Journal of Agricultural and Food Chemistry

Volume 23, Number 1 January/February 1975

Metabolites of *Alternaria* in Grain Sorghum. Compounds Which Could Be Mistaken for Zearalenone and Aflatoxin

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Metabolites, alternariol (AOH) and alternariol monomethyl ether (AME), were isolated from discolored, weathered grain sorghum that had been invaded by *Alternaria* sp. These compounds were found in 1973 spring-harvested (overwintered) sorghum and in weathered sorghum harvested in the fall of 1973. Thin-layer chromatograms and fluorescence of AME and zearalenone are similar. Means of avoiding confusion of AME, AOH, and other fluorescent substances in grain sorghum with zearalenone and aflatoxin are discussed.

Information is scarce concerning detection of mycotoxins in grain sorghum. Shotwell *et al.* (1969) referred to a fluorescing substance in grain sorghum that migrated on thin-layer chromatographic (tlc) plates similarly to aflatoxins G_1 and G_2. They also reported that extracts of "highly damaged" samples contained substances that made interpretation of tlc results difficult. However, "highly damaged" and the nature of the interference in tlc were not defined.

Because of wet weather in the Midwest, during the fall and winter of 1972–1973, much of the sorghum crop was weathered, discolored, and/or mold-invaded. Many Kansas farmers had to salvage grain from their fields in the spring of 1973. This salvaged sorghum was in poor condition and questions were raised as to whether it contained mycotoxins. Farmers and elevator operators sent samples to commercial laboratories in Kansas and surrounding states. Reports from some of the laboratories indicated that high concentrations of aflatoxins were present in some samples. We obtained a sample that a laboratory had reported contained 200 ppb of aflatoxin. Our analysis showed that the sample did not contain aflatoxin, but rather several other fluorescent substances which might be confused with aflatoxin in some analytical procedures.

In the salvaged sorghum we found a substance which might be mistaken for zearalenone (Mirocha *et al.*, 1967, 1971). We isolated the substance and identified it as alternariol monomethyl ether (AME), a metabolite of *Alternaria* (Raistrick *et al.*, 1953; Thomas, 1961). The metabolite, alternariol (AOH), was also isolated. These metabolites also were found in weathered sorghum harvested in the fall of 1973. The overwintered and fall-harvested samples with AME and AOH were heavily invaded by *Alternaria*.

In this paper we present evidence for the presence of AME and AOH in weathered sorghum and report means to avoid confusing these compounds and other fluorescent substances in sorghum with zearalenone and aflatoxin.

EXPERIMENTAL SECTION

Routine Analysis of Sorghum. We have applied a screening test procedure originally developed for detection of aflatoxin in corn (Seitz and Mohr, 1974). Analysis can be completed quickly and minimizes solvent and reagent requirements. The procedure will detect aflatoxin at 5 ppb and zearalenone at about 200 ppb. A conservative estimate of the detection limit for AME and AOH is 100 ppb each.

Thin-Layer Chromatography. We used Brinkman SILG-HR-25 precoated plates activated by heating 1 hr at 120°. Usually 25 μl of the final extract was spotted, and the plates were developed with chloroform–acetone (88:12) (CA). However, in further attempts to separate zearalenone from AME, the following solvents were tried: ben-

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zene–methanol–acetic acid (18:1:1) (BMA); hexane–acetone–acetic acid (18:2:1) (HAA); chloroform–ethanol (95:5) (CE); toluene–ethyl acetate–formic acid (5:4:1) (TEA); benzene–ethanol (96:4, v/v, with about 5 drops of aqueous NaOH solution (0.4 *N*) added to the developing tank) (BE). The first four solvents have been used in various analytical procedures for zearalenone (Mirocha *et al.*, 1967; Caldwell *et al.*, 1970; Epply, 1968; Stoloff *et al.*, 1971). The latter solvent was suggested for AME and AOH analysis (Pero *et al.*, 1971a).

Fluorescent spots on tlc plates were observed in a cabinet equipped with long- and short-wave uv lamps.

Standards. A large sample of zearalenone was supplied by Commercial Solvents Corporation, Terra Haute, Ind. Zearalenone standard was also supplied by the Food and Drug Administration, Washington, D.C. Aflatoxin standards were obtained from the USDA–ARS Southern Regional Research Center, New Orleans, La. Samples of AME and AOH were received from Dr. R. Pero of the National Institute of Environmental Health Sciences, Research Triangle Park, N. C.

Isolation of AOH and AME. A scaled-up version of the routine analysis procedure was used to isolate AOH and AME from spring-harvested sorghum. Instead of methylene chloride, benzene was used to extract the aqueous–methanol–ammonium sulfate layer. The benzene extracts were dried over anhydrous sodium sulfate, concentrated, and chromatographed on a scaled-up (3×) silica gel column prepared in proportions recommended by Epply (1968). After addition of the sample, the column was first washed with 250 ml of benzene and then with 250 ml of 5% acetone in benzene to elute AME. Alternariol was eluted with 250 ml of 4% methanol in benzene. Five of these columns were required to accommodate the concentrated benzene extracts from about 15 kg of grain. The combined AME fractions and the combined AOH fractions were each reduced to about 5 ml and placed in a refrigerator for crystallization. Crystals were collected by centrifugation. AME was recrystallized three times from 5–10% acetonitrile in benzene. Each kilogram of grain yielded only about 1 mg of AME and even less of AOH, so further purification was by preparative thin-layer chromatography (CA developing solvent).

Spectroscopic Studies. Infrared (KBr pellet) and ultraviolet (absolute ethanol, 1-cm cells) spectra were recorded with Perkin-Elmer spectrophotometers, Models 257 and 350, respectively. An AEI MS-902 mass spectrometer at Kansas State University was used to record mass spectra at 70 and 18 eV. High-resolution mass spectra were obtained by the Mass Spectroscopy Laboratory at the University of Minnesota. Proton magnetic resonance spectra of AME in DMSO-d_6 (100% D, Aldrich Chemical Co.) were obtained using a Varian Associates XL-100-15 spectrometer equipped with a time-averaging computer. The spectrometer was locked on the deuterium signal from DMSO-d_6. Although a tetramethylsilane (TMS) signal is not shown in Figure 1, DMSO-d_6 containing TMS was used to adjust the offset setting to correspond to TMS at 0 ppm.

RESULTS AND DISCUSSION

On tlc plates, extracts from the routine analysis gave spots near positions expected for zearalenone and aflatoxins. Two moieties, designated A and B, were apparently characteristic of sorghum and were present in all samples analyzed regardless of variety or degree of discoloration. Two additional compounds (AME and AOH) were observed only in certain samples.

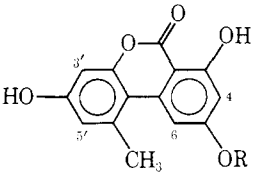
Moieties A and B. Data from tlc of A and B, zearalenone, and aflatoxins are compared in Table I. When the tlc plate was developed with chloroform–acetone (solvent CA) as commonly used for aflatoxin detection, moieties A and B migrated closely together and appeared as blue-green spots under either long- or short-wave ultraviolet light. Moiety B was usually brighter and slightly more blue than moiety A; both were more highly visible under long- than under short-wave light. Their colors suggested that these moieties were more likely to be confused with aflatoxins G_1 or G_2 than with aflatoxins B_1 or B_2. These moieties may represent the substances referred to by Shotwell *et al.* (1969). We have not attempted to chemically identify these substances.

Table I. Thin-Layer Chromatographic R_f Values of Substances Found in Sorghum Grain Compared to Zearalenone and Aflatoxins

	Developing solvents[a]					
	CA	BMA	HAA	CE	TEA	BE
AME	0.65	0.73	0.23	0.73	0.81	0.63
Zearalenone	0.65	0.74	0.26	0.76	0.80	0.68
Moiety A	0.50	0.59		0.60	0.67	0.44
Moiety B	0.47	0.59		0.56	0.69	0.36
Aflatoxin B_1	0.54	0.55				0.34
Aflatoxin B_2	0.48	0.50				0.30
Aflatoxin G_1	0.43	0.44				0.28
Aflatoxin G_2	0.37	0.38				0.22
AOH	0.39	0.49	0.083	0.42	0.74	0.30

[a] CA, chloroform–acetone (88:12, v/v); BMA, benzene–methanol–acetic acid (18:1:1, v/v/v); HAA, hexane–acetone–acetic acid (18:2:1, v/v/v); CE, chloroform–ethanol (95:5, v/v); TEA, toluene–ethyl acetate–formic acid (5:4:1, v/v/v); BE, benzene–ethanol (96:4, v/v, with about 5 drops of aqueous 0.4 *N* NaOH solution added to the developing tank).

Compounds AME and AOH. When extracts of weathered, discolored sorghum were subjected to tlc (CA solvent), spots of similar fluorescence were noted at R_f 0.65 and 0.39. The fluorescence, *i.e.* sky-blue and brighter under short-wave rather than long-wave light, and R_f values of the spots were identical with those of AME (I) (R_f 0.65) and AOH (II) (R_f 0.39) received from Dr. R. Pero. To confirm the identification, the compounds were isolated to allow investigation by uv, nmr, and mass spectroscopy. Quantities were limited because the compounds were present in the grain at about 3–5 ppm or less.



I, alternariol monomethyl ether (AME), R = CH_3
II, alternariol (AOH), R = H

The uv spectra of the R_f 0.65 and 0.39 compounds exhibited maxima at 335–342 (broad band), 301, 290, 257, and 230 nm. These maxima, as well as their relative intensities, matched those reported for AME and AOH by Rosett *et al.* (1957) and Thomas (1961), and were identical with those of AME and AOH received from Dr. R. Pero. The infrared spectrum of the R_f 0.65 compound appeared identical with spectra reported for AME by Pero *et al.* (1971b) and Pero and Main (1970).

Low-resolution mass spectra recorded at 70 and 18 eV exhibited a parent ion peak at *m/e* 272 as expected for AME. High resolution showed *m/e* 272.0659 (calcd 272.0684) and *m/e* 258.0517 (calcd 258.0527) for the R_f 0.65 and 0.39 compounds, respectively.

The 100-MHZ proton magnetic resonance spectrum of the R_f 0.65 compound (Figure 1) also corresponded to that for AME. The spectrum indicated the presence of two pairs of meta-coupled ($J \simeq 2$ Hz) aromatic hydrogens.

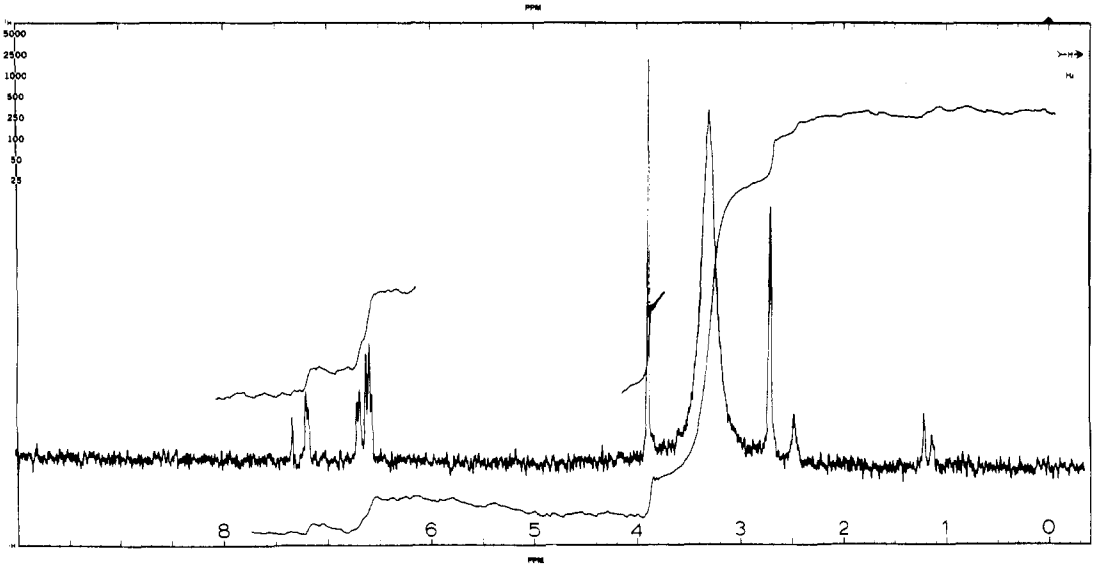


Figure 1. Proton magnetic resonance spectrum of the R_f 0.65 compound (alternariol monomethyl ether) in DMSO-d_6.

Doublets at δ 7.20 and 6.59 were assigned to the 3′ and 5′ hydrogens, respectively, while doublets at δ 6.70 and 6.62 were assigned 4 and 6 hydrogens, respectively. Decoupling experiments supported these assignments. Irradiation at δ 6.6 collapsed the 7.20 doublet to a singlet, while, in turn, irradiation at 7.20 caused appearance of a singlet at 6.59, and thus assignment of the δ 6.59 chemical shift to the 5′ hydrogen. The presence of an aromatic methoxyl group was indicated by the singlet at δ 3.90 and an aromatic methyl group by the peak at 2.71. The phenolic hydrogens were not observed due to exchange with water in the solvent, *i.e.*, the rather broad band at δ 3.3. The multiplet at δ 2.48 was due to undeuterated solvent while the small peaks at δ 1.14–1.22 and 7.35 were apparently impurities (gc analysis confirmed that an unknown impurity was present).

Comparison of AME and Zearalenone Properties. Our data show that AME might be mistaken for zearalenone (Table I). Only the benzene–ethanol solvent effectively separated these compounds. Furthermore, the fluorescence of AME and zearalenone was similar; both exhibited enhanced fluorescence under short-wave light as compared to long-wave uv light. AME fluoresces sky-blue, whereas zearalenone has a more greenish-blue tint, but the color difference is slight. Treatment with $AlCl_3$ enhances the fluorescence of AME and of zearalenone, and since the colors (bright blue) are essentially identical, this reagent is of no value in distinguishing between the two compounds. Upon exposure of the tlc plate to ammonia fumes, AME appears slightly more greenish than zearalenone. Spraying the tlc plate with 50% H_2SO_4 and heating at 130° for 10 min produced a bright yellow and a pale green fluorescence from zearalenone and AME, respectively. Uv spectrophotometry, however, is a more reliable means of making the distinction.

Cautions in the Use of Aflatoxin Procedures. The fluorescence and tlc properties of AME and AOH differ from those of the aflatoxins, especially B_1, and confusion among these compounds is unlikely if tlc is used on the final extract and the plate is developed to a height of 10 or 12 cm. Also, the developed plate should be observed under both long and short-wave uv light. Minicolumn procedures for detection of aflatoxin in corn, soybeans, peanuts, etc. (Holaday, 1968; Cucullu *et al.*, 1972; Holaday and Barnes, 1973; Screening Methods for Corn—Official First Action, 1973a,b) should not be used for sorghum grain unless interferences from AME and AOH and from moieties A and B have either been removed or accounted for. Of the minicolumn methods, that of Velasco (Velasco, 1972; Screening Methods for Corn—Official First Action, 1973b) would appear suitable because the ferric gel cleanup can remove phenolic compounds. When Epply's screening method for zearalenone, aflatoxin, and ochratoxin (Epply, 1968) is used, AME will be in the "zearalenone" fraction and AOH in the "aflatoxin" fraction. The rapid qualitative test for aflatoxin described by Knake *et al.* (1972) should be used only if the final extract is subjected to tlc.

Occurrence of AME and AOH. We found AME and AOH in sorghum grain which was weathered and discolored; they were either absent or present in barely detectable levels in good quality, normal-colored sorghum. A pair of white sorghum samples obtained from a farmer in Riley County, Kan., represented the extremes. One sample was harvested in early fall 1972 before wet weather set in. This early-harvested grain was in good condition and was not discolored or weathered. The second sample was not harvested until April of 1973 because of wet wether. The fall-harvested grain did not contain AME or AOH, whereas the badly weathered sample harvested in April did.

We first noticed AME and AOH in Kansas sorghum harvested in the spring of 1973 after having been in the field all winter. However, AME and AOH are not unique to spring-harvested (overwintered) sorghum, nor to Kansas. We have found the compounds in samples of the 1973 crop from Texas and Kansas. Much of the 1973 crop in these states was exposed to wet conditions during or shortly after ripening of the grain. Results of a study of grain sorghum from Kansas in 1973, *i.e.*, occurrence of alternariol and storability of the grain, will be reported in a subsequent publication.

Suggested Analysis Scheme. The following approach is suggested to determine which of the compounds mentioned above is present in a grain sorghum sample.

First, develop the tlc plate in CA solvent and examine under short- and long-wave uv light. If there is no blue or greenish-blue fluorescence, at or near R_f 0.65 and 0.39, which is brighter under short- than long-wave light, then AME, AOH, and zearalenone are not present. If sky-blue fluorescence is observed at both R_f 0.65 and 0.39, the presence of AOH and AME is suggested since these metabolites are produced concurrently by most *Alternaria* isolates. If greenish-blue fluorescence is observed only at R_f 0.65, the presence of zearalenone should be suspected. To differentiate between zearalenone and AME, the final extract and standards should be respotted and the plate

developed in BE solvent. If any doubt remains, uv spectroscopy should be used to confirm identity.

Examine the plate developed in CA solvent further under long-wave light to determine if aflatoxin is present. The presence of aflatoxin B_1 is indicated by a distinctly blue fluorescence near R_f 0.54, or at the same position as an aflatoxin B_1 standard. Moieties A and B, which are expected slightly below aflatoxins B_1, are usually quite faint and greenish in color and should not be confused with aflatoxins G_1 and G_2. If aflatoxin B_1 is absent, aflatoxins B_2, G_1, and G_2 are not expected. Assignment of any distinctly blue spot near R_f 0.54 to aflatoxin B_1 should be confirmed by cochromatography with an aflatoxin standard. If AME and AOH are present, the CA solvent is recommended for quantitation of the B aflatoxins even though a slight contribution from moieties A and B might occur. If AME and AOH are absent, the BMA solvent could be tried to move moieties A and B above the aflatoxin region and therefore allow for better quantitation.

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Received for review June 28, 1974. Accepted September 27, 1974. Contribution No. 624, Department of Plant Pathology, Kansas State University. Reference to a company or product does not imply approval or recommendation of the product by the U. S. Department of Agriculture to the exclusion of others that may be suitable.

Factors Affecting Chemical Stimulation of Uredospore Germination in Pustules of Crown Rust of Oats, Common Corn Rust, Stem Rust of Wheat, and Leaf Rust of Wheat

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The stimulatory activity of nonanal and related compounds on uredospore germination, previously reported on *Puccinia graminis* F. sp. *tritici* (stem rust of wheat), also has been observed on *P. coronata* F. sp. *avenae* (crown rust of oats), *P. recondita* (leaf rust of wheat), and *P. sorghi* (common corn rust). The effects of type of compound, concentration, and temperature on germination of uredospores in pustules were examined. Volatile chemical stimulators at concentrations of less than 1 ppm by volume, applied during exposure of rust pustules to dew in a sealed chamber, induced germination of spores of *P. coronata, P. sorghi, P. graminis,* and *P. recondita,* over a temperature range of 15–25°. 1-Nonanal, 1-nonanol, 1-octanol, and 6-methyl-5-hepten-2-one stimulated germination of these rust spores floated on water. Germination in pustules of *P. coronata* and *P. graminis* was induced by all four compounds; spores of *P. sorghi* and *P. recondita* did not respond in the pustule to methylheptenone under the conditions examined. Nonanal was most effective in stimulating germination of uredospores of *P. recondita* in the pustule.

1-Nonanal, an endogenous spore-germination stimulator (French and Weintraub, 1957), was identified in *Puccinia graminis* uredospore distillates. Rines *et al.* (1974) found nonanal and 6-methyl-5-hepten-2-one in *P. graminis* spore distillates and in moist air streams passed through fresh spores. In addition, nonanal was found in distillates of uredospores of *P. coronata, P. sorghi, P. recondita,* and other rusts. Of those rust uredospores studied, only these three species were chemically induced to germinate by methods then in use. Germination of spores of *P. graminis* in the pustule by action of stimulators in a closed chamber under dew-forming conditions had been reported by French and Gallimore (1972b). The object of this research was to examine several species of rust for the capability of being stimulated by nonanal and related compounds, and to check for stimulation of germination over a broad temperature range, so that the limitations of in-pustule germination as a practical technique of disease control might be further evaluated.

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MATERIALS AND METHODS

Uredospores of *Puccinia coronata* Cda. F. sp. *avenae* (crown rust of oats), *Puccinia recondita* Rob. ex Desm. f.