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Appendix I

Determination of Aflatoxins

(Eppley, 1968)

Chemicals and solvents

1) Standard aflatoxin B_1, B_2, G_1 and G_2.
2) Chloroform, n-hexane and anhydrous diethylether
3) Silica gel G type 60 (70-230 mesh)
4) Silica gel G-HR
5) Chloroform:methanol (97:3, v:v)

   Chloroform:acetone (95:5, v:v)

Quantitation analysis of aflatoxin in crude toxin

1) Place a ball of glass wool in the bottom of 2.2x30 cm chromatography column, and add anhydrous sodium sulphate (5 gm) to give an even base for silica gel (type 60, 70-230 mesh).
2) Add chloroform until the column is about half full.
3) Add silica gel G (type 60, 70-230 mesh) 10 gm and made into a slurry with chloroform.
4) When the silica gel (type 60, 70-230 mesh) has settled and then add slowly anhydrous sodium sulphate (15 gm) on the surface of the silica gel G (type 60, 70-230 mesh).
5) Drain off the chloroform to the top of the sodium sulphate. This column chromatography is ready to use.
6) Crude toxin (50 mg) is dissolved in minimal amount of chloroform and add to the top of the column using a Pasteur pipette. Wash the flask with small amount of chloroform and add the washing to the column as before.

7) Drain off the chloroform to the top of the sodium sulphate and wash the column, elute the crude toxin with hexane (150 ml) and followed by anhydrous diethylether (150 ml).
8) Finally, elute the aflatoxins from the column with 150 ml of chloroform-methanol (97:3, v:v). Collect the fraction from the time the chloroform-methanol is added until the flow stop.
9) Reduce the volumn of the chloroform-methanol eluate in rotary vacuum evaporator at 40°C and subsequently dry by nitrogen gas.
10) Dissolve the residue in a known volume of chloroform for thin layer chromatography.
11) Apply 5, 10, 15,20 µl of this solution to the thin layer chromatographic plate (silica gel G-HR, 0.25 mm. in thickness), along with 3, 5, 7 µl of standard aflatoxins.
12) Develope the plate in a chromatographic tank containing chloroform-acetone (95:5, v:v) as solvent.
13) Remove the plate from tank and allow the solvent to evaporate.
14) Illuminate the plate with a long wave uv lamp in darkened room and observe the pattern of the fluorescent spot compare the fluorescence intensities of the B_1 unknown aflatoxin with those of the standard aflatoxins and determined which of the sample spot matches one of the standard aflatoxins.
15) If the sample spot intensity is found to be between those three of the standard spot the actual intensity should be estimated.

16) If the spot of the smallest volume of sample are too intense to match the standard aflatoxins the sample should be diluted and re-chromatographed.

Calculation Aflatoxin B_1 (µg/kg rice) = $\frac{S \times Y \times U}{W \times Z \times 1000}$

where S = µl of aflatoxin B_1 equal to that of material being evaluated on the plate.

Y = Concentration of aflatoxin B_1 in µg/ml

W = Weight, in mg of crude toxin used.

Z = µl of sample spotted to give fluorescence intensity equal to S, the B_1 standard

V = µl of solvent required to dilute final extract.

U = amount of crude toxin in mg taken from moldy rice 1 kg.

The concentration of the other aflatoxins may be calculated in a similar manner.

Qualitative estimation

1) Small amount of crude toxin (1-2 mg) was dissolved in 0.1 ml chloroform and spot on thin layer chromatographic plate (silica gel G-HR, thick 0.25 mm) along with standard aflatoxin.
2) The plate was developed in the same solvent as describe above
3) The chromatograme patterns were estimate under UV lamp compare with standard.

Appendix II

Determination of Oxalate

(Hodgkinson and William, 1972)

Materials

1) Electrolytic zinc wire, diameter of 3 mm is cut into short lengths of approximately 5 mm and weighing appriximately 250 mg. Immediately before use the zinc is cleaned by immersing briefly in freshly prepared 10 N HNO_3. After washing in distilled water the zinc is ready for use.

2) Chromotropic acid solution. Dissolve 1 gm of 4,5-dihydroxynaphthalene, 2,7-disulphonic acid, disodium salt in 100 ml of water. Store at $4^{o}C$ and prepare freshly once a week.

3) Stock oxalic acid standrd. Dissolve 1.0231 gm of potassium oxalate monohydrate in 100 ml of water. Store at $4^{o}C$ and prepare freshly once a month. This solution contain 5 mg of anhydrous oxalic acid per ml.

4) Working oxalic acid standard. Dilute the stock standard oxalic acid solution 100 times to give the solution containing 50 µg of oxalic acid per ml.

Methods

1) Crude toxin weight 10 mg is grounded into fine granules and mix with 4 ml of 1% HCl.

2) The solution was filtered through filter paper.

3) Transfer 2 ml of filtrate into 25 ml graduated stoppered centrifuge tube and followed by 0.04% bromo-thymol blue indicator solution.

4) Adjust the solution to pH 7.0 by the addition of dilute NaOH or dilute acetic acid solution.

5) Add 2 ml of saturated aqueous solution of calcium sulphate followed by 14 ml of ethanol, mix gently and allow the solution to stand at room temperature for at least 3 hr or preferably overnight.

6) Centrifuge at 2000 rpm for 10 min, carefully decant the supernatant fluid and allow the tube to drain for a few minutes on filter paper.

7) Wipe the mouth of the tube with clean tissue and dissolve the precipitate in 2 ml of 2N H_2SO_4.

8) Add a piece of freshly cleaned zinc and heat in boiling water bath for 30 min. (The tube is left unstoppered to allow evaporation to occur and the final volume should be less than 0.5 ml to ensure full colour development).

9) Remove the zinc with a bent glass rod. Wash the zinc with 0.5 ml of 1% chromotropic acid solution, adding the washings to the tube.

10) Add 5 ml of concentrate H_2SO_4 slowly, with mixing, and heat in boiling water bath for 30 min. (The tubes do not need to be stoppered).

11) Cool, dilute to 20 ml with 10N H_2SO_4 and determine the optical density at 570 nm. The colour is stable for several hours.

Standard curve

1) Prepare six tubes containing 0, 0.2, 0.4, 0.6, 0.8 and 1.0 ml of working standard solution (0, 10, 20, 30, 40 and 50 µg of anhydrous oxalic acid).

2) Add water to make the final volume of 1 ml and followed by 1 ml of 4N H_2SO_4 and, a piece of freshly cleaned zinc, then proceed as described above.

Calculation

Two ml of filtrate came from 5 mg of crude toxin. µg of anhydrous oxalic acid per 100 mg crude toxin.

= Reading from calibration curve x 20

Appendix III

Paraffin section

Preparation of buffered formalin

Formaldehyde (37%)	340.0 ml
NaH_2PO_4	13.78 gm
Na_2HPO_4	22.12 gm
Water up to	1.0 gallon

Preparation of paraffin sections

1. The visceral organs were fixed in 10% buffered formalin (pH 7.4)
2. The trimmed tissues were dehydrated, clear and embedded with the following solutions for 30 minutes per step:
    a. 70% Ethanol
    b. 80% Ethanol
    c. 90% Ethanol
    d. 100% Ethanol, twice
    e. 100% Ethanol and Xylene (1:1, v:v), once
    f. Xylene, twice
    g. Immersed in solf, medium hard and hard paraffin respectively at 60^{o}C under vacuum.
    h. The tissues were embedded with paraffin in plastic holder.
3. The blocks of tissues were cut to provide sections 4-5 microns in thickness.
4. The sections were mounted on glass slides by standard warm water bath technique and dried at room temperature.

Hematoxylin and Eosin staining

1. The mounted sections were held in the following solutions for the indicated times.
    a. 100% Ethanol, 2 minutes
    b. 95% Ethanol, 2 minutes
    c. 80% Ethanol, 2 minutes
    d. 70% Ethanol, 2 minutes
    e. Distilled water, 2 minutes
    f. Harris hematoxylin, 8 minutes
    g. Distilled water, 2 minutes
    h. 80% Ethanol, 2 minutes
    i. Eosin, 5 minutes
    j. 95% Ethanol, 2 minutes, twice
    k. 100% Ethanol, 2 minutes, twice
    l. Xylene, 2 minutes, twice
2. Mounted with Permount and dried overnight at room temperature.

Appendix IV

Purification of the crude toxin

Reagent

1. Silica gel G
2. Silica gel type 60 (70-230 mesh)
3. $CHCL_3$, benzene, methanol
4. $CHCL_3$:Benzene:Methanol (60:30:10, v:v:v)
5. $CHCL_3$:Methanol (90:10, v:v)

Procedure

1. One gram of crude toxin (A. niger, AN-A30-75) was dissolved in 5 ml chloroform and mixed with 5-10 gm silica gel (type 60).
2. The mixture was dried by a rotary vacuum evaporator at $40^{o}C$.
3. The column was packed in similar manner as described in method for determination of aflatoxin, except more silica gel was used (50 gm), and the solvent was benzene.
4. The mixture was loaded on the top of column chromatrography. Fraction of 75 ml each was collected. The eluents were used in various fraction as list below:

fraction 1^{st}-2^{nd}	-	chloroform:benzene (50:50, v:v)
fraction 3^{rd}-4^{th}	-	Pure chloroform
fraction 5^{th}-11^{th}	-	Chloroform:Methanol (97:3, v:v)
fraction 12^{th}-13^{th}	-	Chloroform:Methanol (95:5, v:v)
fraction 14^{th}-25^{th}	-	Chloroform:Methanol (90:10, v:v)
fraction 26^{th}-29^{th}	-	Chloroform:Methanol (50:50, v:v)

5) The toxin eluted out in each fraction were detected by thin layer chromatography (Silicagel G, thick 0.25 mm) and developed in chloroform:benzene:methanol (60:30:10, v:v:v)

6) The chromatogram patterns containing same kind of ingredients were pooled.

7) All of pooled fractions were concentrated by rotary vacuum evaporator and precipitated by adding cooled petroleum:chloroform (90:10, v:v)

8) The precipitate was separated by low speed centrifugation and dry by blowing with nitrogen gas.

9) All together six ingredients were recovered is list before

Ingredient I was recovered from fraction 1^{st}-6^{th}.

" II " " 7^{th}-9^{th}

" III " " 10^{th}-11^{th}

" IV " " 12^{th}-14^{th}

" V " " 15^{th}-20^{th}

" VI " " 21^{st}-29^{th}

10) The toxin elute out in each ingredient was tested for acute toxicity test by administration through intraperitoneal to 7-day-old rats, and observed for 7 day.

11) It was found that toxins from ingredient IV were toxic to rats. Hence this fraction were further purified by thin layer chromatography.

12) Ingradient IV 111 mg was dissolved in 2 ml of chloroform and lined on TLC plate (silica gel G, 0.5 mm thick), 20 mg of ingredient IV per plate, developed in chloroform:benzene:methanol (60:30:10, v:v:v).

13) Observed the chromatographic line under UV lamp.

14) The combine of blue and blue-green fluorescent bands and orange-brown band were scraped using stainless steel spatula.

15) The toxin was extracted from silica gel by using chloroform:methanol (90:10, v:v) and followed by 3 times of pure methanol.

16) Filtered through filter paper and concentrate by rotary vacuum evaporator and precipitated by adding petroleum ether.

17) The two fractions which obtained each was administered intraperitoneally to 7-day-old rats and observed for 7 days.

18) It was found that fraction which contain combined spot of blue and blue-green spots induce toxicity to experimental animal.

19) This fraction were further purified by using thin layer chromatography the same procedure as described above.

VITAE

Miss Kanda Romruen was born on October 23, 1949, in Nonthaburi, Thailand. She attended Triam Udom Suksa High School from 1966 to 1968. From 1968 to 1972 she attended Faculty of Science, Mahidol University, where she majored in Medical Technology. She was appointed as a scientist at department of Pathobiology, Faculty of Science, Mahidol University. The major work was dealing with food Toxicology.