TLC-Spectrophotometric Analysis for Neutral Fraction Flavones in Orange Peel Juice

LYLE JAMES SWIFT

A thin-layer chromatographic system, developed to separate the flavones of the neutral fraction of benzene peel juice extracts, made possible the spectrophotometric determination of the flavone content. This method of analysis was applied to a series of neutral fractions taken over an entire fruit season. The flavones determined were tangeretin, tetra-*O*-methylscutellarein, 3,5,-6,7,8,3′,4′-heptamethoxyflavone, nobiletin, and sinensetin (5,6,7,3′,4′-pentamethoxyflavone).

In a recent paper, Swift (*3*) gave the results of an analytical study on the neutral fraction of a benzene extract of a single sample of orange peel juice. These data were obtained by a column chromatographic method that was not suitable for routine work. Since rather extensive bitterness studies on the neutral fractions were contemplated, a more practicable method of analysis was developed. Considerable effort was expended in finding a combination of thin-layer material and solvent system capable of separating the five flavones which, because of their similarity in structure, could not be expected to differ greatly in their migration rate on thin-layer plates. Fortunately, the task was made easier because all the flavones fluoresced directly under long wave (3660 A.) ultraviolet light, yielding more or less characteristic colors. The combination that worked best was a plate coated with silica gel G and a developing solution of 15% 1-butanol in hexane by volume. This system yields about eight fluorescent bands from a typical neutral fraction, but three of these have not yet been identified and were not evaluated or used in the analytical method. The known flavones that occur in the neutral fraction and for which the procedure has been developed are as follows: tangeretin (5,6,7,8,4′-pentamethoxyflavone), tetra-*O*-methylscutellarein (5,6,-7,4′-tetramethoxyflavone), 3,5,6,7,8,3′,4′-heptamethoxyflavone, nobiletin (5,6,7,8,3′,4′-hexamethoxyflavone), and sinensetin (5,6,7,3′,4′-pentamethoxyflavone).

The work described in this paper is a continuation of a study of the substances that contribute to the bitterness of orange peel (*1–6*). The neutral portion of the benzene extract of orange peel juice is one of the most important fractions in both amount and taste potency; therefore, this particular fraction was studied.

Experimental

The neutral fractions were prepared from orange peel by expression of the juice from the ground peel in a hydraulic press. The juice then was extracted with benzene as long as color was removed. After the volume of the extract was reduced to a convenient size, it was shaken with 1% sodium hydroxide to remove phenolic and other acidic substances; the process continued as long as appreciable color was extracted. The benzene of the remaining solution was evaporated. Volatile substances were eliminated by adding water and distilling. The residue was taken up in aqueous-ethanol mixture to which 5% sodium hydroxide had been added. After the mixture stood for about 2 hours to convert lactones to the corresponding acid salts, several volumes of water were added, and the neutral fraction was exhaustively extracted with benzene. The combined extract was then washed with water and evaporated. The neutral residues still retained some odor, and this was removed by adding ethanol and again evaporating to dryness.

Thin-layer plates—20 × 20 cm.—were prepared by coating with a silica gel G (Research Specialties Co.) layer 250 mμ thick, drying briefly at room temperature, and finally for an hour at 105° C. Subsequent storage in the open air seemed to give about the right activity, humidity apparently not being critical. Reference curves were constructed by preparing flavone solutions containing 20, 40, and 60 μg. in 5 ml. with absolute ethanol. The absorbances at the principal maxima of these solutions were measured in 1-cm. cuvettes and averaged with others similarly obtained (Table I).

The method of analysis consisted of spotting a solution of the neutral fraction along one side—about 1.5 cm. from the edge—of a 20 × 20 cm. silica gel plate using a 100-μl. syringe. The total amount of solution added was calculated to give a total application of 200 to 400 μg. of solute. The plate was then developed by the ascending method with a 15% solution of 1-butanol in hexane by volume. Removing the plates when the solvent front had reached the top, drying them briefly, and redeveloping them in the same solvent in order to secure sufficient zone separation were necessary. Occasionally, this had to be repeated a third time for the same reason. When inspection with the long wave ultraviolet light showed the zones to be sufficiently separated, the five zones were marked under the light and subsequently scraped separately into 15-ml. centrifuge tubes.

U. S. Fruit and Vegetable Products Laboratory, Winter Haven, Fla.

Table I. Absorbance Data for Standard Flavone Reference Curves

Flavone	Maxima at which Absorbancies Were Measured, Mμ	Log ε	Average Absorbancies in 5 Ml. of Ethanol 20 μg.	40 μg.	60 μg.
Tangeretin	323	4.49	0.348	0.697	0.985
Tetra-*O*-methyl-scutellarein	318	4.58	0.423	0.832	...
Heptamethoxy-flavone	341	4.38	0.229	0.458	0.691
Nobiletin	333	4.45	0.298	0.596	0.900
Sinensetin	328	4.43	0.317	0.627	0.932

The gel residues were then stirred with 12 ml. of acetone and centrifuged, and the acetone extract was decanted through a filter into 2 × 17 cm. test tubes. This was repeated with 5-, 5-, and 2-ml. portions of acetone, each time decanting through the same filters as before. The combined acetone solutions were then evaporated by standing the tubes in a beaker of hot water supported on a steam bath. After the residues had been taken to dryness, 5 ml. of absolute ethanol were added to each tube, the contents were shaken, and the absorbances read in 1-cm. cuvettes at the proper maximum for each compound. Occasionally, a dilution was made in order to stay within the accurate range of the instrument—in this instance a Cary Model 14 was used. The absorbances so obtained were then evaluated using the reference curves to determine the micrograms of each flavone present.

Presumably any filter that removes all the suspended gel could be used in the above operation, provided that it could be efficiently washed with the successive acetone extracts. In the present work, lengths of 1-cm. i.d. glass tubing were heated and pulled out to give constricted portions about 20 cm. apart. These were broken apart at the constrictions and midway between the constrictions to give 10-cm. sections, each constricted on one end. After being fire-polished, a pledget of glass wool was forced into each constriction and a portion of Hyflo Super-Cel added to this and pressed down firmly. Finally another pledget of glass wool was added to retain the filter aid. During the filtration, these tubes were supported in the tops of the large receiving tubes by notched or loosely fitting stoppers.

To test the accuracy of the method, two mixtures of the five flavones were made up in ethyl acetate to a definite volume. Known amounts of these mixtures were spotted on silica gel plates, and the above method was applied (Table II).

Further investigation of the validity of the method, particularly to test its internal consistency, was carried out by additional experimental work. An over-all absorption curve was first run on 50 μg. of a whole neutral fraction. A similar curve was obtained for a mixture containing the same amounts and proportions of the five flavones as did the neutral fraction itself as determined by the method. As expected, the latter composite curve maximum in the 318- to 341-mμ region showed a lower absorption than the neutral fraction, so another 50-μg. TLC neutral fraction plate was developed and the bands representing unidentified substances, not normally taken into account, were removed, eluted, evaporated, and made to 5-ml. volumes. The absorbance maxima of these bands in the 318- to 341-mμ region were added graphically to that of the composite flavones to give an over-all maximum which should approximate that of the whole neutral fraction, provided that there were no considerable amounts of absorbing substances not included in the fluorescent bands. To establish the homogeneity of the substances in each of the normally utilized zones, at least as far as fluorescent constituents were concerned, these zone materials were eluted and rechromatographed on plates coated with two different absorbants using several eluent systems with each absorbant. Polyamide resin plates were developed with 20% ethyl acetate in hexane, 2% methanol in benzene, 30% ethyl acetate in cyclohexane, and 15% acetone in cyclohexane. Silica gel plates were developed with 50% ethyl acetate in hexane, 25% benzene in ether, 50% ethyl acetate in benzene, and a mixture consisting of 50% benzene, 40% ethyl acetate, and 10% hexane. These percentages are, as usual, on a volume basis.

Results and Discussion

The average absorbances obtained on the individual compounds along with the maxima at which they were measured are given in Table I. These values were used in constructing reference curves for analytical purposes.

Table II gives the recovery data for the two test mixtures. In general the recoveries were fairly satisfactory for sinensetin and the heptamethoxyflavone, but somewhat less so for nobiletin. No reason is assigned for the

Table II. Recovery Data of Flavones in Known Mixtures

	Sample 1			Sample 2		
	Applied, μg.	Found, μg.	Recovd., %	Applied, μg.	Found, μg.	Recovd., %
Tangeretin	29.2	30.4	104.0	52.5	54.5	103.6
Tetra-*O*-methoxyscutellarein	27.8	25.0	90.0	50.0	46.3	92.7
Heptamethoxyflavone	18.4	17.5	95.2	33.1	32.0	96.7
Nobiletin	15.2	14.7	96.7	27.4	25.6	93.3
Sinensetin	17.0	16.8	98.8	30.6	29.7	97.2

Table III. Band Color under Ultraviolet and R_f Values of Flavones

Flavone	Color under UV	R_f
Tangeretin	Orange (faint)	1.00
Tetra-*O*-methylscutellarein	Pink	0.87
Heptamethoxyflavone	Blue-green	0.77
Nobiletin	Gray-blue	0.66
Sinensetin	Blue	0.43

Table IV. Per Cent Flavones in Neutral Fractions

	Dec. 1963	Jan. 1964	March 1964	Early April 1964	Late April 1964	June 1964
Tangeretin	2.9	2.7	3.2	3.3	3.3	2.8
Tetra-*O*-methylscutellarein	7.4	7.7	7.3	7.9	8.3	7.5
Heptamethoxyflavone	4.2	5.0	5.0	5.5	5.9	5.6
Nobiletin	18.6	20.3	19.3	24.0	22.1	25.5
Sinensetin	20.8	20.0	18.3	20.2	20.8	21.5
Total accounted for, %	53.9	55.7	53.1	60.9	60.4	62.9

low values for tetra-*O*-methylscutellarein and the high values for tangeretin. These substances occur in adjacent positions on the developed TLC plates, but there appeared to be no overlapping of the fluorescent bands, which seemed to be well separated, leaving no ambiguous zone between them.

Since double or triple developments were necessary to obtain adequate separation of these flavones, it was not feasible to quote R_f values. Instead it seemed logical to quote R_f values where tangeretin is given a value of 1.00 and others are expressed proportionately. In Table III, these values are given together with the color of the band under long wave (3660 A.) ultraviolet light. The colors depend somewhat on the concentration in the band and hence some variability must be expected. A little experience permits one to identify the bands with ease.

In the neutral fractions, certain other bands occurred. The most prominent of these is a brilliant blue-white band at R_f 0.55 between nobiletin and sinensetin owing to some substance not yet identified. Two other greenish bands of very low R_f usually occurred also. These are, of course, also fluorescent colors seen only under ultraviolet light.

The absorption maximum obtained by adding the maxima of the extra bands to that of the flavone composite differed from that of the whole neutral fraction by about 0.07 absorbance unit in the working range. This amounts to only about 4 to 5 μg. in terms of the known flavones and is evidence of the internal consistency of the method. It also implies that most of the neutral fraction constituents that absorb in the 318- to 341-mμ region also occur in or among the fluorescent zones themselves on the developed chromatogram. This is not to say, of course, that other absorbing substances may not occur in the bands ascribed to the five flavones. However, rechromatography of these zone substances in various TLC systems failed to reveal new fluorescent bands, so the presence of other flavones or other fluorescent substances is virtually excluded. Any other possible interfering substance would have to be nonfluorescent, absorb in the 318- to 341-mμ region, be neutral in reaction, and migrate chromatographically with the flavones. These limiting requirements make the occurrence of such an interfering substance very unlikely.

Table IV gives the flavone analyses for neutral fractions isolated from peel juices during a fruit season. Each value is an average of two determinations. The percentage of nobiletin in the neutral fractions showed a general upward trend as the season advanced, as did the total flavone percentage but the other flavones maintained a remarkable constancy in spite of the fact that these fractions came from a number of varieties. In December, only Hamlin and Parson Brown oranges are generally available while, in January, these as well as Pineapple and Seedlings are usually processed. During the March through June period, Valencias are used almost exclusively. In general, the analyses agree well with the results obtained by column chromatography in an earlier study (*3*).

Further work is in progress on the flavor potency of the flavones and their contribution to the neutral fraction flavor.

Literature Cited

(1) Swift, L. J., J. AGR. FOOD CHEM. **9,** 298 (1961).
(2) *Ibid.*, **13,** 282 (1965).
(3) *Ibid.*, p. 431.
(4) Swift, L. J., *J. Food Sci.* **29,** 766 (1964).
(5) Swift, L. J., *J. Org. Chem.* **25,** 2067 (1960).
(6) *Ibid.*, **30,** 2079 (1965).

Received for review September 6, 1966. Accepted November 3, 1966. The U. S. Fruit and Vegetable Products Laboratory is a laboratory of the Southern Utilization Research and Development Division, Agricultural Research Service, USDA. Reference to a company or product name does not imply approval or recommendation of the product by the USDA to the exclusion of others that might be suitable.