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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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Author statement

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

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Santina Romani: Conceptualization, Writing - Review & Editing, Supervision

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

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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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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 alterative 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,

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

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

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

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

2. Materials and methods

2.1 Preparation of kidney bean flours

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

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

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

2.2 Microscopy analysis of kidney bean flours

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

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

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

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

2.3 Preparation of biscuit samples

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

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

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

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

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

2.4 Analysis of the main quality parameters

2.4.1 Moisture

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

Three replicates were performed for each sample and baking batch.

2.4.2 Water activity

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

Three replicates were performed for each sample and baking batch.

2.4.3 Weight loss

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

2.4.4 pH

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

Three replicates were performed for each sample and baking batch.

2.4.5 Colour

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

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

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

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

2.4.6 Texture

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

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

2.5 Quantification of asparagine and acrylamide

2.5.1 Sample extraction

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

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

For AA quantification, 4.75 mL of the formic acid extract with 100 µL of 5000 µg/L AA-d_3 solution (CAS No 122775-19-3, Sigma-Aldrich, St. Louis, MO, USA) were clarified with 0.125 mL of Carrez I and 0.125 mL of Carrez II solutions (Sigma-Aldrich, St. Louis, MO, USA). The mixture was vortexed and centrifuged for 3 min at 10000 rpm and 20 °C. For better clarification, 2 mL of supernatant was collected and centrifuged for 10 min at 14000 rpm and 20 °C. For the solid phase extraction (SPE) clean-up, according to Mogol and Gökmen (2014), the Oasis MCX cartridge (Waters, Milford, MA, USA) was activated with 1 mL of methanol (Actu-All Chemicals, Oss, The Netherlands) and conditioned with 1 mL of Milli-Q water with a speed of 1 drop/second. Subsequently, 1 mL of clean extract was passed through to preconditioned cartridge (1 drop/second) into an amber glass autosampler vial. The first 7-8 drops of the sample were carefully discarded to avoid any dilution. The recovery (RE%) of the SPE procedure was 93.3 ± 5.3%, determined by evaluating the analyte signal responses of 1 mL of standard solutions composed of 980 µL of AA (250 µg/L) and 20 µL of AA-d_3 (5000 µg/L) before (SB) and after (SA) passing it through SPE (RE% = (area SA/area SB)×100).

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

2.5.2 LC-MS/MS method

LC-MS/MS analyses were carried with a Nexera UPLC system coupled with an LCMS-8050 triple quadrupole mass spectrometer (Shimadzu Corporation, Kyoto, Japan). The UPLC unit consisted of a SIL-30AC autosampler, an LC-20ADXR solvent delivery module, a DGU-20ASR degassing unit, a CTO-20AC column oven and an FCV-20AH2 valve unit. The HPLC gradient analytical standards such as AA (C_3H_5NO, molecular weight 71.08 g/mol, CAS No 79-06-1) and L-asparagine ($C_4H_8N_2O_3$, molecular weight 132.12 g/mol, CAS No 70-47-3) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

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

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

The chromatographic separation of AA was performed on a Thermo Scientific™ Hypercarb column (5 µm, 2.1 × 50 mm, Thermo Fisher Scientific, Waltham, MA, USA) connected to an OPTI-GUARD® 1 mm Guard C18 column (Sigma-Aldrich, St. Louis, MO, USA). The flow rate was set at 0.2 mL/min and the column temperature at 40 °C. The mobile phases consisted of 0.1% formic acid (solvent A), methanol with 0.1% formic acid (solvent B). MS data were collected for 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. A calibration curve ranging from 15 to 1000 µg/L of AA was used for quantification. Each calibration solution was spiked with AA-d_3 at a concentration of 100 µg/L.

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

The analytical measurements were repeated three times for each extract, reporting the results as µg/kg for AA and mg/kg for asparagine on dry matter basis. Quantification was performed with a significant linear regression (R^2= 0.99) of an external calibration curve in the range of 39.06 - 10000 µg/L for asparagine and of 15.63 - 1000 µg/L for AA spiked with 100 µg/L of AA-d_3. The coefficients of variations (CV%) of calibration curves intra-day were in the range of 7.0-11.5% and of 8.5-12.9% for asparagine and AA, respectively. In addition, the analyte signal for AA of both calibration curve and samples was corrected with the internal standard (AA-d_3). The corrected areas (AA/AA-d_3) of the MS peak signals were plotted on the concentration.

The limit of quantification (LOQ) and of detection (LOD) were calculated from the standard deviation of the intercept (S) and the slope (s) of the calibration line (LOQ = 10×S/s and LOD =

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

2.6 Quantification of glucose, fructose and sucrose

2.6.1 Sample extraction

The sample preparation for glucose, fructose and sucrose determinations in raw doughs and baked biscuits was performed based on the method described by Nguyen et al. (2016) with slight modifications. Ground biscuits (2.5 g) or freeze-dried doughs (2.5 g) were mixed with 25 mL of Milli-Q water and ethanol (VWR Chemicals, Radnor, PA, USA) mixture (1:1, v/v) and vortexed for 1 min. The samples were incubated for 1 h at 50 °C in a water bath and vortexed again before being cooled down for 20 min at room temperature. Then the samples were centrifuged at 3000 rpm and 20 °C for 10 min and 1.5 mL of supernatant was collected. Then the supernatant was centrifuged at 14000 rpm and 20 °C for 10 min and 1 mL was collected into a glass tube. The water/ethanol solvent was evaporated with a sample concentrator (mod. SBHCONC/1, Stuart, Staffordshire, UK) under nitrogen flush at 50 °C for 4.5 h. After solvent evaporated, the sample was reconstituted with 20 mL of acetonitrile and 20 mL of Milli-Q water and vortexed for 1 min. Samples were stored in a freezer at −20 °C until measurement (maximum 1 week). Before analysis 1.5 mL of sample was passed through Ø28 mm and 0.2 µm CA filters (Phenomenex, Torrance, CA, USA) and transferred into an autosampler amber glass vial.

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

2.6.2 UPLC-ELSD method

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

of acetonitrile/Milli-Q water (3:7, v/v) with 0.2% TEA. The flow rate was 0.25 mL/min and the gradient changes with the following elution profile (min/A%): 0.00/100, 6.00/40, 6.01/100 and 18/100. Before the first injection, the column was equilibrated with 100% A, 0.25 mL/min for 30 min. The injection volume was 1.3 µL and the column temperature was 35 °C. Set up a seal wash with acetonitrile/water (1:1), strong needle wash and weak needle wash with acetonitrile/water (8:2) and acetonitrile/water (7:3) respectively. The pressure of ELSD conditions was 40 psi with a drift tube temperature of 40 °C and a data rate of 10 pps. Operating the software was carried out using a Waters Acquity Control console and data processing was performed with Chromeleon Chromatography Data System (version 7.2.10, Thermo Scientific Corp, Waltham, MA, USA). The quantification was done by an external calibration curve ranging from 85-1360 mg/L (sucrose) and 45-720 mg/L (glucose and fructose). The HPLC gradient analytical standard such as sucrose ($C_{12}H_{22}O_{11}$, molecular weight 342.30 g/mol, CAS No 57-50-1), D-(+)-glucose ($C_6H_{12}O_6$, molecular weight 180.16 g/mol, CAS No 50-99-7) and D-(−)-fructose ($C_6H_{12}O_6$, molecular weight 180.16 g/mol, CAS No 57-48-7) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

The analytical measurements were conducted three times for each extract. The results for sucrose content of doughs and baked biscuits were expressed as g/kg on dry matter basis. Quantification was performed with a significant linear regression (R^2= 0.99) of an external calibration curve in the range of 85-1360 mg/L (sucrose) and of 45-720 mg/L (glucose and fructose). The CV% of calibration curves intra-day were in the ranges of 3.0-8.7%, 6.4-12.7%, 9.8-14.7% for sucrose, glucose and fructose, respectively.

The LOQ and LOD were calculated based on the standard deviation of the intercept and the slope of the calibration line. For sucrose, the LOQ was 8.8 ± 3.0 mg/L and the LOD was 2.9 ± 1.0 mg/L, for glucose, the LOQ was 6.8 ± 3.1 mg/L and the LOD was 2.2 ± 1.1 mg/L, while for fructose the LOQ was 0.2 ± 1.1 mg/L and the LOD was 0.05 ± 0.1 mg/L. The ME% was 115 ± 5%, 105 ± 5% and 97 ± 7% for sucrose, glucose and fructose, respectively.

2.7 Data analysis

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

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

3. Results and discussion

3.1 Kidney bean flours characterization

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

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

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

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

3.2 Influence of kidney bean flours on acrylamide content and main quality characteristics of biscuits

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

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

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

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

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

4. Conclusions

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

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

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

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

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

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Figure captions:

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

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

Figure 3. Linear correlation between asparagine concentrations (mg/kg d.m.) and acrylamide levels (μg/kg d.m.) found in biscuits formulated with Damaged Bean Flour (DBF) and Intact Bean Flour (IBF) after 5, 7, 9 and 11 min of baking at 170 °C.

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

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

Ingredient	Damaged Bean Flour (DBF) biscuits	Intact Bean Flour (IBF) biscuits
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

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

Compound	Precursor ion (m/z)	Product ion (m/z)	Dwell time (ms)	Q1 Pre Bias (V)	Q2 (V)	Q3 Pre Bias (V)
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_3	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

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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.

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

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

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Table 4. Weight loss (%), pH, colour and texture proprieties of biscuits formulated with Damaged Bean Flour (DBF) and Intact Bean 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)
DBF biscuits											
0	-	8.6 ± 0.3^{b}	69.8 ± 0.9^{a}	83.9 ± 1.0^{b}	33.3 ± 5.8^{f}	69.8 ± 0.9^{a}	83.9 ± 1.0^{ab}	33.3 ± 5.8^{g}	-	-	-
5	7.4 ± 0.5^{e}	9.3 ± 0.1^{a}	71.3 ± 0.6^{a}	86.3 ± 1.0^{a}	44.5 ± 2.7^{e}	68.8 ± 0.5^{a}	86.4 ± 1.3^{a}	52.9 ± 5.7^{f}	5.6 ± 1.2^{c}	0.9 ± 0.3^{d}	8.4 ± 1.6^{c}
7	11.1 ± 0.4^{c}	9.1 ± 0.1^{a}	65.0 ± 2.0^{b}	80.6 ± 1.4^{c}	69.1 ± 7.3^{c}	62.2 ± 2.9^{b}	83.2 ± 3.4^{b}	84.2 ± 23.2^{de}	21.1 ± 4.6^{b}	1.0 ± 0.3^{cd}	24.1 ± 4.5^{b}
9	13.7 ± 0.3^{b}	8.6 ± 0.1^{b}	57.0 ± 1.4^{d}	76.4 ± 0.8^{e}	89.3 ± 12.2^{b}	51.4 ± 1.6^{cd}	76.1 ± 1.1^{cd}	120.0 ± 21.9^{ab}	24.1 ± 2.7^{b}	1.3 ± 0.4^{bcd}	32.2 ± 6.3^{a}
11	15.2 ± 0.4^{a}	8.0 ± 0.1^{c}	50.6 ± 2.1^{f}	74.4 ± 0.9^{f}	109.3 ± 10.8^{a}	44.2 ± 2.7^{e}	73.3 ± 1.1^{d}	138.6 ± 18.2^{a}	22.2 ± 3.0^{b}	1.5 ± 0.6^{ab}	31.6 ± 8.0^{a}
IBF biscuits											
0	-	8.7 ± 0.1^{b}	71.7 ± 1.7^{a}	84.7 ± 1.1^{b}	34.2 ± 6.7^{f}	71.7 ± 1.7^{a}	84.7 ± 1.1^{ab}	34.2 ± 6.7^{g}	-	-	-
5	6.0 ± 0.6^{f}	9.2 ± 0.1^{a}	70.5 ± 1.1^{a}	86.4 ± 1.1^{a}	41.5 ± 6.7^{e}	68.1 ± 1.5^{a}	85.8 ± 1.7^{a}	54.3 ± 10.7^{f}	6.4 ± 1.6^{c}	0.8 ± 0.3^{d}	8.8 ± 1.4^{c}
7	9.6 ± 0.4^{d}	9.2 ± 0.1^{a}	66.7 ± 1.2^{b}	83.2 ± 1.1^{b}	55.9 ± 8.7^{d}	61.0 ± 4.8^{b}	82.4 ± 1.5^{b}	71.9 ± 13.4^{e}	20.3 ± 3.4^{b}	1.1 ± 0.2^{cd}	23.2 ± 3.9^{b}
9	12.2 ± 0.4^{c}	8.8 ± 0.2^{b}	59.3 ± 1.4^{c}	78.1 ± 0.8^{d}	76.3 ± 8.8^{c}	55.1 ± 5.6^{c}	78.5 ± 1.1^{c}	95.1 ± 24.9^{cd}	29.9 ± 5.8^{a}	1.2 ± 0.3^{abc}	36.1 ± 6.8^{a}
11	14.3 ± 0.3^{ab}	8.3 ± 0.2^{c}	54.8 ± 1.7^{e}	75.4 ± 0.7^{f}	81.1 ± 15.4^{b}	48.5 ± 5.8^{de}	75.4 ± 1.2^{d}	108.6 ± 25.8^{bc}	28.0 ± 3.6^{a}	1.4 ± 0.3^{a}	37.9 ± 9.5^{a}

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

** Bottom surface of the biscuits.

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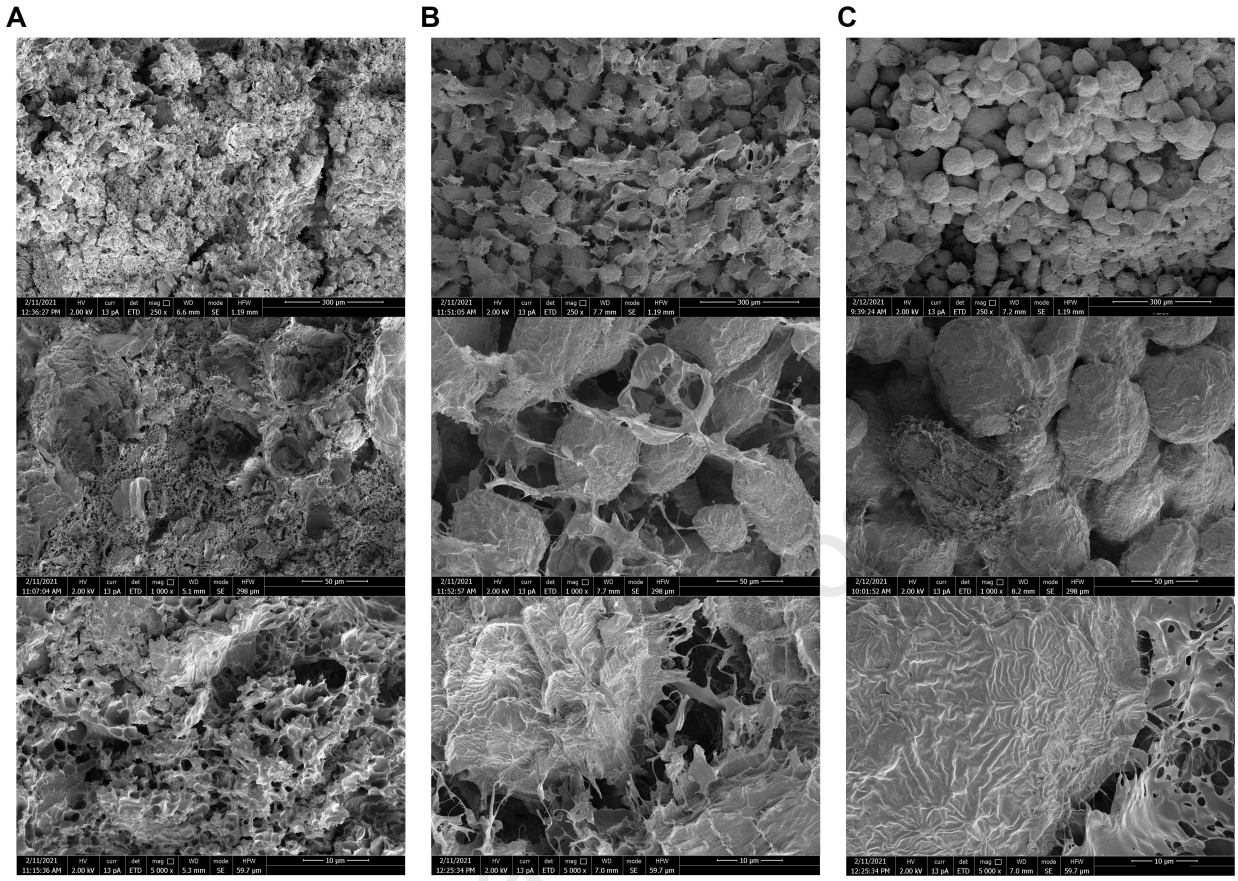

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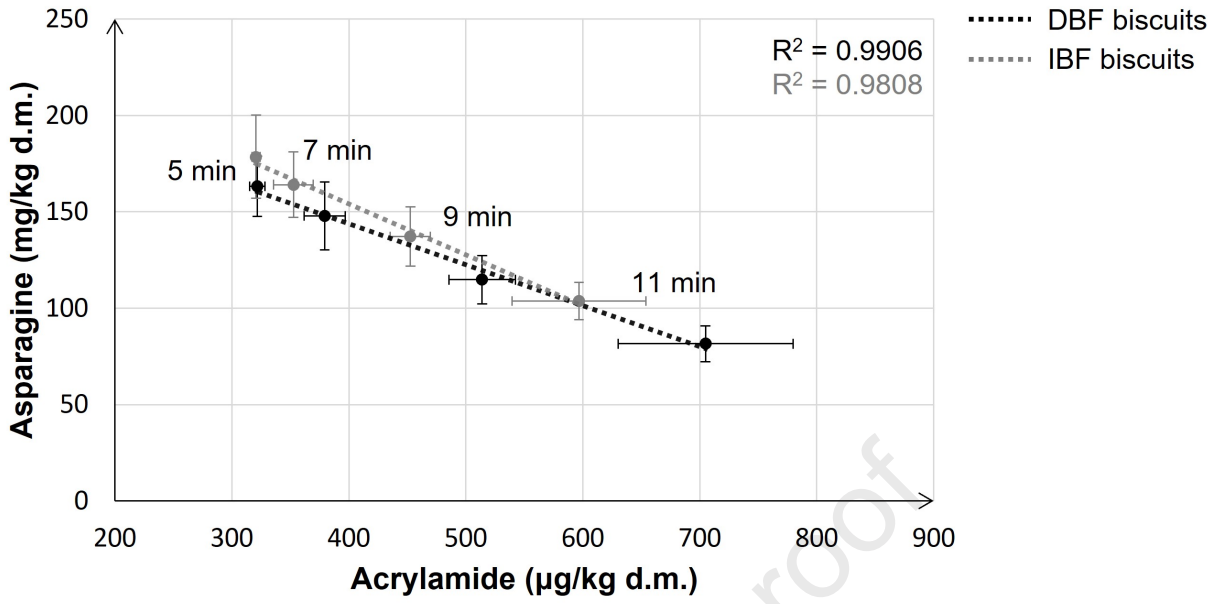

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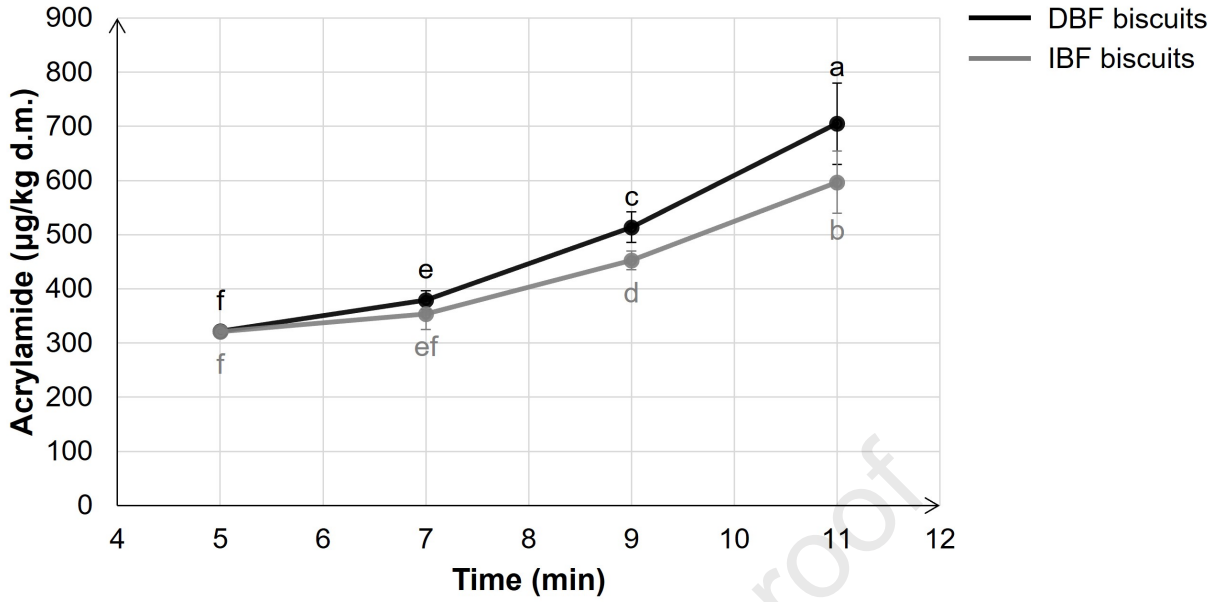

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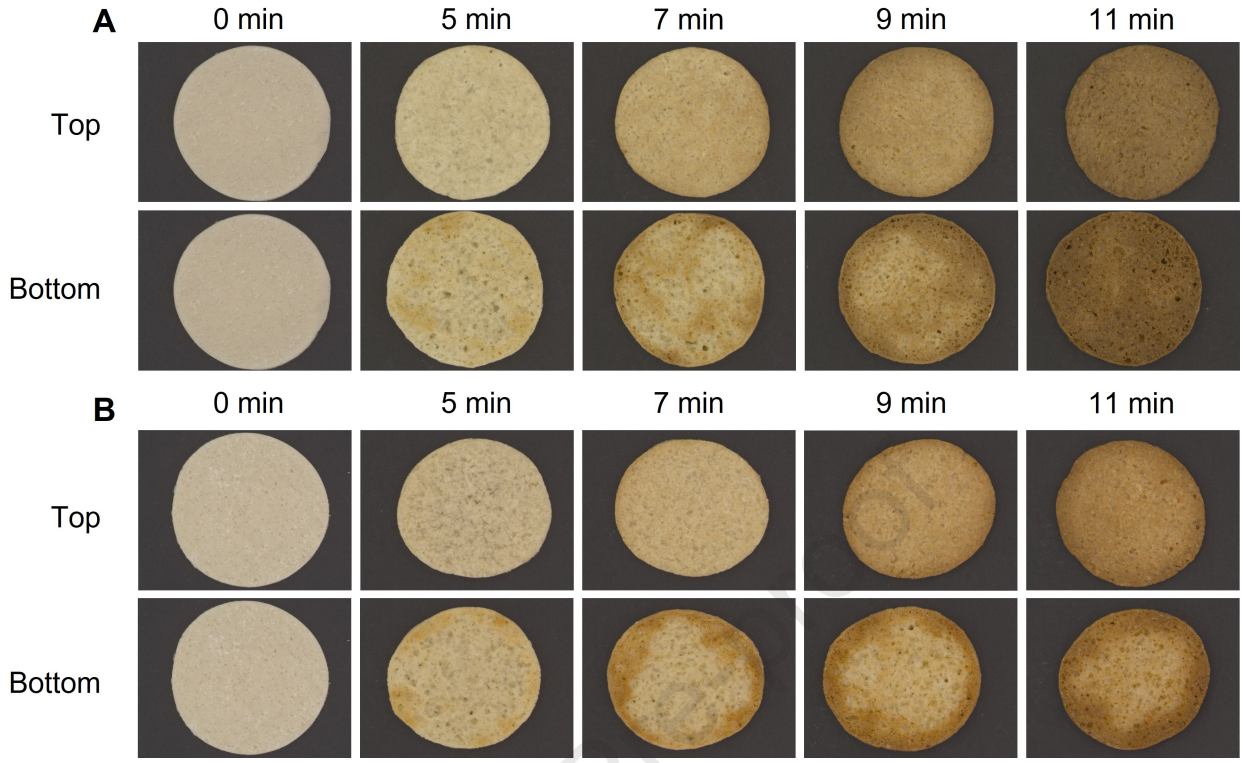

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Highlights:

- 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.

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