Improved Method for Confirmation of Identity of Aflatoxins B₁ and M₁ in Dairy Products and Animal Tissue Extracts

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A method is described for confirming the identity of aflatoxins B₁ and M₁ in dairy products and liver extracts on a thin layer plate. Extracts and standards containing aflatoxins B₁ and M₁ are spotted on 10 × 10 cm plates, which are developed 2-dimensionally in mixtures of isopropanol-acetone-chloroform. After the first development, trifluoroacetic acid-hexane (1 + 4) is sprayed on that part of the plate containing the separated extract components and the undeveloped standard spots of B₁ and M₁, and the plate is heated 6-8 min at 75°C. Then the plate is developed in a second direction, and the reaction products of B₁ and M₁ with trifluoroacetic acid from the extract are compared with the same derivatives of the respective standards. The method has been used successfully on extracts of milk, cheese, and liver containing 0.1 ng B₁ or M₁/g and can be completed in 35-45 min.

Several articles have been published recently about confirmation of identity of aflatoxins with trifluoroacetic acid (TFA) or hydrochloric acid (HCl) (1-5) directly on TLC plates. In some procedures, the reaction is carried out on the extract-spot before development (1-3); in other methods, the reaction is carried out after one (4) and two (5) developments. Either technique permits confirmation of low concentrations of aflatoxins in extracts containing fluorescent impurities. The 2-dimensional HCl spraying technique of Verhúlsdonk et al. (4), which has been tested collaboratively (4), is a reliable technique for confirmation of aflatoxins B₁ and G₁, but it is not applicable for confirmation of aflatoxin M₁. The Dutch modification of the Truckess method (2, 5) for the confirmation of aflatoxin M₁ in dairy products (6) was tested collaboratively and adopted by the AOAC and IUPAC. Although only 12 false negative results were reported for 212 samples, several collaborators encountered difficulties in spotting, zone diffusion, incomplete derivatization, or excessive time consumption. To overcome these problems, the TFA spotting test was modified to a TFA-hexane spraying test. The latter is based on the principles of the HCl spraying test of Verhúlsdonk et al. (4) for confirmation of aflatoxins B₁ and G₁ and the TFA-benzene (1 + 4) spraying test of van Egmond et al. (7) for confirmation of sterigmatocystin. The proposed method will detect aflatoxins in dairy and meat products at concentrations of 0.1 ng B₁ and M₁/g.

METHOD

Reagents
(a) Solvents.—Reagent grade CHCl₃ (0.75% ethanol), acetone, isopropanol, and hexane.
(b) TFA-hexane spray.—Mix trifluoroacetic acid (TFA, ≥95% pure) with hexane to give ratio of (1 + 4). Prepare fresh daily.
(c) Aflatoxin standard solutions.—Prepare separate standard solutions of aflatoxins B₁ and M₁ (0.25 µg/mL each in acetonitrile-benzene (1 + 9) or chloroform).
(d) TLC plates.—0.25 mm thick layer of silica gel (Macherey and Nagel Sil-G-25HR (Duren, GFR) or Merck Kieselgel 60 (Darmstadt, GFR)) on 10 × 10 cm plates, cut from 20 × 20 cm plates.

Apparatus
(a) UV illumination cabinet.—365 nm.
(b) Disposable capillary pipets.—10 and 20 µL, or microsyringes.
(c) Spray unit for thin layer chromatography.—Low volume capacity (5-20 mL).
(d) Air dryer.—Unit capable of providing stream of warm air (40-50°C) to evaporate solvent from TLC plates.

Thin Layer Chromatography
Score 2 straight lines on 10 × 10 cm TLC plate at right angles (3 cm in from each side) (see Figure 1) to limit migration of developing solvent fronts. Spot following solutions on plate, using capillary pipets or microsyringes:
(a) Volume of sample extract equal to volume used for quantitation on point A (normally ca 20 µL).
(b) Volume of standard solution containing ca 2.5 ng M₁ on points B and D.
(c) Volume of standard solution containing ca 2.5 ng M₁ on points C and E (only for animal tissue extracts).
Develop plate in first direction with isopropanol-
Results and Discussion

The described procedure has been successfully used for confirmation of identity of aflatoxins in milk, milk powder, Gouda cheese, and Cheddar cheese. The latter is based on spraying test of Van Egmund et al. (5) if the latter method is carried out properly.

However, from the collaborative study in which this method was investigated (6), it was concluded that the TFA-spotting technique did not always give satisfactory results because diffusion of aflatoxins and poor yields of TFA-reaction product were obtained. Both problems lead to difficulties in interpretation of the chromatogram. Application of the proposed TFA-hexane test produces easily detectable fluorescent zones. Hexane was chosen as the diluting solvent for TFA because it evaporates quickly and does not cause zone diffusion. The test was designed to be carried out with 10 X 10 cm TLC plates, since they develop faster and are cheaper than the normal 20 X 20 cm plates.

For development in the first direction, a TLC solvent was chosen that yields high Rf values for B1 and M1. Consequently, several fluorescent impurities are separated from the aflatoxins. Fluorescent impurities that have approximately the same Rf values as B1 and M1 are separated in...
the second direction, because the TFA-reaction products of B₁ and M₁ have relatively low \( R_f \) values. This makes interpretation of the chromatogram easy, because the M₁ and B₁ derivatives from the sample are, in most cases, well isolated from other fluorescent spots. The polarity of the second solvent was increased to produce higher \( R_f \) values for the B₁ and M₁ reaction products and better differentiate these zones from impurities. It should be noted that the composition of the developing solvents for first as well as second direction were based on types of TLC plates and solvents obtainable in the United States. Since the AOAC-IUPAC collaborative study on aflatoxin M₁ in milk products (6), the authors realize that the ethanol content of chloroform, which influences the polarity of the developing solvent, may differ considerably from one country to another. The same study (6) also showed that some types of TLC plates are not available everywhere, which may mean another source of variability in results. Therefore, the specified compositions of the developing solvents should be checked in the laboratory and adjusted to obtain \( R_f \) values of aflatoxins B₁ and M₁ in the first direction of ca 0.8 and 0.5, respectively, and of ca 0.4 and 0.2 for their respective derivatives in the second direction. The authors noticed that the composition of liver extracts may differ considerably with the geographical region; sometimes interferences occur with the M₁ derivative, if present in low amounts. In these cases, increasing the polarity of the developing solvents may result in better resolution of the M₁ derivative from the interferences; however, it may hamper the simultaneous confirmation of B₁ and M₁ if the B₁ derivative gives too high an \( R_f \) value. Then identities of B₁ and M₁ have to be confirmed separately.

The conversion of B₁ and M₁ to their respective reaction products is not complete. Occasionally, co-reaction products can be seen at \( R_f \) values comparable to those of B₁ and M₁. Several conditions are incorporated in the method that produced optimum conversion of B₁ and M₁. It is important that the first developing solvent be completely evaporated from the plate before the TFA-hexane is applied. Probably isopropanol or water in the isopropanol prevents total conversion. The volume of TFA-hexane sprayed on the plate is important but is difficult to describe or visualize because the hexane evaporates quickly. Generally, 2-3 mL TFA-hexane solution is sufficient for one 10 x 10 cm plate. Conversion was optimal when the plate was covered with a warm glass plate and placed on the oven floor immediately after spraying, so that the critical zones warmed quickly. Ready-made plates are preferred over self-made plates because B₁ and M₁ conversion to their respective derivatives is greater.

The procedure described above is also applicable for aflatoxin G₁ and requires only 35-45 min to confirm a sample for the presence of B₁, G₁, and/or M₁.

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**REFERENCES**