Detection of Rubratoxin B and Seven Other Mycotoxins in Corn

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A method was developed for simultaneous extraction, separation, and qualitative analysis of rubratoxin B, aflatoxin B_1, diacetoxyscirpenol, ochratoxin A, patulin, penicillic acid, sterigmatocystin, and zearalenone in corn. Mycotoxins were extracted with acetonitrile, sequentially eluted from a silica gel minicolumn, and visualized by thin-layer chromatography (TLC) methods. Rubratoxin B was quantitatively measured, using the TLC solvent system acetonitrile/acetic acid (100:2, v/v), with as little as 10 mg of toxin/kg of corn being detected. Extraction and total efficiencies for rubratoxin B were approximately 66 and 31%, respectively. Three new confirmation tests for rubratoxin B are reported: (1) NH_4OH vapor intensified the fluorescence of a heat-produced derivative of rubratoxin B, (2) chlorine/pyrazolinone/cyanide reagent reacted with rubratoxin B to produce a pink spot that rapidly changed to blue, following exposure to heat and NH_4OH, and (3) prolonged heating of rubratoxin B on TLC plates in the presence of NH_4HCO_3 also produced a blue fluorescent derivative.

Mycotoxins are metabolites of fungi that cause illness or death of animals that ingest feeds contaminated with them. Moldy feed has caused mycotoxicoses (Sippel et al., 1953), and frequently more than one toxic fungus was present (Blevins et al., 1969; Burnside et al., 1957). *Aspergillus flavus* and *Penicillium rubrum* are common soil fungi that have been isolated from toxic feed (Burnside et al., 1957). Current multimycotoxin analyses include aflatoxins, the toxic metabolites of *A. flavus* (Durackova et al., 1976; Eppley, 1968; Roberts and Patterson, 1975; Stoloff et al., 1971), but none include rubratoxin B from *P. rubrum*. This omission may be important since the toxicity of aflatoxin B_1 is enhanced by rubratoxin B (Wogan et al., 1971). In previous multimycotoxin analyses, the mycotoxins were extracted with acetonitrile (Roberts and Patterson, 1975; Stoloff et al., 1971). Hayes and McCain (1975) reported that acetonitrile was satisfactory for extracting rubratoxin B from corn. In contrast, however, Emeh and Marth (1977) failed to extract rubratoxin B from corn with acetonitrile. They suggested that possibly no rubratoxin B was present in that portion of corn extracted or that it had been degraded. In view of the report of Hayes and McCain (1975), the first explanation seems most plausible. This new procedure was developed to include an acetonitrile extraction and the detection of rubratoxin B in a multimycotoxin analysis of corn.

MATERIALS AND METHODS

Materials. Mycotoxins were obtained from the following sources: aflatoxin B_1, diacetoxyscirpenol, rubratoxin B, and sterigmatocystin, Makor Chemicals, Ltd., Jerusalem, Israel; patulin, Wale Corp., San Francisco, CA; zearalenone, Commercial Solvents, Terre Haute, IN; and penicillic acid, Northern Regional Research Center, Peoria, IL. Grade 2 yellow corn was obtained from Tallapossa Milling Co., East Tallassee, AL. *Penicillium rubrum* Stoll NRRL 3290 was provided by D. T. Wicklow, Northern Regional Research Center, Peoria, IL.

Extraction. The mycotoxins (aflatoxin B_1, diacetoxyscirpenol, ochratoxin A, patulin, penicillic acid, rubratoxin B, sterigmatocystin, and zearalenone) were added to 50-g samples of ground corn, after which the solvents were evaporated under nitrogen. In other experiments, 50-g portions of sterile, moistened, whole corn were inoculated with *Penicillium rubrum* NRRL 3290 and incubated for 2 weeks at 25 °C and 99% relative humidity (RH) in Blue-M-environmental cabinets. Subsequently, when analyzed, samples were acidified with 20 mL of 1.25% HCl. Acetonitrile (100 mL) was added, and samples were blended for 30 s at low speed in a Waring Blendor. (Precautions were taken to prevent contact with skin or inhalation of acetonitrile.) The mixture was rinsed into a 250-mL flask with an additional 50 mL of acetonitrile and was shaken for 30 min on a platform shaker. The solvent was decanted into a funnel lined with acetonitrile-moistened, coarse filter paper. The residue was shaken for 30 min with an additional 150 mL of acetonitrile and subsequently poured into the funnel. The combined filtrate was defatted by shaking in a 500-mL separatory funnel with two 50-mL portions of isooctane. The isooctane phase was discarded. The acetonitrile phase was dried with sodium sulfate and evaporated to dryness.

A dry minicolumn, modified from that of Eppley et al. (1968), was prepared by placing a plug of glass wool at the constriction of a butte funnel (27 mm × 13 cm), covering it with a thin layer of anhydrous sodium sulfate, and adding 20 g of heat-activated, grade 922 silica gel (200 × 315 mesh). The column was gently tapped to pack it.

The dried extract was dissolved in 5–10 mL of benzene/methanol (99:1, v/v) and 1 g of heat-activated, TLC grade silica gel was added. The slurry was poured on the dry minicolumn. The column was then washed with 50 mL of benzene to elute fraction 1. Fraction 2 was eluted with 100 mL of benzene/acetone (95:5, v/v). A layer of sodium sulfate was next added to the top of the column, which was then washed sequentially with 100 mL of diethyl ether, 200 mL of chloroform/methanol (95:5, v/v), and 200 mL of acetone, thereby eluting fractions 3, 4, and 5. Each of these fractions was collected separately, evaporated to dryness under an air stream, and redissolved in 1.0 mL of the eluting solvent.

Chromatography. Five microliters of each solution, except fraction 5 that contained rubratoxin B, was placed on a TLC plate (0.5 mm thick) beside the external standard (5 μL of a standard solution of the appropriate mycotoxin). The internal standard contained 5 μL of sample plus 5 μL of standard. The plate was developed in toluene/ethyl acetate/88% formic acid (TEF; 6:3:1, v/v/v). Toxins were visualized on TLC plates as previously reported: aflatoxin (Pons et al., 1971), diacetoxyscirpenol (Scott et al., 1970), ochratoxin A (Scott and

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Hand, 1967), patulin (Pohland and Allen, 1970), penicillic acid (Ciegler and Kurtzman, 1970), sterigmatocystin (Stack and Rodricks, 1971), and zearalenone (Eppley, 1968).

Fraction 5 from the minicolumn was examined for the presence of rubratoxin B (Hayes and McCain, 1975). Two 5-μL portions were applied to silica gel TLC plates under a flow of nitrogen. Adjacent to these samples, 0.5, 1, 2, 3, and 5 μg of rubratoxin B standard (0.5 and 1.0 mg/mL of acetone or acetonitrile) were applied to the plate as external standards. On top of one 5-μL sample, 5 μg of rubratoxin B standard was applied to the plate as the internal standard. The plate was chromatographed in acetonitrile/acetic acid (AAA: 100:2, v/v), with the solvent being evaporated under a fume hood. Plates were viewed in a Chromato-Vue cabinet (Model 5, Ultraviolet Products, San Gabriel, CA) under long-wave (365 nm) UV light. Fluorescent spots were marked to avoid confusion with rubratoxin B after heating. Plates were heated 200 °C in a forced air oven for 10 min and cooled to room temperature for 20-30 min.

Samples were examined under long-wave UV light for blue fluorescent spots not present prior to heating. R_f values and fluorescence were compared to those of rubratoxin B standards. The fluorescent intensity of the sample spot was matched with the intensity of one of the external standards and the concentration of rubratoxin B in the sample was then calculated.

Percent Recovery. The percent recovery was determined by adding measured amounts of rubratoxin B to 50-g samples of ground corn. Acetone solutions of rubratoxin B (1 mg/mL) were prepared and appropriate amounts were added to duplicate samples to yield concentrations of 0, 5, 10, 30, or 50 mg/kg. The solvent was evaporated under an air stream and the corn was extracted and quantitated as previously described. For evaluation of the efficiency of rubratoxin B extraction per se, 0.5 mL of extract was removed immediately after extraction, chromatographed on thin-layer plates in chloroform/methanol/acetic acid (80:20:1, v/v/v), and quantitated as previously described (Hayes and McCain, 1975). The remaining extract was processed and rubratoxin B was analyzed quantitatively as previously described, following the minicolumn clean-up procedure. Percent recovery of rubratoxin B was then calculated for the extraction efficiency alone and for the entire procedure.

Detection. In search of improved detection methods for rubratoxin B, TLC silica gel plates were spotted with 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, and 2.0 μg of authentic rubratoxin B, chromatographed in AAA, and examined under long-wave UV light. Plates were heated at 200 °C for 10 min. After cooling the plates to room temperature, half of them were placed in a chromatography tank containing a beaker of concentrated ammonium hydroxide for 20 min. Plates were examined under UV light. TLC silica gel plates spotted with 0.1, 0.3, 0.5, 1.0, 3.0, 5.0, and 10.0 μg of authentic rubratoxin B were chromatographed in AAA and examined under long-wave UV light. A desiccator was heated to 100 °C in a forced air oven. The desiccator was removed from the oven and 6 g of ammonium bicarbonate was placed in the bottom of it. The freshly developed plate was placed in a rack in the desiccator and returned to the oven (Segura and Grotto, 1974). Thereafter at 2-h intervals for 8 h, a freshly developed plate was placed in the desiccator. After the initial plate had been heated 10 h, all five plates were removed from the desiccator, cooled at room temperature, and examined in UV light.

TLC silica gel plates were spotted with 1, 3, 5, 7, and 10 μg of rubratoxin B. Plates were chromatographed and examined under UV light, and half of the plates were heated at 200 °C for 10 min. When cool, plates were examined for blue fluorescence not present previously. All plates were placed in a tank with a beaker of ammonium hydroxide for 25–30 min and then placed under a fume hood for 10–15 min for the excess ammonia to escape. Plates were exposed to chlorine for 20 min, and the excess chlorine was removed by heating for 10 min at 100 °C or by placing the plates under a fume hood for 20–30 min (Ashworth and Bohnstedt, 1966). Spray reagent was prepared each day by mixing equal volumes of a 0.2 M pyridine solution of 1-phenyl-3-methyl-2-pyraxolin-5-one and 1 N aqueous potassium cyanide. Plates were sprayed with the reagent and observed for the development of colored areas.

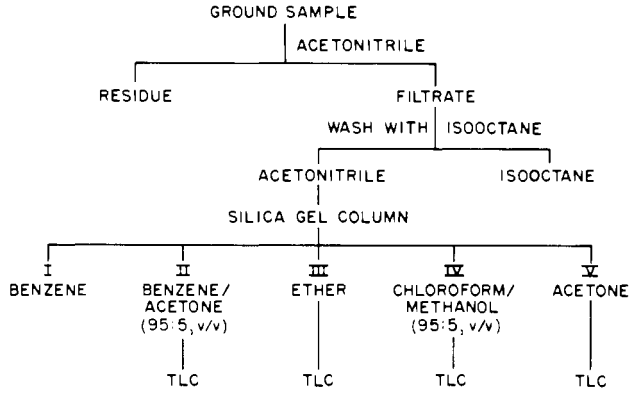

Figure 1. Flow chart of extraction and separation of mycotoxins.

Table I. Efficiency of Extraction of Rubratoxin B from Spiked Corn

toxin added, mg/kg	no. of 50-g samples	extraction effic, % recov	var[a]	SD[b]
5	3	55.7	64.22	±8.0
10	4	47.0	27.0	±5.2
30	4	84.75	323.7	±18.0
50	11	68.9	634.63	±25.2
all levels	22	65.8	379.25	+19.5

[a] Variance. [b] Standard deviation.

RESULTS AND DISCUSSION

Analysis. A procedure was developed in which acetonitrile extracted rubratoxin B simultaneously with seven other mycotoxins. A minicolumn separated the eight toxins into one of five fractions: none in the first fraction, zearalenone and sterigmatocystin in the second, patulin and penicillic acid in the third, ochratoxin A, aflatoxin B_1, and diacetoxyscirpenol in the fourth, and rubratoxin B in the fifth. A summary of the extraction and separation method is presented in Figure 1.

All toxins except rubratoxin B were detected on TLC plates developed in the TEF solvent system (Scott and Hand, 1967). Metabolites of *P. rubrum* obscured the fluorescence of rubratoxin B in that solvent system. However, a new solvent system, acetonitrile/acetic acid (AAA) (100:2, v/v), was developed for rubratoxin B in which the rubratoxin B moved to the upper quarter of the plate, while the interfering metabolites remained in the lower portion of the plate.

The efficiency of the extraction and the total recovery of the analysis were determined from corn samples spiked with rubratoxin B (Tables I and II). Although 67% of the rubratoxin B was extracted, the final recovery was only 31%, whereas Hayes and McCain (1975) reported a re-

Table II. Total Recovery of Rubratoxin B from Spiked Corn

toxin added, mg/kg	no. of 50-g samples	recov, %	var[a]	SD[b]
10	4	26.7	69.2	±8.3
30	4	33.5	416.8	±20.4
50	11	30	72.7	±8.5
all levels	19	31.0	186.2	+12.4

[a] Variance. [b] Standard deviation.

covery of 90% of the rubratoxin B. This disparity is probably due to the length and complexity of the multimycotoxin analysis and the instability of rubratoxin B. Lengthy procedures are unavoidable when analyzing for more than one mycotoxin.

Despite the lower recovery of rubratoxin B by this multimycotoxin method, rubratoxin B was detected when as little as 10 mg/kg was present. However, Hayes and McCain (1975) could only detect rubratoxin B when the level was above 70 mg/kg of corn. Since a pig was killed within 12 h of ingesting an estimated dose of 64 mg/kg (Moss, 1971), a lethal dose of rubratoxin B could be present and remain undetected by the method of Hayes and McCain (1975). Therefore, the improved level of sensitivity of the multimycotoxin method described herein is of practical significance.

Confirmation Tests. The fluorescent derivatives, which were formed from rubratoxin B on a TLC plate after heating at 200 °C for 10 min, were exposed to ammonium hydroxide vapor for 10 min. Examination under long-wave UV light revealed a change in intensity and color of the fluorescence. Rubratoxin was then more easily observed as a light blue spot although the lower detection limit remained the same. Furthermore, fluorescent compounds near rubratoxin B were greatly reduced in fluorescent intensity. This greatly improved the contrast and thereby the ease of detecting rubratoxin B. Also, after the prolonged heating of TLC plates at 100 °C for 2–10 h with ammonium bicarbonate (Segura and Grotto, 1974), rubratoxin B was visible under UV light. The reactions of ammonium hydroxide and bicarbonate with rubratoxin B both produced very similar fluorescent derivatives on TLC plates. The ammonium ion apparently combined with the anhydride derivative of rubratoxin B to produce an amide or imide, which reacted with chlorine/pyrazolinone/cyanide to produce a color reaction. Subsequently, rubratoxin B first turned pink in visible light, very quickly changed to blue, and then changed to brown. The lower detection limit was 10 μg.

Safety Precautions. Diacetoxyscirpenol has dermal toxicity and should not be allowed to contact skin. Aflatoxin B_1 and sterigmatocystin are carcinogens and as such must be used with proper precautions. Laboratory equipment and surfaces should be decontaminated as necessary as described by Goldblatt (1969) and Detroy et al. (1971). Work with chlorine, acetonitrile, and potassium cyanide should be carried out with care under a hood.

LITERATURE CITED

Ashworth, M. R. F., Bohnstedt, G., *Talanta* **13**, 1631 (1966).
Blevins, D. I., Glenn, M. W., Hamdy, A. H., Brodasky, T. F., Evans, R. A., *J. Am. Vet. Med. Assoc.* **154**, 1043 (1969).
Burnside, J. E., Sippel, W. L., Forgacs, J., Carll, W. T., Atwood, M. B., Doll, E. R., *Am. J. Vet. Res.* **18**, 817 (1957).
Ciegler, A., Kurtzman, C. P., *J. Chromatogr.* **51**, 511 (1970).
Detroy, R. W., Lillehoj, E. B., Ciegler, A., in "Microbial Toxins", Vol. 6, Ciegler, A., Kadis, S., Ajl, S. J., Eds., Academic Press, New York, 1971, p 3.
Durackovâ, Z., Betina, V., Nemec, P., *J. Chromatogr.* **116**, 141 (1976).
Emeh, C. O., Marth, E. H., *Z. Lebensm.-Unters.-Forsch.* **163**, 115 (1977).
Eppley, R. M., *J. Assoc. Off. Anal. Chem.* **51**, 74 (1968).
Eppley, R. M., Stoloff, L., Campbell, A. D., *J. Assoc. Off. Anal. Chem.* **51**, 67 (1968).
Goldblatt, L. A., "Aflatoxin", Academic Press, New York, 1969, p 8.
Hayes, A. W., McCain, H. W., *Food Cosmet. Toxicol.* **13**, 221 (1975).
Moss, M. O., in "Microbial Toxins", Vol. 6, Ciegler, A., Kadis, S., Ajl, S. J., Eds., Academic Press, New York, 1971, p 381.
Pohland, A. E., Allen, R., *J. Assoc. Off. Anal. Chem.* **53**, 686 (1970).
Pons, W. A., Jr., Cucullu, A. F., Lee, L. S., *Food Sci. Technol., Proc. Int. Congr., 3rd*, 705 (1971).
Roberts, B. A., Patterson, D. S. P., *J. Assoc. Off. Anal. Chem.* **58**, 1178 (1975).
Scott, P. M., Hand, T. B., *J. Assoc. Off. Anal. Chem.* **50**, 366 (1967).
Scott, P. M., Lawrence, J. W., Walbeek, W. van, *Appl. Microbiol.* **20**, 839 (1970).
Segura, R., Grotto, A. M., Jr., *J. Chromatogr.* **99**, 643 (1974).
Sippel, W. L., Burnside, J. E., Atwood, M. B., *Proc. Am. Vet. Med. Assoc.*, 174 (1953).
Stack, M., Rodricks, J. V., *J. Assoc. Off. Anal. Chem.* **54**, 86 (1971).
Stoloff, L., Nesheim, S., Yin, L., Rodricks, J. V., Stack, M., Campbell, A. D., *J. Assoc. Off. Anal. Chem.* **54**, 91 (1971).
Wogan, G. N., Edwards, G. S., Newberne, P. M., *Toxicol. Appl. Pharmacol.* **19**, 712 (1971).

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