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Separation of Aflatoxin-Contaminated Kernels from Sound Kernels by Hydrogen Peroxide Treatment

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ABSTRACT

Blends (0:1, 1:3, 1:1, and 3:1, wt/wt) of aflatoxin-contaminated and sound peanut kernels were submerged for 1, 2, and 3 min in various concentrations of hydrogen peroxide solution. The effectiveness of these treatments in separating aflatoxin-contaminated kernels from sound kernels was determined. Peanuts that floated (floaters) and those that did not (sinkers) were subjected to aflatoxin analysis. Second order polynomial equations were satisfactorily fitted to the experimental data. Hydrogen peroxide concentrations of 0.075, 0.150, and 0.25% resulted in a reduction in aflatoxin content in the kernels in the sinker fraction by 90% within 1 min, regardless of the initial aflatoxin content. The total aflatoxin content in sinker and floater fractions was approximately the same as that in unfractionated samples, indicating that the low concentrations of hydrogen peroxide in treatment solutions did not degrade aflatoxin. Response surface methodology was used to generate contour plots which revealed optimum treatment conditions for giving a maximum yield of the sinker fraction with low aflatoxin content. For peanuts containing 50 ppb aflatoxin, optimum conditions consist of a 0.08% hydrogen peroxide treatment for 0.7 min. This procedure results in a maximum sinker fraction yield of 85.5% of the original lot with a residual aflatoxin content of ≤5 ppb.

Aflatoxins are secondary metabolites produced by *Aspergillus flavus* and *A. parasiticus*. Because of the mutagenic, carcinogenic, and teratogenic nature of these toxins, consumption of aflatoxin-contaminated foods poses a serious health hazard. The U.S. Food and Drug Administration (FDA) enforces an action level of 20 ppb in foods destined for human consumption. Consumer demand and the world export market for commodities susceptible to aflatoxin contamination, however, are pushing toward zero tolerance.

Researchers continue to investigate methods for removing aflatoxin from contaminated peanuts and other agricultural commodities (*7*). At present, electronic color sorting and handpicking are widely used to separate aflatoxin-contaminated kernels from sound kernels. The efficiency of electronic color sorting, however, is highly variable. An average of only 72% of the aflatoxin contaminated kernels can be removed from a given lot (*2*). Handpicking, on the other hand, is more selective but deemed commercially impractical in the United States. Density-based separation schemes are theoretically feasible but loss of peanuts is high (*5*) and efficiency of separation is highly variable. A water flotation method for separating aflatoxin-contaminated and sound peanut kernels has been patented based on the observation that contaminated kernels are usually less dense than sound kernels (*6*). This procedure has not gained wide commercial acceptance due to a requirement for an additional drying step after the flotation treatment.

Takeuchi et al. (*10*) developed a hydrogen peroxide blanching process for peanuts based on the principle that catalase will react with hydrogen peroxide to yield water and oxygen. The oxygen generated is theorized to form a jacket between the testa and cotyledon, facilitating testa removal during the blanching process. Preliminary studies in our laboratory revealed that aflatoxin-contaminated peanuts floated more rapidly than sound kernels when submerged in hydrogen peroxide solution. Clavero et al. (*1*) demonstrated that *A. parasiticus* produces catalase when grown in peanut milk. It is hypothesized that catalase produced by *A. parasiticus* will react with hydrogen peroxide and promote the formation of oxygen bubbles on the surface of the kernels. The higher the catalase activity, i.e., the more severely infected the peanuts, the more rapid would be the evolution of oxygen, thus causing mold-infected kernels which may contain aflatoxins to float.

This study was conducted to evaluate the feasibility of treatment in hydrogen peroxide solutions for the purpose of separating aflatoxin-contaminated kernels from sound kernels. The development of a model for predicting the hydrogen peroxide concentration and treatment time required for a given lot with known initial aflatoxin content to obtain maximum yield and low aflatoxin content in a segregated fraction was also investigated.

MATERIALS AND METHODS

Treatment in hydrogen peroxide solution

Medium-sized (18/64 in.) peanut kernels (50 kg) (*Arachis hypogaea* L. cv. Florunner) naturally contaminated with aflatoxins (250 to 300 ppb, depending upon the sample) were blended in a rotary mixer with sound kernels (50 kg) in rations of 0:1, 1:3, 1:1, and 3:1 by weight. Seven hundred fifty grams of each blend were evenly deposited in a 28 x 20 x 11.5 cm plexiglass box containing 3 L of 0, 0.075, 0.150, or 0.225% hydrogen peroxide

solution. Peanuts that floated (floaters) after 1, 2, and 3 min were collected using a wire screen. Peanuts that did not float (sinkers) after 3 min were collected by decanting the hydrogen peroxide treatment. To obtain the weight distribution after each treatment time, floaters and sinkers were dried in a forced-air oven (Model 4-148CY, American Instruments Co., Silver Springs, MD) at 30°C to 8-9% moisture. Three replicate experiments were conducted for each concentration of hydrogen peroxide solution for each ratio of the contaminated and sound kernel blends.

Dried peanuts were weighed and the yield, defined as the percent (by weight) sinkers after any given treatment time, was calculated.

Quantitation of aflatoxins

Aflatoxins were extracted using the method of Truckess et al. (*11*) with some modifications. Peanuts (25 g) were combined with 125 ml of methanol-water (7:3, wt:vol). Five grams of NaCl were added and the mixture was blended in a Sorvall Omni mixer (Sorvall, Inc., Newton, CT) for 2 min at medium speed. The slurry was filtered through Whatman No. 4 filter paper and 15 ml of the filtrate was combined with 30 ml of water. A 15-ml sample was passed through an Aflatext-P affinity column (Vicam, Somerville, MA) which contains monoclonal antibodies that bind the aflatoxins to the column packing materials. The column was then washed with 10 ml of water to remove trapped impurities and the aflatoxins were eluted with 1 ml of high-performance liquid chromatography (HPLC)-grade methanol. Water (1.0 ml) was added to the eluate and 20 µl of the mixture was analyzed using HPLC. A HPLC system consisting of two LC-6A solvent delivery modules, a CTO column oven, a SCL-6A system controller module, a Chromatopac C-R5A, and a fluorescence monitor RF-535 (Shimadzu Co., Kyoto, Japan) was used for quantitating aflatoxins. An Econosphere C-18 5 µ reverse phase column (Alltech Associates Inc., Deerfield, IL) was maintained at 60°C. The mobile phase consisted of water-methanol-acetonitrile (60:25:15, vol:vol:vol) with a flow rate of 1 ml/min. To enhance the fluorescence of aflatoxins B_1 and G_1, a postcolumn derivitization with aqueous iodine (*11*) was applied. Saturated iodine solution was prepared by combining 2 ml of methanol and 400 ml of water with 0.8 of iodine crystals followed by constant stirring for 2 h. The iodine solution was then passed through a 0.45-µm filter (Gelman Associates Inc., Ann Arbor, MI), degassed, and added to the eluate through a mixing tee at a flow rate of 0.3 ml/min. The postcolumn reaction temperature was maintained at 90°C. Aflatoxins were detected at an excitation wavelength of 365 nm and an emission cutoff of 450 nm. A calibration curve was constructed by the area normalization method using 20 injections of an aflatoxin solution (10 ppb) prepared from a standard solution containing 5 µg B_1 and G_1 and 1.5 µg B_2 and G_2 (Sigma Chemical Co., St. Louis, MO). The aflatoxin contents in unfractionated peanuts, floaters, and blends were calculated using the following formula:

$$\text{Aflatoxin (ng/g)} = \frac{\text{aflatoxin reading (ng) x dilution factor}}{25\ \text{g}}$$

The sum of concentrations of aflatoxins B_1, B_2, G_1, and G_2 was calculated to give a total aflatoxin content in each test sample. Because the capacity of the affinity column is limited to less than 100 ng of the total aflatoxin per injection, the dilution factor was adjusted depending upon the amount of aflatoxin present in the test sample.

Analysis of data

The PROC RSREG method as defined by Statistical Analysis System (*9*) was used to fit second order polynomial equations described below:

$$R = B_0 + \sum_{i=1}^{3} B_i x_i + \sum_{i=1}^{3} B_{ii} x_i^2 + \sum_{i=1}^{2} \sum_{J=1+i}^{3} B_{ij} x_i x_j$$

Where: R = response: yield and aflatoxin content, and x_i = treatment variables (hydrogen peroxide concentration, treatment time, initial aflatoxin content) and B_0, B_i, B_{ii}, and B_{ij} are regression coefficients.

Response surface methodology with a four-level by three-factor design was used to identify the hydrogen peroxide treatment condition for obtaining a maximum yield and low aflatoxin content of kernels in the sinker fraction segregated from a lot containing a given initial aflatoxin content.

RESULTS AND DISCUSSION

Treatment in hydrogen peroxide solution

A significant reduction in aflatoxin content in the sinker fraction was achieved by treating blends of aflatoxin contaminated and sound peanut kernels in solutions containing various concentrations of hydrogen peroxide for 1 min (Fig. 1). A concentration of 0.075% hydrogen peroxide was sufficient to reduce the amount of aflatoxin in the sinker fractions of blends containing ratios of 1:3, 1:1, and 3:1 (contaminated:sound) kernels by 91.8, 89.9, and 90.5%, respectively. Aflatoxin content in the sinker fractions of the blends treated with 0.15 and 0.225% hydrogen peroxide was not significantly different ($P \leq 0.05$) from those treated with 0.075% hydrogen peroxide. No further reduction in aflatoxin content in the sinker fraction of the various blends treated with 0.075% hydrogen peroxide was observed with increased treatment time (Fig. 2). This observation is significant because effective treatment could be achieved in a short time, hence, minimizing the extent of uptake of water by the kernels. Gnanasekharan (*5*) reported that absorption of water by peanuts occurred most rapidly within the first 2 min. With a hydrogen peroxide treatment of 1 min or less, the time required for an additional drying step would be reduced considerably. No significant differences in the

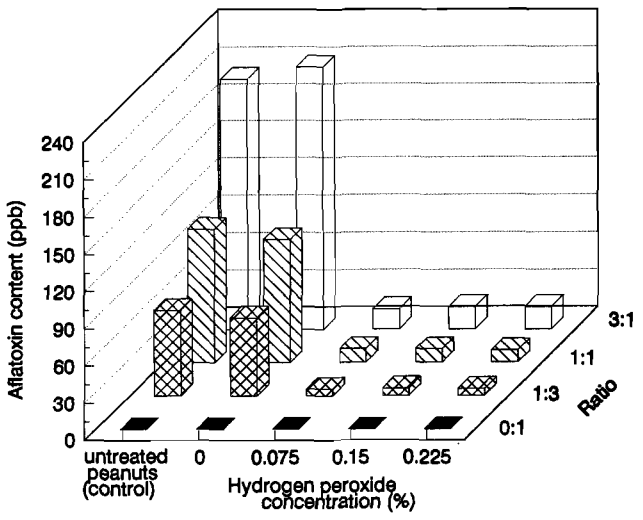

Figure 1. *Aflatoxin content in the sinker fraction of blends (ratios) of aflatoxin-contaminated and sound kernels (0:1, 1:3, 1:1, 3:1) treated with various concentrations of hydrogen peroxide for 1 min.*

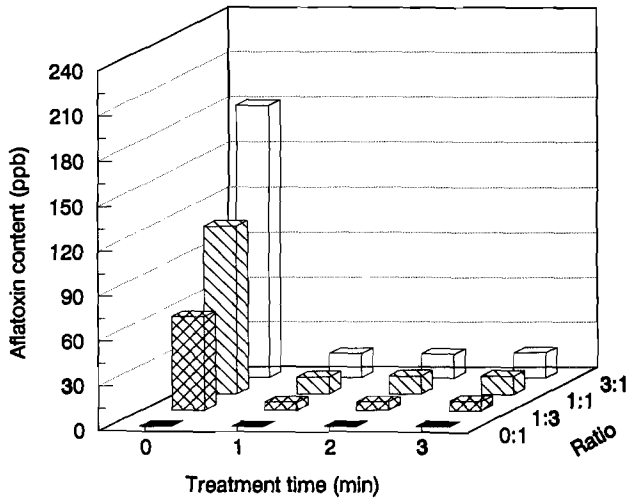

Figure 2. *Aflatoxin content in the sinker fraction of aflatoxin-contaminated and sound kernels blends (ratios) (0:1, 1:3, 1:1, 3:1) treated with 0.075% hydrogen peroxide for 0, 1, 2, and 3 min.*

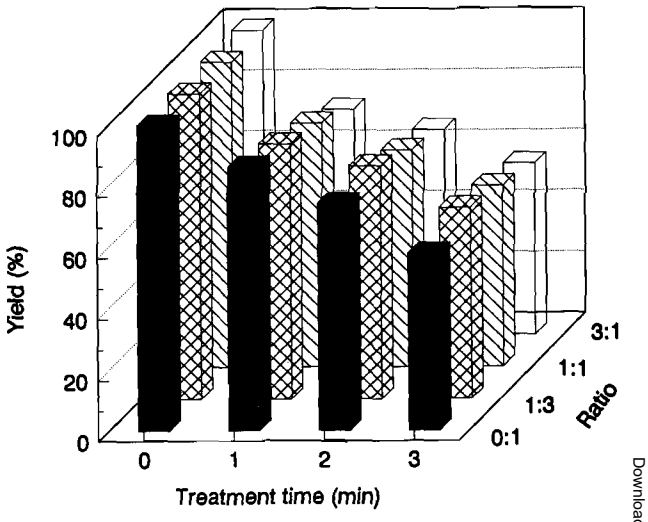

Figure 3. *Yield expressed as percent recovery in the sinker fraction from kernel blends (ratios) (0:1, 1:3, 1:1, 3:1) treated with various concentrations of hydrogen peroxide for 1 min.*

aflatoxin content in the sinker fractions from peanut blends treated with 0.15 and 0.225% were observed as treatment time increased from 1 to 3 min.

At any given hydrogen peroxide concentration, lots containing a high initial aflatoxin concentration yielded sinker fractions containing high concentrations of aflatoxin. This indicates that treatment of peanut kernel blends containing a contaminated to sound peanut kernel ratio of 3:1 or higher (approximately 200 ppb or higher) will yield a sinker fraction with an aflatoxin content greater than the FDA action level of 20 ppb. In this case, the hydrogen peroxide treatment could still be applied, but the treated peanuts could only be marketed as an animal feed, assuming that the aflatoxin content was within tolerance levels.

Segregation of aflatoxin-contaminated kernels from various contaminated:sound kernel blends using water (0% hydrogen peroxide) as the submerging fluid was unsuccessful (Fig. 1). The sinker fraction retained approximately 92% of the initial aflatoxin content of the various blends. This observation does not agree with results from a flotation separation scheme patented by Henderson et al. (*6*) who claimed a reduction of aflatoxin content by 80-90% in the sinker fraction. Mohsenin (*8*) reported that problems such as irregular shape, size and porosity of agricultural products affect measurement of density. Differences in the behavior of peanuts submerged in water which have been observed in various laboratories can be attributed to variability in the size, shape, variety, and maturity of the peanuts used. Gnanasekharan (*4*) also reported that high levels of aflatoxin and low kernel densities are not mutually exclusive and that noncontaminated peanuts can have low densities. Segregation using water, therefore, may not be always feasible.

Yield, expressed as percent recovery in the sinker fraction, decreased as hydrogen peroxide concentration and ratio of contaminated kernels in blends increased (Fig. 3). Blends containing high ratios of aflatoxin-contaminated kernels presumably exhibit a higher catalase activity, such that evolution of oxygen gas due to the action of hydrogen peroxide would be more rapid. This would result in a lower yield of sinkers.

Under treatment conditions of 0.075% hydrogen peroxide for 1 min, yield in the sinker fraction was 83.5, 79.8, and 73.9% for blends containing ratios of 1:3,1:1, and 3:1 (aflatoxin-contaminated:sound kernels), respectively, was achieved when the hydrogen peroxide concentration was increased to 0.15%. Yield was further reduced to 61.0, 57.1, and 60.2% when the respective blends were subjected to 0.225% hydrogen peroxide. An increase in treatment time from 1 to 3 min in 0.075% hydrogen peroxide solution also resulted in a reduction in yield (Fig. 4). For peanut blends containing ratios of 1:3, 1:1, and 3:1 (aflatoxin-contaminated:sound kernels), yield decreased from 83.5 to 62.2%, 79.8 to 59.0%, and 73.9 to 55.8%, respectively, as treatment time was increased from 1 to 3 min. For any

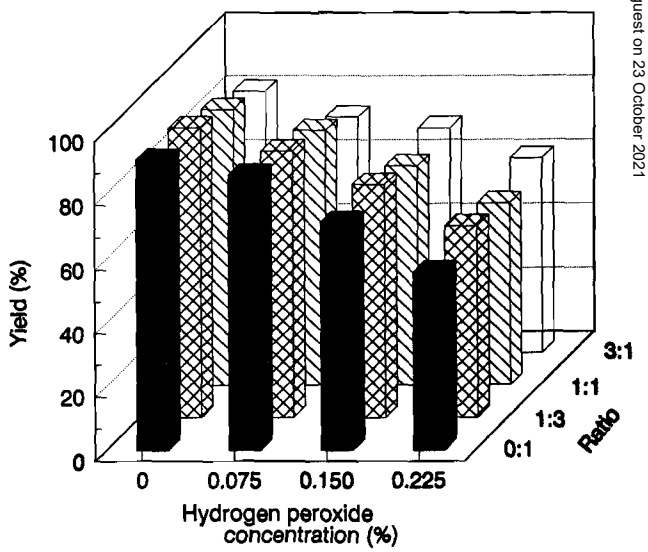

Figure 4. *Yield expressed as percent recovery in the sinker fraction from kernel blends (ratios) (0:1, 1:3, 1:1, 3:1) treated with 0.075% hydrogen peroxide for 0, 1, 2, and 3 min.*

given peanut blend, further reduction in yield occurred as the concentration of hydrogen peroxide solution was increased. Only 15.4, 16.7, and 13.6% yields were obtained when 1:3, 1:1, and 3:1 blends were subjected to treatment in 0.225% hydrogen peroxide solution for 3 min. This indicates that prolonged exposure of peanuts in solution containing a high concentration of hydrogen peroxide results in a substantial loss of sound kernels, i.e., segregation of sound kernels into the floater fraction. A loss in yield also occurred when 100% sound peanuts (0:1 blend) were subjected to hydrogen peroxide treatment. This suggests that excess hydrogen peroxide and prolonged treatment time caused a reduction in yield as a result of the reaction of peanut catalase with hydrogen peroxide. The hydrogen peroxide treatment therefore needs to be optimized to obtain maximum yield and low aflatoxin content in the sinker fraction.

Fitting of models

Second degree polynomial equations fitted to the experimental data are as follows:

$$\text{Residual aflatoxin} = 30.67 - 552.02X_1 - 48.14X_2 + 0.73X_3 + 3963.17X_1X_1 - 126.86X_1X_2 + 21.76X_2X_2 - 4.63X_1X_3 - 0.23X_2X_3 + 0.003X_3X_3$$

$$\text{Yield} = 109.05 - 80.03X_1 - 16.05X_2 - 0.06X_3 + 111.62X_1X_1 - 108.98X_1X_2 + 3.82X_2X_2 - 0.384X_1X_3 - 0.003X_2X_3 - 0.00001X_3X_3$$

Where:

X_1 = hydrogen peroxide concentration (%),
X_2 = treatment time (min), and
X_3 = initial aflatoxin content (ppb).

Analysis of variance revealed that the models appear to be adequate for predicting responses, given the hydrogen peroxide concentration, treatment time, and initial aflatoxin content. R^2 values for yield and residual aflatoxin were 0.98 and 0.80, respectively. Further statistical analysis revealed that all treatment variables had a significant overall effect on both responses.

Localization of optimum conditions

Using the predictive models of yield and residual aflatoxin, computer-generated response surfaces were obtained within the experimental region. The investigation was focused on determining optimum conditions for each blend of contaminated and sound kernels which would yield a sinker fraction with low aflatoxin content and maximum recovery of kernels. A graphical solution was employed by superimposing contour plots of yield and residual aflatoxin. This method reduces the possibility of "unrealistic responses," since only regions within the experimental design were examined (*3*). To demonstrate this procedure, peanuts containing 50 and 100 ppb aflatoxin were considered. In choosing the optimum treatment conditions, preference was given to the shortest treatment time to minimize the extent of water ingress. For peanuts containing 50 ppb aflatoxin, the shaded region in Fig. 5 indicates

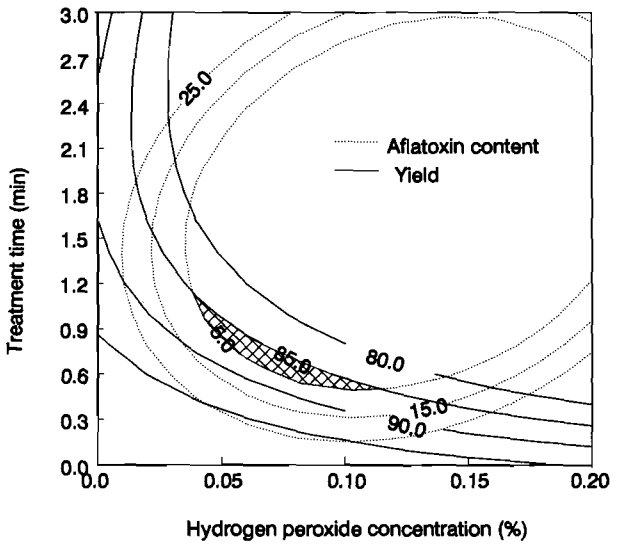

Figure 5. *Superimposed contour plots of aflatoxin content (ppb) and yield (%) in the sinker fraction for peanuts with an initial aflatoxin content of 50 ppb.*

optimum conditions which result in a yield higher than 85% with less than 5 ppb residual aflatoxin in the sinker fraction. The optimum conditions consist of a 0.08% hydrogen peroxide treatment for 0.7 min. This procedure results in a maximum yield of 85.5% of the original lot and an aflatoxin content of 5 ppb in the sinker fraction. Under the same treatment conditions, peanuts containing 100 ppb aflatoxin also resulted in approximately the same yield (84%) (Fig. 6); however, the aflatoxin content in the sinker fraction was much higher (35 ppb) than the FDA action limit. To achieve a sinker fraction with a lower aflatoxin content, then, the hydrogen peroxide treatment time must be increased. The shaded region in Fig. 6 indicates optimum conditions for peanuts containing 100 ppb. Results indicate that optimum conditions consist of treatment in a 0.1% hydrogen peroxide solution for 1 min. This condition

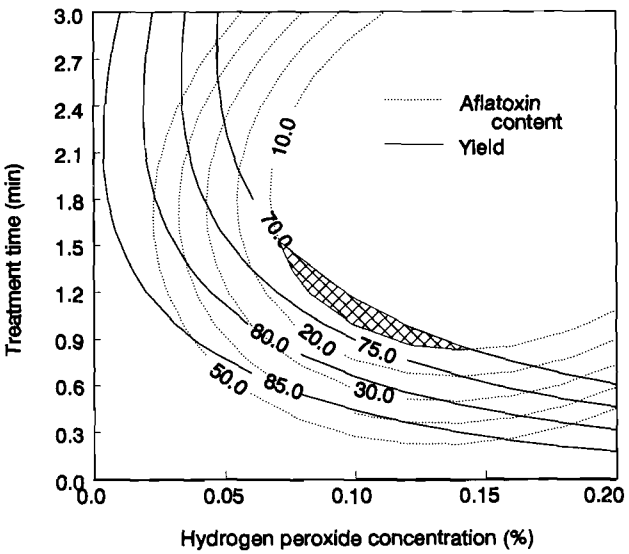

Figure 6. *Superimposed contour plots of aflatoxin content (ppb) and yield (%) in the sinker fraction for peanuts with an initial aflatoxin content of 100 ppb.*

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15. Proctor, V. A., and F. E. Cunningham. 1990. Stability of lysozyme in chemical, food, and pharmaceutical solution. Poult. Sci. 69(Suppl.):107. (Abstr.)
16. Proctor, V. A. 1991. Stability of lysozyme in various food products and preservative effect in meat. Ph.D. Dissertation. Kansas State University, Manhattan.
17. Sandow, A. 1926. The antibacterial activity of egg white. Proc. Soc. Expt. Biol. Med. 24:172-174.
18. SAS. 1991. SAS users guide: Statistics, version 6.01 ed. SAS Institute Inc., Cary, NC.
19. Smolelis, A. N., and S. E. Hartsell. 1952. Factors affecting the lytic activity of lysozyme. J. Bacteriol. 63:665-674.
20. Teotia, J. S., and B. F. Miller. 1975. Destruction of salmonellae on poultry meat with lysozyme, EDTA, X-ray, microwave and chlorine. Poult. Sci. 54:1388-1394.
21. Vakil, J. R., R. C. Chandan, R. M. Parry, and K. M. Shahani. 1969. Susceptibility of several microorganisms to milk lysozymes. J. Dairy Sci. 52:1192-1197.
22. Ward, M. S., and B. Q. Ward. 1967. Initial evaluation of the effect of butylated hydroxytoluene upon *Salmonella senftenberg* 775W. Poult. Sci. 46:1601-1603.
23. Wasserfall, F., and M. Teuber. 1979. Action of egg white lysozyme on *Clostridium tyrobutyricum*. Appl. Environ. Microbiol. 38:197-199.
24. Wooley, R. E., and J. L. Blue. 1975. In vitro effect of EDTA-tris-lysozyme solutions on selected pathogenic bacteria. J. Med. Microbiol. 8:189-194.
25. Yagima, M., Y. Hidaka, and Y. Matsuoka. 1968. Studies on egg white lysozyme as a preservative of sake. J. Ferment. Technol. 46:782-788.
26. Yashitake, S., and A. Shinichiro. 1977. Use of egg white lysozyme in the food industry. New Food Ind. 19:17-23

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resulted in a maximum yield of 73% and an aflatoxin content of 10 ppb.

Response surface methodology is a very useful tool for determining the optimum hydrogen peroxide treatment condition necessary for removing aflatoxin-contaminated Florunner kernels from sound medium-sized kernels. Additional experiments will be required to determine the usefulness of this treatment for separating contaminated and sound kernels of other peanut cultivars. The efficacy of the method as affected by kernel size and temperature of the hydrogen peroxide solution must also be investigated.

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REFERENCES

1. Clavero, M. R. S., Y.-C. Hung, L. R. Beuchat, and T. Nakayama. 1992. Catalase content in peanut milk as affected by growth of *Aspergillus parasiticus*. J. Food Prot. (56:55-57).
2. Dickens, J. W., and T. B. Whitaker. 1975. Efficacy of electronic color sorting and handpicking to remove aflatoxin contaminated kernels from commercial lots of shelled peanuts. Peanut Sci. 2:45-50.
3. Floros, J. D., and M. S. Chinnan. 1988. Computer graphics-assisted optimization for product and process development. Food Technol. 42(2):75-79.
4. Gnanasekharan, V. 1991. Effect of aflatoxin level on the density distribution of naturally contaminated peanuts inoculated with *Aspergillus parasiticus*. M.S. Thesis. University of Georgia, Athens.
5. Gnanasekharan, V., and M. S. Chinnan. 1989. Density characteristics of aflatoxin contaminated peanuts. Am. Soc. Agric. Engr. Tech. Paper No. 89-6510, St. Joseph, MI 49085.
6. Henderson, J. C., S. H. Kreutzer, A. A. Schmidt, and W. R. Hagen. 1989. Flotation separation of aflatoxin contaminated grain or nuts. U.S. Patent No. 4,795,651.
7. Moss, M. O., and J. E. Smith. 1985. Mycotoxins: Formation, analysis and significance. John Wiley and Sons, Chichester, NY. p. 7.
8. Mohsenin, N. N. 1972. Mechanical properties of fruits and vegetables: a review of a decade of research, application and future needs. Trans. ASAE 15:1064-1070.
9. SAS. 1985. SAS user's guide: Statistics, 1985 ed. SAS Institute, Inc., Cary, NC.
10. Takeuchi, Y., A. Kawabata, and Y. Mazumoto. 1970. New skinning process. Peanut J. Nut World 50:12.
11. Trucksess, M. W., M. E. Stack, S. Nesheim, S. W. Page, and R. H. Albert. 1991. Immunoaffinity column coupled with solution fluorometry or liquid chromatography postcolumn derivitization for aflatoxins in corn, peanuts and peanut butter: collaborative study. J. Assoc. Off. Anal. Chem. 74:81-88.