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Determination of mycotoxins

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Abstract - Various approaches exist to the determination of mycotoxins. Chemical assays are of major importance. Generally, all chemical analytical methods for the determination of mycotoxins include the basic steps of extraction, clean-up, separation, detection, quantitation and confirmation of identity. Most widely used are those techniques which include a chromatographic step to separate the mycotoxin of interest (minicolumn chromatography, thin layer chromatography, high performance liquid chromatography and gas liquid chromatography). Immuno-assays, which are bio-chemical separation techniques, are promising but still in an early stage of development for mycotoxin research.

INTRODUCTION

Since the early 1960's, when the outbreak of Turkey X-disease focussed a lot of scientific attention to the until then rather neglected area of mycotoxins and mycotoxicoses, a wealth of information about mycotoxins has been produced. As a matter of course, the availability of methods of analysis has played a keyrole in the development of mycotoxin survey and research programmes. The fact that mycotoxins are usually present in agricultural commodities and products as minor constituents in concentrations ranging from (sub) µg-mg/kg, means that the possibilities to determine mycotoxins are limited to certain trace analytical methodologies. In the period of the initial discovery of the aflatoxins, biological and chemical procedures have been developed, the latter included thin layer chromatography (TLC), at that time a newly found, efficient separation technique. Since then bio-assays have played a role of minor importance and the group of chemical methods was gradually extended with procedures involving minicolumn chromatography, high performance liquid chromatography, gas liquid chromatography, radio-immuno-assay, enzyme-linked immunosorbent assay and several other less frequently used techniques.

A lot of factors may determine and limit the analysts choice of an appropriate assay procedure. Due to the diversity in chemical structures of the few hundred mycotoxins known (for examples see Fig. 1) and the compositional variability of the materials to be analysed, the physical and chemical properties of toxin and matrix may vary considerably. Each toxin in combination with each type of sample poses a unique problem. Moreover, the analyst may have certain requirements or desires about the utility of the method and about the reliability of the analytical data to be generated. The utility of the method is determined by practical characteristics such as the applicability, the cost of performance, the time required, the equipment required and the level of training needed. The reliability is determined by scientific characteristics as precision, accuracy, detectability, sensitivity and specificity. Next to the many differences, there are certain similariaties in the methods of analysis that are being used for the determination of mycotoxins. In the following sections of this paper some attention will be paid to the various analytical approaches and to the merits and limits of different types of methods of analysis. Special emphasis is laid on the determination of the aflatoxins, a group of highly carcinogenic mycotoxins, which have been intensively investigated (see Fig. 1).

ANALYTICAL PROCEDURES

There are two approaches possible for the detection and determination of mycotoxins: biological and chemical. Biological methods may be useful in screening for mycotoxins, especially when the identity of the mycotoxins is not known. As an example, they have played a role of importance in the period of the initial discovery of the aflatoxins (ref. 1). However, if it is known which mycotoxin(s) should be looked for, chemical assays, if available, are to be preferred, because these generally are much more specific, more rapid, more reproducible, and possess lower limits of detection. Hence, chemical assays play a role of major importance in the determination of mycotoxins. Therefore, the bio-assays will not be further

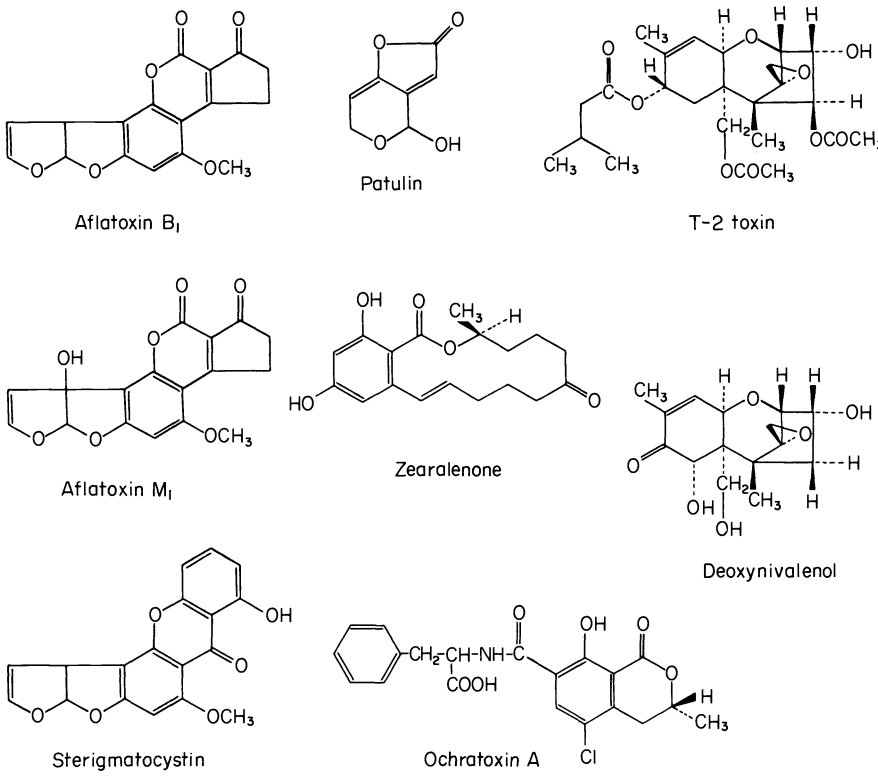


Fig. 1. Chemical structures of some mycotoxins

discussed here and the interested reader is referred to the overviews given by Watson and Lindsay (ref. 2) and Van Egmond (ref. 3).

The limitations of bio-assay techniques to detect and determine mycotoxins led chemists to develop more selective and reliable methods of analysis. Generally, all chemical analytical methods contain the basic steps as outlined in Fig. 2. The first step, sampling, is a most important part of the analysis procedure. However, because sampling and sample preparation are extensively discussed by Professor Dickens in another plenary lecture, no further attention to this subject will be given here.

Homogenized test portions that are taken for analysis usually vary in weight from ca. 20-100 g, a range resulting from a compromise between homogeneity requirements and practical requirements.

Extraction

The following step in chemical analysis involves extraction of the test portion to separate the component of interest from the bulk of the matrix components and to obtain the materials of interest in a manageable form. Contact between solvent and solid substrate is accomplished either for a short period (1-3 min) in a high speed blender, or for a longer period (30 min) by shaking in a flask. Liquids may be extracted in a separatory funnel or absorbed to a hydrophylic matrix which is (pre-)packed in a column, after which extraction is accomplished by eluting the column with an extraction solvent. Examples of the latter extraction techniques are the procedures to determine aflatoxin M_1 in milk in which a self-prepared Celite column is used (ref. 4) or a ready-made Extrelut® column (ref. 5, ref. 6).

The choice of the solvent depends on the chemical properties of the matrix and toxin. Often, mixtures of solvents, or solvents with small amounts of water or acids, are found to be most effective. While the solubility of many mycotoxins in water is low, aqueous solvents may penetrate hydrophylic tissues, leading to a most efficient extraction by the non-aqueous solvents. One of the best-known and practised methods of analysis for aflatoxins, the CB (Contaminants Branch) method, employs a mixture of chloroform and water to extract aflatoxins.

Clean-up

Since mycotoxins are normally only present at very low levels, a strong concentration of the extract is necessary to make detection possible. The frequent presence of lipids and other

substances that may interfere in the final detection make it necessary to clean-up the extract prior to concentration, by column clean-up, liquid-liquid extraction and/or co-precipitation of impurities.

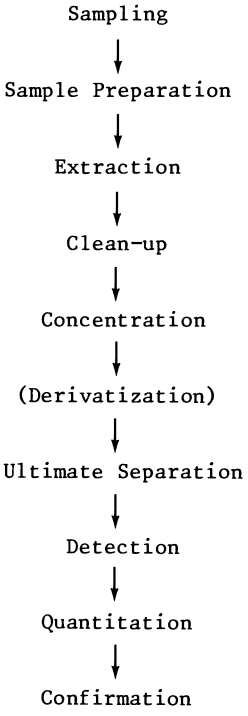


Fig. 2. Analytical procedure for mycotoxin determination

Several column chromatographic clean-up steps are possible with materials such as silica gel, modified silica gel, aluminium oxide, polyamide, Florisil® and Sephadex®. Silica gel is most frequently used. Columns can be packed in the laboratory. However, pre-packed columns are now commercially obtainable, in rather many recently published analytical methods for mycotoxins use is made of these columns. The advantages of such pre-packed columns e.g. SEP-PAK®, Baker® are obvious. Time, needed to prepare the columns is saved, and variations in preparation of columns between analysts are eliminated. On the other hand, variations between lots of pre-packed columns have been reported (ref. 7), and they do not offer the possibility of easily introducing slight variations in the column composition (for instance adjustment of the water content or column size). The sample extract is usually added to the column in an appropriate solvent, after which the column is washed with one or more solvents in which the toxins are insoluble or less soluble than the impurities. Then the solvent composition is changed in such a way that the toxins are selectively eluted from the column and the eluate is collected. Liquid-liquid extraction may also be carried out in separating funnels, for instance pentane against methanol-water. Since most mycotoxins are not lipophylic, fats can be removed in this way without loss of toxin.

In some analysis procedures, precipitating reagents are used. Examples are lead acetate and fresh ferric hydroxide gel to precipitate gossypol pigments in extracts of cottonseed (ref. 8, ref. 9), cupric carbonate to remove chlorophyll (ref. 10), and silver nitrate to remove alkaloids from cocoa extracts (ref. 11).

The above mentioned clean-up techniques are in fact separation procedures in which groups of substances with certain physicochemical properties can be separated from one another. In this way the greater part of the co-extracted material can be removed. The choice of the clean-up procedure may depend on the method used for detection and determination, the required limit of detection, the speed of analysis and the recovery.

Extracts that have been cleaned-up are usually concentrated by evaporating the solvent in a rotary evaporator under reduced pressure, or by using a steam bath, while keeping the extract under a stream of nitrogen. The residue is redissolved in a small volume of solvent, quantitatively transferred to a small vial and brought to a specified volume.

Depending on the toxin and the ultimate separation and detection step to be used, derivatization of the mycotoxin of interest may be necessary to make it measurable or to optimalize its chromatographic behaviour.

Ultimate separation, detection and quantitation

Despite extraction and clean-up, the final extract may contain other co-extracted substances possibly interfering with mycotoxin determination. Several possibilities exist to separate the mycotoxin(s) from the matrix-components in order to allow qualitative and quantitative determination. Chromatographic procedures, which are physical separation techniques, are most often applied and they are used in combination with visual or instrumental determination of the mycotoxin(s) of interest. Immuno-assays which are bio-chemical separation techniques, used in combination with instrumental determination are still in an early stage of development for mycotoxin research. Nevertheless, the latter techniques are promising and it is to be expected that these become of increasing importance.

Chromatographic procedures

In the determination of mycotoxins adsorption chromatography and partition chromatography are the most important types of chromatography. Normally, the phenomenon on which the separation is based is a combination of both types which can be sub-divided into:

a) open column chromatography
b) thin layer chromatography
c) high performance liquid chromatography
d) gas liquid chromatography.

a) Open column chromatography. Open column chromatography has been mentioned already as a technique often used in clean-up procedures. A special design - the glass minicolumn with an internal diameter of ca. 5 mm - can be used for the ultimate separation and detection of some mycotoxins in certain commodities. In the test procedure according to Romer (ref. 12) the minicolumn is packed with successive zones of adsorbents such as alumina, silica gel and Florisil® with calcium sulphate drier at both ends and held in place with glass wool (Fig. 3). A chloroform extract is applied on the top of the column, and drained by gravity. Then, descending chromatography with a mixture of chloroform and acetone is applied, trapping the aflatoxins as a tight band at the top of the Florisil® layer, where they can be detected by their blue fluorescence under U.V.light (Fig. 4). By comparing a sample column with a column containing a known amount of aflatoxins, it is possible to judge whether the sample contains more or less aflatoxins than the standard. Contrary to thin layer chromatographic techniques, the minicolumn method of Romer (ref. 12) does not distinguish between the different aflatoxins.

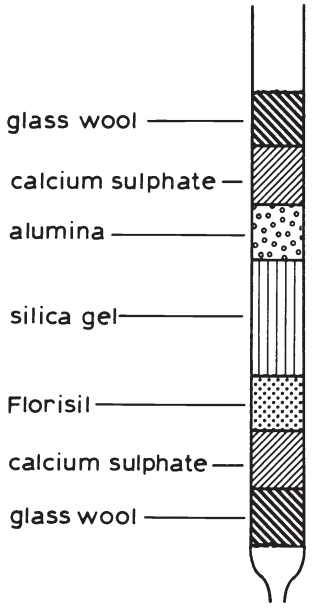


Fig. 3. Packing of a minicolumn according to Romer (ref. 12)

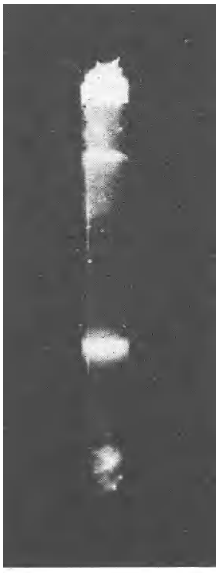


Fig. 4. Adsorption of aflatoxin B_1 on the Florisil® layer of a minicolumn according to Romer (ref. 12)

As well as for the aflatoxins, similar minicolumn procedures have been developed for some other mycotoxins that fluoresce when irradiated with U.V.light, such as ochratoxin A, in a wide range of products (ref. 13) and zearalenone in maize, wheat and sorghum (ref. 14).

Minicolumn methods are "go-no go" methods, which require only little time and expertise and no sophisticated equipment. This makes them particularly useful for field screening tests. However, they are at best semi-quantitative and generally have a higher limit of detection and less sensitivity, separation power and selectivity than is obtained by using thin layer chromatographic and high performance liquid chromatographic procedures.

b) Thin layer chromatography. In the first years of mycotoxin research thin layer chromatography (TLC) became a very common and popular technique to separate extract components and nowadays there are still numerous applications. Initially separations were carried out in one dimension using a single developing solvent. Later two-dimensional TLC was introduced to mycotoxin research (ref. 15). It is a powerful separation technique in which a second development is carried out in a direction at right angles to the first one, using a different developing solvent. This provides a much better separation than one-dimensional TLC and is required especially in those cases where low levels have to be detected, e.g. aflatoxin M_1 in milk, and if extracts contain many interfering substances e.g. feedingstuffs and roasted peanuts.

In thin layer chromatography a wide range of adsorbents and formats can be used. For mycotoxin assays silica gel TLC plates are most often used, as this type of adsorbent generally offers the best possibility of separating the toxin of interest from matrix components. Both pre-coated and self-coated plates can be used.

Thin layer plates can be used in different formats. Most separation problems may be resolved using a square 20x20 cm plate; however the use of 10x10 cm plates and even 7x7 cm self-cut plates will often lead to good results as well. Especially for two-dimensional separation procedures, the use of the smaller sizes saves much time. Examples of analytical procedures in which two-dimensional separations are carried out on small TLC plates are the multi-mycotoxin method of Patterson (ref. 16), the method of Van Egmond for the determination of sterigmatocystin in cheese (ref. 17), the method of Stubblefield for the determination of aflatoxins in animal tissue (ref. 18) and the method of Paulsch for the determination of ochratoxin A in pig kidneys (ref. 19). In two-dimensional TLC the sample extract is spotted at a corner of the TLC plate and two developments are carried out successively parallel to the two sides of the plate using two different developing solvents. The two solvents must be compatible and independent, i.e. there should be little correlation between the retention patterns in both systems, otherwise the spots tend to agglomerate along the bisector of the plate.

An example of the use of two-dimensional TLC is the procedure used in the official EC-method (European Common Market) for the determination of aflatoxin B_1 in feedingstuffs (ref. 20) (Fig. 5): An aliquot of extract is spotted at A and known amounts of aflatoxin B_1 standard are spotted at B. The plate is then developed in the first direction with a mixture of diethyl ether, methanol and water (94+4.5+1.5) and, after drying, the plate is rotated 90° and developed in the second direction with a mixture of chloroform and acetone (9+1). Detection and quantification is carried out under longwave U.V.light (365 nm). In Fig. 6 the result of a two-dimensional TLC separation of an extract of peanut butter contaminated with aflatoxins B_1, B_2, G_1 and G_2 is shown. With the help of the co-developed B_1 standards, the well-separated B_1 spot from the sample can be located. By means of a densitometer the intensities of fluorescence of the B_1 spot from sample and standard can be compared and thus the B_1 concentration in the initial sample can be calculated.

The fortunate characteristic that aflatoxins emit the energy of absorbed longwave U.V.light as fluorescence light enables the analyst to detect these compounds at low levels. Unfortunately not all mycotoxins can be detected by such a simple method. Many do not fluoresce under U.V.light, some show U.V. or visible light absorption, while others do not. If the latter is the case, sometimes the mycotoxin can be made visible by spraying a reagent on the plate, by exposing the plate to reagent vapour or by inpregnating the plate with reagent-solution. An example of such a derivatization is the spraying technique used for the visualization of sterigmatocystin, a toxin sometimes occurring in grains and in cheese. Stack (ref. 21) has found that spraying with an $AlCl_3$-solution followed by heating leads to an Al-complex with the keto- and hydroxyl groups of the sterigmatocystin molecule (Fig. 1), resulting in an enhancement of the fluorescence intensity of ca. 100 times. In addition, the colour of fluorescence changes from brick-red to yellow. Another application of $AlCl_3$ reagent is included in the procedure for the determination of deoxynivalenol (DON) (Fig. 1), where use is made of $AlCl_3$ impregnated silica gel plates (ref. 22). After heating the developed TLC plate, DON appears as a blue fluorescent spot under longwave U.V.light.

In spite of all the clean-up techniques used, there may be still substances which behave in the same manner during TLC separation as the mycotoxins being determined. In order to

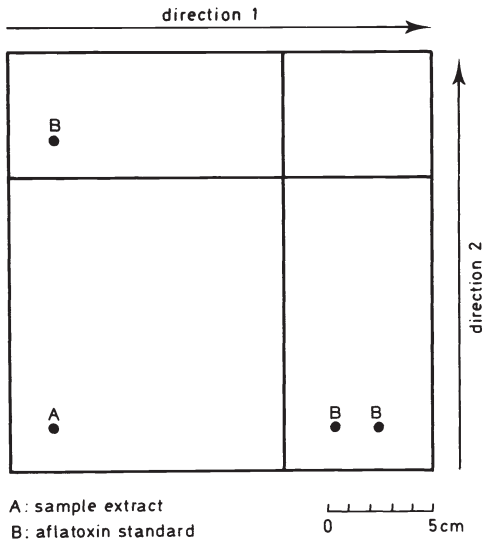


Fig. 5. Spotting pattern for two-dimensional TLC

Fig. 6. Separation of an extract of peanut butter submitted to two-dimensional TLC (level of contamination ca. 5 µg aflatoxin B_1/kg; B_2, G_1 and G_2 also present)

minimize the risk of false-positives, the identity of the mycotoxin in positive samples has to be confirmed. The most reliable method for this purpose is high resolution mass spectrometry (HRMS). HRMS in combination with TLC however is rather time-consuming and not every laboratory is equipped with HRMS apparatus. Therefore more simple techniques have to be applied. Probably the simplest way of confirming the presence of mycotoxins are the use of additional solvents systems, or the application of supplemental chromatography by repeating the TLC procedure, but this time with addition of the suspected mycotoxin as internal standard, superimposed on the extract spot before developing the plate. After completion of TLC, this superimposed standard and the "presumed" toxin spot from the sample must coincide. Another possibility is to spray the developed TLC plate with a reagent, so that the colour (of fluorescence) of the mycotoxin spot changes. An example of the latter possibility is the spraying test with a dilute solution of sulphuric acid (ref. 23), which leads to a change in the colour of fluorescence of aflatoxin spots from blue to yellow. Although the above described tests, if negative, would rule out the presence of the mycotoxin concerned, they do not provide positive confirmatory evidence.

Positive identification can be obtained by formation of specific derivatives with altered chromatographic properties. Both mycotoxin standard and suspected sample are submitted to the same derivatization reaction. Consequently, in positive samples a derivative from the mycotoxin should appear, identical to the derivative from the mycotoxin standard. Confirmatory reactions may be carried out in test tubes, or, preferably, directly on a TLC plate, thus using the separation power of TLC. An example of the latter technique is the confirmation procedure adopted in the official EC-method for the determination of aflatoxin in feedingstuffs and originally published by Verhülsdonk (ref. 24). In this procedure a so-called separation-reaction-separation procedure is carried out (Fig. 7). Hydrochloric acid is sprayed after the first separation run, the reaction takes place. Then a second separation is carried out in the second direction, under identical conditions, after which the isolated blue fluorescent spot of aflatoxin B_{2a} is visible, which can be recognized with the help of a B_1 standard, spotted on the same plate, which has undergone the same procedure. Other (unreacted) components lie on a diagonal line, bisecting the plate, as the separation was carried out in both directions under exactly identical conditions. In Fig. 8 the result of such a confirmatory test applied to feedingstuff contaminated with aflatoxins B_1 and G_1 is shown.

The described technique for confirmation of aflatoxin B_1 is also applicable to aflatoxin G_1 however not to aflatoxins B_2 and G_2 of which the terminal furan ring is saturated. In addition an in situ confirmatory test has been developed for aflatoxin M_1 by Trucksess (ref. 25). In this procedure a reaction is carried out between trifluoroacetic acid and aflatoxin M_1 on the origin spot of a TLC plate, after which the TLC plate is developed.

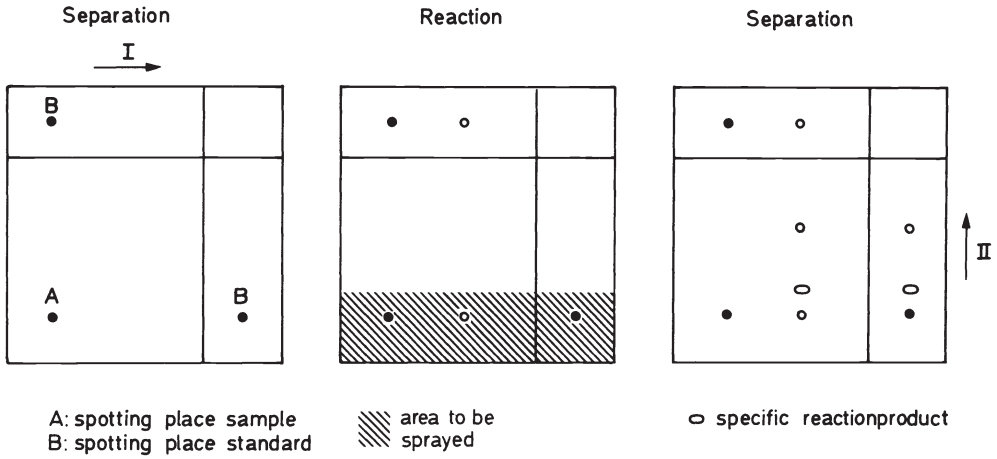


Fig. 7. Schematic representation of the two-dimensional TLC confirmatory test of Verhülsdonk (ref. 24)

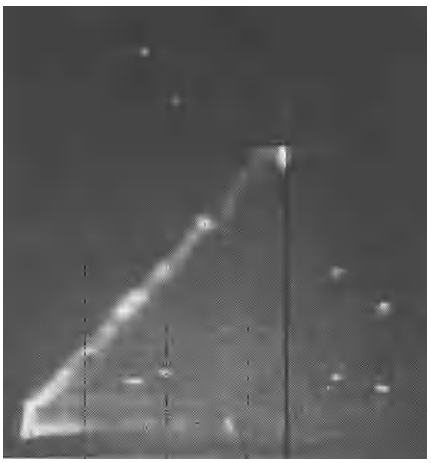


Fig. 8. Result of the TLC confirmatory test of Verhülsdonk (ref. 24), applied to feeding-stuff, contaminated with aflatoxins B_1 and G_1

In situ derivatization procedures on TLC plates followed by TLC of the reaction product(s) to establish the identity of mycotoxins other than aflatoxins are rather scarce. Van Egmond (ref. 17) described a test for the confirmation of identity of sterigmatocystin (see Fig. 1) in cheese extracts. In the test, which is based on the principles of the separation-reaction-separation procedure (see Fig. 7), a mixture of trifluoroacetic acid and benzene is sprayed on the TLC plate. After the reaction and a second development the reaction product is visualized with $AlCl_3$ spray reagent. Paulsch (ref. 19) developed a confirmatory test for ochratoxin A (see Fig. 1) in pig kidneys, based on the findings of Kleinau (ref. 26). Ochratoxin A spots separated after two-dimensional TLC are esterified on the TLC plate with methanol-H_2SO_4 after which the plate is developed for the third time. The methyl ester of ochratoxin A is visible under longwave U.V.light as a fluorescent spot with an R_f value higher than that of ochratoxin A. Just as for ochratoxin A, the methyl ester undergoes the same change in colour of fluorescence from green to blue when the pH of the plate is changed from acid to alkaline by exposing the plate to the vapour of ammonia and this phenomenon can be considered as an additional confirmation of identity.

The use of thin layer chromatography as a technique to separate mycotoxins from matrix components has decreased in recent years in favour of high performance liquid chromatography and to a lesser extent of gas liquid chromatography, a technique of particular value for the determination of trichothecenes. Further analytical developments may be expected in the near future from immuno-assays. Nevertheless thin layer chromatography is a major separation

technique in mycotoxin research, and is particularly recommended to those, who cannot afford to purchase sophisticated analytical instrumentation.

c) High performance liquid chromatography. High Performance Liquid Chromatography (HPLC), or High Pressure Liquid Chromatography as it was initially called, became available for the analysis of foodstuffs in the early seventies and the technique became of rapidly growing importance in the determination of mycotoxins, particularly when several types of column packings and (fluorescence-) detectors became available. The introduction of autosamplers and computerized data retrieval systems made HPLC very useful for large scale analyses.

In the first applications which concerned aflatoxin assays, SiO_2 columns were used in combination with chloroform or dichloro methane containing mobile phases, and detection was by means of U.V.detectors (λ = 254 nm or 265 nm). U.V.detection however is not very selective and also the limits of detection for aflatoxins are much higher than those obtained with TLC. Therefore the use of U.V.detectors for aflatoxin assays was largely discontinued when the more selective fluorescence detectors became available. Initially, the fluorescence detectors had limitations in detectability as well, because aflatoxins B_1 and B_2 do not exhibit strong fluorescence in normal phase solvents and B_1 and G_1 do not exhibit strong fluorescence in reverse phase solvents. Consequently the limits of detection for the mentioned aflatoxins could not compete with those obtained in thin layer chromatography. The following techniques have been introduced to enhance the intensity of fluorescence of the aflatoxins:

- Packing the flow cell for normal phase systems with silica gel (ref. 27, ref. 28). In the adsorbed state, the aflatoxins B_1 and B_2 fluoresce much more intensively than they do in solution.
- The use of a mobile phase which prevents the usual quenching of aflatoxins B_1 and B_2 (ref. 29). A solvent consisting of a mixture of toluene, ethyl acetate, formic acid and methanol has shown to be appropriate.
- Pre-column derivatization by treating extract and standard solutions with trifluoroacetic acid to convert aflatoxins B_1 and G_1 to the respective hemiacetals, B_{2a} and G_{2a} (ref. 30, ref. 31). The hemiacetals fluoresce as strongly as B_2 and G_2 in reverse phase solvents.
- Post-column derivatization with iodine after reverse phase chromatography (ref. 32). The iodine addition enhances the fluorescence of aflatoxins B_1 and G_1 approximately 50 fold without affecting the fluorescence of aflatoxins B_2 and G_2. An advantage of this procedure is that the derivative is formed from an already separated aflatoxin peak, which means that the reaction conditions for sample and standard are the same and the occurrence of multiple reaction products is not important as long as the reaction is repeatable. Additional advantages are the need to derivatize only the portion of the sample to be injected into the liquid chromatograph and the ability to make sequential injections into the liquid chromatograph with and without post-column reagent addition, which confirms the presence or absence of aflatoxins B_1 and G_1. The procedure has proven to be successful for the analysis of samples of maize and peanut butter (contaminated at levels ranging from 0.5 - 2.0 µg/kg) and recently also methods have been developed which allow the determination of aflatoxins in citrus-containing mixed feedingstuffs at a level of ca. 1 µg/kg (ref. 33, ref. 34). (Citruspulp is a popular ingredient in feedingstuffs in Europe and known to lead to strong interferences in many TLC and HPLC procedures). Figure 9 presents a reverse phase HPLC chromatogram as obtained from an extract of feedingstuff containing citruspulp, purified by combined Florisil®/C_{18} SEP-PAK® cartridge clean-up, with application of I_2 post-column derivatization.

HPLC methods have also become available for the analysis of milk and milk products for aflatoxin M_1. Most of these methods use reverse phase HPLC, which does not lead to problems in detectability. Aflatoxin M_1 fluoresces much more intensively in reverse phase solvents than aflatoxin B_1, so that no special provisions or derivatizations are necessary. The limits of detection obtained are comparable with those obtained in (two-dimensional) thin layer chromatography and some of these methods are widely used already in surveillance and monitoring programmes. An example of an excellent HPLC separation of an extract of milk powder, prepared according to the method of Stubblefield (ref. 35), is shown in Fig. 10. In addition to the aflatoxins, HPLC separation procedures have been developed for other mycotoxins. In most of these procedures, U.V.detection is applied, however for various mycotoxins (i.e. ochratoxin A, zearalenone, some ergot alkaloids, and some Alternaria toxins) fluorescence detectors have shown to be useful. It is inappropriate to review here all existing procedures that have been published and interested persons are referred to a comprehensive review, with many technical details, prepared by Scott (ref. 37).

High performance liquid chromatography has partly superceded thin layer chromatography in the analysis of food for mycotoxins. The reasons for this development are obvious. Separations are usually much better than those obtained with one dimensional TLC, HPLC methods generally provide good quantitative information and the equipment employed in HPLC systems can be automated rather easily. Finally, on-line coupling with a mass spectrometer has become possible, although it is too early to estimate the value of the latter technique for the determination and confirmation of mycotoxins.

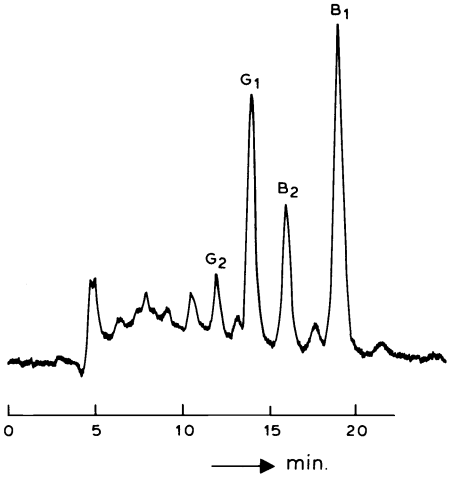


Fig. 9. C_{18} reverse phase HPLC chromatogram of an extract of feedingstuff containing citruspulp, contaminated with aflatoxins B_1, B_2, G_1 and G_2 at ca. 12, 3, 8 and 1 µg/kg respectively, prepared according to the method of Van Egmond (ref. 34)

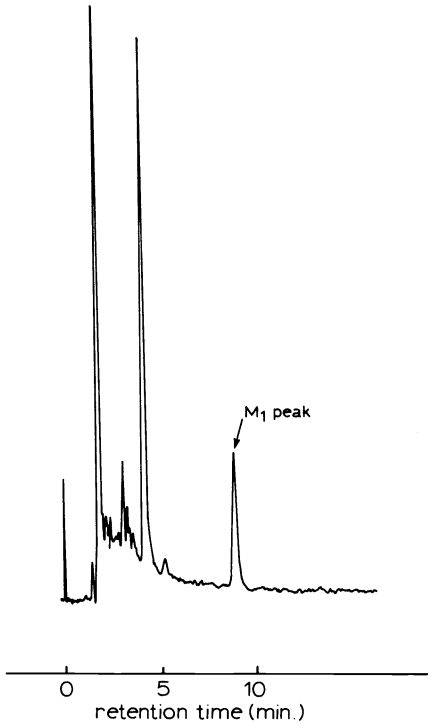


Fig. 10. C_{18} reverse phase HPLC chromatogram of an extract of milk powder, containing ca. 0.4 µg M_1/kg, prepared according to the method of Stubblefield (ref. 35) by Mulders (ref. 36)

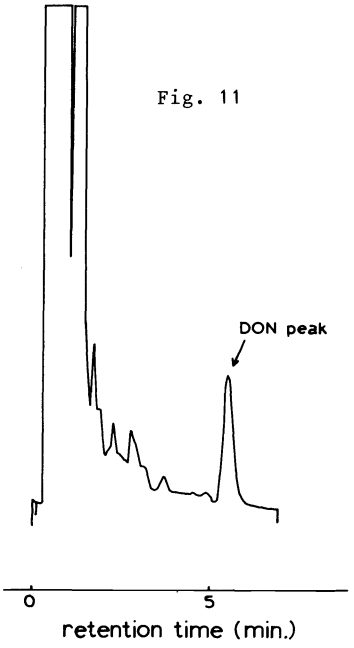
HPLC has limitations as well. The cost of equipment for HPLC is much higher than that for TLC (except when densitometers are used). The extensive experience required to obtain the maximum benefit from an HPLC system constitutes another limitation, whereas TLC can be learned relatively easily. The few published studies in which HPLC has been compared directly with TLC for the determination of aflatoxins in peanuts (ref. 38) and, maize and peanuts (ref. 39) have indicated that both techniques provide results that agree rather well. In addition a collaborative study of 2 HPLC methods for aflatoxins in peanut butter has shown improved limits of detection and better within laboratory precision over current AOAC official TLC methods, but the between laboratory precision was not better (ref. 40). The result of this study was that neither method was submitted for adoption by the Association of Official Analytical Chemists.

In conclusion, it should not be taken for granted that the application of HPLC is always better than TLC in mycotoxin assays. It is the analyst's duty to find out which technique provides the best results in view of his objectives, and not to choose haphazardly a sophisticated technique for sophistication's sake.

d) Gas liquid chromatography. The use of gas liquid chromatography (GLC) in the analysis for mycotoxins has been limited, as most of the mycotoxins are not volatile and must therefore be derivatized before they can be gas chromatographed. In addition, the fact that many of the mycotoxins are readily detected and determined at low levels of concentration using TLC and HPLC techniques, as discussed in the foregoing sections, has not stimulated the development of gas chromatographic assays.

Although some gas chromatographic methods have become available in the 1970's for the detection and determination of patulin (see Fig. 1) in apple juice, penicillic acid in maize and in rice and zearalenone in maize, the only significant advantage over TLC and HPLC techniques is the potentional use of mass spectrometers as highly selective and sensitive detectors. However, this situation is quite different for one important group of the mycotoxins, the trichothecenes (see Fig. 1). Determination of the trichothecenes by TLC and HPLC is difficult due to the fact that these compounds have no fluorescent properties, nor do trichothecenes absorb appreciably in the ultraviolet range. Although TLC methods have been developed using visualization reagents, the detection limits are relatively high when compared with GLC.

GLC permits the detection and quantitation of most of the more common trichothecenes. Trichothecenes can be gas chromatographed as their trimethylsilyl- (TMS) or heptafluorobutyryl- (HFB) derivatives, using non-polar stationary phases such as methylsilicones (SE-30, OV-1), methyltrifluoropropylsilicone (QF-1) or methylphenylsilicone (OV-17), whereas detection relies upon flame ionisation detectors (FID) and electron capture detectors (ECD). The ECD detector is more sensitive and is to be preferred. In Fig. 11 a gas chromatogram is shown of an extract of maize prepared according to the modified procedure of Scott (ref. 41), and analyzed for deoxynivalenol (DON). A packed column has been used in combination with ECD detection of the tris-heptafluorobutyrate of DON.



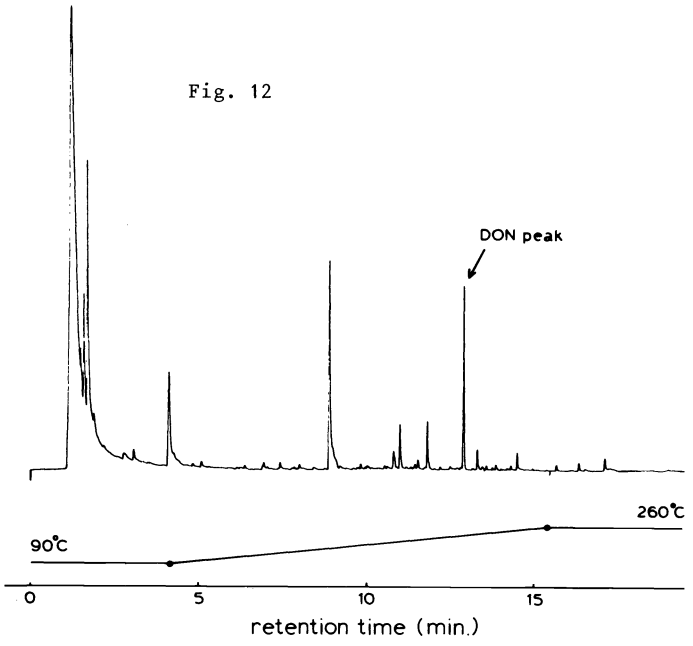


Fig. 11. GLC chromatogram (3% OV-3) of an extract of maize, containing ca. 60 µg deoxynivalenol/kg, prepared according to the modified method of Scott (ref. 41) by Besling (ref. 42)

Fig. 12. Capillary GLC chromatogram (0.12 µm CP® Sil 5CB) of an extract of wheat bran, containing ca. 1.5 mg deoxynivalenol/kg, prepared according to the method of Scott (ref. 40) by Tuinstra (ref. 43)

A newer development in GLC, the use of capillary columns in food analysis, has prompted the development of capillary GLC methods for trichothecenes. Figure 12 presents a gas chromatogram of an extract of wheat bran, prepared according to the procedure of Scott (ref. 41), and analyzed for deoxynivalenol. A fused silica capillary column has been used in combination with ECD detection of the derivative of DON.

In addition to the above mentioned detection systems it is also possible to couple the gas chromatograph directly to a mass spectrometer (MS). When operating in the selected ion monitoring mode, the mass spectrometer is a very selective and sensitive detector, which can be used for quantitative assays of commodities for mycotoxins. GLC-MS employing selected ion monitoring has been reported not only for the determination of trichothecenes, but incidentally for several other mycotoxins as well (ref. 3). However, the practical use of this sophisticated system is limited to those laboratories who can afford to purchase these expensive computer-controlled systems.

Immunochemical procedures

For the determination of mycotoxins, the use of immuno-assays has been limited to date to radio-immuno-assay (RIA) and enzyme-linked immuno-sorbent assay (ELISA), although in principle other labels than radio-isotopes or enzymes could be used. The application of immunochemical procedures is discussed intensively in a separate paper by Professor Chu, therefore details of the techniques will not be given here.

The application of immuno-assays for the determination of mycotoxins is a recent development so that collaborative studies on this subject have not yet been conducted (1985). The present lack of analytical parameters derived from collaborative studies makes it difficult to estimate the value of these new techniques compared to the conventional chromatographic procedures. Incidentally ELISA has been applied to the analysis of samples provided in the 1980 Aflatoxin Check Sample Survey Programme, sponsored by the International Agency for Research on Cancer in Lyon. It was reported (ref. 44) that the results of ELISA, applied to the analysis of samples of peanut meal, deoiled peanut meal and yellow corn meal (naturally contaminated with aflatoxins at estimated levels of ca. 210, 110 and 55 µg/kg respectively) were in excellent agreement with the overall mean values obtained by other collaborators using TLC and HPLC, which offers hopeful perspectives for wider use of ELISA.

RIA and ELISA have been compared in an intra-laboratory study for the determination of aflatoxin B_1 in maize, wheat and peanut butter, aflatoxin M_1 in milk (ref. 45) and T-2 toxin in maize and wheat (ref. 46). It was concluded that ELISA was the preferred method for aflatoxin B_1, because of more consistent data. ELISA was also the preferred method for aflatoxin M_1, mainly because of its simplicity, sensitivity and selectivity. For T-2 toxin both RIA and ELISA seemed to be adequate when maize and wheat were analyzed.

A major advantage of both RIA and ELISA is the possibility of complete automation, making the techniques very valuable as rapid quantitative (screening) procedures. Taking into account the disadvantages of RIA such as the limited shelf-life activity of the radioisotopes, problems of radioactive waste disposal or licensing requirements, it is to be expected that especially ELISA will be of growing importance as an assay technique for mycotoxins.

CONCLUSION

The present state of methodology for determination of mycotoxins in food may be summarized as follows:

- Bio-assays may be useful in tracing sources of known and unknown mycotoxins. However, their use in the surveillance of food and foodstuffs for mycotoxins is of minor importance.
- Chemical assays are of major importance in the determination of mycotoxins. Most widely used are those techniques which include a chromatographic step to separate the mycotoxin of interest from matrix components.
- Minicolumn chromatographic procedures are useful as screening tests for agricultural commodities if quick decisions are needed as to whether accept or reject a lot. They have been developed mainly for aflatoxins.
- Thin layer chromatography, although a veteran in mycotoxin methodology, remains a reliable, practical and relatively simple separation technique with a broad field of application. Its two-dimensional application offers especially good resolution, resulting in low limits of detection.
- High performance liquid chromatography can be an attractive alternative to thin layer chromatography, because it offers the possibility of automating the ultimate separation and quantification steps. However, this more expensive technique has not shown to lead to better results in mycotoxin assays than thin layer chromatography.
- The use of gas liquid chromatography is limited mainly to the analysis of commodities for trichothecenes. Thanks to technical developments gas liquid chromatography has become a powerful technique in determining toxins of this group. Gas liquid chromatography-mass spectrometry with selected ion monitoring is currently the peak of methodological sophistication and can be used to detect and confirm the presence of mycotoxins in the µg/kg range with a high degree of certainty.
- Immuno-assays such as radio-immuno-assay and enzyme-linked immuno-sorbent assay are promising techniques. Although still in their infancy, it is to be expected that these techniques will play an important role in mycotoxin methodology in the near future.

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