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Composition and Variability of Epicuticular Waxes in Apple Cultivars

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Abstract. **Variation in amount and composition of epicuticular wax among several apple (*Malus ×domestica* Borkh.) cultivars was characterized by gas chromatography, thin-layer chromatography, and gas chromatography–mass spectroscopy. Across cultivars, wax mass ranged from 366 to 1038 μg·cm^{-2}. Wax mass decreased during the 30 days before harvest. Ursolic acid accounted for 32% to 70% of the hydrocarbons that make up the epicuticular wax. Alkanes, predominantly 29-carbon nonacosane, comprised 16.6% to 49%. Primary alcohols of the hydrocarbons ranged from 0% to 14.6% of the epicuticular wax. Secondary alcohols of the hydrocarbons were the most cultivar specific, making up 20.4% of the epicuticular wax in 'Delicious' and only 1.9% 'Golden Delicious' strains. Aldehydes and ketones of the hydrocarbons represented a small amount of total wax, ranging from 0% and 6.0%. Percentage of primary alcohol in the epicuticular wax increased as fruit developed. Other components showed no distinct trends with fruit development. Examination of the ultrastructure of cuticular wax using scanning electron microscopy revealed structural differences among cultivars.**

The plant cuticle forms a continuous protective layer on the aerial surface of all terrestrial plants. This layer protects the plant against weathering, abrasions, leaching and water loss, and is also the first line of defense against infection by plant pathogens. In addition, it is the target site for foliar sprays including fertilizers, growth regulators, fungicides, insecticides and herbicides.

The cuticle is composed of two main components: 1) a structural matrix called the cutin and 2) wax. Waxes are embedded in the cutin and form a continuous layer on top of the cutin. Cuticular waxes are the primary component of the cuticle responsible for the wetability and permeability of the cuticle. Composition of the cuticle of some commercially important plants, including apples, has been studied (Holloway, 1984; Lawrence et al., 1985; Martin and Juniper, 1970). However, there is a lack of information on variability of cuticular waxes between apple cultivars. Components of the cuticle have been associated with disease incidence and severity on many plants including apple (Martin et al., 1957), ginko (*Ginko biloba* L.), (Johnson and Sproston, 1965), Beetroot (*Beta vulgaris* L.) (Blakeman and Sztejnberg, 1973), and grape (*Vitis vinifera* L.) (Marios et al., 1987). Understanding the impact of this association could lead to new approaches in disease management.

The purpose of this study was to investigate the composition of apple cuticular waxes and characterize variability among cultivars quantitatively and qualitatively. The objectives of this study were to determine the variability of epicuticular wax (ECW) among cultivars, trees, and fruit; describe the diversity of ECW in a larger number of cultivars; and determine the changes that occurred in ECW during the growing season as fruit developed.

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Materials and Methods

Experiment 1

Six cultivars were selected from a replicated cultivar trial in Raleigh, N.C. Three strains of Delicious—'Starkspur Supreme', 'Starkrimson', and 'Oregon Spur II'—and three Golden Delicious strains—'Lys Golden', 'Pure Gold', and 'Sundalespur Gold'—were evaluated. Three tree replications of each cultivar were selected, and 15 fruit were harvested randomly from each tree replication at commercial maturity on 29 Aug. 1989. Fruit were handled with PVC gloves and placed in trays minimizing contact with fruit. Fruit were taken to the laboratory where maximum and minimum diameters were measured laterally and longitudinally. Diameter measurements were used to estimate fruit surface area by calculating the surface area of a sphere of that diameter.

Wax extraction. Waxes were removed from the apple surface by submerging each apple in three successive 30-s washes of clean chloroform. The three extracts were combined, and the chloroform was removed in a rotoevaporator. Wax residues were transferred in a minimum (10 to 20 mL) of fresh chloroform into preweighed chloroform-resistant vials. The samples were dried over $CaSO_4$ under reduced atmosphere for a minimum of 72 h, and dry mass was determined for wax from individual apples (Ferree et al., 1984).

Preparation of wax for analysis. The gas chromatography (GC) method was adapted from Lawrence et al. (1985). Two to five milligrams of wax was placed in a small glass tube and weighed. Chloroform was added to the tube to bring the concentration of wax to 1 mg·mL^{-1} of chloroform. One milliliter of the dissolved wax mix containing 1 mg of wax sample was transferred into a clean test tube for esterification with diazomethane. Chloroform was removed with a rotoevaporator. The wax sample was redissolved in 1 mL of 10 heptane : 10 ether : 1 methanol. Diazomethane gas was generated by adding 0.2 g of n-methyl-n-nitroso-p-toluenesulfonamide to a tube containing 3 mL of 37% potassium hydroxide, 2 mL 2-(ethoxy-ethoxy ethanol), and 2 mL ether in a steady stream of nitrogen saturated with ether. The diazomethane in gaseous form was then bubbled directly through the sample for 2 min. The reaction was judged complete when the sample attained a persistent yellow color.

Following methylation, the sample was transferred into a 1.6-mL glass sample vial and the solvent was removed under a stream

of air. Silylation was achieved by adding 75 µL BSTFA (bis-(trimethylsilyl)-trifluoroacetamide) and 25 µL anhydrous pyridine. The samples were either heated to 50 °C for 1 h or left at room temperature overnight for completion of the reaction. Samples were again dried and redissolved in 1 mL of chloroform for chromatographic analysis.

GC. Quantification of ECW components was performed on a programmable GC (Varian 3400) equipped with a standard flame-ionization detector. The column was a 15-m × 0.53-mm fused silica capillary column (SPB-1; Supelco, Bellfonte, Pa.). A temperature program was run as follows: initial column temperature was 180 °C, increase at 10 °C/min to 290 °C, then held at 290 °C for 4 min. The injector and detector temperatures were 220 and 320 °C, respectively. Sample volume was 1 µL containing 1 µg of wax and was injected manually. Variations in injection volumes were accounted for by reporting wax components as a percent of the total injection. Comparison of wax components among samples was based on retention time (Rt) and known standards.

GC–MASS SPECTROSCOPY (MS) was used to verify the identity of compounds found in apple wax. Thirteen compounds likely to be found in plant ECW were examined: palmitic acid, stearic acid, stearyl alcohol, tetracosane, oleic acid, lignoceryl alcohol, nonacosane, hexacosanol, octacosanol, nonacosanol, nonacosanoic acid, ursolic acid (UA), and oleanolic acid. All but four of these compounds were found resident in the library of the MS computer. The histograms of nonacosanoic acid and caryphyllin were added to the computer library from 500-mg·L^{-1} injections of the compounds. Standards were prepared in a mix at 100 mg·L^{-1} each. ECWs of 'Red Chief' and 'Lys Golden' were prepared at concentrations of 100 mg·L^{-1} so individual components would be 10 mg·L^{-1}.

Samples in chloroform were filtered through Whatman no. 1 filter paper, dried, and dissolved in 1.0 mL of 10 heptane : 10 ether : 1 methanol. This mix was methylated by adding diazomethane. The sample was dried and redissolved for silylation by 3 BSA : 1 pyridine. Silylation was complete after 1 h at 50 °C.

THIN-LAYER CHROMATOGRAPHY (TLC) was used to determine the family classes of compounds recorded by GC but whose identity was unknown. Commercially prepared plates (Fisher Scientific, Springfield, N.J.) coated with 0.25 mm of silica gel with 1-cm-wide channels etched in the coating were used. Plates were activated by heating at 100 °C for 30 min. Spots of wax were applied on the plates in amounts of 50, 100, or 150 µg of sample per spot. Three concentrations of each sample were replicated on each plate. Samples prepared included the ECWs of 'Royal Gala' and 'NY 61356-22' since they were known to be different by GC analysis. Single applications of three concentrations of nonacosane and UA were included as standards.

A glass TLC tank lined with Whatman no. 3 chromatography paper was used to develop chromatograms. Plates were developed for 30 min in 200 mL of benzene. A nondestructive spray reagent—2',7'-dichloroflourescein (Sigma Co., St. Louis)—was sprayed on the plate for visualization of the compounds using a longwave (350 to 400 nm) ultraviolet light. Spots were marked and the distance from the origin was measured. The intensity of the spots was rated 0 = visible spot, 1 = smallest detectable, through 5 = the largest (15 mm).

A second TLC spray of 4-amino-5-hydrazino-1,2,4-triazole-3-thiol (Sigma Co.), which specifically resolves aldehydes as purple spots on a yellow background, was obtained and applied to a separate duplicate plate. Again, distance from the origin was measured and intensity of the spots was recorded.

Data were analyzed using the general linear models analysis of variance procedure with the Waller–Duncan's mean separation *t* test of SAS (Cary, N.C.). Estimates of variance were calculated.

Experiment 2

Twelve apple cultivars were selected for testing in 1992: 'Coop-17', 'Royal Gala', 'Liberty', 'NY 65707-19', 'NY 61356-22', 'Oregon II', 'Red Chief', 'Stark Red Rome', 'Silverspur', 'Starkrimson', 'Smoothie Golden Delicious', and 'Starkspur Supreme'. Twenty-four single-fruit replications were harvested at maturity from single trees.

Preliminary observations indicated that any physical contact to the cuticle, including gloved fingers, altered the way that water and chemical residues were retained on the damaged area on the fruit. To minimize cuticle damage, untouched fruit, while still on the tree, were skewered with a glass or metal rod in the calyx end of the apple. Then, with the aid of a gloved hand to touch only the stem, fruit were removed from the tree. Fruit were transported to the laboratory in racks fabricated to keep fruit suspended on the rods and separate. Fruit diameter was measured for each fruit and used to estimate surface area as described above. Wax was extracted and analyzed as in Expt. 1. In addition to diameter measurements recorded for Expt. 1, for Expts. 2 and 3, fruit volume was determined by water displacement following wax extraction. Surface area was computed as that of a sphere containing that volume.

Experiment 3

Six apple cultivars were selected from an 8-year-old cultivar trial in Raleigh. There were three 'Golden Delicious' strains—'Lys Golden', 'Pure Gold', and 'Sundalespur Gold', two 'Red Delicious' strains—'Red Chief' and 'Silverspur', and 'Starkspur Red Rome'. Twenty randomly selected single-fruit replicates from each cultivar were tested on each of the three sampling dates: 29 June, which was 60 d before harvest (DBH); 26 July, which was 30 DBH; and 28 Aug., at commercial maturity. Wax extraction and analyses were the same as in Expt. 1. Data were analyzed using a general linear model, and means were separated using the Waller–Duncan's K ratio *t* test (SAS).

Electron microscopy

Samples of fruit cuticle were examined using scanning electron microscopy (SEM). Samples 2 mm in diameter were shaved from the cuticles of undisturbed fruit in the field and fixed to metal microscope mounts with two-sided tape. Samples were shaved thin for SEM to minimize water filled cells and air dried over dry $CaSO_4$ for 24 h before being sputter coated with gold–palladium to a layer 15 Å thick. Micrographs were recorded using a microscope (Phillips 505) with a spot energy of 10 kV. Type 55 Polaroid film was used.

Results

IDENTIFICATION AND ANALYSIS OF WAX COMPOSITION. GC was the most efficient method for quantifying plant ECW. A single injection of 1 µg was sufficient to separate 17 wax components in 6 to 15 min using a temperature program (Table 1).

GC–MS was the best method for positively correlating specific wax components to standards, but there were certain limitations to the technique. Derivatization was necessary to increase the volatility of some components to be within the limitations of the GC column (<320 °C). Compounds that included alcohol, aldehyde, ketone, or ester reaction groups, did not adapt easily to the volatilization requirements of the GC. The derivitization was done in two steps: methylation and silylation. Comparisons of the compounds' molecular weight (MW) and the MW estimate from GC–MS fell into three distinct categories: 1) accurate, 2) 14 units over, and 3) 58 units over. MWs of the alkanes were predicted

Table 1. Apple epicuticular wax components listed in order of retention time from gas chromatography (GC). Components are identified by name or chemical family, along with the method used in identifying each compound [thin-layer chromatography (TLC), mass spectroscopy (MS), or GC–MS]. The percentage of total wax that is attributed to each component averaged over six cultivars is given.

Component no.	Retention time (min)	Component identity	Component identified by	Percent[z]
1	8.88	Hydrocarbon-1	TLC	1.8
2	9.29	Tetracosanol	MS	1.3
3	10.35	Nonacosane	MS	24.0
4	10.69	Hexacosanol	MS	2.0
5	11.59	Secondary alcohol-1	TLC	8.3
6	11.80	Unknown-1	---	2.4
7	11.99	Octacosanol	MS	2.3
8	12.48	Aldehyde-1	TLC	1.6
9	12.65	Unknown-2	---	1.1
10	13.10	Secondary alcohol-2	TLC	5.1
11	13.20	Primary alcohol-4	TLC	1.6
12	13.30	Unknown-3	---	0.8
13	13.45	Primary alcohol-5	TLC	1.3
14	13.80	Aldehyde-2	TLC	0.9
15	14.46	Ketones	TLC	2.5
16	14.62	Ursolic acid-1	MS	15.7
17	15.01	Ursolic acid-2	MS	27.4
			Total GC–MS	72.7
			Total TLC	23.1
			Total	95.8

[z]Percent of compounds as detected by GC. Average of six cultivars.

accurately. The methyl ester derivative matches to the 26- and 28-carbon acids hexadecanoic acid and octadecenoic acid was 14 MW units above the actual MW. This was presumed to be a result of derivitization. Three compounds that contain the primary alcohols tetra-, hexa-, and octacosanol had derivatized MWs 58 molecular units above their actual MW. UA was 14 units above its stated MW. Because of the additions to the molecules by derivation, identification by GC–MS alone was more difficult. However, GC–MS positively correlated the primary alcohols (tetracosanol, hexacosanol, and octacosanol) and UA by comparison to known standards. It also was possible to determine that GC peaks represented single compounds and were not comprised of two or more components sharing a similar retention time.

TLC. Six classes of ECW compounds were previously described by Holloway (1984) on apples. Of these six classes, three class were identified by positive correlation to standards using GC–MS (Table 2). These classes were the alkanes, triterpenols, and primary alcohols, which represented 73% of the total ECW.

Holloway (1984) reported that the solvent benzene resolved compounds including ketones, aldehydes, and secondary alcohols with a minimum relative distance (Rd) of 11%. The inability of benzene to resolve compounds that included primary alcohols from the triterpenols was considered insignificant due to their previous identification by GC–MS. Alkane hydrocarbons easily compared to the internal standard (Table 2). The triterpenol standard did not resolve beyond the origin. However, a clear spot occurred from the extracted apple wax and GC confirmed it as UA.

2',7'-Dichloroflourescein, which resolves only aldehydes, clearly identified only one spot appearing at Rd = 51. GC analysis of the aldehyde spot further separated two aldehyde-like compounds. More importantly, positive identification of the Rd value of the aldehydes ensured that the classes of the components resolving at Rd = 35 were not aldehydes and were in the position of the secondary alcohols. The compounds resolving at Rd = 34, a possible secondary alcohol, matched the compounds that, from GC analysis, appear in large concentrations in 'NY61356-22' (20%) and were almost nonexistent in 'Royal Gala'. Identification of the family with a Rd value of 73 was the least certain. Compounds that included ketones were expected to resolve at Rd = 63, but since there was no spot at Rd = 63, the compounds resolved at that Rd = 73 were probably ketones (Holloway, 1984).

Wax mass of the cultivars used in Expt. 1 ranged from an average of 878 $\mu g \cdot cm^{-2}$ fruit surface for 'Starkrimson' to 1039 $\mu g \cdot cm^{-2}$ for 'Oregonspur' (Table 3). There were no differences in the mass of wax from the different cultivars, because the variation among fruit within cultivar was high.

Table 2. Separation of family classes of compounds using thin-layer chromatography with benzene as a solvent, on a 0.25-mm-thick plate of silica gel.

Cultivar	Predicted[z] Rd[y]	Actual Rd	Spot size rating[x]
Royal Gala			
Hydrocarbons	80	84	4
Ketones	63	71	4
Aldehydes	52	54	2
Secondary alcohols	35	34	0
Primary alcohols	10	11	4
Triterpenols	10	0	---
NY61356-22			
Hydrocarbons	80	81	4
Ketones	63	75	2
Aldehydes	52	54	2
Secondary alcohols	35	33	5
Primary alcohols	10	11	3
Triterpenols	10	0	---

[z]From Holloway (1984).
[y]Rd = distance compound traveled relative to the solvent front.
[x]Rated 0 = none to 5 = greatest.

Table 3. Compositional analysis of apple epicuticular wax by chemical family. Waller–Duncan mean separation *t* test was across cultivars.

Cultivar or strain	Wax mass[y] ($\mu g \cdot cm^{-2}$)	Wax component[z] (%) Ursolic acid	Alkanes	Primary alcohols	Secondary alcohols	Aldehydes	Ketones
Sundalespur Gold	960.7[NS]	54.3 b	36.1 a	4.4 ab	3.3 b	0.1[NS]	1.7[NS]
Pure Gold	882.0	69.8 a	18.9 d	9.1 a	1.1 b	0.0	0.8
Lys Gold	915.7	66.7 a	22.6 cd	7.7 ab	1.3 b	0.0	1.7
Starkspur Supreme	1034.3	46.5 c	29.7 b	0.0 b	22.6 a	0.0	0.6
Oregon Spur II	1038.7	47.0 bc	29.0 b	5.4 ab	17.1 a	0.7	0.0
Starkrimson	877.7	47.0 bc	28.8 bc	0.0 b	21.0 a	0.0	1.2

[z]Wax component means represent 15 fruit per cultivar and are listed as percent of total wax.
[y]Wax mass means represent 45 fruit per cultivar.

Wax composition of cultivars. The triterpenoids, represented by UA, constituted the largest amount of the ECW (Table 3). In Expt. 1, 70% of the ECW of 'Pure Gold' and 67% of 'Lys Golden' was composed of UA. The concentration of UA was significantly more in these cultivars than in 'Sundalespur Gold', 'Starkspur Supreme', 'Oregonspur', or 'Starkrimson'. UA appeared as two separate peaks eluting at retention times of 14.62 and 15.01 min on the GC (Table 4). GC–MS identified both peaks as UA and was unable to distinguish between the two peaks. The two peaks may represent isomers, which were sufficiently different to provide GC separation.

Alkanes constituted the second largest chemical family in ECW, ranging from 18.9% to 36.1% in 'Pure Gold' and 'Sundalespur Gold', respectively (Table 3). The most important alkane was nonacosane (Table 4). Nonacosane is saturated with hydrogens in a straight 29-carbon chain with no double bonds. All compounds, including primary and secondary alcohols, aldehydes, and ketones, found in apple ECW, except for the triterpenoid UA, are derived from this basic nonacosane hydrocarbon chain. Nonacosane accounted for 16.6% to 32.3% of the total ECW. One additional n-alkane, labeled hydrocarbon-1, classified by TLC with a GC retention time of 8.88 min accounted for 1.4%, 2.3%, and 3.7% of the ECW of the Golden Delicious strains 'Lys Golden', 'Pure Gold', and 'Sundalespur Gold', respectively. Golden Delicious strains contained hydrocarbon-1, whereas the three Delicious strains did not contain measurable amounts of this compound.

TLC identified five compounds as those containing primary alcohols by comparison to standards and cross-checked by GC. Three of these were identified by GC–MS as being tetracosanol (Rt = 9.29 min), hexacosanol (Rt = 10.69 min), and octacosanol (Rt = 11.99 min) (Table 4). Hexacosanol accounted for the greatest amount of primary alcohols.

'Pure Gold' had significantly greater amounts of tetracosanol and octacosanol than Red Delicious strains (Table 4). All Golden

Table 4. Compositional analysis of apple epicuticular wax by component from Expt. 1. Waller–Duncan mean separation *t* test was across cultivars.

Cultivar	Wax component[z] (%) Hydrocarbon-1	Tetracosanol	Nonacosane	Hexacosanol	Secondary alcohol-1
Sundalespur Gold	3.7 a	0.3 b	32.3 a	2.4[NS]	0.1 b
Pure Gold	2.3 ab	3.4 a	16.6 b	3.6	0.0 b
Lys Golden	1.4 bc	1.2 b	21.2 b	3.8	0.0 b
Starkspur Supreme	0.0 c	0.0 b	29.7 a	0.5	16.9 a
Oregon Spur II	0.0 c	0.0 b	29.0 a	5.4	11.2 a
Starkrimson	0.0 c	0.0 b	28.8 a	0.0	15.1 a

Cultivar	Octacosanol	Aldehyde-1	Secondary alcohol-2	Primary alcohol-4	Primary alcohol-5
Sundalespur Gold	1.7 b	0.1[NS]	3.1 b	0.0 b	0.0[NS]
Pure Gold	2.1 b	0.0	1.1 c	3.2 a	0.0
Lys Golden	2.7 a	0.0	1.3 c	0.0 b	0.0
Starkspur Supreme	0.0 c	0.0	5.7 a	1.3 ab	0.0
Oregon Spur II	0.0 c	0.7	5.8 a	0.0 b	0.0
Starkrimson	0.0 c	0.0	5.9 a	0.0 b	0.0

Cultivar	Aldehyde-2	Ketone	Ursolic acid-1	Ursolic acid-2
Sundalespur Gold	0.0[NS]	1.7[NS]	16.1 c	38.2 b
Pure Gold	0.0	3.1	22.5 a	47.4 a
Lys Golden	0.0	1.7	20.1 b	46.7 a
Starkspur Supreme	0.0	1.9	12.6 d	33.9 b
Oregon Spur II	0.0	0.0	12.2 d	34.9 b
Starkrimson	0.0	1.2	12.0 d	35.0 b

[z]Means represent 15 fruit per cultivar and are listed as percent of total wax recorded by gas chromatograph.

Table 5. Compositional analysis of apple epicuticular wax by chemical family from Expt. 2. Waller–Duncan mean separation *t* test was across cultivars.

Cultivar or strain	Wax mass[y] ($\mu g \cdot cm^{-2}$)	Wax component (%)[z] Ursolic acids	n-Alkanes	Primary alcohols	Secondary alcohols	Aldehydes	Ketones
Oregon Spur I	686.8 abcde	33.43 de	31.64 cd	4.40 g	23.16 b	3.24 ab	1.60 c
Red Chief	749.5 abc	35.80 cd	31.89 c	5.70 ef	20.52 c	2.20 c	1.59 c
Starkspur Red Rome	793.5 a	37.34 c	30.36 cd	6.83 cd	18.58 d	1.76 c	1.83 c
Silverspur	571.9 de	35.92 cd	30.23 cd	6.07 def	20.86 c	2.41 abc	1.62 c
Starkrimson	638.8 abcde	32.21 de	32.98 c	5.88 def	22.89 b	2.04 c	1.49 c
Starkspur Supreme	768.8 ab	38.42 c	29.00 de	6.31 de	19.78 cd	2.15 c	1.57 c
Coop-17	580.1 Cde	38.22 c	36.47 b	6.55 cde	10.47 f	1.63 cd	2.57 b
Liberty	366.0 f	53.75 a	15.91 g	5.65 ef	15.93 e	3.30 a	2.87 ab
NY65707-19	610.1 bcde	48.27 b	26.23 ef	7.63 c	9.35 f	2.31 bc	2.83 ab
NY61356-22	553.9 e	32.30 de	27.22 ef	4.95 fg	26.72 a	1.91 c	1.51 c
Smoothie Golden	586.3 cde	48.13 b	25.03 f	14.58 a	5.34 g	1.76 c	3.19 a
Royal Gala	732.4 abcd	32.03 e	49.22 a	12.68 b	2.43 h	0.71 d	1.61 c

[z]Wax component means represent 12 fruit per cultivar and are listed as percent of total wax as recorded by gas chromatography.
[y]Wax mass means represent 24 fruit per cultivar.

Delicious strains had more octacosanol than Red Delicious strains. Tetracosanol ranged from 0.3% to 3.4% within the Golden Delicious strains, while none was detected in the Delicious strains. Octacosanol ranged from 1.7% to 2.7% among Golden Delicious strains with none in Delicious strains. The primary alcohol-4 was greatest in 'Pure Gold', with 3.2% and 1.3% in 'Starkspur Supreme'. Primary alcohol-4 (undetermined carbon number) was not observed in the other four cultivars. Primary alcohol-5 (undetermined carbon number) was observed in later experiments but not in Expt. 1.

Two secondary alcohols, detected and classified by TLC by comparison to standards and cross-checked by GC, ranged from 1.1% to 22.6% of total ECW (Table 3). The amount of secondary alcohols-1 and -2 were significantly greater for all three Delicious strains than for that of the three Golden Delicious strains (Table 4). Secondary alcohol-1 was most likely nonacosan-10-ol, (Rt = 11.59). Nonacosan-10-ol is a 29-carbon chain with an alcohol at the number-10 carbon. Because of the differences between cultivars, this component was very useful in distinguishing Golden Delicious strains from other cultivars.

Two compounds containing aldehydes were detected in minor concentrations. The largest amount was in 'Oregonspur' with 0.7% (Table 3). The only other cultivar with a detectable aldehyde was 'Sundalespur Gold', with 0.1%. One ketone was detected (0% to 1.7%), with no significant differences among cultivars (Table 3).

In Expt. 2, wax mass varied significantly among cultivars and strains (Table 5). The range of ECW mass was 366 $\mu g \cdot cm^{-2}$ for 'Liberty' to 793 $\mu g \cdot cm^{-2}$ for 'Red Rome'. There did not appear to be a cultivar pattern, with Golden Delicious and Delicious strains having wax mass values spread throughout the range. Interestingly, 'Liberty', a scab-resistant cultivar, had the least amount of ECW by mass.

Table 6. Compositional analysis of apple epicuticular wax by chemical family. Wax mass and wax component analysis means represent 20 fruit per cultivar in four replications of five pooled fruit samples. Waller–Duncan mean separation *t* test was within cultivar and across time. TI = time of harvest, 1 = 60 d before harvest (DBH), 2 = 30 DBH and 3 = commercial maturity.

Cultivar	Time[y]	Wax mass ($\mu g \cdot cm^{-2}$)	Wax component[z] (%) Ursolic acid	Alkanes	Primary alcohols	Secondary alcohols	Aldehydes	Ketones
Sundalespur	1	545.2 b	48.4[NS]	41.3[NS]	4.1 b	2.6 b	0.9 ab	2.0[NS]
Sundalespur	2	651.6 a	41.6	41.2	6.3 ab	4.0 a	1.5 a	4.4
Sundalespur	3	621.6 ab	42.9	42.8	8.8 a	2.3 b	0.7 b	1.6
Lys Golden	1	467.9 c	58.7[NS]	31.2[NS]	5.3 b	1.8 b	0.4[NS]	1.9[NS]
Lys Golden	2	663.7 a	56.4	32.0	7.1 b	1.9 b	0.6	0.9
Lys Golden	3	603.7 b	53.8	26.4	13.8 a	2.7 a	0.4	1.6
Pure Gold	1	520.7 b	64.9[NS]	24.7[NS]	5.2 b	1.7[NS]	0.4 a	2.0[NS]
Pure Gold	2	607.1 a	57.3	28.7	8.2 b	2.3	1.1 a	1.1
Pure Gold	3	599.4 a	56.3	22.1	14.7 a	2.3	0.3 a	2.2
Stark Red Rome	1	582.9 b	58.7[NS]	19.6[NS]	1.7 b	15.3[NS]	1.3 b	1.5[NS]
Stark Red Rome	2	722.7 a	49.3	20.7	3.0 a	14.0	2.7 a	7.4
Stark Red Rome	3	713.6 a	52.1	21.4	4.0 a	16.9	1.6 ab	1.9
Red Chief	1	545.2 b	52.8 a	24.2 b	1.0 c	18.8 ab	1.1 b	1.2[NS]
Red Chief	2	630.5 a	40.1 b	30.1 a	2.7 b	18.3 b	2.5 a	3.6
Red Chief	3	625.3 a	39.3 b	28.7 ab	3.7 a	22.1 a	1.8 ab	1.6
Silverspur Red Delicious	1	609.7[NS]	47.7[NS]	23.6[NS]	1.4 b	22.5[NS]	1.9[NS]	1.0[NS]
Silverspur Red Delicious	2	698.9	36.6	29.5	2.0 b	19.7	3.6	6.0
Silverspur Red Delicious	3	604.6	40.0	26.9	3.6 a	22.1	2.5	1.9

[z]Chemical families are listed as percent of total wax as recorded by gas chromatography.
[y]Time = time of harvest, 1 = 60 d before harvest (DBH), 2 = 30 DBH, and 3 = commercial maturity.

Table 7. Compositional analysis of apple epicuticular wax by component from Expt. 3. Waller–Duncan mean separation *t* test was across cultivars.

Cultivar	Time[y]	Wax component[z] (%) Alkane-1	Tetracosanol	Nonacosane	Hexacosanol	Secondary alcohol-1
Sundalespur	1	6.5[NS]	0.5 b	34.9[NS]	1.2 b	0.7[NS]
Sundalespur	2	5.2	1.0 ab	36.0	1.6 b	0.6
Sundalespur	3	6.1	1.4 a	36.8	3.3 a	0.4
Lys Golden	1	4.8[NS]	0.9 b	26.4[NS]	1.7 b	1.1 a
Lys Golden	2	3.9	1.4 b	28.1	2.2 b	0.3 b
Lys Golden	3	4.0	3.2 a	22.3	5.4 a	0.8 a
Pure Gold	1	4.5[NS]	1.7 b	20.2[NS]	1.7 b	0.8[NS]
Pure Gold	2	4.5	2.7 b	24.2	2.3 b	0.5
Pure Gold	3	3.4	5.5 a	18.8	5.3 a	0.6
Stark Red Rome	1	2.2 a	0.0[NS]	17.4[NS]	0.2 b	12.8[NS]
Stark Red Rome	2	1.5 b	0.1	19.2	0.4 ab	9.8
Stark Red Rome	3	1.7 ab	0.2	19.7	0.9 a	12.3
Red Chief	1	1.4[NS]	0.1 b	22.8 b	0.1 b	17.3[NS]
Red Chief	2	0.6	0.2 ab	29.5 a	0.5 b	14.6
Red Chief	3	0.6	0.5 a	28.1 a	1.0 a	17.2
Silverspur Red Delicious	1	0.5[NS]	0.2 ab	23.1[NS]	0.1 b	20.6 a
Silverspur Red Delicious	2	1.8	0.1 b	27.7	0.3 b	15.5 b
Silverspur Red Delicious	3	0.5	0.7 a	26.4	0.9 a	17.0 ab

Cultivar	Time[y]	Octacosanol	Aldehyde-1	Secondary alcohol-2	Primary alcohol-4	Primary alcohol-5
Sundalespur	1	1.1 b	0.5[NS]	2.0 b	0.9 b	0.5[NS]
Sundalespur	2	1.4 b	0.9	3.5 a	1.7 a	0.6
Sundalespur	3	2.5 a	0.4	1.9 b	1.1 b	0.4
Lys Golden	1	1.5 b	0.3[NS]	0.7 b	0.7 b	0.5[NS]
Lys Golden	2	1.9 b	0.4	1.6 a	1.3 a	0.3
Lys Golden	3	3.8 a	0.1	1.9 a	1.1 a	0.3
Pure Gold	1	1.2 b	0.1[NS]	0.8 b	0.5 b	0.0[NS]
Pure Gold	2	1.6 b	0.5	1.7 a	1.4 a	0.3
Pure Gold	3	2.9 a	0.1	1.7 ab	0.9 ab	0.0
Stark Red Rome	1	0.5 b	0.6[NS]	2.6 b	1.0[NS]	0.0[NS]
Stark Red Rome	2	1.1 a	1.6	4.2 a	1.4	0.0
Stark Red Rome	3	1.1 a	0.8	4.6 a	1.7	0.1
Red Chief	1	0.3 b	0.7[NS]	1.5 c	0.5 c	0.0[NS]
Red Chief	2	1.0 a	1.4	3.7 b	1.1 b	0.0
Red Chief	3	1.0 a	0.6	4.9 a	1.2 a	0.0
Silverspur Red Delicious	1	0.3 b	0.9[NS]	1.9 b	0.7[NS]	0.0[NS]
Silverspur Red Delicious	2	0.7 a	2.9	5.2 a	0.9	0.0
Silverspur Red Delicious	3	0.8 a	1.2	5.1 a	1.1	0.2

Cultivar	Time[y]	Aldehyde-2	Ketone	Ursolic acid-1	Ursolic acid-2
Sundalespur	1	0.4[NS]	2.0[NS]	15.5[NS]	32.9[NS]
Sundalespur	2	0.6	4.4	18.9	22.7
Sundalespur	3	0.3	1.6	14.4	28.5
Lys Golden	1	0.1[NS]	1.9[NS]	17.9[NS]	40.9[NS]
Lys Golden	2	0.3	0.9	16.9	39.5
Lys Golden	3	0.3	1.6	18.5	35.3
Pure Gold	1	0.2[NS]	2.0[NS]	19.7[NS]	45.2[NS]
Pure Gold	2	0.5	1.1	17.9	39.4 ab
Pure Gold	3	0.2	2.2	19.7	36.6 b
Stark Red Rome	1	0.7[NS]	1.5[NS]	14.3[NS]	44.4[NS]
Stark Red Rome	2	1.1	7.4	16.9	32.4
Stark Red Rome	3	0.9	1.9	16.3	35.8
Red Chief	1	0.4 b	1.0[NS]	14.1[NS]	38.7 a
Red Chief	2	1.1 a	3.6	9.3	30.8 b
Red Chief	3	1.1 a	1.6	12.8	26.4 b
Silverspur Red Delicious	1	1.0[NS]	1.2[NS]	13.2[NS]	34.5[NS]
Silverspur Red Delicious	2	0.7	6.0	17.7	18.9
Silverspur Red Delicious	3	1.3	1.9	13.3	26.7

[z]Wax component analysis means represent 20 fruit per cultivar in four replications of five pooled fruit samples and are listed in percent of total wax recorded by gas chromatography.
[y]Time = time of harvest, 1 = 60 d before harvest (DBH), 2 = 30 DBH, and 3 = commercial maturity.

Table 8. 'Pure Gold' and 'Starkspur Supreme' apples are listed with fruit wax component names and component means in percent. Variance components for fruit-to-fruit variation within each tree and the standard error of the mean are listed for within tree variation. Tree-to-tree variance components within cultivar of fruit wax components are listed along with the probability of a significant F value. Analysis was conducted on six fruit replications per tree, with three trees per cultivar.

Wax component	Mean (%)	Within-tree variation[z]	SE of the mean	Tree-to-tree variation[y]	$P > F$ tree to tree
			Pure Gold		
Ursolic acid	69.8	149.908	2.85	4.957	0.467
Hydrocarbons	18.9	10.201	0.88	5.403	0.036
Primary alcohols	9.1	8.916	0.67	0.975	0.714
Secondary alcohols	1.1	38.303	1.41	3.494	0.644
Aldehydes	0.0	15.825	0.99	2.775	0.163
Ketones	0.8	2.216	0.34	0.119	0.522
Mass[x]	989	64434	67.04	23335	0.071
			Starkspur Supreme		
Ursolic acid	34.6	49.579	1.76	8.771	0.162
Hydrocarbons	26.4	18.683	1.50	30.976	0.001
Primary alcohols	6.2	13.706	1.10	11.290	0.013
Secondary alcohols	22.3	2.671	0.67	7.756	0.000
Aldehydes	4.0	15.671	0.88	2.207	0.858
Ketones	1.9	0.993	0.22	0.124	0.783
Mass	946	18208	65.96	85148	0.001

[z]Within tree variance component = the mean square error term from the ANOVA table.
[y]Tree to tree variance component = [(mean square for tree) – (mean square error)]/number of fruit per tree.
[x]Wax mass units are micrograms/cm^2.

In Expt. 2, composition of waxes was reported by chemical family rather than by individual components. A table of the 14 individual components identified is not presented but can be found in Belding (1996). UA represented the largest component across all cultivars. The cultivar means for UA range from 32.0% for 'Royal Gala' to 53.8% of the ECW for 'Liberty' (Table 5). Primary alcohols were generally low: 4.4 ('Oregon spur') to 14.6 ('Smoothie Golden'). While 'Smoothie' and 'Royal Gala' had relatively large amounts of primary alcohols, they had significantly less secondary alcohols than other cultivars.

The concentrations of secondary-alcohol-containing compounds were consistent for a given cultivar, and there was a wide range of concentrations among cultivars. Statistically, the secondary-alcohol-containing compounds divided the cultivars into seven groups. The highest percentage of secondary-alcohol-containing compounds was found in 'NY61356-22' (26.7%), which followed the Delicious strains, all of which had concentrations >18.6% (Table 5). 'Liberty', 'NY65707-19', and 'Coop-17' were intermediate, ranging from 9.4% to 15.9%.

Aldehydes ranged from a low of 0.7% for 'Royal Gala' to 3.3% for 'Liberty'. Ketones ranged from 1.5% to 3.2% for 'Starkrimson' and 'Smoothie', respectively. There were significant increases in wax mass with time for five of the six cultivars in Expt. 3 (Table 6). In Expt. 3 , UA decreased in 'Red Chief' with time (Table 6).

There was no significant change in the concentrations of total alkanes with time (Table 6). Total alkanes ranged from a low of 19.6% in 'Red Rome' at 60 DBH to a high of 42.8% in 'Sundalespur' at maturity. The concentration of alkanes-1 was similar in all cultivars, ranging from 0.5% in 'Silverspur' to 6.5% in 'Sundalespur', both at 60 DBH (Table 7). Nonacosane remained constant in the six cultivars, except in 'Red Chief', where it increased from 22.8% at 60 DBH to 29.5% and 28.1% at 30 DBH and commercial maturity, respectively (Table 7).

Total primary-alcohol-containing compounds increased in concentration with time in each of the six cultivars (Table 6). Tetracosanol increased about three times in five of the six cultivars (Table 7). Hexacosanol and octacosanol significantly increased in concentration in all six cultivars over time (Table 7).

The concentration of total secondary alcohols significantly changed with time in only two cultivars. 'Lys Gold' increased from 1.8% to 2.7% and 'Red Chief' increased from 18.8% to 22.1%. The secondary alcohol concentrations in the Delicious strains were consistently greater than in the Golden Delicious strains. Secondary alcohol-1 did not change in any cultivar over time. Secondary alcohol-2 increased in concentration over time in four of the six cultivars. Aldehydes and ketones did not change significantly with time.

Variance components. In the first study, samples were replicated by sampling 15 fruit from each tree. Fruit were also sampled from three trees of each cultivar so that a fruit-to-fruit variance component and a tree-to-tree variance component could be defined.

'Pure Gold' had one wax component that was significantly different among trees ($F > 0.05$) and 'Starkspur Supreme' had four components significantly different among trees (Table 8). Despite large variance components, cultivars were still significantly different for each of the wax components. Cuticular waxes are used as biochemical markers for plant identification (Spicer 1989).

Ultrastructure. There were fundamental structural differences in wax ultrastructure among cultivars (Figs. 1–3). However, there was also variation in surface texture within each cultivar examined. For example, wax platelets of 'Granny Smith' appear upright and well defined when grown in the interior shade of a tree, but full sun diminished the distinctions and altered the structure to a less distinct topography (Fig. 1). 'Red Rome' had a unique parallel arrangement of platelets perpendicular to the surface (Fig. 2a); however, the adjacent surface might appear very smooth at similar magnification (Fig. 2b). In these tests in North Carolina, 'Lys Golden' appeared to be similar to 'Red Chief' at all magnifications (Figs. 3 and 4).

Discussion

GC separated apple cuticular waxes into 17 distinguishable compounds; 14 were identified as specific compounds or families

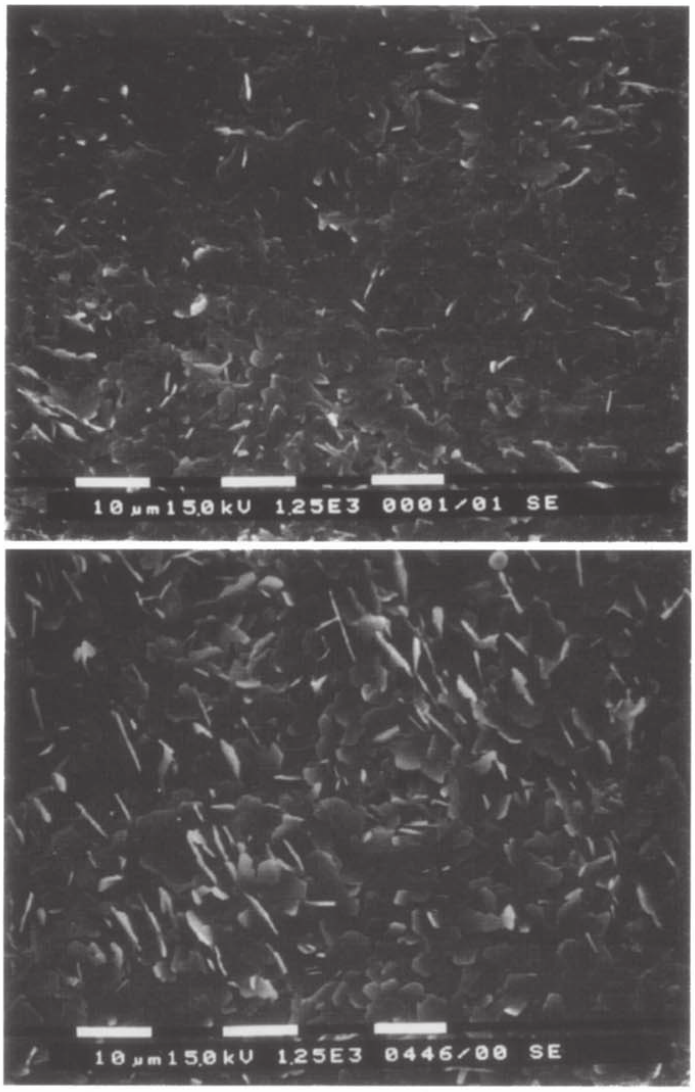

Fig. 1. Electron micrographs of epicuticular wax of 'Granny Smith' apples grown in either full sun (**top**) or partial shade (**bottom**). Bars = 10 µm.

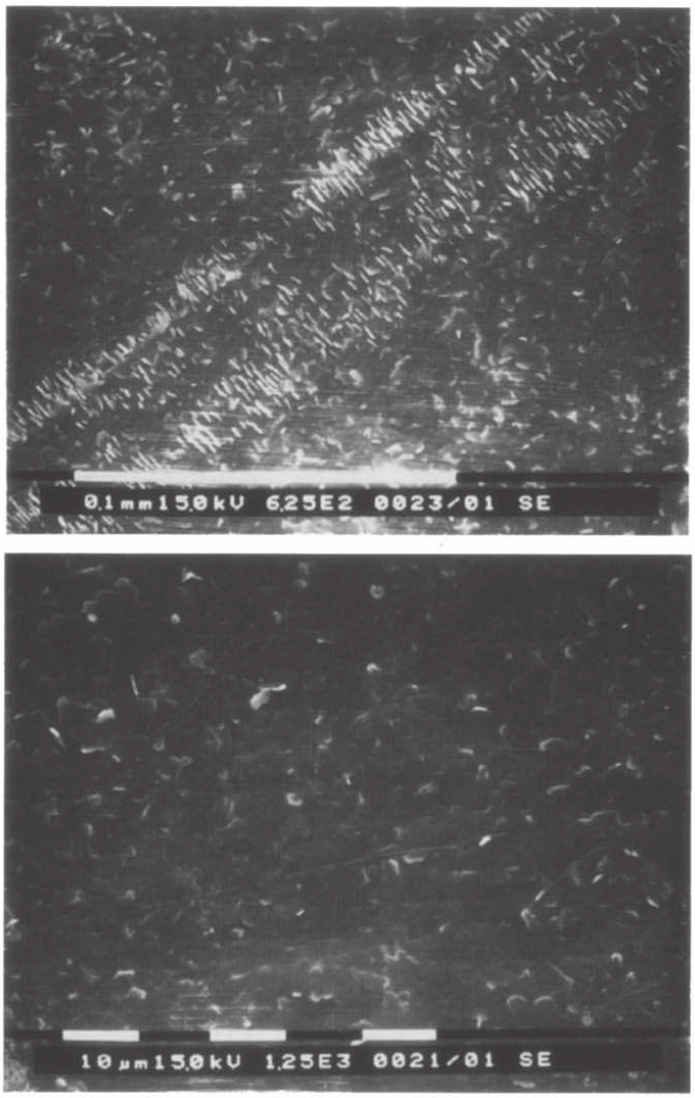

Fig. 2. Electron micrographs of epicuticular wax of 'Red Rome' apples. Bars = 0.1 mm (**top**) or 10 µm (**bottom**).

of identified compounds and represented 96% of the ECW. UA was represented by two GC peaks at 14.6 and 15.0 min. The GC–MS analysis showed these two peaks to be similar in composition and they might represent related isomers.

Common belief suggested that there existed a lack of ECW on Golden Delicious apples since they lose water more rapidly than other cultivars in storage. Wax mass of Golden Delicious cultivars and Delicious cultivars were similar in Expt. 1. Wax mass within Expts. 2 and 3 was significantly different by cultivar. Wax mass increased with fruit development from 60 to 30 DBH. Then, wax mass declined slightly on a per unit area basis from 30 DBH to commercial maturity. Wax either slowed in production or fruit expansion increased, which lead to the apparent decline of ECWs.

UA comprised the largest fraction, representing one-half to two-thirds of the total wax. The n-alkanes were next most abundant, accounting for one-fourth to one-third of the total wax amount. UA, n-alkanes, primary alcohols, secondary alcohols, aldehydes, and ketones were all significantly different as a percent of the total ECW by cultivar. The most distinctive pattern of component composition belonged to the secondary alcohols, which were found to be 20% of the total wax in many cultivars, including 'Delicious' and 'Rome', while comprising a low percentage of wax in the 'Golden Delicious' type apples, including 'Royal Gala'. While the compositional analysis did not turn up any compounds not previously observed on apple, the nature of field experiments allow the potential of contamination by exogenous compounds.

Since epicuticular wax composition varied significantly by cultivar, future work might focus on whether wax amount or composition affects the way plants interact with the environment and how the environment affects wax development. Fruit waxes may determine how fruit leach nutrients and retain moisture, therefore providing a guide to storage and shelf life. It is also conceivable that waxes, being so unreactive and having a low toxicity, may be manipulated genetically to enhance fruit storage and resistance to pathogens and surface disorders.

The chemical composition of ECW and the cuticular microstructure are known to affect wetability and possibly the deposition of chemicals. The chemistry of the wax was found to be related to its crystalline structure in studies of wax recrystalization (McWhorter et al., 1990).

'Red Rome' had the most diverse cuticular structure of the cultivars tested; however, wax composition was similar to other cultivars. This was in contrast to the theory that composition contributes to structure. 'Red Rome' did, however, contain one of the thickest wax deposits of the cultivars tested.

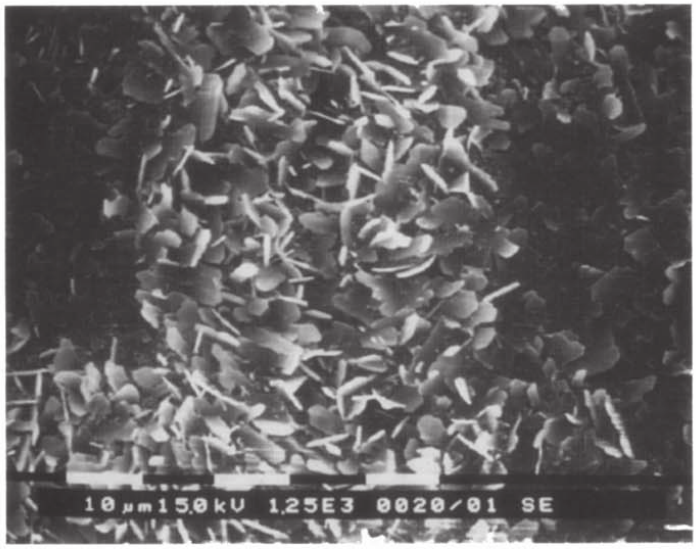

Fig. 3. Electron micrographs comparing high magnification (bars = 10 µm) of the epicuticular wax of 'Lys Golden' (**top**) and 'Red Chief' (**bottom**) apples.

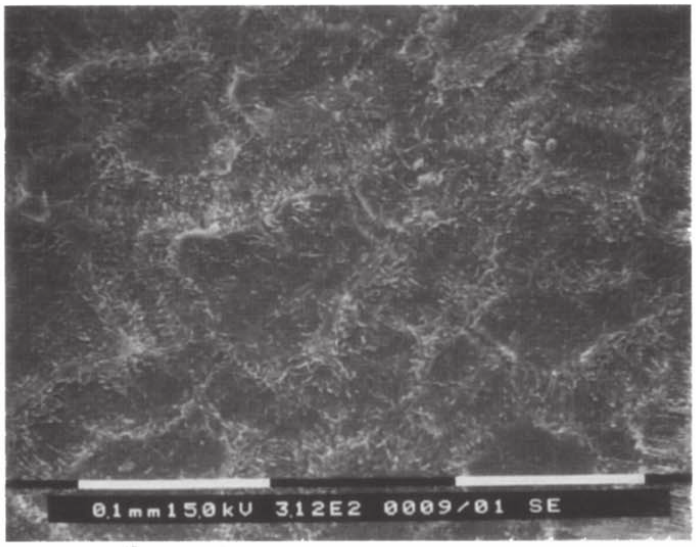

Fig. 4. Electron micrographs comparing low magnification (bars = 0.1 mm) of the epicuticular wax of 'Lys Golden' (**top**) and 'Red Chief' (**bottom**) apples.

It did not appear that the ultrastructure of the ECW produced in a hot, humid climate, such as North Carolina, lends itself to clear classification. Faust and Shear (1972) described the surface of eastern United States-grown 'Golden Delicious' as having a network of cracks early in the season and deepening with maturity. In the same study, western 'Golden Delicious' grown in an arid climate were remarkably free from cracks. In our study, 'Red Delicious' fruit appeared to have the same cracking structure as eastern 'Golden Delicious', but only later in the season, ≈130 d after bloom. There is evidence that the ultrastructure of the wax is strongly affected by environment (Faust and Shear, 1972). We can conclude by comparing these works that the environment in which the fruit are grown has a stronger influence on ultrastructure than the influence of wax composition.

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