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SAMPLING OF AGRICULTURAL PRODUCTS AND THEIR ANALYSIS FOR AFLATOXIN DETERMINATION

Manual

**Centre for International Projects
USSR State Committee for Environment Protection**

Moscow, 1989

**FAO/UNEP/USSR/Tanzanian Project
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"IMPROVEMENT OF MYCOTOXIN CONTROL IN TANZANIA"

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AND THEIR ANALYSIS
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FOREWORD

THIS MANUAL has been primarily designed for use by Tanzania laboratory personnel who participate in the Mycotoxin Monitoring Programme. It is hoped, however, that other specialists with an interest in mycotoxins will find this manual providing a useful introduction to the subject. It reviews, generally, the problems of mycotoxins and, particularly, aflatoxins as well as the methods used at the Government Chemist Laboratory for the analysis of foods and feeds for mycotoxin.

Should any reference to any particular makes of equipment, chemicals or their suppliers be given wherever in this manual it is not intended to imply that these are the only suitable makes or the only secure suppliers of such items but merely that they are the ones officially used in the Mycotoxin Monitoring Programme managed by the Government Chemist Laboratory. Additional information on food sampling and analysis can be provided by the FAO Food and Nutrition Paper "Food Control Manual" No's 14/7, 14/8, 14/9 on "Food Analysis: General Techniques, Additives, Contaminants and Composition", "Food Analysis: Quality, Adulteration, and Tests of Identity", "Introduction to Food Sampling", respectively.

TERMINOLOGY

AT	-	Aflatoxin
MT	-	Mycotoxin
TLC	-	Thinlayer- chromatography
HPLC	-	High-performance liquid chromatography

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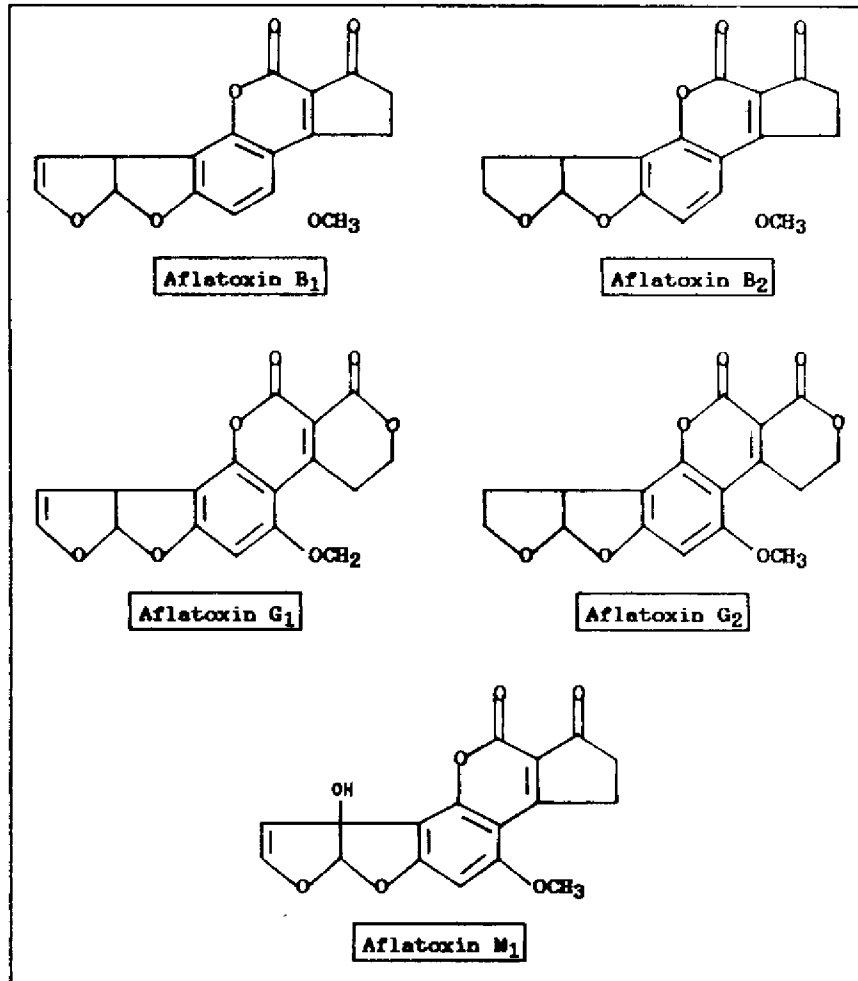
1. INTRODUCTION

Fungal invasion of food can result in marked deterioration in quality and often outright destruction. A meaningful food control programme must therefore include measures to protect food from fungal deterioration. A more subtle but even more compelling reason to control such fungi is that some species are capable of producing highly toxic substances when growing on various organic materials including food. These substances, which are collectively known as **mycotoxins**, have been implicated as causative agents in a number of disease syndromes - **mycotoxicoses** - in humans and animals.

Mould growth on foods is very common, especially in warm, humid climates. It can occur in the field, or in storage after harvest. Mould infection of foods such as grains, seeds and nuts is often localized in pockets, especially in bulk storage. Frequent and adequate sampling for test, therefore becomes a necessity. It is important to remember that all food samples suspected of being contaminated with **mycotoxins**, must be handled with care.

Aflatoxin is probably the most common and widely known **mycotoxin** contaminant. It is produced by the moulds **Aspergillus flavus** and **Aspergillus parasiticus**. In fact the name is a composite word derived from "A. flavus toxin". Foods which are commonly affected include all nuts (especially groundnuts), cottonseed, copra, rice, maize, wheat, grain, sorghum, pulses, figs, oilseed cakes and unrefined vegetable oils. The chemical structures of five aflatoxins are noted below (see p. 7).

Aflatoxins **B₁**, **B₂**, **G₁** and **G₂** refer to toxins which fluoresce blue (B) or green (G) under ultraviolet light and are separable by thin-layer chromatography. Aflatoxin **B₁** is by far the most commonly found of the four toxins. Aflatoxin **M₁** represent the toxin **B₁** which has been metabolized and converted within the body of a lactating animal. Their finding in milk led to their "M" designation.



2. SAMPLING TECHNIQUES

A crucial step in the laboratory control of foodstuff contamination with mycotoxins is the sample collection for analysis. The total error of aflatoxin determination in products includes both the errors due to sampling from a product lot and those related to chemical analysis (Fig. 1).

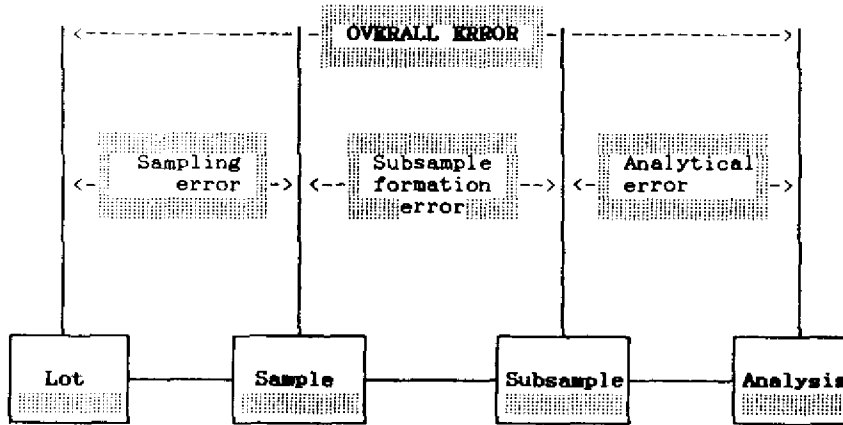


Figure 1. Typical sequence of operations in the evaluation of Aflatoxin concentration and possible contributions to the overall error

While collecting samples of foodstuffs one should pay special attention to a heterogeneous character of mycotoxin distribution. Therefore, the collection of representative samples is indispensable for the correct estimate of the extent of a foodstuff lot contaminated with mycotoxins.

Collection of a sample which is not representative of the whole lot contamination level may result either in the refusal of the entire lot, or otherwise a highly contaminated food lot may be unduly authorized for consumption.

In this context, common requirements have been developed for ensuring a uniform collection of samples. Of these, the representativity of samples appears to be of prime importance. A sample is considered representative if it characterizes the quality of the entire lot. The representative sample is achieved by observing the following principle: the initial sample is formed by combining all individual samples; the average or secondary sample (subsample) is obtained by reducing the quantity of the initial sample; analytical samples or weights are also derived by decreasing the volume of the average sample (subsample).

Usually, the subject for AT analysis is a lot of agricultural produce - and quantity of the product which is homogeneous in quality and intended for a reception, transportation or storage, or else provided with a quality certificate (consignment).

A single sample of a batch is a small quantity of the product taken at a time from a specific spot of the product lot. In order to compose a sample representative of a specific product lot, series of single samples are taken and then combined. The sum of all single samples taken from a specific lot forms the initial or general sample. For determining the level of mycotoxins therein, small amounts of e.g. grain are taken therefrom which are called specimen weights or analytical samples.

When the initial sample collected from important product lots turns out to be too large, the average sample or subspecimen is prepared therefrom. For smaller lots, the initial sample and the average sample may be one and the same (Fig. 2).

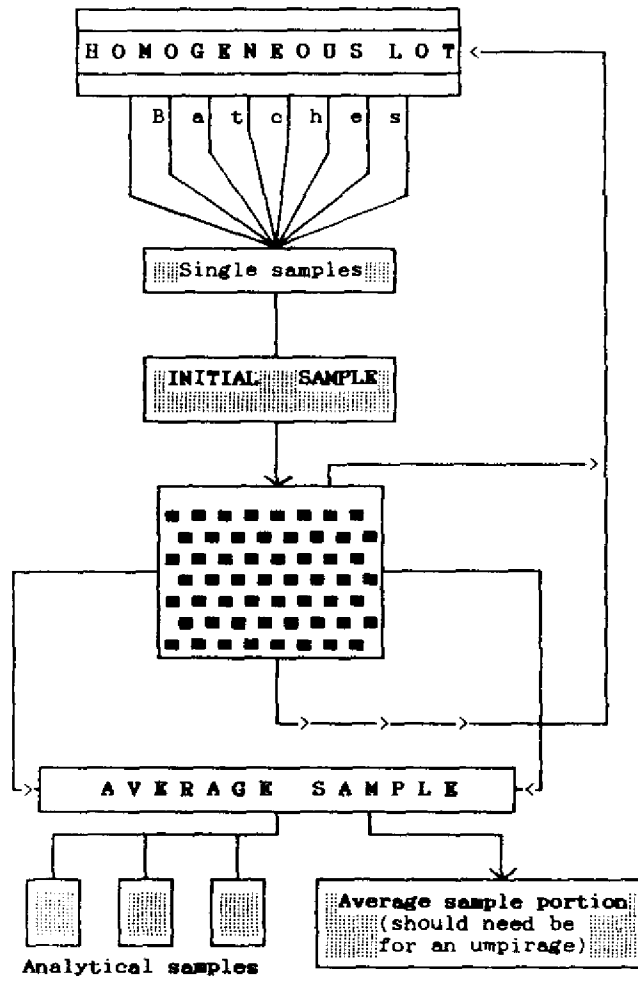


Figure 2. Sampling and specimen preparation scheme

The general (initial) sample is prepared by combining all single samples collected from as many as possible spots of a product lot. For relatively small-sized grains (maize, rice, groundnut, cotton seeds) its mass should not exceed 4-5 kg (Appendix 1). Then the total sample should be thoroughly mixed and fragmented (to ensure the passage of fragments through the 14-mesh sieve). The secondary sample (subsample) is secured by a gradual decrease of the mass of total sample. The mass of subsample is determined depending on the particle size - for coarse fragmentation of the product, its mass should be greater than that of the finer ones (usually a subsample of 1 kg is preferred).

Analytical samples (weights for analysis) are taken only after the subsample has been thoroughly ground (to pass through the 20-mesh sieve), mixed and its mass gradually reduced down to 50 g by quartation.

2.1. TECHNIQUES OF THE SAMPLE COLLECTION FOR MYCOTOXIN ANALYSIS

Samples for mycotoxin analysis in agricultural crops may be collected at different moments: during harvest of crops, storage or processing, since their contamination by mycotoxins may take place in any of these steps. The procedure of sample collection is specific for any of the above cases; however, it is recommended to collect samples when the product under investigation contains greater quantities of small particles, e.g. between corn cobs and grain preference should be given to the latter, whereas crushed maize grains are more advisable for sampling than the whole ones. More representative samples are derived when the sample collection is performed from lots mixed during harvest, loading and unloading operations, transportation and fragmentation, because of the character of mycotoxin distribution. Exporters of the products which may be subject to mycotoxin contamination usually establish direct contacts with their clients and make a joint agreement as regards the procedure of sampling.

2.1.1. Sampling from grain bulks

For collection of grain samples in granaries, the most reliable technique is to perform sampling during loading or unloading operations. Here, various samplers are employed which cross the grain stream at regular time intervals. This sampling method is considered as most accurate, since herein a succession of representative grain stream cross-sections is obtained which forms the basis for establishing the initial (general or bulk) sample. If such a sampler is not available, an operator may be asked to carry out the cross-section sampling using a shovel at regular time intervals. Collection of samples may also be effected by means of mechanical or manual probes which should be long enough to reach the bottom of grain container.

2.1.2. Sampling from a sack-loaded lot

When a grain lot is or will be packed in sacks it is advisable to carry out sampling during the sack filling or emptying. Samples are taken from the top, middle and bottom loads of the sack. When the filled sacks are sealed, single samples are taken using a special probe. The number of sacks to be sampled depends on the lot size and is determined as follows:

Number of sacks in the lot	Minimum number of sacks to be sampled
Up to 10	Each other sack
11 - 100	5 sacks + 5% of the number of sacks in the lot
Above 100	10 sacks + 5% of the number of sacks in the lot

2.1.3. Sampling from trucks

Single samples from trucks with a 3 m-long loaded body are collected from 4 spots, with that of 3.5-4.5 m - from 6 spots and with the body above 4.5 m - from 8 different spots. Spots for sampling are chosen at some 0.5-1.0 m from the front and rear body boards and at 0.5 m from the side ones.

Samples from loaded trailer trains are taken with respect of their dimensions, each trailer being considered as a separate lot.

The total mass of single samples for collection from 4 spots should be under 1 kg, that from 6 spots no less than 1.5 kg, whereas the one from 8 spots should not exceed 2 kg. When the sum of collected samples does not provide the required mass, additional sampling is performed at the same spots from the middle layer of grain bulk.

2.1.4. Sampling from railway cars

Primary samples are usually collected during a goods van loading or unloading, from streams of grain falling from conveyer belts, grain silo outlets, scales, and other places of grain discharge. Sampling is performed either with various samplers or manually.

2.2. HANDLING OF SAMPLES AND IDENTIFICATION

While selecting samples for AT analysis, one should strictly observe the cleanliness of devices and containers used which should be dry and possess no foreign smell. Sampling should be performed in a way that samples themselves and the sampling equipment and containers for their storage are protected from harmful external effects. In view of the fact that samples, depending on specific conditions, are transported or stored for various periods of time (from the instant of sampling to the date of analysis), one should observe measures preventing the mould growth in the interval. It is preferable to reduce the time of sample transportation and to keep samples dry and cool (0°C or below). It is also necessary to do everything possible to avoid the sample moistening during transportation and storage. For this purpose, it is not advisable to keep raw and poorly dried seeds in plastic containers if they are not stored in a cool atmosphere. It is better to use fabric or paper bags. Mould growth and toxin production can take place within a space of several hours; therefore, after their collection, samples should be dried as soon as possible at the temperature of about 80-90°C for three or more hours to reduce the moisture content down to 12-13%. When it is necessary to study the grain microflora, the grain should be dried to the same moisture content at 60°C for a longer time. If a sample intended for analysis was previously cooled, it should be immediately dried and stored dry.

One should also bear in mind that sealed samples cannot be rapidly cooled to 0°C. For this reason the package should be kept open till the grain is cooled. Sample-filled bags should be spacious enough to permit loose grain distribution within a bag for speeding up the cooling process.

It is advisable to analyze samples immediately after cooling or, upon their withdrawal from a cooling chamber, to keep samples in a water-tight package till they reach the ambient temperature.

2.2.1. Restoring lots samples

When collecting a sample from a wholesale or retail source, the inspector must try not to do anything that may jeopardize the dealers possibility of selling the remainder of the lot. If at all possible, especially when collecting a sample from a wholesaler, do not leave slack-filled shipping cases or short weight or short volume containers in the lot after sampling. Back filling is one method of assuring that the dealer has only full cases of merchandise left. Cases from which samples have been collected should be closed and glued shut, barrels and drums properly closed, bags resewn, etc.

2.2.2. Identifying lots sampled

Each shipping case from which a sample unit is collected should be identified in some distinctive way so the lot or any sampled container can be identified if necessary, such as the need to collect an additional sample or if the results of analysis are unfavourable and the remaining product should not be introduced into consumer channels. The identification should not be so conspicuous as to cause undue attention. Industrial or permanent type markers should not be used on containers subject to penetration. Penetration of some ink to the product may interfere with the planned analysis. If water base markers are used the identification should be covered with tape to prevent running.

2.2.3. Identifying marks

Each sample and sub-sample should be identified in a manner that can be related back to the lot from which it was sampled. The identification should also contain some distinctive marks that will indicate a month or even years later that a particular sample was indeed collected. The mark most commonly used for this purpose is the inspectors initials. One way of identifying the subs in a sample is to assign a separate number to each sub. When multiple subs are taken from cases, bales, boxes, etc., in a lot, Arabic numerals and letters in combination may be used. For example: if 2 bags are taken from each van in the lot, the bags may be marked as sub-number 1a, 1b, 2a, 2b, etc., to identify the subs as coming from van number 1, van number 2, etc.

2.2.4. Sample handling

If it is necessary for samples to be shipped to the laboratory the package must be secured with shock absorbing materials for protection from damage en route. Even if the original shipping container is used this does not assure that the sample will be safe. However, often some additional packing with crumpled newspaper will add that extra measure of protection that may be needed. Special precautions must also be taken when shipping products in pressurized containers to avoid possible exposure to excessive heat. Any shippers who use non-pressurized planes may also have special requirements for this type of container to prevent explosion.

3. CHEMICAL METHODS OF AFLATOXIN ANALYSIS

3.1. PRINCIPLES OF AFLATOXIN ANALYSIS

Aflatoxins are colourless, optically active, non-volatile under natural conditions and thermostable crystalline substances which are readily soluble in moderately polar organic solvents and actually insoluble in water.

Under the long-wave UV-radiation (365 nm) aflatoxins B₁ and G₁ produce a blue colour fluorescence (425 nm) while aflatoxins B₂ and G₂ - a green one (450 nm).

Chemical methods of AT analysis are based on the determination of aflatoxins by measuring the intensity of their fluorescence using thin-layer chromatography or high-performance liquid chromatography.

3.2. SAFETY REGULATIONS FOR WORK WITH AFLATOXINS AND AFLATOXINS DECONTAMINATION

Aflatoxins are highly toxic substances and therefore should be manipulated with adequate precautions. By diffusion, solutions of aflatoxins can infiltrate even through rubber and polyvinyl gloves.

Special precaution should be taken when handling crystalline aflatoxins because of their strong electrostatic characters. A metallic spatula should be earthed when used for weighing AT crystals.

After preparation of standard AT solutions one should rinse his mouth with 1% sodium hypochlorite solution; hands should also be rinsed likewise and washed with a soap.

After AT analysis, equipment, instruments and work desks, that could have been in contact with AT, are to be treated with 5% alkaline alcohol solution, then washed with 0.5-1% solution of acetic acid.

The glassware used for AT analysis is to be washed with a potassium dichromate solution in sulphuric acid (one liter of H_2SO_4 is carefully added to 50-60 g of $K_2Cr_2O_7$ mixed with 10 ml of water in a porcelain beaker), then thoroughly washed with tap water and rinsed with distilled water.

For decontamination, AT-containing solutions are totally evaporated; the dry residue is dissolved in 1 ml of methanol, thereafter sodium hypochlorite solution is added and the mixture is kept for two hours and then amended with acetone (5% of the mixture volume).

Decontamination of AT solutions may also be performed by amending successively the dry residue obtained after evaporation with water, conc. sulphuric acid ($d = 2.84 \text{ g/ml}$) and saturated aqueous solution of potassium permanganate (K_2MnO_4 , 0.4 mol/l). After thin-layer chromatography, glass plates containing AT should be treated with 0.8% sodium hypochlorite solution followed by acetone (5% of the total volume).

Nanogram AT quantities may be eliminated by abundant washing with water.

3.3. INSTRUMENTATION AND CHEMICALS

To perform routine analysis for AT it is necessary to have the following equipment and materials:

- Mercury-quartz lamp with a transmission region of 360 nm
- Sample shaker
- Laboratory mill
- Drying cabinet
- Counter balance
- Analytical balance
- Centrifuge with glass inserts
- Rotational evaporator with a trap
- Water bath
- Household refrigerator
- Microsyringes or gauged capillaries
- Chromatographic glass columns (300 x 22 mm)
- TLC chambers with ground caps
- Laboratory propeller mixer

To conduct aflatoxin chemical analysis the following "chemically pure" or "analytically pure" reagents are usually used:

- Chloroform
- Hexane
- Diethyl ether
- Methanol
- Ethanol
- Acetone
- Benzene
- Acetonitrile
- Anhydrous sodium sulphate (calcinated)
- Celite 545 (80-100 mesh) or diatomite Type "Hyflo Super Cel"
- Silica gel for column chromatography, brands L 100/160 μ or L 40/100 μ , Chemapol (CzSSR)
- Silica gel for TLC, L 5/40 μ "Chemapol" or "Merck" or "Silicar" 4G, 7G "Mallinck rodt."
- Ready-made TLC plates "Merck" or "Silufol" (CzSSR)

To prepare aflatoxin standards for chemical analysis one has to take pure aflatoxins B₁, B₂, G₁ and G₂ which are obtainable from the following international sources (list is alphabetical):

1. Aldrich Chemical Co., PO Box 355, Milwaukee, WI 53201 USA.
2. Applied Science Division, Milton Roy Co., 2051 Waukegan Rd., Deerfield, IL 60015 USA.
3. Calbiochem-Behring, PO Box 12087, San Diego, CA 92112 USA.
4. C.Roth, Postfach 1387, 7500 Karlsruhe 1, Federal Republic of Germany.
5. Makor Chemicals Ltd., Box 6570, Jerusalem, 91060 Israel.
6. Myco Lab Co., PO Box 321, Chesterfield, MO 63017 USA.
7. Rijksinstituut voor de Volksgezondheid, P.O. Box 1, 3720 BA Bilthoven, The Netherlands.
8. Senn Chemicals, Laboratorium Guido A. Senn, Postfach 2, CH-8157 Dielsdorf, Switzerland.
9. Sigma Chemical Co., Supelco Inc., P.O. Box 14509, St. Louis, MO 63178 USA.

As a rule the firms provide chrystalline aflatoxins or their solutions.

3.4. PREPARATION AND DETERMINATION OF PURITY AND CONCENTRATION OF AFLATOXINS STANDARD

Analysis of foodstuffs for the presence of aflatoxins requires preliminary preparation of AT standard solutions of preset concentrations. Reliability of the results of chemical analysis is highly dependent on the accuracy of standard solution preparation.

The concentration of standard AT solutions is determined by UV-spectrophotometry. Purity of the prepared standard AT solutions is determined by TLC or UV-spectrophotometry.

3.4.1. Spectrophotometer calibration procedure

Spectrophotometer. ---Capable of measurements from 200 to 400 nm. with 1 cm quartz-face cells.

To calibrate the spectrophotometer it is necessary to prepare three potassium dichromate ($K_2Cr_2O_7$) standard solution in sulphuric acid (H_2SO_4).

- a) Sulphuric acid. ---Approx. 9 mmol/l; dissolve 1 ml H_2SO_4 in 2 liters of water.
- b) Potassium dichromate standard solutions.
 - (1) Approx. 0.25 mmol/l in 9 mmol/l sulphuric acid: weigh accurately 78 mg potassium dichromate and dissolve in 1 liter of 9 mmol/l sulphuric acid. Make measurements of dichromate weight and solution volumes with better than 1% accuracy.
 - (2) Approx. 0.125 mmol/l in 9 mmol/l sulphuric acid: dilute 25 ml of the solution of potassium dichromate 0.25 mmol/l to 50 ml, using the 9 mmol/l sulphuric acid.
 - (3) Approx. 0.0625 mmol/l in 9 mmol/l sulphuric acid: dilute 25 ml of the solution of potassium dichromate 0.125 mmol/l to 50 ml, using the 9 mmol/l sulphuric acid.

Calibrate the spectrophotometer as follows:

- determine the absorbance, A, of the three standard potassium dichromate solution at the maximum absorption near 350 nm, against the 9 mmol/l sulphuric acid as solvent blank;

- calculate the molar absorptivity, ϵ , at each concentration:

$$\epsilon(\text{cm}^2\text{mol}^{-1}) = \frac{A * 1000}{L * C}$$

where: A - absorbance;
C - concentration, mmol/l;
L - 1 cm.

If the three values vary by more than the guaranteed accuracy of the A scale, check the technique and/or the instrument.

- Determine correction factor, F, for instrument and cells, using equation:

$$F = \frac{3160}{\bar{\epsilon}}$$

where: 3160 - value for $\text{K}_2\text{Cr}_2\text{O}_7$;

$\bar{\epsilon}$ - mean value for the molar absorptivities of the three standard potassium dichromate solutions.

If, F, is <0.95 or >1.05, check the technique and/or the instrument to eliminate the cause.

3.4.2. Purity of standard aflatoxin solutions

Before starting the preparation of standard aflatoxin solutions it is necessary to make sure of the purity of the initial preparations.

The purity of initial aflatoxin standards should be analysed by spectrophotometer:

- weigh 1 mg aflatoxin standard to nearest 0.001 mg and transfer quantitatively to 100 ml volumetric flask. Dissolve in and dilute to volume with methanol. Calculate concentration of solution in $\mu\text{g/ml}$.
- Measure the absorbance, A, at the wavelength of maximum absorption (Table 1);

- calculate the molar absorptivities:

$$\epsilon = \frac{A}{L * C}$$

where: C - concentration of aflatoxin in mol/l;
L - 1 cm.

The aflatoxin molecular weights are given in Table 3.

Table 1. Molar absorptivities, ϵ , of aflatoxins in methanol and 95% confidence limits expected from a single absorbance measurement

Aflatoxin	λ (nm)	ϵ (cm ² mole ⁻¹)	95% confidence limits (+)
B ₁	223	22 100	1 600
	285	12 400	800
	360	21 800	1 100
B ₂	222	18 600	1 000
	285	12 100	800
	362	24 000	500
G ₁	216	27 400	2 500
	242	9 800	300
	265	9 800	1 200
G ₂	214	25 300	2 300
	241	10 300	300
	265	9 000	1 100
	362	19 300	800

The values of ϵ obtained must agree with those given in Table 1 to within the cited confidence limits.

For each aflatoxin, calculate the absorbance ratios $A(\lambda)/A(265 \text{ nm})$ at the wavelengths λ , given in Table 2. The ratios obtained must agree with those in Table 2 to within the cited limits.

Table 2. Ratios of Absorbances of aflatoxins in methanol at wavelengths corresponding to major peaks, + 95% confidence limits expected from single spectra

Aflatoxin	λ (nm)	$A(\lambda)/A(265 \text{ nm})$
B ₁	222	1.78 + 0.04
	362	1.78 + 0.04
B ₂	222	1.54 + 0.08
	362	1.54 + 0.08
G ₁	214	2.85 + 0.15
	242	1.88 + 0.02
	362	1.84 + 0.06
G ₂	214	2.91 + 0.13
	242	1.17 + 0.07
	362	2.14 + 0.18

3.4.3. Preparation of standard aflatoxin solutions

(a) FOR AFLATOXIN STANDARDS
RECEIVED AS DRY FILMS OR CRYSTALS.

To containers of dry aflatoxins B₁, B₂, G₁ or G₂ add a volume of benzene-acetonitrile 98:2 to obtain concentrations of 8-10 mg/l.

Use label statement of aflatoxin weight as quid. Vigorously agitate solution 1 minute on Vortex shaker and transfer without rinsing to convenient size glass-stoppered flask. (Dry films on glass are not completely recoverable because of adsorption. Continued contact with solvent may result in slow dissolution).

Do not transfer dry aflatoxin for weighing or other purposes unless facilities are available to prevent dissemination of aflatoxins to surroundings due to electrostatic charge on particles.

(b) FOR AFLATOXIN STANDARDS
RECEIVED AS SOLUTIONS.

Transfer solution to convenient size glass flask. Dilute, if necessary, to adjust concentration to 8-10 mg/l.

3.4.4. Determination of aflatoxins standard concentration

Record UV spectrum of the solution across the wavelength of maximum absorption (Table 3) (from 330 to 370 nm), with solvent used for solution in reference cell.

Table 3. Molecular weights, m , wavelengths of maximum absorption, λ_{max} and molar absorptivities, ϵ , of mycotoxins

Aflatoxin	m^a	Solvent	λ_{max} (nm)	ϵ ($cm^2 \cdot mol^{-1}$)
Aflatoxin B ₁	312	Benzene:acetonitrile (98:2)	350	19 800
Aflatoxin B ₂	314	Benzene:acetonitrile (98:2)	350	20 900
Aflatoxin G ₁	328	Benzene:acetonitrile (98:2)	350	17 100
Aflatoxin G ₂	330	Benzene:acetonitrile (98:2)	350	18 200

Determine concentration, C, aflatoxin solution by measuring absorbance, A, at wavelength of max. absorption close to 350 nm and using following equation:

$$C \text{ (mg/l)} = \frac{1000 * F * m * A}{\epsilon}$$

where: F - correction factor is 0.95 or 1.05;
m - the molecular weight;
 ϵ - the molar absorptivity.

Molecular weights and ϵ values are shown in Table 3.

Return aflatoxin solution to original glass flask. (Normal exposure to UV light during A measurement results in no observable conversion to photoproducts).

3.4.5. Preparation of 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 B₁, B₂, G₁, G₂;
- (b) standard solutions containing a mixture of four aflatoxins (B₁ + B₂ + G₁ + G₂).

Working standard solutions are prepared by dilution of corresponding standard AT solutions. The concentrations of working standard AT solutions for B₁ and G₁ must be equal to 0.5 µg/ml., and for B₂ and G₂ - to 0.1 µg/ml. For the purpose, take an aliquot of the corresponding standard AT solution and bring it to the required volume with benzene-acetonitrile mixture (49:1) when preparing working standard solutions of AT B₁, B₂, G₁, G₂.

For preparing working standard solutions of a mixture of AT B₁, B₂, G₁ and G₂, take aliquots from the corresponding standard AT solutions and dilute to required volume with benzene-acetonitrile (49:1). Aflatoxin concentrations in a standard mixture must amount to 0.5 µg/ml for B₁ and G₁, and to 0.1 µg/ml for B₂ and G₂.

3.4.6. Checking the purity of working standard aflatoxin solutions by TLC

- Apply in succession on a TLC chromatographic plate: (1) 5 µl of working standard AT mixture solution; (2) 5 µl of one of the standard 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.

3.4.7. Storage of standard aflatoxin solutions for use in TLC

- Before storage, after aliquots have been removed for dilution or spotting, weigh flasks containing standard solutions to nearest 1 mg and record weights for future reference. Wrap flasks tightly in aluminium foil and store at 0°C.
- When solution is to be used after storage, reweigh flask and record any change. To avoid incorporation of water by condensation, bring all standards to room temperature before use; do not remove aluminium foil from flask until contents have reached room temperature.
- Recheck concentration of stored standard solution by UV detection and recheck purity each time portion is taken for dilution to spotting concentration. When volume of original solution becomes less than can be employed in standard photometer cell, use microcells (accurately positioned). Instrument must be recalibrated with each set of cells, since calibration includes cell pathlength. Any observed change in concentration should correspond with observed loss in weight due to solution evaporation. Standard solutions of B₁, B₂, G₁ and G₂ are stable for one year.

3.5. DETECTION, IDENTIFICATION AND QUANTIFICATION OF AFLATOXINS BY THIN-LAYER CHROMATOGRAPHY PRINCIPLES

In the course of TLC analysis for aflatoxins, chromatography plates are used where silica gel is as an absorbant. Silica gel has the particle size of 2 to 10 microns.

To prepare plates one has to weigh 30 g of silica gel into 300 ml of glass erlenmeyer, add the amount of water recommended by manufacturer, shake vigorously one minute, and pour into applicator. Adjust the amount of water to obtain best consistency of slurry for spreading, as required by batch-to-batch variation in silica gel. Immediately coat five 20 x 20 cm glass plates with 0.25 mm thickness of silica gel suspension, and let plates rest undisturbed until gelled (about 10 min.). Adjust thickness of spread (and gel weight) to 0.5 mm, if necessary, to provide good resolution of aflatoxins and tightness of spots. Plates are dried

at room temperature and then activated at 100-110°C for one hour. Store the plates in desiccating cabinet with active silica gel desiccant until just before use. The degree of silica gel activity, which depends on the moisture level of the layer, influences considerably the Rf value. The optimum moisture content in the layer is 15-20%.

To prepare the plate for coating, scribe a line 16 cm from the bottom edge as solvent stop; scribe lines about 0.5 cm in from the side or remove 0.5 cm gel from each side to prevent edge effects. Alternatively, use 20 x 20 cm precoated glass plates.

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

The ready-made glass plates coated with a thin layer of silica gel are manufactured by the companies Merck, Customs Service Chemicals, Canag. However, it is more convenient to use silica gel-coated Al foil plates obtainable from Merck (FRG) and Kavalier (CzSSR). The "Silufol" plates produced in Czechoslovakia are flexible silica gel-coated Al-foil plates generally of the size of 5 x 15, 15 x 15 and 20 x 20 cm with starch used as the binding agent. Such plates found wide uses in the analysis of samples for AT by TLC.

The results of TLC analysis of AT are strongly affected by the manner in which the sample is spotted on the thin layer. Before spotting the sample, a reference point is produced at the distance of 15-20 mm from the plate edge. The start 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 the required volume. The mass of the aliquot of extract applied on a TLC plate with a 0.25 mm thick adsorbent should not, however, exceed 5-10 ng. 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 Rf value of the separated components, especially if

chromatography is performed with solvents of low polarity. In addition to this, the solvent should be volatile enough to allow its removal from the plate. An AT-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 Rf values of AT in chloroform can be affected by the admixtures of alcohol used as stabilizer. The dissolving properties of benzene can be improved by addition of 2% acetonitrile; in this case the spots remain compact. The area of the spot at the point of application must be as small as possible, otherwise the quality of separation will be affected. The sample should be applied in small portions; it is also important for the volume applied to be constant each time. To avoid errors in spotting the sample, it is necessary to use an internal standard.

Preparation of chromatography plates for one-dimensional TLC aflatoxin separation is shown in figure 3 (a).

Nowadays two-dimensional TLC is widely used in AT analysis. It ensures a more efficient separation of AT spots from the other extract components that have fluorescence properties and chromatographic mobility close to those of AT. Preparation of the plate for two-dimensional TLC is shown in figure 3 (b).

In the course of TLC they often use the stage of additional cleaning of the sample's extracts from nonpolar impurities. For this purpose the following solvents are used: ether (freed of peroxides), benzene, and their mixtures with hexane (benzene-hexane, 1:1) (benzene-hexane-ether 1:1:1). In this procedure, AT remain on the start line and impurities travel with the solvent front.

To separate AT on the chromatography plate one-dimensional as well as two-dimensional TLC is used.

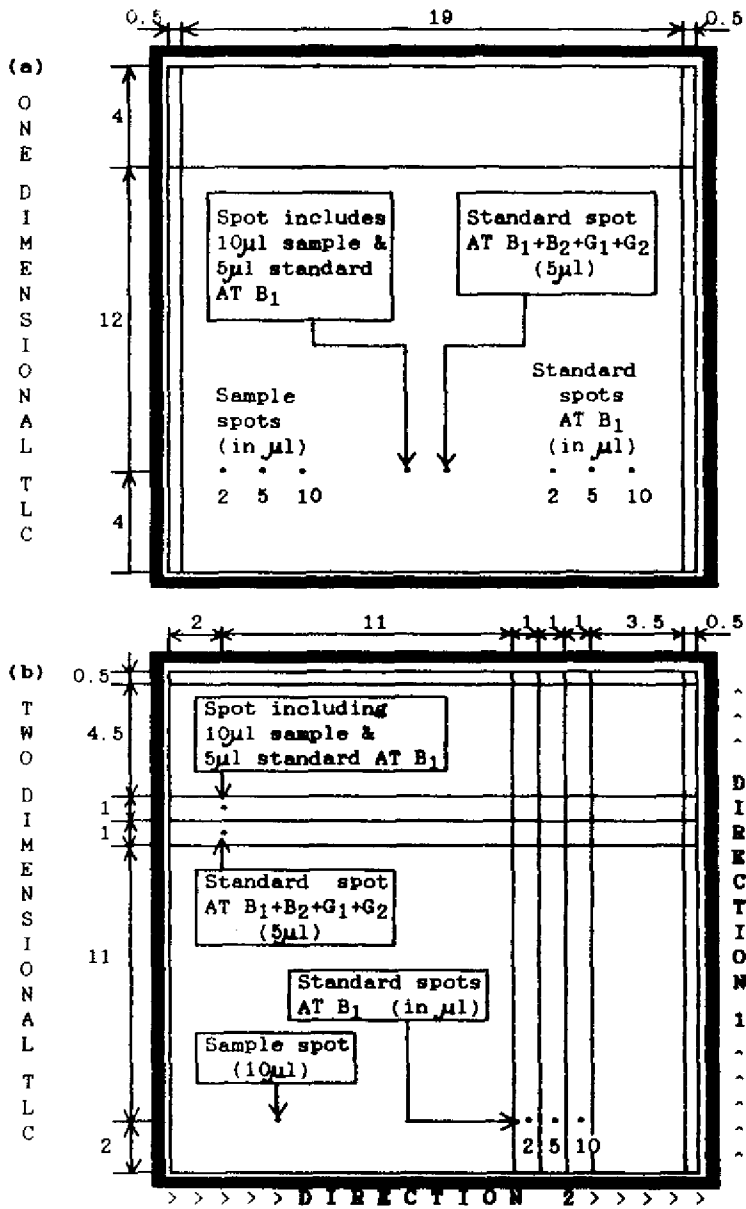


Figure 3. Spotting and scribing pattern TLC plates. (The distances are given for 20cm x 20cm plate)

In one-dimensional TLC different solvent mixtures are used to develop plates: 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 (45:35:19) proved to be a good eluding solvent, but only at optimal humidity and temperature. One of the best systems for AT B₁, B₂, G₁ and G₂ separation is the chloroform-acetone-isopropanol mixture (85:10:5).

It should be noted that the toluene-isoamyl alcohol-methanol mixture (90:32:3) makes it possible to separate distinctly AT B₁ (R_f 0.56), B₂ (R_f 0.48), G₁ (R_f 0.42), G₂ (R_f 0.34), whereas repeated elusion with the same solvent improves the resolution. A series of R_f values in certain solvent systems is shown in Table 4.

In two-dimensional TLC the plates are developed in two directions normal to each other. In practice, more efficient AT separations 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);
- diethyl ether-methanol-water (188:9:11) 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).

Table 4. The Rf x 100 values for some aflatoxins

Aflatoxins	s o l v e n t s							
	A	B	C	D	E	F	G	H
B ₁	28.0*	31.5*	19.5*	80.5*	22.5*	32.5*	31.5*	61.5*
B ₂	30.0	18.0	14.0	80.0	9.0	33.0	20.0	61.0
G ₁	19.0*	19.0*	13.5*	84.0*	12.0*	25.0*	20.0*	50.0*
G ₂	22.5	8.0	8.0	78.0	4.5	17.0	11.0	51.5
* - "tail" formation								
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-ethanol (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 lid, 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 Rf values in the middle of the chromatogram than at the edge of the plate.

After developing the TLC plates, AT are visualized by specific fluorescence in long-wave UV-light (365 nm). AT of the group B have blue fluorescence (425 nm), while the 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.

a) TEST WITH IODINE

A glass plate is uniformly covered with 5% iodine solution in diethyl ether (ether evaporates) and placed over the chromatographic plate (with iodine layer downward) so that the distance between them was 0.5-1.0 cm. The plate is subjected to the action of iodine vapour for 20-30 sec. and then visualized in UV-light. Preservation of the colour and intensity of the spot fluorescence serves as one of the indications to the presence of aflatoxin in a foodstuff.

(b) TEST WITH AN INORGANIC ACID

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

4. QUANTIFICATION OF AFLATOXINS IN CEREALS

For quantification of aflatoxin in cereals, the CB-method is widely used including extraction of aflatoxin from the analyzed sample by chloroform, purification of the extract obtained from lipid components and interfering admixtures by adsorptional column chromatography, TLC and evaluation of the spots produced by visualization (Fig. 4).

4.1. EXTRACTION OF AFLATOXINS

Weigh 50 g of prepared sample into 500 ml glass Erlenmeyer flask. Add 25 ml of water, 25 g of diatomic earth, 250 ml of chloroform and secure stopper with masking tape. Shake 30 min. on wrist action shaker and filter through fluted paper. If filtration is slow, transfer to bunker precoated with a 5 mm layer of diatomic earth and use light vacuum. (Use vacuum filtration only for slow filtering samples since evaporation of chloroform is rapid resulting in concentration of extract). Collect first 50 ml portion of chloroform filtrate and proceed with AT analysis.

4.2. PURIFICATION OF AFLATOXINS BY COLUMN CHROMATOGRAPHY

Place ball of glass wool loosely in bottom of 22 x 300 mm chromatographic tube and add 5 g of anhydrous sodium sulfate to give base for silica gel. Add chloroform until the tube is about 1/2 full; then add 10 g of silica gel. When the rate of settling slows, drain some chloroform to aid settling, leaving 5-7 cm above silica gel.

Slowly add 15 g of anhydrous sodium sulphate. Drain chloroform to the top of sodium sulphate. Add 50 ml sample extract to the column, elude at maximum flow rate with 150 ml of hexane followed by 150 ml of anhydrous ether and discard. Elude aflatoxins with 150 ml methanol-chloroform (3 + 97), collecting this fraction from time of addition until the flow stops. Add a few boiling chips to eluate, evaporate nearly to dryness on steam bath, and quantitatively transfer the residue to vial with chloroform. Add 2-3 boiling chips and evaporate, preferably under gentle stream of nitrogen. Seal the vial with hollow polyethylene stopper and cap. Save for TLC.

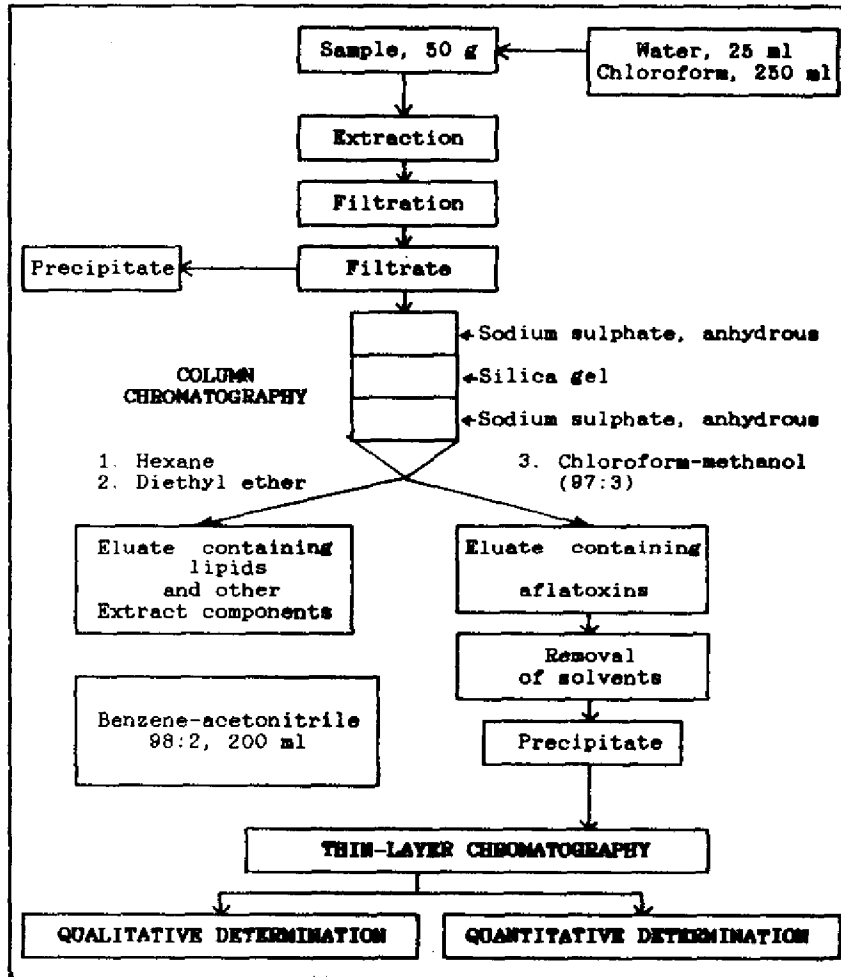


Figure 4. Scheme of AT analysis in cereals by the CB-method

4.3. THIN-LAYER CHROMATOGRAPHY FOR AFLATOXIN ANALYSIS

To determine the concentration of AT in the analyzed sample by TLC method chromatography plates which are prepared as mentioned in point 3.5. are usually used. TLC method includes as a rule two stages: preliminary thin-layer chromatography and quantitative TLC.

4.3.1. Preliminary thin-layer chromatography.

Uncap vial containing the sample extract, add 200 μ l of benzene-chloroform (98 + 2) and reseal with polyethylene stopper. Shake vigorously to dissolve, preferably with Vortex shaking machine. Puncture the polyethylene stopper to accommodate the needle of 10 μ l syringe. In subdued incandescent light and as rapidly as possible spot 2 μ l, 5 μ l and two 10 μ l spots on an imaginary line 4 cm from the bottom edge of TLC plate. Keep the vial for quantitative analysis. On the same plate spot 2 μ l, 5 μ l and 10 μ l aflatoxin standards. Spot 5 μ l standard used on top of one of the two 10 μ l sample origin spots as internal standard. Spot at least 5 μ l resolution reference standard to show whether adequate resolution is attained.

Place 50 μ l of acetone-chloroform (1:9) in trough of unlined developing tank. If the tank is other than Thomas-Mitchell, use volume to provide solvent depth of about 2 cm. Composition of acetone-chloroform can be varied from (5:95) to (15:85) to compensate for variations in silica gel and developing conditions. Use only one plate per tank, placing the trough near one side to permit max. exposure of the coated surface to tank volume. Immediately insert the plate into the tank and seal the tank.

Develop the plate for 40 min. at 23-25°C or until aflatoxins reach Rf 0.4-0.7. Adjust development time to compensate, if different developing temperature is used. Adjust developing solvent if developing time is more than 90 min. Remove from the tank, evaporate solvent at room temperature, and illuminate the plate from below by placing it flat, the coated side up, on long-wave UV lamp in darkened room, or view the plate in Chromato-Vue

cabinet, or illuminate from above. (If illumination requires looking directly at lamps, protect eyes with UV-absorbing filter, such as Eastman Kodak Co.2A). Observe the pattern on four fluorescent spots of resolution reference standard. In order of decreasing Rf, they are B₁, B₂, G₁ and G₂. Note small colour difference (bluish fluorescence of "B" contrasted with slightly green "G" aflatoxins). Examine patterns from the sample for fluorescent spots having Rf close to those of standards and similar appearances.

From this preliminary plate, establish suitable dilution for quantitative TLC analysis. In final calculations, take into account the amount of extract used for preliminary TLC.

Preliminary TLC may be omitted when approximate content is known.

4.3.2. Quantitative thin-layer chromatography

If the preliminary plate shows that new concentration of sample extract is required, evaporate to dryness on steam bath and redissolve in a calculated volume of benzene-CH₃CN (98:2).

Spot successively 3.5, 5.0 and two 6.5 μ l portions of sample extract. All spots should be approximately same size and about 0.5 cm diameter. On the same plate spot, spot 3.5, 5.0 and 6.5 μ l of aflatoxin standards, corresponding to aflatoxins observed on the preliminary plate. Spot 5 μ l of each standard used on top of one of the two 6.5 μ l sample origin spots as internal standard. Spot at least one 5 μ l resolution reference standard to show whether adequate resolution is attained. Proceed as indicated earlier.

INTERPRETATION OF THE CHROMATOGRAM.

For being clearly identified, spots should be visible in resolution reference standard.

Examine the pattern from the sample spot containing internal standard for aflatoxin spots. Rf values of aflatoxins used as internal standards should be same as or only slightly different from those of respective standard aflatoxin spots (since spots from the sample extract are compared directly with standard aflatoxins on the same plate, the magnitude of Rf is unimportant. These may vary from plate to plate).

Compare sample patterns with the pattern containing internal standard. Fluorescent spots in the sample thought to be aflatoxins must have Rf values identical to and colour similar to aflatoxin standard spots when the unknown spot and internal standard spot are superimposed. The spot from the sample and internal standard combined should be more intense than either sample or standard alone.

Compare fluorescence intensities of B₁ spots of the sample with those of standard spots and determine which sample portion matches one of standards. To aid in determination, move the plate away from the lamp to attenuate UV light so any particular pair of spots can be compared at extinction. Interpolate if intensity of the sample spot is between those of 2 of standard spots. If spots of the smallest portion of sample are too intense to match standards, dilute the sample and rechromatograph. Compare B₂, G₁ and G₂ spots in the same manner.

4.4. CALCULATION OF AFLATOXIN CONCENTRATION.

Calculate concentration of aflatoxin B₁ in $\mu\text{g}/\text{kg}$ from the formula:

$$C \mu\text{g}/\text{kg} = \frac{S * Y * V}{X * W}$$

- where: S - volume of spot of B₁ standard with same intensity as sample spot, μl
- Y - concentration of standard B₁ solution, $\mu\text{g}/\text{ml}$
- V - volume of final delution of sample extract, μl
- X - volume of sample spot giving fluorescent intensity equal to B₁ standard spot of volume S, μl
- W - mass of sample applied to the column, g
(10 g if 50 ml of chloroform sample's extract is used)
If final extract dilution does not represent 10 g, calculate correct sample weight and substitute.

5. QUANTIFICATION OF AFLATOXINS IN GROUNDNUTS, GROUNDNUT PRODUCTS AND OTHER OIL SEED PRODUCTS

The method consists of the following stages: extraction of AT 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 chromatography and AT detection and quantification using two-dimensional TLC (Fig. 5). The detection limit of the method is 0.5-1.0 μg AT B₁ per kg product, coefficient of variation - 0.3-5.0, degree of AT B₂ extraction from different products varies within 65-90%.

5.1. EXTRACTION OF AFLATOXINS FROM THE SAMPLE

Grind thoroughly the sample under study in a coffee mill to flour consistency. Weigh 25 g sample and place it in 300-500 ml conical flask, moisten with 25 ml of 10% sodium nitrate aqueous solution and 100 ml of acetone. Shake mixture for 30 minutes, filter the extract through folded paper filter. To speed up filtration, 2-3% Celite can be placed on the filter. Collect 50 ml of filtrate.

5.2. PURIFICATION OF AFLATOXIN EXTRACT USING COMPLEXING AGENTS

Add 50 ml of 10% lead acetate aqueous solution to 50 ml of water-acetone extract and shake. In 10-5 min. filter, withdrawing first 80 ml of filtrate.

5.3. PURIFICATION OF THE AFLATOXIN FRACTION BY LIQUID-LIQUID EXTRACTION

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

5.4. PURIFICATION OF AFLATOXIN EXTRACTS BY COLUMN CHROMATOGRAPHY

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

Drain chloroform to the sodium sulphate surface. Pour Solution of the extract obtained into the column. When the extract level reaches the surface of sodium sulphate add 100 ml of chloroform-acetone (4:1).

Collect the fraction from the beginning of elution to cessation of eluate drainage. Evaporate solvent to dryness. Dissolve the dry residue in 100 μ l of benzene-acetonitrile (98:2).

5.5. THIN-LAYER CHROMATOGRAPHY

Procedures to be followed are the same as in point 4.3.

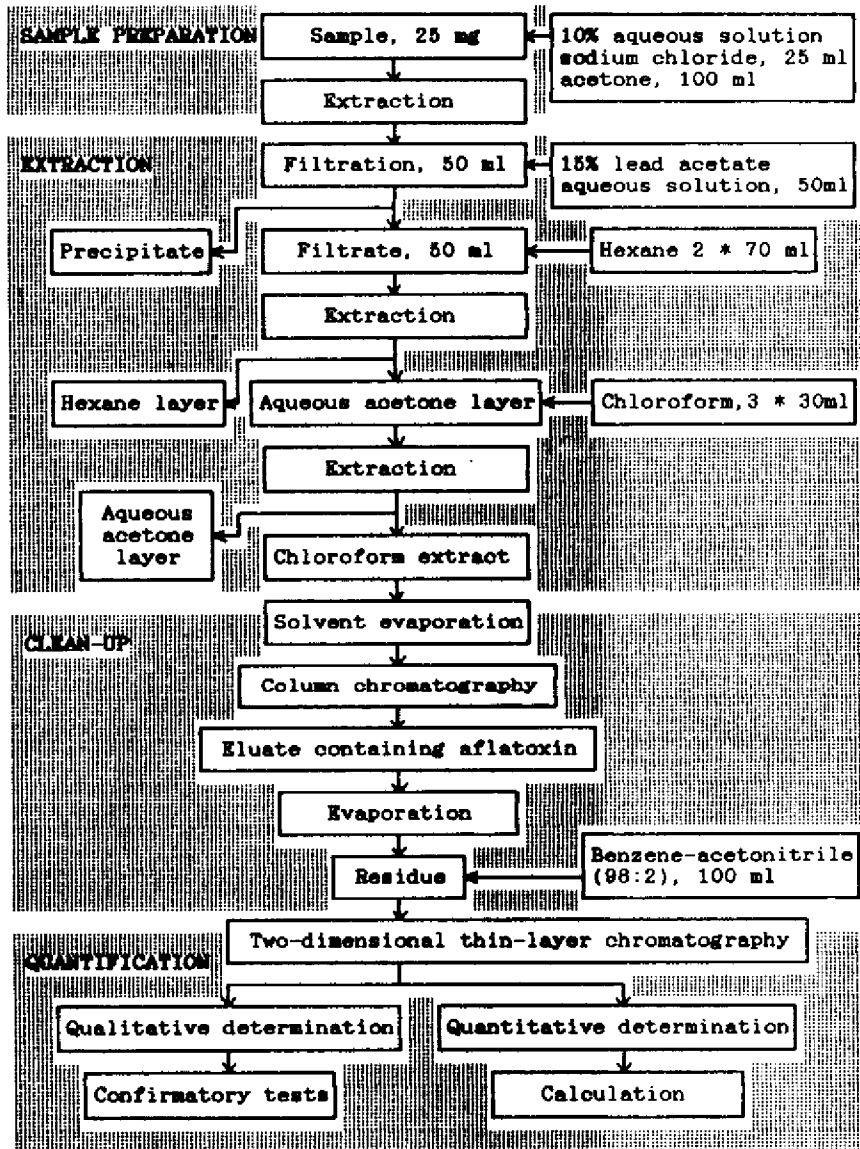


Figure 5. Scheme of AT determination in Groundnuts and other oil seeds

5.6. CALCULATION OF AFLATOXIN CONCENTRATION

Calculate concentration, C, of aflatoxins from the formula:

$$C (\mu\text{g}/\text{kg}) = \frac{V1 * V3 * V5 * m}{V2 * V4 * V6 * M}$$

- where: V1 - volume of water-acetone mixture, ml;
V2 - volume of water-acetone filtrate taken for analysis, ml;
V3 - volume of water-acetone filtrate and lead acetate solution, ml;
V4 - volume of filtrate after purification with lead acetate, ml;
V5 - volume of evaporated purified extract in benzene-acetonitrile mixture before TLC, ml;
V6 - volume of extract applied on a plate, ml;
m - amount of aflatoxin in extract spot on the plate, μg ;
M - weight of the product taken for analysis, kg .

6. QUANTIFICATION OF AFLATOXINS IN COPRA AND ITS PRODUCTS

Extraction and purification of AT are based on the CB-method. Detection and quantification are performed using two-dimensional TLC. The degree of AT B₁ extraction from copra and products of its processing varies within 80-90%, whereas the detection limits of the method are 0.5-10.0 µg AT B₁ per 1 kg product.

6.1. EXTRACTION OF AFLATOXINS FROM THE SAMPLE

The sample under study is thoroughly ground. Place a sample weight of 12.5 g in a 300 ml Erlenmeyer flask, add 1 g sodium chloride, 20 ml of water and 63 ml of chloroform.

The mixture is placed on a shaker for 30 min. Filter the extract through a folded paper filter; 2-3 g of celite can be placed on the filter.

6.2. COLUMN CHROMATOGRAPHY

Place a ball of glass wool loosely in the bottom of 12 x 13 mm chromatographic tube and add 5 mg of anhydrous sodium sulphate to give base for silica gel. Add chloroform until the tube is about 1/2 full; then add 10 mg of silica gel (0.03-0.2 mm), wash sides of the tube with some 10 ml of chloroform and stir to disperse silica gel. When the rate of settling slows, drain some chloroform to aid settling, leaving 5-cm above silica gel. Slowly add 10 mg of anhydrous sodium sulphate. Drain chloroform to the top of sodium sulphate. Add 15 ml of sample extract to the column, elude at max. flow rate with 50 ml hexane followed by 50 ml of anhydrous ether and discard. Elude aflatoxins with 30 ml of methanol-chloroform (3:97), collecting this fraction from the time of addition till the flow spots.

Add a few boiling chips to elude, evaporate nearly to dryness on steam bath, and quantitatively transfer the residue to vial with chloroform. Add 2-3 boiling chips and evaporate, preferably under gentle stream of nitrogen. Seal the vial with hollow polyethylene stopper and cap. Save for TLC.

**6.3. DETECTION AND QUANTIFICATION OF AFLATOXINS
BY TWO-DIMENSIONAL CHROMATOGRAPHY**

Procedures to be followed are the same as in point 4.3.

6.4. CALCULATION OF AFLATOXIN CONCENTRATION

The formula to be used is the same as in point 4.4.

7. STATISTICAL ANALYSIS AND PRESENTATION OF RESULTS

The experimental data should be statistically treated.

Clear and accurate reports on sampling and analysis activities at the laboratory level, are essential.

These data are to be analyzed in a mycotoxin monitoring programme to determine trends and other significant aspects of the problem.

7.1. STATISTICAL ANALYSIS

The experimental data should be statistically treated. The data treatment of sampling and analysis results include the following:

- data treatment of analysis results by laboratory;
- data treatment of various results within mycotoxin monitoring programme.

7.1.1. Data treatment of analysis results by laboratory

Laboratory data treatment is carried out with the aim to obtain true results on levels of aflatoxin contamination of separate agricultural lots. It includes the following statistical parameters necessary to comply the laboratory report:

Number of experiments (n)

This is a number of the studied samples of a single agricultural lot.

Mean arithmetic value (\bar{X} mg/kg)

$$\bar{X} = \frac{\sum_{i=1}^n X_i}{n}$$

where: n - number of analysis;
Xi - contamination level.

Standard deviation (S)

$$S = \sqrt{\frac{\sum_{i=1}^n (X_i - \bar{X})^2}{n - 1}}$$

where: Xi - contamination level;
 \bar{X} - arithmetic mean of a series of results;
n - number of analysis.

Coefficient of variation (CV)

$$CV = \frac{\bar{S}}{\bar{X}} \cdot 100\%$$

where: \bar{S} - standard deviation;
 \bar{X} - arithmetic mean of a series of results.

Check for outlying values

Outlying values have been excluded following the method proposed by Thompson; a value is considered to be an outlier when:

$$\left| \frac{X - \bar{X}'}{S'} \right| > 3.8$$

where: \bar{X}' - arithmetic mean of all non-outlying results;
X - value in question;
S' - standard deviation of all non-outlying results.

7.1.2. Data treatment of various results within mycotoxin monitoring programme

Data treatment of various results within a mycotoxin monitoring programme is carried out with the aim to obtain true results on occurrence and degree of contamination in separate agricultural products.

The following statistical parameters are advisable for inclusion in the summary report: mean arithmetic value, median, mode, 90% percentile, standard deviation, and overall range.

All quantitative parameters showing contamination levels of separate agricultural produce and other sets of features necessary for the statistical analysis should be first and foremost grouped in variational series in the form of tables.

Class	Class boundaries	Occurrence
1	$0 < X \leq h$	f_1
2	$h < X \leq 2h$	f_2
...
i	etc.	f_i

where: f - frequency
(number of values within the i-interval or class);
h - interval value
(should be close to the measuring accuracy).

The intervals should be arranged in the ascending order. The minimum contamination level is considered the lower limit, and the maximum - the upper limit.

$$\text{Midpoint} = \frac{\text{lower limit} + \text{upper limit}}{2}$$

The main parameters of a variational series can be subdivided into two types: measures of location and measures of dispersion.

MEASURES OF LOCATION

Mean arithmetic value (\bar{X})

$$\bar{X} = \frac{\sum_{i=1}^N X_i * f_i}{\sum_{i=1}^N f_i}$$

where: X_i - midpoint interval;
 f_i - frequency;
 N - number of intervals.

Median (Me)

$$Me = b_L + \frac{\frac{n}{2} - F_{m-1}}{f_m} * h_m$$

where: m - is some integer from 1 to i
 b_L - lower boundary of the median class;
 n - number of observations;
 f_m - the number of observations in the median class;
 F_{m-1} - the number of observations in the $m-1$ classes preceding the median class
 h_m - interval value.

$$F_{m-1} = \sum_{i=1}^{m-1} f_i$$

The median interval is determined at the condition that

$$\sum_{i=1}^m f_i > \frac{n}{2} ; \quad \sum_{i=1}^{m-1} f_i < \frac{n}{2}$$

Mode (Mo)

Mode is a midpoint interval of the utmost frequency.

90% Percentile (P90)

It is calculated similar to the median.

$$P_{90} = b_p + \frac{0.9n - F_{p-1}}{f_p} * h_p$$

where: b_p - lower boundary of the percentile interval;

n - total number of observations;

f_p - frequency of the percentile interval

h_p - interval value.

F_{p-1} - the number of observations in the P-1 interval preceding the 90% percentile.

$$F_{p-1} = \sum_{i=1}^{p-1} f_i$$

The percentile interval is determined at the condition that

$$\sum_{i=1}^p f_i > 0.9n ; \quad \sum_{i=1}^{p-1} f_i < 0.9n$$

There are graphical methods to determine the mode, median and percentile.

MEASURE OF DISPERSION

Standard deviation (S)

$$S = \sqrt{\frac{\sum_{i=1}^N (X_i - \bar{X})^2 \times f_i}{\sum_{i=1}^N f_i - 1}}$$

where: X_i - midpoint interval;
 f_i - frequency;
 N - number of intervals.

Overall range

Overall range is the difference between the maximum and minimum contamination levels.

7.2. PRESENTATION OF RESULTS

Results of agricultural produce analysis for mycotoxins contamination should be reported in standardized manner. This can be facilitated by preparing standard form for reporting results and using clear instructions for their compilation.

The statistically treated analysis results are usually generalized in a laboratory report or in a summary report. A summary report should be used to capture data generated by the mycotoxin monitoring programme.

7.2.1. Laboratory report

A laboratory report generalises the data on sampling and analysis of agricultural produce on mycotoxin contamination obtained by a laboratory.

The laboratory report should be submitted to the country's head organization responsible for the mycotoxin monitoring programme.

The laboratory report should include the following:

IDENTIFICATION OF THE SAMPLE

The report should contain all details necessary for complete identification of the sample. In many cases this requirement can conveniently be met by attaching the information supplied on a standardized form by the person who has taken the sample (Appendix 2). If this is not done, then at least the basic information on the nature and origin of the sample, the nature of the container, any identification marks, the date and place of sampling and the name of the person submitting the sample should be recorded. As has already been pointed out, samples for monitoring purposes should be representative. If for any reason the sample is not representative, i.e. is a "suspect" sample, this must be clearly indicated.

ANALYSIS

The way in which the sample unit for analysis has been prepared from the field sample (i.e., portion of sample analysed and sample preparation) should be recorded. The analytical method used should be reported either in detail or, if a standardised published method is used, by giving a reference to the relevant publication. The date and place of the analysis should be recorded, together with the name of the supervising analyst.

LEVELS OF CONTAMINATION

The concentration of aflatoxin contaminants should be expressed as micrograms per kilogram ($\mu\text{g}/\text{kg}$) wet weight or dry weight of the food.

The levels of aflatoxins B₁, B₂, G₁ and G₂ may be expressed in terms of the individual aflatoxins or as the sum of the total aflatoxins. It is important to report the detection limit of the method. It is essential to indicate the concentration of the standard aflatoxin solution used in the method, and the company-manufacturer of the given aflatoxin standard (Appendix 3).

Detailed data on sampling and analysis should be stored at the place where the analysis examination is carried out.

7.2.2. Summary report - Report of the mycotoxin monitoring programme

When several laboratories are taking part in the mycotoxin monitoring programme, the data should be collected, appraised, processed and stored at the head organization of the respective country responsible for the mycotoxin monitoring programme, the so called "Project Data Handling Centre", i.e. the Government Chemical Laboratory.

Sampling and analysis detailed data on the mycotoxin contamination of agricultural produce including any simple statistical parameters are supplied by separate laboratories participating in the mycotoxin control programme to the Project Data Handling Centre, the data are first checked to see, that they are complete and that they meet the agreed criteria as regards sampling and analysis.

The data which satisfy the agreed criteria are classified into groups of agricultural produce and other units according to such features as crop year, region of sampling, etc. Upon statistical treatment the data are arranged in a summary form (Appendix 4). These data are analyzed, the resultant conclusions and recommendations are forwarded to those involved in establishing policy and strategies regarding prevention and control of mycotoxins in food and feeds.

8. QUALITY ASSURANCE PROGRAMME (QAP)

8.1. GUIDELINES FOR QUALITY ASSURANCE PROGRAMME

Quality assurance programme is a comprehensive part in the system of mycotoxin contamination control.

The objective of a quality assurance programme is to achieve precise and accurate results at all times. The importance of obtaining reliable data is emphasized by the fact that errors in analysis can often mean serious economic losses for the food industry or imports, as well as possible illness or injury to the consuming public.

Often, the terms "Quality Control" and "Quality Assurance" are used interchangeably when discussing analytical quality. However there is a certain difference.

QUALITY CONTROL is a planned system of activities whose purpose is to provide a quality product.

QUALITY ASSURANCE is a planned system of activities whose purpose is to provide assurance that the quality control programme is actually effective.

To be successful, quality assurance must be a formal planned activity which is designed to fit the needs of the quality analytical service of the mycotoxin monitoring programme. A formal QAP is prepared by establishing:

- the acceptable quality for a given analytical service function;
- a periodic review to confirm this quality;
- a system of documentation to record the results of the reviews;
- appropriate follow-up to correct any deficiencies found.

QAP should be dynamic and subject to change when needed. Therefore a detailed QA review could be conducted until it is felt that the function under review will continue to operate smoothly, at which time the review could be lessened and another function given in-depth coverage.

The first step in establishing QAP is to decide the functions that should have formal review. The review itself is normally limited to the critical parts of the function under review.

The quality assurance programme shall be documented in a quality manual which is available for use by the laboratory staff. The quality manual shall be maintained relevant and current by a responsible member of the laboratory staff.

When establishing a QAP within the mycotoxin control system priority should be attached to evaluating the analytical service of the mycotoxin control which includes collection of agricultural produce samples and their chemical analysis for the mycotoxin content.

8.2. MAIN CRITICAL PARTS OF THE ANALYTICAL SERVICE IN THE MYCOTOXIN MONITORING PROGRAMME

Every methodology, particularly the analytical one, contains the critical parts where errors are most probable to occur during analysis.

When establishing a QAP within the network of an analytical service in the mycotoxin control there should be a systematic evaluation of fulfilling the following objectives:

- check sample collection and preparation;
- QA of the equipment and instruments used during analysis;
- QA of reference standards;
- check of analytical methodology;
- check of analytical reports.

8.2.1. Check sample collection and preparation

In view of the fact that sampling is one of the most important step in the mycotoxin monitoring programme it should be given priority in the QAP.

Officials responsible for the QAP should systematically check sampling paying special attention to the following:

- accuracy of sampling and preparation of representative samples that should strictly follow the established (official) sampling methods;
- strict (compulsory) observance of transportation and storage regulations for the selected samples for the mycotoxin control;
- correct filling in the adopted certificates (documents) carrying the information on the collected samples (Appendix 2).

The above checks should be organized within a laboratory or among several laboratories. The check results are reflected in the QA protocol and submitted to the head organization responsible for the QA programme.

In case the sampling evaluation has revealed any errors they should be corrected at once.

To improve skills of the personnel directly involved in sampling the agricultural produce training programmes should be periodically launched.

8.2.2. QA of equipment and instruments

Periodic testing of operation and accuracy of equipment used in the mycotoxin analysis is an important component of QAP. It is essential to periodically check the instruments performance. If the performance parameters to be checked are selected carefully, then the analyst has a reasonable measure of whether the instrument is operating properly, on a continuing basis. An individual performance record can be prepared to each instrument. This can be a separate notebook which is kept physically near the instrument, or can be part of the maintenance and repair record kept for each instrument.

If a check result indicates maintenance or repair, then a follow-up check (after the repair, cleaning, calibration, etc.) should be scheduled before the next routine QA check time. Any follow-up action should be recorded in the notebook kept with the instrument.

8.2.3. QA of reference standards

Mycotoxin standards are a major source of analytical error that is most of the time overlooked.

The QAP workplan should envisage periodic QA checking of standards to obtain answers to the following questions:

* RECORDS:

Are they complete and current? Do they reflect the actual condition of the standard? Have appropriate stability tests been run at correct times?

* STORAGE:

Is the storage proper? Are the stored standards accounted for? If not, why?

* PHYSICAL CONDITION:

Where was the standard at the time of the review? Any physical evidence of deterioration or mishandling?

All information on the standard including the test results, date, analyst name and any supporting documents such as spectra of chromatogramme should be recorded in the appropriate QA Review Report (Appendix 5).

The person making the QA review should be a senior analyst or supervisor who has a knowledge and appreciation for problems that improper, contaminated or deteriorated reference standards can create for the analyst.

6.2.4. Check of analytical methodology

The main objective of the check of analytical methodology is obtaining the similar (compatible) quantitative results on the mycotoxin content by all laboratories engaged in the mycotoxin control system. The key role is played by the evaluation of the applied method of chemical analysis in a concrete laboratory. The evaluation procedure can be carried out by means of both (i) spike recovery, and (ii) comparison with an official method.

* SPIKE RECOVERY

In this case the ground sample of agricultural produce without aflatoxin is mixed with the known amount of the standard solution. The method applicability is determined by comparing the standard of aflatoxin added to the sample with the amount of the standard isolated from the sample.

* COMPARISON WITH THE OFFICIAL METHOD

A parallel analysis of a blind sample (with unknown AT concentration) using the method which is to be evaluated and the official procedure - CB method (procedure is the same as in point 5). The evaluated method can be considered satisfactory for use if the results of the comparison show good correlation between the methods.

Check results are registered in a QA protocol (Appendix 6). If in the course of the check of the analytical methodology errors are found measures should be taken to correct them.

8.2.5. Check of analytical reports

The summary report is the culmination of all the laboratory's efforts. A QAP for analytical reports is basically an after-the-fact or retrospective review of a document which has already been through the normal supervisory and management review process. It is designed to detect report write-up and review errors so that appropriate procedures can be instituted to prevent their reoccurrence.

A summary report should be used to capture data generated by the monitoring programme. This form should be kept by the coordinator of the monitoring programme and can be used to summarize the progress of the survey (Appendix 7).

8.2.6. Check analytical service examination

One of the most common ways to evaluate overall individual or laboratory performance in a given analysis is by use of the known check samples. This includes both intra (within) the laboratory and inter (between) laboratories.

A laboratory should demonstrate its proficiency using prototype and non-critical samples, thereby reducing the probability that the data will be generated on critical samples. An intralaboratory quality control programme should include the use of blind samples in addition to check samples whose values are known to the analyst. All analysts should be monitored by the quality control procedure, and any assessment of laboratory performance should include all of the analysts involved, or at least a random selection of those analysts. When an interlaboratory check sample examination programme is carried out the data obtained should be submitted to the organization responsible for this programme. This can be done by any laboratory engaged in the mycotoxin control system.

Check sample examination within the QAP enables detecting weak points and including in the control and monitoring programme actions to improve the analytical services of the mycotoxin control.

In general, 50% of errors in the mycotoxin analysis are due to the personnel poor skills, it is essential to organise training in all parts of analytical services of mycotoxin control.

Many other areas of laboratory operations would also benefit from QA review. Remember that any laboratory function can be subject to QA review.

MYCOTOXIN SAMPLE SIZES

PRODUCT SAMPLE SIZES FOR MYCOTOXIN ANALYSIS

PRODUCT	PACKAGE TYPE	LOT SIZE	NUMBER OF SAMPLE UNITS * minimum	UNIT SIZE min	TOTAL SAMPLE SIZE minimum
Peanut butter-smooth	Consumer or bulk	NA***	24 12	0.2 kg 0.4 kg	4.8 kg 4.8 kg
Peanut butter-crunchy Peanuts-shelled, roasted, or unroasted Peanuts-ground for topping	Consumer or bulk	NA	48	0.4 kg	18.2kg
Tree nuts	Consumer or bulk	NA	INITIAL 10	SAMPLE 0.4 kg	4.0 kg
Pistachio nuts in-shell	Bulk	multiple of 34.000 kg	20% of units	-	22.7 kg for each multiple of 34.000kg or less
Pistachio nuts shelled	Bulk	Same	Same	-	11.4 kg for each multiple of 34.000kg or less
Corn	Consumer or bulk	NA	10	0.4 kg	4.0 kg
Cottonseed	Bulk	NA	15	1.8 kg	27.0 kg
Oil-seed meals-peanut meal, cotton-seed meal	Bulk	NA	20	0.4 kg	8.0 kg
Edible seeds**-melon pumpkin, sesame, etc	Bulk	NA	50	0.4 kg	20.0 kg

(To be continued on next page)

PRODUCT SAMPLE SIZES FOR MYCOTOXIN ANALYSIS
(Continued from prev. page)

PRODUCT	PACKAGE TYPE	LOT SIZE	NUMBER OF SAMPLE UNITS * (minimum)	UNIT SIZE (min)	TOTAL SAMPLE SIZE (min)
Milk-whole, skim, low fat	Bulk Consumer	NA NA	10 --	0.4 kg ---	4.0 kg 4.0 kg
Ginger root - dried, whole-ground	Bulk Consumer	"n" units NA	sq. root "n" 16	--- 0.4 kg	6.8 kg 6.4 kg
Small grains-wheat, sorghum, barley, etc	Bulk	NA	10	0.4 kg	40 kg
Mixtures containing commodities susceptible to mycotoxin contamination -	Consumer	NA			
Commodity particles relatively large			50	0.4 kg	20 kg
Commodity particles relatively small			10	0.4 kg	4.0 kg

NOTE: Containers for samples of unprocessed, intact nuts, seeds, or grains must be sufficiently porous to provide for dissipation of moisture produced by respiration of the nut, seed, or grain.

* To be collected from as many random sites in the lot as possible.

** Optional sampling program for seeds or dried fruit with a low incidence of contamination: Take initial 10 * 1 lb. sample. If any aflatoxin is detected, resample 50 * 1 lb. sample for a determination of contamination level on which to base a regulatory judgement.

*** NA-not applicable

SAMPLING REPORT

1. Name and address of the person collecting the samples: [REDACTED]
2. Date, place and time of sampling: [REDACTED]
3. Reason(s) for sampling - if part of a specific monitoring project, project reference number: [REDACTED]
4. Type of agricultural produce: [REDACTED]
5. Name of the manufacturer, importer, wholesaler, retailer, etc., as appropriate: [REDACTED]
6. Number and size of units constituting the lot: [REDACTED]
7. Number and marking of the lot: [REDACTED]
8. Origin of the lot: [REDACTED]
9. Destination of the lot: [REDACTED]
10. Method of sampling (random throughout the lot, random throughout accessible units, etc.): [REDACTED]
11. Size, number and reference number of field samples: [REDACTED]
12. Date of dispatch and means of transportation to the analytical laboratory: [REDACTED]
13. Name and address of the analytical laboratory: [REDACTED]
14. Analysis to be performed: [REDACTED]
15. Any other relevant information regarding the condition of the lot or the field sample, e.g. details of processing: [REDACTED]

LABORATORY SUMMARY FORM
ON AFLATOXIN CONTAMINATION

1. Name of the analytical laboratory:
2. Name and address of the person responsible for the analysis:
3. Date and time of analysis: :
4. * Number of sample corresponding to the agricultural lot:
5. Type of agricultural produce:
6. Method of analysis (give reference or attach copy):
7. Detection limit (mg/kg):
8. Concentration of the standard AT solution used for analysis, and manufacturer of the standard:
9. Mean arithmetic value for contamination level by aflatoxins B₁, B₂, G₁, G₂ or total AT (mg/kg):
10. Standard deviation:
11. Any other relevant information:

NOTE: * - sample number in item 4 should correspond to the number in the sampling report carrying the necessary information on the sample

SUMMARY FORM
ON MYCOTOXIN OCCURRENCE IN
AGRICULTURAL PRODUCE

1. Name of head laboratory:
2. Date:
3. Type of agricultural produce:
4. Sampling period (month, year):
5. Region of growth/sampling:
6. Sampling represent (domestic, imported, exported):
7. Detection limit (mg/kg):
8. Number of studied samples:
9. Percentage of uncontaminated samples:
10. Mean arithmetic value of the AT contamination level (mg/kg):
11. Median value found (mg/kg):
12. 90th Percentile (mg/kg):
13. Analytical method (give reference or attach copy):
14. Remark on sampling or results:

NOTE: The form should indicate concrete aflatoxins (B₁, B₂, G₁, G₂) or total AT in items 10, 11, 12.

QA REVIEW REPORT

REFERENCE STANDARDS

Reviewer: Date:

Name of laboratory where QA was performed:

Name of person responsible for standard preparation:

Type and source (from where obtained) or reference standard reviewed:

1. Is the record book kept in order? Are the notes in the record book full? (They should reflect method of preparation, time of preparation, test results, analyst name, supporting documents, such as spectra- or chromatograms, etc.)
2. Does the label on the evaluated standard correspond the reference in the record book?
3. Has the application time of the standard expired?
4. Do the standard storage conditions correspond to those contained in storage instructions?
5. Is stability testing performed regularly and correctly?
6. Do the test results obtained by the reviewer correspond to those reflected in the record book?
7. Any other relevant information:

QA REVIEW REPORT

ANALYTICAL METHODS

Name of laboratory where QA was carried out [REDACTED]

1. Date: [REDACTED]

2. Name of QA analysts: [REDACTED]

3. Type of evaluated method (give reference or attach copy) [REDACTED]

4. Method of evaluation: [REDACTED]

 a) spike recovery (level spiked at mg/kg.....) [REDACTED]

 b) comparison with an official or standard method [REDACTED]

Type of official or standard method [REDACTED]

Number of blind sample [REDACTED]

5. Number of results: [REDACTED]

6. Mean aflatoxin: [REDACTED]

7. Any other relevant information: [REDACTED]

QA REVIEW REPORT

SAMPLE EXAMINATION

Name of laboratory where QA was carried out

1. Date:
2. Name of QA analysts:
3. Sample number:
4. Type of analytical method used:
5. Standard concentration (total AT or individual aflatoxins B₁, B₂, G₁, G₂):
6. Number of results:
7. Mean aflatoxin concentration (mg/kg):
8. Standard deviation (mg/kg):
9. Any other relevant information:

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