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PRELIMINARY TESTS FOR DETECTING THE FUNGI-INFECTED GRAIN. SCREENING AS A METHOD OF AFLATOXIN DETECTION BY MINI-COLUMNS

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PRELIMINARY TESTS FOR DETECTING THE FUNGI-INFECTED GRAIN.
SCREENING AS A METHOD OF AFLATOXIN DETECTION BY MINI-COLUMNS

L.S. Lyova, O.D. Doronina

Grain and seeds of many cereals may be contaminated with mycotoxins during harvesting and storing therefore control should be introduced over the presence of mycotoxins and other toxigenic fungi in the products of plant origin.

Since it is rather difficult to identify mycotoxins and aflatoxins, in particular in the infected grain it will be expedient to test those grain lots for toxicity which were either spontaneously heated or were infected with microfungi. The fungi-infected and self-heated grain can be identified by the smell, colour lustre, and the visible fungi growing process.

The identification of the mycotic lesions of grain by a microbiological plate technique is a time-consuming enterprise (6-7 days), which requires sophisticated equipment and skilled personnel. That is why a few simple methods were elaborated for detecting the grain lots, infected with fungi, Aspergillus flavus in particular, or with other unidentified aflatoxins. Apart from these, screening techniques were developed for a quick preliminary test for the presence of aflatoxins in foodstuffs and fodder.
The findings of the North Regional Research Center team, USA, have revealed a direct relationship between the formation of the bright green-yellow fluorescence (BOF) in maize and cotton seeds and the presence of aflatoxins.

Fluorescent green substances are as a result of the interaction between cojic acid, generated by many fungal species and peroxidase of plant tissues. As virtually all the A. flavus strains synthesize cojic acid, the presence of BOF in maize is accompanied by the aflatoxin presence in 70 per cent of cases.

These findings allowed the development of a technique for identifying the maize lots, infected with A. flavus and presumably contaminated with aflatoxins. This method is widely used in the US to control dryground maize. According to this method, BOF should be detected in the course-ground grain as fluorescence may be dulled by grain husks. In terms of quantity, BOF is not an indicator, and, therefore, it cannot be used in determining the aflatoxin level. The presence of merely a single fluorescent particle in a kilogram of grain is evidence of the aflatoxin presence in 70% of cases.

If BOF is traced in a grain shipment, the content of aflatoxins (AT) should be established by express screening techniques. An advantage of these techniques is a fast sampling analysis and the simplicity of the AT-content test. A variation of the screening technique treated henceforth, was proposed by G. Velanki in 1975. The technique includes: extraction of AT from a grain sample by water acetone; purification of the extract from pigments and other fluorescent admixtures by the liquid-liquid extraction and complexing; followed by the nepr-
ration of AT by adsorption chromatography in "mini-columns" (5mm x 200 mm) and the subsequent direct AT concentration test at the Velsako fluorotoxin meter. The device measures total fluorescent intensity of AT, adsorbed inside mini-columns on a florisoril layer. The linear relation between the AT concentration within 10ng/ml - 100ng/ml and the fluorescence intensity was established. The coefficient of variation for this technique is 1.2 - 1.4%.

This mini-column screening technique falls in the category of semi-quantitative methods used in testing food-stuffs for AT content. It should be noted that there are essential shortcomings inherent in the screening technique due to a low resolving power of mini-columns which, in turn, results in low specificity with regard to AT. That is why positive results of the screening test should be checked against the AT content indentified by conventional techniques.

Grain moulding is accompanied by the build-up of green-fluorescent compounds of steroid nature (GPS) formed by the fungi. We have proposed an express chromatographic technique for detecting such compounds which reveal a direct relation between the level of mycotic lesion of grain and the quantity of GPS. The unaffected grain contained 0-20 mg/kg of GPS and the aflatoxin-infected grain contained over 50 mg/kg of GPS.

The technique allows to detect the mycotic lesion of grain at its initial stage.

This paper will treat the following techniques, used in detecting the fungi-infested grain:

(1) Visual detection of bright green-yellow fluorescent grain (BGF):
(2) chromatographic identification of green-fluorescent substance of the fungal origin (GFS)
(3) screening or the mini-column technique of aflatoxin detection by the Veleco fluoroaxin meter.

I. Experimental methods

Identification of bright green-yellow fluorescence (BGY)

1.1. A maize bulk should be inspected and the initial grain sample with a total mass of 2.3 kg should be taken from as many points as possible.

1.2. 1 kg of grain is to be separated from the initial sample with a divider.

1.3. The 1 kg sample is to be ground coarsely with a mill fit with a 1.2 cm diameter sieve.

1.4. The coarse-ground grain is to be spread evenly on a flat surface in a dark room and all bright green-yellow fluorescent (BGY) fragments should be collected in the ultraviolet light. The unaffected maize grain are fluorescent with a dim-violet light.

1.5. In case one or more grains fluorescent with bright green-yellow light are detected in the sample it must be put to aflatoxin test performed with the mini-column screening method.

2. The screening method of aflatoxin detection by mini-columns

2.1. The extraction of aflatoxins from the sample.

2.1.1. The 1 kg grain sample has to be additionally milled (see 1.3) with the mill sieve diameter of 0.8 ml.
2.1.2. The sample is to be stirred either by letting it pass 3 times through the divider or by hand.

2.1.3. 50 gr ± 0.5 gr of the prepared sample are to be weighed and put into a conical flask. Add 250 ml of the aceton-water mixture (17:3) Extract the substance with a special flask shaker for 30 min.

2.1.4. Filter the extract through a folded paper filter into a 100ml graduated cylinder (Fig. 1).

2.2. Purification of the extract

2.2.1. Add the solution prepared in advance (100 ml of distilled water + 10 ml of 10% FeCl₃ + 15 ml of 4.63% NaOH) to 90 ml of the extract and shake the content of the flask well for 30-40 sec.

2.2.2. Filter through a paper folded filter into a graduated cylinder.

2.2.3. Take 180 ml of the filtrate and add 130 ml of distilled water. Place the solution into a separating funnel.

2.2.4. Add 50 ml of chloroform into the funnel and thoroughly stir the substance.

2.2.5. Extract the lower chloroform layer. Remove the solvent in the vacuum of a water-jet pump.

2.2.6. Dilute the residue (15 gr of the initial sample) in 6 ml of the chloroform-methanol mixture (24:1). Thus, 1 ml of the final product corresponds to 2.5 gr of the initial product.

2.3. Preparation of mini-columns

2.3.1. Place a glass wool wad, 2-3 ml thick, on the bottom of mini-columns (5x200 mm).
A sample preparation of (50 gr)

Aflatoxin extraction from bio-substance

Filtering

Sediment

Filtrate (90 ml)

Filtering

Sediment

Filtrate (180 ml)

Extraction

Water layer

Chloroform extract

Concentrating the purified extract

Residue

Benzol-methanol (24:1) 6 ml

1. Applying 1 ml of the solution onto a mini-column
2. Rinsing the column with 1 ml mixture of chloroform-methanol (24:1)

Fig. 1: The sequence of the aflatoxin-text, performed by the screening method
2.3.2. Then add sand (5-7 mm), florisil(5-7mm), sand (5-7mm), silica gel (15 mm), alumina (15 mm) in a consecutive order.

2.4. Column chromatography by a mini-columns

2.4.1. Soak the mini-column, prepared in advance, with chloroform.

2.4.2. Apply 2ml of the final extract on to the mini-column. Let the solvent run down to the surface of the sorbent layer.

2.4.3. Prime the column with the 1 ml mixture of chloroform - methanol (24:1)

2.4.4. Apply 1 ml of the standard solution of aflatoxin B₁, the concentration of which is 50mg/ml, on to the mini-column moistened with chloroform to prepare a standard mini-column.

2.4.5. Prime the column with the 1ml mixture of chloroform-methanol (24:1).

2.5. Aflatoxin concentration test of the sample.

2.5.1. Prime up the fluorimeter for operation.

2.5.2. Calibrate the fluorimeter with a standard mini-column and set an indicator to 20 rrv (μg/l/kg).

2.5.3. Place the mini-column with the tested sample into the Velasko fluritoxin meter.

2.5.4. Measure the aflatoxin concentration in the sample.

3. Testing the intensity of the mycotic lesion of the grain by the content of green-fluorescent compounds of fungi origin
3.1. Weigh and place 20g±0.5g of the prepared sample into a conical flask. Add 100ml of m-hexane. Carry out the extraction for 30 minutes with a flask shaker.

3.2. Filter the extract through a folded paper filter into a 100 ml graduated cylinder.

3.3. Dry 50ml of the filtrate in the vacuum of a rotor evaporator.

3.4. Dissolve the residue in 5 ml of chloroform.

3.5. Dilute the extract 1:10 by adding 9 ml of chloroform to 1ml of extract A. (Extract B).

Dilute the extract 1:100 by adding 9 ml of chloroform to 1 ml of extract B.

3.6. Apply A, B and C extracts onto the "Seelufol" plate. Use the A extract of strongly moulded grain as a standard.

3.7. Develop the chromatogram in the system of petroleum ether-ethoxy ethane (4:1) or in chloroform.

The front path is 12 cm; the chamber is square and non-saturated.

3.8. Take the chromatogram out of the chamber, and remove the residue. Examine the chromatogram through the ultra-violet light (365nm).

Rf of the steroid green-fluorescent substance of the mycotic origin is 0.36-0.40 in the ether system and 0.6-0.7 in chloroform.

The absence of green fluorescent spots or the presence of such spots in extract A is evidence of the unaffected grain. The presence of green fluorescent substance in extract B points to the mycotic lesion and the presence of this substance in extract B is an indication of an intensive mycotic lesion of the grain.
The grain, whose B and, in particular C extracts contain green fluorescent substance must be further tested for aflatoxins by analytical methods.

If need be, the green-fluorescent contents may be quantitatively analysed by measuring the volume of a spot, having the following minimum fluorescence (GFS):

\[
\text{GFS} = \frac{0.013 \times V \times x \times 1000 \times 1000 \times A}{V \times B} \text{ \text{\(\mu\text{g/kg}\)}}
\]

where:

\(V\) - is the volume of a spot in the extract (\(\mu\text{l}\)) having negligible fluorescence (GFS)
\(B\) - is the weight of the grain (\(\text{gr}\))
\(C\) - is the volume of extract A (\(\text{ml}\))
\(A\) - is the dilution factor;
0.013 \(\mu\text{gr}\) is a minimum quantity of GFS detected on the Seelufol plate.

The simplified formula is as follows:

\[
\text{GFS} = \frac{6 \times A}{V \times B} \text{ \text{\(\mu\text{g/kg}\)}}
\]