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

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MULTIDETECTION OF MYCOTOXINS IN GRAIN EXTRACTS AND FUNGI CULTURES

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MULTIDETECTION OF MYCOTOXINS IN GRAIN EXTRACTS AND FUNGI CULTURES

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Plant products may incorporate, at the same time, several mycotoxins, for instance, aflatoxins, kojic acid and zearalenon or ochratoxins and citrinin or other combinations of mycotoxins.

Their presence is stipulated by a whole number of reasons, most important of which are the following:

Mechanical mixing of the lots of grain and seeds, polluted with different mycotoxins;

Possible infection of products with fungi strains, capable of simultaneous production of several mycotoxins;

Development of mycotoxins with the parallel or consecutive development of different fungi-producents of mycotoxins on products.

Specific highly sensitive techniques for identifying certain mycotoxins have been developed and are being developed for the analysis of plant raw materials. In the meantime, rather attractive is the possibility of the simultaneous identification of the most widely spread mycotoxins in one extract. The development of these techniques allows for the identification of a whole number of toxins in the extract of one sample, which essentially reduces the loss of time and funds for the analysis.

Usually, multidetection methods are less sensitive than chemical methods as to the identification of certain toxins, therefore, it is expedient to view them as semi-quantitative screening methods, which enable to identify the presence of toxins. The detected compounds may further be analyzed by quantitatively specific methods, developed for each toxin in particular.

The main stages of the multidetection of mycotoxins include extraction, degreasing, purifying, chromotographic separation, identification and the subsequent semi-quantitative determination.

The specificity of the multidetection of mycotoxins is the universal nature of the technique of extraction and purifying, which allows to avoid the loss of substances with different chemical properties. The necessity of identifying numerous toxins resulted in the development of a whole complex of specific reactions of coloration and the formation of derivatives.

The conditions of growing toxigenic fungi as well as the sequence of extraction by chloroform and the identification of mycotoxins in extracts of cultures of fungi-producents, were developed by Scott P.M. et al., 1970. The proposed sequence of indentifying mycotoxins by their fluorescence with 365 and 254 nm, and also by specific reactions of coloration with an acidic solution of anisaldehyde, phenylhydrazine hydrochloride and ferric chloride, was the basis for many further modifications.

Later, acetonitrile was used for the extraction of mycotoxins, which easily extracts mycotoxins and a comparatively small number of pollutants, Dialysis through membranes, which enables to remove up to 95 per cent of pollutants from the degreased extract was used for a universal purification of extracts. A

reduced output of mycotoxins, which with a high concentration of mycotoxins ranged within 25-90 per cent in certain cases, was compensated by a very high quality of purification.

Another technique of purification is gel-filtration in a column with sephadex LH-20. This method allows to identify five groups of mycotoxins in grain, including aflatoxins, ochratoxin patulin, sterigmatocystine and zearalenon. Mycotoxins are extracted by chloroform, acidified with phosphoric acid and are eluated from the sephadex column with a chloroform-methanol mixture (2:1). The use of gel-filtration provides a very high percentage of toxins' extraction, exceeding 82 per cent.

Recently, widely used is the method of the simultaneous identification of 14 mycotoxins. To extract mycotoxins a mixture of 20 per cent sulfuric acid, 4 per cent solution of calcium chloride and acetonitrile is used in proportion of 1:10:89. Dialysis is substituted by a fourfold extraction with isooctane and purification is performed in the column with silica gel. Neutral mycotoxins are eluated with a mixture of chloroform-methanol (97:3), and acidic mycotoxins - with a mixture of benzene-acetone-acetic acid (15:4:1). The identification of certain mycotoxins ranged within 10-800 mcg/kg, however, it was lower than in specific methods for each toxin. This method is applicable for a mass examination of polished rice, rice, maize, wheat and groundnut.

Recently, the methods of multidetection were introduced, using a selective extraction of mycotoxins with different pH of medium for purification.

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Experimental Part

This is a modification of the methods, proposed by Stoloff L.

(1971) and Scott P.M. et al. (1970), which includes the extraction of mycotoxins with a mixture of acetonitrile and a 4 per cent solution of potassium chloride (4:1), the purification of the extract with lead acetate, the separation of mycotoxins by thin-layer chromatography on "Silufol" plates, their qualitative identification by processing with special reagents and semi-quantitative identification by minimum detected TLC quantities.

Extraction. Extract 25 g of grinded grain (a sieve with 0.8 mm meshes) with 90 ml of acetonitrile and 10 ml of a 4 per cent solution of potassium chloride for 20 minutes, shaking continuously. Filter through a double paper filter. Take 50 ml of the extract and place it into a 250 ml separating funnel.

Purification of the extract. Degrease the extract (50 ml) in the separating funnel with isooctane (25 ml - two times). Take the acetonitrilic layer, add 35 ml of water and 15 ml of a 20 per cent solution of lead acetate to the extract, shake properly and leave for sedimentation for 10-15 min, add 5-10 g of Celite 545, mix and filter the extract. Take 50 ml of the filtrate and place it into the separating funnel and wash with chloroform three times (30, 20 and 20 ml). Filter the chloroform extracts through the paper filter with 5-10 g of anhydrous Na_2SO_4, mix and boil down. Dissolve the dry residue in 200 mcl of the benzene-acetonitrile mixture (49:1 in volume). (Extract A). In case of high concentrations of mycotoxins dilute extract A 5 times (Extract B).

THIN LAYER CHROMATOGRAPHY (TLC)

Ready made "Silufol" chromatographic plates (ČSSR) are usually used for TLC. The mixture of toluene-ethylacetate-formic acid

(6:3:1 in volume, TEF) is used as the main system in analyzing the sample for the content of ochratoxin A, zearalenon, sterigmatocystine, penicillic acid and citrinin. As this system sometimes fail to separate aflatoxins B_2 and G_1, the system of benzene-methanol-acetic acid (24:2:1 in volume, BMA) is use. in identifying the latter. The chamber is unsaturated, the length of the front path of the solvent is 14-14.5 cm.

When ochratoxin A alone is present in the extract, the mixture of chloroform-ethanol (19:1 in volume) is used as an additional system, in which aflatoxins have a bigger R_f value than ochratoxins and the latter are situated on the plate in the area, relatively free of pollutants. The following systems are used to separate citrinin and ochratoxin A: (a) diethyl ether-methanol-water-formic acid (95:4:1:1 in volume); (b) acetone-chloroform-water (8:59:1 in volume). The "Silufol" plates, preliminary impregnated with a 10 per cent solution of the oxalic acid in methanol can be used for identifying citrinin; the plates are developed with the following systems of solvents: (a) chloroform-methanol-hexane (64:1:35); (b) toluene-chloroformathylacetate (5:29:6); (c) toluene-chloroform (13:37).

The following additional systems are used for identifying fusariotoxins: <u>for zearalenon</u>: chloroform-ethanol (95:5); chloroform-acetone (95:5); benzene-chloroform-acetone (45:40:15). - <u>For T-2 toxin, diacetoxyscirpenol and desoxynivalenol</u>: Benzene-acetone (3:2); ethylacetate-hexane (2:1); chloroform-ethanol (95:5). For penicillic acid: toluene-ethylacetate-chloroform-formic acid (7:5:5:2).

<u>Detection and Identification of Mycotoxins</u>

The detection and identification of mycotoxins are perform-

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ed visually by comparing the chromatographic mobility (R_f) of the spots of the analyzed sample with R_f of the spots of the corresponding standards of mycotoxins, and also by the specific fluorescence of the spots and the reactions of coloration with specific reagents (Table 1).

Apply each extract to three chromatographic plates. Develop the first and the second plates with the full set of standards in the two main chromatographic systems - TEF and BMA. Check the presence of fluorescing mycotoxins (aflatoxins, ochratoxin A, zearalenon, citrinin, luteoscirin, sterigmatocystine) in UV light (254 and 366 nm).

Process both chromatograms with n-anise reagent, heat the plate for 5-10 minutes until the full development of spots, preventing the darkening of the background of the chromatogram. Analyze the plate in UV light, drawing special attention to the change and the appearance of fluorescence and the visible coloring of mycotoxins in extracts, when compared with corresponding standards of mycotoxins (Table 1).

Check the results of the detection of mycotoxins on the first two chromatograms by the third chromatographic plate, using for the development of the plate (or its part) the reagents, specific for each toxin.

Process the lower part of the chromatogram with a solution of ferric chloride for the development of the kojic acid.

Spray the upper part of the third chromatogram, situated higher the spot of ochratoxin A with an alcoholic solution of aluminium chlorids, heat at 80°C for 5 minutes and analyze in UV light (254 nm). Sterigmatocystine forms stable derivatives, fluorescing with lemon-yellow color. The spraying with a solu-

tion of aluminium chloride changes the grey-sky blue fluorescence of zearalenon into a blue-violet one.

Process the medium part of the third chromatogram, where patulin, penicillic acid and fusariotoxins are situated, depending on the assumed presence of mycotoxins. Keep the plate for 10 minutes in the vapors of ammonia to confirm the presence of the penicillic acid; the penicillic acid manifests itself in a bright blue spot.

Develop patulin with phenylhydrazine hydrochloride or MBTH, with which it forms bright yellow hydrozones.

The presence of T-2 and diacetoxyscirpenol can be identified by processing the chromatogram with a 20 per cent sulfuric acid with the subsequent heating at 80°C; the toxins develop in the visible light as grey-brown spots with grey-sky blue fluorescence. Desoxynivalenol after such a procession gives a dark-brown spot (black in the UV light). When processed with aluminium chloride desoxynivalenol forms blue-fluorescing derivatives. Ochratoxins are identified on the basis of a characteristic change in the fluorescence color - from sky blue-green to blue under the effect of ammonia, and also on the basis of forming derivatives with a methanol complex of boron trifluoride.

The main reactions of coloration, characteristic fluorescence and R_f values of a number of mycotoxins are given in Table 1. It should be noted that due to a thin layer of silica gel and the use of metal base and the presence of starch, the "Silufol" plates should be less heated during the development as compared with usual plates. For instance, after spraying with anisaldehyde, it will be sufficient to heat the chromatogram for 3-5 minutes at 80°C.

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Semi-quantitative Identification of Mycotoxins

After the detection and identification of mycotoxins by TLC, their content may be identified semi-quantitatively by comparing with quantitative standards of mycotoxins and also by the minimum detected amounts of mycotoxins on the chromatogram.

The use of minimum detected amounts of mycotoxins by TLC is expedient in the semi-quantitative assessment of the content of mycotoxins (Table 2).

Apply with a microsyringe 2, 4, 5, 8, and 10 mcl of extracts A or B to the chromatogram or their dilutions, and also 3 spots of standards (5 mcl each) of the identified mycotoxins.

Develop the plate and detect mycotoxins on the chromatogram, using the above-mentioned technique. Choose a spot of the extract on the chromatogram, containing minimum detected amounts of mycotoxins.

Estimate the content of mycotoxins by the following formula:

$$X = \frac{L \cdot F \cdot 10^6}{A \cdot W} \text{ mcg/kg}$$

where: L - minimum detected amounts of mycotoxins by TLC, mcg;

F - general factor of dilution (extract A - 0.8; extract B - 4.0);

A - the volume of the spot of the extract, containing minimum detected amounts of mycotoxins;

W - mass of the sample, g.

Preparation of reagents for the identification of mycotoxins

a) acidic solution of - anisaldehyde: dissolve 0.5 ml of - anisaldehyde in 100 ml of the mixture - methanol-acetic acid-sulfuric acid (34:4:1);

b) phenylhydrazine hydrochloride: prepare a 4 per cent solution of phenylhydrazine;

c) ferrous chloride: prepare a 1 per cent solution of ferrous chloride in ethanol;

d) aluminium chloride: dissolve 20 g of aluminium chloride (hexaaqueous) in 100 ml of ethanol;

e) potassium hydroxide: dissolve 20 g of potassium hydroxide in 100 ml of methanol;

f) sulfuric acid: prepare a 50 per cent solution of H_2SO_4;

g) sulfuric acid: prepare a 20 per cent solution of H_2SO_4 in methanol;

h) MBTH: dissolve 0.5 g of 2-hydrazone-2,3-dihydro-3-methylbenzothiazol hydrochloride in 100 ml of water.

Table 1. Peculiarities of the chromatographic behaviour of certain mycotoxins on "Silufol" plates (L'vova et al., 1978)

Mycotoxins	Concentrations of standards, mck/ml	R_f in systems:		Colour		Colour after spraying with the solution of anisaldehyde		Additional reagents
		TEF	BMA	visible light	UV-light (365 nm)	in visible light	in UV light	
1	2	3	4	5	6	7	8	9
Citrinin	5	0-0.39	0-0.34	-	yellow-green	-	yellow-green	-
Luteoscirin	500	0-0.46	0-0.29	yellow or dull-yellow		orange-yellow	yellow	-
Kojic acid	500	0-0.1	0-0.1	no light-brown	-	-	-	Brown-cherry colour with ferrous chloride
Aflatoxin G_2	0.1	0.21	0.31	-	green	grey in high concentration	sky blue	All four aflatoxins change fluorescence to yellow when sprayed with 50 per cent sulfuric acid
Aflatoxin G_1	0.5	0.28	0.35	-	green	same	-"-	Formation of derivatives with trifluoride acetic acid
Aflatoxin B_2	0.1	0.31	0.42	-	sky blue	orange in high concentration	orange-rosy	-
Aflatoxin B_1	0.5	0.37	0.46	-	-"-	same	-"-	Formation of derivatives with trifluoride acetic acid

Table 1. (Cont'd)

1	2	3	4	5	6	7	8	9
Desoxynivalenol	500	0.32	0.28	-	-	yellow	dull yellow	Brown-black non-fluorescing spots with a 20 per cent sulfuric acid, blue fluorescence with aluminium chloride
Diacetoxyscripenol	500	0.36	0.30	-	-	bright-rosy	sky blue	Sky blue fluorescence with 20 per cent sulfuric acid
Toxin T-2	50	0.40	0.33	-	-	grey-lilac	dull-sky blue	Dull sky blue fluorescence with 20 per cent sulfuric acid
Zearalenon	20	0.64	0.53	-	dull sky blue bright sky blue (254 nm)	brown-orange	brown-yellow	Bright sky blue fluorescence in long-wave UV with aluminium chloride
Patulin	100	0.36	0.24	-	-	light-brown	yellow-grey	Forms bright-yellow hydrazones
Penicillic acid	100	0.41	0.25	-	-	from greenish-brown to lilac and violet	from sky blue to sky blue-rosy and crimson	Sky blue fluorescence when treated with ammonia
Ochratoxin A	5	0.55	0.42	-	sky blue-breen	-	sky blue-green	When treated with ammonia the colour of fluorescence changes to blue. Formation of derivatives with a methanol complex of boron trifluoride

1	2	3	4	5	6	7	8	9
Sterigmato-cystine	20	0.67	0.86	-	red brick	grey-green in high concentrations	dull yellow-green	Spraying with aluminium chloride results in the bright-yellow fluorescence (short-wave UV)

Table 2. The minimum detected amount of mycotoxins on the plates with silica gel

Mycotoxins	Technique of development	Minimum detected amounts, mcg
Aflatoxin B_1 or G_1	UV 366 nm	0.0004
Aflatoxin B_2 and G_2	Sulfuric acid/UV 366 nm	0.0002
Ochratoxin A and ethers	UV 366, 254 nm; ammonia; aluminium chloride, sodium bicarbonate	0.01
Citrinin	UV 366 nm	0.01 0.0008-0.001 [1]
Zearalenon	UV 254 nm; aluminium chloride	0.02
Sterigmatocystine	UV 366,254 nm; aluminium chloride	0.01
Diacetoxyscirpenol	Sulfuric acid/UV 366 nm	0.05
Toxin T-2	Sulfuric acid/UV 366 nm	0.02
Patulin	MBTH (visible light)	0.05
Patulin	MBTH (UV 366 nm)	0.01
Penitrem A	Ferrous chloride (visible light)	1.0
Penicillic acid	Anisaldehyde (visible light)	0.05
Penicillic acid	Anisaldehyde (UV 366 nm)	0.03

[1] Chromatography on the plates, treated with oxalic acid.

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