Determination of aflatoxins B_1, B_2, G_1, and G_2 in foodstuffs A-12.4

Key words

Instrumental HPTLC - quantitative analysis - densitometry - prechromatographic derivatization - mycotoxins - aflatoxin B_1, B_2, G_1, G_2 - paprika, curry, nutmeg, peanuts, almonds

Scope

Formed by the metabolism of the mold fungi Aspergillus flavus and parasiticus, aflatoxins are carcinogenic and waken the immune system. The most important naturally occurring aflatoxins are B_1, B_2, G_1, and G_2. Of these the commonest and most potent is aflatoxin B_1. The limit concentrations to be monitored in spices, for example, are 5 ppb for aflatoxin B_1 and 5 ppb for the sum of aflatoxins B_2, G_1, and G_2. In nuts the limits are 1 ppb for B_1 and 5 ppb for the sum of B_2, G_1, and G_2.

The mycotoxins are isolated from the matrix by liquid/liquid extraction. In the case of critical matrices a solid phase extraction step is employed. They are chromatographed on silica gel, simultaneously eluted matrix being first removed by prechromatography with diethyl ether, after which the mycotoxins are separated with chloroform - acetone - water. Densitometric evaluation is performed by fluorescence measurement at 366/>400 nm. Positive results can be confirmed by additional prechromatographic derivatization. [1-3]

The Natural Resources Institute [4] uses quantitative HPTLC to monitor aflatoxin/mycotoxin contamination in different Asian countries because this procedure is more robust than HPLC in coping with variable matrix contents while offering comparable accuracy and precision. It is also more cost-effective besides being faster and easier to use.

Advantages of instrumental HPTLC in performing this analytical task

- High sample throughput, low operating costs
- Robust procedure largely unaffected by high matrix content
- Prechromatographic derivatization readily performed for additional confirmation

Chemicals

Hexane
Diethylether, peroxide-free, dried
Petroleum ether
Toluene
Dichloromethane
Chloroform
Acetone
Acetonitrile
Methanol
Water, dist.
Acetic acid, conc.
Trifluoroacetic acid, 99%
Sodium chloride
Sodium sulfate
Paraffin oil, subliquidum
RP-18 cartridge, 6 mL/1g sorbent
Silica gel cartridge, 3 mL/0.5g sorbent

Standard: Aflatoxin B_1, B_2, G_1 and G_2 (Aflatoxin Standard Kit, e.g. Biochemica Art. No. 70040, 1 mg each)

Sample preparation for spices*

- Grind or homogenize sample and mix 5.6 g with 100 mL methanol for 3 min.
- Add 40 mL water, mix for 4 min, leave to stand for 10 min, then filter.
- Shake 20 mL filtrate with 20 mL NaCl solution (10%) and 20 mL petroleum ether for 2 min and leave to separate for 10 min (extraction of matrix in petroleum ether).
- Shake aqueous phase with 50 mL dichloromethane for 1 min and leave to separate (extraction of aflatoxins in dichloromethane).
- Dry dichloromethane phase with 5 g sodium sulfate, filter and evaporate to dryness.
- Dissolve residue in 0.5 mL toluene - acetonitrile 98:2. Use extract (= 0.8 g sample) for application to the HPTLC layer.

For some critical matrices such as paprika, it is advisable to dissolve the residue in 2 mL toluene - acetonitrile 98:2 and to perform further purification:

1 Purification of the extract on a silica gel cartridge: Rinse resp. condition the sorbent with 6 mL toluene - acetonitrile 98:2. (Do not let the sorbent run dry). Elute extract and rinse remaining matrix with 20 mL toluene - acetic acid 9:1 and 20 mL hexane - diethyl ether - acetonitrile 6:3:1 (dry the sorbent between and in the end). Elute the aflatoxins fractionated with 7 and 4 mL dichloromethane - acetone 3:1 direct in a pear shape flask (dry sorbent between and in the end).

2 Evaporate eluate to dryness and take up the residue in 0.5 mL methanol.

3 Purification of the extract on a RP-18 cartridge: Rinse sorbent with 2 mL methanol, dry and condition with 4 mL methanol - water 2:8 and 2 mL water. (Do not let the sorbent run dry). Elute extract and rinse remaining matrix with 5 mL methanol - water 2:8, dry for 1 min. Elute the aflatoxins fractionated with 4 x 2.5 mL methanol - water 5:5 direct in a pear shape flask (dry sorbent between and in the end).

4 Shake aqueous phase for 1 min with 20 mL NaCl solution (10%) and 18 mL dichloromethane and leave to separate for 5 min (extraction of aflatoxins in dichloromethane). Separate dichloromethane phase. Repeat extraction of the aqueous phase with 2 mL dichloromethane.

5 Evaporate eluate to dryness and take up the residue in 0.5 mL toluene - acetonitrile 98:2.

6 Use extract (= 0.8 g sample) for application to the HPTLC layer.

* For other foodstuffs use a higher weighted amount (e.g. 80 g for nuts) if necessary and adjust the amounts of solvent, etc. accordingly.

Standard solution

Make up a standard mixture of aflatoxins B_1, B_2, G_1, and G_2 in toluene - acetonitrile 98:2 containing 200 pg/µL each of aflatoxins B_1 and G_1 and 100 pg/µL each of G_2 and B_2.

Layer

HPTLC plates or sheets silica gel Merck 60 F_{254}, 20x10 cm or 20x20 cm

Sample application

Apply bandwise with CAMAG Linomat, distance from lower edge of sheet 10 cm (for plates 6 cm) , band length 8 mm, distance between tracks 4 mm, distance from left edge 15 mm = 15 applications.

Application pattern:

S1 U U U U S1 U U U U S1 ...
S1 = standard mixture 5 µL each, U = sample of 100 µL each

Chromatography

Double development (in opposing direction) in CAMAG Twin Trough Chamber

1. For the first development, which removes the matrix from the start zone, fill the chamber to a depth of 5 cm with peroxide-free, dried diethyl ether and place the sheet or plate (6 cm free side downwards) in the chamber: migration distance 50 mm (sheet) and 40 mm (plate), respectively.

   View sheet or plate under UV 366 nm; the fluorescent aflatoxins should have migrated little or not at all from the start zone. Cut off the top 85-90 mm (sheet) and 25-30 mm (plate), respectively and turn the plate or sheet through 180°.

2. For the second development, which separates the aflatoxins, charge the chamber normally (to a depth of about 8 mm) with chloroform - acetone - water 140:20:0.3 and insert plate or sheet; migration distance 80 mm (sheet) and 60 mm (plate), respectively.

Densitometric Evaluation

CAMAG TLC Scanner with CATS software, fluorescence measurement at 366/>400 nm, single-level calibration via peak height confirmed by a multilevel calibration (see fig. 1).

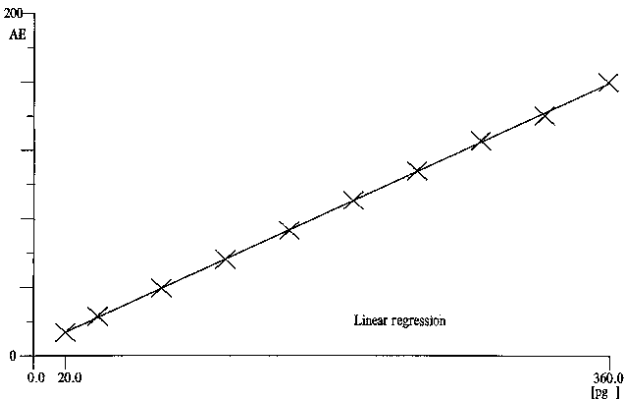

Fig. 1 Calibration function of aflatoxin B_1 (peak height) after dipping in paraffin oil - n-hexane

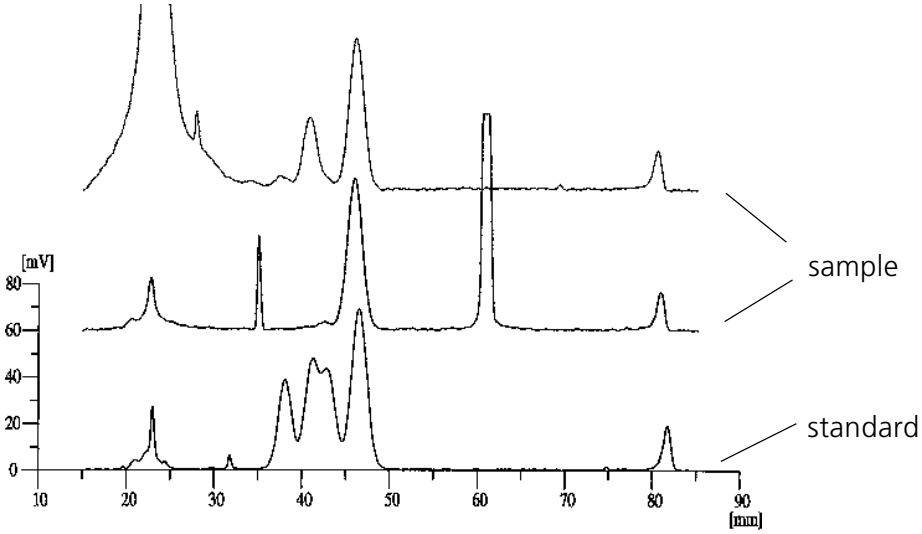

Fig. 2 TLC chromatogram with standard mixture and aflatoxin extracts from different types of paprika (extract additional purified)

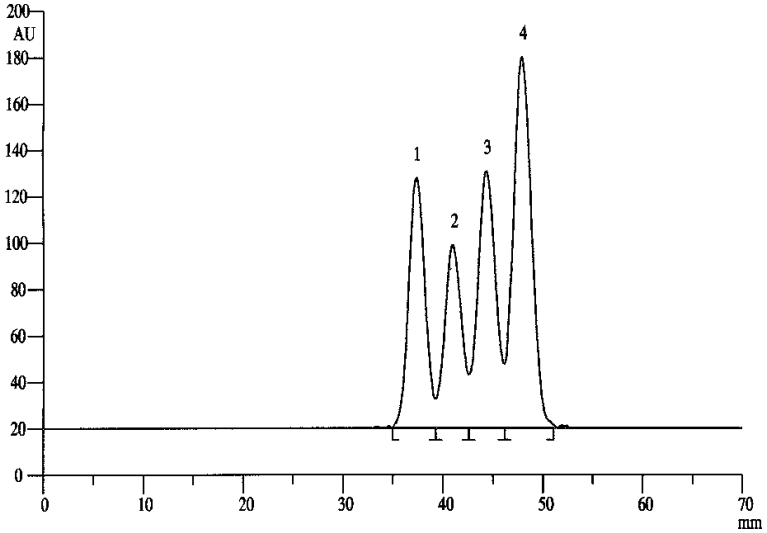

Fig. 3 HPTLC chromatogram with standard mixture (1 = G_2; 2 = G_1; 3 = B_2; 4 = B_1)

Discussion

Recovery is between 70 and 100%. The detection limit is 10 pg for aflatoxins B_1 and G_1 and 5 pg for B_2 and G_2. This can be improved 2 to 3-fold by dipping in paraffin oil - n-hexane 2:3.

Positive results can be confirmed by development after prechromatographic derivatization. For this purpose additionally apply 5 mL trifluoroacetic acid to the start zones, leave for 5 min, then heat for 2 min at 35-40°C on a plate heater. The derivatives of aflatoxins B_1 and G_1 are now polar and stay behind at the start; B_2 and G_2 lie in the medium R_F range.

Notes

- ☞ Avoid contact of aflatoxins with your skin.
- ☞ Aflatoxins are sensitive to light and oxidation. Store chromatographed HPTLC plates or sheets, standards, extracts etc. in the dark at about 5°C.
- ☞ Verify the concentration of aflatoxin stock or standard solutions regularly by photometry.
- ☞ Place contaminated materials at least for 30 min in 6% Javel water.
- ☞ Aflatoxins are able to accumulate at synthetic material and falsify results. Avoid contact with such materials, e.g. plastic tip of an Eppendorf pipette.

Literature

[1] SOP A9024.01B, Kantonales Laboratorium Aargau, 13.03.1997

[2] AOAC Official Methods 970.44, 1990 edition (2 volumes)

[3] H. Jork, W. Funk, W. Fischer, H. Wimmer: Thin-Layer Chromatography, volume 1a, VCH Weinheim, 1990.

[4] J.M. Nagler (Natural Resources Institute, NRI, Central Avenue, Chatham, Kent, ME4 4TB): The Application of HPTLC to the Control of Aflatoxin in Philippine Copra, paper presented at the TLC-Symposium in Guildford, 3rd -5th June 1996.