# Rapid, Non-Destructive Selection of Peanuts for High Aflatoxin Content by Soaking and Tandem Mass Spectrometry

THOMAS F. SCHATZKI* AND WILLIAM F. HADDON

Western Regional Research Center, Pacific West Area, Agricultural Research Service, USDA,
800 Buchanan Street, Albany, California 94710

Peanut lots are subject to aflatoxin levels high enough to cause concern to health agencies and trade channels. A possible solution would be to mechanically sort out high aflatoxin nuts from the process stream. Only highly contaminated nuts would need to be removed. However, there exists at present no sorting mechanism which meets commercial needs of adequate reduction and product preservation. To build such a sorter requires knowledge of the properties that can be used for sorting. The first step in the design is to select on the order of one hundred undamaged contaminated nuts which can be compared with noncontaminated ones. Because contaminated nuts are rare, a very large number of nuts needs to be examined nondestructively. We present a method to rapidly carry out such a selection. The method is based on dipping nuts into extraction fluid and examining the resulting fluid by tandem MS without preliminary cleanup. This method has been applied to examine over 65,000 nuts, yielding approximately 120 nuts, each containing more than 250–43000 ng/g aflatoxin (depending on process stream).

**KEYWORDS: Peanut; aflatoxin; nondestructive analysis; tandem MS; spatial distribution of aflatoxin in peanuts; aflatoxin extraction by dipping; high-speed analysis; elimination of interfering peaks by statistics; peanut training set for aflatoxin sorting**

## INTRODUCTION

Peanuts, along with tree nuts, grains, some spices, and coffees, are among the foods subject to mold attack, both pre- and post-harvest. Some strains of the attacking molds, in particular some strains of subspecies of *Aspergillus,* i.e., *A. flavus, A. parasiticus*, and A. *nomius*, can produce the particularly deleterious toxin aflatoxin. In fact, on a weight basis, aflatoxin is the most potent liver carcinogen known (*1*). Acute toxic effects have also been reported (*2*). Aflatoxin is the generic name for a number of similar compounds, of which aflatoxin $B_1$ is the most potent, and, coincidentally, the most common in nuts. As a result, strict limits on total and $B_1$ aflatoxin levels allowed in foods have been placed by governmental agencies and the market. In the U. S. the FDA uses an action level of 20 ng/g total aflatoxin for foods for human consumption, except for milk (*3*). Foods having higher levels are prohibited from import. In a recent monograph (*4*) Henry et al. argued that aflatoxin was synergistic with hepatitis B for liver cancer. On this basis, and the much lower consumption of mold-damaged foods in the developed countries, they argued that little public health advantage would be gained by reducing acceptance levels from 20 to 5 ng/g. They pointed out that the situation in the underdeveloped world would be quite different, of course. The European Union, a major market, is currently (as of January 1, 1999) setting acceptance levels of 2 ng/g for $B_1$ and 4 ng/g for total aflatoxins for nuts ready for human consumption (*5*). More importantly, consumers and news media are conscious of the carcinogenic nature of aflatoxin (*6*). As a result, markets will shy away from a product if any appreciable fraction of shipments by a given supplier tests above the acceptance level. Whatever the public health implications are, it is clear that the current acceptance levels are in fact being driven by market forces.

Among nuts overall, peanuts probably form the most important food source worldwide, both as a source of oil and of protein. This is particularly true among certain impoverished populations, such as those of West and East Africa, India, and China. Even in industrialized nations peanuts serve this purpose in part for children in forms such as peanut butter. U. S. tree nuts rarely exhibit average aflatoxin levels in commerce above 1.5 ng/g (the actual value depends on year of harvest and method of processing) (*7*). Although average levels in domestic commercial peanuts are not generally available, a comparison can be made for exported nuts. To be suitable for export into the European market tree nuts need to have aflatoxin levels as low as 0.15–0.3 ng/g to achieve commercially viable acceptance rates (*8, 9*). In practice, they generally do. Peanuts shipped to Europe seem for a large part to fall within a 2–5 ng/g range. Although much of this is below the acceptance level, an appreciable fraction will test above 4 ng/g, partly on statistical grounds (*8*), thus requiring reprocessing overseas. The reduction of aflatoxin in peanuts is thus of considerable importance.

* To whom correspondence should be addressed via e-mail: tom@ pw.usda.gov.

Current pre- and post-harvest practice reduces aflatoxin in peanuts to some extent, particularly in florunner peanuts. When presented for sale by farmers, peanuts are inspected and segregated into two classes, seg 1 and seg 3, based in part on visible mold. Only seg 1 nuts are acceptable for human consumption. Irrigation (natural or artificial) reduces aflatoxin and many shellers will keep seg 1 dry-field peanuts apart. Following shelling of the pods, peanuts are routinely sorted by size and appearance into subpopulations (labeled jumbos, mediums, no. 1s, and OKs in order of decreasing size; DKs for damaged, misformed, and/or darkened kernels; and LSKs for typically very small kernels which fall out of pods before shelling). Aflatoxin tends to increase with decreasing size and increasing damage (*10, 11*). In the U.S. only the larger sizes are allowed to be sold for human consumption. It is thus clear that routine sorting reduces average aflatoxin (*12*).

Such sorting is not sufficient by itself. A number of additional methods for peanut sorting within sublots have been tested. None of these is able to reduce aflatoxin by 90% while rejecting less than 2% of product, a goal based on industry costs. Electronic color sorters depend on the color of reflected visible light (*13*). Using the knowledge gained from tree nuts and from corn, fluorescence of the blanched kernel (so-called BKY fluorescence at 490 nm) has been suggested as a way of detecting kojic acid, a co-metabolite with aflatoxin (*14*). A demonstration sorter was built (*15*), but it was found to be inferior to color sorting (*16*). A thorough study of fluorescence in peanuts has not been done, although it was done for pistachios (*17*). Dielectric (*18*) and microwave techniques (*19*) have also been used to detect aflatoxin, but these methods simply measure moisture; the relation to aflatoxin arises because the mold *A. flavus* produces aflatoxin only when the moisture content is high enough (approximately 12%). An interesting approach is to use kernel density, both of shelled kernels (*20, 21*) and unshelled pods (*22*). In the former case one may be detecting the gap between cotyledons, as it is known that *A. flavus* grows and sporelates preferentially in this gap (*23*); in the latter case one is in part detecting immaturity, i.e., small kernels. Maturity detection by machine vision to detect the irregular surface of immature kernels has also been tested (*24*). Finally, mold detection, per se, using transmission NIR in the 1100 nm region has been incorporated in a commercial instrument (*25*). Definitive data on aflatoxin removal are not available, although preliminary work in this laboratory suggests it will not work for aflatoxin. Among these methods, electronic color sorting, and occasionally density sorting, are in common use.

With respect to pistachios, which exhibit the most serious problem among U. S. tree nut crops, statistical work and the measurement of the aflatoxin distribution among individual nuts in various pistachio subpopulations has shown that substantially all the aflatoxin occurs in approximately 1/10,000 of the nuts (*26, 27*). This suggests that removal of a small fraction of the product by sorting could reduce aflatoxin significantly without an excessive loss of product. Although it is not possible to remove only the highly infected nuts, it was shown that removal of 5% of the product could result in a 10-fold aflatoxin reduction (*27*). Whether the removal of a few peanuts containing high aflatoxin content will suffice to reduce the overall aflatoxin levels in peanuts as well is not known. What will be needed to ensure this is knowledge of the details of the aflatoxin distribution at the high end of the distribution function, i.e., at high aflatoxin concentrations, which is not available at this time. There is available the work of Whitaker and co-workers (*28–30*) who measured the aflatoxin concentration of a number of

samples from assorted lots of farmers' stock peanuts. Unfortunately, the sample sizes were too large, and the number of samples was too small to achieve a direct inversion of these results into an individual nut distribution (*26*), even from the raw data (*30*). What is clear, however, is that the probability of a nut having a given aflatoxin level is much larger than that in pistachios, at least among the lots consider by Whitaker et al. and the one reported on here. Overall contamination rates may reach 10 times that which was observed in pistachios, with highly contaminated nuts appearing at frequencies of 1/1000. The actual levels will depend on the lot and the cultivation conditions used (*31*).

It will be assumed here that highly contaminated nuts may be defined as containing more than 1000 ng/g of aflatoxin, that such nuts occur in 1/1000 of all nuts in the lot, and that the removal of such nuts from the lot will be adequate to reduce the average aflatoxin level sufficiently. The actual values will differ somewhat among the sublots, and adjustments were made as data were obtained, but these values suffice to indicate the scope of the problem. The purpose of the present research is then to collect a training set of undamaged peanut kernels which may be used, in subsequent work, to test for features to distinguish aflatoxin-contaminated kernels from others by a high-speed sorting machine. Assuming it might take a half-dozen or so features to accomplish this, pattern recognition experience suggests that a training set consisting of 100 undamaged contaminated and 100 undamaged uncontaminated nuts will be required to accomplish this.

To obtain 100 contaminated nuts will require nondestructive testing of 100,000 nuts if their frequency is only 1/1000. Assuming a testing rate of 100 samples/day, this would require approximately 4 years, if each sample contains a single kernel. By using a larger sample, containing a number of kernels, the total number of samples can be reduced significantly, even taking into account the required retesting to find the kernel of high aflatoxin content. This must be balanced against sensitivity. In the present case a sample size of 20 nuts was found to be adequate except for the DK sublot. This would reduce the testing time to only 2 months. Lack of professional staff time and the possibility of hiring nonprofessional staff during the summer months then led to the following research goals:

(1) Develop a nondestructive method of testing peanut kernel for aflatoxin which would identify kernels containing >1000 ng/g among up to 100,000 nuts in the space of two summers, using nonprofessional staff. (2) Develop the analytic tests necessary to test these peanuts. If possible, these analytical tests should be fully automated, requiring no concurrent professional staff.

## MATERIALS AND METHODS

**Provenance.** The material used in this study consisted of a 1020-lb lot of farmers' stock runner-type peanuts, grown in 1998 on a lot with no supplemental irrigation, which graded seg 1, but on subsequent aflatoxin analysis (of the LSK and OK portions of the test sample) tested at 920 ng/g. This material was cleaned, shelled in part, and sized in a pilot shelling plant at the ARS National Peanut Research Laboratory at Dawson, GA. The resulting sublots consisted of 317 lb unshelled material, 142 lb of jumbos, 206 lb of mediums, 22 lb of no. 1s, 10 lb of split kernels, 26 lb of OKs (including broken kernels and riding over a 14/64-in. slit screen), and 45 lb OKs falling through the screen (referred here as smOKs). In addition, LSKs were recovered from unshelled material riding under a 16/64-in." slotted screen. DKs were hand sorted from the OKs (jumbos and mediums appeared to have very few DKs among them). The large majority of the larger, unsplit fractions consisted of peanut-with-skin, the remainder was skinned. At Albany

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the peanuts were initially stored dry and after some months stored at −35 °C, to protect from insect attack, for up to 1 year. In addition, for some preliminary work discussed below, a 1050-lb lot of farmers' stock, seg 3, runner-type peanuts was obtained. These nuts came from the 1997 crop and had been warehoused for almost one year. They tested at 500 ng/g total aflatoxin and were processed by the ARS Dawson, GA lab in the same manner as the seg 1 nuts discussed above. Only DKs (hand-selected from no. 1s) and LSKs were used.

**Nondestructive Testing by Dipping.** To obtain a rapid, nondestructive test, the standard destructive method of aflatoxin measurement (which involves grinding the nut to a powder and extraction of aflatoxin by a fluid) was replaced by one in which nuts are dipped in standard extraction fluid (60 v% MeOH/40 v% water) for a fixed amount of time. This was followed by filtration of the resulting "dip fluid" through an Acrodisk (Gelman Sciences, Ann Arbor, MI) 0.45-$\mu$ filter, followed by analysis of the dip fluid for aflatoxin. The wet nuts were dried by vacuum pumping.

For this method to be successful, the dip fluid concentration must be monotonic with the nut aflatoxin concentration. A separate set of tests was run, using a limited number of DK and LSK seg 3 peanuts kernels. Except for visible mold, there was no reason to believe that seg 3 nuts would behave differently from seg 1 nuts in such dipping, while randomly chosen LSK and DK kernels could be expected to cover an extended aflatoxin concentration range. The seg 3 kernels were dipped repeatedly in fresh extraction fluid for 1 min. The dip fluid was analyzed by HPLC, and after up to 6 dips the kernels were ground and analyzed by HPLC as well. In this way the amount extracted in each pass and the total aflatoxin present originally were established. From the extraction history an optimal extraction time could be derived. These tests were repeated for kernels of an extended range of initial aflatoxin levels. Details are given in the Results section, where some additional conclusions about aflatoxin in peanuts are also discussed.

To collect enough contaminated nuts for a training set for building a sorter, extraction was carried out on sublots DK, LSK, smOK, OK, medium, and jumbo. Although only nuts from the last two sublots may be sold for human consumption, we extended our range to obtain more information regarding the effect of size and damage. Nuts from the segregation 1 lot were used. Except for DKs, 20 nut samples were placed in a 30-mL beaker with 10 mL of extraction fluid, enough to cover LSK, smOK, and OK nuts. Larger volumes of fluid were needed for mediums (15 mL) and jumbos (20 mL). The time of extraction was set at 2.5 min, following the calibration results. The temperature was room temperature (20 °C) in an air conditioned lab, similar to that used for standard extraction methods. DKs were extracted in a test tube, using 1-nut samples. Use of 20-nut samples sped up the analysis and was justified by Poisson statistics, as it was highly unlikely that more than a single contaminated nut would appear in such a sample at an average rate of 1/100. The contamination rate for DKs was too high to allow this. It is assumed that this treatment is gentle enough to ensure that the features indicative of aflatoxin are not destroyed by the dipping. After extraction, the wet nuts in the 30-mL beakers were vacuum pumped for 20−40 h in a roughing vacuum at about 45 °C for drying and subsequent storage. Subsequent pumping of previously pumped nuts showed that the original pumping had left around 2% moisture in the nuts, far less than would allow formation of aflatoxin (*32*). For DK nuts the residual moisture content appeared somewhat higher, around 5%, which was still in the safe range. A rate of 100+ samples/ day could be maintained. Two high school students, working with minimal supervision, managed to test 65,000 kernels in about 14 weeks, over two summers.

**Analysis of the Dip Fluid by Tandem MS.** To accomplish the desired rapid, automated analysis of the dip fluid concentration, several candidate methods were considered, including HPLC, TLC, and tandem MS. Standard HPLC was too slow and required an analyst for the procedure. TLC could probably avoid the majority of problems, but its dynamic range was limited, requiring the dilution of a number of samples, again a nontrivial task for untrained staff. A fully automated tandem MS, with autosampler and injection at atmospheric pressure, was available. The atmospheric pressure injection would remove the carrier dip fluid, and passage through an acrodisk 0.45-$\mu$ cartridge filter would be the sole sample preparation required. MS has a very wide dynamic range. The tandem MS would select ions corresponding to the mole weight of $B_1$ (312 daltons) in the first stage. In the second stage these would be fragmented, allowing selection of only those ions corresponding to $B_1$ while removing interfering ions. As will be seen below, this intention was not fully satisfied; at least one additional molecule survived the double MS selection. Removal of this interference required statistical treatment of the results, with considerable loss of sensitivity. In the present case, this lack of sensitivity was not serious. Had it been so, a MS-MS-MS selection might have been attempted, but was not done here.

Several reports in the literature describe the analysis of aflatoxins in complex mixtures using mass spectrometry (*33−36*). Tandem mass spectrometry (MS−MS) was chosen for this project because of its ability to reduce interference. Peanut extracts, prepared as described above in methanol−water solution, during the main part of our work were analyzed for aflatoxin $B_1$ by use of an ion trap MS−MS spectrometer using atmospheric pressure chemical ionization (APCI). Experimental details of the MS−MS experiments were as follows. The ion trap mass spectrometer was an LCQ-Classic with a TSP-4000 Autosampler injection system (Thermoquest, Inc., San Jose, CA). Using the autosampler, 10 $\mu$L aliquots of peanut extract from LSK, OK, DK, and smOK samples, or 20-$\mu$L aliquots from medium and jumbo samples, were introduced to the APCI source of this instrument at 0.2 mL/min flow rate in 40 v% water/60 v% methanol (0.25 mL/min for medium and jumbo samples). The solvent reservoir had the same composition, but was fortified with approximately 1% acetic acid to promote ionization. MS−MS spectra, representing the ion decomposition products specific to the 313 MH$^+$ ion generated by APCI, were recorded continuously as solution entered the mass spectrometer from the autosampler. The collision energy was 38%. The capillary sample introduction tube was at 150 °C, the ion source temperature was 450 °C, and the APCI discharge voltage was 8 kV. A sheath gas flow of 50 mL/min of nitrogen was used to optimize the introduction of sample from the discharge region into the ion trap. After injection of the sample, 144 mass spectra were collected in 60 s (for OK, LSK, DK, and smOK) or 120 spectra were collected in 90 s (for medium and jumbo nuts). The mass range of the MS−MS spectrum was $m/z$ 240−320.

Ion intensities for the $m/z$ 285 (MH$^+$−CO) and $m/z$ 295 (MH$^+$−H$_2$O) daughter ions of the $m/z$ 313 (MH$^+$) parent were integrated using a program written in Microsoft Visual Basic 6.0 that allowed adjustment of the integration parameters, subtraction of a baseline, and processing of multiple samples.

**Computation of Dip Fluid Concentration from Tandem MS.** The use of MS−MS allowed detection of aflatoxin directly in the peanut extracts. **Figure 1A** and **B** shows the APCI spectra (not MS−MS) scanned between $m/z$ 280 and $m/z$ 400 for a peanut extract containing no measurable aflatoxin (**Figure 1A**) and 100 ng/g of aflatoxin $B_1$ by spiking (**Figure 1B**). The peak heights at $m/z$ 313 are not significantly different. However, the MS−MS spectra for these same samples (**Figure 1C** and **D**) clearly show the presence of the 100 ng/g spike (**Figure 1D**). Aflatoxin $B_1$ is indicated by the increased height of the $m/z$ 285 ion in the MS−MS spectrum. The reference spectrum for pure $B_1$ is shown in **Figure 2**. (MS spectra are not absolute in height. Ratios are definitive. The illustrations in **Figures 1** and **2** have been chosen to indicate roughly corresponding heights between spectra. The magnitude of the largest peak is scaled to 100.) It is proposed to use $m/z$ 285 to quantify $B_1$ in these samples. To obtain quantitative data, however, it was necessary to correct for interference at $m/z$ 285 from nonaflatoxin matrix components in the samples.

The strategy to correct for matrix interferences arose from the recognition that ions at $m/z$ 295, representing loss of 18 daltons, H$_2$O, from 313, had low abundance in the MS−MS spectrum of aflatoxin $B_1$ (**Figure 2**). The ratio of peak intensities at 295 and 285 remained relatively constant in the extracts of 20-nut samples of peanuts, provided the average aflatoxin content is low (**Figure 1C**). Therefore, it appeared that mass 295 ions might serve as an approximate internal standard for the method.

In the following equations $A$ stands for MS−MS area. If followed by a single number it is the area at that $m/z$. If followed by a number and a symbol, it stands for the area attributable to molecules represented by the symbol. Thus $A(285)$ = total area at $m/z$ = 285. $A(285, B_1)$ stands

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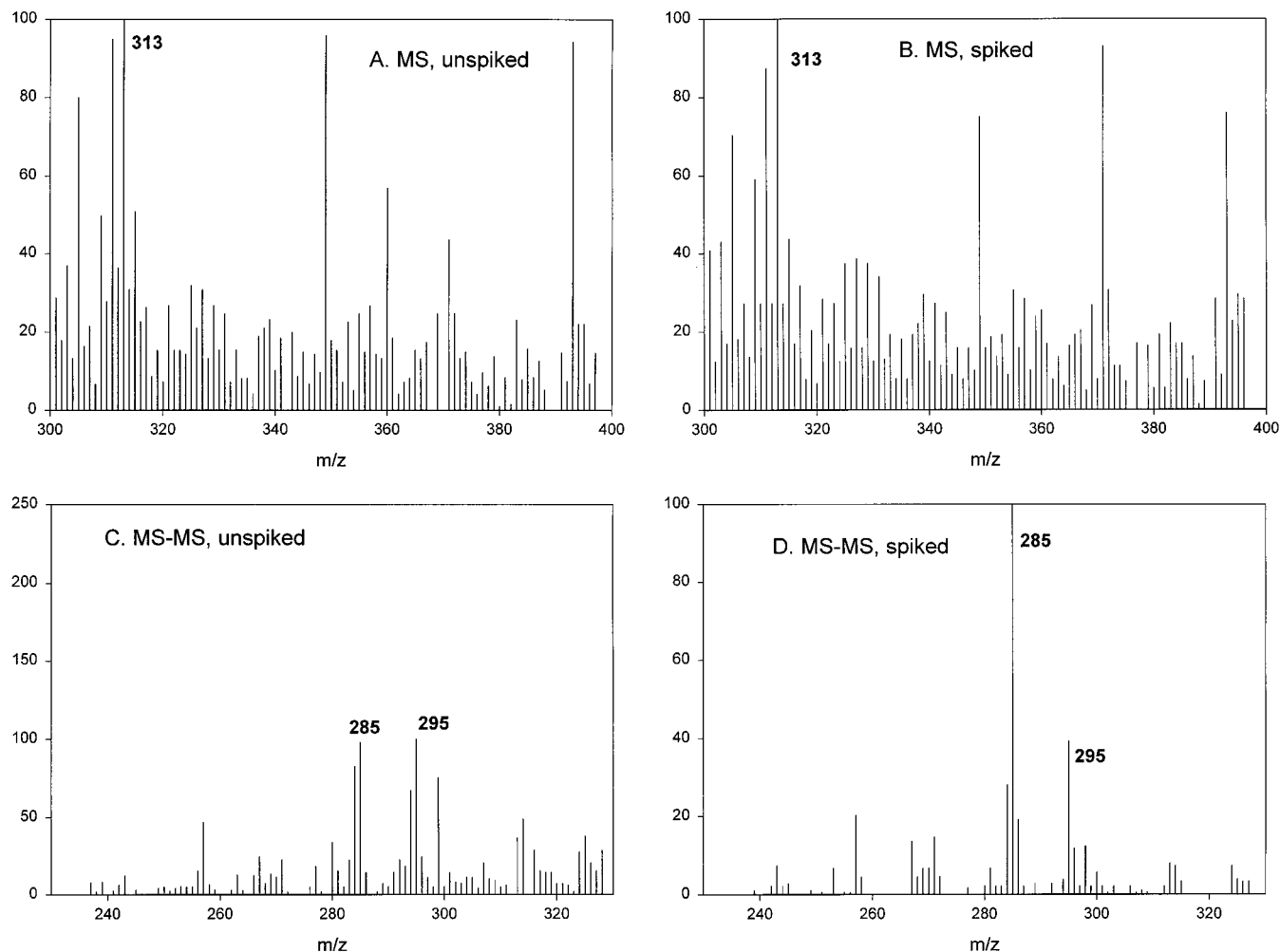
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**Figure 1.** APCI spectra of a MeOH/water (60:40, vol %) dipping extract of peanuts. A,B, MS spectra; C,D, MS–MS spectra. A,C, unspiked extract of nuts of zero or low aflatoxin $B_1$ content; B,D, same after spiking extract to 100 ng/$\mu$mL with aflatoxin $B_1$.
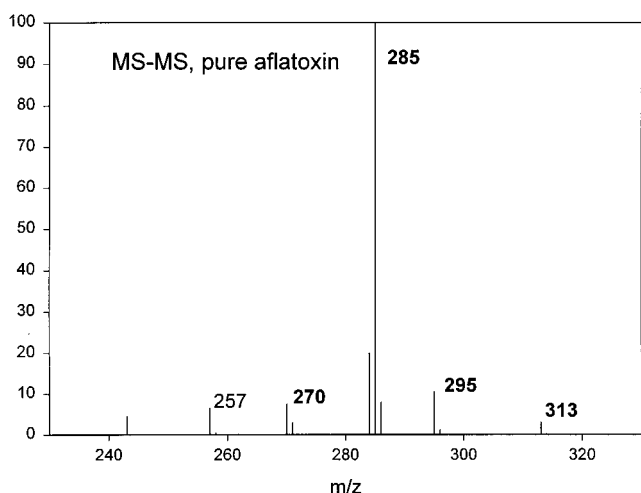


**Figure 2.** APCI MS–MS spectrum of *m/z* 313 for pure aflatoxin $B_1$.

for the part attributable to $B_1$ at $m/z = 285$. $C$ ($B_1$) or $C'(B_1)$ stands for the concentration of $B_1$; $k_1$ and $k_2$ are constants; $\cong$ stands for approximately equal to; and $\equiv$ stands for defined as. Because the mass spectra are linearly additive, the $m/z$ 285 peak can be broken down into the contribution from the aflatoxin 285 ions, and the contribution of all of the matrix ions:

$$A(285) = A(285, B_1) + A(285, \text{matrix}) \qquad (1)$$

Considering MS peaks are proportional to the concentration of source ions, one has

$$C(B_1) = k_1 \times A(285, B_1) \qquad (2)$$

The matrix contribution to the two MS peaks can be expressed as a ratio

$$k_2 = A(285, \text{matrix})/A(295, \text{matrix}) \cong A(285, \text{matrix})/A(295) \qquad (3)$$

Combining the equations one has

$$C(B_1) \cong k_1 \times [A(285) - k_2 \times A(295)] \qquad (4)$$

The calibration constant $k_1$ is obtained by taking a matrix dip fluid extract and spiking it at increasing levels of $B_1$. Solutions to prepare calibration standards were obtained from about 20 OK extracts on which the measurements of individual batches had been completed to verify very low aflatoxin content. A linear response was obtained. The slope yields $k_1 = 1.0272 \times 10^{-5}$, which was found not to vary from extract to extract or between subpopulations. The vertical offset did differ, however, indicating that $k_2$ depended on extract, and thus was matrix dependent. This prevented the use of eq 4 directly to obtain $C(B_1)$.

**Frequency of Selected Kernels.** The matrix correction constant, $k_2$, will vary from sample to sample. An average value, $\langle k_2 \rangle$, for samples of low aflatoxin content (the vast majority of samples) can be determined from averaging the ratio $A(285)/A(295)$ on measurements on peanut extracts by excluding the high values of this ratio. This empirically determined value for $\langle k_2 \rangle$ under constant mass spectrometry conditions differed for different subpopulations of peanuts: OK 1.54;
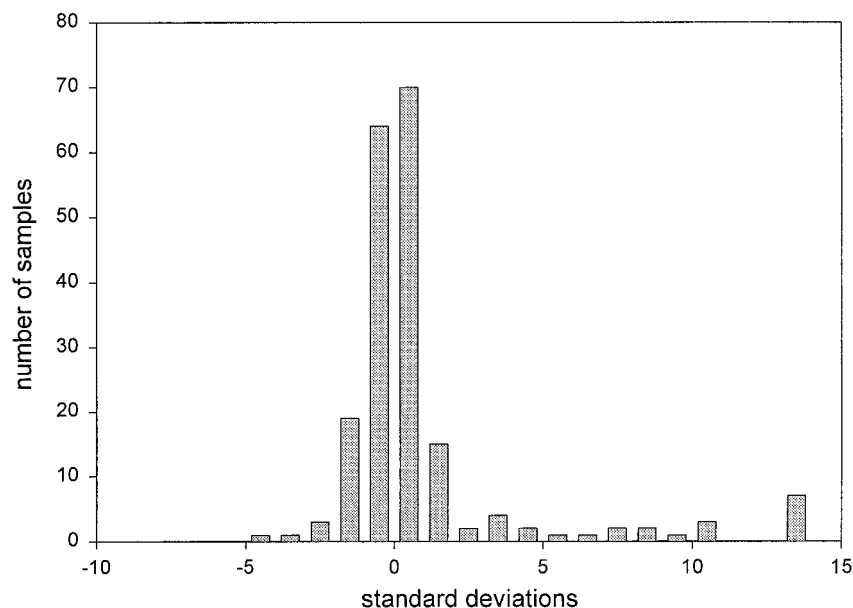
**Figure 3.** Computed dip fluid concentrations, $C'(B_1)$, for all LSK samples.

LSK, 1.12; smOK, 1.06; DK, 1.03, medium and jumbo, 1.65; as determined on a subset of samples from each subpopulation, usually consisting of 100 or more samples. Rewriting eq 4 in terms of $\langle k_2 \rangle$ one has

$$C'(B_1) \equiv k_1 \times [A(285) - \langle k_2 \rangle \times A(295)] \quad (5a)$$

$$= C(B_1) + k_1 \times (k_2 - \langle k_2 \rangle) \times A(295) \quad (5b)$$

Equation 5a indicates that $C'(B_1)$ is an experimentally accessible quantity which can be evaluated for every dip fluid. According to eq 5b, $C'(B)$ for a large number of samples will be distributed as the sum of two distributions. There will be a distribution due to actual aflatoxin concentration. From our knowledge of aflatoxin distribution among individual peanut kernels, this distribution will be nonnegative, start at large frequency for small $C(B_1)$, and rapidly drop off as $C(B_1)$ increases. In addition there is a distribution due to matrix components, which will have positive and negative contributions, depending on the sign of $k_2 - \langle k_2 \rangle$. For a well chosen $\langle k_2 \rangle$ the $C'(B_1)$ distribution should peak at or near $C'(B_1) = 0$ due to the nature of the $C(B_1)$ distribution. From the observation that $k_2$ does not vary much between samples, it follows that the matrix distribution of eq 5b should be narrow. Hence, $C'(B_1)$ for large absolute values should fall outside the range of the matrix distribution. On the assumption that the matrix distribution is symmetric about $C'(B_1)$ the part for $C' < 0$ can be used to estimate the matrix contribution for $C' > 0$. Any remaining part, which should be positive, can then be assigned to aflatoxin content, i.e., can identify dip fluid of high concentration. Put another way, the interference of the matrix distribution acts as a background to aflatoxin measurements, preventing the determination of small values. Large values rise above this background. These concepts can be tested by applying the method to a set of dip fluids of varying aflatoxin contents and comparing the results by measuring these same solutions by HPLC.

There are, in fact, two distinct aflatoxin concentrations which are of interest. The concentration $C(B_1)$ is the concentration in the extraction fluid after extracting aflatoxin from whole or ground nuts. Its units are ng/mL. The more familiar concentration in the whole kernel, in units of ng/g, will be designated $K(B_1)$. The conversion is straightforward, $K(B_1) = C(B_1) \times v/w$, where $v$ is the volume of extraction fluid and $w$ is the weight of kernels extracted. Which units are more appropriate and familiar will depend on context, and will be clear from the units used.

## RESULTS AND DISCUSSION

**Calculation of the Dip Fluid Concentration from Tandem MS.** When a $C'(B_1)$ distribution is plotted, using eq 5a, one

**Table 1.** Parameters of the Random Component of the Distribution of Computed Concentration Values Obtained by Tandem MS

| subpopulation | no. of samples | no. of nuts/sample | vol of dip fluid, mL | mean c, ng/mL | std dev, ng/mL |
|---|---|---|---|---|---|
| OK | 446 | 20 | 10 | 0.05 | 11.30 |
| LSK | 1364 | 20 | 10 | −2.03 | 27.55 |
| DK | 1035 | 1 | 1 | 3.67 | 43.27 |
| smOK | 179 | 20 | 10 | 1.41 | 25.75 |
| medium | 562 | 20 | 15 | 7.0 | 3.85 |
| jumbo | 657 | 20 | 20 | 5.78 | 3.80 |
| total | 4243 | | | | |

finds for all subpopulations a bell-shaped distribution, differing in detail among the subpopulations. Each has a positive tail added, as expected. An example is shown in **Figure 3** for LSKs. Excluding the tail, these bell-shaped distributions can be tested for normality by using the nonparametric Kolmogorov–Smirnov test (*38*). The bell-shaped distributions cannot be distinguished from normal ones at $p = 0.05$ (0.01 for the medium population). Again excluding the positive tail, the parameter $\sigma$ of the corresponding normal distribution can then be computed. If the distribution were truly normal, one would expect to see substantially no samples outside a limited range, which amounts to $\pm 3\sigma$ for 1000 samples (the exact coefficient depends on the sample size). No samples should fall below that range, and samples above it must arise from the concentration component, i.e., they must be samples of truly high aflatoxin content. In actual fact, 1–2 samples/process stream fall below, indicating that the bell-shaped distribution is not truly normal. Significant parameters are listed in **Table 1**. It follows that for values of $C'(B_1) > 3$ or $4\sigma$, $C'(B_1)$ can be used as an estimator of $C(B_1)$, the true aflatoxin concentration, while for lower values the true value falls within the matrix distribution, i.e., the noise. It is noted that the $\sigma$ values for the jumbos and mediums (i.e., the results of the second summer's work) are considerably smaller than those for the other four subpopulations. The reason for this is not clear.

It would appear that a distribution in terms of $K(B_1)$, the kernel concentration, would be a better predictor of outliers than $C'(B_1)$. However, conversion to $K(B_1)$ did not result in a narrower distribution, as might have been expected if this were the case (i.e., if the distribution in weights between samples
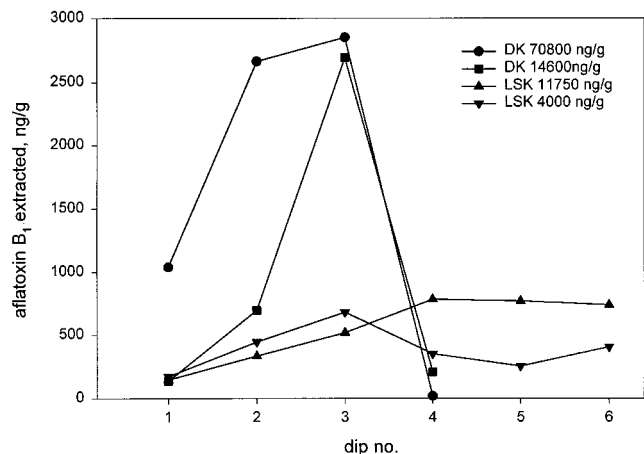
**Figure 4.** Aflatoxin removed by dipping for sequential 1 min dips. DK and LSK kernels of various initial aflatoxin content.

**Table 2.** Comparison of Aflatoxin in Dip Fluids by Tandem MS and HPLC, ng/mL

| method | aflatoxin, ng/mL | | | | | | |
|---|---|---|---|---|---|---|---|
| tandem MS | 12 | 32 | 41 | 46 | 47 | 81 | 124 |
| HPLC | 8 | 38 | 51 | 60 | 24 | 60 | 79 |

contributed to the matrix distribution). Accordingly, the calculations were carried out in terms of $C'(B_1)$.

In practical terms the aflatoxin level can be estimated even below $3\sigma$, although with less confidence. This is shown in **Table 2** which shows a number of positive samples of smOK peanuts in the range 12−124 ng/mL (0.5−5 $\sigma$) which were co-analyzed by HPLC and MS−MS. The data shown in **Table 2** also validate the measurement of aflatoxin solutions by mass spectrometry.

**Relation of Aflatoxin in Nuts and the Amount Extracted.** To establish the relationship between the amount of aflatoxin $B_1$ extracted by a single dip and the total aflatoxin $B_1$ present in the unextracted nut, a number of individual DK and LSK nuts, seg 3, were repeatedly dipped in 1 mL of fresh dip solution for 1 min (followed by drying each time) up to 4−6 times, with analysis for $B_1$ in the dip solutions after each dip. The dipping experiments were followed by grinding and extracting the nut and analyzing it for aflatoxin $B_1$ as well. Adding all the extracts back in yielded the original amount present. Our observations are interpreted on the hypothesis that small quantities of aflatoxin will tend to be present on the surface of the virgin nut, but for high levels of contamination the toxin penetrates the entire nut. We observed the following:

(1) When the initial aflatoxin was high several dips were required to remove most of the extractable toxin. At small initial levels (100 ng/g or less) only the first dip removed any aflatoxin. Some examples for high-aflatoxin-level nuts are shown in **Figure 4**. It seems that at high concentrations the fluid first swells the nut allowing more efficient extraction, whereas at low concentration the toxin is simply removed from the surface. A time of about 2.5 min appeared to be a good compromise which removed enough toxin from the nut to be *indicative of its total content*. This time was subsequently adopted for all experiments.

(2) The amount of aflatoxin extracted in 2.5 min was found to be monotonic with total content (**Figure 5**). This *monotonicity* is, of course, essential if dipping is to be used as a measure of aflatoxin content. It is particularly gratifying that the low aflatoxin LSK data ties in so well with the high toxin DK data. The data for high aflatoxin in **Figure 5** are quite limited, as there were but few such nuts. As our purpose was to collect such high aflatoxin nuts, and the tests to produce **Figure 5** were destructive, we were forced to limit the data at high concentration. For present purposes this appeared adequate, but clearly more extensive work is called for to obtain more definitive results.

(3) As can be seen from **Figure 5**, the fraction of aflatoxin extracted from nuts by dipping decreases as the aflatoxin level of the nut increases, being around 100% at 2 ng/g but dropping to 10% around 4000−10000 ng/g. Thus, the dipping method appears to be largely *nondestructive* of aflatoxin $B_1$ for the highly contaminated nuts which are of primary interest. Furthermore, if one wishes to select solely nuts above, say 500 ng/g, for the training set, one needs solely to collect nuts for which the dip fluid concentration exceeds about 250 ng/g.

One concludes that selection by dipping should be adequate for the present purposes of selecting high aflatoxin peanut
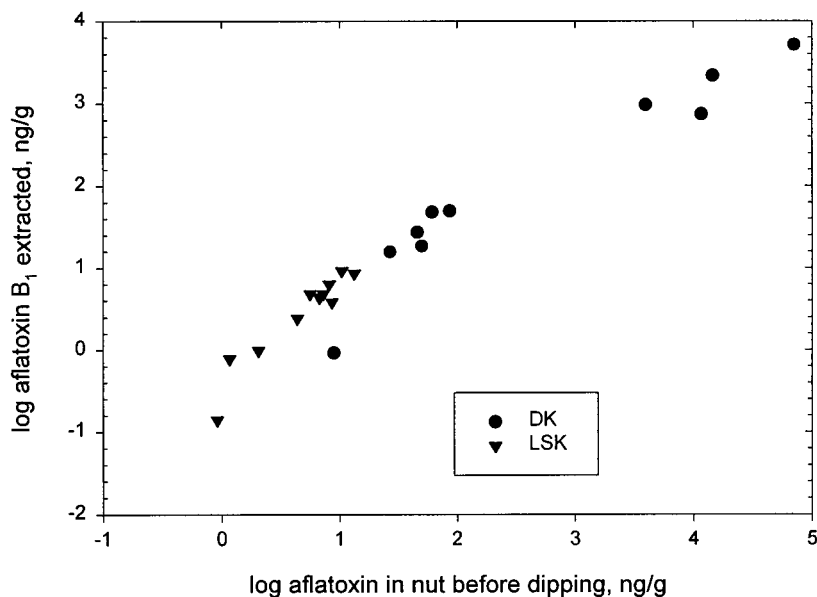


**Figure 5.** Concentration of aflatoxin $B_1$ extracted by dipping (2.5 min) as a function of the concentration in the undipped nut.

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**Table 3.** Selected Peanut Kernels: Lowest Detectable Concentration (ng/g), Number of Kernels Detected, and Probability of Occurrence in Subpopulation[a]

| | $C(B_1) > \langle c \rangle + 3\sigma$ | | | $C(B_1) > \langle c \rangle + 4\sigma$ | | |
|---|---|---|---|---|---|---|
| | | no. of | | | no. of | |
| | $K(B_1)$, min | samples | prob. $\times 10^3$ | $K(B_1)$, min | samples | prob. $\times 10^3$ |
| OK | 3800 | 18 | 2.0 | 4300 | 8 | 0.9 |
| LSK | 25100 | 41 | 1.5 | 43500 | 26 | 1.0 |
| DK | 245 | 45 | 43.5 | 400 | 37 | 36 |
| smOK | 25100 | 23 | 6.4 | 42000 | 16 | 4.5 |
| med | 1000 | 7 | 0.6 | 1860 | 2 | 0.2 |
| jmb | 950 | 17 | 1.2 | 1860 | 11 | 0.8 |
| total | | 143 | | | 100 | |

[a] See Frequency of Selected Kernels section for calculation.

kernels. The results also suggest that any on-line inspection system, planned for future work, must *penetrate into the nut interior*.

**Figure 5** gives the required relation between nut and dip solution $B_1$ content for the LSK and DK subpopulations. It is assumed here that the remaining subpopulations follow the same curve. This assumption is not necessarily valid. It is possible that more compact nuts, such as jumbos or mediums, are not always penetrated by the extraction fluid or are penetrated much less. Were this the case, corresponding data points would fall well below the curve shown, particularly for high aflatoxin nuts. There are at least two nuts in **Figure 5** which do fall below the rest of the data. Thus, false negatives may occur, at least for some subpopulations. For the present work this is not serious, unless it is very common; all one obtains is fewer nuts for the training set than one would like.

**Frequency of Selected Kernels.** The outliers of **Figure 3** are precisely the dip samples which contain high concentrations of aflatoxins. The number of high aflatoxin kernels which were detected by this method, their aflatoxin level, and their frequency may be computed from the number of outliers and the data in **Table 1**. It uses the fact that the high aflatoxin level in such a dip fluid is contributed largely by a single kernel among the sample. This is obvious for the DK samples which consist of only a single kernel. It is also true for 20-kernel samples on statistical grounds with $p > 0.95$ (*26*). The concentration extracted from this single kernel is obtained by conversion to $K(B_1) = C(B_1) \times$ v/w; the initial concentration of aflatoxin in the kernel being extracted is obtained, in turn, by use of **Figure 5**. Because the smallest amount which can be reliably detected in the dip fluid amounts to 3 or 4 $\sigma$ (strictly $C(B_1) > \langle c \rangle + 3\sigma$ or $4\sigma$), use of this value yields the lowest aflatoxin contamination which can be selected by this method. This quantity is listed in **Table 3** as $K(B_1)$, min.

The total number of nuts represented by the 4243 samples amounts to 65195. The frequency with which high-aflatoxin-level kernels occur among all kernels of a subpopulation was computed as the number of positive dip fluid samples ($C(B_1) > \langle c \rangle + 3\sigma$ or $4\sigma$)/total number of samples/(number of kernels/sample). These results are shown in **Table 3** as well. For the total lot a contamination frequency of $143/65195 \cong 0.002$ is obtained, not far from the original estimate of 1/1000. There are now but $(143-46)20 + 46 = 1986$ nuts which need to be retested, rather than 65195. Slightly under 2000 nuts can be tested individually, albeit with some effort. The total number of nuts selected is somewhat less than the desired 200 for a training set, but probably was still adequate to establish the properties, if any, which distinguish these nuts from noncontaminated peanuts.

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