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Decontamination of Red Pepper Using Cold Atmospheric Pressure Plasma as Alternative Technique

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Abstract

Background and objective: Non-thermal methods are suggested for decontamination of spices to preserve safety and quality of the products. In this study, effects of atmospheric pressure floating-electrode dielectric-barrier discharge plasma were investigated on red pepper powder, compared to gamma irradiation.

Material and methods: To achieve the optimum time of treatment for decontamination, *Escherichia coli*, *Bacillus cereus* and *Aspergillus flavus* as microorganisms in red pepper were exposed to atmospheric pressure floating-electrode dielectric-barrier discharge plasma for 10, 20 and 30 min and the structural changes in microorganisms were investigated using scanning electron microscopy and DNA measurement following exposure. The red pepper was exposed to plasma for 20 min (optimum time) and 10 KGy gamma irradiation. Microbial count, color measurement and sensory evaluation of the samples were assessed before and after treatments.

Results and conclusion: Results indicated that the density of surviving bacterial strains decreased when time of exposure increased and this decrease was significant after 10 min ($P \leq 0.05$). The complete decontamination was carried out within 20 min. The deformation of cells and destruction of cell wall structures were seen in bacteria and mold following exposure. Data revealed that cold floating-electrode dielectric-barrier discharge plasma for 20 min inactivated red pepper microorganisms as well as gamma irradiation. As a conclusion, floating-electrode dielectric-barrier discharge plasma is an appropriate method to decontaminate the red pepper powder (regardless of color change) and can replace traditional methods without changes in the product quality and taste.

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1. Introduction

Spices and herbal products are used as flavors, aromas and colors in food products. Spices and herbs may contain large quantities of bacteria and molds. If many bacteria are present in spices, they can result in food poisoning and rapid spoilage of foods. Red pepper (*Capsicum annuum* L.) is used worldwide in dried powder or fresh form as food additives [1]. Red pepper powder is one of the most valuable and widely used spices, because of its distinctive flavor and aroma and medicinal and therapeutic properties [2,3]. This spice is often contaminated with high levels of micro-organisms. Pathogenic microorganisms found on the surface or inside of the red pepper include *Escherichia* (*E.*) *coli*, *Aspergillus* (*A.*) *flavus*, *Bacillus* (*B.*) *cereus*, *Clostridium* (*C.*) *perfringens* and *Staphylococcus* (*S.*) *aureus* [4,5]. This can potentially create public health risks and problems. Because of the importance of red pepper, use of an effective decontamination method without changing in quality of the spice is necessary.

Conventional methods for sterilization such as fumigation with ethylene oxide, irradiation with ionizing radiation and treatment with super-heated steam and ultraviolet (UV) are used in red pepper powder decontamination [6]. However, each of these methods includes disadvantages such as oxidation of most aromatic components of the spices, loss of flavor and color and decreased qualities of fatty acids and vitamins [7,8]. One

of the sterilization methods recently used is atmospheric-pressure low-temperature plasma. Cold plasma sterilization technique is used to decontaminate a wide variety of heat-sensitive instruments [9]. Low-temperature plasma consists of highly energetic species, free radicals, UV photons, electrons, negative and positive ions, excited atoms and molecules and UV radiation, which are able to deactivate and kill bacteria, viruses and other microorganisms without significant temperature effects [10-12].

Floating-electrode dielectric-barrier discharge (FE-DBD) is a convenient plasma source for the generation of non-thermal plasma at atmospheric pressure. Recently, atmospheric pressure plasma is used in decontamination of spices, herbs, seeds and dehydrated vegetables [13]. Therefore, objectives of this experimental study were to 1) investigate microbial inhibition effects of FE-DBD plasma on *A. flavus*, *B. cereus* and *E. coli* as microbes found in red pepper powder; 2) optimize treatment conditions; and 3) microbial decontamination of red pepper powder using atmospheric pressure FE-DBD plasma, compared to gamma irradiation as conventional method.

2. Materials and methods

1.1. Microbiological analysis and colony counting

Two strains of bacteria and a fungus were provided by Persian Type Culture Collection of the Iranian Research Organization for Science and Technology (IROST), including *E. coli* PTCC 1399, *B. cereus* PTCC1247 and *A. flavus* PTCC 5004. The microbial strains were cultivated in sterile media and incubated at appropriate time and temperature. Then, the microbial biomass was centrifuged at 1860 ×*g* for 15 min. The microbial cells were treated with FE-DBD plasma (output voltage of 205-240V AC and frequency of 13.56 MHz). The microbial count was recorded before and after treatments with DBD plasma (at 10, 20 and 30 min intervals). The *E. coli* was cultured in eosin-methylene-blue agar at 37ºC for 24 h, according to ISO No. 7251 standard [14]. The *B. cereus* was prepared by growing cells in mannitol yolk polymyxin at 30ºC for 24 h, according to ISO No. 7932 standard [15]. The *A. flavus* was prepared by growing cells in dichloran-glycerol selective media at 25ºC for 5-7 days, according to ISO No. 21527-2 standard [16]. The colony forming units was calculated per milliliter of each sample based on the following equation of $N = \sum C / \{(n_1 \times 1) + (n_2 \times 0.1)\} d$; where, N was the number of colonies per milliliter of the product, $\sum C$ was sum of all colonies in all plates counted, n_1 was number of plates in lower dilution counted, n_2 was number of plates in higher dilution counted and d was dilution level corresponding to first count.

1.2. Scanning electron microscopy

The Scanning electron microscopy (SEM) was used to investigate possible effects of FE DBD plasma on cell structure of the experimental microorganisms. Briefly, *E. coli*, *B. cereus* and *A. flavus* samples were cultured in Brain Heart Infusion broth and then centrifuged at 6,000 ×g for 15 min. After centrifugation, the aqueous phase layer was collected and the pellet was transferred to a cover glass and exposed to FE-DBD plasma for 20 min. The control sample was not subjected to plasma treatment. Control and treated bacterial pellets were fixed in 2.5% glutaraldehyde for 24 h and dehydrated in series of increasing alcohol concentrations (30, 50, 70 and 95 for 15 min and 100 for 1 h). After drying, samples were coated with gold and SEM was carried out according to a protocol described by Lia et al. [17].

1.3. DNA measurement

The DNA concentration was calculated to assess the bacterial destruction rate. Concentrations of double-stranded DNA in supernatant of the bacteria were suspended in normal saline before and after the exposure to atmospheric pressure FE-DBD plasma using Nano Drop 1000 Spectrophotometer (Thermo Scientific, USA). To calculate concentrations of DNA, 50 µl of the bacterial suspension with a concentration of 0.5 McFarland were mixed with 100 µl of normal saline and was analyzed using Nano Drop 1000 Spectrophotometer (Thermo Scientific, USA).

1.4. Exposure system

The instrument used to produce low-temperature atmospheric-pressure plasma consisted of an electrode (diameter of electrode was 5 cm and electrode charge was negative) and a radiofrequency power supply. The atmospheric pressure FE-DBD plasma was generated using a radio frequency discharge of 13.56 MHz, variable voltages and output powers from 0 to 1000 W.

1.5. Exposure protocol

In general, 1 g of the red pepper powder was spread on a round paper as a single layer and exposed to the plasma for 20 min. To investigate the colonies, the red pepper powder was mixed with 9 ml of sterile normal saline in a tube and vortexed vigorously. A serial dilution of 10^{-1}-10^{-4} of the supernatant was used for the culture. Then, 1 ml of each dilution was cultured on nutrient agar using pour plate method. Number of colonies was counted after 24 h of incubation at 37°C based on ISO 4833: 20139 [18]. *E. coli* was enumerated in Eosin-Methylen-Blue Agar for 24 hours at 37ºC based on ISO 7251[14]. *B. cereus* was prepared in Mannitol Yolk Polymyxin agar according to ISO No. 7932 [15]. *A. flavous* was enumerated in Dichloran-Glycerol selective medium according to ISO No. 21527-2[16]. Enumeration of coliforms was cultured in Crystal Violet Neutral Red Bile Lactose agar based on ISO No. 4832: 2006[19]. *C. perfringens* was cultured in Egg-yolk-free

Tryptose Sulfite Cycloserine agar according to ISO No.7937: 2004 [20].

1.6. Gamma irradiation

To compare results of FE-DBD with gamma radiation, 1 g of the red pepper powder packaged in a vacuumed plastic zipped pack was exposed to 10 KGy gamma radiation using gamma cell 220 with a cobalt irradiation source of 60 and then colony formation was investigated.

1.7. Color measurement

The sample color was measured using digital camera (Canon Power Shot A540, Japan). The color value of samples was analyzed five times. Three parameters of the color, including lightness (L*), redness (a*) and yellowness (b*), were analyzed to evaluate color changes in the samples. The L* ranged from black to white (0-100), a* ranged from green (negative value) to red purple (positive value) and b* ranged from yellow (positive value) to blue (negative value). To analyze L*, a* and b* parameters and compare sample colors in Photoshop Software, digital images were captured in a constant and uniform condition of lightning and angle of camera (low voltage halogen lamp with reflector) and stored in bitmap graphic format with 8-bit pallet (28 = 256 colors). The total color difference (ΔE) was calculated from ΔL (L-L*), Δa (a-a*) and Δb (b-b*) values based on the following equation of $\Delta E_{L^*a^*b^*} = [(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2]^{1/2}$. The L*, a* and b* were associated to color parameters of untreated red paper powder samples. Color changes in the red-green-blue color model were calculated using image analysis [21].

1.8. Sensory evaluation

Sensory analysis was evaluated by 15 trained panelists using 5-point hedonic scales (1 was the lowest and 9 was the highest score). The panelists evaluated treated spice samples for overall acceptance [22].

1.9. Statistical analysis

Statistical analysis was carried out using SPSS Statistical Software v. 16.0 (SPSS, Chicago, IL, USA). Comparison of data between the control and exposed samples was carried out using one-way analysis of variance test. Each experiment was repeated five times. Data were expressed as mean ±SD (standard deviation). A P-value ≤ 0.05 was considered statistically significant.

3. Results and discussion

In this study, effects of atmospheric pressure FE-DBD plasma on red pepper powder were investigated, compared to gamma irradiation as conventional method. To calculate the best atmospheric pressure FE- DBD plasma treatment time, average microbial counts of *E. coli*, *B. cereus* and *A. flavus* were calculated after treatment. As shown in Fig. 1, density of the surviving microorganisms decreased when the time of exposure increased. The microbial count decreased significantly after 10 min (P≤0.05). A much higher decrease was achieved for *E. coli*, a Gram-negative bacteria, while *A. flavus* showed further resistance. Complete decontamination of *E. coli*, *B. cereus* and *A. flavus* was achieved within 20 min (optimum time). Effects of plasma on microbial log decrease of *E. coli*, *B. cereus* and *A. flavus* at 10 min is shown in Fig. 1. Results indicated that the density of surviving microorganisms significantly decreased (P≤0.05). The lowest microbial log decrease was seen in *A. flavus*, while *E. coli* was the most susceptible microorganism to plasma treatment (Fig. 1). Cell wall of the Gram-positive bacteria includes a thickness of nearly 20-80 nm, while Gram-negative bacteria include a relatively thin cell wall (<10 nm), including an outer membrane with a complex layer. The difference in cell walls of Gram-positive and Gram-negative bacteria induces different properties, especially in response to external stresses such as plasma treatment. Plasma disrupts the structure of peptidoglycans in the cell wall of Gram-positive bacteria, while inactivation of Gram-negative bacteria can be due to the destruction of membrane lipid layers by plasma bombardment. Leakage of the cellular components such as potassium, nucleic acids and proteins is happened following the cell wall destruction [23]. The *A. flavus*, a microbial contaminant of paprika, is able to produce aflatoxin, a mycotoxin with carcinogenic effects [24]. The current results showed that the plasma could deactivate *A. flavus* within 20 min. Charged and excited species, reactive neutrals and UV radiations are factors that determine plasma sterilization process. Furthermore, several other factors can affect the killing process, including type of exposure, contribution of UV, operation of gas mixture, type of media, species of microorganism and number of cell layers.

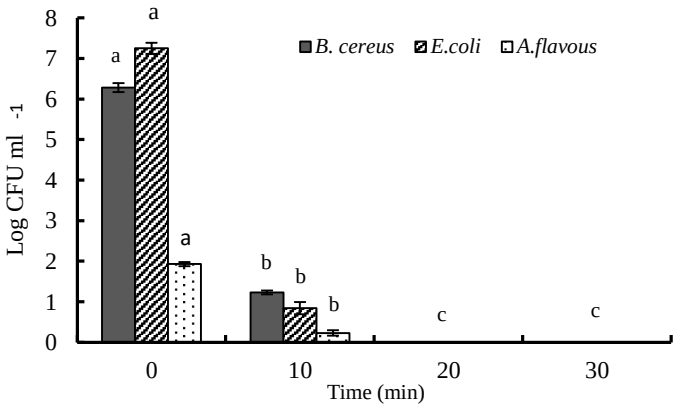

Figure 1. Microbial counts of *Escherichia coli*, *Bacillus cereus* and *Aspergillus flavus* after treatment with FE-DBD plasma for 0, 10, 20 and 30 min.
Different letters show significant differences (P≤0.05).
FE-DBD plasma =Floating-electrode dielectric-barrier discharge

Table 1. Measurement of DNA concentration after exposure to Floating-electrode dielectric-barrier discharge plasma, compared to control

Exposure time (min)	*E. coli*	*B. cereus*
0	52.5 ± 2.4[a]	47.3 ± 1.4[a]
10	67.4 ± 6.9[b]	149.4 ± 9.8[b]
20	132.3 ± 5.7[b]	197.4 ± 7.2[b]

Data are presented as mean ±SD from five separate experiments. Different letters in columns show significant differences (P≤0.05). *E. coli= Escherichia coli, B. cereus= Bacillus cereus*

In this study, SEM was used to investigate morphological changes in microorganisms following the exposure to EF-DBD plasma. Figure 2 (a-i) shows SEM images of *E. coli*, *B. cereus* and *A. flavus* after exposure to cold atmospheric FE-DBD plasma for 10 and 20 min, compared to controls. The images of *E. coli* and *B. cereus* and *A. flavus* supported results of microbial analysis, showing deformation and destruction of bacterial and mold cells after treatment with plasma. Morphological changes and wrinkling of the cell wall structure of bacteria and molds were seen after 10 min of plasma treatment. However, tendency of bacteria and molds to aggregate was seen after 10 min of treatment with plasma. Some of the cells were completely destroyed following exposure to plasma for 20 min. FE-DBD plasma treatments for 20 min resulted in complete rupture of the bacterial and mold membranes, leakage of the cytoplasm materials and agglutination of the cells. Cell membrane deformations and leakage of the bacterial chromosome were observed in microbial cells treated with atmospheric pressure plasma [23]. Hueso et al. reported that exposure to Ar-NO plasma could lead to destruction and lysis of *E. coli* [25]. It is well established that pulsed electric fields induce electroporation of the cell membranes. Furthermore, it seems that plasma acts in a similar way to induce membrane perforations in microorganisms [26-28]. In the present study, bacterial and mold cells appeared intact and separated from each other before treatment with plasma, while bacterial and mold cells exposed to plasma appeared to be aggregated. Aggregation of the bacterial cells may be a result of the response to stress due to antibacterial agents or deformation of the cell surfaces subjected to treatment with plasma. Morphological changes in *E. coli* cells treated with lemon grass oil were reported by Tyagi and Malik [29]. They reported the aggregation of bacterial cells following leakage of the cytoplasm. Tang et al. investigated aggregations in *E. coli* and *Shewanella oneidensis* after addition of fullerene compounds [30].

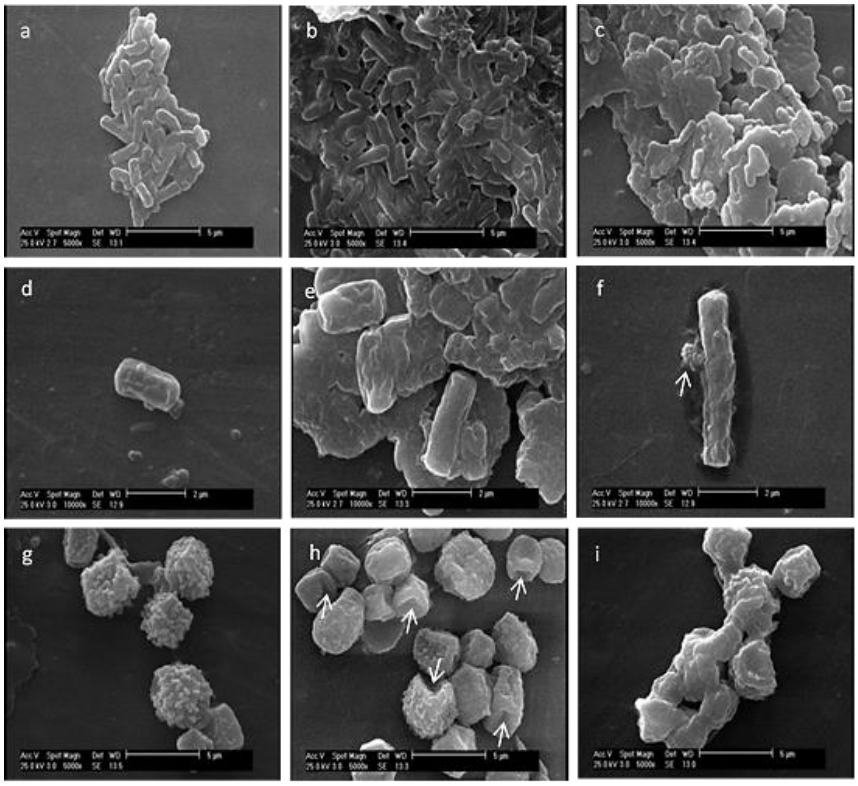

Figure 2. Scaning electron microscopy images of *E. coli*, *B. cereus* and *A. flavous* before and after exposure to FE-DBD plasma. **a,** *E. coli* without exposure; **b,** *E. coli* after 10 min of exposure; **c,** *E. coli* after 20 min of exposure; **d,** *B. cereus* without exposure; **e,** *B. cereus* after 10 min of exposure; **f,** *B. cereus* after 20 min of exposure (a rupture of the bacterial membranes and leakage of the cytoplasm materials are shown by arrows); **g,** spores of *A. flavus* without exposure; **h,** spores of *A. flavus* after 10 min of exposure (morphological changes and wrinkling of the mold cell wall structure are shown by arrows); **i,** spores of *A. flavus* after 20 min of exposure

E. coli= Escherichia coli, B. cereus= Bacillus cereus, A. flavous= Aspergillus flavus
Floating-electrode dielectric-barrier discharge= FE-DBD plasma, Scaning electron microscopy =SEM

The current results showed that the density of surviving microorganisms in red pepper powder decreased to acceptable levels under the exposure of FE-DBD plasma. To assess the membrane destruction rate of bacteria caused by the plasma treatment, concentrations of DNA in supernatant of the bacteria were suspended in normal saline before and after treatments. Concentrations of DNA after treatment with plasma for 10 and 20 min are shown in Table 1 in comparison to controls. The DNA concentrations were calculated from the absorbance at 260 nm. The present results demonstrated that the concentrations of DNA increased after 10 and 20 min of treatments with plasma ($P \leq 0.01$). These results supported results from SEM. It can be concluded that increase of DNA concentration may be resulted from bacterial cell wall destruction by electrostatic force and leaked cytoplasm materials.

Table 2. Logarithm of the microbial population of red pepper powder under exposure to plasma for 20 min and gamma radiation

Microorganism	Control	Cold plasma (20 min)	Gamma irradiation
E. coli	2.86 ± 0.25^{A}	0 ± 0^{B}	0 ± 0^{B}
B. cereus	1.02 ± 0.2^{A}	0 ± 0^{B}	0 ± 0^{B}
A. flavus	1.53 ± 0.65^{A}	0 ± 0^{B}	0 ± 0^{B}
Coliform	3.47 ± 0.15^{A}	0 ± 0^{B}	0 ± 0^{B}
C. perfringens	0 ± 0^{A}	0 ± 0^{A}	0 ± 0^{A}

Data are presented as mean ±SD from five separate experiments.
Different letters in rows show significant differences ($P \leq 0.05$).
E. coli= Escherichia coli, B. cereus= Bacillus cereus, A. flavus= Aspergillus flavus, C. perfringens = Clostridium perfringens

Direct effects of external electric fields on the sterilization and inactivation of microorganisms in plasma are usually negligible, while effects of electric fields associated with movement and accumulation of charged particles are important [31]. Accumulation of charged particles plays a significant role in breaking outer membranes of the bacterial cell electrostatic forces produced by charged particles and stored on the external surfaces of cell membrane. These particles produce strong stretches and thus rupture the membranes [32]. In comparison, gamma irradiation can cause to ionize compounds and produce free radicals, which can break macromolecules such as DNA, thereby killing microorganisms [33]. Table 2 shows the logarithm of microbial population in red pepper powder under the exposure of plasma at 20 min and gamma radiation (10 KGy), compared to controls. Results also showed that the complete decontamination was carried out within 20 min; similar to gamma radiation. As previously reported by Abdel-Khalek [34], a gamma irradiation dose of 10 kGy completely inhibited bacterial flora to increase shelf life of spices during storage. Therefore, it can be concluded that FE-DBD plasma can be used for the sterilization of spices such as red pepper powder. The color change assessment of red pepper powder is necessary after treatment with FE-DBD plasma to show the extent; to which, the treatment affects the powder color. Table 3 illustrates the average of total color changes in red pepper powder after plasma treatment for 20 min and gamma radiation, compared to controls. It can be seen in the table that parameters L and a significantly decreased and parameter b significantly increased ($P \leq 0.05$) under the exposure of plasma at 20 min and gamma radiation, compared to controls. As results indicate, plasma treatment and gamma radiation led to decreased L*(lightness) and a* (redness) and increased b* (yellowness) significantly ($P \leq 0.05$). This may be linked to the alteration in carotenoid pigments of the red pepper during exposure to FE-DBD plasma. The major factor determining commercial quality of red peppers is the red color [35]. Red color of the red pepper is due to the presence of Capsanthin, a major carotenoid pigment in red peppers [36]. Decreased red color of the red pepper after 5 min of treatment with plasma was reported by Hertwig et al. [37]. Furthermore, results were similar to those reported by Rico et al. [38], which have shown that redness of pepper powder decreased after gamma irradiation. Sensory evaluation of the red pepper powder is shown in Table 4. Sensory evaluation results showed that FE-DBD plasma and gamma radiation included no effects on odor and taste of the red pepper powder, while color change was observed by the panelists. Similarly, Abdel-Khalek [34] reported that gamma irradiation (10 kGy) included no effects on flavoring materials.

Table 3. Changes in red pepper powder color after 20 min of treatment with plasma, compared to gamma radiation

Treatment	L*	a*	b*	ΔE
Control	57.33 ± 0.33^{a}	20.33 ± 0.33^{a}	40.33 ± 0.33^{a}	1.41 ± 0.00^{a}
Cold plasma (20 min)	55.33 ± 0.33^{b}	18.5 ± 0.33^{b}	42.66 ± 0.33^{b}	$3.93 \pm .085^{b}$
Gamma irradiation	53.33 ± 0.33^{c}	17.66 ± 0.33^{b}	44.66 ± 0.33^{c}	6.52 ± 0.44^{c}

Data are presented as mean ±SD from six separate experiments. Different letters in columns show significant differences ($P \leq 0.05$).
L*= lightness, a*= redness, b*= yellowness
ΔE= The total color difference

Table 4. Sensory evaluation under exposure to plasma compared to gamma radiation

Treatment	Taste	Odor	Color	Overall acceptance
Control	3.40 ± 0.25[a]	4.53± 0.13 [a]	4.20 ± 0.11 [a]	4.46± 0.13 [a]
Cold plasma (20 min)	3.46 ± 0.22 [a]	4.53 ± 0.13 [a]	3.73 ± 0.12[b]	4.33 ± .16 [a]
Gamma irradiation	3.40 ± 0.23 [a]	4.53 ± 0.16 [a]	3.80 ± 0.14[b]	4.40 ± 0.53 [a]

Data are presented as mean ±SD from six separate experiments. Different letters in columns show significant differences ($P \leq 0.05$).

4. Conclusion

As a conclusion, FE-DBD plasma is a cost effective and appropriate method for the decontamination of red pepper powders regardless the color change of this spice. Costs of the gamma irradiation treatment are higher than those of conventional methods due to the transportation of food products to and from the irradiators and use of special packaging. Therefore, FE-DBD plasma can be replaced with traditional methods that alter quality and taste of spices. Furthermore, this method can be carried out as an economically continuous method for decontamination of spices.

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6. Conflict of interest

The authors declare no conflict of interest.

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آلودگی‌زدایی فلفل قرمز با استفاده از پلاسمای سرد فشار اتمسفری به عنوان روشی جایگزین

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چکیده

سابقه و هدف: برای آلودگی‌زدایی ادویه ها و به منظور حفظ کیفیت و ایمنی آنها روش های غیر حرارتی توصیه می شوند. در این پژوهش، اثر پلاسمای فشار اتمسفری تخلیه سد-دی‌الکتریک الکترود شناور[۱](FE-DBD) بر پودر فلفل قرمز در مقایسه با پرتوی گاما مورد بررسی قرار گرفت.

مواد و روش ها: به منظور تعیین زمان بهینه تیمار برای آلودگی زدایی *اشرشیاکلی*، *باسیلوس سرئوس* و *آسپرژیلوس فلاووس*، به عنوان میکروارگانیسم یا ریزاندامگان های موجود در فلفل قرمز، به مدت ۱۰، ۲۰ و ۳۰ دقیقه تحت تابش پلاسمای FE-DBD قرار گرفتند و متعاقب آن تغییرات ساختاری میکروارگانیسم‌ها توسط میکروسکوپ الکترونی روبشی و اندازه‌گیری DNA بررسی شد. فلفل قرمز به مدت ۲۰ دقیقه (زمان بهینه) در معرض پلاسما و ۱۰کیلوگری پرتوی گاما قرار گرفت. شمارش میکروبی، رنگ و ارزیابی حسی نمونه‌ها قبل و بعد از تیمارها مورد بررسی قرار گرفت.

یافته‌ها و نتیجه‌گیری: نتایج اشاره بر این دارند که تراکم گونه های باکتری زنده مانده با افزایش زمان پرتودهی کاهش یافت و پس از مدت ۱۰ دقیقه این کاهش معنی‌دار ($p \leq ۰/۰۵$) بود. آلودگی‌زدایی کامل پس از زمان ۲۰ دقیقه انجام شد. دگرشکلی[۲]سلول ها و تخریب ساختار دیواره سلولی در باکتری ها و مخمر پس از در معرض قرار گرفتن مشاهده شد. داده ها نشان داد که پرتودهی به مدت ۲۰ دقیقه با پلاسمای سرد تخلیه سد-دی‌الکتریک الکترود شناور[۳]میکروارگانیسم های فلفل قرمز را همانند پرتودهی با پرتو گاما غیرفعال می کند. به عنوان یک نتیجه‌گیری، پلاسمای تخلیه سد-دی‌الکتریک الکترود شناور روش مناسبی برای آلودگی زدایی پودر فلفل قرمز (صرفنظر از تغییر رنگ) است و می تواند جایگزین روش های سنتی، بدون تغییر در طعم و کیفیت محصول باشد.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

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واژگان کلیدی

- *آسپرژیلوس فلاووس*
- *باسیلوس سرئوس*
- آلودگی‌زدایی
- *اشرشیاکلی*
- پلاسمای فشار اتمسفری تخلیه سد دی‌الکتریک
- پودر فلفل قرمز

***نویسنده مسئول**

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