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Neapolitan Pizza: Technological, Nutritional and Health Aspects

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Riassunto

La pizza napoletana è uno dei prodotti più noti della tradizione culinaria italiana ed è considerata il miglior cibo consumato in tutto il mondo. Recentemente, è stato emanato un Disciplinare di Produzione della pizza napoletana che, definisce gli standard per le materie prime e i parametri tecnologici (UE Reg. 97/2010). Inoltre, l'importanza dell'arte del "*pizzaiuolo*" è stata riconosciuta come "Patrimonio Culturale Immateriale dell'Umanità" dall'UNESCO (Jeju, Corea del Sud, 7 dicembre 2017). Tuttavia, la comunità dei ricercatori ha prestato pochissima attenzione sulla pizza napoletana e pochi dati si trovano nella letteratura scientifica. Questa mancanza di informazioni ha generato alcuni fraintendimenti sulla relazione tra il consumo frequente di pizza e la salute, creando così un allarme ingiustificato, con un impatto negativo sull'economia del settore. La pizza è un prodotto a media densità energetica e ad alto contenuto di carboidrati. È una buona fonte di proteine, minerali e vitamine del gruppo B. L'amido è il componente più abbondante delle farine di frumento, poiché i carboidrati costituiscono circa il 70-80%, seguiti dalle proteine (10-14%) e lipidi, fibre, vitamine e minerali, che rappresentano una piccola percentuale. La farina di frumento, rispetto ad altri cereali, viene utilizzata perché sue le proteine del glutine hanno proprietà uniche, come la capacità di formare un impasto viscoelastico quando la essa viene idratata e impastata (Kłosok et al., 2021). Durante il processo di produzione della pizza si verificano diversi cambiamenti strutturali e biochimici. Il primo passo fondamentale nella preparazione dell'impasto è quello di mescolare le materie prime di partenza (farina di frumento, acqua, sale e lievito), imprimendo energia meccanica per formare un impasto elastico con una forte rete glutinica (Ooms and Delcour, 2019), e granuli di amido intrappolati all'interno. Questa matrice complessa ha la capacità di trattenere la CO_2 generata dalla fermentazione del lievito e permette al volume dell'impasto di espandersi durante la lievitazione. Nello studio della formazione dell'impasto, ci sono diverse limitazioni nell'osservare gli eventi fisici su macroscala, a livello sovra-molecolare (Stauffer, 1999). Molte tecniche sono state adattate per lo studio degli impasti, ad esempio l'analisi a raggi X, la risonanza magnetica nucleare (NMR), la calorimetria a scansione differenziale (DSC), la microscopia elettronica a scansione (SEM), ma l'interpretazione dei risultati è molto difficile data la complessità della matrice. Le fasi di lievitazione e cottura degli impasti per pizza provocano alterazioni nella struttura del prodotto finale, influenzandone la digeribilità. Il tempo di lievitazione può contribuire ad aumentare la digeribilità riducendo la coesione tra amido e proteine dell'impasto e aumentando l'accessibilità dell'amido all'α-amilasi (Garcia-Hernandez et al., 2022). Infatti, la cottura permette la penetrazione dell'acqua nei granuli di amido favorendone la gelatinizzazione, e questo aumenta la velocità dell'idrolisi facilitando la disponibilità dell'amido all'azione dell'α-amilasi. Inoltre, ad alte temperature, la superficie della pizza non coperta dal condimento è esposta alla reazione di Maillard, che porta alla formazione di acrilammide, un composto tossico classificato come "probabile cancerogeno per l'uomo" (IARC, 1994). Sebbene manchi una letteratura sul livello di acrilammide nella pizza cotta, interventi mirati per la riduzione di questa sostanza tossica sono sempre auspicabili.

Tutti questi fenomeni devono essere presi in considerazione per ottenere una pizza desiderata: i) corretta lievitazione che porti a un volume e a una consistenza adeguata

dell'impasto; ii) buona digeribilità e qualità alimentare del prodotto finito; iii) lavorazione sicura per ridurre i problemi sulla salute. L'obiettivo di questa tesi di dottorato è stato quello di studiare la pizza napoletana sotto il profilo tecnologico, nutrizionale e salutistico. In particolare, sono stati svolti studi della struttura dell'impasto durante la lievitazione, della digeribilità dell'amido e del contenuto di acrilammide nella pizza cotta a legna.

Il primo caso studio ha avuto come obiettivo quello di indagare i cambiamenti strutturali dell'impasto per pizza nel corso di una lunga lievitazione (0÷48 h) attraverso analisi fisico-chimiche, e come i parametri reologici possano essere correlati alla digeribilità dell'amido della pizza cotta a legna. Un lungo tempo di lievitazione, dell'impasto per pizza, ha mostrato minori e deboli interazioni della rete glutinica, attribuibili all'attività amilolitica e proteolitica del lievito. Ciò si traduce in una maggiore estensibilità che consente di ottenere un diametro maggiore dell'impasto per pizza applicando una minore forza di compressione. Inoltre, i fenomeni fisici e chimici che si verificano con una lunga lievitazione hanno portato a un aumento dell'indice di digeribilità dell'amido e del glucosio rapidamente disponibile.

Il secondo caso studio mirava a confrontare l'effetto di un lungo tempo di lievitazione (0÷48 h) sull'impasto per pizza preparato con batteri lattici e lievito selezionato, in termini di indice volumetrico, prodotti di fermentazione e idrolisi enzimatica. Sono state riscontrate differenze significative tra i campioni per quanto riguarda la digeribilità dell'amido in relazione alla microstruttura degli impasti e dei rispettivi campioni di pizza cotti a legna, confermando che più la struttura è compatta, minore è la frazione di amido rapidamente digeribile (RDS).

Il terzo caso studio ha avuto lo scopo di individuare il tempo di lievitazione ottimale (0÷24 h) per il congelamento degli impasti per pizza, al fine di ottenere panetti scongelati e lievitati con un comportamento reologico simile rispetto al controllo non congelato. Il congelamento, a qualsiasi tempo di lievitazione, ha evidenziato un danneggiamento all'amido e al glutine, prodotto principalmente dalla formazione di cristalli di ghiaccio durante la conservazione. L'impasto congelato prima della lievitazione (0 h), ha mostrato proprietà cinetiche di lievitazione e viscoelastiche simili al controllo, ma con differenze significative nelle proprietà termiche e nella risposta al test di stress-relaxation.

Il quarto caso studio è stato utile per studiare la riduzione della formazione dell'acrilammide (AA) durante la cottura trattando l'impasto per pizza con l'enzima L-asparaginasi. Alla farina di frumento è stato aggiunto l'enzima commerciale durante la fase di miscelazione degli ingredienti. I risultati hanno mostrato che l'uso della L-asparaginasi potrebbe svolgere un ruolo chiave nel mitigare la formazione di acrilammide nel processo di cottura delle pizze fritte e cotte al forno a legna, senza apparentemente alterare le caratteristiche tecnologiche dell'impasto.

Un'altra strategia per la mitigazione dell'AA è stata quella di testare i grani di frumento selezionati con un basso contenuto di asparagina per la produzione di impasti per pizza. Questo studio ha dimostrato chiaramente che la mitigazione della formazione di AA nella pizza può essere ottenuta riducendo il contenuto di asparagina nel grano, con una riduzione di circa il 47-68%, rispetto a una farina commerciale. Inoltre, la pizza preparata con farine integrali presentava un basso tasso di idrolisi dell'amido e una rapida disponibilità di glucosio rispetto alle farine raffinate.

Infine, nell'ultimo caso studio sono stati sviluppare modelli di calibrazione e validazione per la determinazione rapida dei livelli di acrilammide nei campioni di pizza attraverso la spettroscopia FT-NIR. In generale, è stata riscontrata una buona correlazione lineare tra i livelli di acrilammide previsti nella matrice solida con il metodo NIR e i valori effettivi di acrilammide misurati con UHPLC nei campioni di pizza estratti.

Tutti questi studi hanno permesso di esaminare diversi aspetti legati alla pizza napoletana con un approccio scientifico, e di colmare le lacune in letteratura su questo argomento. In particolare, è stato approfondito l'aspetto strutturale degli impasti durante lunghe lievitazioni e le conseguenze sulla digeribilità della pizza. Inoltre, sono state anche valutate strategie di mitigazione dell'acrilammide per rendere più sicuro questo alimento tanto amato e apprezzato in tutto il mondo.

Summary

Neapolitan pizza is one of the mostly known product of Italian culinary tradition and it is considered as the best food consumed all over the world. Recently, a Production Regulation for Neapolitan pizza has been issued that defines standards for raw materials and technological parameters (EU Reg. 97/2010). Moreover, the importance of the "art" of making Neapolitan pizza has been recognized as "Intangible Cultural Heritage of Humanity" by UNESCO (Jeju, South Corea, 7th December 2017). However, the research community has focused very little attention on Neapolitan pizza, and few data can be found in the scientific literature. This lack of information has generated some misunderstanding on the relationship between frequent consumption of pizza and health, thereby creating unjustified alarm, with negative impact on the economy of pizza business area. Pizza is a medium-energy-density, high-carbohydrate product. It is a good source of protein, minerals, and B vitamins. Starch is the most abundant component of wheat flours, since carbohydrates constitute about 70-80 %, followed by proteins (10-14 %), and lipids, fibre, vitamins and minerals, accounted for a small percentage. Wheat flour is used because the gluten proteins in wheat have unique properties, as the ability to form a viscoelastic dough when wet and kneaded, rather than other grains (Kłosok et al., 2021). Several structural and biochemical changes occur during the pizza making process. The starting raw materials, wheat flour, water, salt and yeast, are mixed in order to be combined to form the dough. The first basic step in dough preparation is to mix water with wheat flour, imparting mechanical energy to form an elastic dough with a strong gluten network (Ooms and Delcour, 2019), with entrapped starch granule inside. This complex matrix has the ability to retain the CO_2 generated by the yeast fermentation and allows for the dough volume to expand during leavening. In studying dough formation, there are several limitations to observe physical events on a macroscale, at the supra-molecular level (Stauffer, 1999). Many techniques have been adapted for the study of dough, e.g., X-ray Analysis, Nuclear Magnetic Resonance (NMR), Differential Scanning Calorimetry (DSC), Scanning Electron Microscopy (SEM), but the interpretation of the results is very difficult given the complexity of the matrix. The leavening and baking stages of pizza dough provoke alterations in the structure of the final product, thus affecting the digestibility. Leavening time can contribute to increase digestibility reducing the cohesion between starch and dough protein and increasing the accessibility of starch to α-amylase (Garcia-Hernandez et al., 2022).

Indeed, baking allows the water penetration in the starch granules promoting its gelatinization, and this increases the rate of the hydrolysis by facilitating the starch availability to α-amylase action. In addition, at high temperatures, the pizza surface not covered by topping is exposed to the Maillard reaction, which leads to the acrylamide formation, a toxic compound classified as a "probable human carcinogen" (IARC, 1994). Although there is a lack of literature on the level of acrylamide in cooked pizza, targeted interventions for the reduction of this toxic substance are always welcome.

All these phenomena must be considered to achieve the desired baked pizza: i) right leavening leading to adequate dough volume and consistency; ii) good digestibility and food quality in the finished product; iii) safe processing for reduction of health concerns.

The aim of this PhD thesis was to investigate on Neapolitan pizza: technological, nutritional and health aspects. Specifically, study on dough structure during leavening, starch digestibility and acrylamide content of wood oven baked pizza.

The first case study aimed to investigate structural changes over long leavening time (0÷48 h) of pizza dough through physico-chemical analysis, and how rheological parameters may be related to the starch digestibility of the wood oven baked pizza base. Long leavening time of pizza dough showed fewer and weaker interactions of gluten network, attributable to amylolytic and proteolytic activity of yeast. This results in greater extensibility that allows a larger pizza dough diameter by applying less compression force. Moreover, physical and chemical phenomena occurring at long leavening time led to an increase in digestibility index of starch and rapidly available glucose.

The second case study aimed to compare the effect of long leavening time (0÷48 h) on pizza dough prepared with selected lactic acid bacteria and yeast, in terms of volumetric index, fermentation and enzymatic hydrolysis products. Significant differences among samples were found for starch digestibility connected with the microstructure (SEM) of dough and respective wood oven baked pizza samples, confirming the more compact the structure, the less rapidly digestible starch (RDS) was found.

The third case study aimed to identify the optimal leavening time (0÷24 h) for freezing pizza dough, in order to obtain thawed and leavened dough balls with a similar rheological behaviour compared to the unfrozen control. Freezing, at any leavening time, presented a starch and gluten damage, mainly produced by the formation of ice crystals during storage. The frozen dough before leavening (0 h), showed leavening kinetic and viscoelastic properties similar to the control, but with significant differences in thermal and stress-relaxation properties. The fourth case study aimed to reduce the acrylamide (AA) formation during cooking by treating pizza dough with L-Asparaginase enzyme. Wheat flour was added with commercial enzyme during mixing for the production of pizza dough. Results showed that the use of L-Asparaginase could possibly play a key role in mitigating acrylamide formation in the cooking process of fried and wood oven baked pizza bases, without apparently altering the technological characteristics of the dough. Another strategy to mitigate AA formation was to test selected wheat grains with low asparagine content for making pizza dough. This study clearly showed that mitigation of AA formation in pizza can be achieved by reducing asparagine content in wheat, with a reduction of about 47-68 %, compared to a commercial flour. Further, pizza prepared with wholemeal flours had low starch hydrolysis rate and rapidly available glucose than refined flour.

Finally, last case study aimed to develop a calibration and validation models for the fast determination of acrylamide levels in pizza samples through FT-NIR spectroscopy. Overall, good linear correlation was found between the predicted acrylamide levels in solid matrix by NIR method, and the actual acrylamide values measured by UHPLC in extracted pizza samples.

This study was useful to examine different aspects related to Neapolitan pizza with a scientific approach, and to fill the gaps in the literature on this topic. Specifically, the structural aspect of the dough during long leavening and the consequences on the digestibility of the pizza were investigated. In addition, AA mitigation strategies were also evaluated to make this much loved and appreciated food safer.

Introduction

Neapolitan pizza preparation: mixing, leavening, rolling out and baking

Neapolitan pizza is one of the most popular products of Italian gastronomy. Moreover, pizza is one of the pillars of the catering industry which, only in Italy, counts 61000 pizzerias, 150000 employees and sales near 20 Giga euro per year. Furthermore, the importance of the "art" of making Neapolitan pizza has been recognized as "Intangible Cultural Heritage of Humanity" at Jeju, South Corea on 7th December 2017 (Ganguly, 2017). In reality, Neapolitan "pizzaiuolo" art comes from far away and it is deeply linked to the idea of community in the South of Italy. According to some food historians, it would even derive from the preparation of *mensae*, the oven-baked wheat flatbreads that the ancient peoples of *Mare Nostrum* used to rest food on. A testimony of this ancient food was given by *Virgilio*, the most important Latin poet, in a famous episode of the Aeneid (Canali and Paratore, 2015). The most popular and famous pizzas were "marinara", born in 1734, and "margherita", from 1796-1810, which was offered to the Queen of Italy on a visit to Naples in 1889 precisely because of the colour of its toppings (tomato, mozzarella, and basil) that remind the flag of Italy. The term "Pizza Napoletana" over the centuries has spread so widely that everywhere, even outside Europe, from USA to Central and South America to Asia. According to EU Regulation n. 97/2010, Neapolitan pizza "TSG Traditional Speciality Guaranteed" is presented as a disc of dough topped with tomato, i.e., a round baked product with a variable diameter that must not exceed 35 cm, with a raised rim and with the central part covered by the topping. The central part must be 0.4 cm thick with a permitted tolerance of ± 10%, the pizza rim 1-2 cm. The pizza should be soft, elastic, easily foldable into a "wallet" shape. The basic raw materials for Neapolitan pizza preparation are wheat flour, brewer's yeast, natural drinking water, peeled tomatoes and/or fresh cherry tomatoes, table salt, and extra virgin olive oil. Other ingredients that can be used in the pizza preparation are garlic and oregano, Mozzarella di Bufala Campana DOP, fresh basil and Mozzarella TSG. The flowchart of pizza making operation is shown in Figure 1.

In accordance with EU Regulation n. 97/2010, the dough is prepared by mixing flour, water, salt and yeast. One liter of water is poured into the mixer, a quantity of sea salt between 50 and 55 g is dissolved in, 10 % of the flour is added to the total amount provided, then 3 g of brewer's yeast are dissolved, the mixer is started and 1800 g of flour with 220-380 W (strength) is gradually added until the desired consistency, called "dough point", is reached. This operation should take 10 min. The dough should be worked in the mixer preferably with a fork for 20 min at low speed until a single compact mass is obtained. For optimal dough consistency, the amount of water that the flour is able to absorb is very important. The dough should feel non-sticky, soft and elastic to the touch. The dough, after being taken out of the kneading machine, is placed on a worktable where it is left to rest for 2 hours, covered with a damp cloth. Once the 2 hours of leavening have passed, the dough balls are formed, which must be done by the pizza maker exclusively by hand. With the help of a spatula, a portion of leavened dough is cut and then shaped into a dough ball (250 g). This is followed by a second leavening phase, in which the formed balls take place in food boxes at room temperature.

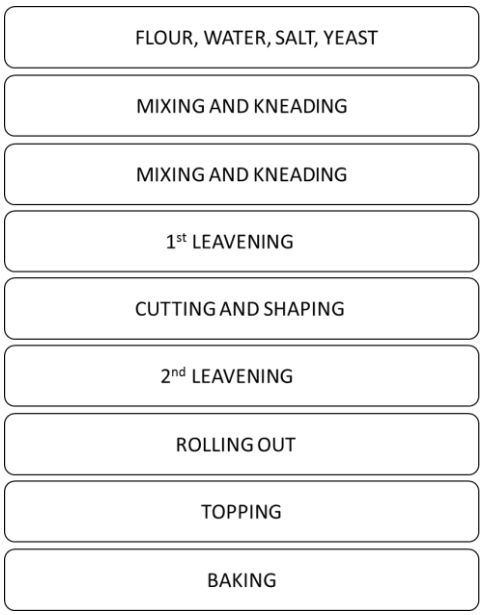

Figure 1 – Flow chart for the production process of Neapolitan pizza "TSG".

Subsequently, each dough ball is taken out of the box with a spatula and placed on the pizzeria counter on a light layer of flour to prevent the dough from sticking to the workbench. With a movement from the center outward and with the pressure of the fingers of both hands on the dough, which is turned over several times, the pizza maker forms a disc of dough thinner in the centre and raised at the edge. After the topping, the pizza maker transfers the pizza onto a wooden (or aluminum) shovel, with a little flour and with a rotary motion, and then lets it slide onto the oven floor with a quick wrist movement such that the topping is prevented from spilling out. “Neapolitan pizza” is baked exclusively in wood oven, where an oven temperature of 485 °C is reached.

Several structural and biochemical changes occur during the pizza making process. The starting raw materials are mixed in order to be combined to form the dough, which will subsequently undergo other changes during both leavening and baking phases. The first basic step in dough preparation is to mix water with wheat flour, imparting mechanical energy, to form an elastic dough with a strong gluten network (Bushuk, 1985; Hoseney, 1985), with starch granule entrapped inside. The mechanical behaviour of the dough is highly dependent on its water content (Ooms and Delcour, 2019). Wheat flour is used, because the gluten proteins in wheat have unique properties; as the ability to form a viscoelastic dough when wet and kneaded, rather than other grains (Shewry, 1999). Gluten proteins, in fact, consist of polymeric glutenin proteins and monomeric gliadins. Differences in the structure of glutenin and gliadin proteins give them different functionalities during dough formation. It is generally established that glutenin proteins build the polymeric protein network that provides cohesion and elasticity to the dough, while gliadins act as plasticizers of the glutenin network and contribute to the viscosity and extensibility of the dough (Ooms and Delcour, 2019). In fact, the absorption capacity of flour depends on higher or lower presence of gliadins. Gliadins and glutenins are insoluble proteins characterized by high amounts of cysteine, proline, and glutamic acid, amino acids that contain side groups allowing the formation of hydrogen bonds with water molecules, bonds that are critical for the hydration of gliadins and glutenins during

kneading and subsequent gluten formation (Kłosok et al., 2021). Starch is the most abundant component of flour and consists of macromolecules, which are two polymers of α-D-glucose: amylose and amylopectin. Amylose is a linear macromolecule consisting of α-1,4-D-glucose units, while amylopectin is a highly branched molecule consisting of α-1,4-D-glucopyranose chains connected with α-1,6 bonds. Typically, wheat starch consists of 25-28% amylose and 75-78% amylopectin (Singh et al., 2010). The chemical and physical properties of starch depend on the relative proportions of amylose and amylopectin and the degree of glucose chains polymerization. Amylose and amylopectin are assembled within starch into water-insoluble, three-dimensional, semicrystalline (partially ordered) structures called granules, and they influence the elasticity of the dough by their presence in the total matrix. Amylopectin is responsible for the formation of crystalline regions and the structure of starch granules, while amylose is present in an amorphous form (Shevkani et al., 2017). In addition to starch in flour, there are other polysaccharides, specifically, pentosans, that are non-starch polysaccharides, in fact, they are hemicelluloses formed from sugars, namely pentoses. They are classified into soluble, if extractable with hot water, and insoluble if extractable with alkaline agents. About 30 % of total pentosans are soluble and consist mainly of arabinoxylans and in small amounts of arabinogalactans. Pentosans affect water-absorbing capacity and contribute to improved dough consistency and viscosity and increase gas retention during the leavening phase (Michniewicz et al., 1992). Many substances, such as salts and sugars, influence the conformational stability of gluten network (McCann and Day, 2013). Alcohol could control the degree of swelling and solubility of gluten proteins, the partition coefficient of lipids and other components between dough phases, the conformational stability of globular proteins, and contribute to the increase of gas bubble pressure and volume (Toistoguzov, 1997). Several phenomena occur during kneading that go into changing the structure of pizza dough. The initial action of mixing is to speed up the conversion of protein bodies into a soft, hydrated protein dispersion, which is then further modified during the development of the gluten network. At the same time, the damaged starch granules absorb water and the water-soluble components of the flour with salt dissolve (Stauffer, 1999). Two protein containing phases are formed in dough: the first is the concentrated protein viscoelastic phase formed by gluten, and the second coexisting phase is a mixed viscous solution of albumins, globulins, neutral and charged polysaccharides. The oxidation of free thiol groups (SH) into disulphide bonds (SS), which results in an increase in the molecular weight of glutenin aggregates, as well as SH-SS exchange reactions, are of utmost importance for the construction of a three-dimensional gluten network during mixing (Kłosok et al., 2021). Other covalent bonds have also been suggested as important for gluten network formation. The high levels of glutamine in gluten proteins allow the formation of intermolecular and intramolecular hydrogen bonds. Although they are much weaker than covalent bonds, their high number and ability to exchange under stress make them major determinants of gluten network properties. Further, the dough weakening effect of hydrogen bond-breaking agents such as urea and the dough strengthening effect of heavy water compared to that of ordinary water illustrates the importance of hydrogen bonds in the structure of the gluten network. Hydrophobic bonds also contribute to the structure of the gluten network. They result from interactions of nonpolar groups in the presence of water. Their functionality in the gluten

network is probably similar to that of hydrogen bonds, although their overall contribution is thought to be less (Ooms and Delcour, 2019). In studying dough formation, we are limited to observing physical events on a macroscale, at the supra-molecular level (Stauffer, 1999). Many techniques have been adapted for the study of dough, e.g., X-ray Analysis, Nuclear Magnetic Resonance (NMR), Differential Scanning Calorimetry (DSC), Scanning Electron Microscopy (SEM), but the interpretation of the results is very difficult given the complexity of the matrix. Brewer's yeast (*Saccharomyces cerevisiae*) is the main leavening agent in the pizza making process. During leavening, yeast metabolism switches from respiration to fermentation, converting fermentable sugars into ethanol, carbon dioxide and other metabolites. The alcoholic fermentation metabolic pathway can be simplistically described by the reaction:

$$C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2\ CO_2.$$

During fermentation, colloidal and biochemical processes continue, the yeast cells produce CO_2, which partly dissolves in the aqueous phase of the dough and forms weakly ionizable carbonic acid that slightly lowers the pH of the dough. When this phase is exhausted, the carbon dioxide subsequently produced may vaporize into the environment or pass into the air nuclei formed within the dough during the mixing phase (Autio and Laurikainen, 1997). As the CO_2 passes into the cores, they expand due to the increase in pressure, generating an overall increase in the volume of the dough and the formation of gaseous alveoli (Cooper and Reed, 1968). During this phase, the properties of the gluten change: the gluten protein molecules continue to swell, absorbing carbon dioxide produced by the yeast, stretch and bind together, making the dough spongier (Garcia-Hernandez et al., 2022). In addition, the proteolysis reaction takes place, which makes the dough more malleable. In fact, at the same time, ripening is observed, in which a set of enzymatic processes take place that break down complex structures, such as proteins and starches, into simpler elements, as amino acids and fermentable sugars for yeast metabolism (Yiltirak et al., 2021). The enzyme α-amylase attacks starch with subsequent production of reducing sugars, this phenomenon, makes the dough structure less interconnected, and thus the dough is less elastic and more digestible. In fact, our body is unable to assimilate these long chains and needs them to be broken down into simple sugars (Struyf et al., 2017). A pizza made with a ripe dough will require less effort, from our digestive system, due to this earlier simplification of starches. Good coordination of leavening and maturation ensures the perfect outcome of the final product. The functionality of yeast in baking is not limited to gas production, however, as yeast produces several other metabolites that can affect the final quality of the product. In addition to the primary metabolites CO_2 and ethanol, significant amounts of secondary metabolites, such as succinic acid, acetic acid, and glycerol, have also been detected in fermented dough. Contrary to common belief, the drop in pH associated with fermentation is not caused by the dissolution of CO_2 in the aqueous phase of the dough, but mainly results from the accumulation of succinic acid (Jayaram et al., 2013). Some studies have shown that these metabolites had a clear impact on the tensile strength and extensibility of the (unfermented) dough and found that glutathione, which can end up in the dough matrix through escape from dead yeast cells, significantly softens the dough (Verheyen et al., 2014; Meerts et al., 2018). For instance, the macro-properties of dough change with time. At the end of the mixing process, the dough has certain viscoelastic characteristics, then, during the resting time, changes these properties and

makes the dough more pliable (relaxed). Splitting and rounding partially reverse this phenomenon and the dough appears more elastic (less relaxed). An intermediate leavening time decreases the elasticity, allowing good molding into the shape of a round disc (Stauffer, 1999). An important quality factor for a good pizza is the dough volume that depends on the ability to retain gas (carbon dioxide), generated during fermentation (leavening), in the form of numerous small gas cells; a proper balance of viscous flow and elastic strength so that the dough can expand adequately during leavening and the early stages of baking.

Freezing process and frozen storage are used in the bakery to extend shelf life and preserve freshness of the product. Generally, commercial products depend on the time of the freezing step in manufacturing process: unfermented and/or prefermented frozen dough and baked or partially baked frozen products (Lorenz and Kulp, 1995). Several studies (Angioloni et al., 2008; Meziani et al., 2012; Wang et al., 2015) determined differences on structural changes induced in the gluten network of the dough during process of dough freezing, by the formation of ice crystals during storage.

Baking is associated by evaporation of water, denaturation of protein, and gelatinization of starch (Masi, 1989). This radically changes the distribution of water between the dough phases. Starch is the main (~70 % by weight) component of the dough and is more hydrophilic than protein. Since starch granules are a filler of both the liquid and the gelatinous protein phase of the dough, starch gelatinization can cause dehydration of both dough phases (Dreese et al., 1988). The glass transition of the glutinic phase fixes its structure, shape and volume, and delays deterioration (Toistoguzov, 1997). During the gelation process, water breaks down the crystallinity of the starch, the granules swell, amylose diffuses out of the granules, leaving most of the amylopectin behind, and the granules eventually collapse and are held in an amylose matrix as part of a gel network (Jekle et al., 2016). High temperatures denature proteins, and can undergo extensive cross-linking, particularly through the formation of disulphide bonds, thus forming a continuous protein network. When proteins and starch are in contact with each other, stable complexes can develop by formation of a protein/starch matrix, where hydrogen and covalent bonds as well as charge-charge interactions can be found (Marshall and Chrastil, 1992). Another very important phenomenon that occurs during baking is the volume increase of gas bubbles (alveoli) present in the dough and the conversion from a closed cell structure to an interconnected open cell structure. This phenomenon is related to several factors ranging from thermal expansion of the gas present in the bubbles, increased enzymatic activity of the yeast with further gas production, evaporation of the water, ethanol and volatile organic compounds, and reduction in the solubility of the gaseous substances dissolved in the liquid phase (Masi et al., 2015). Rapid heating leads to rapid expansion of all gaseous substances, and the alveoli may expand due to increased internal pressure (Garcia-Hernandez et al., 2022). This phase is called oven rise. When the temperature reaches about 50 °C, the yeasts die and most of the enzymatic activity stops. The gases in the dough continue to expand, carbon dioxide, ethanol and other components, move from the aqueous to the gaseous phase and migrate inside the bubbles, while water, in the form of vapor, enters the bubbles and inflates them. This transition leads to another increase in volume, called oven spring. In addition, at high temperatures and in areas not covered by topping, the Maillard reaction takes place, which leads to the formation of certain aromatic

compounds, typical of the pizza smell, and causes the appearance of the typical brown colour of baked foods (Mottram et al., 2002). This reaction occurs only in the areas not covered by topping, because temperature is about 180 °C, at which the deamidation reaction of glutamine and asparagine is triggered, with release of ammonia that starts the reaction. All these considerations must be taken into account if the dough is to have the optimal handling (workability), leavening and baking (dough volume), and finished product (appearance, food quality) characteristics to achieve the desired baked product (pizza).

Nutritional aspect: starch digestibility and glycemic index

The cereal products most frequently consumed in Europe are white wheat flour baked foods, such as bread (Kulp and Ponte, 2000) and pizza, which causes a very rapid rise in blood glucose level. In recent years, in the context of worldwide health concerns, a great deal of interest has emerged in the prevention and control of glucose absorption due to the ingestion of starchy foods. Baked food products can be classified according to their digestibility, which is generally characterised by the rate and the duration of the glycemic response. Since its introduction in the 1980s, the glycemic index (GI) has become accepted as the basic comparative parameter for evaluating the physiological responses to the ingestion of carbohydrates derived from different foods, despite some controversies. Foods with a low (<55) or moderate (<70) GI have been considered healthier, particularly with regard to reducing the risk of metabolic diseases, such as obesity and type 2 diabetes, and cardiovascular disease (Jenkins et al., 2002).

Pizza is a medium-energy-density, high-carbohydrate product. It is a good source of protein, minerals, and B vitamins. Starch is the most abundant component of wheat flours, since carbohydrates constitute about 70-80 %, followed by proteins which are about 10-14 %, and a small percentage accounted for by lipids, fibre, vitamins and minerals. Starch is formed of amylose molecule, that is linear polymer of α-D-glucose units linked by α-1,4 glycosidic linkages and amylopectin molecule, which is branched polymer of α-D-glucose units linked by α-1,4 and α -1,6 glycosidic linkages (Singh et al., 2010). In most conventional baked products, the starch present is rapidly digested and absorbed, thus causing a high GI. The concept of glycemic index has been introduced to classify foods on the basis of their postprandial blood glucose response. The glycemic index is defined as the postprandial incremental glycemic area after a test meal, expressed as the percentage of the corresponding area after an equi-carbohydrate portion of a reference food such as glucose or white bread (Jenkins et al., 1987; Goñi et al., 1997;). The problems associated with the difficulty of using the glycemic index as an indicative parameter are the high cost, time and complex methodology of analysis. For this reason, there have been several proposals in recent years of *in vitro* methods for measurement of the glycemic response (Englyst et al., 1992; Granfeldt et al., 1992; Goñi et al., 1997; Englyst et al., 2000; Angioloni and Collar 2011). The starch hydrolysis index has been considered a good predictor of starch digestibility and, therefore, of the glycaemic response. The *in vitro* starch digestibility method is very useful because it had good correlations when compared with the glycemic response to food *in vivo* GI tests (Anderson et al., 2010; Ronda et al., 2012). Specifically, the method is based on the *in vitro* digestion of starch by simulating stomach and intestinal conditions and measuring glucose release at different times. In the

case of cereal derivatives, the method developed by Englyst et al. (1992) and its subsequent modifications (Englyst et al., 1999, 2000) have been widely used for this estimation, in which starch are classified into three different fractions: rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS). Resistant starch has been defined as the portion of starch that is not hydrolysed by the enzymes in the small intestine and passes to the large intestine (Asp, 1992). RDS and SDS values exhibited a good relationship with GI in humans (Englyst and Englyst 2005), confirming the method reliability. The Starch Digestion Rate Index (SDRI) represents an indicator of the *in vitro* starch digestibility. The rapidly available glucose (RAG) index is a predictor of the potential glycemic response derived from the ingestion of these food items (Englyst et al., 1996). Other components present in flour, such as proteins, lipids, and non-starch polysaccharides, can affect the enzymatic digestibility of starch, considering possible changes in the interactions between these substances during the various stages of dough preparation (Jenkins et al., 1987). The digestible starch is mainly hydrolyzed by the enzymes into glucose through several steps. Salivary α-amylase acts on starch at the beginning of digestion, after which the starch is rapidly degraded in the acidic environment of the stomach. However, starch is most degraded by pancreatic amylase, which is released into the small intestine through the pancreatic duct. The α-amylase (1,4 α-D-glucanohydrolase, EC 3.2.1.1) catalyzes the hydrolysis of α-1,4 glycosidic bonds in amylose and starch amylopectin (Lehmann and Robin, 2007). α-Amylases lack specificity for α-1,6 branching bonds in amylopectin, so their ability to break α-1,4 bonds adjacent to the branching point is reduced mainly because of steric hindrance (Gray, 1992). The hydrolysis end products of amylose digestion are mainly maltose, maltotriose, and maltotetraose (Yook and Robyt, 2002). Whereas the hydrolysis products of amylopectin consist mainly of dextrins or branched oligosaccharides. These oligosaccharides further undergo enzymatic hydrolysis in the intestine. In past years, many studies have focused on different methods of starch digestibility *in vitro*, such as varying sample preparation, use of amylases and proteases. Table 1 shows some recent studies on starch digestibility of different starch and food composition. Indeed, the rate of starch hydrolysis varies greatly among different foods, from the lowest for lentils to the highest for boiled potatoes. Thus, the nature of starch in foods and the size of starch granules also affect digestibility and consequently the glycemic response (Kaur et al., 2010). In general, most food starches contain 25-30% amylose and 70-75% amylopectin (Hoover and Zhou, 2003). Higher amylose content reduces starch digestibility, as there is a positive correlation between amylose content and resistant starch formation. In addition, the larger starch granules showed a lower hydrolysis rate than the medium and small granule fractions. This is due to a lower specific surface area of the large granules, which reduces enzyme binding compared to the small granules (Tester et al., 2006). Furthermore, wheat starch compared to other type of starches has more pores in the granule that facilitate the entry of amylases for digestion. The stages of dough leavening and baking cause an alteration in the structure of the final product, affecting the digestibility of starch. It has been shown that there is an increase in the hydrolysis of starch as dough is baked, in fact, when starch molecules are heated in the presence of water, the water molecules bind with a hydrogen bond to the exposed hydroxyl groups of amylose and amylopectin, causing an increase in the swelling and solubility of the granules, which is determinant for the degree of starch

digestibility (Anguita et al., 2006). Indeed, baking increases the rate of hydrolysis by gelatinizing the starch and making it more readily available for enzymatic attack, because it facilitates starch availability for water penetration and consequent α-amylase action. During the gelatinization of starch, the crystalline structure of amylopectin disintegrates, and the polysaccharide chains take up a random configuration thus causing the swelling and rupturing of the starch granules (Singh et al., 2006). In addition to processing, another factor that influences starch digestibility and glycemic response is the presence of proteins on the surface of starch granules. The protein network may inhibit the rate of hydrolysis in the lumen of the small intestine. Moreover, protein fractions such as albumin, globulins, and glutenins contribute to bonding protein bodies into a matrix surrounding the starch granules that can act as a barrier to starch digestibility (Hamaker and Bugusu, 2003). *In vitro* digestion studies showed that the concentration of total starch digestion products was significantly lower for white bread than for gluten-free bread (Jenkins et al., 1987).

Table 1 – Digestibility of different starch and starch-based foods (Singh et al., 2010)

Source	Starch digestibility	Reference and method
Cooked potatoes	>90[a]	Mishra *et al.* (2008); *in vitro* simulated gastric and small intestinal digestion using pancreatin and amylogucosidase
Sorghum meal	60–85[b] and 40–47[c]	Wong *et al.* (2009); *in vitro* starch digestion with pepsin and without pepsin pre-treatment using pepsin (porcine stomach mucosa) and α-amylase (bacterial; porcine pancreas; human saliva)
Cooked rice noodle dough	43[d] and 33[d]	Koh *et al.* (2009); *in vitro* digestion using α-amylase from *Aspergillus oryzae*
Waxy maize starch	100[e]	Han and BeMiller (2007); *in vitro* digestion by the method of Englyst *et al.* (1999) using pepsin, pancreatin and amyloglucosidase
Chemically modified waxy maize starch	76–87[e]	
Normal maize starch	99[e]	
Chemically modified normal maize starch	71–96[e]	
Potato starch	96[e]	
Chemically modified potato starch	67–76[e]	
Wheat flour	72[f]	Englyst *et al.* (1999); *in vitro* digestion using pepsin, pancreatin and amyloglucosidase
Corn flakes	81[f]	
Cooked rice	70–80[g]	Frei *et al.* (2003); *in vitro* digestion by the method of Goni *et al.* (1997) using pepsin, α-amylase and amyloglucosidase from *Aspergillus niger*
Extruded amaranth seeds	93[h]	Capriles *et al.* (2008); *in vitro* digestion by the method of Goni *et al.* (1997) using pepsin, α-amylase and amyloglucosidase
Amylose-lipid complexes	48–71[i]	Crowe *et al.* (2000); *in vitro* digestion using α-amylase and amyloglucosidase
Legume starches	80–90[j]	Hoover and Zhou (2003); treatment with porcine pancreatic α-amylase
Extruded beans	290–306[k]	Alonso *et al.* (2000); *in vitro* digestion with pancreatic amylase
Autoclaved legumes	87–89[l]	Rehman and Shah (2005); *in vitro* digestion with pancreatic α-amylase

[a] Expressed as rapidly and slowly digestible starch (%).
[b] Expressed as mg reducing sugar/h (with pepsin pre-treatment).
[c] Expressed as mg reducing sugar/h (without pepsin pre-treatment).
[d] Expressed as mg of maltose equivalent liberation per g of dough.
[e] Expressed as digestibility (%).
[f] Expressed as total glucose after 120 min incubation with enzymes.
[g] Expressed as digestible starch (%).
[h] Expressed as hydrolysis index.
[i] Expressed as conversion to glucose (%).
[j] Expressed as hydrolysis (%).
[k] Expressed as starch digestibility (mg of maltose g-1).
[l] Expressed as starch digestibility (%).

The physical texture of the cereal products can also influence the digestion of starch and the absorption of hydrolysis products (Tester et al., 2006; Dona et al., 2010). For example, the long fermentation time may expand the gluten network in which the starch is embedded, whereby the reduction in cohesion between starch and dough protein may increase the accessibility of starch to α-amylase, resulting in increased digestibility (Garcia-Hernandez et al., 2022). On the contrary, dietary fibre has been suggested as the primary factor influencing the slower rate of

glucose release in foods through its high viscosity which slows down gastric emptying absorption of digested products in the small intestine. Nowadays much attention is being given to SDS of starches for the development of foods low in rapidly digestible starch and hence of low glycemic index. SDS is in fact considered to be beneficial for the slow and prolonged release of glucose in metabolic disorders such as diabetes or glycogen storage diseases (Zhang and Hamaker, 2009), and satiety (Lehmann and Robin, 2007). There are several strategies for lowering the glycemic index of baked products including the use of whole grains and/or cereal flours other than wheat such as rye, barley, or oats, enrichment in soluble fibre, the use of resistant starch, or the addition of amylose in the formulation (Bello-Perez et al., 2020). In a paper, Östman et al. (2006), show how it is possible to reduce the glycemic index of bread by about 50 percent using different technological approaches simultaneously, such as the use of fibre and sourdough leavening. In some studies, however, it is suggested that the hypoglycemic impact of breads made from acidic dough, compared to traditional bread, is attributable, at least in part, to an effect of volatile organic acids formed during the leavening process. The presence of lactic acid in the dough involves interactions, established during heat treatment (baking), between starch and gluten at the microstructural level, which can reduce the digestibility of starch (Östman et al., 2002). Moreover, many studies on sourdough fermentation of flours showed an improved *in vitro* protein digestibility and reduced the glycemic index (GI) (Coda et al., 2010; Gabriele et al., 2019; Rizzello et al., 2019). Finally, in the study by Marangoni and Poli (2007), the glycemic responses of two types of bread prepared with wheat flour and with wheat flour supplemented with a fibre mix were evaluated. Analysis of the glycemic curves showed that bread made with fibre-enriched flour leads to a 21% reduction in the glycemic index. Experimental evidence has revealed that low GI diets have a potential preventive and therapeutic effect not only in diabetic subjects, in whom they predominantly result in improved insulin sensitivity, with significant reductions in basal blood glucose and glycosylated hemoglobin values, but also in subjects with dyslipidemia, and even in healthy subjects (Jenkins et al., 1987), by reducing cholesterol and triglyceride levels and increasing insulin sensitivity.

Human health risk: mitigation of acrylamide formation

Acrylamide (AA), or 2-propenamide, is an organic compound, with molecular formula C_3H_5NO. It presents as a crystalline solid, white in colour and has a molecular weight of 71.08 kDa. Its melting point is about 84.5±0.3 °C, it has a low vapor pressure (0.007 mmHg at 25°C) and a high boiling point, corresponding to 136 °C at 3.3 kPa/25 mmHg. In addition, its solubility in water is extremely high (215.5 g/100 mL at 30 °C) (Keramat et al., 2011). Appreciable levels of acrylamide can be found not only in the smoke released from cigarettes, but also as an environmental contaminant, since acrylamide polymers find a wide range of applications as flocculants for wastewater treatment, and as adjuvants in the textile and cosmetic industries (Esposito et al., 2017). Acrylamide is a difunctional monomer in that it exhibits both a highly reactive double bond and an amide group. In terms of its chemical behavior, it exhibits both weakly acidic and basic character. In fact, the carboxyamide group, which attracts electrons to itself, activates the double bond and, consequently, makes possible the attack of

nucleophilic agents, through 1,4 - addition reactions. Many of these reactions are reversible and, their rate, depends on the strength of the nucleophilic agent. Examples are the addition of ammonia, amines and bisulfites, and, under alkaline conditions, the addition of ketones and alcohols could also occur (Habermann, 1991). The presence of AA in food was first reported by the Swedish National Food Agency and Stockholm University in April 2002, who reported that some starchy foods such as potatoes and cereals, treated at high temperatures, had elevated levels of acrylamide (Keramat et al., 2011). In fact, it has been shown that acrylamide is not present in raw native ingredients, such as raw potatoes or cereals but, is formed in carbohydrate-rich foods that are treated at high temperatures such as those encountered during frying, grilling or baking, and roasting (Tareke et al., 2002). Heat-treated food products derived from plant ingredients such as potatoes and cereals tend to contain the highest amounts of acrylamide, the concentration of which, in some cases, exceeds 500 µg/kg (Lineback et al., 2012). The extensive research conducted on the formation of acrylamide in food products treated at high temperatures has concluded that the main mechanism of formation of this organic compound, involves the Maillard reaction (MR), and has amino acids and reducing sugars as precursors. Asparagine was found to be the main aminoacid involved in the formation of acrylamide in food products, processed at high temperatures and in the presence of reducing sugars (Mottram et al., 2002). The mechanism (Figure 2) involving asparagine, begins with the formation of a Schiff base, produced from the reaction between the carbonyl source and the α-amino group of free asparagine.

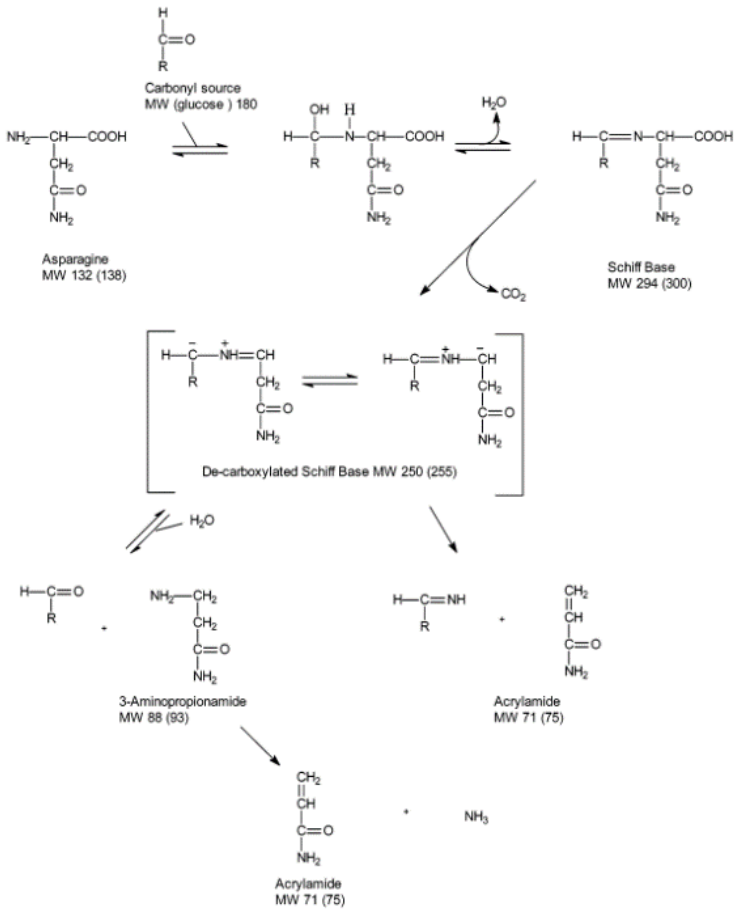

Figure 2 – Mechanism of acrylamide formation from asparagine (Zyzak, 2003).

During heating, there is decarboxylation of the Schiff base, which leads to the formation of an intermediate product that can react in two ways (Zyzak et al., 2003): (1) by hydrolysis, it can give rise to 3-aminopropionamide, which, in turn, can degrade further by removal of ammonia to form AA; and (2) by decomposition, it can directly give rise to acrylamide by elimination of an imine. Although the dominant route of formation of acrylamide in foods is through the reaction between asparagine and reducing sugars, there are alternative pathways that could lead to its formation. In fat-rich foods, such as French fries, a precursor to acrylamide could be acrolein or 1-propenal (C_3H_4O). Acrolein is formed by dehydration of glycerol, which is released during the process of lipid oxidation, when foods of both animal and plant origin, are treated at high temperatures (Umano and Shibamoto, 1987). Acrolein, in turn, can react by oxidation to generate acrylic acid which, in the presence of a nitrogen source, can result in the formation of AA. The nitrogen source, in addition to asparagine, can also be represented by glutamine and, both amino acids, can rapidly produce a three-carbon atom unit (Yasuhara et al., 2003). In foods high in reducing sugars, lipid oxidation has little influence on acrylamide formation whereas, the effect of oxidation, is greater in matrices with low water activity and low carbohydrate concentration. Furthermore, it has been shown that the addition of antioxidants, such as catechin, could prevent acrolein oxidation and, consequently, mitigate acrylamide formation in fat-rich products (Capuano et al., 2010). For the determination of acrylamide, most laboratories use HPLC methods. Paleologos and Kontominas (2005) developed a method using normal-phase HPLC with UV detection for the analysis of acrylamide and methylacrylamide in foods such as potatoes, cookies, cocoa, and Greek coffee. HPLC techniques possess advantages of versatility and lower cost than mass spectrometry techniques (Wang et al., 2008).

AA can be introduced into the body either by oral intake or by inhalation and, once absorbed, is converted to glycidamide, a reactive epoxide metabolite believed to be responsible for the genotoxic effects associated with acrylamide exposure. In fact, both acrylamide and glycidamide, can react with macromolecules, such as hemoglobin and DNA, to form adducts. It is precisely the formation of adducts with DNA, which is believed to be responsible for the toxic and carcinogenic potential of acrylamide (Sarion et al., 2021). In fact, in 1994, acrylamide was classified as a "possible human carcinogen" by the International Agency for Research on Cancer (IARC, 1994). Since a correlation between AA exposure and a possible health risk has been reported, numerous estimates have been made of dietary intake levels in different populations, and great variability has been found depending on the dietary habits of different individuals constituting the populations. The World Health Organization estimates a daily dietary intake of AA of 0.3-2.0 mg/kg body weight for the general population and up to 5.1 mg/kg body weight for the 99th percentile of consumers (WHO, 2005; Capuano et al., 2011). In 2015, the Panel on Contaminants in the Food Chain (CONTAM Panel) of the European Food Safety Authority (EFSA), issued an opinion on acrylamide. Based on animal studies, the Authority confirms that AA in food can increase the risk of developing cancer for consumers in all age groups, although, children appear to be the most exposed population group, based on body weight, likely due to high consumption in acrylamide-rich foods, such as potato chips (EFSA, 2015). In addition, experts estimated the dose range within which acrylamide is likely to cause a slight but measurable incidence of cancer or other potential adverse effects; the

lower limit of this range was set at 0.17 mg/kg body weight per day (EFSA, 2015). Considering the conclusions reached by EFSA, regarding the carcinogenic effects of acrylamide, food companies should establish appropriate mitigation measures in order to reduce AA levels. These measures can be made by applying procedures based on the principles of hazard analysis and critical control points (HACCP). As indicators to verify the effectiveness of mitigation measures, the European Commission has highlighted reference levels for acrylamide in various food products. The most recent ones are shown in Table 2 and were established in EU Regulation 2017/2158. The Regulation applies to food business operators (FBOs) producing and placing on the market the following products: bread and baked foods, breakfast cereals, baby food, and processed cereal-based food products for young children. In conclusion, FBOs covered by the Regulation, must identify, depending on the type of activity carried out, the processing steps in which AA production may occur and must determine, as part of their risk analysis, appropriate containment measures (Sarion et al., 2021).

Many attempts have been put into practice to mitigate the formation of toxic MR products in processed foods, particularly the selection of suitable raw materials, changes in recipe formulation, and optimization of process technologies (Kumar et al., 2014). The enzymatic approach to modify AA formation reaction pathways was first proposed by Amrein et al. (2004), who used asparaginase to hydrolyse asparagine into aspartic acid and ammonia, thereby successfully reducing acrylamide levels in carbohydrate-rich products. This approach is considered effective because asparagine is not considered an important factor in the overall flavour and colour of cooked foods (Parker et al., 2012), so its hydrolysis does not result in a change in the sensory properties of the product.

Table 2 – Reference levels of acrylamide in food the European Commission Regulation 2017/2158.

Food	Benchmark level [µg/kg]
French fries (ready-to-eat)	500
Potato crisps from fresh potatoes and from potato dough Potato-based crackers Other potato products from potato dough	750
Soft bread (a) Wheat based bread (b) Soft bread other than wheat based bread	 50 100
Breakfast cereals (excl. porridge) — bran products and whole grain cereals, gun puffed grain — wheat and rye based products (1) — maize, oat, spelt, barley and rice based products (1)	 300 300 150
Biscuits and wafers Crackers with the exception of potato based crackers Crispbread Ginger bread Products similar to the other products in this category	350 400 350 800 300
Roast coffee	400
Instant (soluble) coffee	850
Coffee substitutes (a) coffee substitutes exclusively from cereals (b) coffee substitutes from a mixture of cereals and chicory (c) coffee substitutes exclusively from chicory	 500 (2) 4 000
Baby foods, processed cereal based foods for infants and young children excluding biscuits and rusks (3)	40
Biscuits and rusks for infants and young children (3)	150

(1) Non-whole grain and/or non-bran based cereals. The cereal present in the largest quantity determines the category.
(2) The benchmark level to be applied to coffee substitutes from a mixture of cereals and chicory takes into account the relative proportion of these ingredients in the final product.
(3) As defined in Regulation (EU) No 609/2013.

Asparaginase (L-asparaginase amidrolase EC 3.5.1.1) is a widely distributed enzyme in animals, plants, and living organisms (Wriston and Yellin, 1973). Asparaginase has been shown to catalyze the hydrolysis of asparagine into aspartic acid and ammonia by hydrolyzing the amide group in the asparagine side chain (Hendriksen et al., 2009). Most asparaginases are quite specific for asparagine, and optimal activity is usually achieved under conditions of pH 5-7 and temperature of 37 °C. Crystallographic studies have shown that both types of asparaginases, asparaginase and glutaminase-asparaginase, have the same basic structure and same catalytic mechanism, but differ under the optimal conditions of activity, such as pH and incubation temperature (Xu et al., 2016). In 1976, Murthy and Knox performed small-angle X-ray diffraction studies on solutions of L-asparaginase extracted from *E. coli*, which showed that the enzyme had a radius of rotation of 34.0±0.5 ANG at pH 7; from these studies, it was shown that the enzyme is a tetramer, and each monomer has the shape of a prolate ellipsoid. The three-dimensional model of the enzyme is shown in Figure 3. Most bacterial L-asparaginases show tertiary and quaternary structures (Ramya et al., 2011). Asparaginase has been found in both Gram-positive and Gram-negative bacterial species from both terrestrial and marine environments (Izadpanah Qeshmi et al., 2014), although Gram-negative asparaginases have been given more consideration than Gram-positive asparaginases (Asthana et al., 2003). The asparaginases of most Gram-negative bacteria can be categorized into two main categories: L-asparaginase I and L-asparaginase II. Asparaginase I possesses enzymatic activity on both glutamine and asparagine, while asparaginase II has specific activity only on asparagine and functions only under anaerobic conditions (Sanches et al., 2007). Along with bacteria, fungi are also a potential source of L-asparaginase: fungal asparaginase has gained importance on the basis that it is an extracellular enzyme, thus very easy to purify (Batool et al., 2016). Another source of L-asparaginase is yeasts: asparaginases extracted from yeasts such as *Saccharomyces sp.*, *Candida sp.*, *Pichia sp.*, and *Spobolomyces sp.* have shown antitumor activity (Nagarethinam et al., 2012).

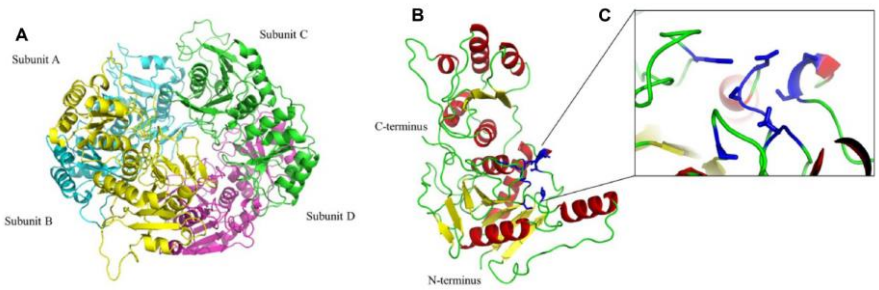

Figure 3 – Three-dimensional structure of L-asparaginase. (A) Tetramer of L-Asparaginase with identical subunits coloured in cyan, yellow, green and pink. (B, C) A representation of the L-Asparaginase monomer: α-helices are shown in red and β-sheets in yellow. The active site residues are shown in blue (Lee et al., 2016).

Treatment of several food products with the asparaginase enzyme in pure form, such as ginger bread dough, resulted in a nearly 70-75% decrease in free asparagine with 50-55% reduction in acrylamide in the finished baked product (Muneer et al., 2020). Haase et al. (2003) studied acrylamide formation in baked foods and identified the degree of flour refining and baking temperature as important factors influencing the final acrylamide concentration in bread. In

Kumar et al. (2014) experiment, L-asparaginase enzyme was mixed into the dough such that it hydrolyzed L-asparagine present in the flour, making it no longer available for MR. The results of analysis of the baked bread showed that when the bread was prepared with the addition of enzyme there is a reduction of acrylamide in the product, in fact, the reduction increases as the concentration of the enzyme increases. In the untreated bread, the crust had an acrylamide content of 729 μg/kg and the crumb 143 μg/kg; in the dough treated with 50 U of enzyme, there was a 73 percent reduction in acrylamide formed in the crust, while in the dough treated with 100 U of enzyme, the reduction was 92 %.

Cereals are one of the main foods consumed around the world and, in addition, are the basis to produce many baked foods such as bread, pizza, cookies, and crackers. Wholemeal flours are rich in fibre, which can slow the release of glucose into the bloodstream, associated with the optimal maintenance of glucose-insulin balance (Aune et al., 2016). It has been shown that, a higher intake of whole grains in the diet, is associated with a reduced risk of incidence of cardiovascular disease, type 2 diabetes and obesity. However, dietary fibre is concentrated in the outermost layers of the caryopsis, namely the bran, which turns out to be the main resource to produce acrylamide precursors in processed whole grain foods. In fact, it has been seen that, under the same baking conditions, bread made with wholemeal wheat flour has a higher concentration of acrylamide, compared to bread made with refined flour, thus devoid of bran (Capuano et al., 2009). As mentioned earlier, the precursors of acrylamide in grain products are the aminoacid asparagine and reducing sugars. The latter are abundant in grains and, consequently, the free asparagine content is the limiting factor for acrylamide formation in baked foods (Keramat et al., 2011). Numerous strategies to mitigate acrylamide formation have been studied and included in the "Acrylamide Toolbox" (acrylamide reduction guidelines) published by FoodDrinkEurope in 2019, and among them is the use of grain varieties with low asparagine content. It has been shown that, asparagine accumulates in wheat grains mainly in response to various stressor stimuli such as disease, lack of water and nutrients, particularly when nitrogen is abundant, but not so much to ensure adequate protein levels, due to deficiency of the other minerals (Lea et al., 2007). In fact, the use of nitrogen fertilizers is suggested to be reduced to an absolute minimum, during the production cycle of wheat grains but, the use of sulfur fertilizers is recommended, to avoid deficiencies, and the use of fungicides to prevent possible diseases (FoodDrinkEurope, 2019). An excess in nitrogen affects the expression of the gene that regulates asparagine production in many wheat grain species, and, in addition, the massive application of nitrogen fertilizers compromises both food security and numerous environmental aspects. Overall, crop management strategies are crucial to keep asparagine levels as low as possible, although, the accumulation of this aminoacid, is influenced, not only by management factors such as, growing conditions, harvest time and postharvest storage conditions, but also by variety and genotype. Thus, a good asparagine mitigation strategy in wheat grains could be the integration of proper management techniques and genotype selection (Oddy et al., 2020).

References

- Amrein, T.M., Schönbächler, B., Escher, F., and Amadò, R. (2004). Acrylamide in gingerbread: critical factors for formation and possible ways for reduction. Journal of agricultural and food chemistry, 52(13), 4282-4288.
- Anderson, G.H., Cho, C.E., Akhavan, T., Mollard, R.C., Luhovyy, B.L., and Finocchiaro, E.T. (2010). Relation between estimates of cornstarch digestibility by the Englyst in vitro method and glycemic response, subjective appetite, and short-term food intake in young men. The American journal of clinical nutrition, 91(4), 932-939.
- Angioloni, A., Balestra, F., Pinnavaia, G.G., and Dalla Rosa, M. (2008). Small and large deformation tests for the evaluation of frozen dough viscoelastic behaviour. Journal of Food Engineering, 87(4), 527-531.
- Angioloni, A., and Collar, C. (2011). Physicochemical and nutritional properties of reduced-caloric density high-fibre breads. LWT-Food Science and Technology, 44(3), 747-758.
- Anguita, M., Gasa, J., Martın-Oru, S.M., and Perez, J.F. (2006). Study of the effect of technological processes on starch hydrolysis, nonstarch polysaccharides solubilization and physicochemical properties of different ingredients using a two-step in vitro system. Animal Feed Science and Technology, 129, 99-115.
- Asp, N.G. (1992). Resistant starch. Proceeding from the second plenary meeting of EURESTA: European FLAIR concerted action n. 11 on physiological inplications of the consumption of resistant starch in man. European Journal of Clinical Nutrition, 46, S1.
- Asthana, N., and Azmi, W. (2003). Microbial L-asparaginase: A potent antitumour enzyme. Indian Journal of Biotechnology, 2:184-194.
- Aune, D., Keum, N., Giovannucci, E., Fadnes, L.T., Boffetta, P., Greenwood, D.C., Norat, T. (2016). Whole grain consumption and risk of cardiovascular disease, cancer, and all cause and cause specific mortality: Systematic review and dose-response meta-analysis of prospective studies. British Medical Journal, 353, 2716.
- Autio, K., and Laurikainen, T. (1997). Relationships between flour/dough microstructure and dough handling and dough handling and baking properties. Trends Food Science and Technology, 8, 181–185.
- Batool, T., Makky, E.A., Jalal, M., and Yusoff, M.M. (2016). A comprehensive review on L-asparaginase and its applications. Applied biochemistry and biotechnology, 178(5), 900-923.
- Bello-Perez, L.A., Flores-Silva, P.C., Agama-Acevedo, E., and Tovar, J. (2020). Starch digestibility: Past, present, and future. Journal of the Science of Food and Agriculture, 100(14), 5009-5016.
- Bushuk, W. (1985). Flour proteins: Structure and functionality in dough and bread. Cereal Foods World, 30, 447–51.
- Canali, L., and Paratore, E. (2015). La Grande Biblioteca dei Classici Latini e Greci: Virgilio, Eneide. Fabbri Centauria S.r.l., Milano.
- Capuano, E., Ferrigno, A., Acampa, I., Serpen, A., Açar, Ö. Ç., Gökmen, V., and Fogliano, V. (2009). Effect of flour type on Maillard reaction and acrylamide formation during toasting of bread crisp model systems and mitigation strategies. Food Research International, 42(9), 1295-1302.
- Capuano, E., Oliviero, T., Açar, Ö., Gökmen, V., and Fogliano, V. (2010). Lipid oxidation promotes acrylamide formation in fat-rich model systems. Food Research International, 43, 1021-1026.
- Capuano E., and Fogliano V. (2011). Acrylamide and 5-hydroxymethylfurfural (HMF): A review on metabolism, toxicity, occurrence in food and mitigation strategies. LWT-food science and technology, 44(4), 793-810.
- Coda, R., Cassone, A., Rizzello, C.G., Nionelli, L., Cardinali, G., and Gobbetti, M. (2011). Antifungal activity of Wickerhamomyces anomalus and Lactobacillus plantarum during sourdough fermentation: Identification of novel compounds and long-term effect during storage of wheat bread. Applied and Environmental Microbiology, 77, 3484–3492.

- Commission Regulation (EU) 97/2010. Entering a name in the register of traditional specialities guaranteed (Pizza Napoletana (TSG). Official Journal of the European Union, 34, 7–13.
- Commission Regulation (EU) 2017/2158. Establishing mitigation measures and benchmark levels for the reduction of the presence as acrylamide in food. Official Journal of the European Union, 60, 24–44.
- Cooper, E.J., and Reed, G. (1968). Yeast fermentation: effects of temperature, pH, ethanol, sugar, salt and osmotic pressure. Bakers Digest, 42(6), 22–29.
- Dona, A.C., Pages, G., Gilbert, R.G., and Kuchel, P.W. (2010). Digestion of starch: In vivo and in vitro kinetic models used to characterise oligosaccharide or glucose release. Carbohydrate Polymers, 80(3), 599-617.
- Dreese, P.C., Faubion, J.M. and Hoseney, R.C. (1988). Dynamic rheological properties of flour, gluten and gluten-starch doughs. I. Temperature-dependent changes during heating. Cereal Chem. 65, 348–353.
- Englyst, H.N., Kingman, S.M., and Cummings, J.H. (1992). Classification and measurement of nutritionally important starch fractions. European journal of clinical nutrition, 46, S33-50.
- Englyst, H.N., Kingman, S.M., Hudson, G.J., and Cummings, J.H. (1996). Measurement of resistant starch in vitro and in vivo. British Journal of Nutrition, 75(5), 749-755.
- Englyst, K.N., Englyst, H.N., Hudson, G.J., Cole, T.J., and Cummings, J.H. (1999). Rapidly available glucose in foods: an in vitro measurement that reflects the glycemic response. The American journal of clinical nutrition, 69(3), 448-454.
- Englyst, K.N., Hudson, G.J., and Englyst, H.N. (2000). Starch analysis in food. Encyclopedia of analytical chemistry, 4246-4262.
- Englyst, K.N., and Englyst, H.N. (2005). Carbohydrate bioavailability. British Journal of Nutrition, 94(1), 1-11.
- Esposito, F., Nardone, A., Fasano, E., Triassi, M., and Cirillo, T. (2017). Determination of acrylamide levels in potato crisps and other snacks and exposure risk assessment through a Margin of Exposure approach. Food and Chemical Toxicology, 108, 249-256.
- EFSA, (2015). Outcome of the public consultation on the draft Scientific Opinion of the EFSA Panel on Contaminants in the Food Chain (CONTAM) on acrylamide in food. EFSA Supporting Publications, 12(6), 817E.
- FoodDrinkEurope (2019) Acrylamide toolbox 2019. FoodDrinkEurope, Brussels
- Gabriele, M., Sparvoli, F., Bollini, R., Lubrano, V., Longo, V., and Pucci, L. (2019). The impact of sourdough fermentation on non-nutritive compounds and antioxidant activities of flours from different Phaseolus vulgaris L. genotypes. Journal of Food Science, 84, 1929–1936.
- Ganguly, M. (2017). Naples' pizza spinning given UNESCO 'intangible heritage' status. http://edition.cnn.com/2017/12/07/europe/naples-unesco-pizza-intl/index.html.
- Garcia-Hernandez, A., Roldan-Cruz, C., Vernon-Carter, E.J., and Alvarez-Ramirez, J. (2022). Effects of leavening agent and time on bread texture and in vitro starch digestibility. Journal of Food Science and Technology, 59(5), 1922-1930.
- Goñi, I., Garcia-Alonso, A., and Saura-Calixto, F. (1997). A starch hydrolysis procedure to estimate glycemic index. Nutrition research, 17(3), 427-437.
- Granfeldt, Y., Björck, I., Drews, A., and Tovar, J. (1992). An in vitro procedure based on chewing to predict metabolic response to starch in cereal and legume products. European Journal of Clinical Nutrition, 46(9), 649-60.
- Gray, G.M. (1992). Starch digestion and absorption in nonruminants. Journal of Nutrition, 122, 172-177.
- Haase N.U., Matthaus B., Vosmann K. (2003). Acrylamide formation in foodstuffs-Minimising strategies for potato crisps. Deutsche Lebensmittel-Rundschau, 99(3), 87-90.

  Habermannn, C.E., (1991). Acrylamide. In: E. Kirk-Othmer Enzyclopedia of Chemical Technology, J. J. Kroschwitz, and M. Howe-Grant, ed., J. Wiley & Sons, New York, 4 (1), 251-266.

- Hamaker, B. R., & Bugusu, B. A. (2003, April). Overview: sorghum proteins and food quality. In Workshop on the proteins of sorghum and millets: enhancing nutritional and functional properties for Africa (Pretoria: South Africa).
- Hendriksen, H. V., Kornbrust, B. A., Østergaard, P. R., & Stringer, M. A. (2009). Evaluating the potential for enzymatic acrylamide mitigation in a range of food products using an asparaginase from Aspergillus oryzae. Journal of Agricultural and Food Chemistry, 57, 4168-4176.
- Hoover, R., and Zhou, Y. (2003). In vitro and in vivo hydrolysis of legume starches by a-amylase and resistant starch formation in legumes e a review. Carbohydrate Polymers, 54, 401-417.
- Hoseney, R.C. (1985). The mixing phenomenon. Cereal Foods World, 30, 453–7.
- IARC (1994). Acrylamide. In Some Industrial Chemicals. International Agency for Research on Cancer, 389–433.
- Izadpanah Qeshmi, F., Javadpour, S., Malekzadeh, K., Tamadoni Jahromi, S., and Rahimzadeh, M. (2014). Persian gulf is a bioresource of potent L-asparaginase producing bacteria: Isolation & molecular differentiating. International Journal of Environmental Research, 8(3), 813-818.
- Jayaram, V.B., Cuyvers, S., Lagrain, B., Verstrepen, K.J., Delcour, J.A., and Courtin, C.M. (2013). Mapping of Saccharomyces cerevisiae metabolites in fermenting wheat straight-dough reveals succinic acid as pH-determining factor. Food Chemistry, 136 (2), 301-308.
- Jekle, M., Mühlberger, K., and Becker, T. (2016). Starch–gluten interactions during gelatinization and its functionality in dough like model systems. Food Hydrocolloids, 54, 196-201.
- Jenkins, D.J.A., Thorne, M.J., Wolever, T.M.S., Jenkins, A.L., Rao, A.V., and Thompson, L.U. (1987). The effect of starch-protein interaction in wheat on the glycemic response and rate of in vitro digestion. American Journal of Clinical Nutrition, 45, 946-951.
- Jenkins, D.J., Kendall, C.W., Augustin, L.S., Franceschi, S., Hamidi, M., Marchie, A., and Axelsen, M. (2002). Glycemic index: overview of implications in health and disease. The American journal of clinical nutrition, 76(1), 266S-273S.
- Kaur, M., Sandhu, K.S., and Lim, S.T. (2010). Microstructure, physicochemical properties and in vitro digestibility of starches from different Indian lentil (Lens culinaris) cultivars. Carbohydrate polymers, 79(2), 349-355.
- Keramat J., LeBail A., Prost C., Soltanizadeh N. (2011). Acrylamide in foods: chemistry and analysis. A review. Food and bioprocess technology, 4(3), 340-363
- Kłosok, K., Welc, R., Fornal, E., and Nawrocka, A. (2021). Effects of physical and chemical factors on the structure of gluten, gliadins and glutenins as studied with spectroscopic methods. Molecules, 26(2), 508.
- Kulp, K., and Ponte, J. G. (2000). Breads and yeast-leavened bakery foods. In Handbook of cereal science and technology, 539-573.
- Kumar, N.M., Shimray, C.A., Indrani, D., Manonmani, H.K. (2014). Reduction of acrilamide formation in sweet bread with L-asparaginase treatment. Food and bioprocess technology, 7 (3), 741-748.
- Lea, P.J., Sodek, L., Parry, M.A., Shewry, P.R., and Halford, N.G. (2007). Asparagine in plants. Annals of Applied Biology, 150(1), 1-26.
- Lee, S.J., Lee, Y., Park, G.H., Umasuthan, N., Heo, S.J., Zoysa, M.D., and Oh, C. (2016). A newly identified glutaminase-free L-asparaginase (L-ASPG86) from the marine bacterium Mesoflavibacter zeaxanthinifaciens. Journal of Microbiology and Biotechnology, 26(6), 1115-1123.
- Lehmann, U., and Robin, F. (2007). Slowly digestible starch- its structure and health implications: a review. Trends in Food Science and Technology, 18(7), 346-355.
- Lineback, D.R., Coughlin, J.R. and Stadler, R.H. (2012). Acrylamide in Food: A Review of Science and Future Considerations. Annual Review of Food Science and Technology, 3 (1), 15-35.
- Lorenz, K., and Kulp, K. (1995). Freezing of doughs for the production of breads and rolls in the United States. Frozen and Refrigerated Doughs and Batters, 135– 153.

- Marangoni, F., and Poli, A. (2008). The glycemic index of bread and biscuits is markedly reduced by the addition of a proprietary fibre mixture to the ingredients. Nutrition, Metabolism and Cardiovascular Diseases, 18(9), 602-605.
- Marshall, W.E., and Chrastil, J. (1992). Interaction of food proteins with starch. Biochemistry of Food Proteins, 3, 75-97.
- Masi, P. (1989). Study of the influence of temperature on the rheological behaviour of gluten by means of dynamic mechanical analysis. Food Properties and Computer-Aided Engineering of Food Processing Systems. 357-362.
- Masi, P., Romano, A., and Coccia, E. (2015). The artisanal production process of Neapolitan Pizza, in The Neapolitan Pizza: a scientific guide bout the artisanal process, 67–96. Doppia Voce ed. Naples, Italy.
- McCann, T.H., and Day, L. (2013). Effect of sodium chloride on gluten network formation, dough microstructure and rheology in relation to breadmaking. Journal of Cereal Science, 57(3), 444-452.
- Meerts, M., Cervera, A.R., Struyf, N., Cardinaels, R., Courtin, C.M., and Moldenaers, P. (2018). The effects of yeast metabolites on the rheological behaviour of the dough matrix in fermented wheat flour dough. Journal of Cereal Science, 82, 183-189.
- Meziani, S., Jasniewski, J., Ribotta, P., Arab-Tehrany, E., Muller, J. M., Ghoul, M., and Desobry, S. (2012). Influence of yeast and frozen storage on rheological, structural and microbial quality of frozen sweet dough. Journal of Food Engineering, 109(3), 538-544.
- Michniewicz, J., Biliaderis, G.G., and Bushuk, W. (1992). Effect of added pentosans on some properties of wheat bread. Food Chemistry, 43(4), 251-257.
- Mottram, D.S., Wedzicha, B.L. and Dodson, A.T. (2002). Acrylamide is formed in the Maillard reaction. Nature, 419, 448-449.
- Muneer, F., Siddique, M.H., Azeem, F., Rasul, I., Muzammil, S., Zubair, M., and Nadeem, H. (2020). Microbial L-asparaginase: purification, characterisation and applications. Archives of Microbiology, 202(5), 967-981.
- Murthy, N.S., and Knox, J.R. (1976). Small-angle X-ray scattering studies of Escherichia colil-asparaginase. Journal of Molecular Biology, 105(4), 567-575.
- Nagarethinam, S., Naik, A.N., Udupa, N., Rao, V.J., and Vanathi, M.B. (2012). Microbial L-Asparaginase and its future prospects. Asian Journal of Medical Research, 1(4), 159-160.
- Oates, C.G. (1997). Towards an understanding of starch granule structure and hydrolysis. Trends in Food Science and Technology, 8, 375-382.
- Oddy, J., Raffan, S., Wilkinson, M. D., Elmore, J. S., and Halford, N. G. (2020). Stress, nutrients and genotype: understanding and managing asparagine accumulation in wheat grain. CABI Agriculture and Bioscience, 1(1), 1-14.
- Ooms, N., and Delcour, J.A. (2019). How to impact gluten protein network formation during wheat flour dough making. Current opinion in food science, 25, 88-97.
- Östman, E.M., Nilsson, M., Elmståhl, H.L., Molin, G., and Björck, I.M.E. (2002). On the effect of lactic acid on blood glucose and insulin responses to cereal products: mechanistic studies in healthy subjects and in vitro. Journal of cereal science, 36(3), 339-346.
- Östman, E.M., Frid, A.H., Groop, L.C., and Björck, I.M.E. (2006). A dietary exchange of common bread for tailored bread of low glycaemic index and rich in dietary fibre improved insulin economy in young women with impaired glucose tolerance. European Journal of Clinical Nutrition, 60(3), 334-341.
- Paleologos, E.K., Kontominas, M.G. (2005). Determination of acrylamide and methacrylamide by normal phase high performance liquid chromatography and UV detection. Journal of Chromatography A, 1077(2), 128-135.
- Parker, J.K., Balagiannis, D.P., Higley, J., Smith, G., Wedzicha, B.L., Mottram, D.S. (2012). Kinetic model for the formation of acrylamide during the finish-frying of commercial French fries. Journal of Agricultural and Food Chemistry, 60(36), 9321-9331.

- Ramya, L.N., Doble, M., Rekha, V.P.B., and Pulicherla, K.K. (2011). In silico engineering of L-asparaginase to have reduced glutaminase side activity for effective treatment of acute lymphoblastic leukemia. Journal of pediatric hematology/oncology, 33(8), 617-621.
- Rizzello, C.G., Portincasa, P., Montemurro, M., Di Palo, D.M., Lorusso, M.P., De Angelis, M., et al. (2019). Sourdough fermented breads are more digestible than those started with baker's yeast alone: An in vivo challenge dissecting distinct gastrointestinal responses. Nutrients, 11, 2954.
- Ronda, F., Rivero, P., Caballero, P.A., Quilez, J. (2012). High insoluble fibre content increases in vitro starch digestibility in partially baked breads. International Journal of Food Sciences and Nutrition, 63(8), 971-977.
- Sanches, M., Krauchenco, S., and Polikarpov, I. (2007). Structure, substrate complexation and reaction mechanism of bacterial asparaginases. Current Chemical Biology, 1(1), 75-86.
- Sarion, C., Codină, G.G., and Dabija, A. (2021). Acrylamide in bakery products: A review on health risks, legal regulations and strategies to reduce its formation. International Journal of Environmental Research and Public Health, 18(8), 4332.
- Shevkani, K., Singh, N., Bajaj, R., and Kaur, A. (2017). Wheat starch production, structure, functionality and applications—a review. International Journal of Food Science and Technology, 52(1), 38-58.
- Shewry, P.R. (1999). The synthesis, processing, and deposition of gluten proteins in the developing grain Cereal Foods World, 44 (8), 587-589.
- Singh, J., McCarthy, O.J., and Singh, H. (2006). Physico-chemical and morphological characteristics of New Zealand Taewa (Maori potato) starches. Carbohydrate Polymers, 64, 569-581.
- Singh, J., Dartois, A., and Kaur, L. (2010). Starch digestibility in food matrix: a review. Trends in Food Science and Technology, 21(4), 168-180.
- Stauffer, C.E. (1999). Principles of dough formation. Technology of breadmaking, 262-295.
- Struyf, N., Verspreet, J., Verstrepen, K.J., Courtin, C.M. (2017). Investigating the impact of α-amylase, α-glucosidase and glucoamylase action on yeast-mediated bread dough fermentation and bread sugar levels. Journal of Cereal Science, 75, 35-44.
- Tareke, E., Rydberg, P., Karlsson, P., Eriksson, S., and Törnqvist, M. (2002). Analysis of acrylamide, a carcinogen formed in heated foodstuffs. Journal of agricultural and food chemistry, 50(17), 4998-5006.
- Tester, R.F., Qi, X., and Karkalas, J. (2006). Hydrolysis of native starches with amylases. Animal Feed Science and Technology, 130, 39-54.
- Toistoguzov, V. (1997). Thermodynamic aspects of dough formation and functionality. Food Hydrocolloids, 11(2), 181-193.
- Umano, K. and Shibamoto, T. (1987). Analysis of acrolein from heated cooking oils and beef fat. Journal of Agricultural and Food Chemistry, 35, 909-912.
- Verheyen, C., Jekle, M., and Becker, T. (2014). Effects of Saccharomyces cerevisiae on the structural kinetics of wheat dough during fermentation. LWT-Food Science and Technology, 58(1), 194-202.
- Wang, H., Lee, A.W., Shuang, S., and Choi, M.M. (2008). SPE/HPLC/UV studies on acrylamide in deep-fried flour-based indigenous Chinese foods. Microchemical Journal, 89(2), 90-97.
- Wang, P., Jin, Z., Xu, X. (2015). Physicochemical alterations of wheat gluten proteins upon dough formation and frozen storage e A review from gluten, glutenin and gliadin perspectives. Trends in Food Science and Technology 46, 189-198.
- WHO (2005). Summary report of the sixty-fourth meeting of the Joint FAO/WHO expert committee on food additive (JECFA). Rome, Italy: The ILSI Press International Life Sciences Institute. Washington, DC, 1-47.
- Wriston, J.C., and Yellin, T.O. (1973). L-asparaginase: a review. Advances in Enzymology - and Related Areas of Molecular Biology, 39, 185-248.
- Xu, F., Oruna-Concha, M.J., Elmore, S. (2016). The use of asparaginase to reduce acrylamide levels in cooked food. Food Chemistry 210, 163-171.

- Yasuhara, A., Tanaka, Y., Hengel, M., and Shibamoto, T. (2003). Gas chromatographic investigation of acrylamide formation in browning model systems. Journal of Agricultural and Food Chemistry, 5(14), 3999–4003.
- Yiltirak, S., Kocadağlı, T., Çelik, E.E., Kanmaz, E.O., Gökmen, V. (2021). Effects of Sprouting and Fermentation on Free Asparagine and Reducing Sugars in Wheat, Einkorn, Oat, Rye, Barley, and Buckwheat and on Acrylamide and 5-Hydroxymethylfurfural Formation during Heating. Journal of Agricultural and Food Chemistry, 69, 9419-9433.
- Yook, C., and Robyt, J.F. (2002). Reactions of alpha amylases with starch granules in aqueous suspension giving products in solution and in a minimum amount of water giving products inside the granule. Carbohydrate Research, 337, 1113-1117.
- Zhang, G. Y., and Hamaker, B.R. (2009). Slowly digestible starch: concept, mechanism, and proposed extended glycemic index. Critical Reviews in Food Science and Nutrition, 49, 852–867.
- Zyzak, D.V., Sanders, R.A., Stojanovic, M., Tallmadge, D.H., Eberhart, B.L., Ewald, D.K. and Villagran, M.D. (2003). Acrylamide formation mechanism in heated foods. Journal of agricultural and food chemistry, 51(16), 4782-4787.

Chapter 1

Effect of leavening time on rheological, textural, thermal and chemical properties of dough and wood oven baked pizza base

Submitted paper

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1. Abstract

Research objective was to investigate how different leavening times affect the structure of pizza dough during fermentation and how rheological and biochemical parameters may be related to the starch digestibility of the baked product. Pizza dough was prepared at high hydration (60.5 %) and proofed for 48 h at controlled temperature and humidity. Leavening kinetics were monitored by digital image acquisition from which the volume of leavened pizza dough was obtained. Pizza dough at selected leavening times was subjected to the stress-relaxation test because they are viscoelastic materials, and it was evaluated how the relaxation of the structure evolves over time. Elastic modulus was determined in the temperature range of 30 to 90 °C and vice versa, using a dynamic rheometer. Results showed that at low leavening times the dough had a higher elastic component, while for longer times it approached a viscous behavior, which results in greater extensibility that allows a larger pizza diameter by applying less compression force. Elastic modulus determination confirmed gluten network formed after baking has fewer and weak interactions at longer leavening times. Furthermore, thermal analyses (DSC and TGA) of dough and morphological (SEM) analyses of dough and pizza were performed. Dough and wood oven baked pizza base samples were also characterized biochemically, through determination of an increase in reducing sugars and free amino groups during leavening time. These results can be attributed to the amylolytic and the proteolytic activity of yeast, and for further characterisation, the levels of digestible and resistant starch will also be determined so that a correlation with digestibility and glycemic index can be assessed.

Keywords: digestible starch, elastic modulus, gluten, stress-relaxation, starch gelatinization

1.1 Introduction

Despite being a widely consumed product, Neapolitan pizza has rarely been the subject of scientific studies. In 2010, the European Union Commission passed a regulation governing the production of the “Pizza Napoletana TSG”, and in the prelude to that document the appearance of "Pizza Napoletana" was traced back to a historical period between 1715 and 1725. Neapolitan pizza spinning is considered an art and, on December 7, 2017, received recognition by UNESCO (United Nations Educational, Scientific and Cultural Organization) as an "Intangible Cultural Heritage of Humanity": this made the art of making pizza of global significance (Ganguly, 2017).

Wheat dough is a complex, viscoelastic mixture, and its composition plays an important role regarding the dough’s processability, the gas holding capacity during fermentation and the baking performance (Janssen et al., 1996). The formation of the gluten network is also fundamental during pasting in which starch and water are included and can go to affect subsequently the development of the dough during leavening.

Ripening is a set of enzymatic processes that progressively break down the more complex structures, proteins, and starches, into simpler elements, namely peptides/amino acids and fermentable sugars for yeasts. Brewer's yeast is the main leavening agent in the pizza-making

process. During leavening, yeast (*S. cerevisiae*) metabolism switches from respiration to fermentation, converting fermentable sugars into ethanol and carbon dioxide. During fermentation, yeast cells produce CO_2, which partly dissolves in the aqueous phase of the dough and forms weakly ionizable carbonic acid that slightly lowers the pH of the dough. When this phase is exhausted, the carbon dioxide subsequently produced may vaporize into the environment or pass into the air cores formed within the dough during the mixing phase. As the carbon dioxide passes into the cores, they expand due to the increase in pressure, generating an overall increase in the volume of the dough and the formation of gaseous alveoli (Masi et al., 2015).

Baking is assisted by evaporation of water, denaturation of protein, and gelatinization of starch. During gelatinization process, water breaks down the crystallinity of the starch, the granules swell, amylose diffuses out of the granules leaving most of the amylopectin behind, and the granules eventually collapse and are held in an amylose matrix as part of a gel network. High temperatures denature proteins, and as a result of denaturation, proteins can undergo extensive cross-linking, particularly through the formation of disulfide bonds, thus forming a continuous protein network. When proteins and starch are in contact with each other, stable complexes can develop by formation of a protein/starch matrix, where hydrogen and covalent bonds as well as charge-charge interactions can be found (Marshall and Chrastil, 1992). Hence, rheological analysis is commonly used to assess the properties of dough, and to gain knowledge of the functions of dough ingredients and structure (Song and Zheng, 2007). To this aim, a stress-relaxation test, applying a strain to the dough, could be used to see how the dough relaxed over the time, while the dynamic oscillatory rheometer can be useful to describe the molecular structure of starch and gluten during baking and cooling.

In cereal science, scanning electron microscopy (SEM) could be used to show the morphology of the dough structure at different leavening time (Srikaeo et al., 2006).

The purpose of our study was to investigate the change in the structure of pizza dough during leavening and baking through physico-chemical analyses and to correlate the dough structure at selected leavening time (0, 4, 8, 16, 24, 48 h) with the digestibility of pizza base baked in the wood oven. For each considered leavening time, physical properties such as rheological, thermal, and morphological were studied, and the biochemical aspect was evaluated by determining reducing sugars, free amino groups and *in vitro* starch digestibility.

1.2 Materials and Methods

1.2.1 Chemicals and Pizza Dough Ingredients

Chemical reagents were of analytical grade from standard chemical companies. Digestible and Resistant Starch Assay Kit was purchased from Megazyme Ltd.

The soft wheat flour used for the experiments was the commercial refined type “00” (Caputo rossa) kindly provided by Mulino Caputo (Antimo Caputo, Srl, Naples, Italy), whose proximal composition shown on the label was: 70% carbohydrate, 13% proteins, 12% moisture, 3% fibre, 1.5% lipids and 0.5% ash. Fresh brewer's yeast (Lievital, Italy) and salt (common fine table food-grade sodium chloride) were purchased into a local supermarket (Portici, Italy).

1.2.2 Dough and pizza making

Pizza dough was prepared following the Commission Regulation (EU) n.97/2010 to obtain a product recognised as "*Pizza Napoletana TSG*" and kneaded with the traditional ingredients (60.35% flour, 37.72% water, 1.88 % salt and 0.04% yeast). After resting for 20 min at room temperature, the dough was divided in 250 g balls which were leavened at 22°C and 80 % relative humidity (RH) for several leavening time (0, 4, 8, 16, 24, 48 h), and then rolled and baked for 60 seconds into a wood oven with at about 485±30°C (Chapter 4, § 4.2.5).

1.2.3 Image analysis of leavening kinetic

The leavening of the pizza dough balls (250 g) was monitored for 48 h by using a digital camera (Go Pro Hero 5) positioned to always frame the dough at the same distance for the entire leavening period (Figure 1.S1) and scheduled to take pictures every hour. The obtained images were used for the measurement of the height (h) and width (l) of the pizza dough through an image analysis software (Image J). The geometry of the dough ball has been assimilated to that of an oblate semi-spheroid and thus, the volume (V) was determined according to the following formula:

$$V = \frac{\frac{3}{4}\pi\left(\frac{l}{2}\right)^2 h}{2} \qquad (1.1)$$

The calculated volume at each hour of leavening was expressed as the V_t/V_0 index, where V_t is the volume measured at time t and V_0 is the initial volume of the dough and plotted as a function of leavening time.

1.2.4 Stress-relaxation test

Pizza dough at various leavening times was subjected to the stress-relaxation test using a dynamometer Instron (mod. 5900R, USA) equipped with two plates with Ø 30 cm (Figure 1.S2), in which the pizza dough is compressed until a final thickness of 2 mm is reached; the plates were specially designed to simulate the dough rolling by the pizza maker. The tests were conducted in air at 23±2 °C by applying a compressive force at a constant speed of 50 mm/min with 3 kN load cell. Once the set thickness was reached, the test was continued without applying any other compression, in order to analyse the relaxation stage of dough. Force versus time curves (Figure 1.S3-A) were recorded with Bluehill 3 software, which was also used for the calculation of the area under the force curve, corresponding to the work (J) performed by the crosshead to deform the dough to the set thickness.

The relaxation graphs were obtained by plotting F_t/F_0 as a function of time (Figure 1.S3-B), where F_t is the force measured at time t while F_0 is the force measured at the starting point of the relaxation test, immediately after the end of the compression stage. The extent of relaxation (a, %) and the speed decay (b, N s^{-1}) during relaxation were calculated trough the mathematical processing of the raw data following the equation (2) (Peleg, 1979):

$$\frac{tF_0}{F_0 - F_t} = \frac{1}{ab} + \frac{t}{a} \qquad (1.2)$$

For each sample 8 specimens were analysed, and, at the end of the test, the diameter of the stretched dough was measured for all analysed samples in 4 replicates. All values were expressed as mean ± SD (Standard Deviation).

1.2.5 Elastic modulus determination

The samples at various rise times (0÷48 h) were analysed for elastic modulus determination by performing the dynamic-mechanical test using the rotational rheometer (Haake™Mars™, Thermo Fisher Scientific, USA) with controlled stress with 80 mm flat and parallel plate geometry. The distance between the two plates (gap) was set at 2 mm and 3 replicates were performed for each sample. 8 g of freeze-dried and ground dough samples (grain size <0.5mm) were weighed and 4.8 mL of H_2O were added to the powder to obtain a rehydration at 60%. All samples were kneaded for 2 min and placed on the parallel plate of the rheometer forming a dough disc covering the whole surface, then the upper plate was brought closer to the lower plate at a distance of 2 mm to ensure that the sample adhered well to the surface of the plates and excess sample was removed with a spatula, after which the edges of the sample were sealed with vaseline ointment to prevent drying of the dough during the test. The elastic modulus was measured using the sweep temperature test with an oscillation frequency of 1 Hz and a strain of 0.1 %. The specimens were heated from 30 to 90 °C and then cooled from 90 to 30 °C with a variation of 10 °C/min (Masi, 1989). The heating rate was designed in a way to follow the thermal history of pizza-dough during baking.

1.2.6 Thermal analysis

Thermal properties of dough at different leavening times were measured by differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA). DSC (Q200, TA Instruments, USA) was used under a nitrogen atmosphere (50 mL/ min). Different fresh dough samples (ca. 40 mg) were placed in DSC aluminum pans and sealed; sample and empty pans were put in the DSC testing cell and heated in the temperature range from 20-100 °C with a heating rate of 10 °C/min (Zhang et al., 2020, Lapčíková et al., 2019). Initial (T_i), gelatinization (T_g) and end peak (T_e) temperatures, and gelatinization enthalpy (ΔH) were calculated from DSC thermograms through the TA Universal Analysis software (TA Instruments, USA). Results were analysed from three replicates.

For TGA analysis, fresh dough samples (ca. 60 mg) were put in oxidized aluminum pans and loaded in a thermogravimetric instrument (TGA7, PerkinElmer, USA); then a ramp temperature in the range of 30-550 °C, with a heating rate of 10 °C/min, was applied under nitrogen atmosphere (50 mL/min). The thermograms curves were derived to obtain the graphs of the weight loss rate (%/°C), from which the weight loss (%) in the interval between 100 and 200°C was calculated which corresponds to the area of the 1st peak. Data processing was conducted with the dedicated software Pyris Manager (USA). Each measurement was performed in triplicates and expressed by mean ± SD.

1.2.7 Reducing sugars and free amino groups analysis

For each leavening time, freeze-dried and ground samples of dough and wood oven pizza base were weight and H_2O_d (1:10) was added for the extraction of soluble substances. Next, the samples were vortexed for 3 min at room temperature (20-25 °C) and then sonicated for 15 min in an ultrasonic bath at room temperature at 240 Watt (cycle 0.5). Subsequently, samples were centrifuged (Microfuge 18 Centrifuge, Beckman Coulter) at 9000×g for 10 min at 25 °C to separate the soluble fraction from the insoluble fraction. The supernatants were stored at -20 °C until subsequent quantitative reducing sugars and free amino groups determinations. The 2,4-dinitrosalicylic acid (DNS) assay for reducing sugars such as glucose was used, according to the method of Miller (1959). Determination of free amino groups was performed using the o-phthaldialdehyde (OPA) method (Lee et al., 1978). 20 µL of supernatant were used in both reaction assays. Absorbance was measured through a spectrophotometer UV/VIS (V-730, Jasco, Sud Africa) at 540 nm and 340 nm for DNS and OPA assay, respectively. 4 measurements were made for each sample analysed. A calibration curve was built for each assay using glucose (0÷0.16 mg/mL) and leucine (20-300 mg/L) as standard. Reducing sugars were expressed as g of glucose equivalent/100 g of sample on dry weight basis (GE, g/100 g_{dw}), while free amino groups were referred to g of leucine equivalent/100 g of sample on dry weight basis (LE, g/100 g_{dw}).

1.2.8 *In vitro* digestible and resistant starch

Determination of digestible and resistant starch, on dough and pizza samples, was carried out following AOAC method 2017.16 (Digestible and Resistant Starch Assay Kit, Megazyme, Ireland) with some modification. 0.5 g of lyophilised sample were weighed and 0.5 mL of 96 % (v/v) ethanol and 17.5 mL of 50 mM sodium maleate buffer, pH 6.0, containing 2 mM calcium chloride were added. The suspension was incubated in a water bath at 37 °C for 5 min with a magnetic stirring at 600 rpm. Then, 1 mL of pancreatic α-amylase and amyloglucosidase (AMG) solution, provided by the kit, and 1.5 mL of sodium maleate buffer were added. The sample was again incubated at 37 °C at 600 rpm. For the determination of digestible starch, 1 mL of incubation solution was taken after 20 (rapidly digestible starch, RDS) and 120 (slowly digestible starch, SDS) min of incubation and placed in 20 mL of 50 mM acetic acid. After mixing, 2 mL of each solution were centrifuged at 15500×g for 5 min (Microfuge, Eppendorf), then, 0.1 mL of supernatant (in duplicate) were added to 0.1 mL of AMG (100 U/mL) and incubated at 50 °C for 30 min at 200 rpm in an orbital shaker (Forma Scientific). Finally, 3 mL of glucose oxidase/peroxidase (GOPOD) reagent were added, and the sample was incubated again at 50 °C for 20 min at 110 rpm. After incubation, the absorbance value at λ = 510 nm was measured in the UV/Vis spectrophotometer. The following formula was used to calculate digestible starch, DS % (w/w) (SDS, RDS):

$$\mathrm{DS}\ (\%) = \Delta\mathrm{A} \times \mathrm{F} \times \frac{EV}{W} \times 0.0189 \qquad (1.3)$$

where ΔA = absorbance of reaction read versus blank after 20 min (RDS) and after 100 min (SDS); F = factor to convert absorbance values to µg D-glucose; EV = sample extraction volume; W = sample weight; 0.0189 adjustment from free D-glucose to anhydro D-glucose.

For the determination of resistant starch (RS), after the enzymatic incubation of the sample for 240 min in 50 mM sodium maleate buffer/2 mM calcium chloride (pH 6.0), 4 mL of solution were taken and mixed with 4 mL of 96 % (v/v) ethanol and then centrifuged at 11510×g for 10 min (Hermle centrifuge, Z 326 K). The supernatant was removed, and the pellet was resuspended twice in 8 mL of 50% (v/v) ethanol and centrifuged in the same conditions. After that, the pellet was resuspended in 2 mL of cold 1.7 M NaOH, and the solution was left to stir at 800 rpm in ice-water bath for 20 min. Finally, 8 mL of 1.0 M sodium acetate buffer (pH 3.8) and 0.1 mL of AMG (3300 U/mL) were added, and the solutions were incubated at 50 °C for 30 min and then centrifuged at 15500×g for 5 min. At this step, the expected samples with an RS > 10% were diluted in water to a final volume (FV) of 100 mL. Then 2 mL of solution, as such or diluted, were centrifuged in the above conditions, and the supernatant (0.1 mL) was mixed with 3 mL of GOPOD reagent and treated as previously described. The following formula was applied to calculate resistant starch RS % (w/w):

$$\mathrm{RS}\ (\%) = \Delta A \times F \times \frac{EV}{W} \times FV \times 0.000225 \qquad (1.4)$$

where ΔA = absorbance of reaction read versus blank; F = factor to convert absorbance values to µg D-glucose; EV = sample extraction volume; W = sample weight; FV = final volume; 0.000225 adjustment from free D-glucose to anhydro D-glucose.

RDS, SDS and RS values were expressed with respect to dry matter in order to avoid the influence of water content on the results of the determined starch fractions.

Starch Digestion Rate Index (SDRI) was calculated as RDS divided by Total Starch (TS=RDS+SDS+RS) and represents an indicator of *in vitro* starch digestibility. Rapidly available glucose index (RAG) was determined as a predictor of the potential glycemic response derived from the ingestion of these food items (Englyst et al., 1996).

1.2.9 SEM of dough and pizza samples

SEM analyses were conducted for the evaluation of dough samples at selected leavening times. Lyophilised dough slices (Sluková et al., 2018) were placed on specimen holders and coated with gold by means of DC sputtering (Sputter and Carbon Coater Agar Scientific B7340) in order to make the sample conductive. The microstructure of samples was observed at a magnification of 2000× with LEO EVO 40 SEM (Zeiss, Germany) with a 10 kV acceleration voltage. Representative micrographs from all the samples were selected. Microstructure of lyophilised wood oven pizza base samples were observed at a magnification of 2000× by Phenom XL SEM (Thermo Scientific, USA) equipped with Ion Sputter Coated.

1.2.10 Statistical analysis

All experiments and analytical measurements were run in triplicate and the data were expressed as mean ± SD. Means of each parameter were analysed by analysis of variance (ANOVA) using Post Hoc Tukey's test. Statistical analyses were performed using XLSTAT software (version 2014.5.03). Differences between treatments at 5% level ($p<0.05$) were considered as significant.

1.3 Results and Discussion

1.3.1 Dough volume during leavening

Leavening kinetic of pizza dough were monitored during 48 h by digital image acquisition, from which the volume of leavened dough was obtained through an image analysis software.

The variation of the volume index (V_t/V_0) over time shows a typical sigmoid kinetic curve, characterised by three phases: in the 1st phase the curve has a very slight positive slope and the V_t/V_0 just reaches 1.3 after 8 h of leavening; the 2nd phase is the real growing phase in which the volume increases in logarithmic way until about the 30th h, by tripling its initial value; the 3rd phase correspond to stationary phase, when the volume index runs along the asymptote of the described curve (Figure 1.1-A). The increase in volume has been linked to the production of CO_2 during leavening. At the mixing of ingredients, air bubbles are introduced into the dough, which will be then replaced during the leavening by CO_2 produced by the fermentation of yeast (*S. cerevisiae*). During fermentation, the sugars in the dough are converted by the yeast mainly into CO_2 and ethanol.

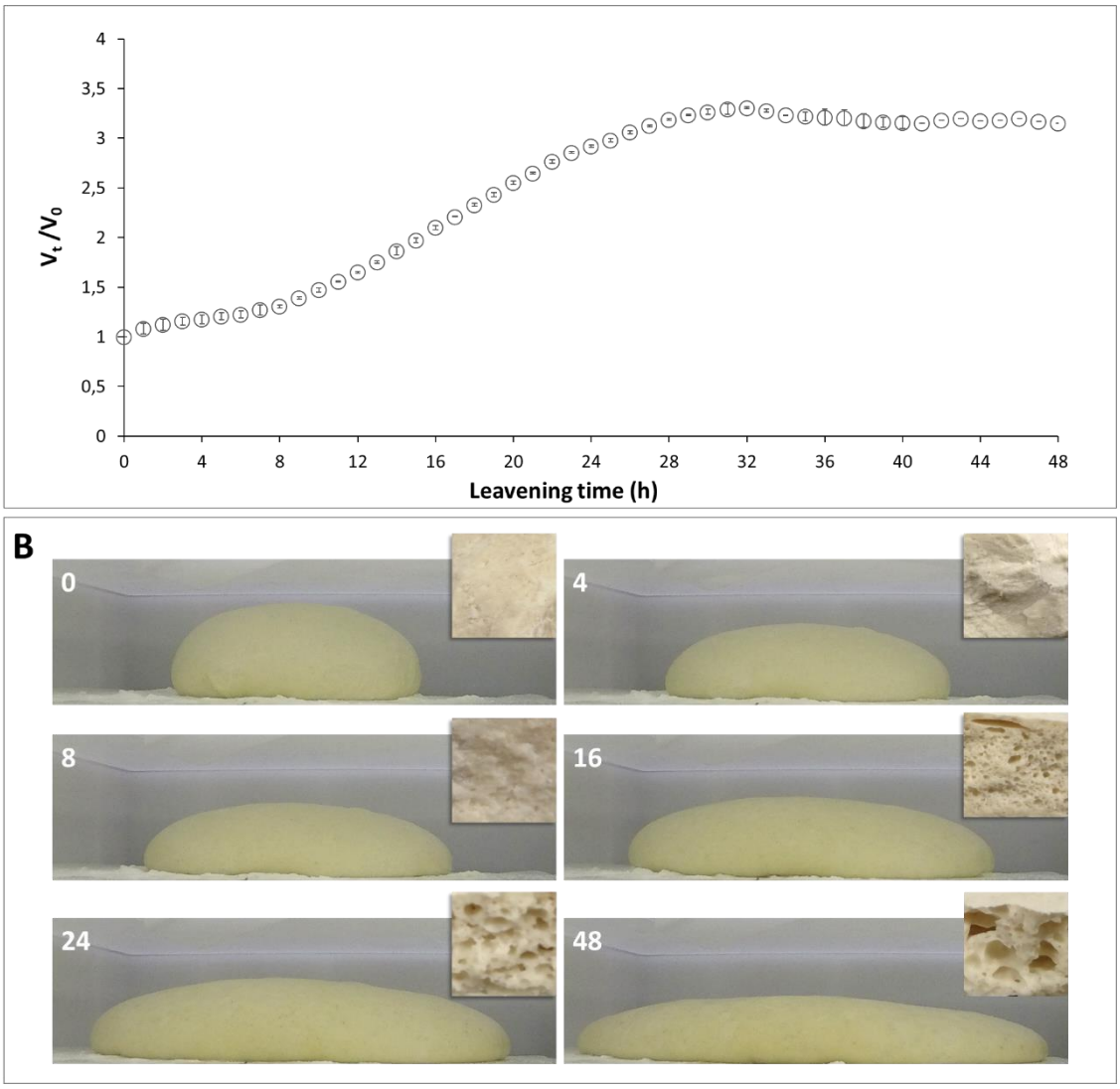

Figure 1.1 – Leavening kinetic of pizza dough. A, Variation of volume index (V_t/V_0) over time; B, images taken at 0, 4, 8, 16, 24 and 48 h. The image inserts at the top right of each photo refer to sections of dough at the corresponding leavening time.

The amount of CO_2 produced depends on the fermentable substrates in the dough (Pyler and Gorton, 2009). The content of free fermentable sugars in wheat flour is too low to support optimal gassing power by yeast cells. The sugars that are consumed during fermentation are generated by enzymatic hydrolysis of damaged starch (Struyf et al., 2017). The size and density of the bubbles can change the texture and sensory properties of the finished product (Scanlon and Zghal, 2001). It is worth noting that in the early stage of leavening, a change in the shape of the dough ball is observed, which is more flattened by gravity and enlarged at the base, but the overall volume remains constant (Figure 1.1-B, images at 4 and 8 h). At later times, as the CO_2 produced by the yeast increases, the volume increases (Figure 1.1-B, images at 16 and 24 h) until the stationary phase. In fact, during this time, the cell structure is definitely changed as the size of the bubbles increases, which results in stretching of the dough matrix due to bubble growth (Figure 1.1-B, image inserts at 16 and 24 h) (Romano et al., 2007; Turbin-Orger et al., 2012). In the stationary phase, the total volume did not change but the dough undergoes flattening phenomena that are very evident at the late stage (Figure 1.1-B, image 48 h). This behavior is typical of viscoelastic materials that relax during leavening, due to destructuring phenomena of the gluten network, with consequent CO_2 loss through the enlarged meshes of the dough (Figure 1.1-B, image insert at 48 h). In fact, increasing the leavening time of pizza dough up to 48 h can result in increased starch hydrolysis that leads to the destructuring of the gluten both for losing the physical support of starch granules and weakening of protein network.

1.3.2 Rheological properties of pizza dough

The viscoelastic properties of solid foods, combining the properties of a purely viscous fluid and an elastic solid, have frequently been studied by stress-relaxation test, consisting in applying a compressive force to the specimen until a final fixed thickness, and then removing it for the relaxation stage assessment (Peleg, 1979; Launay and Michon, 2008; Yang et al., 2019). From the relaxation phase graph of pizza dough balls at different leavening times (Figure 1.S3-B), it is evident that dough samples have a similar behaviour to viscoelastic materials, and it can infer that dough has a more elastic component at early times (4 and 8 h), while for longer times it approaches viscous behaviour. Nevertheless, mathematical elaboration of stress-relaxation curves, registered on, allowed to obtain several parameters, reported in Table 1.1 together with the diameter of the disc dough at the end of the test. The compression work corresponds to the work that the crosshead must apply to the dough in order to deform it. Relaxation degree (a, %) and speed decay (b, N s^{-1}) account for viscoelastic characteristics of the dough. An ideal elastomer which, after a stress, has the ability to recover the original structure instantaneously, displays a relaxation degree of 0 %. On the contrary, an ideal viscous material (i.e. water) shows a relaxation degree of 100 % (Yang et al., 2019). Moreover, longer the relaxation time is, more visible the elastic deformation is, otherwise, the viscous deformation is more apparent. Theoretically, when viscoelastic solids are concerned, the lower value of speed decay the slower stress relaxation. In the relaxation curve, a higher

speed decay (b) value expresses a steeper descent of the relaxation curve toward the residual value (Peleg, 1979).

As shown in Table 1.1, at time 0 h, the deformation of the dough required the minimum work (6.59 59±0.41 J), compared to other leavening times, and gave rise to a flat disc with a diameter of 21.50±2.07 cm, the lowest at all (Table 1.1). This means that, before leavening, the dough is less elastic, thus opposes less resistance to deformation; therefore, it is also less relaxed as it reaches a relaxation degree of 82 % after the stress, with a speed decay of 0.387±0.014 Ns^{-1} (Table 1.1). At time 4 h, the compression work increases almost 5 times, and the diameter of the formed disc is the highest of all. This is related to flattening occurring in the early stage of leavening, which changes the internal structure of the dough by making it more compact as well as more extensible and elastic, as confirmed by the significative decrease of relaxation degree (79.7±0.3 %) and increase of speed decay (0.409±0.007 Ns^{-1}) values (Table 1.1). Therefore, more elastic dough structure represents a strong gluten network structure (Fan et al., 2021) which could be associated to the high value of work. Li et al. (2003) reported the study of gluten relaxation that corresponds to a structure made of a network in which proteins are bound together and a large number of interactions between protein chains leads to an increase in dough strength, as in the case of the dough at 4 h of leavening.

Table 1.1 – Compression force expressed as work (J), relaxation percentage (a), speed decay (b), and diameter reached by pizza dough after stress-relaxation test at selected leavening time.

Leavening time (h)	Compression W (J)	Relaxation a (%)	Speed decay b (N s^{-1})	Diameter (cm)
0	6.59±0.41^{a}	82.5±0.7^{a}	0.387±0.014^{a}	21.50±2.07^{a}
4	29.96±2.52^{b}	79.7±0.3^{b}	0.409±0.007^{b}	29.25±0.53^{b}
8	16.16±0.69^{c}	78.9±0.4^{b}	0.342±0.008^{c}	26.06±1.05^{c}
16	19.72±1.10^{d}	80.8±0.3^{c}	0.359±0.015^{c}	27.50±0.46^{cd}
24	15.97±1.43^{c}	80.9±1.0^{c}	0.347±0.010^{c}	28.37±0.44^{bd}
48	7.65±0.40^{a}	85.2±0.3^{d}	0.283±0.013^{d}	28.06±0.42^{bd}

Values are mean ± SD (n=8). Different letters indicate samples significance calculated by ANOVA statistical test with Post Hoc Tukey ($p<0.05$).

At the 8th hour of leavening, the elastic component remained almost the same, but a huge drop of compression work was observed, accompanied by a significative speed decay and diameter reduction, compared to the previous time step. This means that the structure of dough was still elastic, but the elastic deformation was less visible (elasticity: 0 h < 4 h = 8 h). During the growing phase of leavening, the relaxation degree and the final diameter increased again, while the speed decay was similar (Table 1.1, times 16 and 24 h), indicating important increases in the viscous component. In this phase, the fermentative activity of yeasts is responsible for the CO_2 release by consumption of the reducing sugars, which leads to the filling of the air cells that increase the volume of the dough. The structure become porous because of stretching of the gluten network in which the CO_2 is retained (Figure 1.1-B, times 16 and 24 h). At 16 h, the work value is higher than at 8 h just for the presence of CO_2 in the dough that increases its resistance to deformation (Table 1.1). As the leavening progresses

towards the stationary phase, the work drops significantly, going down to 15.97 ± 1.43 J at 24 h, and to 7.65±0.40 J at 48 h (Table 1.1), due to the further changes in the structure. In fact, the accumulation of CO_2 pushes the gluten mesh to widen so much that at a certain point the gas cells coalesce; simultaneously the hydrolytic activity of enzymes (endogenous or produced by yeast) degrades starch and weakens the gluten bonds. These two phenomena also explain the loss of elasticity and the increase of the viscous component, testified by the highest value of the relaxation degree (85.2±0.3 %) and the simultaneous lowest value of the speed decay registered at 48 h (Table 1.1).

The behaviour of viscoelastic materials is associated with the structural characteristics of the protein component. Indeed, it is well known that gliadins are associated with dough extensibility, they increase the viscous component (G'') of dough, while glutenin proteins contribute to the increase in the elastic component described by the elastic modulus (G') (Goesaert et al., 2005; Song and Zheng, 2007). In addition to the proteins, that play a key role in the relationship with its viscoelastic properties, starch and its damage during leavening also interferes in the model dough. Interactions between the components of the flour, in particular gluten and starch, which in consequence become visible in the viscoelastic characteristics, can be evaluated by macroscopic examination in dynamic oscillation measurements (Rosell and Foegeding, 2007). Information regarding alterations in the gluten network was obtained from determination of the elastic modulus (G') of selected dough (Connelly and McIntier 2008, Marchetti et al. 2012). In addition, dynamic rheological measurements have been useful in understanding how starch and gluten interact with each other. In fact, starch not only acts as a filler in the gluten network but also interacts with gluten proteins and is involved in determining the viscoelastic behaviour of the dough (Miller and Hoseney, 1999). During the interaction phase between these compounds, water plays a key role; in fact, the interactions that are formed depend on the ability of starch granules to swell during the gelatinization phase and on the ability on part of these molecules to interact with water itself during their structural changes.

Experimental results for studying the G' parameter in the dough at different leavening times are shown in Figure 1.2. During heating from 30 °C to about 60 °C, G' slightly decreased as the temperature increases, with a linear stretch having an almost parallel slope in all samples except in the dough at 4 and 48 h of leavening (Figure 1.2-A). This reduction of elastic modulus is probably due to water freed from damaged starch in the early stage of heating (Salvador et al., 2006). As heating continues above 60°C, two events occur: 1) the gluten proteins denature, providing greater flexibility to the network; 2) the starch starts gelatinization. In this phase, the G' curves still decreased along the temperature rising, until reach a minimum value in the range of 70-80 °C (Figure 1.2-A), which correspond to the overall denaturation of gluten proteins and to the transition of starch from solid to gel state, resulting in a reduction in viscosity (G'' modulus, data not shown). The viscosity changes during gelatinization are well known for starch containing materials. During baking phase, starch assumes an important role in rearranging the network (Jekle et al., 2016). At higher temperatures (>70-80 °C) the trend of G' curves showed a sudden increase. In accordance with Masi (1989), the obtained G' curves are typical for thermal denaturation of gluten in a temperature sweep test. It has been shown that the G' and G" moduli decrease when the temperature increases until to the critical point

after which there is a reversal of the behaviour of these materials, with the increasing both moduli (Masi, 1989). Other studies showed the same behaviour of the viscoelastic dough, they also found an abrupt change between 48 and 56 °C, where G' reached a minimum before starting to increase. This phenomenon has been mainly attributed to starch gelatinization (Dreese et al., 1988; Launay and Michon, 2008).

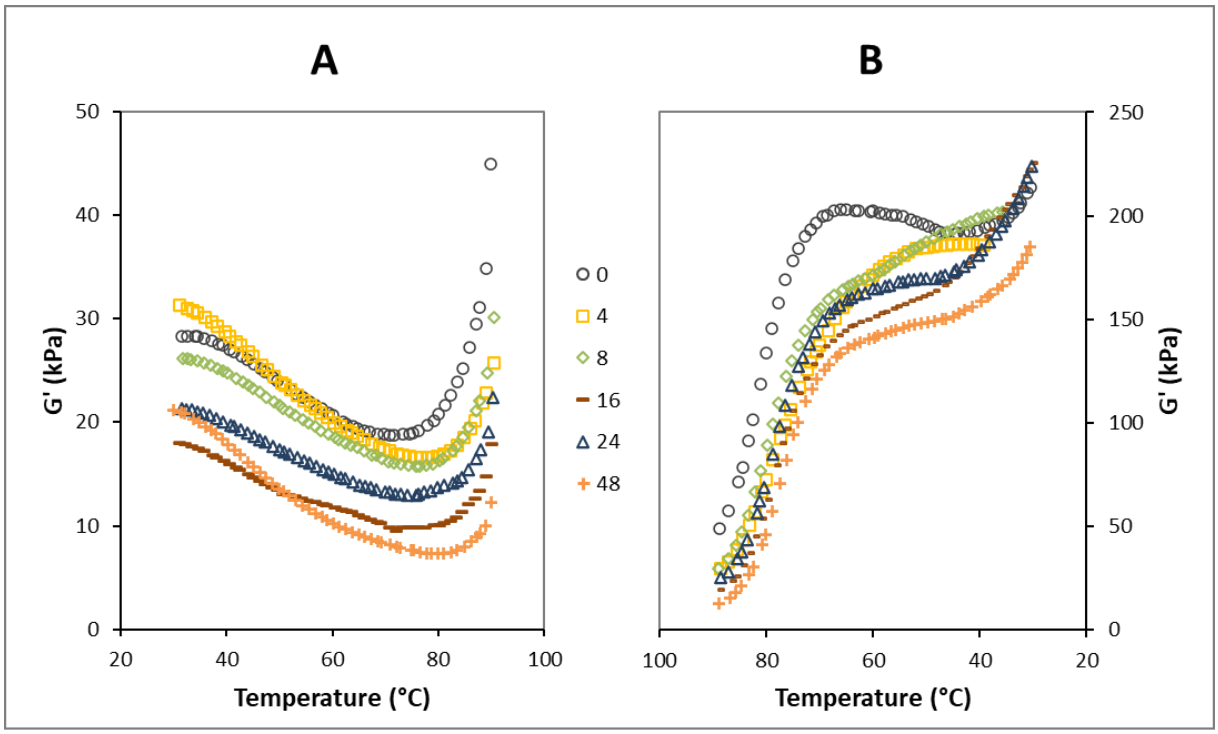

Figure 1.2 – Elastic Modulus (G') of pizza dough at selected leavening time during heating (A) and cooling (B) phase.

Over the years, several models for the structure of the gluten network have been proposed, often focusing on understanding the viscoelastic properties of the dough. Elongation of the gluten network would result in deformation of protein regions (Ooms and Delcour, 2019). The functionality of gluten proteins is highly dependent on the specific dough recipe, as water and other typical dough components, such as salt, influence the formation of the gluten network (Bernklau et al., 2017). Yeast and, in particular, its metabolites produced during fermentation also has an impact on the gluten network. Bernklau et al., 2017, concluded that the level of free (accessible) SH groups and protein surface hydrophobicity are the main determinants of the co-protein network with gluten during heating.

The G' curves in the cooling phase could be a useful way to study the gluten network interaction along the leavening time after baking. As shown in Figure 1.2-B, during the cooling phase from about 90 to 65 °C, a steep increase in G' modulus was visible for all sample, that can be attributed to the strengthening of the gluten network, through the formation of additional cross-links, such as disulphide bonds, leading to stabilization of the dough structure (Masi et al., 2015). At temperature lower than 65 °C, the G' curves advanced much more smoothly, with higher slope values for the dough at 4 and 8 h of leavening, and an almost flat shape for the unleavened dough (time 0) and long leavening samples (16, 24 and 48 h), the

latter showing a reduction in the elastic modulus compared with all other samples (Figure 1.2-B). This result indicates that after a long leavening, the gluten network formed after baking has fewer and weaker bonds, and therefore fewer interactions, compared both to initial dough (time 0 h) and leavened samples for 4 and 8 h. In all cases, the viscous modulus (G") exhibit the same trend as the elastic one G' over the leavening time (data not shown).

1.3.3 Thermal properties

Wheat flour dough is a heterogeneous system in which thermo-dynamically incompatible polymers coexist in separate aqueous phases, and thermal property analysis can help unravel the nature and evolution of the interactions between water and these polymers over time (Fessas and Schiraldi, 2001). The heating to which the dough is subjected during the thermal analysis mimics what happens during the baking of the pizza. From a physical point of view, during heating the dough undergoes a significant loss of water, accompanied by the transition of the starch from the crystalline form to the gelatinized one. At the same time, the proteins that make up the gluten network rearrange themselves in new interactions following the gelatinization of the starch. The thermogravimetric profiles of dough at the selected leavening time, and corresponding 1st derivatives graphs are shown in Figure 1.3-A and -B. After an initial section characterised by a gentle descent of 10 % until about 100 °C, TGA curves showed two main weight loss steps: a first drop from 100 to 250 °C, that led to the reduction of another 32 % of weight, and a further loss of about 28 % in the temperature range between 250 °C and 400 °C (Figure 1.3-A). These two steep steps of the thermogram draw two high peaks in the graph of the first derivative which identify the weight loss rate values, associated with two distinct types of events (%/°C) (Figure 1.3-B). It can be assumed that up to 250 °C the recorded phenomena concern the movement of water molecules between the two polymeric phases of the dough, and between these and the outside. In fact, the heat supplied leads to an increase in the kinetic energy of the molecules, so the free water diffuses more easily according to the gradient from one polymeric phase to another. The result is that the starch tends to hydrate and pass into a gel phase, while the gluten network stretches under the swelling pressure of gelatinized starch, and only a small part of the free water manages to evaporate up to 100 °C producing a 10 % weight loss.

At temperatures higher than 100 °C, the water acquires more and more mobility and, not only the free one, but also the bound one manages to evaporate; this determines the first important drop in weight of the sample of 32 % (Figure 1.3-A) characterised by a huge peak in the derivative curve at around 150 °C (Figure 1.3-B). Fessas and Schiraldi (2001) have shown that the bound water fraction, which mainly interacts with gluten, is released with a maximum rate reached at 125 °C. Wehrli et al. (2021) also found that water tightly linked to gluten is removed at temperatures between 100 and 300 °C, and highlighted that, around 250 °C, phenomena of pyrolysis of the matrix material occur, with the release of CO_2 groups and volatile hydrocarbon species, due to the decarboxylation and degradation of proteins. Therefore, the second important weight loss recorded in the TGA curves is due to the thermal decomposition of the dough proceeding up to 400 °C and beyond (Wehrli et al., 2021).

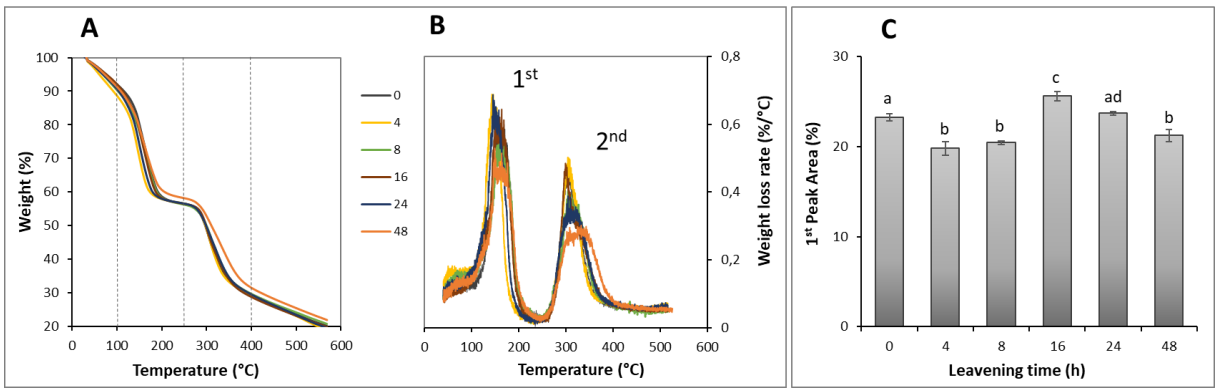

Figure 1.3 – Thermogravimetric analysis (TGA) of pizza dough at increasing leavening time. A, TGA curves as function of temperature. B, first derivate of TGA curves. C, 1st peak area from the derivate curves. Different letters indicate samples significance calculated by ANOVA statistical test with Post Hoc Tukey ($p<0.05$).

Therefore, most of the water in the dough which, taking into account the recipe and the initial moisture of the flour, represents about 50 % of the final dough weight, is removed in the temperature range between 100 and 250 °C, and the measurement of the area under the first peak of the derivative curve expresses the extent of the weight loss rate for this water removal. The values of the weight loss rate calculated for the dough at the selected leavening time are shown in Figure 1.3-C, and are related to the dough structure. Experimental evaluations revealed that after 4 and 8 h of leavening, the water removal rate decreases compared to the unleavened sample (0 h), while it increases significantly at 16 h. At the time of 24 h, it remains higher, to then return to the initial value at the time of 48 h (Figure 1.3-C). Increases in weight loss rate suggest a more open and weak gluten structure, while decreases could be due to a more compact and stronger gluten network (Khatkar et al., 2013). These results agree with the above considerations regarding the volume during the leavening kinetic, confirming that the more alveolar structure of the dough at 16 h is associated to a more rapid evaporation of the water molecules.

DSC analyses agreed with the current literature (Khatkar et al., 2013; Jekle et al., 2016): all samples exhibited a single endothermic transition, with corresponding temperatures and enthalpies shown in Table 1.2. A significative difference between the 16 h leavened sample compared to almost all the others, in terms of initial (T_i) and gelatinization (T_g) temperatures, as well as in the ΔH value, was found (Table 1.2).

Table 1.2 – DSC analysis of pizza dough, at selected leavening time, expressed as initial, gelatinization, ending temperature (T_i, T_g, T_e) and enthalpy variation (ΔH).

Leavening time (h)	T_i (°C)	T_g (°C)	T_e (°C)	ΔH (J/g)
0	68.17±0.01^{a}	75.40±0.01^{a}	85.15±0.33^{a}	0.613±0.015^{a}
4	68.09±0.47^{a}	75.12±0.30^{a}	86.40±0.42^{a}	0.709±0.056^{b}
8	68.08±0.65^{a}	75.53±0.03^{a}	86.70±1.12^{a}	0.805±0.045^{b}
16	66.54±0.29^{b}	74.14±0.27^{b}	87.46±0.42^{a}	0.975±0.013^{c}
24	68.29±0.27^{a}	75.39±0.22^{a}	88.00±0.67^{a}	0.917±0.036^{d}
48	67.65±0.63^{ab}	74.36±0.08^{b}	86.94±1.68^{a}	0.730±0.090^{b}

Values are mean ± SD (n=3). Different letters indicate samples significance calculated by ANOVA statistical test with Post Hoc Tukey ($p<0.05$).

Interactions between the starch and gluten proteins during the heating phase affect the initial, gelatinization, final temperatures (T_i, T_g, T_e) and ΔH. The lower temperatures of the endothermic transition for the 16 h sample (T_i = 66.54±0.29, T_g = 74.14±0.27) indicate a greater ease of starch hydration which could be due to a less ordered microstructure, with fewer bonds. So, during the heating, starch granules start to swell quickly, thus decreasing the content of free water in the surrounding matrix. This happens in a well-bubbled dough following the distension of the gluten network thanks to the expansion of CO_2 produced by yeast. Further, the barrier effects of gluten on the surface of the starch granules it is the major obstacle to the starch hydration and, therefore, to its gelatinization (Jekle et al., 2016).
The increase in ΔH value as leavening time increases to 16 h corresponds to a higher gelatinization degree of starch, in turn due to a greater availability of free water. At longer leavening, the reduction in ΔH could be attributed to a competition for water between gluten and starch molecules which become more damaged due to hydrolytic activities of flour and yest enzymes (Addo et al., 2001).

1.3.4 Reducing sugars, free amino groups and digestible starch in dough and pizza

Extractable reducing sugars in water were detected in almost similar amounts in dough and pizza samples along the leavening time interval, with no significant differences for the considered sample at the same time, except at 4 h (Figure 1.4-A).

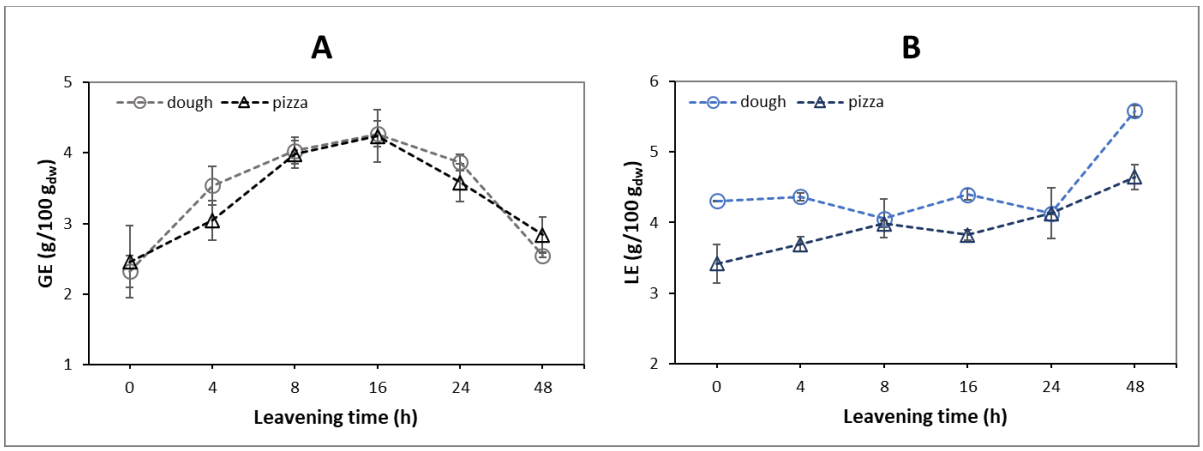

Figure 1.4 – Reducing sugars (A) and free amino groups (B) of dough and wood oven baked pizza base, at selected leavening time. Values are mean ± SD (n=4). GE, glucose equivalent reducing sugars; LE, leucin equivalent free amino groups.

Reducing sugars are expected to increase during leavening as a result of amylolytic activity by both endogenous flour and yeast enzymes, but some of these sugars are used as fermentable substrates by yeasts, so the measured values correspond to residual sugars net of yeast metabolic activity (Yiltirak et al., 2021). Thus, an increasing trend of reducing sugar level was found up to 16 h of leavening, followed by a reduction at 24 and 48 h with significative differences compared to 0 h (unleavened) and to 48 h samples for the dough (Figure 1.4-A). Meerts et al. (2018) reported that the highest yeast microbial activity occurs up to 6 h of leavening, after which it settles. This agrees with the results of this study, which show an increase in sugars up to 4 h and then remain almost constant. Evaluation of peptides present

in aqueous extracts of dough and pizza samples was done indirectly by determination of free primary amino groups. The results, expressed as g Leucine equivalents/100 g of sample on a dry weight basis, showed comparable values in the first 8 h in the case of dough samples, with a slight increase at following hours of leavening (Figure 1.4-B). Several studies have explained this trend as a result of the proteolytic activity of the flour (Yiltirak et al., 2021). Probably this increase is due to alterations in the glutinic network due to the metabolic activity of microorganisms during leavening. Nielsen et al., 2001 suggest that OPA method is used to determine the degree of hydrolysis in hydrolysed proteins in foods. In this sense, it can be said that free peptides, had an upward trend during the leavening of dough, as a result of endogenous proteolytic activity and lactic acid bacteria naturally present in flour (Di Cagno et al., 2002). For pizza samples, however, a slight tendency was observed from 3.42±0.27 g/100 g_{dw} of sample at time zero to 4.64±0.18 g/100 g_{dw} after 48 h of leavening. In each case, the measured values were lower with respect to dough, indicating that baking results in matrix modification because at high temperatures amino acids and peptides tend to react with sugars according to the Maillard reaction. In addition, proteins aggregate more stably, making it more difficult to release the amino ends.

For nutritional purposes, starch has been classified according to the rate of digestion into rapidly digestible starch (RDS, 20 min), slowly digestible starch (SDS, 120 min), and resistant starch (RS, not digested after 240 min) (Englyst et al., 1992). The rate and degree of digestion is widely depending on the structure (Romano et al., 2016). RDS is rapidly and completely digested in the small intestine and is associated with more rapid elevation of postprandial plasma glucose, whereas SDS is more slowly digested in the small intestine and is generally the most desirable form of dietary starch (Jenkins et al., 1981). Results for *in vitro* starch digestion of dough and pizza samples are reported in Table 1.3.

The results obtained from the dough samples showed similar amount of RDS than the amount SDS in 120 min of enzymatic digestion. Specifically, in dough, RDS is equal between 0 and 8 h of leavening, and in subsequent hours it decreases. No significant difference in SDS values were found for all the samples, except for 48 h where it turns out to be less (Table 1.3). In wood oven baked pizza base samples, a significant difference between time 0 and 4 h of leavening was observed, with an increase in RDS and decrease in SDS. From time 8 h onward, there was a clear increase in RDS at the expense of SDS. These results strengthen the findings that pizza is a high glycemic index (GI) food; in fact, after baking all the starch becomes rapidly digestible at long leavening time, while SDS value tends to almost zero, in contrast to the dough samples where the SDS levels were found to be almost similar to the RDS values (Table 1.3). The rapidly available glucose index named RAG, has been determined as a predictor of the potential glycemic response derived from the ingestion of a food and is nothing more than the RDS (Englyst et al., 1996). In fact, increased RDS and decreased SDS is also associated with increased glycemic response (Rosin et al., 2002; Ronda et al., 2012), and the importance of the relative content of the two starch fractions, RDS and SDS, also becomes evident in other studies conducted by Ells et al. (2005). Indeed, they showed that postprandial glucose and insulin varied significantly after consumption of foods with higher or lower RDS and SDS. In this sense, the SDRI parameter was the most useful tool in predicting the GI of pizza samples with different water and TS contents. From the results shown in Table 1.3-B, it can be stated

that in baked pizza samples, starting from 8 h of leavening, an increase in starch digestibility occurs, which correlates with an increase in the glycemic index after gastric digestion, as predicted by RAG values (Table 1.3-B). The resistant starch (RS) levels in the dough settled around 40 percent, decreasing from 44.07±1.75 to 36.89±0.33 % between 0 and 4 h, and then slightly increasing for the following leavening time to 47.99±0.43 % at 48 h (Table 1.3-A). In the case of pizza samples, however, the results showed an almost negligible amount of resistant starch, and this is a further confirmation that pizza is a high glycemic index food.

Table 1.3 – *In vitro* digestible starch of dough (A) and wood oven baked pizza base (B) at selected leavening time.

A

Leavening time (h)	RDS	SDS	RS	TS	SDRI	RAG
	(%)					
0	12.15±0.96^{a}	12.48±0.70^{a}	44.07±1.75^{ac}	68.71±3.42^{a}	--	--
4	11.81±0.07^{a}	12.06±0.54^{a}	36.89±0.33^{b}	60.76±0.29^{a}	--	--
8	13.29±0.54^{a}	11.43±1.44^{a}	38.59±1.05^{bc}	63.32±3.04^{a}	--	--
16	9.76±0.36^{b}	12.22±2.02^{a}	38.83±0.62^{bc}	60.81±2.28^{a}	--	--
24	7.25±0.12^{c}	12.12±1.27^{a}	46.78±2.93^{a}	66.16±4.33^{a}	--	--
48	9.25±0.39^{b}	8.72±0.59^{b}	47.99±0.43^{a}	65.96±0.23^{a}	--	--

B

Leavening time (h)	RDS	SDS	RS	TS	SDRI	RAG
	(%)					
0	46.06±3.01^{a}	26.08±5.87^{a}	0.58±0.05^{a}	72.72±2.82^{a}	63.46±6.60^{a}	51.18±3.44^{a}
4	53.25±1.07^{b}	11.53±5.79^{b}	0.50±0.01^{a}	65.28±4.73^{a}	81.84±7.57^{b}	59.17±1.19^{b}
8	65.65±0.75^{c}	1.62±0.76^{c}	0.48±0.03^{a}	67.75±1.55^{a}	96.91±1.11^{c}	72.95±0.83^{c}
16	67.15±0.87^{c}	1.63±0.87^{c}	0.44±0.02^{a}	69.23±0.02^{a}	97.00±1.28^{c}	74.61±0.97^{c}
24	64.58±3.93^{c}	1.67±1.13^{c}	0.45±0.07^{a}	66.71±2.87^{a}	96.78±1.73^{c}	71.76±4.37^{c}
48	59.02±2.19^{c}	3.22±0.76^{bc}	0.49±0.02^{a}	62.73±1.45^{a}	94.07±1.32^{c}	65.58±2.44^{c}

RDS, after 20 min; SDS, after 120 min; RS, after 240 min.
Values are mean ± SD (n=3). Different letters indicate samples significance calculated by ANOVA statistical test with Post Hoc Tukey ($p<0.05$).

1.3.5 Morphology during leavening

SEM analyses allowed to describe the morphology of dough and pizza samples over leavening time (Figure 1.5). At 0 h, in the dough sample disordered porous structure were visible: the holes can be attributed to the presence of air incorporated during the kneading of the ingredients. After 4 h, the cavities disappeared and the structure was much more compact with the wheat starch granules spherical or oval in shape in foreground (Ee et al., 2020). At 8 h the gluten network, developed under the CO_2 pressure, started to cover the starch granules; from 16 to 24 h of leavening, the protein filaments characteristic of a flaking network appeared and the gluten structure was broken and discontinuous. Finally, at 48 h the network collapsed completely, and the structure returned to being compact (Figure 1.5-A).
According to our result, Yan et al., 2020, reported that leavened dough with starter for 4 h showed conspicuous starch granules and a firm and dense gluten network. The starch granules were embedded in the gluten and combined rigidly in the first few hours of leavening. It has been also demonstrated that, as the leavening time increases, depolymerization of macromolecule proteins and the formation of gluten proteins with a fibrous structure and

greater continuity is observed (Marti et al., 2014). Moreover, other studies have shown the interaction between gluten and starch matrices as evidenced by smooth and uniform character of the contact interface (Peng et al., 2017; Lapčíková et al., 2019). Previous studies reported that at high leavening time the dough loses the ability to hold gas, resulting in products with poor elasticity (Yan et al., 2020), both due to the pH lowering that lead to degradation of polymers, promoting the opening of the gluten network, and the disruption of intermolecular disulphide bonds between glutenin and gliadin which makes starch grains more exposed to the action of hydrolytic enzymes (Di Cagno et al., 2014).

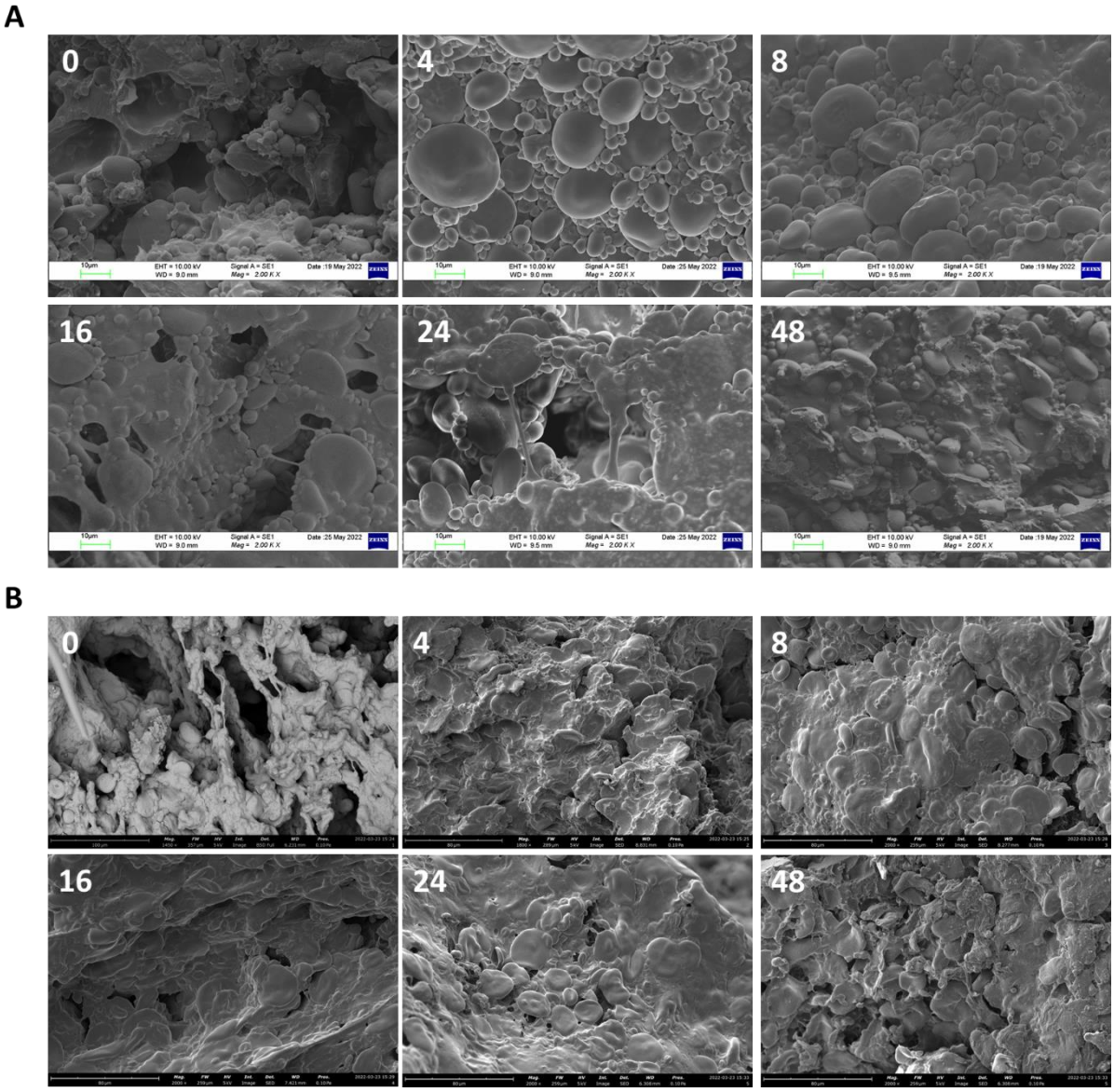

Figure 1.5 – SEM images of dough (A) and wood oven baked pizza base (B) at selected leavening time. Magnification: 2000 ×.

SEM observation of wood oven baked pizza base samples highlighted that, even after baking, the unleavened sample (0 h) presented a porous structure in which protein filaments and starch granules were evident (Figure 1.5-B). After 4 and 8 h of leavening, a disappearance of the holes was found with obvious formation of protein-starch bonds, confirming the results

obtained with mechanical-dynamic rheological tests (§ 1.3.2). Indeed, starch not only acts as a filler in the gluten network but also interacts with gluten proteins. As the leavening time increased, the proteins formed a "sheet" layer that covered the starch granules as was most evidenced at time 16 h. At 24 and 48 h of leavening a "stretching" of the gluten network was observed, with starch granules more exposed but swollen and burst because of gelatinization during heating process (Figure 1.5-B). Lindsay and Skerritt (1999) also showed in earlier studies that gliadins were uniformly dispersed within gluten filaments throughout the dough, consistent with a "space-filling" role.

1.4 Conclusions

During the leavening, variations in the rheological behaviour and biochemical characteristics of the dough are observed. Compression Work (J) increases up to 4 h and then decreases in the following times due to the diffusion of the CO_2 produced by the yeasts in the gluten network. In the relaxation phase, during the long leavening times, an increase in the viscous component is observed to the detriment of the elastic one. Modulus of elasticity (G') decreases as a result of the weak interactions between gluten proteins and starch that are established during heating and consolidated during cooling. Damage to starch, by enzymatic hydrolysis, improves its ability to gelatinize and is reflected in an increase in reducing sugars. SEM analyses showed a gluten network stretching starting from 8 h which allows a better access by the digestive enzymes.

1.5 References

- Addo, K., Xiong Y.L., Blanchard, S.P. (2000). Thermal and dynamic rheological properties of wheat flour fractions. Food Research International 34, 329-335
- Bernklau, I., Neußer, C., Moroni, A. V., Gysler, C., Spagnolello, A., Chung, W., Becker, T. (2017). Structural, textural and sensory impact of sodium reduction on long fermented pizza. Food Chemistry, 234, 398-407.
- Commission Regulation (EU) 97/2010. Entering a name in the register of traditional specialities guaranteed (Pizza Napoletana (TSG). Official Journal of the European Union, 34, 7–13.
- Connelly, R.K., and McIntier, R.L. (2008). Rheological properties of yeasted and nonyeasted wheat doughs developed under different mixing conditions. Journal of the Science of Food and Agriculture, 88(13), 2309-2323.
- Di Cagno, R., De Angelis, M., Lavermicocca, P., De Vincenzi, M., Giovannini, C., Faccia, M., and Gobbetti, M. (2002). Proteolysis by sourdough lactic acid bacteria: effects on wheat flour protein fractions and gliadin peptides involved in human cereal intolerance. Applied and environmental microbiology, 68(2), 623-633.
- Di Cagno, R., Rizzello, C.G., and Gobbetti, M. (2014). Adverse reactions to gluten: Exploitation of sourdough fermentation. In Wheat and Rice in Disease Prevention and Health, 171-177.
- Dreese, P.C., Faubion, J.M. and Hoseney, R.C. (1988). Dynamic rheological properties of flour, gluten and gluten-starch doughs. I. Temperature-dependent changes during heating. Cereal Chem. 65, 348–353.
- Ee, K.Y., Eng, M.K., and Lee, M.L. (2020). Physicochemical, thermal and rheological properties of commercial wheat flours and corresponding starches. Food Science and Technology, 40, 51-59.

- Ells L.J., Seal C.J., Kettlitz B., Bal W., Mathers J.C. (2005). Postprandial glycaemic, lipaemic and haemostatic responses to ingestion of rapidly and slowly digested starches in healthy young women. British Journal of Nutrition, 94, 948-955.
- Englyst, H.N., Kingman, S.M., and Cummings, J.H. (1992). Classification and measurement of nutritionally important starch fractions. European Journal of Clinical Nutrition, 46, S33-50.
- Englyst H.N., Kingman S.M., Hudson G.J., Cummings J.H. (1996). Measurement of resistant starch in vitro and in vivo. British Journal of Nutrition, 75, 749-755.
- Fan, J.L., Han, N., and Chen, H.Q. (2021). Physicochemical and structural properties of wheat gluten/rice starch dough-like model. Journal of Cereal Science, 98, 103181.
- Fessas, D., and Schiraldi, A. (2001). Water properties in wheat flour dough I: classical thermogravimetry approach. Food Chemistry, 72(2), 237-244.
- Ganguly, M. (2017). Naples' pizza spinning given UNESCO 'intangible heritage' status. http://edition.cnn.com/2017/12/07/europe/naples-unesco-pizza-intl/index.html.
- Goesaert, H., Brijs, K., Veraverbeke, W.S., Courtin, C.M., Gebruers, K., and Delcour, J.A. (2005). Wheat flour constituents: how they impact bread quality, and how to impact their functionality. Food Science and Technology, 16, 12-30.
- Janssen, A.M., van Vliet, T., and Vereijken, J.M. (1996). Fundamental and empirical rheological behaviour of wheat flour doughs and comparison with bread making performance. Journal of Cereal Science, 23(1), 43-54.
- Jekle, M., Mühlberger, K., and Becker, T. (2016). Starch–gluten interactions during gelatinization and its functionality in dough like model systems. Food Hydrocolloids, 54, 196-201.
- Jenkins, D.J., Wolever, T.M., Taylor, R.H., Barker, H., Fielden, H., Baldwin, J.M., and Goff, D.V. (1981). Glycemic index of foods: a physiological basis for carbohydrate exchange. The American journal of clinical nutrition, 34(3), 362-366.
- Khatkar, B.S., Barak, S., and Mudgil, D. (2013). Effects of gliadin addition on the rheological, microscopic and thermal characteristics of wheat gluten. International journal of biological macromolecules, 53, 38-41.
- Lapčíková, B., Burešová, I., Lapčík, L., Dabash, V., and Valenta, T. (2019). Impact of particle size on wheat dough and bread characteristics. Food chemistry, 297, 124938.
- Launay, B. and Michon, C. (2008). Biaxial extension of wheat flour doughs: lubricated squeezing flow and stress relaxation properties. Journal of Texture Studies 39, 496-529.
- Lee, K., Drescher, D.G. (1978). Fluorometric amino-acid analysis with o-phthaldialdehyde (OPA). International Journal of Biochemistry, 9, 457-467.
- Li, W., Dobraszcsyk, B.J., Schofield, J.D. (2003). Stress relaxation behavior of wheat dough, gluten, and gluten protein fractions. Cereal Chemistry, 80, 333-338.
- Lindsay, M.P., and Skerritt, J.H. (1999). The glutenin macropolymer of wheat flour doughs: structure–function perspectives. Trends in Food Science & Technology, 10(8), 247-253.
- Marchetti, L., Cardós, M., Campaña, L., and Ferrero, C. (2012). Effect of glutens of different quality on dough characteristics and breadmaking performance. LWT-Food Science and Technology, 46(1), 224-231.
- Marshall, W.E., and Chrastil, J. (1992). Interaction of food proteins with starch. Biochemistry of Food Proteins, 3, 75-97.
- Marti, A., Torri, L., Casiraghi, M.C., Franzetti, L., Limbo, S., Morandin, F., Pagani, M.A. (2014). Wheat germ stabilization by heat-treatment or sourdough fermentation: Effects on dough rheology and bread properties. LWT-Food Science and Technology, 59(2), 1100-1106.

- Masi, P. (1989). Study of the influence of temperature on the rheological behaviour of gluten by means of dynamic mechanical analysis. Food Properties and Computer-Aided Engineering of Food Processing Systems. 357-362.
- Masi, P., Romano, A., and Coccia, E. (2015). The artisanal production process of Neapolitan Pizza, in The Neapolitan Pizza: a scientific guide bout the artisanal process, 67–96. Doppia Voce ed. Naples, Italy.
- Meerts, M., Cervera, A.R., Struyf, N., Cardinaels, R., Courtin, C.M., and Moldenaers, P. (2018). The effects of yeast metabolites on the rheological behaviour of the dough matrix in fermented wheat flour dough. Journal of Cereal science, 82, 183-189.
- Miller, G.L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry, 31, 426-8.
- Miller, K.A., and Hoseney, R.C. (1999). Dynamic rheological properties of wheat starch-gluten doughs. Cereal Chemistry, 76(1), 105-109.
- Nielsen, P.M., Petersen D., Dambmann C. (2001). Improved Method for Determining Food Protein Degree of Hydrolysis. Journal of Food Science, 66(5), 642-646.
- Ooms, N., and Delcour, J.A (2015). How to impact gluten protein network formation during wheat flour dough making. Current Opinion in Food Science, 25, 88-97.
- Peleg, M. (1979). Characterisation of the stress relaxation curves of solid foods. Journal of Food Science. 44, (1) 277-281.
- Peng, B., Li, Y., Ding, S., and Yang, J. (2017). Characterisation of textural, rheological, thermal, microstructural, and water mobility in wheat flour dough and bread affected by trehalose. Food Chemistry, 233, 369-377.
- Pyler, E.J., Gorton, L.A. (2009). Fundamentals and Ingredients. Baking Science & Technology, 1(4).
- Romano, A., Toraldo, G., Cavella, S., Masi, P. (2007). Description of leavening of bread dough with mathematical modelling. Journal of Food Engineering. 83 (2), 142-148.
- Romano, A., Mackie, A., Farina, F., Aponte, M., Sarghini, F., and Masi, P. (2016). Characterisation, in vitro digestibility and expected glycemic index of commercial starches as uncooked ingredients. Journal of Food Science and Technology, 53, 4126-4134.
- Ronda, F., Rivero, P., Caballero, P.A., Quilez, J. (2012). High insoluble fibre content increases in vitro starch digestibility in partially baked breads. International Journal of Food Sciences and Nutrition, 63(8), 971-977.
- Rosell, C.M., and Foegeding, A. (2007). Interaction of hydroxypropylmethylcellulose with gluten proteins: small deformation properties during thermal treatment. Food Hydrocolloids, 21(7), 1092-1100.
- Rosin, P.M., Lajolo, F.M., and Menezes, E.W. (2002). Measurement and characterisation of dietary starches. Journal of food composition and analysis, 15(4), 367-377.
- Salvador, A., Sanz, T., and Fiszman, S. M. (2006). Dynamic rheological characteristics of wheat flour–water doughs. Effect of adding NaCl, sucrose and yeast. Food Hydrocolloids, 20(6), 780-786.
- Scanlon, M.G., and Zghal, M.C. (2001). Bread properties and crumb structure. Food Research International (10), 841-864.
- Sluková, M., Levková, J., Michalcová, A., Horáčková, Š. and Skřivan, P. Effect of the dough mixing process on the quality of wheat and buckwheat proteins. Czech J. Food Sci. 2017, 35(6), 522–531; doi: 10.17221/220/2017-CJFS.
- Song, Y., and Zheng, Q. (2007). Dynamic rheological properties of wheat flour dough and proteins. Trends in food science & technology, 18(3), 132-138.

- Srikaeo, K., Furst, J.E., Ashton, J.F., and Hosken, R.W. (2006). Microstructural changes of starch in cooked wheat grains as affected by cooking temperatures and times. LWT-Food Science and Technology, 39(5), 528-533.
- Struyf, N., Verspreet, J., Verstrepen, K.J., Courtin, C.M. (2017). Investigating the impact of α-amylase, α-glucosidase and glucoamylase action on yeast-mediated bread dough fermentation and bread sugar levels. Journal of Cereal Science, 75, 35-44.
- Turbin-Orger, A., Boller, E., Chaunier, L., Chiron, H., Della Valle, G., Réguerre, A.L., (2012). Kinetics of bubble growth in wheat flour dough during proofing studied by computed X-ray micro-tomography. Journal of Cereal Science, 56, 676–683.
- Wehrli, M.C., Kratky, T., Schopf, M., Scherf, K.A., Becker, T., and Jekle, M. (2021). Thermally induced gluten modification observed with rheology and spectroscopies. International journal of biological macromolecules, 173, 26-33.
- Yan, B., Yang, H., Wu, Y., Lian, H., Zhang, H., Chen, W., Fan, D. and Zhao, X. (2020). Quality Enhancement Mechanism of Alkali-Free Chinese Northern Steamed Bread by Sourdough Acidification. Molecules, 25, 726.
- Yang, Y., Guana, E., Zhang, T., Li, M., Bian, K. (2019). Influence of water addition methods on water mobility characterisation and rheological properties of wheat flour dough. Journal of Cereal Science 89, 102791.
- Yiltirak, S., Kocadağlı, T., Çelik, E.E., Kanmaz, E.O., Gökmen, V. (2021). Effects of Sprouting and Fermentation on Free Asparagine and Reducing Sugars in Wheat, Einkorn, Oat, Rye, Barley, and Buckwheat and on Acrylamide and 5-Hydroxymethylfurfural Formation during Heating. Journal of Agricultural and Food Chemistry, 69, 9419-9433.
- Zhang, Y., Hong, T., Yu, W., Yang, N., Jin, Z., and Xu, X. (2020). Structural, thermal and rheological properties of gluten dough: Comparative changes by dextran, weak acidification and their combination. Food chemistry, 330, 127154.

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1.6 Supplementary materials

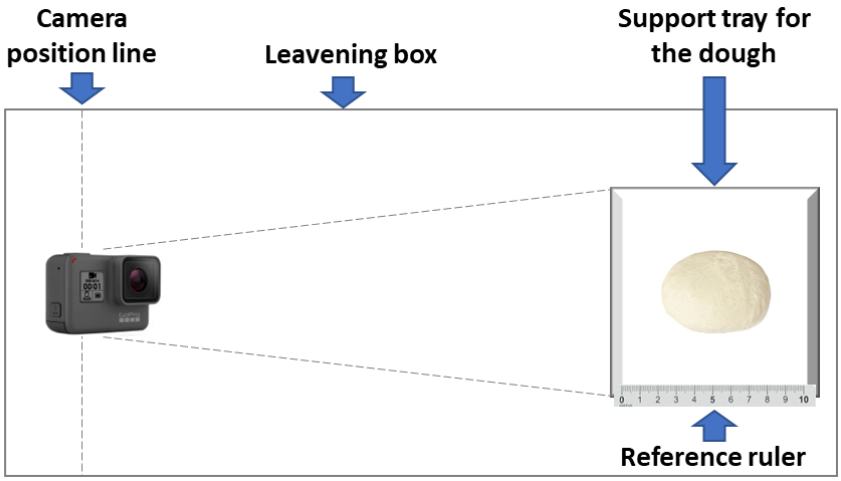

Figure 1.S1 – Schematic representation of camera position respect to the dough for image acquiring during leavening in the *ad hoc* plastic box.

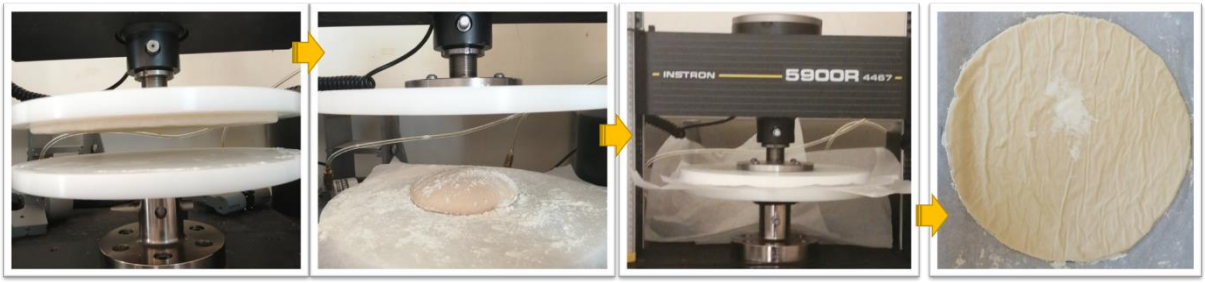

Figure 1.S2 – Flow chart of compression of pizza dough in the two mounted plates (Ø 30 cm) of the dynamometer (Instron, mod. 5900R, USA).

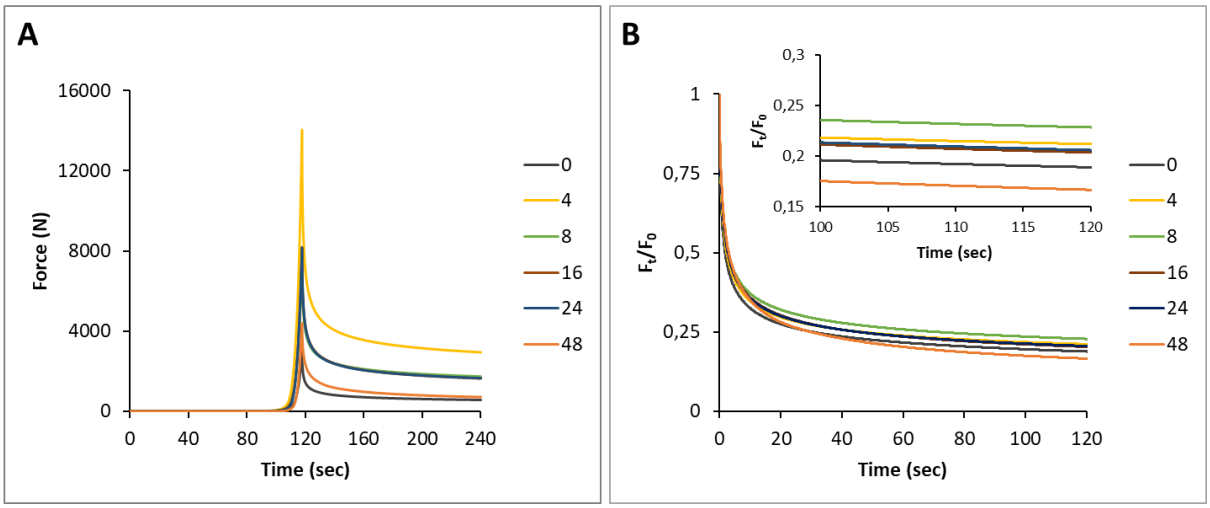

Figure 1.S3 – Stress-relaxation (A) and elaborated relaxation graph (B) of pizza dough at selected leavening time.

1.6.S1 Glucose release from pizza base after *in vitro* simulated gastro-intestinal digestion

In order to evaluate the impact of the leavening time of baked pizza on the total amount of glucose released into the intestine after digestion, *in vitro* simulated digestion experiments were conducted. Wood oven baked pizza base samples prepared at different leavening times (0, 4, 8, 16 24 and 48 h) were subjected to *in vitro* simulated gastro-intestinal digestion following the static method reported by Minekus et al. (2014). Simulated Salivary Fluid (SSF), Simulated Gastric Fluid (SGF), and Simulated Intestinal Fluid (SIF) were prepared according to the established conditions. All digestion steps were carried out in a shaking incubator at 37 °C and 170 rpm. For the oral phase, a little slice of pizza base (0.5 g) was broken up and then suspended in 1 mL of SSF (containing 1500 U/mL of human salivary amylase) and incubated for 2 min. Then, oral digesta were mixed with 1.3 mL SGF containing 40 µL of phospholipid lysosomes (10 mg/mL). The pH was adjusted to 2.7 and 0.16 mL of porcine pepsin (3000 U/mg) at a concentration of 15 mg/mL were added. Samples were incubated for 2 h at 37 °C. Pepsin hydrolysis was stopped by raising the pH to 7.0 with 1 M sodium bicarbonate. The duodenal digestion was carried out 2 h at 37 °C after incorporating 3 mL of SIF, bile salts (80 mg), porcine pancreatic lipase (5 mg), trypsin (3.5 mg, 100 U/mg as TAME activity), α-chymotrypsin (1.5 mg, 40 U/mg) and pancreatic α-amylase (5.5 mg, 10 U/mL). A final step of intestinal digestion was performed with 0.5 mL of BBM (13 µU/µL) preparation after adjusting pH to 7.2 with 1 M sodium bicarbonate. After 4 h at 37 °C peptidases were inactivated by immersion in boiling water for 5 min. Digesta were then centrifuged for 10 min at 13000×g and the supernatants were analysed for glucose determination by enzymatic reaction. For this purpose, 50 µL of 1:10 supernatant dilutions were added to 1.5 mL of GOPOD reaction mix (D-Glucose Assay Kit, GOPOD Format, Megazyme) and incubated at 50 °C for 20 min at 110 rpm. After incubation, the absorbance value at λ = 510 nm was measured in the UV/Vis spectrophotometer V-630 (JASCO, Japan). A calibration curve was built using glucose as standard (0÷50 µg). Samples were analysed in triplicate and freed glucose in the supernatant after digestion was expressed as mean ± SD on dry weight basis (mg/g_{dw} of pizza base). The obtained results are reported in Figure 1.S4. As can be observed, the highest amount of glucose was released in wood oven pizza base samples leavened for 16 h, that reached the value of 43.3±1.2 mg/g_{dw}. At others leavening time, both lower and higher, the amount of freed glucose was assessed around 35 mg/g_{dw}, except for the unleavened product (time 0) which showed the lowest level of glucose release (30.81±0.18 mg/g_{dw}).

From these results it can be deduced that leavening increases the digestibility of starch during simulated digestion. In fact, the unleavened pizza base is the one that releases the least amount of glucose. By comparing the glucose levels recorded after simulated digestion with the content of reducing sugars in the pizza after baking (Figure 1.4-A), a similar trend can be observed, with a maximum peak after 16 h of leavening.

Leavened dough already has higher initial content of reducing sugars (presumably glucose and maltodextrin) than unleavened dough, due to the action of endogenous and yeast amylases during fermentation. These sugars remain after baking and then the maltodextrins are completely hydrolysed to glucose by digestive enzymes, to which is added the glucose released from the starch during simulated gastrointestinal digestion (Figure 1.4-A).

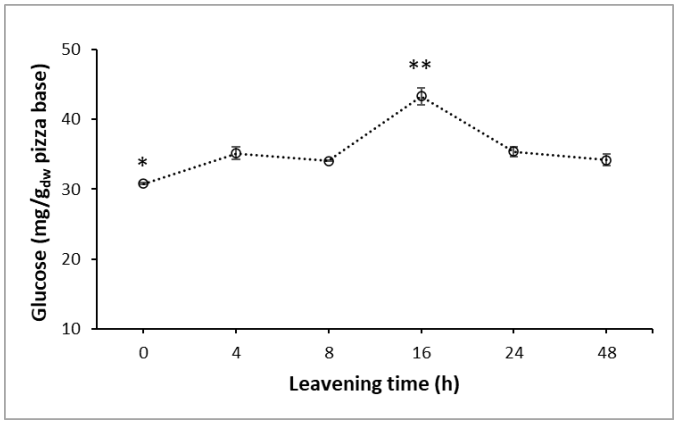

Figure 1.S.4 – Freed glucose after *in vitro* simulated gastro-intestinal digestion. Values are mean ± SD (n=3). Samples marked with asterisks are significatively different by ANOVA Post Hoc Tukey-test ($p<0.05$).

1.6.S2 Acrylamide levels in pizza base leavened for different time

Acrylamide (AA) is formed mainly in the Maillard reaction from asparagine and carbonyl sources, and this reaction occurs mostly at above 120 °C (Friedman 2003; Wang et al., 2017). Several factors influence acrylamide formation, such as reducing sugars, asparagine and fibre contents, damaged starch, fermentation time, moisture of the sample. As a starchy product baked at high temperature in wood oven, Neapolitan pizza is considered a risky food for AA content. In the literature there are no studies reporting the AA content in Neapolitan pizza baked in a wood oven. An in-depth study for the reduction of AA levels in pizza bases using asparaginase enzyme has been conducted in Chapter 4 (Covino et al., 2023). In that study it is reported that the average AA content in a wood oven baked pizza base is around 2000 µg/kg$_{dw}$. To evaluate the impact of the leavening time on the formation of this toxic substance, wood oven baked pizza base samples prepared at different leavening times (0, 4, 8, 16 24 and 48 h) were tested for AA content. The details of the AA extraction and determination protocols are reported in Chapter 4, from § 4.2.6 to § 4.2.9. For the analysed samples the recovery of AA extraction was in the range of 86-92 %.

From the experimental data, the levels of AA varied from 634 to 1703 µg/kg$_{dw}$, with no significative differences between 4 and 48 h of leavening (Figure 1.S5).

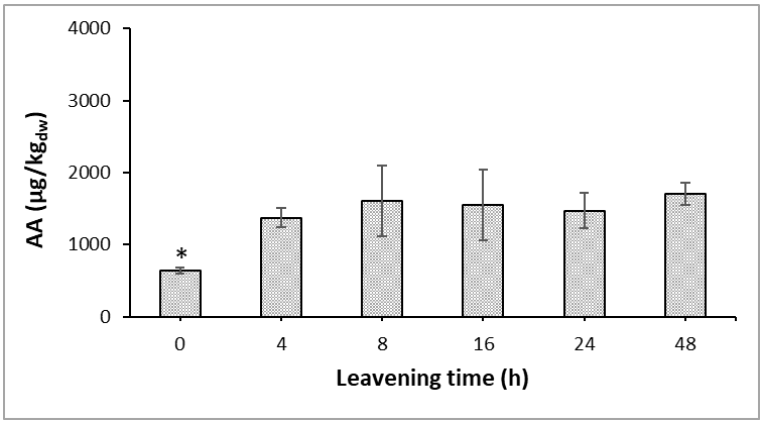

Figure 1.S5 – AA content in wood oven baked pizza base leavened for 0, 4, 8, 16, 24, 48 h. Values are mean ± SD (n=3).

These results can be explained by considering the levels of both reducing sugars and free amines, taken as reference for free amino acids (including asparagine), during leavening (Figure 1.4), the two important precursors in the formation of AA. In fact, between 0 and 4 h, the reducing sugars in the dough increase significantly by 1 point, after which they remain within a range of 3.5-3.8 g/100g_{dw} up to 24 h, to then return to the starting value after 48 h of leavening. The levels of free amino groups, on the other hand, fluctuate around a value of 4.3 g/100g_{dw} for the entire leavening period, with a significant increase at 48 h to around 5.6 g/100g_{dw}. Therefore, given that the most important variation of reducing sugars is recorded between 0 and 4 h, the formation of AA also increases in this time interval and the values do not undergo significant differences for the rest of the leavening. Unlike what expected, despite the collapse of reducing sugars at 48 h, no lowering of the AA value was observed probably due to a simultaneous increase in free asparagine in the dough which keeps the value high (Figure 1.4). Finally, it can be hypothesized that in the unleavened product the AA value is significantly lower also due to issues related to baking. As soon as the dough is placed in the oven, water evaporates very fast from the surface layers, resulting in much lower water content than at the core. As the water content decreases in the external part of the pizza base, the temperature can exceed 100 °C, which supports reactions such a caramelization, carbonization and Maillard reaction, responsible for the browning coloration (Gökmen and Palazoğlu, 2008; Dessev et al., 2020). Therefore, unleavened dough is less malleable and stretchable and than the resulting pizza disc is more compact and the migration of the water during baking is not favoured by the structure of the dough matrix. Since water is an obstacle for the formation of AA, a smaller amount of AA is produced in the unleavened pizza due to the persistence of a certain hydration during baking process in the wood oven. On the other hand, at 48 h of leavening, with the same reducing sugar values as at time 0, the AA level reached is higher because the dough allows for a rapid removal of water during baking, and this triggers the Maillard reaction which leads to the formation of AA.

Reference

- Covino, C., Sorrentino, A., Di Pierro, P., Aiello, A., Romano, R., and Masi, P. (2023). Asparaginase enzyme reduces acrylamide levels in fried and wood oven baked pizza base. Food Chemistry Advances, 100206. https://doi.org/10.1016/j.focha.2023.100206.
- Dessev, T., Lalanne, V., Keramat, J., Jury, V., Prost, C., and Le-Bail A. (2020). Influence of Baking Conditions on Bread Characteristics and Acrylamide Concentration. Journal of Food Science and Nutrition Research, 3, 291-310.
- Friedman, M. (2003). Chemistry, biochemistry, and safety of acrylamide. A review. Journal of Agricultural and Food Chemistry, 51, 4504-4526.
- Gökmen, V., and Palazoğlu, T.K. (2008). Acrylamide Formation in Foods during Thermal Processing with a Focus on Frying. Food and Bioprocess Technology, 1, 35-42
- Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., … and Brodkorb, A. (2014). A standardised static in vitro digestion method suitable for food–an international consensus. Food & function, 5(6), 1113-1124.
- Wang, S., Yu, J., Xin, Q., Wang, S., and Copeland, L. (2017). Effects of starch damage and yeast fermentation on acrylamide formation in bread. Food Control, 73, 230-236.

1.7 Appendix: Biochemical phenomena involved in the leavening of pizza dough: electrophoresis profile of soluble and gluten protein

1.7.A1 Sample preparation

The preparation of dough and wood oven baked pizza bases, used for this study, were thoroughly described in §1.1.2 section. The leavening times at which the dough was taken and subjected to freeze-drying for subsequent analysis, or baking the pizza were 0, 1, 2, 4, 6, 8, 16 and 24 h.

1.7.A2 Extraction of water-soluble substances

For each selected leavening time, freeze-dried and ground samples of dough and wood oven baked pizza base were weight (0.2 mg) and H_2O_d (1:10) was added for the extraction of soluble substances. Next, the samples were vortexed for 3 min at room temperature (20-25 °C) and then sonicated for 15 min in an ultrasonic bath at room temperature at 240 Watt (cycle 0.5). Subsequently, samples were centrifuged at 9000×g for 10 min at 25 °C to separate the soluble fraction (supernatant) from the insoluble fraction (pellet). The supernatants were stored at -20 °C until subsequent electrophoretic analysis, and the pellets were resuspended in 2 mL of H_2O_d in the presence of thermostable α-amylase (1:100, 20000 U/mL). The resuspended pellet was incubated at 90 °C for 1 h in a thermoblock, vortexing every 15 min. Then, the solution was centrifuged at 14000×g for 10 min at room temperature. The centrifuged pellet was stored at -20 °C for subsequent electrophoretic analysis.

1.7.A3 Isolation of protein by modified Osborne method

The Osborne method was used for the extraction of gluten proteins (gliadin and glutenin) with some modifications (Kumar et al., 2021). Freeze-dried and ground samples of dough and pizza baked in the wood oven were weight and H_2O_d (1:25) was added for the extraction of soluble substances. 40 mg of powder sample was weighed and 1.6 mL of H_2O_d was added. Samples were vortexed for 1 min and placed in the orbital shaker (Forma Scientific) for 2 h at 30 °C with shaking at 250 rpm. Subsequently, the samples were centrifuged at 18000×g for 10 min at 25 °C and the pellet was resuspended again in H_2O_d (thrice). The supernatants taken after each centrifuge represent the albumin (not analysed). After removal of the albumins, a second extraction was performed by adding 10% NaCl to the pellets, following the same procedure as previously described. The supernatants removed after this extraction represent the globulins (not analysed). Finally, a third extraction was performed by resuspending the pellet in 70% ethanol using the procedure described above. The supernatants collected represent gliadins in ethanol, which were concentrated by means of a Speed Vac centrifuge (Eppendorf) to a volume of approximately 200 µL, i.e., concentrated 8-fold, and then brought to a final volume of 500 µL with 70% ethanol, and stored for subsequent analysis. The pellets, on the other hand, represent glutenins and were resuspended in "glutenin buffer" and shaken in the orbital shaker for 2 h at 200 rpm. Subsequently, the samples were centrifuged at 18000×g for 15 min at room temperature. The resulting pellets were again resuspended in "DTT buffer," agitated for 1 h at 150 rpm, and subsequently centrifuged (Weiss et al., 1993). Supernatants obtained

after suspension in glutenin and DTT buffer were concentrated in a Speed Vac centrifuge to a final volume of 450 µL and stored at -20 °C for subsequent analysis.

1.7.A4 SDS-PAGE

After extraction of water-soluble substances from dough and pizza samples, supernatants and pellets were analysed by SDS-PAGE using a system (Bio-Rad Laboratories, Inc., USA). Proteins were separated by electrophoresis on 15 % polyacrylamide gel containing 10 % SDS, under reducing and denaturing conditions, according to the method described by Laemmli 1970. Protein samples were dissolved in Laemmli's buffer (Tris 15.6 mM, SDS 0.5 %, glycerol 2.5 %, β-mercaptoethanol 1.25 % and bromophenol blue 0.05 %) and boiled for 5 min. 16 µL were loaded onto the gels for the supernatants; while 8 µL of sample was added to each electrophoresis cell for pellets obtained after treatment with the enzyme. Electrophoretic analysis was conducted for 30 min at a constant potential difference of 80 V and for about 1 hour at 120 V. At the end of electrophoretic migration, protein bands were visualized by staining the gel with Coomassie blue R-250 and subsequent destaining of 10 % methanol and 10 % acetic acid solution (Wang et al., 2019). The same electrophoretic analysis was used to profile the gluten proteins isolated by the Osborne method, specifically representing gliadins and glutenins. For all extracted gliadin samples 12 µL were loaded onto the gels for electrophoretic run. For the samples obtained from the extraction of glutenins, after "glutenin" and "DTT" buffers, the volumes of the solutions after concentration were measured and the volumes of supernatant to be used to have the same concentration on the gel for all samples analysed were calculated. 12 µL of sample was loaded onto the gels, as for gliadins.

1.7.A5 Results and Discussion

The aqueous extracts of lyophilised dough and pizza samples and the water-insoluble component were subjected to electrophoretic analysis by SDS-PAGE at 15% under reducing and denaturing conditions (Figure 1.A1). However, the pellet obtained after extraction of the water-soluble substances from the dough and pizza samples was first subjected to treatment with the enzyme α-amylase to remove all the solubilized components as a result of starch hydrolysis, after which the remaining pellet, containing the insoluble component, was used for electrophoretic analysis (Figure 1.A1-B). From the protein profile, it can be seen that in the supernatants, the protein bands range from 75 to 10 kDa, and the most pronounced bands are visible around 15, 37 and 45 ~ 60 kDa (Figure 1.A1-A). These protein species correspond to the water-soluble proteins found in wheat flour, and in the specific, albumins and globulins that were identified by Eckert et al. (2006) with a molecular weight (MW) of 11 kDa. Regarding the electrophoretic analysis of the pellets, i.e., the nonwater-soluble component, it can be observed that the protein bands are higher MW (Figure 1.A1-B) and thus could be related to high MW glutenins between 250 and 100 kDa (Singh et al., 1991) and low MW glutenins and gliadins that are distributed in the 75 to 25 kDa range (Weiss et al., 1993). Electrophoretic analysis shows in particular that, at time 24 h, corresponding to a reduction in water-soluble protein (Figure 1.A1-A, lane 24) there is a greater intensity of protein bands in the corresponding pellet (Figure 1.A1-B, lane 24). Presumably, a greater degree of proteolysis of water-soluble proteins occurs at this leavening time, and that is in agreement with the results

on the determination of primary amine groups, which reach the highest levels at high leavening time (Figure 1.4-B). Moreover, it can be hypothesized that due to the deconstruction of the gluten network, the extractability yields in denaturing buffer of protein species from the water-insoluble component are improved, which explains the increased intensity of protein bands in the 24 h pellet samples.

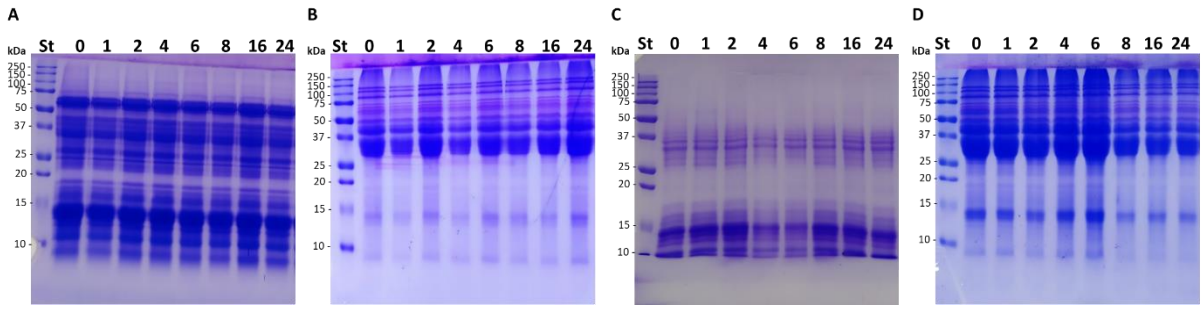

Figure 1.A1 – Electrophoretic profile in 15% SDS-PAGE of dough (A, B) and pizza (C, D) samples at selected leavening time. Soluble proteins in aqueous extracts (A, C), and insoluble proteins after α-amylase treatment of pellets (B, D) are analysed. The numbers on the lanes correspond to the leavening time (h). St, Precision Prestained Protein Standards (Biorad).

The profile of water-extractable proteins was also evaluated in pizza samples baked in a wood oven and compared with what was observed for raw dough (Figure 1.A1-C *vs* Figure 1.A1-A). After baking, the water-soluble protein species decrease and, in particular, only bands below 37 kDa and between 15 and 10 kDa are visible. Furthermore, a reduction in the intensity of protein electrophoretic bands at the leavening time of 4 and 6 h was showed (Figure 1.A1-C). In parallel, for the pellets after treatment with α-amylase, an increase in higher molecular weight protein bands (>37 kDa) is observed especially in the first hours, which is also more pronounced at 4 and 6 h of leavening (Figure 1.A1-D). Starting from 8 h, however, the intensity of proteins extracted from the pizza sample pellets also decreases. This phenomenon indicates that baking tends to cement the proteins into the gluten network making them less soluble in water. In fact, proteins manage to be extracted from the matrix only after hydrolysis of starch with α-amylase. However, at high leavening times (8, 16 and 24 h), the amount of protein extracted from the pellets is significantly reduced, which could mean that from time 8 h onward, the protein network is being affected by the proteases of the microorganisms resulting in increased release of polypeptide chains into the aqueous phase. This is reflected in a concomitant increase in the intensity of the bands in the supernatant of both the first aqueous extraction (Figure 1.A1-C, lane 8, 16 and 24), and the liquid fraction obtained after starch hydrolysis (data not shown).

For a more accurate assessment of the gluten component, the constituent proteins, gliadins and glutenins, were isolated by the Osborne method from dough and pizza samples. For the raw dough samples, gliadins show a majority band between 25 and 37 kDa, and a less intense band at about 45 kDa (Figure 1.A2-A), in agreement with Weiss et al. (1993) and De Angelis et al. (2006). Glutenins, on the other hand, are distributed along a wide MW range (25÷250 kDa) (Figure 1.A2-B and C). Wieser et al. (2007), state that the molecular weight distribution of glutenins has been recognised as a major determinant of dough properties and baking

performance. SDS-PAGE analysis of gluten proteins from the dough showed no particular differences at the low leavening time, however, differences are visible in both gliadins and glutenins for high leavening times, i.e., 16 and 24 h, where the intensity of the bands is slightly lower than in the samples at the other times. This phenomenon can be attributed to a partial hydrolysis of these proteins as a result of the metabolic activity of microorganisms (De Angelis et al., 2016). It is noteworthy that the largest portion of glutenins was solubilized in SDS-containing buffer in the absence of reducing agents (Figure 1.A2-B), while only a smaller portion is released in SDS+DTT-containing buffer (Figure 1.A2-C), indicating that the interactions between these proteins in the slurries are predominantly noncovalent in nature.

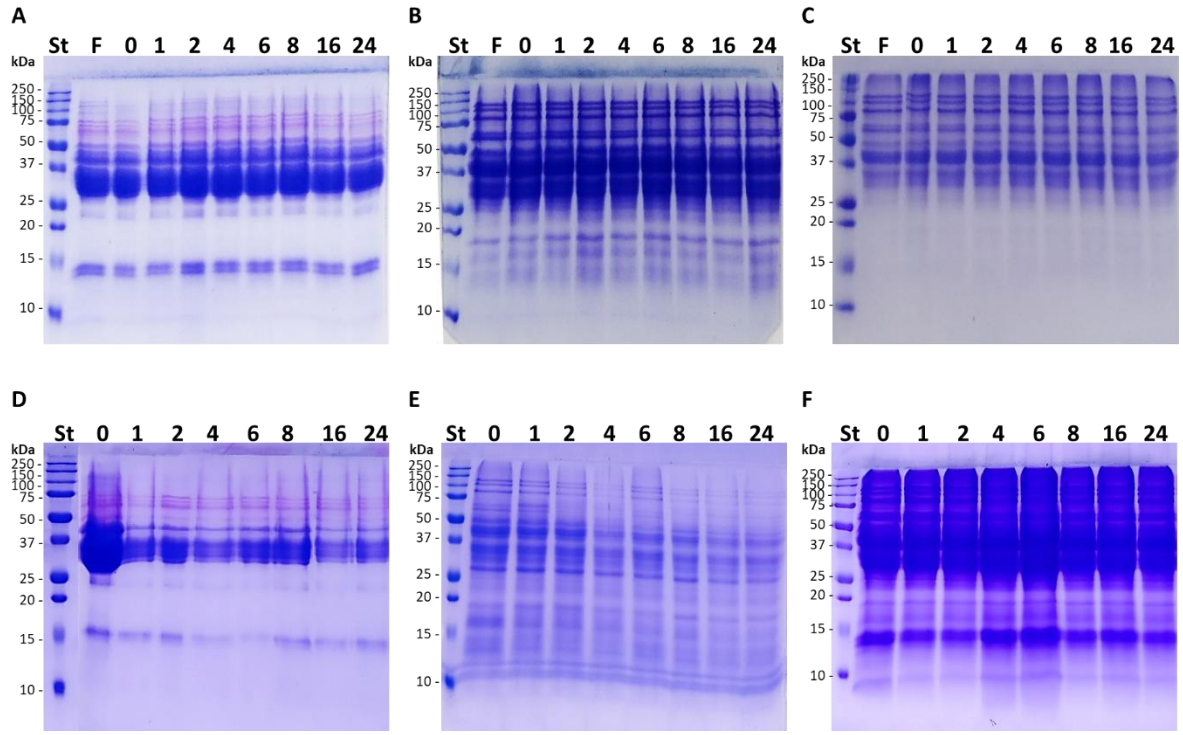

Figure 1.A2 – Electrophoretic profile in 15% SDS-PAGE of protein fractions from dough (A, B, C) and pizza (D, E, F) samples at selected leavening time after isolation by Osborne method. Gliadins (A, D), and glutenins sequentially resuspended in buffer containing SDS (B, E) and SDS+DTT (C, F) are analysed. The numbers on the lanes correspond to the leavening time (h). Lane F, Protein extracted from 00 flour used as control. St, Precision Prestained Protein Standards (Biorad).

The profile of gluten proteins extracted by the Osborne method from pizza deviates significantly from that of dough (Figure 1.A2). After baking, both gliadins and extractable glutenins decrease as the leavening time (0÷24 h) increase, as observed by the obvious decrease in the intensity of the bands (Figure 1.A2-D and -E). This result can be explained by considering that baking results in a stabilization of the protein structure with the formation of disulfide (SS) bonds between gluten proteins. The consequence is an increased compactness of the protein scaffold that can be broken down only in the presence of reducing agents such as DTT (Figure 1.A2-F). Also, the presence of a band at about 15 kDa whose intensity is slight in the profile of gliadins (Figure 1.A2-D) and which instead becomes very pronounced in the profile of glutenins extracted in SDS+DTT (Figure 1.A2-F), a sign that this protein is covalently

bound in the gluten network due to the high temperatures reached during baking. This band is virtually absent in the profile of SDS+DTT-extracted glutenins of the dough (Figure 1.A2-C). Ooms and Delcour (2015) state that several types of reactions and interactions are crucial for gluten network formation. In this regard, intermolecular disulphide (SS) bonds between glutenin polymers with gliadins are crucial.

1.7.A6 References

- De Angelis M., Rizzello C. G., Fasano A., Clemente M. G., De Simone C., Silano M., De Vincenzi M., Losito I., Gobbetti M. (2006). VSL#3 probiotic preparation has the capacity to hydrolyze gliadin polypeptides responsible for Celiac Sprue. Biochimica et Biophysica Acta ,1762, 80-93.
- Eckert R., Berghofer E., Ciclitira P. J., Chirdo F., Denery-Papini S., Ellis H. J., Ferranti P., Goodwin P., Immer U., Mamone G., Méndez E., Mothes T., Novalin S., Osman A., Rumbo M., Stern M., Thorell L., Whim A., Wieser H. (2006). Towards a new gliadin reference material–isolation and characterisation. Journal of Cereal Science, 43, 331–341.
- Kumar A., Nayak R., Purohit S. R., Rao P. S. (2021). Impact of UV-C irradiation on solubility of Osborne protein fractions in wheat flour. Food Hydrocolloids, 110, 105845.
- Laemmli U. K. (1970). Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. Nature, 227.
- Ooms N., Delcour J.A (2015). How to impact gluten protein network formation during wheat flour dough making. Current Opinion in Food Science, 25, 88–97.
- Singh N. K., Shepherd K. W., Cornish G. B. (1991). A Simplified SDS-PAGE Procedure for Separating LMW Subunits of Glutenin. Journal of Cereal Science, 14, 203-208.
- Wang, Y.R., Zhang, B., Fan, J.L., Yang, Q., Chen, H.Q., (2019). Effects of sodium tripolyphosphate modification on the structural, functional, and rheological properties of rice glutelin. Food Chem. 281, 18–27.
- Weiss W., Vogelmeier C., Angelika Gorg A. (1993). Electrophoretic characterisation of wheat grain allergens from different cultivars involved in bakers' asthma. Electrophoresfs, 14, 805-816.
- Wieser H. (2007). Chemistry of gluten proteins. Food Microbiology, 24, 115-119.

Chapter 2

Comparative study of pizza dough prepared with selected lactic acid bacteria and yeast: effect of long leavening time

Under submission

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Chapter 3

How freezing and thawing techniques affect leavening kinetic and physico-chemical characteristics of pizza dough

Paper in preparation

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Chapter 4

Asparaginase enzyme reduces acrylamide levels in fried and wood oven baked pizza base

Published paper
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4. Abstract

The possibility to reduce the acrylamide (AA) formation during cooking by treating pizza dough with L-Asparaginase enzyme was explored. Wheat flour was added with commercial enzyme Preventase® in three formulations: W, M and XR-BG during mixing to produce pizza doughs. AA content was evaluated in the obtained pizza bases through UHPLC-UV analysis. Enzymes concentration (0.15÷12 U/100 g of flour) and water content (58÷64 g/100 g of flour) during mixing were considered for pizza doughs intended for frying. In the fried pizza base, the untreated sample (without Preventase®) showed an AA level of 3150±554 $\mu g/kg_{dw}$. Preventase® treatment was able to reduce AA up to non-detectable levels (M and W) or 89 % (XR-BG). The enzymatic treatment up to 3-6 U/100 g did not affect the dough handling. Preventase® W (1.5÷6.0 U/100 g of flour), which showed the best performance in fried pizza samples, with a lower amount of enzyme and optimal dough consistency, was applied in the preparation of the wood oven baked pizza bases. In these experiments, the AA level of the untreated pizza base sample was 2210±205 $\mu g/kg_{dw}$, and the enzymatic treatment resulted in a maximum AA reduction of approximately 50 %, without apparently altering the technological characteristics of the dough. Results showed that the use of L-Asparaginase could possibly play a key role in mitigating acrylamide formation in the cooking process of fried and wood oven baked pizza bases.

Keywords: Acrylamide mitigation, Maillard reaction, asparaginase enzyme, Preventase®, pizza base, UHPLC-UV.

4.1 Introduction

Acrylamide (AA) is a toxic substance, carcinogenic and dangerous to human health defined a chemical hazard for the food chain by EFSA due to the its presence in cooked food (EFSA 2015:EN-817). AA is a low molecular weight, highly water soluble, organic compound which forms from the naturally occurring constituents asparagine (Asn) and sugars in certain foods when prepared at temperatures typically higher than 120 °C and low moisture during the Maillard reaction (Mottram et al. 2002, Stadler et al., 2002). Maillard reaction and caramelization are the most important chemical events occurring during the manufacture of bakery and fried cereal products. The World Health Organization (WHO) considers 0.5 µg/L the maximum level for AA in water, however foods such as french fries, baked potato chips, crisp breads were found to contain AA between 50 and 1000 µg/kg (WHO, 2011). The benchmark for EU is 50 µg/kg for wheat based bread and 100 µg/kg for soft bread other than wheat based bread (EU Reg 2017/2158). In 2011 a Joint FAO/WHO expert committee on food additives, based on dose-response modeling data to evaluate the exposure-related effects of AA in laboratory animals, established a maximum daily consumption limit of 180 µg/kg body weight (b.w.), on the risk of cancer development (WHO, 2011). In European Countries, EFSA lowered the threshold to 170 µg/kg b.w. per day, and clarified that this is not a "recommended value", but a reference point to calculate the margin of exposure (MOE) (EFSA 2015:EN-817). However, both the authorities recommend to devote great effort into implementation and

development of mitigation methods for acrylamide in foods in order to reduce the levels of this substance as much as possible, at least in foods of major importance for dietary exposure, because of its probable carcinogenic effects (WHO, 2011; EU Reg 2017/2158). Thus, nowadays, the scientific world focuses on mitigation strategies for this substance in cooked foods, especially fried and starchy foods such as cereals (Jia et al 2021; Sarion et al., 2021). Pizza is a tasty and appetizing food, widespread and consumed all over the world. Its dough is made up of simple ingredients: flour (generally wheat flour), water, salt and yeast. Wheat flour is a strongly starchy matrix and contains Asn in a wide range of 7.4 to 66.4 mg/100 g (Claus et al., 2006; Stockmann et al., 2018). For these characteristics, pizza doughs are optimal candidates to develop AA during cooking. Furthermore, in the Neapolitan tradition, pizzas are eaten both fried and baked in a wood oven, two high-temperature cooking methods that favor the formation of AA. Therefore, pizzas fall into the product category of major importance for dietary exposure.

One of the measures applied by food business operators to reduce AA in food is the use of the asparaginase enzyme as suggested by the European Commission Regulation in 2017. L-Asparaginase catalyzes the conversion of the Asn, which is the reaction substrate, to aspartic acid and ammonia, preventing the formation of acrylamide (Rottmann et al., 2021). The enzymatic activity of asparaginases is optimal in applications with a water content of 30 % at least where good enzymatic activity can be reached. Enzymatic action occurs in two steps: (1) the enzyme needs to find its substrates, usually water and another substrate such as asparagine and convert them into other products; (2) the enzyme needs to release these converted products so that it is ready to start the conversion of more substrates (Xu et al., 2016). Furthermore, the enzyme activity is influenced by the contact with the substrate, which in turn might be affected by the food matrix composition and structure (Anese et al., 2011). With regard to the chemical composition, sufficiently high water contents may favor mobility of the enzyme towards the substrate, promoting hydrolysis of asparagine and thus acrylamide mitigation (Amrein et al., 2004; Hendriksen et al., 2009). From these considerations, it becomes clear, that the presence of substrate, the water content and the mixing regime are the three factors that determine the speed of the enzymatic process. For bakery products, such as bread or biscuits, this incubation time can easily be included in the leavening step (Xu et al., 2016).

Asparaginase effect on the acrylamide formation was investigated by Capuano et al. (2009) that reduced AA formation up to 88 % in toasted bread. Kumar et al. (2014) also studied the reduction of acrylamide formation in bakery products, such as sweet bread, by enzyme treatment. With increase in L-Asparaginase level, the acrylamide formation was reduced upon 97 % and 73 % reduction of acrylamide formation in the crust and crumb regions of bread, respectively. Matouri & Alemzadeh (2018), confirmed the benefit of asparaginases in the subsequent baking process at higher temperatures by analysing decreased AA levels compared to dough without an enzyme treatment. More recently, the use of a 1 % asparaginase solution, namely Preventase® L, decreased the acrylamide concentration by 59 % in fried potato (Rottmann et al., 2021). However, there is a lack of studies regarding the levels of AA in pizza in the literature. In this study, AA content in pizza bases was evaluated. Moreover, the use of Preventase®, asparaginase enzyme for food sector, was investigated for

reducing AA content in pizza base both fried and wood oven baked. Preventase® W, M or XR-BG were applied at increasing concentrations in pizza doughs prepared with standard recipe and with different percentage of water. The resulting acrylamide levels were evaluated in the fried pizza bases and compared to the untreated control. The best performing enzyme (Preventase® W) was subsequently used for pizza bases cooked in a wood oven. Finally, a discussion was addressed on the prediction of AA intake levels related to the consumption of a fried or wood oven baked pizza.

4.2 Materials and Methods

4.2.1 Chemicals

AA standard (electrophoresis >99 %) was purchased from Biorad company. Methanol, Water (HPLC-grade), Hexane mixture of isomers were purchased from Carlo Erba. All other chemicals and solvents used were of analytical grade and were procured from standard chemical companies. Ultra-pure water (Milli-Q system, USA) was used throughout the experiments. The reverse-phase C18 column used for acrylamide determination is the Nucleodur C18 Gravity 150 x 3 mm 3 µm and cellulose acetate filters with porosity 0.45 and 0.20 µm were purchased from Macherey-Nagel.

4.2.2 L-Asparaginase Enzyme

L-Asparaginase enzyme (L-asparagine amidohydrolases EC 3.5.1.1) produced from *Aspergillus niger*, was generously donated by the DSM company (NL) in 3 different formulations in pale white powder form: Preventase® W (containing wheat flour), M and XR-BG (containing maltodextrins). These enzymatic preparations have been specially developed for use in the main food industry applications by DMS Company. One Unit of enzymatic activity (U) was defined as the amount of asparaginase that catalyzes the release of one mmol of ammonia from L-asparagine per min under standard conditions (37 °C/pH=5.0). The specific activity of the three commercial asparaginase preparations was calculates to be as follow: Preventase® W = 1.55 U/g, Preventase® M = 1.89 U/g, Preventase® XR-BG = 1.71 U/g. Further details are available in Supplementary materials section S1. The enzyme was added to the flour in substitution of the same amount of flour, in order to maintain the overall quantity of solids in the recipe. In the first experiments, the three different enzyme preparations were tested, then Preventase® W was chosen for the following experiments.

4.2.3 Wheat Flour and Pizza Dough Ingredients

Commercial refined wheat flours, Casillo (12 % moisture, 0.5 % ash, 10 % proteins, 73.5 % carbohydrate, 2 % fibre, 2 % lipids) and Caputo (12 % moisture, 0.5 % ash, 13 % proteins, 70 % carbohydrate, 3 % fibre, 1.5 % lipids), were used for the experiments. Caputo dry yeast (freeze-dried brewer's yeast *Saccharomyces Cerevisiae*, containing sorbitan monostearate, E491, as emulsifier; cell viability on PDA medium > 10 log cfu/g), water and salt (common food-grade sodium chloride) were purchased in a local supermarket (Portici, Italy).

4.2.4 Farinograph analysis

The rheological characteristics of wheat flour as it is or in the presence of different amounts of L-Asparaginase enzyme (Preventase® W, M, or XR-BG) were determined using Brabender farinograph (Brabender® GmbH & Co KG, Duisburg, Germany), fitted with 50 g mixing bowl, according to AACC (1999) methods. The Brabender Units value (BU) was determined at different % of water and the results were expressed as the average value of three replicates for each sample.

4.2.5 Preparation of fried and wood oven baked pizza bases

Several trials of pizza doughs in triplicate were performed with L-Asparaginase. For fried pizza bases, Casillo flour (250 g), added with each of the three different enzyme preparations (Preventase® W, M and XR-BG) at 0 (control, ctr), 0.15, 0.30, 1.5, 3.0, 6.0, 9.0, 12.0 U/100 g of flour, was mixed with the other ingredients (58 % water, 1 % salt, 1 % dry yeast based on the flour weight) inside a Farinograph-E bowl (300 g) (Brabender® GmbH & Co KG, Duisburg, Germany) and kneaded with speed of 63 min^{-1} at 26 °C until the dough development time occurred (which is defined as the time required to achieve the peak value of BU in the farinogram graph) and for as long as the curve on the farinogram remains constant, indicating the maximum consistency and stability of the dough, before the structure breakdown (15 min). In parallel experiments, other doughs were prepared in the same conditions with increasing amount of water (60, 62 and 64 g/100 g of flour) and 3 U/100 g of Preventase®. The obtained untreated and enzyme-treated doughs were fermented in a leavening chamber maintained at 30 °C and 80 % of relative humidity (RH) for 2 h, and then rolled out to form a circular-shaped disc, and cooked by frying in sunflower oil at 190-200 °C for 1 min. The image of a fried pizza base is shown in Figure 4.S2.1 in supplementary material section S2.

For the wood oven baked pizza base, Caputo flour was added with Preventase® W (0, 1.5, 3.0, 6.0 U/100 g of flour) and then used for dough preparation following the Neapolitan pizza TSG recipe (EU Reg 97/2010). Briefly, 250 g flour were kneaded together with 61 % water, 2.8 % salt, 0.17 % dry yeast (on the flour weight basis) in the previously described conditions. The leavening was carried out at 22 °C and 80 % RH for 8 h, after which the doughs were rolled out until a circular-shaped disc with a central thickness of about 2-3 mm and a higher border (~ 10 mm) was formed. The obtained pizza bases were baked for 1 min at 485±30 °C directly placed on the floor of a handcrafted wood oven for pizzeria, made of refractory bricks, equipped with an internal chamber with a circular base (diameter 90 cm) and a vaulted roof with a maximum central height of 40 cm.

During baking, the temperature of the wood oven was monitored with a thermal imaging camera (FLIR E95 42, FLIR System OU, Estonia) and the position of the pizza was rotated with respect to the floor of the oven, following the specification of the Neapolitan pizza TSG. A wood oven baked pizza base is shown in Figure 4.S2.2 in supplementary material section S2.

4.2.6 Processing of samples

The obtained pizza samples were cut into pieces for subsequent experiments. The processing of pizza pieces was carried out according to Kumar et al. (2014), with some modifications. The fried pizza samples were preliminarily defatted by shaking in hexane (1:3 w/v) for 30 min at 25 °C and 170 rpm in an orbital shaker (Forma Scientific). After the evaporation of hexane, the samples were frozen, and then, together with the previously frozen wood oven baked pizza pieces, were lyophilised and finely ground at 40-mesh with a coffee grinder. The obtained powder was used for the following experiments of AA extraction.

4.2.7 Acrylamide Standard Preparation

A standard stock solution (1.0 mg/mL) was prepared by dissolving 50.0 mg of the acrylamide standard in 50 mL of HPLC water by using a volumetric flask. From the stock solution, calibration standards at different concentrations (50, 100, 500, 1000, 5000 and 1000 µg/L), were prepared, respectively. All series of standard solutions were stored in glass dark bottles (light-resistant) at 4 °C until using. Values reported were averages of three determinations.

4.2.8 Acrylamide extraction

Two parallel tubes were set up for each sample, one was used as sample test tube, and the second one as recovery test tube in the AA extraction experiments. One gram (on dry weight basis) of freeze-dried pizza powder was placed inside each tube and was defatted with 5 mL of hexane in the same conditions as above described (Kumar et al., 2014). AA from defatted samples was then extracted in water following the protocols reported by Wang et al. (2013) and Al-Asmar et al. (2019), with some modifications. Briefly, AA standard at a final concentration of 5000 µg/L was absorbed on pizza defatted powder in the recovery test tubes, and then 10 mL of HPLC water were added in each tube. The samples were incubated at 25 °C and 170 rpm in an orbital shaker for 30 min, then centrifuged at 8000×g for 10 min at 4 °C to allow precipitation of solids. The extraction was repeated twice, and both the supernatant were filtered consecutively through 0.45 and 0.20 µm cellulose acetate filters and stored in refrigerated conditions at 4 °C until UHPLC-UV analyses.

4.2.9 Acrylamide content determination

The AA content of the pizza samples was determined by using UHPLC (Jasco, Japan) equipped with a reverse phase C-18 column (Nucleodur C18 Gravity 150 x 3 mm, 3 µm particle size). According to Capuano et al. (2009), Wang et al. (2013), and Kumar et al. (2014), with some modifications, the chromatographic separation was performed at 30 °C using Milli-Q water (solvent A) and Methanol (solvent B), both containing 0.1 % formic acid, as mobile phases. The following elution program was applied: 0-3 min 0 % B, 4-8 min from 0 to 7 % B, 9-12 min from 7 to 100 % B (Capuano et al., 2009) at a flow rate of 0.25 mL/min. Absorbance was monitored at 210 nm (PDA detector MD-4010, Jasco). The retention time of authentic AA standard was 4.9 min under given conditions (Figure 4.1-A). Peak areas generated by 10 µL of standard AA at different concentrations (50, 100, 500, 1000, 5000 and 10000 µg/L) were used to build the

calibration curve (Figure 4.1-B) from which the AA concentration of pizza samples was extrapolated. All analyses were performed in triplicate, and the average results are expressed as µg/kg_{dw} (dry weight) of sample. The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated from the standard deviation of the lowest AA standard (50 µg/L) (Magnusson & Örnemark, 2014) and resulted 23 µg/L, and 78 µg/L, respectively.
Percentage of AA recovery was determined according to Al-Asmar et al. (2018) with the following formula:

$$Recovery\ (\%) = \frac{AA\ (detected\ in\ sample\ spiked\ with\ standard\ AA) - AA\ (sample)}{AA\ (standard\ added)} \times 100 \qquad (4.1)$$

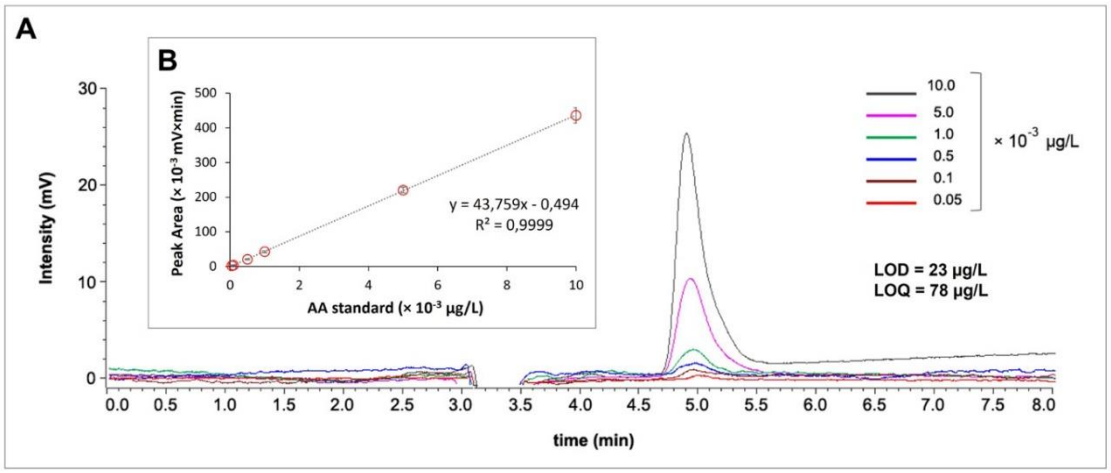

Figure 4.1. Chromatograms of Standard AA at different concentrations (50, 100, 500, 1000, 5000 and 10000 µg/L) by UHPLC-PDA (Jasco, Japan) (A) and calibration curve obtained from the peak area (B). LOD (23 µg/L) and LOQ (78 µg/L) values were calculated from the standard deviation of the lowest AA standard (50 µg/L).

4.2.10 Statistical analysis

All experiments and analytical measurements were run in triplicate and the data were expressed as mean ± Standard Deviation (SD). Means of each parameter were evaluated by analysis of variance (ANOVA) using Post Hoc Tukey's test. Statistical analysis was performed using XLSTAT software (version 2014.5.03). Differences between treatments at 5 % level ($p < 0.05$) were considered as significant.

4.3 Results and Discussion

4.3.1 Asparaginase treatment in fried pizza base

The AA content was determined in the untreated (control, ctr) and L-Asparaginase treated fried pizza bases; results are shown in Table 4.1. The AA level in the ctr sample was 3150±554 µg/kg_{dw} and represented the highest value registered for all the analysed samples. Indeed, the AA content in the treated fried pizza bases decreased with increase of enzymatic units for all the types of commercial Preventase®. Specifically, the AA levels in the enzyme-treated samples were in the range of 1928±109 ÷ 383±184, 1632±194 ÷ 374±228, and 2054±142 ÷ 332±47 µg/kg_{dw} for W, M and XR-BG respectively (Table 4.1), with the difference that the Preventase® W and M preparations, used at the maximum concentration of 12 U/100 g, were

able to bring the AA to undetectable levels (n.d.), while the XR-BG preparation, at the same quantity, still allowed an AA residue of 332±47 μg/kg_{dw}.
Analysing the data numbers in Table 4.1, it was observed that the estimate of the AA value for Preventase® XR-BG 12 U/100 g was not perfectly accurate, as the concentration of analyte in the extract used for the determination was 33.2±4.7 μg/L, and therefore lower than LOQ (78 μg/L), but still detectable because it is higher than LOD (23 μg/L). Furthermore, the estimates of the M-6 U/100 g and XR-BG-9 U/100 g samples were also very close to the LOQ threshold. Instead, the minimum quantities of AA measured in the W and M samples at 9 U/100 g, are certainly lower than the LOQ but are even at the border of the LOD threshold, considering their high standard deviation.

Table 4.1. AA content and Recovery test in fried pizza bases untreated (ctr) and treated with several increasing amount of Preventase® W, M and XR-BG.

sample	Preventase® (U/100 g of flour)	AA (μg/kg_{dw})	Recovery (%)
Ctr	0	3150±554^{a}	99.8
W	0.15	1928±109^{bA}	98.7
	0.3	1577±3^{cA}	95.2
	1.5	1462±66^{cA}	92.2
	3.0	1295±9^{dA}	96.4
	6.0	1129±65^{deA}	93.3
	9.0	383±184^{eA}	77.3
	12.0	n.d.	n.d.
M	0.15	1632±194^{bB}	99.0
	0.3	1611±187^{bA}	98.9
	1.5	1440±130^{bcA}	92.9
	3.0	1227±58^{cA}	98.4
	6.0	801±85^{dB}	94.2
	9.0	374±228^{eA}	89.1
	12.0	n.d.	n.d.
XR-BG	0.15	2054±142^{bA}	97.5
	0.3	2032±141^{bB}	96.1
	1.5	1857±131^{bB}	96.9
	3.0	1640±119^{bcB}	96.2
	6.0	1204±95^{cdA}	95.7
	9.0	768±71^{deB}	93.6
	12.0	332±47^{e}	95.9

Data represent the mean ± SD of three replicates (n=3); nd, not detectable.
Letters indicate samples significance calculated with ANOVA statistical test with Post Hoc Tukey ($p<0.05$). Different lower case letters indicate significant differences between samples treated with the same enzyme preparation at different U/100 g. Different capital letters indicate significant differences between different enzyme preparations used at the same U/100 g.

To confirm the validity of the AA determinations, the extraction efficiency was evaluated by calculating the recovery of the analyte conducted in the presence of 5000 μg/L of AA standard. Analysing the recovery percentage of AA, it was observed that for most of the samples the value was between 92.2 and 99.0 %, and only for the samples prepared with 9 U/100 g of W and M enzyme preparations, the recovery % drops to values of 77.3 and 89.1 % respectively (Table 4.1). However, these are also two of the samples with the lowest AA levels and the

highest standard deviation. Probably, at such low levels of AA the matrix effect in the extraction efficiency was more pronounced. However, despite the low accuracy of AA level estimates for samples with high enzyme concentrations, this does not mean that the differences with other samples are not significant. Definitely, for all the samples in Table 4.1, it can be said that the determined AA values are statistically compatible with the experimental variations.

The enzymatic treatment was, thus, effective in reducing AA formation, and the performance was relatively consistent. The percentage of AA reduction with respect to the control, with increasing enzyme units used in the dough, is shown in Figure 4.2-A. The results showed that the XR-BG enzymatic preparation causes a lower percentage reduction of AA at all concentrations used, reaching a maximum reduction of 89 % respect to the control without enzyme. On the other hand, W and M preparations exhibited a very similar trend and were able to completely break down the formation of AA in samples with 12 U/100 g (Figure 4.42-A). This result is somewhat controversial, given that the same units of enzyme activity were used for the three available enzyme preparations. However, it must be considered that the assays to determine the specific activity of the enzymes were carried out in aqueous solution, with the enzyme and the substrate (free asparagine) dissolved in the reaction buffer, therefore in optimal conditions for the functioning of the asparaginase. In the case of application in the dough, the reaction conditions are certainly different, but the only change in the recipe is the addition of a different enzyme preparation. To justify the different performance of Preventase® XR-BG, it could be assumed a lower mobility of the enzyme in the matrix of the dough due to the presence, in the commercial preparation, of other components which can interfere with the dispersibility of the enzyme in the dough during mixing. Alternatively, there may be other elements present that may act as inhibitors of the enzyme when released into the matrix, compared to the other two preparations.

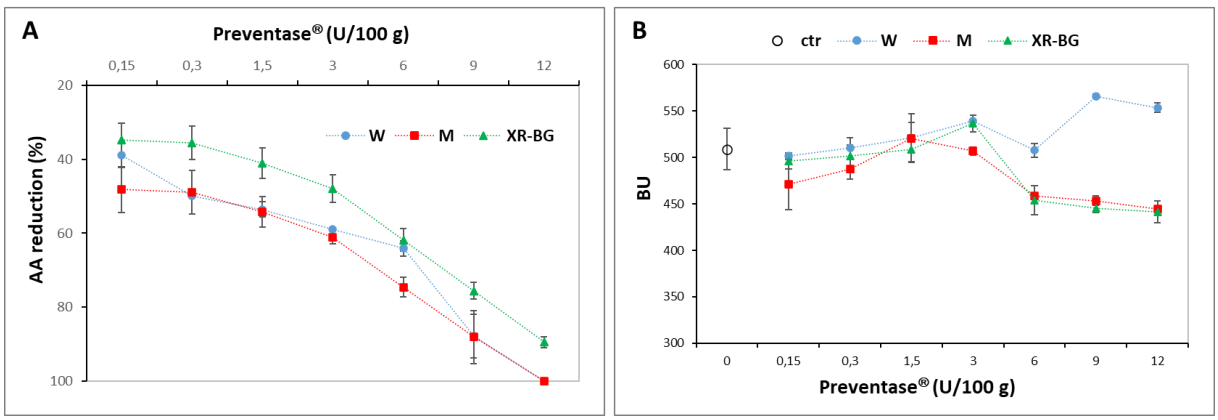

Figure 4.2. AA reduction respect to the untreated control sample (ctr) (A) and Brabender Units values (B) of fried pizza bases treated with several increasing amount of Preventase® W, M or XR-BG. Values are expressed as mean ± SD of three replicates (n=3).

However, these remain only hypotheses since, unfortunately, the composition of these preparations is covered by a patent, and the only information available on the three commercial forms is that, in addition to the enzyme, the powder also contains wheat flour in the case of Preventase® W. and maltodextrins in the case of Preventase® M and XR-BG.

In all cases, Preventase® treatment was able to sensibly reduce AA content in all kinds of fried pizza base samples. The effectiveness of the Preventase® in reducing the formation of AA in other fried products has also been described by other authors: Rottmann et al. (2021) reported an AA reduction of about 60 % in pre-treated potato chips with Preventase®. In general, according to our results, the asparaginase enzyme has been proposed as an AA mitigation strategy in different types of products. Vass et al. (2004) experienced already that the addition of asparaginase in two different crackers was able to decrease the amount of AA formed by 70 %. Capuano et al. (2009) found a reduction of AA content in the range of 70 – 88 % in bread crisps baked at 160 °C and 180 °C.

4.3.1.1 Impact of the addiction of the enzyme preparation in the dough on its technological properties

The extent of the AA reduction is not the only factor to be taken into account for choosing the optimal amount of enzymatic preparation to be added to the product in the formulation phase. It is also necessary to consider whether the addition of a technological adjuvant, with its own composition, does not alter the physico-chemical properties of the product. In fact, it is important not to affect the sensory attributes and overall acceptability by consumers. As regards to the kneaded products, a very important parameter for the quality of the dough is the Brabender Units value (BU), determined by the farinograph, which represents a measure of the rheological characteristics of the dough. For kneaded products, such as bread and pizza, an optimal dough should reach 500 BU (Rosell et al., 2001). The replacement of wheat flour with the enzymatic preparation can lead to the variation of the BU, and, consequently, to the modification of the viscosity of the dough. This feature is a very important parameter for the manipulability of the pizza dough which can, subsequently, affect its extensibility and elasticity. Figure 4.2-B shows BU values measured for doughs of fried pizza, prepared with the different amounts of enzymes using a constant water level adjusted for reaching 500 BU in the control dough. As can be seen in the figure, at high concentrations of enzyme significant deviations from the threshold of 500 BU are observed for all enzymatic preparations, while BU are acceptable up to 3 U/100 g of flour for preparations M and XR-BG, and up to 6 U/100 g for Preventase® W type. In particular, at 3 U/100 g of Preventase®, the average reduction of AA for the three enzymes is around 50-60 %, with the best performance for the M and W enzymes.

4.3.1.2 Effect of water content on BU of doughs and AA reduction in pizza base

This amount of Preventase® was chosen for subsequent experiments in which the fried pizza base samples were prepared at higher hydration degree (60, 62 and 64 g of water added to 100 g of flour, compared to 58 g of the control). From the literature it is known that the water content of the products, during cooking, can influence the formation of AA (Zyzak et al., 2003; Masatcioglu et al., 2014). In fact, as soon as the dough is placed in the hot oil or in the oven, water evaporates very fast from the surface layers, resulting in much lower water content than at the core. As the water content decreases in the external part of the pizza, the temperature can exceed 100 °C, which supports reactions such a caramelization,

carbonization and Maillard reaction, responsible for the browning coloration (Gökmen & Palazoğlu, 2008; Dessev et al., 2020). These reactions belong to the non-enzymatic or non-oxidative browning category. The Maillard reaction is the main responsible for colour development at temperatures below 150 °C. Caramelization and carbonization reactions take place at temperatures above 150 °C. The Maillard reaction is influenced by composition in reducing sugar and amino acids, but also by temperature, pH and water content (Zanoni et al., 1995). The water content seems to play an important role in any such degradation process: acrylamide content decreases simultaneously with water content increasing (Sadd et al., 2008; Masatcioglu et al., 2014). This phenomenon is explained by considering that the dehydration phase is a crucial step in the formation of AA; the increase in water content represents a limitation with consequent inhibition of the reaction (Zyzak et al., 2003).

To investigate the combined effect of the asparaginase in pizza bases with a higher hydration degree, doughs with a water content increasing from 58 g/100 g of flour (used in the previous recipe) to 64 g/100 g of flour were prepared, keeping the Preventase® concentration fixed at 3U/100 g. The content of AA determined in these samples is shown in Table 4.2. All measured values are above the LOQ except those with water content of 64 g/100 g of flour for all enzymatic preparations used, which, however, do not fall below the LOD threshold. A lower concentration of AA is confirmed in the enzymatically treated pizza bases (W, M and XR-BG) compared to the untreated controls (ctr), at all the considered hydration percentages. Moreover, acrylamide levels measured for each typology of pizza base decreased with increasing of water content in the corresponding doughs, with significant differences especially at hydration levels equal to or greater than 62 g/100 g of flour (Table 4.2).

Table 4.2. AA content and Recovery test in fried pizza bases, untreated (ctr) and treated with 3 U/100 g of Preventase® W, M, and XR-BG, prepared at different hydration degree.

sample	Water (g/100 g of flour)	AA ($\mu g/kg_{dw}$)	Recovery (%)
ctr	58	3150 ± 554^{aA}	99.8
	60	2641 ± 119^{aA}	96.9
	62	2190 ± 94^{bA}	97.8
	64	1779 ± 15^{cA}	98.6
W	58	1295 ± 9^{aB}	96.4
	60	1334 ± 130^{aB}	99.5
	62	951 ± 20^{bB}	100.3
	64	306 ± 52^{cB}	101.6
M	58	1227 ± 58^{aB}	98.4
	60	1226 ± 136^{aB}	99.8
	62	987 ± 9^{bB}	100.3
	64	516 ± 45^{cC}	101.2
XR-BG	58	1640 ± 119^{aC}	96.2
	60	1329 ± 77^{bB}	99.6
	62	1067 ± 172^{bB}	100.1
	64	562 ± 257^{cBC}	101.1

Values are mean ± SD (n=3).
Letters indicate samples significance calculated with ANOVA statistical test with Post Hoc Tukey ($p<0.05$). Different lower case letters indicate significant differences between samples treated with the same enzyme at different % of water. Different capital letters indicate significant differences between samples treated with different enzymes at the same % of water.

The increase in the water content of the dough has proved to be a very effective method to reduce the AA in fried pizza bases, in fact in the untreated sample there was a reduction of 16, 30 and 44 percentage points passing from 60 to 64 g/100 g of flour of hydration compared to the 58 g/100 g of flour. In enzymatically treated samples, the performance of the 3 enzymatic preparations was practically comparable from 60 g/100 g of flour onwards, and the extent of the reduction even exceeds 80 % with only 3 U/100 g of enzyme, at the maximum hydration considered (Figure 4.3-A). Such a percentage of reduction, in samples at 58 g/100 g of flour hydration, can only be achieved by using an amount of enzyme at least 3 times higher (Figure 4.2-A).

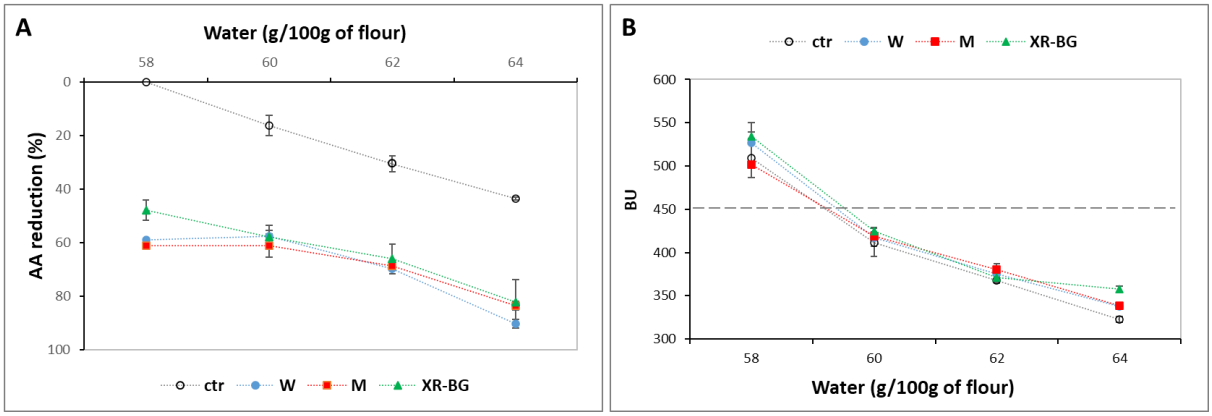

Figure 4.3. AA reduction (A) and Brabender Units values (B) of fried pizza base prepared at different hydration degree (58, 60, 62 and 64 g of water/100 g of flour), untreated (ctr) and treated with fixed amount (3 U/100 g) of Preventase® W, M or XR-BG (3 U/100 g). Values are expressed as mean ± SD of three replicates (n=3).

Other authors have reported a reduction in AA levels in products with higher moisture. In a study published in the 2007, Ahrné and coll. measured the AA content in the bread crust and showed that the amount of AA in the inner crust fraction (the one close to the crumb that has a water content between 6-10 %) was 25-75 % of that in the outer fraction (containing 2-4 % of water). More recently, Masatcioglu et al. (2014) achieved a reduction of 54 and 85 % in corn extrusions by increasing the feed moisture from 22 to 24 and 26 %, respectively.

The obtained results agree with the literature, but also highlight the absence of a synergistic effect of the water content and asparaginase activity on the inhibition of the AA formation reaction. In fact, the reduction curves of AA in the absence and presence of Preventase® seem to proceed in parallel. In this experiment, for the control sample, the AA reduction rate has practically a linear trend with an $R^2 = 1$ (Figure 4.S3.1-D). Even in the samples containing 3 U/100 g of Preventase® the reduction rate shows a certain linearity, that extends for the whole considered range for the XR-BG samples (Figure 4.S3.1-C) and the samples W and M with a hydration degree from 60 to 64 g/100 g of flour (Figure 4.S3.1-A and B). The slopes of these lines fall within a very narrow interval (11÷16 percentage points), suggesting a parallel trend of the AA reduction phenomena. In the presence of synergistic phenomena, a greater slope of the lines corresponding to the samples treated with the enzyme, compared to that of the control, would have been expected. However, this did not occur, indicating that, in all the samples, the increase in the reduction of AA is essentially due to the higher percentage of water present (Figure 4.3-A).

Although it has proved to be an effective AA mitigation strategy, increasing the percentage of water in the doughs is not a viable formulation change for pizza dough processing, as hydration alters the technological parameters of the doughs, starting with viscosity. As a demonstration of this, a collapse of the BUs from 500 to about 300-350 is observed for all the samples passing from 58 to 64 g/100 g of flour hydration (Figure 4.3-B). This means that the dough loses consistency and becomes stickier and more difficult to handle.
Thus, in view of an intervention for the reduction of the AA in fried pizzas, in order not to affect the technological characteristics of the dough, it is preferable to resort to the use of asparaginase, especially the Preventase® W for which up to 6 U/100 g of flour can be used without significantly altering the BU compared to the control (Figure 4.2-B).

4.3.2 AA reduction in wood oven baked pizza base

The action of asparaginase in reducing AA was also evaluated in pizza base samples cooked in a wood oven. In this experiment, 1.5, 3 and 6 U/100 g of Preventase® W were used in the preparation of pizza dough. These quantities were chosen on the basis of previous experiments, because they proved to be effective on average in bringing the reduction of AA to values higher than 50 % in the fried pizza samples (Figure 4.3-A). Furthermore, Preventase® W has been selected among the three, because it contains wheat flour in its composition, and therefore its use does not involve the addition of other ingredients to the recipe, while the other two preparations contain maltodextrins that can interfere with the methods and times of dough development.
Results of the pizza base AA content and reduction, and the BU of the corresponding doughs are shown in Figure 4.4. All measured AA values were higher then LOQ, but unfortunately the recovery values were between 88.0 and 90.2 %, and therefore significantly lower than those of fried pizza bases, which for the same enzyme units were greater than 92.2 %. Anyway, compared to the fried untreated sample, the pizza base cooked in a wood oven showed a lower AA level (2210±205 *vs* 3150±554 $\mu g/kg_{dw}$). This is not surprising, as it is known that frying processes are characterized by higher levels of Maillard reaction with all the consequences, compared to oven baking (including the wood oven) (Danowska-Oziewicz et al., 2007; Zhang & Zhang, 2008; Friedman & Levin, 2008). Moreover, it must also be considered that the doughs for the pizza base baked in the wood oven were produced with a different flour than those of the fried pizza bases, and also the leavening process, before reaching the cooking, was longer (8h *vs* 2h) because the recipe of the TSG specification for Neapolitan pizza was followed. Consequently, the amount of reducing sugars and free Asn in the two different doughs at the moment of cooking could be different both for the different composition of the starting flours and for the different metabolic activity of the yeasts (Fredriksson et al., 2004; Claus et al., 2008; Wang et al., 2017).
However, as observed for the fried pizza bases, also for the wood oven baked ones, the AA levels decreased with increasing amounts of asparaginase in the dough (Figure 4.4-A), and the performance of the enzyme seemed to be comparable to that observed in previous experiments, at least at 3 and 6 U/100 g where a maximum of 46-49 % of reduction was

reached. Only at 1.5 U/100 g there is a lower efficiency of the Preventase® W with a reduction of AA of 12 % (*vs* 54 %).

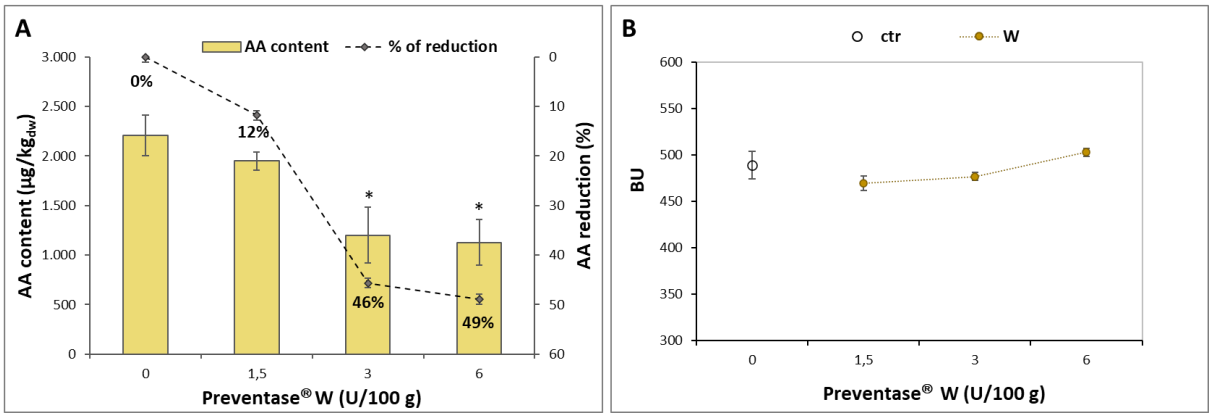

Figure 4.4 AA content and reduction (A) and Brabender Units values (B) of wood oven baked pizza base treated with 1.5, 3 and 6 U/100 g of Preventase® W. Values are expressed as mean ± SD of three replicates (n=3).

As regards the technological aspects of the dough for pizza base TSG, also in this case, the addition of Preventase® W to the ingredients, replacing the homologous quantities of flour, did not lead to an excessive variation of the BU (Figure 4.4-B). Based on the obtained results, it can be stated that Preventase® W was effective in the strategies for mitigation of acrylamide also in products cooked with methods other than frying, such as the wood oven baking.

4.3.3 Safety issues and consumer concerns

Since its discovery in food, the organizations responsible for food safety have been committed to evaluating toxicity studies of acrylamide and supporting information campaigns to increase consumer awareness. For European countries, EFSA has established a limit threshold for the daily intake of AA equal to 170 µg/kg of body weight (EFSA 2015:EN-817). This means that an average person weighing 70 kg must not exceed a maximum dose of AA of 11900 µg in one day. From the results reported in this study, it can be deduced that for a pizza base of 250 g, considering a dry substance of 65 %, the AA content is equal to about 512 µg for a fried pizza base, and about 360 µg for a base pizza baked in a wood oven. In both cases, the values are far below the threshold established for an individual weighing 70 kg, and precisely the dose limit/dose taken ratio is 23 and 33 respectively. There is a further consideration to be made: fried pizza is seasoned after cooking, therefore the entire surface of the product is exposed to the formation of AA; on the contrary, for pizza cooked in a wood oven, the AA content can even drop below the value of 360 µg calculated for the pizza base. In fact, wood oven baked pizza is generally cooked in the presence of topping that covers the upper surface of the dough disc, and this reduces the exposure to the Maillard reaction except on the pizza rim. Thus, the AA is essentially distributed on bottom and the rim surfaces of the pizza.
Considering that a 250 g dough ball produces a circular pizza with an average diameter of 27±1 cm, the total surface area of the pizza base can be calculated as the area of two circles 2 (bottom disc + upper disc) and corresponds to 2x 573±60 cm^2 (about 1146 cm^2) (Figure 4.S4.1). Therefore, in a pizza base baked in a wood oven, a distribution of AA equal to 0.31 µg/cm^2 can

be deduced. Since in a pizza garnished with sauce, the upper disc is exposed to the formation of AA only in the part of the rim, which for a Neapolitan pizza is about 2 cm (Reg. EU 2010), eliminating the surface of the central disc with a diameter of 23±1 cm (416±51 cm^2), it can be calculated that the pizza surface affected by the presence of AA is about 730±69 cm^2 (Figure 4.S4.1), and that the AA content is reduced to about 226±21 μg. For this value, the dose limit/dose taken ratio is around 53. Finally, it is noteworthy that in a real case of pizza baking, even the presence of the topping can affect the AA formation, by slowing down the increase of pizza temperature.

The use of 3 U/g of Preventase® W can lead to a reduction of about 60 % and 40 % of AA in the fried and wood oven baked pizza bases respectively. Consequently, the intake of AA also drops to about 204 μg and 136 μg, with a dose limit/dose taken ratio of 58 and 87.

In any case, it should be remembered that AA is contained in many foods that are consumed daily such as bread, biscuits, crackers, french fries etc. and this is the reason why EFSA encourages the food industry to develop processes that can reduce the AA content in food as much as possible.

4.4 Conclusions

To our knowledge, this is the first time that the asparaginase enzyme has been applied in the production of pizza dough, especially in the Neapolitan style TSG. The experimental results reported in this study demonstrated its effectiveness. In particular, the most performing preparation was Preventase® W which, with 3 U/100 g of flour, allowed to obtain a maximum reduction of AA of 61 % in fried pizza bases and 46 % in those cooked in a wood oven. In this way, for an individual of 70 kg the calculated dose limit/dose taken ratios, for fried and wood oven baked pizza, pass from 23 and 52 to 58 and 87 respectively. Moreover, at this enzyme concentration, there were no significant deviations from the threshold of 500 BU in the produced doughs, indicating a low interference of the technological adjuvant on the rheological properties of the product. Due to the wheat flour based composition of Preventase® W, this enzymatic preparation is particularly suitable for direct addition to the flour intended for the production of pizza dough. However, other aspects regarding the rheological properties of doughs and the physico-sensory characteristics of cooked pizza need to be investigated.

4.5 References

- AACC International Approved Methods of Analysis (1999). Method 02–52.01 (hydrogen ion activity (pH) Method), method 44–15.02 (moisture air-oven method). St. Paul, MN, U.S.A.
- Al-Asmar, A., Giosafatto, C. V. L., Panzella, L. & Mariniello L. (2019). The Effect of Transglutaminase to Improve the Quality of Either Traditional or Pectin-Coated Falafel (Fried Middle Eastern Food). *Coatings*, 9, 331, 2–14.
- Al-Asmar, A., Naviglio, D., Giosafatto, C. V. L. & Mariniello L. (2018). Hydrocolloid-Based Coatings are Effective at Reducing Acrylamide and Oil Content of French Fries. *Coatings*, 8, 147, 2–13.

- Ahrné, L., Anderssona, C.-G., Floberga P., Rosé J., Lingnert H. (2007). Effect of crust temperature and water content on acrylamide formation during baking of white bread: Steam and falling temperature baking. LWT 40, 1708–1715.
- Amrein, T. M., Schonbahler, B., Escher, F., & Amado, R. (2004). Acrylamide in Gingerbread: Critical Factors for Formation and Possible Ways for Reduction. *Journal of Agricultural and Food Chemistry*, 52, 4282−4288.
- Anese, M., Quarta, B., Peloux, L., & Calligaris, S. (2011). Effect of formulation on the capacity of L-asparaginase to minimize acrylamide formation in short dough biscuits. *Food Research International*, 44 (9), 2837-2842.
- Capuano, E., Ferrigno, A., Acampa, I., Serpen, A., Açar, Ö.Ç., Gökmen, V., & Fogliano, V. (2009). Effect of flour type on Maillard reaction and acrylamide formation during toasting of bread crisp model systems and mitigation strategies. *Food Research International,* 42,1295–1302
- Claus, A., Mongili, M., Weisz, G., Schieber, A., & Carle, R. (2008). Impact of formulation and technological factors on the acrylamide content of wheat bread and bread rolls. Journal of Cereal Science, 47(3), 546–554.
- Claus, A., Schreiter, P., Weber, A., Graeff, S., Herrmann, W., Claupein, W., Schieber A., & Carle R. (2006). Influence of Agronomic Factors and Extraction Rate on the Acrylamide Contents in Yeast-Leavened Breads. *Journal of Agricultural and Food Chemistry*, 54, 8968–8976.
- Commission Regulation (EU) 2017/2158. Establishing mitigation measures and benchmark levels for the reduction of the presence as acrylamide in food. *Official Journal of the European Union*, 60, 24–44.
- Commission Regulation (EU) 97/2010 entering a name in the register of traditional specialities guaranteed (Pizza Napoletana (TSG). *Official Journal of the European Union*, 34, 7–13.
- Danowska-Oziewicz, M., Karpińska-Tymoszczyk, M., & Borowski J. (2007). The effect of cooking in a steam-convection oven on the quality of selected dishes. *Journal of Foodservice*, 18(5), 187–197.
- Dessev, T., Lalanne, V., Keramat, J., Jury, V., Prost, C., & Le-Bail A. (2020). Influence of Baking Conditions on Bread Characteristics and Acrylamide Concentration. *Journal of Food Science and Nutrition Research*, 3, 291-310.
- EFSA supporting publication 2015:EN-817. Outcome of the public consultation on the draft Scientific Opinion of the EFSA Panel on Contaminants in the Food Chain (CONTAM) on acrylamide in food. *European Food Safety Authority*, 2–95.
- Fredriksson, H., Tallving, J., Rosén, J. M., & Åman, P. (2004). Fermentation Reduces Free Asparagine in Dough and Acrylamide Content in Bread. *Cereal Chemistry*, 81(5).
- Friedman, M., & Levin, C. E. (2008). Review of Methods for the Reduction of Dietary Content and Toxicity of Acrylamide. *Journal of Agricultural and Food Chemistry*, 56, 15, 6113–6140.
- Gökmen, V., & Palazoğlu, T. K. (2008). Acrylamide Formation in Foods during Thermal Processing with a Focus on Frying. *Food and Bioprocess Technology*, 1, 35–42
- Hendriksen, H. V., Kornbrust, B. A., Østergaard, P. R., & Stringer, M. A. (2009). Evaluating the potential for enzymatic acrylamide mitigation in a range of food products using an asparaginase from Aspergillus oryzae. *Journal of Agricultural and Food Chemistry*, 57, 4168–4176.
- Jia, R., Wan, X., Geng, X., Xue, D., Xie Z., and Chen, C. (2021). Microbial L-asparaginase for Application in Acrylamide Mitigation from Food: Current Research Status and Future Perspectives.
- Kumar, N. S. M., Shimray, C. A., Indrani, D., & Manonmani, H. K. (2014). Reduction of Acrylamide Formation in Sweet Bread with L-Asparaginase Treatment. *Food and Bioprocess Technology,* 7:741–748.

- Magnusson, B., & Örnemark, U. (eds.) Eurachem Guide (2014): The Fitness for Purpose of Analytical Methods – A Laboratory Guide to Method Validation and Related Topics, (2nd ed.). ISBN 978-91-87461-59-0. Available from www.eurachem.org.
- Masatcioglu, M. T., Gokmen, V., Ng, P. K. W. & Koksel, H. (2014). Effects of formulation, extrusion cooking conditions, and CO_2 injection on the formation of acrylamide in corn extrudates. *Journal of the Science of Food and Agriculture*, 94(12), 2562–2568.
- Matouri, M., & Alemzadeh, I. (2018). Suppressed acrylamide formation during baking in yeast-leavened bread based on added asparaginase, baking time and temperature using response surface methodology. *Applied Food Biotechnology*, 5(1)
- Mottram, D. S., Wedzicha, B. L., Dodson, A. T. (2002). Acrylamide is formed in the Maillard reaction. *Nature*, 419, 448–449
- Rosell, C. M., Rojas, J. A., & Benedito de Barber, C. (2001). Influence of hydrocolloids on dough rheology and bread quality. *Food Hydrocolloids,* 15, 75–81.
- Rottmann, E., Hauke, K. F., Krings, U., Berger, R. G. (2021). Enzymatic acrylamide mitigation in French fries – An industrial-scale case study. *Food Control* 123, 107739.
- Sadd, P. A., Hamlet, C. G., & Liang, L. (2008). Effectiveness of methods for reducing acrylamide in bakery products. *Journal of Agricultural and Food Chemistry*, 56 (15), 6154–6161.
- Sarion, C., Codină, G. G., and Dabija, A. (2021). Acrylamide in Bakery Products: A Review on Health Risks, Legal Regulations and Strategies to Reduce Its Formation. *International Journal of Environmental Research and Public Health*, 18(8), 4332. https://doi.org/10.3390/ijerph18084332
- Stadler, R. H., Blank, I., Varga, N., Robert, F., Hau, J., Guy, P. A., Robert, M. C., & Riediker, S. (2002). Acrylamide from Maillard reaction products. *Nature*, 419, 449–450.
- Stockmann, F., Weber, E. A., Mast, B., Schreiter, P., Merkt, N., Claupein, W., & Graeff-Hönninger, S. (2018). Evaluation of Asparagine Concentration as an Indicator of the Acrylamide Formation in Cereals Grown under Organic Farming Conditions. *Agronomy*, 8(12), 294.
- Vass, M., Amrein, T. M., Schönbächler, B., Escher, F., & Amadò, R. (2004). Ways to Reduce the Acrylamide Formation in Cracker Products. *Czech Journal of Food Sciences,* 22, 19–21.
- Wang, H., Feng, F., Guo, Y., Shuang, S., Choi, M. M.F. (2013). HPLC-UV quantitative analysis of acrylamide in baked and deep-fried Chinese foods. *Journal of Food Composition and Analysis*, 31, 7–11.
- Wang, S., Yu, J., Xin, Q., Wang, S., & Copeland, L. (2017). Effects of starch damage and yeast fermentation on acrylamide formation in bread. *Food Control*, 73, 230–236.
- WHO 2011 (Ed): Evaluation of certain contaminants in food, Seventy second report of the Joint FAO/WHO expert Committee on food additives. *World Health Organization*, 959, 1–115.
- Xu, F., Oruna-Concha, M. J., & Elmore, J. S. (2016). The use of asparaginase to reduce acrylamide levels in cooked food. *Food Chemistry* 210, 163–171.
- Zanoni, B., Peri, C., & Bruno, D. (1995). Modelling of browning kinetics of bread crust during baking. *Lebensmittel-Wissenschaft und-Technologie*, 28, 604-609.
- Zhang, Y., & Zhang, Y. (2008). Formation and Reduction of Acrylamide in Maillard Reaction: A Review Based on the Current State of Knowledge. *Critical Reviews in Food Science and Nutrition*, 47(5), 521–542.
- Zyzak, D. V., Sanders, R. A., Stojanovic, M., Tallmadge, D. H., Eberhardt, B. L., Ewald, D. K., Gruber, D. C., Morsch, T. R., Strothers, M. A., Rizzi, G. P., & Villagran, M. D. (2003). Acrylamide formation mechanism in heated foods. *Journal of Agricultural and Food Chemistry*, 51, 4782–4787.

4.6 Supplementary materials

4.6.S1. Asparaginase specific activity determination

Asparaginase catalyzes the hydrolysis of asparagine in aspartic acid and ammonia (NH_3). In acqueous environment, ammonia is immediately protonated and appears as ammonium ion (NH_4^+) which can be measured by the Berthelot reaction (Weatherburn, 1967): the NH_4^+ reacts with phenol, nitroprusside and alkaline hypochlorite resulting in a blue colour that can be quantified by reading the absorbance at 625 nm. A calibration curve was built with 4, 10, 16, 20 and 40 nmol of NH_4^+ as standard (Figure 4.S1.1).

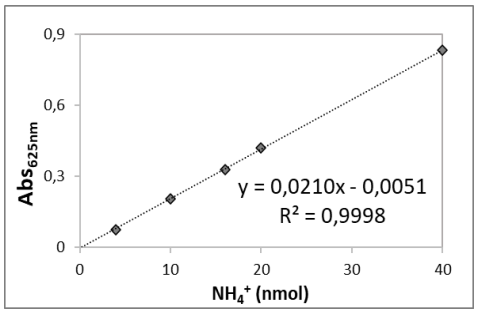

Figure 4.S1.1. Calibration curve obtained with 4, 10, 16, 20 and 40 nmol of NH_4^+ as standard determined by Berthelot reaction (Weatherburn, 1967).

For the preparation of the enzymatic assay, 5, 10, 50 and 100 µg of asparaginase (either Preventase, W, M and XR-BG) were added to a 10 mM asparagine solution (ASN) in 100 mM sodium acetate buffer (pH 5), for a final volume of 1 mL. The reaction mixtures were incubated in a thermostatic bath at the optimum temperature for the enzyme of 37°C for 60 min, after which, the enzyme was deactivated by boiling the samples in a water bath for 10 min. The amount of ammonia produced during the asparaginase reaction was determined by Berthelot assay and extrapolated by calibration curve of Figure 4.S1.2.

The extent of enzymatic activity (nmol of NH_4^+/min) for each preparation of Preventase was reported as function of the µg of enzyme (Figure 4.S2.2). The slope of the regression curves represents the specific activity. 1 unit of enzymatic activity was defined as the amount of asparaginase that release 1 mmol of NH_4^+/min from L-asparagine at 37°C and pH 5.0.

Thus, the calculated specific activity was 1.55, 1.89 and 1.71 U/g for Preventase W, M and XR-BG respectively.

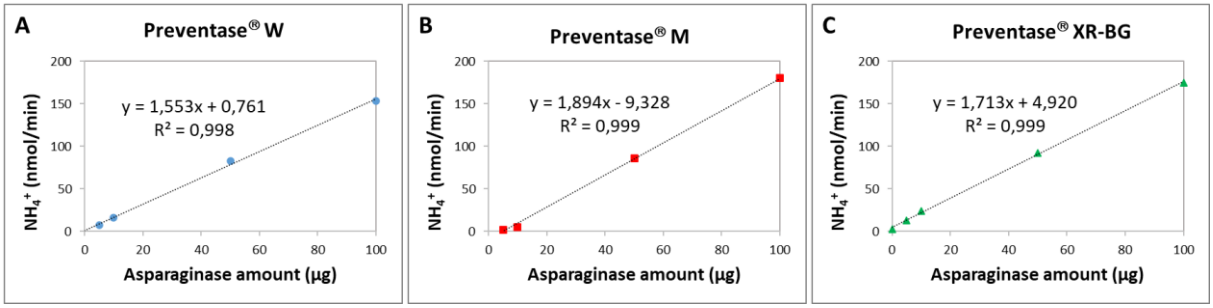

Figure 4.S1.2. Graphs for determining specific activity of asparaginase preparations (Preventase W, M, XR-BG).

Reference

- Weatherburn, M.W. (1967) Urease-Berthelot Colorimetric Method for in Vitro Determination of Urea. Analytical Chemistry, 39, 971-974.

4.6.S2. Global aspect of fried and wood oven baked pizza bases

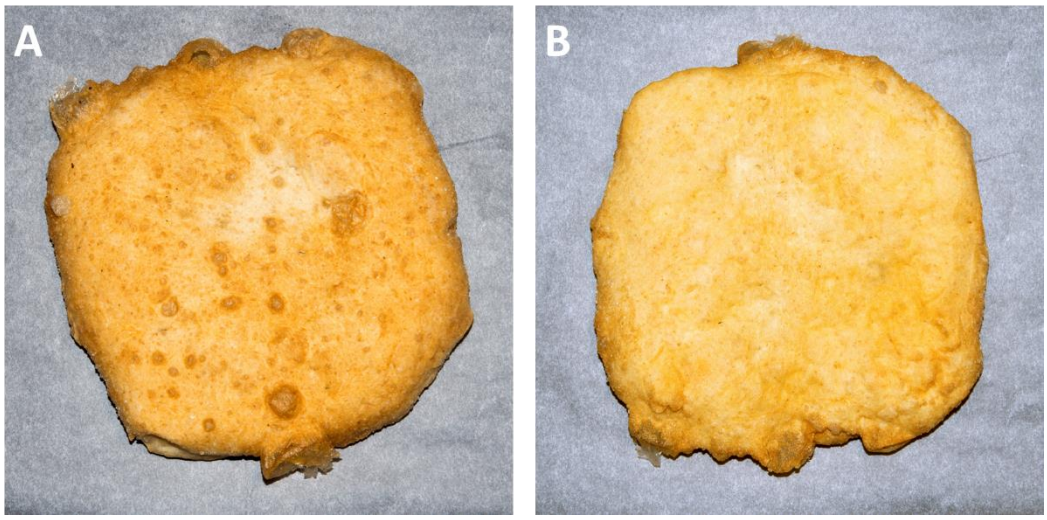

Figure 4.S2.1. Appearance of a fried pizza base. A, top; B, underside.

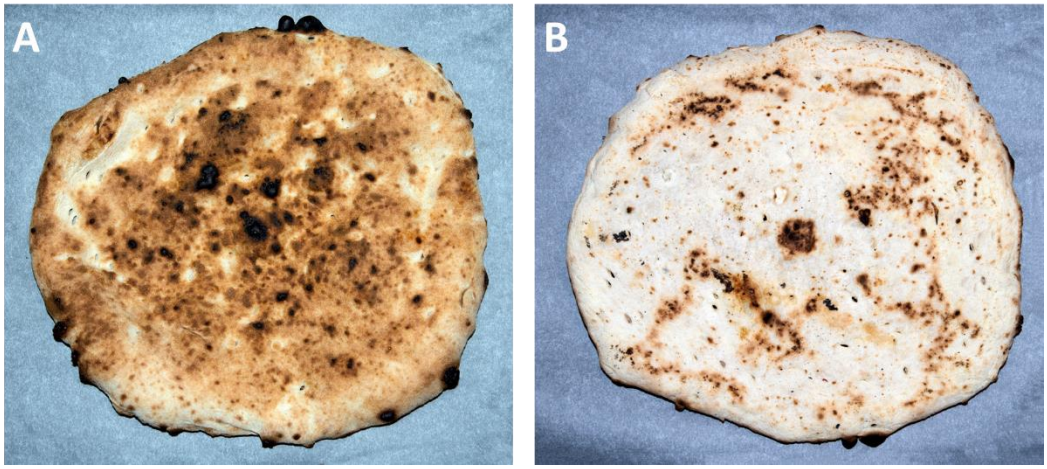

Figure 4.S2.2. Appearance of a wood oven baked pizza base. A, top; B, underside.

4.6.S3 – Acrylamide reduction as function of water content

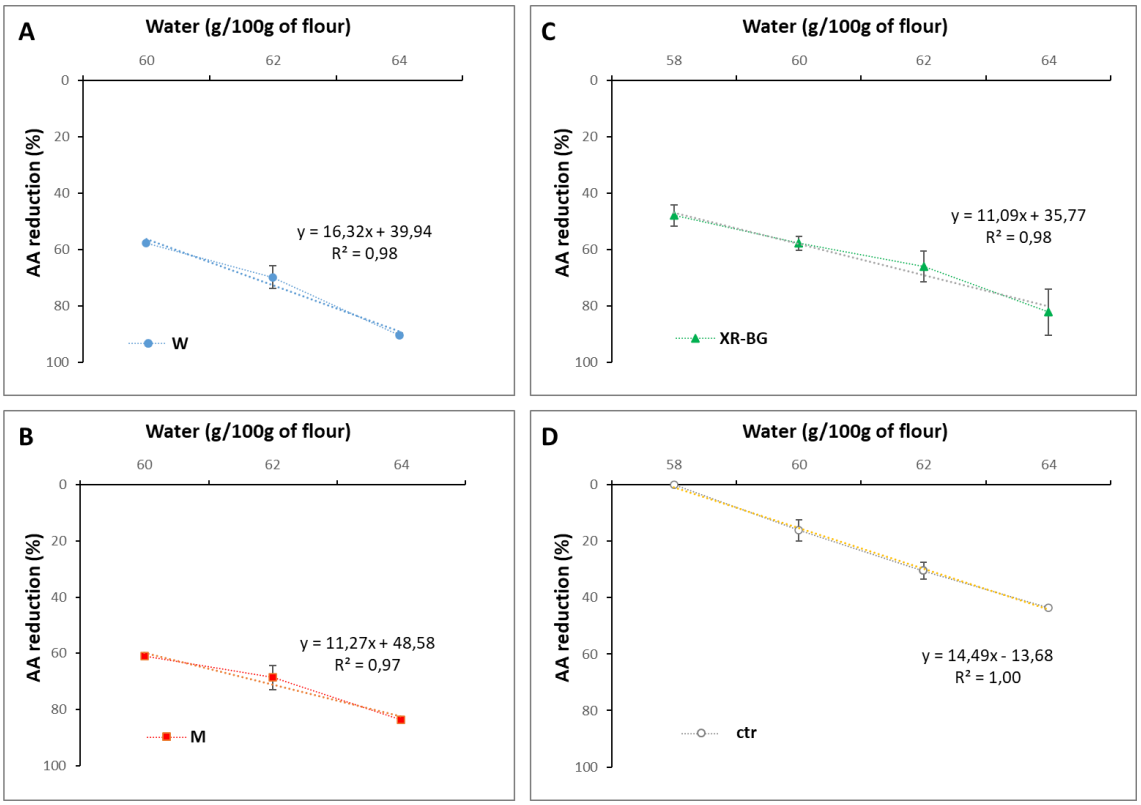

Figure 4.S3.1. Linear interpolation of the AA reduction curves for fried pizza bases prepared at different percentage hydration degree (58, 60, 62 and 64 g of water/100 g of flour), untreated (ctr) and treated with fixed amount (3 U/100 g) of Preventase® W, M or XR-BG (3 U/100 g). Values are expressed as mean±SD of three replicates (n=3).

4.6.S4 – Geometry of a Neapolitan wood oven baked pizza

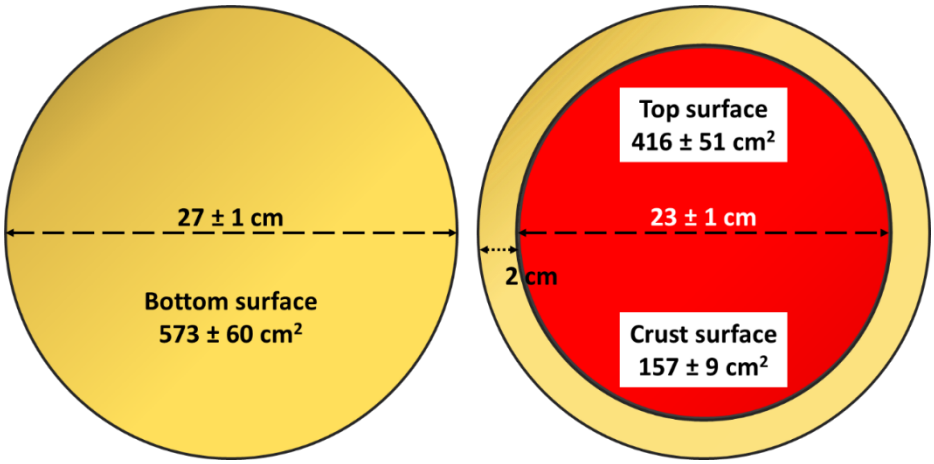

Figure 4.S4.1. Schematic representation of the surface of a Neapolitan wood oven baked pizza.

Chapter 5

Mitigation of acrylamide formation in wood oven baked pizza base using wheat grain with low asparagine content

Under submission

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Chapter 6

Development of Calibration and Validation Models for the Determination of Acrylamide Levels in Pizza Samples through FT-NIR Spectroscopy

Pubblished paper
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6. Abstract

Analytical methods to quantitatively detect acrylamide (AA) in food are expensive, laborious, time consuming and require costly scientific instruments, as LC-MS/MS and GC-MS. Near-Infrared (NIR) spectroscopy is a reliable technique, easy to use and able to quantify chemical components, and therefore could represent a fast tool for acrylamide screening in cooked foods. The aim of this study was to develop a new and innovative method to predict AA content in pizza, using a non-destructive NIR spectroscopy. Specifically, NIR reflectance spectra (1000-2500 nm) of freeze-dried pizza samples, with a known acrylamide level, previously measured by UHPLC, were accurately captured. The recorded spectra were processed to design calibration, by chemometric methods for quantitative analysis as Partial Least Squares (PLS) regression, and validation models for the prediction of acrylamide in cooked pizza samples. Spectral range and the number of PLS factors were examined and the lowest Standard Error of Calibration (SEC) and highest Correlation Coefficient of Determination (R2) were selected. The optimized calibration was applied in scanning the NIR spectra of a new set of pizza samples to validate the created method. Results showed that NIR spectroscopy technique is a screening tool capable of rapidly predicting, with reasonable accuracy, the AA content in pizza. Overall, good linear correlation was found between the predicted acrylamide levels in solid matrix by NIR method, and the actual acrylamide values measured by UHPLC in extracted pizza samples.

Keywords: acrylamide, FT-NIR spectroscopy, PLS regression, pizza, UHPLC

6.1 Introduction

Pizza is one of the most consumed foods in all over the world. Pizza has been produced and consumed in Italy since the 16th century and after World War II, due to the large scale emigration of Italians in the world, it was produced and distributed in all other countries (Caputo and Pugno, 2016). However, being a product derived from flour-based dough, pizza can also be a source of acrylamide (AA), a substance that even taken in modest quantities has harmful effects on health. In fact, acrylamide (H2C=CH-CO-NH2) is a toxic substance formed during cooking at high temperatures by reaction between free sugars, like glucose, and specific amino acids, such as asparagine, which are naturally present in the flour (Linke, 2015). It is well known that AA is present in commonly consumed carbohydrate-rich foods such as bread, pizza, and biscuits and produced at high temperatures during frying, baking, and roasting via the Maillard reaction (Zhang and Zhang, 2007). Acrylamide concentration in processed food products has become a very serious health issue, and thus EFSA recommends do not exceed the dose of 0.17 mg/kg b.w. per day (EFSA 2015:EN-817). Benchmark levels of acrylamide in bakery products are of 50-800 μg/kg while for fried potatoes and coffee 750-4000 μg/kg are accepted. Even if AA levels do not reach daily human reference limits, EFSA suggests adopting mitigation strategies for acrylamide formation and allowing monitoring of acrylamide content in finished food products (EU Reg. 2017/2158). However, there is a lack of literature about the level of acrylamide in cooked pizza nowadays, and therefore studies on

the determination of this substance are always welcome. Current methods for detecting and quantifying acrylamide in food are LC-MS or GC-MS chromatography. However, their use involves long sample preparation, time consuming, dependent on expensive scientific instrumentation and trained operators, which limits their use for monitoring acrylamide content during processing or on finished products (Wenzl et al., 2006), thus they are not suitable for quality control during the manufacturing process or for screening large numbers of samples (Ayvaz and Rodriguez-Saona, 2015). Therefore, a quick and simple method for routine acrylamide analysis is required. Near Infrared (NIR) spectroscopy is inexpensive, fast, easy to use, and applicable to acrylamide detection. As a method, NIR utilizes electromagnetic radiation at wavelengths in the range 780–2500 nm that contain information about the primary structural components of organic molecules, such as C–H, N–H and O–H bonds (Cozzolino et al., 2001), obtaining spectra derived from fundamental absorptions. It allows to quickly (15-90 s) predict with a quantitative method the content of substances such as proteins, amino acids, moisture, fat, starch, sugars and fibres or, in the case of an identification method, if there is the presence or absence of a substance, using a small amount of sample non-destructively and with minimal sample preparation. The main advantages of the NIR technique are the high time savings over the number of samples analysed, low cost, and simultaneous quantification of several traits in an individual sample measurement. Quantitative values can be calculated using mathematical models capable of correlating the NIR spectra, obtained from the scanned sample, with data derived from chemical analysis, and thus the goodness of the created method also depends on the use of appropriate analytical methods. The disadvantage is that it cannot determine low amounts of substances in samples and there is some question about the sensitivity of NIR for measuring acrylamide in food (Adedipe et al. 2016). Anyway, this technique has been used for numerous food applications related to quality control and safety, providing information in the fingerprint region. In fact, it has been utilized to estimate quality parameters such as starch, amylose, protein, moisture, and fat content in grains, seeds, fruits, tubers, wood, meat, fish, and other products (Diaz et al., 2014). Several studies employing these devices have been published, including the quantitation of carotenoids in intact watermelon (Tamburini et al., 2015a), sucrose levels in infant cereals (Lin et al., 2014), fatty acid value during storage of wheat flour (Jiang et al., 2020). For the food quality control, Delwiche and Weaver (1994) studied the quality of wheat flour via NIR technique, following several parameters such as water adsorption, dough mixing time, dough mixing tolerance, dough height, internal grain appearance, and protein level, and/or starch damage. Other recent studies have demonstrated the use of NIR in quantification of acrylamide in potato chips (Pedreschi et al. 2010). The purpose of this study is to establish a standard method for quantitative detection of acrylamide in pizza using NIR spectroscopy. Acrylamide content will be measured from pizza samples using NIR coupled with Partial Least Squares computational software and a standard curve. A standard acrylamide curve in solid matrix will be made. Acrylamide from pizza will be monitored by NIR and the results validated using UHPLC. Finally, the best way to predict the levels of acrylamide in pizza using NIR spectroscopy will be evaluated in order to ensure the safety of consumers.

6.2 Materials and Methods

6.2.1 Pizza preparation

Pizza doughs were prepared and kneaded with the traditional ingredients (flour, water, yeast and salt). The doughs were leavened at 22°C and 80 % relative humidity (RH) for 2h (37 samples) or 8 h (32 samples) and then cooked by frying in sunflower oil (37 samples) or in a wood oven (32 samples). The obtained pizza samples were cut into pieces and subjected to freeze-drying and grinding to obtain a powder with a particle size ≤0.5 mm, which was used both for the following AA extraction and NIR spectra analyses.

6.2.2 Quantification of acrylamide through UHPLC-UV

AA from powder samples was extracted as described by Kumar et al. (2014) with minor modifications and estimated through UHPLC-UV (LC-4000, Jasco, Japan) detection. Chromatographic separation of AA was performed at 30°C on a reverse phase C-18 column (Nucleodur C18 Gravity 150 x 3 mm, 3 µm), using water and methanol as mobile phases, both containing 0.1% formic acid, at a constant flow rate of 0.25 mL/min. Applying a 3 min elution program at 100% water followed by a 5 min methanol gradient from 0 to 7% (Capuano et al. 2009), the AA retention time was estimated to be 5 min. The AA sample peak are as were used to estimate the AA content in pizza samples by extrapolation from a calibration curve ($y = 43759x - 494$; $R^2=0.9999$) obtained with AA standard in the range of 0.05-10 mg/L. The calculated limits of detection and quantification were respectively LOD= 0.034 mg/L and LOQ= 0.113 mg/L.

6.2.3 NIR spectra measurement

Spectra of pizza samples were collected using a NIRFlex® N-500 (Büchi, Switzerland) equipped with the solids cell (Büchi, Switzerland) used for Steriplan Petri dishes (Duran Group, Czech Republic) with a volume of 31.4 cm3. Freeze-dried powder samples were placed on the glass surface of the 100x20 mm diameter by height plate using a spatula. FT-NIR (Fourier Transform Near Infrared) diffuse reflectance spectra were recorded using NIRWare 1.4 (Büchi, Switzerland) and capturing the full range, from 10000 to 4000 cm^{-1}, at 4 cm^{-1} intervals. The spectrometer is suitable for rapid non-destructive analysis of powder samples. 5 measurements were taken to get a complete analysis on the whole sample. Each measurement of freeze-dried pizza sample was achieved by scanning 32 times to have an average spectrum and a good signal-to-noise ratio; therefore, the total measurement time for each sample was 2 min and 30 s. Acquisition of the internal reference was obtained with each spectrum acquisition to optimize the baseline of the spectrum.

6.2.4 NIR analyses: calibration, internal and external validation

NIRCal 5.6 (Büchi, Switzerland) was used to perform the chemometric analyses including math pretreatments, calibration and validation. Raw spectra and spectra preprocessed, first-derivative, standard normal variate (SNV), and multiplicative scatter correction (MSC) transformations were applied to develop the calibration models, in order to minimize the

multiplicative interferences of scatter and surface roughness (Porep et al., 2015). 29 fried pizza and 25 wood oven cooked pizza samples were used to calibrate and cross-validate the FT-NIR technique for acrylamide content. 7 and 9 outliers were found to reach the best calibration, for fried pizza and wood oven pizza samples, respectively. Finally, 22 fried pizza (110 spectra) and 16 wood oven pizza (80 spectra) samples were considered for the calibration and internal validation.

Calibration models for AA content were determined using the blockwise cross-validation approach, which is a default software procedure, by randomly choosing 36 out of 110 fried pizza spectral samples as the validation while the remaining 74 were used as the calibration. In the same way, 26 out of 80 wood oven cooked pizza spectral samples were randomly selected by NIRCal software for the validation curve while the remaining 54 spectra were used as the calibration.

Calibrations were developed using all data points of the calibration in the full spectrum of NIR. Partial Least Squares (PLS) Regression was used to correlate the spectral information of the samples and AA contents determined by reference method. The number of factors used in the equation to calculate the analyte concentration was also considered by the software before selecting the equation for use.

Squared Pearson correlation coefficient (R^2), standard error of calibration (SEC), and standard error of prediction (SEP), Bias (tendency to deviate from the mean value), and the ratio of performance to deviation (RPD=SD/SEP) of the prediction sets were considered in selecting the best quantitative model. The quality of calibration was described by the Q-value calculated by the NIRCal 5.6. Q-statistic was calculated, considering various combinations of wavelength ranges and data pretreatments. The Q-value qualifies all calibrations with a number between 0 (useless) and 1 (ideal) based on the statistic results. A calibration is defined as good when it shows a Q-value between 0.45 and 0.75. When a Q-value greater than 0.75 is obtained, the calibration gives excellent reliable results. LOD was calculated as 3 times the standard deviation of the intercept/slope, and LOQ was calculated as 3 times the LOD (Adedipe et al. 2016).

A second validation (external validation) of the calibrated FT-NIR technique was also carried out as confirm of a good calibration models built. For this purpose, 8 random fried pizza (40 spectra) and 7 wood oven cooked pizza (35 spectra) samples were taken for spectra acquisition using the same instrumentation of the calibration set. External validation was performed comparing NIR predicted data and reference data with Microsoft® Excel (office 365) and the prediction accuracy was calculating in terms of squared Pearson correlation coefficient (R^2).

6.3 Results and Discussion

6.3.1 AA content and NIR spectra

AA content in pizza samples was chromatographically determined by UHPLC-UV instrument and the obtained values were used for the calibration and cross validation dataset on NIR instrument. In fried pizza samples the AA concentration varied from 0.99 and 3.775 mg/kg, while for the wood oven pizza samples the AA levels were in the range of 0.66÷2.46 mg/kg.

The powder pizza model system was used to determine whether NIR tecnique is able to detect acrylamide in the above described pizza samples and evaluate the limit of detection in such a matrix. A total number of 145 and 125 original raw spectra were recorded for fried and wood oven cooked pizza's powder samples respectively. The analysis of the NIR spectrum is aimed at identifying characteristic peaks for which it is possible to hypothesize a direct correlation with the presence and concentration of a specific analyte. The typical trend of a raw spectrum is the wide increase in the reflectance signal generated by the diffusion of light that hits the sample made up of complex and non-homogeneous structures, and therefore has characteristics of surface roughness. Thus, raw spectra could be affected by interferences generated by light scattering and background absorptions caused by other O-H containing molecules, as well as other systematic errors associated with intrinsic factors in the acquisition of spectra. In order to minimize these noises, stabilize the baseline shifts and improve the quality of the minor spectral variations, specific pretreatments were applied by the software, consisting of MSC full and first derivative for fried pizza spectral samples and SNV (Standard Normal Variate) for the wood oven cooked pizza spectra. Pretreated spectra from respective samples, were automatically divided in calibration and validation spectra by NIRCal software (Figure 6.1). The intensity of the NIR region is low so that the peaks in the spectrum are not clearly visible and are very broad. Using NIR spectroscopy, it is impossible for these peaks to correspond to a single vibration. The NIR spectrum (750-2500 nm) reads overtones and low energy vibrations of C-H, O-H and N-H bonds. The absorbances read at 5000-5500 cm^{-1} corresponded to N-H bond and 6000-6500 cm^{-1} were associated with carbonyl (C=O) for the amide functional group of acrylamide (Skinner et al., 2021).

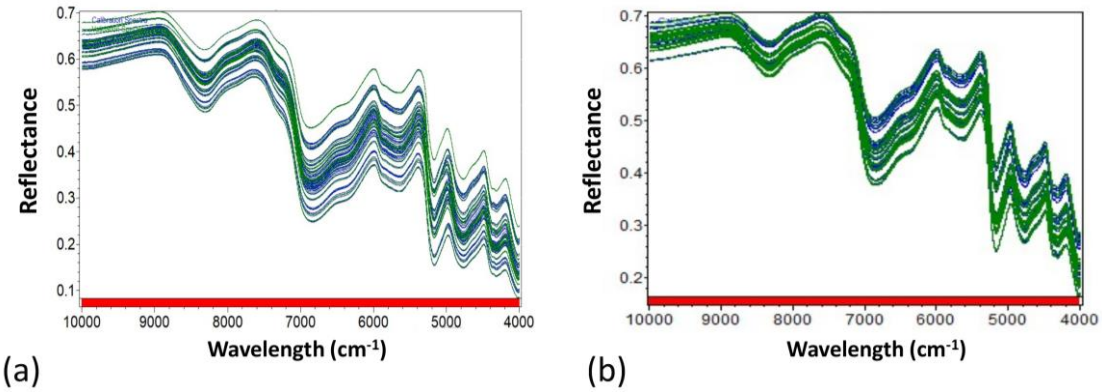

Figure 6.1 - NIR pretreated spectra of fried (a) and wood oven cooked pizza samples (b). NIRCal® software automatically divided original spectra in calibration (blue) and validation (green) set.

6.3.2 Calibration and internal validation of AA models

It is well known that the reliability of a NIR calibration is restricted to the range of values considered. Pretreated spectra have been elaborated with the NIRCal program to build a calibration and internal-validation model for AA prediction in pizza samples.

For the case of fried pizza, 35 outliers out of the 145 spectral samples were observed during the calibration procedure. On the other side, 45 outliers were found out of the 125 wood oven cooked pizza spectral samples. After eliminating the spectra of the outlier samples, the

calibration procedure was repeated with the remaining spectra taking into account 4 and 6 PLS factors for fried and wood oven cooked samples respectively. No outliers were observed during calibration using the data set of 110 samples for fried pizza and 80 samples for wood oven cooked pizza. The calibration curves and the internal validation results are given in Figure 6.2.

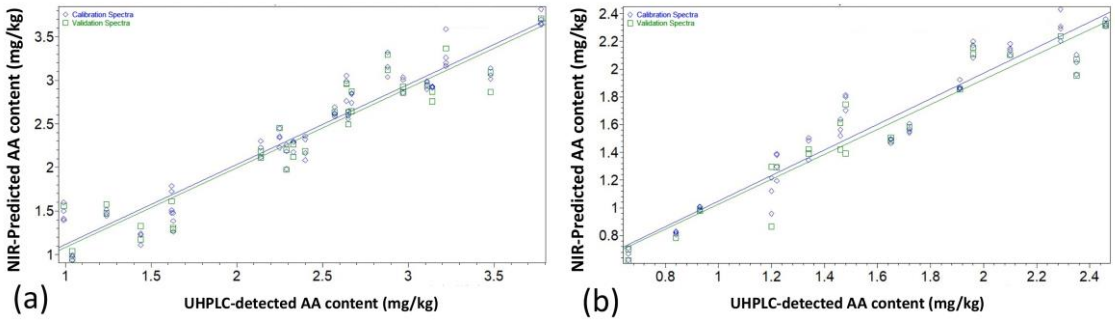

Figure 6.2 - NIR predicted versus measured values of AA content, in fried (a) and wood oven pizza samples (b).

The robustness of calibration model can be evaluated by the statistical parameter of the Q-value which refers to a "good" calibration when it is in the range of 0.45÷0.75 and to an "excellent" calibration if it is > 0.75 (Tamburini et al., 2015a). For fried pizza the Q-value was 0.71, while for the wood oven cooked pizza Q-value was equal to 0.76 thus supporting the robustness of the models built. Moreover, for the calibration set, SEC and the R^2 were 0.2244 and 0.9176, respectively for fried pizza samples and 0.1503 and 0.9219 respectively for wood oven cooked pizza samples. However, since it is a multivariate calibration model, the consideration of the SEC alone is not a reliable test for assessing the accuracy of the model. Therefore, it is necessary to introduce a set of validation samples for which it is possible estimate the SEP parameter, that is, a calculation of the variability of the differences between the predicted and reference values. For the internal validation set of the 36 fried pizza spectral samples, SD, SEP and R^2 were 0.7373, 0.2584 and 0.8851, respectively, while the same parameters were equal to 0.5179, 0.1598 and 0.9173 for the 26 wood oven cooked pizza spectral samples. It is interesting to note that for both calibration models built, a very low Bias value (0.03428 and 0.03428 for fried and wood oven cooked samples) was observed between the measured and NIR predicted AA values during internal validation, indicating a good adherence of the samples used for validation compared to those on which the calibration was built. Another statistical parameter useful for evaluating the accuracy of the prediction, taking into account the standard deviation of the reference data, is the RPD or ratio of performance to deviation, which represents a standardization of the SEP (Herold et al., 2009). In the AA's internal calibration-validation model in fried pizza samples, the RPD was 2.85, while for pizza cooked in a wood oven RPD = 3.26. These values indicate that the built models have a good but not excellent degree of accuracy. In fact, in a good calibration model, the internal validation should be characterized by values of SEP much lower than SD in order to obtain an RPD = 5. However, RPD values around 3 can also be considered acceptable, while suggesting an implementation of the model's prediction capability to make predictions more reliable (Tamburini et al., 2015b).

LOD and LOQ parameters were calculated from calibration curves and resulted 0.64 and 1.94 mg/kg for fried pizza, and 0.42 and 1.27 mg/kg for wood oven cooked pizza samples, respectively. These results are encouraging for the determination of AA in pizza using NIR spectra, although they are considered insensitive for the detection of analytes in food present at levels below 0.100 mg/kg.

6.3.3 External validation of AA models

The predictive ability of the calibration model must be verified through an external validation, i.e., using a set of samples that were not used in the construction of the calibration model nor for internal validation (Fujiwara & Murakami, 2007). The latter, in fact, is carried out using spectra randomly selected from the calibration set and for this reason, it is not considered a sufficient test in complex and inhomogeneous matrices such as pizza samples. Therefore, after obtaining satisfactory internal validations, the performance (robustness and stability) of the calibrations for the prediction of AA levels have been tested with other samples showing an AA concentration between 1.41÷3.77 mg/kg, and 0.78÷2.42 mg/kg for fried or wood oven cooked pizza respectively. In particular, further 40 (fried) and 35 (wood oven) spectral samples were recorded by scanning 8 and 7 pizza powder samples and AA content data predicted by NIR calibration were compared to reference chemical results obtained by UHPLC-UV chromatography. The performance of the effective predictive capacity of NIR calibration models was evaluated by plotting the AA data predicted by NIR as function of the corresponding reference analyses. As shown in Figure 6.3, the predicted results by NIR were positively correlated with chemical results for both calibration models related to fried and wood oven cooked samples. The Correlation Coefficient R^2 was higher than 0.9 for both models (0.9086 and 0.947), thus suggesting a fairly accuracy of predicted values.

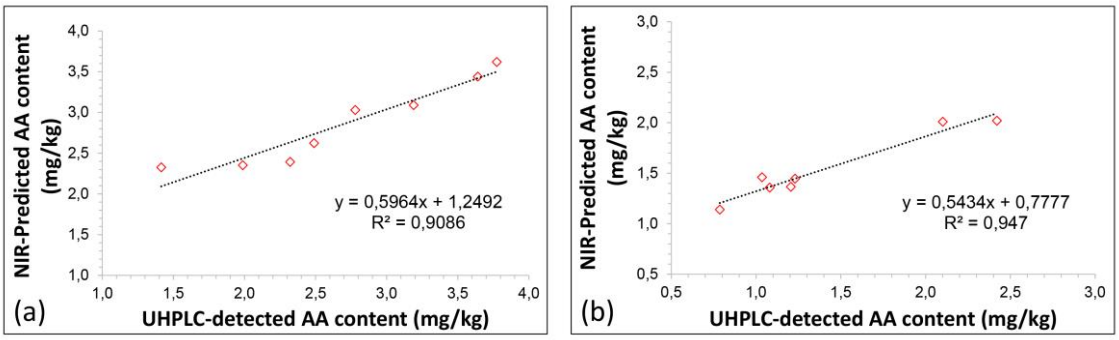

Figure 6.3 - Correlation between NIR predicted and reference measured values for fried (a) and wood oven cooked pizza samples (b) during the external validation.

6.4 Conclusions

For the first time, a non-destructive NIR spectroscopy model has been developed for the prediction of acrylamide levels in pizza samples, certainly a structured and complex matrix associated with a fairly high intrinsic variability. However, for both fried pizza and wood-fired pizza samples, the processing of NIR spectra allowed to build calibration models with internal validation that present an acceptable degree of reliability, supported by RPD values close to 3

(2.85 and 3.26 for fried and wood-fired samples respectively). The robustness of the calibrations was confirmed by the high correlation found between the levels of AA predicted by NIR spectroscopy and those measured at UHPLC for the samples used in the external validation of the models built, with R^2 values equal to 0.9086 and 0.947 for the two developed models. Surely both models can be further improved by increasing the number of samples scanned to obtain spectra, to strengthen the validity of the calibration and minimize prediction errors. In conclusion, this method could lay the groundwork for faster screening of acrylamide levels in order to contribute to food safety, security, and competitiveness within European and U.S. markets.

6.5 References

- Adedipe O.E., Johanningsmeier S.D., Truong V.D., Yencho G.C., 2016, Development and Validation of a Near-Infrared Spectroscopy Method for the Prediction of Acrylamide Content in French-Fried Potato, Journal of Agricultural and Food Chemistry, 64, 1850−1860.
- Ayvaz H. and Rodriguez-Saona L.E., 2015, Application of handheld and portable spectrometers for screening acrylamide content in commercial potato chips. Food Chemistry, 174, 154–162.
- Capuano E., Ferrigno A., Acampa I., Serpen A., Açar Ö.Ç., Gökmen V., Fogliano V., 2009, Effect of flour type on Maillard reaction and acrylamide formation during toasting of bread crisp model systems and mitigation strategies, Food Research International, 42, 1295–1302.
- Caputo W. and Pugno L., 2016. La pizza al microscopio. In: Storia, fisica e chimica di uno dei piatti più amati e diffusi al mondo. San Giovanni Lupatoto, Gribaudo.
- Cozzolino D., Fassio A., Gimenez A., 2001, The use of near-infrared reflectance spectroscopy (NIRS) to predict the composition of whole maize plants, Journal of Science of Food and Agriculture, 81, 142-146.
- Delwiche S.R. and Weaver G., 1994, Bread Quality of Wheat Flour by Near-Infrared Spectrophotometry: Feasibility of Modeling, Journal of Food Science, 59, 2, 410-415.
- Diaz J.T., Veal M.W., Chinn M.S., 2014, Development of NIRS models to predict composition of enzymaticallyprocessed sweetpotato, Industrial Crops and Products, 59, 119–124.
- EFSA 2015:EN-817, Outcome of the public consultation on the draft Scientific Opinion of the EFSA Panel on Contaminants in the Food Chain (CONTAM) on acrylamide in food.
- EU Regulation 2017/2158, Establishing mitigation measures and benchmark levels for the reduction of the presence as acrylamide in food, Official Journal of the European Union, 60, 24–44.
- Fujiwara T. and Murakami K., 2007, Application of near infrared spectroscopy for estimating available nitrogen in poultry manure compost, Journal of Soil Science and Plant Nutrition, 53, 102–107.
- Herold, B., Kawano, S., Sumpf, B., Tillmann, P., Walsh K.B., 2009, VIS/NIR spectroscopy. In M. Zude (Ed.), Optical monitoring of fresh and processed agricultural crops, 141–249.
- Jiang H., Tong L., Quansheng C., 2020, Quantitative detection of fatty acid value during storage of wheat flour based on a portable near-infrared (NIR) spectroscopy system, Infrared Physics and Technology, 109, 103423.
- Kumar N.S.M., Shimray C.A., Indrani D., Indrani D., Manonmani H.K., 2014, Reduction of Acrylamide Formation in Sweet Bread with L-Asparaginase Treatment, Food Bioprocess Technology, 7, 741-748.

- Lin C., Ayvaz H., Rodriguez-Saona L.E., 2014, Application of portable and handheld infrared spectrometers for determination of sucrose levels in infant cereals, Food Analytical Methods, 7, 1407–1414.
- Linke D., 2015, Enzymatic prevention of health risks in food, Current Opinion in Food Science, 1, 21–27.
- Pedreschi F., Segtnan V.H., Knutsen S.H., 2010, On-line monitoring of fat, dry matter and acrylamide contents in potato chips using near infrared interactance and visual reflectance imaging, Food Chemistry, 121, 616.
- Porep J.U., Kammerer D.R., Carle R., 2015, On-line application of near infrared (NIR) spectroscopy in food production, Trends in Food Science & Technology, 46, 211–230.
- Skinner M.M., Seale J.T., Cantrell M.S., Collins J.M., Turner M.W., McDougal O.M., 2021, Instrumentation for Routine Analysis of Acrylamide in French Fries: Assessing Limitations for Adoption, Foods, 10, 2038.
- Tamburini E., Castaldelli G., Ferrari G., Marchetti M.G., Pedrini P., Aschonitis V.G., 2015b, Onsite and online FT-NIR spectroscopy for the estimation of total nitrogen and moisture content in poultry manure, Environmental Technology, 36,18, 2285–2294.
- Tamburini E., Ferrari G., Pedrini P., Donegà V., Malavasi M., Marchetti M.G., 2015a, Non-destructive quantification of carotenoids in intact watermelon (citrullus lanatus) using on-line near infrared spectroscopy, Proceedings of the ICNIRS, <10.17648/nir-2015-34177>
- Wenzl T., Karasek L., Rosen J., Hellenaes K., Crews C., Castle L., Anklam E., (2006), Collaborative trial validation study of two methods, one based on high performance liquid chromatography–tandem mass spectrometry and on gas chromatography–mass spectrometry for the determination of acrylamide in bakery and potato products, Journal of Chromatography A, 1132, 211–218.
- Zhang Y. and Zhang Y., 2007, Formation and reduction of acrylamide in Maillard reaction: A review based on the current state of knowledge, Critical Reviews in Food Science and Nutrition, 47, 521–542.

General conclusion

Despite the worldwide popularity and its economic relevance, Neapolitan pizza is a topic that has attracted little interest from the scientific community. The pizza making process, according to the European Regulation n.97/2010, is abundantly described in the "Introduction" session of this PhD thesis. The study of dough is difficult, because dough is a complex biological system in which there are many factors that influence leavening performance, such as the selection of raw materials (different flours), the use of yeast or lactic acid bacterias, the technological process parameters (time, temperature, humidity) that can be related to the quality of the final product, like pizza.

The aim of this PhD thesis was to investigate technological, nutritional and health aspects on Neapolitan pizza. Specifically, study on its dough structure, starch digestibility, and acrylamide content deriving from the fast baking at very high temperature in the traditional wood oven. Several structural and biochemical changes occur during the pizza making process.

In "Chapter 1", it is largely described how different leavening times (0, 4, 8, 16, 24, 48 h) affect the structure of pizza dough during fermentation and how physical and biochemical parameters may be related to the starch digestibility of the wood oven baked pizza base. The dough has certain viscoelastic characteristics that it can be changed over leavening stage. Long leavening time decreased the elastic component (more relaxed) of the dough, improving the extensibility of the pizza disc by facilitating the action of the pizza maker during the rolling out phase. During heating, starch and gluten protein interactions are established and consolidated during cooling of pizza dough, in which elastic modulus decreased over leavening time, as a demonstration of enlargement of gluten network due to the volumetric expansion of the dough by CO_2, also associated with a reduction of compression force at long leavening time. This effect was demonstrated by the phenomena of destructuring of glutinic network and starch damage, by protease and amylase enzymes over time.

Furthermore, in the Appendix of "Chapter 1", SDS-PAGE profiles of gluten proteins confirmed this hypothesis. Damage to starch, by enzymatic hydrolysis, improves its ability to gelatinize and is reflected in an increase in reducing sugars at 16 h. SEM analyses showed a stretching of gluten network, starting from 8 h, which allows a better access by the digestive enzymes. Indeed, the long leavening time could improve the digestibility by consumers, as digestion enzymes have an easier access to protein and starch matrix due to the breakdown of the structure in the dough. Finally, classic Neapolitan pizza can be associate to a potential high glycemic index food.

In the second case study, doughs were prepared with different starters, commercial or selected yeasts and addition or not of lactic acid bacteria, to further investigate the effect of selected leavening time (0, 4, 8, 16, 24, 48 h), on fermentation products, dough structure and starch digestibility, thoroughly investigated in the "Chapter 2". As leavening time increases, starch and proteins are affected by enzymes, inducing a change in the molecular organization of the components depending on the yeast used and the selected bacteria. ATR-FTIR analysis, confirmed a decreased in amine I and starch band over the leavening time. Volumetric index was minor for dough with selected yeast and lactic acid bacteria, in comparison with the other samples, and the highest amylolytic and proteolytic activity of yeast and lactic acid bacteria

samples was achieved at long leavening time. The same sample dough reached the highest levels of ethanol, lactic and acetic acids over the leavening time. Moreover, pH dropped dramatically in the last hours of leavening for samples with LAB added to the dough. *In vitro* starch digestibility was significantly influenced by the dough structure after selected leavening time (16, 24 and 48 h) and baking. A good correlation was found between microstructure and nutritional properties, thus, more compact is the structure, less rapidly available glucose will have in the bloodstream.

A technological aspect was explored in "Chapter 3", with the aim of assessing the optimal leavening time for freezing pizza dough, to obtain thawed and leavened dough balls with a similar structural and rheological behaviour, compared to the unfrozen control. In this perspective, freezing indicate a reduction in the volume of frozen dough and weakening of the gluten network after thawing of pizza dough, may be caused by the release of reducing substances by yeast during freezing, which results in a reduction of gluten proteins that thus lose their ability to bind water and cross-link gluten. Damaged starch is also influenced by the formation of ice crystals during storage. Freezing dough before leavening (0 h) is the best solution to have leavening kinetics and viscoelastic properties of the dough similar to the unfrozen control.

The last three Chapters of this PhD thesis focused on the determination of acrylamide levels in pizza samples. To our knowledge, this is the first time that different L-Asparaginase enzymes has been applied in the production of pizza dough to reduce acrylamide content, especially in the Neapolitan style TSG.

In the "Chapter 4" the reported experimental results demonstrated its effectiveness. Especially, Preventase W enzyme, with 3 U/100g of flour, allowed to obtain a maximum reduction of AA of 61% in fried pizza bases and 46% in those baked in a wood oven. Moreover, at this enzyme concentration, there were no significant deviations from the threshold of 500 BU in the produced dough, indicating a low interference of the technological adjuvant on the rheological properties of the product.

Another action strategy for mitigation of acrylamide in pizza, was thoroughly described in "Chapter 5". The selected genotypes of wheat grains, have a different potential in the formation of acrylamide in the final product, due to the different levels of free asparagine present in the respective flours. Moreover, it was observed that consumption of products prepared with wholemeal flours could slow the release of glucose into the bloodstream following meal ingestion due to the higher fibre content in wholemeal flours but, at the same time, their use may result in higher levels of acrylamide in wood oven baked pizza bases. Thus, the use of low asparagine wheat grains has been shown to be a good acrylamide mitigation strategy although, it is not only the presence of asparagine that influences acrylamide levels in baked foods, but also other intrinsic factors of flours such as reducing sugar content, the amount of dietary fibre contained in bran, and different flour absorption indices. In both experiments (Chapter 4 and 5), for acrylamide mitigation in pizza samples, the values obtained did not exceed the daily threshold relative to the human body mass. Thus, both wood oven and fried pizza can be described as safe for consumers health.

Further, for the first time, a non-destructive NIR spectroscopy model has been developed for the fast prediction of acrylamide levels in pizza samples, certainly a structured and complex

matrix associated with a fairly high intrinsic variability. In “Chapter 6”, the robustness of the constructed model method calibration/validation is evaluated. To conclude, this method could lay the groundwork for faster screening of acrylamide levels in order to contribute to food safety, security, and competitiveness within European and U.S. markets. However, for all citated Chapters of this PhD thesis, further studies are needed to better investigate the glycemic index in humans with *in vivo* tests and perform sensory analyses of all prepared samples to better assess consumer preferences on taste and texture of this food product, the Neapolitan pizza.

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