Phytochemistry, 1969, Vol. 8, pp. 1771 to 1775. Pergamon Press. Printed in England

# THE CHEMICAL COMPOSITION OF THE SURFACE WAX OF *LOLIUM PERENNE*

R. J. HAMILTON and D. M. POWER*

Department of Chemistry and Biology, Liverpool Regional College of Technology

(*Received* 25 *February* 1969)

**Abstract**—The surface wax of *Lolium perenne* has been separated into three fractions by application of thin-layer and column chromatography using silica gel. Fraction I consists entirely of normal hydrocarbons with $C_{27}$, $C_{29}$, $C_{31}$, and $C_{33}$, predominating. Fraction II is complex and contains high molecular weight esters, some, though not all of which are sterol esters. The alcohol fraction III contains *n*-tetracosanol, *n*-hexacosanol and *n*-octacosanol as the major components.

## INTRODUCTION

THE SIGNIFICANCE of plant waxes in the control of such important biological phenomena as water loss, agricultural spray efficiency and mechanical damage to the leaf is now well established.[1] Surprisingly, however, the literature contains very few reports of the analysis of surface waxes of economically significant plants. In 1931 an investigation of the wax from *Lolium perenne* (Rye grass) was carried out by Chibnall and his co-workers[2] who reported the presence of *n*-hexacosanol and *n*-triacontane; the latter was later shown to be a mixture. More recently a preliminary communication has appeared concerning the surface waxes of the Gramineae.[3]

The findings presented here are concerned with the detailed composition (chemical) of *L. perenne* surface wax as revealed by the application of modern analytical techniques—a necessary preliminary step to the investigation of the biosynthesis of certain wax components.

## RESULTS AND DISCUSSIONS

Rye grass (*Lolium perenne*) was grown for 6–14 days and seedlings were harvested each day throughout the growth period. The surface wax from the leaves was extracted in $CHCl_3$[4] and after removal of the $CHCl_3$ fractionated by column chromatography on silica gel into three fractions (I–III) by successive elution with petrol ether, 2% ether in petrol ether and ether.[3]

Analysis of fraction I by TLC on silica gel G showed the presence of a single spot, $R_f$ 0·87 (petrol ether), identical to that of *n*-alkanes. The absence of unsaturated hydrocarbons was demonstrated by means of argentous TLC.[5] Fraction I showed no absorption at 1720–1740 cm$^{-1}$ or 3600–3400 cm$^{-1}$ in the i.r., confirming the absence of carbonyl- and hydroxyl-containing compounds. The results of analysis by GLC on Apiezon L and OV-1, two non-polar high-temperature stationary phases, are shown in Table 1.

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* Present address: Department of Chemistry and Applied Chemistry, University of Salford, Salford 5, Lancs.

[1] G. EGLINTON and R. J. HAMILTON, *Science* **156**, 1322 (1967).

[2] A. POLLARD, A. C. CHIBNALL and S. H. PIPER, *Biochem. J.* **25**, 2111 (1931).

[3] I. S. TRIBE, J. K. GAUNT and D. WYNN PARRY, *Biochem. J.* **109**, 8P (1968).

[4] J. T. MARTIN, *J. Sci. Food Agri.* **2**, 635 (1960).

[5] V. WOLLRAB, M. ŠTREIBL and F. ŠORM, *Coll. Czech Chem. Comm.* **30**, 1654 (1965).

TABLE 1. HYDROCARBONS IN FRACTION I OF *Lolium perenne* LEAF WAX

| Carbon number | Hydrocarbon percentage composition<br>No. of days after sowing<br>6 | 7 | 8 | 9 | 10 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|---|
| <25 | 15·4 | 9·3 | 4·2 | 3·7 | 6·9 | 1·3 | 3·2 | 3·0 |
| 25 | 2·6 | 2·2 | 2·1 | 1·9 | 1·4 | 1·5 | 1·5 | 1·5 |
| 26 | — | — | 0·3 | Tr | — | Tr | Tr | Tr |
| 27 | 10·8 | 9·5 | 9·6 | 10·1 | 9·6 | 11·8 | 10·8 | 10·2 |
| 28 | 0·4 | 1·1 | 0·8 | 0·2 | 0·3 | 0·4 | 0·5 | 0·3 |
| 29 | 37·6 | 34·4 | 45·8 | 39·2 | 39·8 | 43·8 | 40·0 | 41·1 |
| 30 | 0·8 | 0·6 | 3·0 | 0·4 | 0·5 | 0·5 | 0·6 | 0·6 |
| 31 | 24·1 | 22·5 | 24·6 | 25·5 | 24·4 | 26·4 | 26·8 | 28·3 |
| 32 | — | 1·3 | Tr | 1·0 | 0·3 | Tr | 0·3 | 0·5 |
| 33 | 7·8 | 9·3 | 10·4 | 11·7 | 10·5 | 12·1 | 12·2 | 13·0 |
| 34 | | | | | | | | Tr |
| 35 | | 6·3 | Tr | 6·3 | 4·2 | 2·0 | 3·6 | 1·1 |
| 36 | | | | | | | | |
| 37 | | 2·8 | | | 2·2 | | | |

The major components of fraction I were $nC_{27}$, $nC_{29}$, $nC_{31}$, and $nC_{33}$ alkanes. No branched hydrocarbons were detected. The most striking difference between the hydrocarbon fractions from seedlings of different ages is the sharp decline (15–3·0 per cent of total) in the proportion of material of chain length shorter than 25 carbons during the growth period (6–14 days).

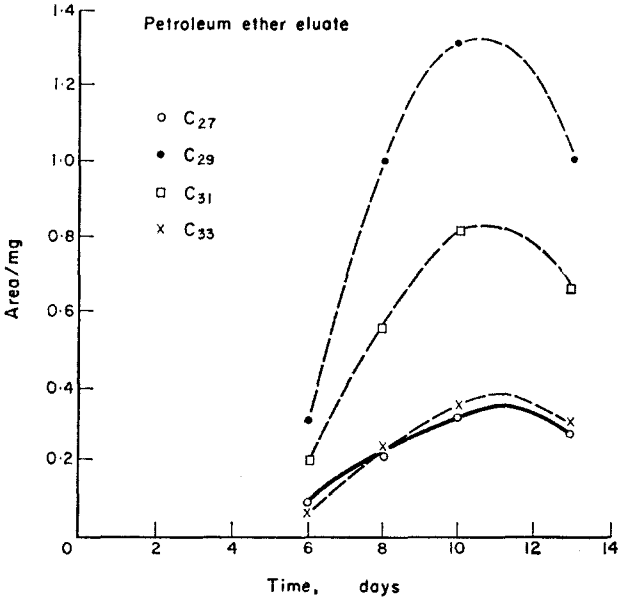

FIG. 1. HYDROCARBON FRACTIONS WERE DISSOLVED IN $CHCl_3$ (1 ml) AND ALIQUOTS (4 $\mu$l) ANALYSED BY GLC ON A 5% APIEZON L COLUMN.

The areas of the four major peaks were measured by triangulation and area of peak mg of hydrocarbon is shown plotted against time of harvesting.

These preliminary results show that some variation in hydrocarbon composition occurs during the early stages of growth (6–8 days) but, after 8 days, a more consistent pattern has emerged (Table 1). The weight of fraction I obtained is approximately constant (10 mg) throughout the growth period. A constant aliquot of each sample was analysed by GLC. The data, represented graphically in Fig. 1, show a decrease in the amount of all four major peaks between 10 and 13 days, even though the percentage composition is approximately the same after these growth times (Table 1). This would suggest that, during the 10 to 13 day period, synthesis of hydrocarbon of chain length very much greater than $C_{37}$ is occurring and that such compounds are not observed under the analytical conditions employed in the work.

The presence of absorption in the i.r. at 3630 $cm^{-1}$ indicates that fraction III consists of alcohols. On TLC fraction III has an $R_f$ of 0·5 (ether–petrol ether, 1:1) identical to that of *n*-primary alcohols.

To identify the individual components of fraction III a series of standard alcohols and their trimethylsilyl ethers were analysed by GLC. The results are shown in Table 2. The retention of an alcohol is recorded as its methylene unit[6] (or carbon number) by comparison with *n*-alkanes. It can be seen (Table 2) that the effect of the primary hydroxyl group of an alcohol is to increase its retention relative to an *n*-alkane by a factor of $+2·7 \pm 0·1$ methylene units, on an OY-1 stationary phase. The corresponding factor ($F_{TMSi}$) for a primary trimethylsilyl ether is $3·5 \pm 0·2$. Thus the change in value on conversion of an alcohol to a trimethylsilyl ether ($\Delta$ OH—TMSi) is $+3·5 \pm 0·2 - 2·7 \pm 0·1 = 0·8 \pm 0·1$.

TABLE 2. GAS–LIQUID CHROMATOGRAPHIC DATA FOR *Lolium perenne* ALCOHOL AND STANDARDS

| Alcohols | No. of carbons in chain | Methylene units | F-X | ΔOH—OTMSi | Column |
|---|---|---|---|---|---|
| *n*-Tetradecanol | 14 | 16·65 | +2·7 | | a |
| *n*-Hexadecanol | 16 | 18·70 | +2·7 | | a |
| *n*-Octadecanol | 18 | 20·70 | +2·7 | | a |
| 3-ethyl-9-ethyl tridecan-6-ol | 17 | 18·00 | +1·0 | | a |
| 2-Hydroxytetradecanol | 14 | 16·00 | +2·0 | | a |
| *n*-Tetracosanol | 24 | 26·70 | +2·7 | | b |
| Cholesterol | 27 | 30·40 | +3·4 | | b |
| *Lolium perenne* major alcohol | | 28·60 | | | b |
| *n*-Tetracosanol | 24 | 27·45 | +3·5 | | c |
| Cholesterol | 27 | 32·30 | +5·3 | | c |
| *Lolium perenne* alcohol | | 29·45 | | | c |
| ***Trimethyl silyl ethers*** | | | | | |
| *n*-Hexadecyl trimethylsilyl ether | 16 | 19·58 | +3·58 | +0·88 | a |
| *n*-Octadecyl trimethylsilyl ether | 18 | 21·50 | +3·50 | +0·80 | a |
| Tetradecyl-2-trimethylsilyl ether | 14 | 17·00 | +3·00 | +1·00 | a |
| *n*-Tetracosanyl trimethylsilyl ether | 24 | 27·50 | +3·50 | +0·80 | b |
| Cholesteryl trimethylsilyl ether | 27 | 31·3 | +4·3 | +0·90 | b |
| *Lolium perenne* alcohol trimethylsilyl ether | | 29·3 | | +0·70 | b |

F-X is the factor due to groupings either OH or OTMSi

Columns (a) 1% OV-1 on Gas Chrom Z (80–100 mesh) at 164°; (b) 1% OV-1 on Gas Chrom Z (80–100 mesh) at 250°; (c) 1% Apiezon L on Gas Chrom Z (80–100 mesh) at 250°.

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[6] E. C. Horning, W. J. A. Vandenheuvel and R. A. Keller, in *Advances in Chromatography* (edited by J. J. Calvin Giddings), Vol. 1, p. 153 (1965).

The *Δ* value for the major alcohol from *L. perenne* of 0·7 therefore indicates a primary alcohol whilst its methylene unit of 28·6 indicates that it is *n*-hexacosanol, in agreement with previous workers.[2] Fraction III was also subjected to mass spectrometric analysis, and its breakdown pattern consisted of peaks at 364 *m*/*e*, 336 *m*/*e* and 308 *m*/*e* which corresponded to values for $C_{26}H_{54}O$ where P-18 = 364 *m*/*e*; P-(18+28) = 336 *m*/*e*; P-(43+31) = 308 *m*/*e*; all typical fragments from *n*-alkanols. In addition to *n*-hexacosanol, fraction III contains two components with retention values of 26·8 and 30·7 methylene units corresponding to *n*-tetracosanol ($nC_{24}$-OH) and *n*-octacosanol ($nC_{28}$-OH). The identity of the components with values of 19·1, 20·0, 25·3 and 30·3 methylene units is unknown, but that with a value of 30·3 is suspected to be a sterol.

Fraction II showed carbonyl absorption in the i.r. at 1740 $cm^{-1}$. When fraction II was analysed by TLC, three spots were revealed, the major of which corresponded ($R_f$ 0·60; 5% ether in petrol ether) to octadecyl stearate. The results of GLC analyses, both under isothermal and temperature programmed conditions, are reported in Table 3. The major component (22·2 per cent) has a retention value of 53·1 methylene units and probably represents a $C_{52}$ ester. The evidence for the existence of such large esters in plant waxes[7] has hitherto been based on analysis of the acid and alcohol fractions obtained after saponification. This is the first report of the direct analysis of such long-chain plant wax esters.

TABLE 3. GLC ANALYSIS OF FRACTION II OF *Lolium perenne*

| Methylene units | Area (%) |
|---|---|
| 26·2 | 0·1 |
| 28·7 | 18·8 |
| 30·7 | 15·7 |
| 32·7 | 8·1 |
| 34·5 | 2·2 |
| 36·5 | 0·5 |
| 38·9 | 0·4 |
| 40·7 | 0·1 |
| 41·7 | 0·3 |
| 43·5 | 3·1 |
| 45·5 | 14·3 |
| 47·4 | 5·8 |
| 49·3 | 5·1 |
| 51·2 | 2·9 |
| 53·1 | 22·2 |

The saponification mixture from fraction II was methylated with diazomethane and separated by TLC into a methyl ester fraction and an alcohol fraction. GLC examination of the methyl esters indicated that $nC_{26}$ was the major acid, with smaller amounts of $nC_{24}$, $nC_{18}$, and $nC_{16}$ acids. The alcohol fraction appeared to be a complex mixture of components with peaks at 18·50, 18·90, 19·30, 24·65, 26·60, 28·60 and 30·55 which indicates the presence of $cC_{24}$, $nC_{26}$, and $nC_{28}$ alcohols. It may be that the peak at 30·55 is due to a sterol since fraction II gave a red colour in the Liebermann–Burchard reaction. Further work is in progress to identify the other alcohol components and to examine the total wax for evidence of polyisoprenols[8] such as dolichol and betulaprenols.

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[7] R. E. CORBETT, M. A. MCDOWALL and S. G. WYLLIE, *J. Chem. Soc.*, 1283 (1964).

[8] R. A. MORTON, *Chem. and Ind.* 1710 (1968).

## EXPERIMENTAL

### *Extraction and Fractionation of Surface Wax*

*Lolium perenne* seeds were sown on seedling compost (Lexington Ltd.). The seeds were liberally watered and the seed boxes were placed inside polythene bags and germinations allowed to take place at 25°. The leaves of the seedlings were harvested at varying time intervals (6–14 days). The leaves (15·3 g wet weight) were dipped in $CHCl_3$ (3 × 200 ml) for 2 min. The solvent was removed under reduced pressure. The last solvent traces being removed in $N_2$ at 40° to yield the surface wax (139 mg). The total surface wax was fractionated on a Silica gel column (15 × 1 in.) by eluting first with petrol ether (40–60°) (300 ml) to give fraction I, then with 2 % ether–petrol ether (400 ml) to give fraction II, and finally with ether (400 ml) to give fraction III.

### *Saponification*

Fraction II (20 mg) was refluxed with dry methanol (20 ml) and Na (30 mg) for 1 hr. A solution of NaOH (0·3 g) in water (2·5 ml) was then added and the solution warmed on a steam bath for a further 30 min. Acidification with conc. HCl gave a mixture of fatty acids and alcohols which were extracted with ether. After esterification with $CH_2N_2$, the mixture was separated on silica gel into methyl esters and alcohols by preparative TLC.

### *Ether Formation*

Fraction III (2 mg) was dissolved in hexamethyldisilazane (100 $\mu$l) and trimethylchlorosilane (5 $\mu$l) as a catalyst and allowed to stand for 30 min. Analyses by GLC were carried out directly on the reaction mixture.

### *Gas–Liquid Chromatography and Mass Spectrometry*

Samples were analysed by gas chromatography on a Pye 104 dual column instrument with columns 5 ft + $\frac{1}{4}$ in. Column packings were: 1 % Apiezon on Gas Chrom Z (80–100 mesh) at 250°, 1 % OV-1 on Gas Chrom Z (80–100 mesh) at 164°, 250° and with temperature programming from 250–320°. Mass spectral analyses were performed on an A.E.I. MS2 spectrometer and on an L.K.B. 9000 GLC/mass spectrometer.

*Acknowledgements*—We should like to thank S.R.C. for a grant to purchase the GLC instrument. We thank Dr. B. A. Knights for helpful discussions and Dr. C. J. W. Brooks for the analyses on the L.K.B. 9000 GLC/mass spectrometer.