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# GAS CHROMATOGRAPHIC-MASS SPECTROMETRIC STUDIES OF LONG CHAIN HYDROXY ACIDS—I.

## THE CONSTITUENT CUTIN ACIDS OF APPLE CUTICLE

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**Abstract**—The constituent cutin acids of apple cuticle have been determined by preparative TLC of the methyl esters followed by combined gas chromatography-mass spectrometry of the TMSi ethers of the methyl esters. This method may be applied to small amounts of cutin and has allowed the detection of a variety of hitherto unreported cutin acids. The principle cutina cids are 10,16-dihydroxyhexadecanoic acid, *threo* and *erythro*-9, 10,18-trihydroxyoctadecanoic, 16-hydroxyhexadecanoic, 18-hydroxyoctadec-9-enoic and 18-hydroxyoctadeca-9,12-dienoic. The location of the double bonds in the unsaturated compounds was determined by a novel method involving hydroxylation followed by GC-MS of the trimethylsilyl ethers of the resulting *vic*-dihydroxy compounds.

## INTRODUCTION

THE aerial parts of plants are covered by a non-cellular protective membrane termed the cuticle. This cuticle comprises[1] the epicuticular waxes and cutin, which is largely composed of interesterified and cross-linked hydroxy acids. The role of the cuticle in the protection of the plant has been reviewed by Martin.[2] The chemistry of cutin has been reviewed by Baker and Martin[3] and they have noted that species variations occur in the cutin composition.[4–8] The observed variations may be explained, in part, by different isolation and identification procedures, but the possibilities of of using species differences as a taxonomic aid should not be ignored.

The methods used so far for the positive identification and determination of the cutin hydrolysis products are not easily applicable to very small amounts of material. We have found that preparative thin-layer chromatography (TLC) of the methyl esters of the cutin acids, followed by gas liquid chromatography (GLC) and combined gas chromatography-mass spectrometry (GC-MS) of the trimethylsilyl (TMSi) ethers gives a convenient analytical method. Its value is shown by the determination of a variety of previously unreported cutin acids. Components present to the extent of as little as 0·01 per cent could be identified using a total cutin acid sample of about 50 mg. Thus for major components (> 5 per cent) this procedure is potentially applicable to samples as small as 500 $\mu$g of cutin.

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[1] G. EGLINTON and R. J. HAMILTON, *Science* **156**, 1322 (1967).
[2] J. T. MARTIN, *Ann. Rev. Phytopathology*, **2**, 81 (1964).
[3] E. A. BAKER and J. T. MARTIN, *Nature* **199**, 1268 (1963).
[4] C. H. BRIESKORN and J. BOSS, *Fette Seifen, Anstrichmittel.* **66**, 925 (1964).
[5] J. T. MARTIN and D. J. FISHER, *Ann. Rep., Long Ashton Res. Station*, 251 (1965).
[6] G. D. MEAKINS and R. SWINDELLS, *J. Chem. Soc.* 1044 (1959).
[7] M. MATIC, *Biochem. J.* **63**, 168 (1956).
[8] C. E. CRISP, Ph.D. Thesis, University of California, Davis, 1965.

TABLE 1. CONSTITUENT CUTIN ACIDS OF APPLE CUTICLE (AS METHYL ESTERS AND TMSi ETHERS). Compounds in each group are given in order of GLC elution

| | | | % of total cutin acids | % of this fraction |
|---|---|---|---|---|
| (a) | Monobasic acids (Fig. 1B) | | | |
| I | Methyl tetradecanoate | | 0·01 | 1·1 |
| II | Unidentified | | 0·01 | 0·7 |
| III | Unidentified | | 0·01 | 1·1 |
| IV | Methyl palmitate | | 0·29 | 23·3 |
| V | Unidentified | | 0·01 | 0·8 |
| VI | Methyl oleate+linoleate | | 0·64 | 51·6 |
| VII | Methyl stearate | | 0·06 | 5·0 |
| VIII | Unidentified | | 0·03 | 2·8 |
| IX | Methyl eicosanoate | | 0·06 | 5·1 |
| X | Methyl docosanoate | | 0·10 | 7·9 |
| XI | Methyl tetracosanoate | | 0·02 | 1·8 |
| | | Total | 1·24 | |
| (b) | Dibasic acids (Fig. 1C) | | | |
| XII | Unidentified | | 0·01 | 2·0 |
| XIII | Unidentified | | 0·03 | 4·1 |
| XIV | Methyl hexadecane-1,16-dioate | | 0·22 | 30·4 |
| *XV | Methyl heptadecadiene-1,17-dioate | } | 0·09 | 11·8 |
| *XVI | Methyl heptadec-9-ene-1,17-dioate | } | | |
| *XVII | Methyl octadeca-9,12-diene-1,18-dioate | | 0·06 | 7·9 |
| *XVIII | Methyl octadec-9-ene-1,18-dioate | | 0·28 | 39·0 |
| *XIX | Methyl octadecane-1,18-dioate | | 0·04 | 4·8 |
| | | Total | 0·73 | |
| (c) | Monohydroxy monobasic acids (Fig. 1D) | | | |
| XX | Methyl 16-hydroxyhexadecanoate | | 8 | 25 |
| XXI | Unidentified | | 5 | 16 |
| *XXII | Methyl 18-hydroxyoctadeca-9,12-dienoate | | 13 | 43 |
| *XXIII | Methyl 18-hydroxyoctadec-9-enoate | | 5 | 16 |
| | | Total | 31 | |
| (d) | *vic*-Dihydroxydibasic acids (Fig. 1E) | | | |
| XXIV | Unidentified | | 0·07 | 24·7 |
| *XXV | Methyl 8,9-dihydroxyheptadecane-1,17-dioate | | 0·18 | 48·5 |
| *XXVI | Methyl 9,10-dihydroxyoctadecane-1,18-dioate | | 0·06 | 16·3 |
| XXVII | Unidentified | | 0·05 | 14·6 |
| | | Total | 0·36 | |
| (e) | Dihydroxymonobasic acids (Fig. 1F) | | | |
| XXVIII | Methyl 10,16-dihydroxyhexadecanoate | | 24 | 58 |
| XXIX | Unidentified | | 7 | 21 |
| XXX | Unidentified | | 7 | 21 |
| | | Total | 38 | |
| (f) | Trihydroxymonobasic acids (Fig. 1G) | | | |
| *XXXI | Methyl 9,10,18-trihydroxyoctadecenoate | | 3 | 11 |
| *XXXII | Methyl *erythro*-9,10,18-trihydroxyoctadecanoate | | 7 | 26 |
| XXXIII | Methyl *threo*-9,10,18-trihydroxyoctadecanoate | | 17 | 63 |
| | | Total | 27 | |

* Indicates previously unreported constituents of apple cutin.

## RESULTS

The cuticle was prepared by the method of Baker, Batt and Martin[9] and was then hydrolysed with methanolic KOH. We used apples (Cultivar, Cox Orange Pippin) because of their ready availability and well formed cuticle. The resulting cutin acids were methylated with diazomethane and the methyl esters separated into fractions by preparative TLC on silica

TABLE 2. $R_f$ VALUES FOR APPLE CUTIN ACIDS, METHYL ESTERS (SILICA GEL G, DIETHYL ETHER/*n*-HEXANE/METHANOL, 40/10/1)

| | |
|---|---|
| (a) Monobasic acids | $R_f$=0·94 |
| (b) Dibasic acids | 0·85 |
| (c) Monohydroxy acids | 0·60 |
| (d) *vic*-Dihydroxydibasic acids | 0·34 |
| (e) Dihydroxymonobasic acids | 0·30 |
| (f) Trihydroxymonobasic acids | 0·16 |

gel. As indicated in Tables 1 and 2, six fractions were obtained and characterized as the methyl esters of (a) monobasic acids, (b) dibasic acids, (c) monohydroxy monobasic acids, (d) *vic*-dihydroxydibasic acids, (e) dihydroxy monobasic acids and (f) trihydroxy monobasic acids. The GLC traces of these fractions (as the TMSi ethers for the hydroxy acids) are shown in Figure 1.

(a) *Monobasic acids* (Fig. 1B). This fraction consisted of the methyl esters indicated in Table 1a. The identity of each methyl ester was determined by GLC co-injection and by its mass spectrum. The presence of normal fatty acids, ranging from $C_{14}$ to $C_{20}$, in cuticle hydrolysate has been reported previously.[9] While these acids may be merely cuticle wax components that have not been fully extracted there is also the possibility that they are actual cutin components, bound by ester linkages to the hydroxy acid network. Electron microscopic studies are held to indicate that some of the epicuticular wax is chemically bound to the cutin.[10] The i.r. spectrum of the extracted cuticle shows strong hydroxyl absorption at about 3400 $cm^{-1}$ which is only partly explicable in terms of bound water, thereby indicating free hydroxyl groups.

(b) *Dibasic acids* (Fig. 1C). Analytical GLC showed this fraction to be composed of the eight compounds given in Table Ib. Only one of these acids, hexadecane-1,16-dicarboxylic acid (XIV), has been previously reported as a cutin component.[4] The GC-MS examination of this fraction gave the mass spectra of the individual components. The spectra of the saturated diesters were compared with published mass spectra.[11] The structures of the other components were established on the following bases: (a) All the compounds in this fraction on TLC have the same $R_f$ value as long chain diesters. (b) For analogous compounds on SE-30 the order of elution is: di-unsaturated; mono-unsaturated; saturated (cf. Fig. 1B, the monobasic acids). (c) The mass spectral fragmentation pattern is similar to, but not identical with, that given by the saturated diesters.

[9] E. A. BAKER, R. F. BATT and J. T. MARTIN, *Ann. Appl. Biol.* **53**, 59 (1964).
[10] P. A. ROELOFSEN and A. L. HOUWINK, *Protoplasma* **40**, 1 (1951).
[11] R. RYHAGE and E. STENHAGEN, *Arkiv Kemi.* **23**, 167 (1964).

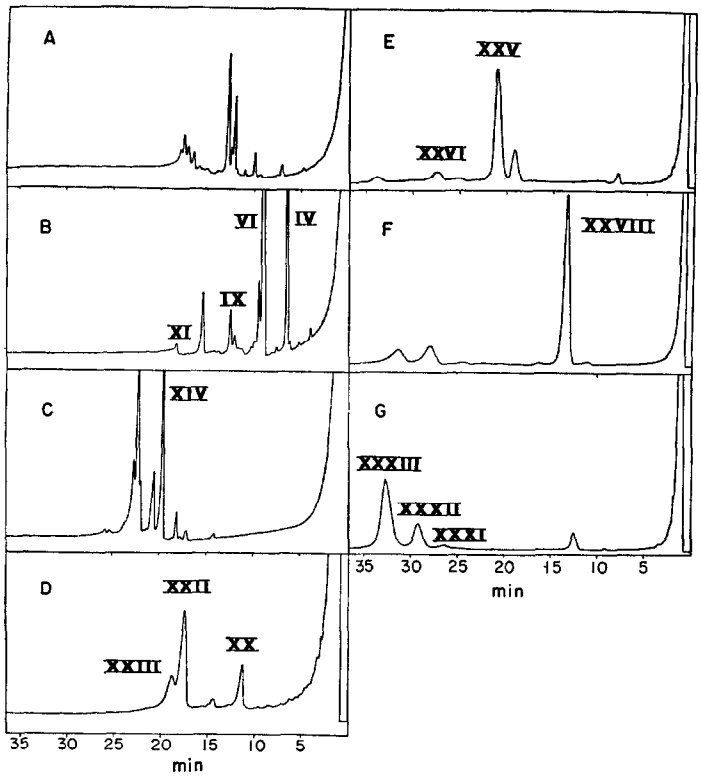


FIG. 1. GAS-LIQUID CHROMATOGRAMS OF APPLE CUTIN ACIDS (AS METHYL ESTERS, TRIMETHYLSILYL ETHERS).
Conditions: Column, 3·4 m × 1·5 mm, 3% SE-30 on Gas Chrom Q, $N_2$ pressure 30 psi. (A) Total cutin acids, temperature programmed at 5°/min from 200–300°; (B) Monobasic acids, temperature programmed at 5°/min from 165°–250°; (C) Dibasic acids, temperature programmed at 5°/min from 120°–250°; (D) Monohydroxymonobasic acids, isothermal at 195°; (E) *vic*-Dihydroxydibasic acids, isothermal at 214°; (F) Dihydroxymonobasic acids, isothermal at 214°; (G) Trihydroxymonobasic acids, isothermal at 214°.
Roman numerals refer to Table 1.

Mass spectra of the methyl esters of unsaturated dibasic acids have not been published. The mass spectra of three of the diesters are shown in Fig. 2: they are methyl octadeca-9,12-diene-1,18-dioate (XVII), methyl octadec-9-ene-1,18-dioate (XVIII) and methyl octadecane-1,18-dioate (XIX). The most striking differences in the mass spectra are provided by the ratios of the peaks in the mass range, m/e 93–98. The m/e 98 peak is dominant in the saturated diesters. In the mono-unsaturated compounds there is an additional strong m/e 95 and in the di-unsaturated there are instead strong m/e 93, 94, 95 peaks. The position of the double bonds cannot be told directly from the mass spectra.[12] Treatment of this fraction with $OsO_4$ followed by silylation gave, in addition to the starting saturated diesters, methy 8,9-dihydroxy-heptadecane-1,17-dioate, diTMSi ether; methyl 9,10-dihyroxyoctadecane-1,18-dioate, diTMSi ether and methyl 9,10,12,13-tetrahydroxyoctadecanoate, tetraTMSi ether. These results show that the originating mono-unsaturated compounds were methyl heptadec-8-ene-1,17-dioate and methyl octadec-9-ene-1,18-dioate, respectively, and that the originating di-unsaturated compound was methyl octadeca-9,12-diene-1,18-dioate.

[12] B. HALLGREN, R. RYHAGE and E. STENHAGEN, *Acta Chem. Scand.* 13, 845 (1959).

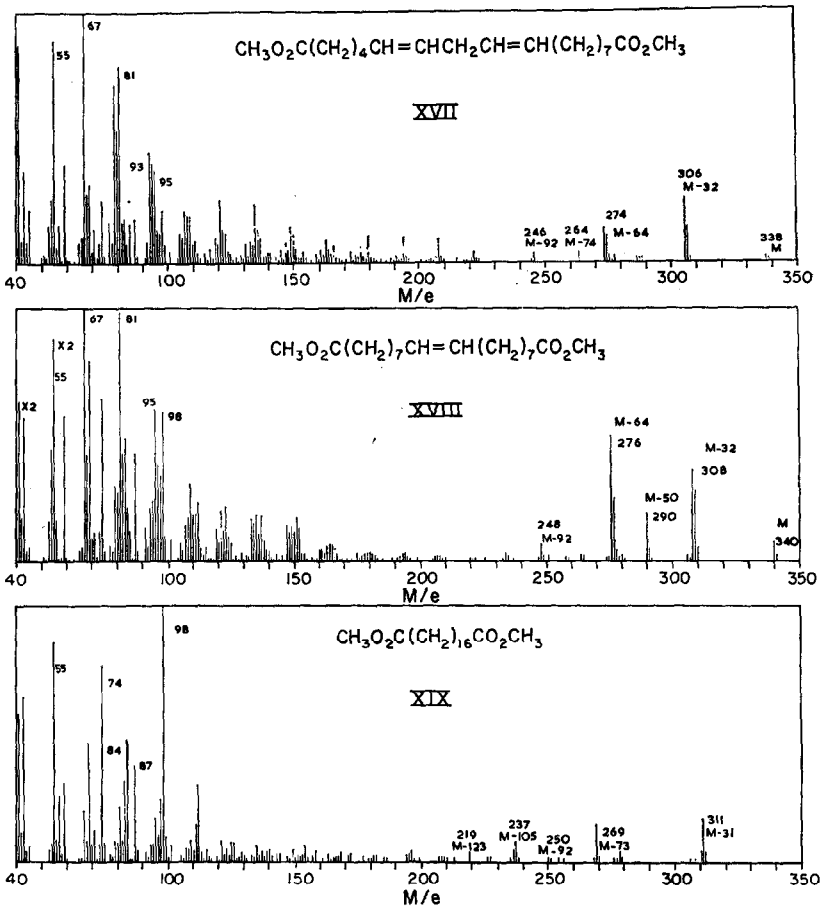


FIG. 2. MASS SPECTRA FROM GC-MS RUN ON THE METHYL ESTERS OF DIBASIC ACIDS (FIG. 1B). FROM APPLE CUTICLE.
Top: methyl octadeca-9,12-diene-1,18-dioate, XVII; Middle: methyl octadec-9-ene-1,18-dioate, XVIII; Bottom: methyl octadecane-1,18-dioate, XIX. Conditions: as in experimental and Fig. 3, temperature programmed from 130–175° at 4°/min.

(c) *Monohydroxy monobasic acids* (Fig. 1D). After removal from the TLC plate this fraction was treated with hexamethyldisilazane to give the TMSi ethers. Analytical GLC showed the presence of four main components as given in Table 1c. The total ion current trace obtained during the GC-MS examination of this sample is given in Fig. 3. Methyl 16-hydroxyhexadecanoate (XX) was identified by the retention time and by comparison of the mass spectrum with that of the authentic compound. The other components were identified by examination of their mass spectra. The mass spectra of the TMSi ethers of methyl 18-hydroxyoctadeca-9,12-dienoate (XXII) and methyl 16-hydroxyhexadecanoate (XX) are shown in Fig. 4. A detailed discussion of the mass spectral fragmentation patterns of these and other hydroxy acids (as the TMSi ethers of the methyl esters) will be published later.[13]

[13] G. EGLINTON, D. H. HUNNEMAN and A. MCCORMICK, in preparation.

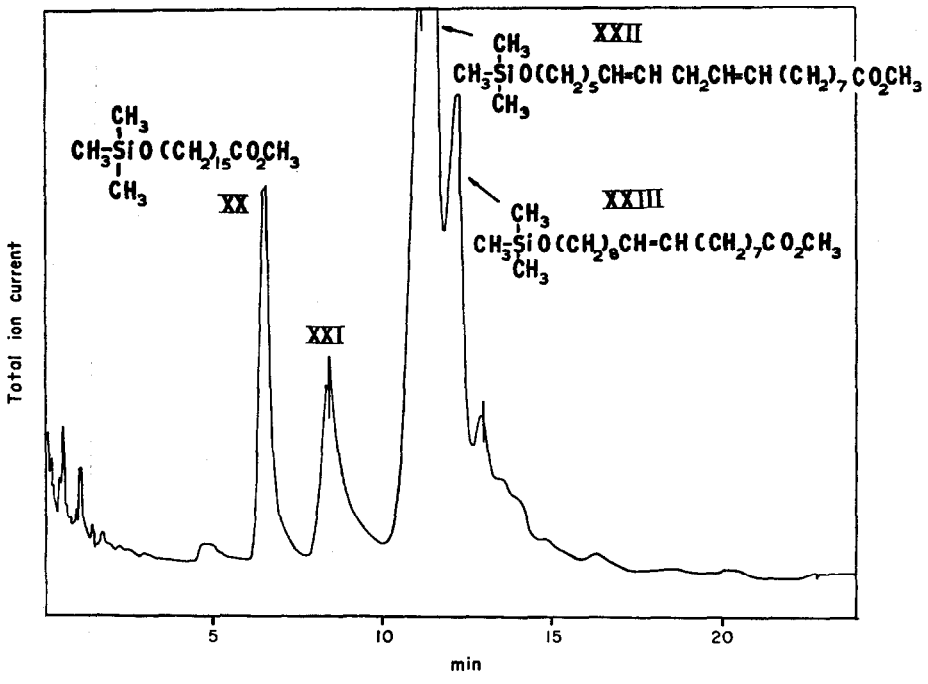


FIG. 3. TOTAL ION CURRENT TRACE FROM GC-MS RUN ON MONOHYDROXYMONOBASIC ACIDS (AS METHYL ESTERS, TMSi ETHERS) FROM APPLE CUTICLE (FIG. 1D).
Conditions: 1% SE-30 column, 3·4 m × 3 mm i.d., He carrier gas at 30 ml/min, isothermal at 205°, 2·5 μl of a 10% (w/v) solution in ethyl acetate. Mass spec. scan time, 2 sec.

As in the dibasic acids, the position of the double bonds in XXII and XXIII could not be decided by simple inspection of the mass spectra. Hydroxylation with $OsO_4$ followed by silylation gave, in addition to the saturated starting materials, the TMSi ethers of methyl 9,10,18-trihydroxyoctadecanoate, and methyl 9,10,12,13,18-pentahydroxyoctadecanoate, showing that the unsaturated compounds were, respectively, methyl 18-hydroxyoctadec-9-enoate and methyl 18-hydroxyoctadeca-9,12-dienoate. The TMSi derivates of the *vic*-diols were used because the most prominent peaks in the mass spectra are those corresponding to the two halves resulting from cleavage between the oxygen bearing carbons.[13] Hence, such spectra are relatively easy to interpret. In fact, this combined procedure of hydroxylation followed by trimethyl silylation of the resulting *vic*-dihydroxy compound and GC-MS of the product represents a method of locating double bonds which is apparently superior to previously reported methods, such as hydroxylation followed by acetonide formation and subsequent GC-MS,[14] both in the ease and rapidity of the procedure and in the simplicity of the mass spectral interpretation. In principal, the known stereospecificity of the osmylation reaction and the demonstrable GLC separation of *threo-erythro* pairs should allow the determination of double bond configuration.

(d) Vic-*Dihydroxy dibasic acids* (Fig. 1E). A fraction running on TLC just a little ahead of methyl 10,16-dihydroxyhexadecanoate was found to contain the methyl esters of at least

[14] R. E. WOLFF, G. WOLFF and J. A. MCCLOSKEY, *Tetrahedron* **22**, 3093 (1966).

two dihydroxydicarboxylic acids, as indicated in Table Id. The identity of methyl 9,10-dihydroxyoctadecane-1,18-dioate (methyl phloionate) was established by GLC coinjection and comparison of the mass spectrum of its TMSi ether with that of the authentic compound. The identity of the other component was established by inspection of the mass spectrum of the TMSi ether.

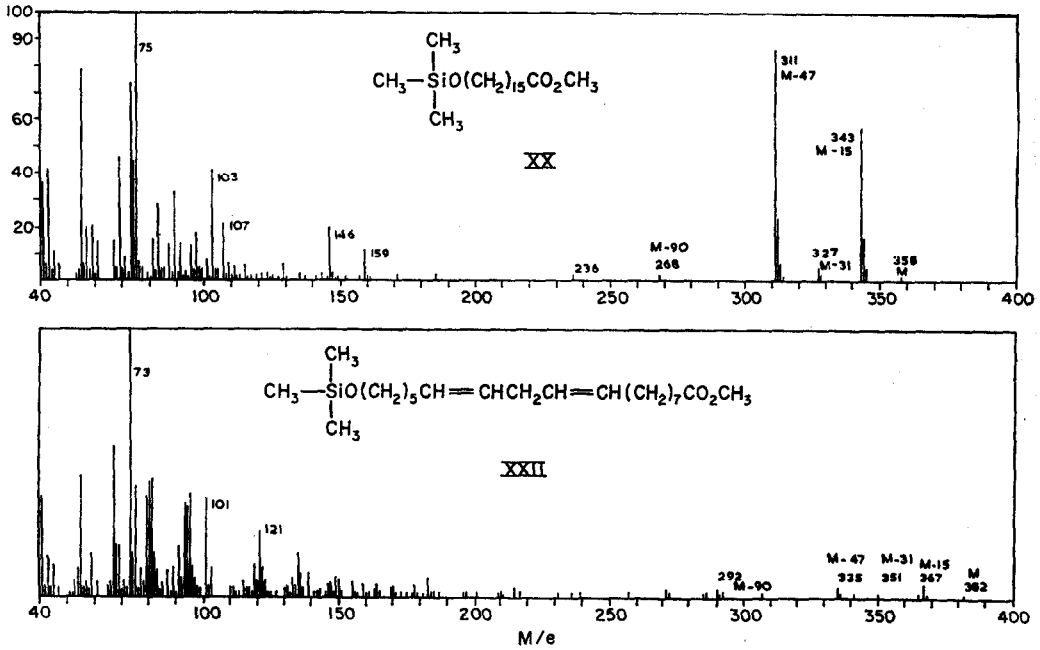


FIG. 4. MASS SPECTRA FROM GC-MS RUN ON MONOHYDROXYMONOBASIC ACIDS (AS METHYL ESTERS, TMSi ETHERS) FROM APPLE CUTICLE.
Top: Methyl 16-hydroxyoctadecanoate, TMSi ether; Bottom: Methyl 18-hydroxyoctadeca-9,12-dienoate; TMSi ether. (see Fig. 1D and Fig. 3). Conditions: as in experimental, see Fig. 3.

(e) *Dihydroxy monobasic acids* (Fig. 1F). This fraction consisted primarily of one compound, methyl 10,16-dihydroxyhexadecanoate, whose identity was established by comparison of the GLC retention time and the mass spectrum of the TMSi ether with those of an authentic sample. The fraction also contained two, as yet, unidentified compounds.

(f) *Trihydroxy acids* (Fig. 1G). This fraction contained some methyl 10,16-dihyroxyhexadecanoate and three other components, one of which (XX) appears to be the monounsaturated ester, methyl trihydroxyoctadecenoate (see mass spectra, Fig. 5). The TMSi ethers of the other two components have the same mass spectrum as that of the triTMSi ether of methyl 9,10,18-trihydroxyoctadecanoate and must then be the *threo-erythro* isomers. The analogous reference compound methyl 9,10-dihydroxyoctadecanoate diTMSi ether has the *erythro* isomer preceding the *threo* under the same GLC conditions and the same isomer assignment was made in this case. This assignment was confirmed by comparison of retention time with authentic methyl *threo*- 9,10,18-trihydroxyoctadecanoate, triTMSi ether.

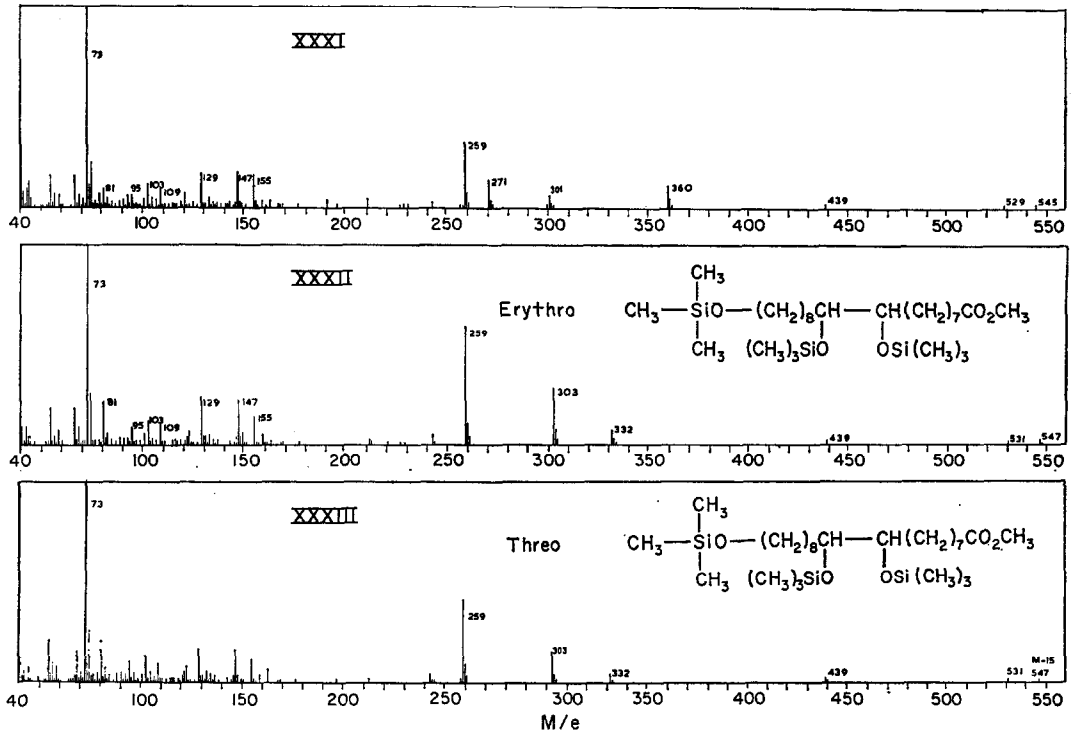


FIG. 5. MASS SPECTRA FROM GC-MS RUN ON TRIHYDROXYMONOBASIC ACIDS (AS METHYL ESTERS TMSi ETHERS) FROM APPLE CUTICLE.
Top: Methyl 9,10,18-trihydroxyoctadecenoate, triTMSi ether, Middle: Methyl *erythro*-9,10,18-trihydroxyoctadecanoate, triTMSi ether, Bottom: Methyl *threo*-9,10,18-trihydroxyoctadecanoate, triTMSi ether. Conditions: as in experimental and Fig. 3 programmed from 150–250° at 4°/min.

## DISCUSSION

Our data, in general, confirm the results of previous workers although we have been unable to confirm the presence of 9,10-epoxy-18-hydroxyoctadecanoic acid[4] or 18-hydroxyoctadecanoic acid and 10,18-dihydroxyoctadecanoic acid.[3] The epoxy compound may well be an artifact and the reported presence of the other two compounds was based on TLC $R_f$ values of the free acids which would not distinguish between them and other compounds.[15] For example, 18-hydroxyoctadecanoic acid would not be distinguished from 16-hydroxyhexadecanoic acid or the unsaturated $C_{18}$ compounds. None of the unidentified components has a mass spectrum resembling those of reference samples of these compounds. The possibility that there are significant changes in cutin composition with the growth of the plant must be considered, however, and these compounds may be present earlier or later in the season.

It has been suggested[16] that the first discrete step of cutin biogenesis is an intracellular hydroxylation, hydroperoxidation or similar oxidation step on the long chain carboxylic acids. This would be followed by hydroperoxidation and cross linking upon exposure of the unsaturated compounds to the atmosphere. It is significant that the individual $\omega$-hydroxy

[15] E. A. BAKER and J. T. MARTIN, personal communication.
[16] W. HEINEN, *Z. Naturforsch.* **18B**, 67 (1963).

monobasic acids occur in approximately the same proportion as the individual unsubstituted monobasic acids of the same chain length and unsaturation (cf. Table 1a and 1c). Terminal oxidation would then seem to be the step occurring intracellularly, while autoxidation would follow on the plant surface. As has been previously pointed out[8] all the cutin hydroxy acids have a terminal hydroxyl group. The 10,16-dihydroxyhexadecanoic acid could be derived from palmitic acid and, by a different route, the 9,10,18-trihydroxyoctadecanoic acid from oleic and/or linoleic acids. The absence of 10,18-dihydroxyoctadecanoic acid and of a trihydroxyhexadecanoic acid might then be attributed to the relatively low abundance of stearic acid and of a hexadecanoic acid.

The greater proportion of the cutin acids are straight chain and even numbered, i.e. $C_{16}$ and $C_{18}$. However, in the *vic*- dihydroxydibasic acid fraction the dominant acid is the $C_{17}$ acid. This curious dominance of an odd-numbered acid might be explicable in terms of chain shortening by terminal oxidation. Similar hydroxy acids are found among the autoxidation products of oleic acid,[17] for example, 9,10-dihydroxyoctadecanoic acid. These acids, however, are not terminally hydroxylated. Furthermore, a liquid 'procutin' has been observed to harden into a varnish-like finish on plant surfaces.[18] Whether or not the further hydroxylation of the cutin acids occurs enzymatically might be decided by examination of the optical rotatory power of certain of the cutin acids; for example, 10,16-dihydroxyhexadecanoic. We shall report the results of such studies when completed.

In the present study we have carried out the preliminary separation and purification of the cutin acid methyl esters by preparative TLC. For survey purposes the rapid examination of the crude cutin acid methyl ester fraction by GC-MS alone would be adequate and would permit fuller studies of the variation of cutin composition with species, season and climate. Extensive chemotaxonomic studies on plant cuticles are therefore feasible. Studies in this area and the associated field of paleochemotaxonomy will be reported in due course.

## EXPERIMENTAL

All mass spectra were obtained on an LKB.9000 combined gas chromatograph-mass spectrometer operating at 70 eV and using a 3·4 m × 3 mm (i.d.) coiled glass column packed with 1% SE-30 on acid washed and silanized Gas Chrom P. This column tested for 3360 theoretical plates with *n*-pentadecane at 100°. The helium flow rate through the column was 30 ml/min. The GLC trace was given by the total ion current at 20 eV. The scan time for each mass spectrum was 2 sec. The background mass spectrum for SE-30 was subtracted from each mass spectrum. Only a few background peaks were evident (at m/e 149, 207, 221, 281, 341, 355, 429, 503) and they did not amount to more than 1–2 per cent relative intensity. Peaks with a relative intensity greater than 1 per cent were tabulated.

Analytical GLC's were run on a Perkin-Elmer F-11 using a 3·4 m × 1·5 mm (o.d.) coiled stainless steel column packed with 3% SE-30 on Gas Chrom Q. This column tested for 3200 theoretical plates with methyl palmitate at 170°. Amounts of material present were determined by photocopying the GLC traces and cutting out and weighing each peak and comparing the results for standard amounts of material, using the external standard method (methyl palmitate and methyl aleuritate, tri-TMSi ether).

Infrared spectra were determined on a Perkin-Elmer 257 grating i.r. spectrophotometer.

### *Preparation of Cuticle*[9]

The peelings from 9 kg of commercially ripe apples (var. Cox Orange Pippin) were extracted with hot $CHCl_3$ to remove surface waxes and then digested with 1% $H_2SO_4$ to remove flesh. The skins were then refluxed with 0·4 per cent oxalic acid—1·6 per cent ammonium oxalate solution, followed by refluxing with $ZnCl_2$—HCl solution. After washing with water and air drying, the cuticles were extracted with methanol for 18 hours in a Soxhlet extractor and then air dried to give crude cutin, 8·1 g (epicuticular wax 8·9 g).

The i.r. spectrum of the cuticle (dipped in nujol/hexane to make it more translucent) showed a strong absorption at 3450 $cm^{-1}$ (OH and $H_2O$) and 1730 $cm^{-1}$ (ester).

[17] H. B. KNIGHT, C. R. EDDY and D. SWERN, *J. Am. Oil Chemist's Soc.* **28**, 498 (1951).
[18] E. RHODES and R. M. WOODMAN, *Proc. Leeds Phil. Soc.* **1**, 27 (1925).

*Solvolysis of Cutin*

To prepared apple cuticle (133·4 mg) were added 30 ml of 3% KOH in methanol. This mixture was refluxed for 20 hr and the insoluble material (26·9 mg, 20·2%) removed by centrifugation. The methanolic solution was evaporated to dryness, acidified and extracted with ether to give the ether-soluble cutin acids plus other ether-soluble materials (99·6 mg, 74·8%). This fraction was then methylated with excess $CH_2N_2$ in ether. The i.r. spectrum ($CCl_4$ solution) of the product showed absorption at: 3620 [$\nu$ (OH), free], 3450 [$\nu$ (OH), intra H—bond], 3000 [$\nu$ (=CH), unsat.], 2930 [$\nu$ (CH), aliph.], 2855 [$\nu$ (CH), aliph.] and 1740 $cm^{-1}$ [$\nu$ (CO), ester].

*Preparative TLC of the Methyl Esters of the Cutin Acid Fraction*

The cutin acids methyl esters (53·5 mg) were chromatographed on silica gel G (20 cm × 20 cm × 0·25 mm) in a solvent system of ether/hexane/methanol (40:10:1). The bands were visualized by exposing briefly to iodine vapours.

Each band was scraped off the plate and eluted with ether. The intermediate areas were also eluted and checked by GLC to establish that there was no significant amount of material present. Except for a small amount of dihydroxy ester which showed up in the trihydroxy ester fraction there was no overlap of bands.

*Monobasic Acids* (methyl esters; Table 1a and Fig. 1B)

An analytical GLC of this fraction is shown in Fig. 1B. The identity of each component was established by GLC co-injection with standards and by the mass spectra which could be compared to published spectra.[19] The large peak just before methyl stearate is an unresolved peak consisting of methyl oleate and methyl linoleate, which was shown by taking mass spectra on both the upside and downside of the GLC peak. The position of double bonds cannot be told directly from mass spectra,[12] but the identity is confirmed by GLC co-injection.

*Dibasic acids* (methyl esters; Table 1b and Fig. 1C)

An analytical GLC of this fraction is shown in Fig. 1C. Structure assignments were made on the basis of GLC, TLC and mass spectral data.

*Hydroxylation and Trimethylsilylation of the Dibasic Acid Fraction* (methyl esters)

One half of the diester fraction (110 $\mu$g) was treated with 30 $\mu$l of pyridine-ether (1:8) and 500 $\mu$g of $OsO_4$ in 10 $\mu$l of ether.[14] The solution was allowed to stand for 1½ hours and then 0·6 ml of a $Na_2SO_3$ suspension (1·5 ml 16% aqueous $Na_2SO_3$ plus 5 ml methanol) was added. The mixture was allowed to stand for 1 hr. After centrifugation the supernatant solution was removed, evaporated, taken up in 20 $\mu$l of ethyl acetate and silylated with 2 $\mu$l of N, O-bis(trimethylsilyl)acetamide.

GLC co-injection revealed one peak to be coincident with methyl 9,10-dihydroxyoctadecane-1,18-dioate, diTMSi ether. This structure was confirmed by the mass spectrum of this peak. Methyl 9,10-dihydroxyheptadecane-1,17-dioate,diTMSi ether and methyl 9,10,12,13-tetrahydroxyoctadecane-1,18-dioate,tetra TMSi ether, were identified on the basis of their mass spectra.

*Hydroxy Acid Fraction* (trimethylsilyl ethers of the methyl esters; Table 1c–f and Fig. 1D–G)

All these fractions were made into the trimethylsilyl ethers by treatment with ½ ml of hexamethyldisilazane and 3 drops of trimethylchlorosilane. After one-half hour the $NH_4Cl$ was separated by centrifugation. The analytical GLC traces of these fractions are given in Fig. 1. The GC-MS total ion current trace for the monohydroxy acid fraction (as the trimethylsilyl ethers of the methyl esters) is given in Fig. 3.

*Hydroxylation and Trimethylsilylation of the Monohydroxy Acid Fraction* (methyl esters)

A portion of the monohydroxy acid fraction (as the methyl esters) was hydroxylated under the same conditions as the diacid fraction. By mass spectra, were identified methyl 9,10,18-trihydroxyoctadecanoate, triTMSi ether, and methyl 9,10,12,13,18-pentahydroxyoctadecanoate,pentaTMSi ether.

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*Note added in proof.* It has come to our attention that the use of hydroxylation and GC-MS of the TMSi ethers for double bond determination has also been studied by Dr. P. Capella, Instituto di Industria Agrarie, University of Bologna, Italy, and this work is soon to be published.