This was determined by utilizing a GLM ANOVA, testing the hypothesis that all tissues in a grapefruit contained the same ppm of limonin vs. the alternate hypothesis that there was an inequality somewhere ($\alpha = 0.05$). A data transformation of log $(x + 1)$ was used to meet the basic assumptions of the model. Differences due to cultivar were partitioned out of the error term in order to test only the differences due to tissue, and the results showed that this was the correct model. The alternate hypothesis was accepted, and a SNK test was performed to locate the statistically significant differences.

The distribution of limonin within the fruit tissue of each cultivar was determined by performing a GLM ANOVA [data transformation of log $(x + 1)$ and $\alpha = 0.05$], testing the hypothesis that all tissues of that cultivar had the same ppm of limonin levels vs. the alternate hypothesis that there was an inequality somewhere. For all cultivars, the alternate hypothesis was accepted, and a SNK test was performed to locate the statistically significant differences. The ranking of the various tissues from each cultivar is presented in Table V.

In all the cultivars studied the highest concentration of limonin was found in the cotyledons and the lowest was in the juice vesicles. With few exceptions the distribution pattern was similar in the other tissues; thus it would appear that the distribution factor showed a similar and quite unifirm pattern of limonin content for all cultivars. This distribution pattern was similar to that already reported for grapefruit (Mansell and Weiler, 1980), and it would tend to support the theory that citrus seeds are the major depository for limonin. If this is indeed the case, then it will be important to determine whether there is a correlation between the number of seeds in a fruit and the percentage of the total limonin found in those seeds. In other words, fruit with fewer seeds would possibly have a greater percentage of the total limonin in the fleshy fruit parts and heavy seeded varieties a lesser percentage. It would also be interesting to determine whether the seeds of "seedless" varieties have more limonin per seed than "seedy" varieties simply because there are fewer storage structures. In future studies it will be important to do a complete three-dimensional analysis of individual fruit from the same branch on a single tree and then compare the results of similar analyses for each of the cultivars.

From the results presented in this study it is clear that a very complex pattern of variation exists with regard to the limonin content in grapefruit. The intrafruit variation pattern suggests that there is a strong distribution factor which results in a wide variation in limonin content even within a single tissue or a single fruit. This variation is somewhat based upon the specific location from which the tissue is sampled.

The intracultivar variation pattern revealed that there is a large variation between the fruit of a given tree, and since the fruit were sampled at random, it cannot be determined whether there is a positional effect, that is, whether the actual location plays no role in determining the final limonin content.

In a related study which has just been comleted in our laboratory (McIntosh and Mansell, 1982), it was found that although the amount of limonin in a single leaf is representative of the branch it was taken from, there was a wide variation in the ppm of the flushing leaves throughout a single tree. This means that each branch is unique in its limonin-producing potential and it might be that the final limonin content in a given fruit is a function of the branch from which it was taken. In this regard it would then be important to test the variation between fruit clusters of a single branch against the fruit of neighboring branches.

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Leaf Hydrocarbons in the Genus *Citrus*

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The composition of whole leaf alkanes for 71 *Citrus* and related biotypes has been determined. No biosystematically consistent grouping was found, and we propose that leaf alkanes seem to function as a noncritical sealing agent in the leaf cuticle.

Long-chain alkanes are believed to be chemically stable terminal products from a sequence of reactions. Their external deposition makes it unlikely that they are part of an active metabolic pool. Since they are ubiquitous in the plant kingdom, their study has elicited much interest in biosystematic investigations.

Early workers (Francis et al., 1930; Malkin, 1930; Garner et al., 1931; Piper et al., 1931; Pollard et al., 1931, 1933; Sahai and Chibnall, 1932) suggested that the *n*-paraffins were made up exclusively of the odd-numbered members of the series, but later work by Waldron et al. (1961) showed that even-numbered members are present as minor

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components and that isoparaffins are also present.

Eglinton et al. (1962) concluded that despite slight seasonal and regional variation, the alkane distribution pattern could be regarded as a species characteristic. However, no generalizations were apparent which would permit (a) monocotyledons to be distinguished from dicotyledons, (b) assignment of a species to a given family, and (c) assignment of species to a particular genus.

Dyson and Herbin (1968) studied the alkane components of *Cupressus* leaf waxes and showed that in east Africa individual trees and species have highly characteristic minor alkane patterns. Species were diagnosed, and hybrids had alkane patterns intermediate those of the parent species.

Hunter and Brogden (1966) reported that a preponderance of low carbon number paraffins and isoparaffins (C_{21} to C_{29} with C_{23} and C_{25} normal and isoalkanes in greatest abundance) present in cold pressed orange oil are characteristic of the orange in the plant kingdom. Herbin and Robins (1968) found that the alkanes which formed only a minor proportion of the leaf waxes were not a useful guide in the genera *Cupressus*, *Juniperus*, *Callitris*, and *Wildringtonia* of Cupressaceae and in the genus *Pinus*.

In 1969 Herbin and Robins found that the dominance of odd over even carbon number chain length tends to disappear when the *n*-alkanes form only a small percentage of the cuticular wax. Alkanes extracted from within the leaf as opposed to cuticular alkanes lack this alternation in chain length. These internal lipid hydrocarbons have a different composition from the external wax hydrocarbons. They found further that alkane production continued throughout the life of the leaf in *Solandra grandiflora* with the chain length increasing with age.

Kolattukudy and co-workers (Kolattukudy, 1970; Kolattukudy and Walton, 1973) reported on the biochemistry of plant wax components. He proposed that in only the epidermal layer of cells, fatty acids that are synthesized in the chloroplast from C_{10} to C_{18} are elongated by the addition of acetate to form the higher acids. These in turn are reductively decarboxylated to give the corresponding alkanes with one carbon less.

In 1972 Calabro and Curro (1972) reported on the paraffins found in the Bergamot orange rind oil. Long-chain hydrocarbons have been isolated from the juice sacs of citrus fruits by Nordby and Nagy (1974). They found that the hydrocarbon profiles from citrus juice sacs are specific for each species and some hybrids; e.g., tangors have a profile that is intermediate between that of the assumed parents. These waxes are synthesized in the juice sacs and extruded to the exterior where they act as the adhesive agent between the invidual sacs (Shomer et al., 1980).

Haas and Schoenherr (1979) investigated the composition of soluble cuticular lipids and water permeability of cuticular membranes from the leaves of a greenhouse grown *Citrus aurantium* tree. No relationship between the composition of soluble cuticular lipids and water permeability was found.

Baker et al. (1975), investigating the composition of leaf and fruit waxes in four *Citrus* biotypes, found the leaf wax deposits consistent during all, except the most juvenile, stages of growth, while the fruit surface waxes showed considerable variation with fruit size and contained in addition to hydrocarbons, primary alcohols, and fatty acids of the leaf waxes also aldehydes as major components. In general, leaf waxes contained a larger proportion of the longer chain constituents and were particularly rich in the C_{31}–C_{33} homologues.

The hydrocarbons from the epicuticular waxes of citrus peels from eight taxa were examined by Nordby and Nagy (1977).

A study on the relationship of rootstock to leaf and juice lipids in *Citrus* showed a small but definite effect on the leaf alkane profile with only a small effect on the alkane patterns from the juice sacs (Nordby et al., 1979a). These authors studied the profiles of 67 *Citrus* cultivars and showed the usefulness of leaf alkanes in the chemotaxonomy of *Citrus* (Nordby et al., 1979b). They showed that alkane profiles changed during the year but were not influenced by the period of spring and fall leaf flush.

We report that our observations on the leaf alkanes from 71 different *Citrus* biotypes show a high degree of similarity, suggesting that the leaf alkane profiles appear to be much less useful than juice sac profiles for taxonomic purposes.

EXPERIMENTAL SECTION

Healthy mature leaves from the previous spring flush were randomly harvested from each tree in October and November of 1975. All trees were grown in the Variety Collection of the Citrus Research Center at Riverside, CA. The trees were sprayed on Sept 12–16 with a narrow-range oil in water (1.25:100) containing a wetting agent and zinc sulfate. No rainfall occurred before the leaves were harvested. The leaves were rinsed in distilled water and dried in a forced-air oven at 27 °C. The amount of leaves extracted ranged from 300 to 900 g dry weight depending upon availability, except for *Poncirus trifoliata*, where 172 g were used.

Petroleum either (30–60 °C), 7.5 L, was passed through the coarsely milled leaf powder in a covered 4-L funnel. The solvent was separated with a Buchi Rotavapor, and the residue was dissolved in a minimum of hot acetone. The hot solution was decolorized with activted charcoal and filtered through super-cel in a preheated Büchner funnel. It was refiltered as necessary until the solution was clear and then cooled in a freezer. All the solids that precipitated were collected by filtration through a cold Büchner funnel. The infrared spectra obtained from some of these were indicative of alkanes. A representative sample of the solid alkanes was dissolved in a minimum of warm isoctane, and 25 μL was injected into a Varian Aerograph series 1520 C gas chromatograph with a thermal conductivity detector.

The oven temperature was programmed from 100 to 340 °C at 2 °C/min with a 304.8 cm long by 0.64 cm o.d. stainless steel column packed with 10% SE 30 on 80–100-mesh Gas-Chrom Q.

RESULTS AND DISCUSSION

Freeman et al. (1979) reported on the changes in the cuticular waxes of developing citrus leaves. The paraffin content usually reached a peak around June and July and then declined and remained reasonably constant during October and November when the leaves for this study were harvested. They reported that the paraffins were generally the second largest fraction in citrus leaf waxes.

The compositions of the various *Citrus* alkanes in our study show a great degree of similarity. The usual observation that the odd carbon numbered molecules are present in higher concentration is observed. However, one observes significantly greater amounts of the even carbon numbered molecules than one would expect from the essential absence of odd carbon numbered precursor fatty acids. We might expect that mixing in the even-numbered carbon chains with the odd-numbered members would cause greater defects in the crystal lattice and render the solid state of the mixture more plastic.

Table I. Composition of Hydrocarbons in Percent[a]

alkane chain length	24	25	26	27	28	29	30	31	32	33	34	35
M. warburgiana (F. M. Bail.) Tan.	0.6	1.3	2.6	7.6	4.1	17.9	7.3	35.6	7.9	11.3	1.9	1.7
P. trifoliata (L.) Raf.	2.2	1.1	0.7	0.7	2.8	29.2	5.3	34.8	7.5	13.3	1.1	1.2
F. obovata Tan.	2.0	1.1	1.2	1.6	1.9	7.2	6.3	38.3	11.0	21.3	4.5	3.5
C. hystrix D.C.	1.1	1.5	1.9	4.5	2.8	8.4	6.7	35.7	12.4	20.3	2.4	2.2
C. macroptera Montr.	0.3	1.0	1.0	2.2	2.9	16.5	7.1	40.5	9.4	14.8	2.5	1.7
C. celebica Koord.	2.5	2.5	2.8	3.8	3.2	9.2	5.6	32.6	12.1	20.6	2.6	2.6
C. latipes Tan.	0.4	0.5	0.8	4.9	4.3	23.4	5.7	35.4	7.6	14.6	1.1	1.3
C. ichangensis Swing.	2.2	1.0	0.8	2.2	1.1	10.3	3.2	38.8	8.2	26.9	3.4	1.8
C. sudachi Hort. ex Sh.	1.0	2.1	1.3	5.8	2.5	26.5	5.3	32.0	5.4	11.9	2.3	3.9
C. hanaju Hort. ex Sh.	2.4	2.5	1.6	4.6	2.8	12.6	4.6	33.6	7.2	17.9	3.1	6.7
C. aurantifolia (C.H.S.) Sw. aggregate												
Mexican lime	2.0	1.6	1.4	1.7	0.9	6.4	2.9	41.2	9.8	27.8	1.7	2.5
thornless Mexican lime	0.2	2.1	0.9	6.9	1.7	13.0	3.9	35.0	9.3	22.9	1.2	2.8
Indian lime	0.5	0.2	0.6	0.5	0.6	5.0	3.0	48.2	8.6	30.2	1.2	1.1
Egyptian lime	0.3	0.2	0.7	2.3	2.2	11.8	7.4	45.8	10.2	15.2	2.2	1.6
Bearss lime	0.2	0.3	0.3	1.6	1.5	6.1	4.9	45.0	11.8	24.3	2.1	2.0
Palestinian sweet lime	0.1	0.8	0.9	2.2	2.1	6.7	4.5	37.5	13.8	27.5	1.3	2.5
sweet lime	0.3	0.7	2.1	2.6	2.4	7.3	5.6	36.5	11.8	23.7	2.4	4.5
C. excelsa West.	0.5	0.9	0.9	1.2	1.4	4.2	4.2	38.2	13.4	30.4	2.8	1.7
C. macrophylla West.	0.2	0.2	0.9	3.1	3.2	15.7	9.2	39.0	10.1	15.0	1.8	1.5
C. limon (L.) Burm. aggregate												
Lisbon	1.4	1.3	1.3	4.2	1.3	11.5	5.3	42.7	9.6	18.3	1.3	1.8
Eureka	2.0	1.6	1.0	3.9	2.2	13.4	4.6	41.6	7.7	18.5	1.6	1.6
Iran sweet lemon	3.3	1.3	1.2	3.3	2.3	12.1	5.3	38.2	9.7	16.7	3.9	2.7
Dorshapo sweet lemon	1.3	1.7	1.4	2.4	2.1	7.3	4.7	38.6	12.9	23.4	2.3	2.1
C. meyeri Y. Tan.	1.9	1.5	1.2	1.9	0.8	8.4	3.1	43.4	9.8	22.8	3.2	2.0
C. jambhiri Lush.												
Indian	1.5	1.2	1.1	1.4	1.2	6.0	3.3	38.9	9.4	29.0	2.6	4.4
Florida	1.4	1.1	0.7	1.5	1.3	5.6	3.4	39.7	9.2	31.1	2.5	2.6
C. megalopsicarpa Lush.	1.4	1.4	1.4	2.4	3.8	7.4	6.0	33.8	14.1	23.5	2.2	2.1
C. assamensis D. et Bhat.	0.4	1.4	1.9	5.5	5.3	18.3	7.2	34.5	8.7	13.9	2.5	0.4
C. karna Raf.	0.3	0.3	1.3	1.6	2.6	8.7	6.4	42.5	9.7	18.1	2.9	5.6
C. medica L.	0.3	0.4	0.5	1.5	1.2	6.7	4.7	43.5	12.4	26.4	1.2	1.1
Corsican citron	0.4	0.5	0.9	1.9	1.9	10.1	8.0	40.4	13.3	19.5	0.8	2.3
C. grandis (L.) Osb. aggregate												
Kao Pan	0.8	1.4	2.1	3.6	4.6	8.9	8.0	29.1	15.0	20.6	3.0	2.9
Siamese	1.7	1.6	1.4	3.6	3.5	10.0	6.2	33.1	11.1	21.6	3.1	3.0
acidless	0.3	0.3	0.5	1.3	2.5	9.5	6.9	34.6	17.5	22.0	2.9	1.7
unnamed	0.3	0.7	1.2	2.4	3.0	12.3	6.8	40.5	10.5	16.2	2.0	4.1
C. pseudograndis Hort. ex Tan.	0.1	0.2	0.5	1.2	1.8	6.6	5.3	35.9	14.3	28.7	2.4	3.0
C. asahican Hort. ex Tan.	1.0	0.7	1.0	3.8	2.1	11.1	4.4	39.4	8.9	22.0	1.6	4.0
C. paradisi Macf.												
Marsh	0.4	0.5	0.4	1.4	1.9	10.1	5.8	40.8	11.2	22.4	2.7	2.5
Duncan	0.2	0.4	0.7	0.8	3.2	9.0	5.5	38.1	12.6	23.4	2.6	3.5
C. glaberrima Hort. ex Tan.	0.5	0.6	0.9	1.3	2.3	10.7	5.7	39.6	11.1	22.1	1.5	3.6
C. natsudaidai Hay.	0.1	0.7	0.5	3.2	1.5	9.0	6.2	39.1	11.7	20.6	2.8	4.4
C. sinensis (L.) Osb. aggregate												
Bahia Navel	2.0	1.7	1.2	4.1	2.0	17.7	4.4	33.9	7.2	19.1	3.9	2.8
Valencia	1.0	1.2	1.3	3.9	2.5	21.0	6.3	36.7	8.2	11.7	3.9	2.7
Shamouti	1.1	1.4	1.3	4.7	4.1	16.7	7.7	32.6	10.3	14.7	3.1	2.2
Torocco	0.5	0.4	0.2	2.8	1.0	20.9	4.3	43.8	6.0	14.2	2.1	3.2
C. funadoco Hort. ex Y. Tan.	0.2	0.2	0.2	1.0	1.1	12.6	5.4	48.3	9.7	16.5	2.6	2.0
C. iyo Hort. ex Tan.	0.7	0.4	1.5	4.1	2.8	21.6	6.8	38.0	7.2	13.8	1.2	1.8
C. sinograndis Hort. ex Tan.	0.5	0.6	3.5	2.8	3.8	10.6	7.8	28.7	12.6	20.5	3.9	4.3
C. aurantium L. aggregate												
var. salicifolia	6.7	3.1	2.1	5.1	3.9	10.5	6.2	26.3	10.5	19.5	1.8	4.2
standard sour	1.8	2.8	1.6	2.0	1.8	10.4	5.3	37.7	11.3	19.4	3.2	2.6
Seville sour	2.3	1.9	1.4	2.6	2.2	11.7	5.3	36.9	9.9	18.9	2.3	4.6
C. rokugatsu Hort. ex Y. Tan.	0.7	1.0	1.0	3.2	2.5	26.5	5.6	37.4	5.1	13.0	1.4	2.8
C. myrtifolia Raf.	0.2	0.8	0.7	2.1	1.7	11.8	5.3	40.7	10.9	19.5	4.1	1.9
C. canaliculata Hort. ex Y. Tan.	1.1	1.7	2.3	6.0	3.5	18.9	7.3	21.1	4.1	26.1	4.6	2.8
C. taiwanica Tan. et Sh.	0.2	0.2	0.2	1.9	1.9	15.0	5.7	51.1	6.4	14.8	0.6	2.2
C. reticulata Blanco aggregate												
Dancy	0.7	3.2	1.7	7.2	2.2	14.7	5.6	34.9	9.3	19.0	0.6	0.7
Willowleaf	0.7	0.6	1.7	4.2	4.1	11.6	7.2	27.9	13.5	17.7	4.4	5.5
Cleopatra	0.9	2.0	1.1	5.5	2.8	19.9	6.6	32.6	18.5	11.6	3.9	4.4
Owari	0.7	2.1	1.1	7.9	3.7	23.5	5.7	29.1	5.8	15.7	0.9	3.8
C. clementina Hort. ex Tan.	1.0	0.9	0.7	2.7	1.5	8.5	4.0	39.0	10.5	23.7	3.6	3.9
C. nobilis And.	0.3	0.8	2.1	3.8	3.2	15.0	6.5	48.3	6.6	11.5	0.9	1.2
C. benikoji Hort. ex Tan.	0.8	1.8	1.9	2.8	2.6	14.4	7.1	38.2	9.8	14.9	2.8	2.8
C. succosa Hort. ex Tan.	0.8	1.3	2.7	5.2	4.2	12.8	7.3	27.5	11.6	21.5	2.0	3.1
C. tachibana (Mack.) Tan.	1.5	1.5	1.4	4.3	2.3	10.0	6.8	39.5	10.1	17.7	2.7	2.1
C. sunki Hort. ex Tan.	1.1	1.3	1.1	7.2	3.6	23.8	5.8	29.5	5.9	16.0	0.9	3.8
C. depressa Hay.	0.3	1.2	1.9	5.6	4.2	34.0	6.8	23.7	4.4	11.6	3.0	3.1
C. amblycarpa Och.	0.9	1.6	1.4	4.8	3.5	15.3	7.0	35.8	9.5	14.8	2.7	2.7
C. mitis Blanco	1.4	1.0	0.8	2.6	1.5	13.5	5.3	38.7	10.0	16.3	4.6	4.3

Table I (*Continued*)

alkane chain length	24	25	26	27	28	29	30	31	32	33	34	35
C. limonia Osb.												
Kusaie	0.3	0.7	0.8	2.4	1.8	14.5	5.7	41.6	8.0	18.5	2.6	3.1
Bakrai	0.2	0.8	1.1	2.8	2.2	11.0	5.2	40.8	9.6	17.1	3.8	5.3
Otaheite	0.3	0.7	0.6	1.8	1.7	10.5	4.3	44.0	9.2	21.9	3.6	1.2

[a] *M., Microcitrus; P., Poncirus; F., Fortunella; C., Citrus.*

The leaf alkanes from the inedible subgenus *Papeda* as well as the leaf alkanes from the related genera *Fortunella*, *Poncirus*, and *Microcitrus* all have a similar composition of the *Citrus* leaf alkanes. The tropical varieties appear to have a similar composition as the subtropical varieties.

The changes in the composition of *Citrus* cuticular leaf waxes that accompany development have been studied by Nordby et al. (1979a). A general trend appeared where the lower alkanes in the range C_{20} to C_{27} are the highest in the immature leaves and diminish with aging, while C_{31} becomes the largest single component in all the mature dark green leaves. The values in this report for whole leaf hydrocarbons for Valencia orange and Marsh and Duncan grapefruits appear to fall between the medium, light green immature stage and the large, dark green mature stage of development. Their value of C_{33} for Dancy mandarin harvested in October at 19.8% is similar to our values of 18.9% (Table I).

In general values for C_{31} in Florida are higher than those in California. However, no generalization with regard to the latitude, climate, or geography or origin of different biotypes is apparent. We believe that the waxes are stable end products in the leaves and therefore presume that the changes in wax composition with age are due to the change in additional material that is deposited as the leaf matures. The major bulk of the hydrocarbons (76–95%) in *Citrus* leaf waxes are present in the narrow range, C_{29} to C_{33}, and many individual differences can be seen. This behavior was reported earlier for the 10 biotypes studied by Nordby et al. (1979b). Although individuals have characteristic alkane compositions, we were disappointed to find that whole leaf alkanes in *Citrus* did not reveal any useful taxonomic groupings.

C. depressa Hay at 34.0% and *P. trifoliata* (L.) Raf. at 29.2% have the highest C_{29} fraction and *C. excelsa* West has the lowest at 4.2%. *C. grandis* (L.) Osb. var. Kao Pan has the highest C_{30} at 8.0% with Mexican lime, *C. aurantifolia* (C.H.S.) Sw., at 2.9% the lowest. *C. taiwanica* Tan. et Sh. has the highest C_{31} at 51.1% and *C. canaliculata* Hort. ex y. Tan. is the lowest at 21.1%. With only two exceptions, *C. canaliculata* and *C. depressa*, C_{31} is the largest single component for all the leaves examined. Cleopatra mandarin, *C. reticulata* Blanco, has the highest C_{32} at 18.5%, and *C. rokugatsu* Hort. ex Y. Tan. has the lowest at 5.1%. Florida rough lemon, *C. jambhiri* Lush., has the highest C_{33} at 29.0% with *C. nobilis* And. at 11.5% and *Microcitrus warburgiana* (F. M. Bail.) Tan. at 11.3% as the lowest.

It appears that the evolution of the elongation–deposition mechanism has reached a stable stage where small changes no longer have any survival significance and fall into the category of random neutral changes. Kolattukudy and Walton (1973) summarized the finding of the investigations of spontaneous and induced mutations in wax synthesis. In general, when synthesis of a particular wax component was apparently blocked, no obvious precursor accumulated. Instead some other component, presumably from a common precursor, accumulated.

We propose that the evolutionarily significant changes occur in the other components of the cuticle and the long-chain alkanes probably function as a noncritical space-filling sealing agent.

LITERATURE CITED

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