Journal of Food Measurement and Characterization (2026) 20:10320–10334
https://doi.org/10.1007/s11694-026-04319-5

ORIGINAL PAPER

Effects of Process Parameters and Storage on the Quality Characteristics and Formation of Potential Hazardous Maillard Reaction Products in Gluten-Free Chestnut (*Castanea sativa* Mill.) Biscuits

Aslı Yıldırım Vardin[1] · Ali Göncü[2] · Ayşe Demet Karaman[1,3]

Received: 15 May 2025 / Accepted: 18 March 2026 / Published online: 1 April 2026
© The Author(s) 2026

Abstract

The aim of this study was to evaluate the effects of baking temperature (170–200 °C), baking time (6–12 min), and chestnut flour: rice flour ratio (1:4, 2:3, 3:2, 4:1) on the formation of Maillard reaction products (MRPs) including acrylamide, 5-hydroxymethylfurfural (HMF), and furan in gluten-free biscuits, as well as to assess the impact of storage (for 3 months at 25 °C in heat-sealed aluminium laminated polyethylene (ALPE) packages) on these compounds and on product quality attributes. Results demonstrated that increasing baking time (6–12 min) led to higher levels of acrylamide (120.85 ± 4.53–508.60 ± 13.72 µg/kg), HMF (6.09 ± 0.80–11.45 ± 0.32 mg/kg), and furan (128.79 ± 15.99–3498.41 ± 12.57 ng/g). When the chestnut flour proportion increased from 20% to 60% under the studied baking conditions, acrylamide, HMF, and furan contents showed an increasing tendency, with maximum values of 508.60 ± 13.72 µg/kg, 11.45 ± 0.32 mg/kg, and 3498.41 ± 12.57 ng/g, respectively. Process parameters affected not only the formation of acrylamide, HMF, and furan, but also total phenolic content, hardness value as well as color, water activity and moisture content of biscuits. Especially with increasing baking temperature, a decreasing trend was observed in total phenolic content, while hardness tended to increase with longer baking time. During storage, acrylamide and HMF levels decreased over time, with the lowest acrylamide content (124.21 ± 4.46 µg/kg) observed in biscuits made solely with rice flour after 90 days. Furan levels also decreased slightly, although not significantly, and HMF was undetectable in all samples after day 7 of storage. Texture analysis revealed an increase in hardness and a decrease in crispness during storage. Following the evaluation of process parameters, optimization was performed to determine the best combination of variables. Overall, both formulation and baking conditions had significant effects on the formation of MRPs and on the bioactive properties of gluten-free biscuits. Higher processing temperatures statistically increased these compounds.

Keywords Acrylamide · Furan · Gluten-free biscuit · HMF · Maillard reaction

Introduction

The Maillard reaction is a non-enzymatic reaction in which the carbonyl group of reducing sugars reacts with amino groups in amino acids or proteins, leading to the formation of Maillard reaction products that contribute various sensory characteristics [1]. During the Maillard reaction, hundreds of different compounds called Maillard reaction (MR) products are formed. Although many desirable components such as flavor and aroma compounds are formed, some undesirable reaction products are also formed. Acrylamide is generated during the Maillard reaction, primarily through the reaction of the amino acid asparagine with the reducing

✉ Ali Göncü
ali.goncu@adu.edu.tr

[1] Department of Food Engineering, Faculty of Engineering, Aydın Adnan Menderes University, Aydın 09010, Türkiye

[2] Department of Food Processing, Çine Vocational School, Aydın Adnan Menderes University, Çine, Aydın 09500, Türkiye

[3] Department of Dairy Technology, Faculty of Agricultural Engineering, Aydın Adnan Menderes University, Aydın 09010, Türkiye

sugars glucose and fructose [2]. The amount of acrylamide formed in foods is influenced by multiple factors, including food composition [3], the concentration of free asparagine [3], the presence of reducing sugars [3], and high carbohydrate content [3]. In addition, processing conditions such as high temperatures [4] pH level [5], the presence of ammonia [5], and the presence of amino acids [3] also play a significant role in acrylamide formation [3–5]. Daily exposure to acrylamide in the human diet has been reported to be 0.3–0.8 µg per kg body weight [6]. Since acrylamide is reported to be formed especially at temperatures of 120 °C and above, biscuits, which are baked at high temperatures, pose a risk in terms of acrylamide formation. The main approaches to reduce acrylamide formation in foods are improving raw materials, adding additives, changing processing conditions, pH, water activity and fermentation conditions [7].

During the thermal processing of foods, various reaction products are formed alongside acrylamide, including 5-hydroxymethylfurfural (HMF) and furan. HMF is primarily generated from the dehydration of hexose sugars under high temperatures and acidic conditions and can also contribute to acrylamide formation due to the carbonyl group in its structure [8, 9]. Its formation is influenced by water activity, sugar type, pH, temperature, and the presence of cations and amino acids, and is strongly affected by both the raw materials and baking conditions, particularly in bakery products [9, 10]. Furan, a highly volatile compound, is formed from the thermal degradation of carbohydrates, amino acids, ascorbic acid, or polyunsaturated fatty acids [11, 12]. Key factors affecting furan formation include temperature, heating time, pH, and oxidation conditions [12, 13]. The formation of HMF, furan, and acrylamide can overlap under high-temperature baking, as Maillard reactions and sugar degradation occur simultaneously. The final levels of these three neo-formed contaminants depend on the composition of the raw materials—particularly sugars and free amino acids, which are the main Maillard reaction substrates—and on processing conditions such as temperature and heating time [14]. Adjusting these parameters can help control their levels.

One effective approach to reduce furan formation is modifying the product formulation, as certain ingredients and their proportions significantly influence its generation. Since there is very limited information on the subject in the literature, there are very few studies on how formulation and processing conditions affect furan formation in biscuits. It is emphasized that more studies should be conducted on the subject [15].

Recent studies have explored the incorporation of chestnut flour into cereal-based products as a means to improve their nutritional and functional properties [16]. For instance, Radhika et al. (2019) developed multigrain, gluten-free cookies and pasta enriched with chestnut flour, demonstrating improvements in both nutritional value and consumer acceptability [17]. Similarly, Ahsan et al. (2023) reported that partial substitution of wheat flour with water chestnut flour in ramen noodles enhanced the product's nutritional characteristics while maintaining acceptable technological and sensory qualities [18]. These findings highlight the potential of chestnut as a value-adding ingredient in the formulation of wheat-based and gluten-free products, supporting its use in the enrichment of conventional cereal-based foods.

Biscuits, as a widely consumed bakery product, have garnered considerable interest in both research and industry, particularly due to their versatility and popularity as convenient snack items. More generally, wheat-based products remain a staple in many diets worldwide, driving ongoing innovation to improve their nutritional profile and accommodate dietary restrictions such as gluten intolerance. Recent studies have emphasized the development of gluten-free biscuits by incorporating alternative plant-based ingredients to enhance their physicochemical, nutritional, and sensory properties. For instance, Saeed et al. [19] investigated the impact of innovative plant sources, including *Cordia myxa* L. fruit and *Phoenix dactylifera* L. biowaste, on the quality characteristics of gluten-free biscuits and they reported that the addition of nutritious gluten free ingredients increased the essential nutritients of biscuits. Similarly, Radhika et al. [17] focused on the formulation and nutritional evaluation of multigrain gluten-free cookies and pasta products, highlighting the growing industrial and consumer interest in healthier, gluten-free bakery alternatives.

To date, limited research has focused on optimizing the Maillard reaction pathway in gluten-free bakery products [20, 21], particularly in formulations based on alternative flours such as chestnut flour, which is naturally gluten-free and rich in reducing sugars and amino acids—key precursors for Maillard reaction products (MRPs) [22]. The application of response surface methodology (RSM) in this study represents a novel and systematic approach to fine-tune the baking parameters in chestnut-based gluten-free biscuits to optimize MRP formation. While RSM has been previously used to improve quality parameters in gluten-free baked goods [23–25] its targeted use for limiting MRPs in a gluten-free, chestnut-enriched matrix has not been reported before. This highlights a critical methodological and application-oriented novelty that distinguishes the present work from earlier studies.In this study, gluten-free biscuits were used as a model food to evaluate the effects of different chestnut flour: rice flour ratios, baking temperatures, and baking times on the formation of acrylamide, HMF, and furan — key Maillard reaction products.

Chestnut is abundantly produced in Aydın, the region of the current study, which contributed to its selection for this research. Additionally, its potential alternative applications further motivated the investigation. In the current study, the effects of baking temperature, baking time, and chestnut flour to rice flour ratio on the levels of acrylamide, HMF, and furan, as well as the physicochemical properties of gluten-free biscuits, were investigated. The biscuit samples produced under optimum conditions were stored for 90 days, and the effects of the storage process on the amounts of acrylamide, HMF, and furan, as well as on the quality and compositional properties of the biscuits, were examined in detail. This study investigated the formation of acrylamide, furan, and HMF in chestnut flour-substituted gluten-free biscuits. Additionally, the effects of baking temperature and time in a microwave-assisted convection oven on the formation of these compounds in chestnut flour-substituted gluten-free biscuits were also examined. This study explores the influence of baking parameters and ingredient ratios on the formation of Maillard reaction products (MRPs) in gluten-free biscuits, offering new insights into how formulation and processing conditions impact food safety and quality.

Materials and methods

Materials

The chestnut and rice flours used in the study were obtained from Naturelka (Aydın/Türkiye) (Table 1). Salt (Billur Tuz/Türkiye, ≥ 99.6%), sodium bicarbonate (Dr. Oetker/Germany, 100%), ammonium bicarbonate (Katkı Dünyası/Türkiye, 100%), skimmed milk powder (Naturdan/Türkiye, 100%), sorbitol (Tito/Türkiye, 100%), xanthan gum (Tito/Türkiye, 100%), sucrose (Carrefour/France, 100%), brown sugar (Irmak/Türkiye, 100%), sodium acid pyrophosphate (Tito/Türkiye, 100%) and glucono delta lactone (Tito/Türkiye, 100%) were obtained from local markets and online. On the other hand, shortening was obtained from Savola Gıda (Ayvalık, Balıkesir/Türkiye), high fructose corn syrup was obtained from Tat Nişasta (Sarıçam, Adana/Türkiye) (Table 1) and deionised water was obtained from Aydın Adnan Menderes University, Faculty of Veterinary Medicine and Faculty of Science and Literature. Black tea extract was obtained from commercially available black tea powder (Lipton first harvest). Chestnut flour and rice flour were supplied by Naturelka (Aydın, Türkiye). Shortening was obtained from Savola Gıda (Ayvalık, Balıkesir, Türkiye), while high fructose corn syrup was purchased from Tat Nişasta (Sarıçam, Adana, Türkiye).

Furan standard was obtained from Ehrenstorfer (Augsburg, Germany), and d4-furan internal standard was supplied by HPC Standards (Borsdorf, Germany). Acrylamide and HMF standards, along with DPPH, gallic acid, methanol, *n*-hexane, hydrochloric acid, sodium bicarbonate, sodium chloride, potassium bromide, hydrobromic acid, ultrapure water, Carrez I and Carrez II solutions, sodium thiosulfate, Folin–Ciocalteu reagent, and triethylamine were purchased from Sigma-Aldrich.

Table 1 Variables and their levels for D-optimal design mixture trial plan specific to gluten-free biscuit production

	Independent variables	Variable levels		
Mixture Variables	Chestnut flour (%)	20 (0)	40 (0.5)	60 (1)
	Rice flour (%)	40 (0)	60 (0.5)	80 (1)
Process Variables	Baking temperature (°C)	170 (-1)	185 (0)	200 (1)
	Baking time (min)	6 (-1)	9 (0)	12 (1)

Methods

Production ofgluten-free biscuits

In the preparation of gluten-free biscuits, the standardized (official) method (Method 10–52) of the American Association of Cereal Chemists [26] was used with modifications. The formulation consisted of 1.0 g salt, 0.096 g xanthan gum, 25.2 g sorbitol, 0.4 g sodium acid pyrophosphate, 0.8 g skim milk powder, 0.4 g black tea extract, and 1.2 g high-fructose corn syrup. The ingredients in the formulation were mixed using a mixer (Karaca Mastermaid, Karaca/Turkey) to form the dough, and then the dough was cut into 5 cm diameter, 0.5 cm thick discs as specified in AACC Method 10–52, followed by baking in a microwave assisted convectional oven. The samples baked only by convectional oven and the samples produced from 100% rice flour were used as controls, during storage period. The baked biscuits were allowed to cool to room temperature and then ground using and electric grinder, and stored in aluminium laminated polyethylene (ALPE) packages at 4 °C until analysis. All production trials were conducted in two replicates, and all analyses were performed in duplicate (two parallels) ($n=4$).

The optimum chestnut flour: rice flour ratio and the optimum baking temperature and time to be used in the production of gluten-free biscuits were using Design Expert 7.1.5.1 (Stat-Ease, Inc., Minneapolis, USA) analysis programme. The lowest (lower limit) and highest (upper limit) values for the ratios of chestnut flour (20, 40, 60%) and rice flour (40, 60, 80%) [27], baking temperature (170, 185, 200 °C) [28] and baking time (6, 9, 12 min) [29] entered into the programme were obtained from the relevant literature findings. The baking process was carried out according to the D-optimal design experimental design considering the predicted value ranges. Variables and their levels for the D-optimal

design mix trial plan specific to gluten-free biscuit production are shown in Table 1. The ranges of the independent variables were tested with preliminary trials. The baking process was carried out in a microwave assisted oven. Chestnut flour, rice flour, baking time and baking temperature were determined as independent variables; minimum amount of furan, acrylamide and HMF were determined as dependent variables. Each experimental condition was conducted in two independent replicates, and each replicate included two parallel analyses, resulting in $n=4$ per treatment. Some experimental runs were intentionally repeated as replicates according to the D-optimal design generated by Design-Expert software.

Production of black tea extract

The method described by [30] was used for the production of extracts. The amount added was deducted from the overall moisture content of the samples.

Determination of moisture, ash, protein and oil content

The moisture, ash and protein content of the samples were determined according to the method of AOAC [31] while oil content was determined according to the official method of AACC 30-10.01 [26]. *n*-hexane was used as solvent.

Determination of water activity

The water activity of the samples was measured using a water activity probe (Testo AG 400, Germany).

pH analysis

The pH values of the samples were measured using a digital pH meter (Hanna Instruments HI 8314, USA) [32].

Measurement of total phenolic content and antioxidant activity

To determine the total phenolic content and antioxidant activity in the raw materials and biscuit samples, firstly, extracts were prepared from the samples according to the method described by Karakoç [33]. The total phenolic content of the samples was measured spectrophotometrically following the method outlined by Karakoç [33]. The results were expressed as µg GAE/g [34]. The antioxidant activity of the samples were determined spectrophotometrically by making some modifications on the method described by Uyrgancı [35]. The results were expressed as TE/100 g.

Gluten analysis

Gluten analysis was performed to ensure compliance that the biscuits were completely gluten-free. The analysis was performed at the Marmara Research Centre of the Scientific and Technical Research Council of Turkey (TÜBİTAK MAM). The gluten content was analysed according to AOAC Method 991.19 [36].

Starch gelatinization

The degree of starch gelatinization of biscuit samples was determined by differential scanning calorimetry (DSC) according to the method described by Sozer [37]. The degree of gelatinization was calculated using the formula given by Ndife [38].

Calculation of carbohydrate and energy values

Based on the data obtained from moisture, protein, fat and ash analyses of the samples, carbohydrate values were calculated: % carbohydrate = 100 - (% moisture + % protein + % fat + % fat + % ash) formula.

Energy values are as follows: Energy (kkal/100 g) = 4 (% carbohydrate + % protein) + 9 (% fat) [39].

Determination of acrylamide content

The method described by Boyacı [40] was used to determine the amount of acrylamide in biscuits. Chromatographic separation was carried out using a DB WAX column (30 m x 0.25 mm x 0.25 µm; J&W Scientific, USA) with GC-MS (Agilent Technologies, California/USA). The temperatures of the injection block, detector, and ion source were set at 240 °C, 250 °C, and 230 °C, respectively. A splitless injection was used, with an injection volume of 2 µl. The column temperature programme was as follows: 50 °C for 1 min, followed by ◊ 180 °C with 20 °C/min increase, ◊ 230 °C with 10 °C/min increase (20 min), 260 °C with 10 °C/min increase (10 min). Helium was used as carrier gas. Mobile phase flow rate was 1 ml/min. Identification was performed in SIM mode and the reference ion was 71. At the end of the analysis, the acrylamide content of the samples was determined using reference standards.

Determination of 5-hydroxymethylfurfural (HMF) content

HMF analysis was conducted using high-performance liquid chromatography (HPLC) (Agilent Technologies, USA) according to the method of Koç [41]. The mobile phase was prepared with 80% distilled water and 20% methanol

was used as the mobile phase at a flow rate of 1 mL/min. Chromatographic separation was carried out using a DAD detector at 285 nm wavelength at room temperature. Characteristics of the column used: C18, particle diameter 3 micrometres, column LxID; 150 × 4.6 [41].

Determination of furan content

The methods of Juaniz [42] and Emektar [43] were taken as reference for the determination of furan content of the samples. Chromatographic separation was carried out with HP PLOT/Q column (15 m x 0.32 mm x 20 µm) with GC-MS (Agilent Technologies, California/USA). The column temperature programme was set as follows: 40 °C for 5 min ◊ 120 °C with 3 °C/min increase ◊ 220 °C with 10 °C/min increase (5 min) Helium was employed as the carrier gas at a flow rate of 1 mL/min, with the split ratio set to 30:1. The reference ions for furan and d4-furan were taken as 68 and 72, respectively. The transfer line temperature was maintained at 270 °C, while the ion source temperature was set at 230 °C. Furan standards in different concentrations and d4-furan were used to plot the calibration curve.

Diameter, thickness and spreading rate measurement

Diameter and thickness of the biscuits were measured using a composing stick. The spreading ratio was calculated by proportioning the diameters of the biscuit samples to their thicknesses [44, 45].

Colour analysis

The colour values (L^*, a^* and b^*) of the biscuit samples were determined with a colour meter (Konica Minolta, Osaka, Japan) after the samples were ground [46].

Texture analysis

Hardness values of the samples were determined with a texturing device (TA.XT2 Texture Analyser). Pre-test speed was set as 2 mm/s, test speed as 2 mm/s and post-test speed as 10 mm/s. Trigger force was set as 30 g [47].

Sensory analysis

Sensory analysis of the biscuit samples was carried out using the method described by Urgancı and Işık [48] with some modifications. The biscuits were evaluated by the panelists according to taste, odour, bitterness, crunchiness, chewiness, aftertaste impression and general liking parameters by scoring between 1 (extremely bad) and 7 (excellent) points on a hedonic scale. Biscuit samples were coded with randomly selected 3-digit numbers.

Storage

Samples produced under optimum conditions (chestnut flour: rice flour ratio of 44.17:55.83; baking temperature of 175.16 °C; baking time of 6 min) were packed in aluminium laminated polyethylene (ALPE) packages and heat sealed, then stored in a refrigerated incubator at 25 °C. For the storage experiments, each replicate was produced as an independent batch at different production times, and all analyses were performed in duplicate (two parallels), resulting in $n = 4$ for each condition. The packaging material consisted of 12 µm polyethylene (PET) + 8 µm aluminium (AL) + 50 µm low density polyethylene (LDPE). During storage, ambient temperature and humidity were recorded daily with a temperature/humidity meter. The temperature was recorded as 25 °C and the relative humidity was recorded as 46.67%. During the storage process, samples were taken on days 0, 1, 3, 5, 7, 14, 28 and months 2 and 3 and storage analyses were performed.

Statistical evaluation

The effects of storage time and processing method on the physicochemical, textural, bioactive, contaminant, and sensory properties of gluten-free biscuits were evaluated using regression-based statistical approaches. Given the longitudinal nature of the storage study and the presence of repeated measurements obtained from the same production batches or sensory panelists, linear mixed-effects models (LMMs) were primarily employed. This approach allows appropriate modeling of non-independent observations by explicitly accounting for random sources of variation [49, 50].

In the mixed-effects models, storage time and processing method were treated as fixed effects, while production batch (or panelist group in the sensory analysis) was included as a random effect. An interaction term between storage time and processing method was incorporated to assess whether the effect of storage duration differed among processing conditions. This modeling framework enables simultaneous evaluation of time-dependent changes and between-batch or between-panelist variability within a single statistical model.

In cases where the data structure did not permit reliable estimation of random effects or where model convergence could not be achieved, ordinary least squares (OLS) regression was applied as an alternative analytical approach. The use of OLS in such situations is consistent with established statistical practice for datasets lacking sufficient hierarchical structure [51].

Parallel analytical measurements were not considered independent experimental units. Instead, measurements obtained from the same production batch and storage time were averaged prior to statistical analysis. Statistical significance was assessed at a confidence level of $p < 0.05$. The regression-based analytical strategy adopted in this study provides a robust and flexible framework for the interpretation of complex, multi-parameter storage data without relying on inappropriate inferential comparisons.

D-optimal integrated design was used to determine the optimum conditions to minimize acrylamide, HMF and furan formation and 20:60 − 40:80 chestnut flour: rice flour ratio was selected as mixture variable, 170–200 °C as baking temperature and 6–12 min as baking time. When the desirability function approach was applied, 5 solutions close to each other were found for the optimum point. Among these solutions determined with the help of the Design Expert program, the ratio of chestnut flour: rice flour in the solution selected as the optimum point was 44.17:55.83, the baking temperature was 175.16 °C and the baking time was 6 min.

It was determined that a second-order polynomial model was the most appropriate model type for predicting acrylamide, HMF, and furan levels. The extent to which the models fitted the experimental data was evaluated by calculating the regression coefficient (R^2) and the adjusted regression coefficient.

Results and discussion

Chemical parameters

The pH values of the biscuit doughs are presented in Table 2, ranging from 5.97 ± 0.02 to 6.75 ± 0.03. The moisture

Table 2 The effects of chestnut flour: rice flour ratio as well as temperature and time on pH values of biscuit doughs and diameter, thickness and spreading ratios of biscuit samples. *Experiments are ordered by flour ratio, temperature, and heating time to improve readability*

Experiment No	Chestnut flour (%)	Rice flour (%)	Temperature (°C)	Time (min)	pH	Diameter (mm)	Thickness (mm)	Spreading ratio
1	20	80	170	6	6.72 ± 0.01	49.84 ± 0.31	7.72 ± 0.13	6.45 ± 0.12
17	20	80	170	9	6.42 ± 0.03	48.43 ± 0.13	6.85 ± 0.11	7.07 ± 0.10
6	20	80	170	12	6.33 ± 0.09	48.09 ± 0.56	7.57 ± 0.36	6.36 ± 0.27
15	20	80	185	6	6.23 ± 0.07	48.97 ± 0.27	6.57 ± 0.13	7.46 ± 0.16
8	20	80	200	6	6.50 ± 0.04	49.74 ± 0.34	7.35 ± 0.17	6.77 ± 0.18
31	20	80	200	6	6.52 ± 0.01	49.38 ± 0.47	7.41 ± 0.32	6.67 ± 0.32
10	20	80	200	12	6.45 ± 0.04	48.41 ± 0.56	7.60 ± 0.22	6.38 ± 0.13
33	20	80	200	12	6.55 ± 0.02	47.43 ± 3.41	7.81 ± 0.11	6.08 ± 0.42
22	30	70	177.5	7.5	6.14 ± 0.04	49.23 ± 0.41	7.39 ± 0.27	6.67 ± 0.24
20	30	70	185	12	6.29 ± 0.07	48.61 ± 0.70	7.35 ± 0.24	6.61 ± 0.18
19	30	70	192.5	9	6.11 ± 0.07	48.08 ± 0.53	7.62 ± 0.24	6.31 ± 0.24
4	40	60	170	6	6.13 ± 0.04	50.26 ± 0.19	7.46 ± 0.27	6.75 ± 0.24
16	40	60	170	9	6.39 ± 0.10	49.34 ± 0.55	7.28 ± 0.18	6.78 ± 0.17
3	40	60	170	12	6.12 ± 0.06	49.57 ± 0.40	7.65 ± 0.11	6.48 ± 0.06
14	40	60	185	6	6.12 ± 0.04	49.55 ± 0.19	7.23 ± 0.15	6.86 ± 0.16
24	40	60	185	9	6.29 ± 0.03	49.60 ± 0.29	7.79 ± 0.19	6.37 ± 0.12
25	40	60	185	9	6.62 ± 0.10	49.71 ± 0.49	7.71 ± 0.12	6.45 ± 0.08
26	40	60	185	9	6.49 ± 0.00	49.38 ± 0.33	7.49 ± 0.13	6.59 ± 0.14
27	40	60	185	9	6.42 ± 0.04	49.20 ± 0.52	7.54 ± 0.23	6.53 ± 0.13
28	40	60	185	9	6.75 ± 0.03	50.05 ± 0.75	7.84 ± 0.03	6.38 ± 0.12
12	40	60	200	6	6.22 ± 0.02	48.89 ± 0.77	6.99 ± 0.24	7.00 ± 0.20
9	40	60	200	12	6.27 ± 0.04	49.90 ± 0.38	7.75 ± 0.13	6.44 ± 0.11
23	50	50	192.5	7.5	6.14 ± 0.10	49.44 ± 0.32	7.51 ± 0.28	6.60 ± 0.27
7	60	40	170	6	6.10 ± 0.08	49.58 ± 0.45	7.09 ± 0.07	6.99 ± 0.08
29	60	40	170	6	6.16 ± 0.05	50.64 ± 0.20	7.51 ± 0.20	6.75 ± 0.19
11	60	40	170	12	6.17 ± 0.02	48.94 ± 0.52	7.40 ± 0.07	6.61 ± 0.05
30	60	40	170	12	6.47 ± 0.04	49.69 ± 0.20	7.88 ± 0.07	6.31 ± 0.03
13	60	40	185	9	6.04 ± 0.07	48.48 ± 0.35	7.50 ± 0.30	6.48 ± 0.26
21	60	40	185	12	6.20 ± 0.07	49.65 ± 0.31	7.49 ± 0.15	6.63 ± 0.15
5	60	40	200	6	6.05 ± 0.02	49.44 ± 0.34	7.20 ± 0.43	6.89 ± 0.40
32	60	40	200	6	6.64 ± 0.02	50.19 ± 0.48	7.77 ± 0.12	6.47 ± 0.13
18	60	40	200	9	5.97 ± 0.02	50.01 ± 0.44	7.61 ± 0.32	6.58 ± 0.26
2	60	40	200	12	6.02 ± 0.06	49.14 ± 0.76	7.48 ± 0.18	6.58 ± 0.24

content of the biscuit samples varied between 0.56±0.10–1.45±0.30% (Table 3). It was determined that the moisture values of the samples tended to decrease with the prolongation of the baking time. The water activity of the samples varied between 0.18±0.00-0.27±0.01 (Table 3).

Physical parameters

The diameter, thickness, and spread ratios of the biscuit samples are presented in Table 2. The diameter of the samples ranged from 47.43±3.41 to 50.64±0.20 mm, the thickness ranged from 6.57±0.13 to 7.88±0.07 mm, and the spread ratio ranged from 6.08±0.42 to 7.46±0.16.

*L**, *a**, and *b** values related to the colour analysis of the biscuit samples were determined and are presented in Table 3. The *L** values of the samples ranged from 37.43±0.00 to 65.98±0.01, the *a** values ranged from 6.49±0.00 to 13.11±0.00, and the *b** values ranged from 15.81±0.01 to 26.09±0.01.

Hardness value is considered as one of the main parameters indicating the textural properties of biscuits and is measured as the highest force required to penetrate into the biscuit [52]. The hardness values of gluten-free biscuits ranged between 238.15±10.38-4276.34±274.82 g (Table 4).

Table 3 Effects of chestnut flour: rice flour ratio, baking temperature and baking time on moisture content, water activity, color values as well as total phenolic content and antioxidant activity of biscuits. *Experiments are ordered by flour ratio, temperature, and heating time to improve readability*

Experiment No	Chestnut flour (%)	Rice flour (%)	Temperature (°C)	Time (min)	Moisture content (%)	Water activity	L*	a*	b*	Total phenolic content (µg GAE/g extract*)	Antioxidant activity (µmol TE/100 g*)
1	20	80	170	6	1.41±0.24	0.26±0.00	60.07±0.02	7.29±0.01	21.49±0.02	386.18±4.84	164.39±1.60
17	20	80	170	9	1.19±0.19	0.27±0.01	56.16±0.02	9.34±0.01	24.98±0.01	110.34±1.09	166.51±1.89
6	20	80	170	12	1.37±0.22	0.25±0.01	49.62±0.01	10.36±0.01	23.16±0.01	184.46±3.58	166.30±1.12
15	20	80	185	6	0.95±0.10	0.25±0.03	57.06±0.01	6.49±0.01	20.21±0.02	144.38±7.00	166.82±0.59
8	20	80	200	6	0.65±0.01	0.20±0.00	59.03±0.01	8.12±0.01	22.68±0.01	145.84±3.42	161.67±2.84
31	20	80	200	6	0.63±0.02	0.20±0.00	65.98±0.01	6.91±0.01	23.75±0.01	105.06±4.47	160.33±3.15
10	20	80	200	12	0.65±0.03	0.18±0.00	45.67±0.02	12.17±0.01	25.44±0.01	263.32±9.96	165.10±0.54
33	20	80	200	12	0.70±0.09	0.18±0.01	48.67±0.01	12.33±0.01	25.16±0.02	134.86±2.81	162.95±0.33
22	30	70	177.5	7.5	0.85±0.02	0.24±0.01	56.06±0.01	8.43±0.01	22.36±0.01	164.59±6.94	165.77±0.48
20	30	70	185	12	0.94±0.10	0.21±0.01	44.95±0.01	10.73±0.01	21.72±0.01	211.30±9.04	165.58±1.13
19	30	70	192.5	9	0.92±0.10	0.23±0.01	51.57±0.01	9.36±0.01	22.35±0.01	164.82±8.36	165.17±0.78
23	50	50	192.5	7.5	0.81±0.11	0.23±0.01	52.01±0.01	9.02±0.01	21.58±0.01	200.34±4.85	164.70±0.42
4	40	60	170	6	1.23±0.19	0.25±0.00	53.25±0.01	8.38±0.01	22.73±0.01	244.95±5.72	165.55±0.11
16	40	60	170	9	1.25±0.11	0.26±0.00	47.51±0.01	8.72±0.01	20.45±0.01	247.89±3.16	168.34±1.05
3	40	60	170	12	1.45±0.30	0.26±0.01	42.76±0.02	10.47±0.01	23.09±0.04	311.76±4.35	164.58±0.59
14	40	60	185	6	0.84±0.13	0.23±0.00	54.15±0.01	7.46±0.01	20.73±0.01	193.55±6.97	166.34±0.90
24	40	60	185	9	0.89±0.16	0.23±0.00	51.07±0.01	10.12±0.01	22.03±0.01	161.67±4.14	164.86±0.32
25	40	60	185	9	0.95±0.12	0.24±0.03	57.14±0.01	9.54±0.01	24.34±0.01	150.75±7.40	165.56±0.64
26	40	60	185	9	0.74±0.12	0.24±0.01	56.58±0.01	10.02±0.01	23.80±0.24	145.84±9.82	163.94±1.83
27	40	60	185	9	0.72±0.09	0.27±0.02	58.66±0.01	9.26±0.01	24.06±0.01	148.60±9.74	161.81±1.79
28	40	60	185	9	0.79±0.09	0.22±0.01	54.35±0.01	10.26±0.01	23.61±0.01	166.82±9.76	163.83±0.50
12	40	60	200	6	0.65±0.17	0.20±0.00	53.91±0.01	7.86±0.01	21.41±0.03	202.04±5.66	164.71±2.12
9	40	60	200	12	0.73±0.11	0.19±0.00	39.97±0.01	11.63±0.01	22.33±0.01	296.89±4.20	164.40±0.67
7	60	40	170	6	1.24±0.09	0.26±0.00	48.90±0.01	8.74±0.01	22.27±0.02	314.26±3.90	165.24±0.41
29	60	40	170	6	1.35±0.20	0.26±0.01	63.54±0.01	6.89±0.01	20.81±0.02	223.98±7.92	166.43±1.36
11	60	40	170	12	1.17±0.11	0.21±0.02	40.88±0.01	9.15±0.01	17.40±0.02	404.86±7.24	165.16±0.89
30	60	40	170	12	1.18±0.13	0.26±0.01	46.79±0.01	13.11±0.01	26.09±0.03	254.37±6.68	166.74±2.93
13	60	40	185	9	0.85±0.02	0.24±0.01	45.86±0.01	10.00±0.01	23.44±0.01	303.12±7.06	166.44±1.72
21	60	40	185	12	0.93±0.13	0.24±0.01	41.45±0.01	8.49±0.01	15.81±0.01	298.29±6.73	166.49±0.95
5	60	40	200	6	0.67±0.00	0.20±0.00	49.92±0.01	8.98±0.01	22.40±0.01	325.25±9.00	165.09±0.59
32	60	40	200	6	0.66±0.01	0.20±0.00	59.45±0.01	8.01±0.01	22.16±0.01	163.29±2.17	165.83±1.58
18	60	40	200	9	0.56±0.10	0.20±0.00	46.45±0.01	10.41±0.01	23.22±0.01	310.44±1.03	166.29±1.84
2	60	40	200	12	0.57±0.11	0.19±0.00	37.43±0.01	11.06±0.02	19.74±0.02	117.46±3.09	162.49±1.26

*The results are presented on a dry matter basis

Table 4 Experimental results obtained according to the D-Optimal Combined Design experimental design. *Experiments are ordered by flour ratio, temperature, and heating time to improve readability*

Experiment No	Chestnut flour (%)	Rice flour (%)	Temperature (°C)	Time (min)	Acrylamide (µg/kg)	HMF (mg/kg)	Furan (ng/g)	Hardness (g)
1	20	80	170	6	120.85±4.53	6.09±0.00	128.79±15.99	518.49±29.73
17	20	80	170	9	166.04±8.36	6.19±0.01	191.68±7.72	2001.39±124.91
6	20	80	170	12	174.95±0.94	6.27±0.01	178.23±12.74	3822.96±306.59
15	20	80	185	6	203.86±0.73	6.42±0.00	345.55±11.55	483.68±60.73
8	20	80	200	6	256.23±12.73	6.80±0.00	1193.92±14.67	727.69±44.66
31	20	80	200	6	254.39±10.32	6.81±0.02	1146.91±26.82	446.11±21.91
10	20	80	200	12	401.53±11.63	8.10±0.29	2060.86±21.67	4276.34±274.82
33	20	80	200	12	402.23±12.19	8.12±0.83	2076.30±16.61	3939.27±45.97
22	30	70	177.5	7.5	199.86±2.91	6.37±0.00	303.20±26.49	1104.91±124.73
20	30	70	185	12	228.04±3.06	6.65±0.01	709.47±16.23	2834.53±94.60
19	30	70	192.5	9	247.34±9.85	6.78±0.02	987.34±14.27	2060.23±132.47
4	40	60	170	6	156.93±4.10	6.11±0.00	162.41±9.30	273.68±3.31
16	40	60	170	9	171.21±1.74	6.21±0.01	224.55±8.85	1513.25±114.28
3	40	60	170	12	180.58±1.17	6.31±0.01	217.05±12.88	2870.22±114.23
14	40	60	185	6	210.70±1.50	6.50±0.01	433.70±16.36	332.32±12.54
24	40	60	185	9	213.80±5.38	6.54±0.01	547.26±25.14	1386.49±60.15
25	40	60	185	9	215.88±5.08	6.58±0.01	596.12±25.29	1259.32±20.33
26	40	60	185	9	211.97±1.54	6.57±0.01	586.60±22.61	803.80±64.40
27	40	60	185	9	217.22±8.53	6.57±0.01	586.27±20.47	816.63±28.60
28	40	60	185	9	216.21±5.91	6.58±0.00	582.85±12.27	1177.45±136.52
12	40	60	200	6	280.45±2.27	6.95±0.00	1308.94±18.63	932.23±45.79
2	60	40	200	12	508.60±13.72	11.45±0.32	3498.41±12.57	2365.01±257.87
9	40	60	200	12	485.57±8.25	9.71±0.48	2976.06±33.11	1973.99±60.63
23	50	50	192.5	7.5	239.38±5.13	6.73±0.01	871.52±16.78	1154.75±45.52
7	60	40	170	6	162.98±8.17	6.15±0.01	181.30±9.10	460.50±22.61
29	60	40	170	6	160.77±10.17	6.15±0.01	180.09±3.02	238.15±10.38
11	60	40	170	12	190.88±11.18	6.38±0.01	244.68±10.99	2584.07±472.93
30	60	40	170	12	190.92±11.61	6.38±0.01	295.72±10.59	1984.07±65.13
13	60	40	185	9	223.26±10.41	6.60±0.01	714.10±15.42	1234.76±42.69
21	60	40	185	12	230.56±1.82	6.70±0.02	794.74±22.35	2232.18±167.30
5	60	40	200	6	300.16±11.22	7.08±0.03	1521.26±15.22	564.13±28.49
32	60	40	200	6	311.60±17.40	7.01±0.02	1532.95±26.63	272.88±41.68
18	60	40	200	9	354.27±6.28	7.94±0.08	1872.40±29.38	1902.87±30.71

Total phenolic content and antioxidant activity in biscuits

Total phenolic content and antioxidant activity of gluten-free biscuit samples are presented in Table 3. The total phenolic content of the samples varied between 105.06±4.47-404.86±7.24 µg GAE/g extract. Especially with the increase in temperature, a decreasing trend was observed in the amount of total phenolic matter. In the study conducted by Joo [53], the total phenolic content of biscuits containing chestnut flour was found to be approximately 40–180 mg GAE/100 g. On the other hand, in the study conducted by Joo and Choi [54], the amount of total phenolic matter in biscuits containing the inner shell of chestnut was determined as approximately 50–90 mg GAE/100 g. The antioxidant activities of the samples varied between 160.33±3.15-168.34±1.05 µmol TE/100 g (Table 3). In the study conducted by Urgancı [35] on the subject, the antioxidant activity values of pomegranate peel substituted biscuits before the storage process ranged between 5.06 and 288.38 µmol TE/100 g.

Acrylamide content of biscuits

The mean acrylamide amounts of gluten-free biscuit samples obtained by following the D-Optimal Design experimental plan ranged from 120.85±4.53 µg/kg to 508.60±13.72 µg/kg (Table 4) The lowest value was determined in the samples containing 20:80 chestnut flour: rice flour ratio and baked at 170 °C for 6 min., while the highest was observed in samples formulated with 60:40 chestnut flour: rice flour and baked at 200 °C for 12 min.

Bakery products are at risk of acrylamide formation because they contain asparagine and reducing sugars, and the baking process takes place at temperatures above 120 °C [55]. Response surface plots and isohypsometric curves were constructed using the model obtained in order to visually determine the effects of the independent variables on acrylamide, HMF and furan. These plots show the infinite number of combinations of the other two variables with one of the variables held constant at the center point of the experimental design.

When the temperature was kept constant, the response surface plot and isohypsometry curve illustrating the change in acrylamide content as a function of the chestnut flour-to-rice flour ratio and baking time are shown in Fig. 1a. As depicted in the figure, acrylamide levels increased, particularly with longer baking times. Similarly, Mesías and Morales (2016) reported that both baking temperature and baking duration contribute to increased acrylamide formation [56]. Amrein et al. [57] reported that the amount of acrylamide increased with the increase in the duration of the baking process during the baking of gingerbread. When flour ratios were kept constant, the response surface plot and isohyps curve showing the change in acrylamide amount as a function of baking temperature and baking time are shown in Fig. 1b. As illusrated in the Figure, especially increasing the baking temperature significantly affected the amount of acrylamide. Similarly, Bråthen and Knutsen [58] reported that baking temperature and time directly affect acrylamide formation and stated that acrylamide formation was at maximum level at about 200 °C. Figure 1c shows the response surface plot and isohypsometry curve for the change in acrylamide content as a function of flour ratios and baking temperature when the baking time was kept constant. Increasing the amount of chestnut flour in the formulation resulted in a slight increase in the amount of acrylamide, while increasing the baking temperature significantly increased the amount of acrylamide. Zhang et al. [59] also reported that foods baked at high temperatures contain very high amounts of acrylamide. Similarly, Haase et al. [60] reported that the amount of acrylamide in biscuits increased exponentially, especially at high temperatures.

HMF content of biscuits

The average HMF amounts of the same samples varied between 6.09 ± 0.00 mg/kg and 11.45 ± 0.32 mg/kg (Table 4). Similar to acrylamide levels, the lowest amount of HMF was found in samples containing chestnut flour and rice flour in a 20:80 ratio, baked at 170 °C for 6 min. The highest HMF content in the present study was observed in samples with a 60:40 chestnut to rice flour ratio, baked at 200 °C for 12 min. This finding is consistent with the understanding that HMF formation is strongly influenced by both the sugar content and thermal conditions during baking. HMF is primarily formed through the dehydration of 3-deoxyglucosone (3-DG), which arises from the 1,2-enolization of glucose and fructose under acidic conditions, resulting in a chain-structured aldehyde compound [61, 62]. As a heat-induced marker, HMF serves as an important indicator of thermal processing in carbohydrate-containing foods [63]. In this study, the elevated HMF levels in the samples with high chestnut flour content can be attributed to the greater availability of reducing sugars and the high baking temperature acting simultaneously, which together accelerate the Maillard reaction These results suggest that both formulation (flour ratio) and baking conditions are critical factors affecting HMF formation.

When the temperature was kept constant, the response surface plot and isohypsometry curve illustrating the change in HMF content as a function of the chestnut flour-to-rice flour ratio and baking time are shown in Fig. 2a. As observed from the figure, HMF levels increased in a manner

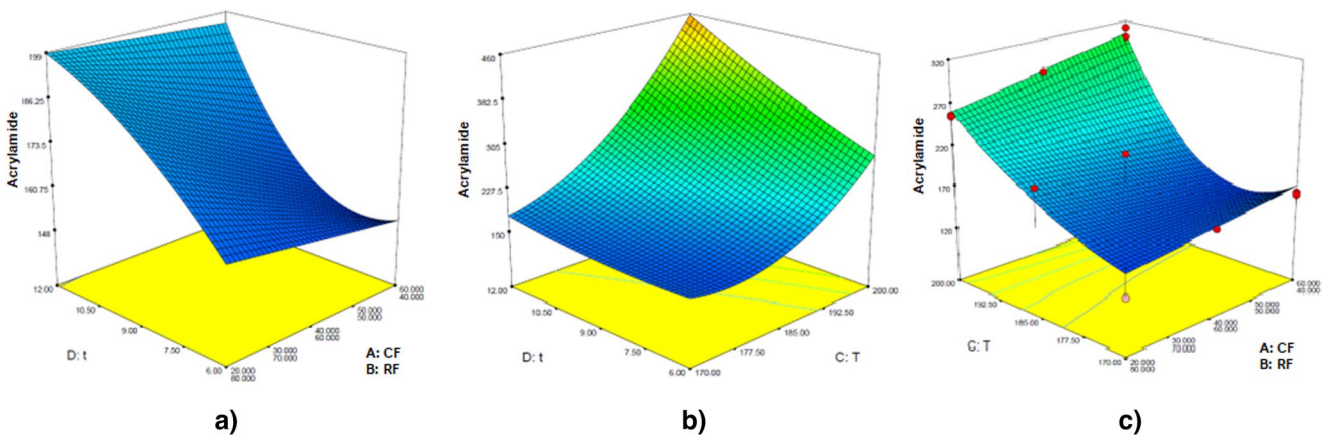


Fig. 1 Response surface graph and isohips curve for acrylamide content (**a**: constant temperature, **b**: constant chestnut flour: rice flour ratio, **c**: constant time)

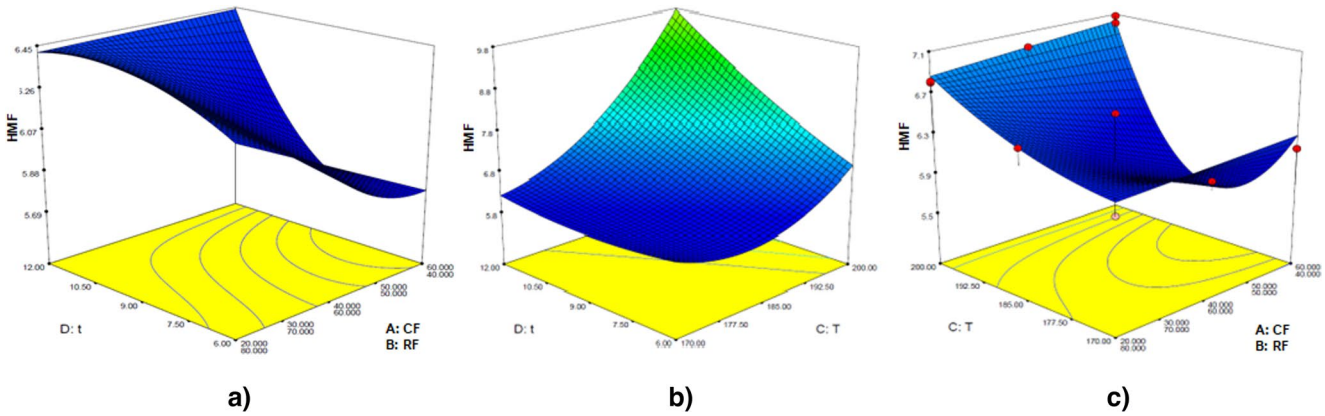


Fig. 2 Response surface graph and isohips curve for HMF content (**a**: constant temperature, **b**: constant chestnut flour: rice flour ratio, **c**: constant time)

similar to acrylamide, particularly with longer baking times. This is consistent with the findings of Hu et al. (2022) who reported that HMF formation in butter cookies is highly sensitive to both baking temperature and duration, highlighting the significant influence of thermal processing conditions on HMF generation in baked products [62]. In the study by Zhang et al. [64], the formation of HMF in cakes baked at different temperatures and times was investigated and it was reported that increasing the temperature and duration of the baking process increased the amount of HMF. Gökmen et al. [65] baked biscuits at different temperatures (180, 200, 210 and 220 °C) and times (10, 15, 20 and 25 min) and reported that high amounts of HMF can be formed especially at temperatures above 100 °C. On the other hand, it has been reported that HMF formation is strongly influenced by the water activity of the samples, with substantially higher HMF levels occurring when the water activity falls below the critical value of 0.4. In the present study, the samples baked at 200 °C for 12 min exhibited lower water activity (0.18 ± 0.01–0.19 ± 0.00) compared to the other samples (Table 3). This markedly low water activity likely contributed to the higher HMF content observed in these samples relative to the others, highlighting the key role of water activity in promoting HMF formation during baking. When evaluating the effect of flour ratios on HMF levels, a general trend of increasing HMF content was observed with higher proportions of chestnut flour in the formulation (Table 4). HMF and other hexose dehydration products are formed as a result of the reaction between hexose and asparagine at high temperatures. On the other hand, HMF reacts with asparagine and leads to acrylamide formation [66]. However, Gonçalves et al. [67] reported that asparagine (143.83 mg/100 g) is the predominant amino acid among the non-essential amino acids in chestnut. Therefore, it is thought that the increase in the amount of HMF with the increase in the ratio of chestnut flour in the formulation may be due to the increase in the amount of asparagine in the formulation.

Figure 2b shows the response surface plot and isohypsometry curve for the variation of HMF content as a function of baking temperature and baking time when flour ratios were kept constant. As demonstrated by the Figure, it was concluded that both baking time and temperature affected the amount of HMF, but especially increasing the temperature of the baking process significantly affected the amount of HMF. In a similar study by Capuano et al. [68], bread was first produced and then sliced and fried at 140, 160 and 180 °C for different times. In the study, it was reported that the amount of HMF and acrylamide increased significantly with the increase in frying temperature and time. On the other hand, in the same study, it was stated that the water activity of the sample is a very important criterion in HMF formation. For the formation of 1 mol HMF, 3 mol water must be removed from 1 mol hexose in an acid-catalyzed environment. Water in the medium can serve as a reactant, partly as a solvent, or as a carrier medium facilitating reactant mobility. However, at extremely high or low water activity levels, the reactivity of the reactants is significantly restricted. This is explained by the decrease in mobility due to the dilution of the medium [69]. In our study, the highest HMF amount was detected in samples containing 60:40 ratio of chestnut flour: rice flour and baked at 200 °C for 12 min, while the lowest HMF amount was detected in samples containing 20:80 ratio of chestnut flour: rice flour and baked at 170 °C for 6 min. Considering the same samples, the moisture and water activity values of the samples baked at 170 °C for 6 min were 1.41% and 0.26%, respectively, while the moisture and water activity values of the samples baked at 200 °C for 12 min were 0.57% and 0.19%, respectively (Table 3). Considering the moisture and water activity values of the samples, it was determined that when high baking temperature and time were applied, reactant mobility was limited and higher levels of HMF were

formed, especially due to low water activity. On the other hand, the samples baked at 170 °C for 6 min showed higher total phenolic content than the other samples. In these samples, HMF content ranged between 6.09 and 6.15 mg/kg. This observation can be interpreted as phenolic compounds limiting HMF formation at these baking temperatures and times due to their antioxidant activity, since it has been reported that phenolics can reduce HMF formation depending on the number and reactivity of hydroxyl groups in their structure [70].

Figure 2c shows the response surface plot and isohypsometry curve of the HMF content as a function of flour ratios and baking temperature when the baking time was kept constant. It was determined that HMF formation was significantly affected by the increase in temperature and HMF formation tended to increase with the increase in temperature. Similarly, Hu et al. [62] reported a significant increase in the amount of HMF with increasing the baking temperature of biscuits.

Furan content of biscuits

In the biscuits produced by following the same experimental plan, furan content ranged between 128.79 ± 15.99 ng/g and 3498.41 ± 12.57 ng/g. The lowest and highest furan amounts showed a similar trend with acrylamide and HMF amounts; the highest and lowest amounts were found in samples containing 20:80 ratio of chestnut flour: rice flour and baked at 170 °C for 6 min, and in samples containing 60:40 ratio of chestnut flour: rice flour and baked at 200 °C for 12 min (Table 4).

Considering the minimum acrylamide, HMF and furan amounts, the optimum chestnut flour: rice flour ratio was determined as 44.17:55.83, the optimum baking temperature was determined as 175.16 °C, and the optimum baking time was determined as 6 min for the optimum point.

When the temperature was kept constant, the response surface plot and isohypsometry curve showing the change in the amount of furan as a function of chestnut flour: rice flour ratio and baking time are shown in Fig. 3a. As presented in the Fig. 3a, it was observed that the amount of furan increased similar to the amount of acrylamide and HMF, especially with the increase in the duration of the baking process. In the study conducted by Ozolina et al. [71], it was noted that the amount of furan derivatives in rye bread increased with the prolongation of the baking process. When the flour ratios were kept constant, the response surface plot and isohypsometric curve showing the change in the amount of furan as a function of baking temperature and baking time are shown in Fig. 3b. According to the Figure, increasing the baking temperature increased the amount of furan significantly. Similarly it has been reported Mariotti et al. [72] that furan formation is significantly affected by temperature and higher levels of furan formation are observed at higher temperatures. Figure 3c shows the response surface plot and isohypsometry curve of the furan amount as a function of flour ratios and baking temperature when the baking time was kept constant. While changing the flour ratios in the formulation did not lead to a notable increase in furan levels, increasing the baking temperature significantly elevated the furan content.

Optimum point verification trial results and properties of gluten-free biscuits produced under optimum conditions

At the optimum point, the acrylamide content of gluten-free biscuits was estimated as 154,03 µg/kg, HMF content as 5.98 mg/kg and furan content as 113,12 µg/kg. The verification of the optimum point results was carried out at the mathematical optimum point determined as chestnut flour: rice flour ratio 44.17:55.83, baking temperature 175.16 °C

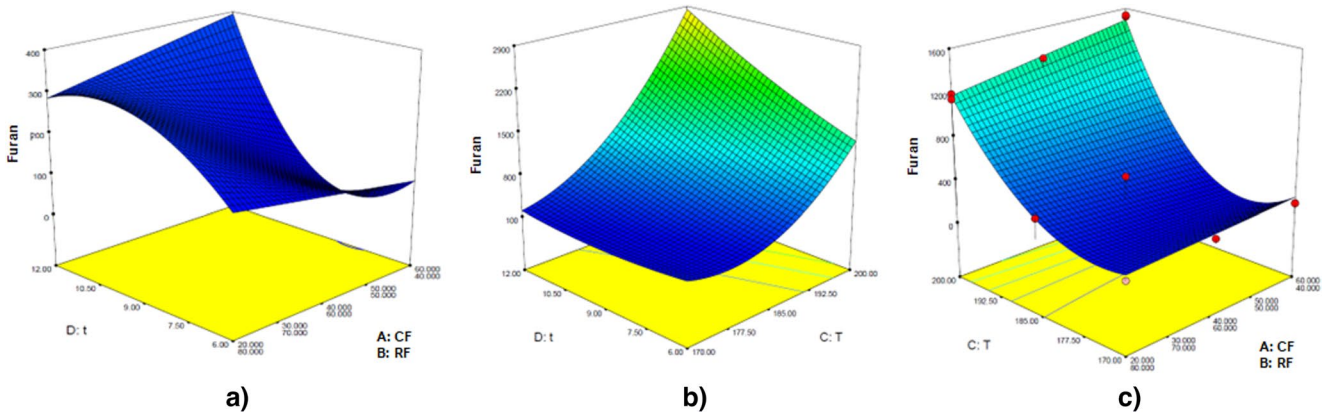


Fig. 3 Response surface graph and isohips curve for furan content (**a**: constant temperature, **b**: constant chestnut flour: rice flour ratio, **c**: constant time)

Table 5 Optimum point verification trial results

Trial No	Acrylamide (µg/kg)	HMF (mg/kg)	Furan (µg/kg)
1	149.65	5.93	125.14
2	164.16	5.91	110.95
3	149.38	6.10	108.43
4	146.32	6.06	112.43
5	170.21	6.13	120.79
Average	155.94	6.02	115.55

Table 6 Comparison of average experimental values at the optimum point with values predicted from the model

Responses	Predicted value	Experimental value[a]	% Error	*p* value
Acrylamide (µg/kg)	154.03	155.94±9.45	1.22	0.706
HMF (mg/kg)	5.98	6.02±0.09	0.66	0.362
Furan (µg/kg)	113.12	115.55±6.34	2.10	0.486

[a] Experimental results were given with standard deviation

Table 7 Physicochemical properties of gluten-free biscuits produced at the optimum point

Parameters	Results
Moisture content (%)	1.38±0.27
Water activity	0.26±0.01
Ash content (%)	3.32±0.16
Oil content (%)	12.31±0.95
Protein content (%)	4.96±0.08
Carbohydrate content (%)	77.86±1.31
Gluten (mg/kg)	<5
pH	7.12±0.06
L	56.03±0.18
a	7.30±0.08
b	20.68±0.20
Total phenolic content (µg GAE/g extract*)	342.33±7.40
Antioxidant activity (µmol TE/100 g*)	162.00±0.17
Energy value (kcal/100 g)	443.63±2.78
Gelatinization degree (%)	54.39±3.38

* Results were expressed on a dry matter basis

and baking time 6 min. The results of 5 verification trials at the optimum point, the average of the results and the values predicted from the model are given in Table 5. And comparison of average experimental values at the optimum point with values predicted from the model is presented in Table 6.

According to the results of the analysis obtained as a result of the validation trials, the analysis results for HMF and furan amounts determined as target criteria and the values predicted from the model predictions and the observed values were very close, with no statistically significant difference between them ($p>0.05$). Moisture, protein and fat, ash and gluten contents; water activity, color, pH, hardness and energy values; starch gelatinization properties; total phenolic matter, acrylamide, HMF and furan amounts; antioxidant activity values and sensory properties of gluten-free biscuits produced under optimum conditions were analyzed. The moisture content of the gluten-free biscuits substituted with chestnut flour was determined as 1.38±0.27%, water activity values as 0.26±0.01, ash, fat, protein and carbohydrate contents as 3.32±0.16%, 12.31±0.95, 4.96±0.08 and 77.86±1.31, respectively (Table 7). On the other hand, *L**, *a** and *b** values of the samples were determined as 56.03±0.18, 7.30±0.08 and 20.68±0.20, respectively. Total phenolic content of the samples was 342.33±7.40 µg GAE/g extract and antioxidant activity values were 162.00±0.17 µmol TE/100 g. The energy values of the samples were determined as 443.63±2.78. The scores of the samples produced at the optimum point in terms of taste, odor, bitterness, crunchiness, chewiness, aftertaste impression and general liking were 5.20±0.87; 4.70±0.90; 5.30±0.64; 5.90±0.94; 5.70±0.90; 4.10±0.70 and 5.30±0.78, respectively (data not shown). The panelists also stated that no significant bitterness was perceived in the samples.

The effect of storage on maillard reaction products and biscuit properties

The effects of storage time and processing method on physicochemical, textural, bioactive, contaminant, and sensory properties of gluten-free biscuits were evaluated using regression-based statistical approaches, primarily linear mixed-effects models, with storage time and processing method treated as fixed effects and batch or panelist group considered as a random effect (Table 8).

Moisture content and water activity exhibited significant changes throughout storage, indicating progressive moisture redistribution within the product matrix. Moisture content was significantly affected by storage time, processing method, and their interaction, suggesting that different processing conditions resulted in distinct moisture loss kinetics during storage. In contrast, water activity was primarily driven by storage time, while the main effect of processing method was not significant; however, the significant interaction effect indicated method-dependent patterns of aw evolution. A significant effect of storage time was also observed for pH, reflecting gradual physicochemical changes during storage, although comparisons among processing methods were not feasible due to data structure limitations.

Color parameters responded differently to storage. The L* value decreased significantly over time, indicating a gradual darkening of the product surface during storage. The a* parameter remained statistically unchanged, suggesting stability in red–green color balance. In contrast, the b* value was significantly influenced by storage time, processing method, and their interaction, highlighting method-dependent changes in yellowness that may be associated with non-enzymatic browning reactions and pigment stability.

Table 8 Effects of storage time and processing method on physicochemical, textural, bioactive, and sensory properties of gluten-free biscuits

Parameter	Statistical model	Storage time (β, direction, p)	Processing method (β, direction, p)	Interaction
Moisture content (%)	LMM	−β, ↓, $p<0.0001$	±β, ↓, $p<0.0001$	$p<0.0001$
Water activity (aw)	LMM	−β, ↓, $p<0.0001$	~0, ns	$p<0.0001$
pH	OLS	±β, ↓, $p<0.0001$	–	–
L*	LMM	−β, ↓, $p<0.0001$	–	–
a*	LMM	~0, ns	–	–
b*	OLS	+β, ↑, $p<0.001$	+β, ↑, $p<0.0001$	$p<0.0001$
Total phenolic content (μg GAE/g extract, d.b.)	LMM	−β, ↓, $p<0.0001$	~0, ns	$p<0.0001$
Antioxidant activity (μmol TE/100 g, d.b.)	LMM	−β, ↓, $p<0.0001$	+β, ↑, $p<0.0001$	$p<0.0001$
Hardness (g)	LMM	+β, ↑, $p<0.0001$	+β, ↑, $p<0.0001$	$p<0.0001$
Acrylamide (μg/kg)	LMM	−β, ↓, $p<0.0001$	−β, ↓, $p<0.0001$	ns
HMF (mg/kg)	OLS	−β, ↓, $p=0.041$	−β, ↓, $p=0.025$	–
Furan (μg/kg)	LMM	−β, ↓, $p<0.0001$	±β, ↓, $p<0.0001$	$p<0.0001$
Sensory: Taste	LMM	−β, ↓, $p<0.0001$	±β, ↑, $p<0.0001$	$p<0.05$
Sensory: Odour	OLS	~0, ns	±β, ↑, $p<0.05$	ns
Sensory: Bitterness	LMM	~0, ns	~0, ns	ns
Sensory: Crunchiness	OLS	−β, ↓, $p<0.0001$	±β, ↓, $p<0.05$	ns

Note: β indicates the direction of the estimated regression coefficient

↑ increase; ↓ decrease; ~0 no meaningful change; ns not significant ($p>0.05$)

LMM linear mixed-effects model, *OLS* ordinary least squares regression

Storage time and processing method were treated as fixed effects, while batch or panelist group was included as a random effect where applicable

Bioactive properties were notably sensitive to storage. Total phenolic content showed a significant dependence on storage time, with additional interaction effects indicating that phenolic degradation or transformation during storage varied according to processing method. Antioxidant activity was strongly influenced by both storage time and processing method, as well as their interaction, suggesting that the preservation of antioxidant capacity during storage is closely linked to the initial processing conditions and subsequent storage-related changes.

Textural analysis revealed that hardness increased significantly during storage and was strongly affected by processing method and interaction effects. This behavior is consistent with moisture loss and structural rearrangements within the biscuit matrix, leading to progressive firming, with different processing methods modulating the extent and rate of this change.

Regarding process-induced contaminants, acrylamide levels were significantly affected by both storage time and processing method, while the interaction effect was not significant, indicating that storage-related changes in acrylamide followed similar trends across processing methods. HMF content was significantly influenced by storage time and processing method, supporting the notion that HMF is a relatively unstable Maillard reaction product that may degrade during storage. Furan content exhibited significant main and interaction effects, indicating that its stability during storage is strongly dependent on both processing conditions and storage duration.

Sensory attributes displayed variable sensitivity to storage and processing. Taste and crunchiness were significantly influenced by storage time and processing method, reflecting the combined impact of physicochemical and textural changes on sensory perception. Odour was primarily affected by processing method rather than storage time, suggesting that aroma-related compounds were more closely linked to processing conditions than to storage duration. Bitterness remained statistically unchanged, indicating sensory stability for this attribute throughout storage.

Taken together, these findings demonstrate that storage time is the principal driver of quality changes in gluten-free biscuits, while processing method modulates the magnitude and direction of these changes for selected parameters. The observed interaction effects for several variables highlight the importance of considering both processing and storage conditions when evaluating product stability.

Conclusions

In conclusion, it was observed that there was a tendency to increase both acrylamide, HMF and furan amounts with increasing baking time in the samples. On the other hand, with the increase in the amount of chestnut flour in the formulation, it was determined that the amount of Maillard reaction products also increased. MRP levels increased accordingly with baking time and temperature. In addition, it was determined that there was a tendency to increase in hardness values, especially with the increase in the ratio of rice flour in the formulation. As a result, it was determined

that the optimum chestnut flour: rice flour ratio in terms of acrylamide, HMF and furan formation was 44.17:55.83, the optimum baking temperature was 175.16 °C and the optimum baking time was 6 min.

The results indicate that storage time is the primary determinant of quality evolution in gluten-free biscuits, whereas the processing method influences the magnitude and direction of these changes for specific parameters. The presence of significant interaction effects underscores the necessity of jointly considering processing and storage conditions when assessing product stability.

The amount of Maillard reaction products in biscuits can be controlled by paying attention to baking temperature and time and by keeping the amount of chestnut flour limited in the formulation. The findings highlight the critical role of the chestnut flour: rice flour ratio, baking temperature, and time in determining acrylamide, 5-hydroxymethylfurfural (HMF), and furan levels. Furthermore, the study reveals how storage conditions affect the degradation of these compounds over time, providing a deeper understanding of the relationship between ingredient composition, thermal processing, and MRP formation in gluten-free baked goods.

Specifically, the results provide important insights for the gluten-free bakery industry by identifying optimal formulation and baking conditions that minimize the formation of harmful Maillard reaction products such as acrylamide, HMF, and furan, which are of significant concern for food safety and quality. The determination of the optimal chestnut flour to rice flour ratio, baking temperature, and time can assist manufacturers in producing safer and higher-quality gluten-free biscuits. Moreover, understanding the effects of storage on these compounds and sensory properties can help in developing strategies to extend product shelf life and maintain consumer acceptability. These findings contribute to the advancement of healthier gluten-free baked products and offer practical guidance for industrial applications.

Acknowledgements The authors thank to TÜBİTAK-TOVAG (Project Number: 222O237) and Aydın Adnan Menderes University Research Fund (Project number: MF-22004) for financial support. The authors also thank to Aydın Adnan Menderes University Agricultural Biotechnology and Food Safety Application and Research Center (TARBİYOMER) for using laboratory infrastructure.

Funding Open access funding provided by the Scientific and Technological Research Council of Türkiye (TÜBİTAK).

Data availability All data supporting the findings of this study are available within the paper.

Declarations

Conflict of interest The authors declare that there is no conflict of interest.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

References

1. S. Liu, H. Sun, G. Ma, T. Zhang, L. Wang, H. Pei, X. Li, L. Gao, Front. Nutr. **9**, 973677 (2022)
2. A. Mousavi Khaneghah, Y. Fakhri, A. Nematollahi, F. Seilani, Y. Vasseghian, Food Rev. Int. **38**(6), 1286–1304 (2022)
3. B. Matthäus, N.U. Haase, K. Vosmann, Eur. J. Lipid Sci. Technol. **106**(11), 793–801 (2004)
4. D. Taubert, S. Harlfinger, L. Henkes, R. Berkels, E. Schömig, J. Agric. Food Chem. **106**(11), 793–801 (2004)
5. R.M. Vinci, F. Mestdagh, B.D. Meulenaer, Food Chem. **133**(4), 1138–1154 (2012)
6. WHO (World Health Organization), FAO/WHO Consultations on the health implications of acrylamide in foods. In Summary report of a meeting held in Geneva. Geneva, (2002, June)
7. T. Krishnakumar, R.J. Visvanathan, Food Process. Technol. **05**(344), 1–9 (2014). https://doi.org/10.4172/21577110.1000344
8. A. Hamzalıoğlu, V. Gökmen, Role of hydroxymethylfurfural on acrylamide formation during thermal processing of foods, 2nd edn., ed. V. Gökmen and B. Ataç Mogol, In Acrylamide in FoodAcademic Press, (2024) pp. 309–329
9. F.C. Martins, G.M. Alcantara, A.F.S. Silva, W.R. Melchert, F.R. Rocha, Food Chem. **395**, 133539 (2022)
10. Z. Zhang, Y. Chen, P. Deng, Z. He, F. Qin, Q. Chen, Z. Wang, H. Pan, J. Chen, M. Zeng, Food Chem. **431**, 137152 (2024)
11. Z. Batool, J.H. Chen, B. Liu, F. Chen, M. Wang, J. Agric. Food Chem. **71**(13), 5093–5106 (2023)
12. Y.H. Huang, T.H. Kao, B.H. Chen, Food Chem. **395**, 133572 (2022)
13. V. Gökmen, Ö.Ç. Açar, H. Köksel, J. Acar, Food Chem. **104**(3), 1136–1142 (2007)
14. D. García-Ríos, F. Pedreschi, M.S. Mariotti-Celis, C. Contreras, D. Campos, J.E. Alvaro, R. Pedreschi, LWT 118398 (2025)
15. B. Kettlitz, G. Scholz, V. Theurillat, J. Cselovszky, R.B. Neil, S. O'Hagan, E. Mavromichali, K. Ahrens, K. Kraehenbuehl, G. Scozzi, M. Weck, C. Vinci, M. Sobieraj, R.H. Stadler, Compr. Rev. Food Sci. Food Saf. **18**(3), 738–752 (2019)
16. M. Mete, D.D. Altıner, Int. J. Food Eng. Res. **3**(1), 9–16 (2017)
17. A. Radhika, M. Virk, P. Kaur, D. Thakur, Q.U.E.H. Chauhan, S. Rizvi, K. Jan, Kumar, Curr. Res. Nutr. **07**(3), 842–853 (2019)
18. M. Ahsan, A. Moin, H. Ashraf, M. Kamran, I. Manzoor, A.M. Giuffrè, Ital. J. Food Sci. **35**(4), 136–146 (2023)
19. S.M.G. Saeed, S.A. Ali, K. Faheem, R. Ali, A.M. Giuffrè, Foods. **11**(15), 2346 (2022)
20. E. Nieto-Mazzocco, A. Saldaña-Robles, E. Franco-Robles, A.K. Rangel-Contreras, A. Cerón-García, C.J. Ozuna, Food Process. **44**(1), e14302, 1–9, (2020)
21. B. Sazesh, M. Goli, J. Food Process. **44**(8), e14563, 1–10 (2020)
22. Q. Yang, L. Kan, Y. Wu, Y. Liu, J. Ouyang, J. Food Process. **43**(10), e14112, 1–9, (2019)

23. M.A. Hernández-Aguirre, J.J. Islas-Hernández, M.E. Sánchez-Pardo, S.L. Rodríguez-Ambriz, P. Osorio-Díaz, J. Food Meas. Charact. **13**(3), 1652–1660 (2019)
24. A.S. Hager, E.K. Arendt, Food. **32**(1), 195–203 (2013)
25. D.F. McCarthy, E. Gallagher, T.R. Gormley, T.J. Schober, E.K. Arendt, Cereal Chem. **82**(5), 609–615 (2005)
26. AACC Approved Methods of the American Association of Cereal Chemists, (2000)
27. B. Seferoğlu, Evaluatıon of bread, cake and biscuıit recipes prepared for celiac patients with chestnut and gluten free flours by sensory evaluation methods. Hacettepe University, M.Sc. Thesis. (2012)
28. I. Davidson, *Biscuit baking technology: processing and engineering manual*, 3th edn. (Academic, 2023)
29. A.K. Shrestha, J. Arcot, S. Dhital, S. Crennan, Food Nutr. Sci. **3**(10), 1445–1452 (2012)
30. F. Zhu, R. Sakulnak, S. Wang, Food Chem. **194**, 1217–1223 (2016)
31. AOAC, *Official methods of analysis*, 18th edn. (AOAC International, Gaithersburg, 2012)
32. M. Anese, B. Quarta, L. Peloux, S. Calligaris, Food Res. Int. **44**(9), 2837–2842 (2011)
33. F.B. Karakoç, The Effects of Addition of Terebinth, Sesame And Flax Seed to Biscuit Formulation on Biscuit Quality and Shelf Life, P.D. Thesis, Necmettin Erbakan University, (2021)
34. N. Gamez-Meza, J.A. Noriega-Rodriguez, L.A. Medina-Juarez, J. Ortega-Garcia, C.-R. Casanova, O. Angulo-Guerrero, J. Am. Oil Chem. Soc. **77**(7), 721 (1999)
35. Ü, Urgancı, Determination of quality characteristics of biscuits fortified with pomegranate peel and packaged under modified atmosphere, Master's thesis, Pamukkale University (2019)
36. AOAC, *Association of Analytical Communities Official Method 991.19. Gliadin as a Measure of Gluten in Foods* (Colorimetric Monoclonal Antibody Enzyme Immunoassay Method, 2001)
37. N. Sozer, L. Cicerelli, R.L. Heiniö, K. Poutanen, J. Cereal Sci. **60**(1), 105–113 (2014)
38. M. Ndife, G. Şumnu, L. Bayındırlı, LWT-Food Sci. Technol. **31**(5), 484–488 (1998)
39. N. Karaağaoğlu, E. Karabudak, S. Yavuz, O. Yüksek, D. Dinçer, G. Tosunbayraktar, F.H. Eren, Çeşitli ekmeklerin protein, yağ, nem, kül, karbonhidrat and enerji değerleri. Food. **33**(1), 19–25 (2008)
40. C.P. Boyacı, Determination of acrylamide exposure arising from the some baby foods used for toddler nutrition, Master's thesis, Akdeniz University (2012)
41. M. Koç, Optimization of honey powder productiion conditions with different drying methods and determining the storage stability, P.D. Thesis, Ege University, (2015)
42. I. Juániz, C. Zocco, V. Mouro, C. Cid, M.P. De, Peña, LWT-Food Sci. Technol. **68**, 549–555 (2016)
43. K. Emektar, M.N. Kantekin-Erdogan, A. Tekin, Food Chem. **386**, 132744 (2022)
44. M. Demir, J. Agric. Sci. **21**(1), 100–107 (2015)
45. T. Alioğlu, The effect of wholemeal sourdough to the quality properties of the short dough biscuit, Master Thesis, Yıldız Technical University (2019)
46. G. Silav-Tuzlu, Z. Tacer-Caba, Food Technol. Biotechnol. **59**(4), 463–474 (2021)
47. H. Yang, L. Li, Y. Yin, B. Li, X. Zhang, W. Jiao, Y. Liang, Food Sci. Biotechnol. **28**(5), 1359 (2019)
48. U. Urganci, F. Işık, Acad. Food. **19**(1), 10–20 (2021)
49. J.C. Pinheiro, D.M. Bates, *Mixed-effects models in S and S-PLUS* (Springer, New York, NY, 2000)
50. G. Verbeke, G. *Molenberghs Linear mixed models for longitudinal data* (Springer, 2000)
51. B.T. West, K.B. Welch, A.T. Galecki, *Linear mixed models: A practical guide using statistical software*, 2nd edn. (CRC, 2014)
52. W. Klunklin, G. Savage, J Food Qual. 2018(1), 8052847 (2018)
53. S.Y. Joo, J. Korean Soc. Food Cult. **18**(2), 165–176 (2013)
54. S.Y. Joo, H.Y. Choi, Korean J. Food Nutr. **29**(2), 177–184 (2012)
55. M. Mesías, F. Holgado, G. Márquez-Ruiz, F.J. Morales, LWT-Food Sci. Technol. **73**, 528–535 (2016)
56. M. Mesias, F.J. Morales, Acrylamide in bakery products. U: Acrylamide in Food: Analysis, Content and Potential Health Effects, 1, 131–157. (2016)
57. T.M. Amrein, B. Schönbächler, F. Escher, R. Amadò, J. Agric. Food Chem. **52**(13), 4282–4288 (2004)
58. E. Bråthen, S.H. Knutsen, Food Chem. **92**(4), 693–700 (2005)
59. Y. Zhang, G. Zhang, Y. Zhang, J. Chromatogr. A **1075**(1–2), 1–21 (2005)
60. N.U. Haase, K.H. Grothe, B. Matthäus, K. Vosmann, M.G. Lindhauer, Food Addit Contam Part A. (2012)
61. J. Degen, M. Hellwig, T. Henle, J. Agric. Food Chem. **60**(28), 7071–7079 (2012)
62. H. Hu, Y. Wang, M. Shen, Y. Huang, C. Li, S. Nie, M. Xie, Curr. Res. Food Sci. **5**, 940–948 (2022)
63. A. Ramirez-Jimenez, E. Guerra-Hernández, B. García-Villanova, J. Agric. Food Chem. **48**(9), 4176–4181 (2000)
64. Y.Y. Zhang, Y. Song, X.S. Hu, X.J. Liao, Y.Y. Ni, Q.H. Li, Food Res. Int. **49**(1), 439–445 (2012)
65. V. Gökmen, Ö.Ç. Açar, A. Serpen, F.J. Morales, Eur. Food Res. Technol. **226**(5), 1031–1037 (2008)
66. V. Gökmen, T. Kocadağlı, N. Göncüoğlu, B.A. Mogol, Food Chem. **132**(1), 168–174 (2012)
67. B. Gonçalves, O. Borges, E. Rosa, J. Coutinho, A.P. Silva, Fruits. **67**(3), 201–214 (2012)
68. E. Capuano, A. Ferrigno, I. Acampa, L. Ait-Ameur, V. Fogliano, Eur. Food Res. Technol. **2282**, 311–319 (2008)
69. J. Michalak, E. Gujska, M. Czarnowska, J. Klepacka, F. Nowak, Plant. Foods Hum. Nutr. **71**(1), 115–122 (2016)
70. A. Albouchi, M. Murkovic, Food Res. Int. **137**, 109444 (2020)
71. V. Ozolina, D. Kunkulberga, B. Cieslak, M Procedia Food Sci. **1**, 1158–1164 (2011)
72. M.S. Mariotti-Celis, R.N. Zúñig, P. Cortés, F Pedreschi J. Food Sci. **82**(1), 232–239 (2017)

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.