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1 **Appendix A. Protocol used for aflatoxin analysis in pistachios**

2

3 A stock solution, containing 100 ng/ml of equal parts of aflatoxin
4 B₁, G₁, B₂, and G₂ (25 ng of each) is maintained in a refrigerator.
5 Each day 1 ml of this solution is used as the "daily stock".

6

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

10

11 1. One can is emptied on tray and hand mixed. Nuts are taken by
12 scoop and weighed and counted to establish the weight of n nuts,
13 where $n = 1, 10, 100$ or 1000 . Nuts are returned to tray.

14 2. Tray content is split into 20 sublots, if enough available to
15 get 20 sublots of n nuts each. Fewer sublots otherwise. For
16 $n = 10000$ approximately two cans are required for each subplot.

17 3. When $n \leq 100$, a scoop is taken from one subplot and n nuts are
18 counted out. If $n \geq 1000$, a weighed amount, equivalent to n , is
19 taken instead. This constitutes a sample. This step is repeated
20 for each of N samples, one from each subplot. Unused material is
21 returned to the tins.

22

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

24

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

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- 1 s, 1 min and 3-4 min) and all material passing a No. 9 screen is
2 collected. Grinding is continued until at least 95% of shells pass
3 screen. (Kernels will grind much faster than shells and all will
4 pass the screen at this point.) No material is discarded. If $n \leq$
5 1000, the sample is blended by sifting thrice. When $n = 10000$
6 blending is done in a sealed V-shaped tumble mixer @ 30 rpm for 10
7 min. Ground samples are typically used within a day or so after
8 grinding, but may be stored and used later.
- 9 5. When $n \leq 10$ the entire sample is extracted (constitutes a
10 single aliquot). When $n > 10$, a ground aliquot weighing as much as
11 10 nuts is removed. Only a single aliquot (subsample) of each
12 sample is run, unless one is checking for reproducibility. In this
13 case aliquots are chosen from different parts of the ground sample.
- 14 6. The subsample is weighed. 20w% of NaCl is added. 5 ml/g of
15 60v%MeOH/40v%aq is added and the slurry is blended for 1 min. in a
16 Waring blender at "high" speed.
- 17 7. Slurry is centrifuged at 4100 rpm, 15.3 cm tip radius, or ~2900
18 g, for 20 min.
- 19 8. Supernatant is filtered twice through Whatman No. 50 filter
20 twice and recovered volume is recorded.
- 21 9. 10v% (as rounded to the nearest ml) of product is reserved.
22 Record reserved volume.
- 23 10. Remainder is diluted with equal volume of water.
- 24 11. Pass through VICAM immunoaffinity column at 1 ml/min with
25 suction.
- 26 12. Flush column with 20 ml water at full vacuum.

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- 1 13. Elude column with 2 ml acetonitrile (AN)
- 2 14. Bring to dryness over nitrogen at 40°C.
- 3 15. Add 200 µl hexane, 200 µl tri-fluoro acetic acid (TFA). Vortex
- 4 to mix.
- 5 16. Let sit 10 min to react for derivatization.
- 6 17. Inspect under fluorescent light in curtained box with eye
- 7 protecting glass or fluorometer and compare with daily stock
- 8 solution which has been carried through steps 14-16 above. If the
- 9 sample shows equal or more fluorescence than this derivatized daily
- 10 stock, go back to step 9, and proceed as follows for as many cycles
- 11 as required:
- 12 The reserve sample from step 9 is diluted with 9 equal parts of
- 13 extraction solvent (60v%MeOH/40v%aq) and a volume equal to the
- 14 reserve volume is removed. The remainder (containing 90% of
- 15 reserve) is analyzed.
- 16 18. Bring to dryness over nitrogen at 40°C.
- 17 19. Add 200 µl (100 µl for n=1 samples) 10%v AN/90v% water.
- 18 20. Filter through 0.2µm Gelman glass microfilter into 200 µl
- 19 insert.
- 20 21. Insert into autosampler of an Hewlett-Packard HPLC (model 1050)
- 21 22. Repeat steps 6-21 for the remaining N samples.
- 22 The HPLC consists of a 200 mm x 4.6 mm reverse phase column (part
- 23 799160D-574), preceded by a 0.45 µm pre-column filter and a 20 mm
- 24 x 4.6 mm guard column (part 79916KT-120_). Mobil phase is 55v%
- 25 aq/35v% MeOH/10v% AN/0.5v% HAc (total 100.5%), at 1.1 ml/min.
- 26 Column temperature is room temp (approx. 27°C). Detection is by

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1 fluorescence, excitation at 365 nm, emission at 455 nm. The model
2 1046A fluorescence detector is used. The system is controlled by
3 HP ChemStation software, running on a 486PC.

4 23. The column is primed before each run by two auto-injections of
5 20 μ l of derivatized daily stock solution and dissolved in AN/aq as
6 per steps 18-21.

7 24. Samples are run in sequence by the autosampler, single
8 injection each of 20 μ l. (Since 120 ng is the calibration limit of
9 the VICAM, and 20 μ l/200 μ l is injected into the HPLC, the
10 calibration limit of 12 ng for the HPLC is equivalent). If $n=1$, use
11 50 μ l injection for sensitivity.

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