in Fig. 1. Chromatograms of a blank plasma sample (A) and a 10-ml plasma sample spiked with 50 ng of tilidine, nortilidine, and bisnortilidine and 500 ng of internal standard (B) are shown in Fig. 2.

The internal standard showed similar extraction properties to tilidine and the metabolites and was consequently well suited for quantitative analysis. The recovery of all substances from plasma ranged from 88 to 92%.

The accuracy and precision of this method are reflected by the data obtained from triplicate analyses of spiked plasma samples at different concentrations (Table I). The sensitivity limit was ~1 ng/ml for all three substances with a 5-ml sample.

The usefulness of an integrator system was checked by a special case regression analysis with the assumption that the line passes through the origin. For tilidine, nortilidine, and bisnortilidine, correlation indexes of 99.74, 99.38, and 99.90%, respectively, were found. In all three cases, the correlation coefficient was above 0.996. The slopes of the regression lines pressed through the origin did not differ from those obtained by a regression analysis with both degrees of freedom. With both methods, slopes of 0.0040, 0.0042, and 0.0029 for tilidine, nortilidine, and bisnortilidine, respectively, were calculated.

Some drugs or their metabolites were responsible for peaks appearing later in the chromatographic run and delayed the analysis. These substances must be eluted by increasing the column temperature prior to the next analysis. Therefore, volunteers who have taken no medication should be used for pharmacokinetic studies.

The described method is well suited for the determination of tilidine, nortilidine, and bisnortilidine in plasma and urine following therapeutic doses of the analgesic tilidine hydrochloride to humans. The results of a plasma level study after a single administration of 50 mg iv of tilidine hydrochloride to a fasting male volunteer (age 32, 70 kg) are given in Fig. 3. Figure 4 shows a typical chromatogram of a plasma sample after oral administration of tilidine hydrochloride to a male volunteer. This method is currently employed for routine analyses of biological samples.

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Toxic Substances Produced by *Fusarium* V: Occurrence of Zearalenone, Diacetoxyscirpenol, and T-2 Toxin in Moldy Corn Infected with *Fusarium moniliforme* Sheld.

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Abstract □ Sweet corn infected with *Fusarium moniliforme* Sheld. (CMI-IMI 204057) while growing in the fields was shown to contain zearalenone, diacetoxyscirpenol, and T-2 toxin. Assays by spectral, chemical, and biological methods established the presence of these substances, with zearalenone being the most abundant. *In vitro* cultures of the fungus also produced the three toxins.

Keyphrases □ *Fusarium moniliforme* mold—infected sweet corn and *in vitro* cultures, toxins isolated and identified □ Toxins—isolated and identified in extracts of sweet corn infected with *Fusarium moniliforme* mold and *in vitro* cultures

Toxicity of natural products from *Fusarium* has received wide attention because of its greater incidence and, perhaps, greater importance than aflatoxins (1, 2). As part of a continuing study on *Fusarium*-induced toxicity of foodstuffs (3–6), this paper reports the isolation, characterization, and quantitation of three mycotoxins, zearalenone, diacetoxyscirpenol, and T-2 toxin, from moldy sweet corn (*Zea mays* Linn., local variety) infected with *Fusarium moniliforme*[1] Sheld. while growing in the fields. The samples were collected from the valley of the Ganges in the Varanasi District of Uttar Pradesh, India, during July 1976, when they were ready for table use.

EXPERIMENTAL

Extraction of Mycotoxins—The ground moldy corn (about 500 g) was macerated in a blender[2] with chloroform (2 liters), and the mixture was kept at 35 ± 5° for 1 week. It then was filtered, and the solvent was removed from the chloroform extract when a thick brown oily liquid was obtained. This substance was dissolved in methanol–water (80:20, 100 ml) and extracted with *n*-hexane (3 × 30-ml portions) to remove the lipid materials.

The aqueous methanol layer was further diluted with water (50 ml) and then extracted with chloroform–ethyl acetate (1:1, 2 × 30-ml portions). Evaporation of the chloroform–ethyl acetate extract gave a light-brown oily substance (1.08 g), which was found to be biologically active when tested according to literature methods (7, 8). It showed several fluorescent and Ehrlich-positive spots on TLC on silica gel G[3]. Benzene–methanol–acetic acid (24:2:1) was used as the developer.

A portion of the oily liquid (0.52 g) was dissolved in chloroform (10 ml) and chromatographed over a column of silica gel[4] (60–120 mesh, 1.8 × 40 cm). Elution was carried out with benzene–ethyl acetate (80:20, 200 ml). Fractions (10 ml) were collected.

Zearalenone—The residue from fractions 4–7, an amorphous solid (8 mg), showed a faint blue fluorescent spot, R_f 0.48, under UV light and an olive-green fluorescent spot when sprayed with concentrated sulfuric acid with subsequent heat treatment (100–110° for 10 min) on thin-layer plates.

Attempts to crystallize the solid from common organic solvents were unsuccessful. The compound fragmented before giving any molecular

[1] The identity of the fungus, Strain CMI-IMI 204057, was confirmed by the Commonwealth Mycological Institute, Kew, England.

[2] Waring.

[3] E. Merck, Darmstadt, West Germany.

[4] British Drug Houses, Poole, England.

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ion peak in its mass spectrum. However, co-TLC behavior with an authentic sample of zearalenone[5] and the UV molar extinction values [λ_{max} (ethyl alcohol) (log ϵ) of the compound from the moldy corn: 235 (4.46), 275 (4.04), and 315 (3.77) nm; λ_{max} (ethyl alcohol) (log ϵ) of zearalenone reported in the literature (9): 236 (4.47), 274 (4.14), and 316 (3.77) nm] suggested that the compound was almost pure zearalenone.

The isolated compound was tested for estrogenic activity (10) in the mice uterus bioassay. An alcoholic solution of the compound, in doses of 40 μg/mouse, was mixed with a ground corn supplement ration and fed to five ovariectomized mice for 3 days. The animals were sacrificed on the 4th day, and their uteri were weighed; a 38–45% increase in uterine weight was recorded. On the basis of the bioassay, the amount of zearalenone present in the moldy corn was estimated as 30 μg/g.

Diacetoxyscirpenol—Fractions 12–15, on evaporation, gave a brown oily liquid (16 mg). It showed an Ehrlich-positive spot on TLC, R_f 0.27. Co-TLC with an authentic sample of diacetoxyscirpenol[6] using ethyl alcohol–ethyl acetate–acetone (1:4:4), ethyl alcohol–chloroform–acetone (1:4:4), and ethyl alcohol–benzene–acetone (1:3:3) suggested that the major component in the brown oily liquid was diacetoxyscirpenol.

The major component was separated by preparative TLC as a colorless amorphous solid (7 mg). It showed $[\alpha]_D^{24}$ +17.8° (c 0.78, ethyl alcohol); mass spectrum: *m/e* 306 (relative abundance 58%), 278 (25), 263 (18), 233 (12), 124 (78), 123 (70), 122 (68), and 105 (100). These values are indistinguishable from those reported in the literature (3).

In the rat skin dermal toxicity test (8), the compound from the moldy corn produced reddish wheals in doses of 200 μg/rat.

T-2 Toxin—The residue from fractions 18–20, another brown oily liquid (11 mg), showed a blue fluorescent spot, R_f 0.08, in addition to three minor gray spots of higher R_f values when the plate was sprayed with concentrated sulfuric acid, treated with heat, and viewed under UV light. The fluorescent component was separated by preparative TLC as a homogeneous gummy material (2 mg). Attempts to crystallize the compound were unsuccessful, presumably because of the scarcity of material.

Co-TLC of the homogeneous gummy material with authentic T-2 toxin[5] in the three different solvent systems suggested that they were identical. The major fragment ion peaks of the compound isolated from the moldy corn were: *m/e* 382 (relative intensity, 11%), 365 (16), 364 (28), 277 (22), 205 (45), 180 (72), 126 (55), 122 (68), and 121 (100). These values were similar to those exhibited by the authentic T-2 toxin[5] and indistinguishable from those reported for T-2 toxin (11).

A chloroform solution of the brown oily liquid, in 400-μg doses, caused necrosis and hemorrhaging in all animals when applied on the shaved skin of four albino rats. Pure T-2 toxin and the homogeneous gummy material from the moldy corn produced similar necrosis and hemorrhaging in albino rats in doses of 100 μg/rat.

Extraction of Intra- and Extracellular Toxins of *F. moniliforme*—The fungus was grown in Richard's solution (200 ml) in a still culture flask (1 liter) at 21° for 21 days. Workup of the intra- and extracellular extractives in the usual fashion (3) afforded a brown oily substance (0.47 g) which, when tested according to published methods, demonstrated emetic activity and dermal toxicity as reported for T-2 toxin (7, 8) and was positive in the hyperestrogenism test as reported for zearalenone (10). It was partitioned in solvents of graded polarity, as already described, and a light-brown semisolid (0.05 g) was obtained.

Identification by TLC indicated that the three toxins, isolated from the moldy corn, were also present in the semisolid substance. From this mixture, the toxins were separated into individual entities by TLC and column chromatography. UV absorption spectra, optical rotation data, and biological assay indicated that the amounts of zearalenone, diacetoxyscirpenol, and T-2 toxin were 5, 3, and 0.8 mg, respectively, from the extra- and intracellular extracts of a 1-liter flask.

RESULTS AND DISCUSSION

Extraction of moldy corn infected with *F. moniliforme* Sheld. with chloroform, followed by partitioning in solvents of graded polarity, column chromatography, and preparative TLC, afforded three recognized mycotoxins—*viz.*, zearalenone, diacetoxyscirpenol, and T-2 toxin, in quantities sufficient for their characterization.

Table I—Toxins Isolated from Infected Sweet Corn

Source	Zearalenone	Diacetoxyscirpenol	T-2
Naturally infected sweet corn	16 μg/g	14 μg/g	4 μg/g
Artificially infected sweet corn[a]	48 μg/g	17 μg/g	5 μg/g
Flask culture	5 mg/flask	3 mg/flask	0.8 mg/flask

[a] In closed polythene bags.

On the basis of chemical, spectral, and biological analyses, the amounts of zearalenone, diacetoxyscirpenol, and T-2 toxin present in the naturally infected moldy corn were estimated as 16, 14, and 4 μg/g, respectively. Zearalenone is known to cause severe genital disorders in dairy cattle (9, 10), diacetoxyscirpenol causes dermal toxicity in albino mice and rats in small doses and death in high doses (8), and T-2 toxin causes necrosis and hemorrhaging in albino rats and demonstrated emetic activity when tested according to published methods (7, 8, 12).

The intra- and extracellular extracts of the fungus, grown in Richard's medium, produced emetic activity in pigeons, dermal toxicity in albino rats, and hyperestrogenism in albino mice when tested according to published methods (7, 8, 10, 12). These observations indicate that the CMI-IMI 204057 strain of the fungus is a producer of diacetoxyscirpenol, T-2 toxin, and zearalenone. The individual toxins from the culture extracts subsequently were isolated and quantitated.

The three toxins were isolated from three sources of fungi, and their relative amounts are shown in Table I. The three toxins were isolated from moldy sweet corn, infected while growing in the valley of the Ganges during July 1976 (a rainy season; temperature 35 ± 5°; relative humidity 41–79%) in India. This is the first report of the toxins being produced in relatively high temperature (35 ± 5°); previously, their occurrence was reported in overwintered corn (9, 12). People in India consume sizable amounts of sweet corn as a vegetable in semibaked form so that toxins would probably not be destroyed in cooking. Prolonged ingestion of the moldy corn presents a high risk of poisoning in humans.

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