# Minor Constituents of the Acetone-Soluble Lipids of Red-Clover (*Trifolium pratense*) Leaves

By R. O. WEENINK
*Fats Research Laboratory, Department of Scientific and Industrial Research, Wellington, New Zealand*

(*Received* 18 *July* 1961)

Weenink (1961) has shown that the major components of the acetone-soluble lipids of red-clover leaves are composed of galactosyl glycerides, but there is no knowledge of the identity of the remaining constituents of the acetone-soluble fraction.

From earlier observations in this laboratory (Weenink, 1961; Shorland, 1961) it appeared that, apart from galactosyl glycerides, the acetone-soluble lipids of leaves of forage plants contained a small amount of lipid material resembling triglycerides in chemical behaviour. The hydrolysis products, however, contained a large proportion of unsaponifiable matter and a correspondingly small amount of fatty acid; and although glycerol could be detected in the aqueous hydrolysates it was not possible to ascertain the nature of the lipids present.

This paper reports the isolation of triglycerides, diglycerides, sterol esters, free sterols and normal hydrocarbons from the acetone-soluble lipids of red-clover leaves.

## EXPERIMENTAL

*Extraction of the lipid.* Red clover (2 kg. wet wt.) was kindly supplied from a pure stock by Dr A. T. Johns, Plant Chemistry Division, D.S.I.R., Palmerston North. The sample was cut on 28 September 1960 and immediately dropped into boiling ethanol (4 l.), cooled and transported to the laboratory within 4 hr. of the time of harvest. The ethanolic extract was decanted and the residue boiled with 1·5 l. of ethanol–ether (3:1, v/v) for 5 min., cooled and again decanted. The residue was dried *in vacuo* at room temperature, chopped into small pieces and again extracted with ethanol–ether. After the extract had been decanted, the residue was dried *in vacuo* and ground to a fine powder in a Christie–Norris mill. Soxhlet extraction of the powdered residue with ether removed the last traces of lipid.

All extracts were combined and then evaporated *in vacuo* on the water bath, leaving an aqueous residue containing the extracted lipid together with water-soluble impurities. The latter were removed by transferring the lipid to a separating funnel with ether (3 l.) and washing with water (7 l. total vol.). The ethereal solution of the lipid was evaporated *in vacuo* in a rotary evaporator at 40°.

*Solvent separation of the lipid.* To remove contaminating material insoluble in light petroleum (b.p. 40–60°) the ether-soluble lipid was dissolved in the minimum quantity of ether and poured into 20 vol. of light petroleum at 5°. After 2 hr. the precipitate was removed by filtration and discarded. The soluble extract was separated into phospholipid and acetone-soluble lipid by three crystallizations from 10 vol. of acetone at 0°. The phospholipid (6·9 g., 2·18% P, 0·42% N) was stored *in vacuo* at −40°, and the acetone-soluble lipid (16·2 g., 0·25% P, 0·75% N) was largely freed from galactosyl glycerides by three distributions between 300 ml. each of *n*-hexane and methanol–water (95:5, v/v) (Carter, McCluer & Slifer, 1956). The combined hexane layers were evaporated *in vacuo* in a rotary evaporator and further purified by dialysis for 4 hr. against light petroleum in a rubber membrane (van Beers, de Iongh & Boldingh, 1957) with two solvent changes each of 1·5 l. The diffusible lipid was isolated as a dark-green oil by evaporation of the solvent *in vacuo* in a rotary evaporator at 40°.

*Chromatographic separation of the diffusible lipid.* The method of Barron & Hanahan (1958) was used, with minor modifications. Silicic acid, especially prepared for the chromatography of lipids (Bio-Rad Laboratories, Richmond, Calif., U.S.A.) was used after activation for 12 hr. at 120°. Column loading was 16 mg. of lipid/g. of silicic acid and running temperature was maintained at 25°. Flow rate was 1 ml./min. and 15 ml. fractions were collected with an automatic fraction collector.

*Methods of analysis.* Lipid samples were saponified by boiling for 2 hr. with ethanolic 0·5 N-KOH (25 ml./g. of lipid). Fatty acids and unsaponifiable matter were extracted with ether as previously described (Weenink, 1961).

Saponification equivalents of the fatty acids were determined in hot ethanolic solution by titration to phenolphthalein with ethanolic 0·1 N-KOH. The soap solutions so obtained were acidified, the fatty acids again isolated and converted into their methyl esters by refluxing for 3 hr. in 5 ml. of 1% (v/v) $H_2SO_4$ in methanol (see Hilditch, 1956).

Fatty acid ester values were determined by the method of Morgan & Kingsbury (1959); phosphorus, nitrogen and glycerol were determined as described earlier (Weenink, 1961).

*Gas–liquid chromatography.* Samples of methyl esters were chromatographed at 207° on a 8 ft. column of Celite impregnated with 20% of its weight of poly(diethylene glycol adipate) as liquid phase. Hydrocarbons were chromatographed in a similar manner except that column packing was changed to 5% (w/w) of Apiezon L–Celite. Column characteristics and sample introduction are described by Gerson (1961).

*Crystallization of unsaponifiable matter.* Unsaponifiable matter was crystallized from 10 vol. of acetone at 0°. The precipitated material was recrystallized twice from acetone at 0° and once from methanol at room temperature. The crystals so obtained were characterized by Lieberman

reaction, melting point and mixed melting point (open capillaries, uncorr.) with authentic β-sitosterol (L. Light and Co. Ltd.). The content of free sterol in the diglyceride fraction was estimated by precipitation with digitonin (Cooke, 1958). Various concentrations of β-sitosterol were run as standards and these were used to calculate the content of unknown samples.

## RESULTS

The extraction of 2 kg. (wet wt.) of red-clover leaves yielded 26·7 g. of ether-soluble lipid together with 213·7 g. of dry residue and 75·2 g. of water-soluble material. Table 1 shows the amounts of lipid contained in various fractions and their yield as a percentage of the dry matter.

It would be expected that the diffusible lipid from the hexane-soluble fraction would contain the simple lipids such as triglycerides and sterol esters if these were to be present in the clover tissues. This lipid fraction was therefore chromatographed on activated silicic acid as described above, the course of fractionation being followed by the fatty acid-ester content. In some cases pigmented fractions obscured colour development in the ester

Table 1. *Yield of lipid fractions from red-clover leaves*

| Fraction | Wt. (g.) | Wt. as % of ether extract | g./100 g. of dry matter |
|---|---|---|---|
| Ether extract | 26·7 | 100·0 | 8·5 |
| Light petroleum-soluble lipid | 23·1 | 94·0 | 8·0 |
| Acetone-insoluble lipid* | 6·9 | 25·8 | 2·2 |
| Acetone-soluble lipid | 16·2 | 60·6 | 5·1 |
| Hexane-soluble lipid† | 3·6 | 13·5 | 1·1 |
| Methanol-soluble lipid†‡ | 12·5 | 46·8 | 4·0 |
| Diffusible§ | 1·7 | 6·4 | 0·5 |
| Non-diffusible§ | 1·9 | 7·1 | 0·6 |

* Phospholipids. † Subfractions of the acetone-soluble lipid.
‡ Galactosyl glycerides. § Subfractions of the hexane-soluble lipid.

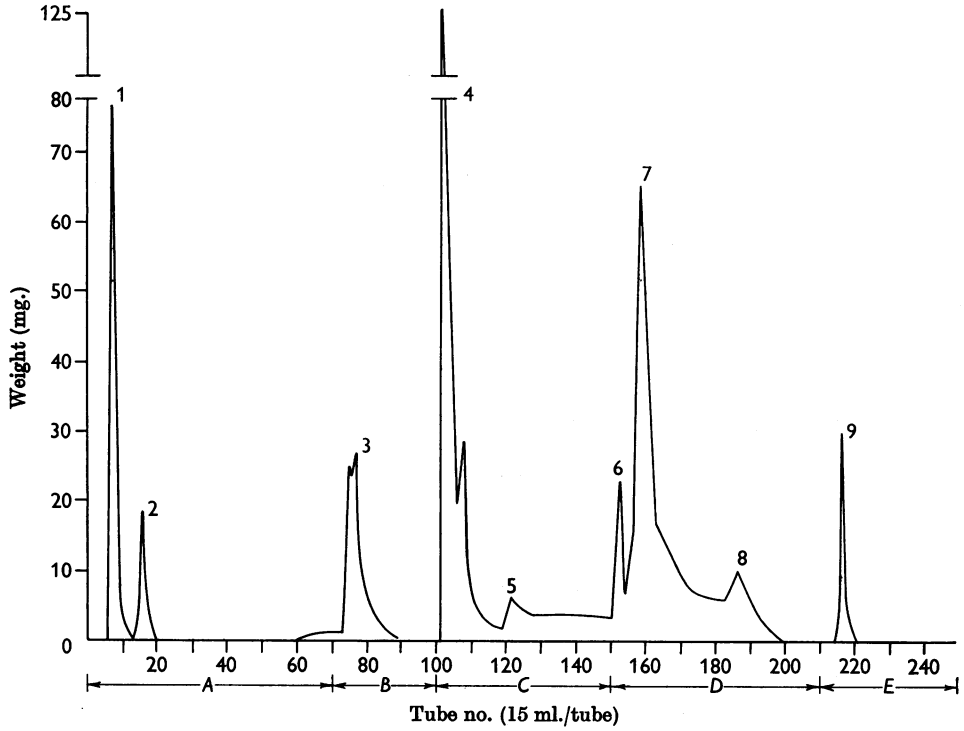


Fig. 1. Elution pattern from the silicic acid column of 1·68 g. of acetone-soluble diffusible lipids from red clover. Column recovery was 91%. Eluting solvents were: *A*, hexane; *B* benzene–hexane (15:85, v/v); *C*, ether–hexane (5:95, v/v); *D*, ether–hexane (20:80, v/v); *E*, ether.

Table 2. *Gas–liquid chromatographic analyses of saturated normal hydrocarbons*

$R_F$ values relative to that of $C_{18}$.

| Carbon no. | $C_{15}$ | $C_{16}$ | $C_{17}$ | $C_{18}$ | $C_{19}$ | $C_{20}$ | $C_{21}$ | $C_{22}$ | $C_{23}$ | $C_{24}$ | $C_{25}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| $R_F$ (standards) | — | 0·49 | — | 1·00 | — | 2·22 | — | 4·92 | — | — | — |
| $R_F$ (red-clover fraction 1) | 0·31 | 0·45 | 0·67 | 1·00 | 1·48 | 2·18 | 3·28 | 4·85 | 7·23 | 10·73 | 16·35 |
| Composition of red-clover hydrocarbons (moles/100 moles) | Trace | 0·34 | 2·42 | 2·78 | 3·46 | 2·78 | 4·50 | 7·26 | 13·80 | 17·30 | 45·00 |

Table 3. *Analyses of lipid fractions*

Results are given as g./100 g.

| Fraction no. | 3 | 4* | 7† |
|---|---|---|---|
| Unsaponifiable matter | 57·2‡ | 18·7 | 59·5 |
| Fatty acid | 40·1 | 80·5 | 37·0 |
| Glycerol | 0 | 7·0 | 4·1 |
| Fatty acid: glycerol molar ratio | — | 3·2 | 2·0 |
| Lieberman reaction | + | +§ | + |

* Free fatty acids washed from this fraction with 10% (w/v) $Na_2CO_3$.

† Free sterol, largely β-sitosterol precipitated with digitonin, constituted 57·8% of this fraction.

‡ β-Sitosterol isolated in small yield.

§ Weak reaction.

determination and it was necessary to weigh the lipid fraction in each tube. A plot of fraction number against weight of lipid present in all tubes is given in Fig. 1.

Peaks 3, 4 and 7 (Fig. 1) contained appreciable proportions of fatty acid esters and these peaks were isolated by bulking the contents of appropriate tubes. The fraction represented by peak 1 was found to contain no ester bonds and, because it comprised an appreciable proportion of the lipid chromatographed, it was also investigated.

*Fraction* 1. The constants of this fraction were wt. 102 mg., m.p. 38–39° (Found: C, 85·8; H, 13·9%). When it was chromatographed on Apiezon L, 11 peaks were observed, some of which coincided with those obtained when authentic saturated normal hydrocarbons were run under the same conditions. A plot of log $R_{n\text{-}octadecane}$ against suspected carbon number produced a straight line, thus indicating a series of saturated normal hydrocarbons.

The $R_F$ values and probable composition are given in Table 2.

*Fractions* 3, 4 *and* 7. These fractions all contained fatty acid-ester linkages and together amounted to 57% of the material applied to the column. From the characteristics of these fractions listed in Table 3 they are identified as sterol ester, triglyceride and a mixture of free sterol and diglyceride respectively. The fatty acid composition of the fractions is given in Table 4 together with the phospholipid (acetone-insoluble) and galactosyl glyceride (methanol-soluble) fractions.

## DISCUSSION

The composition of fractions 3, 4 and 7 given in Table 3 and, in particular, the fatty acid : glycerol molar ratios, appear to establish the presence of sterol esters, triglycerides, diglycerides and free sterols in the acetone-soluble diffusible fraction of red-clover leaves. The triglyceride fraction, however, is not pure, as shown by the high fatty acid: glycerol ratio and the presence of 18·7% of unsaponifiable matter. Since free fatty acids have been washed from the triglyceride fraction with 10% (w/v) sodium carbonate it may be assumed that the excess of fatty acid was combined with the unsaponifiable matter as a sterol ester which was more polar than the sterol esters eluted from the silicic acid column in fraction 3.

The occurrence of minor amounts of triglycerides in leaves of forage plants has been indicated previously (Garton, 1960; Shorland, 1961), but the analytical results were insufficient to establish definitely their presence. In the present work the total diffusible fraction amounts to only 6·4% of the total lipid (Table 1) and, since 23% of this is estimated to be triglyceride, it may be calculated that it is present to the extent of only 1·5% of the total lipid. This is approximately one-half of the amount found by Garton (1960) for dried mixed pasture.

The sterol esters isolated in the present study may contain mixtures of sterols since the melting point of the first precipitate from the crystallization of unsaponifiable matter was 130–132°, rising on subsequent crystallizations to that of β-sitosterol (m.p. and mixed m.p. 136·5°).

Sterol esters have not previously been reported in red-clover lipids although free sterols including β-sitosterol and stigmasterol were found by Pollard (1936) in pasture grasses. The content of sterol esters isolated in the present work amounts to 0·57% of the total lipid.

The combustion analyses of fraction 1, together with its rapid elution from the silicic acid column (Fig. 1), show that this fraction consists of hydrocarbons. Gas-chromatographic analyses showed that the fraction was composed of a homologous series of saturated odd- and even-numbered normal hydrocarbons from $C_{15}$ to $C_{25}$, the last being in greatest concentration (Table 2).

Table 4. *Fatty acid composition of fractions from silicic acid column*

Results are given as moles/100 moles. tr., Trace.

| Carbon no.* ... ... Fraction | <10 | 12–0 | 14–0 | 14–1 | 15–0 | 16–0 | 16–1 | 17–0 | 18 br | 18–0 | 18–1 | 18–2 | 18–3 | 20–1 | 20–2 | 20–3 | 20–4 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sterol ester | 0·2 | 0·5 | 3·7 | — | 0·3 | 24·9 | 0·3 | 0·3 | 0·4 | 13·9 | 12·1 | 27·2 | 16·2 | — | — | — | — |
| Triglyceride | 3·8 | 0·6 | 8·1 | — | 0·2 | 11·5 | 0·4 | tr. | tr. | 1·7 | 3·4 | 37·3 | 31·3 | — | 1·7 | — | — |
| Sterol diglyceride | — | 3·1 | 2·1 | — | tr. | 55·8 | 2·6 | tr. | tr. | 3·0 | 3·6 | 20·7 | 9·1 | — | — | — | — |
| Phospholipid† | — | — | 0·4 | 0·2 | 0·6 | 45·3 | 3·7 | 0·3 | 0·2 | 2·9 | 3·1 | 24·8 | 14·9 | 0·4 | 0·8 | 1·1 | 1·3 |
| Galactosyl glycerides† | — | — | 0·1 | — | — | 1·8 | tr. | — | — | 0·3 | 0·6 | 2·5 | 94·7 | — | — | — | — |

* Figures designate carbon number followed by number of double bonds or branched (br).

† See Table 1.

The work of Waldron, Gowers, Chibnall & Piper (1961) revealed the presence in cocksfoot grass of normal hydrocarbons ranging from $C_{27}$ to $C_{33}$. These hydrocarbons were isolated earlier from the acetone-insoluble fraction of cocksfoot (Pollard, Chibnall & Piper, 1931), whereas those now studied were soluble in cold acetone.

The existence of diglycerides as natural components of leaf lipids is open to question. Their presence could be attributed either to enzymic breakdown of phospholipids during manipulation and extraction (Kates, 1953) or to their being intermediates in phospholipid synthesis (Kennedy, 1956). Support for either conclusion is found in the similarity in fatty acid composition between the phospholipid and diglyceride fractions (Table 4). Palmitic acid and linoleic acid predominate in both these fractions, whereas the sterol esters and triglycerides contain less palmitic acid and more linolenic acid. The galactosyl glycerides, on the other hand, contain almost pure linolenic acid (Weenink, 1961).

Others working with leaf lipids have identified such complex lipids as a sulpholipid (Benson, Daniel & Wiser, 1959), inositol and polyglycerophospholipids (Lepage, Mumma & Benson, 1960; Benson & Maruo, 1958), galactosyl glycerides (Benson & Maruo, 1958; Benson *et al.* 1959), a complex glycoside (Kates, 1959), complex phospholipids (Wheeldon, 1960) and two monoglycerides (Zill & Harmon, 1959). These complex lipids appear to have different fatty acid compositions. Whereas the sulpholipid (Benson *et al.* 1959) contained only oleic acid and the galactolipids had only linolenic acid as their main fatty acid constituents, Wheeldon (1960) found that many different fatty acids were present in the phospholipids of cabbage leaves. Therefore glycolipids may be distinguished from phospholipids by the much simpler fatty acid composition of the former group.

## SUMMARY

1. The acetone-soluble lipids of red-clover (*Trifolium pratense*) leaves have been shown to contain minor amounts of sterol esters, triglycerides, diglycerides, free sterols and hydrocarbons. These lipids are present to the extent of 0·57, 1·50, 0·60, 0·80 and 0·38% of the total extract respectively.

2. The free and combined sterols contain $\beta$-sitosterol and the hydrocarbons consist of members of a homologous odd- and even-numbered series, $C_{15}$ to $C_{25}$, the $C_{25}$ compound predominating.

3. The fatty acid compositions of fractions show that the diglycerides contain over 50% of palmitic acid, whereas the sterol esters and triglycerides have linoleic acid and linolenic acid as their main constituents.

## REFERENCES

Barron, E. J. & Hanahan, D. J. (1958). *J. biol. Chem.* **231**, 493.
Benson, A. A., Daniel, H. & Wiser, R. (1959). *Proc. nat. Acad. Sci., Wash.*, **45**, 1582.
Benson, A. A. & Maruo, B. (1958). *Biochim. biophys. Acta*, **27**, 189.
Carter, H. E., McCluer, R. H. & Slifer, E. D. (1956). *J. Amer. chem. Soc.* **78**, 3735.
Cooke, R. (1958). *Cholesterol*, p. 486. New York: Academic Press Inc.
Garton, G. A. (1960). *Nature, Lond.*, **187**, 511.
Gerson, T. (1961). *J. Chromat.* (in the Press).
Hilditch, T. P. (1956). *The Chemical Constitution of Natural Fats*, p. 575. London: Chapman and Hall.
Kates, M. (1953). *Nature, Lond.*, **172**, 814.
Kates, M. (1959). *Biochem. biophys. Res. Commun.* **1**, 238.
Kennedy, E. P. (1956). *Canad. J. Biochem. Physiol.* **34**, 334.
Lepage, M., Mumma, R. & Benson, A. A. (1960). *J. Amer. chem. Soc.* **82**, 3713.
Morgan, D. M. & Kingsbury, K. J. (1959). *Analyst*, **84**, 409.
Pollard, A. (1936). *Biochem. J.* **30**, 382.
Pollard, A., Chibnall, A. C. & Piper, S. H. (1931). *Biochem. J.* **25**, 2111.
Shorland, F. B. (1961). *J. Sci. Fd Agric.* **12**, 39.
van Beers, G. J., de Iongh, H. E. & Boldingh, J. (1957). *Int. Conf. Biochem. Probl. Lipids.* Oxford, p. 43.
Waldron, J. D., Gowers, D. S., Chibnall, A. C. & Piper, S. H. (1961). *Biochem. J.* **78**, 435.
Weenink, R. O. (1961). *J. Sci. Fd Agric.* **12**, 34.
Wheeldon, L. W. (1960). *J. Lipid Res.* **1**, 439.
Zill, L. P. & Harmon, E. A. (1959). *Fed. Proc.* **18**, 359.


*Biochem. J.* (1962) **82**, 527

# Quantitative Separation of Human Brain Glycolipids: Isolation of an Aminoglycolipid


BY L. A. FAILLACE AND S. BOGOCH

*The Neurochemical Research Laboratory,* Massachusetts Mental Health Center (Boston Psychopathic Hospital), and the Department of Psychiatry, Harvard Medical School, Boston, Mass., U.S.A.*


(*Received* 4 *July* 1961)

In recent years a number of methods have been used for the qualitative separation and purification of mammalian brain glycolipids. However, methods by which human brain glycolipids could be quantitatively separated and recovered would be useful for comparison of their concentrations in normal as well as in pathological states.

Since silicic acid had been effective as an adsorbent for the fractionation of selected synthetic and native lipid mixtures (Weiss, 1956; Hirsch & Ahrens, 1958), this was tried as a means of separating a complex mixture of human brain lipids. Quantitative recoveries were obtained which permit comparisons of specimens from discrete areas of the same brain and from different brains. Further information was also obtained about the macromolecular aminoglycolipids (Bogoch, Belval & Winer, 1961) structurally related to a preparation of bovine brain ganglioside previously studied (Bogoch, 1958).

## METHODS

Dialysis was performed with prewashed cellophan. Phosphorus was determined by the method of Sperry (1942), nitrogen by a modification of the method of Koch & McMeekin (1924) as described by Bogoch (1958), hexose by a modified orcinol-$H_2SO_4$ method (Sorensen & Haugaard, 1933) and neuraminic acid by a modification of the Bial's orcinol reaction (Bohm, Daüber & Baumeister, 1954). Hexosamine was determined after hydrolysis in a sealed tube with N-HCl for 16 hr. by a modified Elson & Morgan (1934) reaction (Bogoch, 1958).

*Extraction of complex lipid mixture.* Human cerebral hemispheres, 6–12 hr. *post mortem*, were freed of investing meninges and blood vessels, separated into grey and white matter and extracted with hot methanol (Bogoch, 1958). The filtered hot methanol extract of cerebral material was kept at 4° for 48 hr. The precipitate formed was filtered in the cold, taken up at room temperature with chloroform–methanol (2:1, v/v), approx. 0·5 ml./g. wet wt. of cerebral tissue, and partitioned with 12·2% of water. The upper and lower phases were removed and the lower phase was partitioned with 5·6% of water. The combined upper phase contained the bulk of the brain ganglioside and other hexosamine-rich aminoglycolipids (Bogoch *et al.* 1961) and the resultant lower phase contained other glycolipids and phospholipids. The lower-phase material was dried by vacuum distillation at 60° and taken up with minimal amounts of chloroform, and kept at 4° until chromatographed. The total yield of lower-phase material obtained by this method is 23·1 (±0·13) (range: 21·0–24·7) mg./g. wet wt.

*Preparation and charging of column.* The size of the column was dependent upon the amount of material to be chromatographed. A ratio of 8 mg. of material for every gram of silicic acid was the maximum amount that could

* Now Foundation for Research on the Nervous System, 36 The Fenway, Boston, Mass., U.S.A.