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APPLICATION OF COMBINED GAS–LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY TO THE IDENTIFICATION OF STEROLS IN OAT SEED

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Abstract—Using a combination GLC-mass spectrometer the presence of saturated C_{27} (I), C_{28} (II) and C_{29} (III) sterols together with the corresponding Δ^5- (IV–VI) and Δ^7-monounsaturated (VII–IX) sterols, both 24-methylene-Δ^5- (X) and 24-methylene-Δ^7-3β-ol (XI), the corresponding 24-ethylidene compounds (XII–XIII) and stigmasterol (XIV) in oat seed has been demonstrated.

INTRODUCTION

THE fungus *Phytophthora cactorum* when grown on oat meal produced mature oospores, whereas when grown upon a basal medium no oospores were formed. The material in oat seed responsible for this phenomenon was found to be the sterol fraction,[1,2] and this has led to a reinvestigation of the major components of this mixture.[3] Earlier it had been found that the principal component of the oat seed sterol fraction was β-sitosterol.[4] In addition two other major components were characterized (Δ^5- and Δ^7-avenasterols), stigmasterol was identified as being present in small amount, and from u.v. absorption data ergosterol was presumed to be present. During the reinvestigation[3] the Δ^5-avenasterol was shown to be 29-isofucosterol (XII) and from gas chromatographic data the Δ^7-avenasterol was presumed to be $\Delta^{7,24(28)}$-stigmastadien-3β-ol (XIII). Using GLC several other components were shown to be present including cholesterol (IV) and campesterol (V) and one compound had mobilities corresponding to Δ^7-cholesten-3β-ol (VII). In order to elucidate the structure of these minor components further investigation using a combined GLC-mass spectrometer has been undertaken.

RESULTS AND DISCUSSION

The sterol fraction, obtained from oat seed as previously described,[3] was subjected to the scheme of analysis indicated in Fig. 1, and the results obtained are summarized in Table 1. Data in mass spectra (1–3) suggested the presence of saturated sterols (M+2) and a small

[1] C. G. ELLIOTT, M. E. HENDRIE, B. A. KNIGHTS and W. PARKER, *Nature* **203**, 427 (1964).
[2] J. ANTONIS LEAL, J. FRIEND and P. HOLLIDAY, *Nature* **203**, 545 (1964).
[3] B. A. KNIGHTS, *Phytochem.* **4**, 857 (1965).
[4] D. R. IDLER, S. W. NICKSIC, D. R. JOHNSON, V. W. MELOCKE, H. A. SCHUETTE and C. A. BAUMANN, *J. Am. Chem. Soc.* **75**, 1712 (1953).

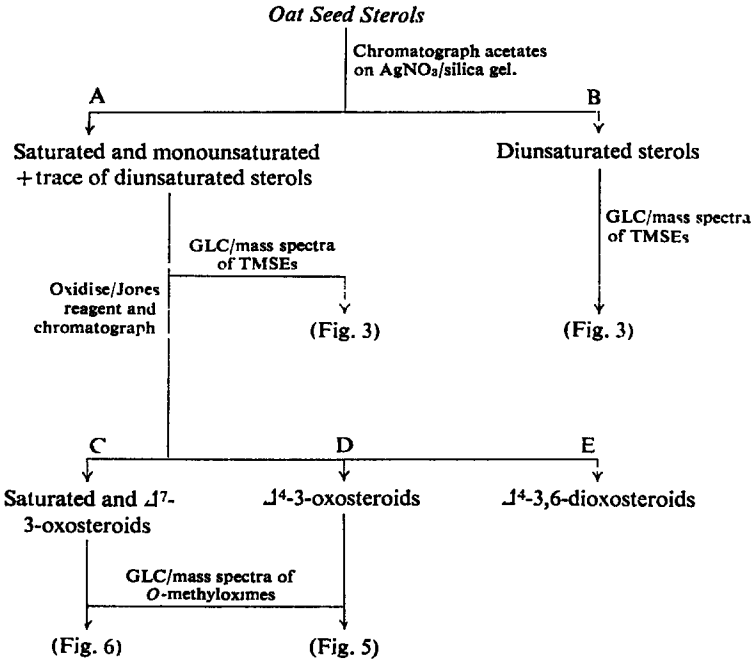

FIG. 1. SCHEME OF ANALYSIS OF STEROL FRACTION FROM OAT SEED.

TABLE 1. SUMMARY OF RESULTS OBTAINED IN SCHEME OUTLINED (FIG. 1)

Fraction	Mass spectrum number	Derived from original compound (Fig. 2)
A	(1)	(IV)
	(2)	(V)
	(3)	(VI)
B	(4)	(X)
	(5)	(XI)
	(6)	(XII)
	(7)	(XIII)
C	(8)	(I)
	(9)	(VII)
	(10)	(II)
	(11)	(VIII)
	(12)	(III)
	(13)	(IX)
	(14)	(XIII)
D	(15)	(IV)
	(16)	(V)
	(17)	(XIV)
	(18)	(VI)

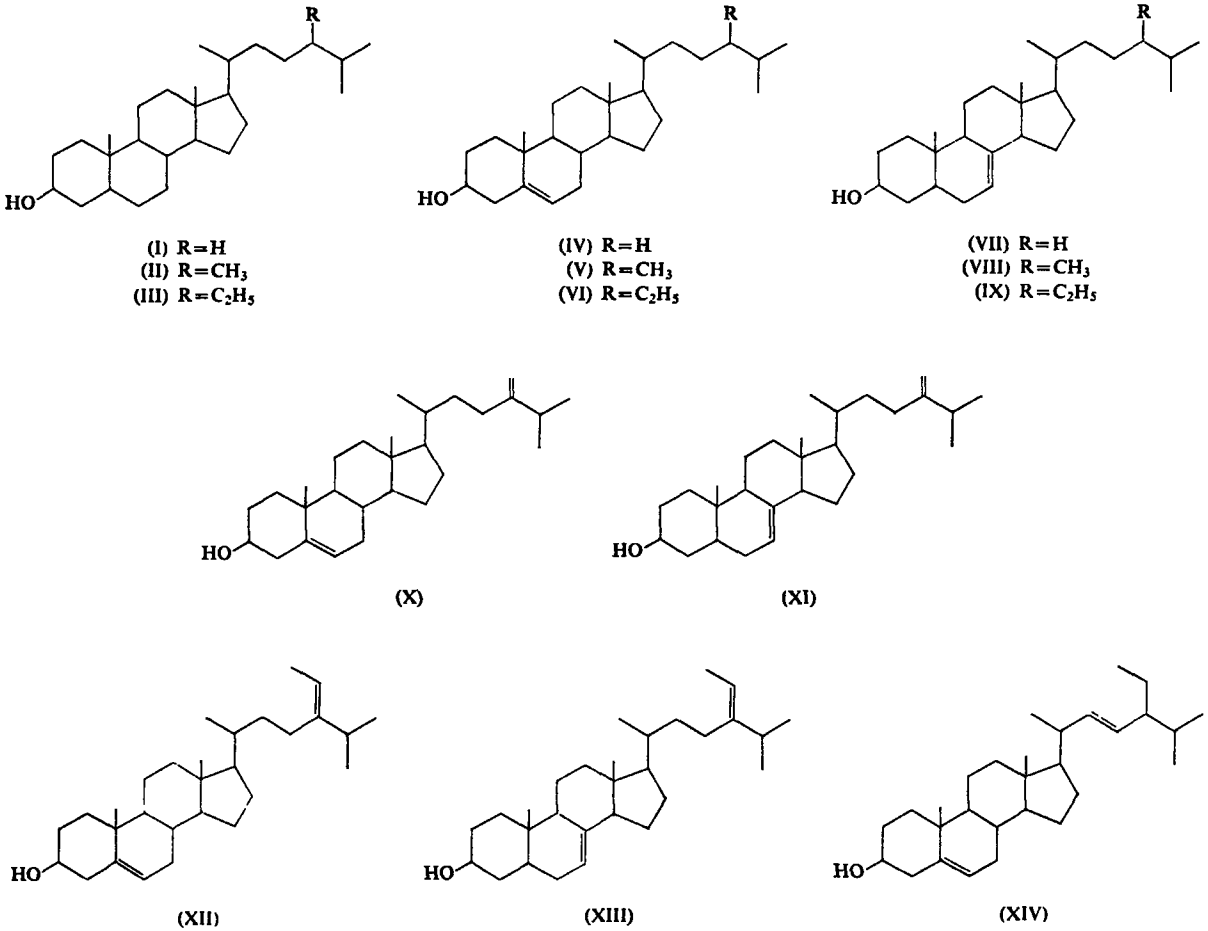

FIG. 2. (I) 5α-CHOLESTAN-3β-OL; (II) 24-METHYL-5α-CHOLESTAN-3β-OL; (III) 24-ETHYL-5α-CHOLESTAN-3β-OL; (IV) CHOLESTEROL; (V) CAMPESTEROL; (VI) β-SITOSTEROL; (VII) Δ^7-CHOLESTEN-3β-OL; (VIII) 24-METHYL-Δ^7-CHOLESTEN-3β-OL; (IX) 24-ETHYL-Δ^7-CHOLESTEN-3β-OL; (X) 24-METHYLENE-CHOLESTEROL; (XI) 24-METHYLENE-Δ^7-CHOLESTEN-3β-OL; (XII) 24-ETHYLIDENECHOLESTEROL (29-ISOFUCOSTEROL); (XIII) 24-ETHYLIDENE-Δ^7-CHOLESTEN-3β-OL; (XIV) STIGMASTEROL

amount of diunsaturated sterol (M-2) in fraction A in addition to the parent molecular ion (M) which corresponded to monounsaturated sterols. From the GLC data for fraction A it seemed probable that Δ^7-sterols were present in the mixture (compounds (VII–IX) in Fig. 2). In order to separate these compounds from Δ^5-sterols, the mixture was subjected to oxidation with Jones reagent,[5] the saturated sterols being converted to the corresponding 3-oxocompounds, the Δ^7-sterols to the Δ^7-3-oxosteroids and Δ^5-sterols to a mixture of Δ^4-3-oxo- and Δ^4-3,6-dioxosteroids.[6] The mixture was then separated by column chromatography (alumina) and the nonpolar fraction C further purified by TLC on silica gel. This procedure for separating plant sterols was used by Rowe to detect saturated sterols in species of pine.[7]

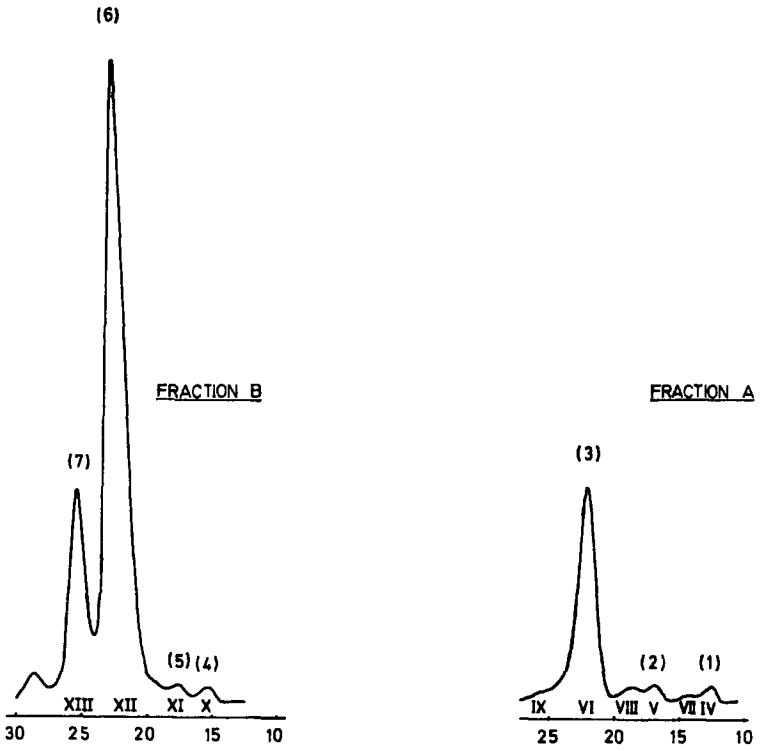

FIG. 3. GLC OF FRACTIONS A AND B, 1% F-60. ARABIC NUMERALS INDICATE POSITIONS CORRESPONDING TO WHERE MASS SPECTRA WERE TAKEN. ROMAN NUMERALS REFER TO STRUCTURES (FIG. 2) FROM WHICH COMPOUNDS WERE DERIVED.

Fractions A and B (Fig. 3) were converted into trimethylsilyl ethers (TMSE) and afforded mass spectra (1–3) and (4–7) respectively. Fractions C (Fig. 6) and D (Fig. 5) were converted into *O*-methyloximes[8] prior to analysis and afforded spectra (8–14) and (15–18) respectively. In addition *O*-methyloximes were prepared from authentic samples of 5α-cholestan-3-one, Δ^7-cholesten-3-one and Δ^4-cholesten-3-one and subjected to analysis in the same instrument. The resultant spectra are shown in Fig. 4.

[5] K. BOWDEN, I. M. HEILBRON, E. R. H. JONES and B. C. L. WEEDON, *J. Chem. Soc.* 39 (1946).
[6] L. F. FIESER and M. FIESER, *Steroids*, p. 202. Reinhold, New York (1959).
[7] J. W. ROWE, *Phytochem.* 4, 1 (1965).
[8] H. M. FALES and T. LUUKKAINEN, *Anal. Chem.* 37, 955 (1965).

Mass spectra (1–3) from fraction A were obtained under previously described conditions[9,10] for sterol TMSEs. on a combination GLC mass spectrometer designed and built by Dr. R. Ryhage at the Karolinska Institutet, Stockholm. They were found to be identical with spectra published[9] for cholesterol (IV), campesterol (V) and β-sitosterol (VI). Additional confirmation for these structures was obtained from the mass spectra from fraction D. Spectrum (15) was found to be identical, except for the relative intensities of some of the ions,

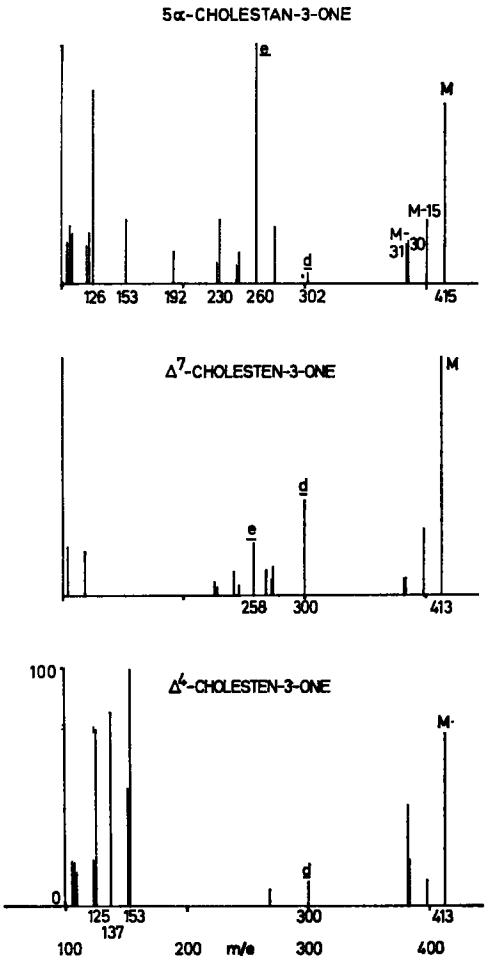

FIG. 4. MASS SPECTRA OF *O*-METHYLOXIMES DERIVED FROM AUTHENTIC 5α-CHOLESTAN-3-ONE, Δ^7-CHOLESTEN-3-ONE AND Δ^4-CHOLESTEN-3-ONE.

with the spectrum from Δ^4-cholesten-3-one (Fig. 4) obtained using an LKB 9000 spectrometer. Spectra (16) and (18) were identical with spectrum (15) except for the parent molecular ion M, and the ions M-15, M-30 and M-31. These ions were increased by 14 and 28 mass units respectively in spectra (16) and (18) compared with spectrum (15), as would be expected for compounds having one and two extra carbon atoms in the side chain. This result is

[9] P. ENEROTH, K. HELLSTRÖM and R. RYHAGE, *J. Lipid Res.* **5**, 245 (1964).
[10] P. ENEROTH, K. HELLSTRÖM and R. RYHAGE, *Steroids* **6**, 707 (1965).

consistent with these compounds being formed from compounds (IV), (V) and (VI) by oxidation as outlined in Fig. 1 and thus affords additional evidence for the presence of compounds (IV)–(VI) in fraction A. β-Sitosterol (VI) has previously been isolated from this mixture.[4]

Mass spectrum (17) was found to be similar to (18) except that the ions for M, M-15, M-30 and M-31 were all two mass units less—indicating an additional double bond in the molecule —and an additional peak for M-43 was observed. The latter peak is a consistent feature of

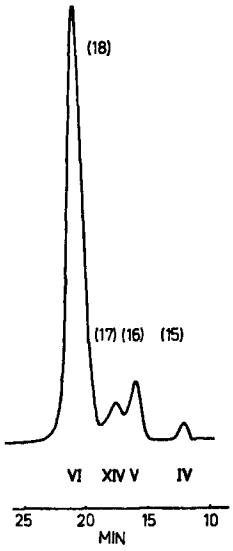

FIG. 5. GLC OF FRACTION D, 1% SE-30.

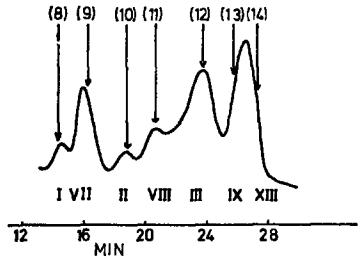

FIG. 6. GLC OF FRACTION C, 1% SE-30.

Δ^{22}-sterols such as stigmasterol (XIV) and the position of the compound in the GLC trace of this mixture (Fig. 5) is as expected for $\Delta^{4,22}$-stigmastadien-3-one. Thus evidence confirming the previous finding of stigmasterol in oat seed[4] has been obtained.

GLC mass spectrum analysis of mixture C (Fig. 6) produced spectra (8–14). Spectrum (8) was found to be identical with that from 5α-cholestan-3-one *O*-methyloxime (Fig. 4) and (9) was found to be identical to that from Δ^7-cholesten-3-one *O*-methyloxime indicating the presence of compounds (I) and (VII) respectively in fraction A. The ions for M, M-15, M-30 and M-31 in spectra (10) and (12) exhibited the same relationship to those in (8) as did the corresponding ions in spectra (16) and (18) to (15), and an identical relationship between

spectra (9), (11) and (13) was also observed. These results strongly suggest the presence of the three saturated sterols (I–III) and the three Δ^7-monounsaturated sterols (VII–IX), related to the Δ^5-sterols cholesterol (IV), campesterol (V) and β-sitosterol (VI), in fraction A. This result is consistent with the currently held view of the biosynthesis of plant sterols,[11] and demonstrates that in oat seed the biosynthetic sequences involving conversion of Δ^7-sterols to Δ^5- and to saturated sterols takes place about as readily in the C_{27} series as in the C_{28} and C_{29} series.

In Fig. 7 are shown mass spectra (13) and (14) obtained at the points indicated in Fig. 6 and these serve to indicate that nearly homogeneous mass spectra may be obtained from steroids not apparently separated by GLC, a result which has been observed for other

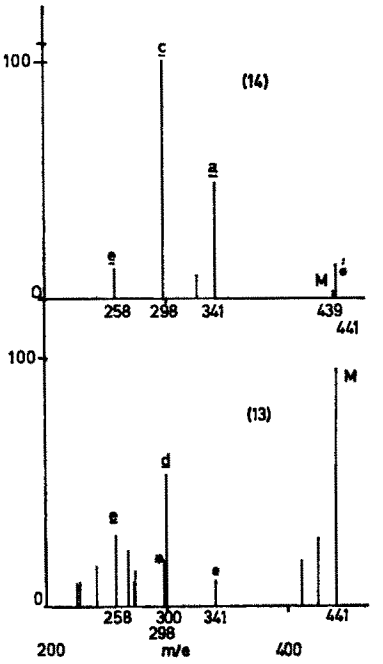

FIG. 7. MASS SPECTRA (13) AND (14) (FIG. VI). * INDICATES PEAKS OVERLAPPING FROM THE OTHER COMPONENT OF THE MIXTURE.

compounds.[12] The mass spectrum (14), showing ions for M-98 and M-141, corresponds to a diunsaturated steroid having an ethylidene group at C-24, the ion M-98 (Fig. 8 fragment *a*) being observed in the mass spectra from citrostadienol and fucosterol[13,14] and 29-isofucosterol.[3] The ion M-141 (side chain+2H: fragment *c*) is apparently only strong in Δ^7-sterols although an examination of the *meta*-stable ions in the mass spectrum from citrostadienol showed the ion to be derived by loss of 43 from the ion M-98.[13] Thus it appears that $\Delta^{7,24(28)}$-stigmastadien-3β-ol is also present in fraction A.

[11] R. B. CLAYTON, *Quart. Rev.* **19**, 201 (1965).
[12] A. E. BANNER, R. M. ELLIOTT and W. KELLY, *Gas Chromatography* (Edited by A. GOLDUP), p. 180. Institute of Petroleum, London (1964).
[13] J. BERGMAN, B. O. LINDGREN and C. M. SVAN, *Acta. Chem. Scand.* **19**, 1661 (1965).
[14] P. BENVENISTE, L. HIRTH and G. OURISSON, *Phytochem.* **5**, 31 (1966).

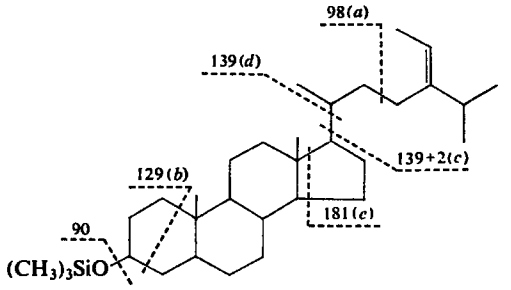

FIG. 8. FRAGMENTATIONS PRODUCED BY ELECTRON IMPACT UPON PLANT STEROLS, ILLUSTRATED FOR 29-ISOFUCOSTEROL (XII).

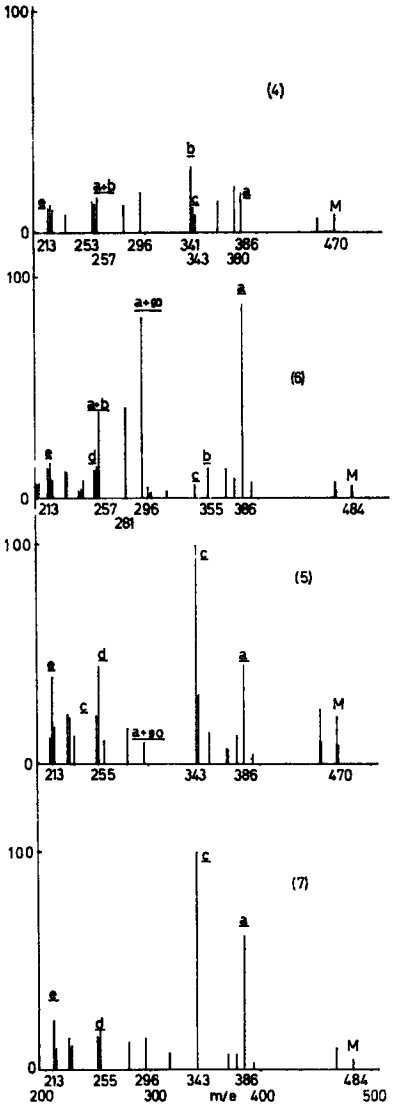

FIG. 9. MASS SPECTRA (4)–(7).

Mass spectra (4)–(7) are shown in Fig. 9, and the principal ions above m/e 200 are listed in Table 2. The molecular ions correspond to diunsaturated sterol TMSE derivatives having respectively 28 (M = 470) and 29 (M = 484) carbon atoms. In spectra (4) and (5) ions for M-84 and M-127 (*a* and *c* respectively) indicate the presence of a 24-methylene group in the side chain[14] and the corresponding ions for 24-ethylidene groups are observed in spectra (6) and (7). The ions M-129 and m/e 129 in spectra (4) and (6) are characteristic of TMSE derivatives of 3β-hydroxy-Δ^5-sterols.[9] The ions at m/e 255 and 213, common to all four spectra, represent fragmentations *d* and *e*,[15] and are also represented by ions in the mass spectra from the *O*-methyloximes of 5α-cholestan-3-one and Δ^7-cholesten-3-one. The difference in relative intensities of these fragments in the *O*-methyloxime spectra (Fig. 4) supports the belief that the Δ^7-double bond strongly inhibits fragmentation *e* and may serve to explain the difference in intensity of the fragmentation *c* between Δ^5- and Δ^7-sterol derivatives.[13,14] The principal component of fraction B has been shown to be 29-isofucosterol (XII) and spectrum (6) is in

TABLE 2. PRINCIPAL IONS ABOVE M/E 200 IN MASS SPECTRA (4)–(7)-FRACTION B

	Ion	Mass spectra (4)	(5)	(6)	(7)
(1)	Molecular ion	470	470	484	484
(2)	M-15	455	455	469	469
(3)	M-90	380	380	394	394
(4)	Cleavage *a*	386	386	386	386
(5)	M-90+15	365	365	379	379
(6)	*a*+15	371	371	371	371
(7)	Cleavage *b*	341	—	355	—
(8)	Cleavage *c*	343	343	343	343
(9)	*a*+90	296	296	296	296
(10)	*a*+90+15	281	281	281	281
(11)	*a*+*b*	257	—	257	—
(12)	*d*+90	255	255	255	255
(13)	*c*+90	253	253	253	253
(14)	*e*+90	213	213	213	213
(15)	*b*	129	—	129	—

agreement with this structure. The second most abundant sterol in fraction B has been presumed to have structure (XIII)[3] and spectrum (7) is consistent with this assumption, and that some of the same compound was also present in fraction A is indicated by spectrum (14) from fraction C. From the GLC data and the corresponding mass spectra data it is evident that 24-methylenecholesterol (X) and 24-methylene-Δ^7-cholesten-3β-ol (XI) are both minor components of fraction B.

The fourteen sterols described above as being present in oat seed are all known to occur naturally, but several have not previously been described as present in higher plants. These are Δ^7-cholesten-3β-ol (VII), the corresponding C_{28} compound (VIII) and 24-methylene-Δ^7-cholesten-3β-ol (XI), although the latter compound has been reported as present in fungi. In higher plants 24-methylenecholesterol (X) has only reported as present in pollen.[16–18]

[15] H. BUDZIKIEWICZ, C. DJERASSI and D. H. WILLIAMS, *Structure Elucidation of Natural Products by Mass Spectrometry*, Vol. 2. Holden Day, San Francisco (1964).
[16] M. BARBIER, M. F. HUGEL and E. LEDERER, *Bull. Soc. Chim. Biol.* **42**, 91 (1960).
[17] M. F. HUGEL, W. VETTER, H. AUDIER, M. BARBIER and E. LEDERER, *Phytochem.* **3**, 7 (1964).
[18] E. LEDERER, *Biochem. J.* **93**, 449 (1964).

$\Delta^{7,24(28)}$-Stigmastadien-3β-ol (XIII) has not apparently been described in any other plant species than oat although it constitutes approximately 10 per cent of the unfractionated mixture. The presence of both Δ^5- and Δ^7- compounds and both monounsaturated and diunsaturated sterols in this mixture suggests that several other compounds may be present although probably in very low percentage. These include a diunsaturated C_{27} sterol such as desmosterol, the C_{28}-$\Delta^{5,22}$-diunsaturated sterol brassicasterol and C_{28} and C_{29}-$\Delta^{7,22}$-diunsaturated sterols. Although a large number of sterols are present in this mixture, three compounds (VI, XII and XIII) constitute 80–85 per cent of the mixture, and it may well prove possible to use oat (*Avena sativa*) to study the interconversion of the three compounds and to study the competition between processes involved in the biosynthesis of plant sterols.

EXPERIMENTAL

Isolation of sterols. Extraction of seed and isolation of sterols were carried out as previously described.[3]

Chromatography of sterols. TLC was carried out on silica gel G using 5% ethyl acetate in petroleum ether as developing solvent. Spots were detected using 2,4-dinitrophenylhydrazine sulphate or ceric sulphate, the plates being heated until colours developed in the latter case. When used for preparative separations prior to GLC, zones were detected on TLC plates by spraying with dichlorofluorescein and scanning under a u.v. lamp (350 mμ). Indicated zones were cut out and steroids eluted with anhydrous ether.

GLC was carried out as previously described for analytical runs.[3] For GLC-mass spectrometry two instruments were used, one designed and built by Dr. R. Ryhage in the Karolinska Institutet, Stockholm, and the other was an LKB 9000 installed in the University of Glasgow Chemistry Department. Analyses were carried out using a 1% SE-30 column at 230° and 250° and at electron energy of 70 eV.

Oxidation of sterols. This was carried out in ether/acetone solution (1:2) by adding Jones reagent[5] dropwise until a permanent brown coloration was imparted to the mixture. Ketones were isolated by partition between water and ether and were purified by chromatography on alumina and then by TLC.

O-*methyloximes* were prepared by dissolving the steroid (3–5 mg) in pyridine (1 ml) adding an equal weight of methoxy amine hydrochloride (Eastman Kodak) and leaving the mixture to stand overnight. The pyridine was evaporated in a stream of nitrogen and the residue triturated with ether. The derivatives left on evaporation of the ether were further purified by sublimation prior to GLC.

Other derivatives were prepared as previously described.[3]

Acknowledgements—Thanks are due to Drs. J. Sjövall and P. Eneroth for mass spectra determined in Stockholm and to Miss P. M. Pellitt and Miss H. Humphrys for mass spectra determined on the LKB 9000 mass spectrometer. Thanks are also due to the S.R.C. for a generous grant to enable the purchase of a combined GLC-mass spectrometer and to the D.S.I.R. for a grant to one of us (B. A. Knights) during the tenure of which part of this work was accomplished. The awarding of a maintenance grant by Glasgow Education Authority to W. Laurie is acknowledged.