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Appendix A. Protocol used for aflatoxin analysis in pistachios

A stock solution, containing 100 ng/ml of equal parts of aflatoxin B_1, G_1, B_2, and G_2 (25 ng of each) is maintained in a refrigerator. Each day 1 ml of this solution is used as the "daily stock".

Lot is received as 10-300 lbs. Lot is stored in closed tin cans, holding approximately 15 lbs. each. No mixing between cans is carried out.

1. One can is emptied on tray and hand mixed. Nuts are taken by scoop and weighed and counted to establish the weight of *n* nuts, where *n* = 1, 10, 100 or 1000. Nuts are returned to tray.
2. Tray content is split into 20 sublots, if enough available to get 20 sublots of *n* nuts each. Fewer sublots otherwise. For *n* = 10000 approximately two cans are required for each sublot.
3. When *n* <= 100, a scoop is taken from one sublot and *n* nuts are counted out. If *n* >= 1000, a weighed amount, equivalent to *n*, is taken instead. This constitutes a sample. This step is repeated for each of N samples, one from each sublot. Unused material is returned to the tins.

The following steps are repeated for each of the N samples.

4. The sample is ground in a Waring blender, model 7100S (shells are included). Grinding is stopped frequently (typically after 10

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s, 1 min and 3-4 min) and all material passing a No. 9 screen is collected. Grinding is continued until at least 95% of shells pass screen. (Kernels will grind much faster than shells and all will pass the screen at this point.) No material is discarded. If $n <= 1000$, the sample is blended by sifting thrice. When $n = 10000$ blending is done in a sealed V-shaped tumble mixer @ 30 rpm for 10 min. Ground samples are typically used within a day or so after grinding, but may be stored and used later.

5. When $n <= 10$ the entire sample is extracted (constitutes a single aliquot). When $n > 10$, a ground aliquot weighing as much as 10 nuts is removed. Only a single aliquot (subsample) of each sample is run, unless one is checking for reproducibility. In this case aliquots are chosen from different parts of the ground sample.

6. The subsample is weighed. 20w% of NaCl is added. 5 ml/g of 60v%MeOH/40v%aq is added and the slurry is blended for 1 min. in a Waring blender at "high" speed.

7. Slurry is centrifuged at 4100 rpm, 15.3 cm tip radius, or ~2900 g, for 20 min.

8. Supernatant is filtered twice through Whatman No. 50 filter twice and recovered volume is recorded.

9. 10v% (as rounded to the nearest ml) of product is reserved. Record reserved volume.

10. Remainder is diluted with equal volume of water.

11. Pass through VICAM immunoaffinity column at 1 ml/min with suction.

12. Flush column with 20 ml water at full vacuum.

13. Elude column with 2 ml acetonitrile (AN)

14. Bring to dryness over nitrogen at 40°C.

15. Add 200 µl hexane, 200 µl tri-fluoro acetic acid (TFA). Vortex to mix.

16. Let sit 10 min to react for derivatization.

17. Inspect under fluorescent light in curtained box with eye protecting glass or fluorometer and compare with daily stock solution which has been carried through steps 14-16 above. If the sample shows equal or more fluorescence than this derivatized daily stock, go back to step 9, and proceed as follows for as many cycles as required:

The reserve sample from step 9 is diluted with 9 equal parts of extraction solvent (60v%MeOH/40v%aq) and a volume equal to the reserve volume is removed. The remainder (containing 90% of reserve) is analyzed.

18. Bring to dryness over nitrogen at 40°C.

19. Add 200 µl (100 µl for *n*=1 samples) 10%v AN/90v% water.

20. Filter through 0.2µm Gelman glass microfilter into 200 µl insert.

21. Insert into autosampler of an Hewlett-Packard HPLC (model 1050)

22. Repeat steps 6-21 for the remaining N samples.

The HPLC consists of a 200 mm x 4.6 mm reverse phase column (part 799160D-574), preceded by a 0.45 µm pre-column filter and a 20 mm x 4.6 mm guard column (part 79916KT-120_). Mobil phase is 55v% aq/35v% MeOH/10v% AN/0.5v% HAc (total 100.5%), at 1.1 ml/min. Column temperature is room temp (approx. 27°C). Detection is by

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fluorescence, excitation at 365 nm, emission at 455 nm. The model 1046A fluorescence detector is used. The system is controlled by HP ChemStation software, running on a 486PC.

23. The column is primed before each run by two auto-injections of 20 µl of derivatized daily stock solution and dissolved in AN/aq as per steps 18-21.

24. Samples are run in sequence by the autosampler, single injection each of 20 µl. (Since 120 ng is the calibration limit of the VICAM, and 20 µl/200 µl is injected into the HPLC, the calibration limit of 12 ng for the HPLC is equivalent). If *n*=1, use 50 µl injection for sensitivity.

All glassware after use is rinsed in 5% NaOCl solution (Clorax or equivalent) after use to render remaining aflatoxin inert.