The HPLC procedure gave excellent resolution at ambient temperatures. Repeated injections of the same samples were consistent within ±3%. Duplicate samples agreed within ±10% on both individual and total nucleotide concentrations. Column performance was remarkably stable over a 6 to 9 month period and duplicate columns were practically identical. Columns used showed 3500 to 4500 theoretical plates for GMP at initial program conditions.

At the present time nucleotide concentrations in more than 150 samples of fresh and processed juice have been measured. Three orange, one mandarin, and two grapefruit varieties are included. All fruit tested had attained commercial maturity. Typical total nucleotide concentrations in the samples tested range from 3.4 to 7 mg/100 ml of fresh juice. These values are somewhat higher than the 2.61 to 5.77 mg/100 g pulp reported by Barmore and Biggs (1972) for juice vesicles of the same type of fruit.

Table I shows concentrations of individual nucleotides found in several of the juice samples investigated. These values differ from the estimated quantities reported by Barmore and Biggs (1972). We report higher diphosphate and triphosphate concentrations. This may be due to differences resulting from the use of juice as opposed to juice vesicles. More probably however it reflects the increased efficiency of the Partisil column and shorter analysis times made possible by the high-pressure liquid chromatograph. This has significantly reduced the possibility for hydrolysis which plagues conventional LC methods.

Using the luciferin–luciferase system of Buslig and Attaway (1969), Barmore and Biggs (1972) reported ATP concentrations of 0.52, 0.55, and 1.33 mg/100 g of pulp in Pineapple oranges. The average ATP concentration in ten Pineapple orange samples tested is 0.44 ± 0.09 mg/100 ml of fresh juice.

The major advantages of the method over that previously reported (Barmore and Biggs, 1972) are improved resolution, ease and simplicity of sample preparation, and a 90% reduction in the time a sample spends on the anion exchange column. No data on percent recovery from fortified samples are available for comparison with the values found in this study.

As the structural units of the nucleic acids, nucleotides are valuable indicators of the metabolic state and maturity of fruit. Furthermore, the nucleotides identified have been shown to be related to sugar content (Hassid, 1967), organic acid levels (Buslig, 1970), and possibly flavor (Schinneller, 1972) of citrus juice. Luh and Chen (1969) suggest that nucleotides act in combination with other compounds to influence the taste of some fruits and vegetables.

Preliminary work indicates that after harvesting the nucleotide concentrations in citrus fruit are influenced by storage, handling, and processing. Thus, flavor changes that occur during these procedures may be in part related to changes in nucleotide concentrations. The method developed to measure such nucleotide concentrations is sensitive, convenient, and accurate.

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LITERATURE CITED

Barmore, C. R., Biggs, R. H., *J. Food Sci.* **37**, 712 (1972).
Buslig, B. S., Ph.D. Dissertation, University of Florida, Gainesville Fla., 1970.
Buslig, B. S., Attaway, J. A., *Proc. Fla. State Hortic. Soc.* **82**, 206 (1969).
Hartwick, R. A., Brown, P. R., *J. Chromatogr.* **112**, 651 (1975).
Hassid, W. Z., *Annu. Rev. Plant Physiol.* **18**, 253 (1967).
Khym, J. X., First Chemical Congress of the North American Continent, Mexico City, Mexico, Nov–Dec, 1972, ANAL 34.
Kuninaka, A., *J. Agric. Chem. Soc. Jpn.* **34**, 489 (1960).
Luh, B. S., Chen, K. C., *Fruchtsaft Ind.* **14**(4), 146 (1969).
P-L Biochemicals, Inc., Milwaukee, Wis., Circular OR-18, 1968.
Schinneller, D. J., M.S. Thesis, University of Florida, Gainesville, Fla., 1972.
Scholar, E. M., Brown, P. R., Parks, R. E., Jr., Calabresi, P., *Blood* **41**, 927 (1973).

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Separation of Pigments, Flavonoids, and Flavor Fractions from Citrus Oils by Gel Permeation Chromatography

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Concentrated cold-pressed oils of citrus and a hexane extract of orange peel were separated into three fractions, color, flavonoid, and volatile flavor, by gel permeation chromatography. Yields of pigment from the cold-pressed oils and peel extract, respectively, were as high as 116 and 350 mg/kg peel. Components of the color fractions from cold-pressed oils and peel extract were separated by thin-layer chromatography (TLC) and compared. Constituents of the flavonoid fractions were identified by comparison of TLC data with those of authentic samples. Twenty-three components of the volatile flavor fraction from tangerine oil were identified by gas–liquid chromatography. Three esters, citronellyl acetate, decyl acetate, and 1,8-*p*-menthadien-9-yl acetate, not previously reported as tangerine oil constituents, were among the identified components.

Analysis of high-boiling components of natural product mixtures, such as citrus essential oils, has been difficult because adequate means for their rapid separation into manageable fractions free of interfering compounds have not been available. The most commonly used technique, distillation, is inadequate for separating high-boiling components of citrus oils because they decompose at high temperatures, even under high vacuum (Moshonas, 1971). Adsorption chromatography (Moshonas, 1971) is often

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used as a preparative preseparation step for gas–liquid chromatographic (GLC) separation of the volatile flavor components (Moshonas and Shaw, 1974; Shaw and Coleman, 1974). This technique does not separate, in a recoverable form, the high-boiling or relatively nonvolatile materials, such as color pigments and flavonoids.

The reported use of lipophilic gels in gel permeation chromatography (GPC) for fractionating alcoholic beverages and some citrus oils, by high performance liquid chromatography (Schmit et al., 1973), fatty acids, triglycerides, aromatic hydrocarbons, and carotenoids (Svega, 1973; Suzuki and Hasegawa, 1974) suggests that GPC might be applicable as a preparative preseparation technique for citrus oils. Since GPC separates according to molecular size, the high-boiling components of a citrus oil, such as carotenoid pigments and flavonoids, might be separated from the volatile flavor compounds in a form suitable for further analysis or for use in foods or pharmaceutical preparations. Color is one of the most important physical characteristics affecting consumer acceptability of orange juice (CECO Marketing Consulting Research, Inc., 1965). Thus, one of the standards for determining orange juice quality is color (U.S. Standards for Canned Orange Juice, 1969). If separable from the oils and peel by satisfactory methods, citrus pigments might be used to color citrus juices and other products. Color pigments extracted from citrus peel by solvent have been purified by column chromatography (Ting and Hendrickson, 1968) and by precipitation from alcohol–water solvent mixtures (Berry et al., 1972). Although several methods for recovering color pigments from citrus peel have been developed, more rapid and efficient commercial methods are needed for their purification (Wilson et al., 1975). GPC is potentially a more rapid and efficient method than those previously tried.

Flavonoids from citrus were shown recently to have pharmaceutical properties (Robbins, 1966, 1975). Current methods for obtaining quantities of these flavonoids are time consuming, and only limited quantities can be obtained readily (Tatum and Berry, 1972). GPC might enable the rapid recovery of mixtures of citrus flavonoids, useful either as is or as ready sources for the isolation of relatively large quantities of individual flavonoids.

A third fraction obtainable from citrus oils by GPC is a concentrated mixture of volatile flavor components free of interfering high molecular weight components. Such a concentrated flavor fraction would be useful to the flavor and perfume industry.

We report the use of GPC to separate the components of citrus oils and citrus peel into color pigment, flavonoid, and volatile flavor fractions. Total carotenoid levels were estimated, flavonoids were identified qualitatively, and some volatile flavor compounds were identified.

EXPERIMENTAL SECTION

Oil Samples and Peel Extracts. Twenty-fold concentrates (oils reduced from 20 to 1 by volume) of cold-pressed oils from tangerines and mid-season and Valencia oranges were prepared by distillation of the oils in a rotary evaporator at 40 °C and 1 mmHg pressure. For the preparation of crude peel extract, 1 kg of Valencia orange peel was stirred with hexane (1:1, w/w), for 10 min. The mixture was filtered, and the filtrate was concentrated in a rotary evaporator to afford 5 g of peel extract.

Gel Permeation Chromatography. The column was calibrated (Figure 1) with pure compounds common to citrus essential oils. The elution volume of most compounds in citrus oils can be accurately predicted from this curve. Five-gram samples of the concentrates and peel extract were separated on a glass column (120 × 2 cm) packed with Sephadex LH-20 (Pharmacia Fine Chemicals, Piscataway, N.J.). The column was developed with tetrahydrofuran (THF) at a flow rate of 0.8 ml/min. Each sample was separated into three fractions on the basis of appearance and aroma. The volumes for the first fraction (total color pigments) were 60 and 85 ml for orange and tangerine, respectively. Fractions two (flavonoids) and three (flavor volatiles) for both orange and tangerine were eluted with about 40 and 75 ml of THF, respectively.

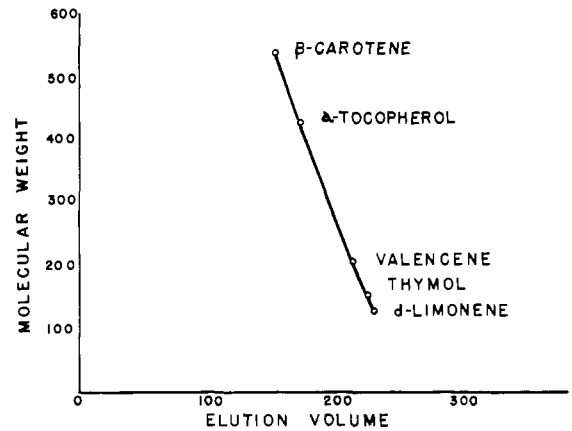

Figure 1. Calibration curve for Sephadex LH-20 column: 120 × 2 cm; flow rate, 0.8 ml/min; solvent, tetrahydrofuran.

Table I. Thin-Layer Analysis of Orange and Tangerine Pigment Fractions Separated by GPC

TLC band no.	R_f (× 100)	Epoxide test (HCl)	Carbonyl ($NaBH_4$)
1	64[a]		
2	57	+	
3	50	+	
4	42	+	
5	36[b]	+	+
6	27	+	
7	16	+	
8	13	+	
9	8		

[a] UV λ_{max} 420, 448, and 475 nm. [b] UV λ_{max} (orange) 410, 445, and 475 nm; (tangerine) 425 and 475 nm.

The recovery of color pigments separated from cold-pressed peel oil or peel extract was determined after the solvent was evaporated by a rotary evaporator, and the residue dried under vacuum to constant weight.

Analysis of Pigment Fraction. Pigment fractions were separated by thin-layer chromatography (TLC) on silica gel G with hexane–acetone (90:10, v/v) as the developing solvent. The TLC plates were then exposed to HCl vapors for the detection of epoxide groups (Cross et al., 1971). For the detection of carbonyl-containing pigments, 50 μl of the concentrated pigment solution was mixed with 50 μl of sodium borohydride solution (0.5 g of sodium borohydride in 50 ml of absolute ethanol) before TLC as above. Change in the appearance of the pigment mixture upon borohydride addition and the absence of some spots on TLC indicated the presence of carbonyl derivatives (Cross et al., 1971). The pigments with R_f values of 0.64 and 0.36 (TLC bands no. 1 and 5; Table I) were eluted and then ultraviolet (UV) absorptions in absolute ethanol were recorded with a Beckman DB recording spectrophotometer.

Analysis of Flavonoid Fraction. The flavonoid fractions were spotted on silica gel G plates with authentic standards, and the spots were developed with benzene–acetone–acetic acid (86:10:4, v/v/v) (Tatum and Berry,

1972). The flavonoids were identified by comparison of their R_f values with those of authentic samples.

Analysis of Flavor Fraction. The volatile flavor constituents of orange and tangerine oils were separated into five zones on preparative plates of silica gel HF by the hexane–acetone mixture. The TLC fractions were further separated by gas–liquid chromatography (GLC) on an F&M Model 700 gas chromatograph equipped with a 0.20 in. i.d. × 20 ft stainless steel column packed with 40% Carbowax 20M on 60/80 mesh Gas-Chrom P, and a thermal conductivity detector. The detector temperature was 250 °C, and on-column injection was used. The He flow rate was 100 ml/min, and samples were temperature programmed from 100 to 210 °C at 1 °C/min. Samples were collected in glass capillary tubes with liquid nitrogen as the coolant. The compounds were identified by comparison of their infrared (IR) and mass spectra (MS) with those of authentic compounds. Infrared spectra were taken as thin-liquid films on a Perkin-Elmer, Model 134, infrared spectrometer, and mass spectra were obtained with a Bell & Howell, Model 21-490, mass spectrometer.

Color Evaluation. The evaporated pigment fractions were added at 1/6000 final dilution to low color frozen orange concentrate (FCOJ) for color evaluation. The samples of one lot of FCOJ (50° Brix) which were prepared from early season Hamlin oranges were then diluted to 12.5° Brix with water. The color of reconstituted FCOJ with and without added pigment was compared with USDA standard color tubes (Wilson et al., 1975).

Flavor Evaluation. For flavor evaluation of color pigments at 1/6000 dilution, 0.25 g of color pigment and 0.041 g of cold-pressed Valencia orange oil were mixed with 367.5 g of 50° Brix evaporator pumpout (concentrated orange juice to which no peel oil, single-strength juice, or other flavor fractions have been added), and the mixture was adjusted to 12.5° Brix with water. A control sample with added oil only was prepared from the same evaporator pumpout. Samples were evaluated by a paired comparison test. Twelve trained panelists were each given two presentations for a total of 24 judgments.

RESULTS AND DISCUSSION

TLC separated the pigment fractions from orange and tangerine oils and orange peel extract into nine bands (Table I) of comparable R_f values, even though some of them probably did not have the same composition. Most of the pigments in TLC bands 1–3 (Table I) had been eluted in the first 20 and 36 ml of eluates from the GPC of orange and tangerine, respectively. The UV spectrum of band 1, after elution from a preparative TLC plate, suggested it to be composed of a mixture of carotenes. When chromatographed on the same TLC plate authentic β-carotene and band 1 had virtually the same R_f. The UV absorption maxima for band 5 from tangerine suggested that it was probably the ester of β-citraurin (Stewart and Wheaton, 1973). UV data indicated that band 5 for orange was different from that of tangerine and was probably a mixture of pigments.

Thin-layer chromatography showed the color fractions from orange oil or peel extract were relatively free of pigmented high molecular weight compounds. In tangerine, however, flavonoids and some high molecular weight flavor compounds began to appear after the first 75 ml of eluate was collected. For both orange and tangerine, TLC bands 2–8 tested positively for epoxides with HCl. Band 5 was shown to shift to a lower R_f value on TLC of the pigment with sodium borohydride mixture. Thus, it appeared to be a mixture of carbonyl derivatives. As determined from the cold-pressed oils, yields of pigment per kilogram of peel for tangerine and mid-season and Valencia oranges were 116, 95, and 116, respectively. The yield of solvent extracted orange pigment amounted to 350 mg/kg peel, which is about three times the yield from cold-pressed oil.

Table II. Thin-Layer Analysis of Orange and Tangerine Flavonoid Fractions Separated by GPC

TLC band no.	Flavones
1	5-Hydroxy-6,7,8,3′,4′-pentamethoxy
2	5,6,7,8,4′-Pentamethoxy
3	3,5,6,7,8,3′,4′-Heptamethoxy
4	5,6,7,4′-Tetramethoxy
5	5,6,7,8,3′,4′-Hexamethoxy
6	5,6,7,3′,4′-Pentamethoxy
7	5,7,4′-Trimethoxy[a]
8	5,7,8,4′-Tetramethoxy[b]
9	5,7,8,3′,4′-Pentamethoxy[b]

[a] In tangerine only; Tatum (1975). [b] Identified by TLC of fraction isolated from 20-fold cold-pressed oil by high-performance liquid chromatography on microstyragel (Wilson et al., 1974).

All color pigments added at 1/6000 dilution to FCOJ of substandard color improved color scores by at least one color grade. Addition of total color pigment of orange either from the cold-pressed oils or from the extract to evaporator pumpout produced a product with acceptable flavor (no noticeable change from control, $p = 0.001$). The total color fraction of tangerine could not be used without further purification because it imparted a mandarin-like flavor to evaporator pumpout. However, when only the first 75 ml of fraction 1, which contained about 20% of the pigments, was added to evaporator pumpout the flavor of the product was not noticeably different from control ($p = 0.001$). Although the pigment fractions separated by GPC effectively raised the color scores of reconstituted orange juices, Federal and State regulations do not provide for the addition of such fractions to citrus products.

The second fraction from GPC consisted primarily of flavonoids (Table II) (Tatum and Berry, 1972). However, that of tangerine oil, which could not be separated as cleanly as orange oil, contained some color pigments and high molecular weight flavor compounds. The flavonoids identified from orange and tangerine oils are listed in Table II. The only qualitative difference in composition noticed was that compound 7 (5,7,4′-trimethoxy flavone) was not in orange oil. Compound 7 was identified (Tatum, 1975) as a constituent of tangerine peel, but has not previously been reported. All of the methoxy flavones reported by Tatum and Berry (1972), with the exceptions of 5-hydroxy-3,7,8,3′,4′-pentamethoxy, 5-hydroxy-5,6,7,8,3′,-4′-hexamethoxy, 3,5,6,7,3′,4′-hexamethoxy (orange), and 3,5,7,8,3′,4′-hexamethoxy flavone (tangerine) were identified by TLC as constituents of these orange and tangerine flavonoid fractions.

Because of the strong commercial interest in tangerine aroma and flavor, we studied the volatile flavor fraction of tangerine in greater detail than that of orange. The main components of the volatile tangerine fraction are listed in Table III in order of their GLC retention times. With the exception of citronelly acetate, decyl acetate, and 1,8-*p*-menthadien-9-yl acetate, these compounds have been previously identified in tangerine (Moshonas and Shaw, 1974). The components of the orange volatile fraction were not extensively studied, except for direct comparison of their GLC elution order with that of known compounds. The volatile orange flavor fraction appeared to consist of components previously reported (Shaw and Coleman, 1974).

Table III. Volatile Flavor Components of Tangerine Flavor Fraction

Myrcene	α-Sinensal
Limonene	Citronellyl acetate[a]
γ-Terpinene	Neryl acetate
Cymene	Geranyl acetate
β-Elemene	Decyl acetate[a]
Δ-Elemene	1,8-*p*-Menthadien-9-yl acetate[a]
Octanal	Octanol
Decanal	Linalool
Dodecanal	Thymol methyl ether
Neral	Thymol
Geranial	Elemol
Citronnellal	

[a] Newly reported tangerine oil component.

Thus, GPC provided a method for separating a fraction of citrus color pigments which may be useful for enhancing color of food products, a flavonoid fraction, and a flavor fraction free of high boiling materials.

LITERATURE CITED

Berry, R. E., Wilson, C. W., III, Bissett, O. W., *J. Food Sci.* **37**, 804 (1972).
CECO Marketing Consulting Research, Inc., Florida Citrus Commission, Lakeland, Fla., 1965.
Cross, J., Gabai, M., Lifshitz, A., *J. Food Sci.* **36**, 466 (1971).
Moshonas, M. G., *J. Agric. Food Chem.* **19**, 769 (1971).
Moshonas, M. G., Shaw, P. E., *J. Agric. Food Chem.* **22**, 282 (1974).
Robbins, R. C., *Int. J. Vitam. Nutr. Res.* **36**, 10 (1966).
Robbins, R. C., *Int. J. Vitam. Nutr. Res.* **45**, 163 (1975).
Schmit, J. A., Williams, R. C., Henry, R. A., *J. Agric. Food Chem.* **21**, 551 (1973).
Shaw, P. E., Coleman, R. L., *J. Agric. Food Chem.* **22**, 785 (1974).
Stewart, I., Wheaton, T. A., *Phytochemistry* **12**, 2947 (1973).
Suzuki, T., Hasegawa, K., *Agric. Biol. Chem.* **35**, 871 (1974).
Svega, A. B., "Sephadex LH-20, Chromatography in Organic Solvents", Pharmacia Fine Chemicals, Piscataway, N.J., 1973, pp 1–14.
Tatum, J. H., U.S. Citrus and Subtropical Products Laboratory, Winter Haven, Fla., 1975, private communication.
Tatum, J. H., Berry, R. E., *Phytochemistry* 11, 2283 (1972).
Ting, S. V., Hendrickson, R., *Proc. Fla. State Hortic. Soc.* **81**, 264 (1968).
U.S. Standards for Canned Orange Juice, U.S. Department of Agriculture, Consumer and Marketing Service, Washington, D.C., 1969.
Wilson, C. W., Shaw, P. E., Kirkland, C. L., Citrus Chemistry and Technology Conference, U.S. Citrus and Subtropical Products Laboratory, Winter Haven, Fla., 1974.
Wilson, C. W., Shaw, P. E., Kirkland, C. L., *Proc. Fla. State Hortic. Soc.* **88**, 314 (1975).

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Relationship of Alkane and Alkene Long-Chain Hydrocarbon Profiles to Maturity of Sweet Oranges

Harold E. Nordby* and Steven Nagy

Juice sacs from early-, mid-, and late-season orange cultivars were collected monthly over an 11-month growing period. Degrees Brix and acid values were assayed so that the maturity dates of the three cultivars could be ascertained. Long-chain hydrocarbons from the freeze-dried juice sacs were extracted with hexane and separated into alkanes and alkenes by $AgNO_3$–thin-layer chromatography. Alkane and alkene profiles were determined by gas chromatographic analyses. Relationships between the hydrocarbon profiles and the maturity dates of the three cultivars were determined.

Defining the legal maturity of citrus fruits for marketing has been important to the citrus industry. Because of the numerous varieties of citrus fruit and the differing times at which they mature, specific criteria were developed for regulating the time at which each could be legally harvested. In Florida, five standards are used to define legally mature fruit (USDA, 1969), viz. (1) color break, (2) minimum juice content, (3) minimum percentage of total soluble solids (TSS), (4) minimum acid content, and (5) total soluble solids/acid ratio (degrees Brix/acid).

Citrus fruit, unlike fruit from deciduous trees, ripen very little after being removed from the tree. Often the interior of the fruit will meet all legal requirements for maturity while the peel is still green. Under these conditions the peel, for aesthetic purposes, may legally be degreened by treatment with chemicals, e.g., ethylene. Ethanol, a metabolite in the fruit, has been shown to be quantitatively related to maturity of the fruit (Davis, 1970). The question remains unanswered as to whether the legal standards of fruit maturity can be related to chemicals other than ethanol.

We have shown that lipids extracted from citrus juice sacs contain long-chain hydrocarbons. These were primarily saturated (alkane) and monounsaturated (alkene) in nature (Nagy and Nordby, 1971). Gas–liquid chromatography (GLC) showed that these hydrocarbons might be used as markers for chemical classification of citrus fruit (Nordby and Nagy, 1974). A further study showed alkanes and alkenes to be present in the epicuticular waxes of citrus peel and leaf (Nagy et al., 1975). In these two tissues, hydrocarbon profiles changed during tissue maturation.

Citrus juice sacs are held together by an epicuticular wax which can easily be extracted with such solvents as hexane or chloroform (Fahn et al., 1974; Shomer and Ben-Gera, 1975). Preliminary studies indicated that this wax contains essentially all of the long-chain hydrocarbons reported present (Nagy and Nordby, 1971) in the juice sac. Furthermore, we determined that these long-chain alkanes and alkenes could be uniformly extracted from either freshly sectionized fruit or from freeze-dried juice sacs.

The present study was undertaken to determine whether the composition of epicuticular hydrocarbons changes

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