II

(Acts whose publication is not obligatory)

COMMISSION

COMMISSION DIRECTIVE 92/95/EEC

of 9 November 1992

amending the Annex to the Seventh Directive 76/372//EEC establishing Community methods of analysis for the official control of feedingstuffs

THE COMMISSION OF THE EUROPEAN COMMUNITIES,

Having regard to the Treaty establishing the European Economic Community,

Having regard to Council Directive 70/373/EEC of 20 July 1970 on the introduction of Community methods of sampling and analysis for the official control of feedingstuffs (1), as last amended by the Acts of Accession of Spain and Portugal, and in particular Article 2 thereof,

Whereas the Seventh Commission Directive 76/372/EEC of 1 March 1976 establishing Community methods of analysis for the official control of feedingstuffs (2), as amended by Directive 81/680/EEC (3), prescribes the methods to be used for the determination of aflatoxin B_1 ;

Whereas there are grounds for adapting these methods in the light of advances in scientific and technical knowledge ; whereas it is advisable particularly to have available a method of controlling the very low limits for aflatoxin fixed by Council Directive 74/63/EEC of 17 December 1973 on the fixing of maximum permitted levels for undesirable substances and products in animal nutrition (4), as last amended by Directive 91/132/EEC (5) ;

Whereas it is also advisable to have available a method of determining aflatoxin B_1 in the presence of interfering substances, such as citrus pulp ;

Whereas the measures provided for in this Directive are in accordance with the opinion of the Standing Committee for Feedingstuffs,

HAS ADOPTED THIS DIRECTIVE :

Article 1

The Annex to Directive 76/372/EEC is amended in accordance with the Annex to this Directive.

Article 2

Member States shall bring into force not later than 1 October 1993 the laws, regulations and administrative provisions necessary to comply with the provisions of this Directive. They shall fortwith inform the Commission thereof.

When Member States adopt these provisions, they shall contain a reference to this Directive or shall be accompanied by such reference at the time of their official publication. The procedure for such reference shall be adopted by the Member States.

Article 3

This Directive is addressed to the Member States.

Done at Brussels, 9 November 1992.

For the Commission

Ray MAC SHARRY

Member of the Commission

(1) OJ No L 170, 3. 8. 1970, p. 2.
(2) OJ No L 102, 15. 4. 1976, p. 8.
(3) OJ No L 246, 29. 8. 1981, p. 32.
(4) OJ No L 38, 11. 2. 1974, p. 31.
(5) OJ No L 66, 13. 3. 1991, p. 16.

ANNEX

I. Under Part A 'One-dimensional thin layer chromatographic method' the text at point 1 'Purpose and scope' is replaced by the following text:

'1. **Purpose and scope**

The method makes it possible to determine the level of aflatoxin B_1 in raw materials and straight feedingstuffs. This method can not be applied in the presence of citrus pulp. The lower limit of determination is 0,01 mg/kg (10 ppb).

In the presence of interfering substances it is necessary to repeat the analysis using method B (high performance liquid chromatography).'

II. Under Part B 'Two-dimensional thin layer chromatographic method' the text is replaced by the following:

'B. DETERMINATION OF AFLATOXIN B_1. HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD

1. **Purpose and scope**

This method is for the determination of aflatoxin B_1 in animal feedingstuffs including those containing citrus pulp. The lower limit of determination is 0,001 mg/kg (1 ppb).

2. **Principle**

The sample is extracted with chloroform. The extract is filtered and an aliquot portion is purified on a Florisil cartridge followed by a C_{18} cartridge. The final separation and determination is achieved by high performance liquid chromatography (HPLC) using a reversed phase C_{18} column, followed by post-column derivatization with iodine in water, and fluorescence detection.

Note:

mycotoxins are extremely toxic sustances. Manipulations should be performed in a designated fume cupboard. Special precautions should be taken when toxins are in a dry form because of their electrostatic nature and resulting tendency to disperse in working areas.

3. **Reagents**

3.1. Chloroform, stabilized with 0,5 to 1,0 % of ethanol, by mass. See observation 10.2.

3.2. Methanol, HPLC grade for preatation of 3.6.

3.3. Acetone.

3.4. Acetonitril, HPLC grade.

3.5. Eluting solvents: Prepare one day before use, or remove air in the solvents ultrasonically.

3.5.1. Mixture of acetone (3.3) and water, 98 + 2 (v + v).

3.5.2. Mixture of water and methanol (3.2), 80 + 20 (v + v).

3.5.3. Mixture of water and acetone (3.3), 85 + 15 (v + v).

3.6. Mobile phase for HPLC

Mixture of water, methanol (3.2) and acetonitril (3.4), 130 + 70 + 40 (v + v + v).

NB The composition of the mobile phase solvent may need to be adjusted, depending on the characteristics of the HPLC column used.

3.7. Saturated iodine solution: add 2 g of iodine to 400 ml of water. Mix for at least 90 min and filter through a membrane filter (4.15). Protect the saturated solution from light to prevent photodegradation.

3.8. Acid washed Celite 545, or equivalent.

3.9. Florisil cartridge (Waters SEP-PAK), or equivalent.

3.10. C_{18} cartridge (Waters SEP-PAK), or equivalent.

3.11. Inert gas e.g. nitrogen.

3.12. Aflatoxin B_1 standard solution in chloroform, concentration 10 µg/ml. Check the concentration of the solution as follows: determine the absorption spectrum of the solution between 330 and 370 nm by means of the spectrophotometer (4.23). Measure the absorbance (A) at the maximum near 363 nm. Calculate the concentration of aflatoxin B_1 in micrograms per millilitre of solution from the formula:

$$\text{Concentration } (\mu g/ml) = \frac{312 \times A \times 1000}{22\,300} = 13{,}991 \times A$$

3.12.1. Aflatoxine B_1 stock standard solution in chloroform.

Transfer quantitatively 2,5 ml of the aflatoxin B_1 standard solution (3.12) to a 50 ml volumetric flask and adjust to the mark with chloroform (3.1). Store this solution in a cool place (4 °C) in the dark, well sealed and wrapped in aluminium foil.

3.13. Aflatoxin B_1 calibraltion solutions HPLC.

NB Use alcid-washed glassware for preparation of these solutions (see 4, Apparatus).

3.13.1. Calibration solution 4ng/ml.

Allow the volumetric flask with stock standard solution (3.12.1) to warm up to room temperature in the aluminium foil (a few hours). Transfer 400 µl of the stock standard solution (200 ng aflatoxin B_1) into a 50 ml volumetric flask, and evaporate the solution to dryness in a current of inet gas (3.11).

Dissolve the residue obtained in approximately 20 ml of water/acetone mixture (3.5.3), make up to the mark with the water/acetone mixture and mix well.

3.13.2. Calibration solution 3 ng/ml.

Transfer quantitatively 7,5 ml of the calibration solution (3.13.1) into a 10 ml volumetric flask, make up to the mark with the water/acetone mixture (3.5.3), and mix well.

3.13.3. Calibration solution, 2 ng/ml.

Transfer quantitatively 25 ml of the calibration solution (3.13.1) to a 50 ml volumetric flask, make up to the mark with the water/acetone mixture (3.5.3) and mix well.

This solution is also referred to as the reference standard, to be used for repetitive injection during HPLC (5.5).

3.13.4. Calibration solution 1 ng/ml.

Transfer quantitatively 2,5 ml of the calibration solution (3.13.1) to a 10 ml volumetric flask, make up to the mark with the water/acetone mixture (3.5.3) and mix well.

3.14. Ampoule containing a mixture of aflatoxins B_1, B_2, G_1 and G_2 concentrations approximately 1, 0,5, 1 and 0,5 µg/ml respectively, in 1 ml chloroform.

3.14.1. Chromatographic test solution.

Transfer the content of the ampoule (3.14) into a glass-stoppered test-tube or screw-capped vial. Transfer 40 µl of this solution into a glass-stoppered test-tube (acid-rinsed) (4.22). Evaporate the chloroform in a stream of inert gas (3.11) and redissolve into 10 ml of the water/acetone mixture (3.5.3).

3.15. Reagents for confirmatory test (6).

3.15.1. Sodium chloride saturated solution.

3.15.2. Sodium sulphate, anhydrous, granular.

4. Apparatus

Caution: Use of non acid-washed glassware for aqueous aflatoxin solutions may cause losses. Particular care should be taken with new glassware and disposable glassware such as autosampler vials and Pasteur pipettes. Therefore laboratory glassware coming into contact with aqueous solutions of aflatoxins should be soaked in dilute acid (e.g. sulfuric acid = 2 mol/l) for several hours, then rinsed well with distilled water to remove all traces of acid (e.g. three times, check with pH-paper). In practice, this treatment is necessary for the round bottomed flask (4.4), the volumetric flasks, measuring cylinders, vials or tubes used for calibration solutions and final extracts (particularly vials for autosamplers), and Pasteur pipettes, if these are used to transfer calibration solutions or extracts.

4.1. Grinder-mixer.

4.2. Sieve of aperture size 1,0 mm, (ISO R 565).

4.3. Mechanical shaker.

4.4. Rotary vacuum evaporator, equipped with a 150 to 250 ml round bottomed flask.

4.5. High performance liquid chromatograph, injector with a loop suitable for the injection of 250 ml. See the manufacturers instructions for partial or complete loop filling.

4.6. HPLC analytical column : 3 µm or 5 µm C_{18} packing.

4.7. Pulse-free pump for delivery of the iodine post-column reagent.

4.8. Valco zero dead volume Tee, stainless steel (1/16" × 0,75 mm).

4.9. Spiral reaction coil ; Teflon or stainless steel. Dimensions of 3 000 × 0,5 mm to 5 000 × 0,5 mm have been found to be appropriate in combination with 5 µm or 3µm HPLC columns.

4.10. Thermostatically controlled water-bath adjusted to 60 °C, capable of temperature regulation to better than 0,1 °C.

4.11. Fluorescence detector, with excitation at 365 nm and emission at 435 nm wavelenths. (For filter instrument : emission wavelength > 400 nm). Detection of at least 0,05 ng aflatoxin B_1 shall be possible. Some back pressure may be advisable (e.g. restrictor, Teflon or stainless steel coil connected to the outlet of the detector), to suppress air bubbles in the flow-cell.

4.12. Strip chart recorder.

4.13. Electronic integrator (optional).

4.14. Fluted filter paper diameter : 24 cm, Macherey-Nagel 617 1/4 or equivalent.

4.15. Membrane filter with a pore size of 0,45 µm, Millipore HAWP 04700 or equivalent.

4.16. 500 ml glass stoppered conical flask.

4.17. Glass column (internal diameter approximately 1 cm, length approximately 30 cm) equipped with a Luer tip.

4.18. Luer chloroform-resistant stopcock (e.g. Bio-rad 7328017, Analytichem A1 6078, J.T. Baker 4514 or equivalent).

4.19. Chemically resistant syringe, 10 ml Luer connector.

4.20. Syringe suitable for HPLC injection of 250 µl (see 4.5).

4.21. 100 µl microsyringe for preparation of calibration solutions (check that the accuracy is within 2 % by weighing).

4.22. 10 ml glass stoppered calibrated tubes.

4.23. Spectrophotometer, suitable for making measurements in the UV region of the spectrum.

4.24. Equipment for confirmatory test (6).

4.24.1. Acid-rinsed 100 ml separating funnel with Teflon stopcock.

4.24.2. Heating block, 40 to 50 °C.

5. Procedure

5.1. *Preparation of the sample.*

Grind the sample so that it passes through the sieve (4.2).

5.2. *Test portion.*

Weigh 50 g of the prepared test sample into the conical flask (4.16).

5.3. *Extraction*

Add 25 g of Celite (3.8), 250 ml of chloroform (3.1) and 25 ml of water to the test portion (5.2). Stopper the flask, and shake for 30 minutes on a mechanical shaker (4.3). Filter through a fluted filter paper (4.14). Collect 50 ml of the filtrate. If necessary, take an aliquot of the filtrate and dilute to 50 ml with chloroform so that the concentration of aflatoxin B_1 is not greater than 4 ng/ml.

5.4. *Clean-up* (the procedure should be carried out without significant interruptions).

Caution :

— protect the laboratory, where the analyses are done, adequately from daylight. This can be achieved effectively by using :

(i) UV absorbing foil on the windows in combination with subdued light (no direct sunlight);

(ii) Curtains or blinds in combination with artificial light (fluorescent tubes are acceptable);

— Aflatoxin containing solutions must be protected from light as much as possible (keep in the dark, use aluminium foil).

5.4.1. Florisil SEP-PAK purification

5.4.1.1. Preparation of the column-cartridge assembly

Attach a stopcock (4.18) to the shorter stem of a Florisil cartridge (3.9) (see figure 1). Wash the cartridge and remove aid by taking 10 ml chloroform (3.1) and passing 8 ml via the stopcock rapidly through the cartridge using a syringe (4.19). Attach the longer stem of the cartridge to a glass column (4.17) and pass the reamining 2 ml chloroform through the cartridge into the column. Close the stopcock. Remove the syringe.

5.4.1.2. Purification

Add the filtrate collected in 5.3 to the column-cartridge assembly and drain by gravity. Rinse with 5 ml of chloroform (3.1), followed by 20 ml of methanol (3.2). Discard the eluates. During these operations, ensure that the column-cartridge assembly does not run dry.

Elute aflatoxin B_1 with 40 ml of the acetone-water mixture (3.5.1) and collect the whole of the eluate in the round bottomed flask of the rotary evaporator (4.4). Concentrate the eluate on the rotary evaporator at 40 °C to 50 °C until no more acetone is distilled. (*NB* approximately 0,5 ml of liquid remains in the flask at this point. Experiments have shown that further evaporation is not harmful and that when 0,5 ml of liquid remains, there is then no significant amount of acetone. Residues of acetone might lead to losses of aflatoxin B_1 on the C_{18} cartridge) Add 1 ml of methanol (3.2), swirl the flask to dissolve aflatoxin B_1 on the sides of the flask, add 4 ml water, and mix. Disconnect and discard the cartridge. Rinse the glass column with water and retain for the C_{18} purification step.

5.4.2. C_{18} SEP-PAK purification

5.4.2.1. Preparation of the column-cartridge assembly.

Attach a stopcock (4.18) to the shorter stem of a C_{18}-cartridge (3.10) (see figure 1). Prime the cartridge and remove any air by passing 10 ml methanol (3.2) via the stopcock rapidly through the cartridge with a syringe (4.19) (Air bubbles in the cartridge are visible as light spots on the otherwise greyish background). Take 10 ml water, and pass 8 ml through the cartridge (Avoid introduction of air into the cartridge, when switching from methanol to water). Attach the longer stem of the cartridge to a glass column (4.17) and pass the remaining 2 ml water through the cartridge in the column. Close the stopcock. Remove the syringe.

5.4.2.2. Purification

Transfer the extract collected in 5.4.1.2 quantitatively to the glass column (4.17), rinsing the flask twice with 5 ml water/methanol mixture (3.5.2) and drain by gravity. During these operations, ensure that the column-cartridge assembly does not run dry. (When air bubbles develop in the constriction near the cartridge, stop the flow and tap the top of the glass column, to remove the air bubbles. Then continue). Elute with 25 ml water/methanol mixture. Discard the eluate. Elute the aflatoxin B_1 with 50 ml water/acetone mixture (3.5.3), and collect the whole of the eluate in a 50 ml volumetric flask. Make up to the mark with water and mix : the resulting test solution is used for chromatography (5.5).

Caution : Filtration of the final extract prior to HPLC is normally not necessary. If considered necessary, cellulose filters are not to be used, because they may lead to losses of aflatoxin B_1. Teflon filters are acceptable.

5.5. *High performance liquid chromatography*

(See Figure 2 for setting-up of the equipment). Allow sufficient time for conditioning and stabilizing the instruments.

Note 1 :

The flow-rates given for the mobile phase and the post-column reagent are indicative only. They may need to be adjusted depending on the characteristics of the HPLC column.

Note 2:

The detector response to aflatoxin B_1 depends on the temperature, therefore compensation should be made for drift (see Figure 3). By injecting a fixed amoung of aflatoxin B_1 reference standard (3.13.3) at regular intervals (i.e. every third injection), the aflatoxin B_1 peak values between these reference standards can be corrected using the mean response, provided that the difference between responses of consecutive reference standards is very small (< 10 %). Therefore injections must be made without interruptions. If interruption is necessary, the last injection before interruption and the first injection after interruption must be the reference standard (3.13.3). Because the calibration curve is linear and passes through the origin, the amounts of aflatoxin B_1 in the sample extracts are determined directly by reference to the adjacent standards.

5.5.1. HPLC pump settings

Set the HPLC pump (4.5) to give a flow of 0,5 or 0,3 ml/min for a 5 µm or a 3 µm HPLC column (4.6) respectively using the mobile phase (3.6).

5.5.2. Post-column pump settings

Set the pump (4.7) to give a flow of 0,2 to 0,4 ml/min of the iodine-saturated water solution (3.7). As a rough guide: Flows of approximately 0,4 or 0,2 ml/min are advisable in combination with flows of 0,5 and 0,3 ml/min of the mobile phase (3.6) respectively.

5.5.3. Fluorescence detector

Set the fluorescence detector (4,11) to exc. = 365 nm and em = 435 nm (filter instrument; > 400 nm). Adjust the detector attenuator to obtain approximately 80 % full scale deflection of the recorder pen for 1 ng of aflatoxin B_1.

5.5.4. Injector

For all solutions, inject 250 µl amounts following the instructions of the manufacturer of the injector.

5.5.5. Check of chromatographic separation

Inject the chromatographic test solution (3.14.1). Valleys should be less than 5 % of the sum of peak heights of the adjacent peaks.

5.5.6. Check of the stability of the system

Before each series of analyses, respectively inject the reference standard (3.13.3), until stable peak areas are achieved (*NB* Peak responses for aflatoxin B_1 between consecutive injections should not differ by more than 6 %). Proceed without delay with the check of linearity (5.5.7).

5.5.7. Check of linearity

Inject the aflatoxin B_1 calibration solutions (3.13.1 to 3.13.4). Every third injection use the reference standard (3.13.3), for correction of drift in response (*NB* Peak responses for this reference standard must not differ by more than 10 % in 90 minutes). Correct for drift according to the formula in 7. The calibration graph should be linear and pass through the origin, within twice standard error of Y-estimate. Values found must not differ by more than 3 % from the nominal values. If these requirements are fulfilled, continue without delay. If not, identify and correct the sources of the problem before continuing.

5.5.8. Injection of sample extracts

Inject the purified sample extracts (5.4.2.2). After every two sample extracts repeat the injection of the reference standard (3.13.3) according to the following sequence: reference standard, extract, extract, reference standard, extract, extract, reference standard etc.

6. Confirmatory test

6.1. *Further treatment of the extract (5.4.2.2)*

Add 5 ml sodium chloride solution (3.15.1) to the final extract obtained at 5.4.2.2.. Extract three times each with 2 ml chloroform (3.1) for one minute, in the separating funnel (4.24.1). Pour the combined chloroform extracts over approximately 1 g sodium sulphate (3.15.2) into a 10 ml test tube. A small funnel (diameter: 4 cm) can be used with a piece of cottonwool in the constriction, covered with a approximately 1 g sodium sulphate.

Wash the sodium sulphate layer with a few ml of chloroform and collect the washing in the same test tube. Evaporate the chloroform extract to dryness in the same test-tube using the heating block (4.24.2) and redissolve in 1 ml of chloroform.

6.2. *Preparation of derivative and thin layer chromatography:*

See Annex to Council Directive 76/372/EEC method A, point 5.6.2.

7. **Calculation of results**

Calculate the aflatoxin B_1 content (µm/kg) present in the sample, using the formula:

$$\text{aflatoxin } B_1 \text{ content in } \mu g/kg = \frac{m \times V_{ext}}{V_m \times M \times \frac{V_f}{V_c}}$$

where:

m = amount of aflatoxin B_1 in ng represented by the B_1 peak of the sample, calculated as follows:

$$m = \frac{P(\text{sample})}{P(st_1) + P(st_2)} \times 2\, r(st)$$

P(sample) = peak area of aflatoxin B_1 for the sample

$P(st_1)$ = peak area of aflatoxin B_1 resulting from the preceeding injection of reference standard (3.13.3)

$P(st_2)$ = peak area of aflatoxin B_1 resulting from the following injection of reference standard (3.13.3)

r(st) = injected amount of aflatoxin B_1 in the reference standard (3.13.3) in ng

V_m = volume of the injected sample extract in ml

V_{ext} = final volume of sample extract in ml, allowing for any dilution that was made (5.3)

M = mass of sample in g

V_f = volume of filtrate transferred to Florisil cartridge (5.4.1.2) in ml

V_c = volume of chloroform, used for the extraction of the sample in ml

If the procedure is followed as in this protocol, the formula reduces to:

aflatoxin B_1 content in µg/kg = 20 × m.

7.1. Calculations of the results may also be done by peak height measurement.

8. **Repeatability:**

see under 10.1

9. **Reproducibility:**

see under 10.1

10. **Observations**

10.1. *Precision*

A collaborative study (1), carried out at the international level on mixed feeding stuffs gave the results for repeatability and reproducibility indicated in Table 1. The term repeatability (r) used here is defined as the largest ratio which is not significant at the 95 % probability level for comparison of two readings of the same sample in the same laboratory under similar conditions. The term reproducibility (R) is similarly defined for comparing two different laboratories. In accordance to ISO 3534 — 1977, 2.35 (2) and Commission Decision 89/610/EEC (3) r and R are also given in Table 1 in terms of coefficients of variation.

Table 1

Repeatability (r) and reproducibility (R) expressed as ratios and corresponding coefficients of variation

(15 laboratories)

Level	r	R	CV_r(*)	CV_R
(µg/kg)			(%)	(%)
8 & 14	1,4	1,7	11	18

(*) CV = coefficient of variation

(1) Egmond, H.P. van, Heisterkamp, S.H. and Paulsch, W.E. (1991). *Food Additives and Contaminants* 8, 17-29.
(2) ISO 3534-1977.
(3) OJ No L 351, 2. 12. 1989, p. 39.

10.2. *Stabilization of chloroform (3.1)*

The adsorption characteristics of the florisil cartridge may be changed if stabilizers other than ethanol are used. This should be verified in accordance with 10.3 when the chloroform described is not available.

10.3. *Accuracy*

The correct applications of the method shall be verified by making replicate measurements on certified reference materials. If these are not available, the performance of the method should be verified by recovery experiments made on the fortified blank samples. The deviation of the mean from the actual value, expressed as a percentage of the actual value, shall not lie outside the limits −20 to +10 %.

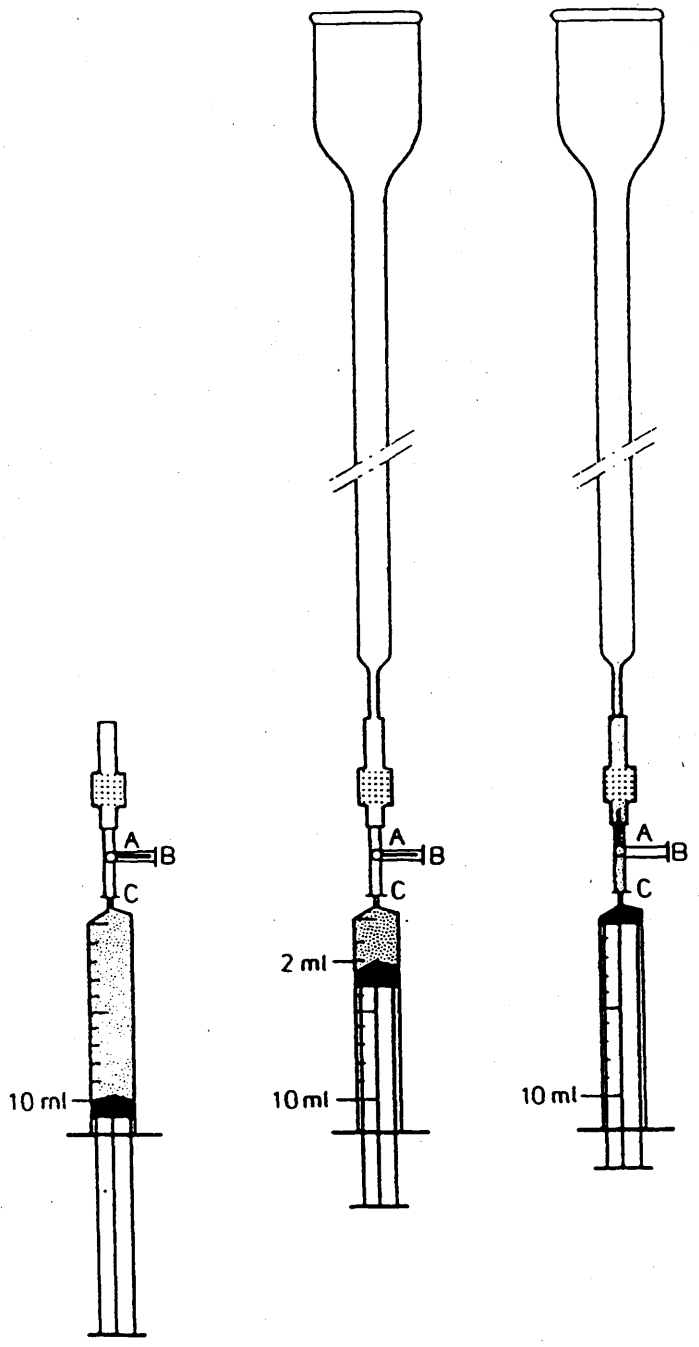
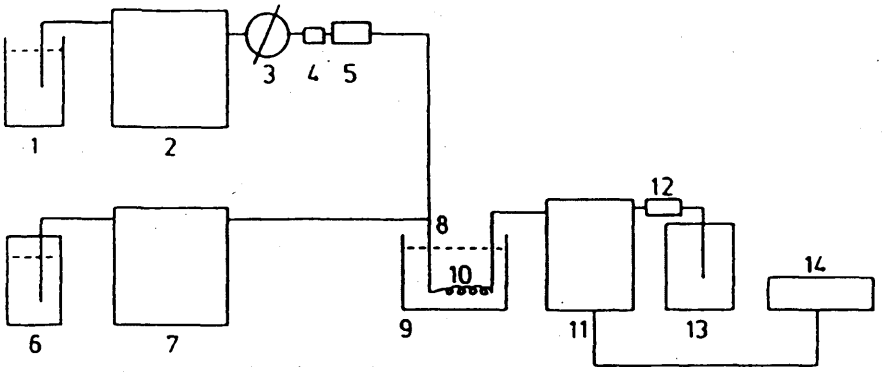


Figure 1: Column-cartridge assembly



1. Mobile phase
2. Pump
3. Injection valve
4. Guard column
5. HPLC analytical column
6. Saturated iodine solution
7. Reagent pump
8. T-joint
9. Thermostatically controlled bath
10. Spiral reaction coil
11. Fluorescence detector
12. Restrictor
13. Waste
14. Strip chart recorder/integrator

Figure 2: Flow diagram of the LC system with iodine post-column derivatization

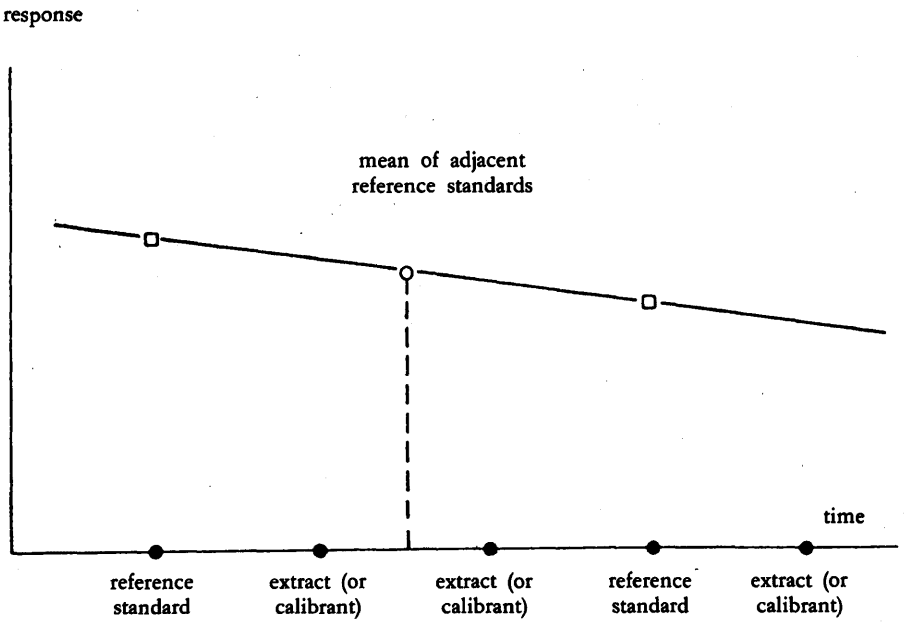


Figure 3: Compensation for drift in aflatoxin B_1 response by injecting reference standard (3.13.3) at regular intervals'