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

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UP-TO-DATE METHODS OF ANALYSIS OF FOODSTUFFS AND FEED FOR ZEARALENONE

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UP-TO-DATE METHODS OF ANALYSIS OF FOODSTUFFS AND FEED FOR ZEARALENONE

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Zearalenone /F=2 toxin/ is a mycotoxin produced by Fusarium Roseum,F.Graminearum moulds and some other species of the Fusarium genus.

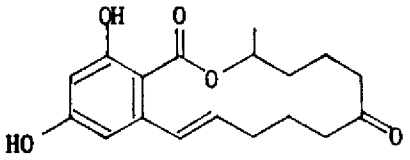
Zearalenone has a pronounced estrogenic effect on farm animals,especially on pigs ,and causes infertility. In high concentrations it produces teratogenic effect. No convincing data on unfavourable effect of zearalenone on man are presently available.

Estrogenic syndrome is observed in pigs even when a 1 mg dose of zearalenone (approximately 0.02 mg/kg of body weight is administered daily during 8 days. The presence of zearalenone at a level of 100 mg/kg of feed in the diet of sows for a period of one week results in infertility. The teratogenic effect on rats is observed at a zearalenone at a dose of 10 mg/kg of body weight.

As a rule, zearalenone-producing fungi affect cereals in the field (root rot).Contamination of grain with zearalenone occurs either during its ripening in the ear or during storage of unthreshed grain. The formation of zearalenone has never been observed during storage of threshed grain even with high moisture content. Most often contamination with zearalenone is observed in maize cobs infected with Fusarium fungi.

As to its chemical structure,zearalenone is lactone of 6-/10^{1} -hydroxy-6^{1} -oxo-trans-1^{1} -undecenyl -β-resorcy-

lic acid with empirical formula $C_{18}H_{22}O_5$ and molecular mass 318. It is a white crystalline substance with a melting point of 164–165°C, slightly soluble in water (up to 2 mg in 100 ml of water) and n-hexane (up to 50 mg in 100 ml) and readily soluble in alcohols, acetone, chloroform, and acetonitrile.



Zearalenone has three UV absorption peaks, at 316, 274, and 236 nm. The data on molar extinction coefficients (ε) of zearalenone in methanol solution are presented in Table 1.

Table 1

Molar extinction coefficients of zearalenone in methanole

Wavelength, nm	Molar extinction coefficients (ε)
236	29 512 ± 376
274	13 225 ± 15
316	6 162 ± 61

The measurement of UV absorption of zearalenone in methanol solutions is used for the determination of the concentrations of standard solutions. Most precise quantitative results are obtained by determining the concentration on the

basis of absorption at 274 nm. The direct UV spectrophotometric determinations of the concentratinns of zearalenone solutions in benzene (often used as standards) are performed on the basis of absorption at 316 nm.

Zearalenone and its alcohol solutions have greenish--blue fluorescence when exposed to long-wave UV light (360nm); the intensity of fluorescence grows under short-wave UV light (254 nm) and the colour of fluorescence changes to green. This phenomenon is used as an additional confirmatory test in thin-layer chromatography (TLC) of zearalenone. The excitation maximum of zearalenone fluorescence is at about 275 nm while emission maximum is observed at 465 nm.

METHODS OF ANALYSIS OF FOODTUFFS AND FEEDS FOR ZEARALENONE

a) Extraction of zearalenone

As a rule, the solvents, used for extraction of zearalenone from samples contain an aqueous component. Before adding the organic component to the sample, it is first wetted with the aqueous component to ensure a more complete extraction of zearalenone. Solvents most frequently used for zearalenone extraction are listed in Table 2.

Table 2

No.	Solvent	Ratio of solvent volume to sample weight	Tested product
1.	Chloroform-water (10:1)	1:5	Plant tissues
2.	Chloroform-enthanol (4:1)	1:10	Feed
3.	Methanol	1:5	Animal tissues
4.	Methylene chloride-water	1:4	Liver
5.	Dichlormethane-2-propanol (9:1)	1:4	Plasma

b) Purification of zearalenone fraction

Normally, the extract contains ,in addition to zearalenone, a number of impurities whose chromatographic and fluorescent properties are close to those of zearalenone. These substances hamper the interpretation of results therefore, the extract purification stage is obligatory in testing the sample. To purify zearalenone fraction, one of the following methods or their combination is usually applied,depending on the object of testing: liquid-liquid extraction in separating funnels column chromatography, preparative thin-layer chromatography,and by partition in liquid phases with different pH-values.

c) Identification and quantitative determination of zearalenone

For the identification and quantitative determination of zearalenone in a purified extract, TLC on silica gel is basically used as well as instrumental methods of analysis:

fluoro-densitometry, gas-liquid chromatography (GLC) and high--performance liquid chromatography (HPLC).

Zearalenone is a sufficiently conveniet object for analysis with the help of the abovementioned methode since it features distinct absorption peaks in the UV region intensive fluorescence which favours its visual detection in TLC analysis capable of sensitive colour reactions with specific spray reagents, easily forms volatile derivatives (in GLC). The selection of the method depends on the required accuracy of the analysis. TLC method is most often used for routine analyses.

The TLC detection of zearalenone by fluorescence at shortwave UV illumination enables the determination of 50 hg minimum value of the substnace in the spot; after spraying with $AlCl_3$ solution, the detection limit may shift to 40ng of zearalenone in the spot. On account of insufficient sensitivity of zearalenone identification by direct fluorescence measurement , another method of zearalenone detection will be used in the laboratory: the spraying of chromatographic plates with bis-diazotized benzidine, which makes it possible to detect as low as 3hg of zearalenone in the spot.

LABORATORY WORK

Determination bf zearalenone content in maize,barley,and other cereal crops

The laboratory work is based on the method of analysis for zearalenone comprising extraction of the sample by chloroform-water system,purification of the extract by column chromatography on silica gel and liquid-liquid partition between hexane and acetonitrile, identification of zearalenone by one-dimensional TLC.

I. For routine analyses for zearalenone in foodstuffs,the following apparatuses,instruments, glassware,and materials are required:

1. UV-spectrophotometer (for example, Hitachi,model 124,
2. Mercury quartz lamp with a 360 nm filter.
3. Test tube shaker
4. Sample pulverizer or coffee grinder.
5. Drying cabinet
6. Technical balance.
7. Analytical balance.
8. Rotary evaporator with trap
9. Water bath with electric heater or electric bath
10. Household refrigerator
11. Microsyringes or calibrated capillaries
12. Chromatographic glass columns,300x22mm
13. TLC chambers with ground stoppers
14. Yellow light filters for protecting eyes from UV radiation

15. Chromatographic plates(for example,"Silufol",Czechoslovakia) with aluminium foil as substrate and silica gel as sorbent.
16. 500,250 and 100 ml conical flasks with ground stoppers No,14.
17. 50 and 100 ml boiling flasks.
18. 100-250 ml separating funnels
19. Glass funnels.
20. Funnels for loose materials.
21. 100,250 ml measuring cylinders
22. Sprayer (with bulb) for liquids.
23. Scissors.
24. 5-10 ml syringe.
25. Filter paper
26. Surgical cotton wool
27. Black paper to protect vessels with zearalenone from light.

II. Reagents for analysis of foodstuffs and feed for zearalenone

Solvents and chemicals should be Chemically Pure, Analytical Reagent ,or ACS grade.

1. Crystalline zearalenone.
2. Medicinal chloroform
3. Ethyl alcohol
4. Acetone.
5. Benzene
6. Acetonitrile
7. Anhydrous calcinated sodium sulphate
8. Celite 545
9. Silica gel for column chromatography,100/160 or 40/100 grade.

10. Acetic acid

11. Aluminium chloride

12. Benzidine

13. Potassium nitrite

14. Concentrated hydrochloric acid

15. Hexane.

III. Preparation of bisdiazotized benzidine spray reagent

The work is carried out in a fume hood.

1. Preparation of Component A.

Weigh 0.5 g of benzidine and place into a 150 ml conical volumetric flask. Add 20 ml of water with 1.5 ml of concentrated HCl (till dissolved). As soon as benzidine is dissolved, add water to 100 ml.

2. Preparation of Component B

Prepare a 10% solution of potassium nitrite in water: weigh 10 g of potassium nitrite, transfer to a glass vial with ground stopper, add water to 100 ml.

3. Directly before spraying the chromatographic plates put the vials with prepared components A and B into a refrigerator at 0° - 6°C. As soon as first crystals of ice appear quickly join the cooled solutions. Warm the reagents to room temperature, filter through cotton wool or paper filter , if necessary .

Preparation of a standard solution of zearalenone

The reliability and accuracy of the whole chemical analysis depends to a large extent on the thoroughness of preparation of the standard solution of zearalenone.

1.Calibration of spectrophotometer (See Seminar 2,item III).

2. Preparation of a standard solution of zearalenone with a concentration of approximately 50 μg/ml:

a) weigh 2.4–2.6 mg of crystalline zearalenone on an electronic balance;

b) transfer the weighed amount to a 50 ml volumetric flask and dissolvet in 20–30 ml of benzene,bring the contents of the flask up to the graduation mark and mix thoroughly.

3. Checking of zearalenone standard purity:

a) take 5 ml of the prepared solution with a pipette and transfer to a 25-30 ml pear-shaped or round-bottomed flask, evaporate to dryness in rotary evaporator;

b) dissolve the dry residue in 15 ml of methanol taken by measuring pipette;

c) record the UV spectrum of zearalenone in methanol; compare molar extinction coefficients at 236,274,and 316 nm (see Table 1).

4. Determination of the concentration of the standard solution of zearalenone in benzene:

a) transfer 3 ml of the standard solution of zearalenone in benzene to the spectrophotometer cell;

b) measure optical density D at 316 nm;

c) calculate the concentration of zearalenone with the formula:

$$C = \frac{D \cdot 100 \cdot M}{E}$$

where: M - molecular weight of zearalenone;
D - optical density;
E - molar extinction coefficient of zearalenone in benzene solution at λ = 316 nm (E = 6060).

5. Preparation of the working solution of zearalenone.

The working solution of zearalenone with a concentration of approximately 5 µg/ml is prepared by a ten-fold dilution of the standard benzene solution of zearalenone. For this purpose:

a) transfer 5 ml of the standard solution of zearalenone to a 50 ml volumetric flask;

add benzene to the graduation mark.

PROCEDURE

1. Extraction of zearalenone from sample

1. Grind a 75 g sample thoroughly in coffee grinder or sample pulverizer.

2. Put 50 g of ground sample into a 500 ml conical flask, add 25 ml of distilled water, and mix thoroughly until the sample is completely wetted. Pour 250 ml of chloroform and add 25 g of Celite, close the flask tightly with a stopper.

3. Carry out extraction in mechanical shaker for 30 minutes.

4. Transfer the extract into a funnel with a folded paper filter. Collect first 50 ml of the filtrate to a measuring cylinder (50 -100 ml).

Alternative procedure: evaporate collected 50 ml of the extract in a round-bottomed flask in rotary evaporator to 2-4 ml.

2. Purification of the zearalenone fraction by column chromatography

1. Place a cotton wool pad on the bottom of a 300 x 22 mm chromatographic tube and add 5g of anhydrous sodium sulphate.

2. Transfer 10 g of a suspension of silica gel in chloroform into column and wash the column with 30 ml of chloroform.

3. Let silica gel settle completely, pour 20-30 ml of chloroform, and add immediately 15 g of anhydrous sodium sulphate.

4. When the chloroform level reaches the top of sodium sulphate, add 50 ml of the filtrate (alternative : add 2-4 ml of the evaporated filtrate).

5. When the upper level of the filtrate reaches the top of sodium sulphate, pour 150 ml of hexane into the column. Discard the hexane eluate.

6. Elute the zearalenone fraction from the column with 250 ml of a benzene-acetone (95+5) mixture.

7. Evaporate the collected eluate to dryness in rotary evaporator.

III. Purification of the zearalenone fraction by of liquid-liquid extraction

1. Transfer the residue (see item II.6) to a small separating funnel and rinse the flask with hexane (45 ml).

2. Rinse additionaly with 10 ml of acetonitrile and transfer to the same separating funnel.

3. Shake the funnel vigorously and let phases separate.

4. Separate the lower acetonitrile layer.

5. Re-extract the hexane layer with 5 ml acetonitrile.

6. Combine the acetonitrile fractions and evaporate to dryness in rotary evaporator.

7. Dissolve the residue in 300 µl of chloroform and close tightly with a stopper.

IV. Qualitative detection of zearalenone

1. Apply 3,6,10 ul spots of the tested solution (item III. 7) onto a Silufol plate (Fig.2) using microsyringe. Try to apply solution in small portions and to obtain spots no more than 5 mm in diameter.

2. Apply 1,3 and 5 µl spots of the working solution of zearalenone about 5 mg/ml to the same plate.

3. Develop the plate in one of the following solvent systems:

a) ethanol-chloroform (5+95);

b) ethanol-chloroform (3.5+96.5);

c) acetic acid-benzene (5+95);

d) chloroform-benzene-acetone (4+1+1).

4. Take the chromatographic plate from the chamber and dry it in a fume hood protecting from bright light.

5. To preliminarily detect zearalenone, examine the plate under long-wave and short-wave UV light. Zearalenone spots have a greenish-blue fluorescence in short-wave UV light.

6. Spray the chromatographic plate with the bis--diazotized benzidine reagent and after 5–10 min compare visually the intensities of fluorescence of the extract and the standard.

V. Quantitative analysis for zearalenone in sample

1. Apply 2,4,6 and 8 µl spots of the studied solution to the chromatographicplate (Fig.2).

2. Apply 2,4 and 6 µl (10,20 and 30 ng) spots of the working solution of zearalenone to the same plate.

3. Develop the plate in a chloroform-benzene-ace-

tone system (4+1+1).

4. Take the plate from the chamber and dry it in a fume hood protecting from bright light.

5. Spray the plate with the bis-diazotized benzidine reagent. Compare visually the colouring intensities of the extract and the standard spots after 5-10 min.

6. Calculate the concentration (C) of zearalenone in the sample with the formula (µg/kg of grain):

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

where S - volume of the standard solution of zearalenone, µl;

Y - concentration of the standard solution of zearalenone , µg/mL;

V - final volume of the sample extract before spotting the plate, µl;

x - sample extract volume giving fluorescence intensity equal to that of S, µl;

W - weight of the analized sample corresponding to the fraction of the chloroform extract applied on the column,g.

If the fluorescence of zearalenone in the extract spot is too intense to match the standards (corresponding to 6 µl of the standard solution), a smaller portion of the extract (X) should be applied to the plate. If the fluorescence of the spots of the extract is again too intense to match the standards,dilute the sample solution (V)·2,4,and more times, taking the dilution into account when calculating the concentration of the sample.

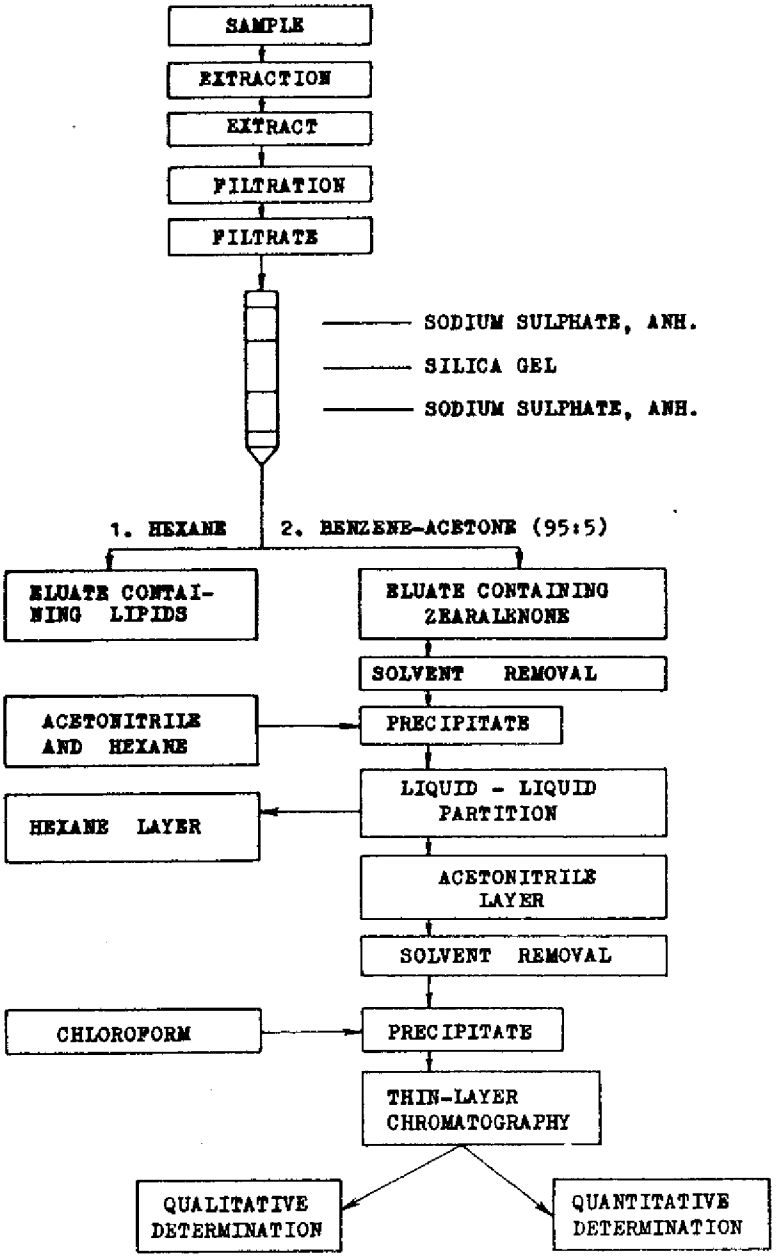
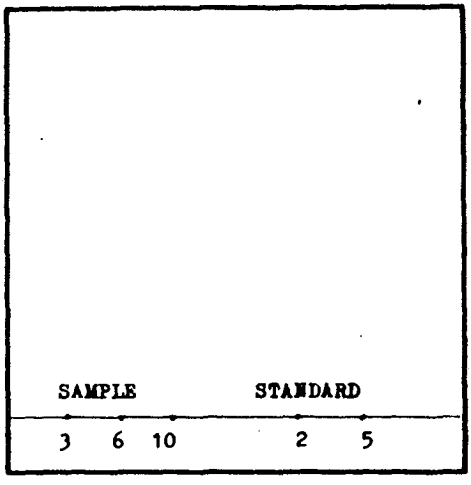
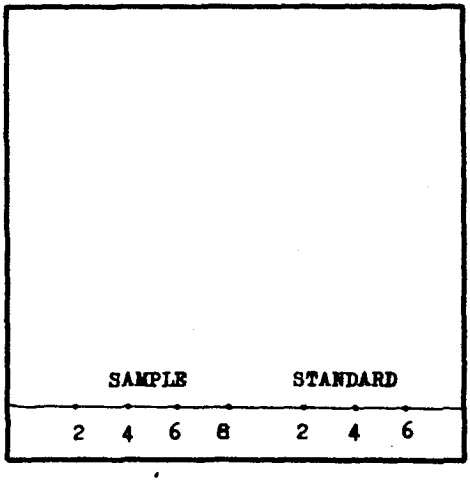


Fig.1. Scheme of analysis of foodstuffs and feed for zearalenone.



Spotting pattern for the qualitative detection of zearalenone.



Spotting pattern for the quantitative determination of zearalenone.

Fig.2.

Зак. 1449 ПИК ВИНИТИ