

Effect of UVC Irradiation on Aflatoxins in Ground Nut (*Arachis hypogea*) and Tree Nuts (*Juglans regia*, *Prunus dulcis* and *Pistachio vera*)

FARHAT JUBEEN,¹IJAZ AHMED, BHATTI*, MUHAMMAD ZARGHAM KHAN
ZAHOOR-UL-HASSAN AND MUHAMMAD SHAHID

¹Department of Chemistry and Biochemistry, University of Agriculture, Faisalabad, Pakistan
ijazchem@yahoo.com*

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Summary: The objective following the present study was to investigate the effect of ultra violet irradiation on aflatoxins in ground and tree nuts. Samples of nuts were randomly selected from the retail market of Faisalabad. The moisture contents of the nuts were artificially increased to 10±3% and 16±3% to facilitate the mold growth. The samples were stored at a room temperature of 25-30 °C for 12 weeks. The stored nut samples were checked after a storage period of 12 weeks, the fungi were found growing in all nuts along with considerable aflatoxins production. Aflatoxins and mold contaminated samples were exposed to UVC radiations of 265nm for 15, 30 and 45 minutes. The fungicidal activity of UVC radiation was more pronounced in nuts adjusted at high moisture level. The order of sensitivity of nuts for fungal disinfection by UVC irradiation was walnut> almond=pistachio> peanuts. There was a proportional decrease in aflatoxins level with increase in exposure time. Complete elimination of aflatoxin G₂ was achieved in all nut samples after 15min. exposure, while aflatoxin G₁ showed 100% degradation only in almond and pistachio. After 45 min. exposure to UVC, aflatoxin B₁ showed maximum reduction of 96.5% in almond and pistachio. The degradation of total aflatoxins as well as that of aflatoxin B₁ by UVC irradiation was found to follow first order kinetics.

Key words: Aflatoxins, Tree nuts, Ground nut, UV irradiation.

Introduction

Nuts are inspired worldwide for their nutritional, sensory and health promoting attributes. In the Middle East and Asia, nuts are cultivated for use as oil crops and are imperative sources of energy as well as necessary dietary nutrients in addition to the phytochemical importance [1]. Walnut (*Juglans regia*), almond (*Prunus dulcis*) and pistachio (*Pistachio vera*) are suggested as tree nuts and peanuts (*Arachis hypogea*) as ground nuts. Exceptional nutrient profile of the nuts constructively affects lipids and lipoproteins [2]. There are a number of reports about aflatoxin contamination of nuts [3-6].

The potential of toxicity, carcinogenicity and mutagenicity of aflatoxins is categorized as B₁ > G₁ > B₂ > G₂. Aflatoxin B₁ (AFB₁) has the ability to be metabolized by constitutive cellular enzymes and this property is thought to be related to its noxious and carcinogenic effects. The metabolism of AFB₁ results in oxidative derivatives including hydroxylated species such as aflatoxin M₁ (AFM₁) and aflatoxin Q₁ (AFQ₁) in addition to covalent macromolecular adducts with DNA and proteins. Biological punishment of oxidative metabolism of AFB₁ extends from cellular alteration to cell death [7]. On the molecular level, aflatoxins metabolize to epoxides which bind to guanine in DNA, and lead to lipid peroxidation by robustly generating reactive oxygen species (ROS) and release mutagenic malondialdehyde as well as direct cell injury [8, 9].

Several studies have revealed that without using detrimental chemicals or without causing momentous losses in functional and nutritive value of food, it is unworkable to remove mycotoxins. A comparatively recent perception offers a substitute to the use of chemical or thermal managements for postharvest control of surface disinfection is the use of ultra violet C (UVC), a component of electromagnetic radiation below 280 nm [10].

Aflatoxin B₁ (AFB₁) absorbs ultraviolet (UV) radiation at 222, 265 and 362 nm with the maximum absorption at 362 nm. Activation of AFB₁ by irradiation at 362 nm amplifies its inclination to degradation. It is described to be very sensitive to UV radiation at a pH of less than 3 or greater than 10 [11].

Results and discussion

Effect of Ultra Violet Light on Fungal Load in Nuts

The antimicrobial action of ultra violet (UV) light is renowned and has been utilized to lessen microbial infection in hospitals, the pharmaceutical industry, community buildings, water treatment, fresh food products and agricultural products. Ultra violet irradiation is widely used in the food industry for disinfection of air, control of contamination on the surface of plant and packaging materials or in post harvest storage of fruits and vegetables [12].

*To whom all correspondence should be addressed.

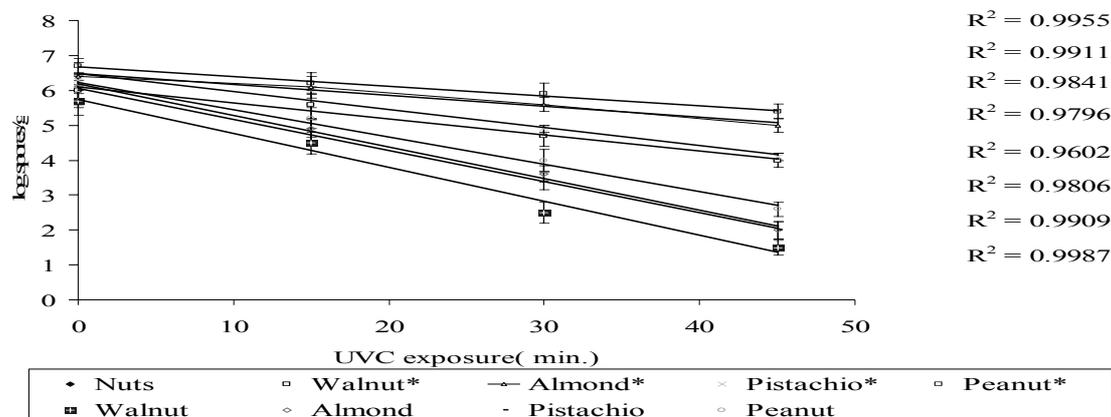
The mycoflora were detected in all samples of tree and ground nuts immediately after collection from market. The identification of fungal spores by microscopy showed the presence of fungi belonging to *Aspergillus flavus*, *Aspergillus parasiticus* and *Penicillium*. Pistachio and peanut samples were found contaminated with *Aspergillus flavus* and *Aspergillus parasiticus* where as walnut and almond showed the incidence of *Penicillium* along with the two species of *Aspergillus*. Our finding was found to be in agreement with previous studies [5, 13]. The fungal count was significantly eminent at high moisture level (16±3%) in comparison to that at low moisture level (10±3%) in all samples of ground and tree nuts analyzed.

The germicidal potential of UVC irradiation for the fungal species detected in nuts was analyzed for 0, 15, 30 and 45 min. exposure time and is presented in Fig. 1. Exposure of 12 week stored, adjusted at 16±3% moisture level, infected walnut to UVC radiation for 15 min. resulted in > log 10 reduction in fungal count. After 30 min., there was 1.3 log 10 reduction and 2 log 10 reduction after 45 min. exposure. For the same nut adjusted at low moisture level, there was 1.2, 3.2 and 4.2 log 10 reduction after 15, 30 and 45 min. exposure to UVC light, respectively. Among the nuts adjusted at low moisture level, peanuts and almonds showed similar level of infectivity with 6.1 log 10 fungal spores. Pistachio stored under the same conditions showed 6 log 10 spores / g. The food matrix of walnut was found to be most sensitive towards fungal disinfection by UVC. Almond and pistachio showed similar level of sensitivity, however, peanut showed highest resistance with 3.5 log 10 and 1.3 log 10

reductions at low and high moisture levels respectively.

The similar reduction of fungal load by UVC has been reported by a number of scientists [10, 12-16]. In our study, the nuts infected with species of *Aspergillus parasiticus*, *Aspergillus flavus* and *Penicillium* were exposed to UVC light and the exposure times were 15, 30 and 45 min. The reduction observed in our nuts exposed to UVC was comparable to that observed for *Aspergillus flavus*, *Aspergillus niger*, *Penicillium corylophilum* and *Eurotium rubrum* by [12]. The exposure time reported by the author was 60, 120 and 180 seconds and the spores of the fungal species were suspended in liquid media. The reason for obtaining the same level of fungal reduction with longer exposure time in our analyzed samples can be explained by the easy availability of the toxins and fungal species in model system rather than natural occurrence [17]. Fungal species in their natural occurrence also develop defense mechanism against the germicidal agents or radiations. A single-celled slightly pigmented conidia and hyaline mycelium are produced by some *Penicillium* species as protective mechanisms against UV radiation [18].

The formation of aflatoxins by species of *Aspergillus* follows polyketide pathway. The earliest precursors are acetyl and malonyl units. Both averufin and versicolorin A are experimentally identified precursors of aflatoxin B₁. Polyketide pathway in response to UV light shows a coordinated decrease in aflatoxins formation. There can be an entire increase or decrease in the activity of one or more of the electron transfer pathways since respiratory enzymes frequently have light captivating prosthetic groups [19, 20].



*Nuts with star are adjusted at 16±3% and those without star are adjusted at 10±3% moisture levels.
* Y-error bars are representing standard deviation

Fig. 1: Effect of UVC exposure on fungal count in twelve week stored ground and tree nuts.

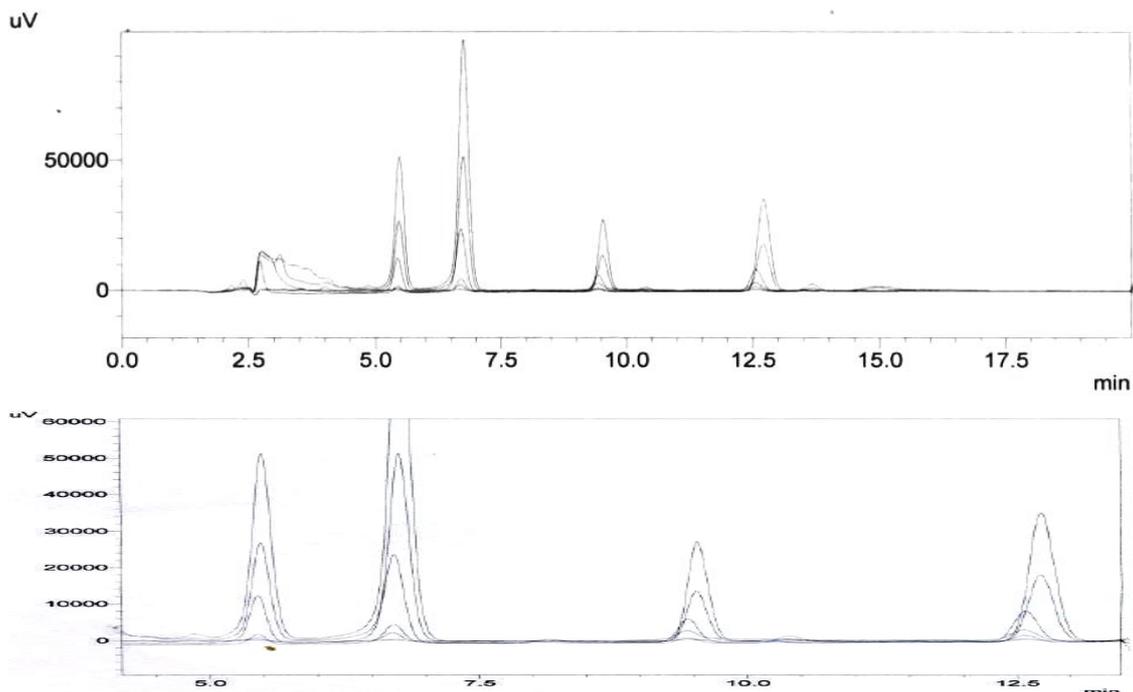


Fig. 2: Chromatographs of standard calibration solutions showing excellent repeatability and reproducibility for AFG₁, AFB₁, AFG₂ and AFB₂ respectively.

Method Validation Parameters for Aflatoxins Analysis

The standard calibration curves were linear over the range of concentrations of aflatoxins injected into the liquid chromatograph. Standard calibration solutions showed excellent repeatability and reproducibility for AFG₁, AFB₁, AFG₂ and AFB₂ respectively as shown in Fig. 2. For aflatoxin G₁ (AFG₁), the concentration range between 0.05-20 ng mL⁻¹, for aflatoxin B₁ (AFB₁) 0.05-150 ng mL⁻¹, for aflatoxin B₂ (AFB₂) 0.02-20 ng mL⁻¹ and for aflatoxin G₂ (AFG₂) 0.02-6.0 ng mL⁻¹, was found linear. The recoveries were determined by spiking aflatoxins to control samples of nuts at concentrations levels 125.5 µg kg⁻¹ of AFB₁, 15.3 µg kg⁻¹ AFG₁ and AFB₂, and 6.3 µg kg⁻¹ of AFG₂. Triplicate samples were analyzed for each toxin level. Estimation of limit of detection (LOD) was carried out as signal to

noise ratio (S / N) = 3 and for limit of quantification (LOQ) as (S / N) = 10. The correlation coefficients and results of recoveries of aflatoxins are shown in Table-1.

Effect of Ultraviolet Light (UVC) on Aflatoxins in Nuts

Ultraviolet C (UVC) light was examined for its effect on aflatoxins, AFB₁, AFB₂, AFG₁ and AFG₂, detected during a storage period of 12 weeks, in ground and tree nuts maintained artificially at two different moisture levels. The nuts were exposed to UVC radiation for 0, 15, 30 and 45 min. It was found that UVC radiation significantly (P<0.05) reduced aflatoxin levels in nuts. The degradation of aflatoxins by UVC radiation was found to follow first order rate kinetics as shown in Fig. 3 for AFB₁ and Fig. 4 for AFG₁ and AFB₂ respectively.

Table-1: Validation of aflatoxin determination by HPLC analysis.

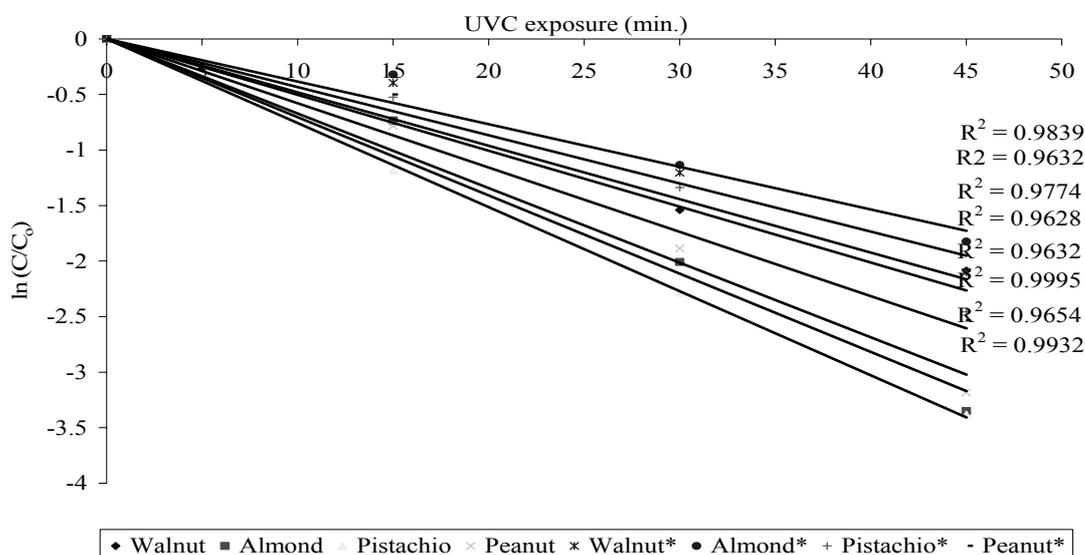
Aflatoxin	LOD ^a (ng mL ⁻¹)	LOQ ^b (ng mL ⁻¹)	Calibration curve	R ²	Recovery(%) ^c	Mean (µg kg ⁻¹) ± RSD(%) ^d
AFB ₁	0.02	0.05	y = 68983x + 34942	0.9997	97.6	125.3± 9.12
AFB ₂	0.01	0.02	y = 104767x - 6094	0.9995	91.2	15.3± 2.01
AFG ₁	0.02	0.05	y = 32045x + 2780	0.9996	97.6	15.3± 1.44
AFG ₂	0.01	0.02	y = 61801x - 85.618	0.9991	91.2	6.3± 3.42

^a Limit of detection.

^b Limit of quantification.

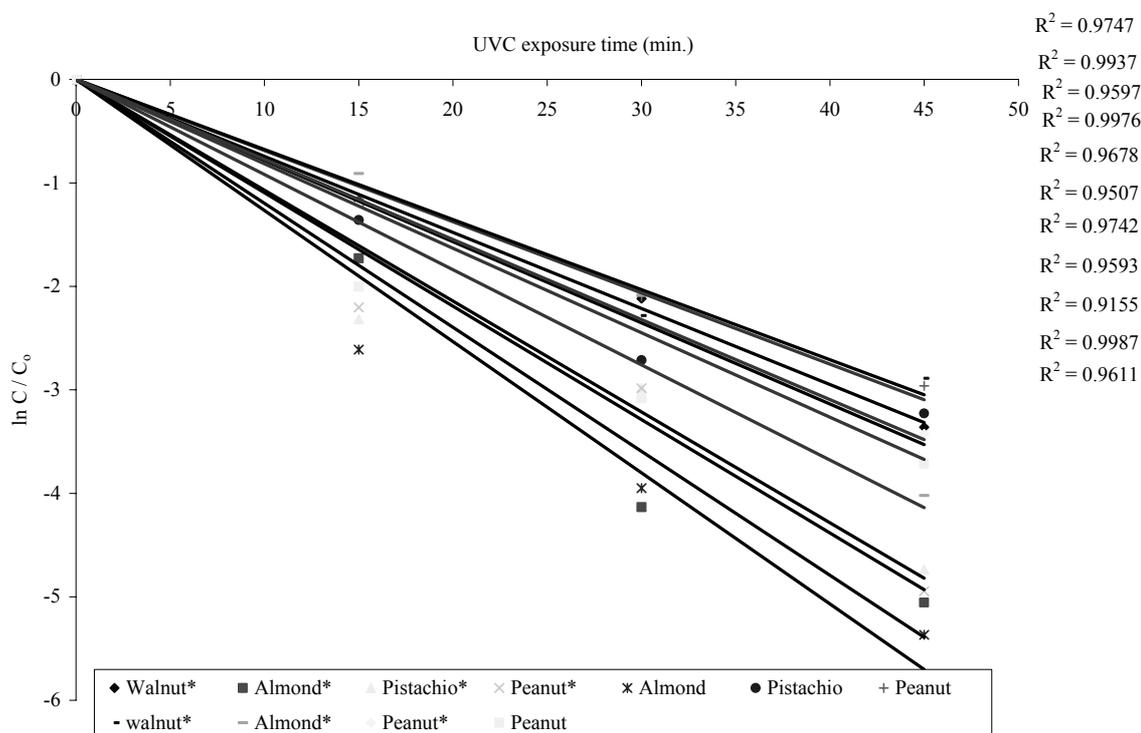
^c Accuracy was determined by the determination of the recoveries of aflatoxins. By spiking 125.5 µg kg⁻¹ AFB₁, 15.3 µg kg⁻¹ AFG₁ and AFB₂ and 6.3 µg kg⁻¹ AFG₂ to the samples (uninfected ground and tree nuts).

^d Multiple analysis of each spiked sample were used to determine the accuracy, expressed as Mean (µg kg⁻¹) ± RSD(%).



* Commodities marked with star are adjusted at 16±3% and those without star are at 10±3% moisture levels.

Fig. 3: Degradation kinetics for AFB₁ following UVC exposure in nuts at different moisture levels.



* Commodities with star are adjusted at 16±3% and those without star are at 10±3% moisture levels.

* Red coloured lines are of AFG₁ and black ones are of AFB₂.

Fig. 4: Degradation kinetics for AFB₂ and AFG₁ following UVC exposure in nuts at different moisture levels.

The degradation kinetics was studied by plotting the exposure time against $\ln(C/C_0)$. The plot of $\ln(C/C_0)$ against UVC exposure time showed a straight line passing through the origin. This finding is in harmony with [10] who reported first order kinetics for aflatoxin degradation by UVC irradiated hazelnuts and model system. The degradation rate constants, k , (min^{-1}) were calculated for all UVC treated nuts. In all UVC treated nut samples, residual AFB₁ level did not fall below limit of detection (LOD). In some nut samples the levels of AFG₁ and AFB₂ fall under LOD, but the levels up to which these toxins were detected, the degradation followed first order kinetics. The values of k are not reported for those aflatoxins which fall below LOD.

The total aflatoxin content ($27.29 \mu\text{g kg}^{-1}$) of walnut adjusted at low moisture level was reduced to $12.62 \mu\text{g kg}^{-1}$ after 15 min. UVC exposure with more than 50% degradation as shown in Table-2. After 30 and 45 min. UVC exposure for the same nut samples, total aflatoxin content reduced to 5.75 and $3.32 \mu\text{g kg}^{-1}$ respectively with 78.93 and 87.83 % degradation. For walnut adjusted at high moisture level almost similar percent degradation was calculated with total residual aflatoxin content of $7.79 \mu\text{g kg}^{-1}$ after 45 min. UVC treatment. In case of walnuts, the degradation rate constant for AFB₁ was in the range of -0.021 to -0.055 min^{-1} as shown in the Table-6. The maximum degradation (87%) of AFB₁ was observed after 45 min. UVC exposure. This observation is in accordance with [11], who reported that AFB₁ absorbs UV radiation at 222, 265 and 362 nm, hence resulting in an increased susceptibility towards degradation.

Following AFB₁, the second major contributor towards total aflatoxin content was AFB₂. There was a proportional decrease in AFB₂ with increase in UVC exposure time. The levels of AFG₁ and AFG₂ were quite low in all untreated nuts at both the moisture levels. From the values of degradation rate constants, it is revealed that AFB₁ was most resistant towards UVC degradation potential and AFG₂ was most sensitive. The highest sensitivity of AFG₂ towards UVC may be attributed to the lower initial concentration in untreated nuts. However, following 45min. UVC exposure, AFB₁ and total

aflatoxins content in walnuts did not fall up to the regulatory limits of $2 \mu\text{g kg}^{-1}$ and $4 \mu\text{g kg}^{-1}$ respectively.

In almond and pistachio sustained at $10 \pm 3\%$ moisture level, AFB₁ level was 35.53 and $34.02 \mu\text{g kg}^{-1}$ with total aflatoxin content 44.01 and $35.77 \mu\text{g kg}^{-1}$ respectively. At high moisture level, 53.12 and $72.99 \mu\text{g kg}^{-1}$ AFB₁ was found in almond and pistachio respectively. After 15 min. UVC exposure, the respective levels were reduced to 24.60 and $43.18 \mu\text{g kg}^{-1}$ with up to 50% degradation respectively. The degradation rate constant was found out to be -0.021 and -0.034 min^{-1} for these decontaminations by UVC treatment. Greater reduction in total and individual aflatoxin levels was observed at 30 and 45 min. UVC exposure. In both almond and pistachio at both moisture levels, AFG₂ was not detected at any UVC exposure time. This finding is in accordance with [21, 22]. The degradation rate constants for AFB₂ in almond and pistachio were found intermediate between those of AFB₁ and AFG₁. From the values of degradation rate constants, it can be inferred that the sustainability of AFB₁ towards UVC exposure was more in comparison to AFB₂, AFG₁ and AFG₂ as observed in walnut. The similar sensitivity order of aflatoxins towards UV irradiation has also been reported by [23].

Peanut ($16 \pm 3\%$ moisture level) was prominent with its highest AFB₁ level ($158.68 \mu\text{g kg}^{-1}$) among the nuts under investigation as shown in Table-5. This level was reduced to $12.56 \mu\text{g kg}^{-1}$ having -0.071 min^{-1} degradation rate constant indicating 95.86% reduction after 45 min. exposure to UVC radiation. The level of AFG₁ and AFB₂ were reduced more than 95% at both the moisture levels, however, AFG₂ was not detected after any UVC exposure. This finding is comparable with the work of [23], who reported 74% degradation of AFG₁ in wheat grains following 60 min. exposure to 254nm UV light. The difference in absolute values of percentage degradation with [23] may be due to difference in wavelength of UV radiation, as the radiation source used in our work was emitting UV light of 265 n

Table-2: Effect of ultraviolet (UVC) light on aflatoxins in twelve week stored walnut adjusted at two moisture levels.

Treatment time (min.)	Moisture Contents (%)									
	10±3Aflatoxins ($\mu\text{g kg}^{-1}$)				16±3Aflatoxins ($\mu\text{g kg}^{-1}$)					
	AFG ₁	AFB ₁	AFG ₂	AFB ₂	Total	AFG ₁	AFB ₁	AFG ₂	AFB ₂	Total
0	0.13 ± 0.01	26.60±0.5	0.25±0.02	0.31±0.01	27.29±1.1	1.16±0.02	63.01±0.9	0.29±0.03	0.40±0.06	64.87±1.5
15	0.02 ± 0.01	12.48±0.2	n.d.	0.13±0.01	12.62±0.9	0.38±0.04	42.42±0.80	n.d.	0.13±0.02	42.92±1.1
	(70.25)	(68.25)	(100)	(68.96)	(53.75)	(67.37)	(32.68)	(100)	(68.95)	(33.83)
30	n.d.	5.69±0.2	n.d.	0.048±0.03	5.75±0.6	0.13±0.01	18.89±0.3	n.d.	0.048±0.01	19.07±0.7
	(100)	(79.00)	(100)	(78.96)	(78.93)	(88.81)	(70.00)	(100)	(87.96)	(70.60)
45	n.d.	3.30±0.03	n.d.	0.014±0.04	3.32±0.5	0.06±0.01	7.71±0.51	n.d.	0.014±0.01	7.79±0.4
	(100)	(87.76)	(100)	(96.52)	(87.81)	(94.44)	(87.76)	(100)	(96.52)	(87.99)

* Mean ± standard deviation (n = 3), *The values in parentheses are representing percent decrease of the aflatoxins immediately above these.,

* n.d. is not detected

Table-3: Effect of ultraviolet (UVC) light on aflatoxins in twelve week stored almond adjusted at two moisture levels.

Treatment time (min.)	Moisture Contents (%)									
	10±3 Aflatoxins ($\mu\text{g kg}^{-1}$)					16±3 Aflatoxins ($\mu\text{g kg}^{-1}$)				
	AFG ₁	AFB ₁	AFG ₂	AFB ₂	Total	AFG ₁	AFB ₁	AFG ₂	AFB ₂	Total
0	3.88 ± 0.51	35.53 ± 0.92	0.27 ± 0.01	4.32 ± 0.23	44.01 ± 1.20	7.04 ± 0.61	53.12 ± 0.97	0.11 ± 0.06	16.81 ± 0.37	77.09 ± 1.1
15	0.063 ± 0.01 (98.37)	17.04 ± 1.0 (52.04)	n.d. (100)	0.32 ± 0.02 (92.65)	17.42 ± 0.25 (60.42)	1.74 ± 0.02 (75.28)	26.08 ± 0.51 (50.90)	n.d. (100)	13.06 ± 0.01 (22.31)	40.88 ± 1.4 (46.97)
30	n.d. (100)	4.77 ± 0.41 (86.57)	n.d. (100)	0.083 ± 0.02 (98.07)	4.85 ± 0.25 (88.970)	0.34 ± 0.01 (95.17)	12.98 ± 0.21 (75.56)	n.d. (100)	4.61 ± 0.02 (72.57)	17.93 ± 0.55 (76.74)
45	n.d. (100)	1.43 ± 0.01 (96.49)	n.d. (100)	0.02 ± 0.01 (98.37)	1.45 ± 0.04 (96.71)	0.033 ± 0.01 (99.57)	6.51 ± 0.02 (87.44)	n.d. (100)	0.02 ± 0.01 (99.88)	6.53 ± 0.02 (91.52)

* Mean ± standard deviation (n = 3)

* Values in parentheses are representing percent decrease in aflatoxins of the values immediately above these

* n.d. is not detected

Table-4: Effect of ultraviolet (UVC) light on aflatoxins in twelve week stored pistachio adjusted at two moisture levels.

Treatment time (min.)	Moisture Contents (%)									
	10±3 Aflatoxins ($\mu\text{g kg}^{-1}$)					16±3 Aflatoxins ($\mu\text{g kg}^{-1}$)				
	AFG ₁	AFB ₁	AFG ₂	AFB ₂	Total	AFG ₁	AFB ₁	AFG ₂	AFB ₂	Total
0	n.d.	34.20 ± 0.47	n.d.	1.56 ± 0.01	35.77 ± 1.1	0.43 ± 0.08	72.99 ± 1.4	n.d.	4.63 ± 0.51	78.06 ± 1.1
15	n.d. (40.84)	10.47 ± 0.59 (73.78)	n.d. (100)	0.40 ± 0.01 (91.32)	10.87 ± 0.24 (69.61)	0.06 ± 0.01 (86.04)	43.18 ± 0.42 (68.43)	n.d. (100)	0.45 ± 0.04 (16.51)	43.69 ± 0.76 (44.03)
30	n.d.	3.47 ± 0.05 (73.78)	n.d.	0.13 ± 0.02 (94.92)	3.61 ± 0.05 (89.91)	0.02 ± 0.01 (95.34)	19.14 ± 0.84 (88.42)	n.d.	0.23 ± 0.05 (93.50)	19.40 ± 0.25 (75.14)
45	n.d.	1.16 ± 0.01 (91.42)	n.d.	0.06 ± 0.02 (99.120)	1.22 ± 0.02 (96.58)	n.d. (100)	6.26 ± 0.18 (95.27)	n.d.	0.04 ± 0.02 (96.03)	6.30 ± 0.51 (91.92)

* Mean ± standard deviation (n = 3)

* Values in parentheses are representing percent decrease in aflatoxins of the values immediately above these.

* n.d. is not detected

Table-5: Effect of ultraviolet (UVC) light on aflatoxins in twelve week stored peanut adjusted at two moisture levels.

Treatment time (min.)	Moisture Contents (%)									
	10±3 Aflatoxins ($\mu\text{g kg}^{-1}$)					16±3 Aflatoxins ($\mu\text{g kg}^{-1}$)				
	AFG ₁	AFB ₁	AFG ₂	AFB ₂	Total	AFG ₁	AFB ₁	AFG ₂	AFB ₂	Total
0	1.02 ± 0.11	46.78 ± 1.3	0.02 ± 0.01	3.99 ± 0.21	51.82 ± 0.96	1.17 ± 0.01	158.68 ± 1.3	0.03 ± 0.02	4.62 ± 0.71	164.51 ± 1.11
15	0.14 ± 0.02 (86.27)	21.36 ± 0.55 (52.04)	n.d. (100)	1.26 ± 0.08 (92.64)	22.76 ± 0.74 (56.08)	0.35 ± 0.04 (90.01)	96.08 ± 0.87 (27.46)	n.d. (100)	0.51 ± 0.03 (94.79)	96.94 ± 1.50 (41.07)
30	0.05 ± 0.03 (95.09)	7.09 ± 0.91 (86.57)	n.d. (100)	0.49 ± 0.03 (98.07)	7.63 ± 0.99 (85.2)	0.11 ± 0.02 (98.20)	20.68 ± 1.20 (86.84)	n.d. (100)	0.23 ± 0.01 (98.39)	65.91 ± 1.42 (59.94)
45	0.03 ± 0.01 (97.06)	1.94 ± 0.31 (96.49)	n.d. (100)	0.21 ± 0.05 (98.370)	2.17 ± 0.04 (95.7)	0.03 ± 0.01 (99.55)	12.56 ± 0.77 (89.530)	n.d. (100)	0.03 ± 0.01 (99.36)	12.64 ± 1.01 (92.31)

* Mean ± standard deviation (n = 3)

* Values in parentheses are representing percent decrease in aflatoxins of the values immediately above these.

* n.d. is not detected

The degradation of individual and total aflatoxins by UVC exposure in nuts was found in consistency with already reported literature [10, 24, 25 and 26]. Our results are partially inconsistent with Basaran, 2009 who reported that a UV light of 254 nm did not affect AFG₂ and AFB₂ but significantly reduced AFG₁ and AFB₁. This may be due to difference in natural contamination in our analyzed nut samples and a little variation in the wavelength of light source. To our knowledge, there are no reports about the degradation rate constants for aflatoxins following UVC exposure in model system, feed or food.

Following UVC irradiation, presence or absence of toxins in food may be attributed to chemical modification of the toxin which alters the known formula of the compound [23]. Irradiation by UVC activates aflatoxin B₁ and enhances its susceptibility to degradation. Aflatoxin B₁ is reported to be highly sensitive to UV radiation. The structure

of terminal furan ring is affected by UV radiation thus the active site for binding is eliminated [11]. The production of 12 new UV degradation products of AFB₁ has been suggested [27]. The formation of degradation products of aqueous solution AFB₁ subsequent to UV irradiation has been suggested using TLC, UV and IR spectroscopy and final degradation products has been identified by [28] by using LCMS and UPLC-quadrupole time of flight mass spectrometry.

Experimental

Chemicals

All the reagents used were of HPLC-grade. Acetonitrile and methanol (LAB-SCAN, ANALYTICAL SCIENCES), acetic acid (Riedel-deHaën), aflatoxin standards were purchased from sigma chemical company, USA, potato dextrose agar and sabrod liquid (Oxoid Ltd. BASINGSTOKE, HAMPSHIRE, England).

Samples

Aflatoxin free samples of walnut, almond, pistachio and peanut with initial moisture content of 0.38%, 0.68%, 0.54% and 0.71% respectively were randomly collected from local market of Faisalabad, Pakistan. Moisture content was determined by drying replicate portions of 5-10 g of ground nuts at 106 °C nuts for 24h and then subsequently up to constant weight, loss in weight was expressed as percentage calculated on wet weight basis [29].

Storage Conditions for Mold Growth

The conditions for storage of nuts were adjusted according to [30] with little modifications. In our analyzed samples, the inhabiting fungal flora in the nuts produced AFB₁, AFB₂, AFG₁ and AFG₂, whereas in the method [30], the selected fungal species were inoculated into maize samples and they produce only B-aflatoxins (AFB₁ and AFB₂). The moisture content of the samples was adjusted to 10 ± 3% and 16 ± 3% and stored in wooden containers. The containers were placed at a store room with proper ventilation at a temperature of 25-30 °C for a period of 12 weeks. After 12th week of storage, the nuts were placed under a 1000 mg ethylene oxide gas environment for 3h to hinder multiplication of microorganisms.

Irradiation of Nuts

The 12 weeks old stored ground and tree nuts at different moisture levels were irradiated with ultra violet light for mould decontamination and aflatoxin degradation. For this purpose, about 200-250 g sub-sample of each stored nut was randomly selected from the lot. The samples were exposed to UV source at a distance of about 25cm ahead packed individually in transparent polythene pouches with thickness of about 1cm to avoid self absorption. The pouches were irradiated in a closed box equipped with six UV-lamps producing a wavelength of about 265 nm. The uppermost surfaces of pouches were exposed to UV radiations at 108 J/m² for 0, 15, 30 and 45 min. at room temperature. The experiments were carried out for three replicates of each radiation dose and each nut at 10 ± 3% and 16 ± 3% moisture levels. Three control replicates were assigned for irradiation dose. The irradiated ground and tree nuts were kept at a temperature of 2 ± 2 °C until further analysis.

Enumeration of Fungal Load

The ground and tree nuts were analyzed for the fungal count following the method of [31]. All the reagent solutions used for fungal load were autoclaved before use and all the glass ware after

proper washing was dried in oven at 180 °C for 3 h. One gram of each finally ground nuts was transferred into 10mL of autoclaved (120 °C for 15 min.) 2% peptone water in a conical flask. Wash water was collected for further analysis after vigorously shaking for 5 min. and filtering through Whatman filter paper No.1.

After extracting microorganisms from samples with 2% peptone water, these were plated on pre sterilized Petri plates in hot air oven at 180 °C for 3 hours containing nutrient agar potato dextrose to obtain fungal biomass and pure cultures were obtained using streaking method. Fungal species were identified by microscopy in the department of Microbiology, University of Agriculture, Faisalabad. Pure cultures isolated from the 12 weeks stored nuts were diluted up to 10¹ spores/ mL and absorbance was taken at 620 nm at u-quart Bio tech. USA. Average standard curve was drawn for pure isolates plotting spores / mL versus optical density. The fungal count of the stored and irradiated nuts was calculated using spread plate method and direct observation method. The data presented is the average count in three Petri dishes for each sample.

Extraction and Purification of Aflatoxins

The method for aflatoxins extraction in nut samples was through following the procedure of [32] with little modifications. Samples of tree and ground nuts were randomly selected from the lot during 12th week of storage. Samples were ground in a laboratory mill (culatti, JANKE & KUNKEL, GmbH) and weighed 25g in Erlenmeyer. Aflatoxins were extracted using 80 mL of a mixture of ACN : H₂O (84:16) by shaking for 30min. The extract was filtered through whatman (Maidatone, UK) filter paper (NO.3). From the filtrate 9 mL was taken in a glass Vial, acidified with 70 µL acetic acid and vortex. The acidified mixture was then passed through a mycosep # 226 Aflazon⁺ column (Romerlabs) with a flow rate of 2mL/min. The pure aflatoxin solution (2 mL) was then dried through stream of N₂ and the residue was dissolved in 2 mL of mobile phase.

Table-6: Degradation rate constant, k (min.⁻¹), for AFB₁ degradation by UVC in nuts.

Nuts	UVC exposure time (min.)		
	15	30	45
Walnut	-0.051	-0.051	-0.046
Almond	-0.049	-0.061	-0.075
Pistachio	-0.039	-0.075	-0.075
Peanut	-0.052	-0.067	-0.071
Walnut*	-0.026	-0.041	-0.047
Almond*	-0.021	-0.037	-0.041
Pistachio*	-0.034	-0.045	-0.055
Peanut*	-0.033	-0.029	-0.048

* Nuts marked with star are adjusted at 16±3% and those without star are at 10±3% moisture levels.

Table-7: Degradation rate constant, k (min.^{-1}), for AFG₁ degradation by UVC in nuts.

Nuts	UVC exposure time (min.)		
	15	30	45
Walnut*	-0.081	-0.076	-0.064
Almond*	-0.061	-0.069	-0.089
Peanut*	-0.079	-0.079	-0.076
Peanut	-0.131	-0.102	-0.082

* Nuts marked with star are adjusted at 16±3% and those without star are at 10±3% moisture levels.

Table-8: Degradation rate constant, k (min.^{-1}), for AFB₂ degradation by UVC in nuts

Nuts	UVC exposure time (min.)		
	15	30	45
Almond	-0.174	-0.132	-0.119
Pistachio	-0.091	-0.091	-0.071
Peanut	-0.077	-0.069	-0.066
Walnut*	-0.078	-0.071	-0.075
Almond*	-0.115	-0.137	-0.112
Pistachio*	-0.154	-0.099	-0.105
Peanut*	-0.146	-0.099	-0.109

* Nuts marked with star are adjusted at 16±3% and those without star are at 10±3% moisture levels.

Aflatoxin Derivatization for Detection up to ppb Level

Sensitivity of UV detectors for aflatoxins is up to ppm level whereas that of fluorescent detector is up to ppb level as AFB₁ and AFG₁ are less fluorescent so post column derivatization was carried out to convert into AFB_{2a} and AFG_{2a} respectively (enhancing their fluorescence). Derivatization was carried out using [33]. Hexane (200 μL) was added to redissolve aflatoxins purified mixture and vortex 50 μL TFA was added and again vortex for 30 sec. The added 1.95 mL deionized H₂O : ACN (9:1), vortex and allowed to stand for a while for the separation of two layers were allowed to separate. The lower aqueous layer containing aflatoxins was removed and filtered through 0.54 μm syringe filter and then injected to HPLC.

HPLC Conditions For Aflatoxins Analysis

All analysis of aflatoxins were performed on (Prominence™, Shimadzu®, Japan) HPLC apparatus equipped with Mediterranea C-18 @ 5 μm 25cm (Shimadzu, Japan) column oven and (LC-20AT® (Shimadzu, Japan) pump. For the determination of aflatoxins in nuts, isocratic mobile phase consisting of methanol: acetonitrile: water (22.5: 22.5: 55) was used with a flow rate of 1 mL / min with an injection volume of 20 μL . The eluate was detected using fluorescence detector RF-10A_{XL}® (Shimadzu, Japan) set at emission 440nm and excitation at 360nm.

Degradation Kinetics of Aflatoxins

The degradation of aflatoxins by irradiation was kinetically studied according to the previous

studies of [34]. To calculate degradation rate constant, absorbed dose at a unit time was converted to irradiation time. The correlation coefficients (R) were calculated from the plot of logarithm of the concentration of aflatoxins in nuts versus irradiation time. The degradation rate constant (k , min.^{-1}) was calculated from the following equation: $k = -\ln(C/C_0)/t$, where t , irradiation time (min.), C , aflatoxins concentration after irradiation and C_0 , aflatoxins concentration before irradiation.

Statistical Analysis

Experimental data including fungal count, aflatoxin detection and degradation by UVC irradiation was subjected to analysis of variance. Means and standard errors were calculated using descriptive statistics.

Conclusion

Tree and ground nuts available in local markets of Faisalabad, Pakistan were sufficiently contaminated with the fungal flora, which can lead to aflatoxins contamination if suitable temperature and moisture level are available for the proliferation of fungal spores. The formation of AFB₁ is always higher than AFB₂, AFG₁ and AFG₂. The physical technique, UVC, has the potential to improve the microbiological value of food in addition to substantial aflatoxins detoxification.

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