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A STUDY OF THE LIPID CONSTITUENTS OF BIRD'S-FOOT TREFOIL (*L. CORNICULATUS*)*

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Abstract—The neutral and other non-acidic lipids of bird's-foot trefoil, *Lotus corniculatus*, have been isolated and identified. Among them are sterol esters, fatty acid methyl esters, triglycerides, free fatty acids and diglycerides. The fatty acids of these lipids along with the fatty acids from the total phospholipid fraction have been analyzed and a comparison made of the first and second harvest.

INTRODUCTION

INFORMATION on the nature of plant lipids and on the type of fatty acids associated with individual lipid fractions are limited. More detailed studies have been made possible as methods of analysis have become faster, more precise and more refined and as a result the lipids in a number of grasses and vegetables have been partially analyzed. Results from the studies of various plant materials indicate the presence of many neutral lipids such as sterol esters diglycerides and triglycerides; [1–6, 11] phospholipids such as lecithin, phosphatidylethanolamine, phosphatidylinositol, phosphatidylglycerol and polyglycerol phosphatides; [1,4,7–9] glycolipids such as mono-digalactosyldiglycerides; [1,9–12] sterol glycosides; sulfolipids; [13–16] and cerebrosides [16]. The use of chromatographic techniques permits resolutions of these and other lipids. Gas–liquid chromatography enables the identification of the various fatty acids that are associated with lipids.

Using the combined techniques of column, thin-layer and gas–liquid chromatography the neutral lipids (sterol esters, methyl esters, triglycerides, free acids and diglycerides) of bird's-foot trefoil, *Lotus corniculatus* var. *viking*, have been investigated. Details of this work

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and a comparison of the lipids from the first and second harvest is reported in this paper. This distribution of the fatty acids attached to the different lipids is also discussed.

RESULTS AND DISCUSSION

Leaves and stems of *Lotus corniculatus* were oven dried (30°), milled, and again dried. Based on thin-layer chromatographic assay, the tissue yielded the same classes of lipid component when extracted with chloroform–methanol, ethyl ether or chloroform–isopropanol. The total weight of the fatty acids from the different solvent extracts were also similar. The lipid extract was washed with water, the solvent evaporated *in vacuo*, and the residue dissolved in petroleum ether for column chromatography.

The column was packed with minus 325 mesh silicic acid and eluted into three fractions (1, 2 and 3) with solvents of increasing polarity. By comparison with known compounds on thin-layer chromatography, *fraction* 1 was shown to consist of sterol esters, fatty acid methyl esters, triglycerides, and free fatty acids; *fraction* 2 to consist of free fatty acids, diglycerides, sterols and green pigments; and *fraction* 3 to consist of phospholipids and green pigments. *Fractions* 1 and 2 were rechromatographed to give *fractions* 1*a*, 1*b*, 2*a*, and 2*b*. *Fraction* 1*a* contained sterol esters and fatty acid methylesters; 1*b*, triglycerides and free fatty acids; 2*a*, free fatty acids, diglycerides and a small amount of sterol; and 2*b*, sterols and green pigments.

Further separation of *fractions* 1*a*, 1*b* and 2*a* by column chromatography was very tedious with several solvent mixtures. However, thin-layer chromatography yielded good resolutions of the different lipids and successfully separated them individually. *Fraction* 1*a* was resolved with a solvent mixture of light petroleum–ethyl ether–acetic acid (90:10:1 v/v). *Fractions* 1*b* and 2*a* were resolved by first using a solvent mixture of isopropyl ether–acetic acid (94:6 v/v) and allowing the solvent to move half-way up the chromatography plate. At this stage the plates were removed, air dried and placed in a second solvent mixture of light petroleum–ethyl ether–acetic acid (90:10:1 v/v). After each lipid class was isolated it was purified by rechromatographing on thin-layer plates.

The purified lipid fractions including the phospholipid fraction were saponified and the fatty acid salts were removed by extraction with water. The fatty acid methyl ester fraction was not saponified. The alcohol portions of the esters were tested for sterols and glycerol. The salts from each fraction were acidified, extracted with ethyl ether and weighed. Fatty acids from the total lipid fraction were also obtained and weighed. Percentages of fatty acids from each lipid fraction are shown in Table 1 along with total amount present. The fatty acids

TABLE 1. FATTY ACIDS PRESENT IN EACH LIPID FRACTION*

Fraction	Fatty acids %	
	1st harvest	2nd harvest
Sterol esters	16·2	8·0
Methyl esters	9·7	4·8
Triglycerides	12·5	8·8
Free fatty acids	19·2	22·4
Diglycerides	11·8	7·2
Phospholipids	30·6	48·7

* Total fatty acids for 1st and 2nd harvests were 715 and 788 mg, respectively, per 100 g of dried sample.

from the individual lipid fractions varied extensively in their concentration patterns. Using the concentration of the fatty acids as being proportional to the lipids; sterol esters, fatty acid methyl esters, triglycerides, and diglycerides decreased in concentration in the second harvest while the free acids and phospholipids increased markedly as compared to the first harvest.

The fatty acids from each fraction were methylated with boron trifluoride–methanol reagent and analyzed by using gas–liquid chromatography. A comparison of the first and second harvest is given in Tables 2 and 3. The acids present were: lauric, myristic, myristoleic, plamitic, palmitoleic, stearic, oleic, linoleic, linolenic, arachidic, behenic and lignoceric. No attempt was made to identify acids of chain length less than lauric acid. In the first harvest there was 38% saturated acids and 62% unsaturated acids while in the second harvest these values were 92% and 108% of the values of the first harvest, respectively. Although the total unsaturated acids increased, the concentration of linoleic in the second harvest was 85% of the value for the first. Similar values for palmitic and linolenic acids were 86 and 117%, respectively. Palmitic, linoleic and linolenic acids were the major acids in both samples.

Fatty acid composition of the individual lipid classes were markedly different. In the sterol esters, palmitic, linoleic, and linolenic acids were the major components. For the first harvest palmitic comprised 38% of the total fatty acid of this lipid, linoleic 17% and linolenic 24%, however, the values for the second harvest as a percentage of the values of the first were palmitic 64, linoleic, 87, and linolenic, 184%. Myristic, palmitic, linoleic and linolenic predominated in the fatty acid of the methyl ester fraction. In the second harvest palmitic acid decreased 41% and linolenic acid increased 31% while the other major acids remained approximately the same in both cases. Fatty acid distribution in the triglyceride fraction did not follow the pattern of the above two components. Myristic, palmitic, linoleic, and linolenic acids were in greatest concentration in the first harvest while palmitic, stearic, and linoleic acids predominated in the second harvest. Unlike the two latter fractions the saturated acids were in excess of the unsaturated acids in the triglyceride fraction. The fatty acid composition of the free fatty acid fraction was very similar for both the first and second harvest forages. The principal acids were palmitic, linoleic and linolenic. These acids made up over 80% of the total acids. In the diglyceride fraction the saturated acids again predominated with myristic acid comprising 50% of the total acid present for the first harvest. In the second harvest there was a 30% increase in the proportion of myristic acid. The phospholipid portion of the first harvest contained 44% myristic and 39% linolenic acids, but for the second harvest it contained 30% palmitic and 49% linolenic acids. There was no analysis made for the individual phospholipids in the phospholipid fraction.

Any nutritional implication of these findings must of necessity be speculative at this time. However, these results indicate that biologically active fatty acids vary in the different harvests of this and probably other forages and that their proportion varies considerably between different lipid fractions.

EXPERIMENTAL

Two samples of *L. corniculatus* was obtained from Michigan State University farms and prepared as discussed under the section on results. The ground tissue was extracted with a mixture of chloroform–methanol (2:1)[17] and the solvent evaporated *in vacuo*. The lipid

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TABLE 2. FATTY ACID COMPOSITION IN VARIOUS LIPID FRACTION FROM THE FIRST HARVEST OF *Lotus corniculatus*

	Fractions						
% Acids*	Sterol esters	Methyl esters	Triglycerides	Free fatty acids	Diglycerides	Phospholipids	Total lipids†
Lauric	2·0	—	Trace	2·3	9·6	—	0·6
Myristic	10·1	14·3	17·1	9·4	49·8	44·5	1·8
Myristoleic	0·7	—	—	Trace	—	—	0·50
Palmitic	37·8	38·8	33·1	25·0	10·6	17·8	27·2
Palmitoleic	3·1	Trace	1·5	3·0	7·8	Trace	1·2
Stearic	0·8	4·5	5·0	1·5	Trace	—	4·2
Oleic	3·5	5·2	5·5	3·5	4·8	Trace	3·3
Linoleic	17·4	18·5	15·3	16·3	5·4	9·58	17·8
Linolenic	24·8	18·7	22·4	39·0	11·9	39·1	38·6
Arachidic	—	—	—	—	—	Trace	1·5
Behenic	—	—	—	Trace	—	—	1·5
Lignoceric	—	—	—	Trace	—	—	1·5

* The percentage is based on the total acid per fraction as represented on a gas–liquid chromatogram.
† Weight of the total fatty acids was 715 mg per 100 g of dried tissue.

TABLE 3. FATTY ACID COMPOSITION IN VARIOUS LIPID FRACTIONS FROM THE SECOND HARVEST OF *Lotus corniculatus*

	Fractions						
% Acids*	Sterol esters	Methyl esters	Triglycerides	Free fatty acids	Diglycerides	Phospholipids	Total lipids†
Lauric	2·7	—	1·37	2·5	Trace	Trace	0·8
Myristic	5·3	16·4	4·16	7·6	65·0	2·7	3·0
Myristoleic	—	4·4	Trace	0·6	—	—	0·3
Palmitic	24·0	22·8	50·6	24·2	11·4	30·3	23·4
Palmitoleic	2·5	5·3	2·63	2·1	4·6	1·9	1·4
Stearic	1·5	Trace	13·0	1·9	Trace	2·3	3·8
Oleic	3·4	9·0	4·6	3·7	3·7	2·4	3·3
Linoleic	15·1	17·6	19·95	13·2	4·1	10·5	15·2
Linolenic	45·6	24·6	4·55	44·0	11·5	49·6	45·2
Arachidic	—	—	Trace	—	—	Trace	1·2
Behenic	—	—	—	Trace	—	—	1·2
Lignoceric	—	—	—	Trace	—	—	1·7

* The percentage is based on the total acid per fraction as represented on a gas–liquid chromatogram.
† Weight of the total fatty acids was 788 mg per 100 g of dried tissue.

extract was washed in a separatory funnel with a mixture of chloroform–methanol–water (8:4:3)[18] and the solvent again taken off *in vacuo*. The washed extract was then taken up in light petroleum (B.P. 50–65°) in preparation for column chromatography. The lipid extract was not very soluble in petroleum and great care was necessary in order to obtain a quantitative transfer to the column. Extraction of the tissue was also done with chloroform–isopropanol (2:1) by the same method used for the chloroform–methanol extraction.[17] Other extractions for comparative purpose were also done with a Soxhlet extraction apparatus for 24 hr using chloroform–isopropanol (2:1) and ethyl ether.

Column chromatography. A column 2·5 cm inside diameter was packed with 36 g of minus 325 mesh silicic acid.[19] The lipid extract from 10 g of tissue was eluted in three fractions. The first, *fraction* 1, was eluted with 750 ml light petroleum containing ethyl ether, 1% followed by 4% ether; *fraction* 2 was eluted with 650 ml of ethyl ether, and *fraction* 3 with 850 ml of methanol. No pressure was applied to the columns. Further fractionation of *fraction* 1 was done on a 1 cm inside diameter column packed with 18 g of minus 325 mesh silicic acid. The two fractions obtained were eluted with 350 ml of 1% ethyl ether in light petroleum and 400 ml of ethyl ether. *Fraction* 2 was further fractionated in a similar column eluting with 350 ml of 4% ethyl ether in light petroleum and 350 ml of ethyl ether. All fractions were rechromatographed after being dried *in vacuo* and redissolved in a small amount of petroleum ether.

Thin-layer chromatography. Thin-layer plates with silica gel G were used for qualitative work and were made 0·25 mm thick. Plates with silica gel H (0·5 mm thick) were used for quantitative work, for isolation and for recovery of individual lipids. Standard compounds were used to obtain the R_f values of the lipids. Phosphomolybdic acid and 2′,7′-dichlorofluorescein were used as indicators. For recovery of the lipids one end of the plate was spotted with the reference sample and the other portion of it was streaked with the lipid extract. After the plate was developed, only the end with the reference compounds was sprayed (2′,7′-dichlorofluorescein). Individual lipids on the other portion of the plate were scraped off according to the R_f obtained from the references and extracted from the gel with chloroform. Each lipid fraction isolated was rechromatographed. The solvents mixtures used to resolve the lipid fractions have been described in the section on results.

The individual lipids were saponified by refluxing for 8 hr with 10 ml of 1·5 N methanolic KOH per lipid fraction. After cooling, ether was added to the reaction mixture and the potassium salts of the fatty acids were extracted with water. The ether from the ether–alcohol layer was removed by evaporation and the residual alcoholic fraction tested for glycerol and sterols.

Test for sterols. (Liebermann–Burchard reaction.) One ml of the alcoholic fraction was evaporated to dryness and the residue dissolved in 2 ml of chloroform. To this solution were added a few drops of acetic anhydride and the contents mixed before the addition of a few drops of sulfuric acid. A final green color was considered a positive test for sterols.[20]

Test for glycerol. To 1 ml of the alcoholic fraction was added 1 ml of 95% ethanol. Several drops of 0·5 M copper sulfate solution were added then added until there was approximately a 2:1 ratio of copper ions to the glycerol. A few additional drops of 0·1 N NaOH caused a green precipitate.[20]

Determination of fatty acids by gas–liquid chromatography. The fatty acid salts were

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acidified, methylated with boron trifluoride–methanol reagent[21] and dissolved in chloroform for chromatographic analysis. The column was 6 ft long and 4 mm inside diameter and packed with 15% diethylene glycol succinate (LAC-728, F and M Scientific Corporation, Pa.) on diatoport S, 80–100 mesh. The temperature for the oven was 195°, injection port 240° and detector 220°. The rate of flow for the carrier gas was 50 ml/min. Fatty acid methyl ester standards were obtained from Applied Science Laboratories, State College, Pa.

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