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

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

PII: S2772-5022(25)00542-6
DOI: https://doi.org/10.1016/j.afres.2025.101237
Reference: AFRES 101237

To appear in: *Applied Food Research*

Received date: 15 May 2025
Revised date: 1 August 2025
Accepted date: 2 August 2025

Please cite this article as: Shpresa Musa , Kristína Kukurová , Zuzana Ciesarová , Karsten Eisenhardt , Katharina Anne Scherf , Asparaginase Treatment for Acrylamide Reduction in Wheat and Oat Flakes: From Laboratory- to Pilot-Scale, *Applied Food Research* (2025), doi: https://doi.org/10.1016/j.afres.2025.101237

HIGHLIGHTS

- Asparaginases were used to reduce acrylamide in oat and wheat flakes
- Asparaginases reduced acrylamide by up to 77% in oat flakes and 58% in wheat flakes
- Minimal effect on color and texture after asparaginase treatment
- Up to 9% reduction in wheat bran flakes produced on pilot-scale
- Process optimization is necessary for asparaginase application on pilot-scale

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

Shpresa Musa[1,5], Kristína Kukurová[2], Zuzana Ciesarová[2], Karsten Eisenhardt[3], Katharina Anne Scherf[1,4,5]*

[1] Department of Bioactive and Functional Food Chemistry, Institute of Applied Biosciences, Karlsruhe Institute of Technology (KIT), Karlsruhe, 76131, Germany

[2] National Agricultural and Food Centre, Food Research Institute, Bratislava, 82475, the Slovak Republic

[3] Technology & Process Development, H. & J. Brueggen KG, 23568 Lübeck, Germany

[4] Leibniz Institute for Food Systems Biology at the Technical University of Munich, Freising, 85354, Germany

[5]Technical University of Munich, TUM School of Life Sciences, Professorship of Food Biopolymer Systems, Freising, 85354, Germany

***Correspondence:** Katharina A. Scherf, Leibniz Institute for Food Systems Biology at the Technical University of Munich, Lise-Meitner-Str. 34, Freising, Germany, Email: k.scherf.leibniz-lsb@tum.de

ORCID for Shpresa Musa: 0009-0001-6847-1920

ORCID for Kristína Kukurová: 0000-0001-9091-9683

ORCID for Zuzana Ciesarová: 0000-0003-1009-1121

ORCID for Katharina A. Scherf: 0000-0001-8315-5400

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.

Keywords

breakfast cereals, color, enzymes, texture

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 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., 2024b, 2024a), 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 Schälmühlenwerk 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-D_3-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 PUR1TE 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., 2024b, 2024a).

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 (H-H) 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 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 CCell (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. CCell 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 CCell. 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. 17009). 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-D_3-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, Frisenette 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 x 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 (N_2) 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). 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

Figure 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 (Figure 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 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; Szafrańska 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., 2024b, 2024a) 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 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 hours 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 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, CCell and Color Muse were used to obtain L*a*b* color values. Figure 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 CCell measurements of wheat flakes, except for a

decrease compared to the control in the a*-value for the R1 and A3 treatments (Figure 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 (Figure 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., 2024b, 2024a). 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., 2024b, 2024a), and the data in the current paper further confirms that Color Muse is suitable for color analysis of flakes. However, CCell 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 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., 2024b, 2024a). 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 fracturablity 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.

Acknowledgments

The authors thank Dr. Peter Müller (Novonesis) for providing the asparaginases and offering valuable support for the scale-up experiments and Jana Horváthová (National Agricultural and Food Centre) for her technical assistance with the amino acid analysis.

Funding sources

This IGF project of the FEI was supported within the programme for promoting the Industrial Collective Research (IGF) of the Federal Ministry of Economic Affairs and Climate Action (BMWK), based on a resolution of the German Parliament. Project 21798 N. This project was further supported by the Committee on Cereal Research of the Arbeitsgemeinschaft Getreideforschung e.V. (AGF), Detmold. We also thank the German Academic Exchange Service (Deutscher Akademischer Austauschdienst, DAAD) for providing a scholarship to Shpresa Musa. This work is related to COST Action ACRYRED, CA21149, supported by COST (European Cooperation in Science and Technology).

Compliance with ethical standards

Conflict of interest. The authors SM, KK, ZC and KAS declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. KE is an employee of H. & J. Brueggen KG.

Compliance with ethics requirements. Not applicable.

Informed consent. Not applicable.

Author contributions

Shpresa Musa: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Visualization, Writing – Original draft

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: Conceptualization, Supervision, Project administration, Funding acquisition, Resources, Writing – review & editing

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.

References

Anese, M., Quarta, B., & Frias, J. (2011). Modelling the effect of asparaginase in reducing acrylamide formation in biscuits. *Food Chemistry*, *126*(2), 435–440. https://doi.org/10.1016/j.foodchem.2010.11.007

Arigo Biolaboratories. (n.d.). *GainData, Arigo's ELISA Calculator*. https://www.arigobio.com/elisa-analysis

Calabrese, M., De Luca, L., Basile, G., Sorrentino, G., Esposito, M., Pizzolongo, F., Verde, G., & Romano, R. (2024). Reducing the acrylamide concentration in homemade bread processed with L-asparaginase. *LWT*, *209*, 116770. https://doi.org/10.1016/j.lwt.2024.116770

Ciesarová, Z., Kukurová, K., Jelemenská, V., Horváthová, J., Kubincová, J., Belović, M., & Torbica, A. (2023). Asparaginase Treatment of Sea Buckthorn Berries as an Effective Tool for Acrylamide Reduction in Nutritionally Enriched Wholegrain Wheat, Rye and Triticale Biscuits. *Foods*, *12*(17), 3170. https://doi.org/10.3390/foods12173170

Ciesarová, Z., Kukurová, K., Torbica, A., Belović, M., Horváthová, J., Daško, Ľ., & Jelemenská, V. (2021). Acrylamide and 5-hydroxymethylfurfural in thermally treated non-wheat flours and respective breads. *Food Chemistry*, *365*, 130491. https://doi.org/10.1016/j.foodchem.2021.130491

Claus, A., Schreiter, P., Weber, A., Graeff, S., Herrmann, W., Claupein, W., Schieber, A., & Carle, R. (2006). Influence of agronomic factors and extraction rate on the acrylamide contents in yeast-leavened breads. *Journal of Agricultural and Food Chemistry*, *54*(23), 8968–8976. https://doi.org/10.1021/jf061936f

Dourado, C., Pinto, C. A., Cunha, S. C., Casal, S., & Saraiva, J. A. (2020). A novel strategy of acrylamide mitigation in fried potatoes using asparaginase and high pressure technology. *Innovative Food Science & Emerging Technologies*, *60*, 102310. https://doi.org/10.1016/j.ifset.2020.102310

EFSA. (2015). Scientific Opinion on acrylamide in food EFSA Panel on Contaminants in the Food Chain (CONTAM). *EFSA Journal*, *13*(6), 4104. https://doi.org/10.2903/j.efsa.2015.4104

European Union. (2017). *COMMISSION REGULATION (EU) 2017/2158 of 20 November 2017 establishing mitigation measures and benchmark levels for the reduction of the presence of acrylamide in food*. https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32017R2158. Date of upload: 21.11.2017

Foster, S., Beck, E., Hughes, J., & Grafenauer, S. (2020). Whole Grains and Consumer Understanding: Investigating Consumers' Identification, Knowledge and Attitudes to Whole Grains. *Nutrients*, *12*(8), 2170. https://doi.org/10.3390/nu12082170

Gazi, S., Göncüoğlu Taş, N., Görgülü, A., & Gökmen, V. (2023). Effectiveness of asparaginase on reducing acrylamide formation in bakery products according to their dough type

and properties. *Food Chemistry*, *402*, 134224. https://doi.org/10.1016/j.foodchem.2022.134224

Gökmen, V., Açar, Ö. Ç., Arribas-Lorenzo, G., & Morales, F. J. (2008). Investigating the correlation between acrylamide content and browning ratio of model cookies. *Journal of Food Engineering*, *87*(3), 380–385. https://doi.org/10.1016/j.jfoodeng.2007.12.029

Gold Standard Diagnostics. (2021). *Enzyme-Linked Immunosorbent Assay for the Determination of Acrylamide in Food Samples: Https://www.goldstandarddiagnostics.us/media/15629/ug-21-046-rev-01-abraxis-acrylamide-elisa_515680.pdf*. https://www.goldstandarddiagnostics.us/media/15629/ug-21-046-rev-01-abraxis-acrylamide-elisa_515680.pdf. Date of upload: 16.08.2021

Hendriksen, H. V., Kornbrust, B. A., Østergaard, P. R., & Stringer, M. A. (2009). Evaluating the potential for enzymatic acrylamide mitigation in a range of food products using an asparaginase from Aspergillus oryzae. *Journal of Agricultural and Food Chemistry*, *57*(10), 4168–4176. https://doi.org/10.1021/jf900174q

IARC. (1994). *Acrylamide. IARC monographs on the evaluation of carcinogenic risks to humans.* https://monographs.iarc.who.int/wp-content/uploads/2018/08/14-002.pdf. Date of upload: 20.08.2018

Kukurová, K., Ciesarová, Z., Mogol, B. A., Açar, Ö. Ç., & Gökmen, V. (2013). Raising agents strongly influence acrylamide and HMF formation in cookies and conditions for asparaginase activity in dough. *European Food Research and Technology*, *237*(1), 1–8. https://doi.org/10.1007/s00217-013-1976-z

Kumar, M., Shimray, C. A., Indrani, D., & Manonmani, H. K. (2014). Reduction of Acrylamide Formation in Sweet Bread with l-Asparaginase Treatment. *Food and Bioprocess Technology*, *7*(3), 741–748. https://doi.org/10.1007/s11947-013-1108-6

Ly, B. C. K., Dyer, E. B., Feig, J. L., Chien, A. L., & Del Bino, S. (2020). Research Techniques Made Simple: Cutaneous Colorimetry: A Reliable Technique for Objective Skin Color Measurement. *Journal of Investigative Dermatology*, *140*(1), 3-12.e1. https://doi.org/10.1016/j.jid.2019.11.003

Mottram, D. S., Wedzica, D. L., & Dodson, A. T. (2002). Acrylamide is formed in the Maillard reaction. *Nature*, *419*(6906), 448–449. https://doi.org/10.1038/419448a

Musa, S., Becker, L., Oellig, C., & Scherf, K. A. (2024a). Asparaginase Treatment to Mitigate Acrylamide Formation in Wheat and Rye Cookies. *LWT - Food Science and Technology*, *203*. https://doi.org/10.1016/j.lwt.2024.116365

Musa, S., Becker, L., Oellig, C., & Scherf, K. A. (2024b). Influence of Asparaginase on Acrylamide Content, Color, and Texture in Oat, Corn, and Rice Cookies. *Journal of Agricultural and Food Chemistry*, acs.jafc.4c06175. https://doi.org/10.1021/acs.jafc.4c06175

Mustafa, A., Andersson, R., Rosén, J., Kamal-Eldin, A., & Aman, P. (2005). Factors influencing acrylamide content and color in rye crisp bread. *Journal of Agricultural and Food Chemistry*, *53*(15), 5985–5989. https://doi.org/10.1021/jf050020q

Oddy, J., Addy, J., Mead, A., Hall, C., Mackay, C., Ashfield, T., McDiarmid, F., Curtis, T. Y., Raffan, S., Wilkinson, M., Elmore, J. S., Cryer, N., de Almeida, I. M., & Halford, N. G. (2023). Reducing Dietary Acrylamide Exposure from Wheat Products through Crop Management and Imaging. *Journal of Agricultural and Food Chemistry*, *71*(7), 3403–3413. https://doi.org/10.1021/acs.jafc.2c07208

Pangarkar, T. (2024, July 4). Breakfast Cereals Market Evolve with Consumer Demands. *Market.Us News*. https://www.news.market.us/breakfast-cereal-market-news/

Pesce, F., Ponzo, V., Mazzitelli, D., Varetto, P., Bo, S., & Saguy, I. S. (2024). Strategies to Reduce Acrylamide Formation During Food Processing Focusing on Cereals, Children and Toddler Consumption: A Review. *Food Reviews International*, *40*(1), 185–211. https://doi.org/10.1080/87559129.2023.2164896

Serpen, A., & Gökmen, V. (2009). Evaluation of the Maillard reaction in potato crisps by acrylamide, antioxidant capacity and color. *Journal of Food Composition and Analysis*, *22*(6), 589–595. https://doi.org/10.1016/j.jfca.2008.11.003

Szafrańska, A., Podolska, G., Świder, O., Kotyrba, D., Aleksandrowicz, E., Podolska-Charlery, A., & Roszko, M. (2024). Factors Influencing the Accumulation of Free Asparagine in Wheat Grain and the Acrylamide Formation in Bread. *Agriculture*, *14*(2), 207. https://doi.org/10.3390/agriculture14020207

Williams, P. G. (2014). The Benefits of Breakfast Cereal Consumption: A Systematic Review of the Evidence Base1234. *Advances in Nutrition*, *5*(5), 636S-673S. https://doi.org/10.3945/an.114.006247

Yaylayan, V. A., Perez Locas, C., Wnorowski, A., & O'Brien, J. (2005). Mechanistic Pathways of Formation of Acrylamide from Different Amino Acids. In M. Friedman & D. Mottram (Eds.), *Chemistry and Safety of Acrylamide in Food* (pp. 191–203). Springer US. https://doi.org/10.1007/0-387-24980-X_15

Yoo, H. C., Yu, Y. C., Sung, Y., & Han, J. M. (2020). Glutamine reliance in cell metabolism. *Experimental & Molecular Medicine*, *52*(9), 1496–1516. https://doi.org/10.1038/s12276-020-00504-8

Žilić, S., Aktağ, I. G., Dodig, D., Filipović, M., & Gökmen, V. (2020). Acrylamide formation in biscuits made of different wholegrain flours depending on their free asparagine content and baking conditions. *Food Research International*, *132*, 109109. https://doi.org/10.1016/j.foodres.2020.109109

Zyzak, D. V., Sanders, R. A., Stojanovic, M., Tallmadge, D. H., Eberhart, B. L., Ewald, D. K., Gruber, D. C., Morsch, T. R., Strothers, M. A., Rizzi, G. P., & Villagran, M. D. (2003). Acrylamide Formation Mechanism in Heated Foods. *Journal of Agricultural and Food Chemistry*, *51*(16), 4782–4787. https://doi.org/10.1021/jf034180i

Tables

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 minutes.

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.

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

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.

Figure legends

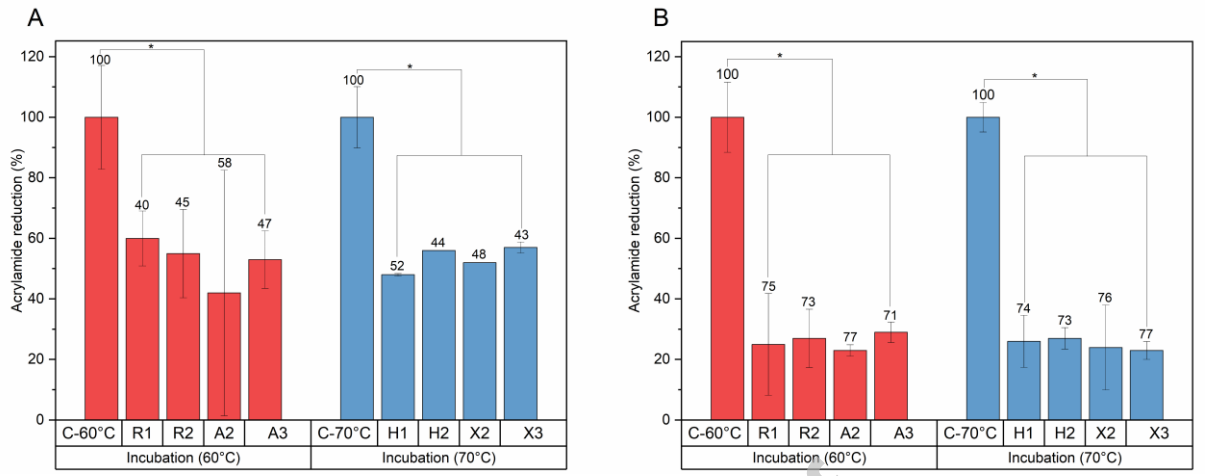

Figure 1. Reduction of acrylamide in wheat (A) and oat (B) flakes produced on laboratory-scale. Values are given as means ± standard deviation (error bars) and asterisks indicate a significant difference compared to the respective control (ANOVA with Dunnett's *t*-test, $p \leq 0.05$). The numbers show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

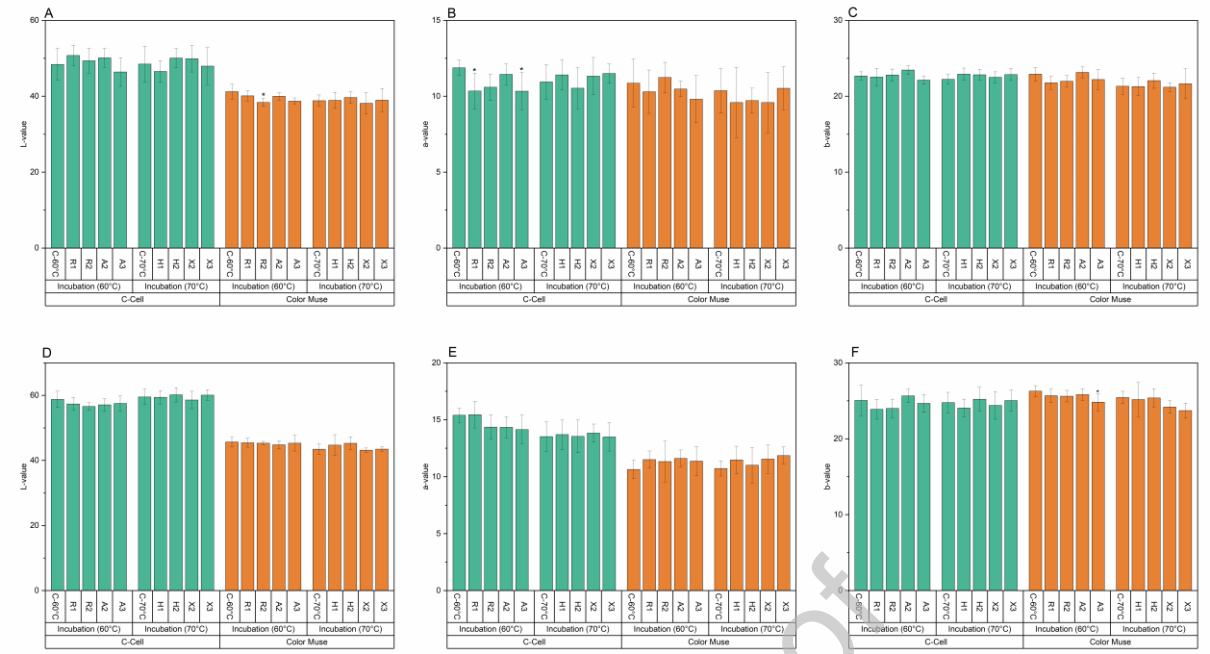

Figure 2. Color analysis of wheat (A, B, C) and oat (D, E, F) flakes baked at 220 °C for 15 min using C-Cell and Color Muse. Color parameters are expressed as L* (lightness), a* (green-red), and b* (blue-yellow). Values are given as means ± standard deviation (error bars) and asterisks denote significant 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.

Ethical statement

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

Declaration of interests

☒ 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.

☐ The author is an Editorial Board Member/Editor-in-Chief/Associate Editor/Guest Editor for *[Journal name]* and was not involved in the editorial review or the decision to publish this article.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: