

Determination of Aflatoxins in NeemAzal and NeemAzal-T/S

HPLC-Method

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General Information about Aflatoxins

Neem seed kernels are the source of a natural insecticide named Azadirachtin. These kernels may be infected by *Aspergillus* spp. during collection and storage.

The concentrations of aflatoxins in NeemAzal technical are analysed routinely during the production process. Also each batch of the formulated product NeemAzal-T/S is analysed to comply with the maximum residue limit (MRL) for foodstuffs, which is 4 µg/kg for the sum of aflatoxins B₁, B₂, G₁ and G₂.

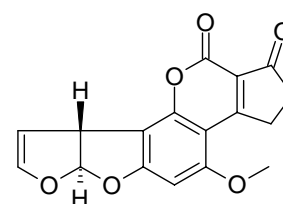
The method used is based on DIN EN 14123 and was peer validated.

Aflatoxin B₁

CAS-No.: 1162-65-8

Structure:

Molecular formula: C₁₇H₁₂O₆

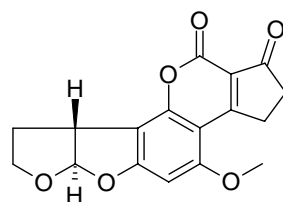


Aflatoxin B₂

CAS-No.: 7220-81-7

Structure:

Molecular formula: C₁₇H₁₄O₆

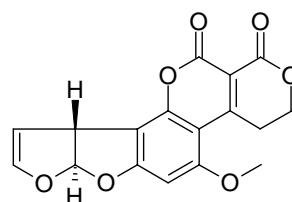


Aflatoxin G₁

CAS-No.: 1165-39-5

Structure:

Molecular formula: C₁₇H₁₂O₇

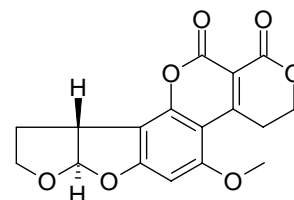


Aflatoxin G₂

CAS-No.: 7241-98-7

Structure:

Molecular formula: C₁₇H₁₄O₇



1. Aflatoxins B₁, B₂, G₁ and G₂ (in NeemAzal technical)

Sampling. Take at least a 50 g sample of NeemAzal technical.

OUTLINE OF METHOD.

Aflatoxins are determined by RP-HPLC using post-column derivatisation and fluorescence detection.

REAGENTS*General*

Use only reagents of recognized analytical grade unless otherwise stated.

Reference material - Aflatoxin stock solutions of known content

Acetonitrile - HPLC grade

Methanol - HPLC grade

Water - HPLC grade

Toluene

Potassium bromide

Nitric acid, HNO₃ = 4 mol/l

Dilution solvent for aflatoxin standard solutions

Toluene + acetonitrile, 98 + 2 (v/v)

Phosphate buffered saline (PBS)

Dissolve 0.2 g KCl, 0.2 g KH₂PO₄, 2.92 g Na₂HPO₄·12 H₂O and 8.0 g NaCl in 0.9 l water and adjust to pH 7.4 with HCl (0.1 mol/l) or NaOH (0.1 mol/l) as appropriate. Make up to 1 l with water.

Immunoaffinity column. Contains antibodies raised against aflatoxins B₁, B₂, G₁ and G₂.

Mobile phase

Water + acetonitrile + methanol, 6 + 2 + 3 (v/v) Add 120 mg KBr and 350 µl HNO₃ per litre of mobile phase. Degas solution prior to use.

Extraction solvent

Methanol + water, 8 + 2 (v/v)

Tween 20[®] - 10% in water

Calibration stock solutions

Prepare a stock solution of each aflatoxin (B₁, B₂, G₁ and G₂) at a concentration of 10 µg/ml each in the dilution solvent. Since the aflatoxins are photolabile, wrap the flasks tightly with aluminium foil and store them below 4°C.

Prepare a mixed aflatoxin stock solution containing 1000 ng/ml of aflatoxins B₁ and G₁ and 200 ng/ml of aflatoxins B₂ and G₂ by appropriate dilution (with dilution solvent) of the single stock solutions. Pipette exactly 2 ml of this solution into a 20 ml volumetric flask and fill to the mark with the dilution solvent. Shake well and keep the solutions also tightly wrapped in aluminium foil and store them below 4°C.

Calibration standard solutions

Pipette volumes of the mixed aflatoxin stock solution into 10 ml volumetric flasks as given below:

Standard solution	Volume of diluted mixed stock solution [μ l]	Mass concentration of standard solution [ng/ml]			
		B ₁	B ₂	G ₁	G ₂
1	10	0.1	0.02	0.1	0.02
2	50	0.5	0.10	0.5	0.10
3	100	1.0	0.20	1.0	0.20
4	150	1.5	0.30	1.5	0.30
5	200	2.0	0.40	2.0	0.40
6	250	2.5	0.50	2.5	0.50
7	300	3.0	0.60	3.0	0.60

Evaporate the toluene + acetonitrile solutions just to dryness under a stream of nitrogen at room temperature. To each flask add 5 ml methanol. After dissolving the aflatoxins, fill to the mark with water and shake well.

Spiking solution

Prepare a spiking solution by pipetting volumetrically 2 ml of the mixed aflatoxin stock solution (containing 1000 ng/ml of aflatoxins B₁ and G₁, and 200 ng/ml of aflatoxins B₂, and G₂) into a 10-ml calibrated volumetric flask. Evaporate the toluene/acetonitrile solution just to dryness under a stream of nitrogen at room temperature. Dilute to the mark with methanol and shake well. The aflatoxin concentrations of this spiking solution is 200 ng/ml of B₁ and G₁, and 40 ng/ml of B₂, and G₂. Wrap the flask tightly in aluminium foil and store it at less than 4°C. Before use, do not open the flask until the contents have reached room temperature to avoid incorporation of water by condensation.

APPARATUS

General

Wash all glassware coming into contact with aqueous solutions of aflatoxins with acid solution prior to use, e.g. with sulphuric acid (2 mol/l) for several hours. Rinse well with water to remove traces of acid and check the absence of acid with pH paper.

Volumetric glassware (flasks and pipettes). Class A.

Reservoir. 10 ml with luer tip connector for immunoaffinity column (IAC).

High performance liquid chromatograph equipped with a fluorescence detector with a wavelength of 360 nm excitation filter and a wavelength 420 nm cut-off emission filter; sample injector; a valve with a 200- μ l loop is recommended.

Integrator or other data system.

Column. any RP-C18 column with an appropriate guard column (with the same packing material as the column). Flow and retention times vary due to column dimension.

Balance. 0.01 mg accuracy

Post-column derivatisation system. Kobra Cell[®] - System for derivatisation with electrochemically-generated bromine.

Calibrated microlitre syringes or microlitre pipettes. 10 to 500 μ l

Manifold

Disposable filter units (optional). Prior to usage, verification that no aflatoxin losses occur during filtration is necessary (recovery testing).

PROCEDURES

(a) *Chromatographic conditions*

<i>Column temperature</i>	room temperature
<i>Flow rate</i>	depending on column
<i>Wavelength</i>	360 nm excitation filter and above 420 nm cut-off emission filter, or equivalent
<i>Injection volume</i>	200 μ l
<i>Retention times</i>	order of elution: G ₂ , G ₁ , B ₂ , B ₁ . Retention times depend on the column.

(b) *Linearity check.* Check the linearity of the detector response by using the mixed aflatoxin standard solutions as described in the previous Section on Reagents. These solutions cover the range of 0.1 ng/ml to 3.0 ng/ml for B₁ and G₁ and also the range of 0.02 ng/ml to 0.6 ng/ml for B₂ and G₂.

If aflatoxin contents in the sample are outside the calibration range, prepare diluted or concentrated sample solutions with aflatoxin contents appropriate for the established calibration curve.

(c) Preparation of the sample solution.

Weigh (to the nearest 0.1 g) 5 g of NeemAzal into a 100 ml volumetric flask and fill up with the extraction solvent to the mark. Shake thoroughly by hand. Take 5 ml of the extract and dilute it with 20 ml of Tween 20[®] solution. Add the diluted sample extract to a 10-ml reservoir connected to the conditioned immunoaffinity column and proceed as described below.

Immunoaffinity cleanup.

Follow different conditioning procedures according to the manufacturer's instruction.

Connect the column to a manifold and attach a 10-ml reservoir to it. Take 9 ml of the sample solution and transfer it to the reservoir and let it pass through the column at a flow rate of approximately 3 ml/min. Do not exceed a flow rate of maximum 5 ml/min. Wash the column three times with 5-ml portions of water and dry it by applying little vacuum for 5 to 10 seconds or passing air through the column by means of a syringe.

Elute the aflatoxins in a two-step procedure by applying 0.75 ml methanol to the column and letting it pass through by gravity. Collect the eluate in a 3-ml volumetric flask and repeat the elution into the same flask with another portion of 0.75 ml after 1 minute. Collect most of the methanol by passing air through the column. Make up with water and shake well. If a clear solution is obtained, use directly for HPLC analysis, but otherwise use a disposable filter prior to injection.

(d) HPLC determination. Inject 200 µl of each sample solution in duplicate. The aflatoxins are separated by RP-phase at room temperature. The recommended flow rate is 1.0 ml/min for a column of inner diameter of 4.6 mm and may be adjusted according to the column dimension used. The aflatoxins elute in the order G2, G1, B2 and B1 and should be base-line resolved (typical chromatograms are enclosed).

(e) Post column derivatisation

When using a KOBRA cell[®] (electrochemically-generated bromine), follow the instructions for installing the cell as supplied by the manufacturers, and operate with a flow rate of 1.0 ml/min for the mobile phase and a current of 100 µA.

(f) *Spiking procedure*

For recovery determinations, carry out the spiking procedure using the methanol spiking solution of aflatoxin standards. The spiking level must be within the calibration range (preferably mean value). Take care that not more than 2 ml of the spiking solution are added and that the subsequent evaporation takes place in the dark and should last 30 min to 2 h.

(g) *Calculation.* Calculate the concentration of aflatoxins in ng/g from the established calibration curve using linear regression.

$$\text{Content of aflatoxin} = \frac{C_{Afla} \cdot V_e \cdot V_{final}}{m_S \cdot V_{iac}} \text{ ng/g}$$

where:

C_{Afla} = concentration of aflatoxin in the injection solution calculated from linear regression, in ng/ml

V_e = volume of the extraction solvent in ml ($V_e = 100$ ml)

V_{final} = final volume after elution from IAC in ml (3 ml)

m_S = mass of the sample taken for analysis in g ($m_S = 5$ g for NeemAzal)

V_{iac} = volume of the sample extract taken for IAC in ml (9 ml)

(h) *Recovery.* The recovery (R) is calculated using the following equation:

$$R(\%) = \frac{R_{fortified}}{F} \cdot 100\%$$

$R_{fortified}$ = residues measured in the fortified specimen, in $\mu\text{g/kg}$

F = fortification level, in $\mu\text{g/kg}$

2. Aflatoxins B₁, B₂, G₁ and G₂ (in NeemAzal-T/S)

Sampling. Take at least a 50 ml sample of NeemAzal-T/S.

Follow details as for aflatoxins in NeemAzal technical except for the following:

(c) *Preparation of the sample solution.*

Weigh (to the nearest 0.1 g) 20 g of NeemAzal-T/S into a 100 ml volumetric flask and make up with the extraction solvent. Proceed as described for NeemAzal technical.

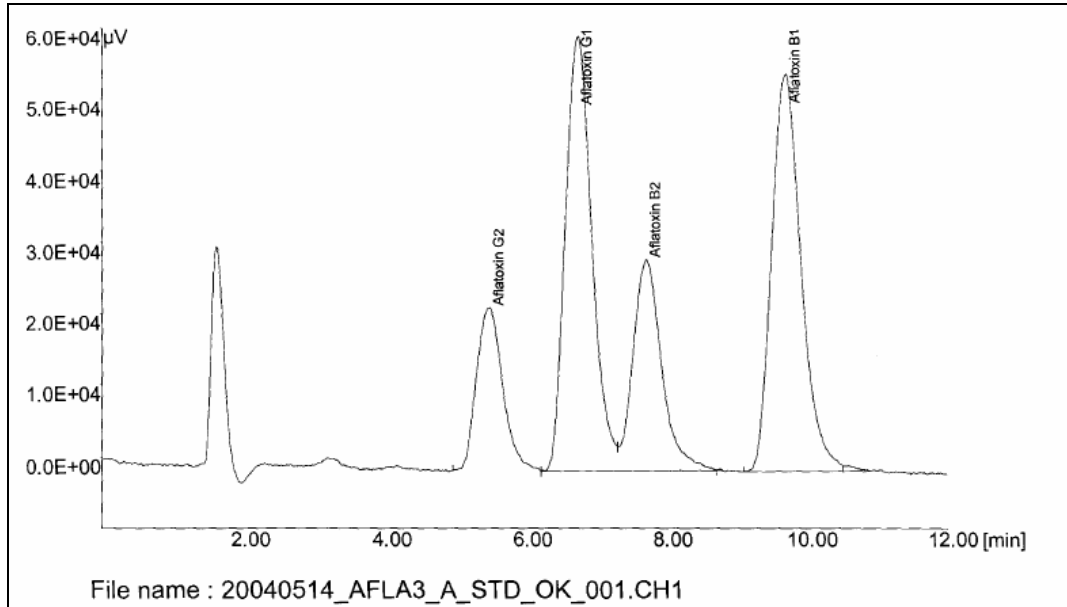
Representative chromatograms

Fig. 1: Chromatogram of a multiple aflatoxin reference standard (1 ppb).

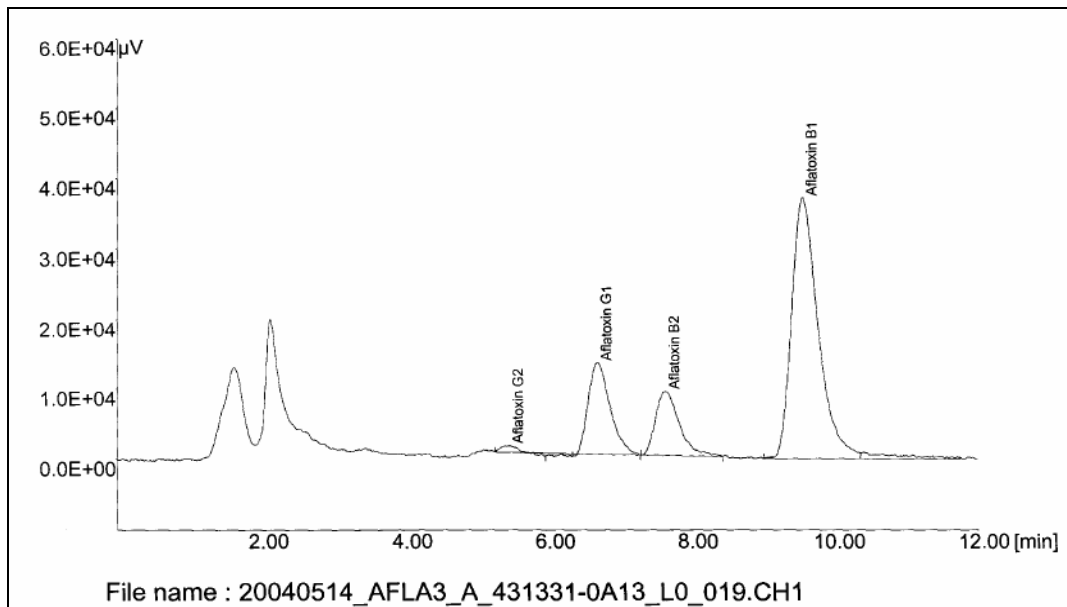


Fig. 2: Chromatogram of a sample extract of NeemAzal technical (TK)

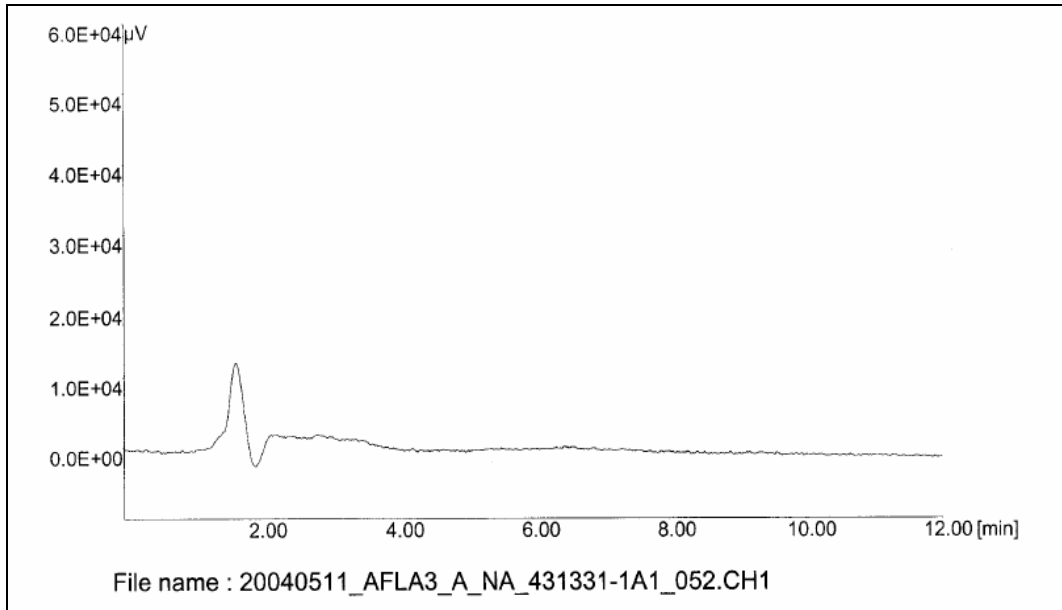


Fig. 3: Chromatogram of a sample extract of NeemAzal-T/S (EC)