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Characterization of Two Isomers of 8′-Hydroxyzearalenone and Other Derivatives of Zearalenone

Richard A. Jackson, Stuart W. Fenton, Chester J. Mirocha,* and George Davis

Two naturally occurring derivatives of zearalenone synthesized by *Fusarium roseum* were shown to be isomers of 8′-hydroxyzearalenone. Their structure was proven by comparing their fragmentation pattern (mass spectroscopy) with the parent compound (zearalenone). The two derivatives differed from zearalenone by two unique fragments, *i.e.* *m/e* 95 and 110 *vs.* *m/e* 97 and 112 for the parent compound. The major fragments of the mass spectrum were followed by labeling the aromatic ring with Br. In addition to spectrometric evidence, the reduced form of the parent compound (zearalenone) was synthesized from both isomers. The two isomers of 8′-hydroxyzearalenone, also known as F-5-3 and F-5-4, were not biologically active in the rat uterotropic bioassay for estrogens.

Fusarium roseum (Cke) snyd. & Hans., "Graminearum" (*Gibberella zeae* (Schw.) Petch), is a storage fungus which under certain conditions of moisture and temperature infects maize and produces a mycotoxin called F-2 or zearalenone. When such corn is fed to swine, it causes the estrogenic syndrome which involves primarily the genital system; in the prepuberal gilt, the vulva becomes swollen and edematous and sometimes, in severe cases, it may progress to vaginal prolapse; the uterus is enlarged, edematous, and tortuous, with some atrophy of the ovaries. The young males may undergo a feminizing effect, with atrophy of the testes and enlargement of the mammary glands.

The nature and effects of this mycotoxin have been investigated by Stob *et al.* (1962), Christensen *et al.* (1965), and Mirocha and Christensen (1971).

The structure of zearalenone (I) (Figure 1) was determined by Urry *et al.* (1966) as 6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)resorcylic acid lactone. Mirocha and Christensen (1971) and Mirocha *et al.* (1968) reported seven other naturally occurring derivatives of zearalenone, two of which are described here, namely F-5-3 and F-5-4 (II). Bolliger and Tamm (1972) reported on the structure of II plus two other derivatives, 5-formylzearalenone and 7′-dehydrozearalenone.

Elemental analysis and high-resolution mass spectra established the formula $C_{18}H_{22}O_6$ and a molecular weight of 334 for isomers of II (Figure 2D, E), insoluble in water, benzene, and petroleum ether and slightly soluble in methylene chloride, and ethyl acetate, acetonitrile, acetone, ethyl alcohol, and diethyl ether.

The objectives of this research were to determine the chemical structures of F-5-3 and F-5-4 and study the mass spectroscopy of the zearalenone.

Department of Chemistry (R.A.J., S.W.F.) and Department of Plant Pathology (C.J.M., G.D.), University of Minnesota, Minneapolis and St. Paul, Minnesota 55101.

MATERIALS AND METHODS

Spectrometric Examination. Mass spectra were obtained with an AEI MS-30 double focusing mass spectrometer. Low-resolution spectra were taken at a resolution of 1000 and a scan speed of 10 sec/decade. The source was heated to 200° and the sample probe was heated as required to get a sufficiently high ion current. Unless otherwise specified, the spectra were obtained at 70 eV. High-resolution mass spectra were obtained using an internal perfluorokerosene reference at a resolution of 5000 and a scan speed of 30 sec/decade. Mass spectral data were calculated with an on-line "Digital pdp8" computer. Gc-mass spectral data were obtained on an LKB-9000 gc-mass spectrometer. Gc-mass spectral data as well as low-resolution mass spectra were plotted by hand.

Infrared spectra were obtained on Beckman IR-12, Perkin-Elmer 521, or Perkin-Elmer 257 infrared spectrophotometers. Melting points were obtained with a Fischer Johns melting point apparatus. Analytical tlc was done on unactivated Eastman ±6060 Chromagram silica tlc plates containing a fluorescent indicator. All of the compounds which contained an aromatic moiety appeared either as dark purple spots against an orange fluorescent background when viewed under an ultraviolet light, or as intense blue or green fluorescent spots against an orange fluorescent background.

Biosynthesis and Preparation of Zearalenone Derivatives. The isomers of II are chemically similar to zearalenone and have been isolated from cultures of *F. roseum* "Graminearum," an isolate designated as Mapleton No. 10. Spores of the fungus were seeded onto previously autoclaved rice at a moisture content of 40%, and then incubated at 25° for 2 weeks followed by 4 weeks at 12°. The culture was dried, ground in a Stein Mill, and moistened with water (15% w/v) before extracting with ethyl acetate. The ethyl acetate was concentrated under vacuum, and the residue partitioned between equal volumes of petroleum ether (bp 30–60°) and acetonitrile. The acetonitrile

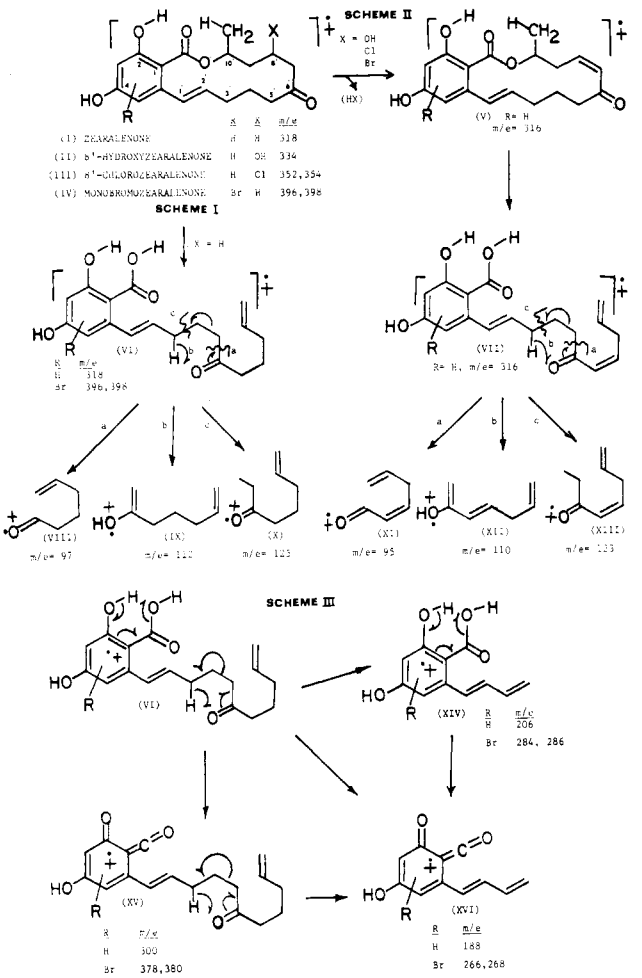

Figure 1. Mass spectral fragmentation schemes of zearalenone. When X = OH, Cl, or Br, then Scheme II is followed; when X = H, then Scheme I is followed. Scheme III describes the general fragmentation of the molecular ion VI.

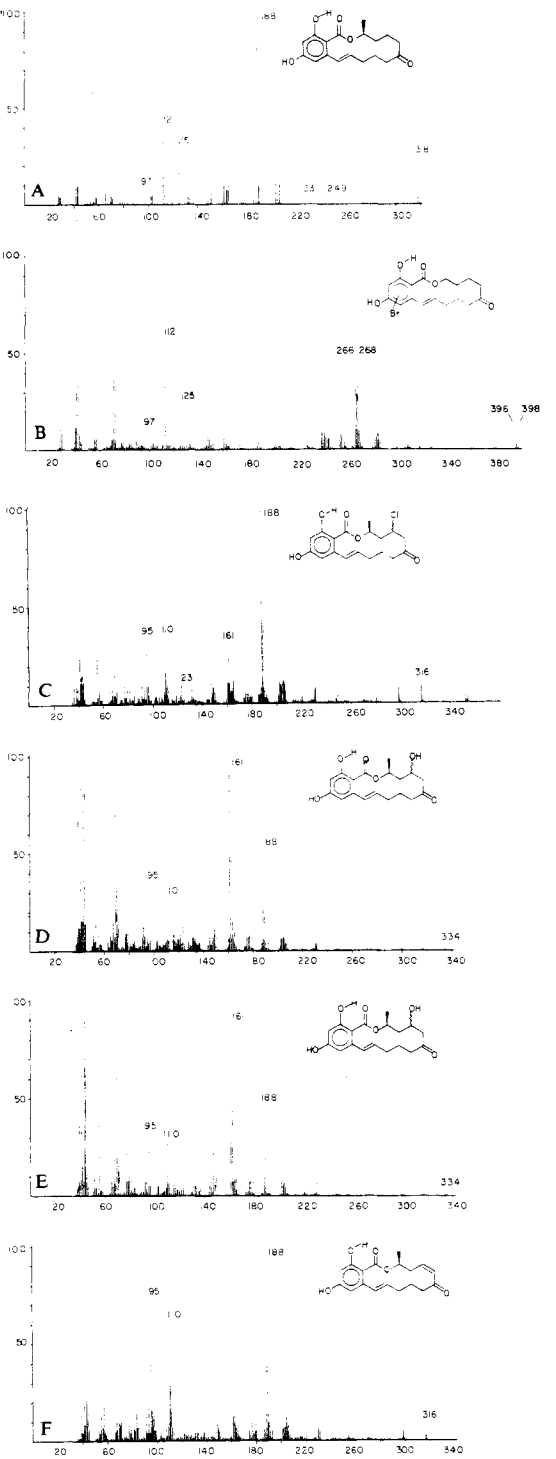

Figure 2. Mass spectra of various derivatives of zearalenone: (A) zearalenone; (B) monobromozearalenone; (C) 8′-chlorozearalenone; (D) 8′-hydroxyzearalenone (F-5-3); (E) 8′-hydroxyzearalenone (F-5-4); (F) 1′,7′-zearaldienone.

layer was concentrated and chromatographed on a silica gel column. As solvent polarity was increased, zearalenone (F-2) was eluted first followed by a mixture of zearalenone, F-5-3, and F-5-4. The fractions containing F-5-3 and F-5-4 were concentrated and were chromatographed on preparative thin-layer silica gel plates using multiple elutions with a 97% chloroform–3% ethanol system. Substances in bands corresponding to F-5-3 and F-5-4, identified by their intense blue fluorescence in ultraviolet light, were eluted and chromatographed again on preparative thin-layer plates. After elution and concentration, these fractions were crystallized repeatedly from ethyl acetate–isooctane mixtures to obtain white crystals with a melting point of 198–199 for F-5-3 and 168–169° for F-5-4.

Reaction of F-5-3 with $SOCl_2$ and Pyridine. F-5-3 (1.5 mg) was dissolved in 0.2 ml of pyridine and 50 μl of thionyl chloride was added. After 1 hr at room temperature, the brown solution was cooled in an ice bath and 4 ml of diethyl ether and 4 ml of water were added. The mixture was shaken and the layers were separated. The ether layer was washed with three 1.5-ml portions of 0.5 *N* HCl and then evaporated under a stream of nitrogen. The residue was redissolved in 0.1 ml of acetone and streaked on a 7 × 10 cm Eastman ≈6060 tlc plate. After drying, the plate was developed with ether. Three components were present, two of which were blue fluorescent and one of which was nonfluorescent. One of the blue fluorescent components had a R_f 0.38 and was unreacted F-5-3. The other blue fluorescent component (III) had R_f 0.73. The mass spectrum of III showed a molecular ion at M^+ = 352 with an M + 2 peak of approximately one-third the intensity of the M^- peaks indicating that III contained one chlorine atom and that the conversion of F-5-3 to III involved replacing a hydroxyl group by a chlorine atom (Figure 2C). The nonfluorescent component (V) had R_f 0.61 and its mass spectrum showed a molecular ion at M^+ = 316 indicating that V had been formed from F-5-3 by dehydration.

Reaction of F-5-4 with $SOCl_2$ and Pyridine. F-5-4 (1.2 mg) was allowed to react with thionyl chloride and pyridine and the reaction products separated under conditions identical with those used for the reaction with F-5-3. Three components were separated by tlc. Unreacted F-5-4

was present as a blue fluorescent component with R_f 0.36. A blue fluorescent compound (III) showed a molecular ion at M^+ = 352 with an M + 2 peak indicating the presence of one chlorine atom and replacement of a hydroxyl in F-5-4 by a chlorine atom (Figure 2C). The other component was a nonfluorescent compound with R_f 0.59, the mass spectrum of which showed a molecular ion at M^+ = 316 indicating that V has been formed by dehydration of F-5-4.

Preparation of Zearalanone from F-5-3. Initial experiments on the reduction of $\Delta^{1',7'}$-zearaldienone (V) prepared from F-5-3 yielded products which were shown by mass spectroscopy to be a mixture of zearalanone (I saturated in the 1',2' position) and another product which could not be separated by tlc.

To circumvent this problem, F-5-3 (3.8 mg) was hydrogenated at atmospheric pressure over 3.9 mg of prereduced platinum in 5 ml of absolute ethanol for 2 hr. The mixture was filtered and the catalyst was washed with 2 ml of ethanol. Evaporation of the filtrate *in vacuo* gave 3.9 mg of residue which was shown by mass spectroscopy to be only partially reduced. The residue was reduced a second time over 4.4 mg of prereduced platinum for a period of 1 hr and worked up as before. The mass spectrum showed the absence of F-5-3 in the residue. Analytical tlc developed with EtOAc showed the presence of one major component (R_f 0.67) and trace amounts of three other components (R_f 0.0, 0.44, and 0.84). The major component was isolated by preparative tlc on a silica gel GF-254 plate developed with EtOAc (R_f 0.88). The mass spectrum showed the expected molecular ion at M^+ = 336 for dihydro-F-5-3 (8'-hydroxyzearalanone).

Dihydro-F-5-3 (2.2 mg) was dissolved in 1 ml of pyridine and 50 μl of thionyl chloride was added. After 1 hr at room temperature, the reaction mixture was added to 40 ml of ether in a separatory funnel. The mixture was washed with three 10-ml portions of 0.5 *N* HCl. Evaporation of the ether layer *in vacuo* yielded 1.3 mg of light yellow residue which was dissolved in 0.1 ml of $CHCl_3$ and streaked on a 7 × 10 cm Eastman =6060 plate. After developing to 2.0 cm four times with ether to sharpen the origin, the plate was developed to 6.0 cm with ether and then air dried and developed to the top of the plate with $CHCl_3$. Five components were separated, one of which was dihydro-F-5-3 (R_f 0.30). Components were also present at R_f 0.0, 0.56, 0.65, and 0.90. The mass spectrum of the component at R_f 0.65 showed a molecular ion at M^+ = 354 with a peak at m/e 356 of one-third the intensity indicating the replacement of the hydroxyl of dihydro-F-5-3 by a chlorine. The component at R_f 0.56 showed a molecular ion at M^+ = 318 which was the expected value for the dehydrated product ($\Delta^{7'}$-zearalanone).

The dehydrated product ($\Delta^{7'}$-zearalanone) was hydrogenated in 5 ml of absolute ethanol at atmospheric pressure over 4 mg of prereduced platinum for 1 hr at room temperature. The mixture was filtered and the filtrate evaporated *in vacuo*. The residue was compared to zearalanone (I, reduced at the 1':2' position) by tlc. The major component had the same R_f as zearalanone when the analytical tlc plates were developed with benzene, CH_2Cl_2, $CHCl_3$, or ether. The product (zearalanone) was isolated on a 7 × 10 cm Eastman =6060 tlc plate developed with ether. The mass spectrum was identical with that of authentic zearalanone.

Reaction of F-5-3 and F-5-4 with Periodate. F-5-3, F-5-4, and ethylene glycol were allowed to react with H_5IO_6 semiquantitatively by the procedure of Weiss (1970). To each of 2.3 mg of F-5-3, 2.4 mg of F-5-4, 1.4 mg of ethylene glycol, and a blank were added 3.0 ml of $CHCl_3$ and 3.0 ml of 0.00219 *M* periodate solution. The samples were placed in a 40° bath for 90 min and then removed; 10 ml of ice cold water was added to each sample, and the samples were shaken for 30 sec. Three milliliters of 0.6 *N* H_2SO_4 and 2.0 ml of 2% KI solution were added. The samples were then titrated with 0.0201 *N* thiosulfate to the starch end point. The blank, F-5-3, and F-5-4 each required 2.70 ml of thiosulfate, whereas the ethylene glycol sample required 2.03 ml of thiosulfate solution. Excess amounts of F-5-3, F-5-4, and ethylene glycol were present based on the periodate. If all the periodate was consumed (*i.e.*, converted to HIO_3), the theoretical amount of thiosulfate required is 0.75 times the amount in the blank or 2.10 ml. Since F-5-3 and F-5-4 did not consume any periodate, they cannot be α-hydroxy ketones.

Uterotropic Activity. The test for uterotropic activity was essentially that as described by Umberger *et al.* (1958), but conducted on 21-day old, white weanling female rats provided by the Holtzman Co., Madison, Wis. The test compounds were incorporated into a nutritionally balanced rat diet and fed to the animals over a period of 5 days. The rats were sacrificed at the end of the fifth day and their uteri excised and weighed.

RESULTS AND DISCUSSION

Analytical thin-layer chromatography on silica gel G, developed three times with a 97% chloroform–3% ethanol system, yielded a single spot for each compound tested with an intense blue fluorescence under ultraviolet light. Each spot was initially bright yellow when sprayed with methanolic sulfuric acid. After the third development with this system, F-5-3 had an R_f value of 0.27; F-5-4, 0.35; and zearalenone, 0.64.

Gas–liquid chromatography of the trimethylsilyl (TMS) ethers of the compounds was carried out using a Varian-Aerograph Model 1500 series, equipped with a hydrogen flame ionization detector. The flow rate of the carrier gas (nitrogen) was 20 ml/min. The temperature of the injector port and the detector cell was 275°. The TMS ethers were separated on a 1.2-m stainless steel column packed with 3% QF-1 on Gas-Chrom Q (100–120 mesh) and gave single peaks with retention time ratios (based on 1.0 for trimethylsilylzearalenone) of 0.82 for TMS F-5-3 and 0.95 for TMS F-5-4. On a 2.4-m column packed with 3% OV-1 on Gas-Chrom Q (80–100 mesh), the TMS derivatives of both compounds had identical retention time ratios of 1.20 when compared to 1.0 for trimethylsilylzearalenone.

Mass spectroscopy of the TMS derivatives of F-5-3 and F-5-4 separated by glc showed molecular ions at M^+ = 550 for both compounds compared to a molecular ion at M^+ = 462 for the TMS derivative of zearalenone. This showed that three protons were replaced by trimethylsilyl groups in F-5-3 and F-5-4 compared to two protons being replaced in the TMS derivative of zearalenone.

The ultraviolet spectra of F-5-3 and F-5-4 (max 236, 274, and 316 nm) were identical with that of zearalenone in respect to the position of absorption maxima, and differed only slightly in intensity. The extinction coefficients determined in neutral ethanol were as follows: F-5-3, 29,400, 12,200, 5680; F-5-4, 30,000, 12,000, 5100; zearalenone, 29,700, 13,900, 6020.

Infrared spectra of the compounds in KBr disks showed the close relationship of F-5-3 and F-5-4 to zearalenone and other similar β-resorcylic acid lactones. F-5-3 had absorption maxima at 3300–3500 (H-bonded OH), 3530 (nonbonded OH), 1695 (ketone), 1645 (lactone carbonyl), 1615, and 965 cm^{-1} (trans double bond). F-5-4 had absorption maxima at 3290 (H-bonded OH), 3530 (nonbonded OH), 1680 (ketone), 1645 (lactone carbonyl), and 1615, and 980 cm^{-1} (double bond). Zearalenone had absorption maxima at 3300 (H-bonded OH), 1688 (ketone), 1645 (lactone carbonyl), 1615, and 965 cm^{-1} (trans double bond).

Because of solubility properties of the compounds, acetone-d_6 or pyridine-d_5 were used for nuclear magnetic resonance studies. Resonance peaks for F-5-3, F-5-4, and zearalenone corresponding to either the phenolic or aliphatic hydroxyl protons were not detected when these

Table I. Resolution by Thin-Layer Chromatography of Zearalenone Derivatives on Unactivated ≈6060 Eastman Silica Chromagrams

Derivatives	R_f values						
	Trichloroethylene	Benzene	CH_2Cl_2	$CHCl_3$	Et_2O	EtOAc	97% $CHCl_3$–3% EtOH
I, zearalenone	0.03	0.04	0.14	0.20	0.70	0.74	0.70
II, 8′-hydroxyzearalenone (F-5-3)	0.00	0.00	0.01	0.02	0.38	0.63	0.35
II, 8′-hydroxyzearalenone (F-5-4)	0.00	0.00	0.01	0.03	0.36	0.63	0.44
III, 8′-chlorozearalenone (derived from F-5-3)	0.05	0.06	0.15	0.20	0.73	0.75	0.70
III, 8′-chlorozearalenone (derived from F-5-4)	0.04	0.06	0.15	0.19	0.73	0.74	0.70
IV, monobromozearalenone	0.20	0.14	0.39	0.51	0.59	0.65	0.76
V, 1′,7′-zearaldienone (derived from F-5-3)	0.01	0.01	0.05	0.13	0.61	0.72	0.66
V, 1′,7′-zearaldienone (derived from F-5-4)	0.01	0.01	0.04	0.12	0.59	0.75	0.66

compounds were dissolved in low concentrations of these solvents. The rest of the spectra were nearly identical for all compounds with only slight shifts from that of zearalenone in deuteriochloroform. The coupling constants for the methyl doublet and the olefinic hydrogen α to the aromatic ring were the same for all three compounds.

Since very little has been reported in the literature on the mass spectra of compounds related to zearalenone, it was felt that it would be helpful in elucidating possible mass spectral fragmentation schemes to compare the mass spectra of zearalenone with a derivative containing a functional group which is easily recognized and which would be expected to remain with that portion of the molecule to which it was attached. Since ^{79}Br and ^{81}Br are naturally occurring in approximately equal quantities, the presence of a doublet two mass units apart of approximately equal intensity is readily apparent and suggestive of monobromo substitution. In addition, a bromine substituted on an aromatic ring would not be expected to be readily lost during fragmentation in the mass spectrometer. For these reasons, IV was prepared and its mass spectrum compared to that of zearalenone.

Comparison of the mass spectra of zearalenone (Figure 2A) to that of IV (Figure 2B) suggests that three major schemes occur in the mass spectral fragmentation of this class of compounds, two of which are shown here in Figure 1. High-resolution mass spectroscopy confirmed this assignment of fragments for zearalenone.

The McLafferty rearrangement at the lactone carbonyl in Scheme I of Figure 1 is similar to that suggested by Scott *et al.* (1968) for the fragmentation of ethyl salicylate and by Jensen *et al.* (1972) for dihydrozearalanone.

A major effect is seen in the spectra due to the influence of the ketone carbonyl group at C_6. In particular, fragments IX (m/e 112) and VIII (m/e 97) are formed from the underivatized zearalenone as shown in Scheme I of Figure 1. Comparison of these two fragments to those formed when the two diasteroeoisomers, F-5-3 and F-5-4, are subjected to ionization is important in determining the structure of the latter. F-5-3 and F-5-4 give rise to fragments XII (m/e 110) and XI (m/e 95) as shown in Scheme II of Figure 1. Such fragmentation could only arise if the undecenyl ring of zearalenone was substituted at the 8′ position, giving rise to fragments as shown in Figure 1. Further evidence for the structures of F-5-3 and F-5-4 (Figure 2D,E) was obtained by the reaction of each isomer with $SOCl_2$ and pyridine. Each of the compounds yielded its respective chloro derivative (Figure 2C) and its respective α,β-unsaturated derivative, *i.e.* 1′,7′-zearaldienone (Figure 2F). The mass spectra obtained from the 8′-chloro derivatives showed $P^+ = 352$ ($C_{18}H_{21}ClO_5$). The ratios of the parent to the P + 2 peaks for both isomers confirmed the presence of one chlorine atom in each compound. This corresponds to the replacement of the OH by Cl in the conversion of II to III. The mass spectra of V (1′,7′-zearaldienone), obtained from both isomers, showed $P^+ = 316$ ($C_{18}H_{20}O_5$), which was analogous to the loss of H_2O from F-5-3 and F-5-4 (Figure 1, Scheme II). Again, fragments at m/e 95 and 110 rather than 97 and 112 were observed. The uv spectra obtained from V showed a broadening of the 236-nm maxima which would be predicted for an α,β-unsaturated ketone moiety being present in the molecules.

The mass spectra of the 8′-chlorozearalenone showed peaks at m/e 95 and 110, rather than at m/e 97 and 112, as predicted by Scheme II with HCl being lost in the initial fragmentation rather than H_2O. The mass spectra of V (Figure 2F) obtained from both isomers showed $P^+ = 316$ ($C_{18}H_{20}O_5$), corresponding to the loss of H_2O from F-5-3 and F-5-4. Again, fragments at m/e 95 and 110 rather than 97 and 112 were observed (Figure 1, Scheme II).

Scheme III of Figure 1 shows the fragmentation of VI to XVI, the base peak (m/e 188). The base peak is formed either directly from VI or through fragments XIV and XV which accounts for its abundance. Loss of water from fragment VI yielding fragment XV is confirmed by the presence of a metastable peak at 283.1 in the mass spectrum of zearalenone and metastable peaks at 361.0 and 363.0 in the mass spectrum of monobromozearalenone (Figure 2B).

The possibility of F-5-3 and F-5-4 having the OH at 7′ rather than 8′ was excluded by negative periodate tests.

Analytical tlc's of the various derivatives were run on ≈6060 Eastman silica chromograms with solvents of increasing polarity. The results of this study are shown in Table I.

Both stereoisomers were compared for uterotropic activity with zearalenone and the two zearalanol isomers (mp

Table II. Comparison of Uterotropic Activity of F-5-3 and F-5-4 with Other Derivatives of Zearalenone in Virgin, Female White Rats

Derivative	No. rats	Wt. gain, g	Uterine wt, mg
F-5-3 (1 mg)	2	22 ± 1	42 ± 4
F-5-4 (1 mg)	2	20 ± 1	50 ± 4
Zearalanol, HMP[a] (1 mg)	2	23 ± 0	196 ± 4
Zearalanol, LMP[b] (1 mg)	2	18 ± 2	98 ± 2
Zearalenone (1 mg)	2	21 ± 4	72 ± 13
Control	6	24 ± 2	44 ± 6

[a] HMP = high melting point isomer (178–180°). [b] LMP = low melting point isomer (146–148°).

178–180°; mp 146–148°) by the rat uterotropic bioassay. The data shown in Table II indicate that at a total dose of 1 mg administered *per os* over a period of 5 days, the two isomers are relatively inactive.

In summary, comparison of the fragmentation pattern of zearalenone with F-5-3 and F-5-4 shows the latter to be stereoisomers differing from the parent compound (zearalenone) only by a hydroxyl group in the 8′ position. Formation of the 8′-chloro derivatives of both F-5-3 and F-5-4, followed by dehydrochlorination to 1′,7′-zearaldienone, and finally reduction of the latter to zearalanone, demonstrate the two isomers to be 8′-hydroxyzearalenone. Compounds F-5-3 and F-5-4 did not consume any periodate when reacted with H_5IO_6, demonstrating that both compounds are not α-hydroxy ketones.

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Gas Chromatographic–Mass Spectrometric Determination of *N*-Nitrosodimethylamine Formed in Synthetic and Human Gastric Juice

Richard P. Lane,[1] Roy H. Rice,[2] and Milton E. Bailey*

An analytical procedure for determination of *N*-nitrosodimethylamine in human gastric juice is described. *N*-Nitrosodiethylamine is used as an internal standard to quantitate *N*-nitrosodimethylamine and high-resolution mass spectrometry is used to detect and confirm identity of the nitrosamine. This procedure could be useful for estimating levels of nitrosamines at 0.1 ppb. Glycine competed for nitrite in a reaction mixture containing nitrite, *N*-nitrosodimethylamine, and glycine in synthetic gastric juice. A mixture of amino acids retarded nitrosamine formation at pH 2.5, but not at other pH values studied. Meat extract from cooked pork also retarded nitrosamine formation at pH 2.5 and 4.5.

As a result of the extreme toxicity (Ender *et al.*, 1964) and carcinogenicity (Druckery *et al.*, 1969) of *N*-nitrosamines it is necessary to evaluate the possibility of the occurrence of these compounds in the human environment. It has been suggested that nitrite and secondary amines, though not abundant, may react in various stages of food processing (Ender and Ceh, 1970) or during gastric digestion of nitrite treated foods (Lijinsky and Epstein, 1970). Though results of several studies have demonstrated the formation of *N*-nitrosamines from uncommonly large amounts of both precursors during processing (Ender and Ceh, 1970; Sen *et al.*, 1970) and in gastric digestion (Sen *et al.*, 1969; Greenblatt *et al.*, 1971; Alam *et al.*, 1971) it has not been demonstrated that a significant health hazard exists as a result of nitrite in the various cured food products at levels currently permitted by law.

Recent advances in analytical methodology (Howard *et al.*, 1970; Fazio *et al.*, 1971b; Crosby *et al.*, 1972) have enabled detection and confirmation of various *N*-nitrosamines at trace levels in a number of different food products. At present there have been a number of positive identifications of these compounds in various nitrite and non-nitrite-treated foods at levels ranging from 4 to 130 ppb (Fazio *et al.*, 1971a,b; Crosby *et al.*, 1972; Wasserman *et al.*, 1972). Recently, Sen *et al.* (1972) confirmed the presence of 120–450 ppb of *N*-nitrosodimethylamine in samples of toxic fish meal though neither nitrite nor nitrate was used in processing.

Increased analytical capabilities in *N*-nitrosamine research have been largely a result of development of efficient extraction and clean-up procedures, and sensitive detection methods. This has enabled a practical detection limit, with positive confirmation, of 10 ppb for certain *N*-nitrosamines (Fazio *et al.*, 1971a; Wasserman *et al.*, 1972). Crosby *et al.* (1972) increased this detection limit to 1–4 ppb by increasing sample size and using high-resolution mass spectrometry.

Considering these recent advances in analytical methodology and the lack of information concerning gastric formation of *N*-nitrosamines from the occasional low amounts of nitrite in the human diet, it was desirable to develop an analytical method suitable for analysis of trace levels of *N*-nitrosodimethylamine isolated from human gastric juice.

Described here is an analytical method capable of detection and confirmation of *N*-nitrosodimethylamine at levels as low as 0.1 ppb in aqueous solutions of human gastric juice following the incubation of nitrite and dimethylamine. We found the "modified thermionic detector" (alkali flame detector) described by Howard *et al.* (1970) to be of great significance in the routine analysis of gastric juice extracts from DMNA due to the large number of interfering compounds. High-resolution mass spec-

Department of Food Science and Nutrition, University of Missouri, Columbia, Missouri 65201.
[1] Present address: Warner Jenkinson, St. Louis, Mo. 63106.
[2] Present address: Station Chemical Laboratories, University of Missouri, Columbia, Mo. 65201.