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COMPOSITION AND INHERITANCE OF FLAVANONES IN CITRUS FRUIT

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Abstract—Early work on the chemosystematics of citrus is briefly reviewed. A short summary of the confusion existing in citrus taxonomy is given with special reference to the source of nomenclatural errors which have appeared in the chemical literature. A tabulation is given of literature citations, through 1966, of flavanone compounds reported to occur in various *Citrus* species. A thin-layer chromatographic survey was made of 41 citrus varieties representing 18 recognized species of citrus. In addition, 49 hybrids of 18 different crosses were also surveyed. An analysis of the data has shown that a qualitative and quantitative consistency of flavanone composition, with minor variations, is characteristic of individual *Citrus* species and crosses. Rules governing the inheritance of citrus flavanones were deduced from the composition of known hybrids. These rules, coupled with compositional data, were used to evaluate the probable relationships of various citrus varieties and species. The use and limitations of flavanone composition in citrus taxonomy and breeding are discussed briefly.

INTRODUCTION

SWINGLE,[1] in his comprehensive work on citrus taxonomy, recognized the possible usefulness of a knowledge of flavonoid composition in serving as an aid in making taxonomic decisions. He comments that "the nature of the characteristic glucoside [*sic*] present in the tissues of a species of *Citrus* may be of definite taxonomic significance in distinguishing that species from another species to which it may have superficial resemblances." Swingle cites the then understood differences in flavonoid composition between the sweet and sour orange as a part of the argument in favor of naming them as distinct species, respectively, *Citrus sinensis* and *C. aurantium*. Swingle also noted the presence of naringin in *C. grandis* as being "an important chemical difference" which in addition to morphological differences serves to make *C. grandis* one of the most distinct species of *Citrus*.

As knowledge of citrus flavonoids expanded, it became apparent to Kefford[2] that citrus fruits contained a much more complex mixture of flavonoids than was formerly suspected. Kefford grouped some of the citrus fruit (sweet oranges, mandarins, lemons and citrons) together due to the presence of hesperidin as their principal flavonoid; and others (pummelo and grapefruit) in a group where naringin was the principal flavonoid. He considered the presence of both hesperidin and naringin in the Natsudaidai (a suspected mandarin-pummelo hybrid) to be significant as it represented a possible combination of the two groups.

Horowitz,[3] in discussing the occurrence of flavanone rutinosides and neohesperidosides in citrus, was led to conclude that it was likely that most citrus species contained either all

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[1] W. T. SWINGLE, in *The Citrus Industry* (edited by H. J. WEBBER and L. D. BATCHELOR), Vol. 1, pp. 129–474. University of California Press, Berkeley (1943).

[2] J. F. KEFFORD, *Advan. Food Res.* **9**, 285 (1959).

[3] R. M. HOROWITZ, in *Symposium on Biochemistry of Plant Phenolic Substances* (edited by G. JOHNSON and T. A. GEISSMAN), pp. 1–8, Colorado State University, Fort Collins, Colorado (1961).

rutinose derivatives (lemons, oranges, citrons, tangerines) or all neohesperidose derivatives (grapefruit, pummelos, Ponderosa lemons, trifoliate oranges). He considered that at that time (1961), *C. aurantium* was the only well-authenticated example of a fruit that contains both classes of glycosides. He recognized the importance of substantiating these findings to aid in helping establish a chemical taxonomy of citrus.

Although Swingle, Kefford, and Horowitz were the first to relate the occurrence of specific flavonoid compounds to different taxa of citrus, other authors, by using color reactions, have employed less precise chemical differences to aid in characterizing species. Judging from the nature of the reagents they employed and the colors produced, it is possible that flavonoid components in the tissue under test could have contributed to the distinctiveness of the resultant color. Thus Selle[4] found that extracts of different citrus rootstocks gave distinctive patterns when a drop, mixed with $AlCl_3$, is dried on filter paper and viewed in u.v. light. Color tests with $FeCl_3$, $(NH_4)_2MoO_4$, or Almen reagent were conducted by Krishnamurthy *et al.*[5] on bark tissue from eight species of citrus. It was possible to differentiate between such similar species as *C. limon* and *C. jambhiri* (not given species status by Swingle), and between *C. aurantifolia* and *C. limettioides* Tanaka (not given species status by Swingle), and between *C. grandis* and *C. paradisi*. Swingle considers the second member of each aforenamed pair to be a possible hybrid, with the first member of the pair as one parent. Colorimetric tests have been employed by others,[6–9] to distinguish citrus species.

Despite the early recognition of the possible usefulness of flavonoids as taxonomic markers in citrus, no systematic study on a wide selection of citrus species and varieties has been made. However, some of the non-flavonoid components of citrus have been investigated for their relationship to taxonomic classification. Scora and co-workers[10, 11] have investigated the possibility of using gas chromatographic analyses of steam-distilled essential oil from various organs of citrus to aid in classification and identifying taxa. Pieringer *et al.*[12] and Kesterson *et al.*[13] found gas chromatography and i.r. and u.v. spectrophotometry useful in correlating steam-distilled leaf oils with the identification of citrus species. Mackinney[14] has attempted to differentiate between *C. aurantium*, *C. sinensis* and *C. reticulata* by reference to their carotene composition. Singh and Schroeder[8, 9] have employed rootstock susceptibility or tolerance to virus infection as an aid in distinguishing *Citrus* species.

Citrus Taxonomy

Students of citrus taxonomy have been challenged with one of the most perplexing and controversial areas of study to confront a taxonomist. Accidental hybridization and preservation of progeny through nucellar embryony has led to the preservation of hybrid populations which were both geographically and morphologically remote from parental types. This has resulted in the elevation of hybrid progeny to species standing. Morphological characteristics

[4] R. M. SELLE, *Nature* **181**, 506 (1958).
[5] S. KRISHNAMURTHY, R. SINGH and P. V. DEO, *Indian J. Hort.* **17**, 107 (1960).
[6] J. R. FURR, P. C. REECE and G. HRNCIAR, *Proc. Florida State Hort. Soc.* **59**, 38 (1946).
[7] F. F. HALMA and A. R. C. HASS, *Plant Physiol.* **4**, 265 (1929).
[8] D. SINGH and C. A. SCHROEDER, *Proc. Am. Soc. Hort. Sci.* **80**, 291 (1962).
[9] D. SINGH and C. A. SCHROEDER, *Proc. Am. Soc. Hort. Sci.* **80**, 296 (1962).
[10] R. W. SCORA and S. TORRISI, *Proc. Am. Soc. Hort. Sci.* **88**, 262 (1966).
[11] R. W. SCORA, A. B. ENGLAND and W. P. BITTERS, *Phytochem.* **5**, 1139 (1966).
[12] A. P. PIERINGER, G. J. EDWARDS and R. W. WOLFORD, *Proc. Am. Soc. Hort. Sci.* **84**, 204 (1964).
[13] J. W. KESTERSON, A. P. PIERINGER, G. J. EDWARDS and R. HENDRICKSON, *Proc. Am. Soc. Hort. Sci.* **84**, 199 (1964).
[14] G. MACKINNEY, in *The Orange, Its Biochemistry and Physiology* (edited by W. B. SINCLAIR), pp. 302–333, University of California Press, Berkeley (1961).

alone are often not sufficient to identify parent–progeny relationships. Experimental breeding studies are frustrated by nucellar embryony, incompatibility, and self-sterility, as well as the limitations of time, space, and availability of funds necessary to support a lengthy breeding program. However, for the more economically important species of citrus, such breeding programs[15,16] have been undertaken, and have yielded valuable information concerning the taxonomic relationships of citrus.

Two modern "systems" of citrus taxonomy have been published. The most widely cited system is that of Swingle,[1] published in 1943. The other is that of Tanaka[17,18] whose most recent compilation was published in 1961. Hodgson[19] has summarized and criticized these two systems and has suggested the use of an intermediate system for the genus *Citrus* consisting of 36 species instead of the 16 recognized by Swingle or the 157 proposed by Tanaka.

Swingle believed that those forms which exhibit morphological characteristics of known species should be proven to be non-hybrids before being recognized as species. This policy has the advantage of possibily minimizing future modifications in a taxonomic system as new knowledge is accumulated. Although the assignment of a specific epithet to known interspecific hybrids is provided for by the rules of botanical nomenclature, this does not make them a species in the biological sense. Since the parentage of most long-established, suspected citrus hybrids is speculative, these hybrids have not been given a collective specific epithet. Swingle is somewhat inconsistent in that he speculates about the possible hybridity of *C. limon* and *C. paradisi*, yet still assigns them species status, while others are denied even a collective specific epithet when hybridity is considered a possibility. He identifies most forms of hybrid nature by a common (or contrived) name followed by a formula for parentage or suspected parentage and groups these as hybrids of a particular species. In addition, he also discusses selected cultivars which may approach the status of a botanical variety.

Tanaka recognizes essentially the same *Citrus* species as Swingle but uses each species in a more limited sense and assigns additional specific epithets to related forms, even those which are known hybrids. Many forms which are considered by Swingle to be botanical varieties, hybrids, or even cultivars are given species status by Tanaka. Whether such proliferation of species is justified is debatable; a clear definition of the species concept in citrus appears to be lacking. Tanaka's system has provided morphological descriptions for many citrus taxa which otherwise would be wanting.

Species recognized by Tanaka are often employed by citrus breeders and horticulturists since they are less ambiguous and less complex than a designation in conformity with Swingle's system. The concurrent use of names from the two systems, or even older systems, without identification of the system which is being followed has led to confusion and misinterpretation, especially among those unfamiliar with citrus taxonomy.

Nomenclatural Confusion

In reviewing the chemical literature, it becomes apparent that many chemical investigations on citrus have very limited value to chemosystematics due to the ambiguous manner in which the plant material was identified. Some investigators have been content to identify

[15] P. C. REECE, *J. Rio Grande Valley Hort. Soc.* **13**, 18 (1959).
[16] P. C. REECE and C. J. HEARN, *Proc. Florida State Hort. Soc.* **77**, 76 (1964).
[17] T. TANAKA, *Species Problem in Citrus*, Japanese Society for the Promotion of Science, Ueno, Tokyo (1954).
[18] T. TANAKA, *Citrologia Semi-Centenial Commemoration Papers on Citrus Studies*, Citrologia Supporting Foundation, Osaka Pref. University, Osaka (1961).
[19] R. W. HODGSON, in *Proceedings of the Second Conference of the International Organization of Citrus Virologists* (edited by W. C. PRICE), pp. 1–7, University of Florida Press, Gainesville (1961).

plant material by the common name used in their locality, without apparently appreciating the non-specificity of the term to a person more familiar with citrus terminology. Thus, pomelo is a term which has been applied to either the fruit of *C. grandis* or *C. paradisi*.

Terminology in different languages can lead to confusion, e.g. the fruit of *C. limon* is called "citron" or "limon" in French, "zitrone" or "limone" in German and "lemon" in English; while the fruit of *C. medica* is called "cedrat" in French, "zitrone" in German, and "citron" in English. "Limon" and "citron" are defined also as French synonyms for the lime (*C. aurantifolia*).

The use of names without indication of whose taxonomic treatment is being followed may also lead to uncertainty as to exact identity. Thus, *C. sinensis* Pers. is cited by Swingle as having been used at one time as the binomial for *C. aurantium* var. *myrtifolia* Ker-Gawl, while *C. sinensis* Osbeck according to Swingle is the binomial applied to the sweet orange. *C. aurantium* has been used by different authors to designate either the sour or the sweet orange.

Confusion of names has led to unfortunate speculation as to the true identity of the plant material by reviewers of the literature. Mackinney[14] concluded that work reported from Europe on coloring matters of *C. aurantium*, "... almost certainly refers to the sweet orange. He also concludes that the binomial *C. madurensis* refers to a tangerine (*C. reticulata*). While *C. madurensis* is considered by Hume[20] and Swingle[1] to be really a kumquat (*Fortunella* sp.). Hodgson[19] in turn identifies *C. madurensis* Loureiro as being the calamondin in Tanaka's system.

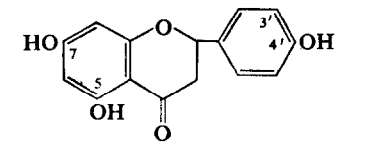
Often nomenclatural synonymy goes unrecognized by reviewers. Dean[21] discusses similar isosakuranetin glycosides reported in *C. trifoliata*, *Pseudaegle trifoliata*, and *Poncirus trifoliata* without apparently recognizing that all three binomials are synonyms for the trifoliate orange. Synonyms are often difficult to establish without careful study of the literature and the plant material. Although Swingle's comprehensive work discusses numerous synonyms, many are not indexed and are therefore often overlooked.

Even when an author gives an unambiguous designation of the plant material the authenticity of the identification may be open to question. The somewhat naïve faith some investigators have in the identification of fruit available on the commercial market may, in part, explain some discrepancies found in the literature on citrus composition. If at all possible, plant material should be obtained from variety plantings used in breeding or from nurseries where accurate records are kept.

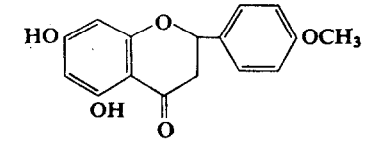
Citrus Flavanones

The chemical structures of the known citrus flavanones are presented in Fig. 1. Figure 2 illustrates the carbohydrate structures found in glucosidic union with the 7-OH of the flavanones. The flavanones which have been reported to occur in various citrus species have been tabulated in three prior reviews.[2, 22, 23] Table 1 summarizes the reported occurrence of flavanones in citrus through the 1966 literature.

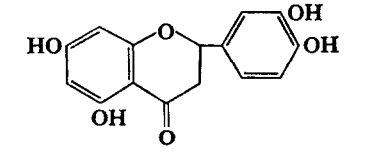
[20] H. H. HUME, *Citrus Fruits*, Macmillan, New York (1957).
[21] F. M. DEAN, *Naturally Occurring Oxygen Ring Compounds*, pp. 333–365, Butterworths, London (1963).
[22] R. M. HOROWITZ, in *The Orange, Its Biochemistry and Physiology* (edited by W. B. SINCLAIR), pp. 334–372, University of California Press, Berkeley (1961).
[23] U.S. Department of Agriculture. Agricultural Research Service, *Chemistry and Technology of Citrus, Citrus Products, and Byproducts*, Agricultural Handbook No. 98, Revised ed. U.S. Government Printing Office, Washington, D.C. (1962).



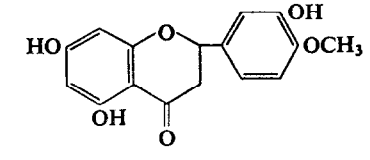
Naringenin



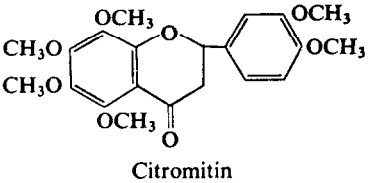
Isosakuranetin



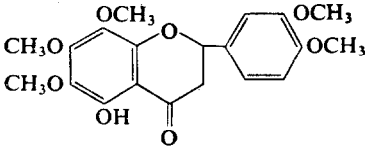
Eriodictyol



Hesperetin



Citromitin



Demethylcitromitin

FIG. 1. FLAVANONE AGLYCONES OF CITRUS.

The indicated occurrence of some of the flavanones listed in the table is likely to be incorrect due to insufficient chemical proof of identity. Other identifications can be reinterpreted in the light of newer knowledge. It is not possible to evaluate the merits of each identification but it is possible to make a few observations on those data which now appear contrary to the known pattern of flavanone distribution in the different citrus species.

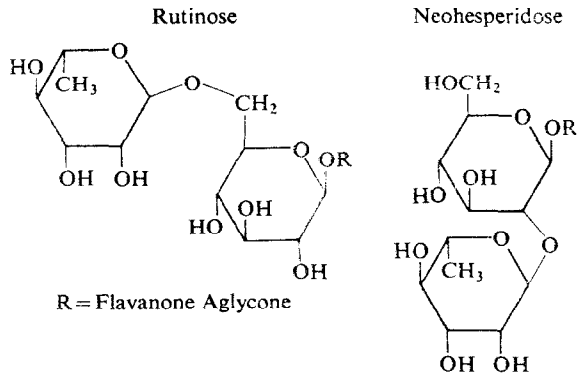


FIG. 2. GLYCOSIDIC CARBOHYDRATES AT 7-OH POSITION OF CITRUS FLAVANONES.

The report of naringin in *C. sinensis* and *C. limon* by Gerngross and Renda[27] should be taken with considerable reservation as their method of identification was wanting in specificity. The chemical evidence described by Dunlap and Wender[26] which led them to report the occurrence of naringin in the peel of *C. sinensis* could also be suggestive of naringenin-7-β-rutinoside, a compound later attributed to the same species by Horowitz.[24]

In view of these interpretations the lack of substantiated flavanone neohesperidosides in *C. sinensis* and *C. limon* is consistent with the report by Horowitz[24] that electrophoretic analysis of ozonized flavonoid glycosides from these same species gave no indication of the presence of neohesperidosides.

Rather than make similar subjective evaluations of prior research concerning the presence or absence of flavanones in the other citrus species, it is more rewarding to apply newer and faster analytical methods to authenticated plant material, as a check on the prior work. The simplified assay method employing polyamide thin-layer chromatography developed by Mizelle *et al.*[32] provides a rapid means of identifying the probable presence of the citrus flavanones in extracts of the plant material. Such a rapid means of flavanone analyses is needed if the chemosystematics of citrus flavanones is to have practical usefulness for the citrus breeder and the taxonomic botanist. A knowledge of the presence of flavanones characteristic of a particular species may also be useful in determining the species composition of citrus products in the processing industry.

RESULTS

Fruit were collected for analysis during November and January in order to obtain the greatest number of fruit varieties at a mature stage of development.

[24] R. M. HOROWITZ, in *Biochemistry of Phenolic Compounds* (edited by J. B. HARBORNE), pp. 545–571, Academic Press, London (1964).
[25] J. W. MIZELLE, W. J. DUNLAP and S. H. WENDER, *Phytochem.* **6**, 1305 (1967).
[26] W. J. DUNLAP and S. H. WENDER, *Arch. Biochem. Biophys.* **87**, 228 (1960).
[27] O. GERNGROSS and N. RENDA, *Ann. Chem.* **691**, 186 (1966).
[28] B. P. CHALIHA, G. P. SASTRY and P. R. RAO, *Bull. Nat. Inst. Sci. India* **31**, 63 (1965); *Chem. Abstr.* **66**, 55,334 (1967).
[29] R. HENDRICKSON and J. W. KESTERSON, *Hesperidin, the Principle Glycoside of Oranges*, University of Florida Agr. Exp. Sta. Bull. 545 (1954).
[30] T. MATSUNO, *Yakugaku Zasshi* **79**, 540 (1959); *Chem. Abstr.* **53**, 18,193 (1959).
[31] T. KARIYONE and T. MATSUNO, *J. Pharm. Soc. Japan* **74**, 363 (1954); *Chem. Abstr.* **48**, 9020 (1954).
[32] J. W. MIZELLE, W. J. DUNLAP, R. E. HAGEN, S. H. WENDER, B. J. LIME, R. F. ALBACH and F. P. GRIFFITHS *Anal. Biochem.* **12**, 316 (1965).

TABLE 1. FLAVANONE GLYCOSIDES REPORTED IN CITRUS FRUIT[a,b]

Citrus taxa	Flavanones													
	7-Rutinosides of:					7-Neohesperidosides of:					Others:			
	naringenin	isosakuranetin	eriodictyol (eriocitrin)	hesperetin (hesperidin)	naringenin-4′-glucoside	naringenin (naringin)	isosakuranetin (poncirin)	eriodictyol	hesperetin (neohesperidin)	naringenin-4′-glucoside	citromitin	demethylcitromitin	eriodictyol glycoside	citronetin-7-rhamnoglucoside
Citrus sinensis (L.) Osbeck	×[24]	×[24]	0[22]	×	×[25]	?[26,27]								
C. reticulata Blanco				×[28]					×[28]		×[28]	×[28]	?	
C. aurantifolia (Christm.) Swing.				?[29]										
C. medica L.				×										
C. limon (L.) Burm.			×	×		×[27]							×	
C. limon (?) "Ponderosa"									×[24]					?
C. grandis (L.) Osbeck						×[28]	×[28]		×[30]	×[25]				
C. tachibana Tan.				×[31]			×[31]							
C. aurantium L.				×		×			×					
C. paradisi Macf.	×[32]	×[32]		×[31]	×[25]	×	×[33]		×[32]	×[25]				
C. jambhiri Lushington[c]				×[28]					×[28]					
C. mitis Blanco[c]				×[34]							×[35]	×[35]		
C. limetta Risso[c]						0[36]								
C. natsudaidai Hayata[c]				×		×			×[37]					
C. tankan Hayata[c]				×										
C. hassaku Tan.[c]				×[31]		×[31]			×					
C. fusca Lour.[c]				×										
C. junos Sieb. ex Tan.[c]				×[31]										
C. kotokan Hayata[c]				×										
C. iyo Tan.[c]				×[31]										
C. luminciana[c]						×								
C. medioglobosa Tan.[c]				×[31]		×[31]								
Poncirus trifoliata (L.) Raf.				×		×			×					

[a] This table was compiled mainly from the reviews of Kefford,[2] Horowitz,[22] and the USDA Agricultural Research Service.[23] Data from other sources are indicated by footnote citations.

[b] Symbols: × = reported present, 0 = reported to be absent, ? = reported to be present but proof is questionable.

[c] These species are not recognized by Swingle.[1]

Six of the flavanones: naringin, naringenin-7-rutinoside, poncirin, isosakuranetin-7-rutinoside, neohesperidin and hesperidin were determined with a high degree of certainty by chromatography on polyamide resin. With this resin and a nitromethane–methanol solvent system it was possible to get good separations of these six flavanones in an area of the thin-layer chromatogram where mostly blue fluorescing coumarins were the only interfering compounds. These coumarins were easily differentiated from the flavanones under long-wave u.v. light since the former possessed a blue fluorescence while the latter were a dull yellow. A further distinction was possible after spraying with a methanolic solution of aluminum

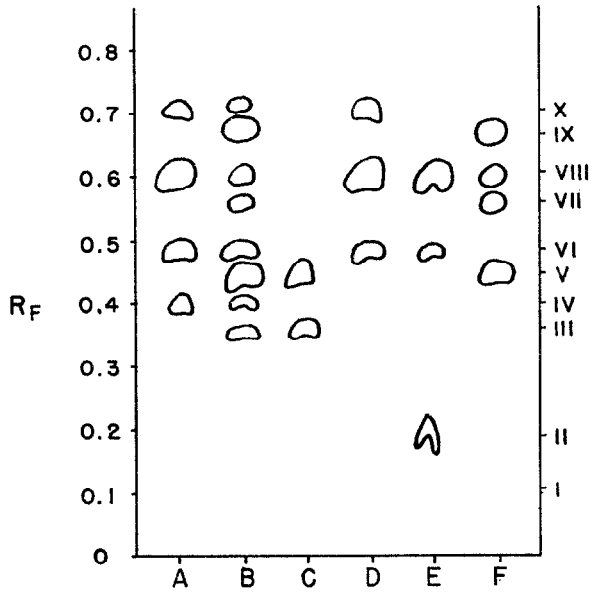


FIG. 3. THIN-LAYER CHROMATOGRAM ON POLYAMIDE OF *Citrus* FLAVANONES. DEVELOPING SOLVENT; NITROMETHANE–METHANOL (5:2).
Key to samples: A, *Citrus sinensis*; B, *C. paradisi*; C, *C. grandis*; D, *C. reticulata*; E, *C. limon*; F, standards.
Key to flavanone positions: 7-neohesperidosides of—I, eriodictyol; III, naringenin-4′-glucoside; V, naringenin; VII, hesperetin; IX, isosakuranetin. 7-rutinosides of—II, eriodictyol; IV, naringenin-4′-glucoside; VI, naringenin; VIII, hesperetin; X, isosakuranetin.

chloride; the flavanones fluoresced a bright greenish-yellow while the coumarins remained unchanged. Flavones were present which also react with $AlCl_3$ to give a yellow fluorescence in the region between naringin and the origin and often prevented an unequivocal assessment of the presence or absence of the eriodictyol-7-rhamnosylglucosides or of the naringenin-4′-glucoside-7-rhanmosylglucosides. These flavones, with Neu's Reagent, generally gave a visible yellow color and exhibited enhanced fluorescence intensity.

Although the decision of the occurrence or non-occurrence of a flavanone compound in a citrus variety was made principally by reference to the polyamide thin-layer chromatograms, these results were checked for consistency with separations obtained on silicic acid plates.

The silicic acid plate was incapable of differentiating between the neohesperidoside or rutinoside analogs of a particular flavanone. However, some of those coumarins which appeared at the same migration distances as the principal flavanones did not occur in the same

region on the silicic acid plates. In all cases where the presence of a flavanone rhamnosyl-glucoside was indicated on the polyamide plates a corresponding indication was also found on the silicic acid plate.

It must be emphasized that the interpretations of flavanone composition by chromatography alone does not constitute adequate chemical proof to unequivocally establish the presence of each component in the plant material. The chromatographic analysis serves only as a convenient method of making analytical estimations on a large number of samples in a short time. More rigorous chemical investigations are necessary to substantiate the data obtained in this survey.

It became apparent upon examining the chromatographic flavanone pattern of the various citrus species and varieties that quantitative as well as qualitative differences existed between individual groupings. The isolation procedure was not conducted with sufficient accuracy to allow quantitative comparisons to be made between samples. However, the relative proportion of each flavanone within any sample could be estimated by examination of the fluorescence intensity of the individual component spots on the chromatogram. Visual estimates of the relative fluorescence intensity of flavanone glycosides on thin-layer chromatographic plates sprayed with methanolic $AlCl_3$ showed good reproducibility.

The most intensely fluorescent flavanone spot among those identified on the chromatogram of a fruit sample was assigned an arbitrary value of ten. All other flavanone spots in the same sample were rated in numerical values of one through ten to express their fluorescence intensity relative to the most intense spot. Those spots whose intensity were below a relative value of one were indicated to occur as a "trace". When the component was below the limits of detectability it was recorded as being "not detected". Hagen *et al.*[38] have observed that there is a different molar fluorescence for the different citrus flavanone glycosides in a dilute methanolic solution of $AlCl_3$. A similar but unknown difference in spot fluorescent intensity also occurs on the thin-layer chromatogram; thus the values of relative fluorescence intensities *within* each fruit sample given on Tables 2 and 3 are *not* to be taken as relative molar quantities. These relative fluorescence intensity values are "*ad hoc*" crude estimates whose only purpose is to illustrate quantitative distribution patterns of flavanones. The consistency of these estimates of relative fluorescent intensity is illustrated by the obtainment of the same estimated values in seven varieties of *Citrus sinensis*.

DISCUSSION

Table 2 summarizes the flavanone patterns found in eight of the 16 *Citrus* species of Swingle's system as well as seven additional ones, most of which are recognized in Tanaka's system.

The failure to find any neohesperidosides in any of the eight varieties of *C. sinensis* which were examined appears consistent with the majority of the prior work on this species as noted in Table 1.

Hesperidin and neohesperidin were the only flavanone glycosides which had previously been reported in *C. reticulata*. The results from this survey find no evidence of neohesperidin,

[33] R. M. Horowitz and B. Gentili, *Arch. Biochem. Biophys.* **92**, 191 (1961).
[34] G. P. Sastry, *J. Sci. Ind. Res. (India)* **19B**, 500 (1960); *Chem. Abstr.* **55**, 16,528 (1961).
[35] G. P. Sastry and L. R. Row, *J. Sci. Ind. Res. (India)* **20B**, 187 (1961).
[36] T. R. Seshadri, *Proc. Indian Acad. Sci. Sect. A* **18**, 201 (1943); *Chem. Abstr.* **39**, 1438 (1945).
[37] T. Nakabayashi, *Nippon Nogeikagaku Kaishi* **35**, 945 (1961); *Chem. Abstr.* **60**, 11,044 (1964).
[38] R. E. Hagen, W. J. Dunlap, J. W. Mizelle, S. H. Wender, B. J. Lime, R. F. Albach and F. P. Griffiths, *Anal. Biochem.* **12**, 472 (1965).

TABLE 2. THIN-LAYER CHROMATOGRAPHIC SURVEY OF FLAVANONES IN CITRUS TAXA

Citrus taxa[b]	Relative fluorescence intensity of flavanones within each fruit sample.[a]									
	7-Rutinosides of:					7-Neohesperidosides of:				
	naringenin	isosakuranetin	eriodictyol (eriocitrin)	hesperetin (hesperidin)	naringenin-4′-glucoside	naringenin (naringin)	isosakuranetin (poncirin)	eriodictyol	hesperetin (neohesperidin)	naringenin-4′-glucoside
Citrus sinensis (7 varieties[c])	4	2		10	1	—	—		—	—
18-A-6	3	1		10	—	—	—		—	—
C. reticulata (4 varieties[d])	4	2		10	—	—	—		—	—
Changsha	3	—		10	—	—	—		—	—
Dancy	3	1		10	—	—	—		—	—
Shekwasha	2	1		10	—	—	—		—	—
SunChuShakat	2	—		10	—	—	—		—	—
Sunki	1	T		10	—	—	—		—	—
C. aurantifolia "Mexican lime"	—	—		10	—	—	—		—	—
C. limon Frost "Lisbon"	2	—	10	10	—	—	—	—	—	—
"Sipora"	9	—	8	10	—	—	—	—	—	—
"Meyer"	1		1	10	—	—	—	—	—	—
? "Ponderosa"	1	—	—	1	—	3	—	—	10	—
C. grandis	—	—	—	—	—	10	—	—	—	1
C. tachibana	1	T	—	10	—	—	—	—		—
C. aurantium (3 varieties[e])	—	—		—		10	—		10	
C. paradisi (2 varieties[f])	4	1		T	1	10	2		T	1
"Oani Kin Kan"	2	T		1	3	10	1		5	3
C. volkameriana Pasquale	1	—		10		—	—		—	—
C. taiwanica Tan. & Shim.	3	T		3		10	2		10	—
C. karna Raf.	7	—		10	2	—	—		—	—
C. limettioides	—	—	—	10	—	—	—	—	—	—
C. excelsa Wester	10	T		10	T	—	—	—		—
C. amblycarpa Ochse	3	1		10	—	3	1		10	—
C. mitis	—	—		10		—	—		—	
Fortunella hindsii (Champ.) Swing.	4	—	10	9	—	4	—	—	—	—
F. crassifolia Swing. "Meiwa"	5	—	4	7	—	6	4	10	—	—
Poncirus trifoliata "Davis B trifoliata"	—	—	—	—	—	10	10	—	—	—

[a] The flavanone with the highest fluorescence intensity was given a value of 10. With this as standard, the relative fluorescence intensities of the other flavanones are indicated by values 1 to 10; T=trace; —=not detected. A blank space indicates no decision was made on the presence or absence of the compound.

[b] When identical data are obtained for more than one variety, these varieties are listed in the footnotes. Numerical designations are code numbers for varieties, strains, or clones not having a varietal name.

[c] "Broward", "Cadena", "Hamlin", "Ovale Sangre", "Parson Brown", "Toregrossa", "Lamb Summer".

[d] "Kara", "Long Huang Kat", "Lau Chang", "Pong Koa.".

[e] "Chinotto", "Bouquet de Fleur", "Sour Orange".

[f] "Duncan", "Foster".

but contain, in addition to the major component, hesperidin, quantities of naringenin-7-rutinoside and isosakuranetin-7-rutinoside in relative amounts similar to those found in *C. sinensis*.

C. aurantifolia is unique among the citrus taxa in that it contains only one compound, hesperidin. Among those taxa where no flavanone neohesperidosides were found hesperidin was the major flavanone component detected; only the eriocitrin of some *C. limon* varieties rivals this dominance.

Even though it was not possible to make definitive decisions concerning the presence or absence of eriocitrin or its neohesperidoside analog in the majority of samples analyzed, it was apparent that if these compounds occurred at all, it was in very small amounts.

Eight species (including *C. limon*, if one discounts the Ponderosa lemon), are found to contain exclusively flavanone rutinosides while only three, *C. grandis*, *C. aurantium*, and *Poncirus trifoliata*, contain only the neohesperidosides.

The chromatographic pattern of known citrus hybrids summarized in Table 3 shows that when a species containing only flavanone rutinosides is crossed with another containing only neohesperidosides, then the progeny contain both types of flavanone glycosides. This is illustrated by the progeny of *C. sinensis* × *P. trifoliata*, *C. limon* × *P. trifoliata*, and *C. grandis* × "Temple orange" (a possible *C. sinensis* × *C. reticulata* hybrid) crosses, where the progeny all contain both rutinosides and neohesperidosides of flavanones. The alleles responsible for the production of these two rhamnosyl-glucoside isomers thus appear to involve additive inheritance in the F_1 generation.

In Table 2 we find four *Citrus* species which contain both the rutinosides and neohesperidosides of flavanones: *C. paradisi*, *C. taiwanica*, *C. amblycarpa*, and *C.* cv. Ponderosa.

Robinson[39] supports Swingle's contention[1] that *C. paradisi* is likely a hybrid between *C. sinensis* and *C. grandis*. Inheritance of rutinoside and neohesperidoside alleles from those parents would explain the heterophenic flavanone pattern in *C. paradisi*. Further confirmation of this flavonoid relationship is provided by Mizelle, Dunlap and Wender[40] who also find that *C. grandis* does indeed contain the same neohesperidosides which have been found in *C. paradisi* and that *C. sinensis* contains the corresponding rutinosides.

Swingle[1] considers that *C. taiwanica* may be a hybrid of *C. aurantium* and some other "long-leaved" citrus species. The presence of neohesperiodsides in *C. taiwanica* is consistent with the probability of *C. aurantium* being one parent. The presence of the rutinosides suggests the other parent may be from among those species containing only rutinosides. The "Willow Leafed Mandarin" may fulfil both the leaf shape and flavanone criteria suggested for the second parent.

Both Webber[41] and Frost[42] discuss the probable hybrid nature of the Ponderosa lemon; *C. limon* and *C. medica* or *C. paradisi* are mentioned as probable parents. Since *C. limon* lacks any neohesperidosides the other parent would be likely to contain neohesperidosides. *C. paradisi* (or *C. grandis*) would fulfil such a requirement. Neohesperidosides have not been demonstrated to occur in *C. medica*. Since *C. amblycarpa* possesses both classes of flavanone rhamnoglucosides, it may be of hybrid origin.

[39] T. R. Robinson, *Econ. Botany* **6**, 228 (1952).

[40] J. W. Mizelle, W. J. Dunlap and S. H. Wender, A chromatographic study of flavanone glycosides of the shaddock and sweet orange (manuscript in preparation).

[41] H. J. Webber, in *The Citrus Industry* (edited by H. J. Webber and L. D. Batchelor), Vol. 1, pp. 475–668, University of California Press, Berkeley (1943).

[42] H. B. Frost, in *The Citrus Industry* (edited by H. J. Webber and L. D. Batchelor), Vol. 1, pp. 817–914, University of California Press, Berkeley (1943).

TABLE 3. THIN-LAYER CHROMATOGRAPHIC SURVEY OF FLAVANONES IN CITRUS HYBRIDS

Citrus hybrids and varieties	Relative fluorescence intensity of flavanones within each hybrid[a]									
	7-Rutinosides of:					7-Neohesperidosides of:				
	naringenin	isosakuranetin	eriodictyol (eriocitrin)	hesperetin (hesperidin)	naringenin-4′-glucoside	naringenin (naringin)	isosakuranetin (poncirin)	eriodictyol	hesperetin (neohesperidin)	naringenin-4′-glucoside
C. sinensis										
× *Poncirus trifoliata* (Citranges)										
"Rustic"	4	4		4		5	10		8	
"Savage"	4	4		1		9	10		T	
"Troyer"	4	4		1		10	10		2	
(*C. sinensis* × *P. trifoliata*)										
× *Fortunella* sp. (Citrangequats)										
"Thomasville"	5	10		1		3	7		8	
"Sinton"	1	2		2		6	10		8	
C. grandis										
× *C. ighangensis*										
"Ichang Pummelo"	—	—		—	—	10	1		—	—
× Temple Orange	3	—		1	—	10	1		9	—
C. paradisi										
× *C. reticulata* (Tangelos)										
"Minneola"	4	2		10	—	—	—		—	—
"Orlanlo"	3	1		10	—	—	—		—	—
"Pearl"	10	T		8	—	—	—		—	—
"Satsumelo"	10	2		T	—	—	—		—	—
18-T-2	5	1		10	—	—	—		—	—
"Sunshine"	1	T		2		4	3		10	—
"Suwannee"	4	2		10	—	—	—		—	—
"Thornton"	10	1		10	—	—	—		—	—
"Webber"	8	T		10	—	—	—		—	—
I-32-14	4	2		10	—	—	—		—	—
C-18-P-1	10	5		10	1	—	—		—	—
18-W-5	2	1		10	—	—	—		—	—
× (*C. paradisi* × *C. reticulata*)										
"Wekiwa"	10	T		10	1	—	—		—	—
× *P. trifoliata* (Citrumelo)										
"Sacaton"	4	1		1	3	10	8		1	3
C. aurantifolia (?)										
× *C. reticulata* (?)										
"Rangpur"	4	—		10	1	—	—		—	—
"Kusaie"	5	—		10	—	—	—		—	—
× *F. japonica*										
"Lakeland"	10	7		9		—	—		—	
C. limon										
× *P. trifoliata* (Citremon)	10	2		2		5	8		1	

TABLE 3. THIN-LAYER CHROMATOGRAPHIC SURVEY OF FLAVANONES IN CITRUS HYBRIDS—*continued*

Citrus hybrids and varieties	Relative fluorescence intensity of flavanones within each hybrid[a]									
	7-Rutinosides of:					7-neohesperidosides of:				
	naringenin	isosakuranetin	eriodictol (eriocitrin)	hesperetin (hesperidin)	naringenin-4′-glucoside	naringenin (naringin)	isosakuranetin (poncirin)	eriodictyol	hesperetin (neohesperidin)	naringenin-4′-glucoside
"Clementine" (*C. reticulata* hybrid ?)										
× (*C. paradisi* × *C. reticulata*)										
"Bowers"	9	5		10	—	—	—		—	—
"Fairchild"	5	2		10	—	—	—		—	—
"Knight"	3	2		10		—	—		—	—
"Ross"	7	2		10		—	—		—	—
6-7-2	3	2		10		—	—		—	—
1-38-1	6	2		10	2					
52-19-4	4	1		10	—	—	—		—	—
× *C. reticulata*										
"Fortune"	4	3		10	—	—	—		—	—
52-85-23	5	2		10	—	—	—		—	—
53-2-46	5	1		10	1	—	—		—	—
53-4-7	5	1		10	2	—	—		—	—
54-2-1	7	1		10	1					
× *C. sinensis*										
53-1-11	2	1		10	—	—	—		—	—
"King" (*C. reticulata* × *C. sinensis* hybrid ?)										
× (*C. paradisi* × *C. reticulata*)										
52-83-16	4	3		10	—	—	—		—	—
52-83-2	10	8		2	—	—	—		—	—
× *C. reticulata*										
"Kincy"	4	2		10	—	—	—		—	—
"Honey"	8	5		10	—	—	—		—	—
52-80-93	9	5		10	—	—	—		—	—
"Wilking" ("King" × *C. reticulata* hybrid)										
× (*C. reticulata* × *C. sinensis*)										
"Dweet"	4	2		10	—	—	—		—	—
C. reticulata "Honey"										
× (*C. paradiai* × *C. reticulata*)										
52-53-1	5	3		10	—	—	—		—	—
× "Umatilla Satsumela"										
52-76-9	7	1		10	—	—	—		—	—
C. reticulata var. *austera*										
× *C. ichangensis*										
"Kansu"	10	—		9	—	2	—		3	—
Murcott "Orange"	10	4		2		—	—		—	

[a] The flavanone with the highest fluorescence intensity was given a value of 10. With this as standard, the relative fluorescence intensities of the other flavanones are indicated by values 1 to 10; T=trace; —=not detected. A blank space indicated no decision was made on the presence or absence of the compound.

Horowitz[24] obtained data which supported his earlier suggestion[3] that citrus fruit could be classed as containing either rutinosyl glycosides or neohesperidosyl glycosides as their main flavonoid components. The present survey further confirms the validity of his observations. Those citrus types which contain both classes of flavanone glycosides, in general, can be considered as being a possible hybrid.

The principal deterrent to considering the possession of all one class of glycoside as a necessary criterion for a species is the considerable amount of literature concerning the flavanone glycosides attributed to *C. aurantium.* Although some workers had reported both rutinosides and neohesperidosides to be present in *C. aurantium,* others have reported one or the other glycoside present. In surveying the literature it is apparent that in the majority of cases where hesperidin was reported as the major glycoside in *C. aurantium* the authors were employing fruit which would be classed as *C. sinensis* by Swingle's system.

In view of the known difficulty[1,41] of differentiating various forms attributed to *C. aurantium* from hybrids and extreme forms of *C. sinensis,* none of the identifications of plant material reported in flavonoid-composition studies of *C. aurantium* appears sufficiently unambiguous. Despite the frequent citation by reviewers of these compositional studies as being indicative of *C. aurantium,* it does not appear that a careful, comprehensive study of authenticated plant material has yet been made which allows any definitive conclusions to be drawn. The three varieties of *C. aurantium* analyzed in the present study appear to contain only the neohesperidoside derivatives. These three varieties, however, represent only a small portion of the diverse forms attributed to *C. aurantium.*

Insufficient data are available on the *Fortunella* species to know if these also have representatives with either all rutinoside or neohesperidosides. It is noteworthy that *F. crassifolia* is no longer considered a valid species in Swingle's last compilation[1] but is regarded as a hybrid. *F. crassifolia* appears to be unique in so far as it may contain a large proportion of both rhamnoglycoside isomers of eriodictyol.

Reference to Table 2 and to those taxa where more than one variety were analyzed appear to support not only the general qualitative consistency of flavanone composition but also the constancy of relative amounts of flavanones within different varieties of the same species.

The relative amounts of flavanones in *C. sinensis* and *C. reticulata* are quite similar except for the lack of naringenin-4′-glucoside-7-rutinoside in the latter species. These same relative values find expression, with minor variations, also among many of the hybrids between varieties of *C. reticulata* or between some backcrosses of interspecific *C. reticulata* hybrids to *C. reticulata.*

In *Poncirus trifoliata* the only two flavanones detected were neohesperidosides of naringenin and isosakuranetin. When *P. trifoliata* is crossed with *C. sinensis,* where hesperetin is the major flavanone moiety along with minor amounts of naringenin and isosakuranetin, the resulting "citrange" hybrids all had hesperetin along with relatively large amounts of naringen and isosakuranetin.

A similar phenomenon is observed in the "citremon", a *C. limon* × *P. trifoliata* hybrid, where the isosakuranetin is introduced into the hybrid by *P. trifoliata* and hesperetin is introduced by *C. limon.* It is also indicated that probably one of the two possible eriodictyol rhamnoglucosides is also present in the hybrid. In the hybrids which were analyzed, no examples were found where an aglycone was present which was not also known to occur in one of the parents. Thus, besides the additivity of alleles responsible for the rhamnoglucoside type, F_1 progeny of interspecific crosses show an additivity of alleles for the nature of the aglycones present.

When one examines the relative fluorescence-intensity values of flavanones among hybrids of *C. paradisi* and *C. reticulata* we find frequent reoccurrences of the values characteristic of *C. reticulata* and *C. sinensis*. If our prior deductions are correct that inheritance of the glycosidic patterns among F_1 generations is based on additivity of glycoside alleles, as well as additivity of aglycone alleles, then the absence of neohesperidosides and the presence of the typical *C. sinensis* and/or *C. reticulata* relative fluorescence-intensity values argue in favor of *C. paradisi* being heterozygous with respect to both the glycosidic and aglycone alleles. One would also deduce from this same evidence that either *C. sinensis* or *C. reticulata* was one of the probable parents of *C. paradisi*. As stated earlier, *C. sinensis* has been considered a probable parent.

Since other *C. paradisi* × *C. reticulata* hybrids (e.g. "Satsumelo") show a relative fluorescence-intensity pattern typical of *C. paradisi* aglycones yet are completely devoid of neohesperidosides it is suggestive of independent transmittance of the glycosidic allele and the aglycone allele(s). Further evidence of the genotypic dihybridity of *C. paradisi* with regard to aglycone and glycosidic components is present in the "Sunshine" tangelo. This has the aglycone fluorescence-intensity pattern typical of *C. sinensis* and/or *C. reticulata* but it still possesses a high percentage of neohesperidosides which apparently were inherited independent of aglycone alleles from *C. paradisi*.

It should not be inferred from the foregoing discussion that the relative-fluorescence-intensity patterns and therefore the relative quantity of the individual flavanone aglycones in citrus are necessarily controlled by the inheritance of a single allele. Indeed, since biosynthetic transformations from one flavanone to another or between a common precursor and different flavanone end-products may require the presence of several different enzymes, it is not inconceivable that each enzyme or enzyme sequence may be controlled by individual genes which are transmitted independently of each other.

Maier and Metzler[43] recently summarized and correlated the interrelationship of phenolic aglycones of *C. paradisi* based on current postulations of biosynthetic pathways. Isosakuranetin, and hesperetin are considered to result from methylation of, respectively, naringenin and eriodictyol. Mairer and Metzler speculate that both methylation reactions are dependent on a para-*O*-methyltransferase enzyme which is specific for flavanones. Evidence in support of the direct methylation of naringenin to produce isosakuranetin is seen in Table 2; in no species does isosakuranetin occur independently of naringenin. Due to the lack of conclusive evidence for the presence or absence of eriodictyol in most of the species a similar consistency for eriodictyol-hesperetin conversion cannot be obtained. Where an eriodictyol derivative is found in large quantities, as in *C. limon* cv. Lisbon and cv. Sipora, hesperidin is also found in about equal quantities; but what is important is that, despite the presence of a naringenin derivative, no isosakuranetin derivative is present. This suggests that perhaps a higher specificity exists for para-*O*-methyltransferase enzymes which distinguish between naringenin and eriodictyol precursors. An alternative explanation is that perhaps the naringenin precursor is in some way tied up so as to make it unavailable to methylation.

Grisebach[44] summarizes the evidence that glycosylation occurs late in the biosynthetic sequence of flavonoids as a step-by-step addition of monosaccharides to flavonoids. The results of the present survey appear consistent with such a hypothesis and also suggest that

[43] V. P. Maier and D. M. Metzler, *Phytochem.* **6**, 1127 (1967).

[44] H. Grisebach, in *Chemistry and Biochemistry of Plant Pigments* (edited by T. W. Goodwin), pp. 279–308, Academic Press, London (1965).

the glycosylation enzyme responsible for the introduction of the rhamnosyl unit is non-specific for the flavanone aglycone.

By employing the general concepts of flavanone inheritance which we have deduced from this survey it may be possible to aid both the taxonomist and plant breeder in deducing the parentage of suspected hybrids by examining their flavanone composition. Any evidence of systematic relationships derived solely from flavanone composition studies should be employed with caution in making taxonomic conclusions.

The taxa and varieties which were examined in this survey were not chosen to aid in solving any specific taxonomic problem; however, it is possible to interpret the data for its compatibility with present theories of parentage of suspected natural hybrids. Some of these interpretations have already been discussed, while others may be derived from a careful study of the data. It would be most rewarding to investigate questions of paternity relationships by applying the technique of TLC analysis for flavanones to the suspected parents, to known hybrids of these parents, and to the variety in question.

One limitation of such investigations is that the flavanone composition of the suspected parents must be sufficiently diverse to allow the progeny to have recognizable contributions from each parent. Thus, from the data in Tables 2 and 3, *C. sinensis* and *C. reticulata* are not sufficiently unique to produce a hybrid whose flavanone composition is distinctive of its hybridity. On the other hand, many other hybrids have flavanone patterns which are unique and representative of their hybridity (e.g. citranges). Where one of the parents itself is a hybrid or a dihybrid, as with *C. paradisi*, the progeny can show considerable individual variations in flavanone composition.

These compositional studies may also be of interest to taxonomists to aid them in deciding whether a pattern of flavanone composition among a particular "variety" is sufficiently unique to provide evidence concerning its taxonomic status. Thus, *C. reticulata* "Shekwasha" is considered by Tanaka as a valid species, *C. depressa* Hayata. It will be noted from Table 2 that this variety does differ slightly from the norm of flavanone composition characteristic of *C. reticulata*. Similar or more pronounced distinctions may be found among other varieties considered for species standing. It is interesting to note that many of those species in this survey which were recognized as species by Tanaka on mainly morphological evidence appear to have unique flavanone patterns.

The efforts by the breeder to produce cold-hardy hybrids by crossing *P. trifoliata* with *C. sinensis* has largely produced citranges with a high degree of bitterness. Since the major cause of bitterness in fresh citrus fruit is due to the presence of flavanone neohesperidosides,[24] this is not surprising. If the cold-hardiness of *P. trifoliata* is inherited independently of the allele responsible for the production of neohesperidosides, then more palatable cold-hardy crosses are likely to result from backcrossing the citranges to *C. sinensis*. It may be possible to exercise some control of bitterness in hybrids by selecting potential crosses with due consideration to their neohesperidoside content.

EXPERIMENTAL*

Plant Material

During the months of November, 1964, and January, 1965, fruit samples were harvested from labeled trees in the variety collection maintained at Rio Farms, Inc., Monte Alto, Texas, or at the Texas Agricultural

* It is not the policy of the Department to recommend the products of one company over those of any others engaged in the same business.

Experiment Station, Substation No. 15, Weslaco, Texas. A summary of the citrus varieties and citrus relatives grown in these and other Texas variety collections has been compiled by Olsen, Maxwell and Shull[45] and was used as a basis for selecting fruit to be analyzed. A representative sample of one or more fruit was collected from each tree and placed in a bag bearing the tree number and variety identification. The fruit was stored at 5° for no longer than 5 days while awaiting extraction.

Isolation Procedure

The fruit were cut into longitudinal pieces, and the portions placed into a beaker of boiling 2-propanol and heating was continued until the solvent resumed boiling. The 2-propanol volume, in ml, was numerically equal to three times the weight of each fruit sample, in g. After reaching a second boil the contents of the beaker was poured into a Waring Blendor cup of 1 gal capacity. Blending was done at the highest speed possible until the slurry appeared homogeneous. The contents of the blender was then filtered under gravity and the filtrate was stored at 5° for at least 1 week. During this period, a pulverulent non-flavanone precipitate was deposited in the flasks of the majoirity of the samples. The clear, amber supernatant was then analyzed by TLC.

Thin-Layer Chromatography

The 2-propanol fruit extracts were analyzed with two separate chromatographic systems developed by Mizelle *et al.*[32] One system employed a 250 μ layer of Woelm polyamide (Alupharm Chemicals, New Orleans, Louisiana) and the solvent mixture nitromethane–methanol (5:2, v/v). The other system employed a 250 μ layer of silica gel G and benzene–acetic acid–water–nitromethane (34:32:5:18).

5 μl of each 2-propanol fruit extract was applied as a spot (5 mm dia.) to an origin line 1·5 cm above the lower edge of the TLC plate. Individual samples were placed at 1 cm intervals along the origin line. Each plate also had one or more sample spots consisting of a mixture of standards: naringin, neohesperidin, hesperidin, poncirin and/or prunin. Development with irrigating solvent was continued until the solvent reached a scribed line 18 cm above the origin. A flavanone extract of *Citrus paradisi* also served as standard since it contained almost the entire compliment of flavanones.

Visualization and Documentation of Chromatographed Components

All chromatograms were allowed to air dry and then were photographed in the dark while being illuminated by a long-wave u.v. light (Blak-Ray Model 100-B, Ultra Violet Products, Inc., San Gabriel, California). Photographs were taken on Polaroid-type 42 film with a Polaroid MP-3 camera (Polaroid Corporation, Cambridge, Massachusetts) fitted with a Kodak 2-A filter (Eastman Kodak Company, Rochester, New York). The exposure time at f. 4·7 was 3 min with the silicic acid plates and 15 sec with polyamide plates when the u.v. light was held 50 cm from the TLC plate and at a 45° angle to the camera axis.

Each plate was photographed three times: (1) following development and drying, (2) after spraying with 1% $AlCl_3$ in methanol, and (3) after spraying with Neu's Reagent (described by Stahl and Schorn[46]), a 1% solution of diphenylboric acid-β-amino-ethyl ester (C. Roth, Karlsruhe, Germany) in methanol.

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[45] E. O. Olsen, N. Maxwell and A. V. Shull, *Citrus Species, Varieties and Hybrids in Texas*, Texas Agr. Exp. Sta. Misc. Publ. No. 549 (1961).

[46] E. Stahl and P. J. Schorn, in *Thin-layer Chromatography* (edited by E. Stahl), pp. 371–391, Springer-Verlag, Berlin (1965).