NOTES

Detection of Mycotoxins by Thin-Layer Chromatography: Application to Screening of Fungal Extracts

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A convenient thin-layer chromatographic screening procedure for the detection of 18 mycotoxins is described.

Examination in our laboratory of fungi isolated from foods and feeds for their ability to produce aflatoxins (15) has been expanded to include other mycotoxins. This has been made possible by thin-layer chromatography (TLC) with suitable general solvent systems and only one initial spray reagent. We can thus detect the following toxins: aflatoxins B₁, B₂, G₁, and G₂ (3); ochratoxin A (14); asperthoxin (8); luteoskyrin (13); zearalenone [F-2 (6)]; 4-acetamido-4-hydroxy-2-butenoic acid y-lactone (18); diacetoxyscirpenol (1) and its 8-(3-methylbutyryloxy) derivative [T-2 toxin (5)]; and nivalenol and its 4-O-acetate (11), in addition to several antibiotics now regarded as mycotoxins, namely gliotoxin, citrinin, patulin, penicillic acid, and sterigmatocystin (4). These mycotoxins are produced mainly by species of Aspergillus, Penicillium, or Fusarium but are not necessarily restricted to any one species or genus.

TLC was carried out in subdued light. Thin layers (0.3 mm) of Adsorbosil 5 silica gel (Applied Science Laboratories, Inc., State College, Pa.) were activated at 110°C for 2 hr. Five-microliter amounts of standard solutions of each toxin in an appropriate organic solvent were spotted and developed for a distance of 15 cm in the following solvent systems, with normal saturation: toluene-ethyl acetate-90% formic acid (6:3:1; TEF) and benzene-methanol-acetic acid (24:2:1; BMA). Toxins were visualized in visible or ultraviolet light (Chromato-Vue cabinet, Ultra-Violet Products, Inc., San Gabriel, Calif.), before and after spraying the plate with a freshly prepared mixture of 0.5 ml of p-anisaldehyde in 85 ml of methanol containing 10 ml of glacial acetic acid and 5 ml of concentrated sulfuric acid (16) and then heating at 130°C for 8 to 20 min. The shorter heating time was better for fluorescence development. Typical RF values and colors observed are shown in Table 1.

The advantage of the acidic solvent systems is that all of the toxins migrate, although nivalenol stays close to the origin and citrinin and luteoskyrin streak. Fluorescence colors of certain mycotoxins in ultraviolet light are well known (10). The anisaldehyde spray allows detection of the nonfluorescent toxins. After the spray treatment, the blue fluorescence of T-2 toxin and penicillic acid in longwave ultraviolet light permits detection of less than 0.2 and 0.01 μg, respectively, on the TLC plate. These limits are much lower than those previously reported (9, 17). Detection limits for nivalenol, nivalenol 4-O-acetate, diacetoxyscirpenol, 4-acetamido-4-hydroxy-2-butenoic acid γ-lactone, and gliotoxin are of the order of 0.2 μg. Replacement of methanol in the spray reagent by ethanol improves the detection of patulin, which then forms a reddish spot (detection limit, 0.1 μg).

Once the order of migration and detection colors are familiar, standard toxins that separate in each of the solvent systems can be mixed and spotted together. This is commonly done with the four aflatoxins.

With the foregoing detection procedure, we evaluated our semimicro culture technique (15)
### Table 1. Thin-layer chromatography of mycotoxins

| Mycotoxin          | Concentration (μg/ml) | RF | Solvent system | Visible light | Ultraviolet light | Color after spray treatment | Alternative spray reagent |
|--------------------|-----------------------|----|----------------|---------------|-------------------|-----------------------------|--------------------------|
|                    |                       |    | TEF | BMA          |                 | Longwave | Shortwave         | Visible light | Longwave ultraviolet light |
| Citrinin           | 10                    |    | 0.16-0.48<sup>b</sup> | 0.0-0.20<sup>b</sup> | Yellow<sup>c</sup> | Yellow | Yellow-green       | Orange | Yellow<sup>e</sup> |
| Luteoskyrin        | 1,000                 |    | 0-0.47<sup>b</sup> | 0.0-0.23<sup>b</sup> | Yellow<sup>c</sup> | Yellow | Orange | Yellow<sup>e</sup> |
| Nivalenol          | 1,000                 |    | 0-0.02<sup>b</sup> | 0-0.01<sup>b</sup> | Green<sup>c</sup> | Faint green | Gray | Light-brown |
| 4-Acetamido-4-     | 1,000                 |    | 0.10 | 0.03 | Green<sup>c</sup> | Faint green | Gray | FeCl<sub>3</sub> |
| hydroxy-2-butenoic |                       |    | 0.16 | 0.03 | Blue<sup>c</sup> | Faint blue | Brown | Brown |
| acid γ-lactone     | 2                     |    | 0.17 | 0.13 | Blue<sup>c</sup> | Faint blue | Blue | Blue |
| Kojic acid         | 0.2                   |    | 0.23 | 0.14 | Green<sup>c</sup> | Faint green | Pink | Pink |
| Asperulosin        | 1                     |    | 0.26 | 0.20 | Blue<sup>c</sup> | Faint blue | Pink | Pink |
| Asperulone         | 0.2                   |    | 0.31 | 0.23 | Blue<sup>c</sup> | Faint blue | Pink | Pink |
| Diacetoxyscirpenol | 1,000                 |    | 0.33 | 0.24 | Bright yellow | Bright yellow | Gray-pink | Green-yellow |
| Aspertonine        | 50                    |    | 0.35 | 0.13 | Bright yellow | Bright yellow | Green | Blue<sup>c</sup> |
| T-2 toxin          | 100                   |    | 0.36 | 0.28 | Faint blue     | Faint brown | Yellow<sup>e</sup> | Blue<sup>d</sup> |
| Patulin            | 200                   |    | 0.41 | 0.21 | Faint blue     | Faint blue | Yellow<sup>e</sup> | Light blue |
| Penicillic acid    | 200                   |    | 0.47 | 0.22 | Green<sup>c</sup> | Green<sup>e</sup> | Faint blue | Faint blue |
| Gliotoxin          | 1,000                 |    | 0.53 | 0.39 | Green<sup>c</sup> | Green<sup>e</sup> | Faint blue | Faint yellow |
| Ochratoxin A       | 10                    |    | 0.55 | 0.35 | Green<sup>c</sup> | Green<sup>e</sup> | Faint blue | Yellow<sup>d</sup> |
| Zearalenone        | 50                    |    | 0.78 | 0.42 | Faint blue     | Blue-green<sup>e</sup> | Faint brown | Faint yellow |
| Sterigmatocystin   | 50                    |    | 0.85 | 0.75 | Red-brown      | Faint blue | Faint brown | Faint yellow | Yello<sup>d</sup> |

* In chloroform solution, except for citrinin and aflatoxins (in benzene-acetonitrile, 98:2), nivalenol (in ethyl alcohol), and kojic acid (in ethyl acetate).

<sup>a</sup> Streak.
<sup>b</sup> Preferred means of detection.
<sup>c</sup> Improved sensitivity if TLC plate still hot.
<sup>d</sup> Color variable.
or its usefulness in screening production of several mycotoxins by representative fungi. Dense spore suspensions from fresh potato-dextrose-agar slopes were inoculated onto 5 ml of 2% yeast extract plus 15% sucrose medium [YES (2)] contained in a 30-ml vial (15). Cultures were incubated for 7 days at 25 C, except for the Fusarium species which usually required special conditions, and then extracted with hot chloroform. Each extract was concentrated to 0.5 ml before TLC. Where mycotoxin production was observed, media were successful, alternative media were rarely used in most cases. Otherwise, the following additional media were tried: Difco mycological broth plus 0.5% yeast extract (MYE), Czapek-Dox broth plus 0.5% yeast extract (CZ plus YE), and moist rice in 250-ml flasks (for zearalenone production). Results are given in Table 2.

Since the fungi were known toxic producers or were of a type expected to produce the given mycotoxins, identification of the compounds was usually limited here to the two TLC solvent systems and one or more spray colors. Aflatoxins B₁ and G₁ from A. sulphureus were confirmed by formation of characteristic fluorescent derivatives on a microgram scale (7).

Although we were unable to obtain production of gliotoxin or zearalenone on YES medium, the value of this medium for screening toxino-

genic fungi by the semimicro culture technique has been indicated.

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tine (P. uredinae), K. Sargeant (A. flavus MRE 2), E. Hoibak and R. L. Moore (other A. flavus strains), H. A. Wallace (A. versicolor 64-45), and R. A. Shoemaker (Cochliobolus nodulonus).

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| Fungus | Strain number | Mycotoxins | Media |
|--------|---------------|------------|-------|
| Penicillium patulum | ATCC 18172 | Patulin | YES |
| P. urticae | NRRRL 1953 | Patulin | YES |
| P. terikowski | HLX 136<sub>a</sub> | Gliotoxin | CZ + YE |
| P. viridicatum | 69-22<sup>b</sup> | Citrinin, ochratoxin A | YES |
| Aspergillus flavus | MRE 2 | Aspertoxin; aflatoxins B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, G<sub>3</sub> | YES, MYE |
| A. flavus (4 strains) | ATCC 18413 | Kojic acid; aflatoxins B<sub>1</sub>, B<sub>2</sub> | YES |
| A. sulphureus | | Penicillic acid; aflatoxins B<sub>1</sub>, G<sub>1</sub>; ochratoxin A | YES |
| A. versicolor | | Sterigmatocystin | CZ + YE, MYE |
| A. versicolor | | Sterigmatocystin | YES, MYE |
| A. versicolor | | Sterigmatocystin | YES, MYE, CZ + YE |
| Cochliobolus nodulosus (Bipolaris state) | | Sterigmatocystin | YES |
| Fusarium sp. | Mapleton 10<sup>d</sup> | Zearalenone | Moist rice<sup>c</sup> |
| Fusarium sp. | 69-15<sup>b</sup> | T-2 toxin | YES<sup>c</sup> |
| Fusarium sp. | 69-17<sup>b</sup> | T-2 toxin | YES<sup>c</sup> |
| Fusarium sp. | 66-57-3<sup>b</sup> | Diaacetoxyscirpenol | YES |

<sup>a</sup> National Research Council of Canada, Halifax.
<sup>b</sup> Canadian Food and Drug Directorate, Ottawa.
<sup>c</sup> Canada Department of Agriculture, Winnipeg.
<sup>d</sup> University of Minnesota.
<sup>e</sup> Fungi cultured for 1 week at 25 C and then 2 weeks at 12 C.
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