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Research Article

Assessment of Acrylamide Levels and Evaluation of Physical Attributes in Bread Made with Sourdough and Prolonged Fermentation

Cristina Lopez-Moreno[1*], Sara Fernández-Palacios[2], Pedro Ramírez Márquez[3], Salvador Jesús Ramírez Márquez[3], Carlos Ramírez Montosa[3], Juan Carlos Otero[1], Juan Teodomiro López Navarrete[1], Rocío Ponce Ortiz[1], María Carmen Ruíz Delgado[1]

[1]Department of Physical Chemistry, University of Malaga, Malaga, Spain
[2]Central Research Support Services, University of Malaga, Malaga, Spain
[3]Salvador Bakery Company, Benajarafe, Malaga, Spain
E-mail: cristinalm@uma.es

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Abstract: Over the past decades, there has been an increasing awareness regarding acrylamide content in cooked foods. The use of natural, homemade, organic and ecological ingredients has been promoted in our society as a strategy to combat the increase in cancer ratios. In this work, the use of sourdough and prolonged fermentation as a mitigation of acrylamide formation in bread were studied. In addition, to accomplish the required standards of industrial production, the dimension and acidity were also assessed. For this purpose, samples were elaborated using diverse fermentation times (0, 60, 120, 180, 240, 300 and 360 minutes) of the dough before baking and different sourdough percentages (0, 20 and 50%). High-performance liquid chromatography/electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) method for acrylamide determination in bread crust was applied. We could corroborate that a decrease in acrylamide levels was observed with fermentation time for samples containing 0 and 20% sourdough, being sharper the decrease for the latter. However, the samples containing 50% sourdough showed a quite different behaviour depending on the fermentation time interval. The physical study revealed that dimension of the pieces increased with fermentation time and sourdough content, until the excessive gluten degradation and acidification of the medium led to a flattening and decrease in elevation of the samples. This study revealed that the samples containing a percentage of 20% sourdough maintained the proper quality of gluten for longer fermentation times than the 50% sourdough samples, delaying the flattening of the samples with fermentation time. Statistical calculations were accomplished in order to elucidate the correlations between the different variables, observing that the titratable index was associated with the fermentation time, the sourdough content, the bulk density and the acrylamide content.

***Keywords*:** acrylamide, Maillard, sourdough, titratable index, fermentation, HPLC-ESI-MS/MS

1. Introduction

Acrylamide is a compound classified as neurotoxic and likely carcinogenic to humans [1]. It forms during the Maillard reaction, a complex process involving reducing sugars and L-Asparagine [2, 3]. The levels of acrylamide are influenced by temperature during production, with elevated heating values resulting in higher acrylamide concentrations

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[4, 5]. Acute health risks associated with acrylamide have been documented, emphasizing the need for strategies to mitigate its presence in food products [6-8].

Recently, several authors [9, 10] have reviewed the strategies to minimize the acrylamide formation in cereal products. All of them were focused on three main ways:

(a) Elimination or replacement of precursor substrates: Substituting invert sugar with sucrose syrup [11], replacing NH_4HCO_3 with $NaHCO_3$ [12] and reducing NaCl concentrations [4, 13] have all shown promise in reducing acrylamide levels in bread. Asparaginase inclusion in bread dough [14, 15], and the use of certain amino acids that compete with asparagine in the Maillard reaction [16, 17] have demonstrated efficacy in decreasing acrylamide levels, however, cost considerations limit their widespread use in the baking industry [15]. Additionally, selecting cereal varieties with low asparagine content will result in a reduction of acrylamide concentration in the final products [10].

(b) Modification of treatment conditions: Acrylamide is primarily generated in the crust, where between 90% and 99% of this compound is found [18] and factors such as low levels of moisture and fermentation duration influence its formation. It is known that at low levels of humidity the formation of acrylamide is favoured, since a lower degree of humidity in the bark favours the triggering of the Maillard reaction [19]. But, on the other hand, an increase in acrylamide has also been observed at very high humidity levels [20, 21], probably due to the increase in the mobility of the precursors and formation of acrylamide [20, 22]. Prolonged fermentation [23, 24] and the use of sourdough, in combination with yeast [20], have been proposed to reduce acrylamide content. More recently, Zhou et al. [25] investigated different strains concluding that Pediococcus Pentosaceus was the most efficient in reducing the content of acrylamide in the final product.

(c) Elimination or reduction of acrylamide after formation: Abedi et al. [15] have lately dept in the use of enzyme engineering to mitigate the formation of acrylamide in foods. Acrylamidase was used to hydrolyze food and environment-born acrylamide into acrylic acid and ammonia. Dietary fiber has also been effective in reducing moisture loss during baking [26], subsequently decreasing acrylamide formation [27, 28].

Despite the importance of decreasing acrylamide in the final product, it is necessary to consider the rheological and organoleptic features, since the bread must meet other requirements established by consumers. The use of natural, homemade, organic and ecological foods has been promoted in our society as a solution to combat the increase in cancer ratios. Sourdough, a natural fermentation agent, offers a promising avenue to reduce acrylamide levels in bread while simultaneously enhancing bread quality. Prolonged fermentation and the inclusion of sourdough have been associated with reduced acrylamide in bread improving also its rheological properties becoming softer, less elastic and therefore, more easily extensible and manageable [29, 30].

During fermentation, dough acidity increases due to microbial metabolic byproducts, influencing the sensory and technical qualities of the bread, as well as its shelf life and nutritional profile [31, 32]. The sourdough induces changes in both the crust and crumb structures, elevating the acidity of the medium and affecting various components crucial to defining the shape of the bread. These components include gluten, starch, arabinoxylans, among others [33]. It is noteworthy that prolonged acid production can inhibit bacteria due to their own metabolites. In terms of rheological properties, doughs with heterofermentative bacteria yield aromatic, slightly elastic textures compared to those with homofermentative bacteria.

Yeast also plays a pivotal role in fermentation. While bacteria transform carbohydrates and amino acids into lactic or acetic acid and other volatile compounds, yeast contributes to alcohol and CO_2 production, enhancing dough volume. The dominance of yeast or bacteria depends on fermentation temperature conditions [34].

In conclusion, the actions of microorganisms during fermentation generate metabolites that influence diverse attributes of the final product [33], including shape and acrylamide content. Consequently, bread quality can be controlled by adjusting fermentation time [30] and the proportion of sourdough used in the recipe.

In that way, this study aims to assess the relationship between fermentation time and sourdough content on both acrylamide levels and the physical attributes of bread (dimensions and acidity). To achieve this, a range of fermentation durations (0, 60, 120, 180, 240, 300, and 360 minutes) were employed prior to baking, along with varying proportions of sourdough (0%, 20%, and 50%).

High-performance liquid chromatography coupled to a high-resolution mass spectrometer with heated electrospray ionization (HPLC-ESI-MS) equipment was employed to determine acrylamide content in the bread crust. To gain deeper insights, statistical analyses were conducted to elucidate Spearman correlations among various variables. Additionally,

a discriminant analysis (DA) was executed to explore sample distribution and clustering based on several factors that could hold significance for enhancing the bread manufacturing process.

2. Materials, reagents and methods

2.1 *Bread samples making*

Firstly, sourdough (referred to as Dough I) was initially prepared using only flour and water. This sourdough was meticulously maintained at optimal temperature and humidity conditions in a designated chamber, undergoing cyclic refreshment through the addition of water, the introduction of fresh flour, and kneading.

Subsequently, various batches of dough (referred to as Dough II) were prepared without the preceding fermentation involving flour and water. To achieve this, 10 kg of wholemeal flour was blended with 6.5 L of water, 60 g of bread improver, and 20 g of yeast powder. Various quantities of Dough I were then incorporated into Dough II, specifically 0, 2, and 5 kilograms, ultimately resulting in a total mass of 10 kg. This process yielded sourdough percentages of 0%, 20%, and 50% in the final bread pieces.

Following the preparation of doughs, a kneading process lasting 6 minutes was conducted at room temperature, resulting in a final sample temperature of 21 °C. After allowing the doughs to rest for 2 minutes, they underwent a shaping process lasting an additional 12 minutes, yielding samples weighing between 60 and 80 g.

These samples were divided into portions and subjected to fermentation in a chamber maintained at 70 ± 2% humidity and a temperature of 30 ± 1 °C for varying durations: 0, 60, 120, 180, 240, 300, and 360 minutes. Finally, they were baked in a shelf oven set to 200 °C for a duration of 23 minutes, during which the internal crumb temperature in the subsamples reached 92 ± 1 °C. Following tempering at room temperature, the samples were sent to the laboratory for further analysis.

2.2 *Preparation of samples for acrylamide analysis*

The methodology outlined in UNE EN 16618:2015 [35] was used for this purpose. The samples were crumbled to enable analysis exclusively of the crust. Subsequently, the crust was crushed with a blade mill and was then subjected to a 48-hour lyophilization process to obtain a dry sample. Following this, they were finely and uniformly ground using a ball mill. Accurately 2 grams of each sample were weighed and deposited into individual 50 mL falcon tubes, where 40 µL of the internal standard Acrylamide-d^3 (Sigma-Aldrich, Wien Austria, 10 ppm in water) was added to each sample. Homogeneous dissolution was achieved through manual agitation, followed by a 15-second vortex mixing. Next, mechanical stirring was conducted for 60 minutes at a speed of 130 revolutions per minute. Following this, the solution was subjected to centrifugation at a temperature of 10 °C and a centrifugal force of 3,600 g for 20 min. From the resulting supernatant, a volume of 10 mL was carefully extracted.

Two solid-phase extraction (SPE) procedures were conducted to remove any potential impurities in the sample that could interfere with the target analyte. In the initial SPE, an Isolute Multimode cartridge was utilized. Initially, the column was equilibrated with 3 mL of methanol (Panreac, Panreac Química S.L.U., Castellar del Vallés, Barcelona), followed by two equilibrations with 6 mL of osmotized water (Millipore Elix 3 Essential, Millipore Corp., Milford, MA, USA). Subsequently, 10 mL of the aqueous extract was passed through the column and collected for further analysis.

For the second SPE, an Isolute ENV+ cartridge was employed. This cartridge was equilibrated with 5 mL of methanol and 5 mL of osmotized water. The 10 mL extract was loaded onto the column, and the eluate was discarded. The column was further rinsed with 4 mL of osmotized water.

To elute the acrylamide, a solution of 2 mL consisting of 60% methanol in water was employed. Finally, the eluate was subjected to evaporation until a constant volume of 500 µL was achieved. This concentrated solution was then transferred into an amber vial for subsequent injection into the high-performance liquid chromatography-mass spectrometry (HPLC-MS) equipment. Results of acrylamide analysis are given in dry crust bread and each sample was prepared in triplicate for acrylamide analysis.

In order to perform the calibration curves, the stock solution of acrylamide standard was prepared by dissolving acrylamide reference (European reference materials ERM-BD272, mass content of acrylamide in crispbread, from

Bundesanstalt für Materialforschung und -prüfung (BAM) with a content of acrylamide of 0.98 ± 0.09 mg/kg) in osmotic water. It was appropriately diluted to prepare working standard solution at 100 ppb and used to prepare the standard addition calibration curve in the range 10-60 ppb. The volumetric materials employed were certified as Class A borosilicate glass.

2.3 *Measurement of bread dimensions*

Given that the weight of the doughs used to make the pieces of bread was approximately the same (76.33 ± 2.9 g), we could quantitatively compare the variation of the dimensions of the pieces in function of the sourdough percentage and the fermentation time of each piece. The measurements were conducted utilizing a scalemeter and the results were expressed in cm. Weighing of the samples was conducted using a Sartorius precision balance with a maximum capacity of 500 g and a tolerance of 0.01 g.

The bread bulk density was determined by dividing the mass (g) by the volume (cm^3) of the bread pieces.

2.4 *Preparation of sample solutions for total titratable acidity analysis*

The titratable index was determined by measuring the volume of 0.1 N NaOH (Panreac, Panreac Química S.L.U., Castellar del Vallés, Barcelona) needed to neutralize the acidity of the sample and achieve a pH of 8.5. This parameter serves as a representative indicator of acidity of the food. The titratable index presents notable advantages over conventional pH measurement by minimizing pH fluctuations induced by variations in environmental temperature. This occurs when comparing sample signals with a calibration derived from temperature-corrected standards.

The tests were conducted in triplicate, using three identical samples of each bread type, prepared and baked under precisely the same conditions and at the same time. The pre-treatment procedure for the samples and the determination of the titratable acidity index followed the method described by Zannini et al. [36]. Initially, each piece was measured and weighed before undergoing milling with an IKA A11 Basic laboratory mill. Three portions of 10 g from the homogenized samples were selected for analysis. Subsequently, 90 mL of deionized water was added to each sample and agitated in an ultrasound bath for 15 minutes, after which they were further homogenized using a magnetic stirrer for 30 minutes. After the sample dilutions were prepared, the titratable acidity index was determined via a volumetric method.

2.5 *Analysis of acrylamide*

In recent times, the application of ESI tandem MS has gained widespread adoption, particularly driven by the growing demand for food composition analysis [37, 38].

The ESI system represents a form of ionization at atmospheric pressure, capable of ionizing molecules in solution in either positive or negative polarity, thereby enhancing mass spectrometric separation. High-performance liquid chromatography (HPLC) combined with electrospray ionization tandem mass spectrometry (ESI-MS) enables the generation of mass spectra encompassing intact molecular ions and fragment ions. The identification of peaks was achieved by matching their mass spectra and retention times with reference compounds [39].

Chromatographic separation was performed using a Thermo Scientifics Ultimate 3000 model HPLC system (Thermo Electron Corporation, Bellefonte, PA) equipped with a Hypercarb (100 × 2.1 mm i.d.) 5 µm analytical column. The Hypercarb porous graphitic carbon (PGC) material consists of fully porous spherical particles and has been demonstrated to offer improvements in applications where there are pH restrictions, a need for polar retention, or enhanced selectivity for closely related compounds, surpassing the capabilities of traditional silica-based stationary phases [40, 41]. The majority of parameter data were set as described in the normative method, with a few exceptions noted here. The sampler temperature and the column temperature were set at 5 °C and 30 °C, respectively. The mobile phases used for the elution gradient were: A (0.1% formic acid in osmotized water), B (methanol) and C (H_2O). The elution gradient was set as follows: 0-10 min, 100% A; 10-13 min, 95% B + 5% C; 13-19.4 min, 100% A, with a flow rate of 0.40 mL/min.

Aliquots of 10 µL of the sample extract were injected into the chromatographic system using the autosampler. The HPLC system was coupled to a high resolution Orbitrap Q-Exactive MS, dotted with a Thermo Scientific ESI source.

The ESI source was operated in positive and negative mode with the spray voltage set at 4 KV, sheath gas (N_2) flow rate at 40 arbitrary units, auxiliary gas (N_2) flow rate at 15 arbitrary units, capillary and auxiliary gas temperatures at 320 °C and 230 °C, respectively. The lens level was set at 50 V and mass range m/z was 50-100. All data were processed using Xcalibur software version 4.5.445.18 (2021) (Thermo Fisher Scientific).

The results of the samples were compared with the reference ERM-BD272 internal standard analysed under identical conditions. Samples quantification was performed by comparison with calibration curves prepared with different dilutions of internal standard.

2.6 *Analysis of titratable acidity index*

The titratable acidity index was determined using a Hanna Instruments pH meter, which was equipped with a refillable combined pH electrode (ref. HI 1131) and a temperature probe (ref. HI 7662-W). Prior to use, the equipment was calibrated at 25 °C using certified Hach standards with pH values of 4.01, 7.00, and 9.21. To determine the titratable index, we utilized a 0.1 N NaOH solution, prepared using technical-grade pellets, and standardized it with a Potassium Hydrogen Phthalate internal standard (Panreac, Panreac Química S.L.U., Castellar del Vallés, Barcelona) with a minimum purity of 99.5%. In the standardization process for NaOH, we employed a solution of phenolphthalein (Panreac, Panreac Química S.L.U., Castellar del Vallés, Barcelona) in water/ethanol at a concentration of 5% w/w as the indicator.

Sample dilutions were prepared using an ultrasound bath and magnetic stirrers. Reagents were weighed using a Mettler Toledo analytical balance with a maximum capacity of 120 g and a tolerance of 0.0001 g.

Whole wheat flour was employed in the sample preparation. The composition of the flour fell within the following percentage ranges: water (13.0-13.5%), starch (63-67%), gluten (10-14%), fat (1.6-2.2%), and sugar (2.0-3.0%). Additionally, the flour improver included wheat flour, wheat enzymes, anti-caking agent (E-170), and antioxidant (E-300).

2.7 *Statistical analysis*

Each measurement was conducted in triplicate and reported as the mean value (± SD, n = 3). Statistical analysis was performed utilizing Origin software, version 2018. The interrelationships among different variables were assessed through two-tailed Spearman's procedure, with a significance threshold set at $p \leq 0.05$. With the aim of making further progress in the statistical analysis, a discriminant analysis (DA) was performed to explore the existing segregation of the samples with different sourdough content according to some features of the bread, such as acrylamide content, elevation, acidity and fermentation time.

3. Results and discussion

3.1 *Dimensions of bread*

Figure 1 depicts the length, width, elevation and section area of samples made with 0, 20 and 50% sourdough at different fermentation times.

As can be observed, the samples dimensions increased until reaching a maximum limit in all cases. An inflection point for length (Figure 1(a)) and width (Figure 1(b)), located in 240 minutes of fermentation time, is reached for pieces with 50% sourdough, after which the slope is no longer so pronounced than was before this point. For samples without sourdough or with 20% sourdough, the maximum length and width were achieved after 300 minutes of fermentation time. The trend of variation in length and width for samples with less than the detailed fermentation times is increasing in all cases. The samples without sourdough reveal the shortest maximum length up to 240 minutes of fermentation, and the samples with 20% of sourdough present the smallest maximum width until the same fermentation time.

Regarding the rise of the pieces over the course of fermentation (Figure 1(c)), there is a noticeable decline in samples made without sourdough after 180 minutes of fermentation. These samples reached at 300 minutes the same elevation than that obtained for samples made without fermentation time. For samples made with 20% and 50% sourdough, the maximum elevation values were reached at 300 minutes of fermentation time.

As can be appreciated, up to 240 minutes of fermentation, the pieces that reached the smallest width are those made

with 20% sourdough, while these same samples reached the highest elevation along the whole fermentation process. The elevation of bread dough is caused by the generation and the proper retention of gas. The maximum elevation values achieved by the bread pieces are as follows: 3.90 ± 0.25 cm for samples without sourdough, 5.40 ± 0.17 cm for samples with 20% sourdough, and 4.80 ± 0.19 cm for samples with 50% sourdough. Based on these measurements, it can be inferred that using 20% sourdough and allowing it to ferment for 300 minutes can result in a 138% increase in maximum elevation compared to pieces prepared without sourdough, which attain their peak elevation after 180 minutes of fermentation. Interestingly, the pieces prepared with 50% sourdough exhibit lower elevation values than those with 20% sourdough.

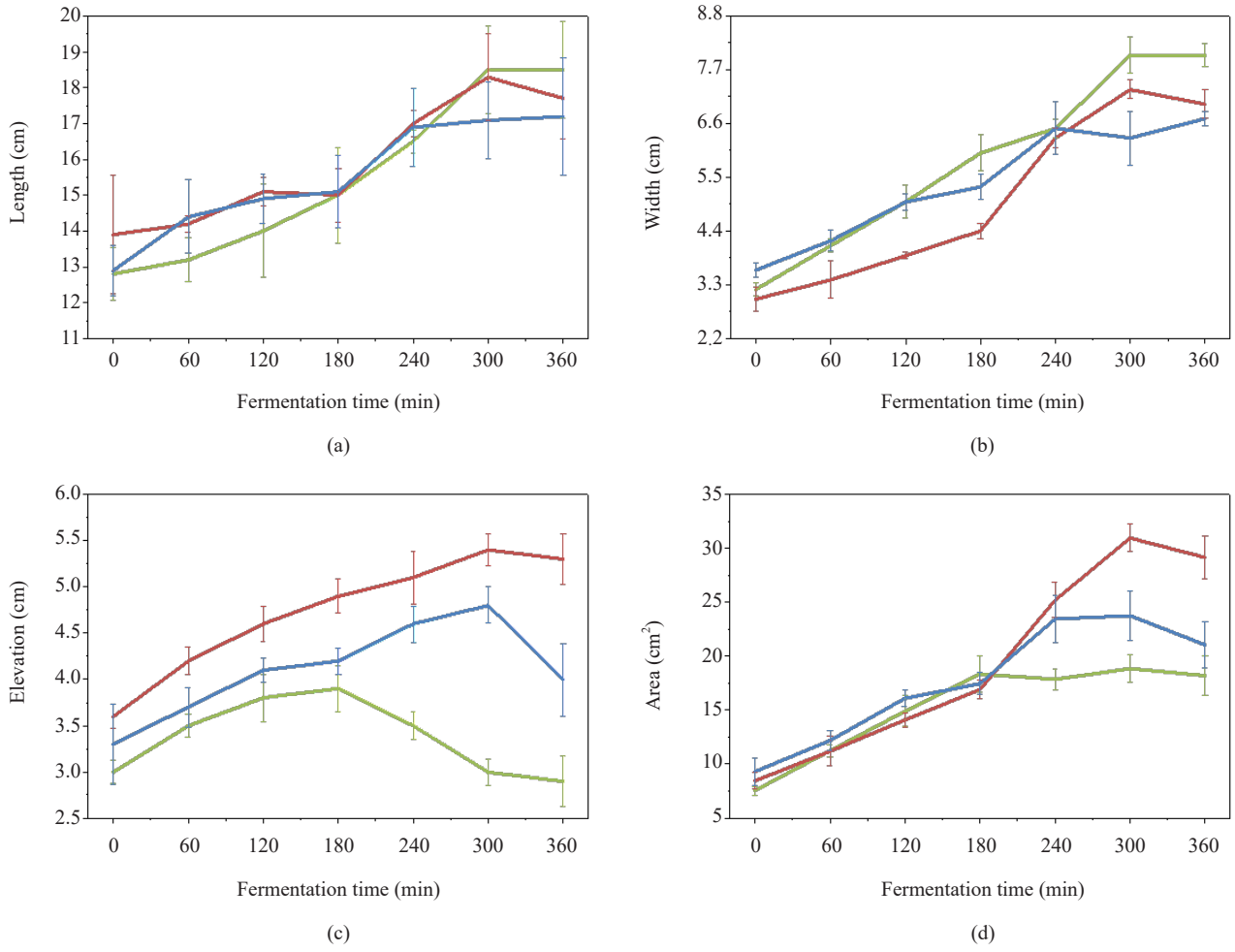

Figure 1. Length (a), width (b), elevation (c) and section area (d) of samples made with 0% (–), 20% (–) and 50% (–) of sourdough and different fermentation times. The standard deviation (SD) bars are also depicted

The variations in the dimensions of the bread pieces can be attributed to the rheological properties of the dough itself, including factors such as consistency, tenacity, extensibility, elasticity, strength, and more. These properties are influenced by several factors, inclusive of the level of gluten degradation, hydration, acidity, sugar content, and the kneading process of the dough. In this context, MacRitchie [42] proposed that gluten degradation arises when relatively inflexible components of secondary structure, such as alpha-helices and beta-sheets, transform into disordered coil structures that do not enhance the overall robustness of the gluten network. As a consequence, this transformation can lead to the production of smaller final products [43].

The degradation of gluten plays a pivotal role in retaining gaseous byproducts and influencing the deformability of dough to accommodate these metabolites. When the dough possesses high permeability, it can release a substantial amount of gaseous byproducts, leading to reduced dimensions in the bread. Conversely, when the dough is excessively sturdy, unyielding, and resistant, its capacity to deform and create air pockets (alveoli) for retaining gaseous metabolites becomes limited. Consequently, the crumb structure becomes denser, also resulting in smaller bread pieces.

Other researchers [33] have highlighted that incorporating sourdough into bread recipes could result in a decrease in bread volume due to gluten degradation caused by the acidity produced by microorganisms. This phenomenon is particularly evident at the end of the fermentation period, where the samples experience a reduction in size.

Analysing the data obtained for the area section in Figure 1(d), we found that samples containing 20% sourdough maintain the quality of gluten for a longer fermentation period compared to the samples made with 50% sourdough.

When comparing these data to those obtained from pieces made without sourdough, it's evident that the latter exhibit lower width and height. Two possible reasons explain this phenomenon: Firstly, there are evidences that prove the presence of a synergistic effect between lactic acid bacteria and yeast, resulting in the generation of higher amounts of gaseous metabolites in samples containing sourdough [44]. Secondly, other researchers have attributed this outcome to the fact that dough does not attain the same level of gluten maturity observed in pieces made with sourdough during longer fermentation periods [43]. These statements also explain the discrepancy in fermentation times required to reach the peak rise in samples prepared with sourdough compared to those made without sourdough. In the latter case, the predominance of yeasts over other microorganisms might imply that the maximum elevation was achieved at a shorter fermentation time compared to samples where lactic acid bacteria (LAB) were the dominant species.

Examining the shape of the bread pieces, Figure 2 presents the variation in bread bulk density after baking as a function of fermentation time for samples with different percentages of sourdough. As depicted in Figure 2, all samples exhibited a decrease in density until reaching 300 minutes of fermentation prior to baking. Beyond this point, the curves for samples containing sourdough began to show an upward trend. This increase can be attributed to a combination of factors: excessive gluten degradation, along with a reduction in the microbiological population responsible for generating these metabolites. This hypothesis helps to explain why the effect appears to be more pronounced in samples with higher sourdough percentages.

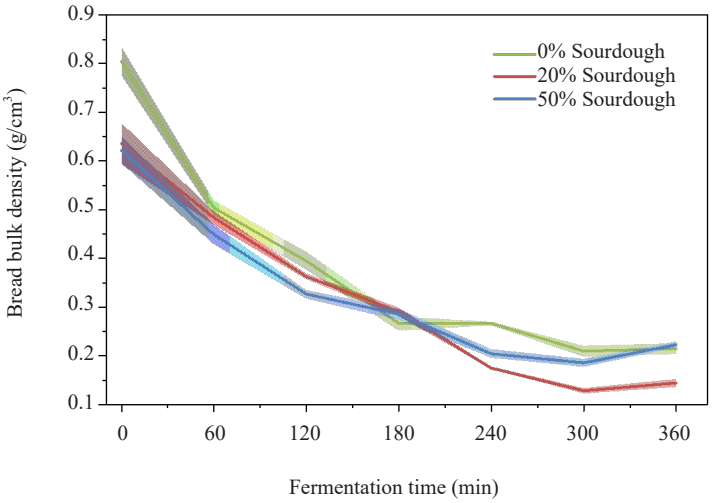

Figure 2. Bread bulk density of samples made with 0, 20 and 50% sourdough and different fermentation times. Filled area under and over the curves represents the SD of the y-axis

3.2 *Acidity of bread*

Under the same fermentation conditions, we assessed the titratable index for samples containing various

percentages of sourdough. Using the data obtained at the 360-minute as a reference point, it was noticed that the titratable index for samples with 0%, 20%, and 50% sourdough content were 6.00 mL, 11.80 mL, and 15.23 mL, respectively. This indicates that acidity increased by nearly 100% and 150% for pieces containing 20% and 50% sourdough, respectively, compared to the reference sample without sourdough. Figure 3 illustrates the titratable index for samples with 0%, 20%, and 50% sourdough content over the course of fermentation. A linear adjustment was indicated by the dashed line. As can be observed, the trend was more pronounced in samples containing a greater proportion of sourdough.

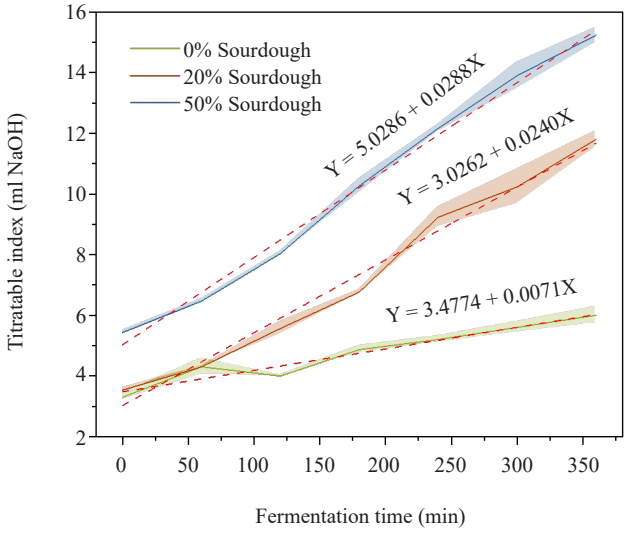

Figure 3. Titratable index of samples made with 0, 20 and 50% sourdough and different fermentation times. Filled area under and over the curves represents the SD of the y-axis. Dash lines represent the regression line of the least square adjustment

As anticipated, an increase in the titratable acid index was observed in the samples with a higher sourdough percentage and longer fermentation time. It's worth noting that certain heterofermentative strains produce lactic acid, acetic acid, alcohols, and carbon dioxide. At 25 °C, the pKa of lactic acid is 1 unit lower than that of acetic acid, indicating that lactic acid is 10 times more prone to deprotonation than acetic acid, leading to higher acidity [20]. Consequently, breads exclusively made with yeast and without sourdough will not exhibit a substantial amount of lactic acid production and will consequently have a higher pH.

The rise in the titratable index with fermentation time was more noticeable in pieces that included sourdough than in those without sourdough. In Figure 3 can be appreciated that the slopes in the adjusted lines of titratable index with the fermentation time were 0.024 and 0.029 for the samples containing 20% and 50% of sourdough, respectively. Both slopes were quite similar; nonetheless, the slope calculated for the samples without sourdough was 0.0071, indicating a 3.7 times steeper decline compared to the slope observed in samples with sourdough.

This outcome can be ascribed to the generation of both lactic and acetic acids by the bacteria present in the sourdough. This phenomenon aligns with findings described by Vrancken et al. [34], who demonstrated that under a fermentation temperature of 30 °C for 6 hours, optimal conditions were established for the proliferation of homofermentative lactic acid bacteria, responsible for lactic acid production. However there could also be some development of other heterofermentative bacteria responsible for acetic acid production, albeit to a lesser extent.

3.3 *Acrylamide assessment*

The schematic process where asparagine reacts with a dicarbonyl from a reducing sugar to form the Schiff base that, after several steps, will lead to the acrylamide final product [45] is shown in Figure 4(a). Additionally, Figure 4(b) presents the content of acrylamide with fermentation time in the crust of baked bread containing 0, 20 and

50% sourdough. As can be appreciated, a reduction in acrylamide levels was noticed over time during fermentation, particularly pronounced in samples containing 0% and 20% sourdough. This discovery aligns with the findings of other authors who have explored the changes in acrylamide levels during fermentation [23, 46, 47]. The decline in acrylamide levels in these samples can be attributed to a combination of several factors. Firstly, it's well-documented that the fermentation process leads to a decrease in the pH of dough due to the production of acid metabolites, primarily lactic and acetic acids, by microorganisms. This increase in the acidity of the medium can protonate the asparagine molecule, hindering its ability to react and form the Schiff base, and therefore the formation of acrylamide [48]. Secondly, yeast and bacteria are responsible for the reduction of asparagine and reducing sugars [17, 23], leading to a decrease in acrylamide concentration in the final product.

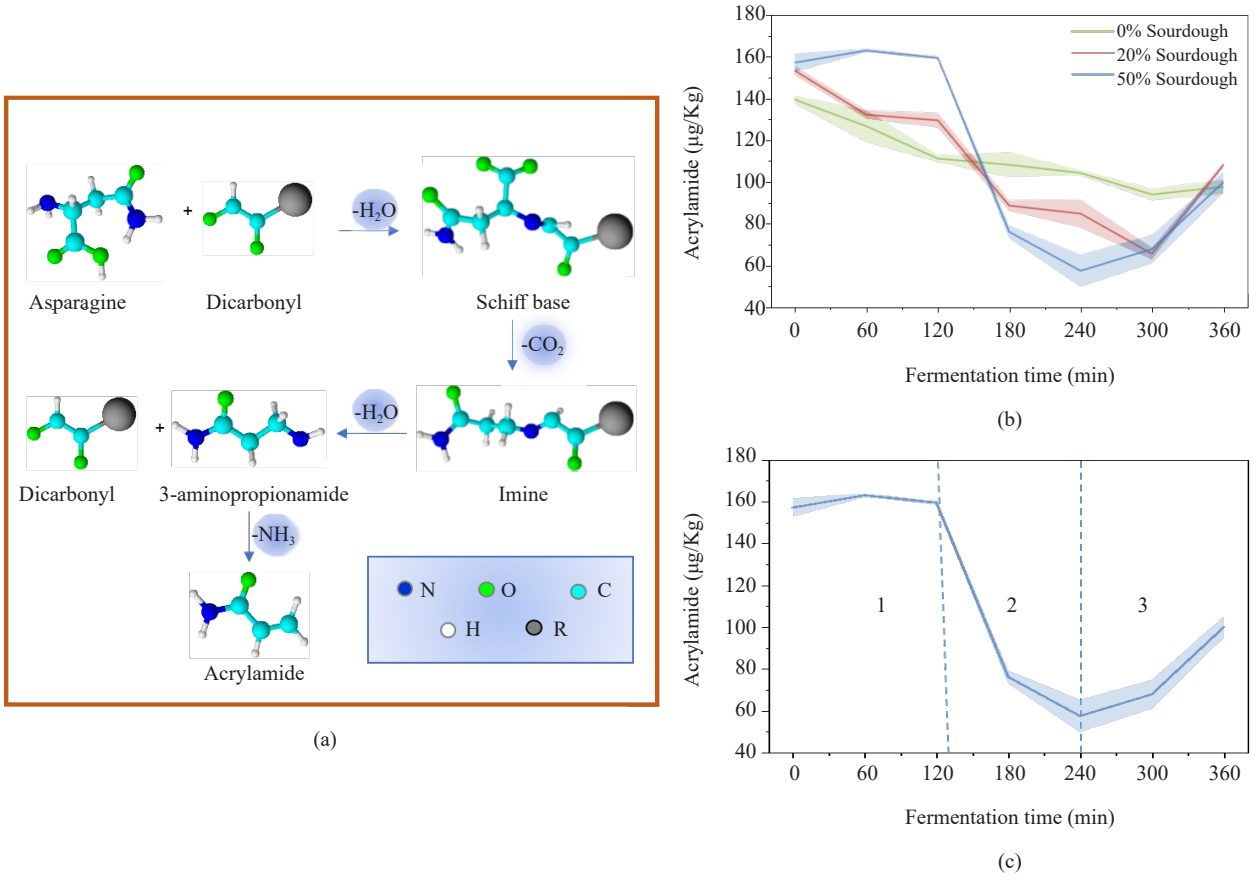

Figure 4. (a) Scheme of acrylamide formation from asparagine precursor. (b) acrylamide content in the crust of baked bread samples containing 0, 20 and 50% of sourdough and different times of fermentation prior to baking. Filled area under and over the curves represents the SD of the y-axis. (c) Detail of the variation of acrylamide in different phases of microorganism biological activity for samples containing 50% sourdough

However, the samples containing 50% sourdough showed a quite different behaviour (Figure 4(c)): In first stage, from 0 to 120 min, the acrylamide level remained apparently steady, followed by a sharp decrease until reaching 240 min, time when the signal rose again. Atypical tendencies in the decrease of acrylamide with fermentation time were also reported by other authors for samples containing high sourdough percentages or excessive fermentation time [23]. The high content of sourdough generates complex reactions by the microorganism development, mitigating and promoting the acrylamide formation depending on the microbiological processes at a certain time. It has been reported that the amylolytic activity and the proteolytic activity of lactic acid bacteria could greatly influence the acrylamide results [49]. The higher the amylolytic activity, the higher the reducing sugar in the medium, resulting in a high level of acrylamide in stage 1. However, there is sharp decrease when the pH of the medium affects the Maillard reaction (stage

2). In the same way, the higher the proteolytic activity, the greater the amount of the amino acids that compete with asparagine in the Maillard reaction [4, 17, 50], which leads to a decrease in acrylamide. These events cause a marked reduction of acrylamide found in the stage 2 of the Figure 4(c).

An increase in acrylamide content at the last stage of fermentation time (240 min for 50% sourdough samples and 300 min for 0 and 20% sourdough samples) was observed for all the cases, highly marked for greater sourdough percentages. This phenomenon was previously documented [23, 24] as an outcome attributed to lactic acid bacteria. These microorganisms appeared to exert a significantly adverse influence on yeast fermentation, resulting in a limitation of yeasts to metabolize asparagine and consequently an elevation in acrylamide concentration. This limitation of the yeast was more accused at lower pH, with samples containing a greater amount of sourdough showing higher growth in acrylamide content (stage 3 in Figure 4(c)). Nevertheless, further research would be desirable in this line.

3.4 *Statistical analysis and correlations*

The correlation between the variables studied in the samples was evaluated using Spearman correlation coefficients. Table 1 exhibits the Spearman correlation between sourdough percentage, fermentation time, acrylamide content, bulk density and titratable index.

Acrylamide content resulted in negative correlation with fermentation time (spearman correlation (SC): -0.69) and titratable index (SC: -0.63) and in positive correlation with bulk density (SC: 0.76). The tendency of the correlations is displayed in Figure 5(a), where the negative correlation between acrylamide and titratable index (a) and the positive correlation with bulk density (b) can be visually appreciated. As can be noticed, in both cases the correlation with the sourdough content was absent.

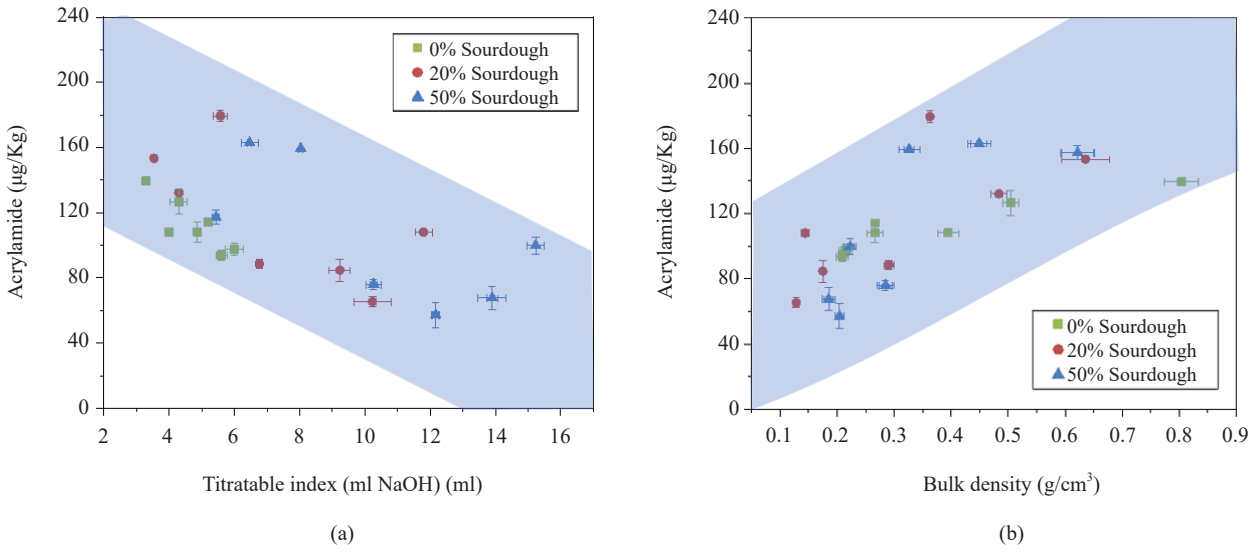

Figure 5. Correlation schemes of acrylamide content with (a) titratable index and (b) bulk density of the samples studied, with error bars

As expected, the titratable index exhibited a positive correlation with both sourdough percentage (SC: 0.66) and fermentation time (SC: 0.72). Conversely, it showed a negative correlation with bread bulk density (SC: -0.76) and acrylamide content (SC: -0.62). Among these correlations, the strongest was observed between the titratable index and bulk density, which is depicted in Figure 6. In this case, the correlation with sourdough was robust and followed an exponential pattern for samples containing sourdough, but not for samples consisting only of yeast. The absence of a correlation for samples lacking sourdough can be attributed to the limited increase in acidity with fermentation time, as illustrated by the slope observed for this group of samples in Figure 3.

Considering the strong correlations among various variables, a statistical multivariate analysis was conducted using

discriminant analysis (DA). Figure 7 illustrates the results obtained in the canonical score plot, which revealed three distinct groups based on four selected factors: fermentation time, acrylamide content, titratable index, and elevation. The data zone with the most negative values on the abscissa scale corresponded to results obtained from samples with 0% sourdough. The other zones corresponded to 20% and 50% sourdough content, respectively, moving progressively toward the positive abscissa axis.

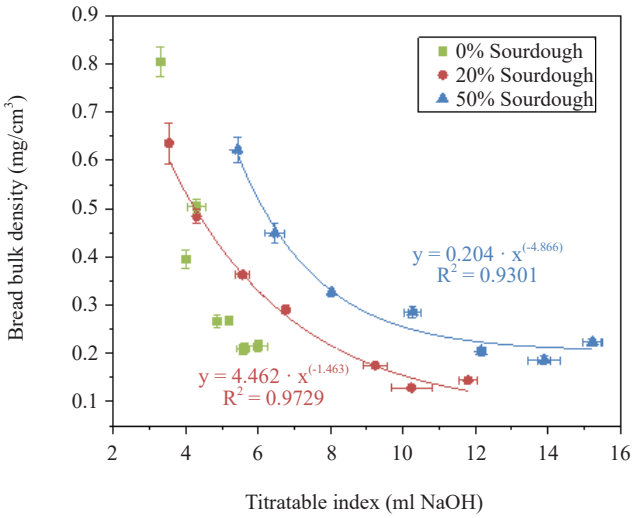

Figure 6. Correlation schemes of bulk density with titratable index, with error bars for different sourdough content in the samples. The lines represent the fitting curves for samples with 20% and 50% sourdough. The mathematical expression of the fitting lines and the R square (coefficient of determination) of the curves are also indicated in the graph

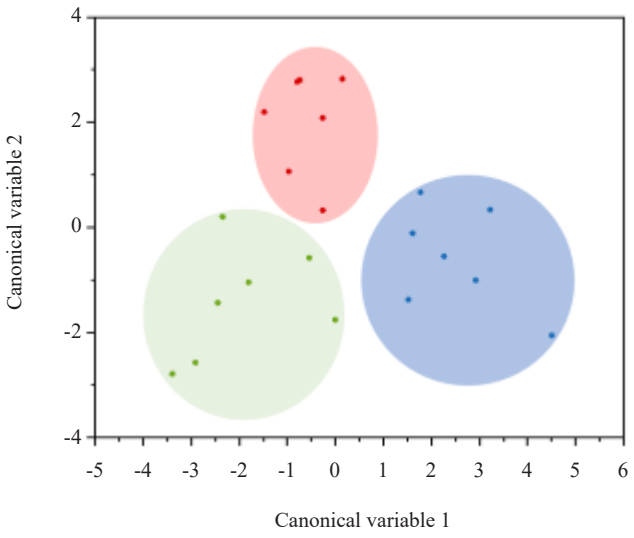

Figure 7. Discriminant analysis of 3 groups of sourdough percentage (• 0%, • 20% and • 50%) according fermentation time, acrylamide, titratable index and elevation

The distribution observed in the DA model enabled us to determine the sourdough percentage of an unidentified sample with 100% confidence, as evidenced by a 0.0% error rate in classifying the training data.

Table 1. Spearman correlation between sourdough percentage, fermentation time, acrylamide content, bulk density and titratable index

	Sourdough	Fermentation time	Acrylamide	Bulk density	Titratable index
Sourdough	1	0	-0.12521	-0.09631	0.65997*
Fermentation time	0	1	-0.69203*	-0.91616*	0.71586*
Acrylamide	-0.12521	-0.69203*	1	0.76364*	-0.63202*
Bulk density	-0.09631	-0.91616*	0.76364*	1	-0.75544*
Titratable index	0.65997*	0.71586*	-0.63202*	-0.75544*	1

* Correlation is significant at $p \leq 0.05$

4. Conclusions

The results revealed that the dimensions of the bread pieces increased with longer fermentation times and higher sourdough content. However, there was a point where excessive gluten degradation and elevated acidity in the medium led to a flattening and reduction in the elevation of the pieces. Samples containing 20% sourdough maintained optimal gluten quality for extended fermentation periods compared to those with 50% sourdough content.

Regarding the titratable index, the positive slopes of the fitted lines for samples with sourdough were quite similar. However, the slope obtained for samples without sourdough indicated a 3.7 times steeper decrease, ascribed to the lower content of microorganism metabolites.

The acrylamide results revealed that the concentration decreased with longer fermentation times, particularly pronounced in samples with 0 and 20% sourdough. However, the behaviour of samples containing 50% sourdough varied depending on the fermentation stage. This variability was attributed to the amylolytic and proteolytic activities of lactic acid bacteria, which significantly influenced the acrylamide results.

To gain further insights, statistical analyses to assess Spearman correlations between different variables were conducted, revealing that the titratable index was associated with fermentation time, sourdough content, bulk density, and acrylamide content.

The canonical score plot from DA revealed three distinct groups based on sourdough content using four selected factors: fermentation time, acrylamide levels, titratable index, and elevation.

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Conflict of interest

The authors declare no conflicts of interest.

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