Detection of Aflatoxin D₁ in Ammoniated Corn by Mass Spectrometry-Mass Spectrometry

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Corn cultured with Aspergillus flavus to produce a high level of aflatoxin was ammoniated at 37°C for 21 days. An extract of the ammoniated corn was separated by thin-layer chromatography, followed by reversed-phase high-pressure liquid chromatography. Examination of the fractions by tandem mass spectrometry led to detection of aflatoxin D₁ as a product of the corn ammoniation process.

Ammoniation of aflatoxin-contaminated corn at ambient temperature and atmospheric pressure(109,362),(851,418)

was an effective process for reducing high aflatoxin levels to below 20 ng/g (2). Long-term feeding studies with rats indicate that the ammoniated corn is nontoxic (7). However, the chemical fate of aflatoxin in this ammoniation process has not been determined. In an earlier investigation, ammoniation in solution at 37°C of a noncarcinogenic model coumarin closely related to aflatoxin B₁ gave rise to three characterized products, but each was formed in only 10% yield or less (4). Two of these compounds are analogous to the major products, aflatoxin D₁ (Fig. 1) and the phenol with a molecular weight of 206, produced the ammoniation of aflatoxin B₁ at high temperature and pressure (3, 6). Therefore, the decomposition pathway of the aflatoxin cyclopentenonecoumarin ring system by ammonium hydroxide solution at ambient temperature and pressure appears to parallel that observed at high temperature and pressure. However, when aflatoxin-contaminated peanut and cottonseed meals are ammoniated at high temperature and pressure, only trace amounts of aflatoxin D₁ are detected by a thin-layer chromatography procedure (5). The relatively recent development of tandem mass spectrometry, or mass spectrometry-mass spectrometry (MS-MS), appears to provide an ideally suited technique for detection of trace amounts of nonvolatile compounds in complex biological mixtures such as those encountered in a moldy grain milieu (8, 9). MS-MS has been used to identify aflatoxin B₁ in corn extracts at nanograms per gram (parts per billion) levels (8a). We report here the application of this technique to identification of one aflatoxin decomposition product, aflatoxin D₁, in ammoniated corn.

We used the procedure described by Wicklow et al. (11) for production of aflatoxin-contaminated corn. The substrate consisted of 10 undamaged corn kernels (3.0 g) placed on double layers of Whatman no. 1 filter paper in each of two glass petri dishes. The kernels were moistened, autoclaved, wounded, and inoculated with Aspergillus flavus Link (NRRL 6554). The dishes were placed in a closed plastic container to prevent moisture loss and were then incubated at 28°C for 12 days. Cultures were then dried in a desiccator and frozen until used. For determination of aflatoxin, the contents of one dish (1.74 g) were extracted in a distilled water (1 ml)—chloroform (10 ml)—Celite (1 g) mixture for 2 h. The extract was removed by filtration, and the residue was washed with chloroform. The combined chloroform extract was dried (Na₂SO₄) and concentrated (134 mg). It contained 2,280 μg/g (ppm) of aflatoxin B₁ by the Contaminants Branch method (1), which is equivalent to 175 ppm in the dried, molded corn kernels.

Ten molded, dried corn kernels (1.70 g) were transferred to a 25-ml flask and treated with concentrated ammonium hydroxide (0.33 ml) and water (0.16 ml) to provide an ammonia concentration of 5%. The flask was sealed and kept at 37°C for 21 days. The ammoniated corn was extracted with dichloromethane-methanol (9:1, 3 x 10 ml). The concentrated extract was defatted by partitioning between hexane-methanol-water (9:1). Concentration of the methanolic phase gave 45 mg of ammoniated corn extract. A solution of the extract (40 mg) in chloroform-methanol (9:1) was applied to a 20- by 20-cm plate of Silica Gel 60 (F-254, 0.25 mm; EM Laboratories). The plate was developed in dichloromethane-methanol (95:5) and viewed under short-wave UV light. The area of silica extending from R₀ 0.33 to 0.44 was removed and extracted with dichloromethane-methanol (9:1, 3 x 10 ml). Concentration of the extract gave 3.5 mg. A solution of the extract in methanol (0.6 ml) was chromatographed in 0.1-ml portions by reversed-phase high-pressure liquid chromatography on a column of Zorbax ODS (4.6 mm by 25 cm) eluted with acetonitrile-methanol-water (17:25:58) at 1 ml/min. Six sequential fractions measuring 8, 2, 1, 2, 2, and 10 ml were collected from each injection for subsequent MS-MS analysis.

Standard aflatoxin B₁ was prepared by reaction of aflatoxin B₂ (2 mg) with concentrated ammonium hydroxide (2 ml) in a sealed flask at 50°C for 14 days. This temperature was used rather than the 37°C used for the corn ammoniation to enhance the conversion to aflatoxin D₁ (4). The reaction mixture was concentrated to dryness, taken up in dichloromethane-methanol (9:1), and applied to a 20- by 20-cm plate of silica gel. The plate was developed and viewed under UV light as above. The major quenching band (R₀ 0.4) was removed and extracted to give aflatoxin D₁ (0.5 mg, 28% yield).

A Finnigan 4535/TSQ triple quadrupole mass spectrometer was used for MS and MS-MS experiments. Electron impact (EI) spectra were recorded at 70 eV. For positive ion (PI) and negative ion (NI) chemical ionization (CI) spectra, isobutane was the reagent gas (0.25 torr). The source temperature was 140°C. Argon was the target gas in collision-activated dissociation MS-MS daughter experiments. The pressure of the collision cell was maintained at 1 x 10⁻³ to

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3 x 10^{-3} torr, and the collision energy was 20 V. Samples were introduced via the direct insertion probe. A 100-ng amount of reference standard was sufficient to produce intense spectra in all modes (EI, CI, and MS-MS). For trace analysis of target compounds in crude samples, as much as several hundred micrograms of material was put on the probe to provide appropriate MS-MS daughter spectra.

A. flavus was cultured on whole corn to obtain an initially high level of aflatoxin B_{1} (175 ppm [175 µg/g]) in the moldy grain and thereby increase the possibility for detection of ammoniation products. Similarly high levels of ca. 1,000 ppm (1,000 µg/g) in spiked and cultured peanut and cottonseed meals were employed in the study of high temperature and pressure ammoniation products of aflatoxin B_{1} (5). Based on our previous study on ammoniation of an aflatoxin analog, the product of greatest stability under the corn ammoniation conditions, and therefore the most likely to be found, should be aflatoxin D_{1} (4).

Mass spectral fragmentation patterns of standard aflatoxin D_{1} produced by EI and MS-MS daughter experiments from the protonated molecule (PICI) and the molecular anion (NICI) are presented in Table 1. The EI spectrum agrees with the previously reported (6). Daughter ion spectra produced by collision-activated dissociation of either protonated aflatoxin D_{1} or the molecular anion are markedly different from the EI spectrum, similar to that found for other aflatoxins (Plattner et al., in press). Both standard PICI and NICI spectra without collision-activated dissociation exhibit only parent ions and no significant fragmentation. NICI is the preferred ionization mode for MS-MS experiments with aflatoxin D_{1} because a greater number of intense daughter ions are produced than are produced by PICI.

<table>
<thead>
<tr>
<th>TABLE 1. Mass spectra of aflatoxin D_{1}</th>
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<td>Mode of fragmentation</td>
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<td>------------------------</td>
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<tr>
<td>Positive ion electron impact</td>
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<tr>
<td>MS-MS of MH^{+} (PICI)</td>
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<tr>
<td>MS-MS of M^{-} (NICI)</td>
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* Produced by argon collision-activated dissociation.

MS-MS analysis for aflatoxin D_{1} in the preparative thin-layer chromatography fraction that migrated from R_{F} 0.33 to 0.44 suggested the presence of aflatoxin D_{1}, but the results were inconclusive because of other ions at m/z 286 contributed by the ammoniated corn matrix. These ions yielded enough "chemical noise" in the daughter spectrum of m/z 286 so that proper ratios of major D_{1} fragment ions were not produced. Therefore, further purification by reversed-phase high-pressure liquid chromatography was required to remove additional interfering matrix components. Fractions from high-pressure liquid chromatography that eluted near the retention volume of authentic aflatoxin D_{1} were collected and subjected to analysis by MS-MS. The daughter ion spectrum of the fraction eluting between 8 and 10 min (Fig. 2) closely matches that of aflatoxin D_{1}. All major m/z 286 daughter fragments produced by this fraction are in good agreement with the relative intensities exhibited by the standard, allowing us to conclude that aflatoxin D_{1} is present in ammoniated corn. Because the aflatoxin D_{1} was spread across several of the high-pressure liquid chromatography fractions, an accurate estimate of its amount was not possible. Based on conversion of the aflatoxin model ketocoumarin to the decarboxylated ketophenol by ammonia at 37°C (4), conversion of aflatoxin D_{1} would not be expected to exceed 10% of the initial aflatoxin B_{1}. Estimates from MS-MS experiments indicate that less than this amount of D_{1} may have been formed. Interestingly, aflatoxin D_{1} could not be detected by a thin-layer chromatography method in cottonseed which had been ammoniated at atmospheric pressure and ambient temperature (10). However, the initial total aflatoxin levels in that study were 7 ppm or less. Most of the aflatoxin ammoniation products in grain are still unaccounted for. This study has shown MS-MS to be a
worthwhile technique for problems of detection or confirmation of minute amounts of target components in small samples by determining the presence of aflatoxin D₁ after ammoniation of only 10 kernels of moldy corn.

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**LITERATURE CITED**


