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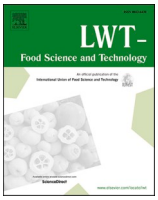

Asparaginase treatment to mitigate acrylamide formation in wheat and rye cookies

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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, Schönbächler, 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; Kukurova 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%. Kukurova 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

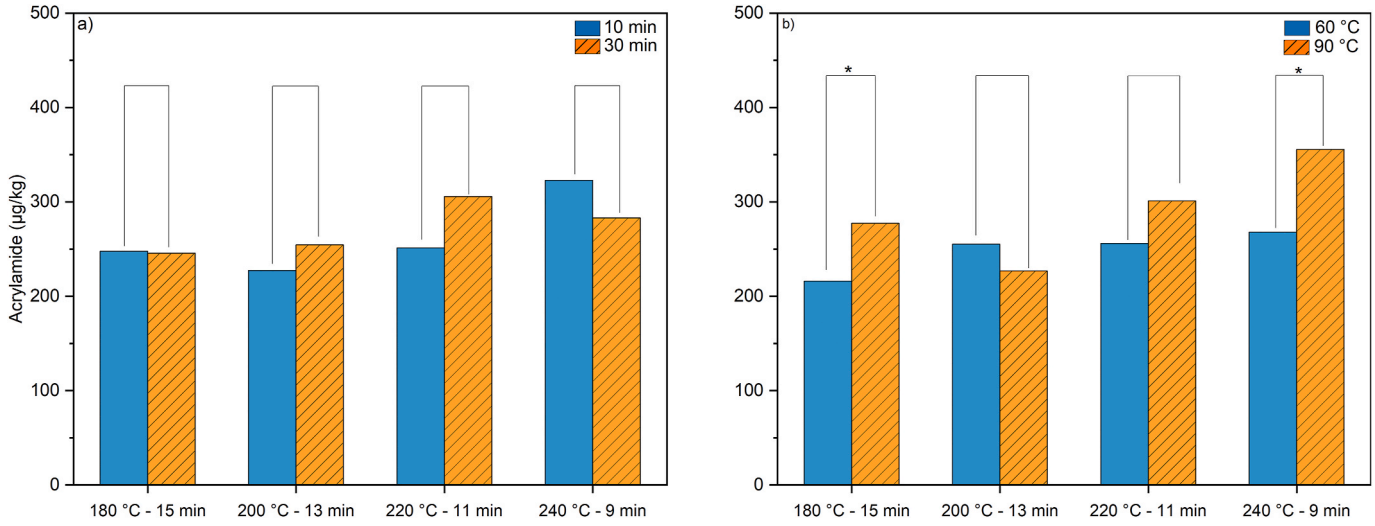

Fig. 1. Effect of dough incubation time (a) and temperature (b) on acrylamide formation in wheat cookies baked at 180 °C, 200 °C, 220 °C and 240 °C, for 15 min, 13 min, 11 min and 9 min, respectively. The data are presented as mean values (n = 3) ± standard deviation and two-sample *t*-test was used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

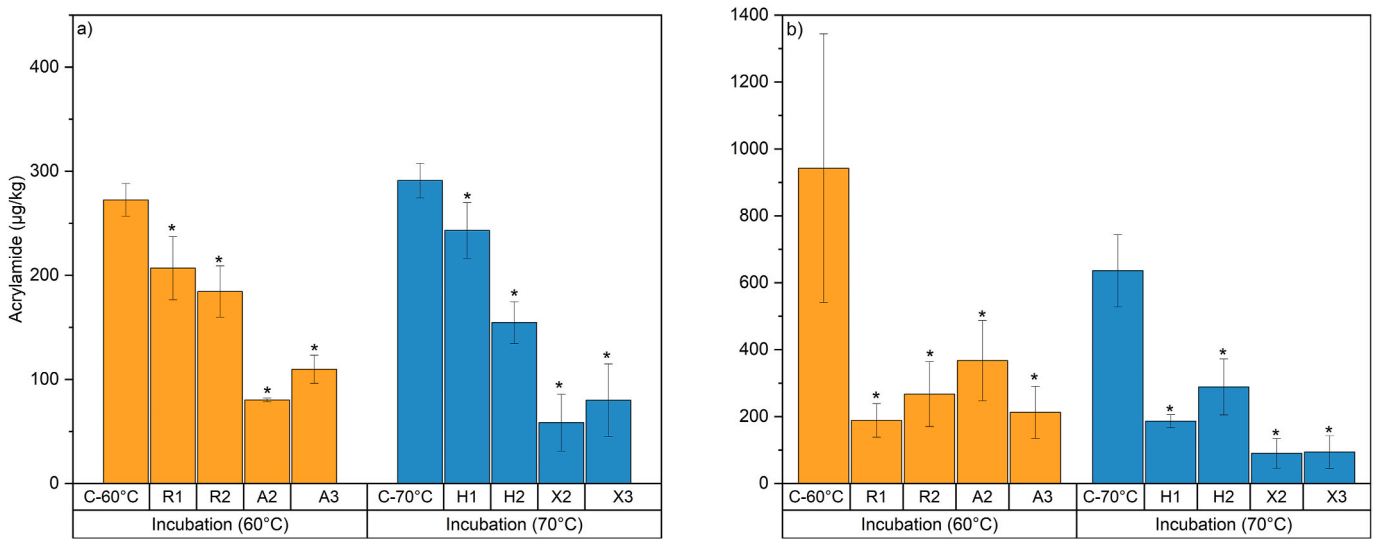

Fig. 2. Acrylamide content using asparaginases in wheat (a) and rye (b) cookies baked at 220 °C for 11 min. Error bars indicate the standard deviation and asterisks indicate a significant difference compared to the respective control (ANOVA with Dunnett's test, $p \leq 0.05$, $n = 3$). Details of p-values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

		Wheat cookies			Rye cookies		
		L	a	b	L	a	b
	Sample ID[a]	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 (Palazoğlu, 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 0.00	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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