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«TRAINING ACTIVITIES ON FOOD CONTAMINATION CONTROL AND MONITORING WITH SPECIAL REFERENCE TO MYCOTOXINS»

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ANALYTICAL METHODS OF DETECTION, IDENTIFICATION AND QUANTITATIVE DETERMINATION OF AFLATOXINS IN FOODSTUFFS AND FODDER

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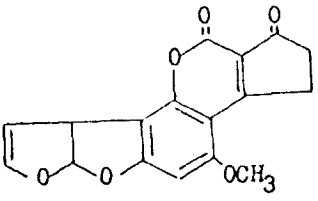
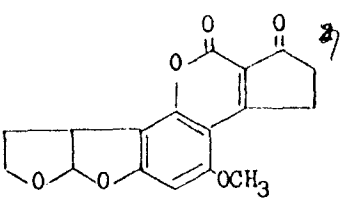
GENERAL INFORMATION ON THE CHEMICAL METHODS OF ANALYSIS OF AFLATOXINS B_1, B_2, G_1, G_2, M_1

Aflatoxins (AT) are secondary metabolites of microscopic molds of the Aspergillus sp. with pronounced toxic, hepatocarcinogenic, mutagenic, and teratogenic properties. AT contaminate grains (wheat, rye, barley, rice, oats, sorghum and millet, buckwheat, corn), pulses (peas, beans, soya), nuts (walnut, pecan, filbert, hazel. almond, pistachio, cashew, Brazil nut, groundnut, marzipan), as well as cocoa and coffee benas, tea, sunflower and cotton seeds, some spices and the products of their reprocessing. Analysis of the products of animal origin has shown that AT B_1 and its hydroxylated metabolite M_1 can be present in the tissues and milk of animals, eggs, of poultry that together with feed receive high doses of AT.

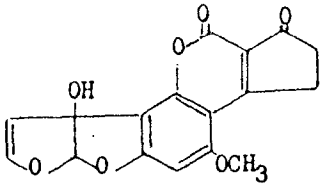
AT belong to the group of bis-furacoumarins and contain in their molecule lactone, a carbonyl and a methoxyl group, a benzene ring and an isolated double bond. The structure of a number of AT and metabolites akin to aflatoxin B_1 is shown in Fig. 1. AT B_2 and G_2 are dihydroderivatives of the initial compounds. AT M_1 and M_2 are hydroxylated metabolites of AT B_1 and B_2, respectively. AT are readily soluble in moderately polar organic solvents (acetone, chloroform, dichloromethane, dimethylsulphoxide, methanol, ethanol, isopropanol), moderately soluble in ether and petroleum ether. AT solubility in water varies within 10-20 mg/l. As pure substances AT are extremely thermostable at heating in air. They are, however, relatively easily decomprosed under the action of light, especially ultraviolet (UV) radiation, and air on thin layer chromatographic (TLC) plates, as well as when being dissolved in highly polar solvents. AT dissolved in chloroform and benzene preserve their stability over a period of several years when stored in the dark and in the cold.



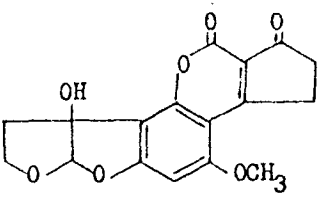
AFLATOXIN B_I



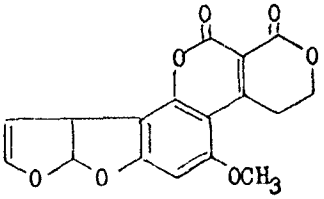
AFLATOXIN B_2



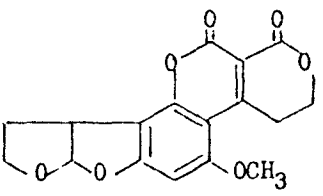
AFLATOXIN M_I



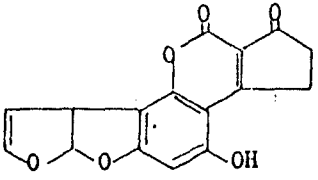
AFLATOXIN M_2



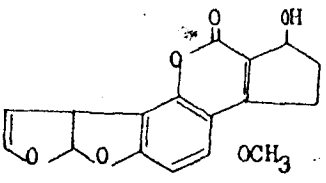
AFLATOXIN G_I



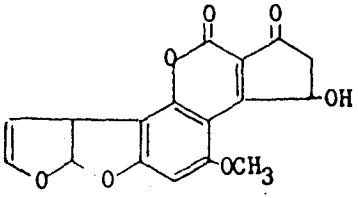
AFLATOXIN G_2



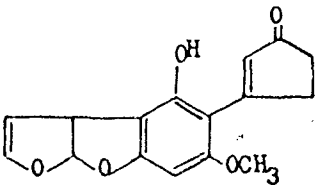
AFLATOXIN P_I



AFLATOXICOLE



AFLATOXIN Q_I



AFLATOXIN D_I

Fig.1. The structure of some aflatoxins.

Long-wave UV radiation induces intensive AT fluorescence. This makes it possible to determine these compounds in extremely low concentrations (about 0.5 ng and less in a spot) by means of the TLC method.

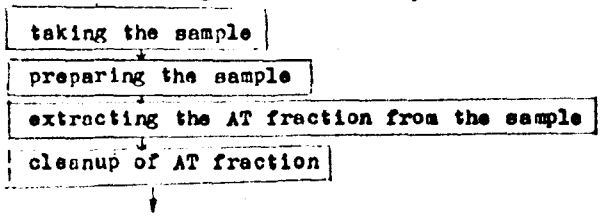
At present more than 500 chemical methods of analyzing AT and their modofications are known, which accounts for more than 40% of the total number of the methods developed for mycotoxin determination. Despite the fact that high-resolution liquid chromatography has recently come to the foreground, the methods based on TLC still occupy the leading place in AT methodology.

The methods of AT determination must satisfy the following basic requirements:

1. Selectivity with respect to aflatoxins
2. High sensitivity
3. Degree of aflatoxin extraction no less than 75%.

The main stages of all possible modifications of the methods are: extraction, extract cleanup, detection, and quantitative determination of toxins. Depending on the nature of the investigated compound some of the stages can be excluded, or some new additional stages introduced. For instance, an enhanced content of lipids in cocoa and groundnut requires additional degreasing, the presence of thrombin in cocoa, of caffeine in coffee, of gossypol in cotton plant - the removal of these components hindering AT detection and determination.

Schematic diagram of the analysis is as follows:



AT detection with TLC
↓
confirmatory tests
↓
quantitative AT determination with TLC

Equipment and materials

To perform routine analyses for AT determination in food products it is necessary to have the following equipment and materials:

1. Mercury-quartz lamp with a transmission region of 360 nm
2. Sample shaker
3. Laboratory mill
4. Drying cabinet
5. Counter balance
6. Analytical balance
7. Centrifuge with glass inserts
8. Rotational evaporator with trap
9. Water bath
10. Household refrigerator
11. Microsyringes or calibrated capillaries
12. Chromatographic glass columns, 300 x 22 mm
13. TLC chambers with ground caps
14. Laboratory propeller mixer
15. Attachment for vacuum filtration
16. Water-jet pump
17. Yellow filters for eye protection
18. Chromatographic plates, 15 x 15 or 20 x 20 cm
19. Bunsen flasks
20. Flat-bottom conical flasks, 250 and 500 ml. NS 29

21. Round-bottom flasks, 200 ml, NS 29
22. Conical flasks, 250 and 100 ml, NS No.14.5
23. Flasks for concentration by evaporation, 50 and 25 ml, NS No. 14.5
24. Volumetric flasks, 25, 50, 100, 250, 1000 ml
25. Separating funnels, 250, 500, 1000 ml
26. Buchner funnels
27. Glass funnels, 150-200 mm dia.
28. Funnels for granular substances
29. Glass bottles with ground (or polyethylene) stoppers, 500 ml
30. Test tubes, 10 and 5 ml, NS No.15.5, with ground stoppers
31. Measuring cylinders, 10, 50, 100, 250 and 500 ml
32. Pipettes, 1, 2 and 5 ml
33. Micropipettes, 0.2 and 0.1 ml
34. Glass rods ca 250 mm long
35. Liquids sprayer with a bulb
36. Exsiccator for plates, 15x15 or 20x20 cm
37. Glass capillaries
38. Glass tubes with ca 44 mm inside dia. for minicolumns
39. Soxhlet extractor, 75 or 100 ml
40. Weighing bottles, 50 and 100 ml with ground covers
41. Petri dishes
42. Glass plates, 20 x 20
43. Vessel for waste liquids, 1000-3000 ml
44. Shears
45. Long metal spatula
46. Surgical scalpel
47. Industrial thermometer, 0-100°C
48. Medical syringe, 2 or 5 ml

49. Filter paper
50. Glass wool
51. Surgical wool
52. General purpose indicator paper
53. Black paper or aluminium foil to protect vessels with aflatoxin solutions from light
54. Plastic ruler
55. Soft lead pencil
56. Pencil for inscriptions on glass
57. Template for TLC
58. Electronic calculator or slide rule

Reagents for the analysis of aflatoxins in foodstuffs

Solvents and reagents have to be of the "chemically pure", "analytically pure" or "ACS" grades.

1. Crystalline B_1, B_2, G_1, G_2, M_1 aflatoxins or their standard solutions
2. Chloroform
3. Hexane
4. Diethyl ether
5. Methyl alcohol
6. Ethyl alcohol
7. Acetone
8. Benzene
9. Acetonitrile
10. Anhydrous sodium sulphate, calcinated
11. Celite 545 (80-100 mesh) or diatomite type "Hyflo Super-Cel"

12. Silica gel for column chromatography, brands L 100/160 μ or L 40/100 μ, "Chemapol" (CzSSR)
13. Silica gel for TLC, L 5/40 μ, "Chemapol" (CzSSR) or "Merck" or "Silicar" 4G, 7G, "Mallinck rodt."
14. Ready-made TLC plates, "Silufol" (CzSSR)
15. Silver nitrite
16. Tetrahydrofuran
17. Florisil (100-200 mesh), "Fisher F-101"
18. Ferric chloride
19. Caustic soda
20. Sand, "Fisher S-25" type
21. Silica gel, "Merck 70-230" type
22. Aluminium oxide (80-200 mesh), neutral, 1st degree of activity, "Fisher A-950" type
23. Isopropanol
24. Toluene
25. Sodium chloride
26. Lead acetate
27. Citric acid
28. Acetic acid
29. Ethyl acetate
30. Formic acid
31. Sulphuric acid
32. Hydrochloric acid
33. Ferric hydroxide
34. Liquid nitrogen
35. Crystalline iodine
36. Trifluoroacetic acid
37. Acetic anhydride

38. Pyridine

39. Calcium carbide bubblers

40. Sodium bicarbonate

41. Oxalic acid

42. Sodium hypochlorite

43. Ammonia solution, 25%

44. Potassium (sodium) hydroxide

Basic safety regulations for those working in a chemical laboratory

Safety rules for handling chemical glassware

To avoid injuries in assembling and dismantling instruments and parts made of glass it is necessary to take the following precautions.

1. Assemble glass instruments or their individual parts carefully. Glass instruments and their individual partes should be protected at the points where they are fixed on the metal rings of racks or holders with elastic pads (asbestos, rubber, leather, etc.)

2. When closing a thin-walled vessel with a stopper hold it by the upper end of the neck as close as possible to the stopper, protecting your hands with a towel. A heated vessel is not to be closed with a ground stopper before complete cooling.

3. When pouring liquids use a funnel placed on a porcelain triangle covering the neck of the vessel. In the absence of a triangle the funnel has to be placed in the ring of the rack or fixed in the lug above the vessel into which the liquid is poured.

4. When mixing or diluting substances that evolve heat

use thermostable glassware, the so-called Pyrex, characterized by a comparatively small coefficient of expansion, high softening temperature and thermal stability.

5. When heating all glass containers avoid sharp changes in temperature, as well as their nonuniform heating at different places.

Safety rules for working with inflammable and explosive substances

1. The principal materials used in chemical laboratories, intermediate products formed in chemical reactions, vapour, gases and dust often constitute combustible, readily inflamable substances. Some of them are explosive compounds, others, in the form of vapour, gases and dust, make with air explosive mixtures capable of igniting or exploding on the introduction of an inflammation source.

2. Highly inflammable and combustible solvents (acetone, diethyl ether, alcohol, benzene, hexane, etc.)must be kept in the laboratory in thick-walled glassware, with ground glass stoppers or screwed-on plastic covers.

3.Diethyl ether should be kept away from other substances in a cold and dark place, because, when it is stored in the light, explosive peroxides are formed.

4. All highly inflammable substances and combustible mixtures are to be worked with in an exhaust hood at functioning ventilation and only when gas burners and electric instruments are switched off. At the working place, inflammable substances can be kept only in the ammounts immediately necessary for work.

5. Combustible waste liquids are collected in special her-

matically sealed containers, to be removed from the laboratory at the end of the working day for regeneration or disposal.

6. In the case of a combustible liquid ignition all the burners must be switched off, the flame covered with an asbestos towel or strewn over with sand. A large flame is quenched with the help of a fire extinguisher.

Safety rules for working with caustic substances

1. Caustic, aggressive substances causing chemical burns (hydrochloric, nitric, sulphuric, hydrofluoric acids, chromic anhydride, as well as concentrated solutions of sodium and potassium hydroxides and ammonia solutions) can seriously injure the skin. Alkalis can cause skin burns in their dry state.

Alkalis are especially dangerous because they can injure the eyes. That is why, to prevent burns, in all cases of working with caustic (aggressive) substances all those working in the laboratory must wear splash goggles (with a leather or rubber rim) and rubber gloves, in certain cases - rubber aprons and boots.

2. To prepare sulphuric acid solutions, the acid must be poured into water in a thin stream with continuous stirring. Pouring water into sulphuric acid is prohibited! Intensive heat liberation causes violent boiling up and splashing of the acid.

3. Waste acids and alkalis should be collected separately into special containers and after neutralization poured into the waste-water disposal system or a specially assigned place.

4. Alkalis should be dissolved by slowly adding small pieces of substance to water with continuous stirring, pieces of alkali must be held with forcepts only.

5. When washing the glassware with a chromium mixture it is necessary to take care it does not come into contact with the skin, clothes and footwear. If it gets on the skin, immediately wash it off with a large amount of water.

6. Liquids that can cause burns or poisonings are to be collected only with the help of pipettes with a rubber bulb.

Safety rules for working with aflatoxins and their decontamination

1. AT are highly toxic substances and, therefore, must be handled with great care. AT solutions are capable of diffusing through rubber and vinyl gloves.

2. When working with crystalline AT one should be especially cautious because of their strong electrostatic properties; when weighing the crystals ground the metal spatula.

3. A person that has performed the analysis and prepared the standard AT solution must rinse the mouth and hands with 1% solution of sodium hypochlorite, and then thoroughly wash the hands with soap.

4. Equipment, instruments, working tables in contact with AT must be treated after the analysis with 5% sodium hypochlorite aqueous solution or with 5% alkali alcohol solution, and then neutralized with 6N hydrochloric acid aqueous solution.

5. The glassware used for analysis should be washed with potassium bichromate solution in sulphuric acid, prepared in the following way: dissolve 50-60 g potassium bichromate in 10 ml water in a porcelain beaker, then carefully pour 1 l concentrated sulphuric acid into it. Wash with a large amount of tap and

distilled water.

6. To decontaminate the solutions containing AT they should be first evaporated to dryness. The dry residue is dissolved in 1 ml methanol, sodium hypochlorite solution is added and left to stand for 2 hours. For the destruction of AT B_1 2,3-dichloroderivatives that can be formed, add acetone (5% of the total volume).

Aflatoxin solutions can also be decontaminated by adding successively to the dry residue: water, concentrated sulphuric acid (ρ = 1.84) and saturated potassium permanganate solution in water (C = 0.4 mol/l).

7. After the analysis TLC plates are to be treated first with aqueous sodium hypochlorite solution (0.8%) and then with acetone (5% of the volume).

8. Nanogram quantities of AT can be removed with a large amount of water.

First aid measures in case of accidents

1. In the case of thermal burns the affected spot should be moistened with ethyl alcohol or potassium permanganate solution, or a dressing applied with a burn ointment. In severe burns the first aid must be performed by medical personnel. If clothes have caught fire, it is first necessary to extinguish the flame by covering them with a woolen or an asbestos blanket, or by some other means, and then to take the clothes off the victim, if necessary putting him on a clean sheet, and immediately call for the doctor.

2. In the case of chemical burns it is first of all necessary to remove from the skin the substance that has caused the

burn, using an appropriate solvent, and then to treat the affected spot with alcohol.

When the burns have been caused by caustic substances soluble in water (acids, alkalis), it is necessary to wash promptly the burnt spot with a large amount of water (in a strong stream), and then to treat it with neutralizing agents. Clothes stained with concentrated acids, alkalis or other caustic substances must be taken off at once, after which they have to be neutralized, dewatered and washed. It is prohibited to put on contaminated clothes.

3. In the case of chemical burns of eyes with an acid or alkali it is necessary, before applying to the medical aid post, to wash the victim's eyes with a large amount of water.

4. When a foreign body gets into the eyes it is necessary to apply to the medical aid post.

5. In the case of an injury caused by electric current, if a person remains in contact with current -carrying parts, the current must be immediately switched off by a master switch, or by unscrewing the fuse plug or chopping the live wire with an insulated instrument. If it is impossible to switch off the electric current at once, the person administering the first aid must insulate his hands with rubber gloves, dry rags, a part of his clothes, and also put on rubber overshoes or stand on a rubber rug, a dry board, dry rags (cloth) and detach the victim from the current-carrying parts with which he is in contact. Do not touch the victim with unprotected hands while he is in contact with a live wire.

If the victim has lost consciousness, immediately, without

wasting time and waiting for the arrival of medical personnel, apply artificial respiration.

6. The most frequent laboratory injuries are wounds - punctured, lacerated and incised, inflicted by different objects. Their main danger is associated with the penetration of pustular microbes into the wound, which can result in infection. Two basic rules are, therefore, to be observed:

a) do not touch the wound with your hands, a handkerchief, part of clothes or paper;

b) under no circumstances wash the wound with either tap or boiled water or medicaments, no matter how dirty the wound is.

It is only necessary to paint the surrounding skin with iodine, to apply a sterile dressing and bandage the wound.

PREPARATION OF SPECIMENS FOR ANALYSIS. REVIEW OF THE METHODS OF AFLATOXIN EXTRACTION FROM VARIOUS FOODSTUFFS. PREPARATION AND STORAGE OF STANDARD AFLATOXIN SOLUTIONS

Preparation of specimens for analysis

The stage of taking and preparing a pecimen is an indispensable and very important part of the analytical method of AT determination. The overall error of the method is made up of the error in taking the aggregate sample, errors in isolating samples for the analysis and the error of analysis. The errors associated with taking a specimen and subspecimen may be so great that the coefficient of variation in the analytical method loses its meaning. The coefficient of variation in sample taking amounts to ca 115% at the contamination level of 20 μg/kg and

ca 145% at the contamination level of 10 µg/kg. In groundnut analysis 98% of the total error of analysis is accounted for by this very stage, and only 2% - by the analysis itself. Traditional rules of taking and preparing a specimen of agricultural product and foodstuffs do not meet the requirements of chemical analysis for mycotoxins. The difficulty of sample taking increases because of the heterogeneity of AT distribution in contaminated untreated products. A commercial lot of groundnut may contain just a few AT-contaminated nuts, but only one affected nut out of 10 thousand investigated contaminates groundnut at a level of 50 µg/kg. In order to obtain a specimen representative for the whole lot it is necessary to take a comparatively large number of samples from different points in the lot (in the U.S.A., according to the criterion for taking groundnut samples, it is prescribed that three 48 lb (21.8 kg) samples be taken from a lot to make up the overall specimen) and then to prepare this sample in an appropriate way to obtain the analytical specimen.

The following products are an exception:

a) powders (flour, oil meal, oil cake, bran)

b) pastes (almond paste, peanut butter, curds)

c) liquids (milk, beer, wort).

For these products it is sufficient to shake them vigorously (liquids), or to stir them in a mixer or blender which assures the homogeneity of a specimen (powders, pastes).

The stages that follow taking the specimens of grain or nuts representative for the whole lot are: stirring this material in a mixer or blender, coarse grinding to the particle size

corresponding to the standard 14 mesh sieve, mixing the sample to obtain homogeneity, and taking portions for finer grinding to obtain fluid material. Specimens with a high oil content (groundnut) or having a viscous texture before grinding must be frozen with liquid nitrogen or solid carbon dioxide ("dry ice"). In some cases the ground specimen (e.g., cocoa powder) should be degreased in Soxhlet apparatus. A 25-100 g analytical specimen is taken from the prepared specimenm depending on the method.

Methods of extracting aflatoxins from various foodstuffs

Extraction is a process of separating a mixture of substances with the help of selective solvents (extractants). The process of extraction consists of three successive stages: the mixing of the initial mixture with the extractant, separation of the two phases formed, removal of the extract (solution of the extracted substance in the extractant).

The systems of solvents used to extract the AT fraction from a sample must contain an aqueous component. The specimen should be thoroughly moistened with the aqueous component before adding the organic part of the extracting mixture. The aqueous components, used to achieve a more complete AT extraction from the specimen and a partial salting out of some kinds of proteins, are water solutions of sodium or potassium chlorides; weak citric acid solutions are used for protein-rich products to break the protein-AT bond; silver nitrates are employed to precipitate theobromine in cocoa analysis. The basic AT extractants are presented in Table 1.

To extract the AT fraction, the extrantat - analyzed specimen mixture is shaken mechanically for 30 min, or in a blender for 1-3 min. The extract is separated from the raffinate by filtering through a folded papel filter. To speed up filtration, the filter is covered with diatomite or Celite, sometimes they are added directly to the extract and the analyzed specimen mixture in the amount equal to the weight of the specimen. When this method of filtration is impossible, vacuum filtration is applied, and in the cases of difficult-to-separate emulsions (e.g., chloroform and milk) - centrifugation.

Preparation and storage of standard solutions

To analyze foodstuffs for aflatoxins, it is necessary to prepare standard working solutions of the corresponding AT of a certain concentration. The reliability of chemical analysis depends on the accuracy of the preparation of AT standard solutions.

The concentration of prepared AT standard solutions is determined on a UV spectrophotometer. The instrument is precalibrated with potassium bichromate solutions having clearly defined absorption maxima. The purity of the obtained standard AT solutions is determined with the help of thin-layer chromatography and UV spectroscopy.

Spectrophotometer calibration

To calibrate the spectrophotometer, it is necessary to prepare three potassium bichromate ($K_2Cr_2O_7$) solutions in sulphuric acid (H_2SO_4) (0.25, 0.125, 0.0625 mm).

Table 1 Solvent systems for aflatoxin extraction

Ser. Nos.	Aqueous component (vol.%)	Organic component (vol.%)	Ratio of extractant volume to weight of specimen
1.	Water (9%)	Chloroform (91%)	5.5:1 (CB)
2.	1.8% NaCL soln (32%)	Methanol (39%)-hexane (29%)	7:1 (BF)
3.	10% NaCl soln (20%)	Acetone (80%)	5:1
4.	10% NaCl soln (20%)	Methanol (80%)	5:1
5.	4% KCl soln (10%)	Acetonitrile (90%)	4:1
6.	4.8% citric acid soln in saturated Na_2SO_4 soln (20%)	Acetone (80%)	4:1
7.	20% $AgNO_3$ soln (9%)	Chloroform (91%)	5:5:1 (CB for cocoa)

Sulphuric acid - 0.018 N soln: Dissolve 1 ml H_2SO_4 (density 1.84) in 2 l distilled water.

Potassium bichromate solutions

a) 0.25 millimolar (mm) $K_2Cr_2O_7$ soln: Weigh 78 mg K_2CrO_7 and dissolve in 1 l 0.018 N H_2SO_4;

b) 0.125 mm $K_2Cr_2O_7$ soln: Bring 25 ml ~~0.125~~ 0.25 mm $K_2Cr_2O_7$ soln up to the volume of 50 ml with 0.018 N H_2SO_4.

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Spe trophotometer calibration is performed as follows:

1. Measure the optical density (D) of the three prepared

solutions in 1 cm quartz cuvettes at the wavelenght (λ) of 350 nm relative to 0.018 N H_2SO_4 soln;

2. Calculate the molar extinction value (E) from the optical density (D) values obtained for each $K_2Cr_7O_2$ soln concentration(C) with the help of the formula

$$E = \frac{D \times 1000}{C}$$

3. From the three molar extinction values for potassium bichromate solutions calculate the mean value of molar extinction (E) and compute the correction factor (K) for the given spectrophotometer and cuvettes from the formula

$$K = \frac{3160}{E},$$

where 3160 is the tabular molar extinction value.

Preparation and storage of standard aflatoxin solutions

Before starting the preparation of standard AT solutions it is necessary to make sure of the purity of the initial preparations. Aflatoxins used as standards must answer the following criteria of purity:

1. The molar extinction value at certain wavelengths must lie within admissible limits (Table 2).

2. The values of molar extinction ratios, $E_{\lambda 1}/E_{\lambda 2}$, at 95% confidence interval for individual AT must correspond to the tabular values (Table 3).

3. They must be chromatographically pure.

Table 2. Molar extinctions of individual aflatoxins in methanol

Aflatoxins	Absorption maximum wavelenght (nm)	Molar extinction (E) in methanol	Deviation limit at 95% confidence interval (±)
B_1	223	22100	1600
	265	12400	800
	362	21800	1100
B_2	223	18600	1000
	265	12100	600
	362	24000	500
	214	27400	2500
G_1	242	9600	300
	265	9600	1200
	362	17700	700
	214	25300	2300
G_2	242	10500	300
	265	9000	1100
	362	19300	800
	226	23100	-
M_1	265	11600	-
	357	19000	-

Spectrophotometric analysis of the purity of initial aflatoxin standards

1. Weight 1 ± 0.001 mg AT standard and transfer quantitatively into a 100 ml measuring flask.

Table 3.

Values of the ratios of molar extinction at maximum absorption

Aflatoxins	B_1	B_2	G_1	G_2	M_1
$E_{\lambda 362}/E_{\lambda 265}$	1.76±0.09	1.98±0.04	1.84±0.07	2.14±0.09	-
$E_{\lambda 265}/E_{\lambda 223}$	0.56±0.04	0.65±0.03	-	-	-
$E_{\lambda 265}/E_{\lambda 242}$	-	-	0.00±0.13	0.86±0.10	-
$E_{\lambda 242}/E_{\lambda 214}$	-	-	0.35±0.01	0.42±0.01	-
$E_{\lambda 357}/E_{\lambda 265}$	-	-	-		0.16
$E_{\lambda 265}/E_{\lambda 226}$	-	-	-	-	0.50

2. Pour 20–30 ml methanol into measuring flask and thoroughly stir to achieve complete dissolution of AT (for faster dissolution you can heat the mixture to 50°C).

3. Bring up the level in measuring flask to the mark with methanol and stir thoroughly.

4. Measure and calculate molar extinction from the formula

$$E = \frac{D \times M \times 1000}{C},$$

where D is the optical density of the solution;

C (μg/ml) is the concentration of the investigated solution;

M is the molecular weight of aflatoxin (see Table 4).

The obtained molar extinction (E) values must correspond to those presented in Table 2 for individual AT.

5. Calculate the molar extinctions ratio (E/E) for every aflatoxin at wavelengths λ given in Table 2. The obtained $E_{\lambda 1}/E_{\lambda 2}$ values must lie within the limits given in Table 3.

If the experimentally observed results correspond to those in the table the initial aflatoxin preparation can be used to prepare standard AT solutions.

Preparation of standard AT solutions

Standard AT solutions are most often prepared from dry crystalline powders of aflatoxins pre-packed in phials obtained from supplier companies.

Standard solutions of individual AT in 10 µg/ml concentrations should be prepared first. AT masses indicated on phial labels can be used for the purpose.

1. Add 3-5 of solvent to phial with crystalline AT and mix thoroughly to achieve complete AT dissolution.

To prepare standard solutions of AT B_1, B_2, G_1 and G_2, the solvent used is a mixture of benzene with acetonitrile (49:1). For the standard solution of M_1 the solvent is benzene-acetonitrile (9:1).

2. Transfer the mixture to a measuring flask whose volume will make it possible to obtain the final concentration of AT solution equal to 10 µg/ml.

3. Thoroughly wash the phial with the corresponding solvent each time transferring the mixtures into the measuring flask. Bring up the volume in the measuring flask to the mark with a corresponding solvent.

When there is no pre-packed initial AT preparation, pre-

pare a sample of AT weighing 0.5 - 0.001 mg and dissolve it in 50 ml of corresponding solvent.

Determining the concentrations of the prepared standard solutions of aflatoxins

1. Take a UV spectrum of prepared standard solutions in the 330-370 nm range with respect to benzene-acetonitrile mixture (49:1) for AT B_1, B_2, G_1, G_2 or with respect to benzene-acetonitrile mixture (9:1) for AT M_1.

2. Measure the optical density (d) of standard AT solutions at 350 nm wavelength using 1 cm quartz cuvettes.

3. Calculate the concentrations of standard solutions from the formula

$$C = \frac{D \times M \times 1000 \times K}{E},$$

where C (μg/ml) is the concentration of standard AT solution,

D is the optical density of standard solution,

M is molecular weight (see Table 4),

K is the spectrophotometer correction factor,

E is the molar extinction of AT (the values of E are given in Table 5).

Preparing the working standard solutions for thin-layer chromatography

After establishing the purity and concentration of every standard AT solution it is necessary to prepare:

a) working standard solutions of aflatoxins B_1, B_2, G_1, G_2, M_1;

b) standard solution containing a mixture of four aflatoxins (B_1, B_2, G_1, G_2).

Working standard solutions are prepared by diluting the corresponding standard solutions of AT. The concentrations of working standard AT solutions for B_1, G_1 and M_1 must be equal to 0.5 μg/ml, and for B_2 and G_2 - to 0.1 μg/ml. For the purpose, take an aliquot portion of the corresponding standard AT solution and bring it up to the required volume with benzene-acetonitrile mixture (49:1) when preparing working standard solutions of AT B_1, B_2, G_1, G_2 or with benzene-acetonitrile mixture (9:1) for AT M_1 working solution.

Working standard solution of the mixture of aflatoxins B_1, B_2, G_1, G_2

Take aliquot portions from the corresponding standard AT solutions and bring up to the required volume with benzene-acetonitrile mixture (49:1). Aflotoxin concentrations in a standard mixture must amount to 0.5 μg/ml for B_1 and G_1 and to 0.1 μg/ml for B_2 and G_2.

Checking the purity of standard AT solutions with the help of TLC

Apply in succession on a "Silufol" chromatographic plate: 1) 5 μl of working standard AT mixture solution, 2) 5 μl of one of the standard AT solutions, 3) 5 μl of the corresponding standard AT solution and 5 μl of working standard AT mixture solution.

Develop the plate in acetone-chloroform (1:9) or diethyl ether-methanol-water (96:3:1) system. Detect AT in UV light.

An individual AT spot must not contain any other AT or any

other fluorescent impurities, manifested as spots near the main AT spot.

Storage of standard solutions

Standard AT solutions (with a concentration of 10 μg/ml) should be kept in ground-stoppered phials wrapped in aluminium foil at the temperature of 0°C. In these conditions standard AT solutions can be stored for a year and used to prepare the working standard AT solutions.

During storage the standard AT solutions may change their concentration slightly because of the solvent evaporation. That is why before using the stored standard AT solutions their concentration has to be controlled with the help of UV spectrophotometry. Prior to determinations, the temperature of standard AT solutions must be raised to 20°C.

Table 4. Molar extinction values for aflatoxins at 35 nm wavelength

Aflatoxins	Molecular masses (M)	Molar extinction (E)
B_1	312	19800
B_2	314	20900
G_1	328	17100
G_2	330	18200
M_1	328	18815

EXPERIMENTAL

Preparation of specimens for analysis

1. From averaged-out coarsely ground specimens of corn, rice, wheat, nuts take a portion of ca 100 g for final grinding

to flour consistency. From the mass obtained take the initial subspecimen (25 g) for analysis.

2. From averaged-out specimens of cocoa and coffee beans take a portion of ca 100 g, freeze in thermostable glassware and grind to flour consistency. From the mass obtained take the subspecimen (25 g) for analysis.

Place the finely ground cocoa specimen in a filter paper bag and degrease in Soxhlet apparatus for three hours. Dry the degreased cocoa powder specimen.

Extraction of the aflatoxin fraction from prepared specimens

1. Place weighed subspecimens of the initial products in 300 ml conical flasks.

2. Moisten the subspecimen with the water component of extracting mixture and then add the organic component in ratios given in Table 5.

3. Extract for 30 min in a dark room at continuous shaking in a shaking apparatus.

4. Filter off the extract through a folded paper filter into a measuring cylinder. Measure the filtrate volume.

PURIFICATION OF AFLATOXIN FRACTION FROM CONCOMITANT COMPOUNDS WITH THE HELP OF COMPLEXING AGENTS, LIQUID-LIQUID EXTRACTION, COLUMN CHROMATOGRAPHY

The most complete purification of the extract from proteins, pigments, alkaloids and some other compounds present in the aflatoxin fraction is achieved by treating the extract with

Table 5. Ratios between the subsample weight and the extraction mixture volume in aflatoxin extraction

Test No.	Foodstuff	Subspecimen weight (g)	Water component volume (ml)	Organic component volume (ml)
1	Wheat	50	water -25	chloroform -250
2	Oils	50	water -25	chloroform -250
3	Groundnut	25	10% NaCl-25	acetone -100
4	Corn	25	10% NaCl -25	methanol -100
5	Cereals	25	10% NaCl-25	acetonitrile -100
6	Dry milk	10	water -100 0.48 g citric acid 4 g NaCl	acetone -100
7	Cocoa	50 predegreased	25% $AgNO_3$-25	chloroform -250
8	Coffee	25	water -12.5	chloroform -125
9	Peanut butter	50	water -112.5	chloroform -137.5 hexane -100

aqueous solutions of complexing agents: lead acetate, zinc acetate, ammonium sulphate, sodium sulphate, cadmium sulphate, silver nitrate.

After adding the above compounds to the AT-containing extract the mixture is left to stand for 10-15 min until a precipitate is formed. The precipitate is centrifuged or filtered off, sometimes with the addition of diatomite or Celite.

Water-acetone and water-methanol extracts are usually purified with the help of liquid-liquid extraction. This is done to

remove lipids, proteins, pigments and other impurities, interfering with AT determination, from the initial extract. Numerous lipids (mostly neutral) and other nonpolar components are extracted with hexane, petroleum ether and isooctane. This is followed by liquid-liquid redistribution of the water-acetone or water-methanol phase with chloroform, where chloroform selectively extracts AT, while polar impurities remain in the water-organic phase.

This method of purification has found extensive application because of its advantages: a high selectivity, simplicity, and universality. A limitation of liquid-liquid extraction is the difficulty of complete AT removal from the initial extract, which necessitates repeated mixing of the interacting phases and their subsequents separation into layers.

Membrane purification of the aflatoxin fraction is based on dialysis, performed after degreasing the initial extract. The extract is evaporated to dryness and dissolved in acetonitrile. The obtained solution is placed in a bag for dialysis, made of cellophane or some other synthetic material. Dialysis is performed against aqueous acetone. AT diffuse through a semipermeable membrane, separating in this way from colloidal particles and macromolecules, such as proteins, lipoproteins, etc. This method of purification has not found wide application because of a number of shortcomings, among them a possibility of AT decomposition and loss in the course of dialysis.

The most widely applied methods of removing small quantities of impurities are based on chromatography - separation of the mixture components by using the differences in their distribution between the two phases (a solid sorbent and a solvent).

Column chromatography is used to purify AT extracts from lipids and polar compounds. As a rule, the sorbent used in column chromatography is silicagel of a general formula SiO_2 x x H_2O, placed into the category of polar adsorbents, its polarity being caused by the presence of surface hydroxyl groups. The most frequent mechanism of adsorption on silica gel is the formation of hydrogen bonds between the adsorbed substance and the surface hydroxyl groups.

When column chromatography is applied to purify the AT extracts of coffee and some dairy products, magnesium silicate (florisil) and cellulose are used as the sorbents.

Selective elution of the lipid fraction is performed with hexane, isooctane, ether; it is also expedient to use benzene or toluene with acetic acid. AT are eluted with chloroform in a mixture with acetone, ethanol or methanol; more polar solvent systems are used to elute AT M_1. Tetrahydrofuran is used to purify the extracts of green coffee from caffeine in a column with florisil. AT are eluted with the methanol-acetone system of solvents.

Experimental

1. Purification of the aflatoxin fraction with the help of complexing agents.

a) Take 50 ml extracts No.3 and No.4 (see Table 5), add 20 ml 15% lead acetate solution to each, shake, leave to stand for 15-20 minutes, filter off through a folded paper filter. Withdraw 90 ml filtrates No. 3, No.4 and No.6, respectively.

b) Take 50 ml extract No.3 (see Table 5), add 50 ml dis-

tilled water, 5.5 ml 10% ferrous chloride solution, 8.3 ml 4.8% sodium hydroxide solution. Shake the mixture obtained. 5 minutes later filter off (filtrate No.3^a).

c) Take 75 ml chloroform extract No.7 (see Table 5), add 7.5 ml 20% silver nitrate solution. Shake vigourously for 3 minutes in a separating funnel. After separation of the phases withdraw 50 ml chloroform layer (extract No.7^a).

2. Purification of the aflatoxin fraction with the help of liquid-liquid extraction.

a) Extract filtrates No.3, No.4 and No.6 (see 1^a) with hexane (2 times x 40 ml) in a separating funnel, each time removing the upper hexane layer.

b) Extract the aflatoxin fraction from water-organic extracts Nos.3, 4, 6 (see 2^a) and No.9 (see Table 5) with chloroform (2 times x 40 ml). Collect the chloroform extracts, dry over anhydrous sodium sulphate.

c) Degrease extract No.5 (see Table 5), extracting in a separating funnel with isooctane (2 times x 40 ml). Remove the isooctane layer. Extract the aflatoxin fraction from the water-acetonitrile phase with chloroform (3 times x 30 ml). Collect chloroform extract No.5, dry with anhydrous sodium sulphate.

3. Membrane purification of the aflatoxin fraction.

Purify chloroform extract No.5 (see 2.c) using dialysis through a cellophane membrane. Filter off the extract through a paper filter, evaporate to dryness, dissolve the residue in 3-5 ml acetonitrile and transfer into a dialysis bag, place the bag in a flask containing 70 ml 30% acetone water solution. Dialyze for 16 hours. Extract AT from water -acetone dialyzate with

chloroform (3 times x 20 ml). Dry the obtained chloroform extract No.5 over anhydrous sodium sulphate.

4. Purification of the aflatoxin fraction by column chromatography.

a) To clean extracts Nos.1,7 (see Table 5) and No.7[a] (see 1.c) prepare the column in the following way: place a small wad of cotton wool on the bottom of a 20 x 300 mm column and add 5 g anhydrous sodium sulphate, then insert the previously prepared suspension of 10 g silica gel in 20 ml chloroform, wash the column with 20 ml chloroform, add 15 g anhydrous sodium sulphate and wash the column with chloroform (10 ml). Insert 50 ml of extract. When the upper level of extract reaches the sodium sulphate layer, elute the column with 150 ml hexane, then with 150 ml ether (previously passed through a column with aluminium oxide). Elute the aflatoxin fraction with 150 ml chloroform-methanol mixture (97:3).

b) To purify the chloroform extract of corn, No.4 (see 2.c) prepare the chromatographic column in the following way: place a small wad of cotton wool on the bottom of a glass column (12-15 x 250-300 mm), add 1-2 g anhydrous sodium sulphate, and then the previously prepared suspension of 2 g silica gel in 5 ml ether-hexane mixture (3:1). After the precipitation of silica gel place 2 g anhydrous sodium sulphate into the column. Wash the column with 1-2 ml of the same mixture, insert extract No.4 evaporated to 2 ml. Elute the column successively with 25 ml toluene-acetic acid mixture (9:1), 50 ml ether-hexane mixture (3:1), 60 ml chloroform-acetone mixture (9:1). Discard the first two eluates, preserve the third eluate, containing AT,

for further investigation.

c) Purification of the chloroform extract of milk, No.6 (see 2.c).

Prepare the chromatographic column as described in par.b, with the exception that the silica gel suspension is prepared in chloroform. Place extract No.6, evaporated to 15 ml, in the column. Elute successively with 1-3 ml chloroform, 25 ml toluene-acetic acid mixture (9:1), 25 ml hexane-ether-acetonitrile mixture (15:3:2). Elute AT M_1 with 25 ml chloroform-acetone mixture (4:1).

DETECTION, IDENTIFICATION AND QUANTITATIVE DETERMINATION OF AFLATOXINS WITH THIN-LAYER CHROMATOGRAPHY

As a rule, silica gel is used as the sorbent in TLC analysis for AT. Silica gel used to prepare the plates has the particle size of 2 - 10 microns. It is applied to the plates in the form of a water suspension; the optimum thickness of silica gel layer is 0.25 - 0.5 mm. Plates are dried at room temperature, and then activated at 100 - 110°C for 1 hour.

The degree of silica gel activity, which depends on the moisture content of the layer, influences considerably the R_f value. The optimum moisture content in the layer is 15-20%.

Heathcote and Hibber have shown the optimum results in the analysis of aflatoxins to be achieved on silica gel of the "SilicAR TLC-7G (Mallinckordt)" brand.

Ready-to-use plates covered with a silica gel layer are manufactured at present. These plates have a more durable layer and save time. They also provide a better reproducibility of

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results. Ready-to-use glass plates with a thin silica gel layer are supplied by Merck, Customs Service Chemicals, Camag companies. It is, however, much more convenient to use ready-made layers applied to aluminium foil (manufactured by Merck, FRG and Kavalier, CzSSR). "Silufol" is manufactured in CzSSR in the form of flexible 5x5, 15x15, 20x20 cm plates with silica gel on smooth aluminium foil and starch used as the binder. These plates are widely applied in TLC analysis of AT.

The results of TLC analysis of AT are strongly affected by how the sample is spotted on the thin layer. Before spotting the sample a reference point is located at a distance of 15-20 mm from the plate edge. The starting line should be marked very carefully so as not to damage the adsorbent surface, since this will distort the shape of the spot. To achieve optimum separation of aflatoxins in the extract it is necessary to follow certain rules. If the analyzed extract is diluted it should be concentrated to a necessary volume. The mass of the aliquot portion of extract applied on a TLC plate with a 0.25 mm thick adsorbent should not, however, exceed 5-10 mg. The sample should be spotted in the form of a solution in the least polar solvent so as to avoid the blurring of the spot at the point of application, which can affect the R_f value of the separated components, especially if chromatography is performed with less polar solvents. In addition to that, the solvent should be relatively volatile so that it can be easily removed from the plate. An AT-containing specimen can be spotted on the plate in the form of its solution in chloroform or benzene. Spotting the sample in benzene has the following advantages: a) because of a higher boiling point of

benzene it is easier to transfer and apply the samples: b) the spots prove to be more compact and have clearly defined boundaries; c) the R_f values of AT in chloroform can be affected by the impurities of alchohol used as stabilizer. The solvent action of benzene can be improved by adding 2% acetonitrile to it; the spots in this case remain compact. The area of the spot at the point of application must be as small as possible because the quality of separation depends on it. The sample should be applied in small portions; it is also important for the volume applied each time to be constant. To avoid errors in spotting the sample, it is necessary to use an internal standard.

When performing one dimensional TLC, AT are eluted with different solvent mixtures: chloroform-methanol (97:3), chloroform-methanol-acetic acid (94:5:5:0.5), acetone-chloroform (9:1 and 3:17), benzene-acetic acid-methanol (90:5:5), as well as chloroform-acetone-2 propanol (33:6:1 and 34:5:1). Experiments have shown the solvent systems that include chloroform and methanol to be sensitive to humidity variations. The benzene-ethanol-water mixture (46:35:19) proved to be a good eluting solvent, but only at optimal humidity and temperature. One of the best systems for AT B_1, B_2, G_1,G_2,M_1 and M_2 separation is the chloroform-acetone-isopropanol mixture (85:10:5). Maximum resolution in M_1 and M_2 separation is obtained with the chloroform-acetone-amyl alcohol mixture (8:1:1), but in this case the other AT also migrate with the solvent front.

It should be noted that the toluene-isoamyl alcohol-methanol mixture (90:32:3) makes it possible to separate distinclty AT

B_1 (R_f 0.56), B_2 (R_f 0.48), G_1 (R_f 0.42), G_2 (R_f 0.34), and that repeated elution with the same solvent improves the resolution.

Good results at repeated elution have also been obtained by using the following systems: methylene chloride-trichloroethylene-n-amyl alcohol-formic acid (80:15:4:1), chloroform-trichloroethylene-n-amyl alcohol-formic acid (80:15:4:1). Also applied in eluting AT on a TLC plate is the xylol-tert.-butanol-acetic acid mixture (94:5:1), used to separate AT by the method of partition chromatography on a layer of H and G-HP silica gel, taken in the ratio of 1:1, saturated with tert.-butanol-formic acid-water mixture (10:1:25)and dried in air for 30minutes.

Two-dimensional TLC is at present widely used in AT analysis. It assures a more efficient separation of the spots of aflatoxins from the other extract components that have fluorescent properties and chromatographic mobility close to those of AT. In two-dimensional TLC the plates are developed in two directions normal to each other.

Chromatograms are developed in the following two ways:

a) The first method is only used to clean the extracts from nonpolar impurities, and AT remain on the starting line. The solvents used for this purpose are: ether (freed of peroxides), benzene, and their mixtures with hexane (benzene-hexane (1:1), benzene-hexane-ether (1:1:1), etc.). The second method is used directly for the separation of aflatoxins.

b) Both methods are used for more efficient AT separation and for the removal of impurities in systems of differing selectivity.

In practice, more efficient AT separation and cleaning are achieved by using different combinations of systems, and among them the following solvent mixtures:

chloroform-acetone (9:1) and diethyl ether-methanol-water (188:9:3);

chloroform-acetone (9:1) and toluene-ethyl acetate-90% formic acid (5:4:1);

benzene-methanol-acetic acid (90:5:5) and chloroform-acetone (9:1);

chloroform-acetone (9:1) and ethyl acetate-isopropanol-water (10:2:1);

ether-methanol-water (188:9:1) and chloroform-acetone-isopropanol (85:10:5);

chloroform-acetone-benzene (4:1:1) and toluene-ethyl acetate-chloroform-90% acetic acid (7:5:5:2).

A series of R_f values in certain solvent systems is shown in Table 6

Table 6 The R_f x 100 values for some aflatoxins

Aflatoxins	A	B	C	D	E	F	G	H
B_1	28.0^x	31.5^x	19.5^x	80.5^x	22.5^x	32.5^x	31.5^x	61.5^x
B_2	30.0	18.0	14.0	80.0	9.0	33.0	20.0	61.0
G_1	19.0^x	19.0^x	13.5^x	84.5^x	12.0^x	25.0^x	20.0^x	50.0^x
G_2	22.5	8.0	8.0	78.0	4.5	17.0	11.0	51.5
M_1	21.5	15.5	10.0	78.5	0	7.5	6.5	64.0

x - formation of "tails"

Solvents:

A - benzene-methanol-acetic acid (24:2:1)

B - toluene-ethyl acetate-90% formic acid (6:3:1)

C - benzene-ethanol (95:5)

D - chloroform-methanol (4:1)

E - chloroform-methylisobutylketone (4:1)

F - chloroform-acetone (9:1)

G - chloroform-acetic acid-diethyl ether (17:1:3)

H - n-butanol-acetic acid-water (4:1:4)

Chromatography can be performed in any vessel of a suitable size, equipped with a hermetically tight cover, as it is necessary to exclude any possibility of solvent evaporation, which disrupts the chromatographic process. The use of a chamber saturated with solvent vapour prevents the undesirable "edge effect", when the same substance has lower R_f values in the middle of the chromatogram than at the edge of the plate.

After developing the TLC plates, AT are vizualized by specific fluorescence in long-wave ultraviolet light (λ 365 nm). AT of groups B and M have blue fluorescence (425 nm), group G - bluish-green (450 nm).

However, for a more reliable identification of aflatoxins in the extracts of foodstuffs, confirmatory tests have to be performed.

Confirmatory tests:

1. Test with iodine

Place chromatographic plate in ground-stoppered desiccator saturated with iodine vapour, or impregnate 20x20 cm glass plate with 5-10% iodine solution if ether. After evaporation of ether place glass plate with thin layer of iodine crystals over TLC plate at a distance of 0.5-1 cm for 20-30 seconds. Determine

visually in long-wave UV light whether colour and intensity of AT spots fluorescence have been preserved in the standard and the extract. The colour and intensity of fluorescence being preserved in spots of the extract confirms possible presence of AT in a foodstuff.

2. Test with an inorganic acid

Spray chromatographic plate with nitric acid solution in water (1:2). If the fluorescence colour of extract spots does not change into yellow, no AT are present in the sample. But if the fluorescence colour of the extract spots, corresponding to AT in their chromatographic mobility, also changes into yellow, this serves as a confirmation of possible AT presence in the investigated foodstuff.

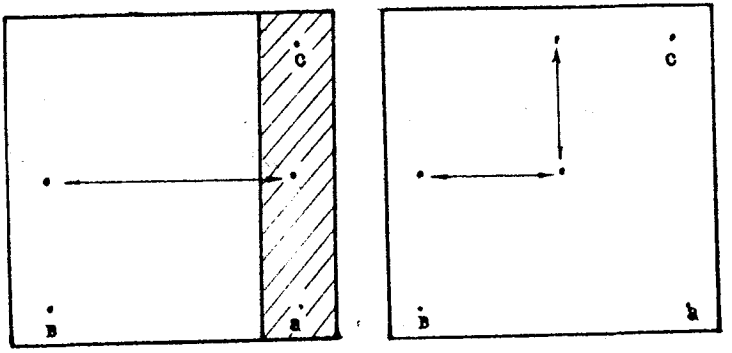
3. Test with trifluoroacetic acid

The test with trifluoroacetic acid (TFAA) is distinguished by a high reliability. It is only applicable for AT B_1, G_1, M_1. The presence of AT in analyzed extracts can be confirmed with the help of TFAA in the following two ways:

First method: Develop chromatographic plate with analyzed extract and standard AT solution, applied according to the pattern for two-dimensional TLC, (see Fig.2A) in first direction in chloroform-acetone-benzene system (4:1:1). Spray the part of TLC plate containing points a and c (hatched area in Fig.2A) with 20% TFAA solution in hexane. After hexane evaporation, cover TLC plate with clean glass plate heated to 75°C. Heat 6-8 minutes at 75°C. Let TLC plate cool, then chromatograph in perpendicular direction in chloroform-acetone-isopropanol system (84:10:16). When spot of extract is detected, corresponding in

R_f to spots of standard AT from point b (Fig. 2B) and standard AT TFAA derivative from point c (Fig. 2B), and also when fluorescence colour of the obtained extract spot coincides with that of standard AT TFAA derivative, it can be concluded that AT is present in the extract.

Fig. 2. Schematic representation of thin-layer chromatogram to confirm aflatoxin identity with the help of trifluoroacetic acid



A - before treatment with TFAA

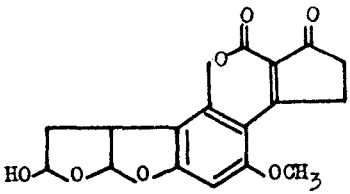
B - after obtaining AT derivative and its chromatography in the 2nd direction

a - point where aliquot portion of sample extract is applied

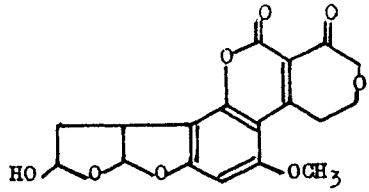
b,c - points where standard AT solution is applied

Second method: Spot consecutively AT standard (5ng), extract, AT standard (5ng), extract on chromatographic plate. Apply 3 µl TFAA to one of extract and AT standard spots. Place cover

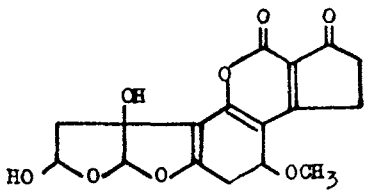
glasses on spots. 10 minutes later remove glasses. After removing unreacted TFAA residues develop plate in chloroform-acetone-isopropanol system (85:10:5). The presence of AT in the extract is confirmed by detecting a TFAA derivative in it, corresponding in its R_f value and fluorescence colour to the TFAA derivative of the AT standard. AT derivatives (B_{2a}, G_{2a}, M_{2a}) obtained in the reaction with TFAA are hydroxylated derivatives of initial AT (B_1, G_2, M_1). The structural formulae of AT derivatives are shown in Fig.3. Owing to the presence of a hydroxyl group in their molecule, AT B_{2a}, G_{2a}, M_{2a} have a higher polarity than the initial AT.



B_{2a}



G_{2a}



M_{2a}

Fig.3. Structural formulae of B_{2a}, G_{2a}, M_{2a} aflatoxins.

Hydroxylated AT derivatives and initial AT are, therefore, separated with the help of sufficiently polar solvent systems, such as: chloroform-acetone (4:1), water-saturated ethyl acetate, chloroform-methanol (95:5), chloroform-acetone-isopropanol (85:10:5). The R_f value of group 2a aflatoxins is lower than R_f of initial AT in standard chromatography conditions, that is why the spots of hydroxylated products fluorescing in long-wave UV light are located on the plate below the initial compounds. The relative chromatographic mobility of aqueous adducts in the reaction of AT B_1, G_1, M_1 with trifluoroacetic acid (Table 7) is a constant value for the concrete TLC conditions (solvent system, adsorbent, saturation of chamber with solvent vapour, humidity, etc.).

Table 7. Chromatographic mobility of B_{2a}, G_{2a}, M_{2a} aflatoxins relative to initial aflatoxin

Solvent system	$R_{f_{B_{2a}}}/R_{f_{B_1}}$	$R_{f_{G_{2a}}}/R_{f_{G_1}}$	$R_{f_{M_{2a}}}/R_{f_{M_1}}$
chloroform-acetone (4:1)	0.25	0.25	-
chloroform-acetone--isopropanol (85:10:5)	0.53	0.49	0.40

4. Test with acetic anhydride (used only for AT M_1).

The presence of AT M_1 in the investigated extract can be confirmed by acetylating the AT M_1 hydroxyl group with acetic anhydride. For this purpose, add 5-10 µl acetic anhydride solution in puridine (1:1) to spot of AT in extract and in standard,

or spray whole plate with this mixture. Place cover glass. Heat plate to 50° for 10 minutes. Remove unreacted acetic anhydride by heating plate without cover glass. Develop plate in chloroform-acetone system (9:1). The obtained AT M_1 acetate displays blue fluorescence in UV light (365 nm), similar to that of AT M_1. The chromatographic mobility of AT M_1 acetate is higher than that of the initial AT M_1 (e.g., in chloroform-acetone (9:1) the R_f of M_1 acetate is 0.5-0.6, R_f of initial M_1 - 0.1 -0.5).

Similar tests with the formation of acetyl derivatives can be performed for AT B_{2a}, G_{2a}, using them as an additional confirmation of the presence of AT B_1 and G_1.

5. Test with 2,4-dinitrophenylhydrazine.

The test with 2,4-dinitrophenylhydrazine is used to distinguish between AT of groups B and G. The test is based on forming stained hydrazones with the pentane ring carbonyl group. To obtain derivatives spray TLC plate with 2,4-dinitrophenylhydrazine solution in acetic acid and heat it to 55°C. At of group G do not show such reaction. This test is seldom used because of its insufficient sensitivity.

6.Test with sodium boron hydride.

The test with sodium boron hydride is used to confirm the presence of AT B_1 and B_2, reduced to AT RB_1 and RB_2, respectively. In elution with chloroform-ethyl acetate mixture (1:3) the R_f values are: B_1 0.54, RB_1 0.71, B_2 0.46, RB_2 0.65.

QUANTITATIVE DETERMINATION OF AFLATOXINS WITH THE HELP OF TWO-DIMENSIONAL TLC

Spot no less than 10% of analyzed extract in lower right-hand corner of 15x15 chromatographic plate. In lower left-hand corner of plate apply, according to pattern, several spots of AT B_1, B_2, G_1, G_2 mixture working standard solution in amounts from 0.5 to 5 ng. Spot 5 ng AT mixture working standard solution in upper right-hand corner. Perform two-dimensional TLC. AT are vizualized by specific fluorescence in long-wave UV light (365 nm). By comparing fluorescence intensity of different quantities of AT standard (from 0.5 to 5 ng) with corresponding spot of AT in extract determine visually AT amount in aliquot portion of extract spotted on plates. The smallest difference in intensity that can be reliably visualized has been found to equal 20%. In different assays the reproducibility of the results of visual comparisons can vary, and the coefficient of variation reaches 15-30%. The method in question is semiquantitative. It has been established that, on the average, the detection limit in fluorescent determination of AT B_1, G_1 and M_1 in a spot amounts to 0.5 ng, and for B_2 and G_2 - to 0.2 ng at the point of application.

PRACTICAL WORK

ANALYSIS OF AFLATOXINS IN CEREAL AND VEGETABLE OIL SPECIMENS BY THE CB METHOD

Widely used for the quantitation of AT in oil-yielding and, partially, cereal crops is the CB method comprising AT extraction from the analyzed sample with chloroform, freeing the obtained extract of lipid components and impurities interfering with fluorescence by means of adsorption column chromatography, TLC, and visual appraisal of the formed spots (Fig.4). The method has the advantages of a sufficiently high degree of extract purification, a high sensitivity and simplicity of determinations. The CB method has at present undergone extensive interlaboratory approbation and is accepted in a number of countries as the official method of AT quantitation.

Experimental procedure

1. Extraction of aflatoxins from the sample.

Grind investigated sample thoroughly in coffee-grinder or laboratory mill. Place 50 g ground sample in conical flask. Add 25 ml distilled water and stir thoroughly to achieve complete moistening of sample.

Pour 250 ml chloroform into flask, add 25 g Celite (the usual total extract volume: sample mass ratio is 5:1) and tightly stopper flask.

Extract 30 min in dark room at continuous stirring in shaking apparatus. Filter off extract through folded paper filter, withdraw first 50 ml filtrate.

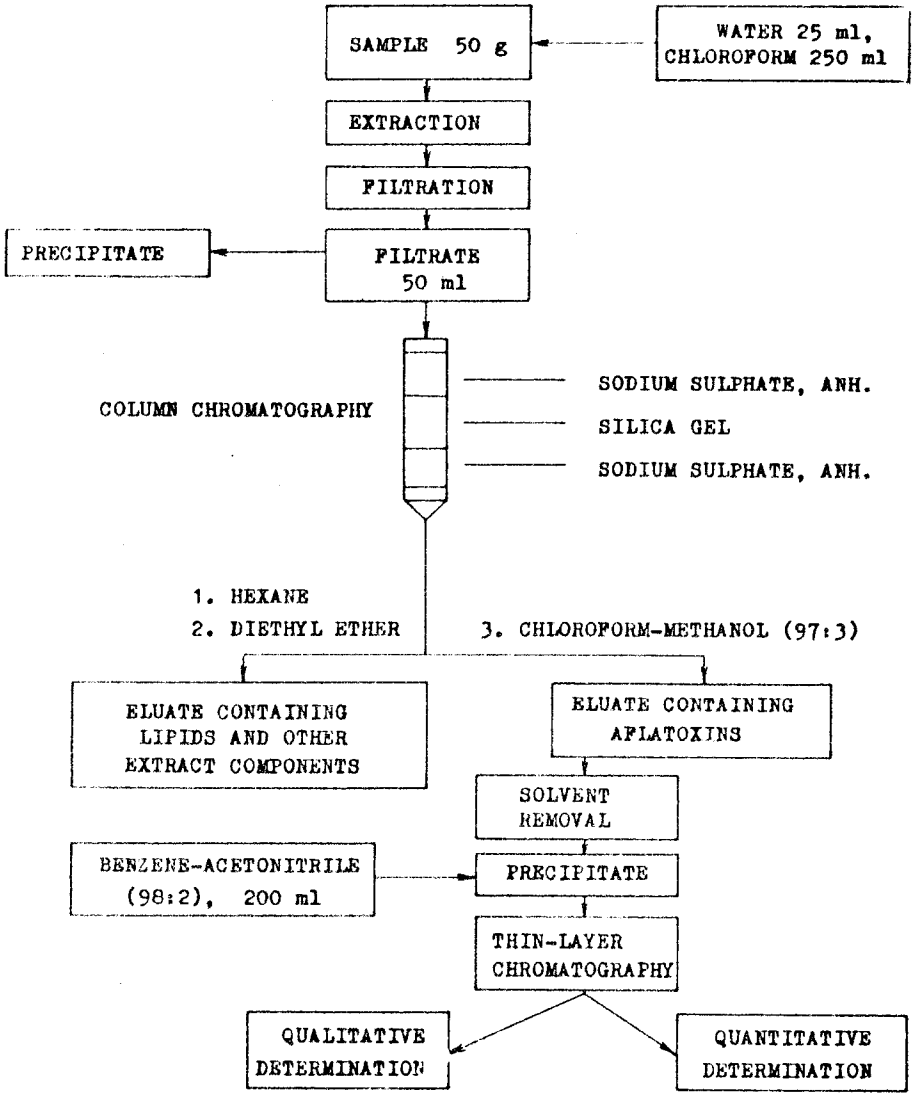


Fig.4. Scheme of the analysis of cereals and vegetable oils for aflatoxins by the CB-method.

2. Purification of the aflatoxin fraction by column chromatography

Place cotton wool plug on bottom of 300 x 22 mm glass chromatographic column. Add 20 ml chloroform and 5 g anhydrous sodium sulphate. Add 10 g silica gel suspension in chloroform and wash column with 20 ml chloroform.

Place 15 g anhydrous sodium sulphate on upper layer of sorbent (silica gel) to provide constant moistening of column while extract is purified. Let chloroform flow down to upper boundary of sorbent layer. Apply 50 ml chloroform extract from analyzed sample on column. Let chloroform flow down to surface of sodium sulphate layer.

To remove lipid components, pigments and other contaminating products, wash column successively with 150 ml hexane and 150 ml absolute diethyl ether.

Elute AT from column with 150 ml methanol-chloroform mixture (3:97). Collect fraction till cessation of eluate outflow.

Withdraw solvent in vacuum of water-jet pump.

Dissolve the obtained residue in 0.2 ml (200 µl) bezene-acetonitrile mixture (49:1).

3. Thin-layer chromatorgraphy of aflatoxins

TLC of AT is performed on ready-to-use chromatograpchic plates "Silufol" (CzSSR).

Qualitative determination

Using microsyringe, spot "Silufol" plate with 2 µl, 6 µl and twice 10 µl analyzed sample. Spots of solutions are applied

on a line 2 cm away from the lower edge of the plate and 1.5-2 cm from each of the lateral sides. Application should be performed in small portions so as to obtain spots of not more than 5 mm diameter on the starting line.

Apply 2.6 µl, 10 µl standard AT B_1 solution with concentration 0.5 µg/ml on the same chromatographic plate. Add 5 µl standard AT B_1 solution to one of analyzed sample spots (10 µl) as internal standard. Spot plate with 5 µl standard solution containing all four AT (B_1, B_2, G_1, G_2) to check whether sufficient separation is achieved after development.

Develop plate in chloroform-acetone system (9:1).

Retrieve chromatographic plate from developing chamber, dry the plate in air, protecting it from bright light.

To detect aflatoxins, illuminate dry developed plate in dark room with long-wave UV lamp (maximum emmission 365 nm). Appraise visually on chromatogram of the sample extract the intensity of spots whose R_f values are similar to those of the standard spots. Choose optimal aliquot portion of analyzed sample for quantitative TLC analysis.

Quantitative analysis

Spot chosen aliquot portion of extract and twice 6 µl investigated extract on "Silufol" chromatorgaphic plate.

Apply to the same plate successively spots of 2.6, 10, 14 µl working standard AT B_1, B_2, G_1, G_2 mixture solution with concentrations 0.5, 0.1, 0.5, 0.1 µg/ml, respectively. Superimpose 10 µl standard on one of 6 µl spots of the sample as an internal standard.

Develop plate in chloroform-acetone system (9:1).

Retrieve plate from developing chamber and dry it, protecting from bright light.

Visually quantitate aflatoxins by comparing fluorescence intensity of spots from analyzed extract with that of spots from pure AT standards of known concentration on developed chromatogram.

Calculate AT concentration in the sample.

AT content (μg/kg)

$$C = \frac{S \times Y \times V}{W \times X},$$

where S is the volume of standard AT solution (μl);

Y is the concentration of standard AT solution (μg/ml);

V is the final volume of sample extract prior to spotting (μl);

X is the volume of extract from the sample, producing in the spot a fluorescence equivalent to that of S (μl);

W is the mass of the analyzed sample, corresponding to the part of chloroform extract applied to the column (kg) (0.01 kg if 50 ml chloroform extract was taken).

ANALYSIS OF AFLATOXINS IN COFFEE AND COCOA BEANS

The chemical method of AT quantitation in coffee and cocoa specimens comprises:

1) AT extraction from the sample with organic solvents;

2) extract purification

3) AT separation with the help of TLC

4) visual appraisal of AT concentration.

It is to be noted that a high oil content in some specimens, e.g., in cocoa, makes it impossible to obtain directly from them a finely ground sample. That is why cocoa beans and coffee are frozen before grinding.

Samples of cocoa beans containing a large percent of lipids are degreased with hexane before extraction.

Cocoa beans are known to contain theobromine (up to 1.8%), and coffee grains - caffeine (up to 1.5%), which, being superimposed on aflatoxins in TLC, interfere with AT quantitation. Therefore, analysis of these products includes a stage of freeing the obtained extracts of these alkaloids. Thus, when aflatoxins are extracted from cocoa beans, a mixture of chloroform with 25% silver nitrate aqueous solution is used to remove theobromine (Fig.5). For chromatographic purification of green coffee extracts, florisil is used instead of silica gel, which makes possible selective elution of caffeine with tetrahydrofuran (Fig.6).

Analysis of cocoa beans for aflatoxins

AT extraction from the sample

Take slightly more than 50 g tested cocoa bean sample, freeze in liquid nitrogen. Grind in frozen state in cofee-mill.

Weigh 50 g ± 0.1 g ground cocoa sample, place in paper cartridge and extract 3 h in Soxhlet apparatus.

Transfer paper cartridge with degreased sample from Soxhlet apparatus to Petri dish and dry the sample in desiccator at 50°C 1 h.

Quantitatively transfer degreased dried sample to conical flask. Add 25 ml 25% silver nitrate aqueous solution and stir

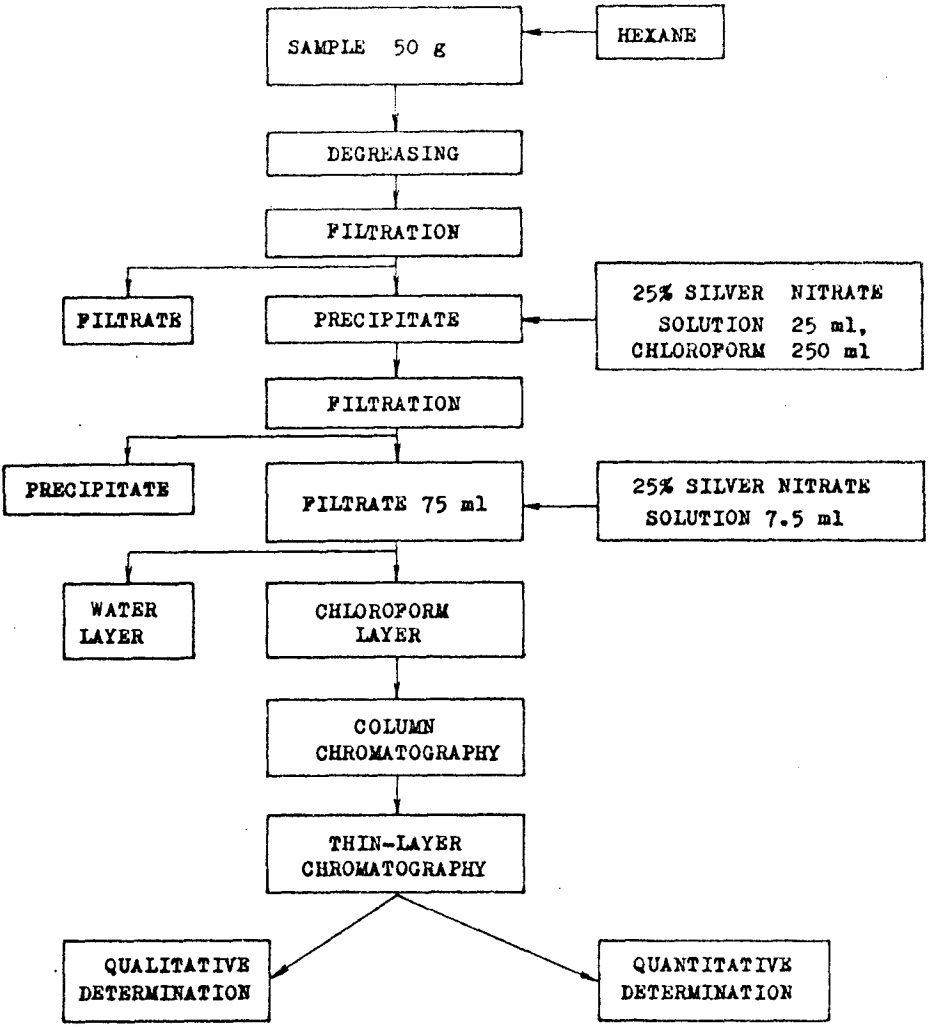


Fig.5. Scheme of determining aflatoxins in cocoa beans.

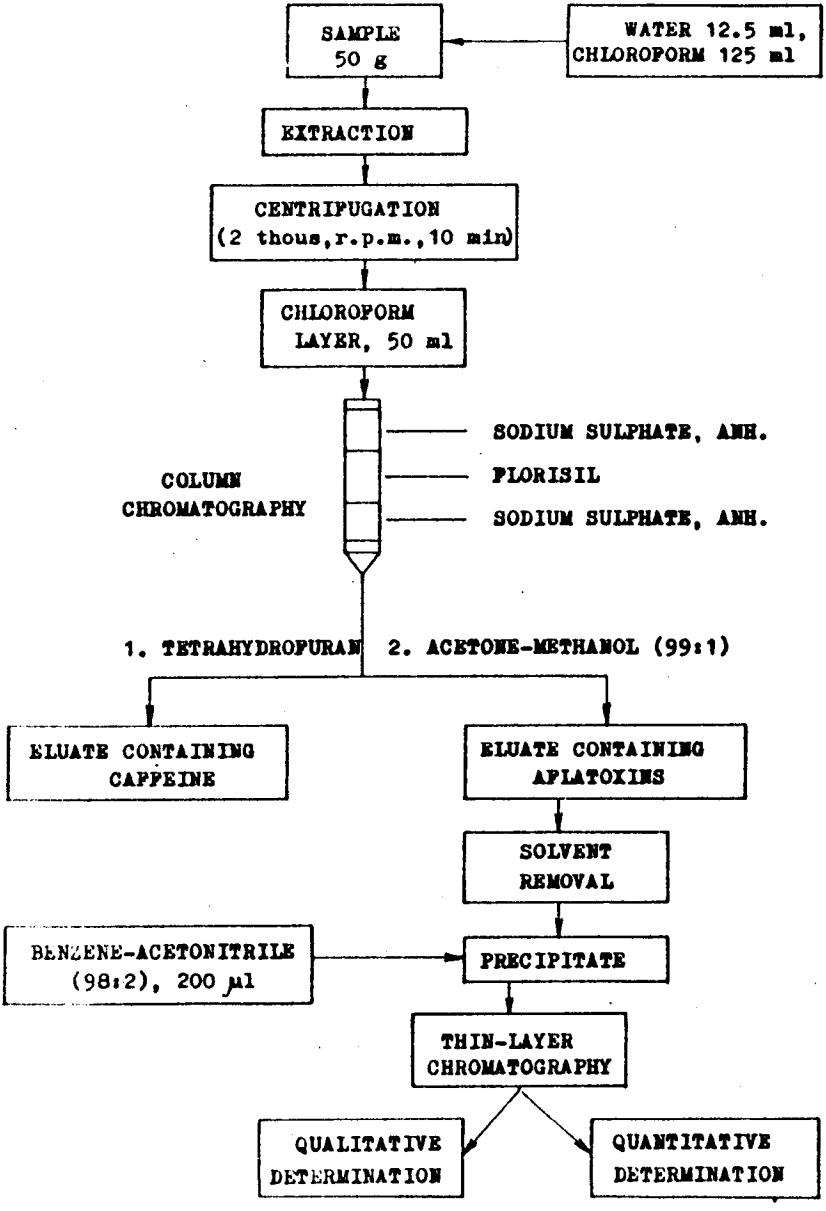


Fig.6. Scheme of determining aflatoxins in coffee grains.

thoroughly to achieve complete moistening of the sample. Pour 250 ml chloroform into mixture. Extract aflatoxins 30 min in darkness at continuous shaking.

Filter off chloroform extract through folded paper filter. Withdraw first 75 ml filtrate and transfer to conical flask, add 7.5 ml 25% silver nitrate aqueous solution. Vigourously shake mixture in flask 15 min.

Transfer mixture from flask to separating funnel. After separation of layers collect 50 ml chloroform extract.

Column chromatography

Proceed as described in practical work "Analysis of aflatoxins in cereal and vegetable oil specimens by the CB method".

Thin-layer chromatography

Proceed as described in practical work "Analysis of aflatoxins in cereal and vegetable oil specimens by the CB method".

Analysis of green coffee for aflatoxins

1. AT extraction from the sample

Freeze tested sample with liquid nitrogen or dry ice and thoroughly grind in frozen state in coffee mill.

Weigh 25 g $\pm$ 0.1 g ground coffee, place in conical flask. Pour in 12.5 ml distilled water and stir the sample with glass rod to achieve complete moistening.

Add 125 ml chloroform to the mixture in flask. Stopper flask tightly. Extract AT 30 min at continuous shaking of the sample in shaking apparatus.

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Transfer mixture from conical flask to centrifuge glass inserts and centrifuge 10 min at 2000 r.p.m.

Collect 50 ml chloroform extract for subsequent sample purification from caffeine by column chromatography on florisil.

2. Column chromatography

Place cotton wool plug on 300 x 22 mm column bottom to retain sorbent. Add 5 g anhydrous sodium sulphate.

Pour in chloroform to approximately 2/3 of column height and add at stirring 5 g previously washed and deactivated florisil.

When florisil settles down add slowly 10 g anhydrous sodium sulphate.

Let chloroform flow down to sodium sulphate layer surface and place 50 ml analyzed extract in column.

Let chloroform flow down to sodium sulphate layer surface and wash column with 150 ml tetrahydrofuran to remove caffeine.

Elute AT from column with 100 ml acetone-methanol mixture (9:1). Collect fraction in separate conical flask till cessation of eluate outflow.

Remove solvent from acetone-methanol eluate by vacuum distillation. Dissolve dry residue in 250 ml benzene-acetonitrile mixture (49:1) to prepare sample for TLC.

3. Thin-layer chromatography

Proceed as in practical work "Analysis of aflatoxins in cereal and vegetable oil specimens by the CB method". In the present work, prior to chromatographic purification of green

coffee extracts on a column with florisil the latter is deactivated as described below.

4. Florisil deactivation

Place 500 g florisil in glass chromatographic column. Wash florisil with 1 l hexane-acetic acid mixture (99:1) and 500 ml hexane to remove residual acetic acid.

Empty florisil from chromatographic column into tray and dry in desiccator overnight (12 h).

Collect 100 g washed and dried florisil in conical flask. Add 10% water (by volume), stir thoroughly and shake in shaking apparatus 60 min. Leave deactivated florisil to stand 24 hours in hermetically sealed vessel. Use prepared portion of flurisil within a week.

Tetrahydrofuran purification

Pass 250 ml tetrahydrofuran through column with 25 g active aluminium oxide.

ANALYSIS OF AFLATOXINS IN CEREAL AND VEGETABLE OIL SPECIMENS

At present the Sanitary and Epidemiological Service of the Ministry of Public Health of the U.S.S.R. uses extensively the fluorescent-chromatographic method for AT detection, identification and quantitation in various foodstuffs of vegetable origin. This method consists of the following stages: extraction with aqueous acetone; freeing the obtained extract of components interfering with AT determination by means of precipitation with complexing agents, liquid-liquid extraction and column chromato-

graphy; AT detection and quantitation with the help of two-dimensional TLC (Fig.7). Detection limit of the method is 0.5-1.0 μg AT B_1 per 1 kg product, coefficient of variation - 0.3-5.0, degree of AT B_1 extraction from different products varies within 65-90%.

Experimental procedure

1. Extraction of aflatoxins from the sample.

Thoroughly grind investigated sample in coffee mill to flour consistency. Weigh 25 g sample and place it in 300-500 ml conical flask, moisten with 25 ml 10% sodium nitrate aqueous solution and add 100 ml acetone.

Shake mixture 30 minutes in shaking apparatus. Filter off extract through folded paper filter. To speed up filtration, 2-3g Celite can be placed on the filter. Collect 50 ml filtrate.

2. Purification of aflatoxin extract with the help of complexing agents

Add 50 ml 10% lead acetate aqueous solution to 50 ml water-acetone extract and shake. 10-15 minutes later filter off, withdrawing first 80 ml filtrate.

3. Purification of aflatoxin fraction by liquid-liquid extraction.

Transfer 80 ml extract to separating funnel. Extract with hexane (2 times x 40 ml). Discard combined hexane extracts. Extract degreased water-acetone layer with chloroform (3 times x 40 ml). Combine chloroform extracts, dry over anhydrous sodium sulphate. Filter off extract through cotton wool wad into round-bottom flask, evaporate in rotary evaporator to 1-2 ml volume.

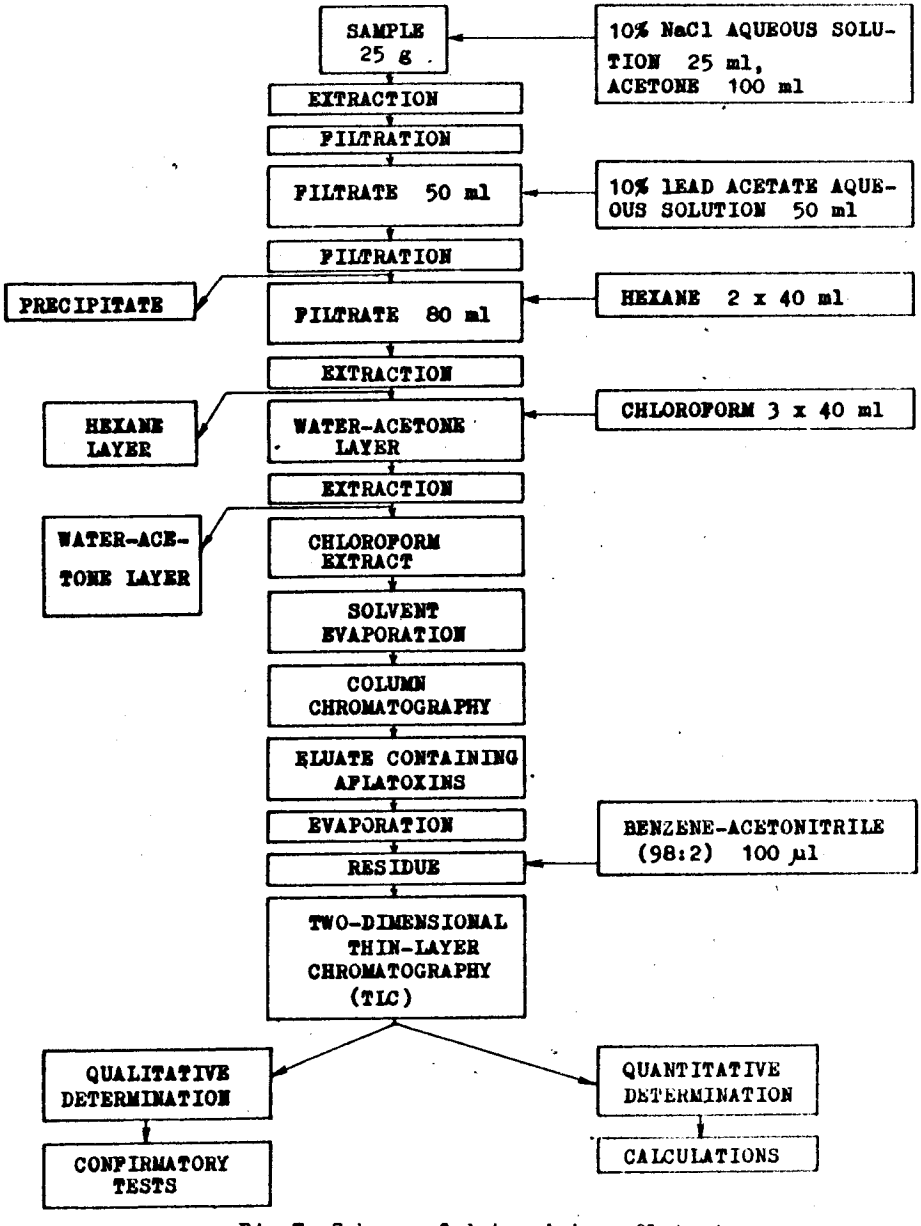


Fig.7. Scheme of determining aflatoxins in cereals and vegetable oils.

4. Purification of aflatoxin extract by column chromatography.

Prepare column as described below. Place cotton wool plug on glass chromatographic column bottom and add anhydrous sodium sulphate (Na_2SO_4 layer height - 3-4 mm). Place in column 2 g silica gel prepared as suspension in 25 ml chloroform. After silica gel precipitation add 2 g anhydrous sodium sulphate.

Let chloroform flow down to sodium sulphate layer surface. Pour solution of obtained extract into column. When extract level reaches upper boundary of Na_2SO_4 add 100 ml chloroform-acetone mixture (4:1). Collect fraction from beginning of elution to cessation of eluate outflow. Evaporate solvent to dryness. Dissolve dry residue in 100 µl benzene-acetonitrile mixture (98:2).

5. Detection and quantitation of aflatoxins in the extract with the help of two-dimensional thin-layer chromatography, qualitative determination.

Apply with microsyringe 10 µl extract to "Silufol" plate in lower right-hand corner 1.5 cm away from the plate edge so that diameter of the spot does not exceed 5 mm.

On the same chromatographic plate apply 10 µl AT B_1, B_2, G_1, G_2 mixture standard working solution in lower left-hand and upper right-hand corners 1 cm away from the plate edge. The size of spots of the standard must correspond to the size of the extract spot at the point of application.

Develop plate in TLC chamber containing chloroform-acetone-benzene mixture (4:1:1), the solvent level must be 1 cm below the applied spots.

Retrieve the plate from the chamber, dry it in exhaust hood. Turn plate 90° and develop it in ether-methanol-water system (96:3:1). Examine plate in long-wave UV light. The presence of spots corresponding in their chromatographic mobility and fluorescence colour to the spots of AT standards indicates possible contamination of the foodstuff with AT.

Quantitation of aflatoxins

If AT have been detected in the extract it is necessary to perform their quantitative determination.

Apply 10 µl extract, with spot diameter not to exceed 5 mm, in lower right-hand corner of "Silufol" plate 1.5 cm away from its edges.

In lower left-hand corner, 1, 2 and 3 cm away from left and 1.5 cm - from lower edge of plate, apply 2.0, 4.0 and 6.0 µl, respectively, working standard AT solution.

Apply 6.0 µl standard solution in upper right-hand corner 1.5 cm away from plate edges.

Perform TLC as described above.

Comparing fluorescence intensity of different quantities of AT standards on plate with that of corresponding spots of extract, visually quantitate AT in extract spot in nanograms (ng).

Calculate AT content in the product from formula

$$C = \frac{V_1 \cdot V_3 \cdot V_5 \cdot M}{V_2 \cdot V_4 \cdot V_6 \cdot M} \text{ µg/kg, where}$$

C is the concentration of aflatoxin in the foodstuff, µg/kg;

V_1 is the volume of water-acetone mixture, ml;

V_2 is the volume of water-acetone filtrate taken for analysis, ml;

V_3 is the volume of water-acetone filtrate and lead acetate solution, ml;

V_4 is the volume of filtrate after purification with lead acetate, ml;

V_5 is the volume of purified evaporated extract solution in benzene-acetonitrile mixture before TLC, μl;

V_6 is the volume of extract solution applied on a plate, μl;

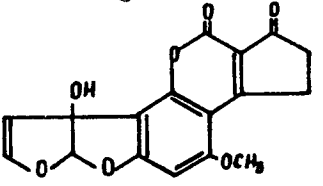
m is the amount of aflatoxin in extract spot on a plate, ng;

M is the weight of product taken for analysis.

If the intensity of AT fluorescence in the spot of the extract is higher than that in the spots of the standards corresponding to 6.0 μl of working solution, a smaller aliquot portion of the extract should be applied to a plate.

ANALYSIS OF AFLATOXINS IN SAMPLES OF DAIRY PRODUCTS

Aflatoxin M_1 is a hydroxylated metabolite of AT B_1 found in the milk of mammals receiving aflatoxin B_1 with the fodder. AT M_1 has the following structure:



Owing to the presence of a hydroxyl group, AT M_1 has a much higher polarity than the other AT. This facilitates the binding of AT M_1 with proteins present in milk in large quantities, which hampers AT isolation. This should be taken into ac-

count when analyzing dairy products. Thus, aqueous acetone containing citric acid and sodium chloride is used to extract AT M_1, and acetic acid mixed with toluene - to clean the extract by means of column chromatography. This facilitates the breaking of AT-protein bonds and, in this way, improves the solvent purification and the completeness of extraction. In addition to this, solvents of higher polarity are used to elute AT M_1 from the chromatographic column.

The chemical method of quantitating AT M_1 in dairy products includes the following stages (Fig.8):

1) AT extraction from the sample;
2) purification with the help of liquid-liquid extraction and column chromatography;
3) AT detection with the help of TLC;
4) visual appraisal of AT in a spot.

1. Aflatoxin extraction from the sample

Place 100 ml milk in Erlenmeyer flask. Add previously prepared solution of 4 g sodium chloride and 0.48 g citric acid in 10 ml water.

When dried milk is analyzed, homogenize 10 g sample in solution of 4 g sodium chloride and 0.48 g citric acid in 100 ml water.

Add 10 g Celite-545 and 300 ml acetone to mixture in flask. Extract AT M_1 30 min at continuous shaking. Filter off extract through folded paper filter into measuring cylinder. Withdraw first 275 ml filtrate.

2. Purification of Extract

Transfer 275 ml filtrate to conical flask and add 20 ml 15% lead acetate aqueous solution. Rinse cylinder with 200 ml water and pour it into the same flask. Stir, leave to settle 10-15 min. Add 10 ml saturated sodium sulphate solution and 10 g Celite-545. Mix thoroughly.

Filter off solution through folded paper filter into previously used measuring cylinder. Transfer 350 ml filtrate to 500 ml separating funnel. Add 100 ml hexane and shake vigourously for 1 min.

Let the layers separate. Collect lower water-acetone layer into clean conical flask. Remove hexane layer from separating funnel.

Transfer water-acetone extract to the same separating funnel. Rinse flask with 50 ml 5% sodium chloride aqueous solution and transfer it to the same separating funnel. Add 100 ml chloroform to separating funnel. Shake 1 min. Let layers separate.

Transfer chloroform extract into clean conical flask.

Repeat extraction procedure using 50 ml chloroform.

Combine chloroform extracts and dry over anhydrous sodium sulphate.

Filter off chloroform extract through folded paper filter. Evaporate extract in rotary evaporator to 15-20 ml volume.

3. Column chromatography

Prepare column as described below.

Place cotton wool plug on bottom of chromatographic column and add 0.5-1 g anhydrous sodium sulphate. Place 2 g silica gel, in the form of suspension in 25 ml chloroform, in column. After

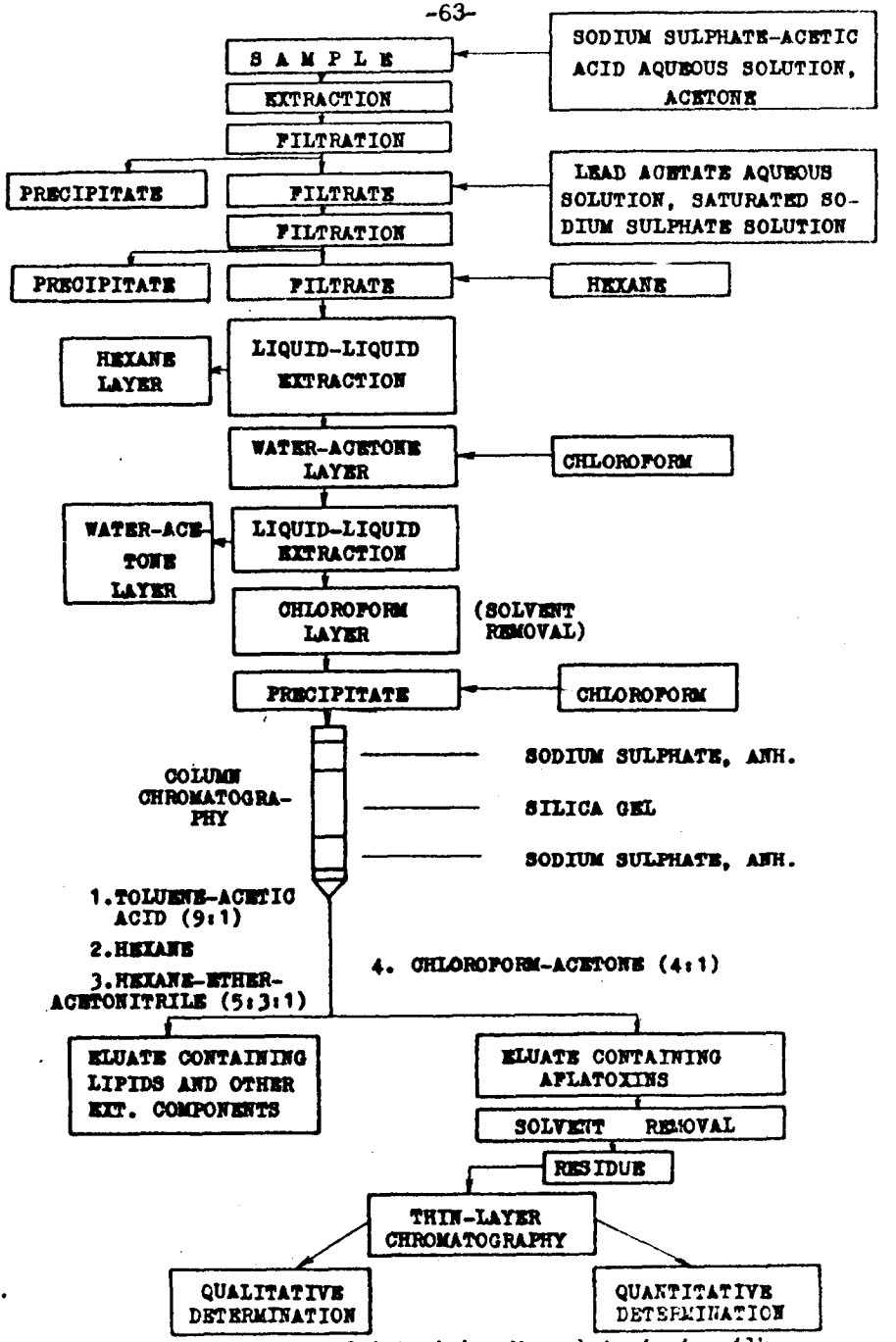


Fig.8. Scheme of detemining M_1 arlatoxin in milk.

silica gel precipitation, add 2 g anhydrous sodium sulphate. Wash column with 25 ml chloroform. Let chloroform flow down to surface of sodium sulphate layer.

Place 15-20 ml chloroform extract of analysed sample in column. Let chloroform flow down to sodium sulphate layer surface. Wash column successively with the following eluents:

25 ml chloroform:

25 ml toluene-acetic acid mixture (9:1);

25 ml hexane;

25 ml hexane-diethyl ether-acetonitrile mixture (5:3:2).

Elute AT from column with 40 ml chloroform-acetone mixture (4:1). Collect fractions from beginning of elution to cessation of eluate outflow. Remove solvent in vacuum. Evaporate to dryness in nitrogen flow. Avoid overheating of dry residue. Dissolve obtained residue in 100 ml chloroform.

4. Thin-layer chromatography

AT M_1 in analyzed material should be quantitated by means of two-dimensional TLC.

Mark "Silufol" plate for two-dimensional TLC. Spot 20 ml solution obtained in the previous section. On the same plate spot 1-4 µl working standard AT M_1 solution, conc. 0.5 µg/ml, so as to obtain 1 ng AT M_1 in 1st direction of chromatogram development and 0.5, 1.2 ng AT M_1 in 2nd direction.

Develop plate in 1st direction in chloroform-acetone-isopropanol system (85:10:7). Retrieve chromatographic plate from developing chamber, dry in air in darkness.

Develop TLC plate in 2nd direction in ether-methanol-water system (94:5:1). Retrieve TLC plate from chamber and dry in air in dark place not less than 5 minutes.

Detect AT M_1 standard spots in light of long-wave UV lamp (maximum emmission 365 nm). Mark spots with pencil.

Through centre of AT M_1 standard spots draw straight lines parallel to the line of application. At their intersection must lie a spot with blue fluorescence corresponding to AT M_1 of sample.

Visually compare fluorescence intensity of corresponding spot from analyzed extract with intensity of spots of pure AT standards of known concentration. Estimate AT M_1 quantity in spot of the sample (in ng).

Quantitate AT M_1 content in 1 l analyzed milk (1 kg dried milk) from formula

$$C = \frac{1}{k} L \frac{W \cdot m}{V \cdot M},$$

where C-is the content of AT M_1 (µg) in 1 l (or in 1 kg) of milk;

W (ml)-is the volume of final extract of sample (TLC);

V (µl)-is the volume of the part of the sample extract applied to a TLC plate;

m (ng)-is the amount of AT M_1 in a corresponding spot of the sample;

M (g or ml)-is the weight or the volume of analyzed portion of milk;

k-is the coefficient of AT extraction from milk (k = 0.7);

L-is the coefficient taking into account the fact that only a part of the initial water-acetone extract is used for analysis, as well as the extract dilution.

This coefficient is calculated from the formula:

$$L = \frac{A\ (B + C)}{B \cdot D}$$, where

A (ml)-is the volume of the initial water-acetone extract of milk (400 ml);

B (ml)-is the volume of the analysed aliquot portion of initial water-acetone extract (275 ml);

C (ml)-is the volume of lead acetate and sodium sulphate aqueous solution (230 ml);

D (ml)-is the volume of the analysed aliquot portion of water-acetone extract after isolation of proteins (350 ml).

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