

Reduction of acrylamide content in cereal products via systematic use of asparaginases, phenolic acids, and salts

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List of papers, presentations, and awards

Papers

Paper 1: Musa, S.; Becker, L.; Oellig, C.; Scherf, K. A. Influence of Asparaginase on Acrylamide Content, Color, and Texture in Oat, Corn, and Rice Cookies. *J. Agric. Food Chem.* 2024, 72, 22875–22882. <https://doi.org/10.1021/acs.jafc.4c06175>.

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Paper 4: Musa, S.; Kukurová, K.; Ciesarová, Z.; Eisenhardt, K.; Scherf, K. A. Asparaginase Treatment for Acrylamide Reduction in Wheat and Oat Flakes: From Laboratory- to Pilot-Scale. *Applied Food Research* 2025, 5. <https://doi.org/10.1016/j.afres.2025.101237>

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Abstract

Acrylamide, a potentially harmful processing contaminant formed during the Maillard reaction in heat-processed foods, poses a significant food safety concern due to its classification as “probably carcinogenic to humans” by the International Agency for Research on Cancer. While asparaginases, phenolic acids, salts, and rosemary extract have shown promise in reducing acrylamide formation, a systematic evaluation of their individual and combined efficacy remains limited. Additionally, there is a gap in research regarding the use of various raw materials, such as wholemeal wheat, oat, rye, corn, and rice flour, alongside acrylamide mitigation in products like cookies, crispbread, and breakfast cereals.

Four different asparaginases were evaluated. Initial experiments were conducted to evaluate the impact of dough incubation time and temperature, and baking time and temperature for cookies on acrylamide content. Increasing the incubation time from 10 min to 30 min showed no significant changes in acrylamide content, regardless of baking temperature. However, the incubation temperature of 90 °C showed a significant increase in acrylamide content compared to 60 °C, for samples baked at 180 °C for 15 min and 240 °C for 9 min. Due to this, a lower incubation temperature of 70 °C instead of 90 °C was selected for further evaluation. Asparaginases were most effective in products made of oat flour with a reduction of up to 97%, while wheat flour showed the lowest reduction with a maximum of 79%. Similarly, oat flakes showed higher reductions when treated with the same asparaginases compared to wheat flakes.

Although phenolic acids and calcium salts slightly reduced acrylamide in cookies, a combination of them with asparaginase achieved higher reductions than using them alone. Rosemary extract showed higher acrylamide reductions in cookies than in crispbread, with up to 50% and 10%, respectively, highlighting the influence of the matrix effect.

While some of the treatments showed a few significant changes in the color and texture of baked products, sensory evaluations revealed no significant changes in cookies for the selected treatments. Evaluation of the new color scanning device, Color Muse, showed similar trends to C-Cell color measurement. Therefore, this may be used for color evaluation of products when a correlation between color and acrylamide content

is desired, or simply for color estimation of baked products. This study showed a correlation ($r = -0.75$; b^* -value) between acrylamide content and color of cookies.

The application of one asparaginase in a pilot scale experiment to make bran flakes showed a low reduction of acrylamide compared to wheat flakes obtained in a laboratory scale. This further emphasizes the need for further research and optimization of incubation time and temperature for asparaginases in larger-scale production.

Overall, this work provides new insights into asparaginase application in different products using different raw materials (flours) as well as a combination of different approaches using asparaginase in combination with phenolic acids and calcium salts. This combination, to our knowledge, was evaluated for the first time. Further, it showed that a cheaper alternative for color estimation may be used to obtain L^* -, a^* -, and b^* -values for color of baked goods, and possibly other food products. To our best knowledge, Color Muse was also tested for the first time in food products.

Zusammenfassung

Acrylamid, eine potenziell schädliche Prozesskontaminante, die bei der Maillard-Reaktion in hitzeverarbeiteten Lebensmitteln entsteht, stellt ein erhebliches Problem für die Lebensmittelsicherheit dar, da es von der Internationalen Agentur für Krebsforschung als „wahrscheinlich krebserregend für den Menschen“ eingestuft wurde. Asparaginasen, Phenolsäuren, Salze und Rosmarinextrakt haben sich zwar als vielversprechend erwiesen, um die Bildung von Acrylamid zu verringern; eine systematische Bewertung ihrer individuellen und kombinierten Wirksamkeit ist jedoch noch begrenzt. Darüber hinaus gibt es eine Forschungslücke in Bezug auf die Verwendung verschiedener Rohstoffe wie Weizen-, Hafer-, Roggen-, Mais- und Reismehl sowie die Verringerung von Acrylamid in Produkten wie Keksen, Knäckebrot und Frühstückscerealien.

Vier verschiedene Asparaginasen wurden untersucht. Zuerst wurden die Auswirkungen der Inkubationszeit und -temperatur des Teigs sowie der Backzeit und -temperatur für Kekse auf den Acrylamidgehalt untersucht. Eine Erhöhung der Inkubationszeit von 10 min auf 30 min zeigte keine signifikanten Veränderungen des Acrylamidgehalts unabhängig von der Backtemperatur. Die Inkubationstemperatur von 90 °C bewirkte jedoch einen signifikanten Anstieg des Acrylamidgehalts im Vergleich zu 60 °C bei Proben, die bei 180 °C für 15 min und 240 °C für 9 min gebacken wurden. Aus diesem Grund wurde für die weitere Untersuchung eine niedrigere Inkubationstemperatur von 70 °C anstelle von 90 °C gewählt. Asparaginasen waren in Produkten aus Hafermehl mit einer Reduktion des Acrylamidgehaltes von bis zu 97% am wirksamsten, während Weizenmehl mit maximal 79% die geringste Reduktion zeigte. Auch Haferflocken zeigten bei der Behandlung mit denselben Asparaginasen eine höhere Reduktion als Weizenflocken.

Obwohl Phenolsäuren und Calciumsalze den Acrylamidgehalt in Keksen leicht verringerten, wurde durch die Kombination mit Asparaginase eine stärkere Verringerung erreicht als durch die alleinige Verwendung. Rosmarinextrakt führte in Keksen zu einer stärkeren Acrylamidreduzierung als in Knäckebrot, und zwar um bis zu 50% bzw. 10%, was den Einfluss des Matrixeffekts verdeutlicht.

Während einige der Behandlungen einige signifikante Veränderungen in der Farbe und Textur der Backwaren zeigten, ergaben die sensorischen Bewertungen keine signifikanten Veränderungen der Kekse bei den ausgewählten Behandlungen. Die

Bewertung des neuen Farbscanners Color Muse zeigte ähnliche Trends wie die C-Cell-Farbmessung. Daher kann dies zur Farbbewertung von Produkten verwendet werden, wenn eine Korrelation zwischen Farbe und Acrylamidgehalt erforderlich ist, oder einfach zur Farbabschätzung von Backwaren. Diese Studie zeigte eine Korrelation ($r = -0,75$; b^* -Wert) zwischen Acrylamidgehalt und der Farbe von Keksen. Die Anwendung einer Asparaginase in einem Pilotversuch zur Herstellung von Kleieflocken zeigte eine geringe Reduzierung von Acrylamid im Vergleich zu Weizenflocken, die im Labormaßstab hergestellt wurden. Dies unterstreicht die Notwendigkeit weiterer Forschung und der Optimierung der Inkubationszeit und Inkubationstemperatur für Asparaginasen in der Produktion im größeren Maßstab.

Insgesamt bietet diese Arbeit neue Einblicke in die Anwendung von Asparaginasen in verschiedenen Produkten unter Verwendung unterschiedlicher Rohstoffe (Mehle) sowie eine Kombination verschiedener Ansätze unter Verwendung von Asparaginasen in Kombination mit Phenolsäuren und Calciumsalzen. Diese Kombination wurde unseres Wissens nach zum ersten Mal bewertet. Außerdem zeigte sich, dass eine kostengünstigere Alternative für die Farbbestimmung verwendet werden kann, um L^* -, a^* - und b^* -Werte für die Farbe von Backwaren und möglicherweise anderen Lebensmitteln zu erhalten. Unseres Wissens nach wurde Color Muse auch zum ersten Mal bei Lebensmitteln getestet.

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1 Introduction

Cereal grains have been part of the human diet for thousands of years and have constantly increased in production year by year since the 1950ies. The Food and Agriculture Organization (FAO) of the United Nations, reported a global production of cereals of 2.841 million tons (FAO, 2024). Cereals are a staple food, and due to their nutritional value, they play a significant role in the human diet. Cereals are defined as the edible grains or seeds of plants belonging to the grass family, Gramineae (Bender, 2006). Some of the main grains that come from this family include wheat (*Triticum*), rye (*Secale*), oats (*Avena*), rice (*Oryza*), and maize (*Zea*) (Figure 1).

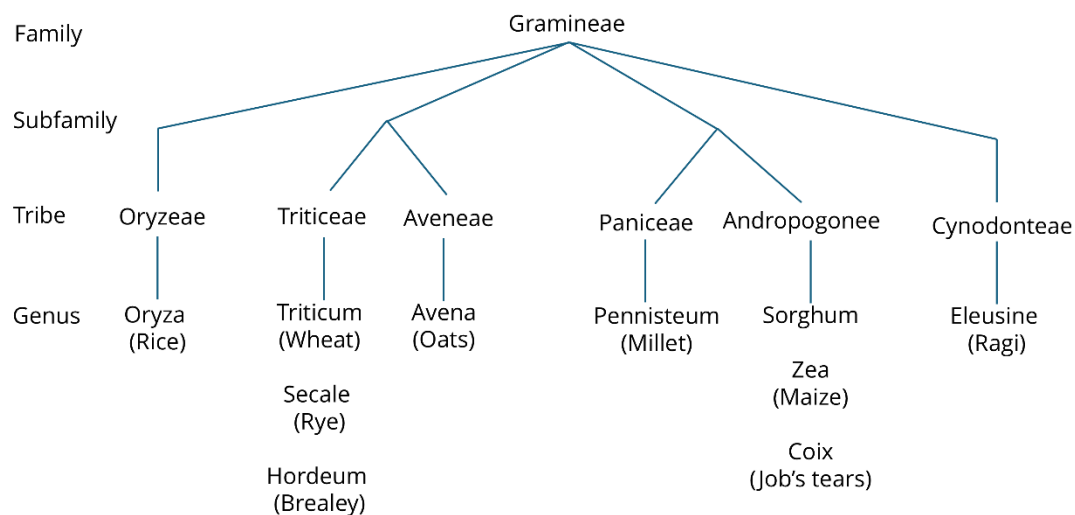


Figure 1. The branch of the Gramineae family, including subfamily, tribe, and genus (adapted from Shewry et al. 1992).

1.1 Nutritional profile of cereal products

The popularity of grains is particularly related to them being a key source of macronutrients, including carbohydrates (starch, dietary fiber), proteins, and fats. Carbohydrates are the major constituents of cereals, making up approximately 75%, and form the main source of energy for humans. Starch represents the highest proportion of carbohydrates with 60–70% (Shewry, 2009). Starch is a polysaccharide that consists of two major components: amylose and amylopectin. Both are made up

of glucose monomers, but they differ in their degree of polymerization and molecular structure. Amylose typically has a degree of polymerization in the range of 500 to 6000 glucose residues, while amylopectin has a much higher degree of polymerization, in the range 3×10^5 to 3×10^6 (Whistler & Daniel, 2000). The ratio of amylose to amylopectin varies among different cereal starches but is generally 20–30% amylose and 70–80% amylopectin (Shewry, 2009). Dietary fiber, another key carbohydrate component, is predominantly concentrated in the bran layer of whole grains (Figure 2). Cereals contribute to up to 50% of human fiber intake (Marcotuli et al., 2020). Dietary fiber has been associated with a number of health benefits primarily related to the prevention of colon cancer, obesity, and glucose response reduction, hence a decreased risk of type 2 diabetes, and cardiovascular diseases among others (Anderson et al., 2009; Montonen et al., 2003). The refining process of grains significantly reduces their fiber content, which is why whole grains are emphasized as an essential part of a healthy diet.

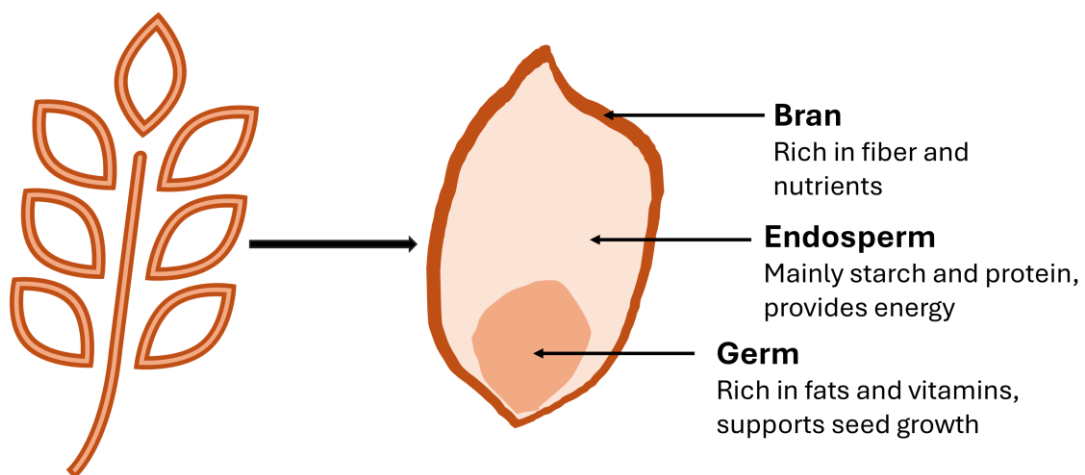


Figure 2. Anatomy of whole grain (own drawing adapted from Garutti et al., 2022).

Cereal proteins are important macronutrients contributing to the nutritional and functional properties of cereals. Storage proteins can make up to 70% to 80% of the total proteins in cereal grains (Wieser et al., 2023).

Table 1. Nutrients present in whole grain maize, oats, rice, rye, and wheat (adapted from Garutti et al., 2022)

Cereals	CHO	Pro	Fat	F _{TOT}	F _{SOL}	Vitamins	Micronutrients
Maize (<i>Zea mays</i>)	75.1	9.2	3.8	2	/	Thiamine 0.36 mg Riboflavin 0.2 mg Niacin 1.5 mg Vitamin A 0.062 mg	Phosphorus 256 mg Magnesium 120 mg Calcium 15 mg Iron 2.4 mg Zinc 2.21 mg Potassium 287 mg Sodium 35 mg Selenium 0.155 mg Copper 0.31 mg
Oats (<i>Avena sativa</i>)	66.3	16.9	6.9	10.6	/	Thiamine 0.763 mg Riboflavin 0.139 mg Niacin 0.961 mg Pantothenic acid 1.35 mg Vitamin B6 0.119 mg	Calcium 54 mg Potassium 429 mg Sodium 2 mg Phosphorus 523 mg Iron 4.72 mg Magnesium 177 mg Zinc 3.97 mg Copper 0.626 mg Manganese 4.92 mg
Rice (<i>Oryza sativa</i>)	80.4	6.7	0.4	1	0.08	Thiamine 0.11 mg Riboflavin 0.03 mg Niacin 1.3 mg	Calcium 24 mg Potassium 92 mg Sodium 5 mg Magnesium 20 mg Phosphorus 94 mg Iron 0.8 mg Copper 0.18 mg Zinc 1.3 mg Selenium 0.01 mg
Rye (<i>Secale cereale</i>)	75.9	10.3	1.63	15.1	/	Thiamine 0.316 mg Riboflavin 0.251 mg Niacin 4.27 mg Pantothenic acid 1.46 mg Vitamin B6 0.294 mg Vitamin A 0.003 mg Vitamin E (alpha-tocopherol) 0.85 mg Vitamin K (phylloquinone) 0.059 mg	Calcium 24 mg Iron 2.63 mg Magnesium 110 mg Phosphorus 332 mg Potassium 510 mg Sodium 2 mg Zinc 2.65 mg Copper 0.367 mg Manganese 2.58 mg
Wheat (<i>Triticum aestivum</i>)	65.2	12.3	2.6	9.7	/	Thiamine 0.42 mg Riboflavin 0.14 mg Niacin 5.4 mg	Calcium 35 mg Phosphorus 304 mg Iron 3.3 mg Copper 0.31 mg Zinc 3.1 mg

CHO: carbohydrates; Pro: proteins; F_{SOL}: soluble fibers; F_{TOT}: total fibers

The proteins of cereals also have a critical role in food structure and quality. For example, wheat gluten proteins are responsible for the viscoelastic properties of the dough, which are important in bread making and preparing pasta and noodles (Shewry & Halford, 2002). The amino acid composition is an important indication of the nutritional value of cereal proteins and is determined by the relative concentrations of the nine essential amino acids: histidine, isoleucine, leucine, lysine, methionine/cysteine, phenylalanine/tyrosine, threonine, tryptophan, and valine. Cereal proteins are a valuable source of energy and contain all essential amino acids. However, they are generally low in lysine, making them less nutritionally complete compared to proteins derived from animal sources (Day et al., 2022; Shewry, 2009). The biological value of white wheat flour is estimated at 52, while in whole-grain wheat flour, it is higher by about 17–26%, estimated at 61–65. This difference reflects the higher contents of gluten proteins in white flour, which is rich in non-essential amino acids such as glutamine and proline, resulting in a lower biological value (Chick, 1942; Scherf et al., 2016).

Cereal lipids are nutritionally significant, with contents lying between approximately 1% to 10% (Garutti et al., 2022). Unsaturated fatty acids, particularly linoleic acid, are part of the lipid profile and these are fundamentally important for maintaining heart health (Dehghan et al., 2017; Stemler et al., 2025). Most lipids are concentrated in the germ and bran fractions, emphasizing the nutritional value of whole grains. Other key micronutrients found in whole grains include the B vitamins thiamine, riboflavin, and niacin and minerals like iron, magnesium, zinc, and selenium (Table 1). The micronutrients are mostly found in the bran and germ, which are often removed during refining. This process significantly reduces the nutritional value of refined cereals. Whole grains also provide bioactive compounds, such as phenolic acids, flavonoids, and lignans (Bach Knudsen et al., 2017; Hosseini & Mazza, 2009). These compounds exhibit strong antioxidant properties and contribute to the prevention of chronic diseases, including cancer, diabetes, and cardiovascular diseases (Adom & Liu, 2002; Slavin, 2004).

1.2 Acrylamide formation

Acrylamide (Figure 3) is a colorless and odorless crystalline powder with a melting point of 84.5 °C that is soluble in water, acetone, and ethanol (Baskar & Aiswarya,

2018; Smith et al., 1996). Acrylamide is formed in carbohydrate-rich foods during heating, e.g., baking, frying, toasting, or grilling. These foods mainly include raw materials made of cereals and potatoes which contain a high amount of free asparagine (precursor) and are produced at temperatures above 120 °C at low moisture (Tareke et al., 2000, 2002). The main pathway leading to acrylamide formation in food is the Maillard reaction (Mottram et al., 2002; Stadler et al., 2002; Zyzak et al., 2003). Although this reaction is responsible for the flavor and color formation during baking, toasting, or roasting of different food products (Mottram et al., 2002), the processing contaminant acrylamide is of concern due to its classification as “probably carcinogenic to humans” class 2A by the International Agency for Research on Cancer (IARC) (IARC, 1994). The Maillard reaction takes place when the amino group of asparagine and the carbonyl group of a reducing sugar react with each other forming the Schiff base, which is decarboxylated under heat.

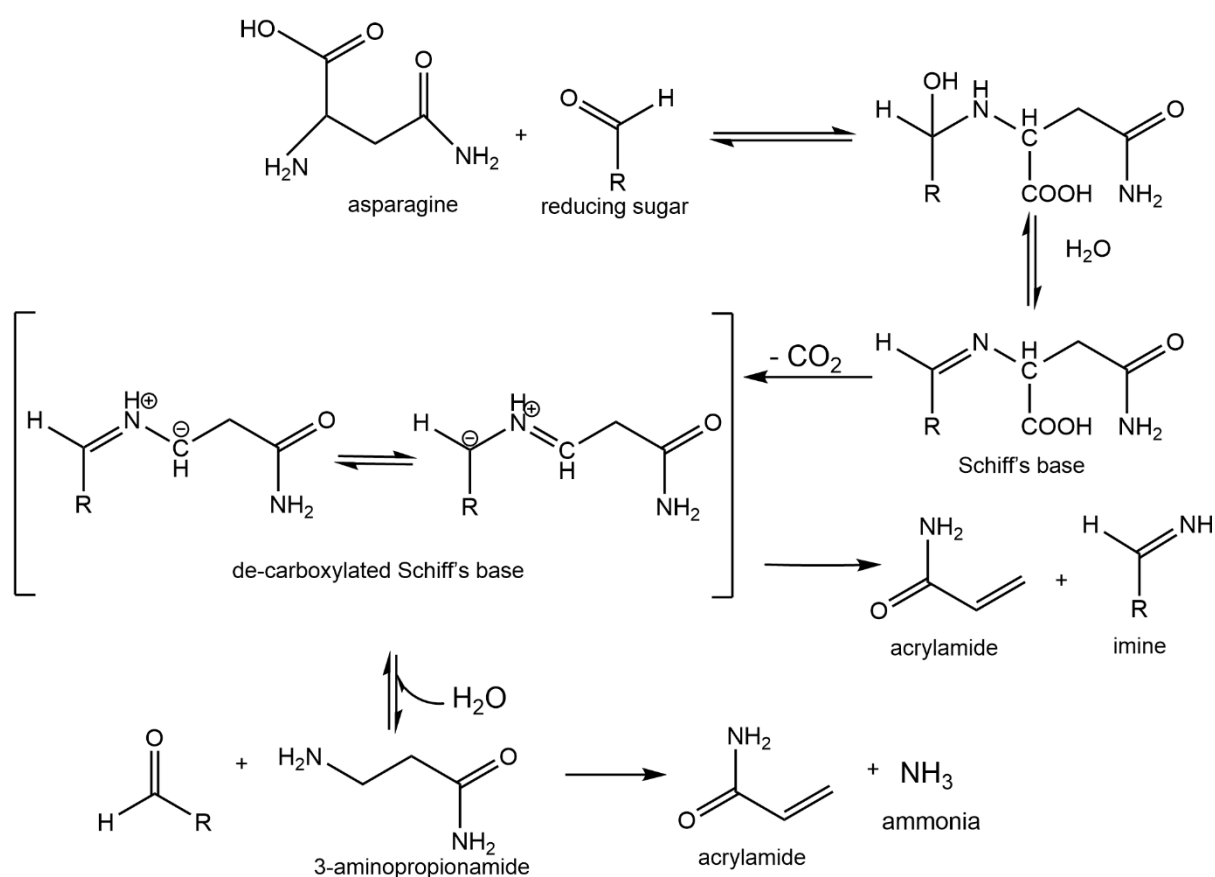


Figure 3. Mechanism of acrylamide formation via the Maillard reaction (adapted from Zyzak et al., 2003).

This reaction can form acrylamide via two ways; it either can decompose and form acrylamide by eliminating the imine or after hydrolysis via 3-aminopropionamide (3-APA) which can further degrade through ammonia elimination to produce acrylamide when subjected to heat (Zyzak et al., 2003). Previously, other pathways have also been described. The concept of oxidative decarboxylation, commonly referred to as Strecker degradation, is introduced as a process that removes the carboxyl group from asparagine and oxidizes it into a Strecker aldehyde. For acrylamide to form, the resulting aldehyde undergoes further reduction and dehydration (Mottram et al., 2002). Asparagine *N*-glycoside was identified as a precursor that forms during the initial reaction between a reducing sugar and an amino acid (Stadler et al., 2002). This compound generates acrylamide in significantly higher amounts compared to more stable Amadori products or a mixture of reducing sugars and asparagine.

The Maillard reaction and the subsequent formation of acrylamide depend on several factors. Key parameters include the type of reducing sugars and amino acids present, the pH of the environment, the thermal input, and other conditions. Keto sugars play a significant role in this process. For example, fructose is more efficient than aldehyde sugars, such as glucose, in generating acrylamide in model systems because of their higher reactivity in the Maillard reaction (Yaylayan & Stadler, 2005). Fructose is less thermally stable under low moisture conditions and its higher reactivity can be attributed to the lower melting point (approx. 126 °C), which facilitates quicker reaction with amines to form the Schiff base (Friedman & Mottram, 2005; Stadler et al., 2002). The amino acid concentration is also an indicator of acrylamide formation. For example, the increase of asparagine further increases the acrylamide content in potatoes, however, other amino acids including glycine, lysine, alanine, glutamine, and glutamic acid show lower formation of acrylamide (Rydberg et al., 2003). The levels of amino acids in raw materials are often correlated with acrylamide formation on the end product (Halford et al., 2012; Muttucumaru et al., 2014; Shepherd et al., 2010). However, this is not always the case as in a previous study, potatoes showed a rather weak correlation between free asparagine and acrylamide formation ($r = -0.32$) (Muttucumaru et al., 2015). The pH value is also an important factor in the Maillard reaction and the formation of acrylamide. The more acidic the environment of potato products, the more the formation of acrylamide is inhibited while an increased pH value promotes the formation of acrylamide in potatoes (Rydberg et al., 2003). Lowering the pH value causes the free, non-protonated amino groups of asparagine to become

protonated, which effectively inhibits the reaction that leads to acrylamide formation. Although the pH value plays a significant role in acrylamide formation, increasing the thermal input and time shows an increase in acrylamide, regardless of the pH value (De Vleeschouwer et al., 2006). In potato strips baked at 200 °C acrylamide increases up to 20 min time; afterward, it decreases due to the degradation of acrylamide (Rydberg et al., 2003). However, when the time is constant (15 min), but the temperature increases from 0 to 220 °C, acrylamide increases gradually. Water activity is crucial for the Maillard reaction as it affects reactant mobility and concentration, with optimal reaction rates occurring at intermediate water activity levels. Acrylamide formation and elimination are enhanced at lower water activity in the model system (Bassama et al., 2011). The role of water activity in acrylamide generation by using aqueous glycerol systems with varying water activity levels demonstrates that decreasing water activity enhances acrylamide formation (Hedegaard et al., 2007), highlighting the significance of low water activity environments in promoting the Maillard reaction and related pathways.

1.3 Health concerns

After the classification of acrylamide as a class 2A carcinogen by the International Agency for Research on Cancer in 1994, the primary concern of human exposure to acrylamide was for levels in drinking water or polymer food packaging (IARC, 1994). This was up to 2002, when acrylamide was found for the first time in food by a group of Swedish researchers who confirmed that acrylamide forms in carbohydrate-rich foods during high-temperature food processing (Tareke et al., 2002). The European Food Safety Authority reports a varying distribution of acrylamide in different foods (Table 2). Several studies link dietary acrylamide intake with carcinogenicity (Hogervorst et al., 2007, 2008; Hogervorst et al., 2009; Larsson, Åkesson, Bergkvist, et al., 2009; Larsson, Åkesson, & Wolk, 2009a; Mucci et al., 2006; Zhivagui et al., 2019) and neurotoxicity effects (Erkekoglu & Baydar, 2014; J. Kumar et al., 2018; Zamani et al., 2017).

Dietary acrylamide intake in men has been reported at an average of 36.1 µg per day, primarily coming from coffee (23%) and wholemeal bread (17%), with other contributions from products like crisp bread, white bread, and breakfast cereals (Larsson et al., 2009c). While some studies have suggested a link between dietary

acrylamide intake and an increased risk of colorectal cancer (Larsson et al., 2009c), others have found no such association (Mucci et al., 2006). The latter study, which adjusted for potential confounders, concluded that common acrylamide-containing foods like coffee, fried potatoes, and crisp bread were not associated with cancer risk.

Table 2. Acrylamide distribution in different food products (source: EFSA, 2015).

Food Category	n¹	LC²	Mean MB³	P95 MB⁴
Potato fried products	1694	14.3	308	971
Potato crisps and snacks	34501	0.2	389	932
Soft Bread	543	48.8	42	156
Breakfast cereals	1230	14	161	552
Biscuits, crackers, crisp bread and similar	2065	18	265	1048
Coffee (dry)	1458	3.5	522	1054
Coffee substitutes	88	6.8	1499	4500
Baby foods, other than cereal-based	416	66.8	24	72
Processed cereal-based baby foods	736	28	73	175

¹n: number of samples; ²LC: percentage of censored results, because the concentrations were below the limit of detection; ³MB: middle bound estimate; ⁴P95 MB: 95th percentile contamination level presented as the middle bound

Further studies have explored the potential links between acrylamide intake and other types of cancer. Larsson et al. (2009b) investigated the risk of epithelial ovarian cancer in Swedish women over an average follow-up period of 17.5 years, finding no relationship between dietary acrylamide intake (mean of 24.6 µg per day) and ovarian cancer risk. Conversely, Hogervorst et al. (2007) reported an increased risk of ovarian cancer in 300 cases over 11.3 years of follow-up. Similarly, no significant association has been found between acrylamide intake and breast cancer risk, even in a large cohort of 2952 cases (Larsson et al., 2009a). Some data show that the risk of cancer may differ in men and women. A study from Hogervorst et al. (2009) found no association between acrylamide intake and lung cancer risk in men, but an inverse association in women, particularly for adenocarcinoma. This points to the possibility that acrylamide might contribute to carcinogenesis through non-genotoxic mechanisms. Furthermore, research indicates a potential association between acrylamide intake and renal cell cancer risk (Hogervorst et al., 2008), though no significant relationships were found with bladder or prostate cancers, and an inverse

trend was observed for advanced prostate cancer in non-smokers. These conflicting results highlight the need for further studies to clarify the potential role of acrylamide in cancer risk. While some studies have provided insight into its possible effects on various cancer types, the underlying mechanisms and the full extent of its carcinogenic potential remain unclear.

The World Health Organization (WHO) has classified acrylamide as a well-established neurotoxin in humans, particularly at high exposure levels. It may cause damage to the nervous system, leading to symptoms such as muscle weakness, numbness, and impaired motor coordination. Although these effects are most apparent at occupational exposure levels or in animal studies, the WHO stresses the importance of minimizing acrylamide levels in food to reduce potential risks for the general population (WHO, 2005). Further research has highlighted the impact of acrylamide on cellular functions. Liu et al. (2015) demonstrated that acrylamide reduces cell viability, impairs mitochondrial function, and triggers mitochondria-driven apoptosis and proinflammatory responses in BV2 microglial cells. This disruption increases oxidative stress and disturbs the redox balance, although these effects were partially mitigated by *N*-acetyl-L-cysteine, which restored mitochondrial function and reduced inflammation. Additionally, acrylamide has been shown to interfere with cellular differentiation in human neuroblastoma and glioblastoma cells, further supporting its potential neurotoxic effects (Chen & Chou, 2015). More research is required to better understand the function of acrylamide and its impact on human health, particularly in neurotoxicity and carcinogenicity.

1.4 Regulatory approach

Council Regulation (EEC) No 315/93 provides a framework for addressing contaminants in food within the European Union, emphasizing that food containing unacceptable levels of contaminants that may pose a risk to human health must not be placed on the market (European Union, 1993). However, this regulation became relevant for acrylamide only after it was identified in food as a processing contaminant which raised significant public health concerns due to its genotoxic and carcinogenic properties (Tareke et al., 2002). After this finding, the European Commission (EC) released the first opinion regarding the presence of acrylamide in food (EC, 2002) and recommended to lower the exposure levels to humans according to the “as low as

reasonably achievable” (ALARA) principle. Further, the European Food Safety Authority (EFSA) statement of the Scientific Panel on Contaminants in the Food Chain (CONTAM) recommended several key actions including monitoring of acrylamide levels in food, risk assessment studies in order to evaluate the possible adverse health effects and mitigation measures (EFSA, 2005). This led to the monitoring of acrylamide levels in the European Union (EU) as outlined in Commission Recommendation 2007/331/EC, followed by Commission Recommendation 2010/307/EU (European Union, 2007, 2010). Further, the EFSA CONTAM panel (2015) highlights the presence of acrylamide as a process contaminant during high-temperature cooking and identified the highest acrylamide levels in coffee substitutes, coffee, and potato fried products with a dietary exposure ranging from 0.4 to 3.4 µg/kg of body weight per day. This opinion provided a comprehensive evaluation of the risks associated with acrylamide risks and emphasized the need for mitigation measures to reduce its levels in food. The EU has established mitigation measures and set benchmark levels for different food categories in the regulation (EU) 2017/2158 (European Union, 2017). The regulation established benchmark values (Table 3) for acrylamide levels in various food categories, such as potato products, coffee, and bakery goods, and outlines best practices for food producers to limit acrylamide formation during processing, including recommendations on temperature, time, and raw material selection. The regulation also requires EU member states to monitor acrylamide levels and report the results to ensure compliance and further reduce the health risks associated with acrylamide consumption.

Different products listed in Table 3 are associated with different benchmark values based on several factors such as food type and composition, cooking methods, and consumer exposure. Some main products include French fries and potato-based products with a benchmark value of 500 µg/kg and 750 µg/kg, respectively, followed by cereal-based products, including breakfast cereals (150–300 µg/kg), biscuits and crispbread (350 µg/kg), roast and instant coffee (400 µg/kg and 850 µg/kg), and baby foods (40 µg/kg). The benchmark values established in Regulation (EU) 2017/2158 are not legally binding threshold levels. Exceeding these benchmark values does not result in legal penalties but serves as an indicator for food producers to check or adapt their manufacturing processes. The purpose is to encourage producers to take appropriate action to reduce acrylamide levels in food to minimize consumer exposure. Although maximum levels are under discussion (Eagle, 2017), until now there are no

updates on the regulation by the European Commission. Due to ongoing efforts to implement Regulation (EU) 2017/2158, the European Commission developed the Acrylamide Toolbox (FoodDrinkEurope, 2019) which provides practical guidelines for food business operators to help them reduce acrylamide in food using different mitigation measures for different food categories. However, it must be noted that this toolbox complements existing regulations rather than imposing new legal requirements.

Table 3. Benchmark levels in different food categories for the presence of acrylamide established from the Commission Regulation (EU) 2017/2158 (source: European Union, 2017).

Food	Benchmark level (µg/kg)
French fries (ready-to-eat)	500
Potato crisps from fresh potatoes and from potato dough	750
Potato-based crackers	
Other potato products from potato dough	
Soft bread	
- wheat-based bread	50
- soft bread other than wheat-based bread	100
Breakfast cereals (excluding porridge)	
- bran products and whole grain cereals, gun puffed grain	300
- wheat and bran products ¹	300
- wheat, oat, spelt, barley, and rice-based products ¹	150
Biscuit and wafers	350
Crackers except for potato-based crackers	400
Crispbread	350
Gingerbread	800
Products similar to the other products in this category	300
Roast coffee	400
Instant (soluble) coffee	850
Baby foods, processed cereal-based foods for infants and young children excluding biscuits and rusks ²	40
Biscuits and rusk for infants and young children ²	150

¹Non-whole grain and/or non-bran-based cereals. The cereal present in the largest quantity determines the category; ²As defined in Regulation (EU) No 609/2013 (European Union, 2013).

The U.S. Food and Drug Administration (FDA) released a guidance document in 2016 that provides non-binding recommendations to food manufacturers on ways to reduce

acrylamide levels in food products. The document outlines strategies to minimize acrylamide formation, such as modifying cooking times and temperatures, choosing specific ingredients, and implementing processing techniques like enzyme treatment. The document does not establish legally enforceable requirements, and the term "should" indicates recommendations, not obligations (FDA, 2016). Also, the state of California has Proposition 65 which requires businesses to warn about significant exposures to chemicals like acrylamide, which is recognized as a potential carcinogen and reproductive toxin (Office of Environmental Health Hazard Assessment, 1986). In addition to regulations in the EU and recommendations by the FDA in the U.S., international organizations also provide information on acrylamide. The Joint FAO/WHO Expert Committee on Food Additives (JECFA), a collaboration between the Food and Agriculture Organization (FAO) and the World Health Organization (WHO), evaluates the effects of food contaminants. During its 2010 meeting, the Committee acknowledged the lack of definitive studies proving that acrylamide exposure increases cancer risk but emphasized the need to reduce acrylamide in food and dietary intake while advocating for further research on its potential health effects (FAO & WHO, 2011).

1.5 Mitigation of acrylamide

Acrylamide is present in different cereal products (Alsharafani et al., 2023; Amrein et al., 2004; Fu et al., 2018; Mesias et al., 2022; Mustafa et al., 2005), potato products (Dourado et al., 2020; Ismial et al., 2013; Mestdag, de Wilde, et al., 2008; Serpen & Gökmen, 2009), and coffee (Bertuzzi et al., 2020; Bortolomeazzi et al., 2012; Dybing, 2003; Granby & Fagt, 2004; Lachenmeier et al., 2018; Soares et al., 2015). As previously mentioned, due to adverse health effects that may come from acrylamide, its reduction is desired. The EFSA reported that potato-fried products are the biggest contributor to dietary acrylamide intake, followed by coffee, biscuits, crackers, and crispbread (Figure 4). Although coffee has the highest distribution as seen in Table 2, in Figure 4 it is listed second. Similarly, breakfast cereals were almost four times higher than soft bread in Table 2, but the dietary intake was 13% lower for breakfast cereals compared to soft bread.

The observed differences highlight how the contribution of food to dietary acrylamide intake depends not only on its acrylamide concentration but also on how frequently

and in what amounts it is consumed. Based on these reports by EFSA, the Acrylamide Toolbox, developed by FoodDrinkEurope, outlines several mitigation measures for the reduction of acrylamide in different cereal products, potato products, and coffee (FoodDrinkEurope, 2019). These measures focus on adjusting raw materials through agronomy, processing conditions, and formulations.

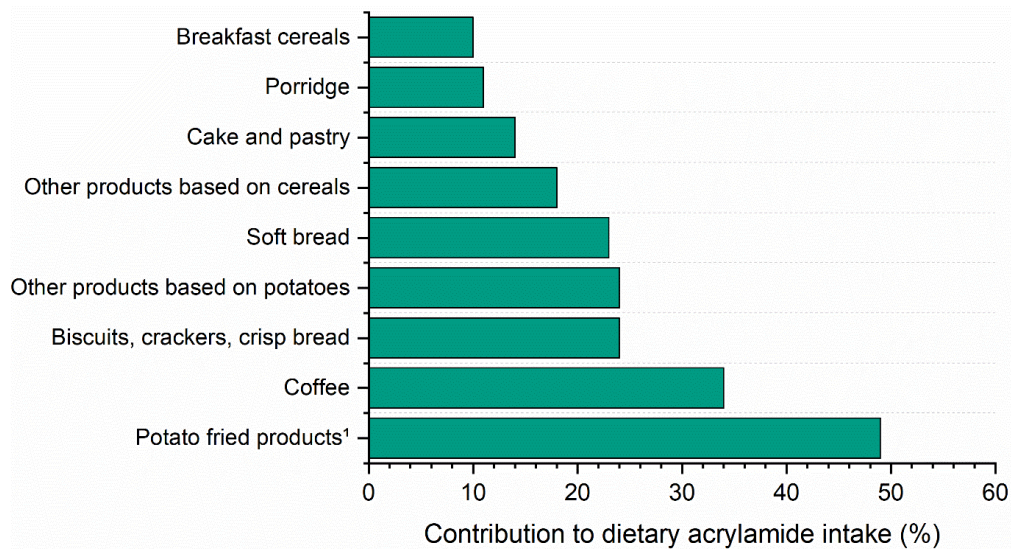


Figure 4. Contribution of different foods to dietary acrylamide intake (%) across adults, elderly, and very elderly derived from EFSA Panel on Contaminants in the Food Chain (EFSA, 2015).

¹ except potato crisps and snacks

1.5.1 Agronomy

Reducing acrylamide formation in food products requires an integrated approach targeting its key precursor, free asparagine. Various agronomic, storage, and genomic strategies have been developed to address this challenge (Curtis et al., 2010; Halford et al., 2012; Oddy et al., 2023; Wilson et al., 2020). The application of sulfur, for instance, has been shown to lower asparagine concentrations in grains and increase yield. However, this yield increase can dilute grain protein concentration, while environmental stressors like heat and drought may elevate free asparagine levels in grain (Wilson et al., 2020). Studies have also reported correlations between asparagine concentration and kernel characteristics such as diameter and weight, (Wilson et al., 2020), which is likely driven by their shared relationship with grain protein

concentration, rather than being a direct causal relationship (Navrotskyi et al., 2018). The acrylamide forming potential and free asparagine relationship was reported by Muttucumaru (2017) who found a higher correlation between acrylamide formation and free asparagine ($r = 0.671$) compared to reducing sugars ($r = 0.516$). Additionally, grains grown in sulfate-deprived soils exhibited higher asparagine content, resulting in higher acrylamide formation when heated (Muttucumaru et al., 2006). Storage conditions also play a significant role in acrylamide formation in the final product. Low-temperature storage of potatoes (e.g., 4 °C) significantly increases reducing sugar levels, enhancing acrylamide formation during processing; however, these storage conditions do not influence the asparagine content in the product (Olsson et al., 2004; Wilde et al., 2005).

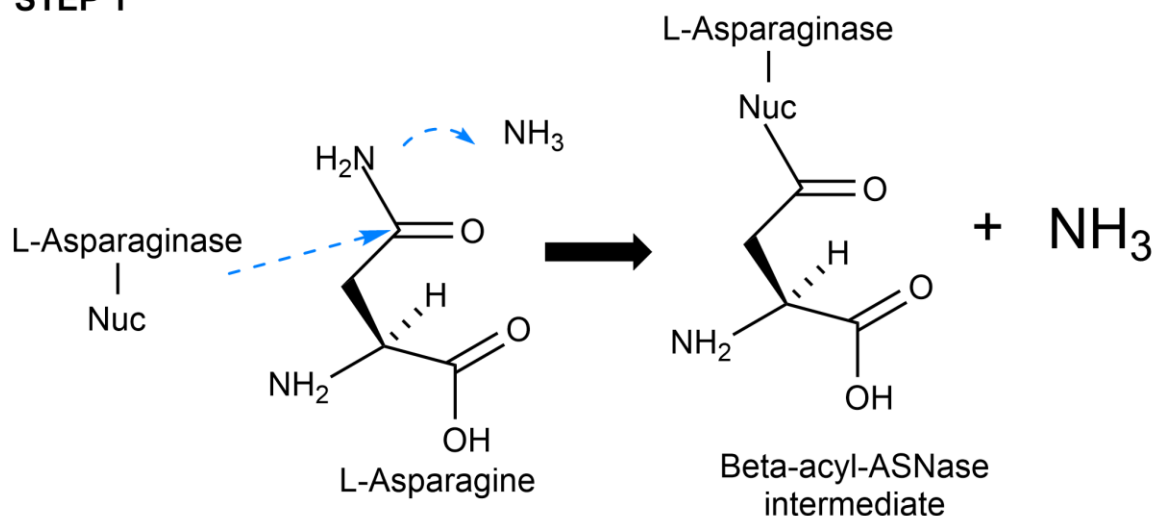
Genomic strategies have also gained attention as innovative solutions to reduce the accumulation of the acrylamide precursor, asparagine. Knocking out the asparagine synthetase gene (TaASN2) in wheat can reduce asparagine levels in grains by 50% without a significant impact on yield or quality while increasing concentrations of glutamate, free glutamine, and aspartate in all gene-edited lines (Raffan et al., 2021). Similar approaches in potatoes, such as silencing Asn1 and Asn2 reduced the asparagine content by 95% without affecting yield under glasshouse conditions (Rommens et al., 2008). Traditional breeding programs have also identified genetic variants naturally associated with lower asparagine levels (Curtis et al., 2009), and both environmental and genetic factors have been recognized as key determinants of asparagine concentration in grains (Halford et al., 2007). Therefore, reducing acrylamide formation in food products required an integrated approach combining agronomic practices, optimized storage conditions, and advanced genomic strategies. These interventions target the key precursor, free asparagine, and offer effective solutions to enhance food safety while maintaining product quality and yield.

1.5.2 Recipe and product design

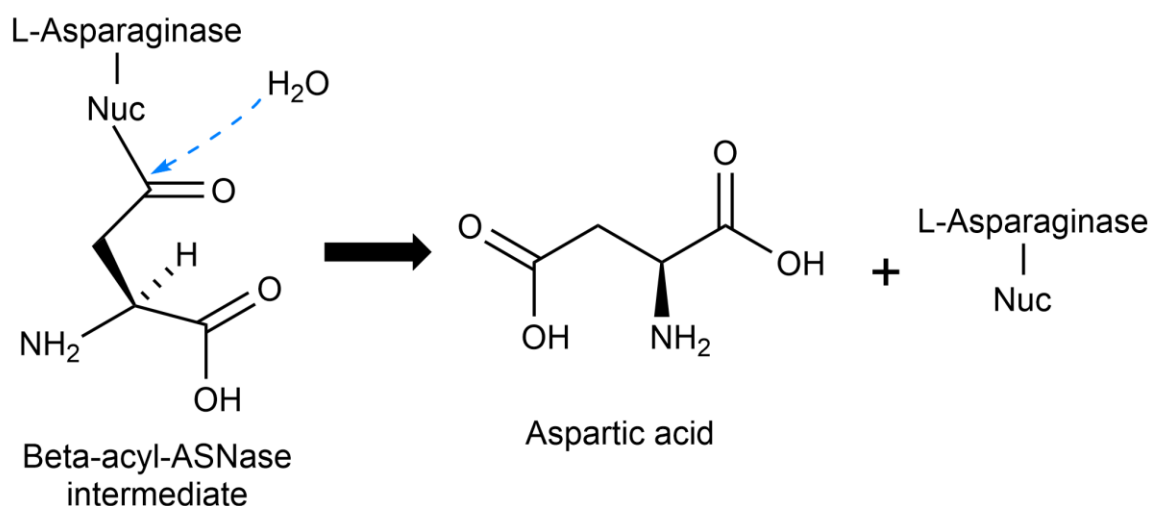
The use of asparaginases is also a novel technique that has been tested in a few studies. The hydrolysis of L-asparagine by asparaginase is done in two steps through a beta-acyl-ASNase intermediate as shown in Figure 5. Initially, the nucleophilic residue of the enzyme attacks the amide carbon atom of L-asparagine, forming the beta-acyl-ASNase intermediate. In the subsequent step, a water molecule activates a

nucleophile that targets the ester carbon atom of the intermediate, resulting in the formation of L-aspartic acid and the release of ammonia (Shakambari et al., 2019).

STEP 1



STEP 2



Global mechanism

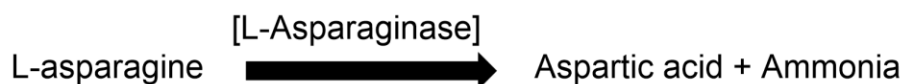


Figure 5. Mechanism of L-asparagine hydrolysis by asparaginase including the formation of the beta-acyl-ASNase intermediate and product release (adapted from Nunes et al. 2020).

Early studies demonstrated the potential of enzymatic treatments, such as using asparaginase, to reduce acrylamide levels significantly. For example, applying asparaginase to microwaved mashed potatoes achieved a 99% reduction of acrylamide and an 88% reduction of asparagine (Zyzak et al., 2003). Similarly, asparaginase reduced acrylamide by 85% in wheat crackers (Vass et al., 2004). The crucial parameters for optimal enzyme activation, including dosage, pH value, incubation temperature, and water content were identified only after a few years of research. *Aspergillus oryzae* derived asparaginase is most effective at pH 6–8 and 37 °C, with an optimal enzyme activity temperature of 60 °C at pH 7. This approach reduced acrylamide by 59% in French fries, 84% in semisweet biscuits, and up to 92% in crispbread (Hendriksen et al., 2009).

The effectiveness of asparaginase is influenced by the moisture content and water activity of the food matrix, which enhance the hydrolytic action of the enzyme on L-asparagine during cooking or baking (Gazi et al., 2023; Hendriksen et al., 2009). Selecting grain varieties with naturally lower asparagine levels and using refined grains instead of whole grains also effectively reduce acrylamide, though the latter approach compromises nutritional quality due to nutrient loss in the germ and bran as discussed earlier in subchapter 1.1 (Mesías et al., 2019; Žilić et al., 2020).

Recipe modifications offer another approach for acrylamide mitigation. Raising agents are widely used in different recipes; however, some of them may impact not only the reduction but they can also promote the formation of acrylamide. For example, the addition of ammonium hydrogen carbonate may increase the formation of acrylamide in cookies, while sodium hydrogen carbonate effectively reduces acrylamide by up to 52 % (Amrein et al., 2004; Kukurová et al., 2013). Ammonium hydrogen carbonate, which is a typical baking agent in gingerbread, promoted acrylamide formation in model systems (Biedermann & Grob, 2003). This was later proven in a gingerbread study where the increased amount of ammonium hydrogen carbonate further promoted acrylamide formation (Amrein et al., 2005). Calcium fortification is also an alternative to reduce the acrylamide content in bread doughs with higher concentrations yielding greater reductions (Sadd et al., 2008).

The application of different natural extracts and antioxidants is also recognized in food not only due to the positive health effects that are associated with antioxidants (Oroian & Escriche, 2015), but also the effect that they have on reducing acrylamide in different food products (Abedini et al., 2024). Green tea extract, for instance, reduced

acrylamide in fried potatoes by 62%, while cinnamon and oregano extracts achieved reductions of 3% and 17%, respectively (G. Morales et al., 2014). However, high concentrations of certain extracts, such as green tea or bamboo leaf antioxidants, can increase acrylamide formation (Zhang & Zhang, 2007). Rosemary extract is another plant extract that has gained attention due to its potential to reduce acrylamide in different food products (Hedegaard et al., 2008; Miśkiewicz et al., 2018; Sarion et al., 2021) and has also shown potential in reducing acrylamide by up to 62% in wheat buns, with further reductions observed in cookies at increased concentrations (Miśkiewicz et al., 2018). Some phenolic compounds may have no reducing effect, or even promote acrylamide formation. In a model study, ferulic acid showed an increase of acrylamide compared to the control, while no reducing effect was observed for coumaric acid, gallic acid, and caffeic acid (Bassama et al., 2010). Gallic acid was found to inhibit acrylamide elimination by scavenging free radicals, providing a new explanation for the increased acrylamide levels observed with certain polyphenols (Liu et al., 2015). However, another study reported that phenolic compounds including protocatechuic acid, ferulic acid, caffeic acid, and gallic acid reduce acrylamide in potato chips, with the highest reduction by up to 98% achieved by gallic acid. Caffeic acid as well as ellagic acid showed a reduction in acrylamide in both, model system and biscuits, respectively (Oral et al., 2014). The differences between different studies increase the need for further investigations of these phenolic acids in different food matrixes.

The choice of co-ingredients, cooking methods, and product shape and size also influence acrylamide formation. For example, carrot strips or potato flakes in bread can increase acrylamide levels depending on their quality and concentration (Swiacka et al., 2024b, 2024a). Likewise, pan-fried potatoes exhibit higher acrylamide levels compared to oven-baked French fries (Hackethal et al., 2023). This is not only related to the baking method but also the shape and size of the product, as the thicker the product the lower the formation of acrylamide (FoodDrinkEurope, 2019).

1.5.3 Processing

One of the focus areas in mitigating acrylamide during food processing has been optimizing thermal input. Gökmen et al. (2006) showed that the temperature and time of frying significantly impact acrylamide levels in potatoes. The increased frying

temperature further increased acrylamide as the moisture decreased due to extended frying time. They reported 40 times higher acrylamide formation in potatoes fried at 190 °C for 3 min compared to 150 °C for 9 min. Similarly, in a model system using wheat and potato starch, acrylamide levels increased as the temperature rose from 140 °C to 180 °C over a 20 min heat treatment. However, the presence of starch appeared to mitigate acrylamide formation to some extent (Ciesarová et al., 2006). These findings underscore the critical role of moisture in controlling acrylamide formation during heat processing (De Vleeschouwer et al., 2008; Pedreschi et al., 2007).

Acrylamide formation during processing is closely linked to the browning and color development resulting from the Maillard reaction. As foods are subjected to higher temperatures, the intensity of browning increases, which often correlates with higher acrylamide levels. Color is represented as lightness (L), redness (a), and yellowness (b). For instance, potatoes fried at 150 °C, 170 °C, and 190 °C exhibited darker color with increased temperature, therefore a linear correlation was observed for L- and a-values ($r^2 = 0.79$ and $r^2 = 0.83$, respectively) (Pedreschi et al., 2006). Similar trends have been observed in cookies, where higher acrylamide levels correspond to darker browning, regardless of the oven type used (Isleroglu et al., 2012).

Saccharomyces cerevisiae also known as baker's yeast, can also impact acrylamide in baked goods. This type of yeast may reduce acrylamide by 80% for a 60 min proof time (Hamlet et al., 2007). The combination of yeast with sourdough can further improve the reduction of acrylamide in whole wheat bread (Nasiri Esfahani et al., 2017). Certain lactic acid bacteria can help reduce acrylamide, such as those used in sourdough fermentation. Sourdoughs prepared with selected cultures demonstrated significant reductions in acrylamide content in bread, ranging from 29.5% (using *Pediococcus pentosaceus* and *Leuconostoc mesenteroides*) to 67.2% (using *P. pentosaceus* and *Lactobacillus curvatus*). These cultures show strong potential for use in mitigating acrylamide levels in bread production (Bartkiene et al., 2017).

2 Hypothesis and aims

As described above, acrylamide formation in cereal-based products poses significant health risks due to its potential carcinogenic properties. Although asparaginases offer a promising mitigation strategy in cereal-based products, we hypothesize that the effectiveness of asparaginases may differ depending on the type of grain used, such as wheat, oat, rye, rice, or corn. Additionally, combining asparaginases with phenolic acids or salts, and rosemary extract (alone) may influence mitigation outcomes differently across grain types. Factors such as the specific type of asparaginase, incubation temperature, and time are expected to affect not only the efficacy of acrylamide reduction but also product characteristics, including color, texture, and sensory attributes. Moreover, the efficacy of these strategies may vary between products such as cookies, crispbread, and breakfast cereals, as well as the type of asparaginases used and its concentration.

Due to these reasons, this work aims to

- (I) evaluate the effectiveness of four different asparaginases, gallic acid, ellagic acid, and rosemary extract in reducing acrylamide formation across various grains and
- (II) evaluate a combination of asparaginase with phenolic acids and salts in cookies, crispbread, and breakfast cereals.
- (III) assess the impact of the treatments by analyzing color and texture and examining potential sensory alterations in cookies.
- (IV) evaluate the transferability of asparaginase applications at a pilot plant scale, and
- (V) evaluate a cost-effective color measurement method for potential industrial application

3 Materials and methods

Asparaginases, phenolic acids, salts, and rosemary extract

Four different asparaginases were used in this study. Two asparaginases were obtained from Novonesis (Bagsvaerd, Denmark), Acrylaway L and Acrylaway HighT, respectively, and asparaginases PreventASe L and PreventASe XR were obtained from DSM (Heerlen, The Netherlands). For Acrylaway L and Acrylaway HighT, 100 and 200 mg/kg concentrations were evaluated, while for PreventASe L and PreventASe XR, 200 and 300 mg/kg were used for each. Phenolic acids tested included gallic acid and ellagic acid, and salts included calcium chloride and calcium carbonate. Each salt and acid were tested using three concentrations, 5 μmol , 50 μmol , and 100 μmol . Additionally, they were tested in combination with Acrylaway L at a concentration of 50 mg/kg. Rosemary extract was tested using concentrations of 0.01%, 0.1%, and 0.2%. Further details are available in the integrated publications (Musa et al., 2024a, 2024b, 2025a, 2025b).

Preparation of cookies, crispbread, and flakes on a laboratory scale

Cookies were prepared using wholemeal wheat, rye, oat, corn, or rice flour. For every 100 g of flour, 48 mL of water, 15.3 g of sugar, 12.6 mL of sunflower oil, and 0.33 g of baking powder were combined. Crispbread was prepared using 77 mL of water and 1.15 g of salt for 100 g of wholemeal rye flour. Wheat and oat flakes were rolled and mixed with 45 mL of water. Further details on preparation and baking conditions are available in the integrated publications (Musa et al., 2024a, 2024b, 2025a, 2025b).

Preparation of bran flakes on a pilot scale

Wheat bran flakes were prepared by incorporating Acrylaway HighT premix (0.5 g/kg or 1.0 g/kg) into the cooker after dosing the dry ingredients. The mixture was cooked for 95 min with saturated steam at 130 kPa, dried at 80 °C, and extruded below 100 °C. Thin flakes were formed using rollers, followed by roasting for < 1 min at 200–270 °C. Further details are available in the integrated publication (Musa et al., 2025b).

Incubation and baking conditions

Wheat cookie doughs were incubated using two different times (10 min and 30 min) and temperatures (60 °C and 90 °C) and baked in a range from 180 °C to 240 °C as outlined by Musa et al. (2024a). Rye, oat, rice, and corn cookies were all incubated at 60 °C or 70 °C for 10 min, respectively, and baked at 220 °C for 11 min (Musa et al., 2024a, 2024b). Crispbread samples that included asparaginase treatment were incubated at 60 °C for 10 min (Musa et al., 2025a). For treatments including phenolic acids or salts alone, and rosemary extract no incubation step was done before baking. Wheat and oat flakes were incubated and baked according to Musa et al. (2025b, unpublished). The baking conditions for each product remained the same regardless of the treatments. Further details for each product are available in the integrated publications (Musa et al., 2024a, 2024b, 2025a, 2025b).

Acrylamide analysis

The acrylamide content in cookies, crispbread, and flakes was quantified using an Acrylamide ELISA kit (Gold Standard Diagnostics, Kassel, Germany). Samples were extracted (2 g) and mixed for 30 min. The clean-up and the derivatization procedure were done according to the manufacturer's manual (Gold Standard Diagnostics, 2021). Acrylamide amounts were determined by measuring absorbance at 450 nm using a photometer (Tecan, i-control infinite 200Pro, Männedorf, Switzerland). Further details are available in the integrated publications (Musa et al., 2024a, 2024b, 2025a, 2025b).

pH value of dough

The dough (10 g) was mixed with 5 mL of acetone and 40 mL of distilled water (Begemann et al., 2016) and homogenized using an Ultra-Turrax (IKA T25, Staufen, Germany). After adding 60 mL of water, the pH value was measured with a pH meter (SI Analytics, LAB 845, Mainz, Germany).

Free asparagine content

The free asparagine content of flours was determined using two different methods. For wheat, rye, oat, corn, and rice flour the free asparagine content was determined according to Hölzle et al. (2023) with small modifications as described by Musa et al. (2024a). Second, for samples tested at the pilot plant, free asparagine was measured

according to Ciesarová et al. (Ciesarová et al., 2021, 2023). Further details are available in the integrated publications (Musa et al., 2024a, 2024b, 2025b).

Color analysis

The color of all baked products was analyzed using C-Cell (Calibre Control, UK) and Color Muse (Variable Inc, USA), which measure L* (lightness), a* (green-red), and b* (blue-yellow) values. C-Cell was calibrated with a calibration card (CC006, Calibre Control, UK), and Color Muse with its white background lid. Bran flakes for scale-up experiments were analyzed only with C-Cell, with six measurements per treatment. Further details are available in the integrated publications (Musa et al., 2024a, 2024b, 2025a, 2025b).

Texture analysis

The texture of cookies and crispbread was measured using a three-point bending rig (HDP/3 PB) and a heavy-duty platform (HDP/90) to break the cookies (American Institute of Baking, 2012). Flakes were analyzed using a texture analyzer (TA.XTplus, Stable Micro Systems, UK) equipped with a Kramer shear cell (A/KS5, Batch No. 17009) and the "Cereal bar shearing" method (CER1_KS5). Each container was filled with 30.0 ± 0.1 g of sample. Further details are available in the integrated publications (Musa et al., 2024a, 2024b, 2025a, 2025b).

Weight loss of flakes

The weight loss of samples was monitored by measuring their weight before and after incubation, as well as after baking, across all three batches (Musa et al., 2025b). Further details are available in the publication (Musa et al., 2025b).

Sensory analysis

To measure the impact of selected asparaginase treatments in wheat and rye cookies (Musa et al., 2024a), as well as asparaginase treatment in combination with calcium chloride and ellagic acid, and rosemary extract alone (Musa et al., 2025a) on sensory properties such as color, hardness, crunchiness, chewiness, taste, aroma, and overall

acceptability of cookies, a quantitative descriptive analysis was performed. The selected treatments are outlined in the following publications (Musa et al., 2024a, 2025a)

4 Results

4.1 Integrated publication I: Asparaginase Treatment to Mitigate Acrylamide Formation in Wheat and Rye Cookies

Authors: Shpresa Musa, Laura Becker, Claudia Oellig and Katharina Anne Scherf

Article: Asparaginase Treatment to Mitigate Acrylamide Formation in Wheat and Rye Cookies

Journal: LWT – Food Science and Technology, Vol. 203, 116365

DOI: <https://doi.org/10.1016/j.lwt.2024.116365>

Currently, the Commission Regulation in the EU 2017/2158 has benchmark levels for cookies of 350 µg/kg of acrylamide. It is recommended that the food industry should maintain these levels. This study hypothesized that acrylamide levels in cookies can vary depending on the baking conditions and the type of flour used. The aim was to systematically examine these factors, along with the effects of different asparaginases and dough incubation temperatures, and their effect on acrylamide reduction and the color, texture, and sensory properties of cookies. The acrylamide content showed an increase when the baking temperature was increased from 180 °C to 240 °C, although the baking time was reduced from 15 min to 9 min, respectively. The dough incubation time of 10 min vs 30 min showed no impact on acrylamide formation, regardless of baking temperature. However, the incubation temperature of 60 °C vs 90 °C, had a higher impact on acrylamide formation. The application of asparaginases showed a significant decrease in acrylamide in both, wheat and rye cookies, respectively. Rye flour had a higher asparagine content compared to wheat flour by 410 µg/kg, therefore, higher acrylamide formation was observed in rye cookies. Asparaginases exhibited higher reductions in rye cookies than in wheat cookies, reaching up to 86% reduction. The incubation temperature had no impact on acrylamide formation in wheat cookies. In contrast, control rye cookies exhibited higher acrylamide formation at an incubation temperature of 60 °C compared to 70 °C, which could be attributed to the distribution of acrylamide between the crust and crumb. We found a rather low correlation between color and acrylamide content in cookies, regardless of which color scanning device was used, C-Cell or Color Muse, respectively. The application of asparaginases showed minimal effects on color, texture, and sensory properties. These findings

highlight the variability of acrylamide formation in different cookie matrices and demonstrate the potential of asparaginases to mitigate acrylamide without significantly altering key quality attributes.

Authorship contribution:

Shpresa Musa: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Laura Becker:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Claudia Oellig:** Writing – review & editing, Supervision, Resources, Project administration, Conceptualization. **Katharina Anne Scherf:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

4.2 Integrated publication II: Influence of Asparaginase on Acrylamide Content, Color and Texture in Oat, Corn, and Rice Cookies

Authors: Shpresa Musa, Laura Becker, Claudia Oellig and Katharina Anne Scherf

Article: Influence of Asparaginase on Acrylamide Content, Color and Texture in Oat, Corn, and Rice Cookies

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To understand better the application of asparaginases in different cereal matrices such as wholemeal oat, corn, and rice cookies, four different asparaginases were applied in two different dosages and incubation temperatures. It was hypothesized that the asparaginase efficacy may show differences in the three flours, which is why cookies were prepared using the same recipe for each. Therefore, the study aimed to investigate the use of asparaginases to mitigate acrylamide in oat, corn, and rice cookies by examining different enzymes, dosages, and dough incubation temperatures. Additionally, the relationship between free asparagine and acrylamide content was evaluated, as well as any impact on the product quality such as color and texture. The study revealed that the asparaginases provide significant reductions of acrylamide in all cookies, regardless of the type of flour used. Reductions of up to 97%, 95%, and 92% were achieved for oat, corn, and rice cookies, respectively. Acrylaway L asparaginase showed that an increased concentration from 100 to 200 mg/kg further decreased acrylamide. However, the other three asparaginases used provided similar reductions regardless of their concentration. The amount of free asparagine in flour was positively correlated with acrylamide formation in cookies ($r = 0.876$). Asparaginase treatment in corn cookies showed a slight decrease in L^* -value, which was statistically significant for most treated samples. However, this was not the case for oat and rice cookies. Also, selected treatments showed small changes in b^* - and a^* -value. A minimal impact on texture was observed. Although these changes were statistically significant, visually they were not evident. Therefore, since reductions of acrylamide are needed to meet the regulatory requirements, application of asparaginases is recommended.

Authorship contribution:

Shpresa Musa: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Laura Becker:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Claudia Oellig:** Writing – review & editing, Supervision, Resources, Project administration, Conceptualization. **Katharina Anne Scherf:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

4.3 Integrated publication III: Mitigation of Acrylamide in Cookies and Crispbread Using Calcium Salts and Phenolic Acids in Combination with Asparaginase as well as Rosemary Extract

Authors: Shpresa Musa, Claudia Oellig and Katharina Anne Scherf

Article: Mitigation of Acrylamide in Cookies and Crispbread Using Calcium Salts, Phenolic Acids, and Rosemary Extract in Combination with Asparaginases

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Acrylamide is formed during high-temperature treatment in foods and presents a significant health and regulatory challenge. This study evaluates the effects of calcium salts and phenolic acids alone or in combination with asparaginase and rosemary extract in wheat cookies and rye crispbread. Acrylamide content, product color, texture, and sensory properties were assessed. When calcium salts and phenolic acids were used alone, acrylamide was reduced by 46 % and 50 % compared to the control. A combination of these with asparaginase resulted in a reduction of acrylamide by up to 89 % using ellagic acid. Specific treatments reduced cookie hardness, but asparaginase addition reversed this effect. Color mainly remained unaffected. Sensory analysis of selected treatments confirmed no significant changes in cookie aroma, taste, color, texture, and acceptability. This work provides a new approach by combining selected treatments to mitigate acrylamide while preserving product quality.

Authorship contribution:

Shpresa Musa: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Claudia Oellig:** Writing – review & editing, Supervision, Resources, Project administration, Conceptualization. **Katharina Anne Scherf:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

4.4 Integrated publication IV: Asparaginase Treatment for Acrylamide Reduction in Wheat and Oat Flakes: From Laboratory- to Pilot-Scale

Authors: Shpresa Musa, Kristína Kukurová, Zuzana Ciesarová, Karsten Eisenhardt and Katharina Anne Scherf

Article: Asparaginase Treatment for Acrylamide Reduction in Wheat and Oat Flakes: From Laboratory- to Pilot-Scale

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Acrylamide is a contaminant in food that is produced during high-temperature processing. There is a growing need for mitigation strategies due to health concerns and regulatory guidelines. This study aims to evaluate the effectiveness of four commercial asparaginases in the reduction of acrylamide in wheat and oat flakes, as well as their impact on product quality. Additionally, scale-up experiments were conducted with one of the asparaginases to evaluate the transferability of laboratory-scale results into pilot-scale production for bran flakes. On a laboratory-scale, asparaginase treatment resulted in a higher reduction of acrylamide in oat flakes by 928 µg/kg using PreventASe XR, compared to the wheat flakes, which reduced acrylamide by 360 µg/kg using PreventASe L. These represent a reduction of up to 77 % and 58 %, for oat and wheat flakes, respectively, compared to the control. This is the first study to compare multiple commercial asparaginases across two whole grain matrices in flakes, providing a direct side-by-side evaluation of their performance. Moreover, we demonstrate the challenges of translating enzymatic mitigation from lab to pilot-scale, where acrylamide dropped by only 76 µg/kg, underlining key practical barriers in industrial applications. Minimal impacts on color and texture were observed. Our results contribute new insights into enzyme-based acrylamide reduction and point to the importance of optimizing processing conditions for the application of asparaginases on an industrial scale, benefiting both producers and consumers.

Authorship contribution:

Shpresa Musa: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Kristína Kukurová:** Methodology, Formal analysis, Writing – review & editing. **Zuzana Ciesarová:** Methodology, Formal

analysis, Writing – review & editing. **Karsten Eisenhardt:** Investigation, Resources, Writing – review & editing. **Katharina Anne Scherf:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

5 Discussion

The reduction of acrylamide in cereal-based products is essential due to its potential adverse health effects when consumed at high dietary levels. Previous studies have demonstrated that asparaginases are a promising solution for mitigating acrylamide formation in various food products (CarolinaVieira-Porto et al., 2024; Gökmen, 2016; Halford & Curtis, 2019; Pedreschi et al., 2011). Additionally, other strategies, such as the incorporation of phenolic acids, salts, and plant extracts, have been investigated for their acrylamide-reducing potential (Göncüoğlu Taş et al., 2023; Heydari Ashkezari & Salehifar, 2019; Mekawi et al., 2019; Mildner-Szkudlarz et al., 2019; Moreau et al., 2009; Tayefe et al., 2025). Despite these advancements, further research is needed to assess the effectiveness of these strategies in different cereal-based products and explore potential synergies between them. Therefore, this discussion will focus on the application of asparaginases in cookies, crispbread, and breakfast cereals, as well as the combined use of multiple mitigation strategies to further reduce acrylamide levels and their impact on product quality.

5.1 Acrylamide reduction in different cereal-based products

Although previous studies investigated the application of asparaginases for acrylamide reduction in different cereal-based products, there is still a lack of information on how the asparaginases behave when a different type of flour, such as wholemeal wheat, rye, oat, corn, and rice flour is used. The hypothesis was that asparaginases applied in different raw materials under the same conditions may have different efficacy in reducing acrylamide. Therefore, the research focused on applying the same four asparaginases using different concentrations in cookies made of the five mentioned flours using the same recipe to assess the impact on acrylamide reduction and product quality. Initial investigations in the integrated publication I (Musa et al., 2024a) were conducted to evaluate the impact of dough incubation temperature and time on acrylamide formation in wheat cookies when samples were baked at four different temperatures, 180 °C, 200 °C, 220 °C, and 240°C, for 15 min, 13 min, 11 min, and 9 min, respectively. This is due to the fact that incubation is necessary in order to activate the enzymes applied in the dough (Hendriksen et al., 2009). However, its impact alone on acrylamide formation should be known. The results showed that the incubation time

of 10 min vs 30 min does not affect acrylamide formation regardless of the baking temperature used. However, the incubation temperature of 60 °C vs 90 °C had a significant effect on acrylamide in wheat cookies. For baking temperatures of 180 °C and 240 °C, increasing the incubation temperature from 60 °C to 90 °C showed an increase in acrylamide formation by 62 µg/kg (180 °C) and 88 µg/kg (240 °C), respectively. Although this increase was significant, differences in acrylamide content within the same batch were also observed across all samples of cookies, crispbread and flakes. This is related to the higher formation of acrylamide in the crust of the product rather than the crumb, resulting in potential issues of homogeneity (Liu et al., 2018). Rydberg et al. (2003) reported that increasing the exposure time to heat further increases acrylamide formation. However, in the present study (Musa et al., 2024a), although an increase of acrylamide was observed with higher baking temperatures, it was not as pronounced. This is because the baking time was reduced, while the baking temperature was increased, thereby reducing the impact of prolonged heat exposure on acrylamide formation. This highlights the complex interplay between time-temperature conditions during baking and acrylamide formation. Future work could investigate whether there is an optimal balance where both acrylamide formation and quality attributes such as texture and color are optimized. Additionally, assessing the acrylamide distribution across different cookie layers could provide further insights into localized mitigation effects, because the impact of baking temperature is mainly on the surface of the product. When using extended frying time, the temperature mainly rises on the surface of the product rather than on the inside, due to a higher amount of water evaporation (Gökmen et al., 2006), which leads to lower formation of acrylamide in the crumb (Swiacka et al., 2024a). This suggests that mitigation strategies should focus on surface modifications, such as adjusting baking conditions, applying protective coatings (Al-Asmar et al., 2018), or using antioxidant-rich ingredients on surface layers to minimize acrylamide formation.

Further experiments were conducted using only two incubation temperatures, 60 °C and 70 °C. The results presented for cookies made of five different flours (Musa et al., 2024a, 2024b) as shown in Table 4 showed that asparaginase efficacy differs across different cookies. Oat cookies showed the highest reduction of acrylamide by up to 97%, followed by corn, rice and rye cookies, with 95%, 92%, and 89%, respectively.

Table 4. Samples with the highest acrylamide reduction including cookies, crispbread, and flakes produced at laboratory-scale and bran flakes produced a pilot-scale.

Study	Product	Mitigation Strategy	Treatment concentration	Acryla- mide Re- duction
Integrated publica- tion I (Musa et al., 2024a)	Wheat cookies	Asparaginase	PreventASe XR 200 mg/kg	79%
	Rye cookies	Asparaginase	PreventASe XR 200 mg/kg	86%
Integrated publica- tion II (Musa et al., 2024b)	Oat cookies	Asparaginase	Acrylaway HighT 100 mg/kg	97%
	Corn cookies	Asparaginase	PreventASe L 200 mg/kg	95%
	Rice cookies	Asparaginase	PreventASe XR 200 mg/kg	92%
Integrated publica- tion III (Musa et al., 2025a)	Wheat cookies	Calcium chloride	CaCl ₂ 100 µmol	46%
		Calcium chloride + Asparaginase	CaCl ₂ 100 µmol + Acrylaway L 50 mg/kg	65%
		Calcium carbonate	CaCO ₃ 100 µmol	41%
		Calcium carbonate + Asparagi- nase	CaCO ₃ 100 µmol + Acrylaway L 50 mg/kg	69%
	Rye Crisp- bread	Gallic acid	Gallic acid 100 µmol	33%
		Gallic acid + Asparaginase	Gallic acid 100 µmol + Acrylaway L 50 mg/kg	65%
		Ellagic acid	Ellagic acid 100 µmol	50%
		Ellagic acid + Asparaginase	Ellagic acid 100 µmol + Acrylaway L 50 mg/kg	89%
		Rosemary extract	Rosemary extract 0.2%	50%
		Calcium chloride + Asparaginase	CaCl ₂ 100 µmol + Acrylaway L 50 mg/kg	37%
Integrated publica- tion IV (Musa et al., 2025b)	Wheat flakes	Asparaginase	PreventASe L 200 mg/kg	58%
	Oat flakes	Asparaginase	PreventASe L 200 mg/kg & XR 300 mg/kg	77%
	Bran flakes	Asparaginase	Acrylaway HighT 1.0 g/kg	9%

On the other hand, the lowest reduction was observed for wheat cookies with a maximum reduction of 79%. The high reduction in oat cookies may be attributed to differences in flour composition, such as fiber, protein, or polyphenol content, which might enhance or stabilize enzyme function. Further investigations into the physicochemical properties of these flours could help determine the key factors driving these differences. Depending on the process to which the asparaginase is applied, the acrylamide reduction may differ. For example, the application of 2000 ASNU [one ASNU/g is defined as the amount of enzyme that produces 1 μ mol of ammonia per minute after hydrolysis of L-asparagine (Lambré et al., 2023)] of asparaginase on rotary-cut biscuit dough, rotary molded biscuit dough, and wire-cut cookie dough, resulted in 65%, 6%, and 0% acrylamide reduction, respectively (Gazi et al., 2023). This may be due to the influence of processing methods on enzyme efficacy, suggesting that dough characteristics such as hydration, sugar content, and fat composition may play a crucial role for asparaginase performance.

The asparaginase efficacy also is dependent on incubation time. Increasing the incubation time from 15 min to 30 min and 60 min improved the reduction of the precursor asparagine in the dough, which will later result in a lower amount of acrylamide during baking (Kukurová et al., 2013). However, while longer incubation times appear beneficial, it remains unclear whether there is a point beyond which further incubation does not yield additional reductions. Differences in asparaginase efficacy in different cereals were also observed in other studies. A study from Ciesarová et al. (2023) showed that the acrylamide content is increased when sea buckthorn pomace powder is added to wheat, triticale, or rye cookies. However, the addition of sea buckthorn pomace powder which was enzymatically treated with Acrylaway L significantly reduced acrylamide. In agreement with our study (Musa et al., 2024a, 2024b), the reduction varied between different cereals, which emphasizes the variation of its efficacy in different cereal matrices. In the study by Ciesarová et al. (2023) the formation of acrylamide but also the reduction of acrylamide was also higher in rye cookies compared to wheat cookies, which is in agreement with the results presented in the integrated publication I (Musa et al., 2024a).

The concentration of asparaginases is one of the main factors in mitigating acrylamide formation (Anese, Quarta, & Frias, 2011). An increased enzyme dosage in biscuits showed a further reduction in acrylamide (Hendriksen et al., 2009). In the integrated publications I and II (Musa et al., 2024a, 2024b) only asparaginases R and H showed

such a pattern, while the other two asparaginases A and X, respectively, showed mainly no effect of asparaginase concentration. A study on potato slices also reported that increasing the enzyme concentration does not always result in a higher reduction of acrylamide (Hendriksen et al., 2009). This further proves that enzyme dosage and acrylamide reductions depend on the type of raw material, asparaginase, and product where it is applied as there are different results across different studies. Therefore, it is recommended to test a wider range of concentrations in the future to assess its impact on acrylamide reduction and determine the optimal dosage. Additionally, research should focus on developing predictive models to determine optimal enzyme conditions based on specific product formulations. Such models could help food manufacturers to implement asparaginase-based strategies more effectively, ensuring both acrylamide reduction and product quality.

Breakfast cereals are also widely consumed and known for their nutritional and health benefits (Williams et al., 2014). However, due to unfavorable high-temperature processing conditions and added fiber or sweeteners during processing, they are prone to the formation of acrylamide. The integrated publication IV investigated the application of asparaginases in wheat and oat flakes. The treatment resulted in significant reductions of acrylamide content, regardless of the asparaginase concentration. Notably, higher reductions were achieved in oat flakes compared to wheat flakes (Table 4), reaching up to 77% and 58%, respectively (Musa et al., 2025b). Currently, the Commission Regulation (EU) 2017/5178 (European Union, 2017) has established benchmark levels for breakfast cereals of 300 µg/kg. In a survey in Spain, the acrylamide content in breakfast cereals ranged from 62–803 µg/kg (Rufián-Henares et al., 2006), with the maximum amount exceeding the benchmark level more than double. These findings highlight the importance of achieving significant reductions in acrylamide to meet regulatory standards. Dietary fiber is often added to breakfast cereals to enhance their nutritional profile. The survey found that products with no dietary fiber added contained less acrylamide (273 µg/kg) than those with added dietary fiber (401 µg/kg). In the integrated publication IV, wheat and oat flakes were produced without the removal of the bran, which contributed to a higher formation of acrylamide (Musa et al., 2025b). However, in general, flakes contain a lower acrylamide content compared to puffed products. This difference is attributed to the preparation process, as puffed cereals undergo higher production temperatures which leads to higher acrylamide formation (Mesías et al., 2019). Bran flakes produced on

pilot-scale showed lower reductions compared to the laboratory-scale with a maximum reduction of 9% as presented in integrated publication IV (Table 4). These results highlight that while asparaginases have proven to be effective in laboratory-scale studies and model systems, further optimization is essential for larger-scale production to achieve the desired reductions. Specifically, improving temperature control during the enzyme incubation process may ensure that the enzyme has sufficient time to be activated and achieve optimal results. Breakfast cereals produced in the industry often contain other co-ingredients, with the most common being sweeteners. A strong positive relationship between added sugars and acrylamide formation in breakfast cereals has been reported. An increase in sugar content further increased hydroxymethylfurfural, which is also an intermediate in the Maillard reaction formed during the dehydration of sugars (Morales et al., 2020). The formation rate of hydroxymethylfurfural is influenced by various factors, particularly the type of sugar involved. Glucose, fructose, and sucrose serve as precursors for hydroxymethylfurfural formation (Capuano & Fogliano, 2011). According to Gökmen et al. (2007), replacing glucose or fructose with sucrose in baked goods results in increased hydroxymethylfurfural levels. A study that used sucrose instead of inverted sugar syrup in sweet biscuits resulted in a significant decrease in acrylamide by 70%. Therefore, co-ingredients play a significant role, and a combination of different strategies is advised to achieve desirable results.

Although asparaginases alone in concentrations of 100, 200, and 300 mg/kg are effective in reducing the acrylamide content in cookies and breakfast cereals, further evaluation of phenolic acids, salts, and rosemary extract alone or in combination with asparaginase using a low concentration of 50 mg/kg asparaginase was investigated. The integrated publication III presented the results of cookies when phenolic acids and calcium salts were used alone or in combination with asparaginase, and the addition of rosemary extract (Musa et al., 2025a). One of the strategies explored in this study to reduce acrylamide levels in baked products was the use of calcium salts, which are commonly employed in flour fortification (BAKERpedia, 2021). In addition to enhancing the nutritional value of flour by increasing dietary calcium intake, calcium salts play a crucial role in acrylamide mitigation. The cation inhibits the formation of the Schiff base during the Maillard reaction, thus preventing the key intermediate responsible for acrylamide formation in the final product (Gökmen & Şenyuva, 2007). Previous studies have shown the potential of salts to reduce acrylamide formation in food products. A

study reported up to 39% reduction of acrylamide in potato slices when they were bleached in a sodium chloride solution (Pedreschi et al., 2010). Increasing the concentration of sodium chloride in a model system from 1% to 10% further decreased acrylamide from 181 µg/g to 160 µg/g, respectively (Kolek et al., 2006). The integrated publication III presents the effect of salt concentration on cookies and crispbread, where the increase in salt concentration further decreased the acrylamide content in the final product (Musa et al., 2025a). For example, in crispbread, the combination of asparaginase with 100 µmol of calcium chloride resulted in a 37% reduction of acrylamide, but when asparaginase was combined with 50 µmol of calcium chloride, only a 10% reduction of acrylamide was observed (Musa et al., 2025a). The addition of different salts has also been shown to reduce acrylamide in the wheat flour dough system, where calcium chloride was more effective than calcium lactate in reducing acrylamide (Göncüoğlu Taş et al., 2023). Calcium carbonate achieved significant reductions when applied to flatbread (Alsharafani et al., 2023), but in the dough model system (Levine & Ryan, 2009) and bread doughs (Sadd et al., 2008) the effect of calcium carbonate on acrylamide reduction was rather low. In the current study, calcium chloride proved to be more effective than calcium carbonate, achieving a maximum reduction of 46% compared to 41% when used alone (Musa et al., 2025a). Calcium chloride achieved up to 90% reductions in the cereal model system (Kukurová et al., 2009). When combined with asparaginase, calcium carbonate resulted in a greater reduction (69%) than calcium chloride (65%) (Musa et al., 2025a).

Food waste extracts have been explored as a pre-treatment to reduce acrylamide formation in potato chips. Soaking potato slices in extracts from potato peels, olive leaves, lemon peels, and pomegranate peels before frying significantly decreased reducing sugars and asparagine content. Lemon and potato peels, which exhibited strong antioxidant activity, were particularly effective, reducing acrylamide levels by 86.1% and 69.7%, respectively (Mohdaly et al., 2022). Further, in the integrated publication III, the application of phenolic acids like gallic acid and ellagic acid was investigated with or without combination with asparaginase. In cookies, gallic acid (100 µmol) in combination with asparaginase achieved the highest reduction of acrylamide by up to 85%, while ellagic acid (100 µmol) in combination with asparaginase achieved higher reductions in cookies compared to crispbread, with up to 89% and 50%, respectively (Musa et al., 2025a). These differences may be attributed to variations in recipes, baking times, and temperatures. Additionally, the lower moisture content in

crispbread could inhibit asparaginase activity, as discussed earlier. Although several studies on the impact of phenolic acids or asparaginases alone are available, to our knowledge this is the first evidence of combining these two mitigation strategies in cereal-based products. Furthermore, when ellagic acid and gallic acid concentrations increased, the acrylamide concentration further decreased in both cookies and crispbread. With 100 μmol as the highest concentration tested for gallic acid and ellagic acid, they achieved 33% and 50% reductions of acrylamide in cookies, respectively (Musa et al., 2025a). Different phenolic compounds have shown differences in acrylamide reduction across different studies. In an emulsion system where ferulic acid, caffeic acid, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, a water-soluble analog of vitamin E), gallic acid, and protocatechuic acid were tested, each achieved significant reductions, and depending on the heating time, different reductions were observed (Kotsiou et al., 2011). Additionally, Kotsiou et al. (2011) showed that the maximum reductions were observed at a heating time of 20 min, with gallic acid specifically achieving 70% reductions. The application of ellagic acid in biscuits reduced acrylamide by up to 19%, while in the model system containing asparagine-fructose acrylamide was reduced by 69% (Oral et al., 2014). Another study, which focused on a variety of polyphenols from plant extracts and their addition in cookies, reported significant acrylamide reductions in cookies, with the highest reductions coming from chokeberry (94%) and elderberry (91%) addition (Borczak et al., 2022). Integrated publication III also investigated the addition of rosemary extract in cookie and crispbread dough using three different concentrations, 0.01%, 0.1%, and 0.2%. The reductions were higher in cookies compared to crispbread. In cookies reductions up to 24%, 42%, and 50% were achieved for concentrations of 0.01%, 0.1%, and 0.2% of rosemary extract, respectively. In crispbread, the highest reduction was up to 10% for the 0.2% rosemary extract (Musa et al., 2025a). Rosemary extract used in potato fries has also shown significant acrylamide reductions when fried in olive oil with 4200 ng/g acrylamide compared to the control (5600 ng/g). However, the addition of rosemary herb in corn oil showed an increase of acrylamide from 1900 ng/g in the control sample to 2500 ng/g acrylamide with rosemary addition (Becalski et al., 2003). In wheat buns, rosemary extract showed a significant reduction with 1% of the extract, but increasing the concentration to 10% did not further improve the reduction of acrylamide (Hedegaard et al., 2008). In our study, higher amounts of rosemary extract (i.e., more than 0.2%) were not tested due to possible changes in the color and possibly

sensory attributes of the products. Similarly, Miśkiewicz et al. (2018) observed a greater reduction of acrylamide levels with increasing concentrations (0.1% to 0.5%) of rosemary extract in shortcrust cookies. The efficacy of the extract varied depending on the baking conditions, with 90% humidity enhancing acrylamide reduction compared to dry air. In our study, while rosemary extract reduced acrylamide in both cookies and crispbread, the reduction in crispbread was relatively low and statistically insignificant. Therefore, higher concentrations of rosemary extract are recommended for crispbread to improve its effectiveness. The phenolic antioxidants which are naturally present in plant extracts, can react with carbonyl compounds through electrophilic aromatic substitution in the Maillard reaction, forming adducts that help limit acrylamide formation (Constantinou & Koutsidis, 2016). Wang and Zhou (2004) suggest that tea catechins interact with wheat proteins, forming cross-links by breaking disulfide bonds; however, although tea catechins indirectly inhibit acrylamide formation, their exact role in the Maillard reaction remains unclear.

5.2 Correlation between free asparagine content in the raw materials and acrylamide content in the product

Another key focus of this study was to investigate the role of free asparagine in acrylamide formation, as it is the primary precursor in the Maillard reaction when reacting with reducing sugars. The free asparagine content of all wholemeal flours used in this study was evaluated, with concentrations of 700 mg/kg in rye, 500 mg/kg in oat, 290 mg/kg in wheat, 220 mg/kg in corn, and 90 mg/kg in rice flour (Musa et al., 2024a, 2024b). Studies have shown that the amount of the precursor asparagine in the grain, and acrylamide formation are significantly correlated (Raffan & Halford, 2019). However, this is not always the case in baked goods. Several factors could influence this discrepancy, such as the influence of other ingredients, baking time, and temperature, which may modulate the Maillard reaction in complex ways. Žilić et al. (2020) showed that while acrylamide formation in biscuits does not always directly correspond to free asparagine levels in flour, cereals with higher free asparagine tend to generate more acrylamide. Hulless oat, durum wheat, and rye flours, which had the highest free asparagine content, produced the most acrylamide after baking and refined bread wheat and wholegrain red maize, with the lowest free asparagine, had the least acrylamide (Žilić et al., 2020). This suggests that selecting cereal flours with low free asparagine can help mitigate acrylamide formation, especially when combined

with lowered baking temperatures and prolonged baking times. Furthermore, the genetic factors influencing free asparagine content in cereals could be considered for breeding programs aimed at reducing acrylamide levels in the final products. Žilić et al. (2020) further concluded that depending on the genotypes of small grains (including bread wheat, durum wheat, soft wheat, hard wheat, triticale, rye, hulless oat, and hulless barely) and maize, the acrylamide formation potential differs. Another study investigated how fermentation time and added asparagine affect acrylamide formation in yeast-leavened wheat bread based on two experiments: one with low asparagine levels (0–0.044 g/100 g flour) and another with high asparagine levels (0.071–0.476 g/100 g flour) (Mustafa et al., 2009). Fermentation time (13–164 min) significantly reduced acrylamide precursors in the dough, especially at low asparagine levels. This was also observed in another study where a longer dough fermentation time significantly reduced acrylamide levels in bread (Longin, 2024). This suggests that optimizing fermentation parameters, alongside ingredient selection, could be a useful tool for reducing acrylamide levels in cereal-based products. Added asparagine increased both dough asparagine and acrylamide levels in bread and crispbread, showing a strong correlation between them (Mustafa et al., 2009).

The current work showed a correlation between free asparagine in flour and acrylamide content ($r = 0.87$) (Musa et al., 2024b). Similar correlation values were also observed in a study that examined acrylamide formation in baked and toasted wheat and rye bread, focusing on asparagine levels in flour, dough, bread, and toast. Acrylamide levels increased significantly in toasted bread (11–161 µg/kg in wheat, 27–205 µg/kg in rye) compared to untoasted bread (<5 µg/kg in wheat, 7–23 µg/kg in rye). This suggests that toasting promotes acrylamide formation, even when the initial levels of free asparagine are relatively low. Moreover, acrylamide concentrations showed a strong correlation with asparagine levels ($r = 0.87$ in wheat bread, $r = 0.88$ in rye bread) (Granby et al., 2008). A study focusing on reducing acrylamide risk along the wheat supply chain by investigating the relationship between free asparagine content and acrylamide formation showed that acrylamide content in puffed durum grains was correlated with the free asparagine content in the flour (Longin, 2024). Further, a study from Oddy et al. (2023) also found a significant correlation between the acrylamide content in biscuits and the free asparagine content in flour. The studies reviewed consistently show a significant correlation between free asparagine content and acrylamide formation in various cereal products. While the relationship may not always

be linear, further research should focus on identifying thresholds of free asparagine content that lead to significant acrylamide formation, as cereals with higher free asparagine levels generally produce more acrylamide upon thermal processing. This highlights the potential for mitigating acrylamide formation by selecting cereal varieties with lower free asparagine content and optimizing processing conditions, such as fermentation time and baking temperature.

5.3 Color evaluation of baked goods and its correlation to acrylamide content

Different acrylamide mitigation strategies that are applied in baked products often compromise the quality of the product, including color. Therefore, this work included color measurements for each treatment applied to cookies, crispbread, and flakes. C-Cell has been previously used in studies to analyze the color of baked products (Musa et al., 2024c). However, to our knowledge, the Color Muse device was tested for the first time in baked goods in this study. This was done to evaluate the applicability of a new alternative color scanning device that provides a cheaper alternative for color measurement compared to C-Cell. Based on the results presented in integrated papers I and II, L^{*}-, a^{*}-, and b^{*}-values measured with C-Cell generally showed lower color values compared to Color Muse. The overall differences in values obtained from C-Cell and Color Muse were higher for the L^{*}-value compared to the a^{*}- and b^{*}-values (Musa et al., 2024a, 2024b). Interestingly, these differences were relatively small in wheat and oat flakes, especially for the a^{*}- and b^{*}-values (Musa et al., 2025b), which may reflect the less complex nature of the color profiles in these products. This could guide future choices for color analysis in baked goods, with Color Muse potentially being more reliable for breakfast cereals, but C-Cell possibly offering better resolution for lightness changes in other products such as cookies and crispbread.

Regardless of asparaginase efficacy in reducing acrylamide, color changes are undesirable as they can affect consumer acceptance. Studies on asparaginase application in different products have shown that this approach has minimal effects on the color of the final products. One study investigated asparaginase addition in *rosquillas*, a deep-fried type of doughnut, in two different concentrations of 100 and 500 U/kg, respectively (Kukurová et al., 2009). The color of the final products showed no significant changes, regardless of asparaginase concentration. Similar observations were also presented when asparaginase was applied in different biscuit formulations regardless of its concentration (Anese et al., 2011; Gazi et al., 2023; Kukurová et al.,

2013). Our study on cookies revealed some small significant changes. For example, asparaginase treatments in wheat and rye cookies showed minimal changes in color as presented in the integrated publication I (Musa et al., 2024a); however, the results presented in the integrated paper II showed more changes in L*-value, especially for oat and corn cookies. Some changes in b*-value were present in all cookies, regardless of which flour was used (Musa et al., 2024b). Although these changes were statistically significant, the visual appearance remained unchanged, as perceived by sensory evaluations discussed in subchapter 5.4. The application of asparaginases in wheat flakes showed no significant changes in color, except for the R2 treatment (L*-value) and R1 and A3 treatments (a*-value). For oat flakes, only A3 showed a slight decrease in b*-value. Other treatments showed no significant change in color regardless of asparaginase concentration (Musa et al., 2025b).

Asparaginase has also been tested in products other than cereals. A study testing 13 Turkish coffee samples using different incubation times and asparaginase dosages showed that regardless of the processing parameters, the color of enzyme-treated samples remained unchanged (Akgün et al., 2021). The application of commercial asparaginase in potato products, such as potato chips showed no changes in color as well (Pedreschi et al., 2011). Further, the application of asparaginase PreventASe L was used in French fries produced on an industrial scale. The results showed a slight decrease in overall color, but none of it was statistically significant (Rottmann et al., 2021). This was also the case for bran flakes produced on a pilot-scale presented in integrated publication IV, where no significant change in color was observed regardless of asparaginase concentration (Musa et al., 2025b).

Other than asparaginases, different mitigation strategies are used to reduce acrylamide in food products. In the integrated publication III, the application of gallic acid, ellagic acid, calcium chloride, and calcium carbonate alone or in combination with asparaginase was evaluated, as well as the application of rosemary extract. For all treatments, the color of baked cookies and crispbread was evaluated. Both gallic acid and ellagic acid increased the L*-value compared to the control. However, when combined with asparaginase, these previously significant effects were no longer observed. Other studies also investigated the addition of different plants rich in phenolic acids. These plants included chokeberry, wild rose, elderberry, rowan, and sea buckthorn. The evaluation of color showed minimal changes in cookies compared to the control (Borczak et al., 2022). Similar to phenolic acids, calcium carbonate and

calcium chloride also showed no significant changes in L*-value when combined with asparaginase (Musa et al., 2025a). However, the addition of calcium chloride significantly increased the L*-value at the 5 μ mol and 50 μ mol concentrations. A similar increase in L*-value was also reported by Açar et al. (2012) in cookies prepared using 0.1%, 0.5%, and 1.0% calcium chloride. Another study showed that dipping potatoes in calcium chloride did not affect color; therefore the potato strips after frying maintained golden yellow color (Gökmen & Şenyuva, 2007). A study where calcium hydroxide was investigated showed that tortilla chips made from nixtamalized corn flour with 1.0, 1.5, and 2.0 g/100 g calcium hydroxide showed no color change. However, the highest color change was observed in chips made with 0.5 g/100 g calcium hydroxide (Salazar et al., 2014).

As previously mentioned, in the integrated publication III, rosemary extract was also tested in cookies and crispbread. For both products, concentrations of 0.01%, 0.1%, and 0.2% were added to the dough. In wheat cookies, significant changes were observed, while in rye crispbread none of the treatments showed any significant color change. This may be due to the darker color of rye flour, which could have influenced the measurement values (Musa et al., 2025a). Changes in color due to the addition of rosemary extract in shortcrust cookies were also reported by Miśkiewicz et al. (2018). Differently from our results, they showed changes not only in L*-value, but a*- and b*-value were also significantly different compared to the control. Other than in cookies, color changes were also evident in bread containing 0.02%, 0.1%, and 0.5% of rosemary extract (Sarion et al., 2021). These changes were both in the crumb and crust of bread. This is because the addition of rosemary extract results in a darker flour color, which resulted in significant changes when adding it to fortified bread samples (Tayefe et al., 2025). When rosemary extract was added to oil which is used for frying potato products, the L*-value showed a difference compared to the control; however, it reduced the yellowing process of oil during the deep frying process (Urbančič et al., 2014). Other plant extracts are also used in cereal-based products to mitigate acrylamide. The addition of green tea extract is one of them. Although studies on the reduction of acrylamide by green tea extract sometimes show a forming or reducing potential, the color change is visible most of the time in products. Fu et al. (2018) reported that bread fortified with green tea extract showed a significant increase in the L*-value in both the crust and crumb. The crust color is influenced by the Maillard reaction and caramelization. The higher L*-value in bread containing green tea extract

may be due to its antioxidant properties, which can inhibit the Maillard reaction by interacting with reactive intermediates, leading to reduced browning.

FoodDrinkEurope (2019) suggests that analyses of the acrylamide content in baked goods can be replaced by the measurement of product attributes (e.g., color) or process parameters if a statistical correlation is found. However, color development in food products is often dependent on the ingredients added and product processing as well. Many studies focus on correlations between acrylamide and color. Gökmen et al. (2008) investigated the ratio of browning using different baking times at baking temperatures of 200 °C and 220 °C in cookies and found a significant correlation between acrylamide and color ($r = 0.946$). In the integrated publication I, the correlation found between acrylamide and color for the L*-value obtained from C-Cell and Color Muse was negative, $r = -0.61$ and $r = -0.62$, respectively. The strongest correlation was obtained for the b*-value ($r = -0.75$) (Musa et al., 2024a), and it was categorized as medium correlation ($0.67 \leq r < 0.78$) (Schuster et al., 2023). Another study also showed a weaker correlation between acrylamide and a*-value in French fries and biscuits, $r = -0.51$ and $r = -0.69$, respectively (Verma & Yadav, 2022). While several studies have explored the correlation between acrylamide content and color in food products, the strength of the correlation varies. These findings highlight the complex relationship between acrylamide levels and color development, influenced by factors like ingredients and processing conditions.

5.4 Texture properties of cereal products

The texture of baked goods is an important parameter in assessing the quality of the products and is also very important for consumer acceptance of food. While mitigation of acrylamide often requires changes in recipe or processing conditions, these can impact the quality of the product. Therefore, a texture analyzer was used to assess the textural properties of cookies, crispbread, and flakes. Asparaginases require a certain incubation time and temperature in order to become activated. Kukurová et al. (2013) reported that no significant changes in texture were present in cookies incubated for 30 min or less. In the integrated publications I and II, the incubation temperature showed a minimal impact on the hardness of wheat, rye, and corn cookies (Table 5). In oat and rice cookies, a higher impact of incubation temperature was observed in control samples (Musa et al., 2024a, 2024b). This indicates that it may be needed to optimize the incubation temperature of other grains and assess the impact on texture

properties since the starch gelatinization temperature varies across cereals (Ubwa et al., 2012), which can lead to changes in texture during the incubation and baking process.

Table 5. Texture analysis for wheat, rye, oat, corn, and rice cookies expressed as mean values for samples baked at 220 °C for 11 min. Asterisks (*) indicate a significant difference compared to the respective control (ANOVA with Dunnett's test, $p \leq 0.05$, $n = 6$).

Texture parameter	Sample ID ¹	Wheat	Rye	Oat	Corn	Rice
Hardness (N)	Control (60 °C)	44.2	62.2	26.8	43.6	31.5
	R1	44.5	62.4	22.3	44.1	35.7
	R2	49.1	58.6	24.2	51.6	37.4
	A2	48.8	55.6	16.7	18.4*	32.6
	A3	49.9	58.7	16.8	17.9*	26.5
	Control (70 °C)	45.9	62.4	18.5	43.4	38.6
	H1	46.8	58.0	26.2*	46.9	43.0
	H2	33.4*	62.0	19.9	46.0	35.5
	X2	31.7*	61.5	22.2	31.1	61.4*
	X3	37.4	34.4*	21.1	29.6*	35.1
Fracturability (mm)	Control (60 °C)	2.4	1.3	0.9	1.7	1.0
	R1	1.4*	1.5	1.3	1.0	1.0
	R2	1.3*	1.6	1.0	1.4	1.0
	A2	1.8	1.8	0.9	1.2	0.9
	A3	1.6*	1.4	1.1	1.4	1.2
	Control (70 °C)	1.8	1.4	1.8	1.2	1.1
	H1	1.8	1.2	1.3	1.1	0.9
	H2	1.5	1.1	0.7*	1.4	1.4
	X2	3.3*	1.7	1.5	1.1	1.6
	X3	3.5*	1.2	1.0*	1.2	1.2

Similar to our study, the application of asparaginase in cookies was also shown to have a low impact on the hardness of wheat biscuits. However, triticale and rye biscuits showed a significant decrease in hardness (Kukurová et al., 2023). They further showed that adding sea buckthorn to biscuits significantly increases the hardness, although it is beneficial for acrylamide reduction. Hardness can also be increased when cookies are enriched with black currant and jostaberry (Molnar et al., 2015), suggesting that such natural additives, particularly those high in phenolic compounds can affect the texture of baked products. Application of asparaginases in bread showed a slight

increase in bread firmness, but this was rather insignificant (Kumar et al., 2014). In potato products, asparaginase application showed an overall minimal impact on the firmness and stiffness of potatoes; however, a higher effect of pressure treatments on textural properties was found, where the increased pressure decreased the texture parameters (Dourado et al., 2020). This suggests that physical treatments, such as pressure, may have a more pronounced effect on potato texture than enzymatic treatments, possibly due to the breakdown of cellular structure under high pressure (Dourado et al., 2020). In potato chips, asparaginase treatment showed an increase in the force needed to break the product (Patial et al., 2022). This increase could be attributed to changes in moisture content, which may affect the crispiness of the product. In the integrated publication III, texture evaluation revealed notable changes in the hardness of the cookies, particularly with treatments involving calcium chloride, calcium carbonate, and gallic acid. Fracturability, also known as the “ability to break food into pieces when it is bitten using the incisors” (Paula & Conti-Silva, 2014), is another important factor in the texture profile of baked goods and it was also influenced by the selected treatments. The addition of salt has been shown to enhance the gluten network, improving dough stability, extensibility, and development time while reducing water absorption (McCann & Day, 2013), which could explain some of the observed textural changes in the cookies. Interestingly, when these treatments were combined with asparaginase R, the hardness of the cookies improved to a level comparable to the control (Musa et al., 2025a). The work presented in the integrated publications I and II also showed minimal or no effect of asparaginases on the textural properties of cookies (Musa et al., 2024a, 2024b), while in the integrated publication IV, neither wheat nor oat flakes showed any changes in the hardness of the final product (Musa et al., 2025b). Fracturability was also influenced by the selected treatments in cookies and crispbread. In the integrated publication I, only wheat cookies showed a significant change in fracturability compared to the control (R1, R2, A2, X2, and X3 treatment) (Musa et al., 2024a); while in the integrated publication II, only treatment H2 and X3 showed a significant decrease in fracturability (Musa et al., 2024b). Ellagic acid, calcium chloride, and rosemary extract showed an increase in the fracturability of cookies (Musa et al., 2025a). Enzyme treatment also showed a minimal impact on biscuits made of wheat, triticale, and rye; a higher impact in fracturability was observed by the addition of sea buckthorn pomace (Kukurová et al., 2023). These changes in texture across different cereals can be a result of interactions between starch and

protein, as well as starch composition and protein content in cereals (Kukurová et al., 2023). The fracturability of potato chips was also significantly affected by asparaginase treatment, which resulted in less crispy chips (Patial et al., 2022). The same authors also found that moisture increased in asparaginase-treated chips, which was also the case in asparaginase-treated bread samples (Kumar et al., 2014). Overall, the enzymatic approach had a lower impact on textural properties, compared to the additional ingredients such as salts and polyphenols. Therefore, further research is needed to explore the interactions between enzyme treatments, grain matrix, and functional ingredients to optimize both safety and textural properties of cereal-based products.

5.5 Impact of acrylamide mitigation strategies on sensory attributes

Sensory properties play a crucial role in consumer acceptance, making it essential to evaluate the impact of acrylamide mitigation strategies on attributes such as appearance, taste, texture, and aroma. While asparaginase treatment and other mitigation strategies have been widely studied for their effectiveness in reducing acrylamide levels, their influence on sensory characteristics remains a key consideration. This section examined how the selected treatments in cookies affect the sensory quality of the final products. In the integrated publication I for wheat and rye cookies most of the treatments showed minimal impact on the sensory properties which were evaluated by 11 participants presented in Figure 6. Only the X3 treatment in wheat showed a significant increase in perceived hardness and crunchiness, but no increase in hardness was detected by measurement with a texture analyzer (Musa et al., 2024a). This highlights that the instrumental methods such as texture analyzers used to assess the product quality often differ from results obtained from sensory panels. However, there are certain challenges when performing sensory tests, as this method is more time-consuming but also has a higher price, especially when using a trained panel (Szczesniak, 1973).

Asparaginase treatment in cookies was also found to have no impact on sensory properties, but the addition of raising agents has a significant impact especially on the taste and aroma of cookies (Kukurová et al., 2013). Asparaginase treatment also showed an insignificant impact on sensory properties in bread as well, regardless of the asparaginase concentration (Ghorbani et al., 2019; Kumar et al., 2014). This was

also observed in coffee treated with asparaginase, in which in some cases the enzyme treatment improved certain sensory attributes (CarolinaVieira-Porto et al., 2024).

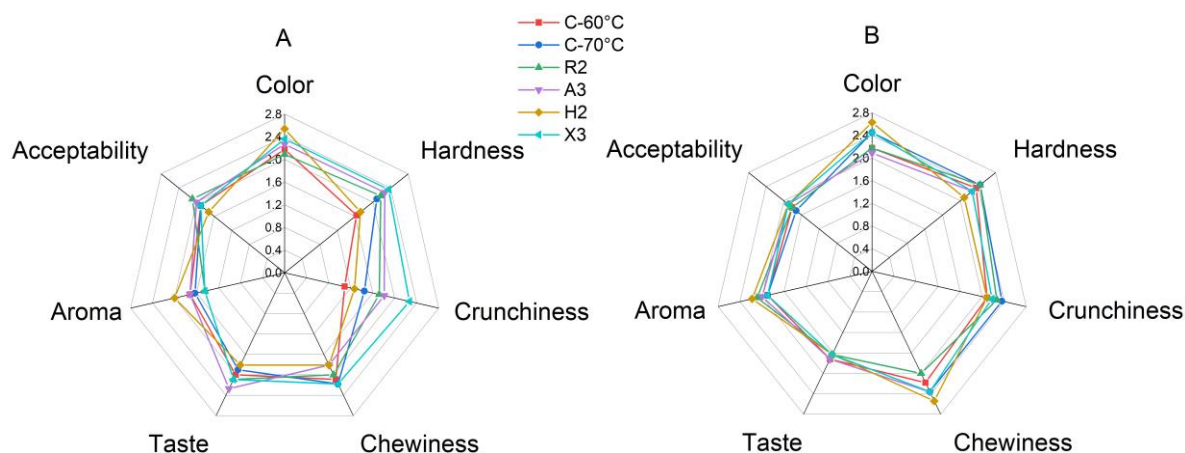


Figure 6. Sensory evaluation including color, texture, taste, aroma and overall acceptability for cookies baked at 220 °C for 11 min. Results are presented as mean values ($n = 11$) for wheat (A) and rye (B) cookies analyzed with the non-parametric Kruskal-Wallis test ($p < 0.05$) and Dunn's test was used as a post-hoc for pairwise comparisons using control and multiple treatment groups. Sample ID: C (control without asparaginase incubated at 60 °C or 70 °C), R (Acrylaway L), A (PreventASe L), H (Acrylaway HighT), X (PreventASe XR), 2 = 200 mg/kg, 3 = 300 mg/kg.

Since texture was previously reported to be influenced by asparaginase treatment in chips (Patial et al., 2022), a sensory evaluation of sweet potato chips revealed a significant change in texture, with enzyme-treated chips exhibiting a softer texture compared to the control (Akwagiobe et al., 2023). Additionally, Akwagiobe et al. (2023) observed changes in the degree of browning, as enzyme-treated chips had a lighter color than the control. However, because the contribution of asparagine to color and flavor during the Maillard reaction is relatively small (Parker et al., 2012), most studies report no significant changes in overall sensory attributes. However, other acrylamide mitigation approaches, such as lowering the baking temperature, have been found to negatively impact sensory properties (Masi et al., 2013).

The effect of salts as an acrylamide mitigation strategy on sensory properties has also been studied. Both calcium chloride and sodium chloride were shown to increase the crispiness of potato crisps, and the overall taste showed an increase when calcium chloride was used (Mestdagh et al., 2008). In the integrated publication III, the impact of the combination of calcium chloride with 50 mg/kg asparaginase on the sensory

attributes was evaluated for cookies. The addition of calcium chloride also showed an increase in taste which was detected by the participants, but other sensory attributes remained unaffected by the salt (Musa et al., 2025a). Similarly, calcium carbonate has been reported to increase the acceptability of cookies (Liang et al., 2014). Different from these observations, Ismial et al. (2013) reported a decrease in the overall acceptability of potato chips when soaked in calcium chloride, while magnesium chloride showed an improvement of the acceptability and taste of the product. The impact of salts on the sensory properties appears to be highly matrix-dependent, as seen in the differing effects of calcium chloride across potato crisps, cookies, and chips. While calcium chloride and sodium chloride enhanced the crispiness of potato crisps, their effect on cookies was limited to taste improvement without altering other attributes. The contrasting findings from Ismial et al. (2013) suggest that salt type and concentration play an important role, with magnesium chloride improving acceptability while calcium chloride reduces it in potato chips. These differences may come from variations in ion interactions with taste receptors, water activity, and starch structure. The concentration of salts is also an important factor that needs to be considered. Açar et al. (2012) showed that increasing the concentration of calcium chloride or calcium salts of lactic acid (Puracal Act 100 and Puracal Act 200) from 0.5% to 1.0% significantly differed from the control. This emphasizes that while varying salt concentrations may differ in their effectiveness in reducing acrylamide, their potential effects on texture, color, and overall acceptability must also be carefully evaluated.

The sensory attributes of phenolic compounds can be primarily described by the two key taste and trigeminal characteristics bitterness and astringency. While these sensations provoke negative consumer responses at high levels, their intensity can be adjusted by incorporating masking agents or altering the concentration and composition of polyphenols (Lesschaeve & Noble, 2005). The integrated publication III also investigated the impact of ellagic acid in combination with asparaginase and of rosemary extract on the overall sensory impact in cookies. Ellagic acid caused a slight but statistically insignificant increase in the crunchiness of cookies, while the other sensory attributes showed a minimal impact compared to the control (Musa et al., 2025a). A study evaluating the effect of rosemary extract concentrations (0.1%, 0.2%, and 0.5%) in shortcrust cookies found that higher concentrations (particularly 0.5%) negatively affected the sensory quality and overall acceptability of the cookies (Miśkiewicz et al., 2018). In integrated publication III, the addition of rosemary extract

was limited to a concentration of 0.1% for the sensory analysis to prevent significant changes to the product. Further, the sensory assessment can also be affected by the baking conditions. When cookies were baked using dry air, a better quality assessment was observed compared to humid air using the same concentration of rosemary extract (Miśkiewicz et al., 2018). The odor and taste were also reported to decrease in fried potatoes when rosemary extract was added alone or in combination with citric acid (Al-Anbari et al., 2019). Beyond rosemary extract, other plant extracts have been explored for their potential to mitigate acrylamide in baked goods. For example, the addition of green tea extract (1.0% and 1.5% concentrations) showed no significant changes in bread quality (Tayefe et al., 2025). These findings emphasize the importance of carefully selecting ingredient concentrations to balance acrylamide mitigation while maintaining desirable sensory properties. Additionally, further research into the addition of phenolic acids in bakery products is recommended, as the existing literature on this topic remains limited.

5.6 Conclusion and Perspectives

While several studies have investigated the addition of different asparaginases, this work expanded on that foundation by systematically exploring the effectiveness of four different asparaginases in reducing acrylamide formation across three different cereal-based products, cookies, crispbread, and breakfast cereals. Further, cookies made from five different wholemeal flours including wheat, rye, oat, corn, and rice were investigated. Asparaginases effectively reduced acrylamide in all products, but oat-based cookies showed the highest reduction and wheat-based cookies the lowest, with up to 97% and 79%, respectively. Oat flakes also showed higher reductions compared to wheat flakes. This confirms that the raw material composition significantly influences acrylamide mitigation under the same processing conditions. This study brings a new perspective on the combination of asparaginases with phenolic acids and salts as an effective approach to mitigate acrylamide in cookies and crispbread. This combined approach was more effective than using phenolic acids or salts individually, demonstrating the potential for multi-strategy approaches. Rosemary extract was tested in small concentrations and showed higher acrylamide reduction in cookies (50%) compared to crispbread (10%) showing that food matrix composition may affect mitigation strategies. Pilot-scale experiments showed significantly lower acrylamide reductions compared to laboratory-made flakes, which emphasizes the need for further optimization in industrial applications. While free asparaginase was found to significantly correlate with acrylamide content, the correlation between color and acrylamide was low for most color values obtained from C-Cell and Color Muse. Color Muse is a new technique that showed potential as a cost-effective tool for color evaluation. This work provides new insights into acrylamide mitigation strategies across various cereal-based products.

While this work represents a comprehensive analysis of acrylamide in different cereal-based products, there is still space for further investigations. The lower acrylamide reduction observed in the pilot-scale bran flakes experiment highlights the need to optimize processing parameters, particularly incubation time and temperature, to enhance the effectiveness of asparaginases in large-scale production. Future research should investigate the stability and activity of different asparaginases under real industrial processing conditions. Future work should further expand the range of raw materials and products. Although this study focused on wholemeal flours from wheat, oat, rye, corn, and rice, further research could evaluate acrylamide reduction in other

cereal-based products and include different processing methods (e.g., sprouting, and fermentation). Investigating additional food matrices could provide deeper insights into how composition influences mitigation strategies. More detailed studies at a molecular level could help to better understand the underlying mechanisms between the interactions of asparaginase with salts and phenolic acids and help optimize their application to enhance acrylamide reduction efficiency. The moderate correlation between acrylamide content and color measurements suggests that imaging techniques or machine-learning approaches could improve the accuracy of this approach. Although sensory evaluations with untrained participants showed mainly minimal impact on product quality, future studies should focus on sensory profiling in greater detail including a larger number of participants, or even a trained panel. Given the potential antioxidant and bioactive properties of phenolic acids and rosemary extract, future studies could assess their broader health impacts and shelf-life extension of cereal products which could add further benefits for the food industry. These findings offer valuable insights for industry applications and regulatory approaches, particularly in the EU, where stringent acrylamide reduction targets continue to shape food safety policies. Further research, particularly at an industrial scale, is essential to refine these approaches and ensure their effectiveness in commercial food production.

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7 Appendix

6.1 Publication I

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Asparaginase treatment to mitigate acrylamide formation in wheat and rye cookies

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ABSTRACT

The Maillard reaction between asparagine and a carbonyl source can lead to acrylamide formation, which is classified as "probably carcinogenic to humans". This study aimed to investigate the impact of varying amounts, incubation times, and temperatures of asparaginases on reducing acrylamide in wheat and rye cookies while preserving sensory attributes. To determine if different asparaginases reduce acrylamide without negative effects on cookie quality, acrylamide was quantified using ELISA, and color, texture and sensory attributes were assessed. Adding asparaginases resulted in a reduction of acrylamide by up to 85%. Incubating cookie dough for 10 or 30 min at 60 °C or 90 °C did not impact acrylamide formation. Acrylamide concentrations were higher by an average of 78 µg/kg for an incubation temperature of 90 °C compared to 60 °C. Rye cookies exhibited higher acrylamide concentrations by 490 µg/kg compared to wheat due to higher free asparagine in rye flour. Asparaginases showed minimal effects on cookie color and texture, with no changes in sensory evaluation. Acrylamide can be reduced using asparaginases, providing a simple and effective means of ensuring safe food for consumers.

1. Introduction

Acrylamide is formed in various baked, fried, roasted, grilled, or deep-fried carbohydrate-rich foods. These mainly include products made from potatoes and cereals, both of which contain the precursor free asparagine and are produced at low moisture and high heating (>120 °C), e.g. French fries, potato chips, bread, cookies, crispbread, rusks, pumpernickel and gingerbread (Tareke, Rydberg, Karlsson, Eriksson, & Törnqvist, 2000). Various reaction pathways are described for the formation of acrylamide, but the main formation pathway is based on the Maillard reaction between free asparagine and reducing sugars via the intermediate 3-aminopropionamide (EFSA, 2015; Granvogl & Schieberle, 2006; Mottram, Wedzica, & Dodson, 2002; Stadler et al., 2002; Yaylayan, Wnorowski, & Perez Locas, 2003; Zyzak et al., 2003). The reducing sugars and asparagine are natural compounds found in plants and plant-derived ingredients used in the preparation of foods (Franek, Rubio, Diblikova, & Rubio, 2014). Among different cereals, rye has the highest free asparagine content, followed by oats, wheat, corn, and rice (Official Journal of the European Union, 2017; Skog & Alexander, 2006).

Several studies link acrylamide as a cancer-causing agent found in baked starchy foods and a range of fried products (Jung, Choi, & Ju, 2003; Mestdagh et al., 2008; Tareke et al., 2000). These reports have caused a worldwide health concern. Even though all cancer-related issues are from experiments in laboratory animals, food agencies have taken measures to prevent and lower the risk of cancer in humans. Due to the classification of acrylamide as a group 2A carcinogen ("probably carcinogenic to humans") by the International Agency for Research on Cancer (IARC, 1994) and by the European Food Safety Authority (EFSA, 2015), minimization guidelines are implemented according to the as low as reasonably achievable (ALARA) principle throughout the EU at all relevant stages of the food chain. Against this background, the European Commission issued the new Regulation (EU) 2017/2158, which is in force since April 11, 2018 (Official Journal of the European Union, 2017). This regulation lays down specific measures to reduce the acrylamide content of certain foods in which acrylamide is present: Potato products (e.g. French fries and chips), bread, breakfast cereals, pastries (e.g. cookies, waffles, crackers, crispbread, and gingerbread), coffee (roasted coffee, instant coffee, and coffee substitutes) and baby food.

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Several mitigation strategies are proposed for the decrease of acrylamide and the European Food and Drink Industry has summarized research progress on the reduction of acrylamide and defined 13 parameters, known as “toolbox” parameters, which include agronomical factors, recipe, processing, and final preparation (Food Drink Europe, 2019). The focus is especially on asparaginases, which convert asparagine into aspartic acid and ammonia, thus removing the precursor. The use of asparaginases is recognized as a possible minimization strategy (Amrein, Schönbachler, Escher, & Amado, 2004; Vass et al., 2004; Zyzak et al., 2003). A few studies in baked goods have already been published where asparaginases are added to mitigate acrylamide formation (Anese, Quarta, & Frias, 2011; Gazi et al., 2023; Hendriksen, Kornbrust, Østergaard, & Stringer, 2009; Kukurowa et al., 2013; Kumar, Shimray, Indrani, & Manonmani, 2014). Hendriksen et al. (2009) focus on the effect of different enzyme dosages, dough resting times, and water content in products such as ginger biscuits, semisweet biscuits, crispbread, French fries and potato chips. The study in semisweet biscuits shows that the increased dosage of enzymes and incubation time at 40 °C reduces up to 84% of total acrylamide content. However, dough incubated for 30 min has higher acrylamide content in control samples when compared to 15 min incubation time. Anese et al. (2011) investigate the addition of asparaginases in short dough biscuits at incubation temperatures of 20 °C–54 °C and a range of incubation times from 10 min to 30 min and show a reduction of acrylamide of up to 71%. Kukurowa et al. (2013) report that incubation at 25 °C for 60 min gives the highest reduction of acrylamide (27%–75%) using asparaginases in cookies. However, this study combines the effect of pH using different raising agents with asparaginase treatment, which can also have a combined impact on acrylamide reduction. With current benchmark levels of acrylamide in cookies (350 µg/kg), the necessary decrease of acrylamide raises the need for further research to mitigate acrylamide in cookies and other products of concern without impairing texture or taste. All studies so far use different baking conditions and also lack the comparison of different enzymes and higher incubation temperatures that can be used to reduce acrylamide. Therefore, the focus of this work was on studying the effect of different amounts of asparaginases on reducing acrylamide. Further, the aim was to test higher incubation temperatures for these asparaginases and investigate the impact on the texture, color and sensory acceptability of cookies. The effect of different enzymes applied in cookies might be different, and this information is currently missing.

The hypothesis suggests that acrylamide formation is influenced by factors such as flour, dough-making and baking conditions. Further, different asparaginases and incubation temperatures might have an effect not only on acrylamide reduction but also on other properties such as color, texture and taste. Therefore, the aim is to systematically investigate the influence of flour types, dough-making processes and baking conditions on the formation of acrylamide in cookies. Additionally, it aims to assess the effects of various asparaginases and incubation temperatures on acrylamide reduction, as well as their impact on the color, texture and taste of the final products.

2. Materials and methods

2.1. Reagents and ingredients

The chemicals and reagents were of analytical grade or higher. Baking powder, sugar and sunflower oil were purchased at a local supermarket. Wholemeal wheat and rye flour were kindly donated (IREKS, Kulmbach, Germany). The asparaginases R (Acrylaway L) and H (Acrylaway HighT) were donated by Novozymes (Bagsvaerd, Denmark) and A (PreventASe L) and X (PreventASe XR) by DSM (Heerlen, The Netherlands). Acrylamide ES ELISA kit and derivatization kit were purchased from Gold Standard Diagnostics (Kassel, Germany). ISOLUTE Multimode 500 mg/3 mL and ISOLUTE ENV+ 200 mg/3 mL solid-phase extraction (SPE) columns were purchased from Biotage (Uppsala, Sweden).

2.2. Asparaginases

Four commercially available asparaginases were used for the baking trials. Before use, each of them was dissolved in water (10 mL). The dosage was determined following the manufacturers' guidelines based on flour weight. Enzymes were named as the following: R (Acrylaway L), T (Acrylaway HighT), A (PreventASe L) and X (PreventASe XR). For both asparaginases R and T, two different concentrations were used, 100 and 200 mg/kg, while for asparaginases A and X, 200 and 300 mg/kg were used. The asparaginase activities are specified in Table 1.

2.3. Cookie dough preparation

For 100 g of wholemeal wheat or rye flour, 48 mL of water, 15.3 g of sugar, 12.6 mL of sunflower oil and 0.33 g of baking powder were added, respectively. All dry ingredients such as flour, sugar and baking powder were mixed for 1 min at low speed using a commercial food processor (Robert Bosch GmbH, Stuttgart, Germany). Then, water only (control) or water mixed with asparaginase was added with the oil. The dough was mixed for 3 min at medium speed and for 30 s at high speed.

2.3.1. Incubation and baking conditions for wheat cookies

After mixing the wheat cookie dough at room temperature (22 °C), preliminary investigations were done on the incubation temperature for asparaginase activation. The dough was incubated at 60 °C and 90 °C, for asparaginases R and T, respectively, and at 70 °C for asparaginase T only using 220 °C for 11 min as baking conditions. A control sample was added for each incubation temperature. The dough was incubated in a proofer (EKA, Padova, Italy) for 10 min and 30 min to investigate the effect of dough incubation time on acrylamide formation. After that, cookies were rolled to a thickness of 0.5 cm and a diameter of 6 cm using a roller pin. Cookies were baked in an oven (UNOX ROSSELL XFT197, Padova, Italy) at four different baking times and temperatures as follows: 180 °C for 15 min, 200 °C for 13 min, 220 °C for 11 min and 240 °C for 9 min. Based on these investigations, all other experiments were conducted using 220 °C for 11 min as baking conditions. Further, the rest of experiments were conducted using an incubation temperature and time of 60 °C for 10 min for asparaginases R and A, while for asparaginases T and X, 70 °C and 10 min were used, respectively, according to the manufacturers' instructions. Detailed information on sample name, incubation time and baking time and temperature, as well as the asparaginases used are found in Table 1.

2.3.2. Incubation and baking conditions for rye cookies

After mixing the rye cookie dough at room temperature (22 °C), the dough was incubated at 60 °C for 10 min for asparaginases R and A, and at 70 °C for 10 min for asparaginases T and X. A control sample was added for each incubation temperature. After incubation, the dough was rolled to a thickness of 0.5 cm and a diameter of 6 cm using a roller pin. The dough was baked at 220 °C for 11 min for all samples.

2.4. Acrylamide analysis

The acrylamide content in cookies was measured using an Acrylamide ES enzyme-linked immunosorbent assay (ELISA) kit in combination with the derivatization kit (Gold Standard Diagnostics, Kassel, Germany). The assay was performed according to the manuals of the ELISA kit (Gold Standard Diagnostics, 2021). The samples were extracted by adding 40 mL of distilled water to 2 g of sample, followed by 30 min of mixing. After 5 min of sedimentation, sample extracts were filtered and centrifuged for 5 min at 13,000×g. The sample clean-up procedure was done using Multimode SPE and ENV + SPE columns. The extracted acrylamide was eluted from the column using 1 mL (2x) methanol/water (60:40, v/v). This eluent underwent derivatization at 50 °C for 60 min. Then, 2 mL of assay buffer was added and analysis following the assay procedure. The absorbance was measured at 450 nm

Table 1

Detailed sample names for wheat and rye cookies baked at 220 °C including the asparaginases used and their concentration and activity, dough incubation time and temperature, and baking time and temperature.

Sample name ^a	Asparaginase	Asparaginase concentration (mg/kg)	Asparaginase activity	Incubation time (min)	Incubation temperature (°C)	Baking time (min)	Baking temperature (°C)
C-60 °C	None (control)	0	None added	10	60	11	220
R1	R	100	3500 ASNU/g	10	60	11	220
R2	R	200	3500 ASNU/g	10	60	11	220
A2	A	200	≥2500 ASPU/g	10	60	11	220
A3	A	300	≥2500 ASPU/g	10	60	11	220
C-70 °C	None (control)	0	None added	10	70	11	220
H1	H	100	6000 TASU/g	10	70	11	220
H2	H	200	6000 TASU/g	10	70	11	220
X2	X	200	47,000–57,500 XRU/g	10	70	11	220
X3	X	300	47,000–57,500 XRU/g	10	70	11	220

ASNU/g: One ASNU/g is defined as the amount of enzyme that produces 1 μmol of ammonia per minute after hydrolysis of L-asparagine (reaction conditions: pH 7.0, 37 °C, 1.5 min) (EFSA CEP Panel et al., 2023).

ASPU/g: One ASPU/g is defined as the amount of enzyme that produces 1 μmol of ammonia per minute after hydrolysis of L-asparagine (reaction conditions: pH 5.0, 37 °C, 30 min) (EFSA CEP Panel et al., 2024).

TASU/g: Thermostable ASparaginase Units/g are used to express asparaginase activity relative to an internal enzyme standard using the same assay as for ASNU/g (EFSA CEP Panel et al., 2023).

XRU/g: one XRU is defined as the amount of enzyme required to release 1 μmol of ammonia per minute from L-asparagine using the XRU asparaginase assay (personal communication with DSM).

^a The same sample ID system is used for both wheat and rye samples.

using a photometer (Tecan, i-control infinite 200Pro, Männedorf, Switzerland) to determine acrylamide amounts.

2.5. pH value of dough

The dough (10 g) was weighed into a 250 mL beaker. Then, 5 mL of acetone and 40 mL of distilled water were added and mixed carefully (Begemann et al., 2016). The sample was homogenized using an Ultra-Turrax (IKA T25, Staufen, Germany) until fully dispersed. The remaining 60 mL of water were added. The pH value was measured using a pH meter (SI Analytics, LAB 845, Mainz, Germany).

2.6. Free asparagine content of wheat and rye flours

The derivatization and subsequent extraction protocol for the measurement of free asparagine was conducted following the procedure described by Hölzle, Becker, Oellig, & Granvogl (2023) with a slight modification. For full details, please see supplementary method M1.

2.7. Color measurement

Two distinct methods were employed to determine color values. The L* (lightness), a* (green-red) and b* (blue-yellow) color values were acquired through the utilization of both CCell (Calibre Control, Warrington, UK) and ColorMuse (Variable Inc, Chattanooga, TN, USA). Prior to use, CCell was calibrated using a calibration card (CC006, Calibre Control, Warrington, UK) and Color Muse using a white background lid that is part of it. For CCell analysis, the samples were placed on a tray with a blue background employed for measurement. For ColorMuse, the samples were placed on a white background and the device was positioned 0.5 cm above the sample. Samples were tested within 4 h after baking to ensure consistency after cooling. Six measurements were taken per sample for both devices.

2.8. Texture measurement

The texture of cookies was measured 3 h after baking using a TA.XTplus texture analyzer (Stable Micro Systems, Godalming, UK) equipped with a 5 kg load cell. The test was performed using a three-point

bending rig (HDP/3 PB) and a heavy-duty platform (HDP/90) to break the cookies (American Institute of Baking, 2012). Hardness (N) and fracturability (mm) of cookies were measured using the method called “Measurement of the hardness and resistance of biscuits/cookies to bend or snap” which is already available in the Exponent software (Stable Micro Systems, Version 6.1.20.0, Godalming, UK). Each batch of samples underwent duplicate measurements, resulting in a total of six measurements.

2.9. Sensory analysis

To measure the effect of asparaginase addition in the cookie recipe, for each treatment, A, R, H and X, respectively, the highest amounts added were tested by sensory analysis using quantitative descriptive analysis with 11 participants. Color, hardness, crunchiness, chewiness, taste, aroma and overall acceptability of cookies were rated. Using a 10 cm line scale, the participants indicated the intensity of each sensory attribute from light to dark, soft to hard, little to very crunchy, little to very chewy, least to most pleasant taste, mild to intense aroma and least to most acceptable, respectively. All participants were asked to rate each characteristic of cookies based on the control. The following wheat and rye samples were tested: C-60 °C, C-70 °C, R2, T2, A3 and X3 (Table 1). The data has been converted to ordinal data by assigning numerical values for evaluation to help facilitate statistical calculations and comparisons.

2.10. Statistical analysis

Means and standard deviations were calculated using Microsoft Excel. For each treatment, three technological replicates (i.e. cookie batches) were prepared. For texture analysis, a total of six cookies (two cookies per batch) were taken, while for color analysis six cookies were scanned on both the surface and the bottom of the cookie, respectively. Origin 2023 (OriginLab Corporation, Northampton, MA, USA) was used for statistical analysis. Additionally, IBM SPSS Statistics (IBM Corp, Version 29.0. Armonk, NY) was used for Dunnett's *t*-test ($p \leq 0.05$) to detect differences between control versus asparaginase-treated samples. For acrylamide analysis, three samples were extracted each and two analytical replicates were performed when pipetting into 96-well plates.

The results were calculated through the online ELISA calculator on the Arigo website (Arigo Biolaboratories, 2014). Sensory analysis was evaluated with the non-parametric Kruskal-Wallis test ($p < 0.05$) and Dunn's post-hoc test was used for pairwise comparisons using control and multiple treatment groups. Pearson's correlation coefficients (r) were calculated using Origin 2023 and were categorized as very weak ($0.41 \leq r < 0.54$), weak ($0.54 \leq r < 0.67$), medium ($0.67 \leq r < 0.78$) and strong ($r \geq 0.78$) (Schuster, Huen, & Scherf, 2023).

3. Results and discussion

3.1. Acrylamide content

To assess the effect of baking temperature and time on acrylamide formation in cookies, experiments were first conducted with wholemeal wheat flour cookies at four different baking temperatures and times with no asparaginase addition (Fig. 1). Even though the baking time was reduced with increasing temperature, the acrylamide content increased. Fig. 1a shows the effect of dough incubation time, regardless of incubation temperature, on acrylamide formation for each baking condition. No differences were observed. Further, the effect of dough incubation temperature on acrylamide formation was also examined (Fig. 1b). There was an increase in acrylamide formation when the higher incubation temperature was used, except for baking at 200 °C for 13 min. However, based on statistical evaluation, there was an increase in acrylamide content only for 180 °C and 240 °C, where higher acrylamide formation was observed for cookie doughs incubated at 90 °C. After these initial experiments, the focus was on investigating only one baking temperature (220 °C) and baking time (11 min), along with two dough incubation temperatures, 60 °C and 70 °C. The incubation temperature of 90 °C was not used further due to the observed increase in acrylamide formation at this temperature (Fig. 1b).

Fig. 2a shows the acrylamide reduction in wheat cookies when using asparaginases. A decrease in acrylamide was observed at both incubation temperatures, 60 °C and 70 °C, respectively. Compared to the control, a decrease of up to 70% was observed when A2 asparaginase was used at an incubation temperature of 60 °C. In comparison, the X2 treatment showed a reduction of up to 80% at an incubation temperature of 70 °C. Both treatments using asparaginases A and X showed a higher reduction in acrylamide compared to R and H. All acrylamide contents were below the 350 µg/kg benchmark level.

Fig. 2b shows the acrylamide reduction in rye cookies. All treatments

showed a decrease of acrylamide, up to 80% with treatment R1 and 85% with treatment X2 relative to the control. Several studies have shown a reduction of acrylamide using asparaginases (Anese et al., 2011; Ciesarová et al., 2014; Hendriksen et al., 2009; Kukurova et al., 2013; Kumar et al., 2014; Xu, Oruna-Concha, & Elmore, 2016). Hendriksen et al. (2009) report that reduction of acrylamide content by 92% is possible using longer dough incubation. A reduction is observed even after 10 min incubation of semisweet biscuit dough but increases between 15 and 30 min. Acrylamide content in short-dough biscuits can be reduced by 70% by treating short-dough biscuits with asparaginase (Anese et al., 2011). Kumar et al. (2014) report a 97% reduction of acrylamide in bread with average concentrations of 729 µg/kg in the crust and 143 µg/kg in the crumb. This explains the high standard deviation in some of the samples presented in Fig. 2 where the control sample of rye cookies C-60 °C (Fig. 2b) had the highest standard deviation with acrylamide concentrations of 1313 µg/kg, 1265 µg/kg, 599 µg/kg and 589 µg/kg. Since cookies were analyzed as a whole, meaning that the cookie was milled all together (crust and crumb), this could lead to higher standard deviations. Both Hendriksen et al. (2009) and Kumar et al. (2014) investigate the effect of asparaginase dosage on acrylamide reduction. They report that increasing the asparaginase dosage further decreased acrylamide. This is in agreement with the current study, even if the dosages were different. Asparaginases R and H (Fig. 2a) showed higher acrylamide reduction when increasing the dosage. However, this was not the case for A and X, where the dosage did not play a role. The sample matrix is also important for the activation and efficacy of asparaginases which makes it very complex to compare our results to those of other studies. A reduction of up to 46 % of acrylamide is reported by Ciesarová et al. (2014) when using asparaginases on the surface of bread. The addition of asparaginases in the dough (not surface) provided even higher reductions of acrylamide. These asparaginases were an effective way of reducing acrylamide below benchmark levels set by the EU (Official Journal of the European Union, 2017; Official Journal of the European Union, 2017). It is necessary to implement mitigating strategies for cereal-based products, as samples such as rye (control) exceeded the 350 µg/kg benchmark level. Asparaginase treatments such as R1, R2, H1, H2, A3, X2 and X3 effectively reduced acrylamide levels below the benchmark levels.

3.2. pH value of dough

Raising agents such as baking powder, which was used in our cookie

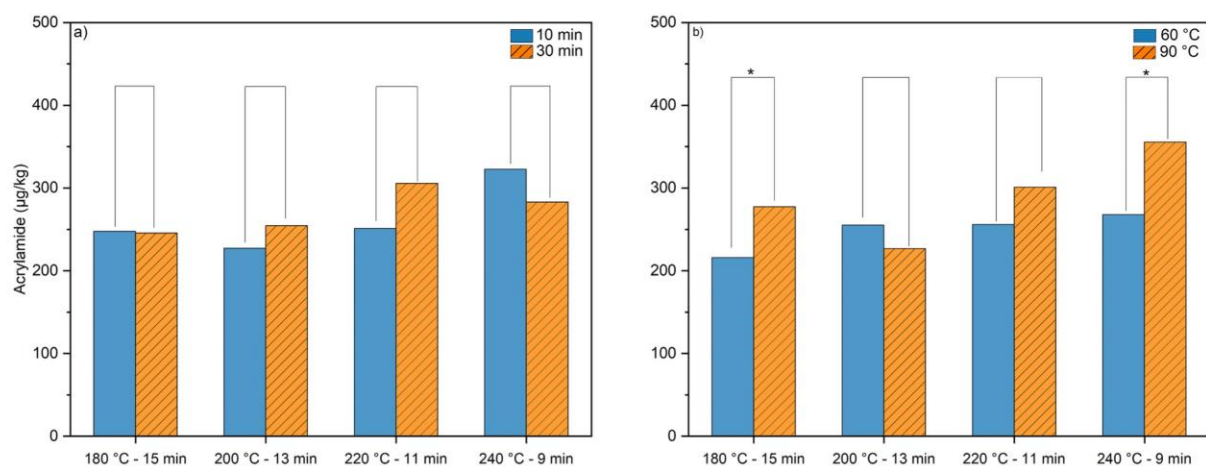


Fig. 1. Effect of dough incubation time (a) and temperature (b) on acrylamide formation in wheat cookies baked at 180 °C, 200 °C, 220 °C and 240 °C, for 15 min, 13 min, 11 min and 9 min, respectively. The data are presented as mean values ($n = 3$) $\pm$ standard deviation and two-sample t -test was used to compare means at $p \leq 0.05$. Details of p -values for each significant (*) difference are found in Table S2.

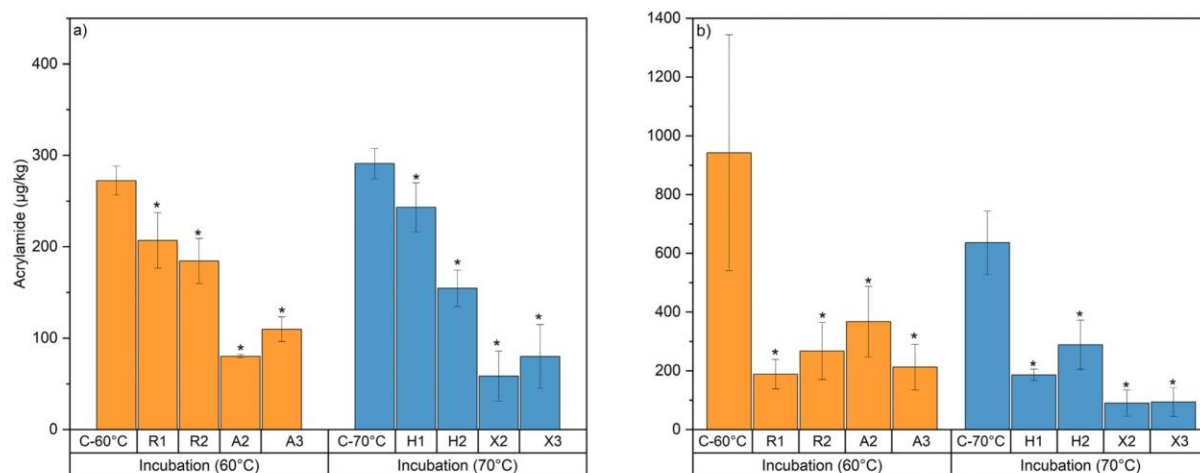


Fig. 2. Acrylamide content using asparaginases in wheat (a) and rye (b) cookies baked at 220 °C for 11 min. Error bars indicate the standard deviation and asterisks indicate a significant difference compared to the respective control (ANOVA with Dunnett's test, $p \leq 0.05$, $n = 3$). Details of p-values for each significant (*) difference are found in Table S2.

recipe, influence the pH value of the dough (Kukurova et al., 2013). Asparaginases depend on the pH environment for their activation with a recommended optimum of 6–9. Lower or higher pH values can lower the activity of asparaginases. Therefore, more free asparagine will remain in the dough. For this reason, analysis of the pH was conducted for wheat and rye cookie dough for the controls and each treatment to see whether the reduction or formation of acrylamide could be affected (Table S1). The pH of the doughs did not differ in wheat nor rye dough and ranged from 5.96 to 6.26 overall. Since only 0.33 g of baking powder was used, the change in pH was not expected and the pH was in the recommended range for asparaginase activity. It is well known that the pH value also influences the Maillard reaction. Chieh Sung and Ya Chen (2017) report that ammonium bicarbonate increases acrylamide more than sodium bicarbonate, and therefore suggest to use sodium bicarbonate to better mitigate acrylamide.

3.3. Free asparagine content of wheat and rye flours

The formation of acrylamide was higher when cookies were made with rye instead of wheat flour. Therefore, the free asparagine content in wheat and rye flour was analyzed. Wheat had a lower free asparagine content compared to rye flour, which was 290 ± 6 mg/kg and 700 ± 8 mg/kg, respectively. The average acrylamide content in rye cookies was up to 942 µg/kg, compared to wheat which had an average of 319 µg/kg at an incubation temperature of 60 °C. Studies report a strong correlation between free asparagine in grain and acrylamide formation in biscuits (Oddy et al., 2023; Žilić, Aktağ, Dodig, Filipović, & Gökmen, 2020). Oddy et al. (2023) suggest that the asparagine content of the flour and the final amount of acrylamide in baked products should be checked for correlation, as the formation of acrylamide in biscuits can be different depending on the ingredients and processing conditions. It is well known that free asparagine is higher when wholemeal flours are used than white flours. The results confirmed the strong relationship of the type of flour used and its free asparagine content and the final acrylamide formation in the product.

3.4. Color measurement

While the Maillard reaction is known to produce a series of potentially hazardous substances in baked foods, it is also responsible for desirable color development during baking, which is an important sensory aspect (Palazoglu, Coşkun, Kocadağlı, & Gökmen, 2012). To

assess the effect of the asparaginase treatments applied in wheat and rye cookies, color was measured using CCell as a reference method and ColorMuse. ColorMuse, similar to CCell, also gives $L^*a^*b^*$ values, but is small, portable, simple to use and cheap. For each sample, the surface color of cookies was given as an average of L^* (lightness), a^* (redness) and b^* (yellowness) values. For wheat cookies, the effect of baking time and temperature on the color of cookies was studied first. Fig. S1 shows the increase or decrease of $L^*a^*b^*$ values depending on the baking condition of both devices, CCell and ColorMuse, respectively. The L-value decreased when using higher baking temperatures, which is to be expected, because lower L-values represent a darker color. a- and b-values did not follow any trend, except for the b-value when measured with ColorMuse, which showed a decrease in yellowness of the sample with increasing baking temperature. Romani, Rocculi, Mendoza, and Dalla Rosa (2009) observe a decrease of L-values when investigating the effect of time and temperature in potato products. Unlike this study, they observe that with the decrease of L-value, a- and b-values increase.

Further, the investigation focused solely on the baking temperature of 220 °C for 11 min for all other wheat and rye samples, as the condition where cookies were not over- or underbaked. The L-value of wheat cookies overall showed no differences to the control when asparaginases were added, except for treatments A3, X2 and X3 (Table 2). a- and b-values were slightly more affected by the treatments applied. Different from the L-value, all differences for a- and b-values were observed in the same way no matter which device was used to measure the color. Even though the values for $L^*a^*b^*$ were different in numbers, they followed the same trend.

The L-value for rye cookies showed a slight decrease for treated samples, but no differences were observed compared to the control, except for the H1 sample measured by CCell. The a- and b-value showed a decrease for A2, A3, X2 and X3 treatments, but the overall change was 35% at most. However, only a few individual values changed and the relative change was small (Table 2). Kukurova et al. (2013) also report some slight differences in colour of cookies prepared with or without asparaginase, but no effect on the final color of the product, while Anese et al. (2011) actually report no impact of asparaginase treatment on the color of cookies.

A considerable interest for the acrylamide topic is also the correlation between color and acrylamide content in baked goods. For wheat cookies baked at a range of 180 °C–240 °C a darker color was observed when higher baking temperatures were used. Therefore, each $L^*a^*b^*$ value taken from either CCell or ColorMuse was examined for

Table 2

Color of wheat and rye cookies expressed as L* (lightness), a* (redness) and b* (yellowness) values obtained from CCell and ColorMuse for samples baked at 220 °C for 11 min. Values are given as mean (n = 6) and pooled standard deviations (SD) are presented. Details of p-values for each significant (*) difference are found in Table S2.

Sample ID ^a		Wheat cookies			Rye cookies		
		L	a	b	L	a	b
		Mean ^b	Mean	Mean	Mean	Mean	Mean
CCell	C-60 °C	21.0	9.61	19.2	27.3	5.4	14.8
	C-70 °C	23.3	10.2	21.5	27.0	5.1	14.5
	R1	20.7	9.1	18.9	19.9	6.9	17.9
	R2	22.8	9.6	19.5	21.1	7.2	18.6
	H1	21.7	9.9	19.7	17.9*	5.3	13.9
	H2	24.0	8.8	18.7*	22.2	7.0	18.3
	A2	23.5	6.1*	15.1*	31.1	3.2*	10.6*
	A3	25.9	6.1*	16.1*	31.7	3.4*	10.7*
	X2	23.2*	8.1*	18.9*	22.5	5.8	15.1
	X3	25.2*	7.5*	17.3*	30.6	3.6	10.0*
	Pooled SD	2.1	1.0	1.4	4.5	1.1	2.3
ColorMuse	C-60 °C	34.2	10.5	20.3	38.3	9.0	21.5
	C-70 °C	39.1	12.2	25.0	39.4	10.2	22.1
	R1	33.4	10.6	21.5	36.1	10.5	23.2
	R2	37.1	11.5	23.3	35.8	10.5	22.2
	H1	36.8	12.2	23.9	35.8	10.7	23.3
	H2	39.6	12.6	25.0*	34.6	10.9	22.3
	A2	34.2	8.5*	19.3*	37.0	7.2	17.1*
	A3	32.8*	9.7*	17.9*	37.2	8.4	17.2*
	X2	34.8	10.6*	21.1*	35.3	7.2*	16.3*
	X3	32.6*	11.5*	21.3*	34.1	9.2	17.5*
	Pooled SD	2.2	1.0	1.2	2.2	1.4	1.1

^a For sample abbreviations, please refer to Table 1.

^b Asterisks indicate a significant difference compared to the respective control (ANOVA with Dunnett's test, $p \leq 0.05$, $n = 6$).

correlation with the acrylamide content in the product. Both CCell ($r = -0.61$) and ColorMuse ($r = -0.62$) showed negative correlations for L-value, meaning that the color values decreased (cookies were darker in color) with increased acrylamide in cookies. The b-value obtained from ColorMuse ($r = -0.75$) showed the strongest correlation. All other correlations were weak or very weak. Other studies also investigate the relationship between color and acrylamide in baked products (Gokmen et al., 2008; Oddy et al., 2023; Parker et al., 2012; Schouten et al., 2022; Verma & Yadav, 2022) and report that there is a strong correlation between biscuit color and acrylamide content. This study had an r-value of -0.75 at most, which is only a medium correlation. However, this was not the main aspect of this study, thereby presenting a limitation as only four different baking conditions were used, resulting in a limited range of color differences.

3.5. Texture measurement

Texture is an important parameter to assess the product quality of cookies. The textural parameters investigated in this study were hardness and fracturability. Table 3 shows that for wheat samples incubated at 60 °C there were no differences between control and the treatments. In comparison, for samples incubated at 70 °C the treatments H2 and X2 showed a decrease in hardness. When looking at the control samples incubated at different temperatures, there was no difference suggesting that the incubation temperature had no effect on the hardness of cookies. In Table 3, the fracturability of cookies showed a decrease for treatments R1, R2 and A3 incubated at 60 °C, and an increase of fracturability was observed for incubation at 70 °C for the X2 and X3 treatments.

In Table 3 the hardness of rye cookies did not show any differences no matter what incubation temperature was used, except for the X3

Table 3

Texture analysis for wheat and rye cookies expressed as hardness (N) and fracturability (mm) for samples baked at 220 °C for 11 min. Values are given as mean (n = 6) and pooled standard deviations (SD) are presented. Details of p-values for each significant (*) difference are found in Table S2.

Sample ID	Hardness [N]		Fracturability [mm]	
	Mean ^a		Mean	
Wheat Cookies	C-60 °C	44.2	2.4	
	C-70 °C	45.9	1.8	
	R1	44.5	1.4*	
	R2	49.1	1.3*	
	H1	46.8	1.8	
	H2	33.4*	1.5	
	A2	48.8	1.8	
	A3	49.9	1.6*	
	X2	31.7*	3.3*	
	X3	37.4	3.5*	
	Pooled SD	6.0	0.5	
Rye Cookies	C-60 °C	62.2	1.3	
	C-70 °C	62.4	1.4	
	R1	62.4	1.5	
	R2	58.6	1.6	
	H1	58.0	1.2	
	H2	62.0	1.1	
	A2	55.6	1.8	
	A3	58.7	1.4	
	X2	61.5	1.7	
	X3	34.4*	1.2	
	Pooled SD	5.6	0.6	

For sample abbreviations refer to Table 1.

^a Asterisks indicate a significant difference compared to the respective control (ANOVA with Dunnett's test, $p \leq 0.05$, $n = 6$).

treatment that resulted in lower hardness. The fracturability of rye cookies (Table 3) showed a slight increase or decrease at both incubation temperatures, but no differences. In some cases, the texture parameters showed high standard deviations within the same treatment. This could be attributed to the baking oven which sometimes showed differences in baking spots resulting in higher standard deviations when analyzing three batches of cookies. When the moisture distribution in samples differs, changes in the hardness of cookies can be an outcome (Palazoglu, Coşkun, Tuta, Mogol, & Gökmen, 2015). They also suggest that a slight difference in baking time can result in cookies with less moisture, which can affect product hardness. Because baking was done individually and manually for each batch, differences in starting temperature at the beginning of baking could also affect the texture of cookies. This will be discussed further in the sensory evaluation (chapter 3.6). Kurková et al. (2013) report that addition of asparaginase with incubation times of 30 min or lower does not affect the texture of cookies, while Kumar et al. (2014) report only a slight impact on textural parameters in other cereal-based products such as bread.

3.6. Sensory evaluation

Sensory analyses are often needed, as the technical methods, such as color-scanning devices and texture analyzers, cannot evaluate the taste of the products and overall acceptability. Table 4 shows the results of sensory evaluations for wheat and rye cookies, respectively. From a sensory point of view, there were no differences in color, chewiness, taste, aroma and overall acceptability of wheat cookies. When control samples were compared to the X3 samples, the treatment increased the hardness and crunchiness of the cookies. Unlike wheat, rye cookies showed no differences ($p \leq 0.05$) in any sensory parameters evaluated. Participants were asked to comment on any general specifics regarding the cookies, and uneven browning on the surface was slightly different in a few samples. As mentioned in chapter 3.5, the uneven heat distribution in the baking oven affected the texture and the color. This also

Table 4

Sensory evaluation for wheat and rye cookies including color, texture, taste, aroma and overall acceptability for cookies baked at 220 °C for 11 min. Results are presented as mean values (n = 11) and pooled standard deviation (SD) and were analyzed with the non-parametric Kruskal-Wallis test ($p < 0.05$) and Dunn's test was used as a post-hoc for pairwise comparisons using control and multiple treatment groups. Details of p-values for each significant (*) difference are found in Table S2.

Sensory evaluation		Wheat Cookies						Rye Cookies					
		C-60 °C	C-70 °C	R2	A3	H2	X3	C-60 °C	C-70 °C	R2	A3	H2	X3
Color	Mean	2.18	2.00	2.09	2.27	2.54	2.36	2.18	2.45	2.18	2.09	2.63	2.45
	Pooled SD	0.44						0.45					
Hardness	Mean ^a	1.63	2.09	2.18	2.27	1.72	2.36*	2.36	2.45	2.45	2.27	2.09	2.27
	Pooled SD	0.51						0.62					
Crunchiness	Mean	1.09	1.45	1.72	1.81	1.27	2.27*	2.09	2.36	2.27	2.18	2.09	2.18
	Pooled SD	0.65						0.65					
Chewiness	Mean	2.09	2.18	2	1.81	1.81	2.18	2.18	2.36	2	2.36	2.54	2.36
	Pooled SD	0.56						0.73					
Taste	Mean	2	1.9	2.09	2.27	1.81	2.09	1.72	1.63	1.63	1.72	1.63	1.63
	Pooled SD	0.55						0.61					
Aroma	Mean	1.72	1.63	1.45	1.72	2	1.45	1.9	1.9	2.09	2	2.18	1.9
	Pooled SD	0.71						0.56					
Overall Acceptability	Mean	1.9	1.9	2.09	2	1.72	1.9	1.81	1.72	1.81	1.9	1.9	1.9
	Pooled SD	0.63						0.55					

^a Numbers with asterisks indicate a significant difference at $p < 0.05$.

explains the color differences in Table 2. The overall acceptability was rated better for wheat compared to rye cookies. This was expected, as the hardness of cookies made with rye flour was higher compared to wheat flour, which was confirmed by sensory evaluation and texture analysis. The hardness of rye cookies tended to decrease when treatments were applied, but the change was not statistically different at $p \leq 0.05$. A decrease in hardness could be a positive aspect of using asparaginases depending on the desired texture of the final product.

No changes in the sensory analyses were a positive result, as the recipe and the baking conditions stayed the same, and desirable results in acrylamide reductions were achieved when using asparaginases. Two other studies also report that adding a commercially available asparaginase does not affect the quality of the final product (Ciesarová et al., 2014; Kukurova et al., 2013).

4. Conclusion

Asparaginase addition in cookie dough prior to baking resulted in a reduction of acrylamide at both incubation temperatures, 60 °C and 70 °C, respectively. Acrylamide formation was higher in rye compared to wheat cookies, attributed to higher free asparagine levels in rye flour compared to wheat flour. While the dough incubation time had no significant effect, the hypothesis that the incubation temperature might affect acrylamide formation was confirmed. An incubation temperature of 90 °C increased acrylamide compared to 60 °C. ColorMUSE, a color scanning device, was introduced for the first time and represents a new method in food sample color analysis. There was a weak correlation between the color of cookies and acrylamide. It is important to note that color also depends on the type of flour, whether it is white or wholemeal flour, and other ingredients. Therefore, acrylamide content must be analyzed for each product separately, as a prediction based on color might not be sufficient. Since only four baking temperatures were analyzed, further investigations are recommended for the relationship between acrylamide and color. Sensory analysis showed that the asparaginases did not affect the properties of the final product while achieving a reduction of acrylamide levels in cereal-based products by up to 85%. Positive sensory evaluations are particularly important for food manufacturers as other acrylamide mitigation strategies often produce undesirable sensory properties. These results will help the industry mitigate acrylamide to comply with specific benchmarks set by regulatory agencies.

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5. Compliance with ethics requirements

Appropriate protocols for protecting the rights and privacy of all participants in the sensory analysis were utilized during the execution of the research.

6. Informed consent

Not applicable.

CRediT authorship contribution statement

Shpresa Musa: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Laura Becker:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Claudia Oellig:** Writing – review & editing, Supervision, Resources, Project administration, Conceptualization. **Katharina Anne Scherf:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the first author used Grammarly in order to improve readability and language. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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APPENDIX A: SUPPLEMENTARY MATERIAL

Asparaginase Treatment to Mitigate Acrylamide Formation in Wheat and Rye Cookies

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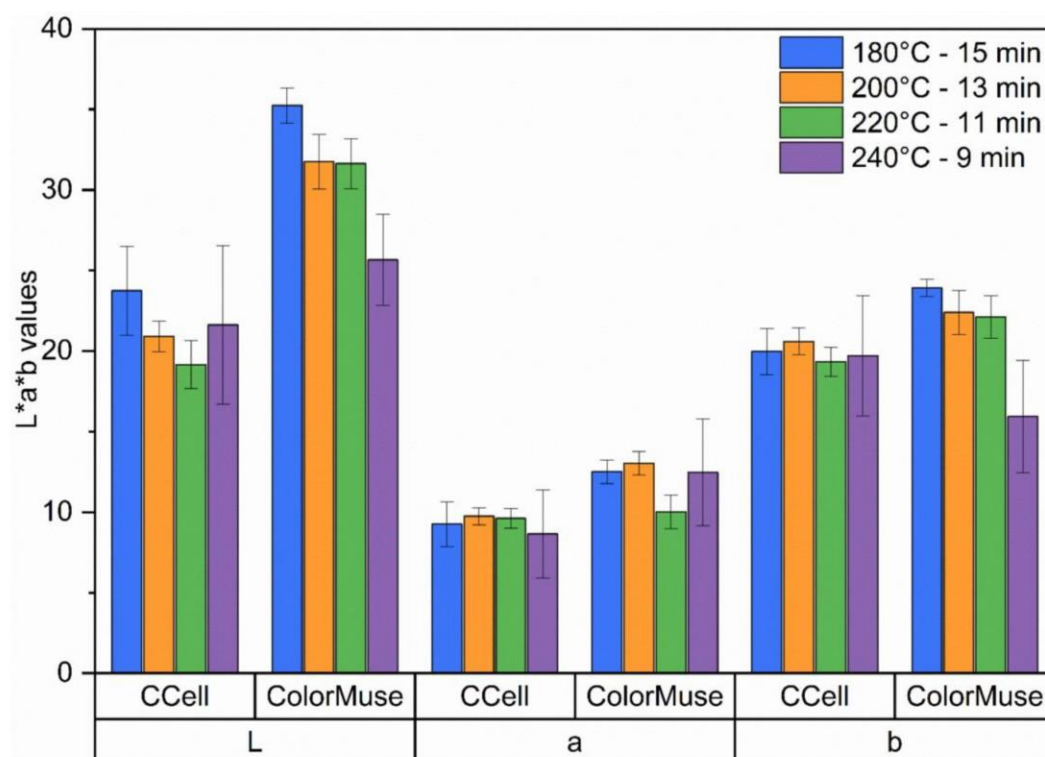


Figure S1. L* (lightness), a* (redness) and b* (yellowness) values obtained from CCell and ColorMuse for wheat cookies baked at 180 °C, 200 °C, 220 °C and 240 °C, for 15 min, 13 min, 11 min and 9 min, respectively. The values are presented as mean ± standard deviation of 6 controls.

Table S1. pH value of wholemeal wheat and rye cookie dough after incubation for 10 min at 60 °C and 70 °C.

	Sample ID	pH of dough
Wheat	Control	6.20 ± 0.01
	R	6.03 ± 0.01
	P	5.99 ± 0.01
	H	5.96 ± 0.01
	X	6.17 ± 0.01
Rye	Control	6.25 ± 0.01
	R	6.13 ± 0.01
	P	6.25 ± 0.02
	H	6.18 ± 0.02
	X	6.26 ± 0.01

Results are presented as means ± standard deviation (n = 3)

Table S2. Detailed p-values for all significant (*) treatments that are presented in Figures 1 and 2, as well as Tables 3, 4, and 5.

Figure/Table	Treatment	p-value
Figure 1b	180 °C - 15 min (60 °C vs 90 °C)	0.004
	240 °C - 9 min (60 °C vs 90 °C)	0.016
Figure 2a	R1, R2, A2, A3, H2, X2 & X3	<0.001
	H1	0.030
Figure 2b	R1, R2, A3, H1, H2, X2 & X3	<0.001
	A2	0.002
Table 3 - Wheat - CCell – L*	A2 & A3	<0.001
Table 3 - Wheat - CCell – a*	X2	0.010
	X3 & A2	<0.001
	A3	0.005
Table 3 - Wheat - CCell – b*	X2	0.002
	H2	0.011
	X3, A2 & A3	<0.001
Table 3 - Wheat - ColorMuse – L*	X2	0.010
	X3 & A2	<0.001
	A3	0.005
Table 3 - Wheat - ColorMuse – a*	X3 & A2	<0.001
	X2	0.002
	A3	0.005
Table 3 - Wheat - ColorMuse – b*	A2, X2 & X3	<0.001
	A3	0.005
Table 3 - Rye - CCell – L*	H1	0.013
Table 3 - Rye - CCell – a*	A2	0.014
	A3	0.033
Table 3 - Rye - CCell – b*	A2	0.029
	A3	0.037
	X3	0.014
Table 3 - Rye - ColorMuse – a*	X2	0.001
Table 3 - Rye - ColorMuse – b*	A2, A3, X2 & X3	<0.001
Table 4 - Wheat - Hardness	H2	0.006
	X2	0.002
Table 4 - Wheat - Fracturability	R1 & X3	0.005
	R2	0.002
	A3	0.025
	X3	<0.001
Table 5 - Wheat - Hardness	X3	0.044
Table 5 - Wheat - Crunchiness	X3	0.003

Supplementary method M1: Free asparagine content

The derivatization and subsequent extraction protocol for the measurement of free asparagine was conducted following the procedure described by Hölzle et al. (2023) with a slight modification. Three grams of milled flour were mixed with 20 mL water, followed by an extracting step on a horizontal shaker (VIBRAX VXR basic, IKA GmbH & Co. KG, Staufen, Germany) for 15 min at 2000 rpm and 22 °C, and in an ultrasonic bath (Sonorex digital 10 P, Brandelin electronic, Berlin, Germany) for 4 min at 22 °C. The extract solution was centrifuged (Multifuge X1R, Thermo Fisher Scientific, Karlsruhe, Germany) for 10 min at 5000 rpm and 22 °C. To an aliquot of the resulting supernatant (0.5 mL), the internal standard [$^{13}\text{C}_4$, $^{15}\text{N}_2$]-Asn (25 µg) was added and equilibrated by vortex (Vortex-Genie 2, Scientific Industries, Bohemia, USA) for 10 s at 22 °C. Subsequently, 1.5 mL of acetonitrile (ACN) was added, and the mixture was homogenized by vortex (Vortex-Genie 2, Scientific Industries) for 10 s before being centrifuged (Biofuge pico, Heraeus, Hanau, Germany) for 15 min at 13000 rpm and 22 °C. An aliquot of the supernatant (1 mL) was mixed with aqueous sodium hydrogen carbonate (0.25 mol/L, 20 mL), with the pH value adjusted to 8.5 ± 0.2 using aqueous sodium hydroxide (1 mol/L). Derivatization of the analyte and internal standard was carried out with benzoyl chloride (2 mg in 5 mL acetone) for 3 h at 22 °C in the dark under stirring. After derivatization, the pH value of the solution was adjusted to 2.5 ± 0.2 using aqueous hydrochloric acid (5 mol/L). Benzoylated Asn and internal standard were extracted by liquid-liquid extraction (LLE) with ethyl acetate for four times (total volume 40 mL) on the horizontal shaker (VIBRAX VXR basic, IKA GmbH & Co. KG), each time for 5 min at 2000 rpm and 22 °C. Finally, the combined organic phases were dried over anhydrous sodium sulfate, and ethyl acetate was removed at 2 MPa and 40 °C on a rotary evaporator (Laborota 4001 efficient; Heidolph, Schwabach, Germany). The residue was re-suspended in 300 µL ACN. An aliquot of the

sample (50 μ L) was diluted with aqueous 0.1% formic acid (w/v) to a total volume of 1 mL and centrifuged (Biofuge pico, Heraeus) at 13000 rpm for 10 min at 22 °C. The resulting supernatant was used for measurement with LC–MS, consisting of a HPLC (1100 Series, Agilent Technologies, Waldbronn, Germany) coupled to a single quadrupole mass spectrometer (G6120, Agilent Technologies). Chromatographic separation of the sample (injection volume 5 μ L) was performed on a Pyramid column (Nucleodur C₁₈ Pyramid, 150 mm $\times$ 4.6 mm i.d., 3 μ m, Macherey-Nagel, Düren, Germany) with the column oven tempered to 30 °C, using a gradient system of the mobile phase (A) 0.1% formic acid in water (w/v) and (B) 0.1% formic acid in ACN (w/v) with a constant flow rate of 0.5 mL/min. Starting from 15% B (0–4 min), the organic content in the eluent was increased to 80% (4–15 min) and then held for 2 min. Within 0.1 min, the initial conditions were set, and re-equilibration of the column was performed until a total runtime of 23 min. The eluate from the time interval of 6.5–10.5 min was directed into the mass spectrometer. Electrospray ionization in positive mode (ESI⁺) was used, with a capillary voltage of 3.5 kV, a drying gas temperature of 300 °C, a drying gas flow rate of 8 L/min and a nebulizer gas pressure of 40 psi. The quadrupole temperature was set to 100 °C. Measurement was performed in selected ion monitoring mode, targeting the molecule ions at m/z 237.1 (benzoylated Asn) and m/z 243.1 (benzoylated [¹³C₄, ¹⁵N₂]-Asn), with a fragmentation voltage of 100 V and a gain of 2.

Reference

Hölzle, E., Becker, L., Oellig, C., Granvogl, M. (2023). Heat-Introduced Formation of Acrylamide in Table Olives: Analysis of Acrylamide, Free Asparagine, and 3-Aminopropionamide. *Journal of Agricultural and Food Chemistry*, 71, 13508–13517. DOI: 10.1021/acs.jafc.3c03457.

6.2 Publication II

Authors: Shpresa Musa, Laura Becker, Claudia Oellig and Katharina Anne Scherf

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Influence of Asparaginase on Acrylamide Content, Color, and Texture in Oat, Corn, and Rice Cookies

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ABSTRACT: The safety of cereal-based baked goods can be compromised by acrylamide, a processing contaminant and class 2A carcinogen. One method to prevent acrylamide formation is by converting asparagine to aspartic acid using asparaginases. Four different asparaginases were tested using two dough incubation temperatures and dosages for oat, corn, and rice cookies. To evaluate the impact of asparaginases on product quality, color and texture were measured. Acrylamide was reduced by up to 97, 95, and 92% for oat, corn, and rice cookies, respectively, compared to the control. Asparaginase treatment resulted in minor changes in color and texture. There was a strong correlation between acrylamide concentrations in cookies and the free asparagine content of the flour. By minimizing the formation of acrylamide while maintaining product quality, the use of asparaginases offers a promising approach to enhancing food safety standards and protecting public health, potentially influencing regulatory guidelines and consumer preferences.

KEYWORDS: asparagine, baking, cereals, color, texture

1. INTRODUCTION

Acrylamide is a toxic compound formed during heat treatment due to reactions between reducing sugars and free asparagine in foods.^{1,2} It is classified as probably carcinogenic to humans (class 2A) by the International Agency for Research on Cancer.³ Numerous investigations have detected the presence of acrylamide in baked starchy foods and various fried products.^{4–6} To protect consumers, the European Food Safety Authority aims to minimize exposure following the “as low as reasonably achievable” (ALARA) principle at every phase of the food supply chain. The European Commission introduced Regulation (EU) 2017/2158, which outlines targeted measures to mitigate the acrylamide content in specific food categories. The affected products include those prone to acrylamide formation, such as potato products, bread, breakfast cereals, pastries, coffee, and baby food.⁷

While various reaction pathways are described for the formation of acrylamide, the primary formation pathway is attributed to the Maillard reaction involving free asparagine and reducing sugars via the intermediate 3-amino-propionamide.^{1,8–10} The formation of a Schiff base between a carbonyl group and the α -amino group of free asparagine is the first step in acrylamide formation. The Schiff base is decarboxylated under the influence of heat and forms acrylamide either directly via elimination of an imine or after hydrolysis via 3-amino-propionamide and elimination of ammonia.⁹ Granvogl and Schieberle proved this mechanism using stable isotope-substituted free asparagine in a model system and in food.¹⁰

The variety of proposed strategies to reduce acrylamide in food are classified into agronomy, recipe, and process-related changes.¹¹ In agronomy, the critical determinant for acrylamide is the content of free asparagine in the raw

material, e.g., cereal grains. The asparagine content varies widely depending on species, cultivars, and growing conditions, so that efforts to control asparagine through breeding hold promise. Strategies involving the recipe focus mainly on the ingredients such as using white flour instead of wholemeal flour, using sodium bicarbonate instead of ammonium bicarbonate as raising agent, changing the type of sugar, and adding asparaginase into the recipe. Processing strategies include fermentation, using low thermal input, or using new heat treatment methods.¹¹

The enzymatic approach to reduce acrylamide was first proposed by Amrein et al.¹² Asparaginases hydrolyze the main precursor asparagine into aspartic acid and ammonia is released during this reaction, resulting in decreased levels of free asparagine, therefore, lowering acrylamide formation during baking.¹³ This is why asparaginase treatment emerges as a prominent strategy to reduce acrylamide.^{9,12–15} Research on baked goods shows that the addition of asparaginases reduces acrylamide by up to 96%.^{13,16–18} Given the existing benchmark levels for acrylamide in cookies (350 $\mu\text{g}/\text{kg}$),⁷ addressing the reduction of acrylamide necessitates additional strategies for mitigating acrylamide in cookies and other products without affecting the texture or color of the final product. There is still a limited number of studies on bakery products and most of the studies available focus mainly on

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Table 1. Sample Names for Oat, Corn, and Rice Cookies, Including the Asparaginase Type, Concentrations, Incubation Parameters, and Baking Specifications

Sample ID ^a	Asparaginase	Asparaginase concentration (mg/kg)	Asparaginase activity ^b	Asparaginase amount in enzyme solution ^c	Incubation time (min)	Incubation temperature (°C)	Baking time (min)	Baking temperature (°C)
C-60 °C	None (control)	0	None added	None	10	60	11	220
R1	R	100	3500 ASNU/g	approximately 3% (w/w)	10	60	11	220
R2	R	200	3500 ASNU/g	approximately 3% (w/w)	10	60	11	220
A2	A	200	≥2500 ASPU/g	50% enzyme solution	10	60	11	220
A3	A	300	≥2500 ASPU/g	50% enzyme solution	10	60	11	220
C-70 °C	None (control)	0	None added	None	10	70	11	220
H1	H	100	6000 TASU/g	approximately 0.8% (w/w)	10	70	11	220
H2	H	200	6000 TASU/g	approximately 0.8% (w/w)	10	70	11	220
X2	X	200	47,000–57,500 XRU/g	50% enzyme solution	10	70	11	220
X3	X	300	47,000–57,500 XRU/g	50% enzyme solution	10	70	11	220

^aThe same sample ID system is used for oat, corn, and rice cookies. ^bASNU/g: 1 ASNU/g is defined as the amount of enzyme that produces 1 μ mol of ammonia per minute after hydrolysis of L-asparagine (reaction conditions: pH 7.0, 37 °C, 1.5 min).³³ ASPU/g: 1 ASPU/g is defined as the amount of enzyme that produces 1 μ mol of ammonia per minute after hydrolysis of L-asparagine (reaction conditions: pH 5.0, 37 °C, 30 min) (FAO, 2008).³⁴ TASU/g: Thermostable asparaginase units/g is used to express asparaginase activity relative to an internal enzyme standard using the same assay as for ASNU/g (EFSA Panel on Food Contact Materials, 2023).³⁵ XRU/g: 1 XRU is defined as the amount of enzyme required to release 1 μ mol of ammonia per minute from L-asparagine using the XRU asparaginase assay (personal communication with DSM). ^cThe composition of the enzyme solutions is obtained from Novozymes (R and H) and DSM (A and X), which can be available upon request for further details on each enzyme preparation.

wheat products and do not target other cereals such as oat, corn, and rice. These three cereals are used for a wide variety of bakery products worldwide, but little is known about their potential to form acrylamide. We hypothesize that the effectiveness of asparaginases may be different depending on the type of cereal. Therefore, this study aims to investigate the addition of asparaginases for mitigating acrylamide in oat, corn, and rice cookies by using different asparaginases, dosages, and dough incubation temperatures. Unlike previous studies, which primarily focus on wheat products, our research explores the impact of asparaginase treatment on a broader range of cereals, specifically oat, corn, and rice. This comprehensive approach will provide new insights into the relationship between free asparagine in different cereal flours and its impact on acrylamide formation. Additionally, any potential changes in the texture and color of cookies that may have been impacted by asparaginase addition will be evaluated. This study aims to fill the gap in current research and offer practical solutions for reducing acrylamide in a variety of cereal-based baked goods.

2. MATERIALS AND METHODS

2.1. Reagents and Ingredients. All chemicals and reagents were of analytical grade or higher. Baking powder, sugar, and sunflower oil were bought from a local supermarket. Wholemeal oat, corn, and rice flours were provided by IREKS (Kulmbach, Germany). Asparaginases R (Acrylaway L) and H (Acrylaway HighT) were donated by Novozymes (Bagsvaerd, Denmark) and A (PreventAse L) and X (PreventAse XR) by DSM (Heerlen, The Netherlands). The Acrylamide ES ELISA kit and Acrylamide derivatization kit were acquired from Eurofins (Eurofins Scientific SE, Luxembourg, No. 515680) and ISOLUTE Multimode 500 mg/3 mL (904–0050-B) and ISOLUTE ENV+ 200 mg/3 mL (915–0020-B) were from Biotage (Uppsala, Sweden).

2.2. Asparaginases. Four commercial asparaginases designated as R, T, A, and X were used. Prior to application, each enzyme was

dissolved in 10 mL of water. The enzymes were named as follows: R (Acrylaway L), T (Acrylaway HighT), A (PreventAse L) and X (PreventAse XR). Two different concentrations were used each for R and T, 100 mg/kg and 200 mg/kg, while 200 mg/kg and 300 mg/kg were used for asparaginases A and X. These dosages were selected according to Musa et al.¹⁵

2.3. Dough Preparation. The recipe used in this study was according to Musa et al.¹⁵ The dry components including flour (100 g) which was either oat (*Avena sativa*), corn (*Zea mays*), and rice (*Oryza sativa*) flour, sugar (15.3 g), and baking powder (0.33 g) were blended for 1 min at low speed using a commercial food processor (Robert Bosch GmbH, Stuttgart, Germany). Subsequently, water alone (as a control) or water mixed with asparaginase (48 mL) was introduced along with the oil (12.6 mL). The dough underwent mixing for 3 min at medium speed and an additional 30 s at high speed.

2.4. Incubation and Baking Conditions. Following the separate mixing of oat, corn, and rice cookie dough at 22 °C, the dough underwent incubation at 60 °C for 10 min for asparaginases R and A, and at 70 °C for 10 min for asparaginases T and X. A corresponding control sample was included for each incubation temperature. After incubation, the dough was rolled out to a thickness of 0.5 cm and a diameter of 6 cm using a rolling pin. Subsequently, all samples were baked at 220 °C for 11 min. An overview of all important parameters for sample preparation is listed in Table 1.

2.5. Acrylamide Analysis. The quantitation of acrylamide in cookies was carried out using an Acrylamide ES ELISA kit in combination with the derivatization kit according to Musa et al.¹⁵ The assay was carried out exactly according to the ELISA kit guidelines.¹⁹ The samples were extracted by adding 40 mL of distilled water to 2 g of the sample and mixing for 30 min. After 5 min of sedimentation, the sample extracts were filtered and centrifuged for 5 min at 13,000g. The sample cleanup technique was carried out with Multimode SPE and ENV+ SPE columns. The extracted acrylamide was eluted from the column using a mixture of methanol/water (60:40 v/v). This eluent was derivatized and analyzed following the assay protocol. Acrylamide levels were determined by measuring the absorbance at

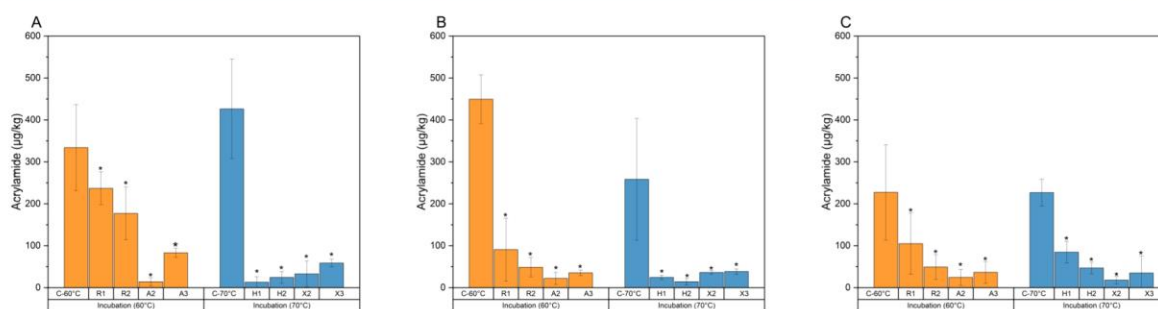


Figure 1. Acrylamide content in oat (A), corn (B), and rice (C) cookies without or with the addition of asparaginases. Error bars indicate the standard deviation of the mean, and asterisks indicate a significant difference compared to the respective control (ANOVA with Dunnett's *t* test $p \leq 0.05$, $n = 6$). For sample abbreviations, please refer to Table 1.

Table 2. Reduction of Acrylamide for Oat, Corn, and Rice Cookies, for Each Treatment Including Both Incubation Temperatures of 60 and 70 °C

Sample ID	Oat cookies		Corn cookies		Rice cookies	
	Acrylamide (µg/kg)	Reduction of acrylamide (%)	Acrylamide (µg/kg)	Reduction of acrylamide (%)	Acrylamide (µg/kg)	Reduction of acrylamide (%)
Control (60 °C)	333.95		449.33		227.31	
R1	236.90	29.1	90.76	79.8	105.04	53.8
R2	177.04	47.0	48.66	89.2	49.19	78.4
A2	14.10	95.8	25.96	94.2	24.51	89.2
A3	83.08	75.1	35.42	92.1	36.56	83.9
Control (70 °C)	426.36		258.48		226.71	
H1	13.07	96.9	24.38	90.6	84.55	62.7
H2	24.46	94.3	14.27	94.5	46.96	79.3
X2	32.90	92.3	36.37	85.9	17.96	92.1
X3	58.72	86.2	38.52	85.1	34.95	84.6

450 nm using a photometer (Tecan, i-control infinite 200Pro, Männedorf, Switzerland). The extraction efficiency using three levels of spiking (low, medium, high) had a recovery rate from 61% to 171%. The Acrylamide ELISA kit has a limit of detection of 50 µg/kg for food samples. The ELISA kit includes six standards with concentrations of 0, 2.5, 5, 10, 25, 50, and 200 ng/mL.¹⁹

2.6. pH Analysis of Cookie Dough. The pH value of cookie dough was measured according to Begemann et al.²⁰ The dough (10 g) was placed into a 250 mL beaker. Then, 5 mL of acetone and 40 mL of distilled water were added and thoroughly mixed until fully dispersed using an Ultra-Turrax (IKA T25, Staufen, Germany). The remaining 60 mL of water were incorporated, and the pH value was determined using a pH meter (SI Analytics, LAB 845, Mainz, Germany).

2.7. Free Asparagine in Flour. The quantitation of the free asparagine content in flour was performed using the method described by Hölzle et al.²¹ In brief, after aqueous extraction of the oat, corn, or rice flours, the internal standard [¹³C₄,¹⁵N₂]-asparagine was added to an 0.5 mL aliquot, followed by the addition of 1.5 mL acetonitrile (ACN) and vortexing (Vortex-Genie 2, Scientific Industries, Bohemia, USA). The sample was centrifuged and 1 mL of the supernatant was used for the derivatization and subsequent extraction procedure. After resuspension of the residue in 300 µL ACN, the sample was diluted with aqueous 0.1% formic acid (v/v). The measurement was performed with LC–MS, consisting of a HPLC (1100 Series, Agilent Technologies, Waldbronn, Germany) coupled to a single quadrupole mass spectrometer (G6120, Agilent Technologies), using a Pyramid column (Nucleodur C₁₈ Pyramid, 150 mm × 4.6 mm i.d., 3 µm, Macherey-Nagel, Düren, Germany) with a gradient system consisting of the solvents 0.1% formic acid in water (v/v) and 0.1% formic acid in ACN (v/v). Electrospray ionization in positive mode (ESI⁺) was used, targeting the molecule ions at *m/z* 237.1 (benzoylated asparagine) and *m/z* 243.1

(benzoylated [¹³C₄,¹⁵N₂]- asparagine) in selected ion monitoring (SIM) mode.

2.8. Assessment of Color Properties. Two distinct methods were employed to determine color values, CCell (Calibre Control, Warrington, UK) and ColorMuse (Variable Inc., Chattanooga, TN, USA), respectively, according to Musa et al.¹⁵ Both devices measure L* (lightness), a* (green-red), and b* (blue-yellow) values. Before use, CCell was calibrated using a calibration card (CC006, Calibre Control, Warrington, UK) and Color Muse using its white background lid. For CCell analysis, the samples were positioned on a blue background during measurement. For ColorMuse, the samples were placed on a white background, and the device was positioned 0.5 cm above the sample. Two measurements were taken per sample for both devices to scan the top surface and the bottom of the cookies. A total of six cookies were analyzed from three batches.

2.9. Assessment of texture properties. The texture analysis of cookies was conducted using a T.A.X.Tplus texture analyzer (Stable Micro Systems, Godalming, UK) equipped with a 5 kg load cell.¹⁵ A three-point bending rig (HDP/3PB) and a heavy-duty platform (HDP/90) were employed to break the cookies. The hardness (N) and fracturability (mm) of the cookies were measured using the "Measurement of the hardness and resistance of biscuits/cookies to bend or snap" method available in the Exponent software (Stable Micro Systems, Version 6.1.20.0, Godalming, UK). Two cookies were analyzed per batch and a total of six samples from three batches.

2.10. Statistical Analysis. Microsoft Excel was used to calculate means and standard deviations. Origin 2023 (OriginLab Corporation, Northampton, MA, USA) was used for statistical analysis and Pearson's correlations and IBM SPSS Statistics (IBM Corp, Version 29.0. Armonk, NY) was utilized for Dunnett's *t* test ($p \leq 0.05$) to identify significant differences between the control and asparaginase-treated samples. For each treatment, three technological replicates (i.e., cookie batches) were prepared. For texture and color analysis, a

total of six cookies were analyzed. For acrylamide analysis, one sample was analyzed for each batch ($n = 3$) and two analytical replicates were performed when pipetting into 96-well plates. The average acrylamide concentrations of the samples were determined using the online ELISA calculator provided by Arigo.²²

3. RESULTS

3.1. Acrylamide Content. To evaluate the impact of different asparaginases on acrylamide reduction in oat, corn, and rice cookies, first, the dough was incubated at 60 and 70 °C for 10 min. The cookies were baked at 220 °C for 11 min. Figure 1 shows the acrylamide content in the control (no addition of asparaginases) and treated samples for oat, corn, and rice cookies. Additionally, in Table 2, the percentage (%) reduction of acrylamide for each treatment for all types of cookies is included. Oat cookies incubated at 60 and 70 °C showed a significant ($p \leq 0.05$) reduction of acrylamide for all treatments compared to the control which contained 333 $\mu\text{g/kg}$ (60 °C) and 424 $\mu\text{g/kg}$ (70 °C) of acrylamide. The treatment with asparaginase R showed a lower reduction ($R1 = 236 \mu\text{g/kg}$, $R2 = 177 \mu\text{g/kg}$) compared to treatments A ($A2 = 14 \mu\text{g/kg}$, $A3 = 83 \mu\text{g/kg}$), H ($H1 = 13 \mu\text{g/kg}$, $H2 = 24 \mu\text{g/kg}$), and X ($X2 = 32 \mu\text{g/kg}$, $X3 = 58 \mu\text{g/kg}$). A higher asparaginase concentration for R resulted in a greater reduction of acrylamide. For asparaginases A, H, and X, the higher dosages did not further decrease acrylamide. All of them resulted in a significant reduction, with the best treatment (H1) resulting in a reduction of acrylamide up to 96.9%. Corn cookies had a higher acrylamide content for control samples incubated at 60 °C (449 $\mu\text{g/kg}$), than for those at 70 °C (258 $\mu\text{g/kg}$). Acrylamide variations were noted within the samples, especially in the controls, primarily attributed to elevated but uneven acrylamide formation on the cookie surface rather than within. Asparaginases applied in corn cookies provided a reduction of acrylamide starting from 79.0% and up to 94.5% using H2 treatment, which resulted in the highest reduction. For asparaginases R and H, the higher asparaginase dosage of 200 mg/kg further decreased acrylamide by 42 $\mu\text{g/kg}$ and 10 $\mu\text{g/kg}$, respectively. The same trend was also observed for rice cookies where an increased dosage of R and H further decreased acrylamide by 56 $\mu\text{g/kg}$ and 38 $\mu\text{g/kg}$, respectively. Control samples for rice cookies showed a lower formation of acrylamide (227 $\mu\text{g/kg}$ and 225 $\mu\text{g/kg}$ for 60 and 70 °C, respectively) compared to oat and corn. All treatments significantly reduced acrylamide for rice cookies, with the X2 treatment achieving the highest reduction by up to 92.1% in comparison to the control. Both oat and corn control samples exceeded the benchmark levels of 350 $\mu\text{g/kg}$ of acrylamide set by the EU, whereas rice control samples were below the benchmark. Rice flour most likely inhibited the formation of acrylamide in the final product due to its lower content of free asparagine (90 mg/kg) compared to oat and corn flours. Because the baking conditions and recipes for the three types of cookies were identical, the reduced level of free asparagine in rice flour resulted in less acrylamide formation. Consequently, the lower availability of this precursor in rice flour directly led to a lower concentration of acrylamide in cookies made with rice flour than in those made with oat and corn flour.

3.2. pH Value of Dough. Since the cookie recipe included baking powder and the pH value influences acrylamide formation, the pH value of oat, corn, and rice cookie dough was measured after incubation. A slightly higher pH was

observed for oat cookie doughs incubated with asparaginase R (pH = 6.15) compared to the control (pH = 6.03). The control for corn (pH = 5.82) and rice (pH = 6.02) had a slightly higher pH compared to the H treatment for corn (pH = 5.75) and rice (pH = 5.94). However, these small pH differences are not expected to significantly affect acrylamide formation in cookies.

3.3. Free Asparagine Content. The content of free asparagine in wholemeal oat, corn, and rice flour was 500 mg/kg, 220 mg/kg, and 90 mg/kg, respectively. There was a significant ($p \leq 0.05$) correlation $r = 0.876$ (Table 3) between

Table 3. Relationship Between Free Asparagine Content and Acrylamide Formation in Flour Samples

Flour type	Free asparagine content in flour (mg/kg)	Acrylamide content in baked cookies ($\mu\text{g/kg}$)	Pearson's r -value
Rye ^a	700 $\pm$ 8.5	790 $\pm$ 254.5	0.876
Oat	500 $\pm$ 9.7	379 $\pm$ 110.7	
Wheat ^a	290 $\pm$ 6.0	276 $\pm$ 12.3	
Corn	220 $\pm$ 2.0	353 $\pm$ 101.1	
Rice	90 $\pm$ 1.1	226 $\pm$ 72.7	

^aWheat and rye flour were investigated earlier¹⁵ and used only for correlation between acrylamide and free asparagine content.

the acrylamide and free asparagine content in flours of the three cereals in this study and wheat and rye, which were tested earlier.¹⁵ The r -value suggests a strong positive linear relationship, meaning that acrylamide content tends to increase as free asparagine in flour increases. This shows that grains with higher asparagine content indeed promote higher formation of acrylamide in the final product.

3.4. Color Measurement. Two color scanning devices, CCell and Color Muse, were used to assess the color of baked cookies. Both devices give $L^*a^*b^*$ values which represent the lightness (L^*), redness (a^*), and yellowness (b^*) of the product. Figure 2 presents all values from each treatment and controls for oat (A), corn (B), and rice (C) cookies. In oat cookies, the L^* value (Figure 2A) was significantly lower for H2 (CCell), X2, and X3 (Color Muse) treatments compared to the control. The a^* and b^* values showed a significant decrease for A2, A3, X2, and X3, while H2 significantly increased for a^* value. Figure 2B shows that treatments R1, R2 and A2 (CCell), and H1 (Color Muse) significantly increased the L^* value for corn cookies, while the b^* value decreased only by A2 and A3 treatments compared to the control. A significant impact on the b^* value was observed in all treatments, except for X2 and X3 treatments. For rice cookies, the L^* value (Figure 2C) showed a significant decrease for all treatments with Color Muse, except X3, while the L^* value measured using CCell showed a significant decrease only for H1 and A2 treatments, with other treatments showing no significant effect. The a^* value was significantly increased for H1 and A2 treatments and the b^* value for H1, H2, and A3 treatments for CCell compared to the control. The color measured with Color Muse increased for A2 and A3 treatments (a^* value) and R1, R2, A2, A3, X2, and X3 treatments (b^* value). Although these values were statistically different, the changes were not visible to the eye after baking for most of the treatments which shows that these significant differences might not be meaningful.

3.5. Texture Properties. The hardness and fracturability of oat, corn, and rice cookies are presented in Table 4. Cookies

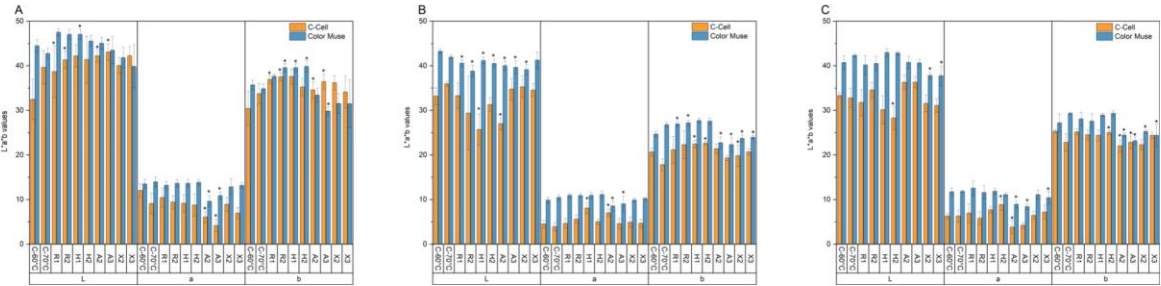


Figure 2. L* (lightness), a* (redness), and b* (yellowness) values obtained from CCell and ColorMue for oat (A), corn (B), and rice (C) cookies baked at 220 °C for 11 min. Error bars indicate the standard deviation of the mean, and asterisks indicate a significant difference compared to the respective control (ANOVA with Dunnett's *t* test *p* ≤ 0.05, *n* = 6). For sample abbreviations, please refer to Table 1.

Table 4. Hardness and Fracturability of Oat, Corn, and Rice Cookies^a

Texture parameter	Sample ID ^b	Oat	Corn	Rice
Hardness (N)	Control (60 °C)	26.86 ± 3.28	43.64 ± 14.18	31.59 ± 6.53
	R1	22.32 ± 5.77	44.17 ± 10.71	35.75 ± 8.13
	R2	24.26 ± 5.04	51.65 ± 8.14	37.41 ± 10.39
	A2	16.79 ± 2–88	18.49 ± 6.41*	32.68 ± 7.29
	A3	16.89 ± 2.30	17.97 ± 7.84*	26.52 ± 5.61
	Control (70 °C)	18.54 ± 3.84	43.43 ± 6.16	38.68 ± 8.46
	H1	26.2 ± 1.92*	46.94 ± 10.73	43.03 ± 13.32
	H2	19.98 ± 2.56	46.07 ± 9.48	35.57 ± 9.04
	X2	22.26 ± 2.84	31.1 ± 9.54	61.44 ± 1.23*
	X3	21.16 ± 2.70	29.62 ± 8.66*	35.19 ± 8.83
Fracturability (mm)	Control (60 °C)	0.97 ± 0.10	1.73 ± 0.14	1.03 ± 0.11
	R1	1.30 ± 0.52	1.08 ± 0.20	1.08 ± 0.16
	R2	1.04 ± 0.14	1.45 ± 0.23	1.05 ± 0.16
	A2	0.95 ± 0.10	1.25 ± 0.52	0.96 ± 0.11
	A3	1.15 ± 0.31	1.47 ± 0.44	1.22 ± 0.33
	Control (70 °C)	1.84 ± 0.41	1.23 ± 0.15	1.16 ± 0.16
	H1	1.31 ± 0.16	1.16 ± 0.21	0.92 ± 0.20
	H2	0.72 ± 0.80*	1.49 ± 0.21	1.47 ± 0.66
	X2	1.56 ± 0.59	1.10 ± 0.50	1.67 ± 0.50
	X3	1.07 ± 0.24*	1.20 ± 0.20	1.20 ± 0.27

^aAsterisks indicate a significant difference compared to the respective control (ANOVA with Dunnett's *t* test *p* ≤ 0.05, *n* = 6, means ± standard deviation) ^bFor sample ID, please refer to Table 1

incubated at 60 °C prior to baking, showed no significant changes in hardness and fracturability compared to the control when asparaginases were applied. Oat cookies incubated at 70 °C showed a significant increase in hardness for the H1 treatment and a significant decrease in fracturability for the H2 and X3 treatments. Texture analysis for corn cookies showed that treatments A2, A3, and X3 significantly reduced the hardness. The treatments applied showed no significant changes in the fracturability of corn cookies. For rice cookies, only the X2 treatment significantly increased the hardness of rice cookies, while other treatments did not affect the hardness or fracturability of rice cookies.

4. DISCUSSION

Cookies from oat, corn, and rice wholemeal flours were investigated to see if these cereals promote acrylamide formation more or less than standard wheat flour which is mainly used for cookies. Both oat and corn cookies without asparaginase addition exceeded the benchmark levels of 350 μg/kg using the recipe and baking conditions described. Our earlier study using asparaginases in cookies made from

wholemeal wheat and rye flours also reported acrylamide exceeding the benchmark level.¹⁵ This underlines the need to use asparaginases or other mitigation strategies to meet the regulatory requirements. Additionally, a reduction of acrylamide by up to 85% was observed.¹⁵ In the current study, a reduction of up to 97%, 95%, and 92% for oat, corn, and rice cookies, respectively, was observed. Another study also investigated the effect of asparaginases in rotary cut biscuits made from wheat flour and reported an acrylamide reduction by up to 99%. An increased dosage of asparaginases in dough is expected to decrease acrylamide further, but in the current study, this was not always the case. One limitation of our work is that the enzyme preparations were used as recommended by the manufacturers and their activity was not normalized for direct comparisons of effectiveness. The dough incubation temperature is often a critical parameter for asparaginase activation. There were no changes in acrylamide content for control rice cookies incubated at 60 and 70 °C, while control samples for oat and corn cookies formed slightly more or less acrylamide, respectively, depending on the temperature. Musa et al.¹⁵ investigated the effect of incubation time and

temperature and reported that there are no significant changes in acrylamide content regardless of incubation time or temperature. Another study also reported significant decreases in acrylamide content when applying asparaginase treatments to wheat biscuits.¹³ They found that the incubation time is one of the key factors in asparaginase efficiency, therefore an increased incubation time from 15 to 30 min further decreased acrylamide. However, in the current study, the effect of incubation time was not evaluated, as 10 min were sufficient in our earlier study.¹⁵ It is known that the ingredients in the recipe also influence asparaginase activity. Žilić et al. reported that biscuits made of hull-less oat flour resulted in the formation of more acrylamide compared to maize flour and this was in line with higher free asparagine concentration in oat.²³ Here, oat (500 mg/kg) had more free asparagine compared to corn flour (220 mg/kg), but acrylamide formation was comparable for oat and corn cookies with an average of 379 $\mu\text{g/kg}$ and 353 $\mu\text{g/kg}$ acrylamide, respectively, as average of both incubation temperatures. In contrast, rice flour contained the lowest free asparagine concentration of 90 mg/kg and also resulted in the lowest average acrylamide formation (226 $\mu\text{g/kg}$). Based on acrylamide reduction (Table 2), we showed that asparaginase treatment has a direct effect on reducing acrylamide in the final baked product, which suggests that also the amount of asparagine after baking might be lower due to the conversion of asparagine into aspartic acid and ammonia. While the final asparagine amount after baking was not determined in this study, this will be considered for future research. Free asparagine in grains is impacted, e.g., by growing conditions, time of harvest, and storage conditions which is why results for asparagine and acrylamide may differ between studies. A study that compared acrylamide formation using different rice and wheat flours reported a higher formation of acrylamide in wheat flour cookies compared to rice.²⁴ To our knowledge, the efficacy of asparaginases in oat, corn, and rice cookies in reducing acrylamide has not been investigated before.

The pH value of dough is known to affect acrylamide formation and enzyme activation. Since baking powder was used in our recipe, the pH of the dough was measured after the incubation and the range of 5.75–6.15 is optimal for asparaginase activation.¹⁷ Kukurová et al.¹⁷ reported that an increase in pH decreased the efficiency of asparaginase treatment. Further, the use of sodium bicarbonate did not adversely affect acrylamide, but they reported higher pH values (8.10) than in the current study, due to higher amounts of sodium bicarbonate (2 g/80 g flour) in their recipe. Lowering the pH of dough is another alternative that has proven to be efficient in mitigating acrylamide, but this method promotes the formation of 3-monochloropropane-1,2-diol (3-MCPD) as a processing contaminant and therefore is not a recommended strategy for acrylamide reduction.²⁵

There was a strong correlation ($r = 0.876$) between free asparagine and acrylamide content depending on the cereal species including oat, corn, and rice flour as well as wheat and rye flour from our earlier study,¹⁵ even if oat and corn were more similar in terms of acrylamide formation than would be expected based on free asparagine. Others also reported that free asparagine in grain is significantly correlated with acrylamide in the final product.^{26,27} Some studies target free asparagine as a strategy to reduce acrylamide, but agronomic methods require field trials and take more time to implement than processing variations such as asparaginase addition.

Two devices were used for color analysis, CCell and Color Muse, respectively. CCell is a well-known method for the analysis of color and other parameters of bakery products, but its price is comparatively high compared to Color Muse which is a small, portable device that is only suitable for color analysis. Even though there were some statistically significant color changes when applying asparaginases, the changes were not visible to the eye. These changes occurred for L^* , a^* , and b^* values, regardless of whether the color was scanned with CCell or Color Muse and no apparent trend was observed for the changes. These results are in agreement with our earlier study on wheat and rye cookies.¹⁵ Other studies did not show any changes in the color of the products when asparaginases were applied.^{16,28} Anese et al.¹⁶ reported no color changes regardless of the asparaginase concentration in cookie dough, but differences when various incubation temperatures were tested, 20 °C, 37 °C, and 54 °C, respectively. In the current study, the incubation temperature did not influence the color of the cookies. Considering that the temperatures only differed by 10 °C in the current study, this could be why color differences were not observed. Furthermore, the significant changes observed for some treatments in oat or corn were not significant for rice cookies or vice versa. It is likely due to the baking oven that some spots were darker on the surface of the cookies depending on the individual batch. Since the color appearance during visual inspection after baking did not change, it is safe to say that asparaginases provide an effective way of reducing acrylamide without altering the color properties of cookies. Further, there was no correlation between the data from CCell and Color Muse, which aligns with our earlier study.¹⁵ Both color scanning devices can detect differences between the control and each asparaginase treated sample and may be used for this purpose. Therefore, Color Muse can be used as a cheaper alternative compared to CCell, if only the color of the product is of interest. Measurement of color is important, not only for detecting quality parameters such as color changes due to the application of different mitigation strategies to reduce acrylamide, but also because there is a strong relationship between color and acrylamide content.^{27,29–32} These studies all agree that the darker the color of the product is after baking the higher the formation of acrylamide is in the product.

Texture results for oat, corn, and rice cookies showed that asparaginases may affect flours from different grains. Considering that the recipe, incubation, and baking conditions were the same, the changes observed for the cookies were different. Only two treatments resulted in larger changes of texture, with the H2 treatment for oat cookies causing a 54% decrease in fracturability and the A3 treatment for corn cookies leading to a 58% decrease in hardness. This is also linked to the processing conditions (human error), as the texture is very sensitive, and even baking for a few seconds less or more can affect both hardness and fracturability. A slight decrease of crumb firmness was reported by Kumar et al.¹⁸ when asparaginase was applied in bread. A different study that also used asparaginases for acrylamide reduction in cookies reported no significant changes in texture, color, and taste,¹⁷ which agrees with our earlier study.¹⁵ Based on the current and other studies,^{15,17,18,28} asparaginase treatments seem to have little or no effect on the color, texture, and taste of bakery products while providing, in most cases, acrylamide reductions below benchmark levels for cookies (350 $\mu\text{g/kg}$). However, the application of asparaginases, such as dosage, dough incubation

temperature, and time, must be product-specific as several factors such as moisture and pH of the product can influence asparaginase activation and are crucial factors for their effectiveness. Therefore, this study recommends the application of asparaginases regardless of what type of wholemeal flour is used due to its efficacy on acrylamide reduction and little or no effect on color and texture properties. The need for acrylamide reduction is immediate for the food industry as the European Commission intends to replace benchmark levels with maximum levels in food. Therefore, easy, fast, and efficient methods such as the application of asparaginases are needed. This study provides more information on acrylamide formation and asparaginase efficacy in different cereals to help the food industry make more effective decisions for acrylamide mitigation.

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S.M.: conceptualization, methodology, formal analysis, investigation, data curation, visualization, and writing (original draft). L.B.: methodology, formal analysis, investigation, and writing (review and editing). C.O.: conceptualization, supervision, project administration, resources, and writing (review and editing). K.A.S.: conceptualization, supervision, project administration, funding acquisition, resources, and writing (review and editing)

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Notes

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6.3 Publication III

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Article: Mitigation of Acrylamide in Cookies and Crispbread Using Calcium Salts and Phenolic Acids in Combination with Asparaginase as well as Rosemary Extract

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Mitigation of acrylamide in cookies and crispbread using calcium salts and phenolic acids in combination with asparaginase as well as rosemary extract[☆]

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ABSTRACT

Acrylamide is formed during high-temperature treatment in foods and presents a significant health and regulatory challenge. This study evaluates the effects of calcium salts and phenolic acids alone or in combination with asparaginase and rosemary extract in wheat cookies and rye crispbread. Acrylamide content, product color, texture, and sensory properties were assessed. When calcium salts and phenolic acids were used alone, acrylamide was reduced by 46 % and 50 % compared to the control. A combination of these with asparaginase resulted in a reduction of acrylamide by up to 89 % using ellagic acid. Specific treatments reduced cookie hardness, but asparaginase addition reversed this effect. Color mainly remained unaffected. Sensory analysis of selected treatments confirmed no significant changes in cookie aroma, taste, color, texture, and acceptability. This work provides a new approach by combining selected treatments to mitigate acrylamide while preserving product quality.

1. Introduction

Acrylamide is formed during high-temperature food preparation, particularly in carbohydrate-rich foods via the Maillard reaction between free asparagine and reducing sugars (Granvogl & Schieberle, 2006; Mottram, Wedzicha, & Dodson, 2002; Stadler et al., 2002; Tareke, Rydberg, Karlsson, Eriksson, & Törnqvist, 2002). Acrylamide was first detected in baked and fried foods in 2002 by researchers in Sweden (Swedish National Food Administration, 2002; Tareke et al., 2002). This finding sparked a significant health concern due to the 2 A classification of acrylamide as probably carcinogenic to humans (IARC, 1994). The European Food Safety Authority (EFSA) found that the daily exposure in the European population of adolescents, adults, the elderly, and the very elderly amounts to 0.4 to 0.9 µg/kg (EFSA, 2015). This is why Commission Regulation 2017/2158 of the European Union established mitigation measures and benchmark levels for various foods (European Union, 2017). Based on this Regulation, a benchmark of 350 µg/kg of acrylamide applies to cookies and crispbread.

Numerous studies have focused on investigating the acrylamide-forming potential in different baked goods (Amrein, Schönbachler, Escher, & Amado, 2004; Fredriksson, Tallving, Rosén, & Åman, 2004; Granvogl & Schieberle, 2006; Hendriksen, Kornbrust, Østergaard, & Stringer, 2009; Jung, Choi, & Ju, 2003; Mestdagh, de Wilde, Delporte, van Carlos, & de Meulenaer, 2008). The three main approaches for acrylamide reduction focus on agronomy, the recipe, and processing conditions (FoodDrinkEurope, 2019). The content of free asparagine in the raw material, such as cereal grains, is the crucial factor in agronomy determining the acrylamide content in the final product. Breeding efforts to control asparagine show promise because the content of asparagine varies greatly depending on species, cultivars, and growing conditions. The use of raw materials with a low asparagine content has been a focus of a few studies (Oddy et al., 2023; Raffan & Halford, 2019; Swiacka et al., 2024). Recipe-related strategies mostly center on the ingredients, such as using white flour instead of wholemeal flour due to the higher acrylamide-forming potential in wholemeal flour associated with the higher free asparagine content in wheat bran, switching to

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sodium bicarbonate as a raising agent rather than ammonium bicarbonate, altering the type of sugar, such as replacing fructose with glucose or non-reducing sugars, and including asparaginase in the recipe. Among the processing techniques are low heat input, fermentation, and novel heat treatment techniques (FoodDrinkEurope, 2019). One of the most common approaches is to reduce thermal input, which has been proven to be an effective way of mitigating acrylamide in cereal-based products (Bråthen & Knutsen, 2005; Mesias, Delgado-Andrade, & Morales, 2024; Mustafa, Andersson, Rosén, Kamal-Eldin, & Åman, 2005; Perestrelo et al., 2024; Wu, Yan, Cao, & Yuan, 2024).

Bakery products are responsible for the highest dietary acrylamide intake (EFSA, 2015). Therefore, many studies have investigated different strategies for reducing acrylamide in cereal-based products. Acrylamide mitigation can be achieved by adapting the recipe, including pH decrease and fermentation, and adding ingredients such as organic acids, calcium salts, and co-ingredients. Some studies showed that the addition of calcium salts could have a positive impact on mitigating acrylamide (Açar, Pollio, Di Monaco, Fogliano, & Gökmen, 2012; Batuwita, Jayasinghe, Marapana, & Jayasinghe, 2024; Gökmen, Açar, Köksel, & Açar, 2007; Kukurová, Ciesarová, Bednáriková, & Marková, 2009; Liang, Wang, & Sung, 2014) with a reduction of up to 60 % in model cookies. Another focus has been on adding polyphenols and plant extracts, representing a promising strategy for mitigating acrylamide (Mildner-Szkudlarz, Różańska, Piechowska, Waśkiewicz, & Zawirska-Wojtasiak, 2019; Oral, Dogan, & Sarioglu, 2014). Furthermore, the addition of asparaginases (Anese, Quarta, & Frias, 2011; Hendriksen et al., 2009; Kukurová, Morales, Bednáriková, & Ciesarová, 2009; Masciola et al., 2025; Musa, Becker, Oellig, & Scherf, 2024a, 2024b; de Souza, Chávez, Fioravante Guerra, Gottschalk, & Freitas-Silva, 2025), replacement of reducing sugars (Amrein et al., 2004), and ammonium salts (Amrein, Andres, Manzardo, & Amado, 2006) has been investigated. However, combining these strategies with asparaginase remains unexplored.

We hypothesized that combining salts and phenolic acids with asparaginase would reduce acrylamide more effectively than asparaginase alone. To test this, the study aimed to evaluate the effectiveness of calcium chloride, calcium carbonate, ellagic acid, and gallic acid, individually and in combination with asparaginase, in mitigating acrylamide formation in wholemeal wheat cookies and wholemeal rye crispbread. Additionally, the potential of incorporating rosemary extract was explored. The effects of these treatments on color and texture were assessed, along with any sensory alterations in cookies. To our knowledge, our study is the first to systematically evaluate the combined effects of these mitigation strategies in cereal-based products, specifically wholemeal wheat cookies and wholemeal rye crispbread. While Regulation (EC) No 1333/2008 lists only calcium carbonate as an additive for cereal-based foods, we further investigated the efficacy of calcium chloride and phenolic acids in reducing acrylamide. These compounds have shown promising results in other food matrices and are either approved for different food applications or recognized for their antioxidant properties. By generating robust scientific data, we aim to provide insights that could support their potential inclusion in future regulatory frameworks, offering additional tools for the industry to enhance food safety and quality (European Commission, 2008).

2. Materials and methods

2.1. Reagents and ingredients

All chemicals and reagents were of analytical grade or higher. Baking powder (consisting of E 450 (diphosphates), E 500 (sodium carbonates), and starch), sugar, salt, and sunflower oil were bought from a local supermarket. Wholemeal wheat and rye flour were provided by IREKS (Kulmbach, Germany). Calcium chloride (CaCl₂), calcium carbonate (CaCO₃), gallic acid (GA), and ellagic acid (EA) were purchased from Thermo Fisher Scientific (Waltham, MA, USA) or VWR (West Chester,

PA, USA). Rosemary extract (RE) was purchased from MIVALIED (Buchholz, Germany). Asparaginase Acrylaway L was donated by Novonesis (Bagsværd, Denmark). The Acrylamide ES ELISA kit and Acrylamide derivatization kit were acquired from Eurofins (Eurofins Scientific SE, Luxembourg), and ISOLUTE Multimode (500 mg/3 mL) and ISOLUTE ENV+ (200 mg/3 mL) were from Biotage (Uppsala, Sweden).

2.2. Addition levels of salts, phenolic acids, rosemary extract, and asparaginases

For cookies, CaCl₂, CaCO₃, ellagic acid, and gallic acid were added to the dough at three amounts: 100 µmol, 50 µmol, and 5 µmol, respectively. Each ingredient was added alone or in combination with 50 mg/kg Acrylaway L (abbreviated as R). For crispbread, only CaCl₂ and ellagic acid were tested using a combination of 100 µmol, 50 µmol, and 5 µmol, with R. CaCl₂ was selected due to its better solubility in water compared to CaCO₃. Ellagic acid was chosen due to better acrylamide reductions than gallic acid in cookies. In cookies and crispbread, rosemary extract was tested without adding asparaginase using three amounts: 0.01 %, 0.1 %, and 0.2 %. The lower levels were selected due to the possible altered sensory properties in baked goods and color changes. For an overview of all samples tested and specific amounts used, please refer to Tables 1 and 2 for cookies and crispbread, respectively.

2.3. Preparation of cookies and crispbread

Cookies were made using wholemeal wheat flour using a recipe reported earlier (Musa et al., 2024a). The dry components, flour, sugar, and baking powder in amounts of 100 g, 15.3 g, and 0.33 g, respectively, were mixed for 1 min at low speed using a food processor (Robert Bosch GmbH, Stuttgart, Germany). Water (48 mL) and sunflower oil (12.6 mL) were mixed with the dry components for 3 min at medium speed and 30 s using high speed. The dough was rolled to a thickness of 0.5 cm, and cookies with a diameter of 6 cm were cut out. Crispbread was prepared using wholemeal rye flour. For 100 g of flour, 77 mL of water and 1.15 g of salt were added. Crispbread dough was mixed at low speed for 30 s, medium speed for 60 s, and high speed for another 60 s. Then, the dough was rolled to a thickness of 0.5 cm and cut into rectangles with a length of 8 cm and a width of 4 cm. When treatments were applied, the amounts of CaCl₂, CaCO₃, ellagic acid, gallic acid, and rosemary extract mentioned under 2.2 were added directly during dough mixing. Cookies and crispbread samples that included a combination of treatment with asparaginase R were incubated at 60 °C for 10 min. Cookies were baked in the oven (Rossella XFT197, Unox S.p.A., Cadoneghe, Italy) at 220 °C for 11 min, and crispbread was baked at 240 °C for 13 min. Wheat flour is typically used for cookies, while rye flour is traditionally preferred for crispbread. Therefore, these flours were selected accordingly for each product.

2.4. Acrylamide analysis

An Acrylamide ES ELISA kit was used to quantify acrylamide in cookies and crispbread. The method was performed according to Musa et al. (2024a, 2024b) and the manufacturer's test method provided in the kit (Gold Standard Diagnostics, 2021). Two grams of the sample were mixed well with 40 mL of distilled water for 30 min. The samples were left to rest for 5 min to allow sample sedimentation after mixing. Then, the sample was filtered and centrifuged for 5 min at 13,000 x g. Using Multimode SPE and ENV+ SPE columns, the sample underwent a clean-up procedure. Finally, acrylamide was eluted using a mixture of methanol/water (60:40 v/v). The eluted sample was then derivatized, and the ELISA was performed using a 96-well plate. The samples were placed in a photometer (Tecan, i-control infinite 200Pro, Männedorf, Switzerland) and measured at an absorbance of 450 nm.

Table 1

Color analysis of wheat cookies using CCell. The color is represented in terms of L^* (lightness), a^* (green-red), b^* (blue-yellow) values and the color difference (ΔE) between control and treatment.

Treatment	Amount	L^* -value	a^* -value	b^* -value	ΔE
Control (C-10) ¹	NA	20.6 ± 4.3	8.5 ± 1.1	18.2 ± 2.0	0
	100 µmol	23.5 ± 4.2	9.2 ± 0.8	19.0 ± 1.6	5.2
	50 µmol	26.1 ± 3.1 [#]	9.1 ± 1.0	19.4 ± 1.3	5.7
CaCl ₂	5 µmol	25.9 ± 2.2 [#]	9.2 ± 1.3	19.7 ± 2.0	5.5
	100 µmol + R	23.5 ± 3.4	9.2 ± 1.2	19.1 ± 1.4	3.8
	50 µmol + R	23.3 ± 5.6	9.0 ± 1.1	19.3 ± 1.9	4.7
	5 µmol + R	23.3 ± 2.4	9.8 ± 0.3 [#]	19.7 ± 1.5	4.4
	100 µmol	22.7 ± 5.5	7.2 ± 0.5	17.7 ± 2.3	4.1
	50 µmol	22.1 ± 7.6	7.4 ± 1.8	16.8 ± 3.4	2.7
CaCO ₃	5 µmol	24.8 ± 4.1	8.6 ± 1.0	19.0 ± 1.3	2.5
	100 µmol + R	23.5 ± 2.0	9.0 ± 0.9	18.9 ± 0.8	6.3
	50 µmol + R	22.9 ± 3.4	8.5 ± 0.6	17.8 ± 0.8	3.9
	5 µmol + R	24.4 ± 2.6	9.4 ± 0.9	19.3 ± 1.2	5.2
Gallic acid	100 µmol	26.9 ± 2.8 [#]	7.6 ± 1.5	17.5 ± 2.4	1.3
	50 µmol	24.4 ± 1.9	8.8 ± 1.0	19.1 ± 1.4	1.4
	5 µmol	25.6 ± 4.9 [#]	7.6 ± 1.2	16.9 ± 2.2	4.3
	100 µmol + R	20.6 ± 3.4	9.2 ± 1.3	19.2 ± 1.7	3.1
	50 µmol + R	20.3 ± 4.1	9.1 ± 1.4	19.3 ± 1.9	5.6
	5 µmol + R	24.4 ± 4.2	7.3 ± 1.5	16.5 ± 2.5	5.6
Ellagic acid	100 µmol	24.4 ± 2.1	9.0 ± 1.5	18.7 ± 1.9	3.1
	50 µmol	25.3 ± 3.6 [#]	8.8 ± 1.0	17.5 ± 0.9	2.9
	5 µmol	24.9 ± 2.5	9.3 ± 1.1	18.8 ± 2.0	3.3
	100 µmol + R	24.6 ± 1.7	8.8 ± 0.9	17.1 ± 0.8	2.5
	50 µmol + R	23.1 ± 2.8	9.3 ± 0.7	17.6 ± 1.4	2.2
	5 µmol + R	22.7 ± 4.9	8.5 ± 1.4	16.9 ± 1.6	4.3
Rosemary extract	Control (C-RE) ²	20.3 ± 4.8	8.7 ± 1.4	18.3 ± 1.8	0
	0.01 %	25.3 ± 2.0 [#]	9.2 ± 0.8	19.9 ± 0.8	3.0
	0.1 %	26.0 ± 2.4 [#]	8.4 ± 0.9	19.0 ± 1.4	2.3
	0.2 %	25.9 ± 5.4 [#]	8.6 ± 1.7	18.6 ± 2.6	4.1

The superscript # indicates a significant difference compared to the control (ANOVA with Dunnett's t-test $p \leq 0.05$, $n = 6$).

NA, no addition; R, 50 mg/kg of asparaginase Acrylaway L.

¹ Control sample incubated at 60 °C for 10 min for treatments including calcium chloride, calcium carbonate, gallic acid, and ellagic acid.

² Control sample without incubation for rosemary extract treatment.

2.5. Color analysis

To evaluate the color properties of baked products, the values L^* (lightness), a^* (green-red), and b^* (blue-yellow), respectively, were obtained using CCell. The device was calibrated using a calibration card (CC006, Calibre Control, Warrington, UK) with two sides, a gray surface

Table 2

Color analysis of rye crispbread using CCell. The color is represented in terms of L^* (lightness), a^* (green-red), b^* (blue-yellow) values and the color difference (ΔE) between control and treatment.

Treatment	Amount	L^* -value	a^* -value	b^* -value	ΔE
Control (C)	NA	29.9 ± 1.5	3.3 ± 0.6	7.9 ± 2.3	0
	100 µmol + R	30.1 ± 1.7	3.0 ± 0.8	9.2 ± 2.6	3.9
CaCl ₂	50 µmol + R	30.3 ± 0.8	3.9 ± 0.9	11.5 ± 2.8	2.2
	5 µmol + R	30.2 ± 1.8	2.6 ± 0.6	8.0 ± 1.9	4.0
Ellagic acid	100 µmol + R	30.3 ± 1.2	3.3 ± 0.4	11.3 ± 1.6	3.4
	50 µmol + R	30.3 ± 2.3	3.7 ± 1.1	9.6 ± 2.7	1.8
Rosemary extract	5 µmol + R	30.8 ± 1.3	4.4 ± 1.5	11.0 ± 2.9	3.4
	0.01 %	29.9 ± 1.4	3.8 ± 1.7	11.8 ± 3.0	1.3
	0.1 %	29.9 ± 1.7	3.4 ± 0.5	10.1 ± 1.7	3.7
	0.2 %	31.0 ± 1.4	4.9 ± 1.5	11.4 ± 3.8	0.8

There were no significant differences compared to the respective control (ANOVA with Dunnett's t-test $p \leq 0.05$, $n = 6$).

NA, no addition; R, 50 mg/kg of asparaginase Acrylaway L.

(neutral hue N8.5) and a ramp surface. After calibration, each sample was placed on a blue background for color measurement. A total of six measurements from three batches of each treatment were performed for each cookie and crispbread, respectively. Finally, the color difference between control and treatment (ΔE) was calculated according to Palazoglu, Coşkun, Kocadağlı, and Gökmen (2012) using the following formula:

$$\Delta E = \sqrt{(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2}$$

For each value, 0 represents the color parameter of the control sample.

2.6. Texture properties

The assessment of hardness (N) and fracturability (mm) was done using a TA.XTplus texture analyzer (Stable Micro Systems, Godalming, UK) equipped with a 5 kg load cell (Musa et al., 2024a). We used a three-point bending rig (HDP/3 PB) and a heavy-duty platform (HDP/90) to break cookies and crispbread and assess textural properties. A method called "Measurement of the hardness and resistance of biscuits/cookies to bend or snap" was used, available in the Exponent software (Stable Micro Systems, Version 6.1.20.0, Godalming, UK) of the texture analyzer. A total of six measurements from three batches were taken for cookies and crispbread.

2.7. Sensory analysis

To evaluate any potential changes in the sensory properties of cookies, three treatments were selected and assessed: calcium chloride (CaCl₂ 100 µmol + R), ellagic acid 100 µmol + R (EA 100 µmol + R), and rosemary extract (RE 0.1 %), respectively. A control sample was included as well. Thirteen untrained panelists were asked to evaluate color, texture (crunchiness, chewiness, and hardness), taste, aroma, and overall acceptability. The participants assessed each sensory attribute using a hedonic scale of 1 to 5 for each sample. They compared it to the reference, using the following range of descriptions: very dark (1) to very light (5) for color, not crunchy (1) to very crunchy (5) for crunchiness, very chewy (1) to not chewy (5) for chewiness, very hard (1) to very soft (5) for hardness, unpleasant (1) to very pleasant (5) for taste, unpleasant (1) to very pleasant (5) for aroma and dislike (1) to like very much (5) for overall acceptability. Finally, participants could add notes in the comment section for any off-taste or other product characteristics not included in the survey. All participants gave informed consent to participate in the sensory study and to use their information.

2.8. Statistical analysis

Microsoft Excel was used to calculate means and standard deviations. Origin 2023 (OriginLab Corporation, Northampton, MA, USA) and IBM SPSS Statistics (IBM Corp, Version 29.0, Armonk, NY) were used for statistical analysis. Dunnett's *t*-test ($p \leq 0.05$) was utilized to identify significant differences between the control and treated samples. Sensory samples were analyzed using the non-parametric Kruskal-Wallis test ($p < 0.05$). Dunn's test was a post hoc method for pairwise comparisons with control and multiple treatment groups. Three technological replicates (i.e., batches) were prepared for each treatment. An online tool by the Arigo website was used to calculate the acrylamide content for each sample (ELISA calculator) (Arigo Biolaboratories, n.d).

3. Results

3.1. Acrylamide content in cookies

To evaluate the impact of salts, phenolic acids, and rosemary extract on the reduction of acrylamide content in cookies, each of these was added using three different amounts (Table 1). Additionally, phenolic acids and salts were tested with asparaginase R (Fig. 1), for CaCl_2 and CaCO_3 , a significant ($p \leq 0.05$) reduction of acrylamide was observed compared to the control (C-10, 538 $\mu\text{g}/\text{kg}$ of acrylamide) when these salts were added to cookies without R, except for 5 μmol of CaCl_2 . However, although the reduction was significant for the amount of 5 μmol of CaCO_3 , this was a relatively low reduction to 498 $\mu\text{g}/\text{kg}$ of acrylamide. Overall, increasing the amounts of calcium salts to 100 μmol reduced acrylamide to 288 $\mu\text{g}/\text{kg}$ (CaCl_2) and 318 $\mu\text{g}/\text{kg}$ (CaCO_3). A combination of each salt with asparaginase R further improved the reduction because even the 5 μmol amounts of CaCl_2 or CaCO_3 + 50 mg/kg R showed a significant reduction of acrylamide to 281 $\mu\text{g}/\text{kg}$ or 239 $\mu\text{g}/\text{kg}$, respectively. However, compared to 50 mg/kg R alone, this reduction was primarily associated with the asparaginase R rather than

the salt. Ellagic acid and gallic acid were also tested alone or in combination with asparaginase R. Gallic acid showed lower reductions of acrylamide compared to ellagic acid. As with the salts, increasing the amount of either acid from 5 μmol to 100 μmol showed an improvement in acrylamide reduction. Ellagic acid reduced acrylamide to 268 $\mu\text{g}/\text{kg}$ and gallic acid to 362 $\mu\text{g}/\text{kg}$ at 100 μmol , to 364 $\mu\text{g}/\text{kg}$ and 384 $\mu\text{g}/\text{kg}$ at 50 μmol , and 472 $\mu\text{g}/\text{kg}$ and 469 $\mu\text{g}/\text{kg}$ at 5 μmol , for ellagic acid and gallic acid, respectively. The addition of asparaginase R in combination with gallic acid or ellagic acid further reduced acrylamide, with the highest reduction to 61 $\mu\text{g}/\text{kg}$ achieved by the treatment with ellagic acid (EA 100 μmol + 50 mg/kg R). Rosemary extract was also tested in cookies using three amounts: 0.01 %, 0.1 %, and 0.2 %, respectively. All amounts achieved significant reductions compared to the control (C-RE, 526 $\mu\text{g}/\text{kg}$), with the highest reduction to 269 $\mu\text{g}/\text{kg}$ of acrylamide achieved by adding 0.2 % of rosemary extract. Comparing the control for phenolic acids and calcium salts incubated for 10 min (C-10) with the rosemary extract control (C-RE), which was not incubated before baking, reveals a negligible difference in acrylamide levels. The acrylamide content was 538 $\mu\text{g}/\text{kg}$ in C-10 and 526 $\mu\text{g}/\text{kg}$ in C-RE. This indicates that incubating the dough at 60 °C for 10 min does not significantly influence acrylamide formation. These findings demonstrate the excellent reproducibility of the baking trials and further validate the reliability of the entire process.

3.2. Acrylamide content in crispbread

In crispbread, only two treatments resulted in a significant reduction of acrylamide (Fig. 2). The combination of 100 μmol CaCl_2 + 50 mg/kg R showed a significant reduction of acrylamide to 547 $\mu\text{g}/\text{kg}$, while 50 μmol or 5 μmol of CaCl_2 with R showed minimal or no reduction at all compared to the control with 872 $\mu\text{g}/\text{kg}$. Like CaCl_2 , ellagic acid caused a significant decrease of acrylamide to 435 $\mu\text{g}/\text{kg}$ only for the combination of 100 μmol + 50 mg/kg R, and minimal or no reduction at all using 50 μmol and 5 μmol , respectively. Rosemary extract showed a

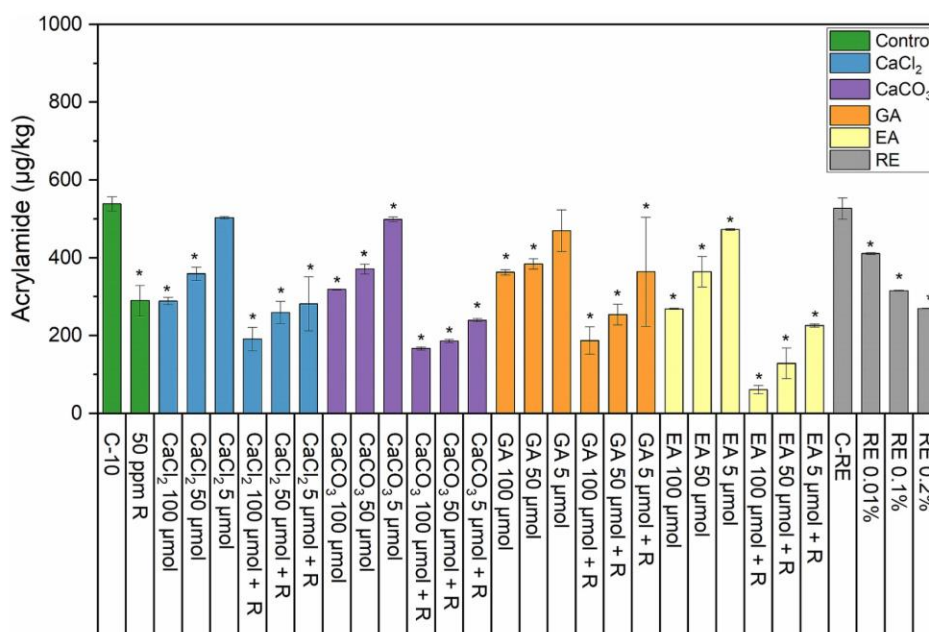


Fig. 1. Acrylamide content of wholemeal wheat cookies with the addition of calcium chloride, calcium carbonate, gallic acid, ellagic acid, and rosemary extract. Error bars indicate the standard deviation of the mean, and asterisks indicate a significant difference compared to the respective control (ANOVA with Dunnett's *t*-test $p \leq 0.05$, $n = 3$). Abbreviations as follows: CaCl_2 (calcium chloride), CaCO_3 (calcium carbonate), GA (gallic acid), EA (ellagic acid), RE (rosemary extract), R (50 mg/kg of asparaginase Acrylaway L).

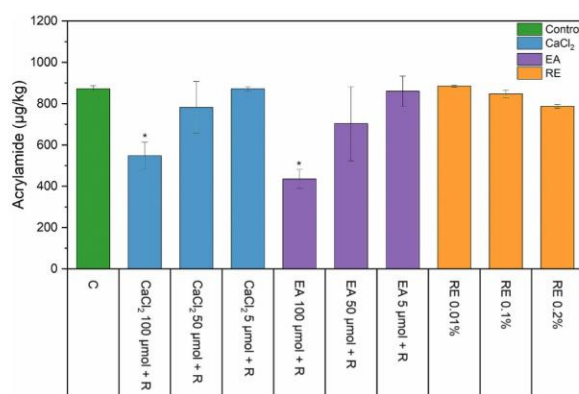


Fig. 2. Acrylamide content of wholemeal rye crispbread with the addition of calcium chloride, ellagic acid, and rosemary extract. Asterisks indicate a significant difference compared to the control (ANOVA with Dunnett's *t*-test $p \leq 0.05$, $n = 2$). Abbreviations as follows: CaCl₂ (calcium chloride), EA (ellagic acid), RE (rosemary extract), R (50 mg/kg of asparaginase Acrylaway L).

minimal decrease at the amount of 0.2 %, but this was statistically insignificant.

3.3. Evaluation of color in cookies and crispbread

To evaluate any effect of the treatments on the color properties of cookies and crispbread, the L^* , a^* , and b^* -values of each treatment were compared to the respective control using ANOVA with Dunnett's *t*-test ($p \leq 0.05$) (Tables 1 and 2). The addition of rosemary extract caused a significant increase in the L^* -value, but there were no significant changes in a^* - or b^* -values. The application of ellagic acid resulted in minor color differences for the cookies. Only 50 µmol of ellagic acid showed an increase in L^* -value, which was small but statistically significant. None of the other treatments with ellagic acid affected the L^* , a^* , or b^* -value. Gallic acid also had a minimal effect on the color of cookies. Only the addition of 100 µmol or 5 µmol of gallic acid showed a significant increase in L^* -value. CaCl₂ showed a slightly larger impact on the color properties of cookies than CaCO₃; however, combining any of the treatments with asparaginase R did not impact any color properties.

Table 2 presents crispbread's L^* , a^* , and b^* -values. Different from cookies, the addition of rosemary extract showed no significant

differences in color compared to the control. Adding ellagic acid and CaCl₂ showed no significant differences in L^* , a^* , or b^* -values.

3.4. Textural properties of cookies and crispbread

The evaluation of textural properties, including hardness and fracturability, was done for all treatments applied to cookies and crispbread. Tables S1 and S2 include the 5 µmol treatment; however, this treatment was excluded from Fig. 3, as the treatment had minimal impact on the texture properties. Overall, most treatments showed minimal cookie changes, which were statistically insignificant. However, four treatments showed a significant decrease in hardness (Fig. 3A): CaCO₃ at 100 µmol and 50 µmol, and gallic acid at 100 µmol and 50 µmol. However, when these treatments were combined with asparaginase R, the hardness showed no significant change compared to the control. Ellagic acid and rosemary extract caused minimal changes in the hardness of cookies, none of which were statistically significant.

Crispbread showed minimal changes in texture compared to the control due to the treatments applied (Fig. 3A). Only adding CaCl₂ 100 µmol + R and EA 50 µmol + R resulted in a significant increase and decrease, respectively. Rosemary extract caused no significant changes in hardness or fracturability.

The fracturability of cookies and crispbread is presented in Fig. 3B. Some treatments showed a significant increase in the fracturability of cookies in comparison to the control. The most impactful treatments regarding the fracturability of cookies were CaCl₂ at 50 µmol + R, CaCO₃ at 50 µmol, and RE 0.1 %, respectively. A high variability between the three batches was found, as shown in the error bars in Fig. 3B. In crispbread, there were only minimal changes in fracturability, none of which were statistically significant.

3.5. Sensory analysis

A sensory evaluation was performed with 13 participants to assess the impact of the treatments applied to baked products. Cookies were selected for the sensory assessment due to their more pronounced sensory attributes, providing a more suitable medium to assess the effects of the treatments. Crispbread, being more neutral in flavor and texture, may not have shown a clear difference, thus limiting the effectiveness of the evaluation in detecting treatment impacts. Three treatments were selected: rosemary extract 0.1 %, ellagic acid 100 µmol + R, and CaCl₂ 100 µmol + R, respectively, and compared to the control sample. The ellagic acid and CaCl₂ treatments (100 µmol + R) were selected because they provided the highest acrylamide reduction, compared to 50 µmol or

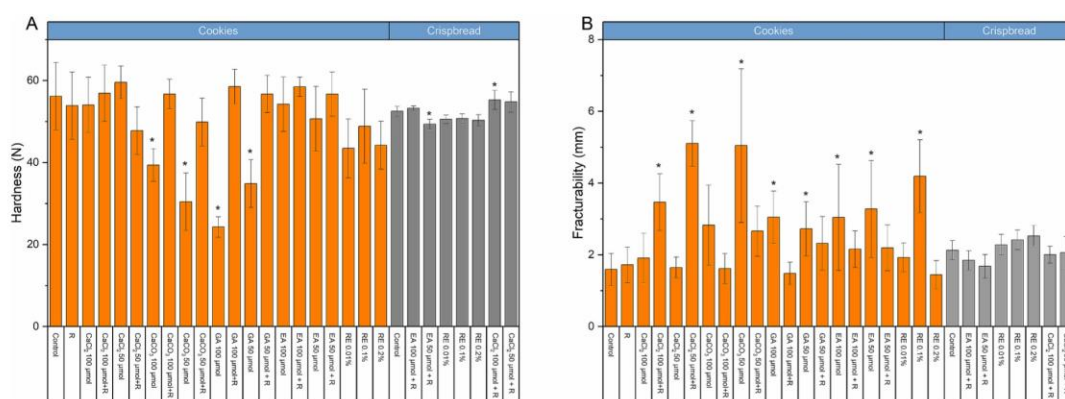


Fig. 3. Hardness (N) and fracturability (mm) of wholemeal wheat cookies and rye crispbread with the addition of calcium chloride, calcium carbonate, gallic acid, ellagic acid, and rosemary extract. Error bars indicate the standard deviation of the mean, and asterisks indicate a significant difference compared to the respective control (ANOVA with Dunnett's *t*-test $p \leq 0.05$, $n = 6$). Abbreviations as follows: CaCl₂ (calcium chloride), CaCO₃ (calcium carbonate), GA (gallic acid), EA (ellagic acid), RE (rosemary extract), R (50 mg/kg of asparaginase Acrylaway L).

5 μmol + R. While rosemary extract at 0.1 % reduced acrylamide slightly less compared to 0.2 %, higher amounts of rosemary extract were avoided as they were assumed to have a more significant impact on the taste of the cookies. This selection aimed to balance acrylamide reduction with the preservation of product quality. The sensory attributes color, hardness, crunchiness, chewiness, taste, aroma, and overall acceptability were evaluated and presented in Fig. 4. Overall, none of the treatments showed any significant changes in sensory attributes compared to the control. The participants also observed that cookies with 0.1 % rosemary extract were slightly darker than the control sample. Ellagic acid showed a slight increase in crunchiness compared to the control sample. CaCl_2 in general, showed improved taste, aroma, and overall acceptability compared to other samples. The participants also noted this in the comment section. Although these slight changes are visible in the spiderweb graph (Fig. 4), statistically, none were significant compared to the control.

4. Discussion

Acrylamide reduction by applying various compounds has reduced or minimized acrylamide in different products (Açar et al., 2012; Miśkiewicz, Nebesny, Rosicka-Kaczmarek, Żyżelewicz, & Budryn, 2018; Narita & Inouye, 2014). While several compounds can be added to lower acrylamide levels in baked products, one of the current study's strategies was using calcium salts because they are often used in flour fortification (BAKERpedia, 2021). Calcium salts enhance the nutritional profile of flour by contributing to the dietary calcium intake of populations consuming baked goods made of fortified flour. Besides, the cations prevent the formation of the Schiff base of asparagine during the Maillard reaction, which is the main intermediate that forms acrylamide in the final product (Gökmen & Şenyuva, 2007). Adding CaCl_2 reduced acrylamide by up to 46 % (CaCl_2 100 μmol) in cookies. Another study that investigated the ability of CaCl_2 to reduce acrylamide in cereal model systems showed that a reduction of up to 90 % is possible (Kukurová, Ciesarová, et al., 2009). In the current study, increasing levels of calcium salts in cookies further decreased acrylamide, considering the three levels tested. However, amounts beyond 100 μmol were not evaluated. Future research should explore higher amounts (e.g., 200 μmol) to investigate whether acrylamide reduction continues. It is also important to consider potential sensory implications, as high levels of calcium salts may introduce off-tastes to the product. Therefore, sensory

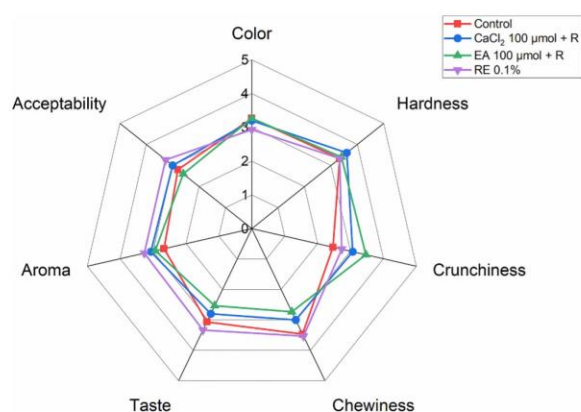


Fig. 4. Sensory evaluation including color, texture, taste, aroma, and overall acceptability for wholemeal wheat cookies. Results are presented as the mean scores of 13 panelists. Samples were analyzed using the non-parametric Kruskal-Wallis test ($p < 0.05$) and Dunn's test was used as a post hoc method for pairwise comparisons with control and multiple treatment groups. Abbreviations as follows: CaCl_2 (calcium chloride), EA (ellagic acid), RE (rosemary extract), R (50 mg/kg of asparaginase Acrylaway L).

analysis at higher calcium salt amounts is essential to ensure product acceptability. In agreement with our study, Açar et al. (2012) also reported a decrease in acrylamide when the amount of CaCl_2 was increased. CaCl_2 also showed a significant reduction of acrylamide in potato products, with a reduction of 95 % in fried potato strips (Gökmen & Şenyuva, 2007).

In the current study, CaCO_3 achieved a slightly lower acrylamide reduction compared to CaCl_2 . At an amount of 100 μmol , CaCO_3 reduced acrylamide by up to 41 %, whereas CaCl_2 achieved a reduction of 46 % compared to the control sample. Increasing amounts of CaCO_3 also showed a significant decrease in acrylamide in white flatbread (Alsharafani et al., 2023). When CaCO_3 was combined with asparaginase R, up to 69 % reduction was achieved (CaCO_3 100 μmol + R). CaCl_2 also improved acrylamide reduction when combined with asparaginase R, with the highest reduction reaching up to 65 % (CaCl_2 + R) in cookies. The combined use of calcium salts and asparaginase resulted in a greater acrylamide reduction than calcium salts alone, highlighting an additive effect. Calcium salts likely modulate the baking environment, inhibiting acrylamide formation, while asparaginase directly reduces the precursor asparagine. Combining CaCl_2 or ellagic acid with asparaginase R gave lower reductions of acrylamide in crispbread compared to cookies. This could be due to crispbread's lower water activity than cookies. Asparaginase requires sufficient moisture to function effectively, as it catalyzes the hydrolysis of asparagine to aspartic acid (Hendriksen et al., 2009). The drier environment in crispbread limits the enzyme's activity, resulting in a less efficient reduction of acrylamide precursors. Further, the higher baking temperature for crispbread promotes acrylamide formation after asparaginase activity has ceased. The free asparagine content of the wholemeal wheat and rye flour used in this study was 290 mg/kg and 700 mg/kg, respectively, as already reported in our previous study (Musa et al., 2024b). Rye flour generally contains more free asparagine than wheat flour (summarized in Food Drink Europe, 2019), leading to a higher potential to form acrylamide caused by the higher content of the main precursor. Furthermore, the precursor and the more intense thermal treatment applied during baking influence the higher acrylamide content in rye crispbread than wheat cookies.

Both phenolic acids, gallic acid and ellagic acid, respectively, achieved significant reductions of acrylamide in cookies. Ellagic acid alone (100 μmol) reduced acrylamide by 50 % (Fig. 1). Oral et al. (2014) showed that plant extracts and polyphenols can inhibit acrylamide formation. Ellagic acid reduced acrylamide by 69 % in the asparagine-fructose model system and 19 % in biscuits. In the present study, ellagic acid further decreased acrylamide when combined with asparaginase R, with a reduction of up to 89 %. Gallic acid alone caused a reduction of up to 33 %; combined with asparaginase R, it was 65 %. A study where a mix of different phenolic compounds, including gallic acid, was used reported a reduction of acrylamide in fried potato chips by 98 % (Ismail, Ali, Askar, & Samy, 2013). Another study combined aqueous rosemary extract with 40 mg of gallic acid in wheat buns (Hedegaard, Granby, Frandsen, Thygesen, & Skibsted, 2008). They reported a significant decrease in acrylamide using the aqueous extract; however, increasing the amount from 1 % to 10 % of the aqueous extract did not further decrease acrylamide. While in our study a combination of rosemary extract with gallic acid was not investigated, gallic acid and rosemary extract alone provided significant acrylamide reductions of up to 33 % and 50 %, respectively, for cookies. The active components in rosemary extract include carnosic acid, carnosol, and rosmarinic acid. Some phenolic compounds serve as carbonyl-trapping agents that inhibit acrylamide formation during the Maillard reaction (Cheng et al., 2009). According to Constantinou and Koutsidis (2016), in Maillard reaction systems, phenolic antioxidants may react with carbonyl compounds to create adducts via electrophilic aromatic substitution reactions and limit acrylamide formation. Miśkiewicz et al. (2018) also reported increased acrylamide reduction with increasing amounts from 0.1 % to 0.5 % of rosemary extract in shortcrust cookies. Differences in extract efficacy were obtained when shortcrust cookies were baked with

or without humid air, and 90 % humidity improved the reductions compared to dry air. While rosemary extract demonstrated an improved reduction of acrylamide levels with increased amounts in both cookies and crispbread in our study, the reductions in crispbread were relatively low and statistically insignificant. Therefore, higher amounts of rosemary extract are recommended specifically for crispbread to enhance its effectiveness. A more detailed investigation of the phenolic compounds present in the rosemary extract will be needed to further clarify its acrylamide-mitigating potential, as some phenolic compounds are known to inhibit acrylamide formation, while others may even promote it. Furthermore, exploring the combination of rosemary extract with ellagic acid or gallic acid offers a promising avenue for future research to identify the most effective strategy for mitigating acrylamide in crispbread. Aside from the regulatory requirements, consumer expectations are important to balance product safety and quality. Therefore, texture, color, and overall sensory properties were evaluated. The texture evaluation showed some significant changes in the hardness of cookies, mainly for the treatment with CaCl_2 , CaCO_3 , or gallic acid, and fracturability was affected by selected treatments (Fig. 3). Adding salt was reported to create a stronger gluten network, which increases dough stability, dough extensibility, and dough development time, and also decreases water absorption capacity (McCann & Day, 2013), which can explain some of the changes observed in the texture of cookies. Interestingly, the hardness of cookies was again similar to the control when the affected treatments were combined with asparaginase R. In previous studies, the asparaginases also had little or no impact on the textural properties of cookies (Musa et al., 2024a, 2024b).

Calcium salts and phenolic acids showed minimal effect on the color of cookies. The addition of CaCl_2 showed an increased L^* -value compared to the control, which was statistically significant for the 5 μmol and 50 μmol levels. The increase in L^* -value was also observed in a study from Açar et al. (2012). The addition of rosemary extract showed significant changes in L^* -value for cookies. Similar results were observed from Miśkiewicz et al. (2018). They showed that the color of shortcrust cookies was significantly affected regardless of rosemary extract amounts. On the other hand, rosemary extract did not significantly affect the color properties of crispbread. This is likely because the naturally dark color of the extract blended well with the darker shade of wholemeal rye flour, which was used as the base for crispbread. As a result, any color changes were less noticeable. The more significant differences observed in cookies compared to crispbread can be attributed to these baked goods' distinct composition, structure, and processing. The cookies made from wholemeal wheat flour had a lighter color and softer texture than crispbread, which was made from inherently darker wholemeal rye flour. Therefore, the changes in hardness, fracturability, and color were more noticeable in cookies compared to crispbread, which also had a denser texture, which can hide the changes in color and texture caused by the same components. Although significant changes in texture and color were detected using a texture analyzer and CCell for selected treatments, sensory evaluations showed little or no impact on the texture or color of the chosen treatments. CaCl_2 treatment (Fig. 4) showed the best overall acceptability compared to the other samples. A study showed that adding CaCl_2 at 0.1 M left a bitter taste in potato crisps (Mestdagh et al., 2008); however, this amount is significantly higher than our study which might be why no off-taste was detected. They also observed a more intense taste when CaCl_2 was applied, similar to our research. Increased acceptability of cookies with CaCO_3 was also reported by Liang et al. (2014). In contrast, Ismial et al. (2013) reported a slight decrease in overall acceptability when CaCl_2 was applied, while adding magnesium chloride in potato chips showed increased taste and overall acceptability. In another study, lower amounts (0.1 % and 0.5 %) of calcium derivatives, including calcium salts of lactic acid and CaCl_2 , had no noticeable effect on sensory attributes like saltiness, sweetness, or bitterness in cookies (Açar et al., 2012). However, significant changes were observed when the amount was increased to 1.0 %. This highlights that while different salt amounts

can vary in their effectiveness at reducing acrylamide, their potential impact on texture, color, and overall acceptability must also be carefully considered. Ellagic acid and rosemary extract (Fig. 4) showed no significant differences in cookies compared to the control sample. Miśkiewicz et al. (2018) also showed that adding rosemary extract in shortcrust cookies at amounts of 0.1 % and 0.2 % baked in dry air did not impact the sensory attributes, while the amount of 0.5 % significantly decreased the sensory properties. Even an 0.2 % amount decreased the sensory assessment for cookies baked in humid air. Since higher amounts than 0.1 % were not assessed using sensory analysis in the current study, further research is needed.

5. Conclusion

This study provides novel insights into combining compounds with asparaginase to reduce acrylamide in cookies and crispbread. Adding ellagic acid without asparaginase R (50 mg/kg of Acrylaway L) achieved the highest acrylamide reduction (50 %) in cookies. At the same time, all compounds showed an improved effectiveness when combined with asparaginase R with a decrease of up to 89 %. This approach shows promise as it requires less enzyme than enzyme-only methods, although further studies are needed to confirm this at larger production scales. Acrylamide reduction varied between cookies and crispbread, possibly due to the differences in moisture content and the amount of asparagine in the flour. Crispbread showed a lower reduction, highlighting the importance of matrix-specific strategies. Further research is necessary for crispbread and other baked products because their unique low-moisture matrices and processing conditions limit the effectiveness of existing mitigation strategies. Identifying optimized combinations or novel approaches can help achieve significant acrylamide reductions without compromising product quality. Most treatments had a minimal effect on color, texture, and sensory properties. While calcium salts influenced textural properties, sensory evaluations maintained product acceptability. Further optimization of combinations, including higher amounts and different matrices, is necessary to balance acrylamide reduction with regulatory requirements and quality maintenance across diverse baked products. A key limitation of this study is the lack of sensory evaluation for the crispbread samples. Future research should address this by assessing consumer-relevant attributes like taste and texture across all product types to ensure practical applicability of the mitigation strategies.

CRediT authorship contribution statement

Shpresa Musa: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Claudia Oellig:** Writing – review & editing, Supervision, Resources, Project administration, Conceptualization. **Katharina Anne Scherf:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Compliance with ethical standards

Conflict of interest. The authors declare no competing financial interest.

Compliance with ethics requirements. Cookies are typically consumed foods and their sensory evaluation does not require ethical permission. Appropriate protocols for protecting the rights and privacy of all participants in the sensory analysis were utilized during the execution of the research. The publication of all sensory research data was subject to the consent of all participants.

Informed consent. All participants in the sensory analysis provided their informed consent to participate in the sensory evaluations and use their information.

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Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fochx.2025.102605>.

Data availability

Data will be made available on request.

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SUPPLEMENTARY MATERIAL

Mitigation of Acrylamide in Cookies and Crispbread Using Calcium Salts and Phenolic Acids in Combination with Asparaginase as well as Rosemary Extract

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Table S1. Hardness (N) and fracturability (mm) of wholemeal wheat cookies with the addition of calcium chloride, calcium carbonate, gallic acid, ellagic acid, and rosemary extract. Abbreviations as follows: CaCl₂ (calcium chloride), CaCO₃ (calcium carbonate), GA (gallic acid), EA (ellagic acid), RE (rosemary extract), R (50 mg/kg of asparaginase Acrylaway L).

Sample ID	Hardness (N)	Fracturability (mm)
CaCl ₂ _100 umol_1	59.85	1.61
CaCl ₂ _100 umol_2	56.87	2.82
CaCl ₂ _100 umol_3	46.19	2.10
CaCl ₂ _100 umol_4	58.43	2.55
CaCl ₂ _100 umol_5	58.23	1.26
CaCl ₂ _100 umol_6	44.64	1.12
CaCl ₂ _50 umol_1	52.28	1.60
CaCl ₂ _50 umol_2	62.00	2.21
CaCl ₂ _50 umol_3	63.58	1.60
CaCl ₂ _50 umol_4	59.70	1.60
CaCl ₂ _50 umol_5	58.47	1.41
CaCl ₂ _50 umol_6	61.38	1.44
CaCl ₂ _5 umol_1	42.54	2.15
CaCl ₂ _5 umol_2	39.83	3.20
CaCl ₂ _5 umol_3	43.67	1.97
CaCl ₂ _5 umol_4	56.48	2.93
CaCl ₂ _5 umol_5	59.36	1.37
CaCl ₂ _5 umol_6	59.30	1.68
CaCl ₂ _100 umol_R_1	59.62	4.11
CaCl ₂ _100 umol_R_2	59.38	4.63
CaCl ₂ _100 umol_R_3	44.64	3.47
CaCl ₂ _100 umol_R_4	61.32	2.85
CaCl ₂ _100 umol_R_5	53.30	2.50
CaCl ₂ _100 umol_R_6	63.06	3.25
CaCl ₂ _50 umol_R_1	50.54	4.17
CaCl ₂ _50 umol_R_2	51.95	5.65
CaCl ₂ _50 umol_R_3	46.33	5.90
CaCl ₂ _50 umol_R_4	50.83	4.67
CaCl ₂ _50 umol_R_5	50.45	5.01
CaCl ₂ _50 umol_R_6	36.62	5.21
CaCl ₂ _5 umol_R_1	54.76	1.43
CaCl ₂ _5 umol_R_2	58.39	1.30
CaCl ₂ _5 umol_R_3	58.65	1.43
CaCl ₂ _5 umol_R_4	57.54	1.16
CaCl ₂ _5 umol_R_5	60.53	2.09
CaCl ₂ _5 umol_R_6	57.37	1.22
CaCO ₃ _100 umol_1	9.83	11.82
CaCO ₃ _100 umol_2	36.24	4.67
CaCO ₃ _100 umol_3	40.59	2.66
CaCO ₃ _100 umol_4	38.70	2.91

CaCO3_100 umol_5	35.85	2.04
CaCO3_100 umol_6	45.68	1.86
CaCO3_50 umol_1	25.71	8.01
CaCO3_50 umol_2	36.73	3.76
CaCO3_50 umol_3	36.80	2.46
CaCO3_50 umol_4	20.64	6.14
CaCO3_50 umol_5	26.47	10.22
CaCO3_50 umol_6	36.25	4.85
CaCO3_5 umol_1	44.50	4.51
CaCO3_5 umol_2	45.89	4.01
CaCO3_5 umol_3	48.12	11.37
CaCO3_5 umol_4	49.27	3.08
CaCO3_5 umol_5	49.16	4.69
CaCO3_5 umol_6	41.56	6.01
CaCO3_100 umol_R_1	61.53	2.09
CaCO3_100 umol_R_2	57.63	1.18
CaCO3_100 umol_R_3	57.11	1.42
CaCO3_100 umol_R_4	51.41	1.38
CaCO3_100 umol_R_5	53.75	2.20
CaCO3_100 umol_R_6	58.90	1.42
CaCO3_50 umol_R_1	61.09	2.00
CaCO3_50 umol_R_2	44.73	1.98
CaCO3_50 umol_R_3	47.39	2.84
CaCO3_50 umol_R_4	49.46	3.46
CaCO3_50 umol_R_5	49.73	2.21
CaCO3_50 umol_R_6	46.73	3.47
CaCO3_5 umol_R_1	50.63	2.35
CaCO3_5 umol_R_2	42.29	1.70
CaCO3_5 umol_R_3	43.09	1.65
CaCO3_5 umol_R_4	54.29	2.18
CaCO3_5 umol_R_5	57.16	3.29
CaCO3_5 umol_R_6	37.95	3.37
GE_100 umol_1	23.84	2.67
GE_100 umol_2	23.01	3.83
GE_100 umol_3	11.70	2.43
GE_100 umol_4	25.87	3.16
GE_100 umol_5	27.55	3.96
GE_100 umol_6	21.22	2.24
GE_50 umol_1	37.12	2.71
GE_50 umol_2	33.26	3.30
GE_50 umol_3	38.09	3.77
GE_50 umol_4	40.37	2.03
GE_50 umol_5	50.01	1.75
GE_50 umol_6	25.52	2.78
GE_5 umol_1	33.45	4.16
GE_5 umol_2	29.34	5.99
GE_5 umol_3	20.24	4.32
GE_5 umol_4	23.54	5.99

GE_5 umol_5	20.54	5.90
GE_5 umol_6	21.97	4.12
GE_100 umol_R_1	58.77	1.32
GE_100 umol_R_2	63.36	1.41
GE_100 umol_R_3	63.40	2.03
GE_100 umol_R_4	57.49	1.22
GE_100 umol_R_5	54.05	1.24
GE_100 umol_R_6	54.04	1.66
GE_50 umol_R_1	58.39	1.51
GE_50 umol_R_2	50.95	3.40
GE_50 umol_R_3	55.96	2.17
GE_50 umol_R_4	61.87	3.00
GE_50 umol_R_5	52.05	2.20
GE_50 umol_R_6	61.04	1.63
GE_5 umol_R_1	38.46	4.62
GE_5 umol_R_2	42.32	6.16
GE_5 umol_R_3	34.05	4.62
GE_5 umol_R_4	38.13	4.71
GE_5 umol_R_5	22.75	4.35
GE_5 umol_R_6	34.73	4.95
EA_100 umol_1	59.16	2.20
EA_100 umol_2	56.81	3.01
EA_100 umol_3	50.11	4.43
EA_100 umol_4	58.13	1.50
EA_100 umol_5	58.70	5.20
EA_100 umol_6	42.52	1.91
EA_50 umol_1	38.77	5.17
EA_50 umol_2	48.89	3.06
EA_50 umol_3	47.99	3.11
EA_50 umol_4	57.24	1.97
EA_50 umol_5	61.40	4.54
EA_50 umol_6	49.73	1.81
EA_5 umol_1	48.87	2.91
EA_5 umol_2	54.39	2.04
EA_5 umol_3	38.98	2.50
EA_5 umol_4	38.64	2.07
EA_5 umol_5	47.52	2.00
EA_5 umol_6	40.17	3.81
EA_100 umol_R_1	57.66	1.29
EA_100 umol_R_2	61.05	2.70
EA_100 umol_R_3	61.76	2.13
EA_100 umol_R_4	56.09	1.91
EA_100 umol_R_5	57.61	2.50
EA_100 umol_R_6	56.65	2.41
EA_50 umol_R_1	47.72	2.15
EA_50 umol_R_2	56.33	1.45
EA_50 umol_R_3	61.23	3.26
EA_50 umol_R_4	55.77	2.54

EA_50 umol_R_5	55.81	1.99
EA_50 umol_R_6	63.18	1.77
EA_5 umol_R_1	47.41	2.28
EA_5 umol_R_2	47.62	3.99
EA_5 umol_R_3	47.12	10.26
EA_5 umol_R_4	61.03	1.74
EA_5 umol_R_5	62.64	2.13
EA_5 umol_R_6	63.44	2.55
RE_0.01%_1	49.74	2.26
RE_0.01%_2	44.97	2.27
RE_0.01%_3	51.14	2.00
RE_0.01%_4	31.51	1.86
RE_0.01%_5	39.46	1.98
RE_0.01%_6	43.92	1.18
RE_0.1%_1	55.14	3.68
RE_0.1%_2	34.31	5.62
RE_0.1%_3	57.30	2.98
RE_0.1%_4	45.93	3.92
RE_0.1%_5	44.25	11.26
RE_0.1%_6	56.16	4.74
RE_0.2%_1	36.63	1.16
RE_0.2%_2	47.67	2.21
RE_0.2%_3	37.91	1.23
RE_0.2%_4	49.51	1.27
RE_0.2%_5	43.58	1.32
RE_0.2%_6	49.97	1.49
Control_1	50.08	1.83
Control_2	53.33	1.56
Control_3	55.53	1.30
Control_4	49.59	1.05
Control_5	50.04	2.93
Control_6	55.45	1.55
Control_R_1	59.23	1.38
Control_R_2	38.54	2.01
Control_R_3	51.50	2.42
Control_R_4	58.43	1.13
Control_R_5	55.21	1.99
Control_R_6	60.43	1.38

Values in grey were identified as outliers.

Table S2. Hardness (N) and fracturability (mm) of wholemeal rye crispbread with the addition of calcium chloride, ellagic acid, and rosemary extract. Abbreviations as follows: CaCl₂ (calcium chloride), EA (ellagic acid), RE (rosemary extract), R (50 mg/kg of asparaginase Acrylaway L).

Sample ID	Hardness (N)	Fracturability (mm)
CaCl ₂ _100 umol_R_1	53.42	1.56
CaCl ₂ _100 umol_R_2	55.87	2.11
CaCl ₂ _100 umol_R_3	53.48	2.20
CaCl ₂ _100 umol_R_4	53.06	1.94
CaCl ₂ _100 umol_R_5	58.73	2.09
CaCl ₂ _100 umol_R_6	57.15	2.12
CaCl ₂ _50 umol_R_1	55.72	2.30
CaCl ₂ _50 umol_R_2	54.28	1.55
CaCl ₂ _50 umol_R_3	56.53	1.91
CaCl ₂ _50 umol_R_4	58.11	2.82
CaCl ₂ _50 umol_R_5	52.60	1.99
CaCl ₂ _50 umol_R_6	51.56	1.80
CaCl ₂ _5 umol_R_1	58.58	2.34
CaCl ₂ _5 umol_R_2	58.22	1.54
CaCl ₂ _5 umol_R_3	54.83	2.13
CaCl ₂ _5 umol_R_4	54.46	2.54
CaCl ₂ _5 umol_R_5	55.33	1.23
CaCl ₂ _5 umol_R_6	56.25	1.72
EA_100 umol_R_1	53.76	1.56
EA_100 umol_R_2	52.72	1.50
EA_100 umol_R_3	53.60	2.02
EA_100 umol_R_4	52.80	1.92
EA_100 umol_R_5	52.62	2.20
EA_100 umol_R_6	53.81	1.87
EA_50 umol_R_1	50.75	2.29
EA_50 umol_R_2	49.37	1.81
EA_50 umol_R_3	49.13	1.59
EA_50 umol_R_4	48.68	1.48
EA_50 umol_R_5	50.59	1.45
EA_50 umol_R_6	47.53	1.46
EA_5 umol_R_1	51.89	2.16
EA_5 umol_R_2	48.32	1.57
EA_5 umol_R_3	52.68	1.86
EA_5 umol_R_4	51.34	2.32
EA_5 umol_R_5	47.50	1.69
EA_5 umol_R_6	51.23	1.91
RE 0.01%_1	50.68	2.30
RE 0.01%_2	52.38	2.12
RE 0.01%_3	49.43	2.85
RE 0.01%_4	50.44	2.18
RE 0.01%_5	50.59	2.03
RE 0.01%_6	49.71	2.19
RE 0.1 %_1	49.31	2.36

RE 0.1 %_2	50.51	2.54
RE 0.1 %_3	50.74	2.77
RE 0.1 %_4	51.77	2.15
RE 0.1 %_5	52.28	2.61
RE 0.1 %_6	49.74	2.08
RE 0.2%_1	49.35	2.75
RE 0.2%_2	50.82	2.21
RE 0.2%_3	49.15	2.65
RE 0.2%_4	49.26	2.89
RE 0.2%_5	50.83	2.46
RE 0.2%_6	52.60	2.17
Control_1	51.83	2.12
Control_2	52.95	1.88
Control_3	53.33	2.47
Control_4	50.42	2.14
Control_5	53.93	2.36
Control_6	52.52	1.78

6.4 Publication IV

Authors: Shpresa Musa, Kristína Kukurová, Zuzana Ciesarová, Karsten Eisenhardt and Katharina Anne Scherf

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Asparaginase treatment for acrylamide reduction in wheat and oat flakes: From laboratory- to pilot-scale

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ABSTRACT

Acrylamide is a contaminant in food that is produced during high-temperature processing. There is a growing need for mitigation strategies due to health concerns and regulatory guidelines. This study aims to evaluate the effectiveness of four commercial asparaginases in the reduction of acrylamide in wheat and oat flakes, as well as their impact on product quality. Additionally, scale-up experiments were conducted with one of the asparaginases to evaluate the transferability of laboratory-scale results into pilot-scale production for bran flakes. On a laboratory-scale, asparaginase treatment resulted in a higher reduction of acrylamide in oat flakes by 928 µg/kg using PreventASE XR, compared to the wheat flakes, which reduced acrylamide by 360 µg/kg using PreventASE L. These represent a reduction of up to 77 % and 58 %, for oat and wheat flakes, respectively, compared to the control. This is the first study to compare multiple commercial asparaginases across two whole grain matrices in flakes, providing a direct side-by-side evaluation of their performance. Moreover, we demonstrate the challenges of translating enzymatic mitigation from lab to pilot-scale, where acrylamide dropped by only 76 µg/kg, underlining key practical barriers in industrial applications. Minimal impacts on color and texture were observed. Our results contribute new insights into enzyme-based acrylamide reduction and point to the importance of optimizing processing conditions for the application of asparaginases on an industrial scale, benefiting both producers and consumers.

1. Introduction

The demand for whole grain foods and breakfast cereals has grown substantially in recent years, which is associated with the increasing trend for consuming healthy food among consumers. Whole grains are recognized for their nutritional benefits, including a high dietary fiber content and their association with a reduced risk of chronic diseases such as diabetes and cardiovascular conditions (Williams, 2014). This aligns with public health recommendations that advocate using whole grains as an integral part of a balanced diet. Despite these benefits, a recent study on whole grain consumption found a significant gap between awareness and actual intake of whole grain foods. The most frequently consumed whole grain foods were bread and breakfast cereals (Foster et al., 2020). The global breakfast cereal market valued at

76.9 billion USD in 2023, is projected to grow significantly in the future due to the increasing consumer preferences for healthier and more nutritious food options (Pangarkar, 2024). Although whole grain breakfast cereals present a healthier alternative compared to refined grains, they are prone to higher formation of acrylamide due to the higher free asparagine content present in whole grains compared to white flours (Žilić et al., 2020). Acrylamide is a processing contaminant that is formed primarily through the Maillard reaction, which involves reducing sugars and free asparagine (Mottram et al., 2002). Although the Maillard reaction is desirable as it is responsible for the formation of flavor and color, one of the by-products is acrylamide, which poses a potential health risk due to its classification as “probably carcinogenic” to humans by the International Agency for Research on Cancer (IARC, 1994). Therefore, the European Union (EU) has established a regulatory

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benchmark to mitigate the presence of acrylamide in different food products. These benchmarks are not strict maximum levels yet, as they only operate as reference levels that encourage food manufacturers to implement measures that minimize acrylamide formation as outlined in Commission Regulation (EU) 2017/2158 (European Union, 2017).

The concentration of free asparagine as acrylamide precursor has been identified as a key factor influencing acrylamide formation in cereal products (Mottram et al., 2002). The correlation between asparagine and acrylamide content after baking is significant, but other amino acids can also influence acrylamide formation and elimination (Ciesarová et al., 2021; Yaylayan et al., 2005). The highest content of free asparagine is typical for rye and oats compared to corn, barley, and wheat grains.

One of the proposed acrylamide mitigation strategies is the use of asparaginases, which reduce acrylamide formation by hydrolyzing asparagine into aspartic acid and ammonia; therefore, contributing to lower acrylamide formation in the Maillard reaction without affecting any of the desirable sensory qualities of food, such as flavor and browning. First described by Zyzak et al. (2003), asparaginases have gained significant attention due to their efficacy and minimal adverse effect on product quality. There have been many studies on the use of asparaginases in different cereal-based products, including asparaginase application in wheat-based products (Anese et al., 2011; Kumar et al., 2014), rye- and oat-based formulations (Ciesarová et al., 2023), and wheat cookies and rye crispbread (Hendriksen et al., 2009; Musa et al., 2024a). Recent studies have further refined the process, optimizing asparaginase use for whole-grain products to address their higher asparagine content (Musa et al., 2024a, 2024b). In a scientific opinion published by the European Food Safety Authority, the distribution of acrylamide in bran products and whole grain cereals was 211 µg/kg (average) and 716 µg/kg (95th percentile). An average of 161 µg/kg of acrylamide in breakfast cereals was reported and 552 µg/kg at the 95th percentile (EFSA, 2015). The benchmark level set by the EU Commission Regulation is 300 µg/kg for breakfast cereals (European Union, 2017). Unlike previous studies that focused on dough-based systems such as cookies (Musa et al., 2024a, 2024b), this work investigates the effectiveness of different asparaginases in low-moisture, flake-type breakfast cereals, which differ substantially in processing, formulation, and enzyme-substrate interaction.

The aim of this study was to investigate the potential of four asparaginase variants to reduce acrylamide formation in whole grain wheat and oat flakes, a product category with limited prior research. Enzyme performance was evaluated across varying concentrations and incubation temperatures. The study further assessed the scalability of optimized conditions by producing wheat bran flakes at pilot-scale (1000 kg) and evaluated key quality parameters, including color and texture, to ensure product acceptability.

2. Materials and methods

2.1. Reagents and ingredients

The chemicals and reagents used in this study were of analytical grade or higher. Wheat groats were purchased from Harries Schälmlenwerk GmbH & Co. KG (Gross Ippener, Germany) and oat flakes were purchased at dm-Drogerie Markt (Karlsruhe, Germany). The asparaginases used in this study were donated by Novonesis (Bagsvaerd, Denmark) which included asparaginases R (Acrylaway L) and H (Acrylaway HighT), and two from DSM (Heerlen, The Netherlands), A (PreventAse L) and X (PreventAse XR), respectively. An Acrylamide ELISA kit together with the derivatization kit was bought from Eurofins (Eurofins Scientific SE, Luxembourg). ISOLUTE Multimode 500 mg/3 mL and ISOLUTE ENV+ 200 mg/3 mL were purchased from Biotage (Uppsala, Sweden). Chemicals for the analysis of free asparagine were L-asparagine ≥ 98 % (Sigma-Aldrich, Steinheim, Germany), 2,4,4-D₃-glutamic acid 97–98 % (Cambridge Isotope Laboratories, Andover, MA,

USA), glacial acetic acid HPLC reagent grade (Fischer Scientific, Loughborough, UK), perfluorooctanoic acid 96 %, acetonitrile and methanol HPLC gradient grade (Sigma-Aldrich, Steinheim, Germany). Deionised water of HPLC purity grade was prepared by PURITE Select system (Oxon, UK).

2.2. Asparaginases

Four commercial asparaginases were evaluated: R, T, A, and X. Each enzyme was dissolved in water before application with dosages following the manufacturers' recommendations. For enzymes R and T, two concentrations were tested (100 mg/kg and 200 mg/kg), while A and X were tested at 200 mg/kg and 300 mg/kg. Asparaginase preparations were used as is with activities specified by the manufacturers as reported before (Musa et al., 2024a, 2024b).

2.3. Preparation of wheat and oat flakes on laboratory-scale

Wheat and oat flakes were prepared in a laboratory setting. Wheat and oat groats were flaked twice using a flaking mill Herculez (Waldner biotech GmbH, Lienz, Austria). For each treatment, 300 ± 1 g of sample was used. The sample was placed on a tray and a total of 45 mL of water only (control) or water containing asparaginase was sprayed and mixed well for even distribution. The samples were then incubated at 60 °C in a proofer (EKA, Padova, Italy) for 10 min for treatments R and A, while for treatments with asparaginase H and X the samples were incubated at 70 °C for 10 min Table 1. The incubation time and temperatures were established as optimal in our earlier study (Musa et al., 2024a). After incubation, samples were baked in an oven (UNOX ROSSELL XFT197, Padova, Italy) at 220 °C for 15 min. After baking, samples were cooled at 22 °C for 30 min before color and texture were measured.

2.4. Preparation of wheat bran flakes on pilot-scale

The process of making wheat bran flakes on a scale of 1000 kg was done by mixing the ingredients (wheat kernels, wheat bran, sugar, malt extract, salt, vitamin mix) with asparaginase H in warm water to create a premix using two dosages of H: low dosage (H-L) 0.5 g/kg and high-dosage 1.0 g/kg. This premix was then added directly into the pressurized rotary cooker with a capacity of 1000 kg after the dry ingredients were dosed, ensuring that the enzyme is evenly distributed throughout the mixture. The ingredients were cooked for 95 min under steam at a pressure of 130 kPa using saturated steam. After cooking, the product was dried at 80 °C to reduce the moisture content. The dried mixture was

Table 1

Samples of wheat and oat flakes prepared on laboratory-scale and incubation time and temperature for samples baked at 220 °C for 15 min.

Sample name ¹	Asparaginase	Asparaginase concentration (mg/kg)	Incubation time (min)	Incubation temperature (°C)
C-60 °C	None (control)	0	10	60
R1	Acrylaway L	100	10	60
R2	Acrylaway L	200	10	60
A2	PreventAse L	200	10	60
A3	PreventAse L	300	10	60
C-70 °C	None (control)	0	10	70
H1	Acrylaway HighT	100	10	70
H2	Acrylaway HighT	200	10	70
X2	PreventAse XR	200	10	70
X3	PreventAse XR	300	10	70

¹ The same sample ID system is used for both wheat and oat flake samples.

then extruded at temperatures below 100 °C, shaping it into the desired form. For extrusion, an intermeshing twin-screw extruder (diameter 125 mm, model MPF 125, APV Baker, Grand Rapids, MI, USA) was used. Next, the extruded material was passed through rollers to create thin flakes, and finally, the bran flakes were roasted for less than 1 min at temperatures ranging from 200 °C to 270 °C. Wheat flakes were selected for pilot-scale testing due to practical considerations, as the industrial partner supporting the scale-up experiments already had wheat flake production established in their processing line, allowing direct integration of the enzymatic treatment without major process adjustments.

2.5. Acrylamide analysis

Acrylamide content was quantified using an Acrylamide ES ELISA kit, combined with a derivatization kit as described by Musa et al. (2024a) according to the instructions provided in the ELISA kit manual (Gold Standard Diagnostics, 2021). For extraction, 2 g of the sample was mixed with 40 mL of distilled water and agitated for 30 min. After allowing the mixture to settle for 5 min, the extract was filtered and centrifuged for 5 min at 13,000 x g. Sample clean-up was performed using Multimode SPE and ENV+ SPE columns. Acrylamide was eluted using methanol/water (60/40, v/v). The resulting eluent was derivatized and analyzed following the assay protocol. Acrylamide levels were determined by measuring absorbance at 450 nm using a photometer (Tecan, i-control infinite 200Pro, Männedorf, Switzerland).

2.6. Color analysis

To evaluate the effect of different treatments, the color of the flakes produced in the laboratory was analyzed using C-Cell (Calibre Control, Warrington, UK) and Color Muse (Variable Inc, Chattanooga, TN, USA). Both devices measure L* (lightness), a* (green-red), and b* (blue-yellow) values and were calibrated prior to use. C-Cell was calibrated using a calibration card (CC006, Calibre Control, Warrington, UK), and Color Muse used a lid with a white background, which is part of the device. Bran flakes produced on pilot-scale were analyzed only using C-Cell. A total of six measurements for each treatment was performed.

2.7. Texture properties

The samples were analyzed using a texture analyzer (TA.XTplus, Stable Micro Systems, Godalming, UK) with the Kramer shear cell fitted with five blades (A/KS5, Batch No. 17,009). The "Cereal bar shearing" method (CER1_KS5) was applied. For each test, the sample container was filled with 30.0 ± 0.1 g of the sample, and the blades were positioned 15 mm above the bottom plate. Each batch was tested in duplicate, providing a total of six measurements.

2.8. Weight loss of samples

To monitor the weight loss of samples, the sample weight was measured before and after the incubation step, as well as after baking for all three batches.

2.9. Analysis of free asparagine, aspartic acid, glutamine, and glutamic acid

Free asparagine, aspartic acid, glutamine, and glutamic acid were analysed according to the method by Ciesarová et al. (2021, 2023) in the pilot-scale samples, including control and asparaginase-treated H-L (0.5 g/kg) and H-H (1.0 g/kg) samples. The sample (2 g), with internal standard addition (2,4,4-D₃-glutamic acid), was extracted using 0.2 mmol/L acetic acid (20 mL) in centrifugation tubes with a vortex mixer (1 min) and a shaker (30 min). The clear supernatant after centrifugation (10 min at 8720 x g at -5 °C) was diluted (10 or 100 times) and filtered through a 0.45 µm nylon syringe filter (Q-Max RR Syringe

Filters, Frisette ApS, Knebel, Denmark) prior to LC-MS/MS analysis. An Agilent 1260 Infinity II system (Agilent Technologies, Santa Clara, CA, USA) coupled to an Agilent 6475 Triple Quadrupole detector equipped with the electrospray ionization (ESI) interface was applied. The analytical separation was performed on a Purospher STAR RP-Sec column (150 × 4.6 mm, 2.7 µm; Merck, Darmstadt, Germany). The mobile phase consisted of 100 mL of acetonitrile, 500 mL of an aqueous solution of perfluorooctanoic acid (0.05 mmol/L) and 1 mL of glacial acetic acid using isocratic elution at a flow rate of 0.5 mL/min at an ambient temperature of 25 °C. The ESI detection was performed in positive mode with the following optimized parameters: drying gas temperature 320 °C, drying gas flow (N₂) 8 L/min, nebulizer pressure 50 psi, capillary voltage 3 kV. For data acquisition, the multiple reaction monitoring (MRM) mode was used for specific mass transitions. The quantification was based on a calibration curve of the standard compound in the range from 0.02 to 4.00 µg/mL using the internal standard at a concentration of 0.5 µg/mL. The time of analysis was 7 min. The LOD of the applied procedure was 6 µg/L and the LOQ was 20 µg/L.

2.10. Statistical analysis

Data analysis was performed using multiple software tools. Microsoft Excel was employed to compute means and standard deviations. Statistical analysis was conducted with Origin 2023 (OriginLab Corporation, Northampton, MA, USA), while IBM SPSS Statistics (Version 29.0, IBM Corp., Armonk, NY, USA) was used to perform Dunnett's *t*-tests ($p \leq 0.05$) to assess significant differences between the control and asparaginase-treated samples. The average acrylamide concentrations in the samples were calculated using the online ELISA calculator available on the Arigo website (Arigo Biolaboratories, 2025). Pearson's correlation coefficients (*r*) were also determined using Origin 2023, with significance set at $p \leq 0.05$.

3. Results and discussion

3.1. Acrylamide content in wheat and oat flakes on laboratory-scale

Fig. 1 presents the percentage of acrylamide reduction for each of the four asparaginases in wheat (A) and oat (B) flakes produced in the laboratory compared to the respective control (set to 100 %). The acrylamide content in the oat and wheat control samples was 1204 µg/kg and 627 µg/kg, respectively. At the incubation temperature of 60 °C, an acrylamide reduction of 58 % at most compared to the control was achieved in wheat flakes using the A2 treatment. Although increasing the concentration of asparaginase from 200 mg/kg (A2) to 300 mg/kg (A3) is expected to further increase acrylamide reduction, this was not the case for wheat flakes. In contrast, the higher concentration of asparaginase R decreased acrylamide slightly more than the lower concentration from 40 % to 45 % for R1 and R2, respectively. Wheat flakes incubated at 70 °C showed the highest acrylamide reduction using the H1 treatment with up to 52 % in comparison to the control. The other treatments also caused a significant reduction of acrylamide with up to 44 %, 48 %, and 43 % for the H2, X2, and X3 treatments, respectively. Asparaginases behaved differently when they were applied to a different cereal matrix such as oat (Fig. 1B). Even though the asparaginase dosage and the incubation and baking conditions were the same for both cereals, acrylamide reduction was higher in oat flakes compared to wheat. All asparaginases efficiently reduced acrylamide in oat flakes irrespective of the dosage and the incubation temperature by 71 % (A3) to 77 % (A2 and X3). The different efficacy of asparaginases in different cereal matrices was also observed in our previous studies in cookies made of wheat, rye, oat, corn, and rice flour (Musa et al., 2024a, 2024b). In cookies made with oat flour, asparaginases had the highest efficacy, while they were least effective in wheat flour cookies. This may be related to the food matrix, as oats typically contains higher levels of the precursor asparagine to wheat. The higher the asparagine content in

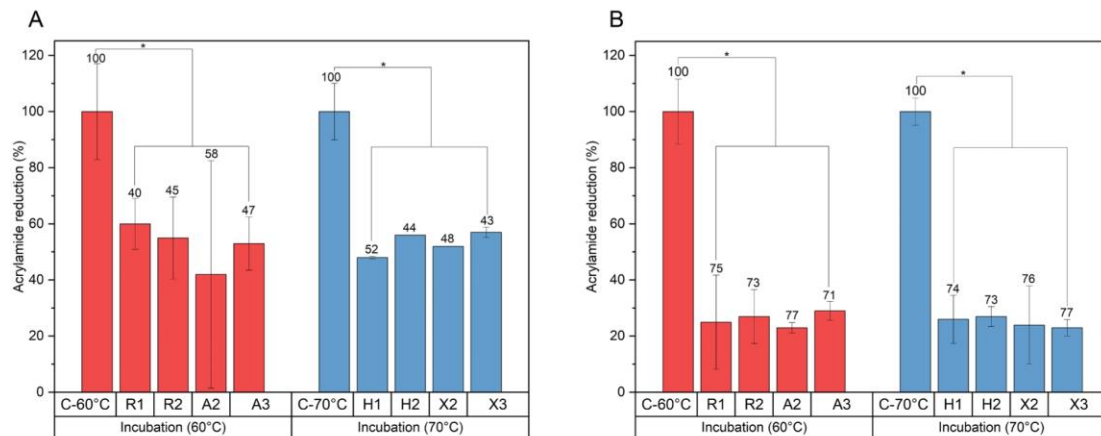


Fig. 1. Reduction of acrylamide in wheat (A) and oat (B) flakes produced on laboratory-scale. Values are given as means $\pm$ standard deviation (error bars) and asterisks indicate a significant difference compared to the respective control (ANOVA with Dunnett's *t*-test, $p \leq 0.05$). The numbers show the percentage of acrylamide reduction based on the respective mean values and compared to the respective control set to 100 %.

C-60 °C: control without asparaginase incubated at 60 °C, C-70 °C: control without asparaginase incubated at 70 °C, R1: sample with asparaginase R at 100 mg/kg, R2: sample with asparaginase R at 200 mg/kg, A2: sample with asparaginase A at 200 mg/kg, A3: sample with asparaginase A at 300 mg/kg, H1: sample with asparaginase H at 100 mg/kg, H2: sample with asparaginase H at 200 mg/kg, X2: sample with asparaginase X at 200 mg/kg, X3: sample with asparaginase X at 300 mg/kg. Please refer to Table 1 for detailed sample information.

the raw material, the higher the formation of acrylamide is, unless asparaginases are added. The correlation between these two factors has been shown also in other studies (Claus et al., 2006; Musa et al., 2024b; Oddy et al., 2023; Szafrńska et al., 2024). Asparaginases were already investigated in a few other studies (Anese et al., 2011; Ciesarová et al., 2023; Gazi et al., 2023; Hendriksen et al., 2009; Kumar et al., 2014; Musa et al., 2024a, 2024b) and reductions of up to 92 % of acrylamide were reported in rye crispbread (Hendriksen et al., 2009). Anese et al. (2011) also report variations in acrylamide concentrations between different batches resulting in high standard deviations. The variability of acrylamide content in three different batches belonging to the same treatment was evident in our study. This could be due to manual processing conditions, such as temperature and time which may differ even when the same protocol is followed. Additionally, sample heterogeneity after baking is a key factor. It was observed that after baking the surface of the batch was slightly darker in color compared to the middle or bottom of the batch. Musa et al. (2024a) discussed that these variabilities may also come due to the higher formation of acrylamide on the surface of cookies, which can result in higher standard deviations in acrylamide within the same samples, especially when samples are milled all together (crust and crumb). A reduction of acrylamide by up to 96 % was achieved in oat cookies (Musa et al., 2024b), whereas the highest reduction in oat flakes was 77 %. Similarly, wheat cookies showed a reduction of up to 79 % (Musa et al., 2024a), while wheat flakes achieved a lower maximum reduction of 58 %. The lower moisture content in breakfast cereals makes it challenging for the asparaginases to act as efficiently as in the cookie dough matrix where it is easier to control time, temperature, and moisture content (Pesce et al., 2024). A further fundamental difference lies in how the enzyme is applied: in cookies, asparaginases act throughout the entire dough, allowing uniform interaction with the substrate. In contrast, for flakes, the enzyme is sprayed onto the surface, restricting its action to the outer layer and leaving much of the asparagine within the matrix untouched. This is why the application of asparaginases needs to be optimized for each cereal matrix and product.

3.2. Acrylamide, free asparagine, aspartic acid, glutamine and glutamic acid in wheat bran flakes on pilot-scale

To further evaluate the impact of asparaginases in pilot-scale production, asparaginase H was added to wheat bran flakes using two different concentrations H-L (0.5 g/kg) and H-H (1 g/kg), and compared to a control sample without asparaginase addition (Table 2). The lower asparaginase concentration resulted in a reduction of acrylamide by 3 %. The higher concentration of asparaginase H decreased acrylamide by 9 %. To better understand this effect, the free asparagine content was measured in control and asparaginase-treated bran flakes. The asparagine concentrations in the control (96 mg/kg), low-dose treatment (H-L, 112 mg/kg), and high-dose treatment (H-H, 94 mg/kg) were similar, with only minor variations. Interestingly, despite the enzymatic activity of asparaginase, which converts asparagine into aspartic acid to reduce the precursor available for acrylamide formation, the asparagine content in the treated samples was not significantly lower than in the control. In fact, the H-L sample exhibited a slightly higher asparagine level compared to the control. This may suggest incomplete conversion of asparagine at lower enzyme concentrations. Furthermore, the difference in asparagine concentration between the control and H-H samples aligns with the observed reduction in acrylamide. This indicates that while asparaginase activity effectively reduces acrylamide formation, the residual asparagine levels might not fully reflect the action of the enzyme, possibly due to asparagine stabilization within the bran or competing interactions in the food matrix. In addition to monitoring the conversion of asparagine to aspartic acid, changes in the content of glutamine and glutamic acid were evaluated, because these amino acids may directly influence acrylamide formation in the process of the Maillard reaction. The sample H-L exhibited the highest levels of both asparagine and aspartic acid. In the H-H sample, a negligible 2 % decrease in asparagine content was observed, from 95.5 mg/kg to 94.4 mg/kg, which was not statistically significant. In contrast, aspartic acid content increased by 8 %, from 46.1 mg/kg to 49.9 mg/kg, and glutamic acid content showed a slight increase, from 42.1 mg/kg to 43.1 mg/kg. The observed statistically significant variations in the content of products resulting from the action of asparaginase, without a decrease in asparagine, indicate that additional enzymatic processes may occur that could be responsible for the accumulation of asparagine e.g. by aspartate transaminase and

Table 2

Characteristics of wheat bran flakes produced on pilot-scale, including the control and samples with Acrylaway HighT low dosage (0.5 g asparaginase/kg, H-L) and Acrylaway HighT high dosage (1.0 g asparaginase/kg, —H-H). The parameters analyzed include acrylamide reduction, content of free asparagine, aspartic acid, glutamine and glutamic acid, as well as color parameters expressed as L* (lightness), a* (green-red), and b* (blue-yellow).

Sample	Acrylamide reduction (%)	Asparagine (mg/kg)	Aspartic acid (mg/kg)	Glutamine (mg/kg) ¹	Glutamic acid (mg/kg)	L*	a*	b*
Control	N/A	95.9 ± 0.9	46.0 ± 1.9	< 20	42.1 ± 0.7	13.0 ± 0.9	5.6 ± 0.3	16.3 ± 1.0
H-L	3	112.6 ± 1.2*	57.2 ± 0.9**	< 20	49.6 ± 0.4**	13.5 ± 0.7	4.9 ± 0.6*	17.0 ± 0.6
—H-H	9	94.3 ± 0.5	49.9 ± 0.7*	< 20	43.1 ± 0.0*	13.5 ± 0.8	5.3 ± 0.2	16.5 ± 1.0

Values are given as means ± standard deviation and asterisks denote significant differences compared to the control (ANOVA with Dunnett's *t*-test, $p \leq 0.05$, $n = 6$). N/A: no addition of asparaginase.

¹ 20 mg/kg is the limit of quantitation.

asparagine synthetase (Ciesarová et al., 2021). Aspartate transaminase catalyzes the reversible transfer of an α -amino group by converting oxaloacetate and glutamate to aspartate and α -ketoglutarate. Asparagine synthase uses aspartate, glutamine and adenosinetriphosphate to produce asparagine, glutamate, adenosinemonophosphate and diphosphate, increasing the amount of asparagine (Yoo et al., 2020). This finding indicates that the elevated aspartic acid content was not predominantly caused by asparagine hydrolysis through the action of asparaginase, suggesting that the effect of the enzyme was not significant. Furthermore, glutamine content in all samples was below the detection limit, indicating its negligible contribution to the reaction pathways associated with acrylamide generation. The application of asparaginase in pilot-scale production demonstrated a much lower percentage of acrylamide reduction compared to laboratory-scale experiments, highlighting the critical need for optimization of processing conditions. While laboratory-scale production allows for precise control of variables such as incubation temperature, enzyme concentration, reaction time, and substrate homogeneity, scaling up introduces several

challenges that can influence the efficacy of the enzyme. In pilot-scale production, achieving uniform distribution of the enzyme within the product matrix can be more difficult due to larger batch sizes and differences in equipment design. The enzyme activity is highly sensitive to temperature and pH value, and maintaining optimal conditions across the entire production process may be inconsistent. In general, an optimum pH values of 6 to 9 is needed, depending on the asparaginase used. For example, the optimal activity of *Aspergillus oryzae* asparaginase has been reported at neutral pH and temperatures of up to 60 °C, with stability maintained between pH 4 and 8 at 37 °C for up to 2 h in buffer, making it suitable for use in food processing applications (Hendriksen et al., 2009). Variations in heat transfer, mixing efficiency, and reaction times can lead to localized differences in enzyme activity, resulting in less effective asparagine conversion and, consequently, lower acrylamide reduction. The differences between laboratory and industrial-scale results underscore the importance of conducting pilot-scale studies to bridge the gap between controlled experimental conditions and real-world manufacturing. Such studies can provide

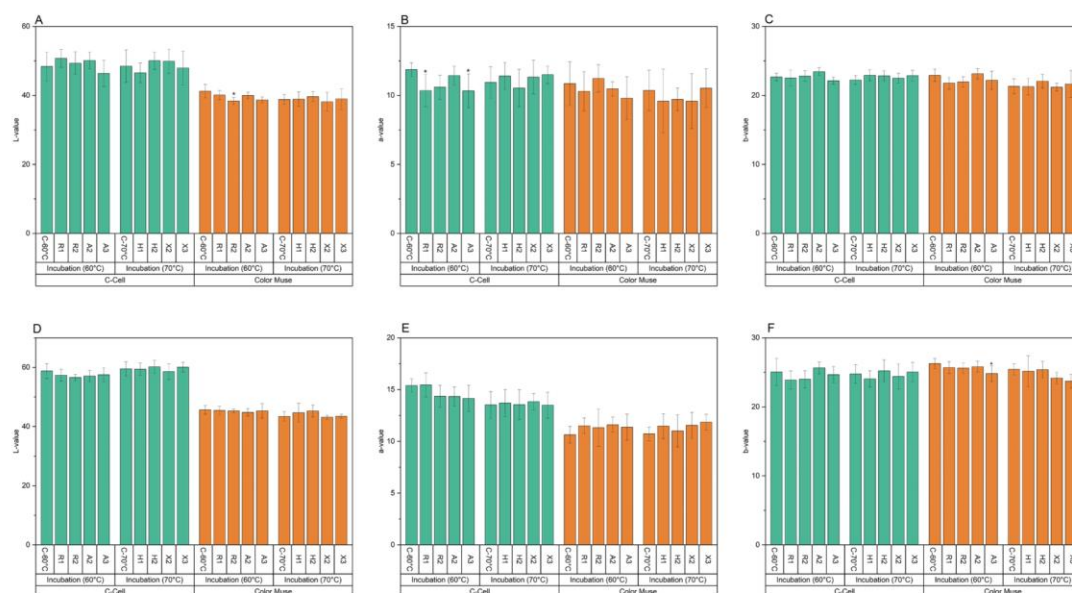


Fig. 2. Color analysis of wheat (A, B, C) and oat (D, E, F) flakes baked at 220 °C for 15 min using C-Cell and Color Muse. Color parameters are expressed as L* (lightness), a* (green-red), and b* (blue-yellow). Values are given as means ± standard deviation (error bars) and asterisks denote significant differences compared to the respective control (ANOVA with Dunnett's *t*-test, $p \leq 0.05$).

C-60 °C: control without asparaginase incubated at 60 °C, C-70 °C: control without asparaginase incubated at 70 °C, R1: sample with asparaginase R at 100 mg/kg, R2: sample with asparaginase R at 200 mg/kg, A2: sample with asparaginase A at 200 mg/kg, A3: sample with asparaginase A at 300 mg/kg, H1: sample with asparaginase H at 100 mg/kg, H2: sample with asparaginase H at 200 mg/kg, X2: sample with asparaginase X at 200 mg/kg, X3: sample with asparaginase X at 300 mg/kg. Please refer to Table 1 for detailed sample information.

critical insights into the adjustments needed for processing parameters, ensuring the consistent efficacy of asparaginase for acrylamide mitigation on a commercial scale, in order to meet the benchmark levels set by the EU commission regulation (European Union, 2017).

3.3. Color analysis

To evaluate the impact of the different asparaginases applied in wheat and oat flakes on laboratory-scale, as well as the bran flakes produced in the scale-up experiments, C-Cell and Color Muse were used to obtain $L^*a^*b^*$ color values. Fig. 2 presents the color values obtained from both devices for wheat (A, B, C) and oat (D, E, F) flakes. The L^* -value represents the lightness of the product with a low value indicating a dark product (0 = black) and a high value indicating a light or bright color (100 = white) (Ly et al., 2020). There were no significant differences in L^* -, a^* -, or b^* -values for C-Cell measurements of wheat flakes, except for a decrease compared to the control in the a^* -value for the R1 and A3 treatments (Fig. 2A-C). Similar results were obtained using Color Muse with no significant changes except for the R2 treatment which showed a slightly darker color compared to the control. For oat flakes (Fig. 2D-F) there were no significant changes in L^* - and a^* -values for either of the treatments applied. For the b^* -value, the observations done using C-Cell showed no significant changes, while for Color Muse, only the A3 treatment showed a small, but significant decrease in the b^* -value. Similar to our study, Gazi et al. (2023) also reported minimal effects of asparaginase addition on the color of different baked products such as rotary molded and cut biscuit dough, wire cut cookie dough, and rotary cut cracker dough. Kukurová et al. (2013) also showed that cookies prepared with asparaginase showed no significant differences in the color of the baked product. Our previous studies on cookies also showed that asparaginases do not have any adverse effects on color with only minimal changes (Musa et al., 2024a, 2024b). A study by Calabrese et al. (2024) further confirmed that the $L^*a^*b^*$ -values of asparaginase-treated bread showed no significant changes in color.

For the bran flakes produced on a pilot-scale, color was measured only using C-Cell. Similar to laboratory-processed flakes, the color of the bran flakes was not affected by asparaginase application (Table 2). Only the a^* -value for the H-L treatment showed a slight, but significant decrease compared to the control. The results demonstrate that the use of asparaginases does not influence the color of the products, regardless of the production scale. This indicates that asparaginase treatment is a reliable process with minimal impact on the visual attributes of the product. Such findings are crucial for industrial applications, where consumer acceptance heavily relies on visual appearance.

Furthermore, the goal of testing two different color scanning devices to obtain $L^*a^*b^*$ -values was to establish a new method for color measurements, which is a cheaper alternative to C-Cell. This was also studied in our previous publications (Musa et al., 2024a, 2024b), and the data in the current paper further confirms that Color Muse is suitable for color analysis of flakes. However, C-Cell provides further quality-related parameters of baked goods, something that Color Muse does not offer. Color evaluations alone are often used in studies related to acrylamide, due to the link between color and acrylamide formation (Gökmen et al., 2008; Mustafa et al., 2005; Serpen & Gökmen, 2009), but also to evaluate any color changes that different acrylamide mitigation strategies might have, as in the current study.

3.4. Texture properties and weight loss

Table 3 presents the hardness and weight loss of flakes produced on laboratory-scale. While some small deviations within the same treatment group were observed, none of the changes were statistically significant, for either oat or wheat flakes, respectively. The deviations may be due to the differences in batches which are inevitable in the baking process. Therefore, the weight loss was monitored after incubation and

Table 3

Hardness and weight loss of wheat and oat flakes prepared on laboratory-scale and baked at 220 °C for 15 min.

Sample ID ¹	Hardness (N)		Weight loss after incubation (%)		Weight loss after baking (%)	
	Wheat Flakes	Oat Flakes	Wheat Flakes	Oat Flakes	Wheat Flakes	Oat Flakes
C-60 °C	58.3 ± 5.5	56.0 ± 5.1	0.1	0.2	16.3	16.0
R1	57.2 ± 5.1	52.2 ± 6.9	0.1	0.1	16.1	16.3
R2	60.4 ± 5.7	53.1 ± 5.8	0.2	0.1	17.6	15.7
A2	54.3 ± 3.9	55.8 ± 5.3	0.2	0.1	17.0	15.8
A3	53.1 ± 6.0	56.6 ± 6.8	0.1	0.1	15.9	15.3
C-70 °C	57.7 ± 4.2	55.1 ± 4.6	0.3	0.2	16.6	16.1
H1	57.9 ± 3.9	55.3 ± 6.3	0.1	0.2	16.9	16.8
H2	55.6 ± 6.1	56.5 ± 6.1	0.1	0.5	17.9	16.3
X2	57.0 ± 6.5	52.2 ± 6.3	0.1	0.2	17.3	16.7
X3	56.9 ± 5.4	55.4 ± 7.0	0.1	0.1	16.7	17.2

¹ Please refer to Table 1 for detailed sample information. Values are given as means ± standard deviation for hardness. There were no statistically significant differences (ANOVA with Dunnett's *t*-test, *p* > 0.05) in these parameters of wheat or oat flakes compared to the control.

baking (Table 3). Measuring the weight loss of flakes during the process is essential to ensure the desired texture and crispiness by controlling moisture content. It also serves as a quality control measure to maintain consistency in the production process by identifying potential variations in baking conditions. The weight loss was low during the 10 min incubation period, due to the low temperature and short time. The weight loss in wheat and oat flakes had an overall difference of 1.9 % at most between the samples. This presents a small deviation between different groups. These results are in agreement with our previous investigation on the effect of asparaginases in cookies (Musa et al., 2024a, 2024b). Both studies evaluated the impact of asparaginase treatment using different raw materials (flour) and several asparaginases in different concentrations. The effect of asparaginases on hardness or fracturability of cookies was minimal. Asparaginases were also tested in potato products. A study by Dourando et al. (2020) on raw potato sticks showed that the application of asparaginases using a time of 0, 5, 10, or 20 min and different pressures showed no substantial difference in firmness or stiffness of the product. A study by Ciesarová et al. (2023) investigated the replacement of whole grain flour with sea buckthorn pomaces powder. This powder was treated enzymatically using Acrylaway L before application, and it showed a significant effect on the textural properties of cookies. However, this study assumes that the changes in texture might be attributed due to the adjusted pH value, rather than the application of enzyme. Similar to our study, Kumar et al. (2014) showed no significant changes using asparaginase treatment in sweet bread. However, recipe formulation often has more impact not only in the formation of acrylamide itself but also on the textural properties of the final product. In the current study, flakes were prepared with asparaginase application after the flaking process, while in the industry often additives and sweeteners are added which influence acrylamide formation and/or asparaginase activity, but also have a higher impact due to different processing conditions. The texture of the bran flakes was not measured, since the samples could not be tested on the same day they were prepared, as the texture can be influenced by the storage conditions as well. Therefore, the evaluation of textural properties for larger-scale production should be further investigated in future studies.

4. Conclusion

This study demonstrates the effectiveness of asparaginases in reducing acrylamide levels in cereal-based products, particularly in wheat and oat flakes. Wheat and oat matrices responded differently to asparaginase treatment; with wheat flakes showing a lower acrylamide reduction than oat flakes, up to 58 % and 77 %, respectively. The evaluation of color and texture properties showed minimal or no impact at all when asparaginases were applied. The study also investigated scaling up the production of flakes and the asparaginase effects. Compared to the laboratory-scale, the reduction was significantly lower. Due to different processing conditions and products as well, the differences are expected. Therefore, further research is needed to optimize methods for the application of asparaginases in pilot- or industrial-scale production to maximize the effectiveness of the enzyme. Application of asparaginases in pilot-scale production showed minimal impact on color, however, sensory analysis and textural properties should also be further evaluated. With further optimization and scale-up measures, the results show promise for broad industrial application, benefiting both producers and consumers.

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Compliance with ethics requirements

Not applicable.

Informed consent

Not applicable.

Declaration of generative AI in scientific writing

During the preparation of this work, the first author used Grammarly in order to improve readability and language. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Ethical statement

This article does not contain any studies with human or animal subjects.

CRediT authorship contribution statement

Shpresa Musa: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Kristína Kukurová:** Writing – review & editing, Methodology, Formal analysis. **Zuzana Ciesarová:** Writing – review & editing, Methodology, Formal analysis. **Karsten Eisenhardt:** Writing – review & editing, Resources, Investigation. **Katharina Anne Scherf:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

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

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Data availability

Data will be made available on request.

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