

codex alimentarius commission



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Agenda Item 11(c)

CX/CF 08/2/10
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**JOINT FAO/WHO FOOD STANDARDS PROGRAMME
CODEX COMMITTEE ON CONTAMINANTS IN FOODS
Second Session**

The Hague, the Netherlands, 31 March - 4 April 2008

**PROPOSED DRAFT SAMPLING PLANS FOR AFLATOXIN CONTAMINATION IN ALMONDS,
BRAZIL NUTS, HAZELNUTS AND PISTACHIOS (N07-2004)**

(At Step 3 of the Elaboration Procedure)

Governments and international organizations are invited to submit comments on the following subject matters no later than 14 March 2008, preferably in electronic format, for the attention of Ms. Tanja Åkesson, the Netherlands Secretariat of the Codex Committee on Contaminants in Foods, Fax No.:+31 70 3786141; E-mail:info@codexalimentarius.nl with a copy to the Secretary, Codex Alimentarius Commission, Joint FAO/WHO Food Standards Programme, Viale delle Terme di Caracalla, 00153 Rome, Italy (Fax +39.06.5705.4593; E-mail: mailto:Codex@fao.org).

Background

1. 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. The Committee also agreed that a working group led by the United States with the assistance of Argentina, Brazil, Iran, EC and the INC would prepare the sampling plans.¹ The 27th Session of the Codex Alimentarius Commission approved the elaboration of proposed draft Sampling Plans for Aflatoxin Contamination in Almonds, Brazil Nuts, Hazelnuts and Pistachios as new work for the Committee (N07-2004).²
2. The CCFAC, at the 37th Session, considered a Proposed Draft Sampling Plan for Aflatoxin Contamination in Almonds, Brazil Nuts, Hazelnuts and Pistachio and noted that data for hazelnuts had been submitted and that additional work on the sampling plan depended upon a maximum level and additional information on aflatoxin distribution in pistachios and Brazil nuts (inshell/shelled).³
3. The Committee returned the Proposed Draft Sampling Plan to Step 2 and agreed to request an electronic working group, led by the United States, to revise the document on the basis of new information becoming available in the future, for circulation and comments at Step 3, and consideration at the next Session.⁴

¹ ALINORM 04/27/12 .para.149.

² ALINORM 04/27/41, para. 88 and Appendix VI.

³ ALINORM 05/28/12, para. 143.

⁴ ALINORM 05/28/12, para. 144.

4. The CCFAC, at the 38th Session, considered a revised Sampling Plan based on data for almonds and hazelnuts using a single 20 kg test sample with an accept/reject limit of 15 ng/g. The Committee agreed to further elaborate the Proposed Draft Sampling Plan once a maximum limit had been established. The Committee agreed to retain the Proposed Draft Sampling Plan at Step 4 awaiting the outcome of the discussion paper on Maximum Levels of aflatoxins in tree nuts.⁵
5. The Codex Committee on Contaminants in Foods (CCCF), at its First Session, discuss the matter and agreed that the Proposed Draft Sampling Plan for Aflatoxin Contamination in Almonds, Brazil Nuts, Hazelnuts and Pistachios be returned to Step 2 for redrafting by an electronic Working Group, led by the United States, with a view to circulate at Step 3 and consideration at Step 4 at the next Session of the Committee. The Committee also agreed that the working document to be considered at its next session should incorporate a revised proposed draft Sampling Plan as well as an explanatory text in support of the consideration of the proposed draft Sampling Plan.⁶
6. This document contains the report of the electronic working group (paragraphs 7 - 15), recommendation (paragraph 16) and the proposed draft sampling plan (Appendix I). A list of the participants to the electronic working group is attached as Appendix II.

Report of the electronic working group

7. Because aflatoxins are considered to be genotoxic and carcinogenic towards humans, it is not possible to identify an intake without risk. About 100 countries have established maximum limits for aflatoxin in food and feeds⁷. The limits are applied at the point of import, and/or at market/retail level. Bulk lots are sampled for aflatoxin to determine if the lot concentration is above or below the maximum limit. If the sample concentration is less than or equal to the maximum limit, the lot is accepted into the trade. Otherwise the lot is rejected and subjected to further processing before it can move into trade.
8. It is difficult to get an accurate estimate of the true lot concentration due to the extreme distribution among contaminated particles in the lot. Research has demonstrated that a small percentage of the particles in the lot are contaminated and the level of contamination on individual particles can vary from low levels to extremely high concentrations⁸. It is important to be able to detect isolated 'hot spots' of contamination, and it is therefore necessary to take a large number of small incremental samples at various places distributed throughout the consignment in order to obtain a representative sample.
9. 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 concentrations greater than the maximum limit will be accepted by the sampling plan and some lots with concentrations below the maximum limit will be rejected by the sampling plan. The performance (risks of misclassifying lots) of a sampling plan depends, in part, on the amount of uncertainty associated with each step of the aflatoxin test procedure and can be described by an operating characteristic curve.
10. The sampling electronic working group recommends that aflatoxin-sampling plans for the four treenuts, almonds, hazelnuts, pistachios, and Brazil nuts, 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.
11. 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. Sampling data have been developed for almonds, hazelnuts, and pistachios. Sampling data for Brazil nuts will be available for statistical analysis at a later date.

⁵ ALINORM 06/29/12, paras 124-126 and para.129.

⁶ ALINORM 07/30/41, para. 62.

⁷ FAO Food and Nutrition Paper 81, 2003

⁸ Cucullu, A., Lee, L., Mayne, R., and Goldblatt, L. 1966. Determination of aflatoxins in individual peanuts and peanut sections. J. American Oil Chemists Society, 43:89-92.

12. 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 (laboratory sample size, sample preparation method (particle size and test portion size), analytical method, and maximum limit). The performance of a sampling plan design is described by an operating characteristic (OC) curve.
13. Results of the sampling studies conducted so far, along with examples of the effect of various laboratory sample sizes, number of laboratory samples, and maximum limits on the performance of aflatoxin sampling plans for each treenut can be found at the following website: <http://www5.bae.ncsu.edu/usda/www/ResearchActDocs/treenutwg.html>
14. An aflatoxin-sampling plan must ensure consumer safety by effectively removing contaminated lots from the market. However, this must be balanced against costs related to restricting trade. There are benefits to minimizing the risks of misclassifying lots to both consumer safety and trade. The operating characteristic curves shown on the website are intended to help the sampling electronic working group define a sampling plan design that will provide a balance between minimizing trade restrictions and maximizing consumer protection.
15. The definitions of several critical terms used in the proposed draft-sampling plan are shown in ANNEX I.

Recommendation of the Electronic Working Group

16. Recommendation of the Electronic Working Group is as follows:
 - The Electronic Working Group has proposed an aflatoxin-sampling plan for ready-to-eat almonds, hazelnuts, and pistachios and these three treenuts destined for further processing. Since a maximum limit has not yet been defined, the sampling plans were designed assuming maximum limits of 8 and 15 ng/g total aflatoxin for RTE and DFP treenuts, respectively. These two maximum limits reflect current discussions in CCCF. Operating characteristic curves were computed to describe the performance of both sampling plans. Sample selection, sample preparation, and analytical methods were suggested to assist CCCF members implement the two aflatoxin sampling plans. Sampling plans will be developed for Brazil nuts when sampling studies are concluded.
 - Consequently, the Electronic Working Group agreed to propose amendments to the document title as follows: “Proposed draft aflatoxin sampling plans for aflatoxin contamination in ready-to-eat treenuts and treenuts destined for further processing: almonds, hazelnuts, pistachios and brazil nuts.
 - The Working Group notes that the First Session of the Committee on Contaminants in Foods agreed that the Proposed Draft Sampling Plan for Aflatoxin Contamination should cover almonds, hazelnuts, pistachios, and Brazil nuts. At the moment, uncertainty and distribution sampling studies on Brazil nuts are underway and the data will be included in this document when available. In addition, the working group notes that while maximum limits of 8 and 15 ng/g for ready-to-eat and destined for further processing almonds, hazelnuts and pistachios, respectively, were used for purposes of designing the proposed draft sampling plan, the final sampling plan will depend on the final maximum limits selected as a given sampling plan is closely linked to maximum limit.

PROPOSED DRAFT AFLATOXIN SAMPLING PLANS FOR AFLATOXIN CONTAMINATION IN READY-TO-EAT TREENUTS AND TREENUTS DESTINED FOR FURTHER PROCESSING: ALMONDS, HAZELNUTS, PISTACHIOS AND BRAZIL NUTS”**i. Sampling Plan Design Considerations**

1. Importers may commercially classify treenuts as either “ready-to-eat” (RTE) or “destined for further processing” (DFP). As a result, maximum limits and sampling plans are proposed for both commercial types of treenuts. Maximum limits need to be defined for treenuts destined for further processing and ready-to-eat treenuts before a final decision can be made about a sampling plan design.
2. Treenuts can be marketed either as inshell or shelled nuts. For example, pistachios are predominately marketed as inshell nuts while almonds are predominately marketed as shelled nuts. If a laboratory sample of inshell nuts is selected from the lot, the aggregate and laboratory sample sizes should reflect the increase in mass of the shell. Information about the hull-kernel mass ratio for each treenut and treenut variety is required.
3. Sampling statistics, shown in ANNEX II, are based upon the uncertainty and aflatoxin distribution among laboratory samples of shelled nuts. Because the shelled nut count per kg is different for each of the three treenuts, the laboratory sample size is expressed in number of nuts for statistical purposes. However, the shelled nut count per kg for each treenut, shown in ANNEX II, can be used to convert laboratory sample size from number of nuts to mass and vice versa.
4. Uncertainty estimates associated with sampling, sample preparation, and analysis, shown in ANNEX II, and the negative binomial distribution⁹ are used to calculate operating characteristic (OC) curves that describe the performance of the proposed aflatoxin-sampling plans (ANNEX III). Brazil nuts will be evaluated once studies have been completed.
5. In ANNEX II, the analytical variance reflects a reproducibility relative standard deviation of 22%, which is suggested by Thompson and is based upon Food Analysis Performance Assessment Scheme (FAPAS) data¹⁰. A relative standard deviation of 22% is considered by FAPAS as an appropriate measure of the best agreement that can be reliably obtained between laboratories. An analytical uncertainty of 22% is larger than the within laboratory variation measured in the sampling studies for the three treenuts. The within laboratory analytical uncertainty for each treenut can be found at the website <http://www5.bae.ncsu.edu/usda/www/ResearchActDocs/treenutwg.html>.
6. The issue of correcting the analytical test result for recovery is not addressed in this document. However, Table 2 specifies several performance criteria for analytical methods including suggestions for the range of acceptable recovery rates.

ii. Aflatoxin test procedure and maximum limits

7. An aflatoxin-sampling plan is defined by an aflatoxin test procedure and a maximum limit. A value for the proposed maximum limit and the aflatoxin test procedure are given below in this section.
8. The maximum limits for treenuts (almonds, hazelnuts, and pistachios) “destined for further processing” and “ready-to-eat” are still under consideration by the Committee. For purposes of the draft-sampling plan, maximum limits of 8 and 15 ng/g total aflatoxin for RTE and DFP treenuts, respectively, are proposed because these limits are currently under discussion for almonds, hazel nuts and pistachios. The maximum level for Brazil nuts will be included after its definition by CCCF.
9. If CCCF agrees on maximum limits other than those used in this discussion paper, the sampling plan designs will be revised in accordance with the new maximum limits and operating characteristic curves in ANNEX III will be replaced with OC curves representing the new maximum limits.

⁹ Whitaker, T., Dickens, J., Monroe, R., and Wiser, E. 1972. Comparison of the negative binomial distribution of aflatoxin in shelled peanuts to the negative binomial distribution. J. American Oil Chemists’ Society, 49:590-593.

¹⁰ Thompson, M. 2000. Recent trends in inter-laboratory precision at ppb and sub-ppb concentrations in relation to fitness for purpose criteria in proficiency testing. J. Royal Society of Chemistry, 125:385-386.

10. Choice of the number and size of the laboratory sample is a compromise between minimizing risks (false positives and false negatives) and costs related to sampling and restricting trade. For simplicity, it is recommended that the proposed aflatoxin sampling use a single 20 kg laboratory sample for all three treenuts. Because pistachios are predominately marketed inshell and have the highest shelled nut count per kg (ANNEX II), it is recommended that the pistachio laboratory sample reflect 20 kg of inshell nuts while the almond and hazelnut laboratory samples reflect 20 kg of shelled nuts. Use of a single 20 kg laboratory sample represents a compromise between high cost of treenuts, shares risks between the exporter (false positive) and importer (false negative) (ANNEX IV), can be easily handled by port personnel, and is consistent with the laboratory sample size used by Codex aflatoxin sampling plan for groundnuts destined for further processing.
11. The two sampling plans (RTE and DFP) have been designed for enforcement and controls concerning total aflatoxins in bulk consignments (lots) of treenuts traded in the export market.

Treenuts destined for further processing

Maximum limit – 15 ng/g total aflatoxin

Number of laboratory samples – 1

Laboratory sample size - 20 kg

Almonds – shelled nuts

Hazelnuts – shelled nuts

Pistachios – inshell nuts (10kg shelled nuts)

Sample preparation – dry grind with vertical cutter mixer type mill and a 50 g test portion

Analytical method – performance based (see Table 2)

Decision rule – If the aflatoxin test result is less than or equal to 15 ng/g total aflatoxin, then accept the lot. Otherwise, reject the lot.

The operating characteristic curve describing the performance of the sampling plan for the three treenuts destined for further processing is shown in ANNEX III.

Ready-to-eat treenuts

Maximum limit – 8 ng/g total aflatoxin

Number of laboratory samples – 1

Laboratory sample size - 20 kg

Almonds – shelled nuts

Hazelnuts – shelled nuts

Pistachios – inshell nuts (10 kg shelled nuts)

Sample preparation – dry grind with vertical cutter mixer type mill and a 50 g test portion

Analytical method – performance based (see Table 2)

Decision rule – If the aflatoxin test result is less than or equal to 8 ng/g total aflatoxin, then accept the lot. Otherwise, reject the lot.

The operating characteristic curve describing the performance of the sampling plan for the three ready-to-eat treenuts is shown in ANNEX III.

Note: Operating characteristic curves describing the performance of sampling plans for maximum limits other than 8 and 15 ng/g total aflatoxin and sample sizes other than 20 kg can be seen at the website <http://www5.bae.ncsu.edu/usda/www/ResearchActDocs/treenutwg.html>.

12. To assist member countries implement these two Codex sampling plans, sample selection methods, sample preparation methods, and analytical methods required to quantify aflatoxin in laboratory samples taken from bulk treenut lots are described in the following sections.

iii. Sample selection

Material to be sampled

13. Each lot, which is to be examined for aflatoxin, must be sampled separately. Lots larger than 20 tonnes should be subdivided into sublots to be sampled separately. If a lot is greater than 20 tonnes, the number of sublots is equal to the lot weight in tonnes divided by 20 tonnes. It is recommended that a lot or a subplot should not exceed 20 tonnes.
14. Taking into account that the weight of the lot is not always an exact multiple of 20 tonne sublots, the weight of the subplot may exceed the mentioned weight by a maximum of 25%.
15. Samples should be taken from the same lot, i.e. they should have the same batch code or at the very least the same best before date. Any changes which would affect the mycotoxin content, the analytical determination or make the aggregate samples collected unrepresentative should be avoided. For example do not open packaging in adverse weather conditions or expose samples to excessive moisture or sunlight. Avoid cross-contamination from other potentially contaminated consignments nearby.
16. In most cases any truck or container will have to be unloaded to allow representative sampling to be carried out.

Incremental Sample Selection

17. Procedures used to take incremental samples from a treenut lot are extremely important. Every individual nut in the lot should have an equal chance of being chosen. Biases will be introduced by sample selection methods if equipment and procedures used to select the incremental samples prohibit or reduce the chances of any item in the lot from being chosen.
18. Since there is no way to know if the contaminated treenut kernels are uniformly dispersed throughout the lot, it is essential that the aggregate sample be the accumulation of many small incremental samples of product selected from different locations throughout the lot. If the aggregate sample is larger than desired, it should be blended and subdivided until the desired laboratory sample size is achieved.

Number of Incremental Samples for Lots of varying weight

19. The number and size of the laboratory sample(s) will not vary with lot (subplot) size. However, the number and size of the incremental samples will vary with lot (subplot) size. Based upon recommendations in Table 1, the incremental sampling rate is one incremental sample per 200 kg when 100 incremental samples are to be taken from a 20,000 kg lot (subplot).
20. The number of incremental samples to be taken from a lot (subplot) depends on the weight of the lot. Table 1 may be used to determine the number of incremental samples to be taken from lots or sublots of various sizes below 20 tonnes. The number of incremental samples varies from a minimum of 10 and to a maximum of 100.

Table 1. Number and size of incremental samples composited for an aggregate sample of 20 kg^a as a function of lot (or subplot) weight.

Lot or Sublot Weight ^b (T in Tonnes)	Minimum Number of Incremental Samples	Minimum Incremental Sample Size ^c (g)	Minimum Aggregate Sample Size (kg)
T<1	10	2000	20
1≤T<5	25	800	20
5≤T<10	50	400	20
10≤T<15	75	267	20
15≤T	100	200	20

a/ Minimum aggregate sample size = laboratory sample size of 20 kg

b/ 1 Tonne = 1000 kg

c/ Minimum incremental sample size = laboratory sample size (20 kg)/minimum number of incremental samples, ie. for T< 1 tonne, 2000 g = 20000/10

Weight of the Incremental Sample

21. The suggested minimum weight of the incremental sample should be approximately 200 grams for lots of 20 metric tonnes (20,000 kg). The number and/or size of incremental samples will have to be larger than that suggested in Table 1 for lots sizes below 20,000 kg in order to obtain an aggregate sample greater than or equal to the 20 kg laboratory sample.

Static Lots

22. A static lot can be defined as a large mass of treenuts contained either in a large single container such as a wagon, truck or railcar or in many small containers such as sacks or boxes and the nuts are stationary at the time a sample is selected. Selecting a truly random sample from a static lot can be difficult because all containers in the lot or subplot may not be accessible.
23. Taking incremental samples from a static lot usually requires the use of probing devices to select product from the lot. The probing devices should be specifically designed for the commodity and type of container. The probe should (1) be long enough to reach all product, (2) not restrict any item in the lot from being selected, and (3) not alter the items in the lot. As mentioned above, the aggregate sample should be a composite from many small incremental samples of product taken from many different locations throughout the lot.
24. For lots traded in individual packages, the sampling frequency (SF), or number of packages that incremental samples are taken from, is a function of the lot weight (LT), incremental sample weight (IS), aggregate sample weight (AS) and the individual packing weight (IP), as follows:

$$\text{Equation 1: } SF = (LT \times IS) / (AS \times IP).$$

25. The sampling frequency (SF) is the number of packages sampled. All weights should be in the same mass units such as kg.

Dynamic Lots

26. Representative aggregate samples can be more easily produced when selecting incremental samples from a moving stream of treenuts as the lot is transferred from one location to another. When sampling from a moving stream, take small incremental samples of product from the entire length of the moving stream; composite the incremental samples to obtain an aggregate sample; if the aggregate sample is larger than the required laboratory sample(s), then blend and subdivide the aggregate sample to obtain the desired size laboratory sample(s).
27. Automatic sampling equipment such as a cross-cut sampler is commercially available with timers that automatically pass a diverter cup through the moving stream at predetermined and uniform intervals. When automatic sampling equipment is not available, a person can be assigned to manually pass a cup through the stream at periodic intervals to collect incremental samples. Whether using automatic or manual methods, incremental samples should be collected and composited at frequent and uniform intervals throughout the entire time the nuts flow past the sampling point.
28. Cross-cut samplers should be installed in the following manner: (1) the plane of the opening of the diverter cup should be perpendicular to the direction of the flow; (2) the diverter cup should pass through the entire cross sectional area of the stream; and (3) the opening of the diverter cup should be wide enough to accept all items of interest in the lot. As a general rule, the width of the diverter cup opening should be about two to three times the largest dimensions of items in the lot.
29. The size of the aggregate sample (S) in kg, taken from a lot by a cross cut sampler is:

$$\text{Equation 2: } S = (D \times LT) / (T \times V),$$

where D is the width of the diverter cup opening (cm), LT is the lot size (kg), T is interval or time between cup movement through the stream (seconds), and V is cup velocity (cm/sec).

30. If the mass flow rate of the moving stream, MR (kg/sec), is known, then the sampling frequency (SF), or number of cuts made by the automatic sampler cup can be computed from Equation 3 as a function of S, V, D, and MR.

$$\text{Equation 3 : } SF = (S \times V) / (D \times MR).$$

31. Equations 2 and 3 can also be used to compute other terms of interest such as the time between cuts (T). For example, the time (T) required between cuts of the diverter cup to obtain a 20 kg aggregate sample from a 20,000 kg lot where the diverter cup width is 5.0 cm and the cup velocity through the stream 30 cm/sec. Solving for T in Equation 2,

$$T = (5.0 \text{ cm} \times 20,000 \text{ kg}) / (20 \text{ kg} \times 30 \text{ cm/sec}) = 250 \text{ sec.}$$

32. If the lot is moving at 500 kg per minute, the entire lot will pass through the sampler in 40 minutes (2400 sec) and only 9.6 cuts (9 incremental samples) will be made by the cup through the lot (Equation 3). This may be considered too infrequent, in that too much product (2,083.3 kg) passes through the sampler between the time the cup cuts through the stream.

Packaging and Transportation of Samples

33. Each laboratory sample shall be placed in a clean, inert container offering adequate protection from contamination, sunlight, and against damage in transit. All necessary precautions shall be taken to avoid any change in composition of the laboratory sample, which might arise during transportation or storage. Samples should be stored in a cool dark place.

Sealing and Labelling of Samples

34. Each laboratory sample taken for official use shall be sealed at the place of sampling and identified. A record must be kept of each sampling, permitting each lot to be identified unambiguously and giving the date and place of sampling together with any additional information likely to be of assistance to the analyst.

iv. Sample preparation

Precautions

35. Sunlight should be excluded as much as possible during sample preparation, since aflatoxin gradually breaks down under the influence of ultra-violet light. Also, environmental temperature and relative humidity should be controlled and not favor mold growth and aflatoxin formation.

Homogenization - Grinding

36. As the distribution of aflatoxin is extremely non-homogeneous, laboratory samples should be homogenized by grinding the entire laboratory sample received by the laboratory. Homogenization is a procedure that reduces particle size and disperses the contaminated particles evenly throughout the comminuted laboratory sample.
37. The laboratory sample should be finely ground and mixed thoroughly using a process that approaches as complete homogenization as possible. Complete homogenization implies that particle size is extremely small and the variability associated with sample preparation (ANNEX II) approaches zero.
38. The use of vertical cutter mixer type grinders that mix and comminute the laboratory sample into a paste represent a compromise in terms of cost and fineness of grind or particle size reduction¹¹. A better homogenization (finer grind), such as a liquid slurry, can be obtained by more sophisticated equipment and should provide the lowest sample preparation variance¹².

Test portion

39. The suggested weight of the test portion taken from the comminuted laboratory sample should be approximately 50 grams. If the laboratory sample is prepared using a liquid slurry, the slurry should contain 50 g of nut mass.

¹¹ Ozay, G., Seyhan, F., Yilmaz, A., Whitaker, T., Slate, A., and Giesbrecht, F. 2006. Sampling hazelnuts for aflatoxin: Uncertainty associated with sampling, sample preparation, and analysis. J. Association Official Analytical Chemists, Int., 89:1004-1011.

¹² Spanjer, M., Scholten, J., Kastrup, S., Jorissen, U., Schatzki, T., Toyofuku, N. 2006. Sample comminution for mycotoxin analysis: Dry milling or slurry mixing?, Food Additives and Contaminants, 23:73-83.

40. Procedures for selecting the 50 g test portion from the comminuted laboratory sample should be a random process. If mixing occurred during or after the comminution process, the 50 g test portion can be selected from any location throughout the comminuted laboratory sample. Otherwise, the 50 g test portion should be the accumulation of several small portions selected throughout the laboratory sample.
41. It is suggested that three test portions be selected from each comminuted laboratory sample. The three test portions will be used for enforcement, appeal, and confirmation if needed.

v. Analytical Methods

Background

42. A criteria-based approach, whereby a set of performance criteria is established with which the analytical method used should comply, is appropriate. The criteria-based approach has the advantage that, by avoiding setting down specific details of the method used, developments in methodology can be exploited without having to reconsider or modify the specific method. The performance criteria established for methods should include all the parameters that need to be addressed by each laboratory such as the detection limit, repeatability coefficient of variation (within lab), reproducibility coefficient of variation (among lab), and the percent recovery necessary for various statutory limits. Analytical methods that are accepted by chemists internationally (such as AOAC) may be used. These methods are regularly monitored and improved depending upon technology.

Performance Criteria for Methods of Analysis

43. A list of criteria and performance levels are shown in Table 2. Utilizing this approach, laboratories would be free to use the analytical method most appropriate for their facilities.

Table 2: Specific Requirements with which Methods of Analysis Should Comply

Criterion	Concentration Range (ng/g)	Recommended Value	Maximum Permitted Value
Blanks	All	Negligible	n/a
Recovery	1 to 15	70 to 110%	n/a
	>15	80 to 110%	n/a
Precision or Relative Standard Deviation RSD _R (Reproducibility)	1 to 120	Equation 4 by Thompson	2 x value derived from Equation 4
	>120	Equation 5 by Horwitz	2 x value derived from Equation 5
Precision or Relative Standard Deviation RSD _r (Repeatability)	1 to 120	Calculated as 0.66 times Precision RSD _R	n/a
	>120	Calculated as 0.66 times Precision RSD _r	n/a

n/a = not applicable

44. The detection limits of the methods used are not stated. Only the precision values are given at the concentrations of interest. The precision values are calculated from equations 4 and 5 developed by Thompson¹³ and Horwitz¹³, respectively.

$$\text{Equation 4: } RSD_R = 22.0 \quad (\text{for } C \leq 120 \text{ ng/g or } c \leq 120 \times 10^{-9})$$

$$\text{Equation 5: } RSD_R = 2^{(1-0.5 \log c)} \quad (\text{for } C > 120 \text{ ng/g or } c > 120 \times 10^{-9})$$

¹³ Horwitz, W. and Albert, R. 2006. The Horwitz ratio (HorRat): A useful index of method performance with respect to precision. J. Association of Official Analytical Chemists, Int., 89:1095-1109.

where:

- RSD_R = the relative standard deviation calculated from results generated under reproducibility conditions
- c = the aflatoxin concentration ratio (i.e. 1 = 100g/100g, 0.001 = 1,000 mg/kg)
- C = aflatoxin concentration or mass of aflatoxin to mass of treenuts (ie. ng/g)

45. Equations 4 and 5 are generalized precision equations, which has been found to be independent of analyte and matrix but solely dependent on concentration for most routine methods of analysis.

DEFINITIONS

Lot - an identifiable quantity of a food commodity delivered at one time and determined by the official to have common characteristics, such as origin, variety, type of packing, packer, consignor, or markings.

Sublot - designated part of a larger lot in order to apply the sampling method on that designated part. Each sublot must be physically separate and identifiable.

Sampling plan - is defined by an aflatoxin test procedure and an accept/reject limit. An aflatoxin test procedure consists of three steps: sample selection, sample preparation and aflatoxin quantification. The accept/reject limit is a tolerance usually equal to the Codex maximum limit.

Incremental sample – the quantity of material taken from a single random place in the lot or sublot.

Aggregate sample - the combined total of all the incremental samples that is taken from the lot or sublot. The aggregate sample has to be at least as large as the laboratory sample or samples combined.

Laboratory sample – the smallest quantity of tree nuts comminuted in a mill. The laboratory sample may be a portion of or the entire aggregate sample. If the aggregate sample is larger than the laboratory sample(s), the laboratory sample(s) should be removed in a random manner from the aggregate sample.

Test portion – a portion of the comminuted laboratory sample. The entire laboratory sample should be comminuted in a mill. A portion of the comminuted laboratory sample is randomly removed for the extraction of the aflatoxin for chemical analysis. Based upon grinder capacity, the laboratory sample can be divided into several equal sized subsamples for grinding, if all results are averaged.

Ready-to-eat treenuts – nuts, which are not intended to undergo an additional processing/treatment that has proven to reduce levels of aflatoxin.

Treenuts destined for further processing – nuts, which are intended to undergo an additional processing/treatment that has proven to reduce levels of aflatoxin before being used as an ingredient in foodstuffs, otherwise processed or offered for human consumption. Processes that have proven to reduce levels of aflatoxin are shelling, blanching followed by color sorting, and sorting by specific gravity and color (damage).

Operating Characteristic (OC) Curve – a plot of the probability of a accepting a lot versus lot concentration when using a specific sampling plan design. The OC curve provides an estimate of good lots rejected (exporter's risk) and bad lots accepted (importer's risk) by a specific aflatoxin sampling plan design.

ANNEX II

Uncertainty, as measured by the variance, associated with sampling, sample preparation, and analytical steps of the aflatoxin test procedure used to estimate aflatoxin in almonds, hazelnuts, and pistachios.

Sampling data for almonds, hazelnuts, and pistachios were supplied by the United States, Turkey, and Iran, respectively.

Variance estimates and the negative binomial distribution¹² were used to compute operating characteristic curves for each treenut in ANNEX III. Sampling, sample preparation, and analytical variances associated with testing almonds, hazelnuts, and pistachios are shown in Table 1 below.

Because of the computational complexities associated with use of the negative binomial distribution to compute operational characteristic (OC) curves for various sampling plan designs, the effect of various laboratory sample sizes, various numbers of laboratory samples, and various maximum limits on the performance (OC curves) of sampling plan designs is provided at the website address <http://www5.bae.ncsu.edu/usda/www/ResearchActDocs/treenutwg.html>.

Table 1. Variances^a associated with the aflatoxin test procedure for each treenut.

Test Procedure	Almonds	Hazelnuts	Pistachios
Sampling ^{b,c}	$S_s^2 = (7,730/ns)5.759C^{1.561}$	$S_s^2 = (10,000/ns)4.291C^{1.609}$	$S_s^2 = 8,000/ns)7.913C^{1.475}$
Sample Prep ^d	$S_{sp}^2 = (100/nss)0.170C^{1.646}$	$S_{sp}^2 = (50/nss)0.021C^{1.545}$	$S_{sp}^2 = (25/nss)2.334C^{1.522}$
Analytical ^e	$S_a^2 = (1/na)0.0484C^{2.0}$	$S_a^2 = (1/na)0.0484C^{2.0}$	$S_a^2 = (1/na)0.0484C^{2.0}$
Total variance	$S_s^2 + S_{sp}^2 + S_a^2$	$S_s^2 + S_{sp}^2 + S_a^2$	$S_s^2 + S_{sp}^2 + S_a^2$

a/ Variance = S^2 (s, sp, and a denote sampling, sample preparation, and analytical steps, respectively, of aflatoxin test procedure)

b/ ns = laboratory sample size in number of shelled nuts, nss = test portion size in grams, na = number of aliquots quantified by HPLC, and C = aflatoxin concentration in ng/g total aflatoxin.

c/ Shelled nut count/kg for almonds, hazelnuts, and pistachios is 773, 1000, and 1600, respectively.

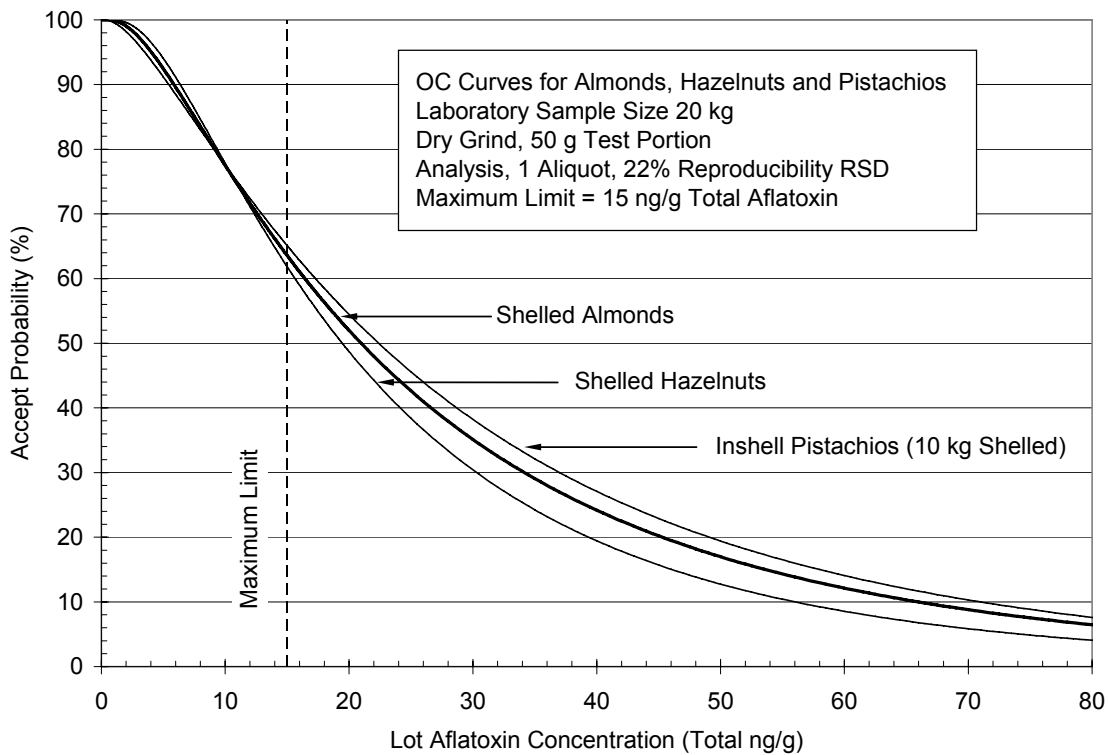
d/ Sample preparation for almonds, hazelnuts, and pistachios reflect Hobart, Robot Coupe, and Marjaan Khatman type mills, respectively. Laboratory samples were dry ground into a paste for each treenut.

e/ Analytical variances reflect FAPAS recommendation for upper limit of analytical reproducibility uncertainty. A relative standard deviation of 22% is considered by Thompson¹³ (based upon FAPAS data) as an appropriate measure of the best agreement that can be obtained between laboratories. An analytical uncertainty of 22% is larger than the within laboratory uncertainty measured in the sampling studies for the three treenuts.

Operating Characteristic Curves describing the performance of draft aflatoxin sampling plans for almonds, hazelnuts, and pistachios.

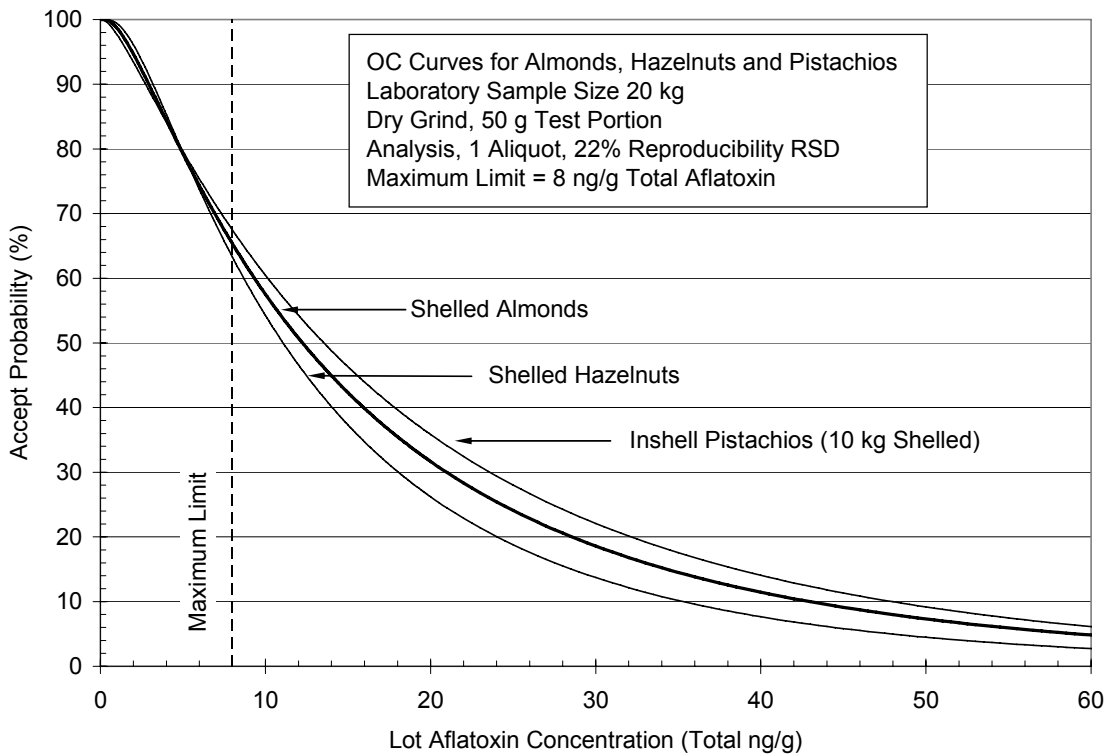
Treenuts Destined for Further Processing

Operating Characteristic curve describing the performance of the aflatoxin sampling plan for almonds, hazelnuts, and pistachios destined for further processing using a single laboratory sample of 20 kg and a maximum limit of 15 ng/g total aflatoxin. The operating characteristic curve reflects uncertainty associated with a 20 kg laboratory sample of shelled nuts for almonds and hazelnuts and a 20 kg laboratory sample of inshell nuts (10kg shelled nuts) for pistachios, dry grind with a vertical cutter mixer type mill, 50 g test portion, and quantification of aflatoxin in the test portion by HPLC.



Ready-to-Eats Treenuts

Operating Characteristic curve describing the performance of the aflatoxin sampling plan for ready-to-eat almonds, hazelnuts, and pistachios using a single laboratory sample of 20 kg and a maximum limit of 8 ng/g total aflatoxin. The operating characteristic curve reflects uncertainty associated with a 20 kg laboratory sample of shelled nuts for almonds and hazelnuts and a 20 kg laboratory sample of inshell nuts (10kg shelled nuts) for pistachios, dry grind with a vertical cutter mixer type mill, 50 g test portion, and quantification of aflatoxin in the test portion by HPLC.



ANNEX IV

Effect of using the proposed ready-to-eat (RTE) and destined for further processing (DFP) aflatoxin-sampling plans on number of lots accepted and rejected, number of lots misclassified (false positives and false negatives), and amount of aflatoxin removed from the trade.

- A. An operating characteristic (OC) curve indicates the probability or percent lots accepted and rejected at a specific lot concentration by a given aflatoxin sampling plan design. The probability of accepting and rejecting lots at a specific lot concentration can be converted to number of lots accepted and rejected if the aflatoxin distribution among lots concentrations (lot distribution) is known¹⁴.
- B. From aflatoxin sampling data provided by the U.S. almond industry for 1496 lots sampled during the 2006/2007 crop year, a lot distribution was constructed and is shown in Table 1. The average aflatoxin among the 1496 lots tested was 1.3 ng/g total aflatoxin.

Table 1. Aflatoxin distribution among U.S. lots of shelled almond harvested in the 2006/2007 crop year.

Aflatoxin Range (total ng/g)	Frequency	Cumulative (%)
$c < 1$	1300	86.88
$1 \leq c < 2$	68	91.45
$2 \leq c < 3$	31	93.54
$3 \leq c < 4$	23	95.09
$4 \leq c < 5$	8	95.63
$5 \leq c < 6$	12	96.37
$6 \leq c < 7$	6	96.77
$7 \leq c < 8$	4	97.04
$8 \leq c < 10$	4	97.31
$10 \leq c < 15$	9	97.91
$15 \leq c < 20$	8	98.45
$20 \leq c < 40$	13	99.33
$40 \leq c < 60$	7	99.80
$60 \leq c < 80$	2	99.93
$c = 231$	1	100.00

- C. The lot distribution in Table 1 was theoretically sampled¹⁷ with the proposed RTE ($1 \times 20 \text{ kg} \leq 8 \text{ ng/g}$) and DFP ($1 \times 20 \text{ kg} \leq 15 \text{ ng/g}$) aflatoxin sampling plans for shelled-almonds using the RTE and DFP OC curves shown in ANNEX III. The results are shown below in Tables 2 and 3 for RTE almonds and Tables 4 and 5 for DFP almonds. For RTE almonds, a good lot has a concentration $\leq 8 \text{ ng/g}$ and a bad lot has a concentration $> 8 \text{ ng/g}$ total aflatoxin. For DFP almonds, a good lot has a concentration $\leq 15 \text{ ng/g}$ and a bad lot has a concentration $> 15 \text{ ng/g}$ total aflatoxin.

Ready-to-Eat

Table 2. Percent lots accepted, rejected, good lots rejected (exporter's risk or false positives), and bad lots accepted (importer's risk or false negatives) when using the proposed RTE ($1 \times 20 \text{ kg} \leq 8 \text{ ng/g}$) aflatoxin-sampling plan to sample the shelled almond lot distribution in Table 1.

Lot Concentration (total ng/g)	Number of Lots Accepted (%)	Number of Lots Rejected (%)	Total Number of Lots Tested (%)
≤ 8	96.07	0.97	97.04
> 8	0.91	2.05	2.96
All Lots	96.98	3.02	100.00

¹⁴ FAO Food and Nutrition Paper 55, 1993.

Table 3. Average aflatoxin among lots accepted, rejected, good lots rejected (exporter's risk or false positives), and bad lots accepted (importer's risk or false negatives) when using the proposed RTE (1x20 kg \leq 8 ng/g) aflatoxin-sampling plan to sample the shelled almond lot distribution in Table 1.

Lot Concentration (total ng/g)	Average Aflatoxin in Lots Accepted (total ng/g)	Average Aflatoxin in Lots Rejected (total ng/g)	Average Aflatoxin in All Lots Tested (total ng/g)
\leq 8	0.3	4.2	0.3
> 8	17.0	37.6	31.3
All Lots	0.5	26.9	1.3

Destined for Further Processing

Table 4. Percent lots accepted, rejected, good lots rejected (exporter's risk or false positives), and bad lots accepted (importer's risk or false negatives) when using the proposed DFP (1x20kg \leq 15 ng/g) aflatoxin-sampling plan to sample the shelled almond lot distribution in Table 1.

Lot Concentration (total ng/g)	Number of Lots Accepted (%)	Number of Lots Rejected (%)	Total Number of Lots Tested (%)
\leq 15	97.41	0.51	97.91
> 15	0.74	1.34	2.09
All Lots	98.15	1.85	100.00

Table 5. Average aflatoxin among lots accepted, rejected, good lots rejected (exporter's risk or false positives), and bad lots accepted (importer's risk or false negatives) when using the proposed RTE (1x20kg \leq 8 ng/g) aflatoxin-sampling plan to sample the shelled almond lot distribution in Table 1.

Lot Concentration (total ng/g)	Average Aflatoxin in Lots Accepted (total ng/g)	Average Aflatoxin in Lots Rejected (total ng/g)	Average Aflatoxin in All Lots Tested (total ng/g)
\leq 15	0.4	7.4	0.4
> 15	25.9	47.7	39.9
All Lots	0.6	36.6	1.3

- D. To compare the proposed aflatoxin sampling plan for treenuts to a sampling plan with lower false negatives and higher false positives, the lot distribution in Table 1 was theoretically sampled using a sampling plan design similar that used by the European Union that requires three (3) laboratory samples of 10 kg each to all test less than 8 total ng/g for RTE almonds and 15 ng/g for DFP almonds for a lot to be accepted. The OC curves for the EU style sampling plan for RTE almonds (3x10 kg \leq 8 ng/g) and almonds DFP (3x10 kg \leq 15 ng/g) are shown at the website address <http://www5.bae.ncsu.edu/usda/www/ResearchActDocs/treenutwg.html>. The results are shown below in Tables 6 and 7 for RTE almonds and Tables 8 and 9 for DFP almonds.

Ready-to-Eat

Table 6. Percent lots accepted, rejected, good lots rejected (exporter's risk or false positives), and bad lots accepted (importer's risk or false negatives) when using the EU style sampling plan (3x10 kg \leq 8 ng/g) for RTE almond with a lot distribution described in Table 1.

Lot Concentration (total ng/g)	Number of Lots Accepted (%)	Number of Lots Rejected (%)	Total Number of Lots Tested (%)
\leq 8	94.17	2.87	97.04
> 8	0.33	2.63	2.96
All Lots	94.50	5.50	100.00

Table 7. Average aflatoxin among lots accepted, rejected, good lots rejected (exporter's risk or false positives), and bad lots accepted (importer's risk or false negatives) when using the EU style sampling plan ($3 \times 10 \text{ kg} \leq 8 \text{ ng/g}$) for RTE almond with a lot distribution described in Table 1.

Lot Concentration (total ng/g)	Average Aflatoxin in Lots Accepted (total ng/g)	Average Aflatoxin in Lots Rejected (total ng/g)	Average Aflatoxin in All Lots Tested (total ng/g)
≤ 8	0.2	3.5	0.3
> 8	13.8	33.4	31.3
All Lots	0.3	17.8	1.3

Destined for Further Processing

Table 8. Percent lots accepted, rejected, good lots rejected (exporter's risk or false positives), and bad lots accepted (importer's risk or false negatives) when using the EU style sampling plan ($3 \times 10 \text{ kg} \leq 15 \text{ ng/g}$) for almond DFP with a lot distribution described in Table 1.

Lot Concentration (total ng/g)	Number of Lots Accepted (%)	Number of Lots Rejected (%)	Total Number of Lots Tested (%)
≤ 15	96.20	1.71	97.91
> 15	0.26	1.82	2.09
All Lots	96.47	3.53	100.00

Table 9. Average aflatoxin among lots accepted, rejected, good lots rejected (exporter's risk or false positives), and bad lots accepted (importer's risk or false negatives) when using the EU style sampling plan ($3 \times 10 \text{ kg} \leq 15 \text{ ng/g}$) for almond DFP with a lot distribution described in Table 1.

Lot Concentration (total ng/g)	Average Aflatoxin in Lots Accepted (total ng/g)	Average Aflatoxin in Lots Rejected (total ng/g)	Average Aflatoxin in All Lots Tested (total ng/g)
≤ 15	0.3	6.0	0.4
> 15	22.2	42.5	39.9
All Lots	0.4	24.8	1.3

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