Phytochemistry, 1970, Vol. 9, pp. 1557 to 1562. Pergamon Press. Printed in England.

THE CONSTITUENT ACIDS OF ANGIOSPERM CUTINS

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(*Received* 13 *October* 1969)

Abstract—The composition of the cutin from the cuticles of twenty-four angiosperm leaves and fruits has been compared by GLC. The most abundant acid found in angiosperm cutin is confirmed as 10,16-dihydroxyhexadecanoic with 9,10,18-trihydroxyoctadecanoic occurring frequently and 16-hydroxyhexadecanoic less frequently. Other hydroxy acids, as yet unidentified, have also been found. Monobasic acids, especially hexadecanoic, are important constituents of cutin. Wide variations of cutin composition occur between species, within species and between leaves and fruits of the same species.

INTRODUCTION

CUTIN is the important structural component of the cuticular membrane of plants.[1] The precise structure of cutin is unknown but it is mainly a polyester of fatty and hydroxyfatty acids. Structural investigations have been based on the identification of the aliphatic acids obtained by alkaline hydrolysis of cuticular membranes.[2-9] GLC has recently been used to identify and assay these acids.[5-7] The hydroxy acids are chromatographed as acetoxymethyl esters[5] or trimethylsilyl ether methyl esters;[6, 7] the latter being useful derivatives for identification by mass spectrometry.[6]

The cutin acids of *Agave* leaf,[4, 5] apple fruit[2, 3, 6] and tomato fruit[7] have been studied in detail. Baker and Martin[8, 9] have also surveyed a range of leaf and fruit cutin acids using paper and thin-layer chromatography. The most characteristic acid of angiosperm cutin appears to be 10,16-dihydroxyhexadecanoic. 9,10,18-Trihydroxyoctadecanoic acid occurs frequently and is also found in the related biopolymer suberin.[10] Other acids, such as 16-hydroxyhexadecanoic and 10,18-dihydroxyoctadecanoic, may also be present.

This paper describes GLC and TLC analyses of the cutin acids of other plant species to examine variations of cutin composition.

RESULTS

Chromatographic data of reported cutin and suberin acids were determined and the results are given in Table 1. The data were used to identify the acids obtained by ether extraction following hydrolysis of the cuticular membranes. TLC fractionates monobasic, dibasic and

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[1] J. T. MARTIN and B. E. JUNIPER, *The Cuticles of Plants*, Edward Arnold, London in press.
[2] E. A. BAKER, R. F. BATT and J. T. MARTIN, *Ann. Appl. Biol.* **53**, 59 (1964).
[3] C. H. BRIESKORN and A. J. BÖSS, *Fette, Seifen, Anstrichm.* **66**, 925 (1964).
[4] M. MATIC, *Biochem. J.* **63**, 168 (1965).
[5] C. E. CRISP, Ph.D. Thesis, University of California, Davis, 1965.
[6] G. EGLINTON and D. H. HUNNEMAN, *Phytochem.* **7**, 313 (1968).
[7] C. H. BRIESKORN and H. REINARTZ, *Z. Lebensmittelunters. u.-Forsch.* **135**, 55 (1967).
[8] E. A. BAKER and J. T. MARTIN, *Nature*, **199**, 1268 (1963).
[9] E. A. BAKER and J. T. MARTIN, *Ann. Appl. Biol.* **60**, 313 (1967).
[10] F. ZETZSCHE and K, WEBER, *J. Prakt. Chem.* **150**, 140 (1938).

ω-hydroxymonobasic acid homologues by class only. When two or more hydroxyl groups are present individual acid homologues are resolved.[11]

For GLC the mono- and dibasic acids were chromatographed as methyl esters and hydroxy acids as trifluoroacetate methyl esters. Trifluoroacetates are rapidly prepared, are more volatile than trimethylsilyl ethers (by a factor of 200 Retention Index units) and do not contaminate the detectors. Two stationary phases of differing polarity were necessary for identification as certain acid peaks overlap.

TABLE 1. CHROMATOGRAPHIC DATA FOR REFERENCE FATTY AND HYDROXYFATTY ACIDS

	T_{rel} tricosane E301	T_{rel} dotriacontane QF1	R_f TLC Me esters	R_f TLC free acids
Monobasic acids*				
dodecanoic	0·07	0·03	0·76	0·90
tetradecanoic	0·06	0·06		
hexadecanoic	0·33	0·11		
oleic	0·57	0·19		
octadecanoic	0·62	0·19		
eicosanoic	1·05	0·35		
docosanoic	1·52	0·52		
α,ω-Dibasic acids*				
tetradecanedioic	0·47	0·30	0·68–0·72	0·77–0·81
hexadecanedioic	0·84	0·48		
octadecanedioic	1·32	0·71		
nonadecanedioic	1·56	0·83		
eicosanedioic	1·83	0·96		
docosanedioic	2·37	1·21		
ω-Monohydroxymonobasic acids†				
16-hydroxyhexadecanoic	0·64	0·42	0·54–0·58	0·68–0·72
18-hydroxyoctadecanoic	1·05	0·63		
20-hydroxyeicosanoic	1·51	0·87		
22-hydroxydocosanoic	2·0	1·22		
Dihydroxymonobasic acids†				
10,16-dihydroxyhexadecanoic	0·79	0·81	0·26	0·25
10,18-dihydroxyoctadecanoic	1·21	1·03	0·32	0·34
Dihydroxydibasic acids†				
threo-9,10-dihydroxyoctadecane-1,18-dioic	1·36	1·27	0·37	0·46
Trihydroxymonobasic acids†				
threo-9,10,16-trihydroxyhexadecanoic	0·78	1·05	0·07	0·06
erythro-9,10,18-trihydroxyoctadecanoic	1·15	1·21	0·13	0·11
threo-9,10,18-trihydroxyoctadecanoic	1·16	1·23	0·13	0·11
9,10-epoxy-18-hydroxyoctadecanoic	1·16	1·23	0·47	0·58

* Analysed as methyl esters by GLC.
† Analysed as methyl esters trifluoroacetates by GLC.

The constituent acids and the relative compositions of the cutins of the plants studied are shown in Table 2. The relative GLC response factors of the principal acid classes were found

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[11] P. J. Holloway, *Proc. Soc. Analyt. Chem.* **6**, 144 (1969).

TABLE 2. RELATIVE COMPOSITION (%) OF THE CUTIN ACIDS OF FRUITS AND LEAVES

Species and organ	Fatty acids		Hydroxyfatty acids			Unidentified acids						
	C_{16}	C_{18}	16OH-C_{16}	10,16OH-C_{16}	9,10,18OH-C_{18}	0·92*	0·97*	1·04*	1·40*	1·45*	1·51*	1·60*
MONOCOTYLEDONS												
Agave americana leaf	1·0	1·0	—	10·5	22·1	—	1·7	1·9	2·3	16·5	21·8	trace
Allium cepa leaf	5·8	3·8	—	24·8	10·4	1·8	5·3	—	16·7	—	17·5	1·2
Bryonia dioica leaf	51·1	8·5	—	5·9	—	1·6	5·5	—	—	—	—	—
fruit	0·9	—	3·9	53·2	10·9	7·2	2·9	5·0	0·9	—	—	—
Chlorophytum elatum leaf	2·4	—	2·0	19·8	12·7	—	3·0	—	7·1	7·6	11·6	12·9
Gasteria planifolia leaf	1·5	—	—	19·3	26·3	—	1·3	—	7·9	15·0	9·2	—
Iris foetidissimal leaf	5·7	—	4·5	31·4	—	—	7·3	2·0	—	3·9	1·6	3·9
fruit	2·1	1·2	—	9·2	28·0	—	6·2	—	8·6	—	9·5	5·9
Sansaveria trifasciata subsp. *laurentii* leaf	1·1	—	1·2	10·9	4·9	1·3	0·7	0·7	—	14·6	21·1	25·3
DICOTYLEDONS												
Beta vulgaris leaf	18·6	—	—	—	—	11·8	—	—	—	6·8	2·4	—
Citrus aurantifolia leaf	6·3	—	—	69·3	—	—	12·6	—	—	—	—	—
Clematis vitalba leaf	24·3	—	—	30·2	7·5	—	2·6	5·9	7·4	—	2·7	—
Euonymus europaeus leaf	2·0	—	2·3	24·2	14·4	2·0	4·1	1·2	4·8	13·5	13·6	trace
fruit	0·6	—	1·0	23·9	17·4	2·4	3·3	1·1	4·6	11·4	15·5	3·1
Ilex aquifolium leaf	1·5	—	3·3	25·5	6·3	4·5	5·6	3·3	1·4	6·1	6·8	9·9
fruit	1·6	—	1·5	11·8	8·6	9·3	1·5	1·4	1·4	8·3	10·4	4·7
Lactuca sativa leaf	51·2	8·4	—	4·2	—	—	2·0	3·5	—	—	5·6	—
Lamium album leaf	6·9	—	—	36·8	—	—	8·2	0·8	1·1	—	4·5	—
Lycopersicon esculentum leaf	31·1	7·5	—	5·5	trace	—	3·6	5·8	—	—	5·5	—
fruit	1·2	—	5·2	71·1	2·6	—	8·1	4·3	—	—	—	—
Malus pumila cv. "Dove" leaf	3·8	—	6·5	36·8	7·0	—	6·8	3·5	5·6	—	11·8	—
fruit	1·4	—	3·0	18·9	6·5	—	5·0	1·0	7·1	—	12·8	13·6
Malus cv. "Golden Hornet" leaf	1·6	—	5·1	39·5	7·6	17·6	2·2	2·2	0·7	2·0	5·2	1·8
fruit	2·4	—	4·3	30·0	11·3	—	3·4	4·2	3·9	8·9	20·0	trace
Malus zumi leaf	5·3	—	6·1	38·2	5·4	5·2	7·3	2·7	6·9	—	15·5	—
fruit	0·7	—	1·6	20·5	26·5	9·8	0·8	1·0	2·5	3·6	7·7	4·3
Rosa canina fruit	1·1	—	1·9	65·3	—	4·2	5·7	4·9	—	—	—	—
Salix scouleriana leaf	11·0	—	—	10·8	3·9	0·7	1·9	2·2	—	—	4·0	1·8
Spinacia oleracea leaf	20·2	6·7	—	2·8	—	—	—	2·7	—	—	3·0	—
Tamus communis leaf	8·5	4·6	—	18·4	5·9	2·0	3·2	1·8	4·6	3·4	11·0	1·2
fruit	3·3	2·5	—	21·9	20·8	11·2	3·3	—	—	10·3	9·1	3·8
Vitis vinifera cv. "Reisling sylvaner" leaf	5·5	—	4·4	21·1	3·6	1·7	12·8	3·6	7·3	—	15·3	—
fruit	1·9	1·4	—	17·9	14·0	2·0	2·0	2·5	7·3	12·0	14·0	3·2
Vitis vinifera cv. "Siebel" leaf	26·7	8·7	—	15·4	3·4	—	3·1	8·9	—	—	11·7	—
fruit	1·2	1·0	—	16·7	7·5	1·9	1·9	2·2	2·3	7·8	11·7	12·5

* T_{rel} values E301.

to be monobasic 155, ω-hydroxymonobasic 111, dihydroxymonobasic 100 and trihydroxymonobasic 101 but they could not be used because unidentified peaks occurred in the cutin acid chromatograms. Wide qualitative and quantitative variations occurred in the cutin acids between species. 10,16-Dihydroxyhexadecanoic acid was confirmed as the most common hydroxy acid and was the major constituent of the cutin of *Bryonia dioica* fruit (53·2 per cent), *Citrus aurantifolia* leaf (69·3 per cent), *Lycopersicon esculentum* fruit (71·1 per cent) and *Rosa canina* fruit (65·3 per cent). However, it was not found in *Beta vulgaris* leaf cutin; the principal acids detected were hexadecanoic (18·6 per cent) and oleic (19·6 per cent). 9,10,18-Trihydroxyoctadecanoic acid occurred in most cutins and was a major constituent of those of *Agave americana* leaf (22·1 per cent), *Gasteria planifolia* leaf (26·3 per cent), *Iris foetidissima* fruit (28·0 per cent) and *Malus zumi* fruit (26·5 per cent). 10,18-Dihydroxyoctadecanoic (4·7 per cent) and 18-hydroxyoctadecanoic (1·9 per cent) acids were found only in the leaf cutin of *A. americana*. 16-Hydroxyhexadecanoic acid, if present, was a minor constituent.

Monobasic acids were major constituents of some cutins, e.g. those of *Bryonia dioica* leaf (59·6 per cent), *Lactuca sativa* leaf (59·6 per cent), *Vitis vinifera* cv. "Siebel" leaf (35·4 per cent), with a consequent decrease in the amounts of hydroxy acids. Hexadecanoic acid occurred in all cutins and octadecanoic much less frequently.

Unidentified acids were found in many of the plant cutins. The most important acids gave peaks of T_{rel} 1·40, 1·45, 1·51 and 1·60 on E301 and of T_{rel} 1·10, 1·14, 1·30 and 1·01 respectively on QF1. These acids also gave spots of R_F 0·37 and 0·52 (TLC of methyl esters). The acids were major constituents of the cutins of *A. americana* leaf (40·6 per cent), *Sansaveria trifasciata* subsp. *laurentii* leaf (61·0 per cent) and *Euonymus europaeus* fruit (34·6 per cent) and were minor constituents of other cutins.

Qualitative and quantitative variations of the cutin acid composition occurred within species but were less marked than those found between species. Similarities in the relative composition of the identified acids were shown by the *Malus* leaf cutins but not by the corresponding fruit cutins. The *Vitis* leaf and fruit cutins were similar in their content of 10,16-dihydroxyoctadecanoic acid but showed qualitative and quantitative differences in the other cutin acids present.

Cutin from the leaf and fruit of the same plant also varied in composition. For example, in *E. europaeus* and *Ilex aquifolium* the two cutins were very similar, in *M. pumila* cv. Dove and *Tamus communis* only quantitative differences occurred whilst in *B. dioica*, *I. foetidissima* and *L. esculentum* marked qualitative and quantitative differences were found.

DISCUSSION

The results obtained for the cutins of *Agave* leaf *Malus* fruit and *Lycopersicon* fruit are in general agreement with published information. The presence of 9,10,16-trihydroxyhexadecanoic acid in tomato fruit[7] and *Agave* leaf[5] was not verified. The commmon occurrence of 10,18-dihydroxyoctadecanoic and 18-hydroxyoctadecanoic acids reported by Baker and Martin[9] was not confirmed. These acids were found only in the cutin of *Agave* leaf. The acid previously reported[8] as 9,10,18-trihydroxyoctadecanoic in *Citrus aurantifolia* leaf cutin was found to be 10,16-dihydroxyhexadecanoic. The importance of monobasic acids as components of plant cutin has been established. They have been previously reported as minor constituents of cutin.[2, 6] Of the acids reported to occur in suberin, only 9,10,18-trihydroxyoctadecanoic was found in significant amounts in cutin. Other suberin acids, 18-hydroxyoctadec-9-enoic and 9,10-dihydroxyoctadecane-1,18-dioic, have been previously reported as minor constituents of cutin.[4,6] The characteristic major acids of suberin, 22-hydroxydocosanoic[12] and docosane-1,22-dioic,[13] were not detected in the cutins studied. The statement that suberin shows a similar composition to cutin[14] was not substantiated.

Many of the unidentified acids (Table 2) are clearly important constituents of plant cutin. Correlations between their GLC retention on E301 and QF1 suggest that they are hydroxylated; aliphatic acids are quickly eluted on QF1. The values of T_{rel} E301 0·92, 0·97 and 1·04 suggest unsaturated ω-hydroxymonobasic acids. The remaining unidentified acids could be higher saturated or unsaturated homologues or more hydroxylated acids, such as 9,10,12,18-tetrahydroxyoctadecanoic reported in *Agave* leaf cutin.[5] It seems likely that these acids, like the identified cutin acids, are of C_{16} and C_{18} chain lengths. Detailed work is in progress to identify them.

Chemotaxonomic correlations have already been attempted using the superficial wax constituents of plant cuticular membranes.[15–17] The value of cutin acids as a taxonomic criterion appears to be doubtful. A limiting factor is that cutins from widely different families may have closely similar cutin acid compositions. No significant differences occur between the cutin of monocotyledons and dicotyledons.

When cutins contained large amounts of monobasic acids, difficulty was encountered in the initial detachment of the cuticular membranes with ammonium oxalate–oxalic acid or pectinase. Such membranes were thin and fragile, possibly due to a lower degree of inter-esterification. Studies of cutin acids may well lead to a better understanding of the formation and structure of plant cuticles.

EXPERIMENTAL

Isolation of Cuticular Membranes

Mature leaves and fruits were first dewaxed with $CHCl_3$ using the method of Fernandes *et al.*[18] 100 2 cm^2 discs (or an equivalent area) were removed and the membranes detached using ammonium oxalate–oxalic acid solution,[19] pectinase[20] or $ZnCl_2$–HCl solution.[21] When ammonium oxalate–oxalic acid or pectinase was used, attached cellulose was removed with $ZnCl_2$–HCl. Isolated membranes were stored in methanol.

Preparation of Cutin Acids

The cellulose-free membranes were hydrolysed with 3% ethanolic KOH and the total acids liberated obtained by ether extraction.[2] The qualitative acid composition was examined by TLC using the method of Baker and Martin.[9] The total acids were methylated with excess CH_2N_2 (freshly prepared). Methylation was carried out as soon as possible after isolation as repolymerization of the liberated acids can occur. The methyl esters were also examined by TLC on Kieselgel HR using $CHCl_3$-EtOAc (7/3) as solvent. The cutin acids were stored as methyl esters.

Preparation of Trifluoroacetates

Aliquots (1–5 mg) from the total cutin methyl esters were taken and refluxed for 10 min with a mixture of $(CF_3CO)_2O$ (100–250 μl) and $CHCl_3$ (50–100 μl). After standing for a further 10 min excess reagents were evaporated under reduced pressure. The cutin acid derivatives were dissolved in hexane (5% solution) for

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[12] L. Duhamel, *Ann. Chim.* **8**, 315 (1963).

[13] F. Zetzsche and M. Bahler, *Helv. Chim. Acta* **10**, 346 (1927).

[14] D. H. Hunneman and G. Eglinton, in *Advances in Organic Geochemistry* 1968, p. 157, Pergamon Press, Oxford (1969).

[15] G. Eglinton, A. G. Gonzales, R. J. Hamilton and R. A. Raphael, *Phytochem.* **1**, 89 (1962).

[16] F. Radler, *Am. J. Enol. Viticult.* **16**, 159 (1965).

[17] G. A. Herbin and K. Sharma, *Phytochem.* **8**, 151 (1969).

[18] A. M. Silva Fernandes, E. A. Baker and J. T. Martin, *Ann. Appl. Biol.* **53**, 43 (1964).

[19] F. E. Huelin and R. A. Gallop, *Australian J. Sci. Res.* **B4**, 526 (1951).

[20] W. H. Orgell, *Plant Physiol.* **30**, 78 (1955).

[21] P. J. Holloway and E. A. Baker, *Plant Physiol.* **43**, 1878 (1968).

GLC injection. Warming was necessary to completely dissolve some samples. The minimum detectable quantity by GLC was about 0·01 μg.

Gas Chromatography

A Hewlett-Packard 5750 gas chromatograph fitted with dual columns and flame ionization detectors and 0–1 mV recorder were used. Retention data were determined using two stationary phases, E301 and QF1 both at a 5% loading on 80/100 mesh Chromosorb W AW DMCS. The chromatographic columns were stainless steel ⅛ in, o.d. 6 ft (E301) and 5 ft (QF1) in length. The carrier gas was nitrogen, flow rate 35 ml/min (50 p.s.i.) for all columns, and the flow rates for each detector were hydrogen 25 ml/min (8 p.s.i.) and air 500 ml/min (33 p.s.i.). The detectors were maintained at 300° and the injection inlet at 250°. Analyses were made using temperature programming at a rate of 2° per min from 190–250° (E301) and 180–230° (QF1). Relative retention times of the peaks were determined using *n*-tricosane (E301) and *n*-dotriacontane (QF1) as standards. Relative peak areas were measured from the chart by the triangulation method.

Acknowledgements—We should like to thank Mr. D. J. Alden for the GLC analyses and Dr. M. Matic, Pretoria, South Africa, Professor I. Ribas-Marques, Santiago de Compostela, Spain, and Dr. L. J. Morris, Unilever Research Laboratory, Sharnbrook, for samples of hydroxy acids.