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Research Article

Elimination of *Aspergillus flavus* from Pistachio Nuts with Dielectric Barrier Discharge (DBD) Cold Plasma and Its Impacts on Biochemical Indices

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In the present research, the effects of different durations (0, 15, 30, 60, 90, 120, 150, and 180 sec) of dielectric barrier discharge (DBD) cold plasma on decontaminating *Aspergillus flavus*, detoxifying pure aflatoxin B1 (AFB1), and the quality attributes of pistachio nuts (total phenolic content, antioxidant activity, chlorophylls, total carotenoids, instrumental color, total soluble protein, and malondialdehyde determination) were studied. The results showed that the viable spore population reduced with the increase of plasma treatment duration, so that after 180 s of the treatment, a decrease by 4 logs was observed in the spore population. Chlorophyll a and b, as well as total carotenoid levels and color parameters, decreased, which led to darker pistachio samples and intensity reduction in soluble protein content and protein bands. Plasma treatment did not alter the total phenolic content but slightly increased the antioxidant activity of pistachio nuts samples. The malondialdehyde values significantly increased all the plasma treatment durations. The maximum reduction of AFB1 was observed after 180 s of the treatment, which was 64.63% and 52.42% for glass slides and pistachio nut samples, respectively. The present findings demonstrated that cold plasma could be used as an efficient decontamination method of food products without inducing undesirable quality changes in nuts.

1. Introduction

Pistachio nut (*Pistacia vera* L.) is a member of Anacardiaceae family and is the most famous tree nuts in the world. Iran, USA, Turkey, China, Syria, Greece, and Italy are the main pistachio-growing countries [1, 2]. Pistachio kernels have been recognized as health promoting food because of their high content of unsaturated fatty acids, proteins, minerals, vitamins, phytosterols, and polyphenols and have been consumed as a salted and roasted snack. They also can be used as the main ingredient of many food products such as confections, biscuits, ice cream, and sausage [2]. Pistachio is the most important nonpetroleum export commodity that plays a vital role in the economy of Iran [3]. Iran is the first and biggest producer and exporter of pistachio nuts in the world. It produced 337,815 tons and exported 78,547 tons in 2019. The total export revenue from pistachio nuts was about half a billion dollars in that year [1]. This valuable commodity is highly susceptible to contamination by one of the most prevalent aflatoxin producing fungi *Aspergillus flavus* during preharvest and postharvest stages, which leads to reduction in quality and nutritional value; finally, it causes economic losses to the producing countries [4, 5].

Aflatoxins are extremely toxic, teratogenic, and carcinogenic secondary metabolites that are synthesized by the polyketide pathway [6]. Aflatoxins B_1 (AFB_1) and B_2 (AFB_2) are two popular toxins made by *A. flavus* [7]. AFB_1 has a high potential for the liver cancer and is one of the most common toxins in pistachios [8, 9]. The strict international laws have been enacted to monitor the level of aflatoxin contamination in pistachios, and the acceptable maximum levels for pistachio nuts are 8 μg/kg for AFB_1 and 10 μg/kg for the total aflatoxins (TotAFs) according to the Iran and European commission regulations [10, 11].

Various physical, thermal, and chemical methods have been studied to prevent the mold growth and mycotoxin decontamination; but using these methods is limited due to potential risks to the human health ,as well as reduced quality, sensory properties (flavor, color, and texture), and nutritional value of the foodstuffs [12, 13]. Hence, choosing reliable alternative decontamination methods to maintaining the quality and safety of food products is of great importance. Cold plasma is a futuristic nonthermal technique, which is considered as the fourth state of matter including radicals, electrons, charged ions, neutral and/or excited particles, molecules, atoms, and UV photons with inactivating properties of microorganisms [4, 14]. Several factors such as type of feeding gas, electric and magnetic field, gas flow rate and pressure, order and shape of electrodes, relative humidity, treatment time, and type of cold plasma system can affect the composition of plasma reactive species [4, 8]. Several studies have been done on applying cold plasma technology in food processing such as modifying wheat flour functionality [15], degrading pesticides residue in vegetables and fruits [16], reducing the cooking time of rice [17], inactivating enzymes [18], and inactivating microbes in various food products [17, 19, 20]. Recently, promising results of *A. flavus* decontamination and aflatoxin detoxification using cold plasma in nuts have been reported [4–9, 12–14, 21].

The purposes of this research are the possibility of using cold plasma in decreasing the *A. flavus* spore population and reducing the amount of AFB_1 in pistachio powder along with the plasma effect on some quality characteristics of pistachios.

2. Materials and Methods

All the chemical materials and solvents were prepared mainly from Sigma-Aldrich (St. Louis, MO, US) and Merck (Darmstadt, Germany) companies. Pure AFB_1 from *A. flavus* was purchased from Sigma-Aldrich (Darmstadt, Germany) Company.

2.1. Pistachio and Sample Preparation. Pistachio nuts (*P. vera* L.), Akbari cultivar, were prepared from Iranian Pistachio Research Centre located in Kerman Province in Autumn 2019. The peel of all the pistachios was removed; the kernels were milled and stored properly in dry conditions in sterile Petri dishes at $4 \pm 1^{\circ}C$ and protected from direct sunlight until further experiments.

2.2. Inoculation of Pistachio with Aspergillus flavus and Aflatoxin B_1. The aflatoxigenic fungus *A. flavus* R5 [22] was grown on potato dextrose agar (PDA) at 28°C for 7 days. Some pistachio kernels were inoculated with pathogen fungal spores or Aflatoxin B_1 (final concertation about 400 ppb) and milled with other pistachio kernels. Concentration of conidial in contaminated pistachio powder was determined by hemocytometer counts using suspended 0.5 g of inoculated pistachio powder in 4.5 ml of sterile saline dilute solution containing 0.5% Tween-80. The population of spores in pistachio powder was set to $(6.18 \pm 1.18) \times 10^5$ spores/g pistachio power. The contaminated pistachio powders were packed in sterile Petri dishes and kept at $4 \pm 1^{\circ}C$ [9].

2.3. Cold Plasma Treatment of Pistachio Powder and Pure Aflatoxin B_1. The nonthermal atmospheric pressure dielectric barrier discharge (DBD) air plasma system consisted of two parallel metallic electrodes separated by a 5 mm gap (Figure 1). The upper electrode had the diameter of 75 mm, was made of stainless steel 316, and was covered from the bottom by the dielectric pyrex glass with the thickness of 3 mm and diameter of 80 mm. The plasma treatment was operated in ambient air as working gas at 15, 30, 60, 90, 120, 150, and 180 s time intervals. The process was done at 130 W power, 20 kHz frequency, and 15 kV voltage, and the distance between the electrodes and sample was 3 mm. Treatments were done in triplicate. About 4 g of pistachio nut samples contaminated with *A. flavus* active spores and/or AFB_1 with the thickness of 3 mm was placed between two electrodes and treated using a DBD plasma device at different time intervals. AFB_1-contaminated glass slides with the size of 24×50 were used alongside with pistachio nut samples for quantifying the AFB_1 residual.

2.4. Determining A. flavus Total Count. After plasma treatment of pistachio samples, each sample was suspended with sterile saline solution (8.5 g NaCl in 1000 ml distilled water) by vortex; after serial dilution preparation, 100 μl of each dilution was inoculated into two Petri dishes comprising Potato dextrose agar (Merck, Germany) with 50 ppm chloramphenicol and 0.033 $g \cdot L^{-1}$ of Rose-Bengal by surface plating and, then, incubated at 28°C for 3–5 days. Fungal colonies were counted visually after incubation, and the results were means of triplicate reported as log colony forming unit per gram (log cfu/g) [23].

2.5. Total Phenolic Content (TPC) and Antioxidant Activity. Samples were analyzed for TPC and antioxidant activity using Folin-Ciocalteu method and DPPH free radical scavenging activity, respectively, according to the procedure used by Hojjati et al. [2]. About 1 gr of each ground pistachio samples was extracted for 15 min with 80% methanol solution (10 ml) and sonicated (Sonorex Digitech DT 1028 H, Bandelin, Germany) two times for 15 min. The mixture was kept at the room temperature for a day and, then, centrifuged (5000 ×g at 4°C for 4 min) (Httich Refrigerated Centrifuge Universal 320R, Germany). The collected supernatants were used for both experiments.

2.6. Malondialdehyde Determination. Lipid oxidation was assessed based on thiobarbituric acid reactive substances (TBARS) analysis according to Papastergiadis et al. [24]. The sample extracts were obtained by mixing about 7 gr of ground pistachio nuts with 15 mL of 7.5% trichloroacetic acid (TCA) (w/v) with 0.1% of ethylenediaminetetraacetic acid (EDTA) (w/v) and 0.1% of propyl gallate (w/v),

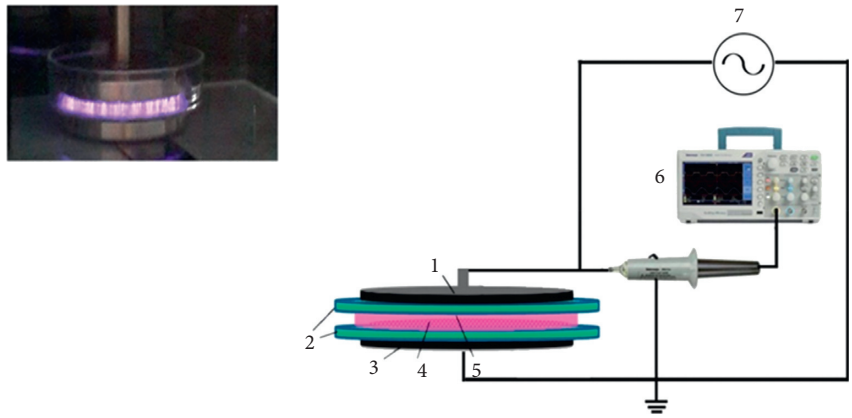


Figure 1: Photograph (a) and schematics (b) of an atmospheric pressure diffuse plasma generated by DBD: (1) discharge electrode, (2) pyrex glass dielectric barrier, (3) ground electrode, (4) pistachio nut sample, (5) cold plasma, (6) oscilloscope, and (7) power supply. The discharge in the photo (a) was driven by kHz sinusoidal high voltage, and the working gas was air. Photo was taken at the author's laboratory.

homogenized at 18,000 rpm for 1 min, and adjusted to 30 mL with TCA. After adding 2.5 mL of the filtrated extract (with 150 mm filter paper) and 2.5 mL of thiobarbituric acid reagent (46 mM) in glacial acetic acid (99%) to a 15 mL tube, the tubes were placed in boiling water bath for 35 min and cooled to the room temperature. The collected supernatant absorbance was read at 532 nm using a spectrophotometer. The blank sample was TBARS reagent in distilled water, and the malondialdehyde (MDA) standard curve was plotted using different MDA standard solutions in TCA solution (7.5%). The results were reported as nanomolar malondialdehyde per gram pistachio powder.

2.7. Chlorophylls and Total Carotenoids. Total carotenoids, as well as chlorophyll a and b, were measured by spectrophotometric method according to Roueita et al. [25]. The ground pistachio nut samples (0.2 g) were mixed with 80% (v/v) acetone (5 ml). The mixtures were left in darkness for 15 min and, then, centrifuged (1500 ×g for 10 min) and filtrated with no. 2 Whatman filter paper. The absorbance of the filtrated extracts was determined at 470 (A_{470}), 663 (A_{663}), and 645 (A_{645}), nm. The contents of carotenoids ($\mu g \cdot g^{-1}$) and chlorophyll a and b ($\mu g \cdot g^{-1}$) were calculated according to the following formulae:

$$C_a = 12.21\left(A_{663}\right) - 2.81\left(A_{645}\right),$$
$$C_b = 21.13\left(A_{645}\right) - 5.03\left(A_{663}\right), \quad (1)$$
$$C_t = \frac{\left[1000\left(A_{470}\right) - 3.27C_a - 104C_b\right]}{229}.$$

2.8. Instrumental Color. The color parameters of pistachio nuts' powder samples were examined by means of a Minolta Colorimeter CR-400 (Konica Minolta, Inc., Osaka, Japan) with a D65 illuminant as a light source and a 10 standard observer at 25°C. This colorimeter is based on International Commission on Illumination (CIE) L^*, a^* and b^* values for describing the accurate location of a color within a three dimensional space, in which parameter L^* indicates the brightness of the color and is between zero (black) and 100 (white), a^* parameter shows the characteristic of red and green color and has negative (green) and positive (red) values, parameter b^* represents yellowness and blueness, and the negative and positive values indicate the colors blue and yellow, respectively; finally, C^* is chroma, and the value of C^* is 0 at the center of a color sphere and increases with distance from the center. For determining the color values, a watch glass was placed on the samples, and the L^*, a^*, and b^* parameters were calculated using a colorimeter. Chroma was determined using [19]

$$C^* = \sqrt{a^{*2} + b^{*2}}. \quad (2)$$

Color analyses of the samples were done in five replicates.

2.9. Total Soluble Protein. Total soluble protein was measured using Bradford method [26]. The pistachio nut powder was mixed well with 10% sodium phosphate buffer solution (pH = 6.8, W/V) using a mortar and pestle; then, it was stirred for 30 min. The mixture was poured into a falcon tube and centrifuged at 5000 ×g for 5 min. The collected supernatant was kept at −20°C for later examination. Also, 5 ml of Bradford reagent was added to 100 μl of the collected extracts, and, after 30 min, the absorbance of the extracts was calculated using the bovine serum albumin (BSA) standard curve at 595 nm. The results were reported as mg of protein per g of pistachio nuts (mg/g).

2.10. Electrophoretic Pattern of Proteins. 250 μ of protein extracts was precipitated with cold acetone and centrifuged (5000 ×g for 4 min at 4°C). The supernatant was gathered and placed in the room to remove the remaining acetone. Then, a certain amount of the samples was prepared in

distilled water and the sample buffer (nonreducing buffer condition; 65 mM Tris, pH 6.8, 10% glycine, 2 SDS, 0.2% Bromophenol blue). On the other hand, the same samples were mixed with 5% 2-mercaptoethanol and heated in boiling water bath for reducing buffer. All the samples were tested for electrophoretic pattern of proteins by SDS-PAGE using a Mini Protean II Cell system (Bio-Rad, Hercules, USA) by adopting Laemmli method [27]. Also, 50 μg of the samples was transferred onto the gel consisting of 12.5% acrylamide separating gel and 5% acrylamide stacking gel. The gels were stained with Coomassie Brilliant Blue R-250 and destained in a methanol-acetic acid-water (1 : 1 : 8 v/v) mixture. The Quantity One 1-D Analysis software and Gel Doc XR + (Bio-Rad, Hercules, USA) were used for capturing gels images, and the relative density, molecular weight, and bands intensity were analyzed by Gel-Pro Analyzer (ver.6.0).

2.11. Aflatoxin Extraction and HPLC Analysis of Aflatoxin B1. In order to extract AFB_1 for HPLC analysis, the sample (1 g pistachio nut powder) was mixed with methanol 80% v/v and shaken well at 150 rpm for 24 h. The mixture was centrifuged at 10000 ×g for 5 min, and the supernatant was purified using an ASPEC 401 immunoaffinity purification column. The immunoaffinity column was washed with PBS (10 ml) to remove impurities; then, it was loaded with the sample and washed again with water (10 ml). Finally, the column-bounded AFB_1 was released by the elution with acetonitrile (1.5 ml). One aliquot of eluate (0.5 ml) was retained in a glass vial, and the remainder was diluted with water (2 ml) before HPLC analysis. 400 ml of the diluted eluate was injected into the HPLC system equipped with a Spherisorb Excel ODS1 (250 × 4.6 mm; 5 μm) C18 reversed column with a guard column (25 × 4.6 mm i.d.). Perkin Elmer LC420 fluorescence detector with excitation at 364 nm and emission at 440 nm were applied to detection. Water: methanol: acetonitrile (56 : 14 : 30, v/v/v) at the flow rate of 0.86 ml/min was used as the mobile phase. Post-column derivatization was achieved with a zero dead volume T-piece and 30 cm × 0.3 mm i.d. PTFE reaction tube. Pyridine hydrobromide perbromide reagent was added at 0.3 ml/min. The retention time for AFB_1 was approximately 13.55 min [13].

2.12. Statistical Analyses. Data analyses were performed using SPSS Software v.26.0 (SPSS Inc., Chicago, IL, USA) using one-way analysis of variance (ANOVA). Differences were significant at 95% confidence level (statistical significance at $p < 0.05$) using Duncan's multiple comparison test. All the experiments were administrated in a completely randomized design, and data presented in this study were the mean ± standard deviation (SD) of the triplicate experiments.

3. Results and Discussion

3.1. Decontamination Effect of Plasma Treatment. Figure 2 shows the effect of different durations of cold plasma on the reduction of *A. flavus* spore population in contaminated pistachio nuts. The results showed that the population of the viable spores significantly decreased with the increase of plasma exposure time ($p < 0.05$). The total count of the plasma-treated pistachio nut samples was incrementally reduced by increasing the treatment duration in comparison to the control (Figure 2(a)). As shown in Figure 2(b), the rate of spore reduction was slow at the treatment duration of 15 s and 30 s. However, the rate of spore reduction gradually increased with increasing plasma treatment up to 180 s duration. After 120 s and 150 s of the treatment, a reduction of 2 and 3 log (CFU/g) in *A. flavus* spore population was achieved, respectively. As the plasma treatment duration raised to 180 s, a 4 log (CFU/g) reduction in spore population was observed, and no detectable spore was found after plating the plasma-treated sample at the treatment duration of 180 s (Figures 2(a) and 2(b)).

The present results were in agreement with the findings by Sohbatzadeh et al. [5] and Ghorashi et al. [8] who have investigated the effect of cold plasma treatment on *A. flavus* population in pistachio nut samples. Dasan et al. [12] also observed the 4 log reduction of *A. flavus* after 5 min of plasma treatment on hazelnut samples. Mošovská et al. [21] studied the cold plasma using different gases (O2, N2, ambient air, synthetic air with or without vapor) on hazelnut samples and observed that the use of synthetic air (with vapor) as plasma working gas reduced the *A. flavus* spore population below the detection limit (4 log) after 180 s of the treatment. Sen et al. [4] also reported that population of *A. flavus* and *A. parasiticus* in hazelnut samples decreased by about 4 log after low pressure (LP) plasma treatment (100 W for 30 min). Bagheri et al. [28] observed that *A. flavus* spores reduced after cold atmospheric plasma (15 V, 10 min, and 3 cm) treatment on contaminated military ration snack samples. Šimončicová et al. [29] stated that cold plasma treatment duration of 180 s showed a remarkable reduction in *A. flavus* growth on agar medium and also observed changes in hyphae structure after plasma treatment durations of 60 s and 180 s. Suhem et al. [30] investigated the inactivation effect of cold atmospheric plasma on *A. flavus* spores in both agar medium and brown rice cereal bars and found that plasma treatment (40 W for 25 min) significantly suppressed the fungi growth on both samples. They observed that cold plasma treatment effectively damaged the fungi structure and broke the conidiophores and vesicles. Several inactivation mechanisms have been suggested for plasma treatment by many authors. The air plasma is composed of oxygen reactive species (ROS), nitrogen reactive species (RNS), charged particles, atoms, excited molecules, and UV radiation, which can lead to spore inactivation by creating a harsh oxidative environment in collision with sample surface and cause denaturation of the proteins of spore coat and loss of spore coat integrity, exposing the center of the spore to plasma reactive species and reducing the cell viability [5, 12, 14]. In addition, plasma reactive species can result in rupturing the fungal cell wall via affecting the lipid bilayers of cell wall and/or accumulating charged particles on the external surface of the cell membrane, which forms electrostatic force between the reactive particles and the cell membrane and leads to fungal deactivation [4, 6]. On the

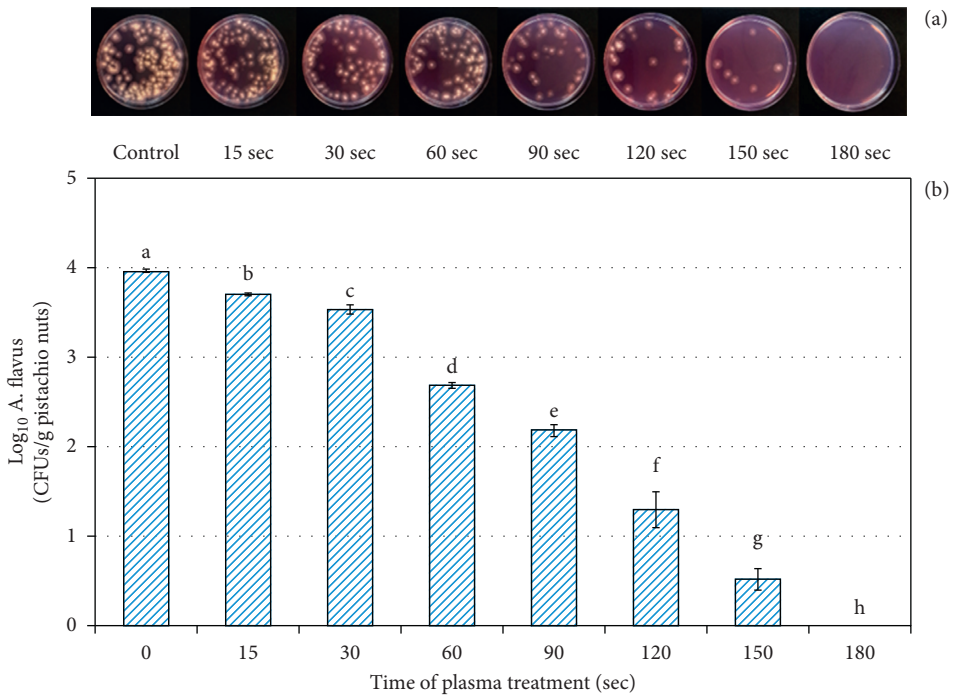


Figure 2: Decontamination of *A. flavus* by different durations of cold plasma on contaminated pistachio nut powders. (a) Cultured Petri dishes in similar dilution. (b) Enumeration of viable spores in untreated (0 sec) and plasma-treated (15–180 s) pistachio nut powders with PDA media after 72 h of incubation at 28°C. Data are means of three replicates. The same letter does not differ statistically according to Duncan's test ($p \leq 0.05$).

other hand, UV photons generated by plasma treatment can damage cell wall and inhibit cell replicate by damaging DNA strand and result in cell inactivation [6]. However, the exact antimicrobial mechanism of action of cold plasma is still unknown and requires further investigation.

3.2. Total Phenolic Content (TPC) and Antioxidant Activity. The results obtained from the effect of different durations of cold plasma on TPC and antioxidant activity of samples are summarized in Table 1. The results indicated that the TPC and antioxidant values of pistachio nut samples were significant at some of the plasma treatment durations. The lowest amount of the TPC of plasma-treated samples was observed after treatment durations of 30 s, 60 s, and 180 s, while the maximum amount of TPC was shown in the samples treated for 120 s and 150 s. No significant difference was found between TPC values at other plasma treatment durations in comparison with the control; plasma treatment had no effect on the amount of TPC of pistachio nut samples. The lowest amount of antioxidant activity of control and treated samples was observed after treatment duration of 15 s. Other plasma-treated samples displayed slight changes in antioxidant activity, and plasma treatment slightly enhanced the antioxidant activity after longer plasma treatment durations. Nevertheless, there was no significant difference between treated samples after treatment durations of longer than 60 s to 180 s. A similar trend of reduction in TPC was observed in the initial part of the plasma treatment (30 s and 60 s) on orange juice, tomato juice, apple juice, and sour cherry juice by Dasan and Boyaci [31] who described this initial decrement of phenolic compounds as the result of interaction between plasma reactive oxygen species (peroxyl radicals, hydroxyl radicals, singlet oxygen, and atomic oxygen) and phenolic compounds known for their free radical scavenging ability. As shown in Table 1, some of the plasma-treated samples were slightly enhanced in antioxidant activity. This increment can be due to the chemical reactions of plasma reactive species (ions, chemically reactive species, and UV irradiation) that lead to cell membrane breakdown and, consequently, collapse the cell membrane-bound phenolic compounds of food product, promoting polyphenols concentration and antioxidant activity after plasma treatment because of their role as antioxidant agents [20, 31]. Bagheri et al. [28] reported that no remarkable differences were found in TPC of military ration snacks after exposure of atmospheric cold plasma treatment. Ramazzina et al. [32] reported that DBD plasma had no effect on TPC and antioxidant activity of kiwifruit. In another study, Ramazzina et al. [33] indicated that TPC and antioxidant activity of apples were not affected by DBD plasma exposure in less than 30 minutes.

3.3. Malondialdehyde Determination. The results showed that different plasma durations significantly affected the MDA content of the plasma-treated pistachio samples (Table 1), and an increase of plasma duration increased the MDA content of all the samples. The lowest and highest MDA values were observed at the treatment durations of 30 s, and 15 s (3%), and 180 s (27.88%), respectively. On the other hand, MDA content of plasma-treated pistachio

Table 1: Effect of different durations of cold plasma on the values of the total phenolic content (TPC), antioxidant activity, malondialdehyde (MDA), pigments, total soluble protein (TSP), and instrumental color parameters of pistachio nuts.

Properties	Time of plasma treatments (sec)							
	0	15	30	60	90	120	150	180
TPC (mg GAE/g)	$1.81^{ab} \pm 0.05$	$1.78^{a-c} \pm 0.08$	$1.67^{c} \pm 0.08$	$1.71^{bc} \pm 0.08$	$1.76^{a-c} \pm 0.03$	$1.84^{a} \pm 0.05$	$1.85^{a} \pm 0.08$	$1.79^{a-c} \pm 0.01$
Inhibitory of DPPH activity (%)	$86.85^{c} \pm 0.58$	$86.43^{c} \pm 0.60$	$87.01^{bc} \pm 0.40$	$87.82^{ab} \pm 0.35$	$87.90^{a} \pm 0.29$	$87.12^{a-c} \pm 0.42$	$87.82^{ab} \pm 0.42$	$87.94^{a} \pm 0.42$
MDA (n mol. g^{-1})	$24.96^{d} \pm 1.34$	$25.77^{cd} \pm 0.39$	$25.71^{cd} \pm 1.14$	$28.31^{bc} \pm 1.66$	$29.04^{b} \pm 1.66$	$28.82^{b} \pm 1.14$	$29.49^{ab} \pm 1.46$	$31.92^{a} \pm 2.61$
TSP (mg/g)	$3.56^{b} \pm 0.22$	$3.40^{b} \pm 0.23$	$4.10^{a} \pm 0.10$	$3.61^{b} \pm 0.10$	$4.30^{a} \pm 0.20$	$3.68^{b} \pm 0.30$	$2.86^{c} \pm 0.08$	$2.50^{d} \pm 0.09$
Pigments ($\mu g \cdot g^{-1}$)								
Chlorophyll a	$25.91^{a} \pm 0.27$	$22.89^{b} \pm 0.39$	$21.83^{c} \pm 0.51$	$18.65^{e} \pm 0.76$	$18.50^{e} \pm 0.53$	$20.16^{d} \pm 0.62$	$20.65^{d} \pm 0.36$	$20.13^{d} \pm 0.60$
Chlorophyll b	$26.44^{a} \pm 0.21$	$23.86^{c} \pm 0.43$	$24.63^{bc} \pm 0.56$	$24.63^{d} \pm 0.76$	$22.17^{d} \pm 0.55$	$24.15^{c} \pm 1.06$	$26.28^{a} \pm 0.61$	$25.83^{ab} \pm 1.02$
Total chlorophyll	$52.35^{a} \pm 0.43$	$46.75^{b} \pm 0.79$	$46.46^{bc} \pm 1.05$	$46.46^{d} \pm 1.39$	$40.67^{d} \pm 1.07$	$44.31^{b} \pm 1.69$	$46.93^{b} \pm 0.97$	$45.96^{bc} \pm 1.60$
Total carotenoid	$24.23^{a} \pm 0.10$	$21.63^{d} \pm 0.28$	$22.42^{b} \pm 0.13$	$18.08^{g} \pm 0.16$	$17.51^{h} \pm 0.08$	$18.77^{f} \pm 0.09$	$19.10^{e} \pm 0.07$	$21.90^{c} \pm 0.04$
Instrumental color								
Lightness (L)	$62.07^{a} \pm 0.38$	$61.03^{b} \pm 0.60$	$60.82^{bc} \pm 0.40$	$61.20^{ab} \pm 0.56$	$61.25^{ab} \pm 0.24$	$59.85^{d} \pm 0.47$	$59.97^{cd} \pm 0.31$	$61.45^{ab} \pm 0.80$
Redness (a^*)	$-5.51^{f} \pm 0.30$	$-4.74^{e} \pm 0.16$	$-4.13^{d} \pm 0.43$	$-3.83^{cd} \pm 0.17$	$-3.54^{bc} \pm 0.15$	$-3.37^{bc} \pm 0.38$	$-3.04^{b} \pm 0.47$	$-0.38^{a} \pm 0.06$
Yellowness (b^*)	$32.04^{a} \pm 0.25$	$30.13^{b} \pm 0.33$	30.48b ± 0.18	$30.77^{b} \pm 0.50$	$30.04^{bc} \pm 0.58$	$30.39^{b} \pm 0.34$	$29.26^{cd} \pm 0.65$	$28.49^{d} \pm 0.57$
Chroma (C^*)	$32.51^{a} \pm 0.29$	$30.50^{b} \pm 0.35$	30.76b ± 0.23	$31.00^{b} \pm 0.52$	$30.24^{b} \pm 0.56$	$30.57^{b} \pm 0.35$	$29.42^{c} \pm 0.70$	$28.49^{d} \pm 0.57$

Values (mean ± SD) in a row without the same superscript letter differ statistically ($p < 0.05$).

samples had no statistically significant differences at the treatment durations of 90 s, 120 s, and 150 s; however, they had greater MDA content than plasma-treated samples at shorter treatment durations. There are a few reports that have investigated the effect of cold plasma on lipid oxidation in nuts and nut products. Bagheri et al. [28] reported an increment of peroxide values of military ration snacks by increase in the voltage of generating the plasma device. Thirumdas et al. [34] indicated that plasma treatment of walnuts and peanuts at different powers and time intervals led to increase in the peroxide value (20%) of the samples. Ahangari et al. [19] found that peroxide value of plasma-treated walnuts (50 W for 15 and 20 min) slightly increased, but no differences were observed between the plasma-treated and control samples. This increase in MDA content can be because cold plasma reactive species can initiate lipid oxidation by attacking various compounds including unsaturated fatty acids and increasing the MDA content of plasma-treated food products [35].

3.4. Chlorophylls and Carotenoids. The pigment content data of pistachio nuts submitted to different durations of cold plasma are set out in Table 1. The results suggested that the amount of different pigments in pistachio samples decreased with the duration of the plasma treatment. The results revealed that chlorophyll a and b significantly reduced after plasma exposure ($p > 0.05$). However, chlorophyll a reduction was greater than chlorophyll b. The lowest amount of chlorophyll a (28%) and chlorophyll b (17.6% and 16.1%, respectively) was observed after 60 s and 90 s of the process, respectively. Longer treatment durations (120, 150, and 180 s) showed lower amount of chlorophyll a than the control sample, but there was no significant difference between the plasma-treated samples. Chlorophyll b showed lower values than the control at the treatment duration of 120 s, but no significant difference was observed after the treatment durations of 150 s and 180 s compared to the control sample. Also, the total chlorophyll content was reduced by the duration of plasma exposure. Total chlorophyll content significantly reduced with the increase of plasma treatment duration of 90 s. Total chlorophyll reduction after treatment durations of 60 s and 90 s was 24% compared to the control. Treatment durations of 120 s showed lower amount of total chlorophyll than the control, but there was no remarkable difference between the treated and control samples at the treatment times of 150 s and 180 s. In general, increasing the plasma treatment durations to at least 90 s resulted in significant reduction in total chlorophyll. From data in Table 1, it is apparent that total carotenoids content of plasma-treated pistachio nuts reduced significantly as the plasma treatment increased to 90 s ($p < 0.05$), and the lowest amount of total carotenoids was observed after the treatment durations of 60 s and 90 s. Generally, the duration of plasma treatment to 90 s led to a reduction in total content of carotenoids. The reduction in total carotenoids after these treatment durations was about 25% compared to the control. Ramazzina et al. [32] reported a 15% decrement in chlorophyll a right after applying an air DBD plasma treatment to the kiwifruit samples. In this study, chlorophyll and carotenoid content showed a significant decrease after storage time. Beyer et al. [20] observed that air plasma treatment reduced the chlorophyll a concentration of spirulina algae powder. It has been suggested that

chlorophyll degradation upon plasma treatment can be related to pigment oxidation by plasma reactive species and/or denaturation of enzymes responsible for chlorophyll catabolism during the plasma treatment [32].

3.5. Impact of Cold Plasma on Instrumental Color. The results of different plasma treatment durations on instrumental of pistachio nuts color are presented in Table 1, showing that the L^* value decreased with an increase in plasma treatment times. This parameter significantly reduced after 120 s and 150 s of plasma exposure ($p < 0.05$), but it remained unchanged at other exposure durations. The b^* index slightly decreased during plasma treatment compared to the control sample, while a^* values increased considerably after treatment durations of longer than 120 s. Parameter C^* or chroma also decreased with the increase of the treatment duration, but this reduction was not significant between the samples treated with treatment durations of less than 120 s. This parameter remarkably decreased after the treatment durations of 150 s and 180 s. This overall color changes during plasma treatment resulted in darker pistachio nuts color at longer cold plasma treatment durations due to Maillard reactions and/or chlorophyll and carotenoids degradation. The breakdown of glycosidic and peptidic bonds during radiation led to the formation of breakdown products such as carbonyl and amino compounds, which induced the Maillard reactions and, finally, formed colored compounds [3]. In a similar study, a decrease in L^* and b^* of walnut samples after plasma treatment (20 min and 40 W) was observed [19]. Hertwig et al. [36] found a decrement in L^*, b^*, and chroma parameters after cold plasma treatment resulted in darker almond samples.

3.6. Total Soluble Protein. The effect of different plasma durations on total soluble protein of pistachio nuts is illustrated in Table 1. All the data were significantly different ($p < 05.0$). Total soluble protein showed an increment with the increase of the plasma exposure time to 90 s, and the highest amount of total soluble protein was observed after 30 s and 90 s. No differences were shown between the protein solubility of control and plasma-treated samples after the treatment durations of 15 s, 60 s, and 120 s. Plasma treatment durations of longer than 90 s resulted in a decrease in protein solubility, so that the lowest protein solubility was observed after 180 s and 150 s compared to the control sample. Nonthermal plasma contains a wide range of reactive species (ROS, RNS, and UV photons) that can attack the protein backbone and lead to alteration and unfolding of the secondary and tertiary structures of the protein. Consequently, the interaction between hydrophobic groups of unfolded proteins resulted in aggregation, followed by coagulation and precipitation inducing changes in chemical properties of the proteins such as decrease in protein solubility [37, 38]. Bubler et al. [38] observed that the protein solubility of *Tenebrio* flour declined after cold atmospheric pressure plasma (CAPP) treatment. Similar results were reported by Ekezie et al. [39]: protein solubility of king prawn samples decreased with increasing the plasma treatment time. In this study, the authors explained that atmospheric pressure plasma jet (APPJ) treatment can expose the hydrophobic groups to the protein surface by unfolding both secondary and tertiary structures of the protein molecules. Interaction of this hydrophobic groups led to protein agglomeration and induced a reduction in protein solubility [39].

3.7. Electrophoretic Pattern of Proteins. Impact of different durations of plasma treatment on soluble protein patterns of pistachio nuts is provided in Figures 3 and 4. In accordance with Figure 3, a variety of protein bands were identified in a molecular weight range from 5 to 204 kDa and 6 to 236 kDa in reduced and nonreduced gels for all the samples, respectively. According to the results and presence of low molecular weight peptides in the reducing condition, the presence of β-mercaptoethanol in reducing conditions led to the breakdown of the polypeptides disulfide bonds and converted them into smaller subunits. Nonreducing condition (without β-mercaptoethanol) did not change the proteins' disulfide bonds (Figure 3). Comparing polyacrylamide gel in both nonreducing and reducing conditions showed slight differences in the pattern of protein band due to plasma treatment. According to the densitometry analyses of protein bands, with the increase of exposure time, the intensity of protein bands in nonreducing gel decreased in comparison with the control sample (Figure 4). Nevertheless, these decreasing changes were not the same in the intensity of all protein bands, and the intensity of some of the protein bands increased as a result of plasma treatment. For example, protein bands in the molecular weight of 7 kDa increased at all the treatment durations. The plasma treatment of 30 s showed the lowest intensity of pistachio protein bands in pistachio samples (Figure 4). These results can be related to the results of protein solubility (Table 1), because plasma reactive species may lead to the alteration of protein structure and/or creation of new proteins through cross-linkage of free amino acids to the protein and protein-protein aggregation and reduce the solubility [40]. Results of a similar study conducted by Alinezhad et al. [3] demonstrated that gamma irradiation (1–6 kGy) altered the pattern and intensity of pistachio protein; the intensity of protein bonds was declined in all pistachio nut samples, while gamma irradiation at dose of 4 kGy showed an increase in the intensity of pistachio protein bands. Similar reports were stated by Meinlschmidt et al. [40] who indicated that CAPP (1, 2.5, 5, 7.5, and 10 min) reduced the intensity of soy bean protein bonds corresponding to Gly m5 allergen. On the contrary, Ekezie et al. [39] reported that ATPJ (2–10 min) had no impact on the intensity of king prawn protein bands in both reducing and nonreducing conditions.

3.8. HPLC Analysis of Aflatoxin B_1. The cold plasma effect on AFB_1 concentration is shown in Table 2. AFB_1 was extracted with 80% methanol solution from glass slides contaminated with the same concentration of pure toxin and was used to determine the AFB_1 residual by means of a HPLC method. The chromatograms of pure AFB_1 (left) and contaminated pistachio nuts (right) treated at different durations (0, 60,

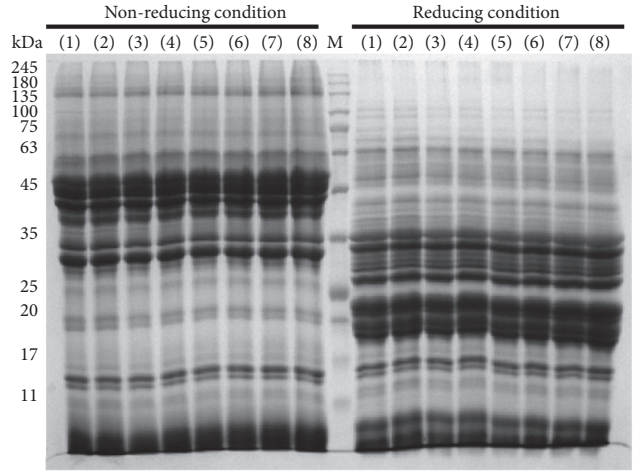
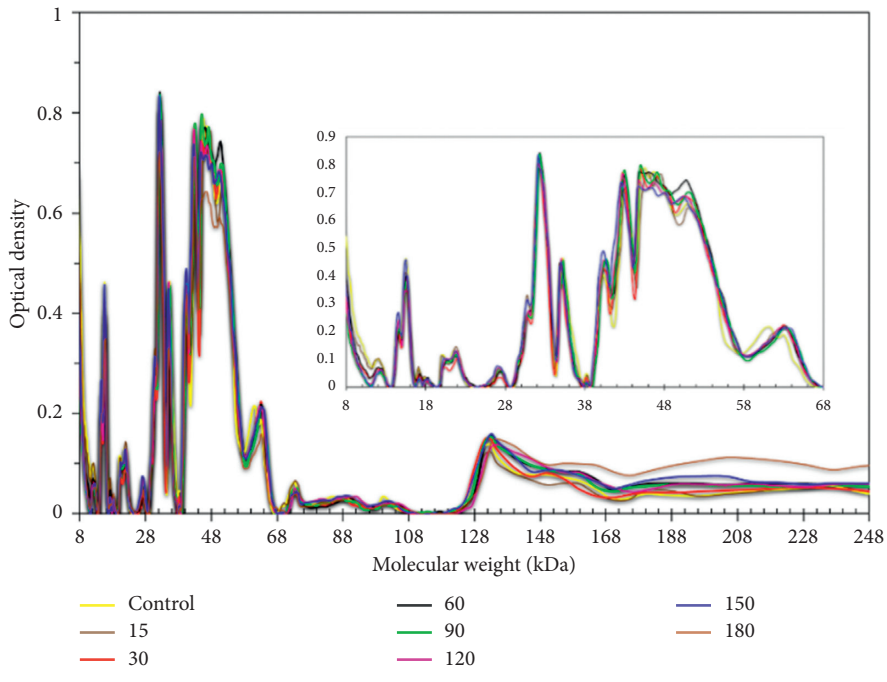


Figure 3: Profiles of proteins from nontreated (lane 1) and treated pistachios with different durations of cold plasma (lane numbers of 2, 3, 4, 5, 6, 7, and 8 for 15, 30, 60, 90, 120, 150, and 180 sec, respectively) under reducing and nonreducing conditions. "M" shows a molecular weight marker (SinaClon BioScience, prestained protein ladder, PR901641).



(a)

Figure 4: Continued.

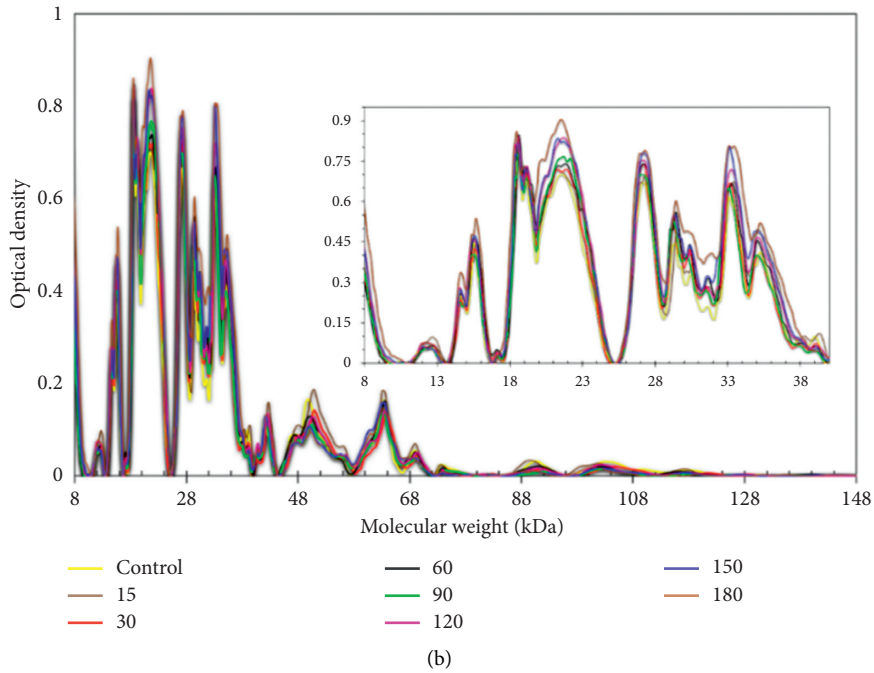


Figure 4: The densitometric analysis of the SDS-PAGE profile of soluble proteins extracted from untreated (0.0 sec) and treated (15–180 sec) pistachios nuts with different durations of cold plasma in nonreducing (a) and reducing conditions (b).

Table 2: Effect of cold plasma (0–180 sec) on aflatoxin B_1 concentration (ppb) in pure toxin and contaminated pistachio nut powder.

Aflatoxin B1 concentration (ppb)	Time of plasma treatments (sec)			
	0	60	120	180
Pure toxin	$710.38^{a} \pm 0.93$	$458.89^{b} \pm 0.88$	$277.27^{c} \pm 1.06$	$251.32^{d} \pm 0.88$
Pistachio nut powder	$383.19^{a} \pm 0.38$	$259.39^{b} \pm 1.11$	$211.64^{c} \pm 1.70$	$182.32^{d} \pm 1.78$

Values (mean ± SD) in a row without the same superscript letter differ statistically ($p < 0.05$).

120, and 180 s) are illustrated in Figure 5. The increment of the plasma exposure time significantly decreased the AFB_1 concentration in plasma-treated samples, as shown in Table 2. The concentration of AFB_1 remarkably decreased after 60 s and 120 s of the plasma treatment. A reduction of 35.41%, 60.97%, and 64.63% was observed in contaminated glass slides after 60 s, 120 s, and 180 s of the treatment, respectively. This rate of reduction in AFB_1-contaminated pistachio powder samples was 32.31%, 44.77%, and 52.42%, respectively. According to the results, the rate of AFB_1 reduction in pistachio nut samples was less than the AFB_1-contaminated glass slides. This could be the result of complex matrix of pistachio nuts compared to the glass slides, which limited the AFB_1 decontamination process by cold plasma treatment. Basaran et al. [13] investigated the effect of LP plasma on the reduction of Aflatoxins on three different nuts (hazelnut, pistachio nut, and peanut). The results of the investigation represented that the AFB_1 concentration in hazelnut samples decreased from 15.75 to 1.74 ng/g (88.95%) after 20 min of air plasma treatment. Sen et al. [9] reported that AP and LP plasma treatments on contaminated hazelnuts reduced the concentration of AFB_1 by about 72% and 73%, respectively. In another report, 83.2% of decrement in AFB_1 after atmospheric nitrogen plasma treatment of contaminated hazelnuts (400 W and 4 min) was reported [7]. Devi et al. [6] observed 65% and 95% reduction in AFB_1 on air plasma-treated groundnuts with the power of 60 W and 12 min and 40 W and 15 min, respectively. Plasma generated reactive species (ROS, RNS, free radicals, excited particles, and UV radiation) can effectively degrade mycotoxins including aflatoxins [9]. There are some factors that affect mycotoxin degradation upon cold plasma treatment such as molecular structure of toxins, plasma chemistry, and interaction between plasma reactive species and toxin molecules [28]. It has been stated that plasma treatment is capable of AFB_1 degradation by the ozonolysis of the AFB_1, and it is due to the opening of terminal furan ring by the reaction of the ozone with C_8=C_9 double band. Also, modifying the lactone ring structure with the opening of lactone ring (disappearance of the C_8=C_9 double band) and blocking AFB_1-exo-8,9-epoxide formation led to complete or partial degradation of the toxin to nontoxic or lower toxic materials [7]. However, in this study, the breakdown products of AFB_1 after DBD plasma treatment were not specified. The results of the present work showed that although cold plasma treatment may not be able to induce complete elimination of AFB_1, it can effectively reduce the AFB_1 concentration in pistachio samples.

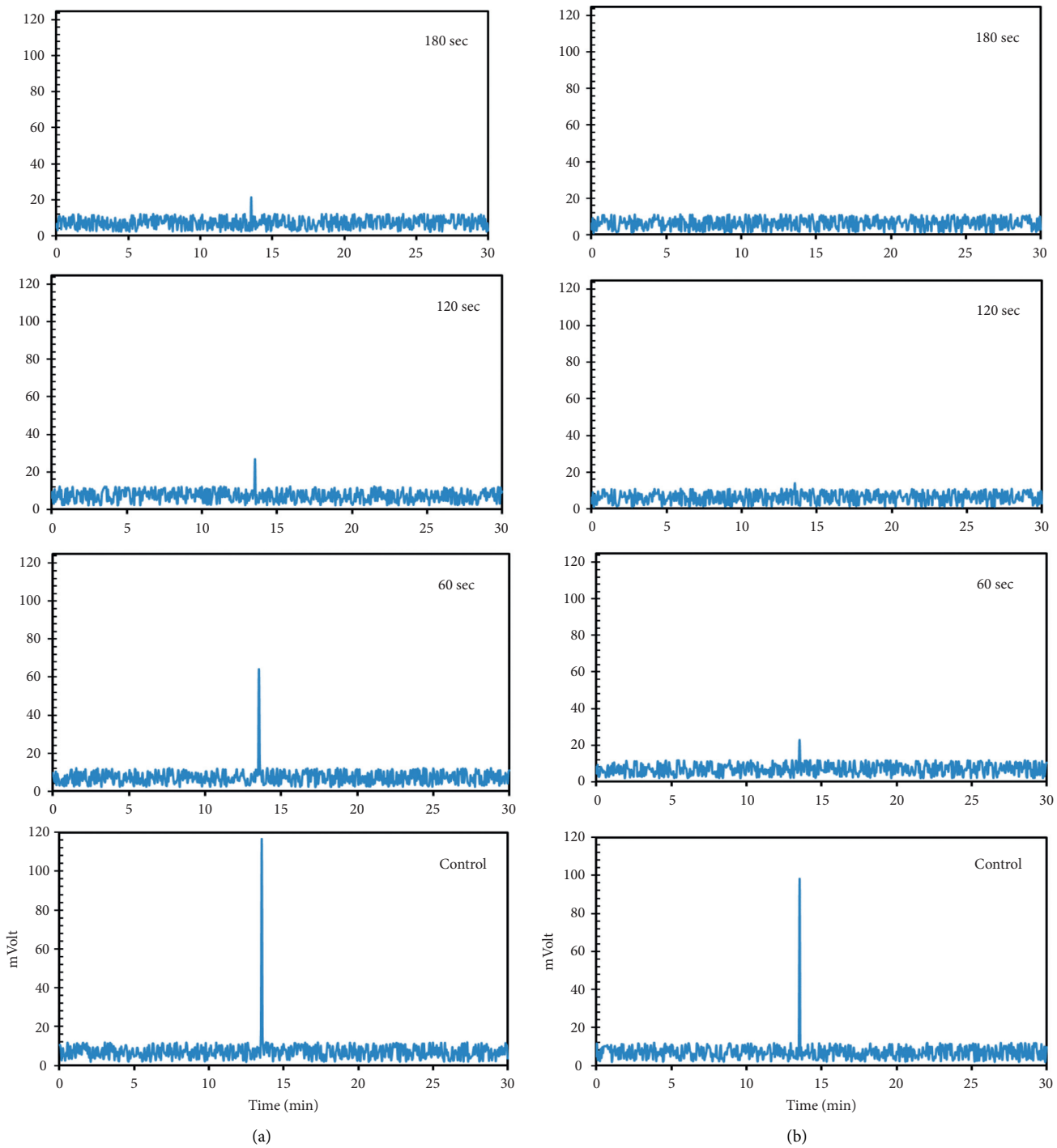


Figure 5: Chromatograms: effect of cold plasma (0–180 sec) on detoxification of pure aflatoxin B_1 (a) and pistachio nut powder (b) contaminated with aflatoxin B_1.

4. Conclusion

Investigating the effect of different durations of DBD cold plasma treatment (15, 30, 60, 90, 120, 150, and 180 s) on the inactivation of *A. flavus*, reduction in AFB_1 mycotoxin, and quality attributes of pistachio nuts demonstrated that cold plasma significantly decreased the viable spore population of *A. flavus* by increase in the treatment duration; after 180 s of plasma exposure, the spores were not detectable (4 log). The TPC of pistachios were not affected by the plasma. The antioxidant activity was marginally promoted, but there was no significant difference between plasma-treated samples at the treatment durations of longer than 60 s. The MDA values raised with the increase of the treatment duration and showed the highest increment after the treatment duration of 180 s. Plasma exposure resulted in lower levels of

chlorophyll a, b, and total carotenoids content. Color parameters were affected by the plasma treatment, and plasma treatment led to darker pistachio nuts. Plasma treatment reduced protein solubility and altered the pattern and intensity of proteins in the pistachio nuts. The maximum reduction in AFB_1 concentration was observed after the treatment duration of 180 s; the reduction rate of this toxin at this time interval for AFB_1-contaminated glass slides and pistachio nut samples was 64.63% and 52.42%, respectively. Plasma treatment had several positive and negative effects on quality characteristics of plasma-treated pistachio nuts. However, plasma treatment induced a smaller amount of quality changes than other decontamination traditional technologies. The results of this experiment showed that cold plasma treatment can be used efficiently for decontamination of nuts. Further work is recommended to investigate the impact of various plasma generating systems with different input power, voltage, frequency, working gas, and flow rate alongside with exposure duration on the decontamination of *A. flavus* or other toxigenic fungus and their toxins, and/or quality attributes of pistachio nuts or other food products.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors notify that they have no conflicts of interest.

Authors' Contributions

M. Makari carried out all the experimental tasks and wrote the first draft of the manuscript. M. Hojjati conceptualized the study, developed the methodology, performed formal analysis and investigation, edited the manuscript, and supervised all the analyses. S. Shahbazi conceived the research and contributed to the design and coordination. H. Askari carried out all the experimental tasks and contributed to designing the research methodology and data analysis.

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