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Food Chemistry

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

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

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

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

Keywords:
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Chemical compounds studied in this article:
Acrylamide (PubChem CID: 6579); Acrylamide-d_3 (PubChem CID: 12209671); Asparagine (PubChem CID: 6267); Sucrose (PubChem CID: 5988).

1. Introduction

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

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

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

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

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

2. Materials and methods

2.1. Biscuit ingredients and chemicals

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

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

2.2 Preparation of biscuit samples

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

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

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

To ensure homogeneous distribution in the dough, asparagine, 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 control 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 for 20 min in a refrigerator at 4 °C. For some subsequent analyses, parts of the raw dough samples were freeze-dried and finely grounded with a mortar.

The raw 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, 8 biscuits were baked in an electrical oven (OV185C, Inventum, Arnhem, The Netherlands) with convection mode at 175 °C for 5, 7 and 9 min. The different baking conditions were chosen in preliminary tests to obtain biscuits that were neither undercooked nor overcooked. The biscuits were placed in the middle position of a baking tray inside the oven; for each baking cycle, the air temperature inside the oven chamber was recorded every 20 s using a digital thermometer equipped with type K thermocouples (Pro 206-3722, RS Components, Corby, UK) to ensure equal temperature exposure between the baking cycles. After baking, biscuits were removed from the oven, placed on a grid and kept cooling at room temperature for about 1 h.

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

2.3 Characterization of flours and biscuits

2.3.1 Proximal analysis

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

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

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

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

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

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

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

All analyses were performed in duplicate for each flour type.

2.3.2 Particle size

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

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

2.3.3 Hydration properties

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

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

2.3.4 pH

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

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

2.3.5 Moisture content

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

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

2.3.6 Water activity

The water activity (a_w) of flours, raw doughs and biscuits was determined at 25 °C with an a_w-meter (LabMaster, Novasina AG, Lachen, Switzerland) setting both time and temperature factors stability at 2 min.

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

2.3.7 Weight loss

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

2.3.8 Colour

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

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

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

2.3.9 Texture

The texture analysis of 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), determined by means of maximum force, fracturability (1/mm), expressed as one/breakpoint distance between the origin of curve till the point where the biscuit breaks, and crispness, evaluated by the linear distance between the first and the last peak registered (Romani et al., 2012).

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

2.4 Quantification of asparagine and acrylamide by LC-MS/MS

2.4.1 Sample extraction

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

For asparagine determination, the formic acid extract (5 mL) was centrifuged for 10 min at 20817 × g and 20 °C (5430 R, Eppendorf AG, Hamburg, Germany). For better clarification, 4 mL of supernatant were centrifuged for 7 min at 20817 × g and 20 °C, then 1 mL of clear supernatant was mixed with 1 mL of acetonitrile and filtered with 0.2 µm PTFE filters (Ø15 mm) into an amber glass autosampler vial. For AA determination, the formic acid extract (4.75 mL) with AA-d_3 solution (100 µL) were clarified with 0.125 mL of Carrez I and 0.125 mL of Carrez II. The mixture was vortexed and centrifuged for 3 min at 10621 × g and 20 °C. For better clarification, 2 mL of supernatant was collected and centrifuged for 10 min at 20817 × g and 20 °C. Solid phase extraction cleaning was carried out according to Mogol & Gökmen (2014) using the Oasis MCX cartridge and collecting the sample in an amber glass autosampler vial.

2.4.2 LC-MS/MS methods

Samples analyses were carried out with a Nexera UPLC system (Shimadzu Corporation, Kyoto, Japan) 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-20AH_2 valve unit.

The chromatographic separation of free asparagine was performed injecting 5 µL of samples on a SeQuant® ZIC HILIC 3.5 µm, 4.6 × 150 mm (Merck KGaS, 64271, Darmstadt, Germany) attached to a SeQuant® ZIC HILIC PEEK coated guard column 20 × 2.1 mm (Merck KGaS, 64271, 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) 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.

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

Positive ionisation mode was used for both MS analyses. The voltage of the turbo ion-spray ionization was 4.0 kV. The temperature of the electrospray ionization probe, desolvation line and heat block was 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 msec, respectively for asparagine and AA, and the most abundant fragment ion was selected for quantitation. The second and third fragments in ion yield were selected as a structural confirmation based on the optimized SRM transition. The precursor/product ion transitions m/z 133.20→74.00, 133.20→87.05 and 133.20→28.15 were monitored for asparagine; 72.00→55.10, 72.00→27.10 and 72.00→44.00 were monitored for acrylamide; 75.25→58.05, 75.25→30.05 and 75.25→44.05 were monitored for acrylamide-d_3. Data

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

The sample extraction was repeated twice for each flour and each batch per sample and the analytical measurements were replicated twice for each extract. The results were expressed as μg/kg for AA and mg/kg for asparagine on dry matter basis.

2.5 Glucose, fructose and sucrose contents

2.5.1 Sample extraction

Raw doughs and biscuits were analysed for glucose, fructose and sucrose contents. The sample extraction process was based on Nguyen et al. (2016) with slight modifications. Grounded biscuit (2.5 g) or freeze-dried dough (2.5 g) was mixed with 1:1, v/v of Milli-Q water and ethanol mixture (25 mL). The samples were incubated for 1 h at 50 °C in a water bath and cooled down for 20 min at room temperature. Then the samples were centrifuged at 1962 × g and 20 °C for 10 min (Heraeus Multifuge X3R, Thermo Fisher Scientific, Waltham, MA, USA). The supernatant (1.5 mL) was centrifuged at 20817 × g and 20 °C (5430 R, Eppendorf AG, Hamburg, Germany) for 10 min and 1 mL was collected into a glass tube. The water/ethanol solvent was evaporated with a sample concentrator (SBHCONC/1, Stuart, Staffordshire, UK) under nitrogen flush at 50 °C for 4.5 h. The sample was reconstituted with acetonitrile (20 mL) and Milli-Q water (20 mL) and stored in a freezer at −20 °C until measurement (maximum 1 week). Before analysis 1.5 mL of sample was passed through CA (Ø28 mm; 0.2 μm) filters and transferred into an autosampler vial.

2.5.2 UPLC-ELSD method

The samples were analysed according to the procedure provided by Waters' technical application notebook with an Acquity UPLC-H Class Plus System (Waters, Milford, MA, USA) equipped with an Acquity Evaporative Light Scattering (ELSD) detector, an Acquity UPLC BEH Amide column (1.7 μm, 2.1 × 100 mm) and 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) while mobile phase B consisted of acetonitrile/water 3:7, v/v with 0.2% TEA. The flow rate was 0.25 mL/min. 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. The pressure of ELSD conditions was 40 psi with

a drift tube temperature of 40 °C and a data rate 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 sample extraction was repeated twice for each batch per sample and the analytical measurement was conducted twice for each extract. The results for sucrose content were expressed as g/kg on dry matter basis.

2.6 Data analysis

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

3. Results and discussion

3.1 Characteristics of flours

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

3.3 Influence of legume flours on some quality characteristics of biscuits

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

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

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

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

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

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

4. Conclusions

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

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

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

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

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

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

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Abstract:

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

Keywords:

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

Chemical compounds studied in this article:

Acrylamide (PubChem CID: 6579); Acrylamide-d_3 (PubChem CID: 12209671); Asparagine (PubChem CID: 6267); Sucrose (PubChem CID: 5988).

1. Introduction

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

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

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

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

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

2. Materials and methods

2.1. Biscuit ingredients and chemicals

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

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

2.2 Preparation of biscuit samples

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

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

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

To ensure homogeneous distribution in the dough, asparagine, 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 control 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 for 20 min in a refrigerator at 4 °C. For some subsequent analyses, parts of the raw dough samples were freeze-dried and finely grounded with a mortar.

The raw 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, 8 biscuits were baked in an electrical oven (OV185C, Inventum, Arnhem, The Netherlands) with convection mode at 175 °C for 5, 7 and 9 min. The different baking conditions were chosen in preliminary tests to obtain biscuits that were neither undercooked nor overcooked. The biscuits were placed in the middle position of a baking tray inside the oven; for each baking cycle, the air temperature inside the oven chamber was recorded every 20 s using a digital thermometer equipped with type K thermocouples (Pro 206-3722, RS Components, Corby, UK) to ensure equal temperature exposure between the baking cycles. After baking, biscuits were removed from the oven, placed on a grid and kept cooling at room temperature for about 1 h.

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

2.3 Characterization of flours and biscuits

2.3.1 Proximal analysis

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

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

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

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

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

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

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

All analyses were performed in duplicate for each flour type.

2.3.2 Particle size

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

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

2.3.3 Hydration properties

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

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

2.3.4 pH

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

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

2.3.5 Moisture content

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

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

2.3.6 Water activity

The water activity (a_w) of flours, raw doughs and biscuits was determined at 25 °C with an a_w-meter (LabMaster, Novasina AG, Lachen, Switzerland) setting both time and temperature factors stability at 2 min.

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

2.3.7 Weight loss

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

2.3.8 Colour

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

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

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

2.3.9 Texture

The texture analysis of 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), determined by means of maximum force, fracturability (1/mm), expressed as one/breakpoint distance between the origin of curve till the point where the biscuit breaks, and crispness, evaluated by the linear distance between the first and the last peak registered (Romani et al., 2012).

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

2.4 Quantification of asparagine and acrylamide by LC-MS/MS

2.4.1 Sample extraction

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

For asparagine determination, the formic acid extract (5 mL) was centrifuged for 10 min at 20817 × g and 20 °C (5430 R, Eppendorf AG, Hamburg, Germany). For better clarification, 4 mL of supernatant were centrifuged for 7 min at 20817 × g and 20 °C, then 1 mL of clear supernatant was mixed with 1 mL of acetonitrile and filtered with 0.2 µm PTFE filters (Ø15 mm) into an amber glass autosampler vial. For AA determination, the formic acid extract (4.75 mL) with AA-d_3 solution (100 µL) were clarified with 0.125 mL of Carrez I and 0.125 mL of Carrez II. The mixture was vortexed and centrifuged for 3 min at 10621 × g and 20 °C. For better clarification, 2 mL of supernatant was collected and centrifuged for 10 min at 20817 × g and 20 °C. Solid phase extraction cleaning was carried out according to Mogol & Gökmen (2014) using the Oasis MCX cartridge and collecting the sample in an amber glass autosampler vial.

2.4.2 LC-MS/MS methods

Samples analyses were carried out with a Nexera UPLC system (Shimadzu Corporation, Kyoto, Japan) 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-20AH_2 valve unit.

The chromatographic separation of free asparagine was performed injecting 5 µL of samples on a SeQuant® ZIC HILIC 3.5 µm, 4.6 × 150 mm (Merck KGaS, 64271, Darmstadt, Germany) attached to a SeQuant® ZIC HILIC PEEK coated guard column 20 × 2.1 mm (Merck KGaS, 64271, 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) 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.

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

Positive ionisation mode was used for both MS analyses. The voltage of the turbo ion-spray ionization was 4.0 kV. The temperature of the electrospray ionization probe, desolvation line and heat block was 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 msec, respectively for asparagine and AA, and the most abundant fragment ion was selected for quantitation. The second and third fragments in ion yield were selected as a structural confirmation based on the optimized SRM transition. The precursor/product ion transitions m/z 133.20→74.00, 133.20→87.05 and 133.20→28.15 were monitored for asparagine; 72.00→55.10, 72.00→27.10 and 72.00→44.00 were monitored for acrylamide; 75.25→58.05, 75.25→30.05 and 75.25→44.05 were monitored for acrylamide-d_3. Data

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

The sample extraction was repeated twice for each flour and each batch per sample and the analytical measurements were replicated twice for each extract. The results were expressed as μg/kg for AA and mg/kg for asparagine on dry matter basis.

2.5 Glucose, fructose and sucrose contents

2.5.1 Sample extraction

Raw doughs and biscuits were analysed for glucose, fructose and sucrose contents. The sample extraction process was based on Nguyen et al. (2016) with slight modifications. Grounded biscuit (2.5 g) or freeze-dried dough (2.5 g) was mixed with 1:1, v/v of Milli-Q water and ethanol mixture (25 mL). The samples were incubated for 1 h at 50 °C in a water bath and cooled down for 20 min at room temperature. Then the samples were centrifuged at 1962 × g and 20 °C for 10 min (Heraeus Multifuge X3R, Thermo Fisher Scientific, Waltham, MA, USA). The supernatant (1.5 mL) was centrifuged at 20817 × g and 20 °C (5430 R, Eppendorf AG, Hamburg, Germany) for 10 min and 1 mL was collected into a glass tube. The water/ethanol solvent was evaporated with a sample concentrator (SBHCONC/1, Stuart, Staffordshire, UK) under nitrogen flush at 50 °C for 4.5 h. The sample was reconstituted with acetonitrile (20 mL) and Milli-Q water (20 mL) and stored in a freezer at −20 °C until measurement (maximum 1 week). Before analysis 1.5 mL of sample was passed through CA (Ø28 mm; 0.2 μm) filters and transferred into an autosampler vial.

2.5.2 UPLC-ELSD method

The samples were analysed according to the procedure provided by Waters' technical application notebook with an Acquity UPLC-H Class Plus System (Waters, Milford, MA, USA) equipped with an Acquity Evaporative Light Scattering (ELSD) detector, an Acquity UPLC BEH Amide column (1.7 μm, 2.1 × 100 mm) and 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) while mobile phase B consisted of acetonitrile/water 3:7, v/v with 0.2% TEA. The flow rate was 0.25 mL/min. 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. The pressure of ELSD conditions was 40 psi with

a drift tube temperature of 40 °C and a data rate 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 sample extraction was repeated twice for each batch per sample and the analytical measurement was conducted twice for each extract. The results for sucrose content were expressed as g/kg on dry matter basis.

2.6 Data analysis

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

3. Results and discussion

3.1 Characteristics of flours

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

3.3 Influence of legume flours on some quality characteristics of biscuits

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

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

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

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

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

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

4. Conclusions

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

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

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

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

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

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

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

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

Table 2_UNmarked

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

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

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

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

Table 2_Marked

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

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

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

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

Table 3

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

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

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

Table 4

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

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

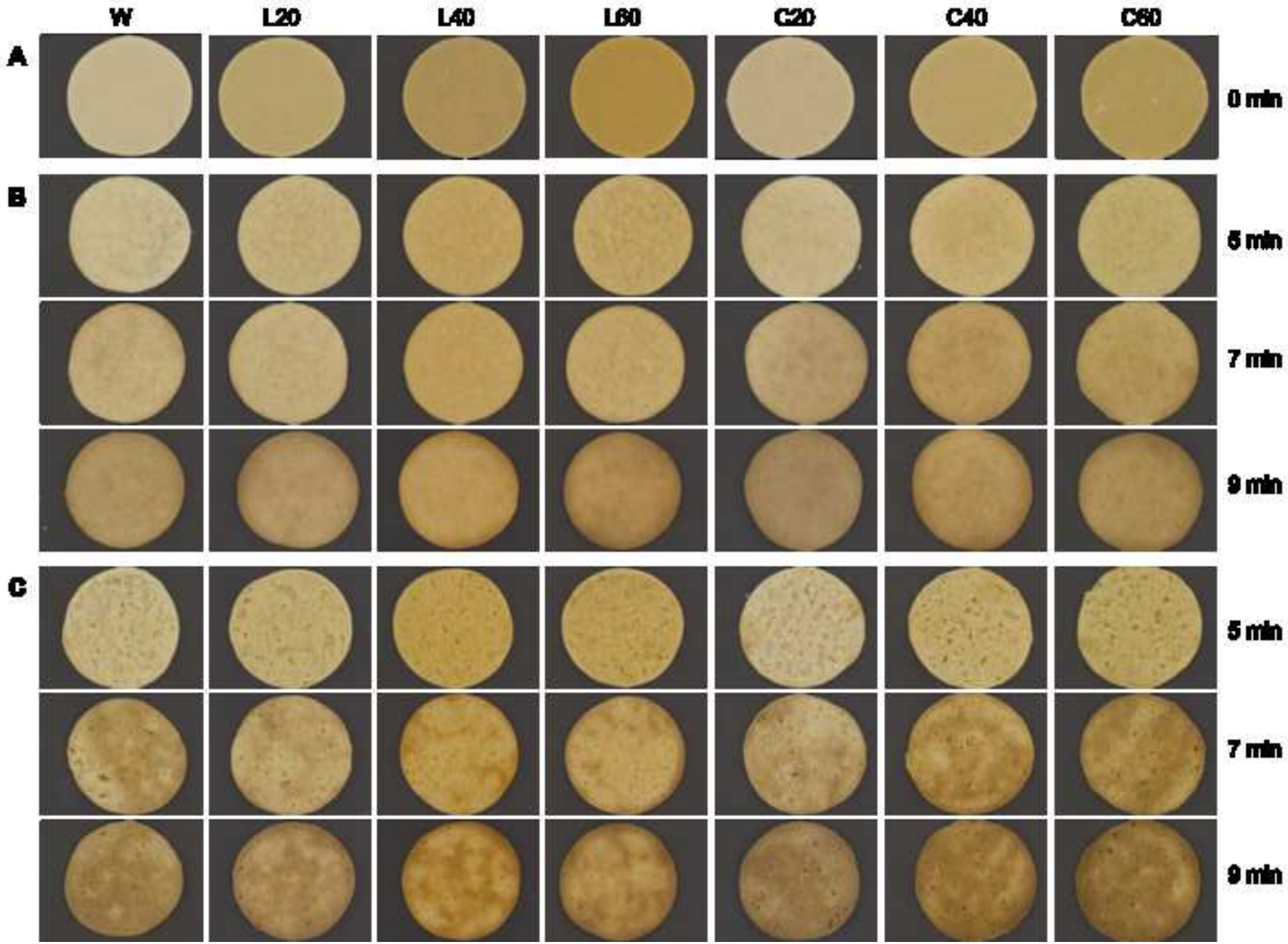
Hardness (N)							
0	-	-	-	-	-	-	-
5	$6.5 \pm 1.0^{c, C}$	$6.9 \pm 1.3^{c, C}$	$9.5 \pm 1.5^{b, B}$	$13.0 \pm 1.7^{c, A}$	$6.3 \pm 1.2^{c, C}$	$5.6 \pm 0.8^{c, D}$	$4.1 \pm 0.6^{c, E}$
7	$21.7 \pm 3.8^{b, D}$	$24.4 \pm 3.4^{b, C}$	$27.1 \pm 3.4^{a, B}$	$33.6 \pm 2.0^{b, A}$	$21.7 \pm 2.0^{b, D}$	$21.4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

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

** Lower surface of the biscuits.

Figure 1

Click here to access/download;Figure(s);Figure 1.png



Graphical Abstract

AIM

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

BISCUIT SAMPLES

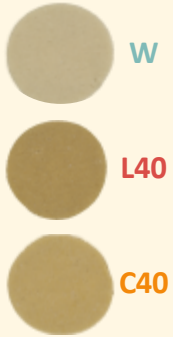
Formulations:

W (control)
100%
Wheat flour

vs

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

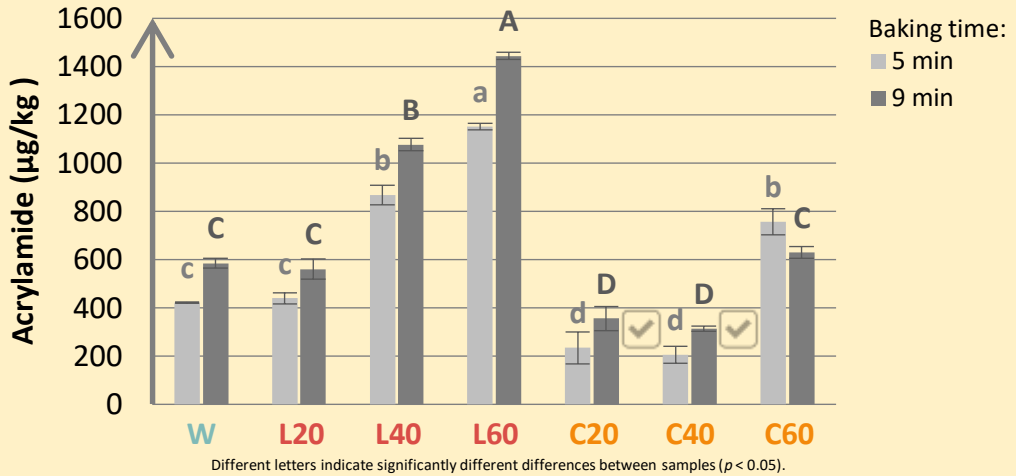
C20, C40, C60
20%, 40%, 60%
Chickpea flour



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

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

ACRYLAMIDE RESULTS



Different letters indicate significantly different differences between samples ($p < 0.05$).

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

Declaration of interests

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

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

Author statement

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

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

Silvia Tappi: Writing - Review & Editing

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

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