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RESEARCH

Investigating the Effect of Glucoamylase Enzyme Treatment and Continuous Ultrasound Application on Quality Characteristics and Aflatoxins Degradation of Hazelnut Paste by Box-Behnken Response Surface Design

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Abstract
Aflatoxins (AFs) are toxic secondary metabolites of filamentous fungi which can reduce the quality of several food commodities like hazelnut and hazelnut products. For the AFs, the data were fitted to polynomial response models using multiple regression analysis, resulting in high coefficients of determination (R^2 values ranging from 0.8917 to 0.9674) for each type of aflatoxins. The optimal conditions for achieving maximum degradation percentages for total aflatoxins (AFT) and AFB1 were determined to be US power of 80W, enzyme treatment of 2.5 U g sample, and 20 min of US application for 12.5 µg kg AFT conditions after graphical and numerical optimizations. The optimum conditions resulted in 44.33% and 45.58% degradation for AFT% and AFB1 respectively, with predicted values of 43.93% and 44.17% for AFT% and AFB1. The data exhibited that, enzyme treatments were not significant for degradation for AFB1% and AFT%, whereas significant for AFB2 and AFG2. Depending on the initial AFT concentration, more than 50% degradation rate was achieved by current design parameters. Study results indicated that US treatment can alter certain quality parameters of hazelnut paste, including changes in aroma, a decrease in browning index, and a slight increase in peroxide value.

Keywords Ultrasound · Glucoamylase · Aflatoxin degradation · Aflatoxin B1 · Box-Behnken response surface design

Introduction

Hazelnut (*Corylus avellana L.*) is a well-liked tree nut, along with cashews, almonds, pistachios and walnuts, that is one of the most commercially cultivated and consumed worldwide with 3rd ranking in terms of production quantity [1]. Even though a large production volume, compared to other tree nuts, hazelnuts have received less attention in terms of their health benefits. Hazelnuts are high in fats, fibers, vitamins (especially fat-soluble ones), minerals and phytosterols. Their thin brown skin is also rich in flavonoids and phenolic compounds [2]. The Black Sea Region, mainly Ordu, Giresun and Trabzon provinces is the largest hazelnut plantation area in Turkey. The average shelled hazelnut production quantity of the world is about 872,000 metric tons between 2001 and 2021 and Turkey compensates 66% of the total production. Italy (13%), USA (4.3%), Azerbaijan (3.7%), and Georgia (2.8%) follow Turkey with lower production quantities [1]. Apart from being consumed as a snack in raw and roasted forms, hazelnut kernels are widely used in the food industry [3]. Hazelnut products such as chopped or sliced, hazelnut flour or hazelnut paste are used in chocolate, bakery and confectionary industries to enhance the flavor, texture or nutritional value of the products [4].

Hazelnut paste (also known as hazelnut puree) is a kind of industrial semi-product produced by grinding of roasted shelled hazelnut kernels to obtain a creamy structure and homogenous consistency. It is generally used as an

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ingredient in chocolate products, chocolate spreads, candies, bakery products, salads and desserts [5, 6]. The general production flowchart of hazelnut paste is given in Figure S1. Briefly, the hazelnut in shells is shelled and sorted according to size. Then, in order to remove inappropriate kernels (such as moldy kernels) from healthy ones, a sorting process is conducted. While relatively bigger size kernels (above to 10 mm diameter) are used for snacks, chocolate production and desserts, smaller kernels (underdeveloped or lower-developed kernels) are used for hazelnut paste production [7, 8]. The aspiration process is applied optionally to the small-sized kernels to remove dark skins (thin husk) after the roasting process. Then the roasted kernels are grounded to mean 20–90 µm by mills. Final hazelnut paste is pasteurized and stored in the large tanks under nitrogen gas until marketing. Final product usually contains more than 55% oil and less than 1% water [5, 9]. Hazelnut paste production is conducted per Turkish standard legislation (TS10938). According to this legislation, hazelnut paste must contain a minimum of 55% oil and a maximum of 6% humidity. The color of the products may vary from pale cream to dark brown depending on the hazelnut kernel quality [10]. Table 1 compares physicochemical properties between hazelnut paste samples used in the current study and reference literature values.

It is generally accepted that the thick hazelnut shells and the dark brown thin husk coating the kernel serve as a good barrier for hazelnut kernels, protecting them from contamination and invasion. However, certain mold species that can produce aflatoxins (AFs) have the potential to contaminate the kernels with AFs at any stage of the production process, especially when the shells are damaged by insects, mammals, or mechanical forces [11]. Due to a shortage of high-capacity modern drying plants in the Black Sea Region, producers are forced to dry their products using traditional methods such as drying under the sun on concrete or grass surfaces. This is because there isn't sufficient capacity to handle the large volume of production. [12]. The weather in the region is typically rainy, and humidity is high during the drying period of shelled hazelnut kernels. If the drying session coincides with rainy and humid weather conditions, it may take longer to reach the appropriate moisture content (around 6%) for the hazelnut kernels, making them more susceptible to invasion by mycotoxigenic fungi [11]. Furthermore, improper storage conditions, such as high humidity and temperature, can lead to mold growth and the formation of AFs. [13, 14].

Aflatoxins are toxic and carcinogenic secondary metabolites produced by certain types of molds, particularly two species of Aspergillus section Flavi: *Aspergillus flavus* and *A. parasiticus* [17]. Due to the consumption of various products contaminated with AFs, the global human population, particularly in developing countries, is frequently subjected to chronic exposure to low levels of these toxins. An estimated 4.5 billion individuals worldwide are affected by this exposure primarily through the ingestion of food that has been contaminated with AFs [18]. While over 18 different types of aflatoxins have been identified thus far, it is AFB1, AFB2, AFG1, and AFG2 that predominantly contaminate agricultural products [19, 20]. Moreover, if the feed is contaminated with AFs, dairy animals can ingest the contaminated feed, and AFB1 and AFB2 are metabolized into their hydroxylated forms, AFM1 and AFM2, which can be detected in their milk [21]. Research has shown that AFs can cause mutagenic, immunosuppressive, hepatocarcinogenic, and teratogenic effects in humans and animals due to their toxigenicity. The International Agency for Research on Cancer (IARC) has classified AFB1 and naturally occurring mixtures of aflatoxins as Group 1 carcinogens [22]. Experimental animal studies provide sufficient evidence for the carcinogenicity of AFB1, G1, and M1, limited evidence for AFB2, and insufficient evidence for AFG2 [23]. It is speculated that AFB1 is the most commonly encountered type of aflatoxin in agricultural commodities, while the other three types (AFB2, AFG1, and AFG2) are generally not observed without the presence of AFB1 [24].

Table 1 Comparison of physicochemical properties between hazelnut paste samples used in the current study and reference literature values

Physicochemical Properties	Initial sample values of the current study	Reference Values from the literature	References
Oil Content (%)	65.5–67.4	58–65	[15]
Hue	61.3–64.1	53–64.5	[15]
Chroma	15.97–25.4	13.43–16.56	[15]
Browning Index (BI)	385–444	ND	
Particle size (µm)	28–30	23–80	[15]
Maximum force during penetration (N)	ND	7.8–9.9	[15]
Maximum force during withdrawal	ND	-3.5- -7.4	[15]
Aroma Index (AI)	29–51	ND	
Peroxide Value	0.01–0.02	0.00–0.08	[16]

ND: No data available

Despite the absence of a globally standardized legal limit, the European Commission has established specific maximum levels for aflatoxins (AFs) in different food products, including hazelnuts. In the case of hazelnuts intended for direct human consumption or use as an ingredients in food stuffs, the European Commission has defined the maximum levels (MLs) for AFB1 and total AFs (the combined sum of AFB1, AFB2, AFG1, and AFG2) as 5 $\mu g\ kg^{-1}$ and 10 $\mu g\ kg^{-1}$, respectively [25].

When examining the Rapid Alert System for Food and Feed (RASFF) reports between 2002 and 2022, it was determined that out of a total of 159,555 notifications in the system, 12,326 (7.7%) were related to mycotoxins, and more than 830 (6.7%) of these mycotoxin-related notifications were related to AFs in hazelnut kernels and hazelnut products originating from Turkey [26]. Moreover, a recent study revealed that, according to the RASFF notifications from 2011 to 2021, approximately 3000 notifications were about “Nuts, Nut products and Seeds”. While 10% (n = 311) of total notifications for “Nuts, Nut products and Seeds” were reported for hazelnuts and of 11 of them were about hazelnut paste [27]. It should be noted that one of the main reasons for the relatively low incidences reported for hazelnut paste products by the RASFF system may be attributed to the mixing of hazelnut paste products containing high levels of AFs with AF-free products. Consequently, this practice ensures that the final product can have lower AF levels than the MLs for AFs stipulated by EU legislation. On the other hand, as a popular ingredient in confectionery industry, hazelnut paste is often subjected to adulteration. Possible methods for adulterating hazelnut paste include the addition of chickpea flour [28], peanuts [29], or the dilution with refined/ non-refined vegetable oils [7]. Thus, certain forms of adulteration, particularly the use of vegetable oils as an adulterant, can not only be used for product fraud but also for reducing the levels of AFs in hazelnut paste.

Moreover, there have been very few studies in the literature regarding the presence of AFs in hazelnut paste. Even though [30] (n = 5), [31] (n = 619) and Şen & Civil (2022) (n = 202) reported quite high incidence of AFs (100% of the samples were contaminated with AFs), very few samples exceeded the maximum permitted limits were reported in those studies (below 2%). These studies (also the RASFF reports) have shown that, higher incidence (but with lower AFs contamination levels below the maximum permitted limits for AFs) for the hazelnut paste samples may be resulted from using lower quality kernels and/or lowering the AFs levels of the product by means of mixing AFs high product with AFs free products or another adulterant.

Pre- and post-harvest approaches has been discussed last few decades for the prevention of aflatoxin contamination in Agro-food products. Preventative approach contains the selection of correct fungal-resistant crop varieties, managing the field, handling the post-harvest treatment (such as sorting or drying) and providing the appropriate storage conditions. However, these prevention methods are very challenging, nestle several variables and do not guarantee to obtain aflatoxin-free products [32]. Nonetheless, achieving aflatoxin-free products is not always guaranteed through these prevention methods, underscoring the significance of post-harvest detoxification techniques in managing aflatoxin contamination. Conventional methods for mycotoxin decontamination include physical techniques like adsorption, cooking, roasting, baking, and frying, as well as chemical approaches involving chemical agents, ammoniation, and ozonation, along with biological methods such as fermentation, microbial metabolization, and enzyme-based treatments [33]. Moreover, in recent studies, several innovative approaches, including microwave treatment, irradiation, pulsed light exposure, pulsed electric field application, ultrasound treatment, and cold plasma technology, have been explored as alternative methods [32].

Ultrasound (US) is a non-thermal food processing technique that has gained attention in recent decades. The term US refers to sound waves with frequencies higher than what humans can hear, typically between 20 kHz and 10 MHz. The sound waves can create cavitation bubbles as they cycle between compression and expansion. Physicochemical processes, such as rectified diffusion, cause microbubbles to grow rapidly until they violently implode at a critical size due to energy absorption limitations [34]. When a cavitation bubble collapses, it creates a temporary hot spot with high localized temperature and pressure. This can increase the chemical reactivity in the surrounding medium, with estimated temperatures and pressures reaching up to 5000 K and 5000 atm [35, 36]. These conditions can lead to faster chemical reactions and strong shear forces due to microbubble collapse, which can break down large molecules into smaller compounds [34, 35, 37]. In such an extreme condition, the contaminants present in the medium are degraded. The use of ultrasonic (US) processing has diverse applications in the food and chemical industries, generating physical and chemical effects like emulsification, homogenization, extraction, cleaning/sterilization, and degradation of contaminants such as mycotoxins [34]. Ultrasound processing is an environment-friendly technology which has shown potential for controlling mycotoxigenic fungi and degrading mycotoxins. It is speculated that during ultrasound application in an aqueous medium, the covalent bonds in the water are broken and OH• radicals are formed. After that, these radicals oxidize the contaminants in the aqueous medium such as AFB1, Deoxynivalenol, Zearalenone and Ochratoxin A [38, 39]. Previous studies on the mycotoxin decontamination using US have shown that the initial concentration of the mycotoxin, solid–liquid ratio, ultrasonic intensity and application time are the crucial parameters for the degradation of AFs [39, 40].

Laccases, peroxidases, reductases and lactonases are the most reported enzymes used for the degradation of mycotoxins in food and feed systems both in vitro and real samples [41–43]. Among these enzymes, laccase has been the most extensively studied enzyme for various applications in the food industry over the last few decades. These applications include beverage processing, wine stabilization, beer stabilization, improving dough consistency in baking, and sugar beet pectin gelation [44]. Moreover, several researchers have proposed that the use of this enzyme can degrade AFs to varying degrees, depending on factors such as the source of the enzyme, pH, temperature of the media, and application time [41, 42, 45, 46]. Even though most of the enzymatic degradation processes remove AFs toxicity by using these enzymes in vitro, except biotechnological applications, their use in real food systems is still far from practical industrial applications [47]. In the context of hazelnut paste, the use of these enzymes in the process may bring certain risks. It is known that the interaction between α-dicarbonyl compounds and amino acids plays a crucial role in the Maillard reaction, contributing to the development of both aroma and color through Strecker degradation [48]. α-Dicarbonyl compounds are significant in initiating polymerization reactions, ultimately resulting in the creation of carbohydrate-based melanoidins. Thus, the role of the α-Dicarbonyl compounds and the amino acids during roasting of hazelnuts for the development of aroma and color was well-established in a previous study [49]. It is known that most of the enzyme types proposed for the degradation of AFs (such as laccases [50], peroxidases [51], and reductases [52]) are also capable of degrading Maillard reaction products. Considering that aroma and color are the main quality parameters of hazelnut paste, using these enzymes may result in a loss of quality in the product inevitably. In the absence of any commercially available AFs degradation enzymes, the efficiency of certain commercial amylase enzymes in decomposing AFs in waste wheat bread was previously tested in the literature [53].

Investigating the effect of US treatment on mycotoxin decomposition in various food categories is a challenging task. It is crucial to determine the optimum parameters for each product to obtain favorable results and successfully implement the study findings in the industry.

Response surface methodology (RSM) is a useful statistical technique that is used to model and optimize complex processes. RSM is often used in various fields such as engineering, chemistry and biology, to identify the optimal conditions for a process to achieve a desired output. The Box-Behnken design (BBD), a RSM tool, is frequently used to optimize experimental trials. The Box-Behnken design excludes extreme points in the cubic region, which may be impractical to test due to physical limitations in experimentation. Box-Behnken design (BBD) is widely used to extract bioactive compounds from different sources for human consumption in industries such as pharmaceuticals, bioprocessing, food engineering, and agrochemicals. These compounds include polysaccharides, phenolic compounds, and proteins [54].

In this study, response surface methodology was used to investigate the effect of amylase enzyme treatment and continuous ultrasound application on the quality characteristics and decomposition of aflatoxins in hazelnut paste.

Materials and Methods

Material

Naturally contaminated hazelnut paste samples (containing approximately 5 µg/kg, 12.5 µg/kg and 20 µg/kg total AFs) were obtained from Yavuz Gıda, Giresun, Turkiye. The samples contained 6.93 µg/kg, 12.85 µg/kg, and 20.82 µg/kg total AFs respectively. Table 2 demonstrates aflatoxin concentrations of the samples belonging to each level. Each lot of samples weighed around 5 kg and was mixed with a Waring blender for 2 min to ensure a homogeneous sample before use.

Chemicals and reagents

Methanol, HPLC-grade acetonitrile, (65%) nitric acid, and analytical-grade NaCl and KBr, diethyl ether, potassium iodide, starch and sodium hydroxide were purchased from Merck (Darmstadt, Germany). The Labostar TWF water purification system from Evoqua Water Technologies in Pittsburgh, USA was used to obtain ultrapure water. Phosphate-buffered saline (PBS) tablets and Whatman No. 4 filter units (pore size 45 µm, size 125 mm) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The immunoaffinity columns (IACs) with product code P07 were purchased from Aflaprep®, Rhone Diagnostics GmbH (R-Biopharm AG, Germany). Mixed standard AFs (Product no. P22) containing 0.25 µg of AFB1, AFB2, AFG1, and AFG2 in 1 ml of acetonitrile were supplied by Rhone Diagnostics GmbH (R-Biopharm AG, Germany). Heat-stable glucoamylase (Amylase® AG XXL, 460 AGU mL^{-1}) provided by Novozymes A/S (Denmark).

Apparatus

A Waring blender (Waring Products Co. in Torrington, Connecticut, USA) was utilized for the extraction procedure. To prevent cross contamination, the blender jars were treated with a 10% hypochlorite solution for 2 h and washed before reuse.

For the analysis of AFs, a high-performance liquid chromatography system from Shimadzu Corp. in Kyoto,

Table 2 Variables and levels used in Box-Behnken design

Variable	Level		
Independent Variables	Low (-1)	Central (0)	High (+1)
US Power (W), A (Power density, W cm^3)	30 (0.46 W cm^{-3})	55 (0.67 W cm^{-3})	80 (0.91 W cm^{-3})
Enzyme Treatment (µL/50 g), B (U enzyme/g sample)	50 (0.5)	150 (1.5)	250 (2.5)
Application time (min), C	10	20	30
Initial total AFs (AFT) concentration (µg/kg), D	6.93	12.85	20.82
Initial AFG2 concentration (µg/kg)	*0.43*	*0.802*	*1.05*
Initial AFG1 concentration (µg/kg)	*2.89*	*4.654*	*7.11*
Initial AFB2 concentration (µg/kg)	*0.38*	*0.968*	*1.32*
Initial AFB1 concentration (µg/kg)	*3.23*	*6.43*	*11.34*
Dependent Variables			
AFT degradation (AFT%), R_1			
AFG2 degradation (AFG2%), R_2			
AFG1 degradation (AFG1%), R_3			
AFB2 degradation (AFB2%), R_4			
AFB1degradation (AFB1%), R_5			
Aroma index change% (ΔAI%), R_6			
Peroxide value change, (ΔPV) R_7			
Browning index change (ΔBI), R9			
Temperature change (ΔT), R8			

Japan was utilized. The system consisted of a RF20A fluorescence detector, a LC20AD pump, a SIL20A auto sampler, and a DGU20A degasser. Separation of the analytes was carried out using an Phenomenex® C18 (5 µm, 250 × 4.6 mm) column (CA, USA). AFB1 and AFG1 fluorescence intensity enhancement was achieved via post-column derivatization using the Kobra® cell system from Rhone Diagnostics GmbH (R-Biopharm AG, Germany).

Experimental design

RSM was used to optimize and evaluate the maximum percentage of degradation in AFs while assessing changes in the main quality characteristics of hazelnut paste. The effects of the four independent variables [US Power 30–80 W at 20 kHz (0.46–0.91 W.cm^{-3}), enzyme treatment [(50–250 µL.50 g^{-1}), 0.5–2.5U enzyme per g sample], application time (10–30 min) and initial total AFs concentrations (5–20 µg. kg^{-1})] on the responses were investigated, and the optimal conditions were check using the Box–Behnken experimental design of RSM to improve maximum AFs degradation percentage (especially at total AFs and AFB1) with relative quality loss in hazelnut paste. BBD of four factors and three levels used to determine the effects on AFs degradation (%), aroma change, peroxide value change, total color difference and temperature change is shown in Table 2.

The best-fit model selected to illustrate the impact of individual factors and their interactions on the responses is a full quadratic polynomial (Eq. 1) model [55, 56]

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i + \sum_{i=1}^{k} \sum_{j=i+1}^{k} \beta_{ij} x_i x_j + \varepsilon \quad (1)$$

β_0, β_i, β_{ii}andβ_{ij} represent the regression coefficients for intercept, $x_i$andx_j are variables and ε represents the residuals. The design was generated using Design Expert® Software 11.0 (Stat-Ease, Minneapolis, USA). The design includes 29 sets of test conditions, each with six levels assigned to factors at high, central, and low levels, along with 11 replicated center points (Table 3). The experiments were randomized to reduce bias, and consistent software was used to analyze the responses for significant effects of each factor. Coefficients were used to develop a model that represents the relationship between the response and the factors. The model's significance was evaluated using analysis of variance (ANOVA). Processing conditions were optimized based on model desirability features using graphical and numerical analyses.

Table 3 Box-Behnken response surface design and response value

Run	US Power(W)	Enzyme Treatment (μL 50 g^{-1})	US app. Time (min)	Initial AFT concentration (μg kg^{-1})	AFT%	AFG2%	AFG1%	AFB2%	AFB1%	ΔAI	ΔPV	ΔE	ΔBI	ΔT
1	55	250	20	5	32.9	32.6	32.9	31.6	33.1	4.1	0.2	1.9	-32.3	82.4
2	30	150	30	12.5	30.0	30.0	30.0	30.0	30.0	5.9	0.1	3.1	-75.9	81.2
3	55	150	10	20	38.0	27.3	33.7	25.9	43.1	6.2	0.1	6.7	-67.5	43.5
4	55	250	30	12.5	42.0	42.0	42.0	42.0	42.0	8.8	0.2	7.1	-153.9	83.9
5	55	50	20	5	30.2	18.6	30.1	26.3	32.2	3.9	0.0	3.5	-57.2	76.0
6	55	150	20	12.5	41.0	41.0	41.0	41.0	41.0	4.4	0.1	2.9	-88.1	74.1
7	80	150	10	12.5	43.0	43.0	43.0	43.0	43.0	5.5	0.1	0.2	14.0	78.7
8	55	150	10	5	29.0	16.3	26.3	42.1	31.6	2.9	0.2	2.8	-45.7	44.5
9	55	150	20	12.5	40.0	40.0	40.0	40.0	40.0	6.2	0.0	2.4	-88.2	75.2
10	55	50	10	12.5	36.9	37.0	36.7	37.0	37.0	2.8	0.1	1.5	-0.7	44.6
11	55	250	20	20	41.2	35.3	37.6	32.0	45.1	9.0	0.3	7.4	-101.0	73.9
12	30	150	20	20	34.5	31.6	29.6	26.0	38.9	7.1	0.2	6.0	-94.4	41.3
13	30	150	20	5	28.3	23.3	36.7	26.3	21.7	3.5	0.2	2.6	-43.8	43.1
14	80	150	30	12.5	46.1	46.4	38.1	59.7	49.8	10.0	0.1	3.8	99.8	109.1
15	80	250	20	12.5	40.1	46.4	30.8	58.7	43.2	5.6	0.2	1.5	-17.6	91.9
16	80	50	20	12.5	45.0	45.0	45.0	45.0	45.0	5.9	0.0	3.4	-78.9	80.6
17	55	150	30	20	37.6	37.4	34.2	30.0	40.7	10.4	0.1	11.2	-122.5	97.1
18	55	50	20	20	36.6	16.0	31.4	26.3	42.9	6.9	0.0	8.0	-109.7	65.3
19	80	150	20	20	45.2	34.4	42.5	33.9	49.2	7.8	0.1	7.5	-109.4	85.5
20	55	150	20	12.5	36.0	36.0	36.0	36.0	36.0	3.9	0.1	0.4	-22.1	65.6
21	30	250	20	12.5	30.0	30.0	30.0	30.0	30.0	2.7	0.2	0.9	-11.6	42.1
22	30	50	20	12.5	29.2	29.2	29.2	29.2	29.2	4.3	0.0	2.7	8.2	41.6
23	55	250	10	12.5	39.5	42.7	44.5	36.4	36.0	2.3	0.2	1.1	-10.8	37.1
24	55	150	20	12.5	36.0	36.2	36.0	36.0	36.0	6.8	0.2	5.8	-143.2	74.3
25	55	150	30	5	45.3	39.5	48.4	26.3	45.5	7.3	0.1	1.8	-41.5	93.8
26	55	150	20	12.5	39.0	39.0	39.0	39.0	39.0	8.8	0.1	0.4	-36.0	76.1
27	30	150	10	12.5	29.1	29.0	29.3	29.0	29.0	1.5	0.2	0.3	-14.26	37.1
28	55	50	30	12.5	37.1	37.1	37.1	37.1	37.1	7.3	0.0	1.1	-30.29	81.9
29	80	150	20	5	56.3	41.9	56.1	65.8	57.3	6.3	0.1	1.7	-40.90	102.4

Preparation of samples for US application

A 100 W ultrasonic processor (Bandelin 4050 HD, Bandelin Electronic GmbH, Berlin, Germany) with a 3 mm probe (MS73) was used for sonication trials. 20 kHz constant frequency was applied for each sample. The energy input was varied to assess the influence of power intensity, and the resulting power intensity values were converted to the average power density of the hazelnut paste samples. (W/cm^3). All the operations were carried out under continuous mode.

To determine the power density of the hazelnut samples, the following formula (Eq. 2) was used to determine the power density (W cm^{-3}) of the samples.

$$\frac{P}{V} = \frac{C\Delta T}{\Delta t} \tag{2}$$

where;

P/V Power density for hazelnut paste (W cm−3)

C Specific heat capacity of hazelnut paste $\frac{J}{kg.K}$ (2090 $\frac{J}{kg.K}$) [57]

ΔT Temperature difference between measurements of temperatures (K)

Δt US treatment time (s)

The ΔT values were determined by taking the mean of the temperature differences observed in the experimental runs conducted at various ultrasonic (US) power levels. Then, power density values were calculated at three distinct power levels: 30 W (0.46 w cm^{-3}), 55 W (0.67 W cm^{-3}), and 80 W (0.91 W cm^{-3}), respectively. The power densities corresponding to the various ultrasonic (US) power levels, which were applied at low, central, and high levels, have been presented in Table 2.

200 g sample were weighed in a volumetric flask and then enzyme was added according to [58]. The samples were stirred at 200 rpm during 3 h at 50 ºC via water bath shaker. Subsequently, each sample was transferred to a US device. Then US power at 20 kHz was applied to each sample according to the experimental design, with varying durations and various US power levels.

Extraction procedure, clean-up, and analysis of AFs

The proposed extraction procedure for hazelnut paste was implemented with slight modifications [6] as follows; a 50 g sample of hazelnut paste was first blended with 100 ml ultra-pure water containing 4 g of NaCl using a Waring blender for 1 min, resulting in the formation of a slurry. Subsequently, 150 mL of methanol was added to the slurry and blended for an additional 2 min. The resulting mixture was then filtered using Whatman No.4 filter paper, with the filtrate collected in a conical flask. To prepare for the immunoaffinity column (IAC) purification step, 5 mL of the clear filtrate was diluted with 15 mL of PBS solution at a pH of 7.3. The IACs were conditioned by passing 10 mL of PBS through them at a flow rate of 2–3 mL min-1. Following this, 20 mL of the diluted extract was passed through the IACs entirely by gravity at a flow rate of 2–3 mL min^{-1}. To ensure cleaning of the columns, 20 mL of ultrapure water was used for washing, followed by drying with air. The target AFs were then eluted from the IACs using a solution of 1000 µL of methanol and 1000 µL of water, allowing it to pass through the columns by gravity. Finally, the eluted samples were stored at temperatures between 4–8 °C until HPLC analysis.

The mobile phase was composed of a mixture of methanol and water (40:60, v/v), containing 120 mg of potassium bromide and 350 µL of nitric acid at a concentration of 4 mol L^{-1} in a methanol–water solvent (40%, v/v). The column oven was maintained at a temperature of 40 °C, with a mobile phase flow rate of 1 mL min^{-1} and an injection volume of 100 µL. The fluorescence detector (FLD) was configured with the following parameters: Excitation (Ex) at 360 nm and Emission (Em) at 430 nm. For method validation, various analytical parameters including linearity, limit of detection (LOD), limit of quantification (LOQ), recovery, and precision were determined. Linearity was assessed using a set of five mixed standard solutions with concentrations ranging from 0.125 to 2 ng mL^{-1} for AFB1, AFG1, AFB2, and AFG2. The LODs and LOQs were established based on recovery experiments. The LODs were calculated as three times the standard deviations (SD) of nine replicate analyses of blank samples spiked with 0.25 µg kg^{-1} of each AFB1, AFG1, AFB2, and AFG2. The LOQs were then determined as ten times the SD of the replicates. To evaluate recovery, blank materials were fortified with AF standards at low (0.625 µg kg^{-1} for each AFB1, AFG1, AFB2, and AFG2), medium (1.25 µg kg^{-1} for each AFB1, AFG1, AFB2, and AFG2), and high (2.5 µg kg^{-1} for each AFB1, AFG1, AFB2, and AFG2) levels in triplicate. The recovery results were used to calculate inter-day and intra-day precision.

The recoveries of fortified samples were determined using the following equation (Eq. 3):

$$Recovery\ (\%) = \frac{measured\ quantity}{fortification\ level} \times 100 \tag{3}$$

Decomposition rate of AFs

Decomposition rate of each AF was determined using Eq. 4.

$$Degradation\ (\%) = \frac{C_i - C_f}{C_i} \times 100 \quad (4)$$

where, C_i is the initial concentration of mycotoxin (which refers to AFB1, AFB2, AFG1, AFG2 and AFT) at initial stage of the experiment and C_f is the final concentration of mycotoxin after US treatment [39].

Determination of quality characteristics of hazelnut paste

The peroxide value (PV) is a widely used objective method for evaluating the level of oxidation of hazelnut paste, which quantifies the formation of hydroperoxides. Peroxide value of the samples was determined with slight modifications according to the method based on colorimetric reaction between iodine and sodium thiosulfate [59]. To extract the hazelnut oil, 100 ml diethyl ether was added to 50 g hazelnut paste, and mixed for 10 min via a rotary shaker (Nuve ST30, Turkey). After that, the mixture was centrifuged at 4000 g for 10 min (Nuve NF800R, Turkey). Diethyl ether was removed under vacuum (Büchi R300, Germany). 1 g of extracted oil was weighed into a 250 mL flask and a 30 mL mixture of acetic acid/chloroform (3/2, v/v) and 0.5 mL saturated potassium iodide were added. The sample was kept in the dark for 1 min. After that, the sample was diluted with 30 mL of water, and 2–3 drops of 0.5% starch solution were added as indicator. The sample was titrated with 0.01 N Sodium thiosulphate and the peroxide value expressed as milliequivalents of active oxygen kg^{-1}.

Color measurements were performed using the HunterLAB Miniscan (EZ-4500L, Hunter Associates Lab. Inc., Reston, VA, USA). The instrument was standardized before each measurement using both a black and a white tile with the following color coordinates: X = 79.90, Y = 84.98, Z = 90.90. The color values of the samples were represented using three dimensions: L (lightness), a(redness/greenness) and b (yellowness/ blueness) values. The hazelnut paste sample was poured into an optically clear 64 mm glass sample cup, filling it up to the 25 mm mark. To eliminate any potential light interferences, measurements were consistently conducted with the black cover of the sample cup under the same conditions. The reported results were the means of five measurements for each dimension. The color changes in hazelnut paste samples from their initial state to after undergoing the ultrasonic (US) processing can also be quantified as total color difference, denoted as ΔE, and Browning Index change (ΔBI). ΔE and ΔBI values can be calculated using Eq. 5 [60], Eq. 6 [61] and Eq. 7 based on the L, a, b values.

$$\Delta E = \sqrt{\Delta L^2 + \Delta a^2 + \Delta b^2} \quad (5)$$

$$BI = \frac{100(x - 0.31)}{0.17} \quad (6)$$

Where;

$$x = \frac{a + 1.75L}{5.64L + a - 3.012b}$$

$$\Delta BI = BI_f - B_i \quad (7)$$

where; ΔBI_f represents final BI value after US treatment, B_i represents initial state of BI value.

Hazelnut volatiles, mainly alkyl pyrazines, were determined according to de Brito and Narain (2003) [62] with slight modifications. A 120 g hazelnut paste sample was mixed with 200 mL of distilled water using a Waring blender for 1 min. Following this, 90 g of the mixture was weighed and transferred into a distillation tube. Alkali distillation was performed using a steam distillation unit (Büchi KjelfexK-360, Germany) with a 25% NaOH solution. The absorbance of the distillate was measured at 279 nm using a spectrophotometer (Shimadzu UV-1800, Japan). The spectrophotometer was calibrated with distilled water before use. The absorbance results were multiplied by 100 and expressed as the aroma index. Aroma index changes in the samples were calculated using Eq. 8 and expressed as ΔAI%.

$$\Delta AI\% = \frac{AI_f - AI_i}{AI_i} \times 100 \quad (8)$$

where; AI_i is the initial aroma index before treatments and the AI_f is the final aroma index value after US treatment.

Results and Discussion

Validation of the AFs determination method

Table 4 provides the method's linearity data and validation results. Highly linear calibration curves were obtained for AFB1, AFB2, AFG1, and AFG2 with R2 values up to 0.999, over a concentration range of 0.5 to 2 μg L^{-1} for each analyte. Based on the results of the individual analytical method, the LOD and LOQ values were fair, ranging from 0.06–0.09 μg kg^{-1} for LOD and 0.17–0.26 μg kg^{-1} for LOQ. These values were sufficient for detecting AFs in hazelnut paste. According to the analysis, the average recovery rates for individual types of AF ranged from 84.1% to 101.5%. Meanwhile, the average recovery rates for total AFs (AFT)

Table 4 Method linearity data and validation results of AFB1, AFB2, AFG1, AFG2, and AFT in hazelnut paste samples with different fortification levels

Analyte	Range (μg L^{-1})	Linearity (y = ax + b)	Coefficient of determination R^2	Fortification level (μg kg^{-1})	LOD (μg kg^{-1})	LOQ (μg kg^{-1})	Average Recovery (%)	Intra-day repeatability, RSD_r%	Inter-day repeatability, RSD_R%	0.66xHorwitz equation
AFB1	0.125–2.0	y = 61044.99x-4.55051	0.9998	0.625	0.09	0.26	101.5	5.7	7.5	32.1
				1.25			88.5	2.2	5.1	28.9
				2.5			86.6	3.77	7.6	26.0
AFB2	0.125–2.0	y = 107208.3x-11.69199	0.9998	0.625	0.05	0.17	97.6	6.5	10.4	32.1
				1.25			89.7	1.5	3.6	28.9
				2.5			86.7	3.8	7.6	26.0
AFG1	0.125–2.0	y = 65861.47x + 39.79701	0.9999	0.625	0.07	0.25	100.5	7.8	13.2	32.1
				1.25			91	2.6	4.4	28.9
				2.5			85.9	3.8	5.1	26.0
AFG2	0.125–2.0	y = 87909.54x-36.005	0.9998	0.625	0.06	0.20	97.2	7.2	10.7	32.1
				1.25			87.1	3.1	3.8	28.9
				2.5			84.1	3.9	5.2	26.0
AFT				1			98	2.6	7.4	36.8
				2.5			99.2	6.6	10.7	32.1
				5			89.0	3.2	3.8	28.9
				10			85.5	3.5	5.2	26.0

varied between 85.5% and 99.2%, based on the fortification levels. According to the standards outlined in Commission Regulation No 401/2006, these results seem satisfactory [63]. The recovery results were expressed using raw data, without correction for AFs recovery values. The precision (RSD) for intra-day repeatability (RSDr%, n = 3) was determined at three spiked levels for individual AFs, and at four fortification levels for total AFs. The RSD ranged from 2.2 to 7.8% for individual AFs, and 2.6 to 6.6% for total AFs. On the other hand, all inter-day repeatabilities (RSDR%, $n = 9$) were smaller than 13.2%, meeting the requirements of Commission Regulation No 401/2006 [63].

Response surface regression analysis

Table 2 displays the design matrix and the response values obtained for the combination of four factors at three levels. The analysis of the responses involved the use of coded units to assess the significance of the four factors. A factor is deemed significant and considered to affect the response when the coefficients significantly deviate from 0, and the p-value is less than 0.05 [56, 64]. Table 5 presents the constants and corresponding p-values for each response.

Degradation of AFs

As shown in Table 5, Total AF degradation percentage (AFT%) was most significantly affected by US power intensity and US application duration with p values of < 0.0001 and 0.022 ($p < 0.05$). While US power level, enzyme treatment and US application duration significantly affected ($p < 0.05$) AFG2 (AFG2%) and AFG1 (AFG1%) degradation percentages, AFB2 degradation percentage (AFB2%) was most affected by US power intensity and enzyme treatment ($p < 0.05$). On the other hand, similar to the AFT%, AFB1 degradation percentage (AFB1%) was significantly affected by US power intensity and US application duration. Equation models for AFT%, AFG2%, AFG1%, AFB2% and AFB1% were fitted as follows:

$$\begin{aligned} AFT\% = {} & 38.6 + 7.88A + 1.88C - 4.33AD - 4.18CD + 4.72A^2 \\ & -2.25D^2 + 3.69B^2D - 7.26A^2B^2 - 6.29A^2C^2 \end{aligned} \tag{9}$$

$$\begin{aligned} AFG2\% = {} & 38.45 + 6.99A + 8.32B + 8.34C - 3.96AD - 3.29CD \\ & +3.18A^2 - 2.81B^2 + 2.89C^2 - 10.2D^2 - 7.78A^2B \\ & -7.24A^2C - 8.49B^2C - 5.67BC^2 - 7.42A^2C^2 \end{aligned} \tag{10}$$

$$\begin{aligned} AFG1\% = {} & 38.40 + 8.07A + 2.71B + 5.67C - 0.1048D - 1.92AB \\ & -2.97AC - 1.62AD - 0.7277BC - 5.41CD + 7.73A^2 \\ & -0.4969B^2 + 2.17C^2 - 4.91D^2 - 4.23A^2B - 8.29A^2C - 5.04A^2D \\ & -2.09AB^2 - 4.19AC^2 - 6.19B^2C - 10.05A^2B^2 - 14.77A^2C^2 \end{aligned} \tag{11}$$

$$\begin{aligned} AFB2\% = {} & 37.25 + 11.30A + 2.48B - 2.92C \\ & +3.22AB + 3.93AC - 7.88AD \\ & +4.97CD + 4.48A^2 - 6.03D^2 \\ & +7.35A^2C - 8.05A^2D + 4.35B^2C \end{aligned} \tag{12}$$

$$\begin{aligned} AFB1\% = {} & 38.54 + 11.48A + 0.5017B + 2.11C \\ & +1.98D - 0.6413AB + 1.44AC - 6.32AD \\ & +0.3189BD - 4.10CD - 1.49B^2 - 0.014C^2 \\ & +2.07D^2 - 4.27AB^2 - 3.04AC^2 + 3.71B^2D \end{aligned} \tag{13}$$

It was observed that certain interaction effects were identified as statistically significant for AFs degradation percentages. In case of AFT%, 2 linear coefficients (A and C), 1 quadratic (A^2) and 5 interactive/interactive quadratic (AD, CD, B^2D, A^2B^2, A^2C^2) coefficients were found to be significant ($p < 0.05$). Similarly, for AFB1%, 3 linear (A, C and D), 1 quadratic (D^2) and 4 interactive/interactive quadratic (AD, CD, AB^2 and BC^2) were also found to be significant ($p < 0.05$). On the other hand, besides linear coefficients, AFG2%, AFG1% and AFB2% were significantly affected ($p < 0.05$) by more than 5 interactive/interactive quadratic equations.

Depending on the experimental conditions, while AFT% responses varied from 28.3% to 56%, AFG2%, AFG1%, AFB2% and AFB1% were varied from 18.6% to 46.4%, 26.2% to 56%, 25% to 65% and 21.7% to 57.3% respectively (Table 3). Figure 11a, 1b and 1c shows that AFT% increased with the increase in ultrasonic power and ultrasonic time. On the other hand, AFT% was not affected by increasing enzyme concentration in the hazelnut paste samples. Torabi et al. (2021) investigated the potential degradation effect of enzymatic hydrolysis of AFs by using α amylase and glucoamylase in hydrolyzed bread waste and they found that amylolytic enzymes were able to remove AFB1 up to 75% in the presence of the initial AFB1 concentration was around 92.5 ng mL^{-1} AFB1 and 95 ng mL^{-1} for total AFs in the medium. On the other hand, according to this study, AFG2 and AFG1 were not detected in the hydrolyzed bread media. Our study results conflicted those results. It's worth noting that the difference observed could be attributed to the varying experimental conditions and the distinct food samples used. It should be noted that, our study is the first study to investigate the degradation fate of AFG1, AFG2 and AFB2 by using glucoamylase. Our study results have shown that glucoamylase treatment significantly affected the degradation process of AFG1, AFG2 and AFB2 in the hazelnut paste samples. It is well known that although laccases, peroxidases, oxidases and reductases are the most investigated enzymes for degradation AFs. Several researchers have monitored microorganisms and fungi that can degrade AFs and have developed procedures to obtain pure enzymes from them. Song et al. (2021) [46] investigated the

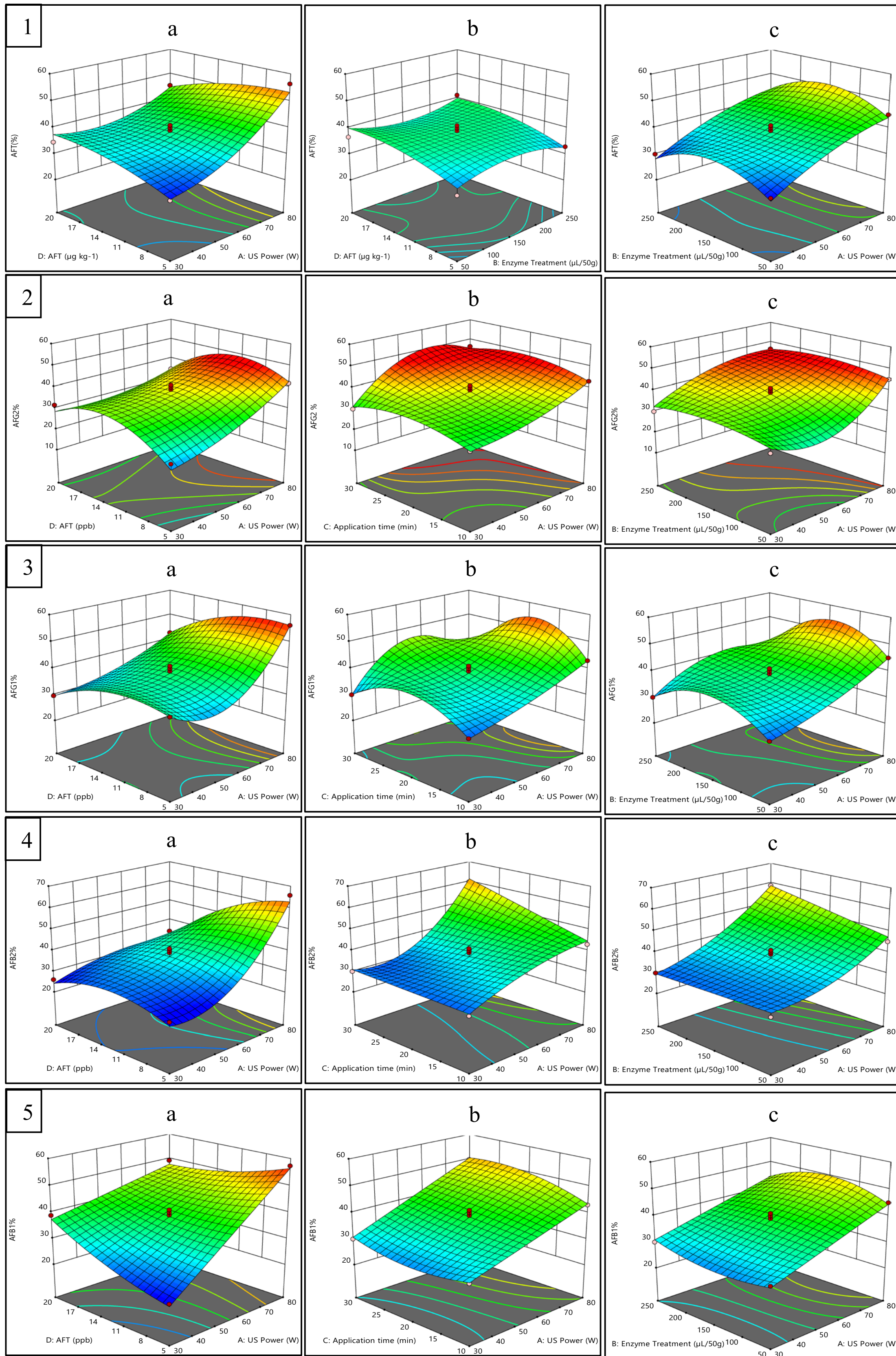
Table 5 Regression coefficients and probability (*p*) values for each response

	R1 (AFT%)		R2 (AFG2%)		R3 (AFG1%)		R4 (AFB2%)		R5 (AFB1%)		R6 (AI%)		R7 (ΔPV)		R8 (ΔE[†])		R9 (ΔBI[¥])		R10(ΔT)	
Term	C	*p*	C	*p*	C	*p*	C	*p*	C	*p*	C	*p*	C	*p*	C	*p*	C	*p*	C	*p*
Constant	38.598		38.449		38.4		37.251		38.545		6.116		0.121		607.039		8.726		69.790	
A	**7.878**	**< 0.0001**	**6.995**	**< 0.0001**	**8.068**	**0.0004**	**11.301**	**< 0.0001**	**11.480**	**< 0.0001**	**1.340**	**0.0007**	-0.025	0.077					**21.82**	**< 0.0001**
B			**8.322**	**< 0.0001**	**2.707**	**0.0208**	**2.478**	**0.011**	0.502	0.399*	0.1089	0.7519*	**0.100**	**< 0.0001**	-0.247	0.973				
C	**1.880**	**0.022**	**8.338**	**< 0.0001**	**5.666**	**0.0032**	-2.925	0.069*	**2.115**	**0.003**	**2.3747**	**< 0.0001**	-0.025	0.077	**23.514**	**0.004**			**21.792**	**< 0.0001**
D					-0.1048	0.912			**1.979**	**0.015**	**1.6146**	**< 0.0001**	0.000	1.000*	**49.596**	**< 0.0001**	**-1.648**	**0.012**		
AB					-1.921	**0.18**	**3.219**	**0.048**	-0.641	0.531*	0.341	0.593*								
AC					-2.975	0.054	**3.928**	**0.019**	1.442	0.171*	0.020	0.975*								
AD	**-4.332**	**0.004**	**-3.961**	**0.007**	-1.162	0.250	**-7.880**	**< 0.0001**	**-6.322**	**< 0.0001**										
BC					-0.728	0.59									**29.341**	**0.029**	**-2.613**	**0.020**		
BD									0.319	0.754*										
CD	**-4.176**	**0.005**	**-3.290**	**0.020**	**-5.406**	**0.004**	**4.970**	**0.004**	**-4.100**	**0.001**					**26.191**	**0.049**				
A^2	**4.724**	**0.008**	**3.176**	**0.021**	**7.730**	**0.004**	**4.479**	**0.001**									**2.116**	**0.013**		
B^2			**-2.806**	**0.018**	-0.497	0.704			-1.486	0.075	-0.843	0.067*								
C^2			**2.887**	**0.033**	2.170	0.128			-0.014	0.985*	0.066	0.891*								
D^2	-2.245	0.093*	**-10.019**	**< 0.0001**	**-4.912**	**0.006**	**-6.035**	**< 0.0001**	**2.066**	**0.019**					**51.049**	**< 0.0001**				
A^2B			**-7.776**	**0.001**	**-4.227**	**0.032**														
A^2C			**-7.242**	**0.001**	**-8.287**	**0.003**	**7.352**	**0.003**												
A^2D					**-5.044**	**0.015**	**-8.045**	**< 0.0001**												
AB^2					-2.089	0.290			**-4.222**	**0.010**										
AC^2					-4.189	0.06			**-3.039**	**0.050**							2.059*	0.061*		
B^2C			**-8.492**	**0.000**	**-6.189**	**0.01**	4.347	0.057									**-3.243**	**0.005**		
B^2D	**3.691**	**0.011**							**3.714**	**0.009**										
BC^2			**-5.668**	**0.007**													**-2.820**	**0.013**		
A^2B^2	**-7.264**	**0.004**			**-10.054**	**0.006**														
A^2C^2	**-6.291**	**0.011**	**-7.416**	**0.004**	**-14.775**	**< 0.0001**														
R^2	**0.8917**		**0.9561**		**0.9632**		**0.9524**		**0.9674**		**0.8000**		**0.7197**		**0.8138**		**0.6566**		**0.8854**	
,R^2_{adj}	**0.8404**		**0.9121**		**0.8530**		**0.9167**		**0.9298**		**0.7206**		**0.6729**		**0.7630**		**0.5630**		**0.8766**	

The p values denote different significant effects. p values with **bold font represent p≤ 0.05**, the p values with normal font represent significance at $0.05 \leq p < 0.1$ and the p values with "*" represents significance at $p \geq 0.1$.

ΔE[†]: Coefficients and p values were obtained after response value transformations.

ΔBI[¥]: Coefficients and p values were obtained after response value transformations.


1
a
AFT(%)
D: AFT (µg kg-1)
A: US Power (W)
b
AFT(%)
D: AFT (µg kg-1)
B: Enzyme Treatment (µL/50g)
c
AFT(%)
B: Enzyme Treatment (µL/50g)
A: US Power (W)
2
a
AFG2%
D: AFT (ppb)
A: US Power (W)
b
AFG2 %
C: Application time (min)
A: US Power (W)
c
AFG2%
B: Enzyme Treatment (µL/50g)
A: US Power (W)
3
a
AFG1%
D: AFT (ppb)
A: US Power (W)
b
AFG1%
C: Application time (min)
A: US Power (W)
c
AFG1%
B: Enzyme Treatment (µL/50g)
A: US Power (W)
4
a
AFB2%
D: AFT (ppb)
A: US Power (W)
b
AFB2%
C: Application time (min)
A: US Power (W)
c
AFB2%
B: Enzyme Treatment (µL/50g)
A: US Power (W)
5
a
AFB1%
D: AFT (ppb)
A: US Power (W)
b
AFB1%
C: Application time (min)
A: US Power (W)
c
AFB1%
B: Enzyme Treatment (µL/50g)
A: US Power (W)

◂**Fig. 1** Three-dimensional plots showing the mutual effect of; **a** initial AFT concentration (μg kg^{-1} or ppb) and US power (W), **b** US application time (min) and US power (W) and **c**) Enzyme treatment (μL $50g^{-1}$ hazelnut paste) and US power (W) on 1) AFT%, 2) AFG2%, 3) AFG1%, 4) AFB2% and 5) AFB1%

degradation properties of AFB1 and zearalenone (ZEN) by Lac2, a recombinant protein produced from the fungus Pleurotus pulmonarius, which was expressed in the Pichia pastoris X33 yeast strain. According to study results, Lac2 degraded AFB1 standard in buffer solution over 90% at 37 °C after 12 h of incubation, depending on the pH of the medium and the mediators used in vitro. Another research study aimed to assess the effectiveness of a laccase (LC) obtained from Pleurotus eryngii and a laccase-mediator system (LMSs) in breaking down mycotoxins like AFB1, fumonisin B1 (FB1), ochratoxin A (OTA), deoxynivalenol (DON), Zearalenone (ZEN), and T-2 toxin in in vitro experiments. It was found that laccase mediators drastically increased the degradation ratio of the mycotoxins and 73% AFB1 degradation (for 1 μg mL-1 AFB1 solution in sodium acetate buffer) were obtained after 72 h incubation at 25 ºC [65]. Moreover, some researchers have also tried to reveal the structure and toxicity of degradation products of AFs after the mentioned enzyme treatments [66, 67]. Unfortunately, the production of these enzymes and their use in the food and feed industry in practice have some struggles because they may have low production yields and production problems in case if the production is done by conventional fermentation and culture techniques [68]. Therefore, investigating the effectiveness of conventional enzymes used in the food industry on aflatoxin degradation is a crucial subject for the practical approach. Our study results have shown that glucoamylase alone was only effective for the partial degradation of AFG2, AFG1 and AFB2 but further research is needed to reveal the structure and the toxicity of the degradation products.

Considering the toxicity of AFs, the decontamination percentage of AFB1 and implicitly AFT, should be more crucial than the decontamination rate of the other three AF types. It is important to highlight that, in comparison to both the power intensity and application duration of ultrasound (US), enzyme treatment did not have a significant impact (Table 4) on the AFB1% and AFT% values ($p > 0.05$). Therefore, based on the p-values from the coefficients table, it can be concluded that the degradation of AFB1 was significantly affected by the initial AFs concentrations ($p < 0.05$), whereas the degradation of the other types of aflatoxins was impacted by enzyme treatments.

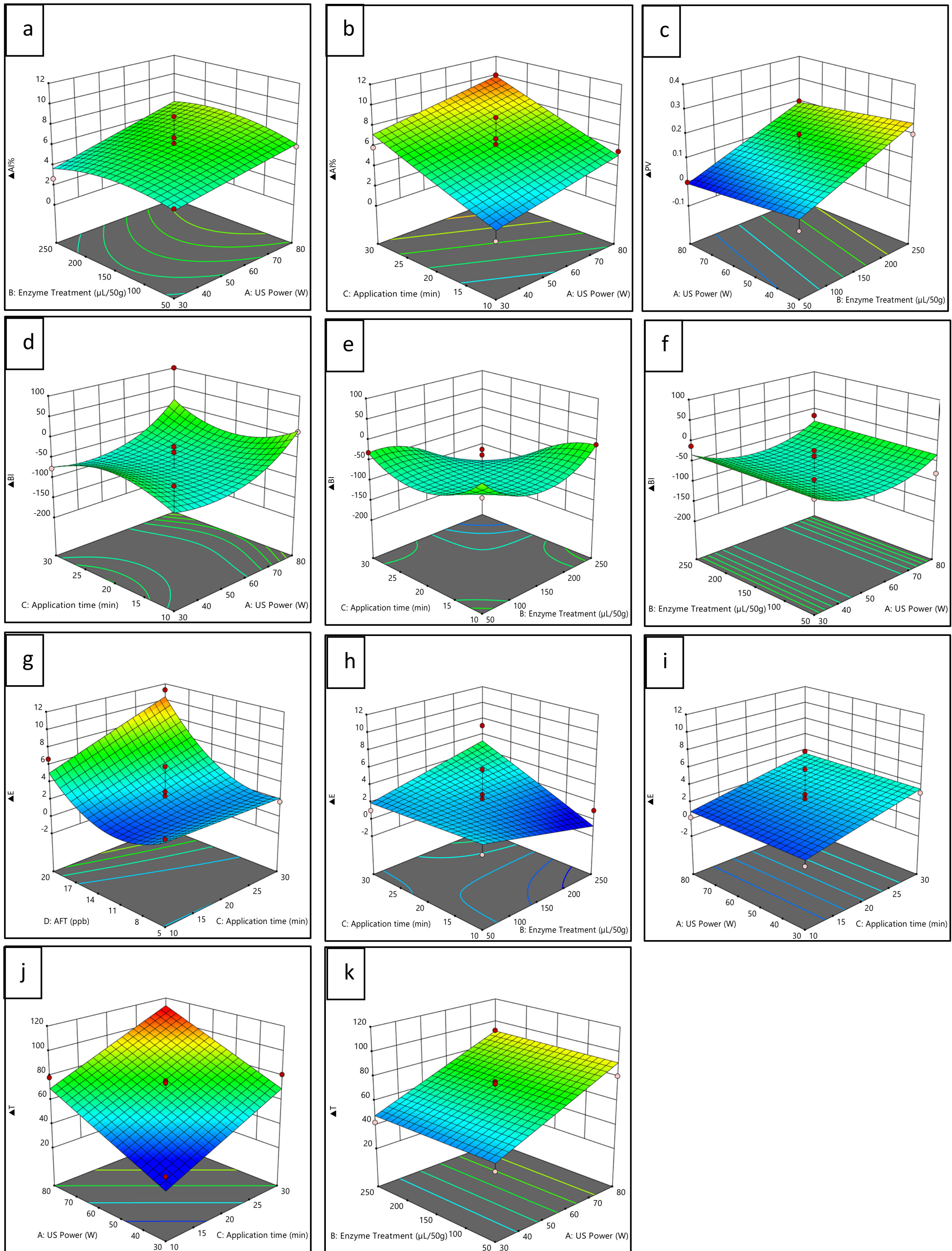
It should be noted that, this study is the first study for investigation of the degradation fate of AFs in hazelnut paste under the US and amylolytic enzyme treatment conditions using BBD. Furthermore, only a limited number of studies have been published that explore the impact of ultrasonication (US) treatment on aflatoxins (AFs) in food products. Liu et al. (2019) [38] investigated the effect of pulsed US at 20 kHz on the degradation of different mycotoxins including AFB1 in an aqueous medium. The study also revealed that AFB1 degradation increased with increasing ultrasound intensity, but initial AFB1 concentration negatively affected the AFB1 degradation efficiency. Our study results were complied with these findings. A recent study has suggested two models for the degradation of AFB1 in aqueous media through ultrasonication. According to the first proposed model, AFB1 may degrade in the aqueous medium due to the presence of hydroxyl radicals and hydrogen peroxide generated from the water molecules. However, in the second model, it has been shown that H2O2 molecules generated by ultrasound cavitation initiate epoxidation, oxidation, and addition reactions, leading to modifications of the lactone ring and methoxy group of AFB1 [38]. On the other hand, it should be noted that hazelnut paste samples used in this research contained less than 1% water. So, the degradation mechanism of AFs might be different than the proposed degradation mechanisms. Considering the high oil content of the hazelnut paste, it is thought that US cavitation might oxidize and degrade some fatty acid structure and initiates oxidation, then produce some radicals such as OH• radicals and those radicals may be used to degrade AFs in the samples. Further study is needed to confirm these findings.

Aroma index change (ΔAI%)

Experimental ΔAI% response of hazelnut paste samples varied from 1.46% to 10.37%. As shown in Table 4, ΔAI% response was significantly affected by linear ($p < 0.05$) terms of US power, enzyme treatment and US application duration, by interaction terms of US power and enzyme treatment and US power and application time ($0.05 \leq p < 0.1$), by quadratic terms of enzyme treatment and US application time ($0.05 \leq p < 0.1$), while other terms were not significant ($p > 0.1$). The regression equation of the model showing the effect of in dependent parameters of US power (A), enzyme treatment (B), US application time(C) and initial AFT concentration (D) on the ΔAI% as follows:

$$\begin{aligned}\Delta AI\% = 6.12 + 1.34A + 0.1089B + 2.37C \\ +1.61D + 0.3411AB + 0.0203AC \\ -0.8435B^2 + 0.0665C^2\end{aligned} \quad (14)$$

Alkyl pyrazines are one of the most important volatile compounds from the industrial point of view because they occur during the roasting process of foods such as coffee, cocoa and roasted nuts such as hazelnuts. It is speculated that heating conditions (temperature and duration) lead to aroma development during the roasting of hazelnuts [69].



a
12
10
8
6
4
2
0
▲AI%
250
200
150
B: Enzyme Treatment (µL/50g)
100
50
30
40
50
60
70
80
A: US Power (W)
b
▲AI%
C: Application time (min)
A: US Power (W)
c
0.4
0.3
0.2
0.1
0
-0.1
▲PV
A: US Power (W)
B: Enzyme Treatment (µL/50g)
d
100
50
0
-50
-100
-150
-200
▲BI
C: Application time (min)
A: US Power (W)
e
▲BI
C: Application time (min)
B: Enzyme Treatment (µL/50g)
f
▲BI
B: Enzyme Treatment (µL/50g)
A: US Power (W)
g
▲E
D: AFT (ppb)
C: Application time (min)
h
▲E
C: Application time (min)
B: Enzyme Treatment (µL/50g)
i
▲E
A: US Power (W)
C: Application time (min)
j
120
100
80
60
40
20
▲T
A: US Power (W)
C: Application time (min)
k
▲T
B: Enzyme Treatment (µL/50g)
A: US Power (W)

Fig. 2 Three-dimensional model showing; **a** mutual effect of enzyme treatment and US power on ΔAI%, **b** mutual effect of US application time and US power on ΔAI%, **c** mutual effect of US power and enzyme treatment on ΔPV, **d** mutual effect of US application time and US power on ΔBI, **e** mutual effect of US application time and enzyme treatment on ΔBI, **f** mutual effect of enzyme treatment and US power on ΔBI, **g** mutual effect of AFT and US application time on ΔE, **h** mutual effect of US application time and enzyme treatment on ΔE, **i**) mutual effect of US power and US application time on ΔE, **j** mutual effect of US power and US application time on ΔT and **k** mutual effect of enzyme treatment and US power on ΔT.

That is why, monitoring the changes in alkyl pyrazines is a wise strategy to evaluate the effect of ultrasound treatment on the aroma development of the hazelnut paste. It was found that ΔAI% was significantly affected by US power intensity, US application duration and the initial AFT concentration ($p < 0.05$) (Table 4). The application of ultrasound technology involves the generation of high-frequency sound waves that, when directed through a liquid, create highly energetic bubbles [70]. The collapse of cavitation bubbles on a product's surface releases an extreme amount of energy to the medium, resulting in a temperature increase and aroma index change. Figure 2a shows that the effect of independent variables on ΔAI% parameter of hazelnut paste. Our study results have shown that US power and application time was the main parameters of the ΔAI% of the hazelnut paste samples. Moreover, *A. flavus*, *A. niger*, *A. glaucus*, *Penicillium*, *Mucor* and *Fusarium* mold species are the most isolated molds from raw and roasted hazelnut kernels [71]. Considering Aspergillus species are the most potent aflatoxigenic fungi, the mold activity might not just increase the aflatoxin concentration, their growth pattern makes some changes to the quality characteristics of the hazelnut kernels. Previous studies indicated that the mold activity results in various quality losses including dry matter, lower oil content, and flavor/aroma loss [72]. Higher initial AFs concentrations in the hazelnut paste samples may indicate higher mold growth in the hazelnut kernels before hazelnut paste production. Indeed, considering the initial aroma index values of the samples, it was observed that the aroma index was inversely proportional to AFs content of the samples. The initial absorbance values at 279 nm were detected as 0.51, 0.34, and 0.29 for the hazelnut paste samples that initial AFT contents of 5, 12.5, and 20 µg kg-1 respectively. Even though the US application intensity and the application duration led to a change 1.5–2 units of the AI, the proposed model based on ΔAI% were extremely affected by the initial AI values ($p < 0.05$).

Peroxide value change (ΔPV)

According to the proposed model variance analysis results, while ΔPV was significantly affected by enzyme treatment ($p < 0.05$), other parameters were not significant (Fig. 2b). Enzymatic applications are widely used in the food industry for controlling quality, modifying physicochemical and rheological properties, or directly used as food additives. Even though there is no research on the effect of enzymatic applications on the physicochemical properties of hazelnut paste in the literature, most of the studies have been focused on the effect of enzyme pretreatments (including hydrolytic enzymes) for the oil extraction of plant tissues. A previous study focused on the pretreatment of cottonseed flakes with amylase for higher oil yield revealed that enzyme treatment significantly increased the PV of the extracted oil samples [73]. Our study result complied with that finding. Linear equation model for ΔPV as follows:

$$\Delta PV = 0.1207 - 0.025A + 0.100B - 0.025C \tag{15}$$

Hazelnut paste may contain more than 50% hazelnut oil, which has a chemical composition similar to olive oil and varies based on the variety, climate, soil, and growing conditions of the hazelnut kernels [9, 74]. Some studies on the effects of ultrasound treatment on oil quality parameters and oxidative stability have produced conflicting results. The studies on olive oil, sunflower oil and the palm oils revealed that short time ultrasonication (below 30 min) had not any significant increase on PV of the samples [75, 76]. On the other hand, a recent study has been conducted to explore the impact of ultrasound pretreatment on the quality parameters of olive oil. The research findings suggest that the oxidative stability of olive oil is significantly influenced by ultrasound pretreatment [77]. Proposed model in this study complied with Jimenez et al. (2007) and Halim and Thoo (2018) but conflicted with Rigane et al. (2020). On the other hand, further research is needed to reveal the fate of peroxides and fatty acid composition change of the hazelnut paste samples under high ultrasonication conditions.

Browning index change (ΔBI) and Total color difference (ΔE)

The color, especially brown color is an important quality parameter in terms of consumer acceptability and preferences for roasted products. It is well known that color development is a complicated phenomenon that is affected by many factors such as pH of the raw material, chemical composition, and roasting conditions such as roasting time and temperature. There was not any proposed model fitted to the ΔBI and ΔE without any transformations of the responses. After that, both responses were transformed by using the transformation interface of the Design Expert program until obtaining the most appropriate fit statistics.

The model equations for ΔBI and ΔE as follows:

$$(\Delta E + 52.6)^{1.6} = 607.04 - 0.2472B + 23.51C + 49.6D + 29.34BC + 26.19CD + 51.05D^2 \quad (16)$$

$$(\Delta BI + 154)^{0.5} = 8.73 - 1.65D - 2.61BC + 2.12A^2 + 2.06AC^2 - 3.24B^2C - 2.82BC^2 \quad (17)$$

According to the Table 4, the variables with the largest effect ΔE parameter were the linear terms of US application time ($p < 0.05$), followed by initial AFT ($p < 0.05$) which was a very significant parameter on ΔE response at the linear and quadratic levels ($p < 0.0001$). Interactions term of enzyme treatment and application time (BC) ($p < 0.05$) and application time and initial AFT (CD) ($p < 0.05$) were significant parameters on ΔE response (Fig. 2c). In here, it should be noted that initial AFT factor was very crucial for both ΔBI and ΔE. It is known that especially in heavy seasons, hazelnut paste is produced using small, low-quality, and relatively immature, defected, or moldy kernels which may lead to high level of AFs in the product. When kernels are moldy or defective, they may have necrotic spots, white–grey mold covering the surface, and dark-brown spots inside the kernel when cut. Completely rotten and black kernels can also be observed [71]. Previous studies have shown that defected/moldy hazelnut kernels may contain AFs more than 400 µg kg^{-1} [78, 79]. Thus, in case of using moldy/defected kernels for hazelnut paste production, the amount of AFs in the product wouldn't be the only quality parameter which is effected by the raw material quality such as color values. This ΔE increase may be attributed to easy fragmentation of cell walls of defected kernels (which has darker colors) by prolonged US effect, thus resulted in higher ΔE values.

Although ΔE is a useful tool for demonstrating the total color changes of the food materials under certain process conditions, it is insufficient to show the direction of the color change. Considering the ΔBI values, while positive ΔBI demonstrating increase in browning, negative ΔBI shows decrease in browning index of the hazelnut paste samples.

It was found that individual effects of US power intensity, application duration and enzyme concentration were not significant ($p > 0.05$) on ΔBI, whereas, initial AFs concentration significantly affected ΔBI values of the hazelnut paste ($p < 0.05$). On the other hand, 4 interactive quadratic coefficients (BC, A^2, B^2C, BC^2) were found to be significant ($p < 0.05$). It is speculated that ultrasonication process may increase the luminosity (L*) value and improve color characteristics of foods. Thus, in our study, while US application increase L* value and decrease the ΔBI (which means obtaining lower brown color after applied process) of the responses. This can be attributed to the precipitation of the unstable dark pigments with US application time and degradation of some pigments during US treatment. The pulp of gulupa, which was treated with low frequency US as a pretreatment to improve shelf life, showed similar changes in color as observed in our study [80]. While the samples treated with the lowest US application duration and with a minimum or maximum amount of enzymes showed minimum ΔBI (Fig. 2d). Moreover, regardless of US power intensity, the samples treated with the highest enzyme concentration and maximum US application duration showed the highest BI change. Plant cell walls consist of polysaccharides like starch, cellulose, hemicellulose (xyloglucans), and pectin. These form a barrier for the release of intracellular substances. Commercial cellulase, amylase, and pectinase enzymes have been investigated for solubilizing the cell wall structure of plants to extract pigment and flavor compounds [81]. In this study, commercial glucoamylase was used to investigate the potential decomposition effects on AFs. It should be noted that glucoamylase treatment before ultrasonication may increase colorant pigment transfer from cell walls of the thin hazelnut skin and kernels to the liquid medium. Regardless of US power intensity and enzyme concentration, short ultrasonication time slightly decreased ΔBI value. However, enzyme treatment at the highest concentrations with long ultrasonication times significantly decreased ΔBI, resulting in a lighter color. Previous studies on hazelnut and hazelnut skins revealed that flavan-3ols was the most abundant flavonoids in hazelnut skin [82]. Considering hazelnut paste production, the hazelnut paste is produced by crushing hazelnut kernels with skin (Fig. 1). Thus, the color of hazelnut paste composed of not just the colorants from hazelnut kernels, but also the colorants from hazelnut skin. Previous studies have also shown that flavan-3ols decomposed by several factors such as high-pressure homogenization [82] and ultrasound processes [83]. The lighter brown color might be explained by decomposition of flavan-3ols and composition of dimers or possibly polymers [84], precipitation of highly polymerized melanoidins and destruction of dark brown hazelnut thin skin after prolonged US treatment.

Temperature Change (ΔT)

Figure 2j and k show the independent variables on the ΔT parameter of the hazelnut paste. Figure 2j shows that ΔT was detected to its maximum level at 80 W US power and 30 min US application time conditions. On the other hand, enzyme treatment contribution was not significant to the ΔT of the responses ($p < 0.05$).

The model equations for ΔT as follows:

$$\Delta T = 69.79 + 21.82A + 21.79C \quad (18)$$

It was found that individual effects of US power and US application time were found to be significant (Table 4), but other parameters were not found to be significant on ΔT ($p > 0.05$). Higher US power levels and prolonged application times led to increase temperature of the samples more than 100 ºC in the hazelnut samples.

It is known that US application can generate cavitation bubbles and when these bubbles collapse, high amount of energy release and this collapses resulted in temperature increase in the medium [39]. Thus, the higher ΔT values at higher US power levels with longer application times can be explained by the higher energy release converting to heat in the samples due to the collapse of cavitation bubbles. It should be noted that to observe how much energy was released and the amount of temperature increase in the samples, there was not any temperature-preventing system used during the study. It is speculated that temperature increases can be prevented by an external cooling system while applying US to the samples.

Model Validation

To optimize AFT% and AFB1% degradation rates for the hazelnut samples which have AFT concentrations just above the MLs, the following parameters of AFT concentration (12.5 µg kg-1), US time (0–30 min), enzyme treatment (50–250 µL 50 g-1 hazelnut paste), application time (0–30 min) were investigated for maximum values of AFT% and AFB1% and simultaneously in-range AFG1%, AFG2%, AFB2%, ΔPV, ΔAI%, ΔBI, ΔE and ΔT values. It is important to noted that weight factor ranges from 0.1 to 10, and higher weight factors emphasized the importance of the criteria [85]. Weight factor 5 is selected for AFT% and AFB1% and a weight factor 3 were selected for other responses. Table 5 indicates the one optimal AFT% and AFB1% degradation conditions. To compare predicted and experimental values of dependent variables with two replicates. Table 6 values demonstrate that the experimental values are in acceptable agreement with predicted values. Therefore, the proposed BBD models can be utilized to predict the percentage of degradation of AFs through ultrasonication, as well as certain quality parameters of hazelnut paste products.

Conclusion

Ultrasound is a promising method for decontamination, with potential applications in the global food processing industry. To our knowledge, our study is the first study to demonstrate the AFs decontamination by using industrial enzyme and ultrasonication together in hazelnut paste. All studies in the literature on the effect of ultrasonication on AF degradation were conducted in aqueous media and few studies were conducted in real food solutions. Thus, further investigation is necessary to determine the degradation products and toxicity of AFs.

Additionally, chemical changes to hazelnut paste, especially changes in oil composition after ultrasonication should be explored.

Even though more than 50% degradation rates were obtained for AFT degradation, our study results have shown that the initial concentration of AFs noticeably affects their degradation rates in the samples. Higher initial AFT concentrations negatively impacted the degradation efficiency, resulting in relatively lower values. Degradation of AFs was greatly affected by ultrasound power (or intensity) and the US application time, whereas, glucoamylase concentration didn't affect the degradation of significant mycotoxins, AFT and AFB1.

Study results have shown that ΔAI% significantly affected from US power intensity and the sonication time applied to the samples. In other words, higher the US intensity and

Table 6 Different levels of factors and their responses for the optimized AFs degradation process

Independent variables	Lower limit	Upper limit	Optimized level	
US Power (W)	30	80	80	
Enzyme treatment (µl 50 g^{-1}) or (U enzyme g^{-1} hazelnut paste)	50 (0.5)	250 (2.5)	250 (2.5)	
Application time (min)	10	30	20	
AFT (µg kg^{-1})	5	20	12.5	
Dependent variables	Constraints	Experimental	Predicted	Residual*
AFT%	Maximum	44.43 ± 2.38	43.94 ± 2.61	-0.49
AFG2%	In range	43.18 ± 2.53	46.35 ± 2.50	3.17
AFG1%	In range	38.17 ± 2.17	43.36 ± 3.15	5.19
AFB2%	In range	56.98 ± 3.02	58.72 ± 3.00	1.74
AFB1%	Maximum	45.59 ± 2.19	44.17 ± 1.99	-1.42
ΔAI%	In range	9.7 ± ± 1.26	7.06 ± 1.25	-2.64
ΔPV	In range	0.20 ± 0.03	0.19 ± 0.04	-0.01
ΔE	In range	2.07 ± 1.36	2.27 ± 1.42	0.20
ΔBI	In range	-44.75 ± 0.10	-32.13 ± 4.55	-12.62
ΔT	In range	96.05 ± 5.92	91.60 ± 7.53	-4.45

Residual* = Predicted value-Experimental value

application time, the more volatile component might occur in the hazelnut paste. Thus, further study is needed to investigate the quality and quantity of those volatiles.

Our study results also indicated that while PV was significantly increased with glucoamylase enzyme treatment, other parameters was not affective on that increase. Considering color characteristics of the hazelnut paste, our study results was shown that using of ΔE and ΔBI terms together for the interpretation of the color changes of the hazelnut paste samples after ultrasonication application might give more healthy conclusions. ΔE and ΔBI were significantly affected by initial AFT concentration of the samples. Moreover, US application clearly decreased ΔBI of the hazelnut paste. Application of higher US power and prolonged application times led to increase temperature up to 100 ℃ in the hazelnut samples. Thus, considering the degradation of AFs using the US in industrial applications, appropriate cooling systems should be improved to control such temperature increases to consider product quality.

In this study, a BBD model successfully applied to predict the AFs degradation rates in hazelnut paste samples by using ultrasonication. The success of degradation is largely dependent on the initial AFT concentration, US intensity, and application time. Although ultrasonication is a promising novel method for using degradation of AFs in hazelnut paste, there are still a lot of unknown parameters to explain. Thus, to reveal the chemical structure and toxicity of the by products from AFs, to detect quality changes such as free fatty acid composition changes after ultrasonication, the further researches are crucially needed.

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