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NEUTRAL LIPIDS OF LEAVES AND STEMS OF *TRIFOLIUM REPENS*

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Abstract—The neutral lipids of white clover leaves and stems have been separated into wax esters, free fatty acids, free fatty alcohols, free sterols, triglycerides and hydrocarbons The wax esters were mainly of C_{18} di- and tri-unsaturated fatty acids and C_{30} fatty alcohol. Linolenic acid was the predominant free fatty acid and triacontanol was the principal free fatty alcohol Of the hydrocarbons, C_{29} and C_{31} were present in the largest amounts

INTRODUCTION

GALACTOLIPIDS and phospholipids are known to be[1,2] the major lipids of a number of forage plants common in New Zealand. Weenink[3,4] identified the principal acyl lipid from *Trifolium pratense* as galactosyl diglyceride and Shorland[5] demonstrated that most of the fatty acids in several other pasture plants were also combined as galactolipids. More detailed analyses of white clover galactolipids were carried out by Roughan and Batt[2] who found they were comprised mainly of mono- and digalactosyl diglycerides in the ratio of about 5:3. However, apart from the surface waxes of certain plants,[6] the composition of the total neutral lipids of most pasture plants has not been reported.

This paper reports the composition of the neutral lipids extracted from the leaves and stems of white clover.

RESULTS AND DISCUSSION

The total lipid extracted from the white clover leaves and stems represent 0·7% on a wet weight basis and is similar to that (0·8%) from red clover.[4] Combinations of silicic acid column and preparative TLC procedures separated the neutral lipids into wax esters (including small quantities of sterol esters) (2·9%), free fatty alcohols (2·5%), triglycerides (2·0%), free fatty acids (1·7%), free sterols (1 6%), carotenoids (1·2%), hydrocarbons (1·1%) and two unidentified components (2·5%) The unidentified substances were thought to be

[1] WEENINK, R O (1964) *Biochem J* **93,** 606
[2] ROUGHAN, P G and BATT, R. D (1969) *Phytochemistry* **8,** 363
[3] WEENINK, R. O (1959) *N Z J Sci* **2,** 273
[4] WEENINK, R O (1961) *J Sci Food Agric* **12,** 34
[5] SHORLAND, F B (1961) *J Sci Food Agric* **12,** 39
[6] HITCHCOCK, C and NICHOLS, B W. (1971) *Plant Lipid Biochemistry*, p 72, Academic Press, London

aldehydes and secondary alcohols which are derived from surface wax,[7] but no further examination was pursued. The neutral lipids represented 15·5% of the total lipid extract, the remainder being mainly galactolipids and phospholipids

TABLE 1 FATTY ACIDS, FATTY ALCOHOLS AND HYDROCARBONS OF WHITE CLOVER NEUTRAL LIPIDS (WT %)

Homologues	Wax esters		Free	Free	Triglyceride	Hydrocarbons
	Fatty acid	Fatty alcohol	fatty acid	fatty alcohol	fatty acids	
<14 0	3 4	-	—	--	4 5	—
14.0	3 5	0 6	1 1	—	8 4	—
14 1	1 1	—	—	—	--	—
15 0	1 9	tr*	0 4	tr	3 7	—
15 1	0 7	—	--	—	0 7	—
16 0	11 9	0 7	13 9	0 2	31 2	--
16 1	1 9	—	0 8	—	1 9	—
17 0	0 4	1 3	0 7	0 6	1 9	—
17 1	0 4	—	0 2	--	0 5	—
18.0	7 4	7 2	4 1	1 4	21 9	—
18 1	5 3	—	2 9	--	11 2	—
18 2	15 7	--	3 1	--	7 5	—
18.3	16 9	—	25 6	—	2 8	—
19 0	0 4	0 2	tr	1 8	0 7	—
20 0	6 0	9 8	0 9	1 9	2 4	--
21 0	0 9	3 1	0 3	2 6	0 5	tr
22 0	4 2	10 9	2 5	2 4	0 2	tr
22 1	-	-	tr	-		-
23 0	0·9	3 1	0 9	2 0	--	0 4
24 0	4 1	6 4	3·1	2 0	-	0 3
25 0	0 5	1 1	0·5	1·4	-	2 2
26 0	2 7	4 2	5 9	3 5	—	1 1
27 0	0 5	0 6	1 2	0 8	—	11 9
28 0	1 9	1 4	17 4	5 1	-	2 9
29 0	tr	tr	1 5	2 0	--	38 8
30 0	7 4	48 0	10 8	69 0	-	4 3
31 0	—	tr	0 4	0 4	—	34 3
32 0	—	1 4	1 8	2 9	-	1 6
33 0	—	—	—	—	—	2 2

* tr = Trace, less than 0 1%

The compositions of the wax esters, free fatty acids, free fatty alcohols, triglycerides and hydrocarbons of neutral lipid fractions were determined by GLC and the results are recorded in Table 1. The identification of unsaturated acids was confirmed by further GLC analyses of their hydrogenated products. In the course of examining the fatty acid methyl esters by TLC, it was found that they were resolved into two bands, the more polar of which contained the n-C_{18} plus lower homologues, and the other band the n-C_{20} and higher homologues This separation facilitated GLC analyses. The same phenomenon was observed[8] in studies on human plasma sphingomyelins when their TLC properties appeared to be governed by their individual fatty acid contents

The wax ester alcohols have a similar composition to those of the free alcohols, the preponderant C_{30} component (triacontanol) representing 48% of the total alcohols in the wax esters and 69% in the free alcohols Waldron *et al*.,[9] in their analysis of the paraffins de-

[7] KOLATTUKUDY, P E (1970) *Lipids* **5**, 398
[8] WOOD, P D S and HOLTON, S (1964) *Proc Soc Exp Biol Med* **115**, 990
[9] WALDRON J D, GOWERS, D S CHURNALL A C and PIPER, S H (1961) *Biochem J* **78**, 435

rived from primary alcohols of white clover leaf waxes, also observed that the C_{30} alcohol was the predominant homologue.

Linolenic acid was the main constituent in both the wax esters (16·9%) and in the free fatty acids (25·6%), but whereas the wax esters also contained 15·7% linoleic acid those of the free fatty acid fraction had only 3·1% of this component. Unsaturated fatty acids constituted 42% of the wax ester fatty acids, linolenic and linoleic acids together representing 32·6% and oleic acid 5·3% The occurrence of substantial amounts of C_{18} polyunsaturated fatty acids in plant wax esters, has not been previously recorded Studies of other plant waxes indicate that both the esterified and free fatty acids are comprised mainly of saturated acids.[10,11] However, recent work on *Lolium perenne*[12] showed that the wax esters contained 5% of esterified oleic acid Work by Tulloch and Hoffman[13] on the leaf waxes of *Triticum durum* showed 15% of monounsaturated fatty acids in the wax esters; these components were 22:1 (docosenoic) and 24:1 (tetracosenoic) acids and both had a *trans* configuration. Animal tissues, on the other hand, contain high levels of unsaturated fatty acids and, to some extent, unsaturated fatty alcohols in their wax esters.[14–17]

Of the three mechanisms proposed for the biosynthesis of wax esters,[18] the third which is related to acyl transfer from phospholipids and other polar lipids[19] is of interest. It has been established[20] that the fatty acids of plant phospholipids, especially those in the β-position, are predominantly unsaturated. The products derived from acyl transferase and polar lipids could therefore provide the free fatty acid pool with a substantial quantity of polyunsaturated C_{18} fatty acids. This was confirmed by GLC of the free fatty acids (Table 1) which showed a relatively high content of linolenic acid (25·6%). It is difficult to understand why wax esters in general comprise mainly saturated fatty acids when an abundance of polyunsaturated fatty acids is available during their biosynthesis. Perhaps the wax esters on the surface are different from those isolated from the whole plant. Alternatively the unsaturated wax esters may be located mainly in the internal tissues.

The chain lengths of the wax esters were determined directly by GLC and comprised C_{48} (78·8%), C_{38} (3·9%), C_{40} (3·2%), C_{44} (2·6%), C_{42} (2·5%), C_{36} (2·5%), C_{46} (1·8%), C_{34} (1·5%), C_{50} (1·4%), C_{51} (1·2%), C_{49} (0·6%) and C_{52} (trace). The composition was in approximate agreement with the combinations predicted from the compositions of the fatty acids and fatty alcohols determined after methanolysis (Table 1). The principal wax ester is the same as that in sweet clover,[21] but the structure of the two esters is markedly different. That of white clover is formed mainly of polyunsaturated C_{18} (linolenic and linoleic) acids and triacentanol whilst that from sweet clover is composed of a saturated C_{22} acid and a C_{26} fatty alcohol.

White clover triglycerides contained mainly saturated fatty acids (75·4%) with palmitic (31·2%) and stearic (21·9%) acids predominating. The principal components of the hydro-

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[18] KOLATTUKUDY, P E (1967) *Biochemistry* **6**, 2705
[19] BUCKNER, J S and KOLATTUKUDY, P E (1973) *Arch Biochem Biophys* **156**, 34
[20] MCKILLICAN, M E (1967) *J Am Oil Chemists' Soc* **44**, 200
[21] EMERY, A E and GEAR, J R (1969) *Can J Biochem* **47**, 1195

carbon fraction were C_{29} (nonacosane, 38·8%) and C_{31} (hentriacontane, 34·3%) which resemble those of many other plants including perennial ryegrass[22] and little club wheat (*Triticum compactum*) leaves.[23] The GLC analysis of the white clover free sterol fraction gave a composition which consisted of β-sitosterol (81·5%), campesterol (8·9%), stigmasterol (8·7%) and cholesterol (0·9%). Little information is available on free sterol composition of pasture plants but these components and their proportions are typical of many commercial vegetable oils[24] from other plant sources

EXPERIMENTAL

Material White clover (*Trifolium repens* L. cv. "Grasslands Huia") was grown under New Zealand agricultural conditions and harvested in early summer (December, 1972). Leaves and stems (400 g) were cut into 2–3 cm lengths and extracted with $CHCl_3$–MeOH (2:1)[25] within 15 min of collection. This yielded 2·75 g of lipid extract.

Column chromatography An aliquot (530 mg) was applied to a glass column (1 cm i.d.) of silicic acid (40 g).[26,27] The first solvent $CHCl_3$ (300 ml) eluted the neutral lipids as 3 fractions (A, B and C). $CHCl_3$–MeOH (9:1) (100 ml) and MeOH (400 ml) eluted the polar lipids (fraction D). The column fractions were monitored by TLC as described previously.[28,29]

Preparative TLC Plates were coated with 0·5 mm "purified" Silica gel G as outlined earlier.[29] Using hexane-Et_2O (95:5),[16] fraction A was sub-divided into four other fractions: hydrocarbons, carotenoids, sterol esters, wax esters and unidentified components. The sterol-wax esters fraction was resolved by TLC on $MgO/CaSO_4$ with hexane-acetone (99:1).[30] Fraction B was fractionated using $CHCl_3$ to yield triglycerides and free fatty alcohols.

Fatty acid Me esters and fatty alcohols derived from the wax esters were prepared by methanolysis with BF_3/MeOH.[14] Those from triglycerides were obtained by direct treatment with BCl_3/MeOH under similar conditions.[31] Free fatty acids were methylated with CH_2N_2.[32] The acetate derivatives of fatty alcohols and free sterols were formed by acetylation with Ac_2O.[14] In all cases, the compounds were purified by silicic acid column chromatography[27] prior to GLC examination.

GLC analyses Fatty acid Me esters, fatty alcohols, fatty alcohol acetates, free sterols and sterol acetates were analysed on both polar (2·1 m × 2·5 mm i.d. with 10% EGSS-X on 60–70 mesh GC Z) and non-polar (1·83 m × 5 mm i.d. with 3% JXR on 100–120 mesh GC Q) columns using a dual FID instrument. Wax esters were examined directly on a short column (65 cm × 2 mm i.d.) packed with 3% JXR and programmed over the range of 150–350°.

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