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Strategies to mitigate acrylamide development in bakery products: Effect of asparagine content in flour and tartaric acid addition in biscuit formulation

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ABSTRACT

This paper explores strategies to mitigate acrylamide formation in bakery goods, with a focus on using tartaric acid as an acid neutralizer in biscuit recipes. Initial analyses involved screening the asparagine content in various flours, aiming to encompass a wide range of samples, including those that presented substantial challenges. Subsequently, experimental biscuits were produced with various treatments (tartaric acid addition, steam release during baking, and both steam/acid). Results showed a correlation between asparagine content in flours and acrylamide formation. Tartaric acid was remarkably effective, achieving a 57%–87% reduction in acrylamide production. Colorimetric measurements assessment demonstrated that tartaric acid treatment reduced browning, while the a_w (<0.15) was not affected even using steam during cooking. Sensory analysis indicated slight sour taste in acid-treated biscuits, but overall acceptability remained high. Texture analysis revealed improved friability, crucial for preventing oral mucosal lesion, especially in children's products. PCA highlighted the impact of treatments on variables like acrylamide content, color, water activity, and sensory attributes. The study suggests that using tartaric acid as a neutralizing agent is a solid strategy to mitigate acrylamide without compromising sensory qualities in biscuits. Further research will include exploring other acids, optimal dosage, and variations in flour composition.

1. Introduction

Acrylamide, recognized as a genotoxic molecule and a probable human carcinogen by the International Agency for Research on Cancer (IARC) (WHO, 1984), is formed mainly by the reaction between reducing sugars and L-asparagine (ASN), an amino acid present in a variety of raw food, including potato-derived products (like French fries and potato chips), bread, cereals, coffee, and certain types of snacks (Mottram et al., 2002; Stadler et al., 2002).

The formation of acrylamide occurs through a branch of the Maillard reaction, which is responsible for the browning and flavor development in cooked food. It is well-known that higher cooking temperatures and longer cooking times tend to increase the levels of acrylamide in food (De Paola et al., 2017).

Dietary exposure to acrylamide affects various age groups, from infants to the elderly (Koszucka et al., 2020). The European Food Safety Authority (EFSA) has not yet established a tolerable daily intake due to the potential cancer risk from genotoxic substances, but, conversely, dose range likely to cause minor tumors or other adverse effects like neurological and reproductive disorders has been determined. The lower limit of this range, called "benchmark dose lower confidence limit" (BMDL10), is 0.17 mg/kg bw/day for tumors and 0.43 mg/kg bw/day for other effects (EFSA, 2015). Precisely to avoid exceeding these limit values, the Eur. Reg n. 2158/2017 has been implemented to establish benchmarks of acrylamide content in selected food categories (European Commission Regulation).

To mitigate acrylamide formation and minimize the contaminant's impact on the population, numerous approaches have already been investigated (Gunduz, 2023). These methods encompass exploration of raw materials with naturally low levels of ASN; use of enzymes capable of neutralizing ASN, specifically L-asparaginase (Yaylayan & Stadler, 2005); reduction of baking temperatures and times; introduction of steam during oven baking; partial or complete substitution of Maillard reaction-promoting leavening agents such as ammonium bicarbonate

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with sodium bicarbonate (Lo Faro et al., 2022). Even though the use of L-asparaginase is a highly targeted strategy with little or no effect on the physical and sensory attributes of baked products, this enzyme is now obtained from genetically modified organisms like *Aspergillus niger* (da Cunha et al., 2019), thus rendering these products ineligible for "organic" certification. Moreover, the use of a "new" protein in food could potentially cause intolerance or allergenic phenomena in sensitive individuals.

One strategy worth exploring to reduce acrylamide formation involves carefully adjusting leavening agents, like alkaline bicarbonate salts combination with acidic neutralizing agents to favor CO_2 release. At the same time, acids partially protonate the amino group of amino acids, including ASN, effectively preventing the Maillard reaction initiation and, in turn, the acrylamide formation.

Graf et al. (2006) introduced tartaric acid into the dough of semi-finished biscuits at concentrations of 244 g/100 kg of dough and 293 g/100 kg of dough, leading to a reduction of acrylamide content by 33% and 44%, respectively. The incorporation of ferulic acid and ascorbic acid into cracker dough (Levine & Smith, 2005) generated a 50% decrease in acrylamide levels, while the inclusion of ascorbic acid showed a more significant reduction of 70%.

A recent study demonstrated that pre-soaking sweet potatoes and carrots in acetic acid before frying led to a remarkable decrease in acrylamide formation up to 99%, while the addition of organic acids like citric acid at a concentration of 0.2% resulted in substantial reductions of 82.2% and 72.8% in acrylamide levels for fried and baked corn chips, respectively (Nguyen et al., 2022).

Building upon the findings from our earlier research (Lo Faro et al., 2022), this study first focused on the concentration of the initial ASN content in biscuit flours. Hence, this research work aimed to investigate the impact of incorporating tartaric acid into biscuit recipes, alongside previously studied methodologies such as lowering baking temperature and steam release during the baking process, on the formation of acrylamide.

Chemical and physical assessments of the experimental biscuit samples were carried out to evaluate the acid neutralizer's effects on consumer-preferred characteristics. These evaluations encompassed color analysis in the CIELab color space, water activity measurement, texture analysis via a test of softening to breaking point of the biscuits by immersing them in hot water, and sensory acceptability assessment.

2. Materials and methods

2.1. Materials

All solvents and reagents used in this study were of analytical grade. Acrylamide, with a purity of 99%, was employed as the external standard, while acrylamide-2,3,3-d3 (acrylamide d3), with a purity of 99%, served as the internal standard. Both acrylamide and acrylamide d3 were purchased from Sigma-Aldrich Merck KGaA (Milan, Italy). Acetic acid, acetonitrile (Chromasolv® Plus purity for LC-MS), L-asparagine, cycloleucine, diethyl ethoxymethylenemalonate (DEEM), methanol, sodium hydroxide, and tartaric acid were also purchased from Sigma-Aldrich Merck KGaA (Milan, Italy).

QuEChERS pouches containing 4.0 g of $MgSO_4$ and 0.5 g of NaCl were purchased from Agilent Technologies Italia S.p.A. (Milan, Italy). Deionized water used throughout the experiment was generated using an Elix 3^{UV} purification system manufactured by Millipore Merck KgaA (Milan, Italy).

Flour samples were provided by Industria Molitoria Denti (Borzano di Albinea, Reggio Emilia, Italy).

- soft wheat flour (Italian type '00', W180) (hereafter referred to as N);
- soft wheat flour (Italian type '1', W210) added with toasted wheat germ (hereafter referred to as 210);
- soft wheat flour (Italian type '2', W230) added with toasted wheat germ (hereafter referred to as 230).
- soft wheat flour (Italian type '0', W350) added with bran and toasted wheat germ (hereafter referred to as P).

2.2. Determination of asparagine in raw materials

The extraction from each sample was made up follow the method described by Benedito de Barber et al. (1989). The determination of ASN was conducted using an HPLC system (PU4180, Jasco Europe Srl, Cremella, Italy) after derivatization with DEEM, as described by Gómez-Alonso et al. (2007).

A 500-μL aliquot of the derivatized sample was transferred to chromatography vials, and 10 μL of each sample was injected through an autosampler into an RP-C_{18} column (Poroshell 120 SB-C_{18}, Agilent Technologies, D.T.O. Servizi Srl, Spinea, Italy). The flow rate was set at 0.6 mL/min with an initial pressure of approximately 22 MPa. The solvent system was made up follow the method described by Montevecchi et al. (2021). Each sample was extracted and analyzed in triplicate.

ASN quantification was performed using the external standard method in the presence of internal standard through a calibration curve built with 7 points. Each point consisted of a constant concentration of the internal standard (cycloleucine) at 30 mg/L and increasing concentrations of pure ASN standard at 10, 20, 30, 35, 40, 60, and 80 mg/L.

2.3. Biscuit formulation

The biscuits were prepared according to the recipe provided by the American Association of Cereal Chemists (AACC International, 2010) with slight modifications. The recipe was modified by removing high-fructose-content corn syrup. This adjustment aimed to eliminate reducing sugars, known contributors to acrylamide formation. In place of corn syrup, sucrose was introduced as a substitute.

An amount of 0.536 g of tartaric acid was added to stoichiometrically neutralize the 0.600 g of sodium bicarbonate in the recipe.

The biscuits were prepared using the creaming method by means of a kneading machine equipped with a leaf-shaped beater (PastaMatic Gourmet 1950 Edition, De Longhi Appliances Srl, Campi Bisenzio, Italy), according to the procedure described in a previous work (Lo Faro et al., 2022). Once the dough was prepared, the biscuits were round-shaped (thickness 2 mm; diameter 50 mm) using a steel biscuit cutter.

The samples were baked in a fan-assisted electric oven (EKF 616 E UD, Tecnoeka S.r.l., Borgoricco, Italy). Each batch consisted of 16 biscuits arranged in the same pattern on a perforated steel pan. The pan was positioned on the third shelf (out of six) from the top in the oven chamber. Two different baking programs (BP) were used, as described by Lo Faro et al. (2022).

BP1: 150 °C for 6 min, then 140 °C for 4 min – used to bake the control samples (C) and the sample treated with tartaric acid (A);
BP2: 150 °C for 3 min, 150 °C and 40% RH for 3 min, 140 °C for 4 min – used to bake samples with the use of steam, both with tartaric acid (SA) and without tartaric acid (S).

For each type of flour, four different biscuit samples were produced, as follow.

- control biscuit (C): BP1, prepared using the modified AACC 10–54 method;
- acid biscuit (A): BP1;
- steam/acid biscuit (SA): BP2;
- steam biscuit (S): BP2.

After baking, the biscuits were cooled at room temperature for 60 min. A portion of the biscuits was used for daily tests, while the remaining biscuits were placed in oriented polypropylene bags, sealed using a sealing bar (FoodSaver, model IFS001X, Jarden Consumer Solutions JCS Europe Ltd, Cheadle, UK), and stored for further analysis.

2.4. Color measurement

Color analysis was performed using a colorimeter (Konica Minolta, Spectrophotometer CM-700d/600d, Milan, Italy) in reflectance mode, covering the visible spectrum from 380 to 770 nm. D65 illuminant and the 10° standard observer (McLaren, 1976) were employed. Measurements were taken on the central biscuit section, assumed to have uniform color. CIELab parameters (L* for brightness, a* for the green-red axis, and b* for the yellow-blue axis) were recorded.

Derived measures included hue (h°), saturation (chroma or C*), and browning index (BI) were calculated as follows:

$$h^{\circ} = \left(\frac{\left(\tan^{-1}\frac{b*}{a*}\right)}{2\pi}\right) \times 360 \quad [1]$$

$$C* = \sqrt{(a*)^2 + (b*)^2} \quad [2]$$

$$BI = \frac{[(X - 0.31) \times 100]}{0.17}, \quad [3]$$

$$\text{where } X = \frac{a* + 1.79 \times L*}{5.645 \times L* + a* - 3.012 \times b*}$$

Color distance (ΔE) was determined following the method described in the literature (Hunt & Pointer, 2011) using the following formula:

$$\Delta E = [(L^*_1 - L^*_2)^2 + (a^*_1 - a^*_2)^2 + (b^*_1 - b^*_2)^2]^{½} \quad [4]$$

where 1 represents the control sample for each flour, and 2 represents each of the three biscuit types.

2.5. Determination of the water activity

Water activity (a_w) measurements were conducted for each batch of biscuits, taking the readings 60 min after the biscuits were removed from the oven to ensure they had completely cooled. The water activity meter used for these measurements was the AcquaLab Series 4 TE (Decagon Devices, Inc. Pullman, WA, USA).

2.6. Test of the softening to breaking point of the biscuits by immersing them in hot water (dipping test)

The test was conducted the day after the production by means of a dynamometer (Z1.0 UTM, ZwickRoell Srl, Ulm, Germany) to determine the number of immersion cycles (IC) required for the biscuits to break. The dynamometer was equipped with a 1 kN load cell and controlled using the testXpert II software. To secure the biscuit onto the load cell, a lab clothespin specifically adapted for the analysis was used. The test was performed with a cyclic load, the point of application of the load during each cycle had a holding time of 2 s, the cycle speed was set at 2000 mm/min, and the immersion of sample was performed for 2.5 cm in a beaker filled with water at 75 °C.

2.7. Extraction and determination of acrylamide

The acrylamide extraction and LC-MS-QQQ determination protocol utilized in this study follows the procedure described by Lo Faro et al. (2022). Quantification was performed employing the external calibration method in the presence of the internal standard (acrylamide d3). A series of increasing concentrations (0.02, 0.04, 0.06, 0.10, 0.20, 0.40, 0.60 μg/mL) of acrylamide in deionized water were prepared, maintaining a constant concentration of acrylamide d3 at 0.40 μg/mL throughout the calibration process.

2.8. Sensory analysis

A descriptive sensory acceptance study was conducted on all experimental biscuits, involving a panel of 12 untrained assessors aged 21–60 years. The sensory analysis comprised 16 biscuit samples, divided into two tasting sessions, each session containing 8 samples.

Assessors were instructed to evaluate four descriptors (color, i.e. intensity of browning; brittleness; crunchiness; sourness) of the biscuits quality on a hedonistic scale ranging from 0 to 5 (0 = absence of the attribute; 5 = pronounced presence of the attribute). In addition to these descriptors, assessors were asked to provide an overall acceptability rating for each biscuit sample.

2.9. Statistical analysis

The data are shown as average values of three replicates (± standard deviations). To analyze the effects among samples, a two-way analysis of variance (two-way ANOVA) using the factors "Type of flour" and "Recipe/Baking method combination" was conducted for acrylamide values.

As for ASN values, color measurements, water activity values, dipping test, and sensory analysis, a one-way ANOVA was used. When significant effects were observed (at least *p-value* $\leq$0.05), post-hoc multiple comparison tests were performed using post-hoc Tukey's method for pairwise comparisons. Prior to performing the ANOVA analysis, homoskedasticity and normal distribution of the data were verified.

In addition, a multivariate analysis, specifically principal component analysis (PCA), was performed on the data matrix. This test aimed to identify any latent variable (i.e. linear combinations of the variables) and assess the arrangement of the samples on a Cartesian plane, thus providing insights into their mutual relationships.

All statistical analyses were carried out using Statistica v8.0 software (previously developed by Stat Soft Inc., now TIBCO Software Inc., Palo Alto, USA).

3. Results and discussion

3.1. Determination of asparagine

During the initial phase of the study, the concentration of ASN was assessed in the four selected types of flour. In Table 1 the results of the ASN determination expressed as mg/kg_{flour} are shown.

The study included a range of flour types with variable concentrations of free ASN, from 80 to 270 mg/kg. The highest values were observed in semi-wholemeal flour "P" (W350), which contained a significant amount of bran and germ fractions, leading to higher levels of

Table 1
Asparagine (ASN) concentration (mg/kg_{flour}) average values (n = 3) ± standard deviation (sd) in the flour samples.

Type of flours	ASN (mg/kg_{flour})	sd (±)
N	80.75 c	8.47
210	207.95 b	23.08
230	225.87 b	2.00
P	272.11 a	9.58

The one-way ANOVA showed a *p-value* $\leq$0.001. Post-hoc Tukey's test (*p-value* $\leq$0.05) was employed. Results are reported using different letters that label average values that are significantly different (a > b > c).
N: soft wheat flour, Italian type '00', W180; 210: soft wheat flour, Italian type '1', W210, added with toasted wheat germ; 230: soft wheat flour, Italian type '2', W230, added with toasted wheat germ; P: soft wheat flour, Italian type '0', W350 added with bran and toasted wheat germ.

free ASN compared to more sifted flours. The sifting level played a crucial role in the variation of ASN content, in fact, among the flour types, N (W180), an Italian type '00' flour, had the lowest ASN content, while P sample, an Italian type '0' flour enriched with roasted wheat germ and bran, showed a significant increase in free ASN content (3.37 times higher than N). Samples named 210 and 230, Italian type '1' (W210) and type '2' (W230) flours respectively, both supplemented with roasted wheat germ, had intermediate values, closer to P flour.

Since correlation between the free ASN content in flours and acrylamide formation is well established (Mesias et al., 2022), considerable efforts are being made to study how the wheat genotype and crop management can affect the ASN concentration in seeds, and, naturally, in milled products (Kaur & Halford, 2023). In recent works (Tafuri et al., 2023), the free ASN content of wholemeal flours, obtained from 18 Italian wheat bread and biscuits grain genotypes over two years of investigation, has spanned from 0.55 to 2.84 mmol/kg dry matter (equivalent to 73–375 mg/kg dry matter, largely overlapping with the range considered in the present study). In an agronomic study (Oddy et al., 2023), two trials of 12 soft (biscuit) wheat varieties have been supplemented with different nitrogen, sulfur, potassium, and phosphorus fertilizer combinations, resulting in the possibility to modulate free ASN levels in the wholemeal flours ranging from 2.5 to 20 mmol/kg (equivalent to 330 and 2600 mg/kg, respectively).

3.2. Acrylamide content in biscuits

In Table 2, the average AA concentrations in experimental biscuits are shown. The control samples, specifically those with P flour and added roasted bran and roasted germ, demonstrated the highest acrylamide levels. This aligns with the observed highest free asparagine levels. Following this, samples 230, 210, and N exhibited progressively lower acrylamide concentrations.

As for experimental samples, the impact of different treatments (S, SA, and A) was evident, showing a significant reduction in AA concentrations. The introduction of steam (S samples) during the baking process led to a 39% decrease in N flour biscuits, approximately 35% in 210 flour, 48% in 230 flour, and 42% in P flour compared to corresponding control biscuits.

These findings align with a previous work of ours (Lo Faro et al., 2022), where a combination of low baking temperature, steam, and reduced ammonium bicarbonate resulted in an 80% AA reduction. Table 2 highlights the effectiveness of tartaric acid (A samples), reducing AA by around 80% in N, 210, and 230 biscuits, while achieving a 57% reduction in P biscuits. The combination of steam baking and acid addition (SA samples) generally did not enhance tartaric acid's impact on AA production, except in P flour samples.

The application of organic acids approved as food additives effectively reduced acrylamide levels in experimental biscuits. This reduction aligns with findings from other studies using similar strategies. Graf et al. (2006) have found that adding 0.30% tartaric acid to 100 kg of dough resulted in acrylamide levels below 100 μg/kg. Similarly, the present study showed that incorporating 0.34% tartaric acid into 157.9 g of dough achieved final acrylamide concentrations well below 100 μg/kg.

While the biscuits in this study target adult consumers, all samples kept AA concentrations below current legal limits for infant products (150 μg/kg) and other food items (100 μg/kg). Notably, C samples with 230 and P flour, lacking steam and acid neutralizer, presented the highest acrylamide levels, thus representing a "worst-case scenario".

The results of the two-way ANOVA analysis, presented in Table 2, clearly indicate the significant impact of the "Recipe/Baking method" factor on the reduction of AA content, with the "Type of flour" factor exhibited a slightly lower effect.

After establishing the significance of these statistical factors, a Tukey's test was conducted to assess the significant differences among samples based on both "Type of flour" and "Recipe/Baking method" statistical factors. The test shows that samples from 210 to 230 flours did not significantly differ. This similarity arises from their nearly identical compositions, differing in the technological parameter W (flour strength). Conversely, samples from N flour and P flour were distinctly separated, both from each other and from the group of samples obtained with 210 and 230 flours. The "Type of flour" factor significantly influenced the distinction among these samples.

Regarding the "Recipe/Baking method" factor, the sample set is distinctly categorized into three groups: C samples, those baked with steam alone (S), and those prepared with the introduction of acid in the recipe, regardless of steam use during baking (A and SA). This fact emphasizes the crucial role of incorporating tartaric acid in the recipe for acrylamide reduction in biscuits. The "Recipe/Baking method" factor emerged as crucial in both the two-way ANOVA analysis and as a fundamental variable for distinguishing between different sample groups.

Fig. 1 illustrates the linear relationships between AA amounts in biscuits and ASN concentration in flours, considering the different treatments.

The coefficients of determination (R^2) of the straight lines of the C biscuits and those obtained through steaming (S samples) were remarkably high, at 0.995 and 0.946, respectively. By contrast, the lowest coefficients were observed in the last two series of biscuits (A and

Table 2
Acrylamide (AA) concentrations (μg/kg ± sd; mean n = 3) in experimental biscuits and percentage reduction.

Type of flour	Recipe/Baking method combination	AA (μg/kg)	sd (±)	AA reduction (%) in comparison with the C for each flour
N	C	38.21	3.52	–
	A	5.51	2.16	85
	SA	15.00	1.37	61
	S	23.29	2.34	39
210	C	89.55	14.84	–
	A	18.24	3.10	79
	SA	19.70	3.11	78
	S	57.94	15.13	35
230	C	100.06	26.15	–
	A	13.24	5.33	87
	SA	21.42	1.91	79
	S	51.66	5.00	48
P	C	112.71	21.55	–
	A	48.74	2.78	57
	SA	34.68	0.78	69
	S	65.19	7.53	42

Two-way ANOVA	*p-value*	Type of flour	Tukey's test	Recipe/Baking method	Tukey's test
Type of flour	***	N	c	C	a
Recipe/Baking method	***	210	b	A	c
Interaction (Type of flour × Recipe/Baking method)	**	230	b	SA	c
		P	a	S	b

Two-way ANOVA results using "Type of flour" and "Recipe/Baking method combination" as statistical factors were shown. The two-way ANOVA results were expressed as *p-value* (** *p-value* ≤0.01; *** *p-value* ≤0.001). Post-hoc Tukey's test (*p-value* ≤0.05) was employed for both statistical factors. Results are reported using different letters that label average values that are significantly different (a > b > c).
Type of flour. N: soft wheat flour, Italian type '00', W180; 210: soft wheat flour, Italian type '1', W210, added with toasted wheat germ; 230: soft wheat flour, Italian type '2', W230, added with toasted wheat germ; P: soft wheat flour, Italian type '0', W350 added with bran and toasted wheat germ.
Recipe/Baking method combination. C: control; A: tartaric acid added; SA: steam + tartaric acid added; S: steam.

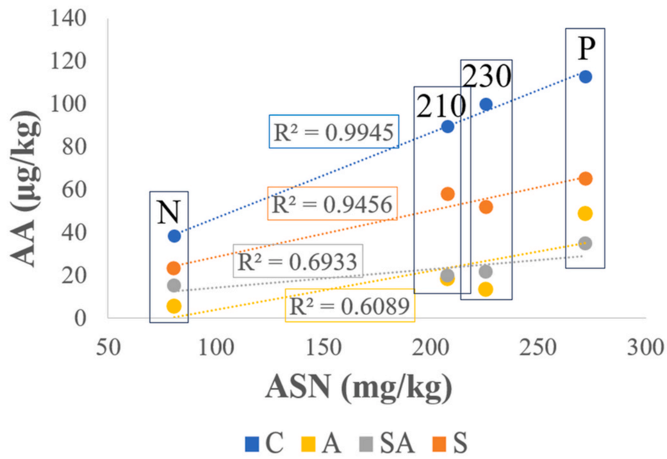

Fig. 1. Scatter plot depicting the linear correlations between acrylamide (AA) concentration (μg/$kg_{Biscuit}$) and asparagine (ASN) concentration (mg/kg_{Flour}). Evaluation of the coefficients of determination (R^2) for each treatment's linear correlation straight line.
Type of flour. N: soft wheat flour, Italian type '00', W180; 210: soft wheat flour, Italian type '1', W210, added with toasted wheat germ; 230: soft wheat flour, Italian type '2', W230, added with toasted wheat germ; P: soft wheat flour, Italian type '0', W350, added with bran and toasted wheat germ.
Recipe/Baking method combination. C: control; A: tartaric acid added; SA: steam + tartaric acid added; S: steam.

SA), produced by adding tartaric acid and steam during baking process. The latter coefficients were 0.6089 and 0.6933, respectively, and these lower values can be attributed to the very low levels of AA determined, which may exhibit higher variability compared to the other two series.

The abundance of steam during baking in SA samples likely lowered surficial temperature by slowing water evaporation. This contrasts with biscuits baked without steam, where rapid water loss allows the outer layers to quickly reach oven temperature, thus promoting the Maillard reaction.

These findings align with the results reported by Oddy et al. (2023), who observed a robust relationship between a broad range of AA levels (from 488 ppb to 13,523 ppb) and ASN levels (from 200 ppm to 2640 ppm). However, it is important to highlight that our study achieved a good correlation despite the very low values of both free ASN and AA.

These significant results allow to estimate, with reasonable approximation, the amount of AA that will be formed in biscuits based on the ASN content present in the flour. This estimation is particularly useful for baking under more severe conditions.

3.3. Colorimetric measurements

Fig. 2 displays the biscuits obtained from various combinations of flours and treatments. To obtain a more objective confirmation of the differences in the colors of the biscuits, the color distance ΔE was used (Fig. 3). Calculated to compare the colors of a pair of samples based on the measured values in the CIELab color space, ΔE serves to ensure that the displayed color closely corresponds to the color perceived by the human eye. Lower values of ΔE indicate higher color accuracy, while higher values of ΔE indicate a mismatch between colors. The difference in ΔE values between two samples provides valuable information for evaluating their color. A standard observer sees the difference in color as follows, according to Mokrzycki and Tatol (2011).

$0 < \Delta E < 1$ - observer does not notice any difference;
$1 < \Delta E < 2$ - only experienced observer can notice the difference;
$2 < \Delta E < 3.5$ - unexperienced observer also notices the difference;
$3.5 < \Delta E < 5$ - clear difference in color is noticed;
$5 < \Delta E$ - observer notices two different colors.

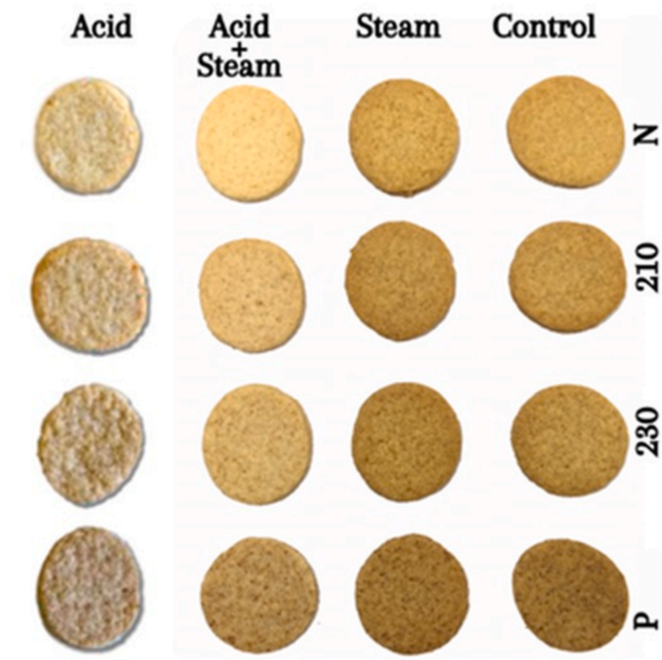

Fig. 2. Picture of complete experimental sample set.
Type of flour. N: soft wheat flour, Italian type '00', W180; 210: soft wheat flour, Italian type '1', W210, added with toasted wheat germ; 230: soft wheat flour, Italian type '2', W230, added with toasted wheat germ; P: soft wheat flour, Italian type '0', W350 added with bran and toasted wheat germ.

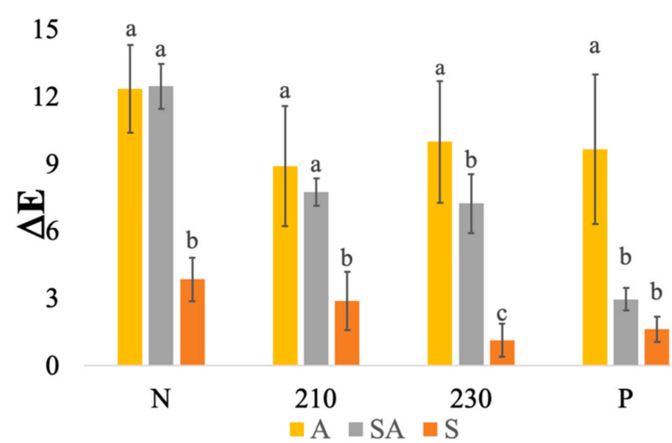

Fig. 3. Color distance (**ΔE**) average values in biscuits obtained for each treatment in comparison with the Control biscuit, within each type of flour. The one-way ANOVA showed a *p-value* ≤0.001. Post-hoc Tukey's test (*p-value* ≤0.05) was employed. Results are reported using different letters that label average values that are significantly different (a > b > c).
Type of flour. N: soft wheat flour, Italian type '00', W180; 210: soft wheat flour, Italian type '1', W210, added with toasted wheat germ; 230: soft wheat flour, Italian type '2', W230, added with toasted wheat germ; P: soft wheat flour, Italian type '0', W350 added with bran and toasted wheat germ.
Recipe/Baking method combination. A: tartaric acid added; S/A: steam + tartaric acid added; S: steam. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Fig. 3 demonstrates consistent ΔE values across all types of flour. The significantly highest ΔE value occurred in biscuits treated with tartaric acid (A samples), followed by biscuits with the combined treatment of acid and steam introduction (SA samples), and then by biscuits baked with steam (S samples).

Table 3 shows the average values (with standard deviation) of the L*,

Table 3
Results of the measurements of CIELab color parameters (L*, a*, and b*) and derived parameters, expressed as average values ± standard deviations (n = 3).

Type of flour	Recipe/Baking method combination	L*	a*	b*	h* (°)	C*	BI
N	C	60.48 ± 1.42 b	3.45 ± 0.61 b	22.59 ± 0.68 a	81.37 ± 1.51 c	22.86 ± 0.69 a	55.06 ± 2.33 b
	A	71.86 ± 2.28 a	−0.46 ± 0.34 c	22.52 ± 2.54 a	91.2 ± 1.05 b	22.53 ± 2.54 a	41.12 ± 4.26 c
	SA	71.19 ± 1.00 a	−1.11 ± 0.21 d	18.18 ± 0.56 b	93.47 ± 0.72 a	18.22 ± 0.55 b	32.43 ± 1.47 d
	S	56.98 ± 1.01 c	4.71 ± 0.62 a	22.29 ± 0.66 a	78.14 ± 1.29 d	22.78 ± 0.75 a	59.81 ± 2.83 a
	p-value	***	***	***	***	***	***
210	C	61.06 ± 1.17 c	3.03 ± 0.60 b	21.36 ± 0.39 a	81.98 ± 1.64 b	21.58 ± 0.54 a	49.56 ± 1.78 b
	A	68.44 ± 3.53 a	1.61 ± 0.46 c	21.17 ± 2.35 a	85.71 ± 1.10 a	21.24 ± 2.35 a	41.61 ± 4.00 c
	SA	66.47 ± 0.79 b	1.36 ± 0.20 c	18.21 ± 0.60 b	85.76 ± 1.13 a	18.27 ± 0.58 b	36.48 ± 1.26 d
	S	58.42 ± 1.79 d	3.63 ± 0.70 a	21.31 ± 0.56 a	80.36 ± 1.89 c	21.63 ± 0.55 a	52.822.05 a
	p-value	***	***	***	***	***	***
230	C	57.92 ± 2.04 c	3.73 ± 0.50 a	20.51 ± 0.46 b	79.72 ± 1.28 c	20.85 ± 0.69 a	51.35 ± 1.61 a
	A	66.9 ± 4.73 a	1.5 ± 0.32 c	21.69 ± 2.97 a	86 ± 0.99 a	21.74 ± 2.97 a	43.61 ± 4.30 b
	SA	64.47 ± 2.62 b	1.97 ± 0.23 b	18.11 ± 0.37 c	83.83 ± 1.05 b	18.22 ± 1.59 b	38.2 ± 1.02 c
	S	57.58 ± 1.75 c	3.5 ± 0.46 a	20.47 ± 0.30 b	80.33 ± 1.30 c	20.77 ± 0.32 a	51.3 ± 1.54 a
	p-value	***	***	***	***	***	***
P	C	54.64 ± 3.11 c	3.91 ± 0.58 b	18.31 ± 0.64 b	77.98 ± 1.83 b	18.73 ± 0.63 b	49.11 ± 2.59 a
	A	63.51 ± 3.25 a	3.09 ± 0.52 c	20.63 ± 2.99 a	81.46 ± 1.39 a	20.87 ± 3.00 a	45.77 ± 5.78 b
	SA	56.46 ± 1.06 b	3.64 ± 0.37 b	16.22 ± 0.72 c	77.37 ± 1.46 bc	16.62 ± 0.48 c	41.63 ± 1.11 c
	S	53.59 ± 1.05 c	4.31 ± 0.46 a	18.23 ± 0.62 b	76.74 ± 1.33 c	18.74 ± 0.61 b	50.49 ± 2.30 a
	p-value	***	***	***	***	***	***

The one-way ANOVA results were expressed as *p-value* (*** *p-value* ≤0.001). Post-hoc Tukey's test (*p-value* ≤0.05) was employed. Results are reported using different letters that label average values that are significantly different (a > b > c > d).
Type of flour. N: soft wheat flour, Italian type '00', W180; 210: soft wheat flour, Italian type '1', W210, added with toasted wheat germ; 230: soft wheat flour, Italian type '2', W230, added with toasted wheat germ; P: soft wheat flour, Italian type '0', W350 added with bran and toasted wheat germ.
Recipe/Baking method combination. C: control; A: tartaric acid added; SA: steam + tartaric acid added; S: steam.

a*, and b* parameters, along with the derived parameters. Combining the findings with the L*, a*, b* values, it was evident that treatments A and SA resulted in less colored and brighter biscuits. This significant effect can be due to the incorporation of the acid neutralizer, which helps mitigate the extent of the non-enzymatic browning reaction in the final products (Sarion et al., 2021). Indeed, the presence of acid inhibits the nucleophilic addition of ASN to reducing sugars and other carbonyl intermediates, thereby preventing the formation of key compounds in the Maillard reaction and, consequently, the formation of melanoidins (Huang et al., 2022).

The average ΔE value calculated for biscuits prepared with N flour and tartaric acid addition was as high as 12.35. When considering the ΔE values of biscuits made with all other flours (which contain higher levels of bran) and the addition of organic acid, the ΔE obtained scores were quite high (ranging from 8.90 to 9.99), although significantly lower than the value obtained for biscuits made with N flour. This indicates that although the color of control biscuits is somewhat affected by the presence of bran, treating them with acid does not alter the color to the same degree when using a finer flour. The presence of proteins, particularly beneath the bran in the aleurone layer, may account for increased Maillard reaction activity in whole-meal samples.

The introduction of steam during the baking process resulted in ΔE scores always below 4. This indicates that steam baking alone could not significantly alter the appearance of the biscuits.

Analyzing the hue angle data of the CIELab color space, it is evident that most biscuits, except for both samples prepared with N flour and tartaric acid addition, had h° angles ranging from 0° to 90° (set in the first quadrant of h°), indicating a red/yellow coloration. By contrast, samples prepared with N flour and tartaric acid addition (A and SA) showed h° values of 93.47 and 91.20, respectively, thus falling in the upper left quadrant (second quadrant of h°), which represents the yellow/green area. This implies a reduced level of surface browning, which aligns with the changes observed in the L*, a*, and b* values, as well as the calculated ΔE for samples A and SA.

The browning index (BI) values decreased significantly in biscuits made with all flours when tartaric acid was added regardless of the introduction of steam into the oven during baking (A and SA samples). There is substantial experimental evidence supporting a strong correlation between the intensity of surface browning and the concentration of AA in various food products. For instance, it has been confirmed that the BI of biscuits can serve as a screening tool, where a high browning index is indicative of a high AA content (Verma & Yadav, 2022; de Borba et al., 2023; Gunduz, 2023).

Fig. 4 shows the relationship between BI and AA concentration values for all the experimental biscuits. However, it is not possible to find a significant correlation between BI values and AA concentration. In particular, the notable anomalous behavior of N_C and N_S samples (Fig. 4), characterized by low AA concentrations and high BI values, deserves further investigation.

Taking into account multiple variables such as various raw materials (flours), the addition of acid, and the utilization of steam in the baking process, it is highly probable that this combination of parameters could

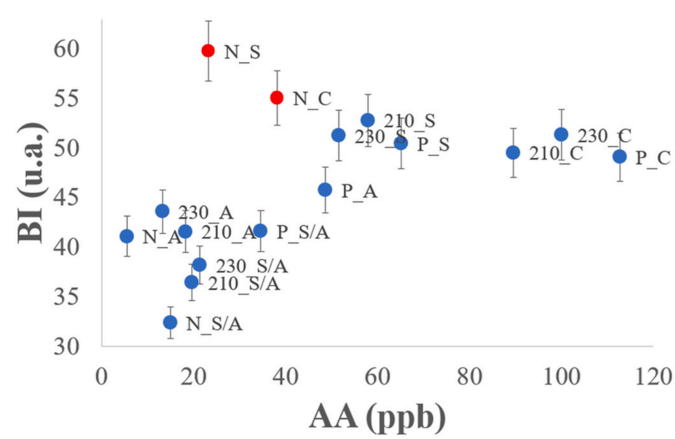

Fig. 4. Scatter plot of the acrylamide (AA) concentrations (μg/$kg_{Biscuit}$) vs browning index (BI) values.
Type of flour. N: soft wheat flour, Italian type '00', W180; 210: soft wheat flour, Italian type '1', W210, added with toasted wheat germ; 230: soft wheat flour, Italian type '2', W230, added with toasted wheat germ; P: soft wheat flour, Italian type '0', W350 added with bran and toasted wheat germ.
Recipe/Baking method combination. C: control; A: tartaric acid added; SA: steam + tartaric acid added; S: steam.

have influenced the results depicted in Fig. 4. Therefore, the viability of using the browning index (BI) as an indicator of AA content in biscuits warrants reconsideration.

3.4. Determination of a_w

Table 4 shows the water activity (a_w) measurements for each type of experimental biscuit. Generally, the a_w values can be classified into two distinct levels due to the two different baking methods employed. One level corresponds to the samples baked without the use of steam, referred to as the control group (C) and the experimental samples prepared with tartaric acid (A). The other level includes samples that were baked with the introduction of steam, both with (SA) and without (S) the addition of acid neutralizer.

The a_w values of the steam-baked biscuits (SA and S) were significantly higher compared to C and A samples. However, it is important to note that these values still remained comfortably below critical thresholds that could potentially compromise sample stability. This is significant in the context of reducing AA levels, with reductions ranging from 35% to 48%, as demonstrated in earlier research by Lo Faro et al. (2022).

3.5. Results of the dipping test

A method based on the dynamometer was utilized to determine the number of immersion cycles (IC) needed for breaking the samples (Fig. 5). IC was related to rheological properties following biscuit dipping and, in turn, to assess the ease of chewing also in relation to the fragile mucous membrane of children. Regarding the type of flour, P exhibited the highest resistance to breakage, particularly in the absence of acid. On the other hand, 210 and 230, which are flours less refined than P and N, showed the most favorable values for breakage (Fig. 5).

The one-way ANOVA applied to the IC data set established a significant difference (*p-value* $\leq$0.001) within each type of flour. In general,

Table 4
Results of measurements of water activity (a_w) expressed as average values ± standard deviations (n = 3).

Type of flour	Recipe/Baking method combination	a_w
N	**C**	0.12 ± 0.02 b
	A	0.11 ± 0.03 b
	SA	0.22 ± 0.01 a
	S	0.23 ± 0.02 a
	p-value	***
210	**C**	0.13 ± 0.04 b
	A	0.13 ± 0.02 b
	SA	0.23 ± 0.03 a
	S	0.20 ± 0.03 ab
	p-value	**
230	**C**	0.13 ± 0.01 b
	A	0.13 ± 0.01 b
	SA	0.19 ± 0.03 a
	S	0.22 ± 0.01 a
	p-value	***
P	**C**	0.11 ± 0.02 b
	A	0.15 ± 0.03 b
	SA	0.21 ± 0.02 a
	S	0.21 ± 0.01 a
	p-value	***

The one-way ANOVA results were expressed as *p-value* (** *p-value* $\leq$0.01; *** *p-value* $\leq$0.001). Post-hoc Tukey's test (*p-value* $\leq$0.05) was employed. Results are reported using different letters that label average values that are significantly different (a > b).
Type of flour. N: soft wheat flour, Italian type '00', W180; 210: soft wheat flour, Italian type '1', W210, added with toasted wheat germ; 230: soft wheat flour, Italian type '2', W230, added with toasted wheat germ; P: soft wheat flour, Italian type '0', W350 added with bran and toasted wheat germ.
Recipe/Baking method combination. C: control; A: tartaric acid added; SA: steam + tartaric acid added; S: steam.

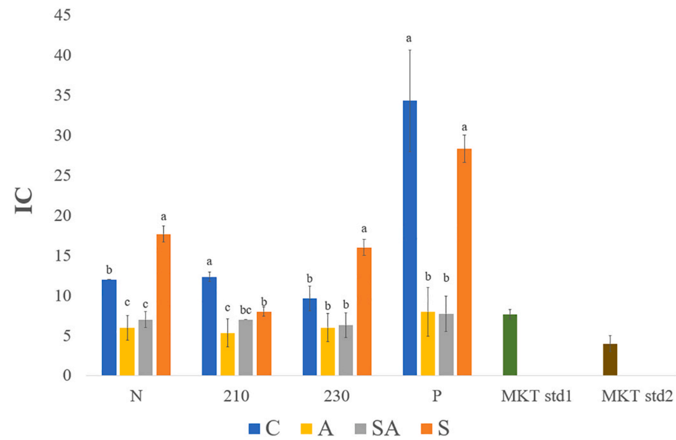

Fig. 5. Average and standard deviation of number of immersion cycles (IC) for all the experimental samples and two standard biscuits collected from the local market.
The one-way ANOVA showed a *p-value* $\leq$0.001. Post-hoc Tukey's test (*p-value* $\leq$0.05) was employed. Results are reported using different letters that label average values that are significantly different (a > b > c).
Type of flour. N: soft wheat flour, Italian type '00', W180; 210: soft wheat flour, Italian type '1', W210, added with toasted wheat germ; 230: soft wheat flour, Italian type '2', W230, added with toasted wheat germ; P: soft wheat flour, Italian type '0', W350, added with bran and toasted wheat germ.
Recipe/Baking method combination. C: control; A: tartaric acid added; SA: steam + tartaric acid added; S: steam.

samples that contained the acid neutralizer in the recipe required a lower number of IC before reaching breakage compared to samples that did not contain it. In addition, the values found in biscuits with acid were comparable with those obtained by testing products on the market (Fig. 5).

Sodium bicarbonate coupled with a neutralizing acid releases CO_2 more easily and contributes more effectively to the formation of a less firm structure with positive effects on the friability and the ability of water to quickly soak the biscuit matrix. It is therefore reasonable that in a shorter time and with fewer immersion cycles, acid-containing biscuits will break. This particular property is highly appreciated, particularly for biscuits intended for children's consumption, where the ease of breaking into hot solutions is essential to prevent any risk of oral mucosal lesion.

3.6. Sensory analysis

The objective color evaluation of the biscuits indicated that, for certain samples (specifically, all biscuits baked with the introduction of steam), despite a significant reduction in AA content, there was no notable difference in color compared to the control biscuits. However, to validate this finding and primarily to ascertain whether the addition of tartaric acid resulted in a perceptible sour taste in the biscuits, it was essential to conduct a sensory evaluation.

Table 5 shows the average scores for the four selected quality descriptors and overall acceptability of biscuits. Notably, samples prepared with acid exhibited reduced perceived surface browning (color), aligning with observed CIELab color parameters. The comparable browning perception between C samples and those baked with steam is noteworthy, indicating their similarity despite a significant reduction in AA, as confirmed by Tukey's test (Table 5). Regardless of the type of flour, samples C and S were consistently identified by the letter 'a' (highest scores).

Acid perception notably increased in N samples (from 0.5 to 4.1), whereas this increase was more limited in other biscuits, particularly P samples. The similar content of butter and sugar suggests that the presence of toasted germ and bran in flour can mask the sour perception.

Table 5
Average scores and deviation standard of sensory analysis.

Type of flour	Recipe/Baking method combination	Color	Friability	Crunchiness	Acidic perception	Overall acceptability
N	**C**	3.27 ± 0.60 a	3.64 ± 0.73 a	3.41 ± 1.00 a	0.55 ± 0.80 c	3.63 ± 1.00 a
	A	2.18 ± 1.00 b	3.27 ± 0.88 a	3.27 ± 1.00 a	4.09 ± 0.70 a	2.18 ± 1.10 b
	SA	1.45 ± 1.10 c	3.32 ± 0.95 a	3.55 ± 0.90 a	2.32 ± 1.30 b	2.82 ± 1.10 ab
	S	3.93 ± 0.70 a	3.18 ± 1.05 a	3.23 ± 1.20 a	0.45 ± 0.80 c	3.55 ± 1.10 a
	p-value	***	*ns*	*ns*	***	***
210	**C**	3.6 ± 0.91 a	3.86 ± 1.13 a	3.18 ± 1.26 a	0.86 ± 0.83 bc	3.73 ± 1.12 a
	A	1.55 ± 0.91 b	2.55 ± 1.41 b	3.36 ± 1.26 a	2.09 ± 1.48 a	2.73 ± 1.32 a
	SA	1.6 ± 0.96 b	2.64 ± 1.14 b	3.36 ± 1.09 a	1.86 ± 1.64 ab	3.05 ± 1.43 a
	S	3.9 ± 0.90 a	3.40 ± 1.10 ab	3.50 ± 1.10 a	0.60 ± 1.00 c	3.00 ± 1.60 a
	p-value	***	**	*ns*	***	ns
230	**C**	3.5 ± 1.00 a	3.80 ± 1.10 a	3.80 ± 1.30 a	0.50 ± 1.10 b	3.00 ± 1.50 a
	A	1.4 ± 0.90 c	3.09 ± 1.27 a	3.27 ± 0.88 a	2.82 ± 1.50 a	1.82 ± 1.22 b
	SA	2.3 ± 0.80 b	3.20 ± 1.00 a	3.40 ± 1.00 a	1.50 ± 1.50 b	3.40 ± 1.10 a
	S	3.8 ± 1.00 a	3.30 ± 1.20 a	3.30 ± 1.30 a	0.80 ± 1.00 b	2.90 ± 1.50 a
	p-value	***	*ns*	*ns*	***	**
P	**C**	3.7 ± 0.80 a	3.70 ± 1.00 a	3.30 ± 1.00 ab	1.00 ± 1.20 b	3.60 ± 1.10 a
	A	1.91 ± 0.68 b	3.18 ± 1.14 a	4.00 ± 0.62 a	1.55 ± 0.80 ab	3.55 ± 1.10 a
	SA	2.3 ± 0.77 b	2.91 ± 1.06 a	3.41 ± 0.85 ab	2.14 ± 1.25 a	3.09 ± 1.06 a
	S	3.91 ± 1.06 a	3.32 ± 1.09 a	3.09 ± 1.02 b	1.14 ± 1.36 b	2.91 ± 1.06 a
	p-value	***	*ns*	**	**	*ns*

The one-way ANOVA results were expressed as *p-value* (** *p-value* ≤0.01; *** *p-value* ≤0.001). ns: not significant. Post-hoc Tukey's test (*p-value* ≤0.05) was employed. Results are reported using different letters that label average values that are significantly different (a > b > c).
Type of flour. N: soft wheat flour, Italian type '00', W180; 210: soft wheat flour, Italian type '1', W210, added with toasted wheat germ; 230: soft wheat flour, Italian type '2', W230, added with toasted wheat germ; P: soft wheat flour, Italian type '0', W350 added with bran and toasted wheat germ.
Recipe/Baking method combination. *C:* control*; A: tartaric acid* added*; SA: steam + tartaric acid* added*; S: steam.*

The texture parameters (friability and crunchiness) appeared unaffected by the applied treatments, maintaining consistently high values. One-way ANOVA confirmed no significant differences for the two texture parameters considered. Finally, the overall acceptability was always >3.0 for all biscuits, with the exception of N with and without acid and 230 with acid, which were scored as less colored and with a clear acidic perception.

3.7. Principal component analysis (PCA)

The entire dataset was subjected to PCA, including variables such as acrylamide content (AA), L*, a*, b*, browning index (BI), h°, C°, a_w, softening to breaking (StB; dipping test). The first three principal components (PC) collectively accounted for 90.01% of the total variance.

Fig. 6a shows the loading plot of PC1 (49.16% of the total variance) vs PC2 (29.05% of the total variance). PC1 was characterized by the concentration of AA, a*, BI, and StB with a negative sign along this PC, while L* and h° weighed on PC1 with a positive sign. PC2, on the other hand, was characterized by b* and C° with a positive sign, while a_w weighed with a negative sign on this PC.

In this loading plot, some linear correlations were evident. The contents of acrylamide, StB, and a* were positively correlated with each other and negatively correlated with L* and h, while C° and b* were positively correlated with each other and negatively correlated with a_w.

The score plot of PC1 vs PC2 (Fig. 6b) shows that samples prepared with acid neutralizer (A and SA) are found exclusively in the positive quadrant of PC1, while P_A and P_SA samples (characterized by the highest ASN concentrations) are located near the border between the positive and negative quadrant. Conversely, samples without the acid in the recipe (C and S) were concentrated in the negative quadrant of PC1.

In Fig. 7a, the loading plot illustrates the relationship between PC1 and PC3 (11.80% of the total variance), where a_w (water activity) emerges prominently with a negative sign. Notably, a fairly high positive correlation between acrylamide content and StB is confirmed; in addition, there is a moderate negative correlation between the acrylamide content and the a_w value of the samples. The latter observation aligns

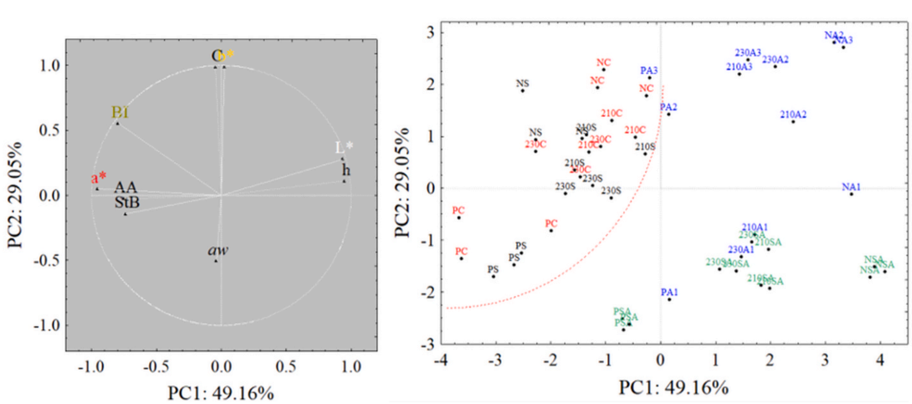

Fig. 6. **a)** Loading plot of PC1 vs PC2; **b)** Score plot of PC1 vs PC2.
Type of flour. N: soft wheat flour, Italian type '00', W180; 210: soft wheat flour, Italian type '1', W210, added with toasted wheat germ; 230: soft wheat flour, Italian type '2', W230, added with toasted wheat germ; P: soft wheat flour, Italian type '0', W350 added with bran and toasted wheat germ.
Recipe/Baking method combination. C: control; A: tartaric acid added; SA: steam + tartaric acid added; S: steam.
AA: acrylamide content; StB: dipping test.

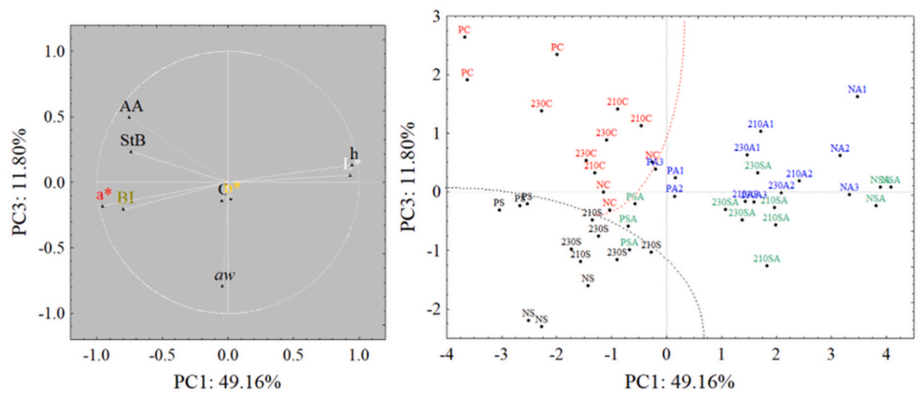

Fig. 7. **a)** Loading plot of PC1 vs PC3; **b)** Score plot of PC1 vs PC3.
Type of flour. N: soft wheat flour, Italian type '00', W180; 210: soft wheat flour, Italian type '1', W210, added with toasted wheat germ; 230: soft wheat flour, Italian type '2', W230, added with toasted wheat germ; P: soft wheat flour, Italian type '0', W350 added with bran and toasted wheat germ.
Recipe/Baking method combination. C: control; A: tartaric acid added; SA: steam + tartaric acid added; S: steam.
AA: acrylamide content; StB: dipping test.

with findings documented in several studies (Mesias et al., 2022; Taeymans et al., 2004) and is consistent with information provided by the Acrylamide Toolbox (FoodDrinkEurope, 2019).

In the loading plot of PC1 vs PC3 (Fig. 7b), the configuration set steam-prepared samples (N_S, 210_S, 230_S, P_S) in the negative quadrant of PC3 and those without steam (N_C, 210_C, 230_C, P_C) in the positive quadrant of PC3 into two rather distinct clusters. Finally, the separation of samples with the acid neutralizer (A and SA) is less arranged in the positive quadrant of PC1.

4. Conclusions

A wide range of asparagine concentrations from 80 to 270 mg/kg flour was found, with distinct influences of wheat types and sifting levels, underscoring the importance of ongoing research in understanding and modulating asparagine levels in wheat-based products for acrylamide mitigation.

The use of specific organic acids – already used as food additives – as a mitigation strategy for acrylamide in experimental biscuits proved to be an excellent alternative to other mitigation systems. The use of tartaric acid in the concentrations used, in addition to the favorable effect on the mitigation of acrylamide, did not significantly alter the main sensory characteristics of the biscuits. This therefore meets not only the requirements of food safety, but also consumer acceptability.

The dipping test revealed an improvement in the friability of biscuits prepared with acid, crucial for preventing the risk of oral mucosal lesion, especially in products intended for children.

Ultimately, this research lays the foundations for further experiments in which other parameters can be included to be evaluated, such as the study of other acids or combinations of them, the optimal dosage of the acid neutralizer in order to find the right compromise between taste, color, and acrylamide content, trials on flours with different concentrations of free asparagine and, finally, the mitigation of acrylamide in high-protein biscuits, in relation to current consumption trends.

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CRediT authorship contribution statement

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Declaration of competing interest

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.

Data availability

Data will be made available on request.

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