Distribution of Aflatoxin in Almonds. 2. Distribution in Almonds with Heavy Insect Damage

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The aflatoxin distribution function in individual insect-damaged NePlus Ultra almonds was determined and found to be the sum of two distributions. Substantially all almonds exhibited a positive aflatoxin level between 0.02 ng/g (the detection level) and 0.3 ng/g, the precise form of this distribution depending on the lot studied. In addition, 1/1000 of the nuts showed contamination between 60 and 600 000 ng/g, independent of the lot. The latter distribution showed a smooth decrease with log concentration in this range, with no evidence of a minimum, as had been found previously for pistachios. No distribution data between 0.3 and 60 ng/g could be obtained. The distribution below 0.3 ng/g was assigned to contamination during post-harvest storage. The distribution above 60 ng/g was tentatively assigned to navel orange worm damage occurring when insects enter the kernel during split hulls late in the growing season. Considerable additional work will be required to verify these assignments.

Keywords: *Almonds; insect damage; aflatoxin; nut distribution functions*

INTRODUCTION

The carcinogenic mycotoxin aflatoxin is produced in a number of foods and feeds, and in particular tree and ground nuts, by the molds *Aspergillus flavus* and *Aspergillus parasiticus*. The resulting distribution of aflatoxin among individual nuts in a lot has been of considerable interest for some time. Sampling theories can be developed from such distributions (Schatzki and De Koe, 1999; Whitaker et al., 1994), and in favorable cases knowledge can be gained as to the source of toxin contamination (Schatzki, 1998) and the effects of product sorting to which all nuts are subject prior to sale (Schatzki and Pan, 1996). As early as 1966 Cucullu et al. showed that in peanut lots, the bulk of the toxin was contained in relatively few nuts. Whitaker et al. (1994), assuming a parametric form for the aflatoxin distribution, obtained information about this distribution mainly in peanuts. A similar skewed distribution was found in tree nuts, particularly in pistachios (Schatzki, 1995b). Schatzki (1995a) pointed out how such distributions could be obtained directly without parametric assumptions and, more importantly, without the excessive labor of measuring the concentration in a very large number of individual nuts. Using these methods, the distributions in five distinct collections of pistachio lots were obtained and found to be remarkably similar (Schatzki, 1998). Since pistachios are generally processed in the United States in such a way as to prevent post-harvest mold growth, the presence of aflatoxin had to be assigned to pre-harvest mold entry and in particular to a process of hull splitting, so-called "early splitting" in the weeks before harvest (Sommer et al., 1986). In fact, Schatzki (1998) was able to explain the shape of the distribution curve on the basis of the early splitting process.

Much less is known about the distribution of aflatoxin in almonds, although it is known that these nuts are subject to *A. flavus* infection. In 1975 Schade et al. reported, on the basis of a limited number of experimental results, that high aflatoxin levels seemed to be only found in almonds with insect damage. Schatzki (1996), on the basis of an industry-wide survey of the 1993 crop, noted that relatively high levels (3 ng/g) appeared to occur only among ground almonds, a product of low quality. Since insects leave unsightly tunnels, one might surmise that insect-damaged nuts had been either removed or ground and that ground nuts should exhibit aflatoxin, in agreement with Schade et al.'s conclusion. The production and processing of almonds in the United States differs significantly from that of pistachios. Almonds dry out on the tree well before harvest, making mold growth unlikely without some outside rehydration. It has been suggested that insect attack could cause such rehydration (Mahoney, 1999). In any event, hull splitting occurs in most nuts on the tree during 2 weeks prior to shakedown. Nuts are then kept on the orchard floor for some time, making post-harvest insect attack much more likely than in pistachios. Finally, nuts are stored under conditions allowing both insect attack and moisture accumulation. It would thus appear that the aflatoxin distribution in almonds should differ significantly from that of pistachios. Furthermore, the recent tightening of aflatoxin import regulations in the European Union increases the need to have almond distributions available for sampling calculations. The present research was undertaken to establish the aflatoxin distribution for such calculations and to learn about the process of aflatoxin growth in almonds.

METHODS AND MATERIALS

Two almond lots, obtained from an almond processor in California, were sampled. Each lot consisted of so-called "oil stock" nuts which had been culled from the process stream

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Figure 1. Navel orange worm (NOW)-damaged almonds.

Table 1. Sample Concentration Distribution among Samples of a Fixed Size

sample size, *n*		no. of samples, *N*	concentration, *c*, ng/g											
			<0.02	0.02−0.03	0.03−0.10	0.10−0.31	0.32−1.00	1.00−3.16	3.17−10.0	10.0−31.6	31.7−100	100−316	317−1000	1000−3162
1	A	20[a]		1	11	4	1	2						
10	A	20		3	11	6								
20	A	20			6	13	1							
100	A	30[a]		2	23	1	2	1						
200	A	99	12	4	35	32	3	3	2	3	0	2	2	1
200	B	200	77	9	36	32	12	11	7	5	6	2	1	2
1000	A	20		1	6	4	2	2	0	1	2	2		

[a] One sample at an undetermined, but high, concentration.

because of perceived surface and tunneling damage due to insects (Figure 1) and consisting principally of the NePlus Ultra cultivar. Sublots of 45 and 90 kg, respectively, were removed from these lots for analysis. Since the lots represented product processed approximately 6 months apart, it was at least possible that the lots, and hence the sublots, might differ either because of pre-harvest or post-harvest handling. Accordingly the sublots and samples taken therefrom were kept separate and designated sublots A and B. A total of 20 samples of 1 nut each, 20 of 10 nuts, 20 of 20 nuts, 30 of 100 nuts, 99 of 200 nuts, and 20 of 1000 nuts were randomly selected from sublot A, and 200 samples of 200 nuts each were taken from sublot B. Samples were individually ground with dry ice to pass a #20 screen (maximum particle size approximately 1 mm) and were analyzed for aflatoxin with HPLC, using the protocol previously described (Schatzki and Pan, 1996). Aflatoxin is reported as total aflatoxin ($B_1 + G_1 + B_2$; G_2 is virtually never observed). The aflatoxin concentrations obtained for each sample size were logarithmically binned into one-half decade size bins, ranging from 0.02 to 3200 ng/g, plus the bin representing aflatoxin below the detection limit of 0.02 ng/g aflatoxin, following the suggestions in Schatzki (1995a). In agreement with other workers, results are reported based on the extraction fluid recovered and no correction was made for the liquid left behind in the mash. As such, they are about 25% lower than corrected values.

RESULTS

The sample concentrations, appropriately binned, are shown in Table 1. Schatzki (1995a) had previously shown that if a large number of samples of fixed size were measured for aflatoxin and if the results were binned in logarithmic bins of one-half decade each, then the distribution of aflatoxin among the individual nuts in the lot could be deduced directly and easily from the sample distribution (using the "sparse" approximation), provided that each bin contained no more than 10% of the total samples. (The fraction of samples in a given bin is an estimate for the sample probability density function, *P*, in the given range of sample aflatoxin concentration, *C*.) As the sample size is increased, the fractions in some of the bins (those near the actual average concentration in the lot) will increase and eventually exceed the 10% limit. On the other hand, if the sample size is decreased, an increasing fraction of samples would appear in the "not-detected" or zero bin, leaving less for the bins of interest and making the determination of the lot distribution function inefficient. The optimal solution then is to use a sample size for which the bin occupancy approaches 10% (for highly peaked distributions it may be necessary to use different sample sizes in different concentration ranges). Inspection of Table 1 indicates that the sparse approximation criterion is met for bins for which $C > 0.3$ ng/g, but not for bins where *C* is smaller than that. Accordingly, the data for $C < 0.3$ ng/g and that for $C > 0.3$ ng/g must be treated separately.

The bulk of the samples contain aflatoxin between 0.02 and 0.3 ng/g, regardless of sample size. This situation is quite different from what was observed in pistachios (Schatzki and Pan, 1996; Schatzki, 1998), where most of the samples contained no detectable aflatoxin. The result obtained here is not at all what would be expected from a distributed Poisson distribution alone (Schatzki, 1995a). Rather, this is what is predicted when the lot distribution is the sum of two distributions: a uniform distribution where all or most of the nuts contain just 0.02−0.3 ng/g aflatoxin each and a distributed Poisson where a very few nuts contain much larger amounts. Considering first the uniform distribution, it remains to be tested whether the two sublots A and B differ significantly in this region of sample concentration. This can be done by application of the nonparametric Kolmogorov−Smirnov test (two-tailed case; Siegel, 1956) to the two 200-nut sample distributions. Indeed, a maximum cumulative probability difference $D = 0.265$ is obtained, which exceeds the critical D even at $p = 0.001$. Thus it is highly unlikely that the two uniform distributions are the same. Sublot B has a significantly larger fraction of samples below 0.02 ng/g. Neither the data nor the mathematical development at the present time is adequate to break down the lot distributions in the 0.02−0.3 ng/g range. All that one can conclude is that most of the nuts contain aflatoxin in that range.

In the above discussion it would appear that the uniform distribution (most nuts having aflatoxin from 0.02 to 0.3 ng/g) would not apply to the samples above 0.3 ng/g. But this is not so; it is perfectly possible and consistent with the data that even in samples which test above 0.3 ng/g all or most nuts fall within that range, while a few nuts contain a higher level. The 0.02−0.3 ng/g level of all nuts would simply raise every sample concentration by that amount (in effect provide a fixed background), which would not be observable. Thus the distributions for $C > 0.3$ ng/g can be analyzed using the methods for distributed Poissons to obtain the lot distributions at higher levels (above and beyond the uniform <0.3 ng/g present in every nut). Only the two sample sets at $n = 200$ contain enough samples for such an analysis; the remaining sample sets will be shown to be consistent with the lot distribution obtained. Again, the two lots are compared using the Kolmogorov−Smirnov test. However, since here the question only concerns the distributions >0.3 ng/g, the bins below that value are combined. One obtains a maximum difference of 0.06, which is well below the critical D of 0.15 at $p = 0.10$. Thus the hypothesis that two distributions do not differ for $C > 0.3$ ng/g cannot be rejected, and the sets of data for the two 200-nut sublots can be summed. The same conclusion is obtained from the use of the Fisher exact test for 2 × 2 groups (SAS, 1988). Grouping lot against fraction for $C < 0.3$ ng/g, one obtains a two-tailed probability of 0.2250, while grouping lot against fraction for $C > 0.02$ ng/g, one obtains 1.8×10^{-6}. (We thank Dr. B. Mackey for pointing this out to us.) The highest estimated sample probability density P amounts to but 0.05, well below the criterion for application of the sparse approximation. Hence one obtains for the lot concentration values $c = C/n = C$*200 ng/g and lot probability densities $p = P/n = P/200$. The resulting values are listed in Table 2. From the values listed there, an average aflatoxin level of the lots can be computed from $\sum p_i c_i$; one obtains 28 ng/g, >100 times that due to the uniform distribution.

Table 2. Lot Distribution of Aflatoxin in Insect-Damaged Almonds

concentration, c, ng/g	probability, p
<0.3	0.79
63−200	2.5×10^{-4}
200−632	2.3×10^{-4}
632−2000	1.5×10^{-4}
2000−6325	1.3×10^{-4}
6325−20000	1.0×10^{-4}
20000−63246	6.7×10^{-5}
63246−200000	5.0×10^{-5}
200000−632455	5.0×10^{-5}

The resulting lot distribution can now be used to predict the sample distribution for any other sample size, n. (Sample distributions can be computed directly from lot distributions without any approximation, simply by using Poisson statistics.) This computation is carried out for $n = 1000$, and the results are compared with the actual experimental distribution in Table 3. (For convenience the bins are shifted slightly and the experimental distribution is re-binned.) The computed and experimental distributions can be compared, again using the Kolmogorov−Smirnov test, now for small samples $N_1 = N_2 = 20$. One finds a maximum cumulative probability difference of 0.2, well below the critical values of 0.4 (one-tailed) or 0.45 (two-tailed). One concludes that the hypothesis that the experimental data at $n = 1000$ is consistent with that at $n = 200$ cannot be rejected. The predicted sample values at $n \leq 100$ are too small to allow a rigorous test, and indeed, the results match that. Thus the data in Table 1 is not inconsistent within itself and the results listed in Table 2.

DISCUSSION

The distribution of aflatoxin in the highly damaged almond lots considered here can thus be summarized as the sum of two distinct distributions. First, almost all the nuts contain a low aflatoxin level in the range of 0.02−0.3 ng/g; the exact distribution appears to depend on the lot chosen. In addition, a small fraction of nuts (less than 1/1000) contain a much higher aflatoxin level, ranging from 60 to at least 600 000 ng/g, which is described in Table 2. This distribution does not differ between the two sublots. The aflatoxin distribution among the nuts from 0.3 to 60 ng/g cannot be discerned from the present data. It is reasonable to assume that these two distributions arise from very different causes.

An aflatoxin infection which is relatively uniform among the nuts would be characteristic of a situation where nuts were touching, where storage conditions were such that some mold growth were possible, and, as a result, where mold could spread from nut to nut as on a matrix. This is the kind of condition under which almonds are stored long-term following harvest. In fact, frequent fumigation is common. In the present case, the level of aflatoxin which seems to be developed in this way is not serious, amounting to <0.3 ng/g. The exact level may well be a function of storage conditions, as it appeared to be here. There is no reason to believe that such infection is caused by insects, at least directly. The situation is quite different from that in pistachios; for while these nuts are stored in mass as well, considerable care is taken to keep the stored nuts too dry to allow mold growth. And indeed, such low level uniform aflatoxin contamination is not seen in pistachios.

Table 3. Sample Distributions for 20 1000-Nut Samples, Based on Table 2

$n = 1000$	concentration, c, ng/g							
$N = 20$	<0.2	0.2−0.6	0.06−2	2−6.3	6.3−20	20−63	63−200	200−630
actual	9	3	1	2	0	2	3	0
predicted	5.1	4.2	3.0	2.6	2.0	1.3	1.0	1.0

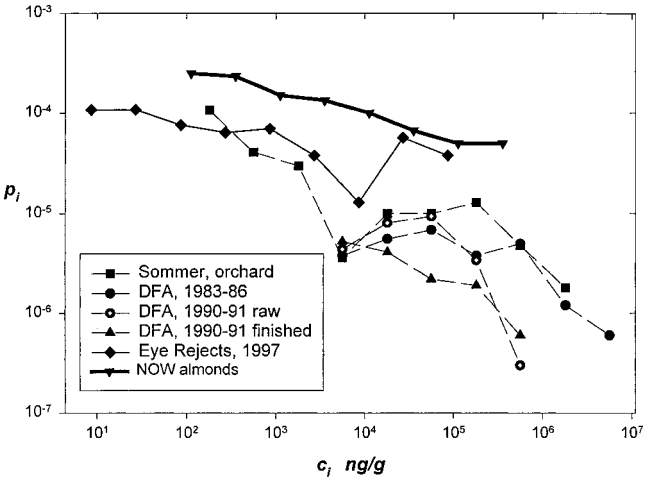

Figure 2. Lot distribution functions (probability of a nut falling in one-half decade of concentration) of pistachios (lower five curves: taken from Schatzki, T. F. *J. Agric. Food Chem.* **1998**, *46*, 2−4) and insect-damaged almonds.

A very different growth regime appears to cause the rare, high-level aflatoxin-infected nuts. Such a distribution would result from nuts isolated from each other, as they are on the tree, and thus not capable of transmitting infection from nut to nut, growing under conditions favorable to mold growth. Infection and (rapid) growth will not occur unless spores can penetrate the shield around the nut to initiate growth. Hence the governing step is shield penetration. In pistachios this step is early hull splitting; although once this step occurs, insect attacks can also contribute. In a previous publication (Schatzki, 1998) it was argued that evidence regarding the governing step could be obtained from the distribution function. Specifically, a plot of log p vs log c could be interpreted as a log probability of aflatoxin infection vs time of infection (based on the assumption of exponential aflatoxin growth). Such a plot is shown in Figure 2 which shows the results previously obtained for five pistachio populations, augmented now by the data from Table 2 for insect-infested almonds. The pistachio data shows a clear minimum around 6000−8000 ng/g for all pistachio populations, along with a maximum around 50 000−100 000 ng/g. The shape of the distribution function was interpreted as evidence of two processes. The data above the minimum was assigned to early hull splitting which occurs during approximately 6 weeks with a maximum 2−4 weeks before harvest (Doster and Michailides, 1995). The data below the minimum might be due to tattering, a disintegration of the hull commencing shortly before harvest. The almond aflatoxin distribution is clearly quite different. First, the level is somewhat higher, which is not surprising since the nuts were specifically selected for insect damage. More important, there is no minimum, i.e., no evidence of two processes. Indeed, it is known that navel orange worm requires a split hull for access to the almond kernel, and such hull splitting occurs in substantially all almonds during the last 2 weeks before harvest (Gradziel, 1999). There is no early hull splitting as in pistachios. Accepting that insect damage is required for the start of aflatoxin growth in almonds, the increased probability at lower c and thus later time could represent more opportunity of insect attack and/or simply more rapid splitting at later times. What does not fit with the pistachio data is the time scale. In pistachios roughly 4 decades in c correspond to about 6 weeks in time. In almonds a similar range in c accounts for but 2 weeks. It is, of course, possible that aflatoxin grows 3 times faster in almonds than in pistachios, and there is evidence that it does grow 1.5 times faster under controlled laboratory conditions (Mahoney, 1999). But whether this applies in field conditions, particularly when the average almond is drying out as it does just before harvest, while pistachios remain quite wet, is far from clear. A more complete answer would require distribution functions or at least lot averages to be determined at different times prior to harvest. Such measurements are beyond the scope of the present project.

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