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Hazelnuts as Possible Substrate for Aflatoxin Production[1]

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ABSTRACT

A study was carried out on the fungal contamination of commercially available hazelnuts, and the effect of different factors (water activity, temperature and presence of potassium sorbate) on fungal growth and aflatoxin production in hazelnuts. All samples (100%) of raw hazelnuts showed fungal contamination. None of the samples showed aflatoxin contamination, but when hazelnuts were inoculated with *Aspergillus parasiticus*, and water activity and temperature were optimal for mold growth, high production of aflatoxin was found. Potassium sorbate at subinhibitory levels seemed to inhibit fungal growth, but enhanced aflatoxin production.

Aflatoxins are mycotoxins produced by strains of the *Aspergillus flavus* group and are associated with certain carcinogenic processes in man and animals *(6,10,11)*. *A. flavus* and other mycotoxin-producing strains are present in dried fruits in their natural state and proliferate through deficiencies in the storage system, and infestation by insect and rodents. Their development constitutes a hazard to human and animal health.

Since Spain is the third greatest producer of hazelnuts in the world, our purpose was to study the presence of fungal contamination in commercially available hazelnuts as well as the incidence of different factors (water activity, temperature and presence of potassium sorbate) on fungal growth and aflatoxin production in hazelnuts.

MATERIALS AND METHODS

Samples

Fifty samples of hazelnuts (25 toasted, 25 raw) were obtained from food manufacturing plants in the provinces of Lleida, Tarragona and Valencia. The samples were taken by the inspection services or directly by personnel from the analytical laboratories in the retail market or from processing plants. The samples were sent to the laboratory as soon as they were collected, and tested on arrival. Otherwise they were stored at 4°C to arrest any aflatoxin formation up to the moment of analysis.

Aflatoxins analysis

Aflatoxin B_1 was determined in samples of hazelnuts by a modification of Gimeno's method *(3)*, which has been in use since 1981 as the official Spanish method of analysis for aflatoxins in foods and their raw materials *(2)*.

Each ground sample was subsampled (30 g) and extracted with acetonitrile-potassium chloride 4% (9:1). A portion of the extract representing 15 g of the subsample was defatted four times with 50 ml of isooctane and cleaned up by washing it with 12.5 ml of distilled water. Toxins were extracted with chloroform. The extracts were sequentially filtered through anhydrous sodium sulfate and evaporated until almost dry. Residue was dissolved in chloroform and transferred to a 10 ml vial. The organic phase was evaporated to dryness under a gentle stream of nitrogen and 50 µl of trifluoroacetic acid (TFA) were added and vortex-mixed 1 min. Four ml of acetonitrile-water-acetic acid (1.8-8.2-0.1 ml) were added and vortex-mixed for another minute. The sample was filtered through MF-Millipore filter (HATF OU300) and 10 µl of clear filtrate were injected into a high performance liquid chromatograph equipped with a µBondapak C_{18} column (30 cm by 3.9 mm) and 360-nm UV detector. The amount of aflatoxin was calculated on a 730 data module integrator (Water Associates Inc.) using external standards (retention time 7.2 min; mobile phase, water + acetonitrile, 70 + 30, flow rate 1 ml/min).

Mycological studies

The study of fungal flora present in the samples was done by taking 50 kernels of each sample, sterilizing them with a solution of 2% sodium hypochlorite, and washing them twice afterwards with sterile water. Ten groups of five of these kernels were placed, under aseptic conditions, in potato dextrose agar (PDA). After incubation for 5 d at 25°C, they were examined for fungal growth, and the molds present on the kernels were later isolated and identified.

The qualitative test for aflatoxin-producing strains was carried out by inoculating strains of *A. flavus* at the center of Hara's medium *(4)* and coconut agar medium *(5)* in glass petri dishes, and incubating them at 28°C in the dark. Plates were examined under long wave UV light (365 nm) until the seventh day of incubation for presence or absence of blue fluorescence in the agar surrounding the colonies. A confirmatory test was

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carried out by TLC of chloroform extracts of both agar media and comparing them with standards of toxins.

Aflatoxin production by A. parasiticus *in hazelnuts*

Without potassium sorbate. A suspension of 2 x 10^6 spores, counted by hemocytometer, of *A. parasiticus* NRRL 2999/ml was obtained by incubation of fungi in potato dextrose agar (PDA) for 7 d at 28°C. One millilitre of this suspension was inoculated into each of three 500-ml Erlenmeyer flasks containing 60 g of hazelnuts and with different water activities (measured by Novasina Humidat IC-I). Aflatoxin production was determined using the following kinds of commercial hazelnuts: ground raw, unground raw, ground toasted and unground toasted, after an incubation period of 15 d at 20, 30 and 40°C.

With potassium sorbate. One millilitre of the *A. parasiticus* suspension, obtained as described above, was inoculated into each of three 500-ml Erlenmeyer flasks containing 60 g of hazelnuts and a known amount of potassium sorbate (1000, 2000 and 3000 ppm) with either water activity of 0.78 in raw samples or 0.81 in toasted ones. Aflatoxin production was determined after an incubation period of 15 d at 30°C.

A. parasiticus *growth estimation in the different tested systems*

A fungal estimation in hazelnut samples was carried out using the amount of ergosterol present on the samples *(8,9)*. Previously *A. parasiticus* had been grown in YES medium, mycelial mass had been recovered by filtration and ergosterol amount in the mycelial mass had been calculated using the same method.

RESULTS AND DISCUSSION

Table 1 indicates the incidence with which the fungi were found in samples of hazelnuts. All samples examined (100%) showed fungal contamination. The raw samples presented the highest mold contamination with a total infection of 85.8% compared to 58.2% in the toasted samples. Thus whereas *A. flavus* was isolated from raw samples, giving 17.1% yield from kernel samples, this yield was down to 4.4% in the toasted ones. While these species were present in all raw samples they were only found in 60% of the toasted ones.

The fungal contamination of the samples was higher than the ones described by other authors *(7)*.

We isolated 59 strains of *A. flavus*, and 25 of these were aflatoxigenic strains. This yield of 50% is very high if we compare it with other results obtained in different foods by other authors *(7)*.

None of the analyzed samples showed aflatoxin contamination, consequently, considering this fact and the high *A. flavus* infection found in these samples, we can conclude that the usual storage conditions were not optimal for fungal growth and aflatoxin production.

To study which conditions favor aflatoxin production in hazelnuts, we carried out different experiments using hazelnut subtrata inoculated with *A. parasiticus*. In Fig. 1 and 2 aflatoxin production by *A. parasiticus* is represented against water activity, temperature of incubation and commercial hazelnuts used. The optimum temperature for aflatoxin production is 30°C. Thus the highest amount of aflatoxin (20 ppm) was found when the mold was grown at 30°C on ground raw hazelnuts with 0.77 water activity.

Aflatoxin production decreased drastically when the temperature rose to 40°C in all the commercially used

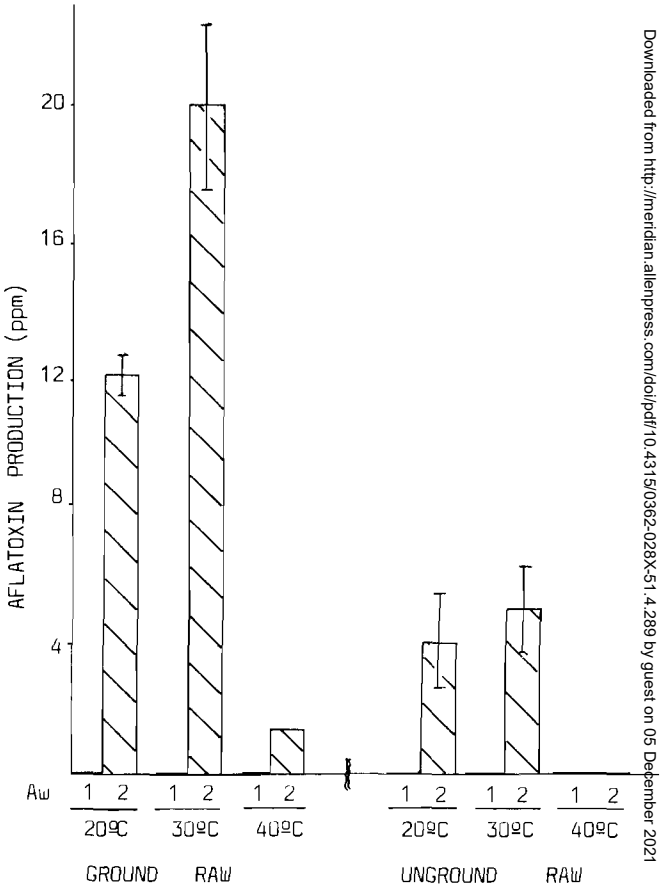

Figure 1. *Aflatoxin production in raw hazelnuts at different temperatures (20, 30, 40°C) and* a_w *(1=0.38; 2=0.78).*

TABLE 1. *Mold infection in hazelnuts*[a].

Molds	Positive samples (%)		Incidence of kernels examined(%)			
	Raw	Toasted	Raw		Toasted	
Aspergillus flavus	100	60	17.1[b]	9.8[c]	4.4[b]	8.1[c]
Aspergillus niger	96	84	40.0	22.8	14.4	19.0
Aspergillus glaucus	76	40	7.6	7.7	2.4	5.5
Penicillium	84	96	31.0	28.4	21.6	19.6
Mucorales	100	100	37.6	3.5	34.3	10.1
Fusarium	0	12				

[a]Number of samples screened, 25 raw, 25 toasted.
[b]Means.
[c]SD.

hazelnuts. Unground toasted hazelnut is the poorest medium showing the lowest production of these toxins when the incubation temperature was 40°C and the water activity 0.81. At 20°C, production was still considerable, but generally lower than at 30°C, water activity being again an important factor to be considered.

Consequently we can conclude that ground raw hazelnut is the best medium for aflatoxin production, whereas unground toasted hazelnut is the worst one when the same temperature and a_w are used. Also, it is important to note that while the usual storage conditions don't favor aflatoxin production, when water activity is 0.78 to 0.81 (amounts usually found in cake products), hazelnuts become a good substrate for production of those mycotoxins.

Fungal growth based on ergosterol production is displayed in Table 2. No total relationship was observed between fungal growth and aflatoxin production (Table 2 and Fig 1 and 2), although optimal fungal growth occurred when the incubation was at 20 or 30°C and water activity also was higher than usual for storage conditions (0.24 for toast material and 0.38 for raw nuts).

Potassium sorbate enhanced aflatoxin production and seemed to inhibit fungal growth when the optimal conditions were used (Table 3). This effect is also described by other authors *(1,12,13).*

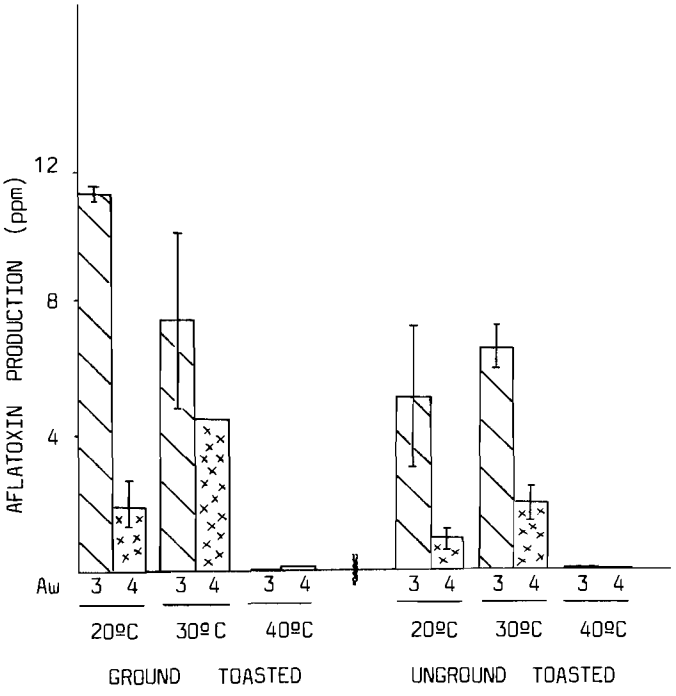

Figure 2. *Aflatoxin production in toasted hazelnuts at different temperatures (20, 30, 40°C) and a_w (3=0.70; 4=0.81).*

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TABLE 2. *Incidence of different factors on* A. parasiticus *growth[a] using hazelnuts as substrate.*

Temperature →	20°C			30°C			40°C		
a_w →	0.38	0.78		0.38	0.78		0.38	0.78	
Raw hazelnuts									
Ground	--	1.6[a]		--	2.4		--	1.6	
Unground	--	1.1		--	0.8		--	0.1	
a_w →	0.24	0.70	0.81	0.24	0.70	0.81	0.24	0.70	0.81
Toast hazelnuts									
Ground	--	2.0	1.5	--	0.4	0.7	--	0.6	0.1
Unground	--	1.3	1.9	--	1.3	0.9	--	--	--

[a]Amounts are given in g of estimated dry mycelium.

TABLE 3. *Influence of amount of potassium sorbate on* A. parasiticus *growth and aflatoxin production in hazelnuts.*

	Potassium sorbate amount[a]							
Samples	0		1000		2000		3000	
Raw hazelnuts a_w=0.78	2.4[b]	0.6[c]	2.7	0.5	1.5	1.3	1.3	1.6
Toast hazelnuts a_w=0.81	0.7	0.4	0.7	0.5	0.5	0.5	0.8	1.5

[a]Amounts are given in ppm.

[b]Amounts are given in g of estimated dry mycelium.

[c]Amounts are given in mg aflatoxin produced/g estimated dry mycelium.

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