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THE CHEMICAL COMPOSITION OF THE CUTICULAR WAX OF CRANBERRY

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Abstract—Cuticular wax of cranberry fruit (*Vaccinium macrocarpon* var. Howes) was analysed by thin layer and gas–liquid chromatography in conjunction with mass spectrometry and infrared spectroscopy. The wax was shown to contain *n*-paraffins (10·7%), *n*-aldehydes (14·3%), *n*-alcohols (6·5%), *n*-fatty acids (9·7%), sterols (5·0%) and pentacyclic triterpene alcohols (5·8%), acetates (6·1%) and acids (30·7%).

INTRODUCTION

THE CUTICLE of plants has broad implications in the production, storage and processing of agricultural commodities and the importance of cuticular waxes in the control of such important phenomena as dehydration, agricultural spray efficiency and mechanical damage to plant tissue is now well established.[1,2] Surprisingly, the literature contains few reports of the analysis of cuticular waxes of economically important plants. In 1934, Markley and Sando[3] investigated the wax extracted from cranberry pomace (air-dried cuticle with adhering tissue and seeds) and reported the presence of nonacosane, hentriacontane and ursolic acid. The unsaturated fatty acids oleic, linoleic and linolenic and the saturated acids from hexadecanoic to hexacosanoic were also reported, both free and as glycerides. More recently, Laakso[4] analysed 'crude ursolic acid' commercially extracted from cranberry pomace and isolated two hydrocarbons, five alcohols and seven acids from this product. Ursolic acid and oleanolic acid were conclusively identified.

The findings presented here are concerned with the detailed chemical composition of cranberry cuticle wax as revealed by the application of modern analytical techniques—a necessary prerequisite to the investigation of the functional properties of cuticle wax or of the biosynthetic origin of these materials.

RESULTS

The cuticle wax from intact commercially ripe cranberries was extracted with $CHCl_3$[5] and, after removal of the solvent, separated by preparative TLC[6] into seven fractions. The composition of the cuticular wax of cranberry as obtained by TLC is shown in Table 1. Wax accounted for 0·21% of the fr. wt. of the berry, occurring at a level of about 340 $\mu g/cm^2$ and a thickness of 3·5 μ (for a s.g. of 0·975).[7]

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[1] G. EGLINTON and R. J. HAMILTON, *Science* **156,** 1322 (1967).
[2] P. MAZLIAK, *Progr. Phytochem.* **1,** 49 (1968).
[3] K. S. MARKLEY and C. E. SANDO, *J. Biol. Chem.* **105,** 643 (1934).
[4] P. V. LAAKSO, *Suomen Kemistilehti* **25B,** 59 (1952), *Suomen Kemistilehti* **31B,** 103 (1958).
[5] J. T. MARTIN, *J. Sci. Food Agric.* **2,** 635 (1960).
[6] P. J. HOLLOWAY and S. B. CHALLEN, *J. Chromatogr.* **25,** 336 (1966).
[7] A. H. WARTH, *The Chemistry and Technology of Waxes*, p. 159. Reinhold, New York (1947).

TABLE 1. COMPOSITION OF CRANBERRY CUTICLE WAX

Fraction	R_f*	Total wax (%)
(1) Hydrocarbons	0·85	10·9
(2) Aldehydes	0·66	14·3
(3) Triterpenyl acetates	0·53	6·7
(4) Triterpene alcohols	0·22	7·0
(5) Alcohols	0·19	6 5
(6) Sterols	0·12	5·5
(7) Triterpene and fatty acids	0·05	40·4

* TLC on silica gel G with benzene–$CHCl_3$ (7:3) developing system.

Fraction 1. TLC indicated that this fraction contained hydrocarbons and the IR spectrum confirmed their identity as long chain paraffins. Lack of absorption at 1720–1740 cm^{-1}, 3600–3400 cm^{-1} or 1100–1250 cm^{-1} confirmed the absence of carbonyl-, hydroxy- or internal oxygen containing compounds. GLC on SE-30 revealed the presence of *n*-paraffins from C_{13} to C_{35} with C_{29} predominating (Table 2). Mass spectroscopy confirmed the identification of the normal paraffins and indicated the presence of a minor

TABLE 2. COMPOSITION OF FRACTIONS FROM CUTICLE WAX OF CRANBERRY

Chain length	Hydrocarbon	Aldehyde	Acid Fraction (%)	Alcohol*
C_8 to C_{15}			< 0·5 each	
C_{16}			1·1	
C_{17}			0·3	
C_{18}			0·9	
C_{19}			0·4	
C_{20}			1·8	
C_{21}			0·2	
C_{22}			1·7	2·1
C_{23}			0·2	0·6
C_{24}			7·0	2·9
C_{25}	1·3		0·3	0·7
C_{26}	2·5	1·1	1·7	5·9
C_{27}	6·5	1·9	0·3	0·9
C_{28}	6·0	10·2	3·8	21·1
C_{29}	56 6	5·2	0·7	1·0
C_{30}	0·9	76·2	2·9	55·2
C_{31}	10·5	0·8	0·2	0·7
C_{32}	3·0	3·9	0·3	3·9
C_{33}	1·7			
C_{34}	1·7			
C_{35}	0·8			
Unidentified	1·7†		0·2	
Oleanolic			27·6	
Ursolic			48·2	
Unidentified			0·2	

* Early fractions less than 1% in each case.
† Tentatively an urs-12-ene.

component (1·7% of Fraction 1) tentatively identified as a cyclic hydrocarbon of the urs-12-ene series. The mass spectrum of this compound showed a base peak at *m/e* 218 corresponding to the retro-Diels–Alder cleavage in ring C.[8] Other prominent ions were noted at *me/* 203 (35% of base), *m/e* 189 (30%) and *m/e* 133 (15%) and were all consistent with a urs-12-ene compound.[8] The molecular ion of this compound was not distinguished although it was probably in the *m/e* 408–*m/e* 410 range. Urs-2,12-diene and olean-2,12-diene eluted slightly before this compound under identical GLC conditions. The mass spectral fragment ratio *m/e* 218:*m/e* 203 tended to indicate that this compound was a member of the urs-12-ene series rather than the olean-12-ene series.[8,9]

Fraction 2. The aldehyde fraction isolated by TLC gave an IR spectrum consistent with the tentative identification 1720 cm^{-1}, (C=O); 2720 cm^{-1}, (H—C=O); 1420 cm^{-1}, (H—C=O). GLC analysis on SE-30 of the aldehydes and of their corresponding alcohols, produced on reduction with $NaBH_4$, gave results shown in Table 2. The mass spectra (both high and low voltage) of components of this fraction confirmed the presence of aldehydes, giving the appropriate ions M, M-28, M-44 and the 68 + 14 n series terminating at M-18.[10]

Fraction 3. The triterpene acetate fraction isolated via TLC gave an IR consistent with this identification[11] and which was essentially superimposable with a spectrum of authentic β-amyrin acetate. GLC separation on OV-22 revealed two major components having identical retention times as authentic α- and β-amyrin acetate. Saponification of this fraction with 0·5 N methanolic KOH afforded the free alcohols which, along with the TMS-ether derivatives, were subjected to GLC analysis. Retention coincidence with authentic standards was again demonstrated. Mass spectral studies of Fraction 3 were performed on subfractions isolated via GLC, and spectra were compared to authentic reference compounds. The identity of α-amyrin acetate (27% of fraction) and β-amyrin acetate (64% of fraction) were thus confirmed.

Several minor components, comprising 9% of the fraction, were noted but were not conclusively identified. The mass spectra of all of these compounds possessed prominent ions at *m/e* 218—the retro-Diels–Alder cleavage of amyrin derivatives.[8] As these subfractions did not appear to be contaminated with amyrin acetates, the *m/e* 218 ion suggests the presence of other amyrin esters.

Fraction 4. The triterpene alcohol fraction isolated via TLC gave an IR spectrum consistent with this identification[11] which was essentially superimposable with that of authentic α-amyrin. GLC separation of this fraction on OV-22 revealed two major peaks whose retentions were identical with authentic α- and β-amyrin. Conversion of a portion of this fraction to the TMS-ether or acetate derivatives followed by GLC again demonstrated retention coincidence with authentic standards. The identities of α-amyrin (51% of fraction) and β-amyrin (32% of fraction) were confirmed by comparison of their mass spectra to those of authentic compounds. Several minor components, comprising 17% of the fraction, were noted, but the mass spectra obtained from them was insufficient for identification.

Fraction 5. The *n*-alcohol fraction isolated via TLC gave an IR spectrum consistent with this identification. No bands due to unsaturation, branching or oxygenation other than hydroxyl were observed in the IR. GLC of the alcohols and their TMS-ethers on SE-30

[8] H. Budzikiewicz, J. M. Wilson and C. Djerassi, *J. Am. Chem. Soc.* **85**, 3688 (1963).
[9] A. F. Thomas and B. Willhalm, *Tetrahedron* 3177 (1964).
[10] K. Christiansen, V. Mahadevan, C. V. Viswanathan and R. T. Holman, *Lipids* **4**, 421 (1969).
[11] C. N. R. Rao, *Chemical Applications of Infrared Spectroscopy*, pp. 192, 381. Academic Press, New York (1963).

gave results shown in Table 2. GC-MS analysis of this fraction, as the free alcohols, confirmed the identification of individual components.

Fraction 6. This fraction gave an IR spectrum consistent with 5-sterols[11] and was superimposable with that of β-sitosterol. GLC separation of this fraction of SE-30 revealed two major peaks, the first (8% of fraction) having a retention time coincident with stigmasterol and the second (83% of fraction) showing coincidence with β-sitosterol. GLC of Fraction 6 acetates revealed identical retentions as the authentic sterol derivatives. The identities of stigmasterol and β-sitosterol were confirmed by GLC trapping followed by mass spectrometry and comparison of spectra to the spectra to the literature[12] and to authentic standards. Several minor components were noted in this fraction but were not identified.

Fraction 7. This fraction was Liebermann-Burchard positive[6] and gave an IR spectrum indicative of hydroxy acids of both aliphatic and cyclic character. The fraction was methylated and on GLC revealed the presence of *n*-fatty acids from C_8 to C_{23} by comparison of retentions with authentic standards. Two large peaks eluted after C_{32} methyl ester and had retentions coincident with methyl oleanolate and methyl ursolate. Quantitative analysis of Fraction 7 is given in Table 2.

The identities of *n*-fatty acid methyl esters were confirmed by GC-MS analysis. The two peaks eluting after C_{32} methyl ester were trapped individually via GLC and their identities confirmed by comparison of their mass spectra with those of authentic triterpene acid standards. Two minor components (less than 0·5% each) were noted in this fraction but available data was insufficient for identification.

DISCUSSION

The results of the present analysis confirmed several of the earlier findings.[3,4] Markley and Sando[3] reported the presence of glycerides and free fatty acids in cranberry wax, and after saponification noted the presence of oleic, linoleic and linolenic acids and the series of saturated acids C_{16} to C_{26}. While the saturated acids are consistent with this report the unsaturated acids and glycerides are not. It seems certain that the glycerides and unsaturated acids reported by these authors[3] were derived from the seed portion of their experimental material. Previous work on cranberry seed lipid composition[13] has demonstrated the presence of sizeable amounts of glycerides, the fatty acid content of which was almost entirely oleic, linoleic and linolenic acids. Although Markley[3] reported that cranberry wax contained no alcohols other than glycerol, this claim was not well substantiated and it has now been shown that long chain alcohols are actually present.

The composition of cranberry cuticle wax is somewhat unusual, even for fruit which are generally noted as containing significant proportions of terpenoid materials. Pear,[14] apple[14] and grape[15] cuticle wax contain considerable amounts of triterpenoid acids. The cranberry, however, contains several triterpenoid classes including acids, alcohols, esters and possibly hydrocarbons. The major triterpenoids found in cranberry cuticle wax are those derived from the amyrins. The biosynthetic origin of these compounds has been reviewed[16,17] although not in connection with their occurrence in cuticular wax. An interesting observation from the present work is that the ratio of the urs-12-enes to olean-12-

[12] B. A. KNIGHTS, *J. Gas Chromatogr.* **5**, 273 (1967).
[13] R. CROTEAU and I S. FAGERSON, *Phytochem.* **8**, 2219 (1969).
[14] A. M. SILVA FERNANDES, E. A. BAKER and J. T. MARTIN, *Ann. Appl. Biol.* **53**, 43 (1964).
[15] F. RADLER and D. H. S. HORN, *Austral. J. Chem.* **18**, 1059 (1965).
[16] I. D. FRANTZ and G. J. SCHROEPFER, *Ann. Rev. Biochem.* **36**, 691 (1967).
[17] C. J. SIH and H. W. WHITLOCK, *Ann Rev. Biochem.* **37**, 661 (1968).

enes in the triterpene acid fraction (ursolic:oleanolic) and in the triterpene alcohol fraction (α-amyrin:β-amyrin) is about 2:1 while in the triterpene:acetate fraction this ratio is reversed.

The *n*-paraffins, *n*-alcohols and *n*-fatty acids are very common wax components and chain length distributions of these components correspond to those often encountered in the literature.[1,2] Other than this report, aldehydes have been found in grape,[15] sugar cane[18] and pea[19] surface wax. This is one of the few reports, however, in which aldehydes were shown to be the major aliphatic component present.

Recently, enzymatic reduction of acyl-CoA to the corresponding aldehyde and subsequent reduction of the aldehyde to the alcohol by an aldehyde reductase has been demonstrated in broccoli and pea leaf acetone powders.[20] If the aldehydes found in plant cuticle wax are intermediates in the conversion of acids to alcohols, the aldehydes would be expected to resemble the alcohols in their chain length distribution. Table 3 shows such a correspondence, which has also been noted in the surface waxes of other plants.[15,18,19] That the aldehydes constitute such a large proportion of the aliphatic waxes in cranberry suggests that the acyl-CoA reductase and aldehyde reductase are not closely coupled.

TABLE 3. CHAIN LENGTH DISTRIBUTION OF ALIPHATIC COMPOUNDS FROM CUTICLE WAX OF CRANBERRY

Chain length	Aldehyde	Alcohol	Acid	Paraffin
C_{22}	—	4	24	—
C_{23}	—	1	6	—
C_{24}	—	5	100	1
C_{25}	—	1	7	2
C_{26}	1	11	24	4
C_{27}	3	1	7	12
C_{28}	13	31	54	11
C_{29}	7	2	10	100
C_{30}	100	100	41	2
C_{31}	1	2	3	19
C_{32}	5	7	4	5

Each fraction is normalized on the basis of the largest component in that fraction. (—) indicates less than 1% of the largest component.

The long chain acids exhibit a bimodal distribution (Table 3) and similar observations have been noted in the literature.[1] As the chain length distribution of compounds synthesized may change with the maturity of the plant,[14,21] the bimodal distribution may represent the mature pattern of biosynthesis superimposed on the accumulated products synthesized at an earlier phase of growth.

Much evidence has accumulated that fatty acids are the precursors of plant paraffins, but a mechanism for this conversion has not been clearly established.[20,22,23] A scheme by

[18] J. A. LAMBERTON, *Austral. J. Chem.* **18**, 911 (1965).
[19] P. E. KOLATTUKUDY, *Lipids* **5**, 398 (1970).
[20] P. E. KOLATTUKUDY, *Lipids* **5**, 259 (1970).
[21] G. A. HERBIN and P. A. ROBINS, *Phytochem.* **8**, 1985 (1969).
[22] P. E. KOLATTUKUDY, *Science* **159**, 498 (1968).
[23] P. E. KOLATTUKUDY, *Ann. Rev. Plant Physiol.* **21**, 163 (1970).

which an intermediate length fatty acid is elongated and then decarboxylated to give a paraffin of one less carbon has been proposed by Kolattukudy.[20,23] Comparison of fatty acid chain length distribution to that of the paraffins (Table 3) shows rough correspondence above C_{26} although such superficial resemblence of proper chain lengths may be biochemically meaningless. One could speculate that C_{30} fatty acid is the key precursor in the biosynthesis of aliphatic substances in cranberry cuticle wax, giving rise to C_{29} paraffin via decarboxylation and to C_{30} aldehyde and C_{30} alcohol by progressive reduction.

Waxy esters are common components of cuticle wax and several mechanisms for waxy synthesis in plants have been demonstrated.[24] Although the required fatty acid and alcohol precursors are apparently available for ester synthesis, no significant amount of waxy esters were found in cranberry wax extracts. A trace of component having the proper TLC R_f value for waxy esters (between paraffin and aldehyde) was noted, but not identified

The primary function of cuticle wax is to waterproof the plant and this function is performed so well that less than 10% of the water loss from a plant takes place by cuticular transpiration.[25] The water permeability (and wettability) of cuticle has been correlated with the gross quantity of surface wax but Silva Fernandes[26] has demonstrated that wax level alone is not a sufficient criterion for predicting this behaviour. Measurement of water loss through artificial membranes treated with various isolated wax components has indicated that the aliphatic components such as hydrocarbons, aldehydes and alcohols are far more effective in reducing transpiration than cyclic triterpenes.[25,27] It is somewhat surprising that the cranberry (with 50 per cent of its wax as triterpenes) should possess such an impermeable surface. This may be explained, however, by features of the physical structure of the wax or by the effects of the absolute amount of cuticle wax, which is rather high and which may contain a great enough proportion of aliphatic components to be effective.

Cuticle wax also provides abrasion resistance to plant tissues and influences the entry of foliar sprays and other chemicals from the environment. These functional properties are dependent on both the quantity and composition of the wax and have generally been associated with the aliphatic substances most commonly found. The effects on these properties of large proportions of terpenoid materials, such as found in cranberry wax, are unknown, and whether the notable hardness of cranberry wax can be attributed to its high terpenoid content is uncertain. A few observations have been made on the interactions of spray chemicals with specific wax components. These studies, however, deal only with waxy esters[26] and aliphatic β-diketones,[28] while it is the cyclic triterpenoids in cranberry cuticle wax that are probably more influential. The terpenoids, especially the triterpene acids, are relatively more polar than most aliphatic wax components and their ability to interact with and solubilize the more polar spray chemicals should be considerably greater.

EXPERIMENTAL

Extraction and fractionation of cutical wax. Cranberries were obtained from the Ocean Spray Co., Hanson, Massachusetts at the commercially ripe stage in October, 1969. Intact berries (1 kg fr. wt., 6040 cm^2 surface area calculated via the mean semi-axes of *n*-prolate spheroids) were immersed for 10 sec

[24] P. E. KOLATTUKUDY, *Biochem.* **6**, 2705 (1967).
[25] M. GRNCAREVIC and F. RADLER, *Planta* **75**, 23 (1967).
[26] A. M. SILVA FERNANDES, *Ann. Appl. Biol* **56**, 297 (1965).
[27] J. SIVADJIAN, *Compt. Rend.* **266**, 353 (1968).
[28] D. H. S. HORN and J. A. LAMBERTON, *Chem. Ind.* 2036 (1962).

in six successive 3 l. portions of $CHCl_3$.[5] The solvent was removed from the wax extract under vacuum and, after filtering, H_2O washing and drying ($CaSO_4$) further concentrated under N_2 to yield surface wax (2·06 g, 340 $\mu g/cm^2$). The wax was fractionated by preparative TLC on silica gel G wedge plates with benzene–$CHCl_3$ (7:3) as developing solvent.[6] Seven prominent bands were located with 0·05% aq. rhodamine G6, scraped from the plates and wax components eluted from the silica gel with dry Et_2O. Areas between bands were also analysed (less than 3% of total wax). Slight overlap of Fraction 4 into Fraction 5 was noted during GLC examination and was corrected for during quantitative analysis.

IR spectroscopy. All samples were solid at room temp. and were run as thin films on the surface of AgCl disks. Spectra were taken with a Perkin–Elmer model 337 spectrophotometer.

Preparation of derivatives. Aldehydes were converted to alcohols by reduction with excess 5% $NaBH_4$ in MeOH for 1 hr at room temp. Micro-methylation with CH_2N_2 was carried out according to Schlenk and Gellerman[29] using *N*-nitrosomethylurea. Acetates were prepared by refluxing with excess Ac_2O for 2 hr. Silylation of alcohols was carried out in bis-(trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane as catalyst using 1 ml reagent to 5–10 mg sample. GLC analyses were carried out directly on the reaction mixture after initial heating to 80° and standing for several hr.

GLC. Samples were analysed by GLC on a Perkin–Elmer 900 dual column instrument with an exit-port splitter using the following column systems. (1) 1·5 m × 3 mm column coated with 5% SE-30 on 60/80 mesh, acid washed and silanized chromsorb W, programmed from 140 to 320° at 10°/min for Fraction 1, 160–300° at 10°/min for Fraction 2 and 6, and 180–310° at 10°/min for Fraction 5 (all at 30 ml/min flow rate). (2) 1·2 m × 3 mm column coated with 3% OV-22 on 60/80 mesh, acid washed and silanized chromasorb W, programmed from 200 at 310° at 10°/min for Fraction 3, 190–310° at 10°/min for Fraction 4 and 180–340° at 10°/min for Fraction 7 (all at 30 ml/min flow rate).

For quantitative analysis, internal standards for each fraction were employed as follows: cholestane (1), cholestanone (2), cholesteryl acetate (3), cholestanol (4,5,6) and methyl lithocholate (7). Peak areas were calculated by triangulation with no corrections made.

Mass spectrometry. Mass spectra were taken with a Hitachi–Perkin–Elmer RMU-6A mass spectrometer. When the GC–MS operational mode was employed, a Varian Aerograph 1200 gas chromatograph was utilized as the inlet system along with a Biemann-type separator. Spectra of Fraction 3 components were taken at both 70 and 20 eV.

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[29] H. Schlenk and J. L. Gellerman, *Analyt. Chem.* **32**, 1412 (1960).