Identification of a Potential Interference in the Analysis of Aflatoxin 2,4-Dimethyl-6-ethoxyquinoline, a Reaction Product of Ethyoxyquin

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Investigation of the chromatography and chemical reactions of ethoxyquin has provided the chemical identity of a reaction product of ethoxyquin which could interfere in the analysis of mixed commercial feeds for aflatoxin. This product occurs in commercial feeds. Chemically it is 2,4-dimethyl-6-ethoxyquinoline. Spectrometric data have been used to confirm its identity.

The presence of interfering nonaflatoxin fluorescent components in aflatoxin thin-layer chromatographic (TLC) systems is well known (Schroeder, 1968; Shotwell et al., 1968; Moss, 1972; Seitz et al., 1975). We have frequently encountered unidentified compounds with the same bluish fluorescence as aflatoxin and similar or differing R_f values, during TLC screening of various feed extracts for aflatoxins and other mycotoxins. In a preliminary investigation aimed at identifying some of these fluorescent components, we investigated the TLC behavior of ethoxyquin, a common feed antioxidant.

It has recently been claimed (Issaq et al., 1977) that ethoxyquin produces the same bluish fluorescence under long wavelength UV irradiation as aflatoxin B_1 in several TLC solvent systems. However, in an earlier report by Stefaniak (1969), it was shown that it was not ethoxyquin (1,2-dehydro-2,2,4-trimethyl-6-ethoxyquinoline, I) itself which exhibited aflatoxin-like blue fluorescence at similar R_f's to aflatoxin B_1, but rather unidentified chemical impurities accompanying this compound. Stefaniak found that ethoxyquin had a higher R_f than the blue fluorescent impurity and aflatoxin B_1, in a number of different solvent systems.

Our investigation led us to the discovery that 2,4-dimethyl-6-ethoxyquinoline (II), an ethoxyquin conversion product and artifact, has similar R_f's and bluish fluorescence on TLC as aflatoxin B_1, and that this conversion product appears in commercial ethoxyquin samples and feeds containing ethoxyquin in their formulation.

MATERIALS AND METHODS

Thin-layer chromatography was carried out on precoated silica gel 60 F-254 plates (E. Merck, Germany) of 0.25-mm layer thickness, or on silica gel G (E. Merck), 0.25-mm thickness, on glass.

Mass spectra were measured for probe samples at 70 eV on a Finnigan Model 4000 mass spectrometer.

Proton nuclear magnetic resonance (^{1}H NMR) spectra were determined in CCl_4 with a Varian HA-100 spectrometer and are recorded as parts per million (δ) relative to tetramethylsilane (internal standard). Melting points were taken on a Kofler hot-stage microscope and are corrected.

Synthesis of 2,4-dimethyl-6-ethoxyquinoline. To a 500-mL three-neck flask fitted with a gas delivery tube, water-cooled condenser, and heating mantel were added 32 mL of ethoxyquin (Feed Specialties Co., Des Moines, IA) and 250 mL of chlorobenzene. Oxygen (1 L/h) was then bubbled through the pale-yellow solution, which was heated under gentle reflux for 18 h. The cooled reaction product was shaken with 200 mL of 1 N HCl and 100 mL of $CHCl_3$ and allowed to stand. The lower chlorobenzene–$CHCl_3$ layer was discarded; the upper aqueous phase was extracted with 2 × 100 mL of petroleum ether. The extracted aqueous phase was then treated with 4 M NaOH solution, until its pH was 11. This basic solution was extracted with 200 mL of $CHCl_3$, and the $CHCl_3$ extracted was washed with 2 × 100 mL of H_2O, dried over anhydrous Na_2SO_4, filtered, and evaporated to give a dark-brown syrup which eventually solidified. The solid product was dissolved in a small volume of ethanol, and to this was added a solution of 100 g of picric acid in 120 mL of ethanol. An immediate dense yellow precipitate of a picrate was obtained. This was filtered off, resuspended in fresh ethanol, and filtered to give 29.3 g of yellow solid.

A portion of the above yellow solid was dissolved in dimethyl sulfoxide (Me_2SO) and eluted through a column of basic aluminum oxide (J.T. Baker and Co., J.J.; Brockman Activity Grade I). The picric acid remained on the column, and a light-tan clear solution eluted from the column: this solution was intensely fluorescent (blue) under UV irradiation. The Me_2SO eluate was partitioned against 100 mL of petroleum ether, and the petroleum ether phase washed with 3 × 50 mL of H_2O. Evaporation of the nearly colorless petroleum ether solution under vacuo gave an almost white crystalline solid. This was dissolved in the minimum volume of petroleum ether and allowed to stand. Colorless needles of 2,4-dimethyl-6-ethoxyquinoline formed, mp 86–87 °C.

Purification of Ethoxyquin. Samples of ethoxyquin (Santoquin, Monsanto, St. Louis, MO) were applied to silica gel G TLC plates (20 × 20 cm, 0.25-mm thickness) and developed in toluene/ethyl acetate/acetone (3:2:1, v/v). This gave a wide yellow band at the top of the plate; also, when viewed under long-wavelength UV, an intensely blue fluorescent band was observed midway up the plate. Both bands were scraped from the plate, and the recovered silica gel eluted with chloroform/methanol (7:3, v/v) in pipets plugged with glass wool. Evaporation of the eluates gave a pale-brown thick liquid identified as pure ethoxyquin (see below) corresponding to the upper band, and a colorless solid identified as 2,4-dimethyl-6-ethoxyquinoline (II) (see below) corresponding to the lower band.

Extraction of Poultry Feed. (*a*) *Poultry Feed Not Containing Ethoxyquin.* A 50-g portion of a typical commercial poultry feed (Wayne Pullet Starter, Medicated M-60, Allied Mills Inc., Chicago, IL) was extracted in the following manner: The weighed feed sample was blended with 200 mL of acetonitrile (180 mL)–4% KCl (20 mL) in a Waring blender at high speed for 2 min. One hundred milliliters of the supernatant phase was then filtered through a glass filter paper (Whatman GF/A) and transferred to a 500-mL separatory funnel fitted with a Teflon stopcock. This extract was defatted by extracting twice with 50 mL of isooctane (2,2,4-trimethylpentane). The acetonitrile–H_2O phase was shaken for 2 min with $FeCl_3$

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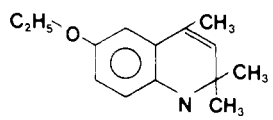
gel (100 mL of distilled H_2O), with 10 mL of 10% $FeCl_3$ solution, and the pH was adjusted to ca. 4.6 with 4% NaOH. After standing, 100 mL of the decolorized extract was drained from the funnel into a beaker. This decolorized extract was extracted with two 50-mL portions of chloroform, and the combined chloroform extracts were evaporated under nitrogen on a water bath, prior to TLC examination.

(*b*) *Poultry Feed Containing Ethoxyquin.* A 50-g portion of Doboy Turkey Start R Jets (Domain Industries Inc., New Richmond, WI), a poultry feed including ethoxyquin in its formulation, was extracted in the above manner. TLC examination of the concentrated extract revealed the presence of several blue fluorescent bands, one of which had identical R_f with synthetic 2,4-dimethyl-6-ethoxyquinoline. This band was scraped from the TLC plate and eluted with chloroform/methanol (7:3, v/v); the concentrated eluate was further examined by TLC and MS.

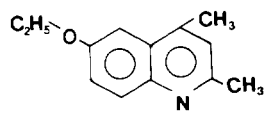
RESULTS AND DISCUSSION

Thin-layer chromatography of a commercial ethoxyquin sample on silica gel G in toluene/ethyl acetate/acetone (3:2:1) gave a wide yellow-colored band at the top of the plate. Also, irradiation by long-wavelength UV light revealed an intensely blue fluorescent band midway up the plate. Elution of the yellow silica gel band allowed isolation of a pale-brown liquid, which could not be induced to solidify. This liquid was identified as ethoxyquin itself, i.e., 6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline (I). Thus its mass spectrum showed a molecular ion $M^{+\cdot}$ at m/e 217, a base peak at m/e 202, and other peaks including a strong signal at m/e 174. These data were in agreement with the mass spectral data recently published (Skaare and Dahle, 1975) for 6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline.

To further confirm the identity of the pale-brown liquid as I, a 100-MHz NMR spectrum was determined in CCl_4. A 60-MHz NMR spectrum has previously been published for ethoxyquin (Tung, 1963; Nekipelova and Gagarina, 1976). However, the 100-MHz spectrum obtained in the present study contained additional fine structure allowing complete and unambiguous assignment of the aromatic substitution pattern and, in fact, is consistent only with structure I. Thus, aromatic signals appeared at δ 6.54 (d,



Structure I



Stucture II

1 H, $J_m \sim 3$ Hz, C-5 H), 6.44 (dd, 1 H, $J_m \sim 3$ Hz, $J_o \sim 8$ Hz, C-7 H), and 6.19 (d, 1 H, $J \sim 8$ Hz). These assignments are completely consistent with the NMR spectra of substituted 1,2-dihydroquinolines, in which the C-5 H lies furthest downfield, and the C-8 H lies furthest upfield in the aromatic region (Batterham, 1973a). Other signals in the 100-MHz NMR spectrum of I included a doublet at δ 5.22 (1 H, $J \sim 1.8$ Hz) due to the vinylic H on C-3 coupled to the vinylic methyl group on C-4 which appeared as a doublet at δ 3.21 (1 H), due to the N-H; a triplet at δ 1.33 (3 H, $J = 7$ Hz) coupled to a quartet at 3.88 (2 H, $J = 7$ Hz) due to the C-6 ethoxyl group protons; and a singlet at δ 1.2 (6 H), due to the C-2 methyl groups.

The blue fluorescent band from the thin-layer chromatogram of ethoxyquin (I) yielded a colorless solid, identified as 2,4-dimethyl-6-ethoxyquinoline (II), in the following manner. First, the mass spectrum of this compound had a molecular ion $M^{+\cdot}$ at m/e 201, a base peak at m/e 173, and other peaks including a strong peak at m/e 144. This spectrum was essentially identical with that of II (Skaare and Dahle, 1975). Next, the 100-MHz NMR spectrum was determined. This spectrum showed some dramatic differences to that of the parent ethoxyquin. Thus, there were only two ring methyl signals present in the spectrum of the fluorescent compound, and these occurred as singlets at δ 2.48 and 2.56. Such downfield positions for methyl singlets indicated that they were substituents in a fully aromatic system, e.g., the methyl signals of 6-ethoxy-2,4-dimethylquinoline (II) appear at δ 2.40 and 2.53 in CCl_4 (Batterham, 1973b). Apart from a triplet at δ 1.45 (3 H, $J = 7$ Hz) and a quartet at δ 4.03 (2 H, $J = 7$ Hz) due to ethoxyl group signals, the rest of the signals of the molecule were in the aromatic region. These signals were interpreted as follows: a doublet at δ 7.80 (1 H, $J_o \sim 8$ Hz), a doublet of doublets at δ 7.17 (1 H, $J_o \sim 8$ Hz, $J_m \sim 3$ Hz), a doublet at δ 6.92 (1 H, $J_m \sim 3$ Hz), and a singlet at δ 6.89 (1 H), belonging to C-5 H, C-7H, C-8H, and C-3H, respectively, in II. Finally, II was synthesized from I by the method of oxidation described by Nekipelova and Gagarina (1976). This remarkable oxidation, accompanied by the loss of a molecule of methane from the starting material, is a known reaction of alkyl-substituted 1,2-dihydroquinolines (Zobian et al., 1964; Vaughan, 1948). In the present study, ethoxyquin was heated in refluxing chlorobenzene whilst oxygen was bubbled through the reaction solution. Purification of the product was achieved by partitioning and extraction, followed by picrate salt formation. The purified picrate salt was then decomposed by chromatography on aluminum oxide, and the quinoline crystallized from petroleum ether to yield colorless needles of II, mp 86–87 °C [cf. 87–88 °C obtained by Nekipelova and Gargarina (1976)]. This synthetic material was identical in all respects with the blue fluorescent compound isolated by thin-layer chromatography of technical ethoxyquin, as described above.

The fluorescent properties and TLC behavior of II confirmed the reports of Stefaniak (1969) and Issaq et al. (1977) that in some aflatoxin analysis systems the aflatoxin-like blue fluorescence could cause interference and confusion. However, as pointed out by Issaq et al., TLC in the solvent system benzene/methanol/acetic acid (90:5:5) clearly resolves aflatoxin B_1 (R_f 0.27) and the fluorescent compound (R_f 0.01). It would appear that salt formation of II in the acid solvent system inhibits migration of II, in comparison to the neutral aflatoxin molecule.

The usefulness of our finding was demonstrated by analysis of commercial poultry feed pellets known to contain ethoxyquin in their formulation. TLC analysis of the regular mycotoxin extract, obtained via a multimycotoxin analysis procedure used routinely in this laboratory (Stahr, 1977) revealed the presence of several blue fluorescent bands, one of which had identical R_f with the synthetic II. This band was recovered from TLC and subject to MS examination, revealing that it was indeed II. Thus, a source of one of the troublesome blue-fluorescent bands obtained during TLC analysis of certain feeds was identified. It should be pointed out that the official AOAC confirmatory procedure (AOAC, 1975) for aflatoxin will confirm that the compound is not aflatoxin. Also, ultraviolet or spectrofluorometry will furnish enough information to eliminate aflatoxin as a possible compound. Thin-layer chromatography will allow these confirmatory procedures. The official AOAC procedure for mixed feeds uses a column, and with that system, these materials will not be differentiated.

It should be noted that there are a number of reports in the literature on the biological activity of ethoxyquin.

The first toxicity studies on ethoxyquin, involving the effects on rats, were reported in 1959 by Wilson and De Eds. More recently, in common with several other feed antioxidants, ethoxyquin has been demonstrated to modify the acute toxicity of certain mutagenic and carcinogenic chemicals to the mouse (Cumming and Walton, 1973) and the rat (Skaare et al., 1977). Also, ethoxyquin potentiated the antimicrobial activity of the antioxidant butylated hydroxytoluene (Turcotte and Saheb, 1978). Most interestingly, significant effects of ethoxyquin on aflatoxin-producing strains of *Aspergillus flavus* and *A. parasiticus* have very recently been reported (Foudin et al., 1978). This latter report indicated that ethoxyquin had the unique capability of selectively altering aflatoxin synthesis in *A. parasiticus* strains which produce both B and G type aflatoxins. The possible importance of the ethoxyquin conversion product 2,4-dimethyl-6-ethoxyquinoline in phenomena such as these is unknown, but clearly should be considered in future studies.

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Diplodiol: A New Toxin from *Diplodia macrospora*

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A toxic metabolite, *trans*-6-ethyl-5-hydroxy-3-hydroxymethyl-5,6,7,8-tetrahydrochromone, trivial name diplodiol, was isolated from *Diplodia macrospora*, a pathogen of corn. It was toxic to day-old cockerels and the calculated LD_{50} was 88.4 mg/kg. The toxin may contribute to chick mortality in Mexico and Central and South America.

Diplodia macrospora Earle, a fungal pathogen of corn, causes ear- and stalk-rot, and under humid conditions, a severe leaf spot. The fungus has been known for many years in the southern corn belt of the U.S. as a minor cause of ear- and stalk-rot, but not often as a leaf pathogen (Eddins, 1930). In the humid tropics and subtropics of Mexico and the countries of Central and South America, we have found *D. macrospora* to be widely distributed, occurring most commonly as a leaf pathogen, but also present as an ear-rotting organism, along with *D. maydis* (Berk.) Sacc., *Fusarium* spp., and other fungi.

In 1970, De León and Perez reported from Mexico on severe maladies in chicks that were fed corn grains infected with *Diplodia* spp., including heart and liver enlargement, effusion of major organs, rupturing of blood vessels in the skin, and premature death. They attributed the injury to a mycotoxin produced by *D. maydis*. Steyn et al. (1972), working in South Africa, isolated and defined the structure of a toxic metabolite from *D. maydis*, "diplodiatoxin". Further work on the toxicity of *D. maydis* to ducklings and rats was reported by Rabie (1977).

We report the isolation of a new toxin from cultures of *D. macrospora*, *trans*-6-ethyl-5-hydroxymethyl-5,6,7,8-tetrahydrochromone (I) (Figure 1), to which we have assigned the trivial name diplodiol, and describe its effect on chicks.

MATERIALS AND METHODS

Production and Purification of the Toxin. *Diplodia macrospora* (ATCC accession no. 36896) was isolated from

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