THE ESTIMATION OF AFLATOXIN : A CRITICAL SURVEY OF THE AVAILABLE PHYSICO-CHEMICAL METHODS

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The outbreak of Turkey-X disease in the UK in 1960 led to an intensive search for the cause of death of the 100,000 or so turkey poults affected. Toxicologists in due course traced the origin of the disease to a toxic component produced by a fungus present in the groundnut meal used to make up the rations fed to the birds. In the course of efforts to establish the chemical structure of the toxic substance, which was given the name of 'aflatoxin', it was found that extraction was optimal with methanol or chloroform and that extracts containing aflatoxin fluoresced in ultraviolet light. Chromatography of the extract on a column of neutral alumina resolved the aflatoxin into two bands, one of which gave a blue-violet fluorescence (aflatoxin B_1 and B_2) and the other a greenish fluorescence (aflatoxin G_1 and G_2) under ultraviolet irradiation.

This preliminary work paved the way for the elaboration of both qualitative and quantitative methods for the estimation of aflatoxin. To date all methods proposed are based on its fluorescence under ultraviolet light.

METHODS

1. The first publication describing a method for the semi-quantitative estimation of aflatoxin was one of Coomes and Sanders (1963)[1] of the Tropical Products Institute, London. The method may be summarized as follows: After defatting, the material to be examined is extracted with methanol. The methanol extract is freed from all traces of fatty material by partition extraction with light petroleum, and the aflatoxin is then removed from the methanol extract by continuous liquid-liquid extraction with chloroform. The chloroform extract is thereafter chromatographed on a column of neutral alumina. After the elution of aflatoxin the portion containing the aflatoxin is subjected to paper chromatography and the chromatogram is then examined under ultraviolet light. The smallest quantity of toxin that can be detected in this way is 0·2 μg.

2. In a second method, proposed by Broadbent, Cornelius and Shone (1963)[2] of the Tropical Products Institute, London, greater precision is obtained by spotting dilutions of the chloroform extract on a thin-layer chromatoplate until fluorescence ceases to show up in the spots. The extract for this method is obtained in much the same way as that described by Coomes and Sanders.[1] After concentration, the extract is spotted in suitable quantities on thin-layer chromatography plates, coated with neutral alumina. The plates are then developed in chloroform containing 1·5% methanol. The developed plate is viewed under UV light at 365 mμ. If aflatoxin is present in the aliquot it shows up as a blue-violet fluorescent spot in the case of aflatoxin B and a greenish spot in the case of aflatoxin G. The smallest quantity of aflatoxin B which is detectable under these conditions is 0·006 μg.

3. In a later publication,[3] the group at the Tropical Products Institute, London, stated that their method could be improved by introducing the following modifications:

(*a*) Substituting kieselgel G for alumina on the TLC plates.
(*b*) Increasing the methanol content of the eluent from 1·5% to 5%.

The higher resolution obtainable with the kieselgel plates makes it possible to detect 0·002 μg. aflatoxin.

4. A provisional description of a third method was published by Nesheim *et al.*[4] in January 1964 and later confirmed in a private communication received from the USA. The basis of the method is as follows: The material to be examined is blended at high speed either with methanol, if it is defatted, or with a mixture of methanol and hexane in the case of undefatted material. A suitable aliquot of the clarified extract is chromatographed on a column of celite 545 and eluted successively with hexane and chloroform to separate the aflatoxin-containing portion from any fatty material present. The resultant extract is chromatographed on thin-layer plates as described under (2) and (3).

5. In an effort to bring the determination of aflatoxin onto a more exact basis, the TPI group (Nabney and Nesbitt, 1964)[5] suggested the following additional modification of their procedure: The final extract is applied to the thin-layer plate as a band on the baseline. After elution of the plate, the band of aflatoxin is scraped off carefully, desorbed and made up to a suitable volume. The fluorescence of this solution can be accurately determined in a spectrophotometer or fluorimeter at 363 mμ.

6. The final method to be discussed is one described in a paper by De Iongh and Van Pelt of the Unilever Research Laboratories in Vlaardigen, and Ord and Barrett of the Uni-

lever Laboratories at Welwyn.[6] The method is basically the same as that proposed by the TPI group, but with the following refinements:

(a) The extraction time with methanol is shortened from 4 hours to 1 hour, and this is followed by extraction with chloroform for one and a half to 2 hours. After evaporation of the chloroform the residue is taken up in methanol and transferred to the methanol extract.

(b) The concentration of the methanol is adjusted to 85% and all traces of lipids are removed by partition extraction with light petroleum. This step ensures that no interfering light blue fluorescent streaks due to lipids will appear on the chromatogram.

(c) Satisfactory separation of the components is obtained by the use of 2% methanol in pure chloroform.

(d) A standard solution of aflatoxin B_1 (0·1 μg./ml.) is used as a reference standard.

DISCUSSION

The merits and demerits of the available methods may be summed up as follows:

The paper chromatography method[1] is rather time-consuming and thus not very suitable for routine analysis. Furthermore, the resolution is unsatisfactory and the sensitivity of 0·2 μg. relatively low.

The method proposed by Broadbent *et al.*[2] has been used extensively in this country. It has two major shortcomings. Firstly, the alumina used for the TLC plates is actually intended for column chromatography and hence gives poor resolution. Secondly, the proposed extraction method leaves traces of fat in the extract which produce interfering streaks of light blue fluorescence. These in turn enhance the intensity of the fluorescence due to aflatoxin.

The shortcomings of the alumina method were largely eliminated when, in their later publication,[3] the TPI group recommended the use of kieselgel on the TLC plates. The sensitivity of the method is improved by this modification but the presence of lipids in the final extract still interferes with the assessment of the chromatogram.

The method described by Nesheim *et al.*[4] is unacceptable for the following reasons: (*a*) Extraction in a Waring blend or with inflammable solvents is dangerous, (*b*) Elution of interfering substances by column chromatography is almost impossible with this method since hexane will not soak into the celite, and (*c*) The method does not have any particular advantage over the others.

The quantitative method proposed by TPI[5] is very time-consuming and is therefore unsuitable for routine analyses. It also lacks sensitivity in the range below 1 μg./G. The authors admit in their summary that 'this method is less sensitive than the one depending on dilution to extinction of fluorescence but it provides a more reliable means of assaying meals containing 1 μg./G or more aflatoxin'.

The method of De Iongh *et al.*[6] has the following definite advantages:

(a) Extraction with methanol followed by extraction with chloroform removes *all* the aflatoxin from contaminated material.

(b) Partition extraction of lipids from the methanol extract yields a chromatoplate free from interfering fluorescent substances.

(c) The use of a standard solution of aflatoxin B_1 enables the operator to evaluate the chromatogram with much greater confidence.

All the methods proposed except that of Nabney and Nesbitt can at best be termed semi-quantitative since the estimation of the toxin content of a sample depends on the naked-eye assessment of the intensity of the fluorescence produced by the toxin. The toxicity level is classified on the basis of the presence or absence of fluorescence at certain Rf values on the chromatoplates after chromatography of (usually) three aliquots of differing concentrations. The results are reported as:

very high	>2·0 μg./G
high	0·5 - 2·0 μg./G
medium	0·1 - 0·5 μg./G
negative	<0·1 μg./G

In view of the greater accuracy, speed and ease of manipulation afforded by the method of De Iongh *et al.* this method is clearly the most satisfactory for the purpose of routine analyses. The NNRI has been applying this method in conjunction with a standard solution of 0·002 μg. pure aflatoxin B_1/10 μl. as a reference standard.

OPSOMMING

Die Bepaling van Aflatoksien

Die kenmerkende blou-violet fluoressensie van aflatoksien B (B_1 en B_2) en die groenerige fluoressensie van aflatoksien G (G_1 en G_2) onder ultraviolet-lig vorm die grondslag vir die bepaling van hierdie stowwe in al die metodes wat tot dusver vir hierdie doel aangewend is. Verskillende metodes wat gegrond is op uitloging, gevolg deur kolom- en/of papierchromatografie en die opsporing van aflatoksien onder ultraviolet-lig word beskryf. Die beste metode is dié van De Iongh e.a. Hierdie metode word tans in gewysigde vorm in verskeie laboratoriums in die Republiek gebruik.

REFERENCES

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