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CUTINS OF *MALUS PUMILA* FRUITS AND LEAVES*

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Key Word Index—*Malus pumila*; Rosaceae; apple; fruit cutin; leaf cutin; fatty and hydroxy-fatty acids; epoxy-fatty acids.

Abstract—The cutins of fruits and leaves of four apple cultivars have been analysed using TLC, GLC and GC–MS. They are similarly composed of saturated, monounsaturated and diunsaturated fatty, hydroxy-fatty and epoxyhydroxy-fatty acids. The most abundant monomers are 18-hydroxyoctadeca-9,12-dienoic, 10,16-dihydroxyhexadecanoic, 9,10-epoxy-18-hydroxyoctadec-12-enoic, 9,10-epoxy-18-hydroxyoctadecanoic and 9,10,18-trihydroxyoctadecanoic acids. The fruit cutins have high contents of epoxides (35–40%) and unsaturated components (>40%) and C_{18} compounds predominate over C_{16}. The leaf cutins contain smaller amounts of unsaturated components than the fruits and higher proportions of C_{16} compounds. The adaxial leaf cutin differs in composition from the abaxial. 10,16-Dihydroxyhexadecanoic and 9,10-epoxy-18-hydroxoctadecanoic acids are the major constituents (each *ca.* 30%) of the adaxial leaf cutin and 10,16-dihydroxyhexadecanoic acid (55–65%) predominates in the abaxial.

INTRODUCTION

THE COMPOSITION of apple fruit cutin has been examined qualitatively[1–11] and quantitatively[12–19] and about 30 constituent acids have already been reported. Some uncertainty about the composition of the cutin still exists, however, because the results obtained are influenced by the method of depolymerization used. Work published before 1971 involved the use of alcoholic KOH and indicated that 16-hydroxyhexadecanoic, 18-hydroxyoctadeca-9,12-dienoic, 10,16-dihydroxyhexadecanoic and 9,10,18-trihydroxyoctadecanoic acids are

* For the previous paper in this series see HOLLOWAY, P. J. and DEAS, A. H. B. (1973) *Phytochemistry* **12**, 1721.

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important constituents. Recent investigations using $LiAlH_4$ reduction[9,19] or methanolysis[11] have shown that large amounts of 9,10-epoxy-18-hydroxyoctadecanoic acid and its Δ^{12} analogue occur among the products of depolymerization, in addition to some of the hydroxy-acids previously recorded. The development of a reliable method for the analysis of plant cutins[11] has prompted a re-examination of apple cutin and the results obtained from the mature fruits and leaves of four cultivars are reported in the present paper.

RESULTS

Cutin Contents of Cuticular Membranes

Fruits and leaves of Jonathan, Cox's Orange Pippin, Golden Delicious and Worcester Permain cultivars were collected from orchard trees when the fruits were ready for picking. The cutin contents of their isolated cuticular membranes are shown in Table 1. The fruit membranes contain from 7–15 times more cutin per unit area than those of the leaves; the Golden Delicious membranes have the heaviest cutin deposits. The cutin contents of the adaxial leaf membranes are invariably higher than those of the corresponding abaxial. The range of cutin contents agrees with values reported by other workers.[20–24] Cutin represents about 50–60% of the weight of the isolated fruit membranes obtained after pectinase treatment and about 80% of the leaf membranes isolated by the same method. Removal of cellulose from the isolated membranes using $ZnCl_2$–HCl[25] or cellulase[7,19] gave no significant increase in the yield of monomers and was therefore not used.

TABLE 1. CUTIN CONTENTS* (mg/cm²) OF THE CUTICULAR MEMBRANES FROM FRUITS AND LEAVES OF APPLE CULTIVARS

Cultivar	Fruit	Leaf		Cultivar	Fruit	Leaf	
		Adaxial	Abaxial			Adaxial	Abaxial
Jonathan	1·05	0·13	0·07	Golden Delicious	1·47	0·18	0·14
Cox's Orange Pippin	0·99	0·11	0·07	Worcester Permain	0·92	0·13	0·09

*Assessed from yield of monomers obtained after depolymerization.

Composition of Fruit Cutins

The products of methanolysis of the cutins were identified using TLC, preparative-TLC, GLC and GC–MS and their quantitative compositions determined by GLC. The monomers of the Jonathan fruit cutin are given in Table 2 and the major constituents of the other fruit cutins in Table 3. The cutins of the four cultivars are similar qualitatively; 35 monomers were identified, comprising saturated, monounsaturated and diunsaturated fatty, hydroxy-fatty and epoxyhydroxy-fatty acids. The major constituents of each cutin are C_{16} and C_{18} hydroxy-fatty acids which account for over 50% of the total monomers, and C_{18} epoxy-

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TABLE 2. MONOMERS OF JONATHAN FRUIT CUTIN

Monomer classes	Acid class (%)	Total monomers (%)
Monobasic acids*		
Hexadecenoic	2·4	
Hexadecanoic	29·0	
‖Octadecadienoic + octadecenoic	46·1	
Octadecanoic	8·7	
Eicosanoic	4·4	
Docosanoic	4·0	1·7
Tetracosanoic	2·4	
Hexacosanoic	0·7	
Octacosanoic	1·0	
Triacontanoic	1·0	
Dotriacontanoic	0·3	
α, ω-Dibasic acids*		
Hexadecane-1,16-dioic	58·4	
Octadeca-9,12-diene-1,18-dioic	16·5	0·9
Octadec-9-ene-1,18-dioic	17·5	
Octadecane-1,18-dioic	7·6	
ω-Hydroxymonobasic acids†		
16-Hydroxyhexadecanoic	13·9	2·8
18-Hydroxyoctadeca-9,12-dienoic	70·7	14·2
18-Hydroxyoctadec-9-enoic	11·9	2·4
18-Hydroxyoctadecanoic	1·5	0·3
20-Hydroxyeicosanoic	1·0	0·2
22-Hydroxydocosanoic	0·5	0·1
24-Hydroxytetracosanoic	0·5	0·1
	Total	20·1
Monohydroxydibasic acids†		
§7-Hydroxyhexadecane-1,16-dioic	80	0·5
§8-Hydroxyhexadecane-1,16-dioic	20	
Monohydroxyepoxymonobasic acids‡		
9,10-Epoxy-18-hydroxyoctadec-12-enoic	64·1	22·3
9,10-Epoxy-18-hydroxyoctadecanoic	35·9	12·5
	Total	34·8
Dihydroxymonobasic acids†		
§8,16-Dihydroxyhexadecanoic	6	
§9,16-Dihydroxyhexadecanoic	18	23·4
§10,16-Dihydroxyhexadecanoic	76	
§8,18-Dihydroxyoctadecanoic	4	
§9,18-Dihydroxyoctadecanoic	16	0·4
§10,18-Dihydroxyoctadecanoic	80	
Trihydroxymonobasic acids†		
9,10,18-Trihydroxyoctadec-12-enoic	20·5	3·1
9,10,18-Trihydroxyoctadecanoic	79·5	12·0
	Total	15·1

* Determined by GLC as corresponding methyl esters.
† Determined by GLC as corresponding methyl ester TMS ethers.
‡ Determined by GLC as corresponding methoxyhydrin methyl ester TMS ether.
§ Determined from MS taken at apex of GLC peak.
‖ Not resolved by GLC.

hydroxy-fatty acids which comprise a further 35–40%. The ratio of C_{16} to C_{18} compounds ranges from 1:2·2 in Worcester Permain cutin to 1:3·4 in Golden Delicious. Unsaturated members predominate among the C_{18} compounds and make up at least 40% of the total monomers.

The major classes of hydroxy-fatty acid present are ω-hydroxymonobasic, dihydroxymonobasic and trihydroxymonobasic. An homologous series of ω-hydroxymonobasic acids (C_{16}–C_{24}) is present with $18:2\Delta^{9,12}$ as the major homologue. This compound usually comprises about 15% of the total monomers. Smaller amounts of the 16 and $18:1\Delta^{9}$ acids occur; the higher homologues (C_{20-24}) probably arises from the russetted (suberized) areas of the fruit cuticle. The major C_{16} acid of the cutin (up to 30% of the total monomers) is dihydroxyhexadecanoic in which the 10,16 isomer predominates together with smaller amounts of the 9,16 and 8,16 isomers. This pattern of hydroxylation in dihydroxyhexadecanoic acid is constant in all four cutins and a similar pattern also occurs in the minor monohydroxyhexadecane-1,16-dioic and dihydroxyoctadecanoic acid fractions of the cutins (Table 2). Two trihydroxymonobasic acids are present; 9,10,18-trihydroxyoctadecanoic which comprises 12–15% of the total monomers and the Δ^{12} analogue (3–4% of total). The epoxyhydroxy-fatty acids are exclusively 9,10-epoxy-18-hydroxyoctadecanoic and its Δ^{12} analogue; more unsaturated compound (up to 30% of total) occurs than saturated (up to 15% of total).

TABLE 3. MAJOR MONOMERS OF APPLE FRUIT CUTINS

Acid	Total monomers (%) Cox's Orange Pippin	Golden Delicious	Worcester Permain
16-Hydroxyhexadecanoic	1·9	2·1	1·1
18-Hydroxyoctadeca-9,12-dienoic	15·1	14·3	6·9
18-Hydroxyoctadec-9-enoic	2·7	2·4	1·8
Dihydroxyhexadecanoic*	20·1	19·7	28·0
9,10-Epoxy-18-hydroxyoctadec-12-enoic	22·1	22·5	28·0
9,10-Epoxy-18-hydroxyoctadecanoic	13·2	15·3	12·9
9,10,18-Trihydroxyoctadec-12-enoic	3·9	4·3	3·4
9,10,18-Trihydroxyoctadecanoic	15·1	14·4	11·9

* Mixture of positional isomers, mainly 10,16-dihydroxyhexadecanoic.

Fatty acids are minor components of the cutins (1–3% of total) and include both monobasic and α,ω-dibasic acids. An homologous series of monobasic acids (C_{16}–C_{32}) with predominantly even numbers of carbon atoms is present in all cutins with hexadecanoic and octadecenoic/octadecadienoic acids as the major homologues. The dibasic acid fraction contains chiefly the C_{16} homologue with smaller amounts of the $18:1\Delta^{9}$ and $18:2\Delta^{9,12}$ homologues.

Composition of Leaf Cutins

The adaxial and abaxial membranes were isolated and analysed separately; the important constituents of their cutins are summarized in Tables 4 and 5. The cutins of the four cultivars are similar qualitatively. The leaf monomers closely resemble those of the fruits, the chief

differences being the absence among the leaf monomers of the higher ω-hydroxymonobasic acids (C_{20}–C_{24}) and the presence of compounds which elute by GLC much later than the other constituents.

TABLE 4. MAJOR MONOMERS OF APPLE ADAXIAL LEAF CUTINS

Acid	Total monomers (%) Jonathan	Cox's Orange Pippin	Golden Delicious	Worcester Permain
16-Hydroxyhexadecanoic	2·7	3·1	1·8	3·0
18-Hydroxyoctadec-9-enoic	0·8	1·7	Tr	1·5
Monohydroxyhexadecane-1,16-dioic*	1·0	Tr	0·9	0·9
Dihydroxyhexadecanoic†	37·1	31·9	32·9	37·3
9,10-Epoxy-18-hydroxyoctadec-12-enoic	5·5	2·9	1·7	3·5
9,10-Epoxy-18-hydroxyoctadecanoic	27·7	26·5	32·4	27·7
9,10,18-Trihydroxyoctadec-12-enoic	Tr	2·2	1·1	2·0
9,10,18-Trihydroxyoctadecanoic	8·2	13·2	13·6	10·7

Tr < 0·5% of total monomers.
* Mixture of positional isomers, mainly 7-hydroxyhexadecane-1,16-dioic.
† Mixture of positional isomers, mainly 10,16-dihydroxyhexadecanoic.

The monomers from the cutin of the adaxial membranes contain C_{16} and C_{18} compounds in approximately equal amounts with 10,16-dihydroxyhexadecanoic and 9,10-epoxy-18-hydroxyoctadecanoic acids as the major components, each comprising about 30% of the total monomers. Unsaturated compounds make up less than 6% of the total and 18-hydroxyoctadeca-9,12-dienoic acid is present only in trace amounts. Significant quantities of 16-hydroxyhexadecanoic, 9,10-epoxy-18-hydroxyoctadec-12-enoic and 9,10,18-trihydroxyoctadecanoic acids occur together with smaller amounts of 18-hydroxyoctadec-9-enoic, 7-hydroxyhexadecane-1,16-dioic and 9,10,18-trihydroxyoctadec-12-enoic acids.

TABLE 5. MAJOR MONOMERS OF APPLE ABAXIAL LEAF CUTINS

Acid	Total monomers (%) Jonathan	Cox's Orange Pippin	Golden Delicious	Worcester Permain
16-Hydroxyhexadecanoic	4·9	4·2	2·1	4·2
18-Hydroxyoctadeca-9,12-dienoic	1·5	3·6	1·6	3·7
18-Hydroxyoctadec-9-enoic	1·5	2·3	1·0	1·9
Monohydroxyhexadecane-1,16-dioic*	Tr	1·0	1·6	0·9
Dihydroxyhexadecanoic†	64·1	58·6	53·9	64·1
9,10-Epoxy-18-hydroxyoctadec-12-enoic	4·1	4·2	5·5	5·3
9,10-Epoxy-18-hydroxyoctadecanoic	11·5	16·2	21·8	11·9
9,10,18-Trihydroxyoctadec-12-enoic	2·0	1·5	2·2	2·3
9,10,18-Trihydroxyoctadecanoic	3·1	3·0	3·7	3·2

Tr < 0·5% of total monomers.
* Mixture of positional isomers, mainly 7-hydroxyhexadecane-1,16-dioic.
† Mixture of positional isomers, mainly 10,16-dihydroxyhexadecanoic.

10,16-Dihydroxyhexadecanoic acid is the major constituent (55–65% of the total) of the cutin of the abaxial membrane and the ratio of C_{16} to C_{18} compounds varies from 1:0·3 in Jonathan cutin to 1:0·6 in Golden Delicious. 9,10-Epoxy-18-hydroxyoctadeca-9,12-

dienoic acid is present in detectable quantities and unsaturated compounds comprise at least 10% of the total monomers. Monobasic and α,ω-dibasic acids make up a very small proportion of the monomers from both adaxial and abaxial membranes.

DISCUSSION

The present work has shown that the cutin of the aerial parts of *Malus pumila* is composed essentially of 18-hydroxyoctadeca-9,12-dienoic, 10,16-dihydroxyhexadecanoic, 9,10-epoxy-18-hydroxyoctadecanoic, 9,10-epoxy-18-hydroxyoctadec-12-enoic and 9,10,18-trihydroxy-octadecanoic acids. The cutin of the fruit differs from that of the leaf mainly in the relative amounts of these constituents. Small variations of composition occur between cultivars. The fruit cutins show a predominance of C_{18} over C_{16} compounds and large proportions of epoxy and unsaturated compounds. The leaf cutins differ from those of the fruit chiefly in increased amounts of C_{16} compounds and much smaller contents of unsaturated monomers.

Most of the compounds now reported in apple fruit cutin have been previously recorded. The C_{18} epoxy acids are identical with those found by Kolattukudy *et al.*[9,19] after depolymerization of the cutin by $LiAlH_4$ and $LiAlD_4$; these workers, however, do not report any reduction products derived from diunsaturated, monohydroxydibasic or dihydroxy-octadecanoic monomers. The fatty and hydroxy-fatty acids found agree in general with those identified by Eglinton and Hunneman,[12,16,18] although their work did not establish the existence of epoxides in the cutin. Methanolysis demonstrates that the fatty acids occur in an esterified form in the apple cuticular membrane since the corresponding methyl esters are obtained after depolymerization; this information is not obtained by other methods of depolymerization.

The presence of 6- and 7-hydroxypentadecane-1,15-dioic,[18] heptadecadiene-1,17-dioic,[12] heptadec-9-ene-1,17-dioic[12] or 8,9-dihydroxyheptadecane-1-17-dioic[12] has not been confirmed; they are probably artefacts from the action of diazomethane and correspond with 10- and 9-hydroxy-16-methoxyhexadecanoic, 18-methoxyoctadecadiene-1,18-dioic, 18-methoxyoctadec-9-ene-1,18-dioic and 9,10-dihydroxy-18-methoxyoctadecanoic acid respectively.[26] Neither could the presence of 9,10-dihydroxyoctadecane-1,18-dioic acid[12] or alkan-1-ols and α,ω-diols[19] be verified. 10,18-Dihydroxyoctadecanoic acid has previously been identified only from an R_f value;[5] its presence as a minor constituent is now established.

Previous analyses using alcoholic KOH have failed to estimate epoxide content and the amounts of *vic*-diol monomers reported from them have included diols derived from the hydrolysis of the corresponding epoxides.[11] In addition several of the cutin monomers are poorly differentiated by the reduction method[19] yielding the same alkanol which can only be determined with difficulty from the mixed MS of the deuterated compounds. Nevertheless certain comparisons are possible regardless of the analytical method used. The amounts of 10,16-dihydroxyhexadecanoic and monobasic, dibasic and ω-hydroxymonobasic acids found in the four fruit cultivars agree with those previously reported.[12,14,17,19] The contents of epoxy and unsaturated monomers, however, are higher than those found earlier.[12,19] The ratio of C_{16} to C_{18} compounds accords with that found by De Vries[13] (1:2·2) and by Walton and Kolattukudy[19] (1:2·3) but is higher than that reported by Eglinton and Hunneman[12,16,18] (1:1·6). Some differences, however, may arise from variations between cultivars and the cutin may change in composition with the growth of the plant. Aldehyde

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components[10,27] have been reported in the cutin of young but not in that of mature tissues. Changes in composition may even occur during the preparation and handling of samples; significant reductions in the amounts of unsaturated compounds, especially 18-hydroxyoctadeca-9,12-dienoic acid, were prevented only by storing membranes or monomers under vacuum.

The occurrence of 16-hydroxyhexadecanoic,[14] 10,16-dihydroxyhexadecanoic[14,28] and 9,10,18-trihydroxyoctadecanoic[14,28] acids as important constituents of apple leaf cutin is confirmed but fatty acids,[14] 10,18-dihydroxyoctadecanoic[28] and 18-hydroxyoctadecanoic[28] acids are present in much smaller amounts than those previously reported. The presence of of epoxide and unsaturated monomers in the leaf cutin has not previously been recorded. The unidentified compounds, especially prominent in the cutin of the adaxial leaf membrane, were not studied in detail but their TLC and GLC behaviour suggest that they are triterpenoid in nature. Ursolic acid is a major constituent of apple leaf wax[29] and previous work[16,30] has suggested that wax constituents may also be involved in the cutin polyester.

The variations in cutin composition in different parts of the apple plant must in turn reflect differences in the amounts of fatty acids available for the biosynthesis of the cutin monomers. Radiotracer studies[9,31] have shown that the epidermis is the most probable site of cutin synthesis and that the C_{16} cutin monomers of the apple fruit are derived from hexadecanoic acid and the C_{18} monomers from octadec-9-enoic and octadeca-9,12-dienoic acids.

EXPERIMENTAL

Isolation of cuticular membranes. Apple (*Malus pumila* Mill.) leaves and fruits from Jonathan, Cox's Orange Pippin, Golden Delicious and Worcester Permain cultivars were collected in October 1972 from orchard trees growing at Long Ashton Research Station. Samples were first dewaxed by immersion in $CHCl_3$, 100 2 cm^2 discs of tissue removed and the cuticular membranes detached by incubation at 35° with 5% Pektolase FL 10 (supplied by Aktielskabet Grindstedvaerket, Denmark) in phosphate buffer at pH 3·5. Adaxial and abaxial leaf membranes were isolated separately. Before depolymerization the isolated membranes were exhaustively extracted successively with hot $CHCl_3$ and MeOH to remove residual lipids.

Depolymerization. Samples (50 cm^2 fruit membranes or 100 cm^2 leaf membranes) were refluxed for 3 hr with NaOMe in MeOH as previously described.[11] A second methanolysis of the residue invariably yielded no further monomers. Cutin content was assessed from the wt of $CHCl_3$ soluble monomers obtained. The minimum time possible elapsed between collection of the samples and depolymerization; the whole procedure was normally completed within 2 days.

Chromatographic and MS analysis. Qualitative and quantitative analysis of the depolymerization products of the cutins was carried out using the analytical TLC, preparative-TLC, GLC and GC–MS methods described in earlier papers.[11,17,30,32] Preparative-TLC on silica gel gave six methyl ester fractions, (a) monobasic acids, (b) dibasic acids, (c) ω-hydroxy monobasic and monohydroxydibasic acids, (d) methoxyhydrins of monohydroxyepoxymonobasic acids and dihydroxyoctadecanoic acids, (e) dihydroxyhexadecanoic acids, and (f) trihydroxyoctadecanoic acids. The constituents of the various fractions were characterized using TLC R_fs, GLC retention data on two stationary phases and MS recorded at the apices of the corresponding GLC peaks. Monobasic acids were identified with the aid of commercially available fatty acids methyl esters and the dibasic and ω-hydroxymonobasic acids by comparison with those previously isolated from *Quercus suber*[32] and *Ribes grossularia*[33] suberins. The chromatographic be-

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haviour and MS of octadeca-9,12-diene-1,18-dioic and 18-hydroxyoctadeca-9,12-dienoic acids, were in agreement with previous work on apple fruit cutin.[12] The monohydroxydibasic acids correspond with 7- and 8-hydroxyhexadecane-1,16-dioic previously identified in coffee leaf cutin,[30] and the constituents of the methoxyhydrin fraction with the methoxyhydrins obtained from 9,10-epoxy-18-hydroxyoctadecanoic acid (identical with authentic sample[11]) and 9,10-epoxy-18-hydroxyoctadec-12-enoic acid (identical with the compound previously identified in *Gasteria planifolia* leaf cutin).[11] The dihydroxyoctadecanoic acids were identified by comparison with the same compounds isolated from *Betula pendula* suberin[32] and the dihydroxyhexadecanoic acids by comparison with published data.[17] The trihydroxymonobasic acids correspond with 9,10,18-trihydroxyoctadecanoic (identical with authentic sample)[32] and 9,10,18-trihydroxyoctadec-12-enoic (identical with compound previously identified in *G. planifolia* cutin).[11]

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