Sample Preparation for Aflatoxin Assay: The Nature of the Problem and Approaches to a Solution[1]

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Abstract

Cases have been reported of individual peanuts, cottonseeds or Brazil nuts so highly contaminated with aflatoxin that, for a 50 g portion to be representative of the whole, the sample preparation procedures should grind each unit to a large number of particles and distribute them uniformly throughout the sample. Assuming uniform contamination of the individual kernel, each 50 g sample should contain 1/100 of that kernel. Even though these extreme cases may be encountered only infrequently, the more usual situation still presents difficulties because of great variability in individual kernel contamination. However, if the extreme can be handled, one can expect to handle the more usual situation. Equipment and procedures to achieve this distribution goal are described. The equipment studied includes a food chopper (Hobart), a nut mill (Thomas Mills), a disc mill (Bauer), a hammer mill (Fitzpatrick Model D comminuting machine), a hammer mill designed specifically for peanut samples (Dicken's subsampling mill), a Polytron homogenizer (Bronwill Scientific), a vertical cutter-mixer (Hobart), and a sample splitter (Jones riffle). Commodities examined were shelled peanuts and in-shell Brazil nuts, walnuts, pecans and almonds. Comminution and mixing effectiveness were determined by particle size analysis, by distribution of kernels made radioactive by neutron activation and by aflatoxin analysis of naturally contaminated products. From the results we conclude that the ultimate in sample uniformity can be achieved with a disc mill, solvent addition to obtain a fluid system and mixing and grinding of the fluid with a dispersion mixer-grinder. A practical uniformity can be achieved in a vertical cutter-mixer with less expenditure of time and effort for the commodities studied.

Introduction

From the first recognition that field infection of individual peanut kernels by aflatoxin-producing molds was a primary source of aflatoxin contamination (1), it was clear that obtaining representative samples of whole kernel nuts for determination of aflatoxin contamination could be a problem. Normal sampling practices are dependent on random distribution of numerous particles containing approximately the same level of the measured entity. The type of infection observed was not likely to be random nor of uniform magnitude. It was not possible to put the problem in quantitative terms until Cucullu et al. (2) reported a study with two selected lots of aflatoxin-contaminated peanuts. From a careful sorting into categories based on appearance, and from assays of bulk sections and of individual nuts, they found contaminated nuts in categories representing 5% of one sample and 0.24% of the other. Measurable aflatoxins were found in approximately half the nuts in these categories, the contamination levels of individual nuts in each group covering more than a 1000-fold spread. Constituent parts of selected nuts were contaminated at varying levels with high/low ratios of about 100 within individual nuts, and no consistent pattern of contamination. Contamination of all the individual nuts examined ranged from a trace of aflatoxins to 1,100 μg/g of nut meat, and averaged 112 μg/g. The average value for the most highly contaminated group of nuts (>100 μg/g) was 500 μg/g. If we take this value as a reasonable basis for judging the sampling problem, one contaminated kernel in 10,000 could result in an aflatoxin level of 50 μg/kg of nut meats. The scattered nature of the contamination was shown by the analysis of two separate 2 kg subsamples of the lot containing the more highly contaminated individual kernels. After each subsample was ground and mixed, one subsample contained 30 and the other 400 μg aflatoxin B_1/kg of nut meat. Similar studies with cottonseed (3), corn (Eppley, personal communication) and Brazil nuts (Lee and Cucullu, personal communication) provide the same quality of results (Table I).

It is evident that whole kernel samples should contain upwards of 10,000 units if, under conditions represented by the figures in the preceding paragraph, each lot sample is to contain at least one contaminated kernel. The study reported here does not consider the problems involved in obtaining the lot sample but is concerned only in the preparation of the lot sample in order to assure a representative analytical sample.

The obvious approach is size reduction and mixing; the problem is selection of equipment and procedures. The usual analytical sample for aflatoxin analysis is 50–100 g. A 50 g sample of shelled Virginia peanuts will contain approximately 100 kernels. Assuming one contaminated kernel in 10,000 and uniform contamination of the kernel, each 50 g sample should contain 1/100 of that kernel for the sample to be representative or, from a viewpoint

TABLE I
Assay of Single Kernels for Aflatoxins

	Commodity and Selection				
	Peanuts (2)	Cotton-seed (3)	Corn (4)		Brazil nuts (5)
Selection	Defects	Random	Defects	Random	Defects
Number of kernels examined	40	150	10	256	100
Number positive for aflatoxin	22	28	5	0	5
Range of aflatoxin found[a] Low	Trace	0.057	Trace	.....	0.05
High	1000	600	8	.....	25

[a] Microgram of total aflatoxins per gram kernel.

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that is independent of commodity, if all kernels are to be equally represented in the sample the 50 g sample should contain a minimum of 10,000 pieces. As a first approach to a determination of the degree of size reduction, let us assume cubic particles of unit density (1 g ~ 1 cm^3). A cube weighing 50/10,000 g (5 mg) would have a linear dimension of 1.7 mm. This is approximately the opening of a U.S. standard 12 mesh screen. Starting with the 500 mg peanut having a short diameter of 8 mm and assuming that the masses of all particles are related through the cube of the linear dimension that allows them to pass a screen opening, we have 1.7 mm as the screen opening, which checks with the previous approach. The size reduction goal should, therefore, be the finest practical grind smaller than will pass a 12 mesh screen. Since mixing can be interrelated with size reduction, and is certainly related to the physical system created by the size reduction process, it will be discussed as part of each size reduction system examined.

Shelled peanuts and in-shell Brazil nuts, pecans, walnuts and almonds were the commodities used in the study. As the mechanisms of the size reduction devices are developed it will be evident that the in-shell nuts provide a cross section of varying shell hardness and firmness of meat. All the nuts have one thing in common. Size reduction and accompanying mechanical action, if carried far enough, render oil from the tissues to form a paste which is difficult to mix. Thus, the sample preparation procedures used by the first investigators attempted to stop short of oiling off so that mixing devices dependent on free granular flow could be used.

Experimental Procedures

There is an inherent imprecision in the aflatoxin assay and there is no simple way to establish the incidence of contaminated nuts in a sample. Therefore, nuts made radioactive by neutron activation were used to establish a desired incidence of contamination for determining the effectiveness of the sample preparation devices studied. The distribution of radioactivity in the selected portions after grinding and mixing provided a sensitive, precise means for measuring sample variability in respect to the added contaminant. After the effectiveness of the devices had been established by particle size and radioactivity measurements, two selected grinding-mixing systems were tested with naturally contaminated samples. One was the system that was judged to be most practical; the other was the system that until now has been used most extensively. Particle size distribution, except where indicated, was determined after the ground sample was defatted in a Soxhlet extractor. The distributions were made with a Tyler Ro-Tap shaker and 8 inch U.S. standard screens.

Radioactivity counts were made with a 3 × 3 in. NaI(Ti) crystal detector connected to a 400 channel pulse height analyzer. The primary radioisotope in the neutron activated peanuts was ^{42}K (12.4 hr half life, 1.52 MeV gamma ray) and the primary radioactive isotope in the neutron activated Brazil nuts, pecans, almonds and walnuts was ^{82}Br (36 hr half life, 0.55, 0.78, 1.04 and 1.32 MeV gamma rays). Initially, the net counts in the characteristic photopeak per unit time were taken to be representative of the amount of radioactive material in each analytical sample. Later it was shown that the net counts in the entire spectrum (0–4 MeV) were an equally reliable measure of the radioactivity distribution. Sample counting periods were chosen so that the statistical counting error for each sample was less than 3% (1000 counts total). Where time allowed, this error was held to about 1% (10,000 counts total). When necessary, corrections were made for the decay of the radionuclide during the period required to count each group of samples.

The following size reduction devices were employed: Thomas Mills nut grinder No. 3 (Thomas Mills Manufacturing Corp., Philadelphia, Pa.); Hobart food cutter, Model 84181-D (Hobart Manufacturing Co., Troy, Ohio); Hobart vertical cutter-mixer, Model VCM-40; Bauer disc mill, Model 148–2–8 in. (Bauer Bros. Co., Springfield, Ohio); Fitzpatrick hammer mill, Model D comminuting machine (The Fitzpatrick Co., Elmhurst, Ill. 60126); a hammer mill specially designed for preparation of peanut samples by W. Dickens (USDA, North Carolina State University, Raleigh); and Willems Polytron, Model 45TE (Bronwill Scientific, Rochester, N.Y.).

The vertical cutter-mixer and the Polytron performed blending and size reduction operations simultaneously, although the Polytron was not used for primary size reduction but only for continued reduction and mixing of the discharge from the Bauer disc mill. For mixing the discharge from the other size reduction operations the following machines were used where appropriate: a sample splitter (Jones riffle sampler, ASTM); a Hobart planetary mixer, Model A 120 or H600T; or a twin shell intensifier-blender, 8 qt (Patterson-Kelley Co., E. Stroudsburg, Pa.). Each of these pieces of apparatus is illustrated in Figure 1.

The following experiments were performed, not necessarily in the order given:

Experiment 1

A 20 kg lot sample of aflatoxin-contaminated, raw, shelled, Virginia peanuts was passed twice through a Thomas Mills nut grinder and mixed in a Hobart planetary mixer. The ground sample was divided by riffling and each half divided again and again until the recovered portions approached analytical size. This dividing provided 32 samples weighing 100+ g and 16 weighing 800+ g. Each of these samples was analyzed for aflatoxins by the popularly named Celite procedure (4,5). The extraction portion of the procedure was scaled up for the 800+ g samples. Half of the 100+ g samples were analyzed by one chemist, the other half and all 16 of the 800+ g samples were analyzed by a second chemist.

Experiment 2

A 3.4 kg lot sample of raw, shelled Virginia peanuts was ground in a Bauer mill set for a fine grind. A single neutron activated nut (0.72 g) was added during the milling operation to provide a dilution factor of $2.13/10^4$. This operation took about 45 min. The resulting paste was liquefied by the addition of 2 L of *n*-heptane (commercial grade) and mixed at medium speed with a Polytron to disperse the nut meat particles. Five accurately weighed portions of the slurry, representing 31–33 g of original peanut meat, were removed for radioactivity counting after 2, 4, 6 and 7.5 min of mixing.

Experiment 3

A single radioactive peanut (0.581 g) was ground and mixed with 5663 g of raw, shelled Virginia

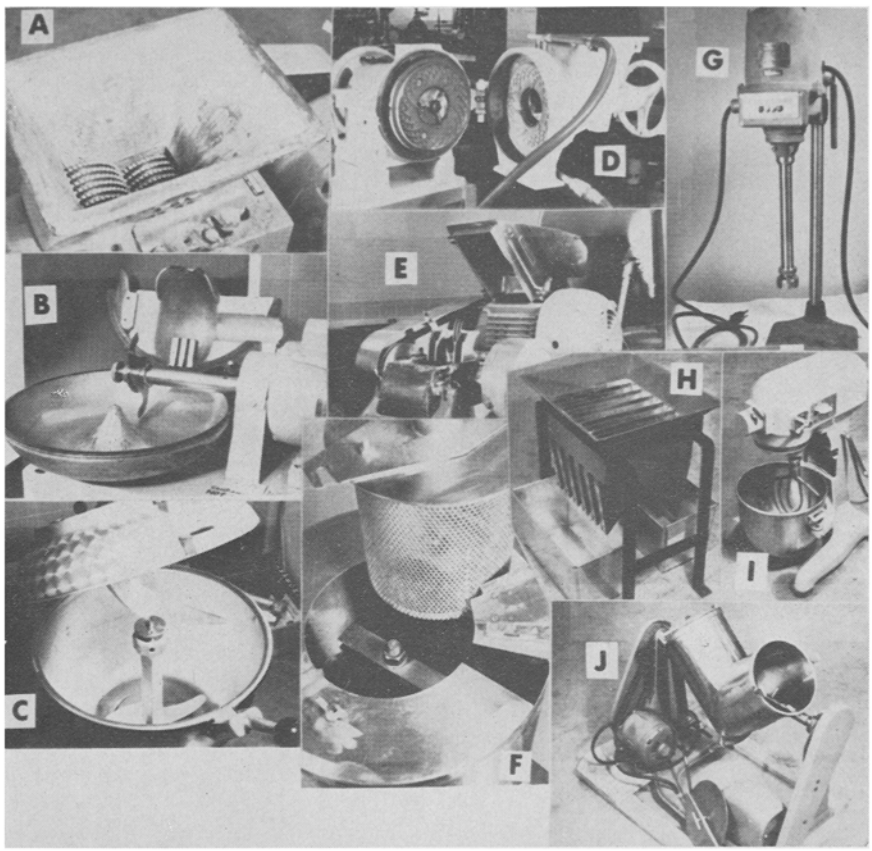

FIG. 1. A, Thomas Mills nut grinder; B, food cutter; C, Hobart vertical cutter-mixer; D, Bauer disc mill; E, Fitzpatrick hammer mill; F, Dickens mill; G, Willems Polytron; H, Jones riffle sample splitter; I, Hobart planetary mixer; J, Patterson-Kelley twin shell intensifier-blender.

peanuts (dilution factor $1.03/10^4$) in a Hobart vertical cutter-mixer equipped with a blunt agitator blade. The machine was operated 1 min at slow speed followed by 2 min at high speed. Twenty 50 g portions were removed for radioactivity measurement.

Experiment 4

This was a duplication of Experiment 3, except that 4 radioactive peanuts (2.207 g) were added to the sample mass (dilution factor $3.89/10^4$).

Experiment 5

A 6.8 kg lot sample of raw, shelled Virginia peanuts was ground and mixed in a Hobart vertical cutter-mixer equipped with a sharp, scimitar-shaped agitator blade. The machine was operated 1 min for determination of particle size distribution only.

Experiment 6

A single radioactive peanut (0.750 g) was ground and mixed with 2000 g of raw, shelled Virginia peanuts (dilution factor $3.75/10^4$) in a Hobart food chopper until judged by eye to be fine enough to pass a 10 mesh sieve. The ground peanuts were transferred to a twin shell intensifier-blender and mixed for 15 min. These steps correspond to the procedure used by the aflatoxin assay laboratories of the Market Quality Research Division (MQRD), USDA. Twenty 50 g portions were removed for measurement of radioactivity.

Experiment 7

This was a duplication of Experiment 6, except that five radioactive nuts (3.24 g) were added into the sample mass (dilution factor $16.21/10^4$).

Experiment 8

A single radioactive peanut (0.65 g) was ground with 6800 g of raw, shelled Virginia peanuts (dilution factor $0.96/10^4$) in a vertical hammer mill designed by W. Dickens, MQRD, USDA to simultaneously grind and split a sample of peanuts. The discharge of the mill is so arranged that approximately 1/20 of the sample stream is shunted off as a portion representative of the main sample body. This smaller, more conveniently transported portion is intended for the analytical laboratory. The shunt sample (436 g, 1/16 of the total) was transferred to a plastic bag and mixed by tumbling and kneading; the main portion of the sample was transferred to a 60 qt bowl of a planetary mixer and mixed 20 min at low speed with a flat beater. Three 50 g portions were removed from the shunt sample and 17 50 g samples from the main portion for radioactivity measurement.

Experiment 9

This was a duplication of Experiment 8 except that five radioactive nuts (3.38 g) were added to the sample mass (dilution factor $4.97/10^4$).

Experiment 10

A single radioactive Brazil nut meat (3.39 g $\sim$ 6.8 g in-shell nut) was ground and mixed with 10.8 kg of in-shell Brazil nuts (dilution factor $6.29/10^4$) in a Hobart vertical cutter-mixer equipped with a blunt agitator blade. The machine was operated as in Experiment 3. Twenty 100 g portions (50 g nut meat) were taken for radioactivity measurement.

Experiment 11

A single radioactive Brazil nut meat (5.48 g $\sim$ 11.0 g in-shell nut) was ground with 11.4 kg of in-shell Brazil nuts (dilution factor $9.65/10^4$) through a Fitzpatrick hammer mill using the blunt edge of the hammers and a screen with ¼ in.

diameter perforations. The ground sample was transferred to a 60 qt bowl of a planetary mixer and mixed 20 min at low speed with a flat beater. Twenty 50 g portions were removed for radioactivity measurement.

Experiment 12

This was a duplication of Experiment 10, except that a single radioactive walnut meat (3.45 g ~ 7.0 g in-shell nut) was added to 11.3 kg of in-shell walnuts (dilution factor $6.25/10^4$).

Experiment 13

This was a duplication of Experiment 10, except that a single radioactive almond meat (1.53 g ~ 3.8 g in-shell nut) was added to 11.1 kg of in-shell almonds (dilution factor $3.45/10^4$).

Experiment 14

This was a duplication of Experiment 10, except that a single radioactive pecan meat (2.98 g ~ 6.2 g in-shell nut) was added to 11.4 kg of in-shell pecans (dilution factor $5.56/10^4$).

Experiment 15

This was a duplicate of Experiment 10, except that a single radioactive peanut (0.54 g) was added to 6.8 kg of raw, shelled Virginia peanuts (dilution factor $0.79/10^4$) together with 6.8 kg of coarse, crushed oyster shell (a commercial chicken feed grit). To determine the particle size distribution of the peanut meat portion of this mixture, a weighed portion of each sieve fraction was ashed at 600 C. Since the oyster shell had a negligible weight loss at this temperature, the proportion of peanut meat in each fraction could be calculated from the weight loss after including a correction for the ash content of the peanuts.

Experiment 16

A 21+ kg lot sample of aflatoxin-contaminated, roast Virginia pickout peanuts was passed through a Thomas Mills nut grinder and divided into three 6.8 kg portions by riffling. (a) One portion was ground through a Bauer mill set for a fine grind. After the experience gained from prior experiments, the time for this operation was reduced to 20 min. The resulting paste was liquefied by addition of 1 L *n*-heptane and mixed at high speed for 2 min with a Polytron to disperse the nut meat particles (see Experiment 2). (b) A second portion was ground in a Hobart vertical cutter-mixer with an equal weight of oyster shell. The machine was operated 1 min at slow speed followed by 2 min at high speed (see Experiment 15). (c) A third portion was screened through a 10 mesh U.S. standard sieve and the course particles reground until all the material passed through the sieve. The screened material was mixed 5 min in a double shell intensifier-blender. Analytical samples were obtained by riffling. These steps correspond to the procedure used by the aflatoxin assay laboratories, MQRD, USDA.

Six 50 g (nut meat basis) and six 1000 g (nut meat basis) analytical samples were removed from each portion. Each sample was analyzed for aflatoxins by the popularly called CB procedure (6,7) except that fluorodensitometry was used for quantitation on the thin layer chromatograms (8). To help establish the variability of the analytical procedure a number of analyses were performed on replicate portions of two of the kilogram sample extracts.

Experiment 17

This was a repeat of Experiment 16 with the following modifications. Samples of raw peanuts selected for the desired level of contamination were composited. They had been ground in a Hobart food chopper to a visual approximation of a particle size reduction to under 10 mesh and actually screened 100% through 6 mesh and 95% through 10 mesh. This ground material was riffled to obtain the three 6.8 kg portions, one of which was used with no further modification for the third portion.

Analytical samples were removed from each portion according to the same protocol as in Experiment 16. Analyses were performed on replicate portions of the kilogram extracts as in Experiment 16, except that one set of replicates was used to establish day to day variability in the fluorodensitometry.

Experiment 18

An 11.4 kg lot sample of naturally contaminated Brazil nuts was ground in a Hobart vertical cutter-mixer, following the procedure used in Experiment 10. Six 100 g and six 1000 g analytical samples were removed from the ground, mixed nuts and were analyzed for aflatoxins in the same manner as in Experiments 16 and 17.

Discussion and Results

The ultimate uniformity of a lot sample is a result of both final particle size and mixing efficiency. We do not have an independent measure of mixing efficiency. Particle size distribution does give an independent measure of size reduction; however, this is an average for all the nuts and does not necessarily give an accurate representation of what has happened to the few contaminated nuts. Only a very general correlation between particle size distribution and sample homogeneity could therefore be expected.

Because there is no statistical device by which the measure of homogeneity can be adjusted for the dilution factor, critical comparisons should be made between samples at approximately the same dilution.

A determination of adequate homogeneity requires that other sources of error and the ultimate interpretation of the analytical data be taken into account. A major source of error in an aflatoxin assay is the analytical procedure itself. Since the squares of the coefficients of variation of the procedure and of the sample can be handled as simple variances, the coefficients of variation (CV) can be related by the function:

$x = \sqrt{a^2 + (ya)^2}$ which can be altered to: $x = a\sqrt{1 + y^2}$ where a = CV of assay procedure, ya = CV of sample, and x = total CV.

By observing how $\sqrt{1 + y^2}$ varies with change in y, the relative effect of the sample error on the total error can be seen (Fig. 2). For instance, if the sample CV is ½ of the method CV, the total CV will be 1.12 times the method CV. Thus a 30% coefficient of variation for the procedure and a 15% coefficient of variation for the analytical sample would be expected to produce an overall 34% coefficient of variation.

The grinding procedure used in Experiment 1 was established before the quantitative information on individual nut contamination was available, and was selected because of the resulting free flowing, non-segregating product. The particle size distribution of a typical grind is shown in Figure 3. The data

TABLE II

Degree of Homogeneity of Lot Samples of a Number of Nut Products Prepared for Analysis by Various Size Reduction and Mixing Procedures

Commodity and Expt. No.	Sample Preparation			Dilution[a]	Through 40 mesh, %	Sample Homogeneity Determined by										
						Radioactivity		Aflatoxin Contamination								
	Mill	Mixer	Procedure variant			Sample, g[b]	CV,[c] %	Sample, g[b]	Mean, μg/kg B1	Mean, μg/kg Total	CV,[d] %	Sample, g[b]	Mean, μg/kg B1	Mean, μg/kg Total	CV,[d] %	Expt. No.
Peanuts, raw Virginia																
1	Thomas	Planetary and riffle	2 passes through mill	.....	4[e]	....	....	100	119	235	60	800	114	194	34	1
3		VCM,[f] blunt rotor	1 min low, 2 min high speed	1.03	30[g]	50	204									3
4		VCM, blunt rotor	1 min low, 2 min high speed	3.89		50	96									4
5		VCM, sharp rotor	1 min low, 2 min high speed	.....	52	....	....									5
8	Dickens	Planetary	20 min mixing	.96	41[g]	50	39									8
9	Dickens	Planetary	20 min mixing	4.97		50	21									9
6	Food chopper	Double shell	to pass 10 mesh, 15 min mixing	3.75	25	50	13									**6**
7	Food chopper	Double shell	to pass 10 mesh, 15 min mixing	16.21	23	50	9									7
15		VCM, blunt rotor	Oyster shell added	.79	75	50	2									15
2	Bauer	Polytron	Fluidized with heptane	2.13	99	32	1									2
Peanuts, roasted Virginia[h,i]																
16c	Thomas	Double shell & riffle	Reground to pass 10 mesh	.....	....	....	....	50	177	413	25	1000	174	356	12	16c
16b		VCM, blunt rotor	Oyster shell added	.....	....	....	....	50	188	424	17	1000	178	406	17	16b
16a	Bauer	Polytron	Fluidized with heptane	.....	....	....	....	50	207	473	8	1000	206	446	12	16a
Peanuts, raw Virginia[i]																
17c	Food chopper	Riffle	Ground to pass 10 mesh	.....	53	....	....	50	18	30	29	1000	27	48	17	17c
17b		VCM, blunt rotor	Oyster shell added	.....	63	....	....	50	27	44	20	1000	24	46	14	17b
17a	Bauer	Polytron	Fluidized with heptane	.....	99	....	....	50	25	45	20	1000	23	46	14	17a
Brazil nuts, in shell[i]																
10		VCM, blunt rotor	1 min low, 2 min high speed	6.29	90[j]	50	3									10
11	Fitzpatrick	Planetary	¼ in. holes in screen, 20 min mixing	9.65	95[j]	50	1									11
18		VCM, blunt rotor	1 min low, 2 min high speed	.....	85[j]	....	....	50	38	61	21	500	45	74	8	18
Walnuts, in shell																
12		VCM, blunt rotor	1 min low, 2 min high speed	6.25	70[j]	50	3									12
Pecans, in shell																
14		VCM, blunt rotor	1 min low, 2 min high speed	5.56	80[j]	50	4									14
Almonds, in shell																
13		VCM, blunt rotor	1 min low, 2 min high speed	3.45	50[j]	50	8									13

[a] Radioactive nuts per 1×10^4 nuts by weight.
[b] Weight of nut meat only.
[c] Coefficient of variation of radioactivity counts, corrected for counter precision.
[d] Coefficient of variation of aflatoxin B1 values uncorrected for assay method variance.
[e] From data on similar grind.
[f] Hobart vertical cutter-mixer.
[g] Composite.
[h] Pickouts.
[i] Naturally contaminated.
[j] Estimated from appearance of sieve fractions.

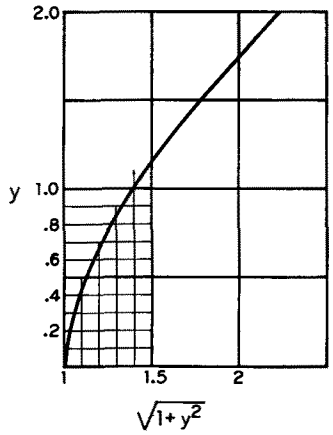

FIG. 2. Effect of sample error on total error as proportions of assay procedure error, from the relation: $x/a = \sqrt{1+y^2}$, where a = CV of assay procedure, y = CV of sample/a, and x = total CV.

on aflatoxin content, which was obtained before the variance of the analytical procedure was known, indicated large differences between analytical samples and was responsible for much of the subsequent work on sample preparation. Later information on within-laboratory precision from a collaborative study of the analytical procedure (4) has allowed us to put these data in proper prespective (Table II). From the collaborative study data we estimate a within-laboratory precision of 32% (coefficient of variation) for the determination of aflatoxin B_1. It can be determined from inspection of the coefficients of variation of the replicate samples that any variance of the 800 g samples is buried within the analytical error, but that there must be a sizeable sample variance for 100 g portions.

The grinding and mixing procedure used in Experiment 2 was devised as an answer to the problem posed in the introduction of this paper. Peanut butter was accepted as representative of the finest practical grind, and equipment simulating that used for commercial manufacture of peanut butter as the most practical for the size reduction. Liquid mixing was expected to be the most effective, so the peanut paste was converted to a liquid by addition of a fat solvent which brought the peanut oil into the liquid system. *n*-Heptane was selected as the solvent on considerations of availability, low cost, low volatility, aflatoxin insolubility and noninterference with the analytical procedure. A Polytron was selected for liquid mixing since it is designed for maximum dispersion of the discontinuous phase in a two phase system. In later experiments (16 and 17) it was found that less heptane is required to achieve a fluid system if the heptane were first blended into the butter with a spatula or paddle. The results in size reduction and particle dispersion fully matched expectations (Fig. 3, Table II).

The Hobart vertical cutter-mixer used in Experiment 3 was originally applied to sample preparation of in-shell Brazil nuts for aflatoxin assay, and the results of Experiments 10 and 18 (Fig. 4, Table II) confirm expectations of adequate homogeneity which had been based on the appearance of the prepared lot sample. The results of Experiments 3 and 4 (Table II), in which shelled peanuts were substituted for in-shell Brazil nuts, demonstrate what was first interpreted as inadequate size reduction (Fig. 3), although later experiments (6 and 7)

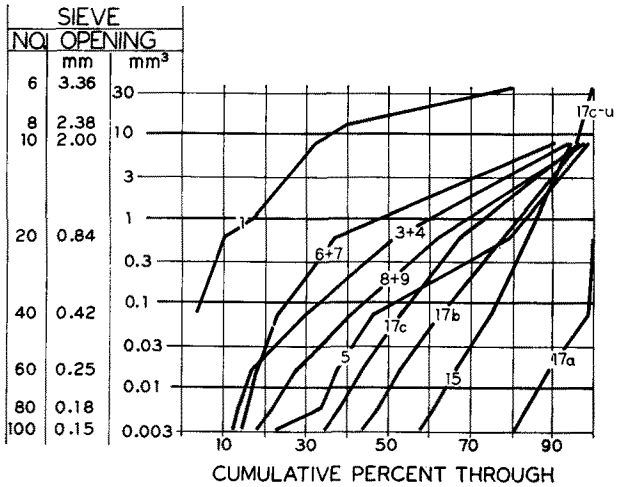

FIG. 3. Particle size distribution of shelled peanuts milled with various devices: 1, one pass through Thomas nut mill; 3,4, Hobart vertical cutter-mixer, blunt rotor; 5, Hobart vertical cutter-mixer with scimitar rotor; 6, 7, 17c, 17 c-u (17c undefatted) Hobart food cutter; 8, 9, Dickens hammer mill; 15, 17b, Hobart vertical cutter-mixer, blunt rotor with oyster shell; 17a, Bauer mill. Key numbers coincide with experiment numbers in text.

indicate this may be only part of the answer. A change from blunt to sharp rotor blades in the equipment (Experiment 5) slightly improved the size reduction (Fig. 3). The discrepancy between shelled peanuts and in-shell Brazil nuts seemed attributable to the presence of the hard shell, which could conceivably act as a grinding aid and dispersant. This conjecture was proved correct in Experiment 15 (Fig. 3, Table II), in which coarse, crushed oyster shell was used with peanuts as a hard shell substitute, and is supported by the experiments with the other in-shell nuts (Experiments 12, 13 and 14). The particle size distribution and the analytical sample variance correspond to the relative hardness of the shells and firmness of the meats, as judged subjectively (Fig. 4, Table II). The nut meat particle size distribution is our concern and since there is no obvious way to separate the meat from the shell after grinding, some other basis for judging the composition of each fraction must be used. The nature of the fragments on the coarse sieves (10, 20

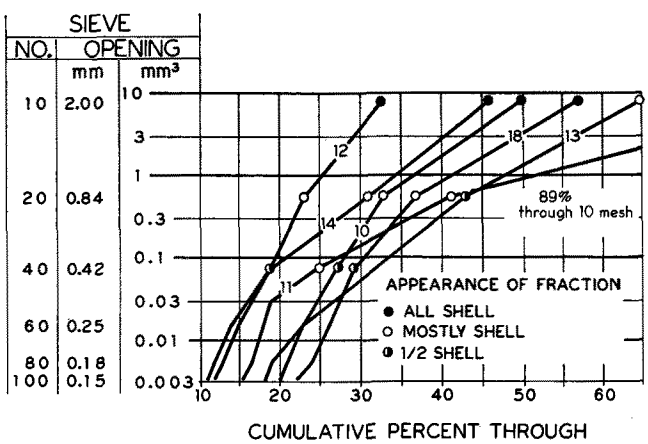

FIG. 4. Particle size distribution of in-shell nuts ground in a Hobart vertical cutter-mixer: 10, 18, Brazil nuts; 12, walnuts; 13, almonds; 14, pecans; and ground in a Fitzpatrick hammer mill: 11, Brazil nuts. Key numbers coincide with experiment numbers in text.

and 40 mesh) was obvious from their appearance and the relative amounts of meat and shell could be estimated roughly. A calculation of the weight ratios of shell and fat-free meat for each of the nuts found the shell to be remarkably close to 75% of the total fat-free weight for all four in-shell nut varieties studied. Where the sum of the fractions, observed to consist of all shell and mostly shell, account for 75% of the total fat-free weight, the estimation of the particle size distribution of the nut meat is apparent. From these considerations it is possible to say that the Brazil nut meats were ground to the finest particle size, followed by the pecans and walnuts, all of which were found finer than to pass a 20 mesh screen. The almond meats were ground to the coarsest particle size but finer than to pass a 10 mesh screen.

A possible greater homogeneity of Brazil nuts was obtained by size reduction in a hammer mill (Fitzpatrick) and mixing in a planetary mixer (Hobart), but with any of the analytical procedures in current use the improvement is inconsequential, and selection of the tested equipment can be made upon considerations of economy or convenience.

The grinding and mixing procedure that had been used by the MQRD laboratories results in a better degree of sample homogeneity (Experiments 6 and 7, Table II) than would have been expected from the comparisons of particle size distribution, but of a magnitude with 50 g samples that could have a discernible effect if added to the analytical error. This conclusion is verified by the experiments with naturally contaminated peanuts (Experiments 16c and 17c, Table II) which will be examined in greater detail later in the discussion. The increase in error is evident even though in these latter experiments there is an apparent improvement in particle size reduction. The anomalous relationship between homogeneity and particle size as seen in Experiments 6 and 7 vs. 8, and 9 vs. 3 and 4 bear out the possibility that the particle size of the composite sample may not reflect what has happened to the few contaminated nuts.

The experiments with the Dickens hammer mill were designed to answer two questions: does the portion in the shunt stream represent the contamination in the total sample, and is the particle size reduction such that, given adequate mixing, the 50 g analytical samples are representative of the whole. The second question is better answered first. The data (Table II) show that, for peanuts at approximately the same level of radioactive contamination, the Dickens hammer mill produces samples with twice the variability of those produced by the Hobart food chopper (21^2 vs. 13^2) and that in the extreme dilution factor situation the sample error could exceed the analytical error. Within the wide limits set by the analytical sample variability, the shunt sample was clearly representative of the total sample. Further size reduction and mixing of the shunt sample would be expected to improve the sample homogeneity.

Since this experiment was carried out, and partially based on these results, the USDA, MQRD has recommended using the entire shunt sample for analysis.

The data from the experiments with the naturally contaminated nuts (16a, b, c; 17a, b, c; 18) cannot be interpreted quantitatively in terms of lot sample homogeneity. The experiments designed to determine the reproducibility of the analytical procedure yielded data with too much scatter to measure small differences in analytical sample variability. The pooled data from all but two of the experiments (16a and 17a, 50 g samples) have close to the same coefficients of variation (15.8 vs. 15.7) and the same range (7.9–21.0 vs. 7.9–22.7) as the analytical procedure. The two odd sets of data produced coefficients of variation (25 and 29%) markedly higher than the others. These data derive from sample preparation technique and analytical sample size that, judging from our previous data with radioactive nuts, would be expected to produce less homogeneous samples. The relative coefficients of variation of these two groups is even in the direction that would be expected from the relative contamination; the sample with the lesser contamination is the less homogeneous.

The data support the original premise that adequate size reduction and mixing are essential to the preparation of a uniform lot sample. The ultimate in uniformity for shelled peanuts can be achieved with an attrition mill, solvent addition to obtain a fluid system and mixing of the fluid with a dispersion mixer. A practical uniformity can be achieved in a single operation, requiring less time and effort by using a grinding and dispersing aid with shelled peanuts in a Hobart vertical cutter-mixer. This equipment is equally effective for in-shell nuts which provide their own grinding aids. This applies particularly to hard shell nuts such as Brazils, walnuts and pecans, and less so to almonds, which have a hard meat and soft shell. The size reduction and mixing procedure, as practiced by the MQRD, USDA, results in some observable degree of sample inhomogeneity. An assumption that all size reduction equipment is equally effective, if each produces the same particle size distribution in the discharge, is probably not warranted. A contaminated particle that is missed by the mill action is not affected by the fineness to which other particles may be reduced. In marginal situations, the type of mill employed could determine whether the sample is likely to be homogeneous, even if the grind appears to be adequate, e.g., burr mill vs. hammer mill vs. food choppers.

Attempts at extrapolation of these data and experience to other commodities should recognize both similarities and differences in physical state and composition.

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REFERENCES

1. Austwick, P. K. G., and G. Ayerst, Chem. Ind. (London) *1963*, 55–61.
2. Cucullu, A. F., L. S. Lee, R. Y. Mayne and L. A. Goldblatt, JAOCS *43*, 89–92 (1966).
3. Whitten, M. E., Cotton Gin and Oil Mill Press, 7–8, Dec. 17, 1966.
4. Campbell, A. D., and J. T. Funkhouser, J. Assoc. Offic. Anal. Chemists *49*, 730–739 (1966).
5. Assoc. Offic. Anal. Chemists, Ibid. *49*, 229–231 (1966).
6. Eppley, R. M., L. Stoloff and A. D. Campbell, Ibid. *51*, 67–73 (1968).
7. Assoc. Offic. Anal. Chemists, Ibid. *51*, 485–488 (1968).
8. Beckwith, A. C., and L. Stoloff, Ibid. *51*, 602–608 (1968).

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