Separation Science and Technology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713708471

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To cite this Article Vandenheuvel, W. J. A.(1968) 'The Gas-Liquid Chromatographic Behavior of the Zearalenones, A New Family of Biologically Active Natural Products', Separation Science and Technology, 3: 2, 151 — 163
To link to this Article: DOI: 10.1080/01496396808053468
URL: http://dx.doi.org/10.1080/01496396808053468

SEPARATION SCIENCE, 3(2), pp. 151–163, April, 1968

The Gas–Liquid Chromatographic Behavior of the Zearalenones, A New Family of Biologically Active Natural Products

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Summary

The gas–liquid chromatographic behavior of a group of zearalenones has been investigated through the use of both selective and nonselective stationary phases. Separation of these compounds can be effected using conditions similar to those employed for steroid work, differentiation being accomplished on the basis of molecular weight, unsaturation, functional groups, and stereochemistry. Functional group alteration has been employed to improve separations and the quantitative aspects of analysis. Separation of a pair of epimeric alcohols proved to be especially difficult, requiring the use of several approaches. The contribution of the phenolic group at the 2 position to retention behavior is considerably smaller than that of the 4-phenol, a difference ascribed to the ability of the former to intramolecularly hydrogen bond to the carbonyl group of the lactone. These methods have been employed to identify the major urinary metabolite of one of the compounds of this series in the sheep.

INTRODUCTION

The zearalenones, members of a series of compounds related to the naturally occurring b-resorcylic acid lactone, (−) zearalenone, I, a fungal fermentation product, are compounds of interesting physiological activity (*1–4*). Although they do not possess the tetracyclic structure of the steroids,* these substances do exhibit estrogenic activity, but lack certain of the side effects of the steroid hormones. Interest exists in determining the effect of the zearalenones upon

* The structure of the parent compound, (−)-zearalenone, is given in Fig. 1. Other members of this series are named as derivatives of this compound.

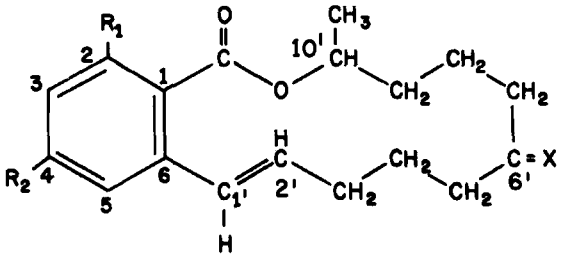
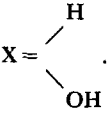
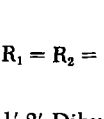


FIG. 1. Structural formula of (−)-zearalenone. I: $R_1 = R_2 = OH$; $X = O$. II: 1′,2′-Dihydro; $R_1 = R_2 = OH$; $X = O$. III: 1′,2′-Dihydro; $R_1 = R_2 = OH$; $X =$  . IV: 1′,2′-Dihydro; $R_1 = R_2 = OH$; $X = H_2$. V: 1′,2′-Dihydro; $R_1 = R_2 = OH$; $X =$  . VI: 1′,2′-Dihydro; $R_1 = H$; $R_2 = OH$; $X = O$. VII: 1′,2′-Dihydro; $R_1 = OH$; $R_2 = H$; $X = O$. VIII: $R_1 = OCH_3$; $R_2 = OH$; $X = O$. IX: $R_1 = OH$; $R_2 = OCH_3$; $X = O$. X: $R_1 = R_2 = OCH_3$; $X = O$.

the growth rate of domestic animals and in investigating what role they might play in animal and human fertility control. Work of this type requires extensive toxicological and metabolism studies, which in turn necessitate the availability of sensitive, reliable methods for the analysis of drug residues and metabolities. Gas-liquid chromatography (GLC) has proved to be valuable in many studies involving the analysis of microgram amounts of biologically active compounds (*5–7*), and we have found it to be well suited to the separation of the zearalenones. GLC has recently been described as a satisfactory method for the detection of an estrogenic compound, clearly a zearalenone, produced by a fungus in stored corn (*8*). It thus seems appropriate for us to report at this time a study of the GLC behavior of the zearalenones.

EXPERIMENTAL

Derivatives of the zearalenones were prepared by established procedures (*5,7,9,10*), and their structures were confirmed by combined GLC–mass spectrometry (*10–12*) using the LKB Model 9000 instrument. The O-methyloxime ditrimethylsilyl (TMSi) ether of 1′,2′-dihydro-(−)-zearalenone and the tri-TMSi ether of one of the corresponding 6′-alcohols (III) were isolated and possessed satis-

factory elemental analyses. Other derivatives were prepared on a submilligram scale and used directly. GLC retention data were obtained with Barber–Colman Model 15 (argon ionization detector) and 5000 (hydrogen flame ionization detector) and F & M Model 402 (hydrogen flame ionization detector) instruments; quantitative data were obtained with the F & M. The column packings [prepared according to Horning et al. (5)] were 1.7% SE-30 (a methylpolysiloxane; General Electric Company), 1.6% F-60 (a methylpolysiloxane containing a few percent of *p*-chlorophenyl groups; Dow-Corning Corp.), and 1.5% QF-1 (a fluoroalkylpolysiloxane; Dow-Corning Corp.) coated on acid-washed and silanized Gas-Chrom P (Applied Science Laboratories). Column conditions: SE-30, 200°C, and 25 ml/min carrier gas; F-60, 235°C and 40 ml/min; QF-1, 210°C and 25 ml/min.

RESULTS AND DISCUSSION

The polyfunctionality and molecular weight range of the zearalenones is not unlike that of the steroids, and the aromatic ring with its phenolic groups is particularly remindful of the estrogens, which were among the first steroids successfully separated by GLC (*13*).* The GLC behavior of I and several closely related compounds is presented in Table 1; cholestane serves as a suitable reference standard. The double bond in I contributes significantly

* The GLC of diethylstilbestrol, a nonsteroidal estrogen, has been reported (*14*).

TABLE 1

Retention Behavior of a Group of Zearalenones

Compound	Relative retention times[a]		
	SE-30		QF-1
	Free	TMSi	TMSi
(−)-Zearalenone, I	0.94	1.17	5.13
I OMO[b]	1.18	1.55	2.89
1′,2′-Dihydro-(−)-zearalenone, II	0.84	1.03	4.52
II OMO[b]	1.01	1.31	2.42
6′-Alcohol from 1′,2′-dihydro-(−)-zearalenone, III	0.99	1.35	2.16
6′-Deoxy-1′,2′-dihydro-(−)-zearalenone, IV	0.48	0.60	1.24

[a] Cholestane = 1.00; column conditions given in the experimental section.
[b] O-Methyloxime.

to the retention behavior of this compound with the "nonpolar" or "nonselective" (5) stationary phase SE-30, for reduction to 1′,2′-dihydro-(−)-zearalenone, II, results in a considerable decrease in retention time. A change in volatility of this magnitude with such a stationary phase suggests that a considerable alteration in molecular geometry is associated with reduction of the double bond. Reduction of the keto group at the 6′ position of II to the corresponding alcohol* (to give compound III) leads to a decrease in volatility. An alcohol is generally considered to be more "polar" (and hence "less volatile") than the corresponding ketone, and the results observed with the zearalenones fit this generalization. Steroidal ketones, however, are usually retained as long as or longer than the corresponding alcohols with SE-30 (5). The 6′-deoxy-1′,2′-dehydro-(−)-zearalenone, IV, is much more volatile than either II or III, a consequence of its lower molecular weight and, to a lesser extent with a nonselective stationary phase, its reduced "polarity."

Derivatives of functional group-containing solutes are employed in GLC to increase separations between closely related compounds. Changes in volatility following derivatization not only often lead to improved differentiation, but the change in retention time ("peak shift") observed for a compound following treatment with a reagent specific for a certain kind of functional group is prima facie evidence for the presence of that functional group. Furthermore, the new retention time is an additional piece of data for identification and characterization, much like the melting point of a derivative in classical organic chemistry. Trimethylsilyl ethers have proved to be valuable derivatives in the GLC of estrogens (*15,16*), and for this reason the GLC behavior of the TMSi derivatives of the zearalenones was investigated. The ethers are formed rapidly and quantitatively, and with SE-30 are eluted more slowly than the parent compounds (see Table 1). Separation of 6′-alcohol, III, from the corresponding 6′-ketone, II, is improved by trimethylsilylation. The TMSi ethers exhibit peaks of very nearly theoretical shape (a high degree of symmetry), indicating excellent chromatographic properties and very little adsorption and concomitant peak "tailing." The separation of a mixture of the TMSi ethers of I, II, III, and IV is shown in Fig. 2. When the peak areas observed for equimolar amounts of III and its tri-TMSi ether with the flame ionization detection system were compared, a considerably greater "response" was found for the derivative (see Table 2). It has been

* Two diastereoisomers are possible; see p. 156.

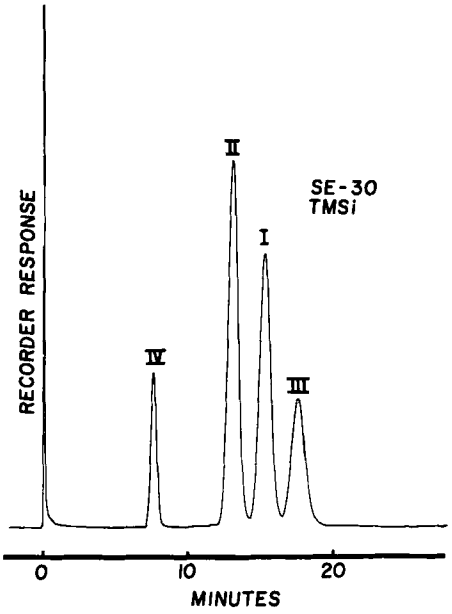


FIG. 2. Gas–liquid chromatographic separation with SE-30 of the TMSi ether derivatives of IV, II, I, and III. Column conditions given in the experimental section.

demonstrated that transformation of a polar hydroxyl group to a hydrocarbon-like TMSi ether reduces irreversible adsorption and results in a greater "detector response" because more compound reaches the detector (*17*). In addition, the hydrogen flame ionization detection system may actually give a greater response to an

TABLE 2

Detector Response (relative to *n*-Octacosane) of Equimolar Amounts of III[a] and III-Tritrimethylsilyl Ether[a]

Compound	Peak area ratio
III	1.05 ± 0.01
III Tri-TMSi ether	2.24 ± 0.03

[a] Samples equivalent to 1 μg of III were analyzed with the F & M instrument. The area ratio values are the averages of four determinations. Measurements of area were height × width at half-height.

equimolar quantity of a higher molecular weight derivative than to the parent compound (*18*). In any event it is clear that etherification leads to greater peak area and hence improved sensitivity.

Derivatives for GLC work may be prepared from keto as well as hydroxyl groups (*9,19*). The 6′-ketone has been found to readily form an O-methyloxime (OMO), a transformation leading to an increase in retention time with SE-30.* When a zearalenone substituted in this manner is subjected to trimethylsilylation conditions, the phenolic groups are etherified to yield an O-methyloxime TMSi ether. These reactions do not affect the lactone group and can provide much information concerning molecular structure.

The use of so-called polar or selective stationary phases as well as phases of the SE-30 type is a widely used means for distinguishing between closely related compounds (*20*). Not only are separation patterns often improved with polar phases, but changes in relative retention behavior with changes in stationary phase may be correlated with the functional group content of solute molecules, leading to more reliable characterization and identification. QF-1, a fluoroalkylpolysiloxane, is very selective for ketones (5), and this property is clearly evident from the greater separation of the TMSi ethers of II and IV with this phase than with SE-30 (see Table 1). Furthermore, whereas with SE-30 a 6′-trimethylsilyloxy group leads to greater retention than a 6′-keto group, this relationship is reversed with QF-1. In contrast to the results seen with SE-30, with QF-1 transformation of the strongly bonding carbonyl group to the OMO effects a sharp increase in volatility.†

As stated earlier, reduction of the 6′-keto group of II can yield two epimeric alcohols. Stereochemistry often exerts a profound influence upon biological activity, and for this reason it was desirable to possess a GLC method for the separation of the two isomers. Table 3 compares the retention behavior of III and its isomer, V, and several of their derivatives with SE-30 and QF-1. The similarity in volatility of the two alcohols with SE-30 is rather expected, since this phase is not known for its ability to distinguish between epi-

* The possibility of formation and separation of isomeric (presumably *syn* and *anti*) OMO derivatives with certain keto groups has been reported (*10*); the suggestion of a "shoulder" on the OMO peaks from I and II may reflect this type of isomerism.

† It is interesting to note that the two ketones are not separable on QF-1, II being only slightly more volatile than I.

TABLE 3

Retention Behavior of the Epimeric 6′-Alcohols and Their Derivatives

Compound	Relative retention time[a]	
	SE-30	QF-1
III	0.99	5.40
V (epimer of III)	1.01	5.50
III Tri-TMSi[b]	1.35 (1.21)[c]	2.16
V Tri-TMSi	1.43 (1.28)[c]	2.27
III Di-Ac TMSi[d]	1.85[e]	9.90
V Di-Ac TMSi	1.98[e]	10.9
III Ac_3[e]	—	22.5
V Ac_3	—	23.2
III Ac_2[f]	—	16.8
V Ac_2	—	17.5

[a] Cholestane = 1.00; column conditions given in the experimental section.
[b] Trimethylsilyl ether.
[c] F-60 column.
[d] 2,4-Diacetyl-6′-TMSi.
[e] Triacetyl.
[f] 2,4-Diacetyl.

meric alcohols (*5,18*). Transformation of hydroxyl groups to TMSi ethers often improves the separation observed between epimeric steroidal alcohols (*5,18*), but when this approach was employed with a mixture of the two epimeric zearalenones, the increase in separation was not of sufficient magnitude to lead to more than a slight bifurcation of the peak with the columns employed (approximately 2500 "theoretical plates"). QF-1 has been shown to be "stereoselective" (*5*), but the separations found for III and V and their TMSi ethers (see Table 3) were no more satisfactory than those with SE-30.

One of the methods for improving separations in GLC is to employ a column of greater "efficiency." Chromatography of the TMSi ethers of III and V on an F-60* column (12 ft) possessing approximately twice the efficiency of the SE-30 column (5 ft) gave a nearly "base-line" separation when a mixture of the two epimeric deriva-

* A stationary phase with partitioning properties very similar to SE-30.

tives were analyzed, although the separation factor or difference in relative volatilities was not improved by the change in column. A second approach to obtaining greater separation is to increase the separation factor between the compounds, either by changing the stationary phase or by preparing derivatives which may exhibit different relative volatilities than the parent compounds. The 2,4-diacetyl derivatives of III and V* showed no improvement in sepa-

* Selective acylation of the phenolic groups can be achieved through use of the theoretical amount of acetic anhydride; D. B. R. Johnston, unpublished results.

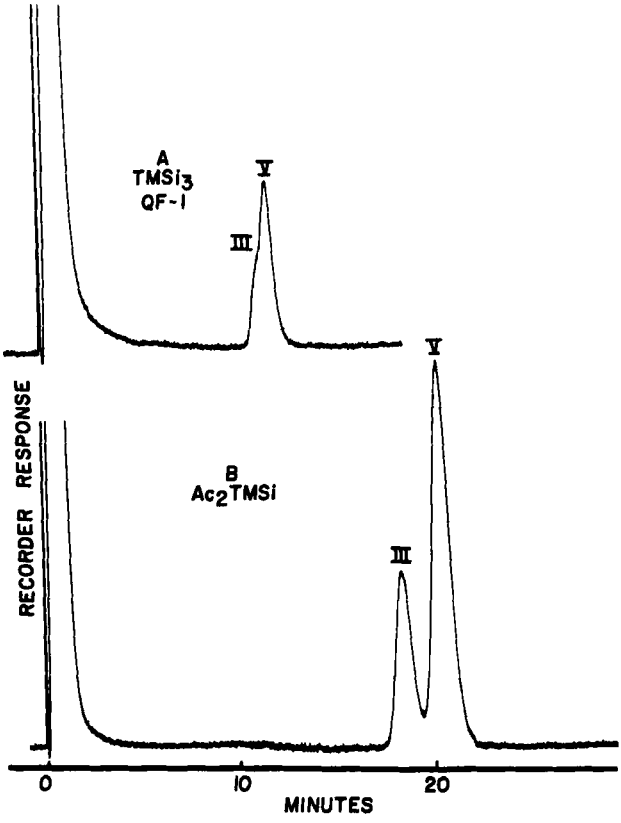


FIG. 3. Gas–liquid chromatographic separations with QF-1 of the 2,4,6′-tri-TMSi (upper chromatogram, A) and the 2,4-diacetyl-6′-TMSi (lower chromatogram, B) derivatives of the epimeric 6′-alcohols, III and V. Column conditions given in the experimental section.

TABLE 4

Retention Behavior of a Group of Zearalenones

Compound	Relative retention time[a]	
	SE-30	QF-1
2-Deoxy-1',2'-dihydro-(−)-zearalenone, VI	0.50	3.74
VI TMSi[b]	0.50	2.70
4-Deoxy-1',2'-dihydro-(−)-zearalenone, VII	0.25	1.90
VII TMSi	0.43	2.38
2-O-methyl-(−)-zearalenone, VIII	1.17	10.7
VIII TMSi	1.00	6.74
4-O-methyl-(−)-zearalenone, IX	0.74	4.61
IX TMSi	1.04	5.51
2,4-Di-O-methyl-(−)-zearalenone, X	0.85	6.54

[a] Cholestane = 1.00; column conditions given in the experimental section.
[b] Trimethylsilyl ether.

ration, and trimethylsilylation of the remaining 6'-hydroxyl group led to only a slightly greater separation for these compounds with F-60. The separation observed for the 2,4-diacetyl-6'-TMSi ethers with QF-1, however, is significantly greater than that for the tri-TMSi ethers (see Table 3), and Fig. 3 illustrates that a 6-ft packed column of "normal efficiency" is sufficient to lead to an excellent differentiation. A comparison of retention behavior shows that acetylation leads to considerable increases in retention time with QF-1 (triacetyl > diacetyl > diacetyl TMSi > parent alcohols > tri-TMSi) and that it is the combination of 2,4-diacetylation and 6'-trimethylsilylation which results in the best separation (diacetyl TMSi > triacetyl ≅ diacetyl ≅ tri-TMSi > parent alcohols).

It is clear from the foregoing that instances exist when it may be necessary to prepare rather exotic derivatives in order to achieve a desired separation. On the positive side, GLC is often able to take advantage of structural differences which, though slight, do have profound influences upon volatility. A case in point is the ability of even the nonselective stationary phase SE-30 to readily distinguish between the isomeric monophenolic 2-deoxy and 4-deoxy derivatives of II (VI and VII, respectively). The remarkable difference in retention behavior between these isomers (see Table 4) can be ascribed to intramolecular hydrogen bonding between the

2-hydroxyl group and the carbonyl oxygen of the lactone in VII and the lack of such an interaction for the 4-hydroxyl group in VI. The latter group is thus completely free to interact with the stationary phase, whereas the 2-hydroxyl group in VII is not fully available for this intermolecular bonding. The dramatic difference in retention times illustrates the fact that even with nonpolar or nonselective stationary phases molecular weight is not necessarily the determining factor for volatility. The same retention relationship holds with QF-1, and with both stationary phases trimethylsilylation sharply decreases the isomer separation; etherification eliminates the possibility of intramolecular hydrogen bonding and the isomeric TMSi ethers, of equal molecular weights, exhibit very similar retention times. It is interesting to note that with the more polar or selective QF-1, derivatization of VI leads to a decrease in retention time, whereas with VII the retention time actually increases. In the former, transformation of the polar hydroxyl group to a nonpolar ether would be expected to result in a decrease in retention time (5); with VII, however, the hydroxyl group is already "derivatized" (intramolecularly hydrogen bonded) and rather than leading to a reduction in retention time due to a decrease in polarity etherification actually results in an increase in retention time and an "unmaking" of the carbonyl group.

Table 4 also presents the retention data for two other isomeric phenols, the 2- and 4-monomethyl ethers of (−)-zearalenone—VIII and IX, respectively. The 4-hydroxyl group again leads to greater retention times than its 2 isomer; indeed, from the behavior of the 2,4-dimethyl ether of (−)-zearalenone, X, it is clear that a 2-methoxy group causes greater retention than the corresponding hydroxyl group.

GLC techniques have been employed to demonstrate that in sheep a major pathway for metabolism of III is oxidation of the 6′-hydroxyl group. The major urinary metabolite of III has been identified as II, the corresponding 6′-ketone. Table 5 shows the excellent correlation between the GLC retention data for the metabolite and the reference standard under a variety of conditions. Figure 4 illustrates the use of derivative formation in the identification of the metabolite. When the trimethylsilylated metabolite was analyzed on the LKB combination gas chromatograph–mass spectrometer, the molecular ion was observed to possess an *m/e* value of 464, in complete agreement with the molecular weight for the di-TMSi ether of II. In addition, this mass spectrum was identi-

TABLE 5

Comparison of Retention Data for II and Metabolite of III

	Relative retention times[a]	
	SE-30	QF-1
Metabolite	0.81	—
II	0.82	—
Metabolite TMSi[b]	1.01	4.52
II TMSi	1.01	4.48
Metabolite OMO[c]	1.03	—
II OMO	1.04	—
Metabolite OMO TMSi	1.29	2.37
II OMO TMSi	1.27	2.34

[a] Cholestane = 1.00; column conditions given in the experimental section.
[b] Trimethylsilyl ether.
[c] O-Methyloxime.

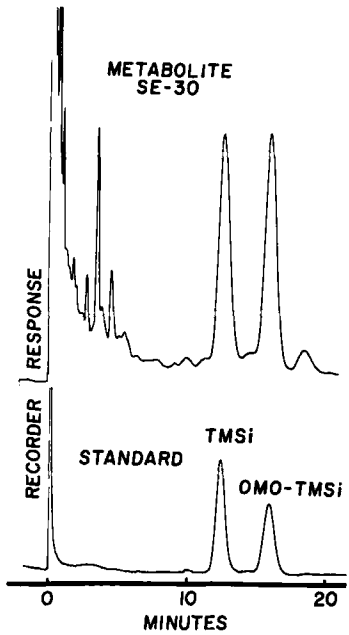


FIG. 4. Comparison of the gas–liquid chromatographic behavior (SE-30) of the 2,4-di-TMSi and the 6'-OMO, 2,4-di-TMSi derivatives of II (lower chromatogram) with that of the two compounds obtained when the major urinary metabolite of III in the sheep was exposed to the two different sets of derivatizing conditions. Column conditions same as in Fig. 2.

cal to that of the authentic di-TMSi ether of II. This article is a further demonstration that conditions may be established for the successful GLC of compounds of considerable structural complexity.

Acknowledgment

These studies were carried out during a cooperative effort between Merck & Co., Inc., and The Commercial Solvents Corporation. The author is most grateful to the latter for generous supplies of (−)-zearalenone, 1′,2′-dihydro-(−)-zearalenone and one of the corresponding epimeric 6′-alcohols (III), 6′-deoxy-1′,2′-dihydro-(−)-zearalenone, 2-O-methyl-(−)-zearalenone, 4-O-methyl-(−)-zearalenone and 2,4-di-O-methyl-(−)-zearalenone. It is also a pleasure to acknowledge the cooperation of a number of Merck scientists in this investigation. R. H. Silber, T. N. Mellin, G. V. Downing, and A. B. White made available the sheep urine metabolite. D. B. R. Johnston and T. B. Windholz provided samples of the other epimeric 6′-alcohol (V) of 1′,2′-dihydro-(−)-zearalenone, of the 2,4-diacetylated epimeric alcohols, and of 2-deoxy- and 4-deoxy-1′,2′-dihydro-(−)-zearalenone.

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Received by editor January 27, 1968
Submitted for publication February 6, 1968