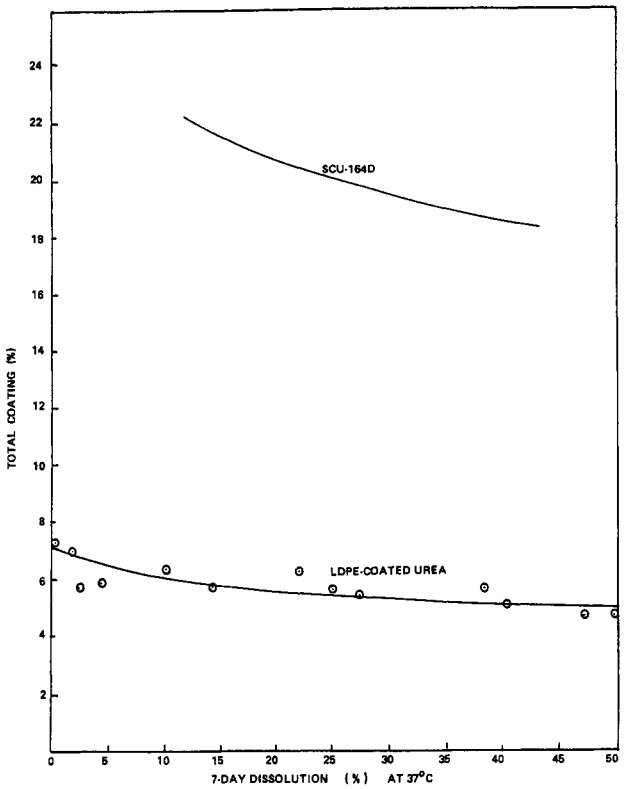

Figure 11. Comparison of the 7-day dissolution of sulfur-coated urea (SCU-164D, TVA) and LDPE-coated urea.

corresponds to a nitrogen content of 36.8% for the former and 43.4% for the latter.

A second advantage of LDPE-coated urea over SCU is its higher crushing strength. The crushing strength for a 1.7-mm prill of LDPE-coated urea is 126 kg/cm^2 (5.8% coating). A granule of SCU with a prill diameter of 1.7 mm has a crushing strength of 37 kg/cm^2. This makes SCU more susceptible to fracture upon handling, increasing the powder content and the dissolution.

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Registry No. Polyethylene, 9002-88-4; vinyl acetate–vinyl chloride copolymer, 9003-22-9; polystyrene, 9003-53-6.

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Occurrence and Chemical Determination of Zearalenone and Alternariol Monomethyl Ether in Sorghum-Based Mixed Feeds Associated with an Outbreak of Suspected Hyperestrogenism in Swine

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Swine fed sorghum-based mixed feeds in South Africa developed clinical signs of hyperestrogenism, a disease known to be linked to zearalenone contamination of feed. Thin-layer and high-performance liquid chromatographic analysis of feed samples indicated the cooccurrence of zearalenone and alternariol monomethyl ether (AME). The major contaminant was found to be AME, present in all samples at levels of 1.2–2.25 μg/g, while zearalenone was found at levels of 0.85–1.25 μg/g. With the optimization of fluorescence detector parameters, existing methods dedicated to the determination of zearalenone and its derivatives were found to be suitable for the coextraction of AME. One of these methods extracted AME at an average recovery of 91.8%, and the limit of detection was found to be in the order of 10 ng/g for both AME and zearalenone. It is concluded that zearalenone was probably responsible for the clinical signs of hyperestrogenism and that AME can easily be mistaken for zearalenone in TLC analysis of sorghum-based mixed feeds.

During 1986 four samples of mixed feeds associated with a field outbreak of suspected hyperestrogenism in swine in South Africa were received for analysis. Clinical signs observed during the field outbreak included pseudoestrus and swollen vulvas in young gilts and reduced lactation in sows with piglets. Only one field outbreak of hyperestrogenism in swine has previously been reported in South Africa, and this was associated with corn infected by *Fusarium graminearum* Schwabe and contaminated with zearalenone (Aucock et al., 1980). In the present case, the mixed feeds were sorghum based and corn free (Table I). Although zearalenone (Figure 1A) has been reported to occur naturally in sorghum in the United States (Schroeder

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Table I. Analyses of Zearalenone and Alternariol Monomethyl Ether in Four Samples of Mixed Feeds Associated with an Outbreak of Suspected Porcine Hyperestrogenism[a]

sample no.	% sorghum	zearalenone, μg/g	AME,[b] μg/g
1	61	0.85	2.10
2	69	1.25	2.25
3	67	0.90	1.20
4	64	1.00	1.90

[a] Analyzed according to the method of Bagneris et al. (1986). [b] AME, alternariol monomethyl ether.

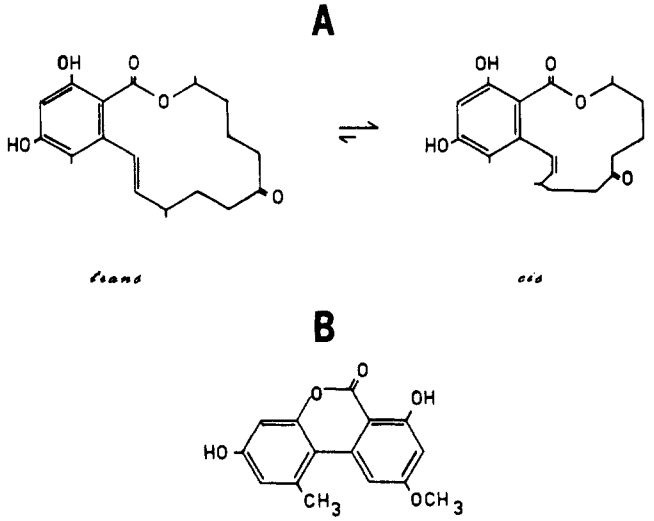

Figure 1. Chemical structures of (A) zearalenone and (B) alternariol monomethyl ether.

and Hein, 1975; Shotwell et al., 1980; McMillian et al., 1983), no reports could be found on the association of zearalenone-contaminated sorghum with field outbreaks of hyperestrogenism in swine.

Samples were screened for zearalenone contamination. However, as heavy invasion of sorghum grains by *Alternaria* have been reported in the United States (Seitz et al., 1975b) as well as in South Africa (Rabie and Lübben, 1984), subsequent analysis included the screening of the samples for the *Alternaria* metabolites alternariol (AOH) and AME as well as for α- and β-zearalenol. Furthermore, Seitz et al. (1975b) reported that the presence of AME (Figure 1B) could, under certain circumstances, interfere with zearalenone determinations. A range of analytical techniques including thin-layer chromatography, high-performance liquid chromatography, gas chromatography, and mass spectrometry have been employed for the determination of zearalenone and its derivatives (Möller and Josefsson, 1978; Ware and Thorpe, 1978; Scott et al., 1978; Diebold et al., 1979; Smyth and Frischkorn, 1980; Cohen and Lapointe, 1980; Gimeno, 1983; Swanson et al., 1984; Trenholm et al., 1984; Chang and De Vries, 1984; Bennett et al., 1985; Bagneris et al., 1986) as well as for the determination of the *Alternaria* toxins AOH and AME (Pero et al., 1971; Seitz et al., 1975a; Seitz and Mohr, 1976; Stinson et al., 1980; Heisler et al., 1980; Chu and Bennett, 1981; Wittkowski et al., 1983; Stack et al., 1985).

The chemical analysis of the sorghum-based mixed feeds associated with an outbreak of suspected porcine hyperestrogenism and the cooccurrence of zearalenone and AME in these feeds are reported in this paper.

EXPERIMENTAL SECTION

Feed Samples. Samples (1 kg) of four sorghum-based mixed feeds (Table I) associated with an outbreak of suspected hyperestrogenism in swine were obtained from a feed supplier in South Africa.

Standards. AME and AOH were isolated from *Alternaria alternata* (MRC 1031), zearalenone and β-zearalenol were obtained from Makor Chemicals, and α-zearalenol was a gift from Dr. C. J. Mirocha, University of Minnesota. Each standard was assessed for purity by TLC and HPLC procedures. Only a single component was detected in each standard.

Chemical Analyses. *1. Extraction Procedures.* Zearalenone and AME were extracted from finely ground subsamples of each mixed-feed sample according to three methods, i.e. AOAC (1984), Chang and De Vries (1984), and Bagneris et al. (1986), which differ considerably with respect to their extraction and cleanup methodologies. The AOAC method (1984) involves extraction with chloroform/water followed by chromatographic separation from lipid-type material on a silica gel column and final partition into acetonitrile prior to quantification by thin-layer chromatography. The method of Chang and De Vries (1984) relies on extraction with dichloromethane/water, precipitation of proteinaceous material with cupric carbonate, and partition into acetonitrile. Bagneris et al. (1986) also use chloroform/water for extraction purposes, followed by primary partitioning into a base solution and secondary partitioning into dichloromethane. Both latter methods use high-performance liquid chromatographic separation coupled with fluorescence detection for quantitation.

2. Qualitative and Quantitative Procedures. (*a*) *Thin-Layer Chromatography (TLC).* Aliquots of the sample extracts (5 and 10 μL) were chromatographed with zearalenone and AME-spiked sample extracts and a range (0.2–1.0 μg) of zearalenone and AME standards, on silica gel 60 TLC plates (without fluorescent indicator, Merck). The plates were developed in benzene/acetone (95:5), dried, and viewed under ultraviolet light at 366 nm. Component color development, relative intensity, and R_f values were noted and used for quantitative determinations. Plates were then sprayed with 20% aluminum chloride solution, heated, and reevaluated. Duplicate plates were developed in a second solvent system (benzene/acetic acid, 90:10) and similarly treated.

(*b*) *High-Performance Liquid Chromatography (HPLC).* Reversed-phase separations of 20-μL aliquots of each sample extract were performed on a 5-μm Nova Pak C_{18} column (Waters; 3.9 mm (i.d.) × 15 cm). Mobile phase (MeOH/H_2O, 70:30, v/v) was delivered by means of a Waters Model 510 liquid chromatographic pump, at a flow rate of 0.5 mL/min. Peak detection was performed on a Perkin-Elmer 650S fluorescence detector set at 320-nm excitation and 445-nm emission wavelengths with slit widths of 15 nm. Quantitative determinations of the zearalenone and AME present in the sample extracts were by comparison of their peak heights to those of verified zearalenone and AME standards. Spiked sample extracts were also chromatographed under the same conditions.

RESULTS AND DISCUSSION

Since the clinical signs observed in the swine suggested hyperestrogenism, the feed samples were initially screened for the presence of zearalenone, according to the AOAC method (1984). The purified extracts were chromatographed on TLC plates with use of benzene/acetone (95:5) as the mobile phase. Primary inspection under ultraviolet light showed that each sample screened contained a number of fluorescent compounds, one of which was highly fluorescent with color and R_f value characteristics similar to those of zearalenone standard. Spraying the plate with

aluminum chloride solution followed by heating enhanced the fluorescence characteristics of both the zearalenone standard and the corresponding spot in the samples. The sample extracts were then diluted 10 times with methanol and reanalyzed by TLC under the same chromatographic conditions. Quantitative assessment of the suspected zearalenone spot against zearalenone standard indicated concentrations of 14–17 $\mu g/g$ of zearalenone in the different samples. For confirmatory purposes, a similarly prepared TLC plate, including sample extracts spiked with zearalenone standard, was developed in a second mobile phase (benzene/acetic acid, 90:10) and viewed under ultraviolet light. Critical inspection showed that two narrowly spaced fluorescent compounds were present. The zearalenone standard chromatographed at R_f 0.27, whereas the major fluorescent compound in the sample (previously identified as zearalenone) chromatographed at a slightly lower R_f value of 0.24. Samples were then screened for the *Alternaria* metabolites AOH and AME, by TLC using the benzene/acetic acid developing solvent. AOH remained on the base line, but the R_f and color characteristics of the AME standard matched those of the unidentified, strongly fluorescent compound present in the sample extracts. The extracts were then analyzed by reversed-phase HPLC. Spiking of the extracts with zearalenone and AME standards showed that both compounds were present in each of the four samples.

One of the samples was also analyzed according to the methods for zearalenone analysis described by Chang and De Vries (1984) and Bagneris et al. (1986). In both cases, AME was coextracted with the target toxin. The method of Bagneris et al. (1986), which is a modification of that adopted by the AOAC following successful collaborative studies reported by Bennett et al. (1985), appeared to give the least "complex" sample extract of the three methods screened. The samples were then analyzed by the method of Bagneris et al. (1986), and the results are given in Table I.

Zearalenol is an estrogenic derivative of zearalenone and exists as two possible diastereomers (α- and β-zearalenol). Mirocha et al. (1979) reported the presence of the α isomer in animal feed, while Bottalico et al. (1985) reported the occurrence of both isomers in corn stems from southern Italy. Hagler et al. (1979) concluded that α-zearalenol was 3 times more estrogenically active than zearalenone in the rat uterus assay, whereas β-zearalenol was equal in activity to zearalenone. It was, therefore, necessary to screen the feed samples for both zearalenol diastereomers, particularly since a well-defined chromatographic peak that eluted prior to that of zearalenone in the sample extract (Figure 2A) could be seen at a retention of ±6 min. However spiking of the same extract with α- and β-zearalenol clearly demonstrated that neither diastereomer was present (Figure 2B).

In order to verify the identity of the AME detected in the feed samples, the compound was isolated and purified from a 1-kg composite sample. The extraction and primary purification were performed according to the AOAC (1984) method, though on a larger scale. The cleaned extract was further purified by two consecutive preparative TLC steps, followed by HPLC separation and collection of the appropriate fractions from several injections (HPLC conditions were the same as previously described). The combined fractions were evaporated to dryness, and the residue was subjected to mass spectral examination. The mass spectrum (Figure 3) showed excellent agreement with that reported for AME (Cole and Cox, 1981). The fluorescence detector response was found to be linear over the range

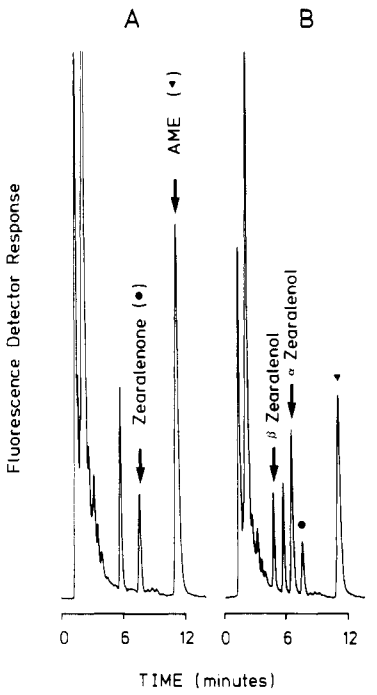

Figure 2. Chromatograms of (A) a sample extract and (B) the same extract spiked with both α- and β-zearalenol, monitored at 274-nm excitation and 418-nm emission wavelengths.

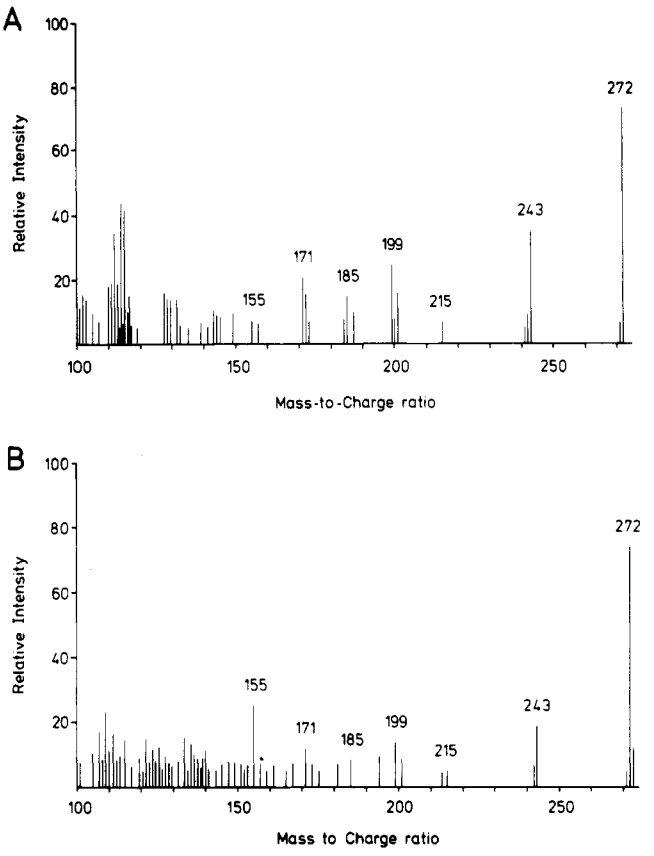

Figure 3. Mass spectra of (A) authentic alternariol monomethyl ether and (B) a compound isolated from feed samples associated with a suspected outbreak of swine hyperestrogenism.

10–45 ng for both zearalenone and AME. Triplicate determinations by the method of Bagneris et al. (1986) on control maize spiked with 90 ng/g of zearalenone and AME gave recoveries of 64% (SD = 1.34%) and 91.8% (SD = 1.68%), respectively. The limit of detection for both compounds was 10 ng/g. Although the recovery of zear-

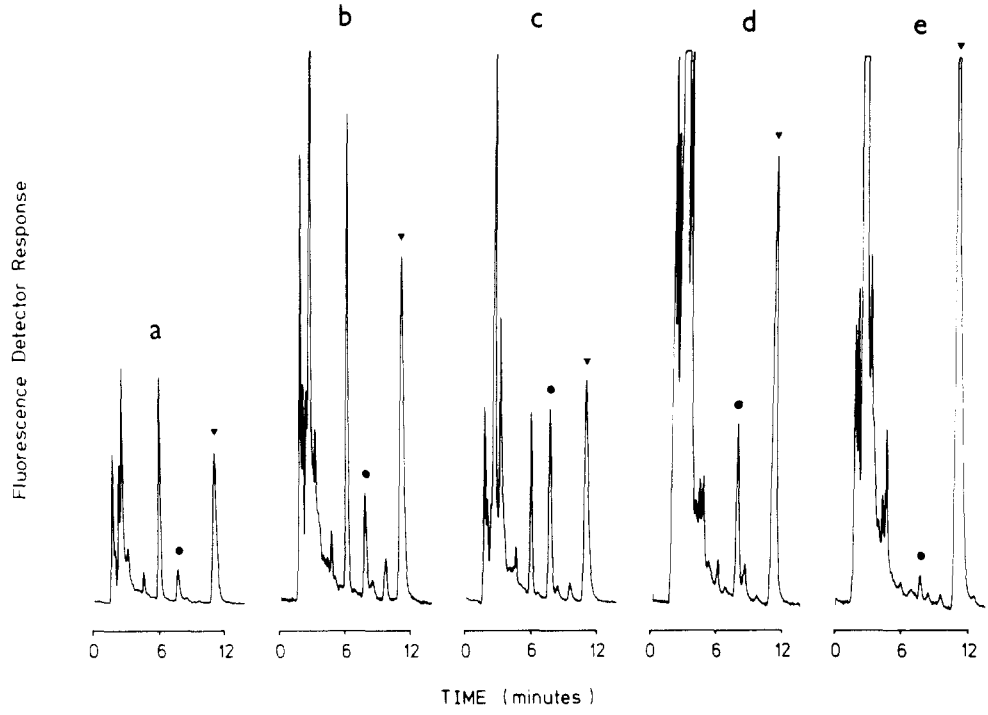

Figure 4. Chromatograms of a sample extract monitored at various wavelengths [excitation wavelength (nm), emission wavelength (nm), reference]: (a) 236, 418, Trenholm et al. (1984); (b) 274, 418, Bennett et al. (1985), Bagneris et al. (1986); (c) 280, 465, Chang and DeVries (1985); (d) 320, 445; (e) 335, 404. Key: ●, zearalenone; ▼, alternariol monomethyl ether.

alenone was low, it falls within the range reported by Bagneris et al. (1986) for a number of substrates. When fluorescence detection is utilized in the determination of zearalenone and its derivatives, the excitation and emission wavelengths prescribed in the literature vary considerably. Figures 4a–c show chromatograms of a single sample extract [isolated according to the method of Bagneris et al. (1986)] screened under identical chromatographic conditions and instrument sensitivity settings but monitored in a range of excitation and emission wavelengths cited in the literature. Figure 4d illustrates the chromatogram of the same extract monitored with the wavelengths employed in this study, while Figure 4e shows the chromatogram obtained using the AME excitation and emission wavelength maxima (as recorded by the authors). Fluorescence detection offers one of the most selective HPLC detection systems available. With all the combinations of excitation and emission wavelengths (Figure 4a–e), both zearalenone and AME could be detected, but the choice of excitation and emission wavelengths substantially influenced the sensitivity of detection.

Dietary levels of 1–5 mg/kg of zearalenone are known to cause hyperestrogenism in young gilts (Mirocha and Christensen, 1974). The levels found in the four mixed feeds (0.85–1.25 μg/g; Table I) are marginal but probably sufficient to have caused the mild clinical signs of hyperestrogenism during the field outbreak. However, the possibility of a synergistic effect between zearalenone and AME may exist and requires further investigation. It should be noted that much higher analytical values for zearalenone in the feed samples would have been obtained if the AME also present in the feeds would have been mistaken for zearalenone.

The levels of AME detected in the mixed feeds during the present investigation (1.2–2.25 μg/g; Table I) are considerably lower than the combined levels of 0.2–7.9 μg/g of AME and AOH that have previously been reported in weathered, discolored sorghum grains (Seitz et al., 1975a,b). The natural occurrence of AME has also been reported at levels ranging from 0.1 to 0.8 μg/g in tomatoes (Stinson et al., 1981), 0.2 to 2.3 μg/g in apples (Stinson et al., 1981), and 0.03 to 2.87 μg/g in moldy olives (Visconti et al., 1986). AME is not toxic to brine shrimp and chicken embryos (Bruce et al., 1984; Griffin and Chu, 1983). No toxic effects could be detected in 1-day-old chicks fed diets containing 100 μg/g of AME (Griffin and Chu, 1983) nor in rats fed dietary levels of 24 μg/g (Sauer et al., 1978) or dosed with 23.4 μg/g body weight (Pollock et al., 1982). However, decreased fetal weights and increased numbers of fetal resorptions have been reported in hamsters treated intraperitoneally with 200 mg/g of AME (Pollock et al., 1982). In view of the low degree of toxicity of AME and the relatively low levels detected in the mixed feeds, it seems unlikely that AME was responsible for any of the clinical signs observed during the field outbreak in swine.

CONCLUSION

In summary, the findings of Seitz et al. (1975b) that AME can easily be mistaken for zearalenone during TLC analysis were confirmed. Seitz et al. (1975b) suggested that separate (similarly spotted) TLC plates be developed in different mobile phases for identification purposes. We further suggest that TLC results should be confirmed by another technique, such as HPLC utilizing either UV or preferably fluorescence detection systems. It has been shown that at least three methods, specifically developed for the determination of zearalenone and its derivatives, are suitable for the coextraction and analytical determination of AME. Therefore, these methods should be useful in the screening and evaluation of different types of feed samples. In reports of zearalenol, the specific diastereomer should be specified, since α- and β-zearalenol produce markedly different responses both analytically and clinically. Finally, when fluorescence detection techniques are utilized, care should be taken with regard to the choice of excitation and emission wavelengths, since these parameters may significantly affect the selectivity and sensitivity of the procedure.

Registry No. AME, 26894-49-5; zearalenone, 17924-92-4; α-zearalenol, 36455-72-8; β-zearalenol, 71030-11-0.

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