A rapid assay procedure was developed for mycotoxin citrinin in corn using liquid–liquid extraction (LLE) cartridges. Ground corn was extracted with methylene chloride and 0.5N phosphoric acid. The extract was added to an LLE cartridge containing a diatomaceous-earth adsorbant, previously impregnated with sodium bicarbonate solution. After aspiration to dryness, the cartridge was eluted with methanol–water (4 + 1), and aliquots were taken for quantitation by reversed-phase liquid chromatography with fluorescence detection. Recoveries of citrinin added to ground corn at 200–1600 ng/g ranged from 71.2 to 86.3%, with coefficients of variation between 4.1 and 10.6%. An indirect enzyme immunoassay was also evaluated, using sodium carbonate solution for extraction. Recoveries of citrinin added to ground corn at 200–2000 ng/g ranged from 53.2 to 67.2%, but the coefficients of variation varied between 18.4 and 51.5%. The LLE cartridge procedure offers the advantages of low solvent consumption and speed, and is amenable to automation.

The mycotoxin citrinin is produced in foods and feeds by several *Aspergillus* and *Penicillium* species (1), and was originally isolated in 1931 (2). Citrinin has been detected in Canadian wheat, oats, rye (3), and durum wheat (4), in Swedish barley (5), in wheat flour from the United Kingdom (6), in American corn (7, 8), in Danish feed grains (9), and in German vegetarian foods (10). Citrinin frequently occurs together with ochratoxin A and, like ochratoxin A, acts as a nephrotoxin (11) and teratogen in test animals. Citrinin and ochratoxin A are thought to act synergistically, and have been tested in combination in renal studies (12, 13), in teratogenicity assessments (14), and in carcinogenicity trials (15).

**Experimental**

**Apparatus**

(a) Sample mill.—Retsch ZM-1 centrifugal mill (Brinkmann, Toronto, Canada) with 12-tooth rotor and screen with 1 mm holes.

(b) Vacuum manifold for LLE cartridges.—12 stations; wetted parts are made of polypropylene (Lida, Kenosha, WI).

(c) Sample reservoir for LLE cartridges.—25 mL polypropylene with adapter (Varian, Harbor City, CA).

(d) LC unit.—Hewlett-Packard 1084B (Hewlett-Packard, Edmonton, Canada) with Hewlett-Packard 79850B digital chromatographic data system, column compartment temperature control, and 10–200 μL variable-volume injector.

(e) Column.—LiChrospher RP-18 column (250 × 4 mm id, 10 μm particle id; Merck, Darmstadt, Germany).

(f) Fluorescence detector.—Schoeffel FS970 (McPherson, Chelmsford, MA).

(g) EIA microtiter plate reader.—SLT 400 AER or equivalent (SLT, Crailsheim, Germany). Sample wavelength was set to 450 nm and reference wavelength to 620 nm.
Standards, Adsorbents, and Reagents

(a) **Citrinin.**—Sigma Chemical Co., St. Louis, MO.
(b) **LLE cartridges.**—Chem Elut 1219-8002 containing diatomaceous earth-type adsorbant, 1 mL sample capacity (Varian).
(c) **Casein (sodium salt).**—Sigma-Aldrich Vertriebs GmbH, Deisenhofen, Germany.
(d) **Polyoxyethylenesorbitan monolaurate (Tween-20).**—Sigma-Aldrich Vertriebs GmbH.
(e) **Anti-rabbit IgG–HRP.**—From goats. Sigma-Aldrich Vertriebs GmbH.
(f) **3,3′,5,5′-Tetramethylbenzidine (TMB).**—Sigma-Aldrich Vertriebs GmbH.
(g) **Other reagents and solvents.**—Analytical reagent grade.

Solutions

(a) **Phosphoric acid solution for LLE extraction.**—0.5N aqueous.
(b) **Sodium bicarbonate solution for LLE.**—1.25% aqueous.
(c) **LLE elution mixture.**—Methanol–water (4 + 1).
(d) **LC elution mixture.**—Acetonitrile–water (2 + 3). The water was acidified by adding 2% formic acid.
(e) **Sodium bicarbonate solution for EIA extraction.**—0.13M, pH 8.4.
(f) **Phosphate-buffered saline (PBS) solution.**—Potassium phosphate 0.01M with 0.1M NaCl, pH 7.3.
(g) **Sodium carbonate buffer.**—0.05M, pH 9.6.
(h) **Casein solution.**—Casein (sodium salt) 2% in PBS.
(i) **NaCl–Tween solution.**—NaCl 0.85% with Tween-20, 250 μL/L.
(j) **Citrinin standard solutions.**—For LLE, citrinin (MW = 250.1) solution was prepared in methanol (ca 40 μg/mL) and the exact concentration was determined by photometric absorbance measurement at 319 nm, using ε = 4700 (24). Standards for LC were prepared by diluting with methanol.

For EIA, a citrinin stock solution, 20 mg/mL in methanol was prepared. Standards were prepared by diluting 25 μL stock solution with 975 μL PBS. Further dilutions were prepared as required using methanol–PBS (2.5 + 97.5).
(k) **Goat anti-rabbit IgG–HRP solution.**—Received as PBS solution and diluted 1:2000 in PBS containing 1% casein (sodium salt).
(l) **Enzyme substrate solution.**—Hydrogen peroxide 3 mM and 1 mM TMB in potassium citrate buffer (0.2M, pH 3.9).
(m) **Sulfuric acid solution.**—1M.

LLE Protocol

Corn was ground to pass through 1 mm aperture screen. To 10 g corn in a 125 mL Erlenmeyer flask, 5 mL phosphoric acid solution and 50 mL methylene chloride were added. The opening was wrapped tightly with aluminum foil and the contents stirred 30 min. Each LLE cartridge was prepared by adding 1.0 mL sodium bicarbonate solution, and a reservoir was attached. After 5 min, 25 mL extract was aspirated through the cartridge slowly so that single drops could be seen. After the entire extract was run through, the cartridge was then aspirated with full available vacuum (ca ~70 KPa) a further 20 min. Each column was then eluted under vacuum with methanol–water (4 + 1) solution, and 5.0 mL was collected in a graduated tube.

Chromatographic Conditions

Aliquots of 25–150 μL were injected into the LC unit. The C18 column was eluted with acetonitrile–water (2 + 3), with the water component containing 2% formic acid, at 1.5 mL/min and 40°C. For fluorescence detection, irradiation wavelength was set to 333 nm, range to 1.0 μA, time constant to 4 s, and appropriate cutoff filter was installed to give emission wavelength >405 nm.

EIA Protocol

Conjugates of citrinin with glucose oxidase (GOX) protein from *Aspergillus niger* van Tieghem were prepared by coupling citrinin to the proteins using the formaldehyde condensation reaction as previously described (23). To each microtiter plate well 100 μL CT–GOX, diluted 1:2000 with sodium bicarbonate buffer, was added. Plates were incubated overnight at ambient temperature in a chamber with >90% relative humidity. The citrinin-GOX solution was removed, and the free protein-binding sites of the wells were blocked with
casein solution for 30 min at ambient temperature. Each plate was washed with NaCl–Tween solution. Serum from a rabbit previously immunized (22) was diluted 1:2000 with PBS, and 50 μL per well was added. To prepare standard curves, citrinin standard solution (50 μL per well) at various dilutions was then added. After incubation for 2 h at room temperature, each plate was washed with NaCl–Tween solution. Goat anti-rabbit IgG–HRP solution, 100 μL per well, was added. After 1 h at ambient temperature, each plate was washed as above, and 100 μL enzyme substrate solution was added per well. After 15 min, the enzyme reaction was stopped with sulfuric acid (1 M) at 37°C. The absorbance was measured at 490 nm.

Recovery Studies with Artificially Contaminated Corn Samples

(a) LLE.—Samples (10 g) of ground citrinin-free corn (Pioneer 3770, crop year 1992) were artificially contaminated with methanol solutions of citrinin to give final concentrations of 0, 200, 400, 800, and 1600 ng citrinin/g corn. Each sample was stirred with 5 mL phosphoric acid solution and 20 mL methylene chloride for 30 min in a sealed flask as described above. Following LLE, aliquots were taken for LC determination.

(b) EIA.—Samples (2 g) of the same citrinin-free corn were artificially contaminated with methanol solutions of citrinin to give final concentrations of 0, 200, 500, 1000, and 2000 ng citrinin/g corn. Each sample was stirred with 10 mL sodium bicarbonate solution for 30 min. The mixture was passed through Whatman No. 1 filter paper, and aliquots were diluted 1 + 3 with PBS. Further dilutions were made as necessary with methanol–PBS (2.5 + 97.5) prior to the indirect EIA described above.

Results and Discussion

Corn seems to be a good substrate for citrinin production under storage conditions that are conducive to mold growth (7, 8), and Pioneer 3770, a fast-maturing feed variety, was used as a matrix for evaluating performance of the LLE and EIA assays for citrinin. LLE afforded a fairly clean extract in which citrinin could be easily separated by LC and measured by fluorescence (Figure 1). Band broadening of citrinin arising from injection volumes of 100 mL or more appeared to be offset by improved mass transfer resulting from running the C18 column at 40°C. Results for artificially contaminated corn samples are presented in Tables 1 and 2, respectively. Using the LLE procedure, recoveries of citrinin at 200–1600 ng/g ranged from 71.2 to 86.3%, with coefficients of variation (CVs) between 4.1 and 7.4% (Table 1). Using the indirect EIA, recoveries of citrinin added to ground corn at 200–2000 ng/g ranged from 53.2 to 67.2%, with CVs between 18.4 and 51.5% (Table 2). Recovery and CV figures for citrinin in ground corn, cited in a previous method (17) using solvent partition and LC, indicated recoveries of 92.0% (CV = 10.6%), 69.5% (CV = 10.9%), and 75.8% (CV = 22.8%) for corn spiked at 200, 1000, and 2000 ng/g, respectively (n = 4). The present LLE procedure produced correspondingly good recoveries of citrinin from corn spiked at comparable levels.

The indirect EIA produced much lower recoveries and much higher CV figures at all spiking levels except 2000 ng/g.

The proposed LLE procedure has some advantages over the earlier solvent partition (17) method. The reduction of solvent volume in the LLE procedure permits more economical processing of samples and much lower solvent disposal costs than the partition method. The compact LLE apparatus and 125 mL extraction flasks take up much less fume hood area than the numerous separatory funnels and 500 mL flasks required for the solvent partition method. Because 12 stations are available on the LLE vacuum manifold, 12 samples can be processed in little more time than required for one sample. The LLE method can also be more readily adapted to robotics and automation than the solvent partition method.

The EIA method underwent some unsuccessful trial modifications in search of higher recoveries at the 1000 and 2000 ng/g spiking levels. Extraction of the Pioneer 3770 corn with pure methanol or acetonitrile rarely gave recoveries above 50% at 1000 ng/g, and extraction of food-grade domestic corn from Germany at 1000 ng/g gave about 40% recovery with the latter solvent. Changing the extraction solvent from sodium carbonate solution to methanol–PBS (1 + 9) adversely affected recovery of citrinin from Pioneer 3770 corn. Recoveries with the latter solvent were 48.1% at 1000 ng/g (n = 3, CV = 7.0%) and 40.7% at 2000 ng/g (n = 5, CV = 20.8%). In previous studies with other grains, the EIA performed much better, giving recoveries of 89–104% (CV = 6.9–13%) for wheat spiked at 200–2000 ng/g (22), and 105–112% (CV = 4.5–12%) for barley spiked at 100–2000 ng/g (23).

<table>
<thead>
<tr>
<th>Added, ng/g</th>
<th>Mean recovery, %</th>
<th>n</th>
<th>CV, %</th>
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<tr>
<td>200</td>
<td>86.3</td>
<td>4</td>
<td>4.1</td>
</tr>
<tr>
<td>400</td>
<td>82.1</td>
<td>3</td>
<td>6.7</td>
</tr>
<tr>
<td>800</td>
<td>79.5</td>
<td>3</td>
<td>10.6</td>
</tr>
<tr>
<td>1600</td>
<td>71.2</td>
<td>3</td>
<td>7.4</td>
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Table 2. Recovery of citrinin from artificially contaminated corn using indirect enzyme immunoassay

<table>
<thead>
<tr>
<th>Added, ng/g</th>
<th>Mean recovery, %</th>
<th>n</th>
<th>CV, %</th>
</tr>
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<tbody>
<tr>
<td>200</td>
<td>53.2</td>
<td>6</td>
<td>51.5</td>
</tr>
<tr>
<td>500</td>
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</tr>
<tr>
<td>1000</td>
<td>60.5</td>
<td>7</td>
<td>22.0</td>
</tr>
<tr>
<td>2000</td>
<td>67.2</td>
<td>6</td>
<td>18.4</td>
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The LLE method, with its advantages of speed and solvent economy, could conveniently be used for assaying corn samples containing citrinin at levels as low as 200 ng/g. For regulatory purposes, confirmation of LC results by an alternative detection method, such as thin-layer chromatographic analysis on silica gel, is advisable. The sensitivity of the indirect EIA, using polyclonal antibodies, would probably not be sufficient to assay citrinin in corn at levels below 2000 ng/g. The immunoassay for citrinin seems adversely affected by the lipid content of the corn matrix (about 4.5%), but an EIA with monoclonal antibodies (10) may show different performance characteristics.

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