# Methods for Characterization of Kernel Density and Aflatoxin Levels of Individual Peanuts[1]

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**ABSTRACT**

A helium pycnometer, specific gravity bottle and a displacement method were evaluated for the determination of individual peanut (*Arachis hypogaea* L.) kernel densities. Three coating materials were tested to minimize water absorption with the latter two methods. The displacement technique with a coefficient of variation (CV) of less than 0.1% was found to be most reproducible and had a total variability of 0.002 g/cm$^3$. Polyurethane was adopted as the coating material of choice. A reverse phase High Performance Liquid Chromatography (HPLC) assay was adapted for quantification of the aflatoxins in individual peanuts. Picogram levels of aflatoxin were quantified with an average recovery of 82%. Characterization of peanut kernel density distributions and relationships between kernel density and aflatoxin level are envisioned with data generated using this method.

Key Words: Physical properties, peanuts, density, aflatoxin.

Aflatoxins, toxic secondary metabolites produced by *Aspergillus flavus* Link and *Aspergillus parasiticus* Speare, have plagued the peanut (*Arachis hypogaea* L.) industry since their discovery in 1960 (2). Currently food materials intended for human consumption in the US cannot contain aflatoxins in excess of 20 parts per billion (ppb). However, the rapid advancement of analytical techniques is pushing the minimum detectable limit lower, and some European countries have already lowered the action limit to 3 ppb. Annually about 6.5 billion square meters (1.6 million acres) of peanuts are grown in the US, translating into approximately 0.6 billion kilograms (1.4 billion pounds) entering the food chain (16). The sheer volume involved makes aflatoxin contamination a serious health hazard and a significant economic concern.

Currently, options for separation of contaminated peanuts are limited to hand sorting, color sorting and density segregation. Hand sorting, currently recognized as the best method is monotonous and time consuming, hence commercially impractical. Color sorting, which is relatively rapid and amenable to automation, has become the industry standard. However, color sorting entails a definite loss of noncontaminated peanuts. Density segregation which has been studied with mycotoxin contaminated agricultural products (1, 6, 7, 8, 13) is a potentially attractive alternative.

Recently, a U.S. patent (5) was granted for a flotation based separation of aflatoxin contaminated nuts. The peanut industry is however, wary of using any liquid based separation process since peanuts absorb most liquids readily. Efficient physical separation exploiting density differences mandates knowledge of the true density distributions of contaminated and noncontaminated peanuts, and also the patterns and distribution of aflatoxins in the kernels. While empirical observations have attempted to satisfy this need (5, 12), a theoretical database has not been documented.

Commercialization of this theoretically feasible concept has been further confounded by the extreme variability both in incidence and levels of aflatoxin. Variability ranging from 1% to 95% has been documented, making representative sampling and sub-sampling at best difficult (3, 17).

The objectives of this study were to develop methods for the specification of the density distributions of contaminated and noncontaiminated peanuts by:

1. Developing a method for individual peanut kernel density determination.
2. Determining a suitable HPLC assay for aflatoxin quantification in individual kernels.

## Materials and Methods

### Test Samples

Florunner peanuts grown under drought stress were obtained from the National Peanut Research Laboratory, Dawson, GA. The peanuts were shelled and separated into commercial size ranges (jumbo, medium, and No. 1) using the official grading screens (8.3 mm, 7.1 mm, and 6.4 mm). Each size range was then treated as a separate population. For this study, samples were drawn from the population of No. 1 peanuts.

### Density Determination

Three methods of density determiniation were studied and will be referred to as methods A (Helium pycnometer), B. (Specific gravity bottle) and C. (Water displacement). The extent of water ingress into peanuts with time was characterized since methods B and C involved contacting peanuts with water. To minimize water absorption three coating materials, paraffin wax, corn zein in ethanol (26.5%, w/w) and clear polyurethane gloss, were evaluated. Penetration of the coating material into the peanut was monitored by sectioning and observation using high powered light microscopy. An Olympus BH2 (Olympus Corporation, Lake Success, NY) light microscope equipped with a video monitor (Panasonic Co., Secaucus, NJ) was used at a magnification of 1000 X.

**Method A** - A helium pycnometer (Micromertitics, Norcross, GA) was evaluated for density determination of nonporous solids and peanuts. This instrument measures the change in pressure of a pure non adsorbing gas, due to a volume change in a closed vessel. The change in pressure is a function of the volume of any solid object also present in the closed vessel. Thus, from the functional relationship, the volume and hence the density

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of the solid object can be determined (14).

**Method B** - A 24 mL specific gravity bottle (Fisher Scientific, Atlanta, GA) was used to determine densities of coated nonporous solids and coated peanuts by the method outlined in (14). The cited procedure was modified by substituting toluene with water as the test medium. To minimize water absorption, the peanuts were coated by dipping in polyurethane gloss (Red Devil Paints & Chemicals, Mount Vernon, NY) for 10 sec, followed by air drying for 11 hours.

**Method C** - This method was based on measurement of water displacement and will be referred to as the displacement method henceforth. Each kernel was weighed using an A-250 electronic analytical balance (Mettler, Hightstown, NJ) to the thousandth of a gram. A stainless steel pin (Extra fine dressmaker pin, J. & P Coats, Stamford, CT) was introdued into the peanut until the tip of the pin was embedded. The peanut was coated with polyurethane gloss as described for the specific gravity bottle method. A mini clothes pin was used to fabricate a sample holder (Fig. 1) and will be referred to as the holder henceforth. Each kernel with its pin was clipped to the holder, suspended from a hook attached to the balance pan, and its weight in air recorded. A 50 mL beaker containing distilled water with a drop of wetting agent (Triton X-100, Octyl phenoxy polyethoxyethanol) (Sigma Chemical Co., St. Louis, MO) was introduced under the holder (Fig. 1). The weight of the peanut in water was recorded after aligning the tip of the holder just above the surface of the water.

Individual kernel density was computed as follows:

(A) Apparent density

$$W_d = (W_w - W_C) - W_p \quad (1)$$

$$V_a = W_d / D_w \quad (2)$$

$$D_a = W_u / V_a \quad (3)$$

(B) True Density

$$V_c = (W_c - W_u) / D_c \quad (4)$$

$$D_t = W_u / (V_a - V_c) \quad (5)$$

Where,

$W_u$ - Weight of uncoated sample, g
$W_c$ - Weight of coated sample, g
$W_d$ - Weight of water displaced, g
$W_p$ - Weight of water displace by pin, g
$W_w$ - Weight of sample in water, g
$D_w$ - Density of water, g/cm$^3$
$D_c$ - Density of coating material, g/cm$^3$
$D_a$ - Apparent sample density, g/cm$^3$
$D_t$ - True sample density, g/cm$^3$
$V_a$ - Apparent sample volume, cm$^3$
$V_c$ - Volume of coating, cm$^3$

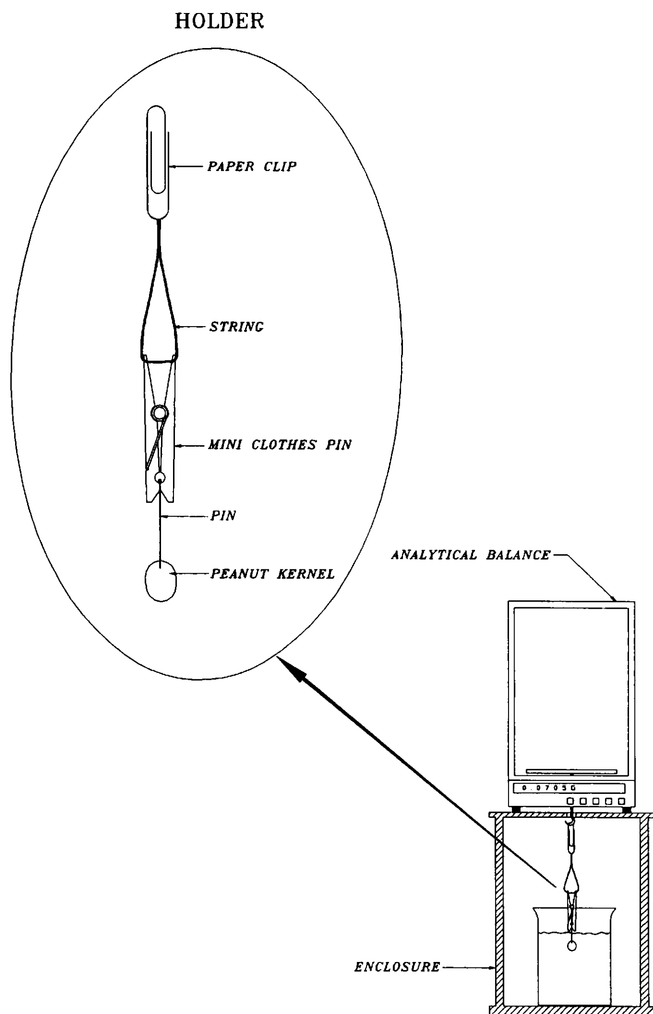


**Fig. 1. Density determination apparatus for the displacement method.**

**Aflatoxin quantification**

The extraction procedure was modified from the method of Dorner and Cole (4) with reaction volumes adjusted for the small mass of individual peanut kernels. Recovery was increased to acceptable levels by reducing extract transfers using an in-house designed and fabricated apparatus (Fig. 2). Derivatization and liquid chromatographic (LC) quantification procedure were modified from that reported by Hutchins *et al.* (11).

The peanut kernel was sliced thinly and blended for 2 min with 12 mL methanol: water (80:20, v/v) in a mini Sorvall blender. An equal volume of salt solution, water: acetic acid: zinc acetate: sodium chloride (11.7:0.05:2:2, v/v/w/w) was added to the blender jar and vortexed for 2 min. The mixture was then quantitatively transferred to a mini Buchner funnel (Fisher Scientific) fitted to a 30 mL separatory funnel (Tudor Scientific Glass Co., Belvedere, SC) and vacuum filtered. Three milliliters of chloroform was added to the filtrate in the separatory funnel; the funnel was capped, shaken for two min, and set aside for layer separation. Two milliliters of the aflatoxin containing chloroform layer (lower layer) was directly collected in a 4 mL amber glass vial and evaporated to dryness under nitrogen flux.

Derivatization was then accomplished as follows: The residue was redissolved in 1 mL of trifluoroacetic aicd: acetic acid:water (1:1:8, v/v) (Sigma Chemical Co.) by vortexing for 1 min. The extract was then incubated at 55 C for 15 min, cooled, vortexed for 1 min and filtered using a 13 mm, 0.2 µm disposable nylon syringe filter (Alltech, Deerfield, IL).

A Shimadzu (Columbia, MA) HPLC consisting of a LC-6A solvent delivery module. SCL-6A system controller, CTO-6A column oven, C-R5A data processor, and RF 535 fluorescence detector was used.

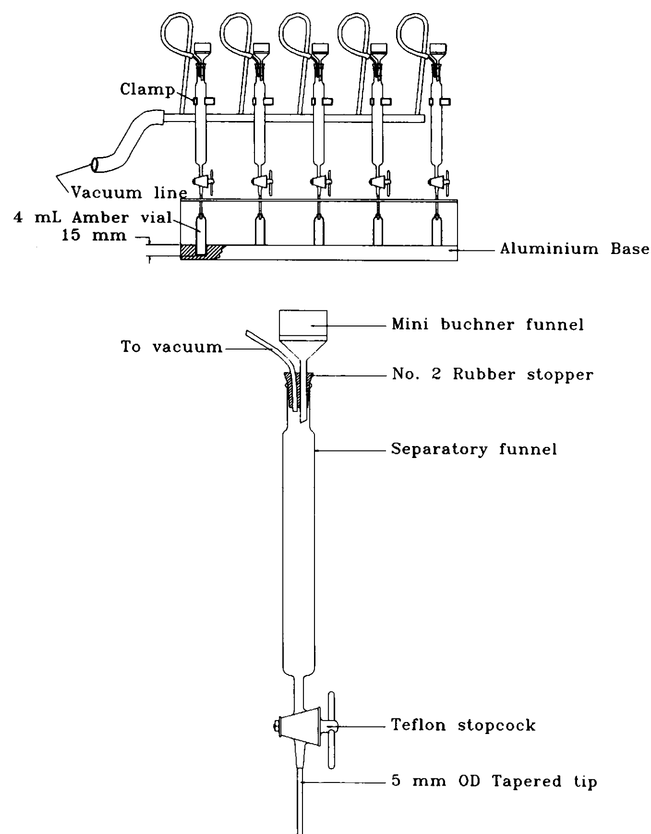


**Fig. 2. Apparatus for aflatoxin extraction.**

Fluorescence detection was carried out at an excitation wavelength of 365 nm and an emission wavelength of 440 nm. An Alltech reverse phase 5 µm Econosphere $C_{18}$ column (250 mm x 4.6 mm) and a mobile phase of acetonitrile:water: tetrahydrofuran (10:84:6, v/v/v) were used, with the column held at 25 C and the flow rate set at 1.5 mL/min. Injection volume was 20 µL. Quantification was carried out with a standard curve developed using aflatoxin standards (commercial mixed standards, Sigma Chemical Co.).

Picogram level aflatoxin detection in single kernels necessitated HPLC detector operation at maximum sensitivity. The inherent fluctuation of detector response at the threshold of sensitivity was offset by the use of a temperature controlled analytical column. To minimize interference by late eluting metabolites and maintain acceptable levels of recovery, pre-injection cartridge cleanup was substituted with programmed gradient elution (flow and temperature). Mobile phase flowrate was increased from 1.5 mL/min to 2.0 mL/min in steps of 0.1 mL/min, simultaneously column temperature was increased from 25 C to 40 C in steps of 3 C. These conditions were maintained for 10 min before reversing flow and temperature gradients to attain normal operating conditions (1.5 mL.min and 25 C). This gradient elution sequence was initiated after elution of all four aflatoxins. High sensitivity induced variation in aflatoxin results was averaged by analyzing all samples in triplicate. Recovery studies were conducted by spiking individual kernels with known amounts of commercial mixed standards.

The large volumes of raw data generated during preliminary work indicated the need for automated data acquisition. We developed software to interface both the HPLC recorder and the analytical balance with a personal computer. The raw data generated was directly analyzed using a mainframe Statistical Analysis System, SAS (15).

## Results and Discussion

### Density determination

A significant increase in weight was observed within the first 2 to 3 min of peanuts being immersed in water (Fig. 3). This suggested that some method of minimizing water ingress was necessary to ensure accuracy of density data. Coating the peanuts prior to density determination was, hence, deemed appropriate.

Wax melting temperature was found to be high enough to displace the air entrapped in the peanut lumen, hence the measured density would be distorted. Several combinations of wax and mineral oil were evaluated in an attempt to lower the melting point. However, the combination that was suitable with respect to melting point was found to be too soft for coating. The corn-zein film, which did dry at ambient temperature (25 C), was soluble in water to an unacceptable extent. Polyurethane coating overcame these limitations. Peanuts coated with polyurethane absorbed negligible amounts of water (Fig. 4); thus polyurethane was selected as the coating material. Furthermore, polyurethane did not

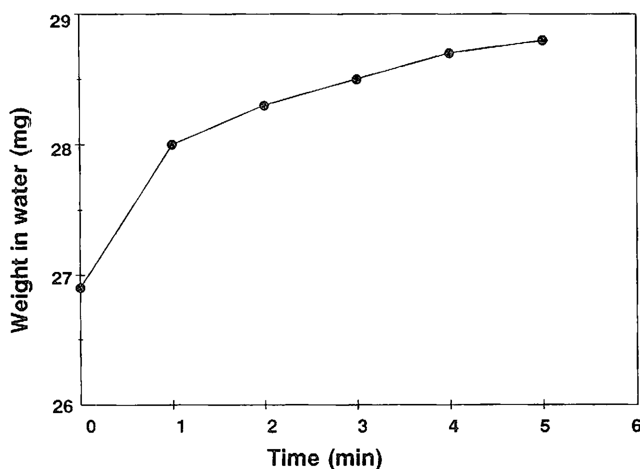


**Fig. 3. Water absorption by peanuts.**

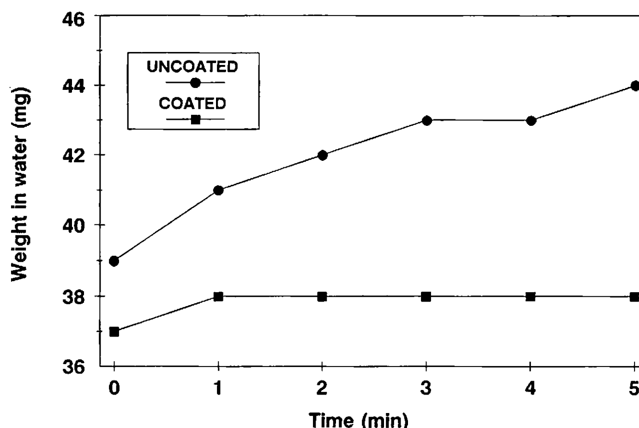


**Fig. 4. Reduction of water absorption after coating peanuts with polyurethane clear gloss.**

chemically interfere with the aflatoxin analysis and did not penetrate the peanut testa to an appreciable extent.

The three methods evaluated for density determination are compared in Table 1 using nonporous solids as samples. Since the size and materials of construction of the samples were different, the coefficient of variation (CV) is the most appropriate criterion for comparison. While the helium

**Table 1. Comparison of methods with standard solids[1].**

| Trial # | Density ($g/cm^3$) using method[2] | | |
|---|---|---|---|
| | A | B | C |
| 1 | 7.0129 | 3.3798 | 3.4014 |
| 2 | 7.7089 | 3.3222 | 3.4006 |
| 3 | 7.7839 | 3.2578 | 3.4007 |
| 4 | 7.8693 | 3.2599 | 3.4001 |
| 5 | 7.6320 | 3.1828 | 3.4015 |
| 6 | 7.8603 | 3.5188 | 3.4018 |
| 7 | 7.7859 | 3.4379 | 3.4002 |
| 8 | 7.7674 | 3.3752 | 3.4018 |
| 9 | 7.8231 | 3.4379 | 3.4010 |
| 10 | 7.7189 | 3.3752 | 3.4003 |
| Mean | 7.6962 | 3.3548 | 3.4009 |
| Std. Dev. | 0.2378 | 0.0955 | 0.0006 |
| Coeff. Var. (%) | 3.09 | 2.85 | 0.02 |

[1] Standard solids were a steel sphere for Method A and a polyethylene ovoid for Methods B and C.

[2] Method A - Helium pycnometer
Method B - Specific gravity bottle
Method C - Displacement

pycnometer was easier to use than the other methods, it was found to be sensitive to factors such as operator technique, level of negative pressure, helium filling time and environmental conditions. Since peanuts are porous and have a variable central cavity (lumen), the extent of evacuation and filling with helium, would probably be considerable sources of variation. Reproducibility was better with the specific gravity bottle than with the helium pycnometer. With this method the primary sources of variation were the weighing steps. The analytical balance used was sensitive enough to detect difference due to a few extra drops of water. It was not possible to fill a specific gravity bottle, close the capillary cap, wipe off excess water and maintain weight reproducibility to a thousandth of a gram. Change in weight due to evaporation loss was another source of variation. The displacement method which had a total variability of 0.002 g/cm$^3$ ($\pm$ 3 standard deviations), and a CV of less than 0.1% was selected as most precise and reproducible.

Table 2 provides descriptive statistics for the densities of a 200 peanut data set, obtained using the displacement method. The extremely small density differences that exist between individual kernels is highlighted by the small range of densities (0.97 to 1.13 g/cm$^3$). Hence, a highly precise and reproducible technique for density determination is desirable.

**Aflatoxin quantification**

The majority of published protocols for aflatoxin analysis are designed for sample sizes of 25 g or more. When these methods were scaled down to accommodate the small mass of a single peanut (0.2 g - 1.2 g), aflatoxin recoveries were extremely poor, about 40%. The extraction protocol cited (4) was modified to yield average recoveries of 73% to 91% from aflatoxin spiked samples (Table 3) conforming to that reported in the original work (71% to 81%). The extraction apparatus designed (Fig. 2) significantly contributed to the increase in recovery by reducing extract transfers to the maximum extent possible. Average extraction time was found to be 15 min.

Precision of precolumn derivatization was checked using retention time and integrator counts (peak areas) of aflatoxin standards as criteria. The results of the precision study using two concentrations of standards with three trials at each concentration are presented in Table 4. On average a CV range of < 1 % to 6 % was observed, conforming to the ranges reported in the literature (9, 10, 11).

**Table 2. Density distribution characteristics of naturally contaminated No. 1 florunner peanuts.**

| Statistic | Density (g/cm$^3$) |
|---|---|
| Minimum | 0.9664 |
| Maximum | 1.1295 |
| Mean | 1.4419 |
| Median | 1.0666 |
| 1st Quartile (25%) | 1.0421 |
| 3rd Quartile (75%) | 1.0833 |
| Standard deviation | 0.0328 |
| CV (%) | 2.27 |

**Table 3. Recovery of aflatoxins from peanuts spiked with high and low concentrations of aflatoxin.**

| Level | Toxin | Concentration (ng) | | Recovery % |
|---|---|---|---|---|
| | | Added | Recovered | |
| Low | $B_1$ | 7.0 | 6.4 | 91 |
| | $G_1$ | 7.0 | 5.6 | 80 |
| | $B_2$ | 2.0 | 1.8 | 90 |
| | $G_2$ | 2.0 | 1.6 | 80 |
| High | $B_1$ | 35.7 | 28.9 | 81 |
| | $G_1$ | 35.7 | 29.9 | 84 |
| | $B_2$ | 10.7 | 7.8 | 73 |
| | $G_2$ | 10.7 | 8.3 | 78 |

**Table 4. Precision of trifluoroacetic acid derivatization at two levels of aflatoxin standards.**

| Total conc. (ng) | Toxin | Retention time | | | Peak area | | |
|---|---|---|---|---|---|---|---|
| | | Mean (min) | Standard deviation (min) | CV (%) | Mean (mV) | Standard deviation (mV) | CV (%) |
| 0.06 | $B_1$ | 5.6 | 0.01 | 0.12 | 41.5 | 0.49 | 1.17 |
| | $G_1$ | 7.4 | 0.02 | 0.22 | 105.5 | 1.10 | 1.05 |
| | $B_2$ | 11.1 | 0.04 | 0.34 | 14.0 | 0.77 | 5.50 |
| | $G_2$ | 16.4 | 0.02 | 0.10 | 40.0 | 0.72 | 1.81 |
| 1.16 | $B_1$ | 5.6 | 0.02 | 0.31 | 514.9 | 1.37 | 0.26 |
| | $G_1$ | 7.4 | 0.01 | 0.14 | 1290.5 | 7.92 | 0.61 |
| | $B_2$ | 11.1 | 0.01 | 0.06 | 172.8 | 0.32 | 0.18 |
| | $G_2$ | 16.4 | 0.01 | 0.09 | 482.2 | 1.00 | 0.21 |

LC conditions were adjusted to obtain reproducible results and achieve a suitable degree of sensitivity. Column temperature control significantly improved reproducibility of component retention times. A lower detection limit of 10 picograms was achieved at the detector. This translates to an average detection limit of 1 ppb in a single peanut (in a 25 g sample the detection limit would be 40 parts per trillion). Table 5 provides descriptive statistics for the aflatoxin levels of a 25 peanut subpopulation, obtained using this method. The well documented variability in occurrence and levels of aflatoxin in naturally contaminated peanuts (3, 17) is reflected in Table 5. Assays on individual peanuts would facilitate establishment of realistic bounds on the variability in levels and occurrence of aflatoxins in peanuts.

Density-aflatoxin data on individual kernels can be used to investigate relationships between these two variables. Correlation coefficient between kernel density and aflatoxin level for a 25 peanut subpopulation was found to be -0.3. The negative sign suggests that aflatoxin contamination is more evident in low density kernels, conforming to

Table 5. Aflatoxin contamination patterns of naturally contaminated No. 1 florunner peanuts.

| Statistic | Aflatoxin concentration (ppb) |
|---|---|
| Minimum | 0.0 |
| Maximum | 6.7 |
| Mean | 0.6 |
| Median | 0.1 |
| 1st Quartile (25%) | 0.0 |
| 3rd Quartile (75%) | 0.2 |
| Standard deviation | 1.4 |
| CV (%) | 233 |

observations by other researchers (5, 12). The small sample size (n=25), high variability in levels of aflatoxin, and the small density differences between individual kernels may have contributed to the poor correlation.

## Conclusions

1. The water displacement method developed was sufficiently sensitive and reproducible (CV: <0.1 %, total variability ($\pm$ 3 s.d.): 0.002 g/cm$^3$) for characterizing the densities of individual peanut kernels.

2. Polyurethane coating prior to density determination significantly reduced water ingress into the peanut, enhancing accuracy and reproducibility.

3. The aflatoxin assay reported was capable of picogram level aflatoxin resolution in individual kernels, while maintaining recovery (73% to 91%).

4. The aflatoxin assay was not affected by the polyurethane used to coat peanuts for density determination.

5. Density and aflatoxin data at the individual kernels level generated using the methods described can be used to map the density distributions of peanuts, characterize patterns of aflatoxin contamination, and investigate possible relationships between kernel density and aflatoxin level.

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