Comparison of Fluorescence Sorting and Color Sorting for the Removal of Aflatoxin from Large Groups of Peanuts

M. J. Pelletier[1] and J. R. Reizner*

ABSTRACT

Fluorescence sorting (by both machine and hand) and color sorting (by both machine and hand) are compared as methods for reducing aflatoxin levels in groups of peanuts. An experimental design is used that allows the four methods to be compared quantitatively. Approximately 1200 photographs of peanut groups were taken, allowing for a more complete understanding of the visual characteristics of contaminated peanuts. Sampling errors are controlled by the experimental design and by including a large fraction of the peanuts in aflatoxin assays. Fluorescence sorting was not effective as an aflatoxin control method. Machine color sorting was shown to be effective in the removal of aflatoxin-contaminated peanuts. Hand sorting was shown to be even more effective than machine color sorting in aflatoxin removal.

Key Words: Aflatoxin, fluorescence sorting, color sorting, manual sorting, hand sorting, sorting

Better methods for removal of aflatoxin-contaminated peanuts from peanut lots are desirable. Fluorescence has been used for decades to quantitate aflatoxin in extracts from nuts and grains. Fluorescence has also been used to detect aflatoxin-producing strains of *Aspergillus flavus* and *Aspergillus parasiticus* growing of agar medium (26). Since aflatoxins fluoresce strongly (10, 41, 42, 51) one possible approach for the removal of aflatoxin-contaminated peanuts from a lot is to remove peanuts having surface fluorescence that is characteristic of aflatoxin.

Bright green-yellow fluorescence has been successfully used to identify aflatoxin-contaminated corn (6, 8, 19, 24, 46, 47), cotton (3, 35, 36, 37), and pistachio nuts (17, 22, 23). In these cases, the fluorescence, called BGY fluorescence in the literature, was not due to aflatoxin. The BGY fluorescence was due to other materials whose presence was strongly correlated with aflatoxin contamination (37). Aflatoxin-contaminated peanuts do not exhibit BGY fluorescence (8). Violet-purple fluorescence has been correlated with aflatoxin contamination on almond kernels (27, 43, 44), but the material responsible for this fluorescence was not identified. Violet fluorescence from peanuts has been correlated with aflatoxin in another study (45) where two peanuts were found with unusually intense violet fluorescence and an aflatoxin concentration of 35 ppb.

The evaluation of potential aflatoxin-removal methods is complicated by the heterogeneous composition of contaminated peanut lots. Aflatoxin contamination in peanut lots is usually due to a few highly contaminated peanuts among a much larger number of uncontaminated peanuts (9, 15, 16, 53,57, 58).

Sampling errors can therefore invalidate the evaluation of a potential aflatoxin-removal method (9, 53, 54, 55, 56, 58). Large numbers of typical peanuts must be used to evaluate a potential aflatoxin-removal method. A large percentage of these typical peanuts must also be included in the supporting aflatoxin assays for sampling errors to be controlled.

It is not enough to show that a new aflatoxin control method can detect and remove aflatoxin-contaminated peanuts. Other issues need to be addressed before the new method can be seriously considered for practical application. Does the new method remove aflatoxin-contaminated peanuts that existing methods do not? Does the new method accept aflatoxin-contaminated peanuts that existing methods do not? How much good product does the new method falsely reject? In addition, it would be desirable to identify contaminated peanuts and characterize their properties (appearance, density, level of aflatoxin, etc.). These data could be used to guide future aflatoxin control work. Unfortunately, the aflatoxin concentration in a set of typical peanuts is always unknown until that set is assayed. The assay is destructive, making further studies on the peanuts impossible. Contaminated peanuts can be made by inoculating peanuts with *A. flavus* spores under controlled conditions (20), but these peanuts cannot be assumed to model naturally contaminated peanuts.

This report describes the evaluation of fluorescence sorting for removal of aflatoxin-contaminated peanuts from peanut lots. The experimental design used in this work allows a quantitative comparison between fluorescence sorting by machine, fluorescence sorting by hand, color sorting by machine, and color sorting by hand. Differences in appearance between uncontaminated peanuts and aflatoxin-contaminated peanuts were recorded photographically. The experimental design used for this evaluation can also be used quantitatively to evaluate other aflatoxin removal methods.

Materials and Methods

Four 22 kg bags of milled, raw, unblanched Southeast peanuts (*Arachis hypogaea* L., cv. Florunner), obtained from a commercial shelling mill were used in this study. The peanuts were obtained from a lot that tested positive by Peanut Administrative Committee procedures (then above 20 ppb aflatoxin). The peanuts were roasted and split-nut blanched at The Procter & Gamble Process Research Laboratory in Cincinnati, Ohio. The aflatoxin concentration in this set of peanuts was determined, after the study, to be 6.86 ppb.

Aflatoxin analysis. The aflatoxin assay procedure (25, 50) used a 60% methanol-water extraction to remove aflatoxin from ground peanuts. Five ml of methanol solvent were used per 1 g peanut tissue, diluted 1 to 1 with water to reduce the methanol concentration. This resulted in a 10 ml diluent to 1 g peanut tissue ratio. Affinity chromatography using Aflatest-P affinity columns (Vicam, Somerville, MA) was then used to isolate the aflatoxins. Bromination solution fluorometry was used for quantitation. For samples weighing more than 50 grams, the entire sample was ground, mixed thoroughly by hand, and a 50 gram sub-sample was extracted. For samples weighing less than 50 grams, the total sample was weighed, ground, and extracted. The aflatoxin concentration in the peanuts before the sorting process was calculated using assay results from all of the peanut subsets produced by the sorting operations.

Fluorescence sorting by machine. Peanut fluorescence sorting by machine was done using a custom-made sorting instrument (39). This

[1]Staff Scientist, The Procter & Gamble Company, Miami Valley Laboratories, P. O. Box 398707, Cincinnati, Ohio 45239-8707.

*Technology Leader, The Procter & Gamble Company, Winton Hill Technical Center, 6071 Center Hill Road, Cincinnati, Ohio 45224-1791.

*Corresponding author.

instrument illuminated the entire surface of each peanut with ultraviolet light at a wavelength of 365 nm. It then analyzed fluorescence emission from 10 to 20 discrete spatial regions that constituted the entire peanut surface (360 degrees, peanut top-to-bottom). Aflatoxin B_1 fluorescence emission was expected at 410 nm (10, 41, 42, 45, 51). Peanut background fluorescence (expected to be independent of aflatoxin B_1 fluorescence) was measured at 490 nm (45). Peanuts having more than 2.5 times as much emission intensity at 410 nm than at 490 nm on any region of the peanut were rejected by the device since they were believed to be contaminated. The factor of 2.5 was based on statistical variation of peanut fluorescence data measured previously (39).

The false acceptance rate of the automatic fluorescence sorter was less than 0.5%, but the false rejection rate was 8 to 10%. These values were determined by programming the fluorescence sorter rejection logic either to accept all peanuts or to reject all peanuts. Error rates were then measured after sorting a batch of peanuts. Peanuts were fluorescence-sorted twice to reduce the effective sorting error rates. Fluorescence sorting by machine, therefore, produced a set of accepted peanuts, a set of rejected peanuts, and a set of once-rejected and once-accepted peanuts. The once-rejected and once-accepted peanuts were classified as accepted peanuts in the data analysis. This was reasonable given the low false acceptance rate and the high false rejection rate.

Color sorting by machine. Peanut color sorting by machine was done with an ICORE 5161 color sorter (Sortex-Scancore, Incorporated, Union City, California). The color sorter was carefully set-up, cleaned, and adjusted for optimal performance using number 8 background plates, number 22 orange filters, and 0.020 inch slits. The pulse that drove the rejection solenoid valve was 5 milliseconds wide. The air pressure at the solenoid valve was 18 kg/cm^2. In addition, the feed rate was kept at approximately 17 kg per hour to ensure good peanut singulation.

Fluorescence sorting by hand. Peanuts were fluorescence sorted by hand using a UVP model C-5 ultraviolet light box (Ultra-Violet Products, Incorporated, San Gabriel, California). The illuminating wavelength of the UVP light box was 365 nm, exactly the same as that used in the machine fluorescence sorter. Small groups of peanuts were held between plates so they could be easily turned over. The top plate was removed while the peanuts were being inspected. Peanuts having areas with intense blue or violet fluorescence were rejected.

Color sorting by hand. Peanuts were color sorted by hand on a glass table. A mirror below the table allowed the top and bottom sides of each peanut to be conveniently observed without moving the peanut. Peanuts that appeared darker than normal peanuts over all or part of their surface were rejected.

Experimental design. The strategy used to measure and compare the different peanut sorting methods is shown in Figure 1. All the peanuts were fluorescence sorted twice by machine. This produced sub-sets of twice-accepted peanuts, of twice-rejected peanuts, and of one-rejected-once-accepted peanuts. All three subsets were separately color sorted by machine, making a total of 6 sub-sets. All 6 sub-sets were separately color sorted by hand, making 12 sub-sets. Of the 12 sub-sets, only those that were twice-rejected by fluorescence sorting were fluorescence sorted by hand. These operations made the 16 sub-sets shown in Figure 1 as subsets A, B, C..., P. The weight of each sub-set was recorded. All the sub-sets that were either twice-rejected or twice-accepted by fluorescence sorting were separately photographed using visible illumination. In addition, all sub-sets that were twice-rejected by fluorescence sorting were photographed using ultraviolet illumination. Each of the 16 sub-sets was then assayed for aflatoxin. This complete strategy was carried out 29 times.

Photography. Top and bottom views of peanuts were photographed using white light illumination after the sorting operations were complete. Peanuts were placed on a glass table. Two separate cameras photographed the peanuts from above and from below the glass. In this way the entire surface of each peanut in the group was recorded. Each group was separately photographed. Up to about 650 peanut halves were included in single photographs. Top and bottom views of 8 peanut groups shown in Figure 1 were also photographed using 365-nm ultraviolet illumination. In this case, peanuts had to be manually turned over to expose the bottom side for photography.

Results and Discussion

The weight and aflatoxin concentration of each of the 16 sub-sets shown in Fig. 1 are given in Table 1. Each entry in Table 1 is the combined result of 29 separate implementations of the strategy shown in Fig. 1. It is immediately clear from Table 1 that the sorting operation described in Fig. 1 was

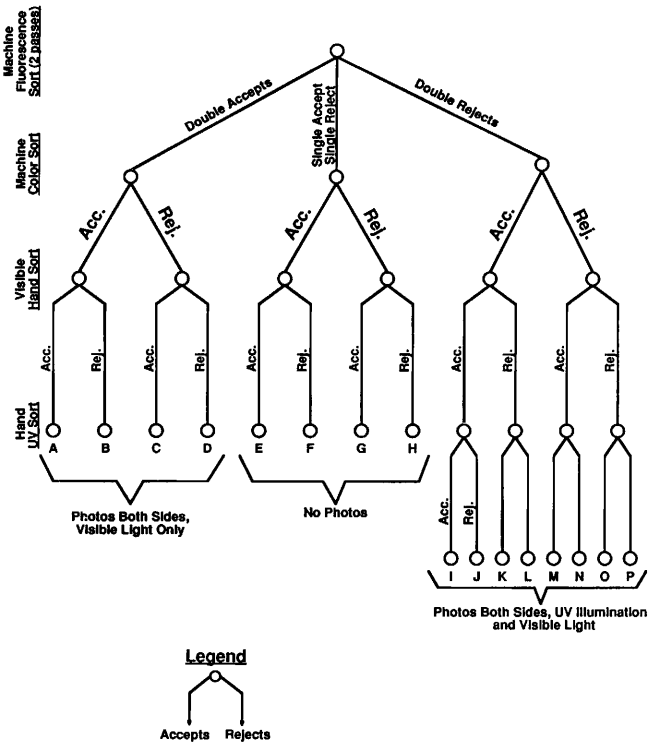

Fig. 1. Peanut Sorting Strategy Using Color and Fluorescence.

Table 1. Sorting Data and Results of Figure 1 Multiple Sort.

Peanut Set	Grams of Peanuts	ppb Aflatoxin	% of total Aflatoxin	% Peanuts Sub-sampled *
A	44958.8	1.4	13.50%	3.2
B	5530.0	8.6	10.51%	25.2
C	303.4	2.0	0.13%	100.0
D	1358.8	89.4	26.72%	100.0
E	9102.7	0.4	0.75%	15.9
F	1594.7	10.5	3.69%	73.3
G	74.8	4.4	0.07%	100.0
H	535.3	0.5	7.48%	100.0
I	1738.1	0.0	0.55%	81.7
J	91.8	1.3	0.02%	100.0
K	528.4	5.3	0.62%	100.0
L	83.4	0.9	0.02%	100.0
M	15.0	1.0	0.00%	100.0
N	2.2	0.0	0.00%	100.0
O	374.3	377.4	31.07%	100.0
P	43.7	508.3	4.88%	100.0
Totals/Avg's.	66335.4	6.86	100.00%	15.5

* "% Peanuts Sub-sampled" is the percent of the "Peanut Set" which were randomly removed from the ground sample for aflatoxin analysis. Excluding Peanut Set 'A',41.3% of all ground peanut material was sub-sampled for aflatoxin analysis.

effective at aflatoxin removal. Sub-set A, the peanuts accepted by fluorescence sorting, color sorting, and hand color sorting, had an aflatoxin concentration of only 1.4 ppb, a factor of 4.9 lower than the starting concentration (6.86 ppb). Sub-set P, the peanuts rejected by all four sorting methods, had an aflatoxin concentration of 508.3 ppb, a factor of 74 higher than the starting concentration.

The data in Table 1 can also be used to quantitatively compare the performances of the sorting methods. Since the

same peanuts were used for each sorting operation, the comparison of sorting methods is not confounded by variation in aflatoxin distribution among the peanuts. Table 1 also shows the fraction of peanuts from a given group that were included in the extraction step of the aflatoxin assays. Sampling error was minimized by ensuring that the entire sample was ground and well mixed, and by using large fractions of the ground sample for extraction as shown in Table 1.

The effectiveness of various sorting strategies can be determined by adding and/or subtracting values from Table 1. For example, the sum of the peanut or aflatoxin weights from sub-sets, C, D, G, H, M, N, O, and P gives the weight of peanuts, or ppb of aflatoxin, that was rejected by color sorting alone. Likewise, the sums from sub-sets, B, F, K, and L give the weight of peanuts or ppb of aflatoxin rejected by color sorting by hand but not by color sorting by machine. This gives a direct measure of any added benefit that color sorting by hand in conjunction with color sorting by machine would have had. Summing operations of this type that are relevant to the interpretation of Table 1 are summarized in Table 2. Numerical values resulting from the operations shown in Table 2 are given in Table 3.

Table 3 shows that the fluorescence sorter removed 37.2% of the aflatoxin from the peanuts by rejecting 4.3% of the peanuts. The aflatoxin concentration in the rejected peanuts was 58.7 ppb, considerably higher than the starting concentration of 6.86 ppb. The aflatoxin concentration of the accepted peanuts was 4.5 ppb.

Color sorting by machine removed 70.3% of the aflatoxin by rejecting 4.1% of the peanuts. The aflatoxin concentration in the peanuts rejected by the color sorter was 118.2 ppb. The aflatoxin concentration of the peanuts accepted by the automatic color sorter was 2.1 ppb.

It is possible that fluorescence sorting by machine removed aflatoxin that color sorting by machine did not. The data in Table 3, however, show that using both methods together is less effective than using color sorting alone. The row labelled "FS not CS" describes the peanuts rejected by fluorescence

Table 2. Calculations Used to Generate Table 3.

Rejected by...	Sample from Fig. 1
FS Reject	IJKLMNOP
CS Reject	CDGHMNOP
VH Reject	BDFHKLOP
UH Reject*	JLNP
FS not CS	IJKL
CS not FS	CD
CS not VH	CGMN
VH not CS	BFKL
(FS and CS)*	MNOP
(FS and VH)*	KLOP

Legend: FS=Machine Fluorescence Sorted
CS=Machine Color Sorted
VH=Manually Color Sorted
UH=Manually Fluoresence Sorted

* Based on the FS set of peanuts (2876.9 grams of peanuts and 169 micrograms of aflatoxin), not on the weight of the entire peanut group.

Table 3. Comparisons of Sorting Methods.

Rejected by...	micrograms aflatoxin rejected	% of total aflatoxin rejected	grams of peanuts rejected	% peanut weight rejected	ppb aflatoxin rejected	ppb aflatoxin accepted
FS	169.0	37.2	2876.9	4.3	58.7	4.5
CS	319.9	70.3	2707.5	4.1	118.2	2.1
VH	386.5	85.0	10048.6	15.1	38.5	1.2
UH	22.4	13.3 *	221.1	7.7 *	101.3	55.2
FS not CS	5.5	1.2	2441.7	3.6	2.3	null
CS not FS	156.4	34.4	2272.3	3.4	68.8	null
CS not VH	0.9	0.2	395.4	0.1	2.3	null
VH not CS	67.5	14.8	7736.5	11.7	8.7	null
FS and CS	163.5	96.7 *	435.2	15.1 *	375.7	2.3
FS and VH	166.4	98.5 *	1029.8	35.8 *	161.6	1.4

Total peanut weight =66335.4 grams
Total micrograms aflatoxin = 454.8
Incoming aflatoxin level = 6.86 ppb

* Based on the FS rejects set of peanuts (2876.9 grams of peanuts and 169 micrograms of aflatoxin) not the weight of the entire peanut group.

sorting, but not by color sorting. Only 1.2% of the aflatoxin was rejected by rejecting 3.6% of the peanuts. The aflatoxin level in the rejected peanuts was 2.3 ppb, well below the initial concentration and close to the aflatoxin concentration of the peanuts accepted by both color sorting by machine and fluorescence sorting by machine (2.1 ppb). Peanuts rejected by fluorescence sorting and not by color sorting should not have been rejected, as far as aflatoxin contamination is concerned.

The B aflatoxins fluoresce strongly in the violet region of the spectrum when excited with 365-nm light. The lack of correlation between fluorescence and aflatoxin contamination *in peanuts* is surprising considering the fact that all peanuts were split with testae completely removed. This allowed the entire cotyledon surface to be visible for inspection. This lack of correlation between fluorescence and aflatoxin contamination in peanuts is contradictory to a study reported earlier (45). However, there is a strong correlation between visible wavelength characteristics on the surface of the cotyledons and aflatoxin contamination, as shown by the efficacy of color sorting by machine and hand.

We included fluorescence sorting by hand in our experimental design to check the performance of the fluorescence sorter and to gain insight about the aflatoxin levels on peanuts exhibiting unusual fluorescence behavior. Fluorescence sorting by hand was only done on the peanuts rejected from the automatic fluorescence sorting operation (4.3% of the peanuts studied) due to the tedious nature of the operation. We also had data showing that very few fluorescing peanuts were accepted by the fluorescence sorter (39). The initial aflatoxin concentration of the peanuts that were fluorescence sorted by hand was 58.7 ppb (row "FS" in Table 3). Row UH in Table 3 shows that fluorescence sorting by hand removed only 13.3% of the aflatoxin by rejecting 7.7% of the peanuts. The aflatoxin concentration of peanuts rejected by fluorescence sorting by hand, 101.3 ppb, was less than a factor of two greater than the aflatoxin concentration before the sorting operation.

We are confident that no strong correlation exists between 365 nm excited fluorescence at any visible wavelength and aflatoxin in peanut cotyledons. Utilizing human vision to look for any type of "unusual" fluorescence gave us the same weak correlation between fluorescence and aflatoxin as did the machine fluorescence technique. Therefore, simply changing the filter wavelength on the machine fluorescence sorter will not improve this correlation.

In contrast, machine color sorting of the same set of peanuts removed 96.7% of the aflatoxin by rejecting 15.1% of the peanuts (row "FS and CS" in Table 3). Like- wise, hand color sorting of these peanuts removed 98.5% of the aflatoxin by rejecting 35.8% of the peanuts (row "FS and VH" in Table 3). Sorting by hand confirms the conclusions made from the machine sorting operations. The correlation of aflatoxin contamination with fluorescence is, at best, weak, but the correlation with discoloration in roasted florunner peanuts is strong.

If there is little or no correlation between fluorescence and aflatoxin contamination, how could the fluorescence sorter remove 37.2% of the aflatoxin from a set of peanuts by rejecting only 4.3% of the peanuts (row "FS" in Table 3)? The answer to this question appears to be in the firmware of the real-time computer in the automatic fluorescence sorter (39). When a peanut is detected by the diode-array trigger in the fluorescence sorter, the real-time computer begins looking for background peanut fluorescence intensity at 490 nm. Once a threshold intensity at 490 nm is exceeded, the real-time computer begins evaluating fluorescence data from the peanut. If the threshold is not exceeded, the real-time computer generates an error signal and activates the reject mechanism. The background fluorescence from dark peanuts is considerably lower than that from normal peanuts. Therefore, the fluorescence sorter behaves as a color sorter when no region on the peanut is bright enough to exceed the threshold. Since the fluorescence sorter does not necessarily reject peanuts having dark spots, its performance as a color sorter is poor.

Approximately 1200 photographs of peanut groups were taken as a part of this study. The photographic records, along with the aflatoxin assay results, allowed us to see what the contaminated peanuts looked like. Generally, normal-looking peanuts that do not have dark regions on the surface are not contaminated with aflatoxin. Peanuts having strong blue or violet fluorescence are usually not contaminated with aflatoxin unless these peanuts also have dark regions on their surface. Peanuts having regions of discoloration are often, but not always, contaminated with aflatoxin. This is reasonable since the discoloration may be due to molds that produce aflatoxin, to molds that do not produce aflatoxin, or to non-mold related damage. A more detailed analysis of the correlation between peanut appearance and aflatoxin contamination in roasted, raw, blanched peanuts will be made in a future article.

We have carried out more limited fluorescence sorting studies on other large groups of peanuts (67 kg. and 38 kg.). We consistently found a small fraction of peanuts having unusually intense blue and/or violet fluorescence. This fluorescence is weakly correlated or even negatively correlated with aflatoxin contamination. There are several chemical compounds produced by molds that grow on peanuts (11, 13, 29, 61), or even by the peanut itself (7, 59) that could be responsible for this fluorescence. Phytoalexins are one of the better-studied compounds known to fluoresce in peanuts. Phytoalexins are low molecular weight secondary metabolites synthesized by plant cells due to metabolic interaction between a host plant and a pathogenic fungus (4, 31, 38). They appear to be involved in disease resistance in peanuts. A peanut attacked by *A. flavus* will produce phytoalexins in response to the mold invasion. The phytoalexins will then attempt to kill or restrict the intracellular development of the mold (7, 12, 48, 59). Phytoalexin production appears to be related to kernel free water (21, 60). Others have described phytoalexin production as a consequence, not the cause of plant resistance to infection (30), but this view is not widely held.

Some of the phytoalexins in peanuts have been reported to be stilbenes. Cis-and trans-resveratrol (3, 5, 4'-trihydroxystilbene) (28), 3, 5,4'-trihydroxy-4-isopentenystilbene derivatives (1), and 3-isopentadienyl-4, 3', 5'-trihydroxystilbene (14) have been isolated. Cis-and trans-resveratrol are known to fluoresce pale blue (4). These phytoalexins may account for some of the slight negative correlation found between peanut fluorescence and aflatoxin contamination.

Roasting may lower the aflatoxin concentration in peanuts (32, 33, 34, 40, 52). It might also interfere with the observation of aflatoxin fluorescence from the peanut surface. We

examined the fluorescence of unroasted peanuts and tried to find a correlation with aflatoxin contamination. The fluorescence background from unroasted peanuts was stronger than that from roasted peanuts. This stronger background fluorescence made the 410-nm fluorescence more difficult to detect, increasing the difficulty of the sorting operation. Again, fluorescence was not useful as an indicator for aflatoxin contamination in raw peanuts, but color was.

By minimizing the effect of sampling error and using a multiple sorting strategy, we can conclude with a high level of confidence that color sorting was effective at removing aflatoxin from the peanuts and fluorescence sorting was not. The color sorting results were also consistent with results reported by other researchers (2, 5, 18, 49). Generalizing the results of this study to the problem of aflatoxin control in peanuts introduces yet another potential source of sampling error. The peanuts chosen for this study were intended to be a representative sample of the set of all peanuts that will be delivered in the future. If future peanuts are critically different from those used in this study, the generalization of results from this study may not be valid. This problem is inherent in any attempt to evaluate a peanut sorting strategy and should be considered when drawing conclusions about a peanut sorting method.

Summary and Conclusions

Color sorting was very effective at aflatoxin reduction from the groups of peanuts used in this study, while fluorescence sorting was not. The multiple sorting strategy described in this report can compare sorting methods quantitatively, and should be useful in the evaluation of other sorting methods.

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