Development of Aflatoxin Sampling Plans for Almonds, Hazelnuts, Pistachio, and Brazil Nuts

Technical information provided by the U.S. Delegation to members of the Electronic Working Group to help recommend aflatoxin sampling plan designs for treenuts to the full committee.

Background

The 36th Session of the Codex Committee on Food Additives and Contaminants (CCFAC) agreed to commence work on the development of sampling plans for aflatoxins in almonds, Brazil nuts, hazelnuts, and pistachios, subject to approval as new work by the Codex Alimentarius Commission.

An aflatoxin-sampling plan is defined by an aflatoxin test procedure and an accept/reject limit. The accept/reject limit is a threshold value that is usually equal to the maximum limit. The aflatoxin test procedure for treenuts consists of a sampling, sample preparation, and analytical steps. Because of the uncertainty associated with each step, the true aflatoxin concentration of a bulk lot can't be determined with 100% certainty. As a result, there is a chance that some lots with concentration greater than the maximum limit will be accepted by the sampling plan and some lots with concentration below the maximum limit will be rejected by the sampling plan. The performance (risk of misclassifications) of a sampling plan depends, in part, on the amount of uncertainty associated with each step of the aflatoxin test procedure.

The United States suggested that aflatoxin sampling plans recommendations for the four treenuts be based upon the measurement of uncertainty and distribution among sample test results for each treenut. From the uncertainty and distributional information, the performance of sampling plans can be estimated, which will help the electronic working group to design aflatoxin-sampling plans for each treenut.

The U.S., Turkey, Iran, and Brazil agreed to conduct sampling studies to determine the uncertainty and distribution among sample test results for almonds, hazelnuts, pistachios, and Brazil nuts, respectively. As of April 2007 (1st session of the Codex Committee on Contaminants in Foods, CCCF), sampling data has been developed for almonds, hazelnuts, and pistachios. Brazil anticipates that sampling data for Brazil nuts will be available for statistical analysis in October 2007.

From the uncertainty and distributional data, the chances of accepting (or rejecting) a lot at a given aflatoxin concentration can be predicted for a specific sampling plan design (sample size, sample preparation method, analytical method, and maximum limit). The performance of sampling plan designs is described by operating characteristic (OC) curves.

Sampling plan design considerations

The design of an aflatoxin-sampling plan is a compromise between costs of the sampling plan and the benefits of minimizing the risks of misclassifying of lots and effectively removing contaminated lots from the market. The previous discussions by the CCFAC sampling-working group considered the following practical aspects:

1. Two separate maximum limits and two separate sampling plans need to be established, one for raw shelled treenuts destined for further processing and one for consumer-ready (also called ready-to-eat) shelled treenuts.
2. Maximum limits need to be defined for raw treenuts destined for further processing and consumer-ready treenuts before a final decision can be made about a sampling plan design.
3. Codex established a 20 kg sample size and a 15 ng/g total aflatoxin maximum limit for raw shelled peanuts destined for further processing.
4. Should the sampling statistics be based upon in-shell nuts or shelled kernels. Either can be used knowing the hull-kernel mass ratio?
5. Treenuts are more expensive than peanuts making the cost of equivalent sampling plans more expensive.
6. There appears to be more uncertainty associated with the sampling step for treenuts than for peanuts requiring larger samples for treenuts to get an equivalent level of performance to peanuts.
7. Can the same sampling plan design be used for two or more type of treenuts? This will depend upon the similarity of uncertainty data for the four treenuts.

Results of Sampling Studies

Uncertainty - The aflatoxin test procedure used for each treenut study along with the sampling, sample preparation, and analytical variance equations are shown in Annex I, Table 1. Where possible, the experimental design was similar (Brazil nut studies have not yet started). Generally, the uncertainty was measured using a 10 kg samples, dry grinding the sample with vertical cutter type mills (VCM), selection of a 25 to 100 g subsample from the comminuted sample, and HPLC analytical methods to quantify the aflatoxin in the comminuted subsample. Almond and hazelnut studies used 10 kg samples of shelled kernels while the pistachio study used 10 kg of inshell nuts, which is equivalent to 5 kg of shelled kernels. The count per unit mass for shelled almonds, hazelnuts, and pistachios is 773, 1000, and 1600 kernels per 1 kg, respectively.

The sampling, sample preparation, and analytical variances were found to be a function of aflatoxin concentrations (C). Variance equations were developed for each treenut showing the effect of aflatoxin concentration and quantity inspected on the magnitude of each variance.

Using the variance equations in Table 1, the sampling, sample preparation, and analytical variance associated with the specific test procedure are shown in Tables 2 and 3 for each treenut when sampling a lot at 8 and 15 ng/g total aflatoxin. Tables 2 and 3 show that the sampling step contributes most of the uncertainty associated with the test procedure. The effect of uncertainty associated with sampling, sample preparation, and analytical steps of the aflatoxin test procedure for all three treenuts are approximately the same magnitude.

Effect of type treenut on the performance of aflatoxin sampling plan design - Because the variance equations associated with the sampling, sample preparation, and analytical steps are slightly different for each treenut (Table 1, Annex I), the performance or OC curves for the same sampling plan design will be slightly different for each of the three treenuts. As an example, three OC curves for the same sampling plan that uses a 20 kg sample, dry grind, 50 g subsample, HPLC analytical method, and 8 ng/g maximum limit is shown in Annex II, Figure 1 using uncertainty data measured for almonds, hazelnuts, and pistachios. Figure 2 shows three OC curves for the same sampling plan design, but for a maximum limit of 15 ng/g total aflatoxin.

The OC curves for hazelnuts and pistachios (either maximum limit) are similar, while the OC curve for almonds is slightly different. The OC curve for almonds reflects more uncertainty in the aflatoxin test procedure than for hazelnuts and pistachios (Table 1, Annex I). Because there appears to be slightly more uncertainty with the aflatoxin test procedure used for almonds, the uncertainty associated with sampling almonds for aflatoxin could be used to predict the performance of aflatoxin sampling plans for all three treenuts. Performance estimates for pistachio and hazelnut sampling plans would be slightly more conservative.

<u>Effects of Sample Size on the Performance of Sampling Plans </u>- To help the electronic working group recommend a sampling plan for treenuts, operating characteristic curves were developed for the following design parameters:

1. Using uncertainty equations for almonds in Table 1, Annex I for all three treenuts;
2. Sampling plans were developed for raw shelled treenuts destined for further processing and for consumer-ready shelled treenuts;
3. Maximum limits of 15 ng/g total aflatoxin for raw shelled treenuts destined for further processing and 8 ng/g total aflatoxin for consumer-ready shelled treenuts;
4. Three sample sizes of 10, 20, and 30 kg;
5. Because the uncertainty of the sampling step accounts for a major portion of the total uncertainty associated the overall aflatoxin test procedure, the following sample preparation and analytical parameters were considered: dry grind, 50 g subsample, and use of HPLC to quantify aflatoxin in one aliquot taken from the subsample/solvent blend.

An OC curve describes the performance or the chances of accepting lots at a given aflatoxin concentration using a specific sampling plan design. Three OC curves representing the performance of sampling plans that use 10, 20, and 30 kg samples of raw shelled treenuts destined for further processing (maximum limit of 15 ng/g total aflatoxin) are shown in Figure 1, Annex III. Three OC curves representing the performance of sampling plans that use 10, 20, and 30 kg samples of consumer-ready shelled treenuts (maximum limit of 8 ng/g total aflatoxin) are shown in Figure 2, Annex III.

The relative effect of sample size on the OC curve (or the performance of a sampling plan design) is the same for both maximum limits of 8 and 15 ng/g. As sample size (ns) increases, the variance (Table 1, Annex I) decreases and fewer lots are misclassified by the sampling plan. In Figure 1, Annex III, as sample size increases, the slope of the OC curve increases. As a result, more good lots (lot <=15 ng/g maximum limit) are accepted and fewer bad lots (lot <= 15 ng/g maximum limit) are accepted by the sampling plan as sample size increases. Increasing sample size has the same effect for consumer-ready shelled treenuts.

Effect of maximum limit on the performance of aflatoxin sampling plans – The effect of using a 4, 8, 10, and 15 ng/g maximum limit on the performance of sampling plans that use either a 10, 20, or 30 kg sample are shown in Annex IV, Figures 1, 2, and 3, respectively. For a given sample size, as maximum limit decreases from 15 to 4 ng/g, the OC curves shift to the left. The results of reducing the maximum limit are both good and bad. As the maximum limit decreases, fewer lots at low concentrations are accepted and fewer lots at high concentrations are accepted. For a given sample size, as the maximum limit decreases, fewer lots are accepted (more lots are rejected), but overall aflatoxin in the accepted lots decreases. Choosing a maximum limit is a balance between not interrupting international trade (rejecting too many lots) and removing contaminated lots to protect public health.

Conclusions

Sampling studies have been completed for almonds, hazelnuts, and pistachios. Several sampling plan designs were evaluated to demonstrate the effect of type treenut, sample size, and maximum limit on the risk of misclassifying lots. As part of the electronic working group, the U.S. delegation can evaluate other sampling plan designs for the electronic working group as maximum limits and sample sizes are discussed.

Acknowledgement

The United States delegation wishes to thank and acknowledge parties in the United States, Turkey, and Iran for conducting the sampling studies and providing the sampling data and statistical analyses so the United States could evaluate the performance of sampling plans for almonds, hazelnuts, and pistachios, respectively, for the CCCF Electronic Working Group.

ANNEX I. Uncertainty associated with sampling, sample preparation, and analytical steps of the aflatoxin test procedure used to estimate aflatoxin in almonds, hazelnuts, and pistachios.

Table 1. Experimental test conditions and uncertainty results for each treenut.

Test Procedure	Almonds	Hazelnuts	Pistachios
Sampling	$S^2_s = (10/ns)5.759C^{1.561}$	$S^2_s = (10/ns)4.291C^{1.609}$	$S^2_s = (5/ns)7.913C^{1.475}$
Sample Prep	$S^2_{sp} = (100/nss)0.170C^{1.646}$	$S^2_{sp} = (50/nss)0.021C^{1.545}$	$S^2_{sp} = (25/nss)2.334C^{1.522}$
Analytical	$S^2_a = (1/na)0.0041C^{1.966}$	$S^2_a = (1/na)0.0028C^{1.990}$	$S^2_a = (1/na)0.0368C^{1.598}$
Sample Product	shelled kernels	shelled kernels	In-shell (5 kg shelled kernels)
Sample size ns kg	10	10	10 kg inshell/ 5kg shelled
Sample Prep (mill)	Hobart (dry grind)	Robot Coupe (dry grind)	Marjaan Khatam (dry grind)
Subsample size nss g	100	50	25
Analytical method	HPLC (na = 1 aliquot)	HPLC (na = 1 aliquot)	HPLC (na = 1 aliquot)
Total variance	$S^2_s + S^2_{sp} + S^2_a$	$S^2_s + S^2_{sp} + S^2_a$	$S^2_s + S^2_{sp} + S^2_a$

Note: All sampling variances reflect shelled kernels. Pistachio sampling study was conducted on 10 kg of in-shell nuts. Hull represents about 50% of the total inshell mass. Sampling data for almonds, hazelnuts, and pistachios supplied by the United States, Turkey, and Iran, respectively. S^2 = variance, ns = sample size in kg, nss = subsample size in g, na = number of aliquots quantified, and C = aflatoxin concentration (ng/g).

Table 2. Uncertainty associated with the aflatoxin test procedure to estimate aflatoxin in bulk lots of almonds, hazelnuts, and pistachios at 8 total ng/g using equations in Table 1.

Test Procedure	Size	Variance			Coeff. of Variation (%)			Variance Ratio (Component/Total)		
		Almonds	Hazelnuts	Pistachios	Almonds	Hazelnuts	Pistachios	Almonds	Hazelnuts	Pistachios
Sample (kg)	10	147.93	121.80	84.99	152.04	137.95	115.24	93.27	99.43	74.78
Sample Prep (g)	50	10.42	0.52	27.64	40.35	9.03	65.72	6.57	0.43	24.32
Analysis HPLC	1	0.24	0.18	1.02	6.18	5.24	12.63	0.15	0.14	0.90
Total		158.60	122.49	113.65	157.42	138.35	133.26	100.00	100.00	100.00

Table 3. Uncertainty associated with the aflatoxin test procedure to estimate aflatoxin in bulk lots of almonds, hazelnuts, and pistachios at 15 total ng/g.

Test Procedure	Size	Variance			Coeff. of Variation (%)			Variance Ratio (Component/Total)		
		Almonds	Hazelnuts	Pistachios	Almonds	Hazelnuts	Pistachios	Almonds	Hazelnuts	Pistachios
Sample (kg)	10	394.66	334.88	214.81	132.44	122.00	97.71	92.90	99.41	74.19
Sample Prep (g)	50	29.33	1.38	71.96	36.11	7.83	56.55	6.90	0.41	24.85
Analysis HPLC	1	0.84	0.61	2.79	6.12	5.22	11.13	0.20	0.18	0.96
Total		424.83	336.87	289.55	137.41	122.36	113.44	100.00	100.00	100.00

Size = 1 for analysis indicates that 1 aliquot was quantified by HPLC

ANNEX II. Comparing performance of sampling plans using almond, hazelnut, and pistachio sampling data.

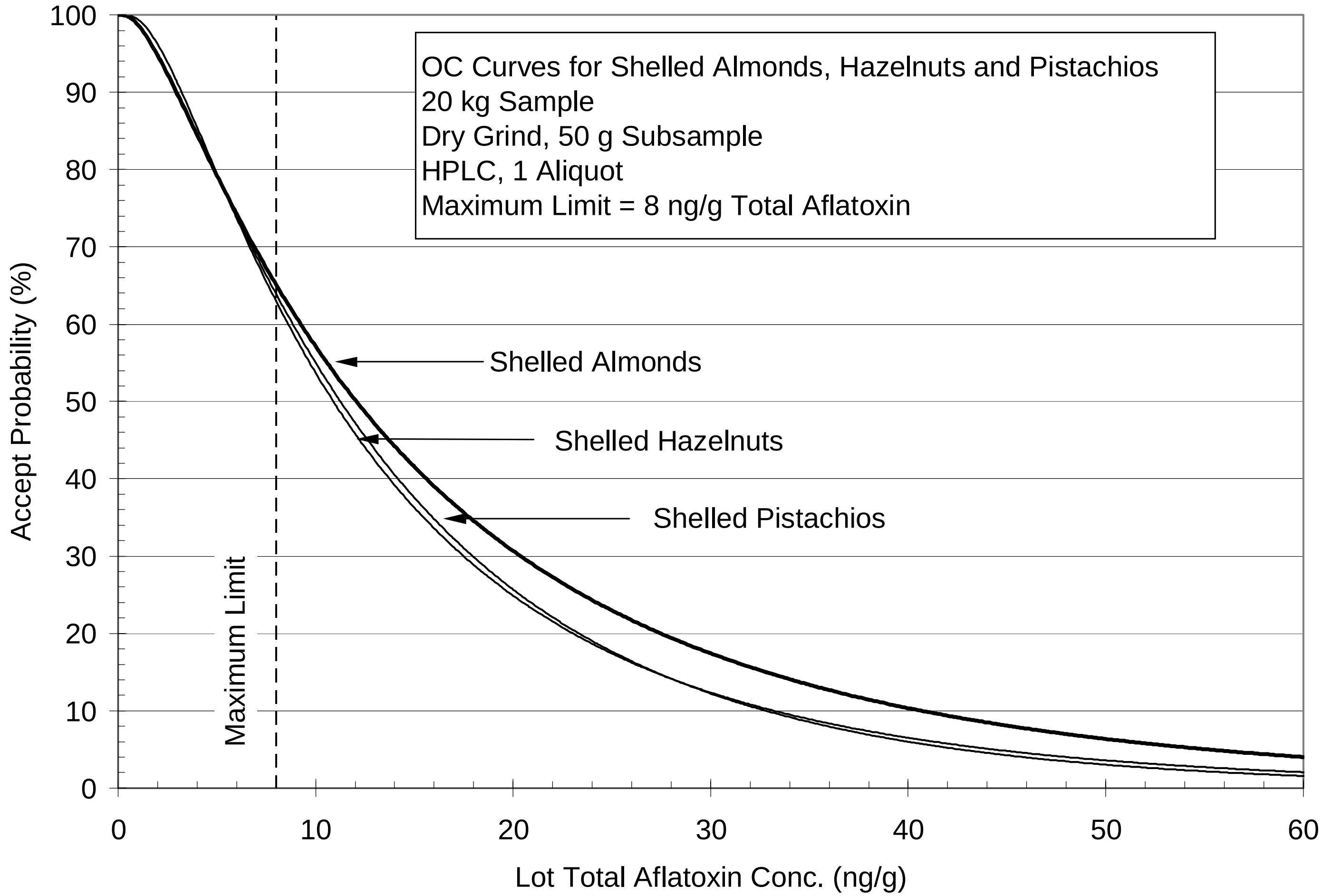

Figure 1. Operating characteristic curves based upon consumer-ready almond, hazelnut, and pistachio uncertainty data for a 20 kg sample, dry grinding, 50 g subsample, using HPLC to quantify aflatoxin in 1 aliquot, and 8 ng/g maximum limit.

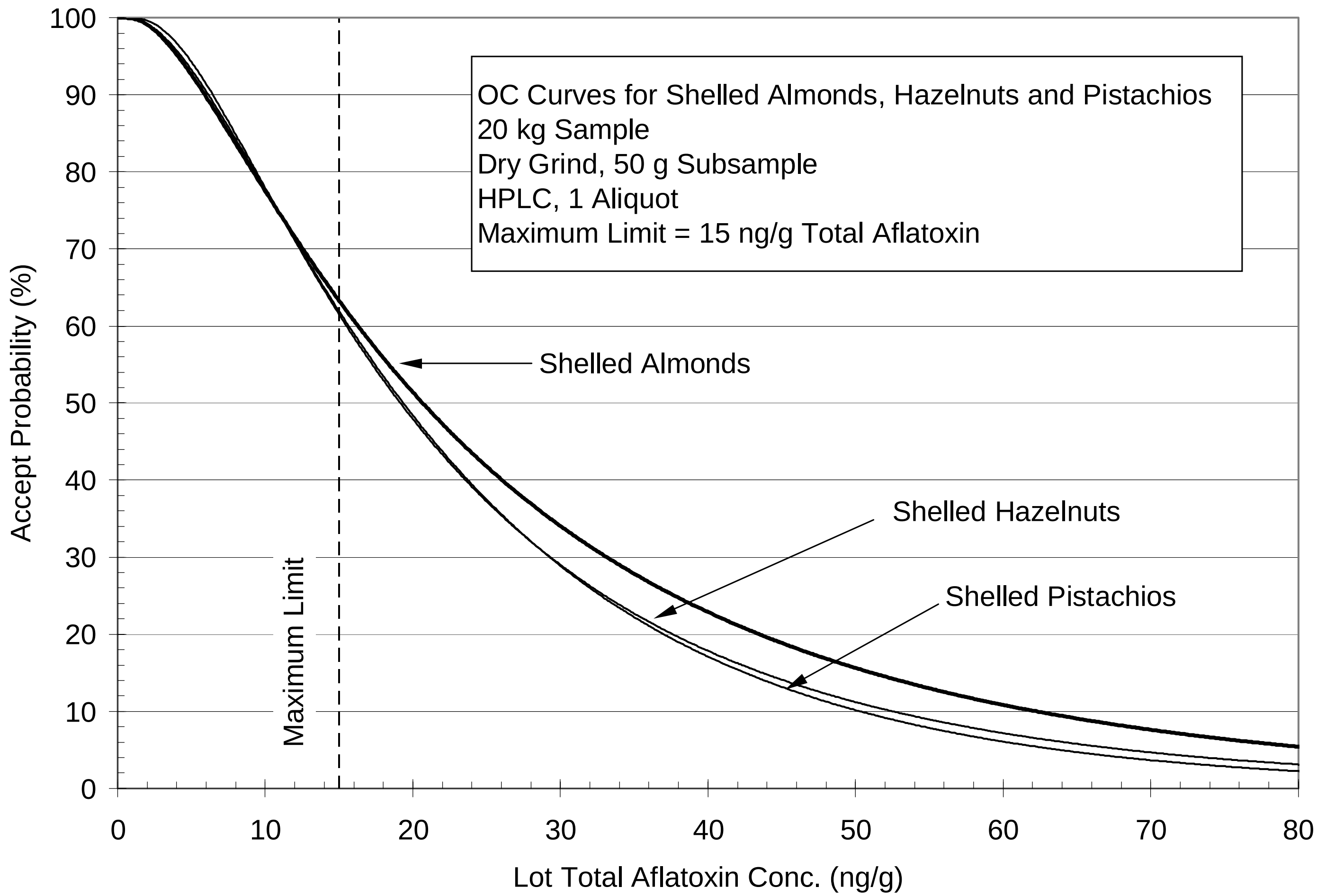

Figure 2. Operating characteristic curves based upon raw shelled almond, hazelnut, and pistachio uncertainty data for a 20 kg sample, dry grinding, 50 g subsample, using HPLC to quantify aflatoxin in 1 aliquot, and 15 ng/g maximum limit.

ANNEX III. Effect of sample size on performance of aflatoxin sampling plans for three treenuts (almonds, hazelnuts, and pistachios).

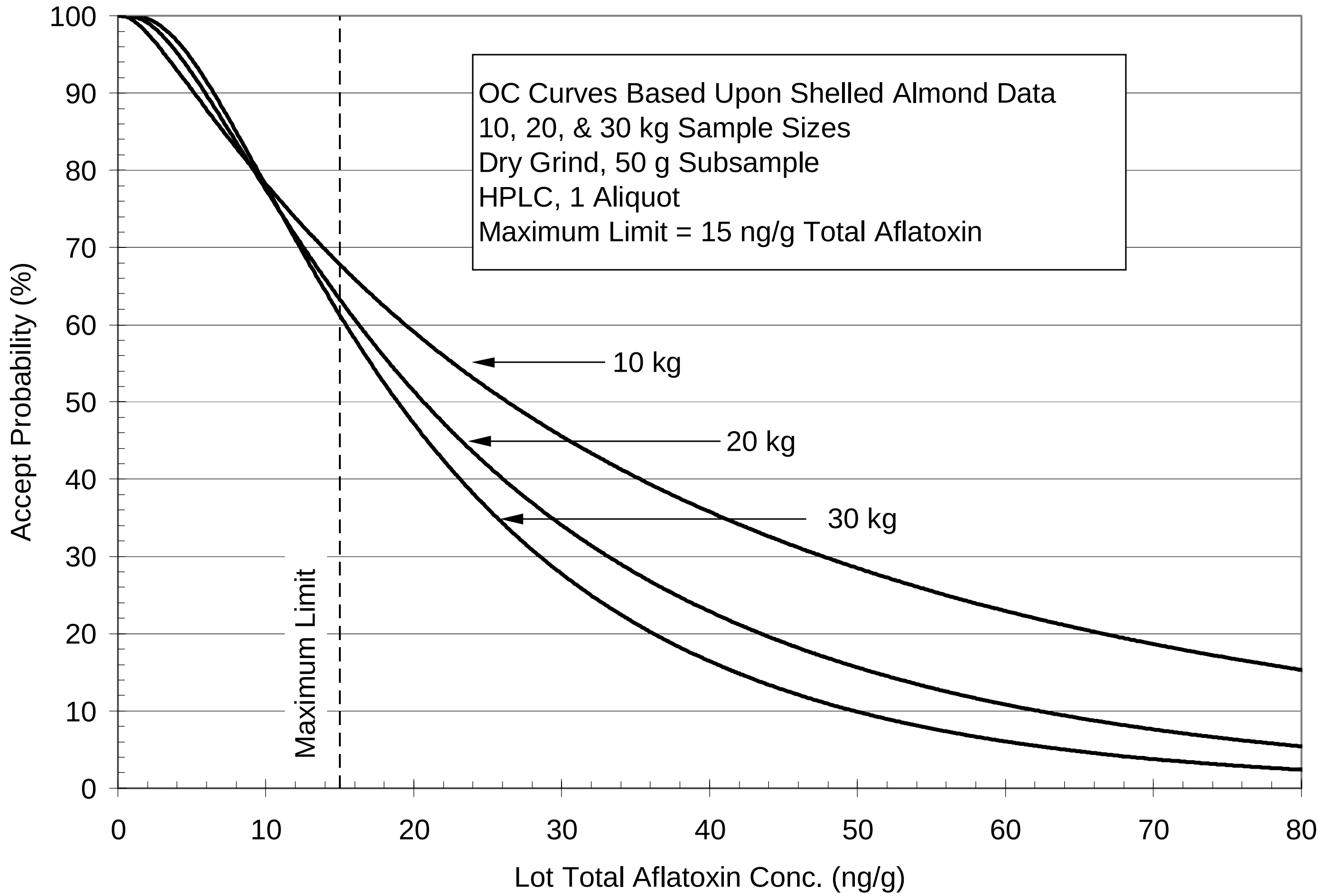

Figure 1. Three OC curves showing the effect of sample size on reducing the risk of misclassifying raw shelled treenut lots destined for further processing. The maximum limit for all three sampling plans is 15 ng/g total aflatoxin.

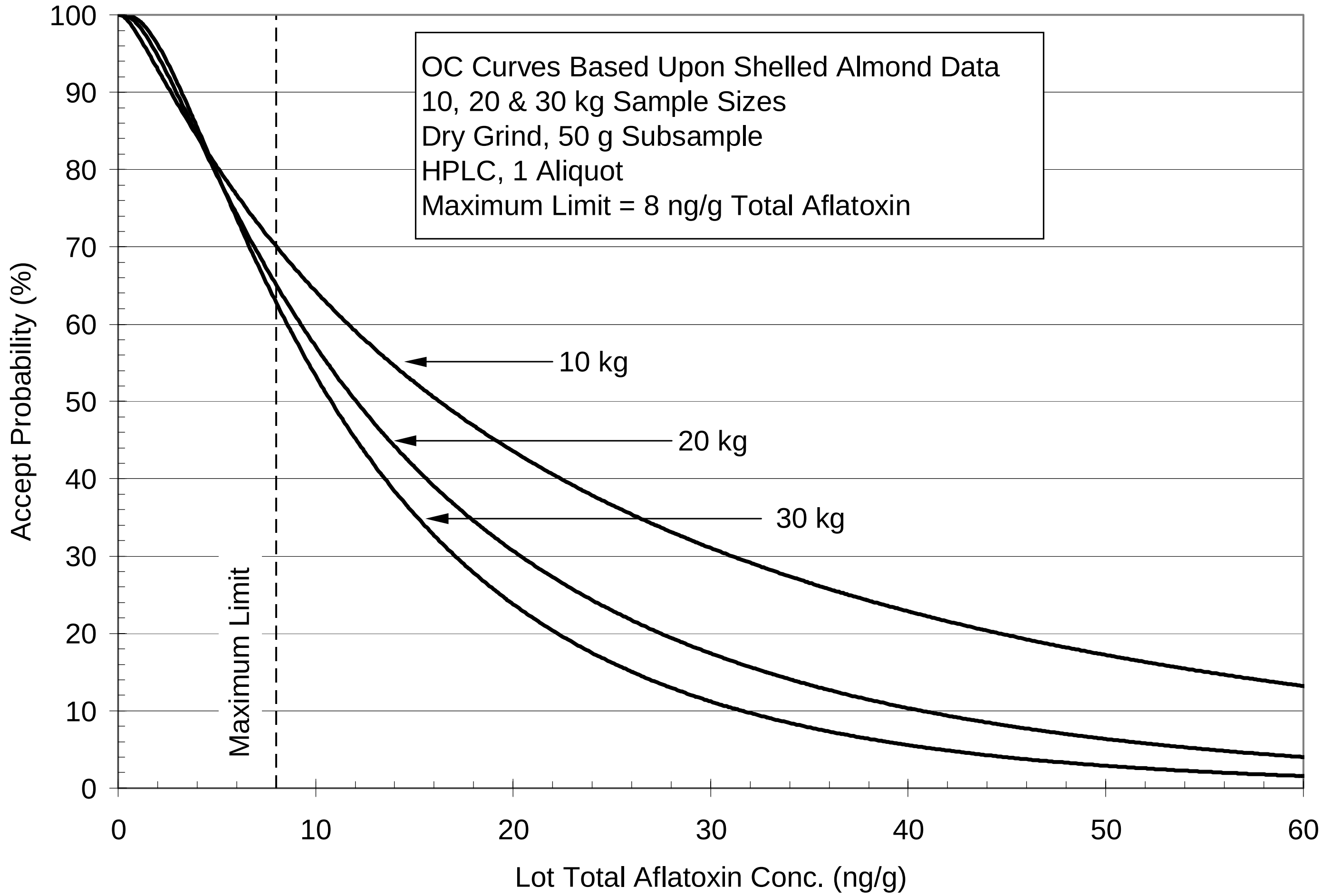

Figure 2. Three OC curves showing the effect of sample size on reducing the risk of misclassifying shelled consumer-ready treenut lots. The maximum limit for all three sampling plans is 8 ng/g total aflatoxin.

Annex IV. Effect of maximum limit on the performance of aflatoxin sampling plans for treenuts.

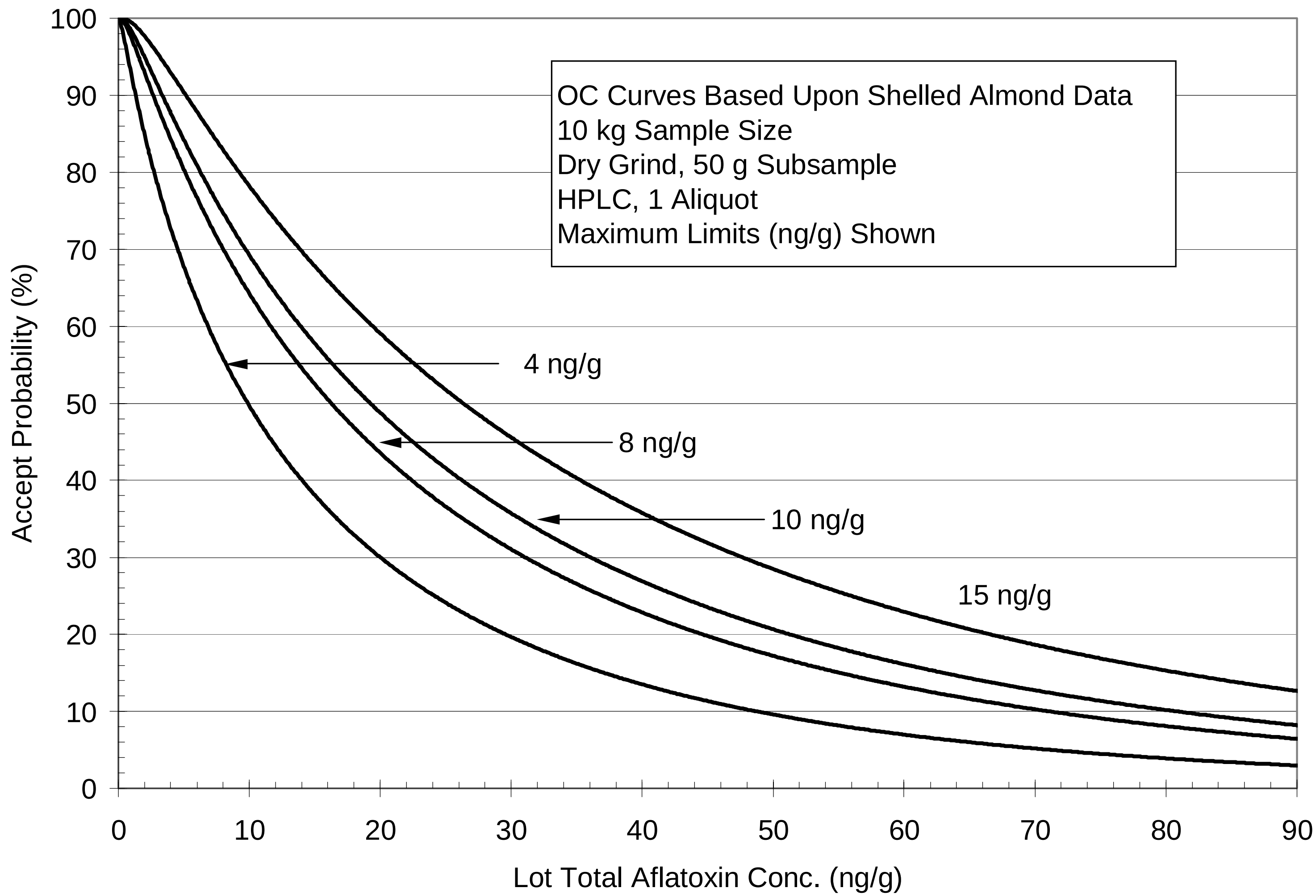

Figure 1. Performance of four-aflatoxin sampling plans for treenuts using 4, 8, 10, and 15 ng/g maximum limits. All sampling plans use a 10 kg sample.

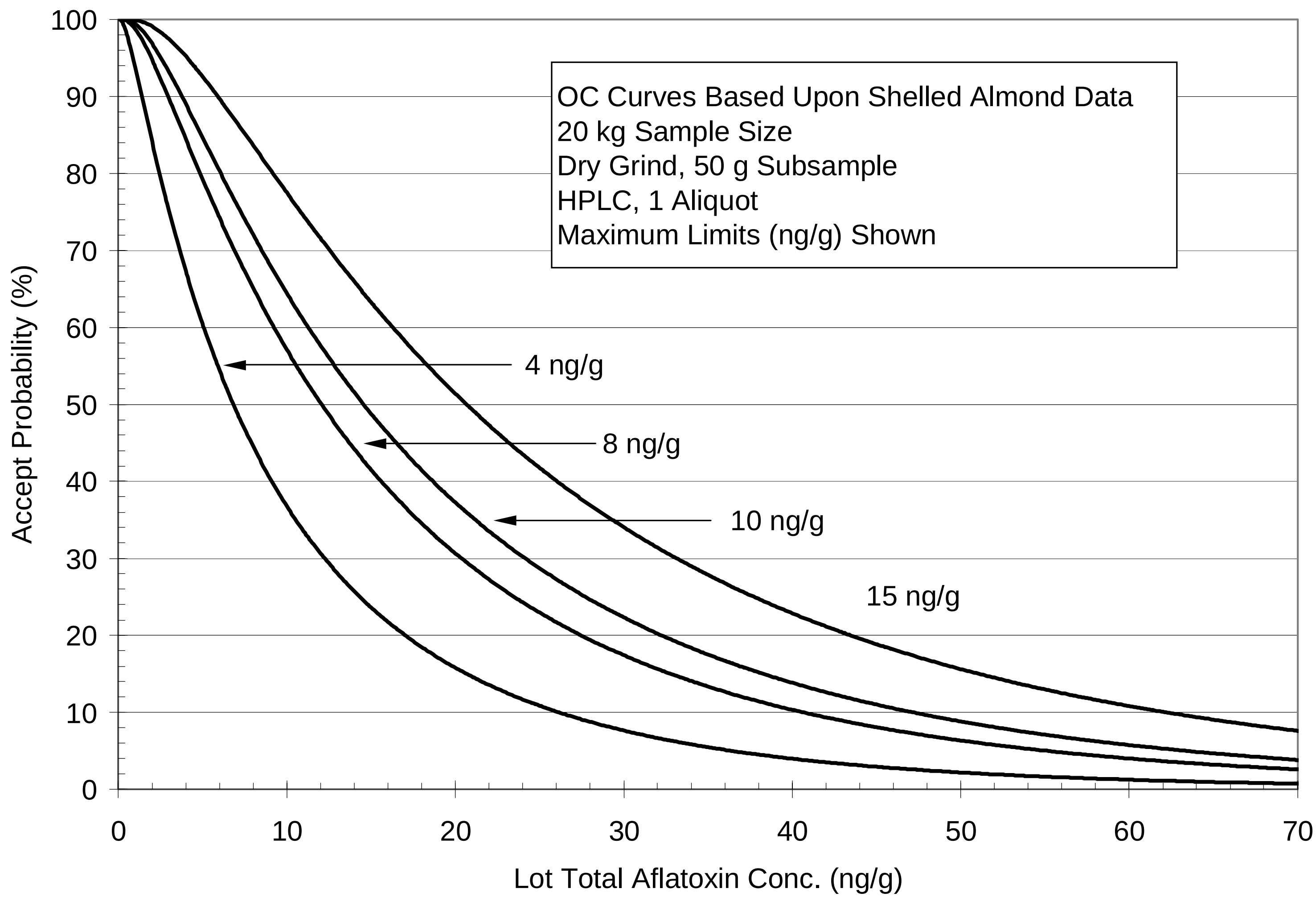

Figure 2. Performance of four-aflatoxin sampling plans for treenuts using 4, 8, 10, and 15 ng/g maximum limits. All sampling plans use a 20 kg sample.

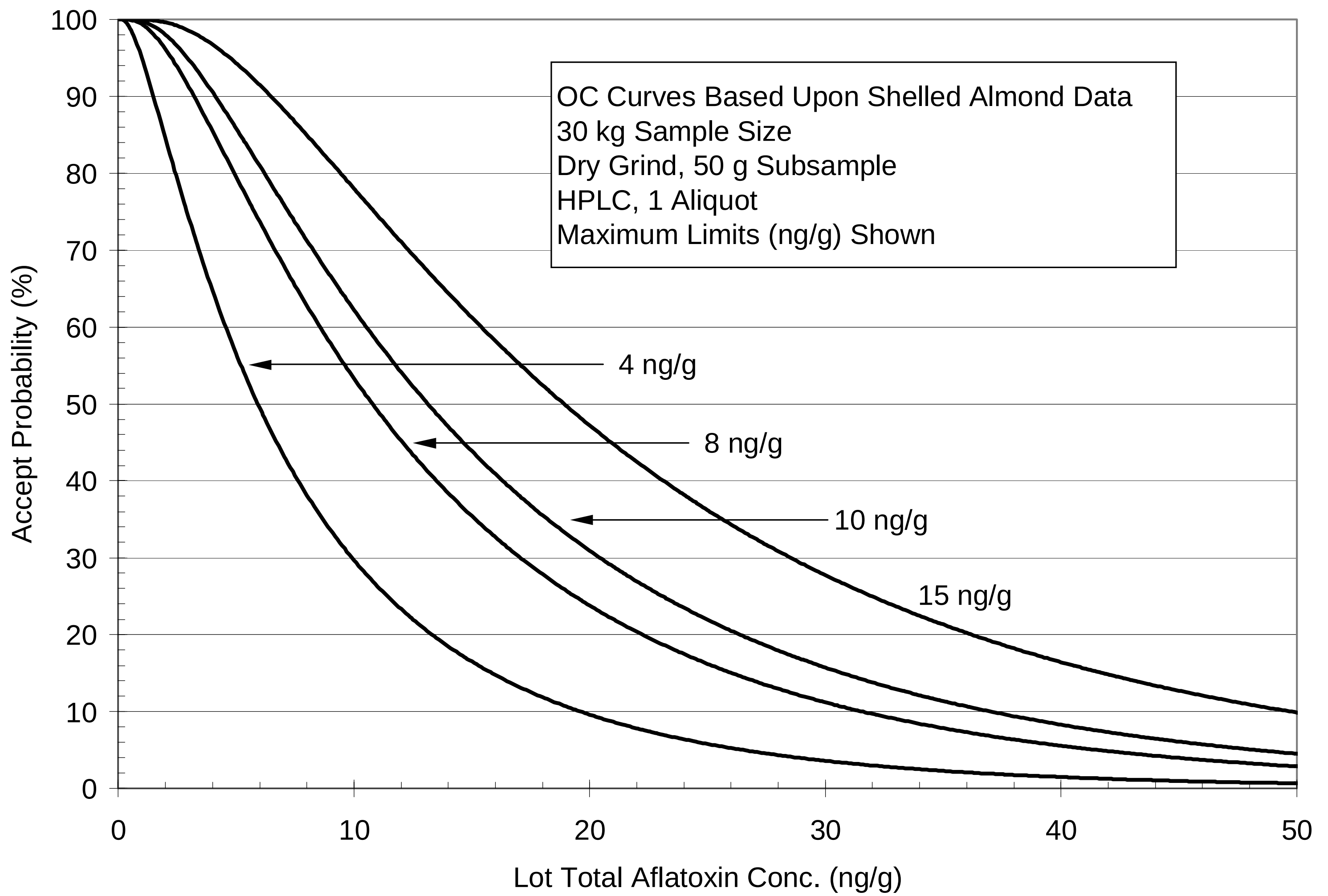

Figure 3. Performance of four-aflatoxin sampling plans for treenuts using 4, 8, 10, and 15 ng/g maximum limits. All sampling plans use a 30 kg sample.