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Optimization of Ozonation Method for Reduction of Aflatoxin B1 in Ground Corn and its Impact on other Mycotoxins

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ABSTRACT

Food and agricultural products can be contaminated by mycotoxins. Many emerging methods, including ozonation, have been used to reduce the level of these contaminants. This study aimed to assess the effects of different treatment times and doses of ozonation on the reduction of aflatoxins in contaminated corn samples. A central composite design (CCD) via response surface methodology (RSM) was used to optimize the ozonation for maximum reduction of AFB_1 contamination level. The variables used in this study were: AFB_1 concentration (X1, 5–50 ng/ml), ozone dose (X2, 200–600 mg/kg), and ozonation time (X3, 100–400 min). Increasing the dose and time of ozonation showed significant effects on initial AFB_1 content. The results have demonstrated that an ozonation dose of 600 mg/kg for 250 min, was sufficient to eliminate at least 96% of the AFB_1 contamination level of corn samples. The obtained results have been validated and showed that ozonation under optimal conditions could be a promising method to reduce aflatoxins, ochratoxin A, zearalenone, and deoxynivalenol contamination in corn.

Keywords: Aflatoxins; Corn; Detoxification; Optimization; Ozonation

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1. Introduction

Food Mycotoxins are identified as secondary fungal metabolites and one of the most significant food contaminants, affecting both primary and final agricultural and food products. These contaminants are produced by several fungal species, mostly from *Aspergillus, Penicillium,* and *Fusarium* genera, that may contaminate food and feed during production or storage (Krstovic, Krulj, Jaksik, Bocarov-Stancic, & Jajic, 2020). These fungi are usually found in commodities such as cereals and their products, different fruits and fruit products, coffee, and animal-derived foods. Consuming mycotoxin-polluted foods causes adverse health effects on humans and animals, such as infections and allergies in the nervous, immune, and reproductive systems (Maresca & Fantini, 2010).

Among more than 20 types aflatoxins, the most leading are aflatoxins B_1, B_2, G_1, and G_2, (AFB_1, AFB_2, AFG_1, and AFG_2, respectively) mostly reported in dry food commodities (cereals, dry fruits, and spices), whereas the metabolic products of aflatoxins, such as aflatoxin M_1 and M_2 (AFM_1 and AFM_2), are found in milk and milk products (Udomkun et al., 2017). These important mycotoxins are carcinogenic, mutagenic, and immunosuppressive agents to the extent that AFB_1 and the mixture of aflatoxins are classified as group 1 carcinogens by the International Agency for Research on Cancer (IARC) (Cancer, 2012). AFB_1 is normally predominant and the most toxic among others. Teratogenicity, cytotoxicity hepatotoxicity, genotoxicity, and growth impairment are additional health adverse effects of aflatoxins (Afsah-Hejri, et al., 2020).

Aflatoxin contamination of foods and feeds remains a great concern to the public health. Therefore, most countries and regions of the world impose strict limits on the level of aflatoxins in foods. The usual maximum residual limits for AFB_1 and total aflatoxins in foods are 5 to 30 μg kg-^{1} (Mahato et al., 2019), while those in European countries are 2 and 4 μg kg^{-1}, respectively (European Commission, 2006).

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Corn is a major cereal and is a crucial agricultural and industrial product, mostly used as staples and ingredients for the production of food and feed. However, corn is more disposed to mycotoxins such as AFB_1 pollution under unsuitable processing and storage conditions than other grain and foodstuffs. Once mycotoxins are emitted in products, their detoxification has as often as possible been a colossal assignment for both the agricultural and food industries. Approximately 25% of post-harvest losses come from the contamination of grains by fungi and mycotoxins, which prevents product sale and consumption (Alemayehu et al., 2020) Considering the sensitivity of corn to contamination with fungi and mycotoxins, many measures and processes (chemical, physical, and biological) have been proposed to hinder the formation or reduce mycotoxins in the corn supply chain. However, the disadvantages of each method, for instance, sensory characteristics change, nutrition loss, and cost of operation and equipment, must be considered (Luo, Wang, Wang, Li, Bian, et al., 2014).

Therefore, to improve consumer safety, mycotoxin-contaminated products should be effectively detoxified and for this purpose, numerous physical, chemical, biological techniques have been developed to reduce or eliminate these toxic compounds in polluted feed and foodstuffs (Adegoke & Letuma, 2013). Physical methods, such as radiation, heating, and the addition of absorbents, are time-consuming. Chemical strategies, such as calcium hydroxide and formaldehyde treatment, ammoniation, peroxide and chlorine treatment, and so on, can result in wholesome misfortunes. Yet, there is insufficient information regarding the mechanisms of detoxification and the toxicity of the related transformation products and new research is being done in this field (Peng, Marchal, & Van der Poel, 2018).

Ozone is one of the most powerful oxidants and is an effective sanitizer for the food industry (Kırış, Velioglu, & Tekin, 2017). In oxidation mode, ozone molecules directly destroy organic molecules. Ozone is approved by the Food and Drug Administration and is generally considered safe in 1997 (Liu, Gao, & Yu, 2006). Ozone has strong oxidation capacity, cheap production cost, and no residue after treatment which makes great potential in the food industry for degradation of pesticides, (Tabakoglu & Karaca, 2015) and mycotoxins (Akbas & Ozdemir, 2006). Ozone can react and quickly degrade various mycotoxins, such as aflatoxins, Ochratoxin A (OTA), Zearalenone (ZEA), and Deoxynivalenol (DON) (Freitas-Silva & Venâncio, 2010). Aflatoxins in corn (Prudente Jr, 2008) peanuts (Proctor, Ahmedna*, Kumar, & Goktepe, 2007), pistachios (Akbas & Ozdemir, 2006) and red peppers (Inan, Pala, & Doymaz, 2007) have also been successfully degraded by ozonation. Ozone degrades AFB_1 and AFG_1 by electrophilic attack of their furan ring's C8–C9 double bonds. The main degradation yields of ozone are then rearranged into monozonide products, yielding various aldehydes, ketones, or organic acids (McKenzie et al., 1997). In expansion, ozone shows satisfactory penetrability and can naturally break down into oxygen without producing harmful residues (Luo, Wang, Wang, Li, Zheng, et al., 2014). Ozone is not harmful to health due to its short half-life of 15–30 min (Freitas-Silva & Venâncio, 2010). However, the effectiveness of ozone detoxification depends on the concentration and time of exposure to ozone gas, as well as the type of food matrix (Trombete et al., 2017). Ozone treatment can be applied in gaseous or aqueous medium, so several studies have demonstrated the benefits of using different forms of ozonation and their effects in the detoxification of contaminated grains, especially corn, wheat, and barley (Obadi et al., 2018; L. Wang et al., 2016).

Optimizing of ozonation method is a technique that evaluates several factors including toxin concentration, ozonation time, and dose which helps to obtain better results and saves costs and production time (Tirado-Kulieva et al., 2021). Therefore, it is necessary to apply mathematical and statistical methods through scientific validity (Malekjani & Jafari, 2020) that help to estimate the change of the desired variables. For simultaneously evaluation the effects and interactions of a high number of varying factors with a limited number of runs a central composite design (CCD) using response surface methodology (RSM) is an important tool. RSM is a perfect method for optimal conditions determination (Anosheh Rahmani, Selamat, & Soleimany, 2011) because it can evaluate several independent variables and even their interaction, important to know their synergic and/or antagonistic effects on one or more responses.

The aim of this study was to optimize an ozonation method for contaminated corn, investigating the effects of three factors including ozonation dose, time, and AFB_1 concentration on the efficiency of ozonation with the aid of a RSM to achieve maximum mycotoxin degradation. Then, the final validated method was applied for the detoxification of aflatoxins, OTA, ZEA, and DON in contaminated corn.

2. Material and Methods

2.1. Chemicals

Analytical standards of mycotoxins including AFB_1, AFB_2, AFG_1, AFG_2, OTA, ZEA, and DON as well as the immunoaffinity columns (IACs) were supplied by Aokin (Germany). The analytical grade solvents were from Merck (Germany), and the pure water for assays was produced by Millipore filters (specify filtration). The aflatoxins mixture was prepared in methanol at 1000µg/mL for AFB_1 and AFG_1 and 200µg/mL for AFB_2 and AFG_2. The stock solution of OTA was prepared in methanol at 1000 µg/mL. The working solutions of aflatoxins and OTA were diluted in the same solvent and put away in glass-stoppered tubes at 0°C.The stock solution of ZEA and DON were prepared in water: acetonitrile at 100000 µg/m. The working solutions of ZEA and DON were diluted in the same solvent and put away in glass-stoppered tubes at 0°C.

2.2. Apparatus

High-performance liquid chromatography (HPLC; Waters, USA) equipped with an auto-sampler system (type 717 plus), quaternary pump and column oven (type 1525), Multi λ fluorescence detector (type 2475), UV detector (type 2487). The chromatographic separation was performed on a reverse phase column (Shanghai, China) C18-WP, 100A, 4.6mm ×250mm, 5µm (Waters). An electrochemically generated bromine (e.g., FARLIB® ECD CELL) has been used for the derivatization of aflatoxins. Ozone generator MOG002 (O3 Tech H.K Limited, Shenzhen, China) was used to generate gaseous ozone.

2.3. Sample Preparation and mycotoxins analysis

Blank corn samples were supplied by Standard Research Institute (SRI, Iran), and they were grounded finely by a miller in the laboratory. Powdered corn was divided into 5 subgroups and was spiked with an appropriate amount of stock standard solution of

AFB_1 according to RSM-designed runs. All subgroups were homogenized completely.

Sample analysis for the determination of AFB_1 was performed by the HPLC method using a modified version of the official AOAC method 991.31 (Horwitz, 2000). Based on this method, 200 ml of methanol 80% was added to 50 g of the sample, homogenized, and stirred for 5 min in a high-speed blend jar. Then aliquot was passed through a glass microfiber filter. To clean up the sample, 10 mL phosphate buffer saline (PBS) was passed through the IAC (Afla test), followed by 70 mL of the filtrate passing through the IAC at a flow rate of 1 drop/sec. The IAC was then washed with 10mL water and dried under a mild vacuum. Finally, the aflatoxins were eluted with 1.5 mL methanol and 1.5 mL pure water, and a volume of 100 µL of final collected and mixed mycotoxin was injected into the HPLC equipped with a fluorescence detector (excitation and emission wavelengths of 365 and 435nm, for aflatoxins) and column oven (40 ºC). The mobile phase was water, methanol, and acetonitrile solution (60:30:20, v/v) at a flow rate of one mL/min. For daily quantification of AFB_1 in samples, a calibration curve with seven points was built for AFB_1, and the linearity of curves was checked. Sample fortification was used for the determination of recovery of analysis. The recovery was determined by using blank corn samples which were spiked with the standard solution to make a contamination level of 5 ng/g for AFB_1. The AFB_1 recoveries ranged between 80 to 93%.

Determination of other mycotoxins- OTA, ZEA, and DON in corn samples was also performed using HPLC methods with immunoaffinity column clean-up. The analysis procedures of OTA, ZEA, and DON were respectively performed according to Iranian National Standard guideline which was based on AOAC official methods (EN14132, 2003; Horwitz, 2000; MacDonald et al., 2005).

Intended for determination of OTA, 100 ml extraction solvent (a mixture of acetonitrile: water at a ratio of 84:16) were added to 25g of sample, homogenized, and stirred for 5 min in a high-speed blend jar. Then aliquot was passed through a glass microfiber filter. To clean up the sample, 10 mL phosphate buffer saline (PBS) was passed through the IAC (OTA test), followed by 55 mL of the filtrate passing through the IAC at a flow rate of 1 drop/sec. The IAC was then washed with 10mL water and dried under a mild vacuum. Finally, the OTA were eluted with 1.5 mL methanol-acetic acid mixture (98 volumes of methanol and 2 volumes of acetic acid) and 1.5 mL pure water, and a volume of 100 µL of final collected and completely mixed extract was injected into the HPLC equipped with a fluorescence detector (excitation and emission wavelengths of 333 and 477nm, for OTA) and column oven (40 ºC).

For determination of ZEA, 100 ml extraction solvent (a mixture of acetonitrile: water at a ratio of 84:16) were added to 25g of sample, homogenized, and stirred for 5 min in a high-speed blend jar. Then aliquot was passed through a glass microfiber filter. To clean up the sample, 10 mL phosphate buffer saline (PBS) was passed through the IAC (ZEA test), followed by 10 mL of the filtrate and 65 mL deionized water passing through the IAC at a flow rate of 1 drop/sec. The IAC was then washed with 10mL water and dried under a mild vacuum. Finally, the ZEA were eluted with 2 mL methanol and 2 mL pure water, and a volume of 100 µL of final collected and completely mixed extract was injected into the HPLC equipped with a fluorescence detector (excitation and emission wavelengths of 275 and 450nm, for ZEA).

To determine DON, 200 ml extraction solvent (water) were added to 25g of sample, homogenized, and stirred for 5 min in a high-speed blend jar. Then aliquot was passed through a glass microfiber filter. To clean up the sample, 10 mL phosphate buffer saline (PBS) was passed through the IAC (DON test), followed by 2 mL of the filtrate passing through the IAC at a flow rate of 1 drop/sec. The IAC was then washed with 10mL water and dried under a mild vacuum. Finally, the DON were eluted with 1.5 mL mobile phase, and a volume of 100 µL of final collected and completely mixed extract was injected into the HPLC equipped with a UV detector (wavelengths of 218 nm, for DON).

2.4. Ozone generation and ground corn decontamination procedure

A corona discharge ozone generator MOG002 (O3 Tech H.K Limited, Shenzhen, China) using medical oxygen has been used for the production of gaseous ozone. According to the ozonation capacity of the device (flow rate 5 g/h), and based on the volume of contaminated corn samples, different times of ozone production from oxygen were calculated. According to the experimental design spiked ground corn samples (200g) were placed in a jacketed reactor and the ozone generator provided ozone, so that the ozone concentration of 64, 200, 400, 600, and 736 mg/kg were delivered. Ozone gas running durations were 0, 100, 250, 400, and 500 min (based on an experimental design by RSM), and ozonized ground corn samples were immediately collected and aflatoxin B_1 was extracted and analyzed after ozone exposure.

2.5. Experimental Design, statistical analysis, and Model validation

In this study, a CCD via RSM was used to fit the models and optimize the ozonation process. evaluating the effects of different factors, the ozone dose (X_1), ozonation time (X_2), and AFB_1 concentration level (X_3), were used as independent variables in the experimental design. To estimate the experimental error, six replicates of the central points were added to the runs. Experiments were carried out in random order. The AFB_1 concentration in samples before and after ozonation from the HPLC analysis was recorded and the reduction percentage of AFB_1 has been calculated as the response of the experiment.

Table 1. Independent variables used in RSM design for ozonation optimization.

Symbol	Independent variable	Coded levels				
		-α	-1	0	+1	+ α
X1	Ozone dose (ppm)	64	200	400	600	736
X2	Ozonation time (min)	0	100	250	400	500
X3	AFB1 contamination level (ng/mL)	4.5	14	28	42	51.5

A Minitab 16 statistical software (State College, PA, USA) was used for data analysis and to determine statistical significance a probability value of 0.05 was considered. Replication in the center points permitted to checking the adequacy of curvature expressed in the response. Analysis of variance (ANOVA) combined with Fishers' statistics test ($p< 0.05$) has been used for the examination of model terms adequacy and significance. Optimized amounts of variables were proposed by the response surface optimizer of Minitab software according to the trial data and experimental responses.

A second-order polynomial equation was used to express the AFB_1 reduction percentage (%) (Y) as a function of the independent variables as follows:

$$Y = a_0 + a_1X1 + a_2X2 + a_3X3 + a_{11}X1^2 + a_{22}X2^2 + a_{33}X3^2 + a_{12}X1.X2 + a_{13}X1.X3 + a_{23}X2.X3 \quad (1)$$

Where Y represents the response, a_0 is a constant, and a_i, a_{ii}, and a_{iij} are the coefficients for linear, quadratic, and interactive terms, respectively.

A two-sample t-test analysis has been used among the predicted and experimentally observed values, using the software (fitted amounts of responses) and of responses for all 20 experiments, to validate the final models, theoretically. As a final point, optimal conditions which are presented by the response surface optimizer, were applied for ozonation treatment. In addition, one sample t-test was used to ascertain significant differences between the probable and the achieved response values of five replicates under optimal conditions.

2.6. Model validation

Two sample T-Test analyses between AFB_1 reduction in experimental and predicted amounts by software demonstrated that there is no significant difference between predicted amounts and experimental amounts (P value=1). Besides, the experimental values of the AFB_1 reduction were obtained in five optimal conditions replicates and predicted responses calculated by equation 1 compared using one sample t-test (A. Rahmani, Jinap, & Soleimany, 2010). The p-value indicates whether is there any significant difference between predicted values fitted by the model and the experimentally obtained amounts or not ($p > 0.05$).

3. Results and Discussion

3.1. Ozonation method optimization

This study explains the optimization method of ozonation by means of the pooled effects of the ozone dose (X_1), ozonation time (X_2), and AFB_1 contamination level (X_3) on the AFB_1 reduction in spiked corn samples. The levels of AFB_1 of contaminated corn treated with ozone are displayed in Table 2. Moreover, the reduction percentage of AFB_1 in different ozonation conditions has been demonstrated.

The greatest reductions in AFB_1 (100% REDUCTION OF AFB_1) were obtained in the treatment using 250 min of exposure to 736 mg/kg ozone (Table 1, run order 13). In addition, using 600 mg/kg ozone for 400 min reduced 97 and 99% of AFB_1 in run orders 15 and 17, respectively. However, 600 mg/kg ozonation for 100 min (run orders 14 and 18) was not able to reduce more than 75 % of AFB_1. A lower ozonation dose (400 mg/kg) was not able to reduce more than 76% of AFB_1 (run order 8). Reduction percentage in similar ozonation conditions was almost comparable for different contamination levels of AFB_1 (run order 10, 20, and center points)

The AFB_1 reduction rate is significantly ($P < 0.05$) increased with an increase in ozone concentration dose (X_1) and ozonation treatment time (X_2). The results were in agreement with the previous researches, in which the detoxification of AFB_1 in corn, red pepper, peanuts, and pistachios was confirmed to be improved with increased ozonation time, and ozone concentrations (Akbas & Ozdemir, 2006; de Alencar, Faroni, Soares, da Silva, & da Silva Carvalho, 2012; Inan et al., 2007; Luo, Wang, Wang, Li, Bian, et al., 2014)

AFB_1 reduction is due to its destruction in the double bonds between carbon number 8 and 9 (C8-C9) by the ozone attack. This double bond is the most susceptible part of AFB_1 to oxidation, which breaks when the ozone molecule forms primary ozonide through binding to the carbons, followed by derivatizing to other molecules such as ketones, aldehydes, and organic acids (Chen et al., 2014; Proctor et al., 2007). As expected, the lower reduction of AFB_1 was observed on using a lower dose of ozone (64 and 200 mg/kg) that was not able to break the C8-C9 double bound in 100 and 250 min ozonation (run order 6,7 and 11), but 400 min ozonation provided a slight reduction of 28 and 31% in AFB_1 (run order 3 and 5).

Statistical parameters obtained from ANOVA are shown in Table 3. The first column of the table shows an analysis of variances of response (AFB_1 reduction) at the full quadratic model. Non-significant terms ($p < 0.05$) were then eliminated from the model and the reduced model was presented in the second column.

Table 3, shows that the ozone dose (X_1) had the greatest effect on the AFB_1 reduction, while the contamination level did not affect this response. Based on the observed response results, the most effective terms were the main and quadratic terms, meanwhile, there was no evidence of a significant effect for interaction terms of factors. The model fitting has been done by excluding the non-significant terms, consequently, the AFB_1 reduction optimization model was presented using the linear and square models. The equation of the fitted model is shown below.

$$Y = 74.33 + 32.96\ X1 + 17.52\ X2 - 9.287\ X1^2 - 13.877X2^2 \quad (2)$$

Where Y represents the response variable (AFB_1 reduction percentage), and X1 and X2 represent the ozone dose and, ozonation time, respectively.

The coefficient of determination R^2, for model fitting was >0.97 in the reduced model, demonstrating that a reduced mathematical model can describe more than 97% of response variability.

The surface plot of the fitted equation model for the interaction between variables (X1, X2 and X3) on response (AFB_1 reduction) are shown in Figs 1 to 3. Ozone dose and ozonation time have a positive significant effect on response ($p < 0.05$) and AFB_1 reduction increased with an increase in both variables.

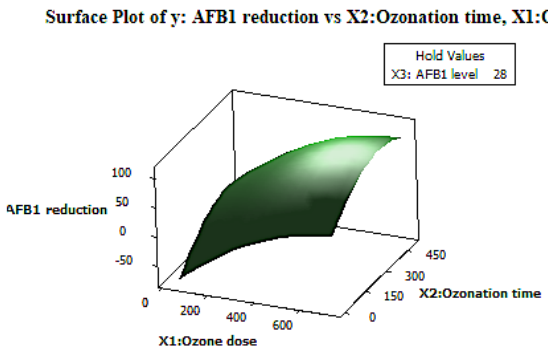

Fig. 1. Surface plot of the response (AFB1 reduction percentage) versus X1: ozone dose, X2: ozonation time.

Figs 2 and 3, demonstrate that X3 (AFB_1 contamination level) had no significant effect on response, likewise Table 3 showed that

Table 2. Design matrix, experimental values, and predicted values in the screening design for ozonation optimization.

Run order	X1 Ozone dose (ppm)	X2 Ozonation time (min)	X3 AFB1 Contamination level (ng/mL)	Ozonation results		
				AFB1 contamination level after ozonation (ng/mL)	AFB1 reduction percentage Experimental (%)	AFB1 reduction percentage Predicted (%)
1 CP	400	250	28	7.0	75	75.14
2 CP	400	250	28	6.5	76	75.14
3	200	400	42	29.0	31	35.05
4 CP	400	250	28	7.0	75	75.14
5	200	400	14	10.0	28	31.79
6	64	250	28	28.0	0	0.00
7	200	100	42	42.0	0	0.00
8	400	500	28	6.5	76	75.96
9 CP	400	250	28	7.0	74	75.14
10	400	250	4.5	1.0	75	74.38
11	200	100	14	14.0	0	1.15
12 CP	400	250	28	7.0	76	75.14
13	736	250	28	0.0	100	100.00
14	600	100	14	3.5	75	70.58
15	600	400	14	0.5	97	97.22
16	400	0	28	28.0	0	4.64
17	600	400	42	0.5	99	97.48
18	600	100	42	13.0	70	65.84
19 CP	400	250	28	7.0	75	75.14
20	400	250	51.5	14.5	72	73.14

CP: Center point

Table 3. Analysis of variance of the regression coefficients of the quadratic equations for ozonation optimization.

Variable	ANOVA of Response in full quadratic mode			ANOVA of Response after excluding non-significant terms		
	Regression coefficient	T- value	P-value	Regression coefficient	T-value	P-value
a_0	75.220	29.068	0.000[a]	74.333	39.330	0.000[a]
Linear						
a_1	32.964	19.197	0.000[a]	32.964	22.327	0.000[a]
a_2	17.524	10.186	0.000[a]	17.524	11.847	0.000[a]
a_3	-0.369	-0.215	0.834[b]	-	-	-
Quadratic						
a_{11}	-9.395	-5.622	0.000[a]	-9.287	-6.495	0.000[a]
a_{22}	-13.783	-8.188	0.000[a]	-13.677	-9.494	0.000[a]
a_{33}	-1.086	-0.650	0.530[b]	-		
Interaction				-	-	-
a_{12}	-1.000	-0.446	0.665[b]	-	-	-
a_{13}	-0.750	-0.334	0.745[b]	-	-	-
a_{23}	-1.250	0.557	0.590[b]	-	-	-
R^2	0.9825	-	-	98.06	-	-
R^2(adj)	0.9668	-	-	97.55	-	-

linear, quadratic and interaction p-value of AFB_1 contamination level on response is not significant.

* a_0 is a constant, ai, aii, and aij are the linear, quadratic, and interactive coefficients of the quadratic polynomial equations, respectively. 1: ozone dose; 2: ozonation time; 3: AFB1 contamination level.
[a] Significant ($p<0.05$).
[b] Not significant ($p>0.05$)

According to previous reports, the reduction rate of AFB_1 in wheat and corn increases with ozonation time and ozone concentration, while the grain mass provided an adverse effect on response. The most significant reduction in AFB_1 contamination was accomplished by utilizing 60 mg/L of ozone for 300 min, for 2 kg samples (Luo, Wang, Wang, Li, Bian, et al., 2014; Porto et al., 2019; Trombete et al., 2017).

In addition, based on the results aflatoxin-contaminated corn showed high cell toxicity while there was no significant difference in toxicity results of ozone-treated contaminated samples and the AFB_1-free culture solution (Luo, Wang, Wang, Li, Bian, et al., 2014).

According to published articles increasing humidity and temperature will provide higher penetration and destruction of aflatoxins (Freitas-Silva & Venâncio, 2010; S. Wang, Liu, Lin, & Cao, 2010). In this research, these two variables were not used to provide the possibility of storing corn grains in silos and avoid the

cost of re-drying or the possibility of quality changes due to the increase in temperature.

3.2. Optimal conditions obtained by the response surface optimizer of the software

The optimum point for the response was as follows: X_1=600 mg/kg, X_2=250 min. Therefore, final optimal ozonation conditions were realized using a 600 mg/kg ozonation dose for 250 min, which could reduce more than 98% of AFB_1 contamination level in contaminated corn samples.

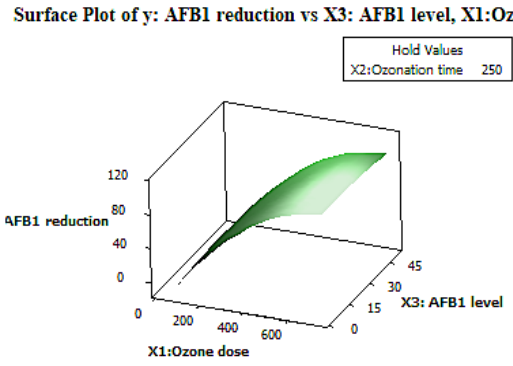

Fig. 2. Surface plot of the response (AFB1 reduction percentage) versus X1: ozone dose, X3: AFB1 level.

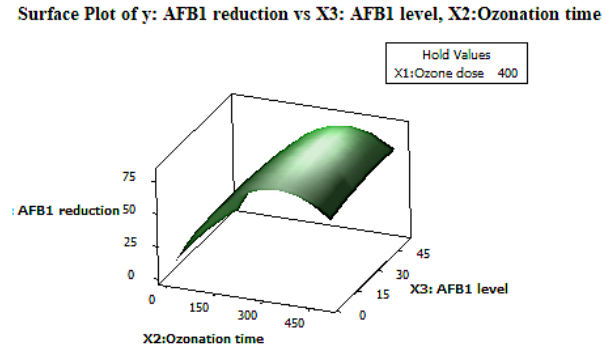

Fig. 3. Surface plot of the response (AFB1 reduction percentage) versus X2: ozonation time, X3: AFB1 level.

3.3. Model Verification and Validation

The agreement between the experimental and predicted value (given by software) was evaluated to examine the precision, predictability, and statistical verification of the model and the obtained optimal conditions. The precision was evaluated by replications of six center points for the response. The mean value and the range of response for 6 center points were 75.17% and 74-76 %, respectively and the coefficient of variance was 1%. The p-values for two sample t-tests used for predicted and observed (actual) responses was 0.942 ($p > 0.05$), signifying that there were no significant differences between the fitted model's predicted amounts and experimental values.

Validation of the optimized method, conducted by regression analysis under obtained optimal conditions in five replications. Naturally-contaminated corn samples (AFB_1: 7.1 and 27.9 ng/g) have been used. The obtained results are shown in Table 4.

Table 4. Ozonation method validation for maximum AFB1 reduction using optimal conditions (ozone dose: 600 ppm, ozonation time: 250 min).

AFB1 contamination level in naturally contaminated corn (ng/g)	AFB1 contamination level after ozonation (ng/g) predicted	AFB1 reduction percentage (%) predicted	AFB1 contamination level after ozonation (ng/g) Experimental	AFB1 reduction percentage (%) Experimental
7.1	0.07	98.9	0.05	99.3
			0.25	96.5
			0.20	97.2
			0.10	98.6
			0.30	95.8
27.9	27.6	98.9	0.30	98.9
			1.20	95.7
			1.40	95.0
			1.10	96.0
			0.15	99.5

There was a worthy agreement between the predicted and observed AFB_1 reduction percentages. Experimental AFB_1 reduction percentage mean and standard deviation for naturally contaminated corn in 7.1 and 27.9 ng/g AFB_1 was 97.48 ± 1.45 % and 97.02 ± 2.03 %, respectively, while the predicted value was 99 %. Because of the p-value ($p>0.05\%$) of one sample t-test conducted between experimental values and their predicted amounts, it could be concluded that there was no significant difference between predicted and observed values for responses. Subsequently, the mathematical model was accurate and valid (statistically, and experimentally) for the ozonation process to reduce AFB_1.

3.4. Investigation of optimized ozonation method on other mycotoxins

An optimized method has been used for the reduction of AFB_1, AFB_2, AFG_1, AFG_2, OTA, ZEA, and DON in spiked corn samples. The obtained results have been demonstrated in Table 5.

Table 5. Mycotoxin reduction using optimal conditions (ozone dose: 600 ppm, ozonation time: 250 min).

Mycotoxins	Mycotoxin contamination level in spiked corn (ng/g)	Mycotoxin contamination level after ozonation (ng/g)	Mycotoxin reduction percentage (%)
AFB1	500	20	96
AFB1	28	0.3	99
AFB2	7	0.2	97
AFG1	28	0.3	99
AFG2	7	ND	100
OTA	50	ND	100
ZEA	200	ND	100
DON	1000	ND	100

Ozonation has been reported as a safe chemical method for aflatoxins detoxification, without producing any toxic leftover because ozone quickly decomposes into oxygen (Akbas & Ozdemir, 2006; de Alencar et al., 2012; Inan et al., 2007; ZorlugenÃ§,

ZorlugenÃ§, Ã–ztekin, & Evliya, 2008). Degradation products of AFB_1 after aqueous ozonation have been identified in a study using ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometers (UPLC Q-TOF MS) and six key degradation product structures (mass 305.1-371.1 m/z) and possible paths for generating fragment ions were proposed. According to those results due to the conjugate addition reaction on the double bond of the terminal furan ring for AFB_1, the toxic effects of the degradation molecules were essentially diminished in comparison with that of AFB_1 (Luo, Wang, Wang, Wang, & Chen, 2013).

The obtained results for other mycotoxins were in agreement with those previously reported for ozonation of corn to decrease OTA, ZEA, and DON, though a higher reduction in those toxins (almost 100% in comparison to 70-90% reduction in other reports) achieved by this presented optimized method (Krstovic et al., 2020; Qi et al., 2016).

The toxicity of the chemical products after degradation of AFB_1 of the ozone-treated contaminated corn has been examined by Luo et al., and results demonstrated that aflatoxin-contaminated corn (ACC) may cause significant changes in various biochemical indexes and physiological characteristics in liver and kidney tissues, but ozone treatment of ACC seem altogether diminish these adverse effects (L. Wang et al., 2017). Yet, gaseous ozone is used for agricultural products without adverse effects on their quality (Diao, Hou, & Dong, 2013). More studies focusing on toxicity tests (in vitro and in vivo) need to be conducted to ensure that ozonized products are safe for humans and animals.

3.5. Investigation of ozonation on physicochemical factors of corn

The main physicochemical factors of corn before and after ozonation is shown in table 6. According to the obtained results, except for the acidity factor, no significant changes had been occurred in physicochemical factors of corn during the ozonation process.

Table 6. Comparison of physicochemical properties of corn before and after ozonation.

No.	physicochemical factors	before ozonation	after ozonation
1	Protein (weight percentage based on dry matter)	11 ± 0.7	10.9 ± 0.8
2	Fat (weight percentage based on dry matter)	5.1 ± 0.4	5 ± 0.3
3	Fiber (weight percentage based on dry matter)	2.1 ± 0.2	2 ± 0.2
4	Ash (weight percentage based on dry matter)	1.1 ± 0.1	1.1 ± 0.1
5	acidity	2.7 ± 0.4	3.4 ± 0.4
6	Color (I)	82.37 ± 1.4	80.09 ± 2.4
	Color (a)	5.8 ± 0.7	6.88 ± 1.9
	Color (b)	30.2 ± 1.1	28.3 ± 2.1

4. Conclusion

The results showed that the application of ozone reduced the mycotoxin contamination in contaminated corn. Among the studied variables ozone dose (X_1) and ozonation time (X_2) provided the greatest effect in mycotoxin reductions, while the contamination level was not significant in the reduction percentage of AFB_1 in the ozonation process. The application of ozone in the 600 mg/kg for 250 min (optimum condition), reduced more than 96% of AFB_1. In addition, using optimal conditions provided an effective method for the reduction of other mycotoxins including AFB_2, AFG_1, AFG_2, OTA, ZEA, and DON to reduce at least 97% of these mycotoxins. Therefore, until now, the use of ozonation has been accepted as a definitive method to reduce mycotoxins. Despite the few articles, investigations are still ongoing to look for the effects of metabolites resulting from the breakdown of mycotoxins in food and possible food safety hazards.

Authorship contribution statement

Masoumeh Mahmoudi-Meymand: Methodology, Data curation, Writing Original draft preparation, Visualization, Investigation, and Validation, Anosheh Rahmani: Conceptualization, Reviewing, Editing, and Supervision, and Reza Haji-Hosseini: Supervision

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Conflict of interest

The authors declare that there is no conflict of interest.

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