Phytochemistry, 1974, Vol. 13, pp. 2201 to 2207. Pergamon Press. Printed in England.

INTRACUTICULAR LIPIDS OF SPINACH LEAVES*

PETER J. HOLLOWAY
Long Ashton Research Station, University of Bristol, Bristol BS18 9AF

(*Received* 25 *March* 1974)

Key Word Index—*Spinacia oleracea*; Chenopodiaceae; spinach leaf; cuticular wax; cutin; fatty acids; hydroxy-fatty acids; epoxy-fatty acids.

Abstract—The cuticular wax and cutin components of the cuticular membranes isolated from the leaves of two spinach cultivars have been determined. The membranes contain about 0·007 mg/cm^2 of cuticular wax which comprises monobasic acids (C_{16}–C_{38}) with hexadecanoic as the major component. The amounts of cutin are comparable with those of cuticular wax and the monomeric constituents are predominantly C_{18} epoxy compounds. The most abundant monomer is 9,10-epoxy-18-hydroxyoctadecanoic acid (up to 63%) together with substantial amounts of 9,10,18-trihydroxyoctadecanoic acid (up to 22%). Also present are 9,10-epoxyoctadecane-1,18-dioic acid (6–7%), dihydroxyhexadecanoic acid (3–4%), and ω-hydroxymonobasic and fatty acid fractions. The tentative identification of two minor components, 18-hydroxyoxooctadecanoic and 9,10-epoxy-12,18-dihydroxyoctadecanoic acids, is also made. Although spinach membranes have a delicate structure their cutin composition is essentially similar to that of much more substantial membranes.

INTRODUCTION

SEVERAL workers have attempted to classify plant cutins according to the chemical composition of their principal monomeric units and in turn to relate differences in composition to the physical characteristics of the cuticular membrane.[1–8] It has been generally agreed that the degree of development of the membrane is related to the numbers of hydroxyl groups in the monomers which governs the extent of cutin polymerization. Thus, thin poorly-developed membranes tend to have a low hydroxyl content and yield mainly monobasic acids[1,9] on depolymerization, whereas those which are thicker and better elaborated have a high hydroxyl content, containing monomers such as dihydroxyhexadecanoic acid, in its isomeric forms, and 9,10,18-trihydroxyoctadecanoic acid. The latter compound is reported to predominate in plants with exceptionally thick membranes, such as xerophytes and certain fruits, although recent work on such plants[6,10,11] has shown

* Part VII in the series "The Composition of Plant Cuttings". For Part VI see DEAS, A. H. B., BAKER, E. A. and HOLLOWAY, P. J. (1974) *Phytochemistry* **13,** 1901.

[1] BAKER, E. A. and HOLLOWAY, P. J. (1970) *Phytochemistry* **9,** 1557.
[2] HOLLOWAY, P. J. and BAKER, E. A. (1970) *Ann. Appl. Biol.* **66,** 145.
[3] BAKER, E. A. (1970) *New Phytologist* **69,** 1053.
[4] BAKER, E. A. (1971) In *Ecology of Leaf Surface Micro-organisms* (PREECE, T. F. and DICKINSON, C. H., eds.), pp. 55–65, Academic Press, London.
[5] HOLLOWAY, P. J., BAKER, E. A. and MARTIN, J. T. (1972) *An. Quim. R. Soc. Esp. Fis. Quim.* **68,** 905.
[6] WALTON, T. J. and KOLATTUKUDY, P. E. (1972) *Biochemistry* **11,** 1885.
[7] KOLATTUKUDY, P. E. and WALTON, T. J. (1972) *Progress in the Chemistry of Fats and Other Lipids* (HOLMAN, R. T., ed.), Vol. 13, Part 3, pp. 121–175, Pergamon Press, Oxford.
[8] MARTIN, J. T. (1973) In *Phytochemistry* (MILLER, L. P., ed.) Vol. III, pp. 134–161, Van Nostrand Reinhold, London.
[9] HOLMAN, R. T. and NICHOLS, P. C. (1972) *Phytochemistry* **11,** 333.
[10] HOLLOWAY, P. J. and DEAS, A. H. B. (1973) *Phytochemistry* **12,** 1721.
[11] HOLLOWAY, P. J. (1973) *Phytochemistry* **12,** 2913.

that it occurs to a large extent in the form of the corresponding 9,10-epoxide, so that the hydroxyl content of these membranes is much lower than was originally thought.

For some time we have regarded the situation of delicate membranes as anomalous because fatty acids alone would be unable to form the usual polyester structure found in other membranes, although esterification with some other component of the membrane, such as cellulose, cannot be ruled out. Also, as most of the analyses on these membranes have been carried out using ethanolic KOH for depolymerization the possibility that free acids are originally present cannot be excluded from the results since the method does not distinguish between free and esterified acids. This is important in view of the recent finding that free fatty acids of similar chain lengths to those reported in delicate membranes occur as major constituents of the cuticular waxes* of several plants.[12,13] However, our recent work using methanolysis has also demonstrated that esterified fatty acids do occur in apple cutins,[11] although they comprise only a small proportion of the total monomers.

The work described in this paper is part of a general reinvestigation of the cutin composition of delicate cuticular membranes and is concerned with the detailed analysis of one example of this type of membrane, namely that present on spinach leaves. The isolation and analysis of the cuticular wax was also carried out at the same time for comparative purposes.

RESULTS

Cuticular wax

The yields of wax obtained from the isolated membranes of both cultivars are similar and amount to about 0·007 mg/cm^2. TLC of the wax showed one major spot corresponding to long-chain monobasic acids and, after methylation, a spot corresponding to methyl esters of monobasic acids. The minor component of the wax had the same R_f value as long-chain primary alcohols.

The total methylated wax, after silylation, was analysed by GLC and an homologous series of predominantly even carbon numbered monobasic methyl esters and primary alcohol TMS ethers were identified. The methyl esters ranged from C_{16} to C_{38} and the main component is hexadecanoate (50·5% of wax) with significant amounts of the C_{18}:1Δ(2·4%), C_{24} (2·6%), C_{26} (8·6%), C_{28}(9%) and C_{30} (1·4%) homologues. The primary alcohols comprised less than 5% of the wax and ranged from C_{24} to C_{30} with hexacosan-1-ol as the major homologue (2·2% of wax).

Cutin

The cutin content of the isolated membranes from both spinach cultivars was low, ranging from 0·007 to 0·010 mg/cm^2, representing about 20% of the weight of the solvent extracted membranes. Non-lipid materials must clearly comprise the major part of the cuticular membranes of such plants; preliminary tests have indicated that the residue from methanolysis is carbohydrate in nature.[14]

The monomers obtained from Yates Fillbasket cutin after methanolysis and PLC are

* Cuticular wax is used to denote wax embedded within the cuticular membrane which is distinct from that found on the surface (the superficial or epicuticular wax).

[12] BAKER, E. A. (1972) M.Sc. thesis, University of Bristol.
[13] BAKER, E. A. (1973) *Rep. Agric. Hort. Res. Stn. Univ. Bristol* **1972**, 99.
[14] DEAS, A. H. B. and HOLLOWAY, P. J. unpublished work.

TABLE 1. FRACTIONATION AND COMPOSITION OF THE METHANOLYSIS PRODUCTS FROM SPINACH (YATES FILLBASKET) CUTIN

PLC fraction	Compounds	Fraction (%)	Total (%)
I	Methyl hexadecanoate	30·6	
	Methyl octadecenoate	17·5	
	Methyl octadecanoate	1·8	
	Methyl eicosanoate	6·3	
	Methyl docosanoate	15·1	
	Methyl tetracosanoate	2·7	0·9
	Methyl hexacosanoate	14·8	
	Methyl octacosanoate	1·2	
	Methyl triacontanoate	0·5	
	Methyl dotriacontanoate	0·5	
	Unidentified compounds	9·0	
II	Dimethyl hexadecane-1,16-dioate	4·7	
	Dimethyl octadec-9-ene-1,18-dioate	85·3	1·0
	Dimethyl octadecane-1,18-dioate	4·1	
	Unidentified compounds	5·9	
III*	Methyl 16-hydroxyhexadecanoate	2·9	0·3
	Methyl 18-hydroxyoctadeca-9,12-dienoate	1·0	0·1
	Methyl 18-hydroxyoctadec-9-enoate	23·5	2·7
	Methyl 18-hydroxyoctadecanoate	0·7	0·1
	Unidentified M^+ 414†	5·0	0·6
	Unidentified M^+ 430†	2·2	0·3
	Compound M^+ 400†	3·3	0·4
	Dimethyl 9-hydroxy-10-methoxyoctadecane-1,18-dioate	61·4	7·1
IV*	‖ Unidentified M^+ 472†474†	5·0	3·4
	‖ Methyl 9,18-dihydroxy-10-methoxyoctadec-12-enoate	1·9	1·3
	‖ Methyl 10,18-dihydroxy-9-methoxyoctadec-12-enoate		
	‖ Methyl 9,18-dihydroxy-10-methoxyoctadecanoate	91·8	63·0
	‖ Methyl 10,18-dihydroxy-9-methoxyoctadecanoate		
	Dimethyl 9,10-dihydroxyoctadecane-1,18-dioate	1·3	0·9
V*	‖ Methyl 10,16-dihydroxyhexadecanoate	41·2†	
	‖ Methyl 9,16-dihydroxyhexadecanoate	25·0†	
	‖ Methyl 8,16-dihydroxyhexadecanoate	31·3†	2·9
	‖ Methyl 7,16-dihydroxyhexadecanoate	12·5†	
VI*	Methyl 9,10,18-trihydroxyoctadec-12-enoate	3·5	0·5
	Methyl 9,10,18-trihydroxyoctadecanoate	86·2	12·9
	Compound M^+ 592†	10·3	1·6

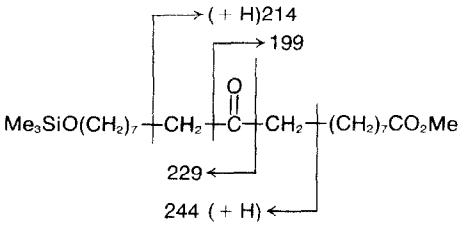
* Fractions analysed as TMS ether derivatives.
† Determined from the MS taken at apex of GLC peak.
‖ Unresolved by GLC.

summarized in Table 1. They show a marked predominance of C_{18} compounds with methoxyhydrin compounds as the major constituents. Fatty acid methyl esters are minor components of the methanolysis products (*ca* 2% of total) and both monobasic (Fraction I) and α,ω-dibasic (Fraction II) esters are present. They are predominantly even carbon numbered and range from C_{16}–C_{32} in the monobasic fraction but are confined to the C_{16} and C_{18} homologues in the dibasic fraction. Hexadecanoic is the major monobasic acid together with substantial amounts of octadecanoic, docosanoic and hexacosanoic; octadec-9-ene-1,18-dioic is the dominant dibasic acid.

The main constituent of mixed Fraction III (7% of total) is dimethyl 9-hydroxy-10-methoxyoctadecane-1,18-dioate which was identified by chromatographic and MS comparison with the compound previously isolated from *Quercus suber* suberin.[10] This compound is the methanolysis product derived from 9,10-epoxyoctadecane-1,18-dioate in the original cutin polymer. C_{16} and C_{18} ω-hydroxymonobasic methyl esters also occur in this fraction with the C_{18}:1Δ^9 compound being the major homologue.

Three other minor components were also present in Fraction III which did not correspond with any known cutin monomers. They were resolved from the other compounds in the fraction by GLC which permitted GC–MS analysis. The MS of the three peaks showed intense ions at *m/e* 73 and 75 and ions corresponding with M^+-15, M^+-31 and M^+-47 at the upper end, confirming that they were TMS ethers. Compounds M^+ 414 and 430, however, could not be identified from their major fragment ions. The former had a base peak *m/e* 71 with strong ions at *m/e* 213, 227, 257 and 271, suggesting a mixed MS of different positional isomers, and an unusual ion corresponding with M^+-47–32 (m* *m/e* 305·8). The MW of compound M^+ 430 corresponds to a mono TMS ether of a saturated C_{18} dimethyl ester but the major fragment ions *m/e* 231 and 271 indicate a different structure (see dimethyl 9-TMS-octadecane-1,18-dioate major fragments *m/e* 259 and 273).

Compound M^+ 400 is tentatively identified as a mixture of positional isomers of methyl 18-hydroxyoxooctadecanoate by MS comparison with the corresponding C_{16} homologues recently identified in lemon fruit cutin.[15] The overall appearance of the spectrum of the spinach compounds was very similar to that from lemon, showing a strong M^+-15 ion together with prominent ions derived from α- and β-cleavage with respect to a keto group (see experimental). The ions at *m/e* 199, 229 (α-cleavage) and 214, 244 (β-cleavage) indicated the presence of the 10-oxo isomer (Scheme 1) and those at *m/e* 185, 243 (α-cleavage) and 200, 258 (β-cleavage) the 9-oxo isomer. An insufficient amount of the compounds was isolated to enable further confirmatory tests, such as $NaBH_4$ reduction, but their TLC R_f and GLC RR_t data are in accordance with the proposed structures.



SCHEME 1. MS FRAGMENTATION OF METHYL 18-TMS-10-OXOOCTADECANOATE.

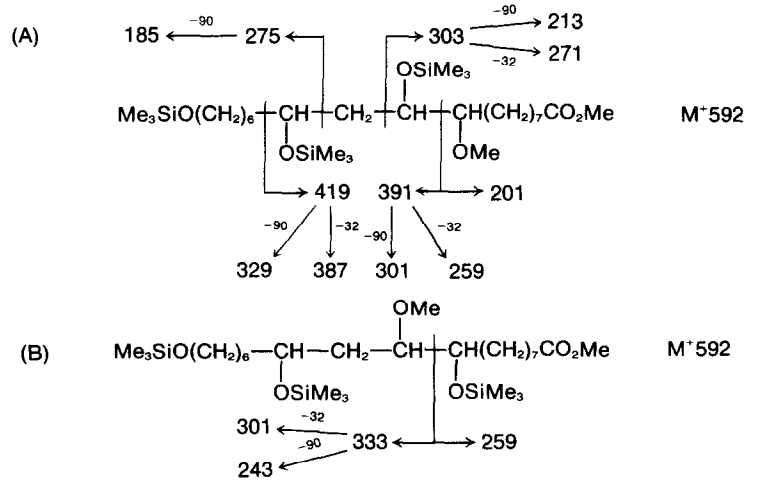
The main component of the spinach methanolysis products (63% of total) occurred in Fraction IV and corresponded with the two methoxyhydrins derived from 9,10-epoxy-18-hydroxyoctadecanoate. The two compounds are unresolved by TLC and GLC but are identifiable from the mixed MS.[10] Much smaller amounts of the methoxyhydrins of the Δ^{12} analogue and dimethyl 9,10-dihydroxyoctadecane-1,18-dioate, the *vic*-diol corresponding with the epoxy compound identified in Fraction III, are present. The major unidentified compound of the cutin (3·4% of total) was also obtained from Fraction IV; it was eluted as a single GLC peak but gave a mixed MS. The MS indicated two compounds

[15] DEAS, A. H. B., BAKER, E. A. and HOLLOWAY, P. J. (1974) *Phytochemistry* **13**, 1901.

M^+ 472 and 474 which were TMS ethers (base peak *m/e* 73 and ions *m/e* 75, 95, 103, 129, 147) and the high end showed ions corresponding with the losses of 15, 31, 47, 90–15 and 90–47 from the M^+. The major fragment ion occurred at *m/e* 275 (rel. intensity 90%) with smaller ions at *m/e* 270 and 277.

Positional isomers of methyl dihydroxyhexadecanoate were the sole constituents of Fraction V. The isomer content determined from the MS[16] showed that the major isomer is 10,16-, together with about equal amounts of the 9,16- and 8,16-isomers and a small amount of the 7,16-isomer.

Fraction VI, which also included the start-line material of the PLC plate, contained mainly methyl 9,10,18-trihydroxyoctadecanoate (13% of total) and a small quantity of the corresponding Δ^{12} analogue. Another component of the fraction, which was eluted as a single GLC peak after these two compounds, was tentatively identified by MS as the two unresolved methoxyhydrin TMS ethers derived from 9,10-epoxy-12,18-dihydroxyoctadecanoate. The MS (see experimental) is similar to those of other long-chain polyTMS ether[17–19] and polymethoxy[20] methyl esters, and shows typical methoxyhydrin TMS ether cleavage[10] between the carbon atoms bearing the methoxy and TMS ether groups (Scheme 2). The fragmentation of the 10,12,18-*tris* TMS-9-methoxy isomer (A) gives ion *m/e* 201 and one at *m/e* 391 of low intensity which yields ions at *m/e* 301 and 259 by the loss of Me_3SiOH and MeOH respectively. The presence of the 10-TMS-9-methoxy methyl ester moiety was confirmed by the rearrangement ion *m/e* 274.[10] Fragment ions of the corresponding 10-methoxy isomer (B) appear at *m/e* 259, 301 (333-MeOH) and 243 (333-Me_3SiOH). Ions common to both isomers are derived from cleavage between C-10 and C-11 [*m/e* 303, 271 (303-MeOH) and 213 (303-Me_3SiOH)] and also from α-cleavage with respect to the 12-TMS ether group [387 (419-MeOH), 329(419-Me_3SiOH), 275 and 185 (275-Me_3SiOH)].



SCHEME 2. MS FRAGMENTATIONS OF (A) METHYL 10,12,18-*tris* TMS-9-METHOXYOCTADECANOATE AND (B) METHYL 9,12,18-*tris* TMS-10-METHOXYOCTADECANOATE.

[16] HOLLOWAY, P. J. and DEAS, A. H. B. (1971) *Phytochemistry* **10**, 2781.
[17] EGLINTON, G., HUNNEMAN, D. H. and MCCORMICK, A. (1968) *Org. Mass Spectr.* **1**, 593.
[18] PERKINS, E. G. and ARGOUDELIS, C. J. (1969) *Lipids* **4**, 619.
[19] BRIESKORN, C. H. and KABELITZ, L. (1971) *Phytochemistry* **10**, 3195.
[20] NIEHAUS, W. G. and RYHAGE, R. (1968) *Analty Chem.* **40**, 1840.

The depolymerization products from the cutin of the Noorman cultivar had an identical qualitative composition to that of Yates Fillbasket but showed some quantitative differences. The main difference occurred in the relative amounts of 9,10-18-trihydroxyoctadecanoate and methoxyhydrins from 9,10-epoxy-18-hydroxyoctadecanoate; this cultivar contained less of the epoxy compound (47% of total) but more of the trihydroxy compound (23% of total).

DISCUSSION

The present work failed to confirm the earlier reports[1] that spinach cutin is composed essentially of monobasic acid monomers. Nevertheless, such acids do occur in the spinach membrane but predominantly as free acids in the cuticular wax and to a very much smaller extent in an esterified form in the cutin polymer. The esterified monobasic acids are similar in chain length to the free acids and hexadecanoic is predominant in both which suggests that esterification of some of the cuticular wax may occur in the cutin matrix. However, no free acids corresponding to the esterified dibasic acids of the cutin occur in the wax indicating that there is no definite chemical relationship between the cuticular wax and cutin. The wax acids of spinach, are similar to those found in the plants examined by Baker.[13]

The previous results may partly be explained by inadequate removal of cuticular wax, the remainder of which was then removed together with the cutin monomers on depolymerization. It should be noted that spinach membranes contain about equal proportions of cuticular wax and cutin; a similar relationship is also found in other membranes.[13]

The monomers of spinach cutin are no different from those of other plants and demonstrate that the cutin composition of a delicate membrane can be very similar to those of a more substantial nature. Although 9,10-epoxyoctadecane-1,18-dioic acid and the compounds provisionally identified as 18-hydroxyoxooctadecanoic and 9,10-epoxy-12,18-dihydroxyactadecanoic acids have not been previously recorded in a cutin, they comprise less than 10% of the monomers and cannot be regarded as having any great structural significance. The epoxy dibasic compound is also found more commonly in suberins.[10]

The quantitative composition of the monomers, on the other hand, is exceptional. They are characterized by a marked predominance (*ca* 90%) of C_{18} compounds; dihydroxyhexadecanoic acid, usually an important constituent of most plant cutins, is present only as a minor constituent. The epoxide content of the Yates Fillbasket monomers (*ca* 70%) is the highest that has been recorded in a cutin and is greater than those of monomers from thick and heavily cutinized membranes (cutin content 0·5–1 mg/cm^2) such as *Agave americana* (45%),[10] *Gasteria planifolia* (60%),[10] *Sansevieria trifasciata* (48%)[10] leaves and *Malus pumila* (35–40%)[11] fruits. The amounts of 9,10,18-trihydroxyoctadecanoic acid in spinach membranes are also equivalent to those of much thicker membranes.[10,11]

With regard to the molecular structure of spinach cutin, its composition signifies that any polyester structure must be essentially linear (estolide) because monomers having secondary hydroxyl groups, which can possibly cross-link the polymer, make up less than 20% of the Yates Fillbasket cultivar and about 25% of the Noorman cultivar. However, the lack of cutinization is the most likely explanation for the delicate nature of the membranes.

EXPERIMENTAL

Plant material. Mature leaves of *Spinacia oleracea* L. cv. Yates Fillbasket and cv. Noorman were obtained from plants grown in pots in a greenhouse. After removal of epicuticular wax by a brief immersion in $CHCl_3$,

1000 discs (2 cm^2) were removed from each cultivar and the cuticular membranes detached enzymatically using a method described previously.[11]

Cuticular wax was obtained by exhaustive extraction of the combined abaxial and adaxial membranes with hot $CHCl_3$ and MeOH followed by evaporation of solvents. Initial TLC was carried out on silica gel G using hexane–Et_2O–HOAc (70:30:1)[21] and the constituents detected with phosphomolybdate. The total wax was methylated with CH_2N_2[22] and the products examined by TLC using C_6H_6.[23] N,O-*bis* (trimethylsilyl) acetamide was used for silylation[24] and GLC analyses were made on an FID instrument using temp. programming on SE30 and OV210 phases.[16] Monobasic acid Me esters and primary alcohol TMS ethers were identified by co-injection with authentic compounds.

Cutin monomers were obtained by methanolysis[10] of the solvent-extracted membranes and examined by analytical TLC, PLC, GLC and GC–MS methods described in preceding papers.[10,11] 6 Fractions (Table 1) were obtained by PLC on silica gel using $CHCl_3$–EtOAc(7:3); the constituents of the various fractions were identified by comparison with authentic compounds and with those previously isolated from other plant cutins or suberins. Mixed MS of compounds tentatively identified as mixture of positional isomers of methyl 18-TMS-oxooctadecanoate, 70 eV *m/e* (rel. intensity): 385 (M^+-15) (36), 369 (M^+-31) (4), 353 (M^+-47) (11), 323·7 (m^* M^+-15 → M^+-47), 315 (4), 313 (2), 288 (2), 287 (4), 286 (3), 279 (7), 273 (6), 272 (5), 258 (5), 245 (5), 244 (6), 243 (5), 229 (7), 227 (5), 215 (5), 214 (11), 213 (5), 201 (7), 200 (9), 199 (9), 187 (7), 185 (10), 143 (14), 130 (19), 129 (17), 111 (20), 103 (25), 97 (28), 95 (28), 83 (40), 81 (28), 75 (100), 73 (88), 69 (40), 67 (28), 59 (24), 55 (76). Mixed MS of compounds tentatively identified as methyl 9,12,18-*tris* TMS-10-methoxyoctadecanoate and methyl 10,12,18-*tris* TMS-9-methoxyoctadecanoate, 70 eV *m/e* (rel. intensity): 577 (M^+-15) (1), 561 (M^+-31) (0·5), 545 (M^+-47) (1), 487 (M^+-90-15) (1), 471 (M^+-90-31) (3), 387 (3), 383 (1), 329 (4), 303 (5), 301 (3), 275 (100), 274 (15), 271 (5), 259 (83), 243 (5), 231 (11), 217 (9), 213 (5), 201 (7), 187 (7), 185 (11), 159 (7), 155 (17), 149 (7), 147 (17), 129 (28), 103 (27), 95 (33), 75 (33), 73 (93).

Acknowledgements—The author is indebted to Dr. R. L. S. Patterson and the Meat Research Institute, Langford for GC–MS facilities, and to Mr. A. H. B. Deas for assistance with the MS analyses.

[21] NICHOLS, B. W. (1963) *Biochim. Biophys. Acta* **70,** 417.
[22] SCHLENK, H. and GELLERMAN, J. L. (1960) *Analty Chem.* **32,** 1412.
[23] BARBER, H. N. and NETTING, A. G. (1968) *Phytochemistry* **7,** 2089.
[24] HOLLOWAY, P. J., DEAS, A. H. B. and KABAARA, A. M. (1972) *Phytochemistry* **11,** 1443.