sup>a</sup>	3.1 ± 0.1 <sup>g</sup>	0.26 ± 0.02 <sup>f</sup>
11	81.5 ± 9.2 <sup>e</sup>	187.7 ± 33.8 <sup>a</sup>	1.7 ± 0.1 <sup>i</sup>	0.16 ± 0.01 <sup>g</sup>
<i>IBF biscuits</i>				
0	173.9 ± 12.8 <sup>a</sup>	233.6 ± 89.5 <sup>a</sup>	16.4 ± 0.4 <sup>a</sup>	0.82 ± 0.01 <sup>a</sup>
5	178.6 ± 21.5 <sup>a</sup>	213.6 ± 66.7 <sup>a</sup>	10.8 ± 0.4 <sup>b</sup>	0.70 ± 0.02 <sup>b</sup>
7	164.1 ± 17.0 <sup>ab</sup>	205.1 ± 84.0 <sup>a</sup>	7.2 ± 0.3 <sup>d</sup>	0.54 ± 0.03 <sup>c</sup>
9	137.2 ± 15.3 <sup>c</sup>	218.2 ± 69.4 <sup>a</sup>	4.5 ± 0.2 <sup>f</sup>	0.35 ± 0.03 <sup>e</sup>
11	103.7 ± 9.6 <sup>d</sup>	214.9 ± 70.8 <sup>a</sup>	2.5 ± 0.3 <sup>h</sup>	0.19 ± 0.04 <sup>g</sup>

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

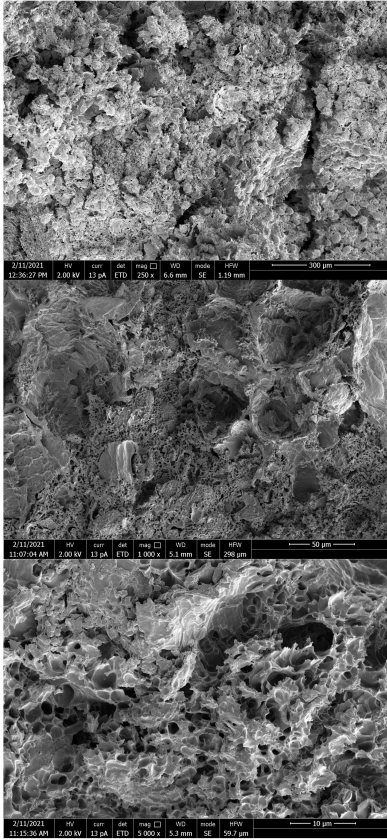
**Table 4.** Weight loss (%), pH, colour and texture proprieties of biscuits formulated with Damaged Bear Flour (DBF) and Intact Bear Flour (IBF) baked at 170 °C for different times.

Baking time (min)	Weight loss (%)	pH	Lightness (L*)	Hue angle (h°)	Browning index (BI)	Lightness (L*) **	Hue angle (h°) **	Browning index (BI) **	Hardness (N)	Fracturability (1/mm)	Crispness (linear distance)
<i>DBF biscuits</i>											
0	-	8.6 ± 0.3 <sup>b</sup>	69.8 ± 0.9 <sup>a</sup>	83.9 ± 1.0 <sup>b</sup>	33.3 ± 5.8 <sup>f</sup>	69.8 ± 0.9 <sup>a</sup>	83.9 ± 1.0 <sup>ab</sup>	33.3 ± 5.8 <sup>g</sup>	-	-	-
5	7.4 ± 0.5 <sup>e</sup>	9.3 ± 0.1 <sup>a</sup>	71.3 ± 0.6 <sup>a</sup>	86.3 ± 1.0 <sup>a</sup>	44.5 ± 2.7 <sup>e</sup>	68.8 ± 0.5 <sup>a</sup>	86.4 ± 1.3 <sup>a</sup>	52.9 ± 5.7 <sup>f</sup>	5.6 ± 1.2 <sup>c</sup>	0.9 ± 0.3 <sup>d</sup>	8.4 ± 1.6 <sup>c</sup>
7	11.1 ± 0.4 <sup>c</sup>	9.1 ± 0.1 <sup>a</sup>	65.0 ± 2.0 <sup>b</sup>	80.6 ± 1.4 <sup>c</sup>	69.1 ± 7.3 <sup>c</sup>	62.2 ± 2.9 <sup>b</sup>	83.2 ± 3.4 <sup>b</sup>	84.2 ± 23.2 <sup>de</sup>	21.1 ± 4.6 <sup>b</sup>	1.0 ± 0.3 <sup>cd</sup>	24.1 ± 4.5 <sup>b</sup>
9	13.7 ± 0.3 <sup>b</sup>	8.6 ± 0.1 <sup>b</sup>	57.0 ± 1.4 <sup>d</sup>	76.4 ± 0.8 <sup>e</sup>	89.3 ± 12.2 <sup>b</sup>	51.4 ± 1.6 <sup>cd</sup>	76.1 ± 1.1 <sup>cd</sup>	120.0 ± 21.9 <sup>ab</sup>	24.1 ± 2.7 <sup>b</sup>	1.3 ± 0.4 <sup>bcd</sup>	32.2 ± 6.3 <sup>a</sup>
11	15.2 ± 0.4 <sup>a</sup>	8.0 ± 0.1 <sup>c</sup>	50.6 ± 2.1 <sup>f</sup>	74.4 ± 0.9 <sup>f</sup>	109.3 ± 10.8 <sup>a</sup>	44.2 ± 2.7 <sup>e</sup>	73.3 ± 1.1 <sup>d</sup>	138.6 ± 18.2 <sup>a</sup>	22.2 ± 3.0 <sup>b</sup>	1.5 ± 0.6 <sup>ab</sup>	31.6 ± 8.0 <sup>a</sup>
<i>IBF biscuits</i>											
0	-	8.7 ± 0.1 <sup>b</sup>	71.7 ± 1.7 <sup>a</sup>	84.7 ± 1.1 <sup>b</sup>	34.2 ± 6.7 <sup>f</sup>	71.7 ± 1.7 <sup>a</sup>	84.7 ± 1.1 <sup>ab</sup>	34.2 ± 6.7 <sup>g</sup>	-	-	-
5	6.0 ± 0.6 <sup>f</sup>	9.2 ± 0.1 <sup>a</sup>	70.5 ± 1.1 <sup>a</sup>	86.4 ± 1.1 <sup>a</sup>	41.5 ± 6.7 <sup>e</sup>	68.1 ± 1.5 <sup>a</sup>	85.8 ± 1.7 <sup>a</sup>	54.3 ± 10.7 <sup>f</sup>	6.4 ± 1.6 <sup>c</sup>	0.8 ± 0.3 <sup>d</sup>	8.8 ± 1.4 <sup>c</sup>
7	9.6 ± 0.4 <sup>d</sup>	9.2 ± 0.1 <sup>a</sup>	66.7 ± 1.2 <sup>b</sup>	83.2 ± 1.1 <sup>b</sup>	55.9 ± 8.7 <sup>d</sup>	61.0 ± 4.8 <sup>b</sup>	82.4 ± 1.5 <sup>b</sup>	71.9 ± 13.4 <sup>e</sup>	20.3 ± 3.4 <sup>b</sup>	1.1 ± 0.2 <sup>cd</sup>	23.2 ± 3.9 <sup>b</sup>
9	12.2 ± 0.4 <sup>c</sup>	8.8 ± 0.2 <sup>b</sup>	59.3 ± 1.4 <sup>c</sup>	78.1 ± 0.8 <sup>d</sup>	76.3 ± 8.8 <sup>c</sup>	55.1 ± 5.6 <sup>c</sup>	78.5 ± 1.1 <sup>c</sup>	95.1 ± 24.9 <sup>cd</sup>	29.9 ± 5.8 <sup>a</sup>	1.2 ± 0.3 <sup>abc</sup>	36.1 ± 6.8 <sup>a</sup>
11	14.3 ± 0.3 <sup>ab</sup>	8.3 ± 0.2 <sup>c</sup>	54.8 ± 1.7 <sup>e</sup>	75.4 ± 0.7 <sup>f</sup>	81.1 ± 15.4 <sup>b</sup>	48.5 ± 5.8 <sup>de</sup>	75.4 ± 1.2 <sup>d</sup>	108.6 ± 25.8 <sup>bc</sup>	28.0 ± 3.6 <sup>a</sup>	1.4 ± 0.3 <sup>a</sup>	37.9 ± 9.5 <sup>a</sup>

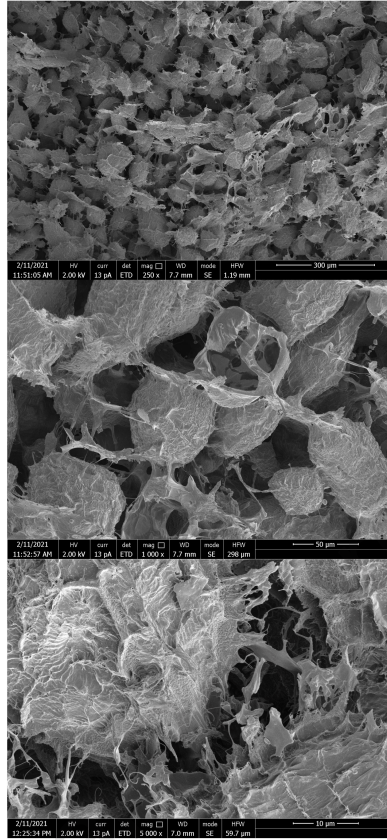
Different letters in the same columns indicate significant differences among samples ( $p < 0.05$ ).

\*\* Bottom surface of the biscuits.

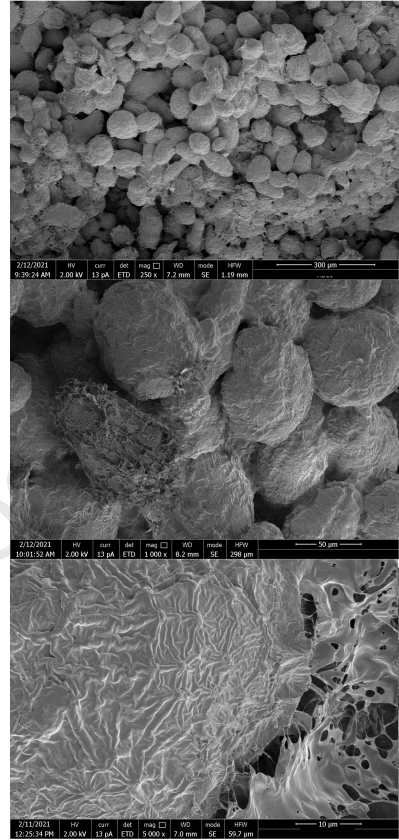
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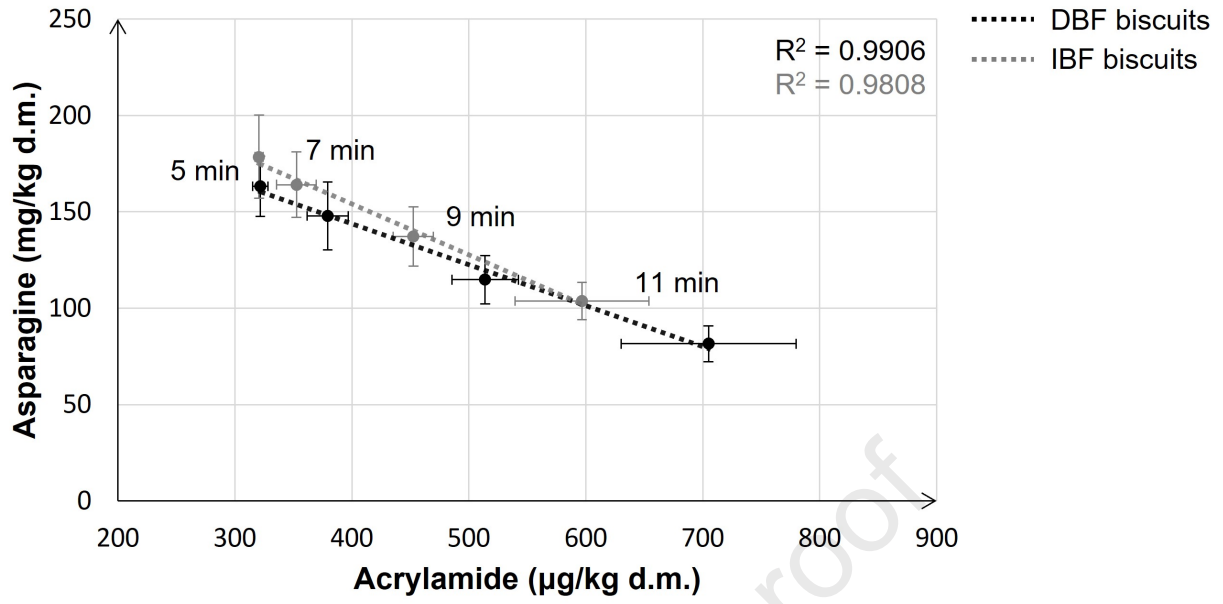


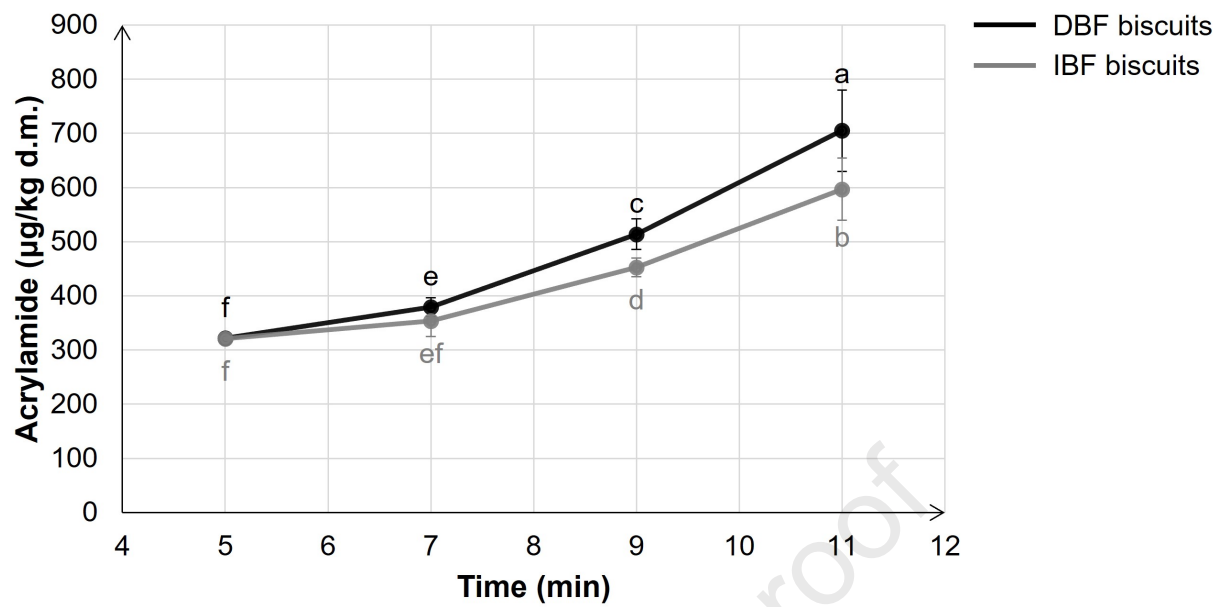
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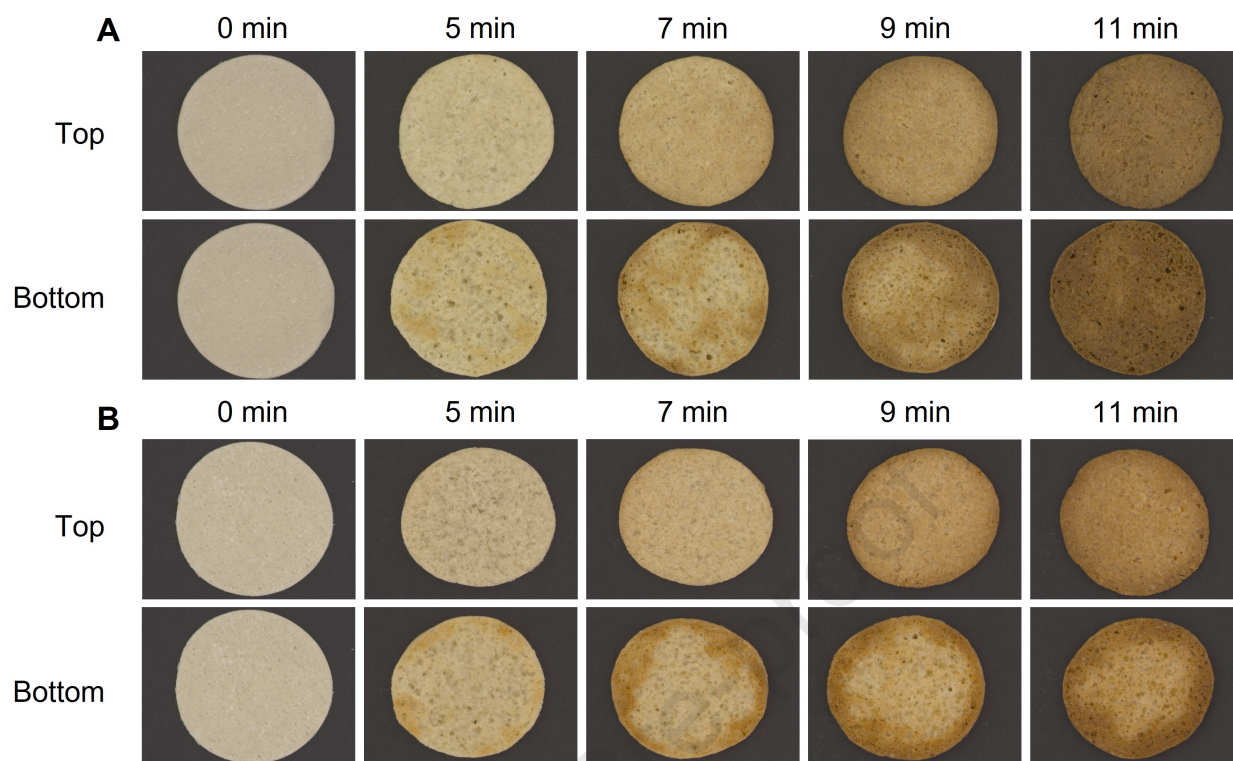
C











**Highlights:**

- The different preparations of the bean flours led to different cell walls integrity.
- Biscuits made with intact bean flours developed the lowest level of acrylamide.
- Asparagine was less available for the acrylamide formation in intact bean flour.
- Biscuits made with damaged bean flours developed the highest level of acrylamide.
- Biscuits made with damaged bean flour dehydrated more quickly during baking.

Journal Pre-proof