Contamination of Tree Nuts by Aflatoxigenic Fungi: Aflatoxin Content of Closed-Shell Pistachios

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Inoculation of fresh, or dried and rehydrated, closed-shell pistachios at the stem end of the shell with spores of *Aspergillus flavus* (*A. flavus*) resulted in aflatoxin contamination of the kernel after incubation. The proportion of contaminated nuts was 48% for the fresh pistachios and 35% for the dried pistachios with 18% and 4%, respectively, having kernels containing aflatoxin levels in excess of 90 μg/kernel, sufficient to contaminate a 10 lb test lot at the 20 ppb guidance level. Closed-shell pistachios batch-rehydrated for 3 h in a bath inoculated with *A. flavus* spores showed aflatoxin levels in the kernels of 170 ppb after 2 days incubation and 87 500 ppb after 6 days. This study demonstrates that the kernels of closed-shell pistachios can be contaminated with aflatoxin, probably through penetration of the stem end of the shell by aflatoxigenic *Aspergillus* species, and the practice of rehydration prior to mechanical splitting should therefore be avoided.

Keywords: *Aflatoxin; Aspergillus spp.; pistachio; Pistacia vera; stem end; aflatoxin analysis; rehydration*

INTRODUCTION

Tree nuts such as pistachios (*Pistacia vera*), almonds (*Prunus dulcis*), and walnuts (*Juglans regia*) are major crops in the state of California, comprising 100% of all U.S. production, with an aggregate 1995 value in excess of $1.26 billion (Moyer and Menke, 1996). Under certain conditions these nuts may be infected by various strains of *Aspergillus flavus* (*A. flavus*) and *A. parasiticus*, resulting in biosynthesis and accumulation of mycotoxins detrimental to quality and food safety. The carcinogenicity in animals of the primary metabolites, the aflatoxins, has resulted in tolerance levels being established for domestic consumption and for export. In the United States a maximum guidance level limit of 20 ng/g (20 ppb) for nuts (shells included) intended for human consumption has been set by the Food and Drug Administration (1996), whereas standards imposed by importing countries may be even more restrictive, with levels as low as 1 ng/g for aflatoxin B_1 (AFB_1, **1**; Figure 1) and 4−5 ng/g total aflatoxins. Rejection of shipments is therefore a potential consequence of aflatoxin detection, since even low levels of fungal infection can result in such mandated levels being exceeded. For the 1995/1996 crop year, a large proportion (41%) of the total U.S. tree nut production was exported, and aflatoxin contamination could have a serious economic impact upon the industry (National Agricultural Statistics Service, 1997).

High levels of aflatoxins in pistachios have been correlated with an increased rate of *Aspergillus* infection associated with insect damage (Sommer et al. 1976, 1986). However, even when such damage does not occur, the phenological development of the nut may still offer significant opportunities for microbial infection. In pistachios, the kernel is formed inside a shell (endocarp) that grows within a hull consisting of outer and inner layers (epicarp and mesocarp). In the course of normal development the shell dehisces from the hull and opens about a month prior to harvest while the hull remains closed, providing a barrier to contamination (Crane and Iwakiri, 1982). Under certain environmental or agronomic conditions "early split" of the hulls may occur due to scission of the shell before dehiscence has taken place, so that fungal spores may then be introduced by airborne transport, on dust particles, or by insect exploration (Doster and Michailides, 1994). Physical damage might also occur when nuts are shaken from the tree during harvest or during transportation to the processing plant, where they are cleaned and dried.

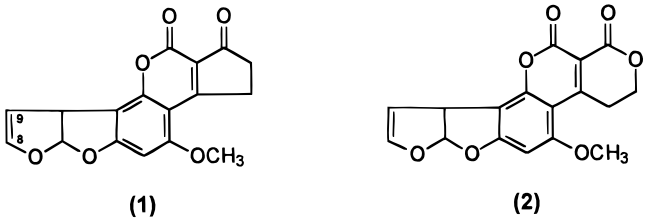

Figure 1. Chemical structures of aflatoxin B_1 (AFB_1, **1**) and aflatoxin G_1 (AFG_1, **2**). The minor metabolite aflatoxin B_2 is 8,9-dihydro-AFB_1.

Although damaged nuts are the primary source of contamination, surveys of processing streams have detected aflatoxins in closed-shell pistachios in which the shell does not split, consequently never exposing the kernel to fungal spores (Schatzki and Pan, 1996). However, Michailides (1989) has shown that the base (stem end) of the fruit remains relatively soft later in the season compared to the rest of the shell and is vulnerable to heteropteran sucking insects, which feed preferentially at this site, so that it seems probable that any fungal attack would also be most likely to occur at the stem end. In addition, preliminary investigations in this laboratory have indicated the presence of ergos-

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terol, a metabolite which arises from fungal metabolism only, in the stem ends of pistachios (King and Mahoney, unpublished). In any event, the general screening technique for aflatoxins by means of the characteristic fluorescence produced on irradiation with UV light (Dickens and Welty, 1975) is such that internal mold growth may result in the toxins not being detected unless the shells are deliberately broken open and the edible portion removed for chemical analysis.

Natural dehiscence is a desirable feature of pistachios since most of the crop is marketed in-shell and the separation enables the shell to be easily removed by the consumer. The undehisced portion of the crop must be sorted and the shells removed before marketing as kernels (Crane and Iwakiri, 1982). Closed-shell pistachios are generally reprocessed overseas by water-soaking and artificial opening using low-cost labor (Schatzki and Pan, 1996). Any nuts sequestering aflatoxigenic *Aspergillus* spores or aflatoxins could thus potentially contaminate the batch during the rehydration process. To investigate the conditions under which such contamination could occur, a study was therefore undertaken to assess the extent of fungal propagation during reprocessing and to attempt to define the point of entry of *A. flavus* giving rise to aflatoxins in closed-shell pistachios.

MATERIALS AND METHODS

Rehydration of Dried Pistachios. A bulk sample of dried and sorted closed-shell pistachios was obtained from a California processor. Pistachios (3 kg) with shells free of any visible cracks or splits were selected and soaked in water (Barnstead Nanopure). Triplicate samples (25 nuts each) were removed every hour up to 9 h, and an additional triplicate sample was removed after 24 h. A control sample of unsoaked nuts was also taken (0 h). Shells were removed by hand, and the kernels were ground in 250 mL blender cups. Triplicate accurately weighed wet pistachio samples were dried under vacuum at 100 °C to constant weight (15 h). Loss in sample weight was calculated as percent moisture.

Preparation of Spore Suspension. *Aspergillus flavus* NRRL 25347, which produces aflatoxin B_1 (AFB_1) with trace levels of AFB_2 (8,9-dihydro-AFB_1) (Figure 1) was grown on potato dextrose agar for 7 days. Spores were collected on a swab and transferred to 0.05% Tween 80. Spore concentration was calculated using a Neubauer counting chamber.

Preparation of Individually Inoculated Pistachios. Fresh pistachios were collected from Winters, CA, and hulled by hand. Closed-shell pistachios (200) with no visible cracks or splits in the shell were selected and placed discontinuously, 5 per Petri dish (20 × 100 mm). Dried, closed-shell pistachios (200) were soaked in purified water (1 L) for 3 h, and then nuts were placed 5 per Petri dish. The fresh and rehydrated pistachios in half (20 each) of the Petri dishes were inoculated on the stem end of each shell with 200 spores of *A. flavus* NRRL 25347 in 0.05% Tween 80 (5 μL). The remaining half (20 each) of the dishes containing fresh and rehydrated pistachios were left uninoculated. All samples were incubated at 30 °C for 6 days.

Preparation of Bath Inoculated Pistachios. Closed-shell, dried, and sorted pistachios (6 kg), with shells free of any cracks or splits, were selected. One batch (3 kg) was soaked in purified water (4 L) for 3 h, and a second batch (3 kg) was soaked in purified water (4 L) containing 4×10^6 spores of *A. flavus* NRRL 25347. From each treatment, 45 nuts were placed in each of 21 Petri dishes (20 × 100 mm). The samples were incubated at 30 °C, with three Petri dishes from each treatment being removed from the incubator for analysis each day for a total of 6 days. Untreated fresh pistachios were also selected, incubated, and analyzed in the same way but with no prior water treatment.

Table 1. Aflatoxin Content of Fresh and Rehydrated Inoculated and Uninoculated Closed-Shell Pistachios

incubation period (days)	aflatoxin B_1[a] (ppb)		
	fresh	rehydrated	
	uninoculated	uninoculated	inoculated
0	0	0	0
1	0	0	0
2	0	0	170
3	0	90	15 400
4	0	540 (also 350 ppb AFG_1)	32 700
5	0	160	57 100
6	0	390	87 500

[a] Average of three samples, 45 nuts/sample; only one sample on day 4 contained aflatoxin G_1.

Analysis of Individual Pistachios for Aflatoxin. Individual, inoculated and uninoculated, pistachios were shelled by hand after incubation and each extracted in MeOH (20 mL) by crushing with a flattened stirring rod. The MeOH was removed from an aliquot (1 mL) by evaporation with N_2 at 40 °C and the residue derivatized by treatment with hexane (200 μL) and trifluoroacetic acid (200 μL) (Pierce Chemical Co.) at room temperature for 10 min. The sample was evaporated to dryness with N_2 at 40 °C and redissolved in H_2O-CH_3CN (9:1; 1 mL). Aliquots (20 μL) were analyzed for aflatoxin by reversed-phase HPLC and detected by fluorescence detection, with excitation at 365 nm and emission at 455 nm (Rodriguez and Mahoney, 1994; Mahoney and Rodriguez, 1996). The lower detection limit was 0.02 μg per kernel. AFB_2 was detected at levels which were insignificant relative to AFB_1 (ca. 0.1%) and was therefore not quantitated.

Analysis of Bath Inoculated Pistachios for Aflatoxin. Each sample (45 nuts) was extracted in a Waring blender with NaCl (10 g) and $MeOH-H_2O$ (60:40; 250 mL) for 1 min. A portion of the extract (100 mL) was centrifuged at 2500 rpm for 5 min, and a supernatant aliquot (10 mL) was filtered through a 0.2μ 45 mm syringe filter (Millipore Millex). An aliquot (100 μL or 5 mL, depending on the aflatoxin concentration) was diluted with an equal volume of H_2O and the aflatoxin retained by passage through an Aflatest P immunoaffinity column (Vicam). Aflatoxin was eluted with CH_3CN (2 mL) and the solvent removed by evaporation with N_2 at 40 °C. Aflatoxin was derivatized and analyzed by HPLC as described above, with a lower detection limit of 1 ppb per 45 nut sample. Values (ppb aflatoxin B_1) for individual samples were as follows (*incubation day*, sample1/sample2/sample3). Noninoculated pistachios: *3*, 190/67/14; *4*, 500(+350 AFG_1)/1100/29; *5*, 9/280/180; *6*, 560/3/610. Inoculated pistachios: *2*, 320/80/110; *3*, 17 200/13 400/15 600; *4*, 37 700/30 800/29 500; *5*, 68 500/50 300/52 600; *6*, 68 200/101 000/93 400. These results are summarized in Table 1.

RESULTS AND DISCUSSION

The rate of rehydration of closed-shell pistachios by soaking in water is shown in Figure 2. Dried pistachios typically have a residual water content of 5.6%. The initial uptake of water was rapid, with a moisture content of 17% being attained in only 3 h, well above the 10−12% range necessary to support active fungal growth. After 8 h the moisture content was 25% but thereafter increased only slowly to 32% on 24 h of immersion, close to the value of 35% typical of fresh pistachios. It is apparent that relatively brief rehydration periods would be sufficient to provide an environment compatible with growth of any fungal spores which may be present and consequent biosynthesis of aflatoxins by aflatoxigenic *Aspergillus* species. Moreover, immersion in a rehydration bath would enable spores, which might be present in only a very small number of nuts, to be dispersed throughout the whole batch.

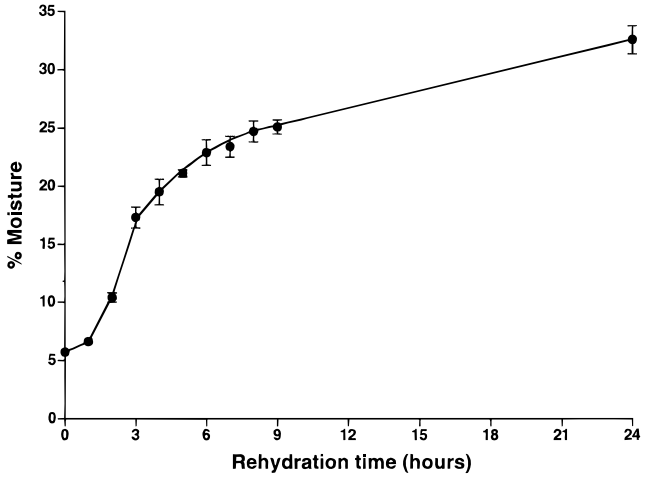

Figure 2. Moisture content of dried, closed-shell pistachios rehydrated by immersion in water, as a function of time.

Contamination of pistachios by *Aspergillus* is normally associated with the "early split" phenomenon or with damage by insects. The kernels of closed-shell pistachios would be anticipated to be physically well-protected from invasion by the fungus. Nevertheless, closed-shell pistachios have been found to contain aflatoxins, so that it must be possible for the fungus to gain access to the interior of the shell under certain conditions. The most probable point of entry is at the base of the fruit, or stem end, an area of about 10 mm^2 which remains softer until later in the season relative to the pericarp in the apical part of the fruit. It has been observed (Michailides, 1989) that certain small heteropterans in the Miridae and Rhopalidae families commence feeding preferentially at this point, and it may therefore be possible, particularly in fresh or rehydrated pistachios, for this area to be vulnerable to fungal penetration also.

To test this hypothesis, groups of 100 fresh and rehydrated pistachios were individually inoculated at the stem end of each shell with a very low level of 200 spores of aflatoxigenic *Aspergillus flavus*. After incubation of these samples at 30 °C for 6 days, the shells were cracked open and the aflatoxin levels of each kernel measured by HPLC. A control group was rehydrated but was maintained under the same conditions without inoculation. The shell did not support any fungal growth, but some of the samples showed slight visual evidence of colonization by *A. flavus* at the stem end.

The control group of noninoculated, rehydrated closed-shell pistachios showed a very low incidence of aflatoxin contamination. Only four nuts (4%) of this group had detectable aflatoxin B_1, with levels of 0.09, 2.4, 10, and 10 μg/kernel, respectively. Nevertheless, the detection of any contamination indicates that aflatoxin most probably was formed in the closed-shell pistachios from the natural presence of spores of an aflatoxigenic *Aspergillus* species, which produced the toxin upon rehydration to a level capable of supporting fungal growth. In the individually inoculated, rehydrated, closed-shell pistachios the degree of aflatoxin contamination was much higher, 35% of the nuts showing detectable aflatoxin, at values ranging from 0.02 to 349 μg/kernel, with 14% showing levels below 1 μg/kernel. The individually inoculated, fresh, closed-shell pistachios had an even higher proportion (48%) of contaminated nuts, with aflatoxin levels in the 0.02−700 μg/kernel range, and a significant proportion (16%) having levels in excess of 100 μg/kernel.

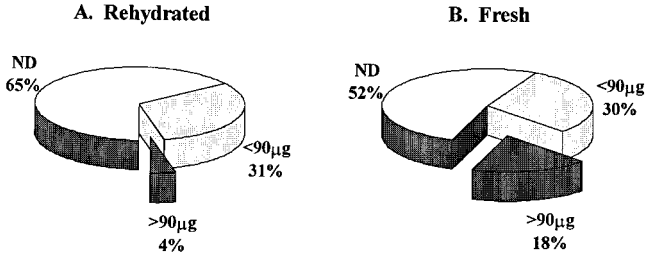

Figure 3. Percentage of kernels containing aflatoxin, at levels greater or less than 90 μg/kernel, from individual closed-shell pistachios inoculated at the stem end of the shell and incubated at 30 °C for 6 days. A level of 90 μg aflatoxin B_1 in a single kernel would contaminate a 10 lb test lot at the maximum guidance level of 20 ppb. ND = none detected. (A) Rehydrated dried pistachios; (B) fresh (undried) pistachios.

To relate these values to the regulatory standards, results for the individually inoculated rehydrated and fresh pistachios are compared in Figure 3. Percentages of contaminated kernels are grouped into ND (none detectable) and into those with levels less than, and exceeding, 90 μg per kernel, since a typical kernel weighing ca. 1 g containing this amount of aflatoxin would be sufficient to contaminate a 10 lb test lot (i.e. 4.54 kg) of shelled nuts at the 20 ppb level. The FDA guidance level is predicated on a shell plus kernel basis, but since all of the aflatoxin is likely to occur in the kernel even in in-shell pistachios, it is assumed that a similar level would be applied to shelled nuts. In the rehydrated closed-shell pistachios, 4% of the kernels contained aflatoxin in excess of 90 μg, while 65% of the kernels showed no detectable aflatoxin. The fresh closed-shell pistachios showed a much higher level of contamination, with 18% exceeding 90 μg aflatoxin/kernel and only 52% having no detectable aflatoxin.

These results support the hypothesis that the fungus can enter closed-shell pistachios through the comparatively soft portion of the shell surrounding the stem. The higher rate of contamination of fresh pistachios relative to rehydrated nuts might well be expected since the drying process is likely to induce lignification of the stem end which cannot be completely reversed by hydration. Nevertheless, the results of this study indicate that rehydration of dried, closed-shell pistachios for the purpose of shell removal may induce fungal growth and consequent contamination of the kernels, giving rise to aflatoxin levels which could make the kernels unmarketable.

Since the previous experiment demonstrated that closed-shell pistachio kernels were capable of being contaminated by aflatoxin through stem end infection by *Aspergillus flavus*, a more realistic simulation of the rehydration process was investigated. Dried, closed-shell pistachios were immersed in a flotation bath inoculated with *A. flavus* NRRL 25347 at 100 spores/mL for 3 h. A control batch was treated in the same manner without inoculation of the water. The nuts were then removed and incubated at 30 °C with samples being removed for aflatoxin analysis each day for a period of 6 days. An additional control, consisting of fresh nuts which were not subjected to flotation, was incubated and analyzed under the same conditions.

As shown in Table 1, neither the inoculated nor uninoculated pistachios showed the presence of aflatoxin on removal from the bath after 3 h or after 1 day of

incubation. However, the inoculated sample showed a level of 170 ppb in the kernels after day 2, and this rapidly increased to 87 500 ppb by the end of the experiment on day 6. In contrast, the uninoculated pistachios did not show any aflatoxin until day 3 when an average level of 90 ppb was recorded. Aflatoxin levels were highly variable in the uninoculated pistachios, both within samples and between days, the maximum level attained being 540 ppb (Table 1). Such variability would be consistent with aflatoxin arising within a few contaminated nuts containing *Aspergillus* spores which germinate on rehydration. Further evidence for this possibility was the occurrence in one sample of 350 ppb of aflatoxin G_1 in addition to aflatoxin B_1. Since aflatoxin G_1 is not a metabolite of *A. flavus* NRRL 25347, the strain used for inoculation in our laboratory, and is structurally distinct from aflatoxin B_1 (Figure 1), it must have arisen from a natural strain endemic to individual nuts in the pistachio sample. In contrast, no aflatoxin was detected in the fresh pistachios which were not treated in a flotation bath, even after 6 days of incubation. This is consistent with the hypothesis that batch contamination arises from exposure to a very few *Aspergillus*-infected nuts during flotation. The fresh nuts were hand-hulled, and therefore no intermingling occurred.

These results demonstrate that closed-shell pistachios inoculated at the stem end can become contaminated with aflatoxin either when fresh or upon rehydration, even though the shell remains unbreached. The vulnerability of the stem end relative to other parts of the shell probably permits fungal penetration and consequent access to the kernel. Moreover, the practice of flotation separation, or rehydration in a water bath prior to mechanical cracking of the shells, can result in dispersion of spores and initiation of fungal growth and aflatoxin biosynthesis, since fresh pistachios which were not subjected to flotation showed no evidence of aflatoxin contamination. Industrial practice in the California pistachio industry involves rapid mechanical drying of pistachios, but closed-shell pistachios which are shipped overseas for hand-cracking are understood to be processed by a "cottage industry". Under such circumstances, processing after rehydration may be slow, and it is probable that the desire to keep costs low would result in air-drying, often in a high humidity environment, rather than mechanical drying of the product. These conditions may well lead to periods of several days of storage at hydration levels capable of supporting *Aspergillus* growth, comparable to our experimental protocol. The aflatoxin contents thus attained are capable of reaching levels far in access of the FDA guidance level of 20 ppb and indicate that pistachios should be dried as rapidly as possible postharvest and rehydration should be avoided in order to prevent aflatoxin contamination of this valuable crop.

ACKNOWLEDGMENT

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