RESEARCH ARTICLE

Frontiers in Advanced Materials Research

DOI: 10.34256/famr2411

Efficacy of Aflatoxin decontamination in red chilli powder using Low or Vacuum -Pressure Cold Plasma Technology

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Received: 09-02-2024, Revised: 17-04-2024, Accepted: 30-04-2024, Published: 15-05-2024

Abstract: Addressing aflatoxin contamination in red chilli powder requires a multi-faceted approach, including prevention, regular monitoring, and stringent regulatory measures to protect consumer health. This study seeks a technological approach to destroy the aflatoxin generated in red chilli powder. The main objective of this study was to evaluate the efficacy of low or vacuum pressure (VCP) plasma on detoxifying aflatoxins in red chilli (Capsicum anuum L.). Vacuum (0.5 mbar pressure) Cold Plasma treatment was applied for aflatoxins contaminated red chilli powder sample at different exposure times: 3, 6, and 9 min at 37 °C. All the types of aflatoxins in red chilli powder samples were significantly reduced, and AFB2, AFG1, and AFG2 were not detected in any treated chilli powder samples. The lowest AFB1 level (1.6µg/kg) was recorded in a 3 min treated sample. Water activity, moisture percentage, and colour of chilli powder significantly decreased with increasing treatment time, highlighting negative effects of VCP treatment. The study reveals the potential for the removal of aflatoxin using VCP.

Keywords: Red chilli powder, Vacuum Cold Plasma, Aflatoxin, Toxin degradation

1. Introduction

Aflatoxins are types of mycotoxins that are carcinogenic, neurotoxic, and toxic to the endocrine or immune system [1]. The aflatoxins display potency of toxicity, carcinogenicity, and mutagenicity in the order of AFB_1>AFG_1>AFB_2>AFG_2 [2]. The International Agency for Research on Cancer (IRAC) has classified AFB_1 as a human carcinogen (group 1) and AFB_2, AFG_1, and AFG_2 as possibly carcinogenic to humans (group 2B) [3]. Mycotoxins can appear in the food chain due to the infected crop, consumed directly by humans or used as livestock feed, appearing in meat, milk, or eggs [1]. Aflatoxins are made mainly by two fungal species:

Aspergillus flavus and *Aspergillus parasiticus*. Favourable environmental conditions for aflatoxin-producing fungi are high temperature and high humidity. Although these mould attacks occur most probably in tropical and subtropical regions under favorable conditions, drought stress, insect damage and poor storage in temperate regions also contribute to this matter (WHO-Department of Food Safety and Zoonoses, 2018).

Sri Lanka follows EU regulations since most of our products are specially exported to European countries. According to the commission regulations (EC) No 1881/2006 of the European Union, Maximum level for total aflatoxins and maximum level for AFB1 in Capsicum spp. (dried fruits thereof, whole or ground, including chillies, chilli powder, cayenne, and paprika) Piper spp. (fruits thereof, including white and black pepper) *Myristica fragrans* (nutmeg) *Zingiber officinale* (ginger) *Curcuma longa* (turmeric) are 10 µg/kg and five µg/kg, respectively ((EC) No 1881/2006).

Up to now, physical strategies, chemical methods, and biological degradation are three major detoxification methods. However, a full and systematic discussion of the detoxification methods for aflatoxins is still unavailable [4]. Cold plasma technology has become a new trend in the food industry since the last decade, and it acts as a powerful non-thermal technique in the industry to inactivate various pathogenic and spoilage microorganisms in food [5-6]. Plasma can be formed by increasing the energy level of the matter from a solid state through the liquid and gaseous state. The final result of this energy increment is plasma formation, which is an ionized state of gas and has unique chemical and physical properties. Many reactive species; free electrons, neutrals, metastable, and ions, are produced during plasma generation [1]. The feasibility of plasma-induced degradation of several mycotoxins, including AFs, ZEN, ENN, OTA, DON, T_2, and Fu, has been explored. The researchers investigated the effects of 0, 12, and 15 min of atmospheric cold plasma treatment (at the power of 40 and 60 W) on the Aflatoxins produced by *A. parasiticus* and *A. flavus*. The results showed that plasma treatment reduced the AFB1 production of *A.flavus* and *A. paraticus* by up to 97% and 95%, respectively.

2. Materials and Methodology

LC-MS-grade Acetonitrile was purchased from Merk, USA, and a 1 mg/L Aflatoxin mix reference standard was purchased from Romer Labs, Singapore. Rbiopharm PuriTox Total Myco-MS (TC-MT3000) cleanup columns were used for the sample cleanup. The research was conducted at the Fruit and Vegetable Processing Laboratory, the Analytical Laboratory of the Food Technology Section, Industrial Technology Institute, Malabe, and the Residual Analysis Laboratory, Industrial Technology Institute, Colombo. VCP treatments were conducted at MAS Innovations Pvt Ltd, Export Processing Zone, Biyagama, Sri Lanka.

A registered customer's sample that was rejected due to Aflatoxin contamination was taken from the Residual Analysis Laboratory, Industrial Technology Institute, and stored in a

refrigerator at 40˚C. The required chilli powder was removed from the refrigerator 30 minutes before the experiment.

2.1 Vacuum Cold Plasma Treatment for red chilli powder

The treatment used a VCP unit (30 L, Low-pressure plasma, Germany). The capacity of the sterilization chamber in the cold plasma unit is 30 L. The applied power was 300 W with a radio frequency of 13.56 MHz. The applied pressure was 0.5 mbar, and normal atmospheric air was used as the gas medium. The contaminated red chilli powder sample was spread as thin layer (approximately 2 mm thick) on the treating plate. VCP Treatment was carried out at 37˚C temperature, and the three different treatment times used were 3, 6, and 9 min. After the VCP treatment, all treated and control samples were stored at 4˚C.

2.2 Aflatoxins Quantification

2.2.1 Extraction & Clean-up

Extraction of Aflatoxins from chilli sample was initiated by adding 20.0 mL of 80% acetonitrile into 2.00 g of treated, untreated, and blank (control) chilli powder samples. Another set of 2.00 g of each sample (both control and treated) was spiked by using 160 µL of Aflatoxin standard stock solution (1 µg/mL each AFB1, AFB2, AFG1, and AFG2 in acetonitrile). To maintain the same volume, 19.84 mL of 80% Acetonitrile was added to spiked chilli powder samples. All the samples were vortexed (Benchmark Multi-tube Vortex mixture) at 2500 rpm for 10 min and centrifuged (Thermo Fisher Scientific Centrifuge) at 4000 rpm for 10 min at room temperature. After that, 10mL of supernatant acidified with100µl of LCMS grade acetic acid. After that, the tubes were vortexed at 2500rpm for 1 minute. The samples were then passed separately through Puritox Clean-up columns (PuriTox Total Myco-MS (TC-MT3000) and 1.5mL of purified extract from each sample was collected separately (A volume of 3ml of purified extract was collected from the blank sample). A volume of 500 µL from the purified extract was diluted with 1.5 mL of LC-MS grade 1% acetic acid solution (4D dilution series). Of each diluted solution (except 4D dilutions of the blank), 500 µL was again diluted with 500 µL of LCMS grade 1% acetic acid in LC-MS grade 20% acetonitrile (2D dilution series).

2.2.2 Preparation of Standard Solution Series

Intermediate solutions of 5µg/L (5 ppb) and 0.5µg/L (0.5 ppb) were prepared by using 1ppm aflatoxin stock solution and 1% acetic acid in 20% acetonitrile solution. Then to get 2.5 µg/L, 2.0 µg/L, 1.0 µg/L and 0.5 µg/L standard solutions, 500 µL 400 µL, 200 µL, 100 µL of 5 µg/L intermediate solution and 0 µL, 100 µL, 300 µL, 400 µL of 1% acetic acid in 20% acetonitrile were added 500 µL matrix blank was added to each amber color vial. To get 0.25 µg/L, 0.1 µg/L, 0.05 µg/L and 0.02 µg/L standard solutions, 500 µL, 200 µL, 100 µL, 50 µL of 0.5 µg/L

intermediate solution and 0 µL, 300 µL, 400 µL, 450 µL of 1% acetic acid in 20% acetonitrile and 500 µL matrix blank were added to each amber color vial respectively. All eight of the amber colour vials were closed and vortex (VWR Digital Vortex Mixture) for 1 minute.

2.2.3 LCMS/MS conditions

The Chromatographic separation of aflatoxins was carried out on an Ekspert Ultra LC 110 HPLC system using a Phenomenex Gemini 3µ 110Å 50×2 mm column. The optimum separation was achieved with a binary mobile phase at a flow rate of 0.35ml/min. Solvent A: 10mM Ammonium acetate in water and B: Methanol. The column oven temperature was 400 °C, and the injection volume was 20 µL. The HPLC system was coupled to an AB Sciex QTRAP 4500 Tandem Mass Spectrometer LC-MS/MS. The Ionization mode was ESI (Electro Spray Ionization), and the polarity was positive. An ionization temperature was used at 600 °C, and an ion spray voltage was used at 5500V. Calculations of aflatoxins content using LCMS/MS results were carried out using the equation below.

$$\text{Aflatoxins content, µg/kg} = \frac{(\text{Analyte Concentration}) \times (\text{Acetonitrile Volume}) \times D}{\text{Sample Weight}}$$

Where,

- D= Dilution factor (8)
- Analyte Concentration in ng/ml
- Acetonitrile Volume in milliliters
- Sample weight in gram

2.3. Microbiological Analysis

Microbiological analysis (aerobic plate count test, yeast and mould count test, and total coliform and E. Coli) was conducted for treated and control samples after one month as described by Sri Lanka Standards (SLS) 516 part 1: section 1: 2013, SLS 516: Part 2: Section 2: 2013, SLS 516 Part: 3: 2013, and SLS 516: Part 12: 2013, respectively. Results were enumerated and calculated according to SLS 1463:2013.

2.4 Water Activity in chilli powder samples

Each control and treated sample were added up to the marked level of the moisture dishes, and samples was analyzed separately using a Water activity meter (AQUALAB 4TE).

2.5 Color Analysis

The colour of the control and treated chilli powder samples was measured using a reflectance Chromameter (KONICA MINOLTA. INC, Chromameter CR-400, Japan) based

on the L* (brightness/ whiteness), a* (redness/greenness), and b* (yellowness/blueness) values. The following two equations were used to calculate the Hue angle (H^0) and Chroma value.

Hue Angle (H^0) = tan-1 (b*/a*)

Chroma Value = $\sqrt{(a*)^2 + (b*)^2}$

2.6 Sensory Evaluation

Sensory evaluation was carried out to compare the consumer appeal of the colour and appearance of the VCP-treated chilli powder and untreated chilli powder. Sensory analysis was carried out with a semi rained sensory panel of 8 members (Food Technology Section, ITI). Each response was marked in a separate ballet paper, and data was statistically analyzed with a Non-Parametric test (Friedman Test) using Minitab-19 software.

2.7 Statistical data analysis

The statistically analysis of data was carried out for all experiments using descriptive statistics and ANOVA to test the significance of each variable (α =0.05) and followed by comparison performed using the Tukey Pairwise Comparison by the statistical software MINTAB 19. One-way ANOVA was used to determine the effect of VCP treatments on aflatoxins level and other parameters for each treated chilli powder samples. Friedman test in IBM SPSS STATISTICS 22 was carried out to analyze the sensory evaluation results.

3. Results and Discussion

3.1 Aflatoxins Quantification

Results of the removal of aflatoxin are tabulated in Table 1.

Table 1. Aflatoxin in red chilli powder after VCP treatment

Sample	AFB_1	AFB_2	AFG_1	AFG_2	Total Aflatoxins
Untreated	32.53±7.29[a]	38.30±9.54	19.87±4.37	34.20±3.64	124.9±20.8
3 min treated	1.60 ±0.00[b]	ND	ND	ND	
6 min treated	2.67 ±1.29[b]	ND	ND	ND	
9 min treated	2.60 ±1.56[b]	ND	ND	ND	

ND- Not Detected (Values are lower than 1.6 µg/kg)

*Data presented as mean value for three replicates ±SD (n=3). Mean in the same column that does not share a letter significantly different at 5% significance level (Tukey HSD test)

Initial untreated red chilli powder sample contained 32.53 µg/kg, 38.30 µg/kg, 19.87 µg/kg and 34.20 µg/kg of AFB_1, AFB_2, AFG_1 and AFG_2 respectively. Its total aflatoxins content was 124.9 µg/kg. Results shows significant reduction of all aflatoxins types when vacuum cold plasma treatment was applied. AFB_2, AFG_1 and AFG_2 were not detected in 3, 6 and 9 min treated samples. The lowest AFB_1 content was reported in 3-minute treated sample. The lowest detectable level of aflatoxins in a sample that can be accurately identified underused LC-MS/MS conditions was 1.6 µg/kg. Values lesser than 1.6 are shown as "Not Detected" since the accuracy level of the values lesser than 1.6 were not determined. As a result of the treatment initial AFB_1 level has reduced by 95%. According to the SLS regulations, the maximum aflatoxin B_1 level is 5.0 µg/kg and the maximum allowable level for total aflatoxin is 10.0 µg/kg (SLS 1563: 2017). Here, all the VCP treated samples aligned with the SLS regulations.

Detoxification of aflatoxins by using cold plasma treatment has also been reported in previously conducted studies. Aflatoxins in hazelnuts have been significantly reduced with the increasing treatment time and applied power used for producing cold atmospheric plasma. According to the authors, it has been reduced by 70% at 1150W power during 12min of cold plasma treatment [7]. Park *et al.*, 2007 investigated that 5 s of microwave induced atmospheric pressure plasma with argon as a carrier gas destroyed three different mycotoxins, AFB_1, DON and NIV that were dried on glass cover slide [8]. Shi et al., 2017 have degraded AFB_1 into six products by using High Voltage (90kV) Atmospheric Pressure Cold Plasma. When treatment time increased, AFB_1 has been gradually decomposed. All degraded products gradually increased in chromatogram except the product suggested as an intermediate reactant product [9]. Wang *et al.* (2015) have shown that cold plasma treatment detoxified AFB1 owing to the break of covalent bonds in the terminal furan ring that is responsible for the toxicity and carcinogenicity of AFB [10].

The aflatoxin degradation efficiency by cold plasma depends on the plasma system used, the operating parameters applied (e.g., working gas, moisture, and energy input), exposure time, and the type of food products [11]. The highest detoxification efficacy has been obtained from a nitrogen or nitrogen-oxygen mixture [7]. In the present study, treatments were carried out using atmospheric air that contains approximately 78% Nitrogen.

The current study used LCMS grade 80% Acetonitrile in the aflatoxin purification step. Since aflatoxins are soluble in moderately polar solvents, they are normally extracted using a mixture of organic solvents such as acetonitrile, chloroform, or methanol. The acetonitrile: water (9:1) mixture was given more satisfactory recoveries, and all the aflatoxins were at least 85% extracted [12]. The extracted aflatoxin solution was diluted eight times to reduce the matrix effect using several dilution steps.

3.2. Microbiological Analysis

The reduction of aerobic bacteria and yeasts, and moulds after VCP is in Table 2.

Table 2. Log survival of Microbial Population of chilli powder samples

Sample	APC (log)	Yeast and Mould (log)
Untreated	5.38 ±0.00[a]	3.44 ±0.01[a]
3min treated	4.66 ±0.01[d]	2.72 ±0.02[b]
6min treated	4.88 ±0.00[c]	2.33 ±0.06[c]
9min treated	4.92 ±0.00[b]	2.64 ±0.03[b]

*Data presented as the mean value for three replicates ±SD (n=3). Mean in the same column that do not share a letter significantly different at 5% significance level (Tukey HSD test)

Microbiological analysis was conducted for treated samples one month after treatment to determine the microbiological stability. Since the presumptive test for total coliform gave negative results, *E.Coli* confirmation test was not conducted. Initial aerobic plate count (2.4×10^5 CFU/g) and initial yeast and mould count (2.8×10^3 CFU/g) have been reduced significantly in cold plasma-treated chilli powder samples even a month after treatment. Results show a log reduction of APC 0.72, 0.5 and 0.46 log cycles after exposing cold plasma treatment for 3, 6, and 9min. respectively. The initial yeast and mould counts were decreased by 0.72, 1.11, and 0.8 log cycles. According to SLS regulations, the maximum allowable level for yeast and mould count in chilli is 10^3 CFU/g and *E.Coli* should be absent. All the treated samples can be said to comply with the SLS regulations by microbiological parameters. Although the microbial count was reduced compared to the initial untreated sample, there is no specific reduction pattern in APC and yeast and mould count between treated samples. Hence, further studies should be conducted to clarify further. Reported study showed that Cold plasma has effectively reduced the populations of native microflora on fresh blueberries, a reduction that persisted throughout the storage period of the study [13]. According to the Hertwig et al., 2015 no moulds and yeasts were detected in 5 min. remote cold plasma-treated black pepper seeds. The total mesophilic aerobic count and the total spore count have significantly reduced after 30 min. of remote cold plasma treatment [14]. Reactive species in plasma directly oxidize the outer layer of microbial cells and damage the Deoxyribonucleic acid (DNA) in the chromosomes [6]. Especially, reactive species interact with water molecules, form OH* around the DNA, and destroy the DNA. Reactive oxygen species (ROS) react with the various macromolecules in the microbial cells. The damage of the lipid bi-layer in the cell membrane causes impaired transportation of molecules in and out of cells. Since microbial cells are continuously exposed to a highly intense radical load, "etching" occurs in microbial cells and inactive the microbes.

3.3 Water Activity

Table 3. Water activity after VCP treatment

Sample Name	Water Activity
Untreated (control)	0.5746 ± 0.00^{a}
3 min treated	0.1890 ± 0.00^{b}
6 min treated	0.1002 ± 0.01^{c}
9 min treated	0.0650 ± 0.00^{d}

Values are given as means with standard deviation; each determination is performed in triplicate. Values within rows with the same letter (a-d) were not significantly different ($p>0.05$)

The untreated sample recorded the highest water activity level (0.5746 ± 0.00) and the lowest water activity value was recorded by the 9-minute VCP treated chilli powder sample (0.065 ± 0.00) among the tested four samples. The water Activity of chilli powder sample has significantly decreased ($p<0.05$) with increasing treatment time. The water activity of the red pepper powder decreased as the treatment time increased at two power levels, 650 and 826 W. This might be due to the evaporation of moisture from the red pepper powder, facilitated by the formation of low-pressure (667 Pa). This evaporated moisture could result in the formation of oxygen-reactive species in the plasma. The lowered water activity benefits the red pepper powder product by enhancing its microbiological stability [15]. Even though reducing water activity in chilli powder is an extra advantage in cold plasma treatment to enhance the keeping quality, it brings a disadvantage for manufacturers due to excessive reduction of the final weight of the product. Since excessive reduction of moisture level results in a high hygroscopic product, treated chilli samples should be stored in packaging with good barrier properties to moisture.

3.4 Color Analysis

Colour analysis data are presented in Table 4. And Figure 1 shows colour degradation with the VCP treatment time. Here, L^*, a^*, and b^* values represent the whiteness (brightness), redness, and yellowness. The L^*, and b^* values for the untreated samples were 21.23 ± 2.94, 17.93 ± 2.62 and 11.98 ± 1.55 respectively. Since L^* value was increased with the increasing treatment time, the brightness of the chilli powder sample was increased significantly. The a^* value represents the redness (+redness, -greenness); the largest a^* value was detected in the untreated sample. When VCP was applied, a value was significantly reduced ($p<.05$). With the treatment time, the b values of the samples were increased. The Yellowness of the sample is represented by the b^* value. When the b^* value is increased, the yellowness of the sample is increased. Hue angle by main colours are recorded as red purple ($h=0^0$), red ($h=20.14^0$), green ($h=164.25^0$), yellow ($h=90.00^0$), and blue ($h=237.53^0$). The table shows that Chroma parameters

showed a less significant increment in cold plasma-treated samples. Hue angle significantly increased ($p<.05$) with the increasing duration.

Colour degradation of food materials due to cold plasma treatment has been reported in previous studies. Colour of the oregano sample altered after remote atmospheric plasma treatment due to the destruction of chlorophyll. Significant colour changes due to the remote plasma treatment have been observed for the red paprika powder independent of the treatment time. The value of a* has decreased while L* and b* values have increased [15]. According to the Hertwig *et al.*, 2015 oxidizing reaction between reactive nitrogen species and carotenoid results in the loss of the red colour of the paprika powder.

Table 4. Colour parameters of chilli powder samples for different treatment time

Sample	*L**	*a**	*b**	Hue Angle	Chroma Value
Untreated (control)	21.23±2.94^{c}	17.93±2.62^{a}	11.98±1.55^{b}	0.58±0.00^{d}	17.83±0.37^{a}
3 min treated	25.60±0.37^{b}	12.15±0.30^{b}	13.05±0.23^{b}	0.82±0.00^{c}	21.60±1.41^{b}
6 min treated	32.73±2.81^{a}	14.31±0.92^{b}	16.18±1.08^{a}	0.84±0.00^{b}	19.43±1.68^{a}
9 min treated	34.63±2.31^{a}	12.50±1.16^{b}	14.88±1.22^{a}	0.87±0.00^{a}	21.56±3.04^{ab}

Values are given as means with standard deviation with each determination performed in triplicate. Values within rows with the same letter (a-c) were not significantly different ($p>0.05$)

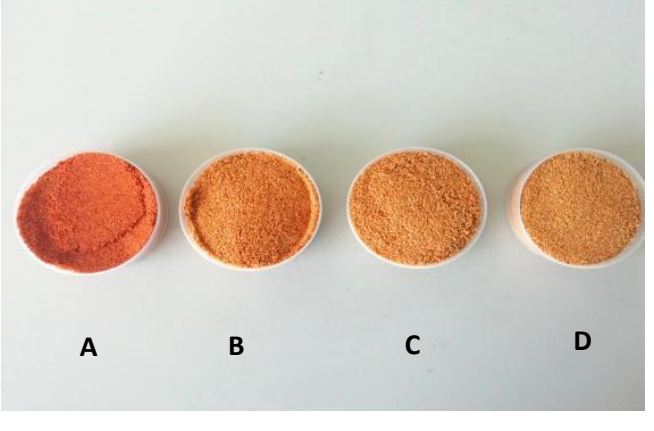

Figure 1. A - Untreated chilli powder sample B – 3 min VCP treated chilli powder sample, C- 6 min VCP treated chilli powder sample and D – 9 min VCP treated chilli powder sample

3.5. Sensory Analysis

Results of sensory evaluation of chilli powder samples are given in Table.5. Since, the original chili powder sample contained high level of aflatoxins, sensory evaluation was conducted for only two sensory parameters: color and appearance. Results of sensory analysis data show a significant difference ($P<0.05$) between the appearance of the reference sample and each of treated chilli powder samples.

Table 5. Results of Sensory Evaluation of Chilli powder samples

Sample	Colour	Appearance
Untreated	4.00	4.00
3 min. treated	2.88	2.44
6 min. treated	1.94	1.94
9 min. treated	1.19	1.63

Data represented as Mean Rank (n=8)

In addition to that, there was a significant difference ($P<0.05$) of appearance between 3 min and 9 min treated chilli powder samples. Sensory evaluation data express a significant difference ($P<0.05$) between the colour of untreated and treated chilli powder samples. There is no significant difference ($P>0.05$) between the colour of 3 min and 6 min treated samples. Sensory evaluation data clearly states that colour and appearance of the Vacuum Cold Plasma treated samples have been degraded compared to the untreated chilli powder samples. The data obtained from sensory evaluation match with colour analysis data which shows that colour of the chilli powder has been degraded due to the cold plasma.

4. Conclusion

VCP treatment has significantly degraded the aflatoxins in chilli powder sample under experimental conditions. The lowest AFB1 level (1.6 µg/kg) was recorded in 3 min VCP treated chilli powder sample and the initial level was reduced by 95%. AFB_2, AFG_1, and AFG_2 were not detected in any treated sample. Our study shows 3 min exposure to vacuum cold plasma (0.5 mbar) at 37 °C is enough to obtain desirable degradation of aflatoxins with minimum effect on the color and appearance of the chilli powder. Both aerobic plate count and yeast and mould count of all the treated samples were significantly reduced compared to the untreated sample. However, Significant reduction of colour can be seen with increasing VCP treatment time.

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Acknowledgements

Indo Sri Lanka Joint Research Project (FP/125), brought together the Industrial Technology Institute (ITI) under the State Ministry of Technology, Government of Sri Lanka, and the Institute of Chemical Technology (ICT) in Mumbai, operating under the Department of Science, Government of India (2017 – 2019).

Conflict of interest: The Authors have no conflicts of interest to declare that they are relevant to the content of this article.