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QUANTITATIVE STUDIES ON FLAVANONE GLYCOSIDES OF THE GRAPEFRUIT

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RICHARD E. HAGEN

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QUANTITATIVE STUDIES ON FLAVANONE

GLYCOSIDES OF THE GRAPEFRUIT

APPROVED BY:

Lyman H. Wender

Leon S. Cieszko

Robert E. Coleri

J. C. Colbert

H. A. Rowley

DISSERTATION COMMITTEE

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CHAPTER I

INTRODUCTION

For many years naringin was thought to be the only bitter flavonoid present in grapefruit, Citrus paradisi. This compound was first isolated in 1857 by De Vry (1) from the flowers of the shaddock, Citrus grandis, of which the grapefruit is thought to be a mutant. During the period 1928 to 1939, the combined efforts of several groups of workers established that naringin was the 7-rhamnoglucoside of naringenin (4', 5, 7-trihydroxyflavanone) (2,3,4,5). In 1963 the rhamnosylglucose portion of the molecule was shown to be identical with neohesperidose, the structure of which was found to be 2-O-α-L-rhamnopyranosyl-D-glucose (6). This information, together with the finding that partial hydrolysis of naringin yielded naringenin-7-β-glucoside (7,8), makes it possible to give the complete structural formula of naringin as naringenin-7-β-neohesperidoside.

Recently, two other bitter flavanone neohesperidosides have been found to occur in small amounts in the grapefruit. These are neohesperidin (7-β-neohesperidoside of hesperetin: 3',5,7-trihydroxy-4'-methoxy-

flavanone (9), and poncirin (7-β-neohesperidoside of isosakuranetin: 5,7-dihydroxy-4'-methoxyflavanone) (10). The structural formulas of the flavanone neohesperidosides are given in Figure 1.

Three tasteless flavanone glycosides have been found to occur in the sweet orange, Citrus sinensis. These are naringenin-7-β-rutinoside (11), hesperidin (12) (7-β-rutinoside of hesperetin), and isosakuranetin-7-β-rutinoside (11). Rutinose, the rhamnosylglucose portion of these compounds, has the structure 6-O-α-L-rhamnopyranosyl-D-glucose (13,14). The structure of these rutinosides are given in Figure 2.

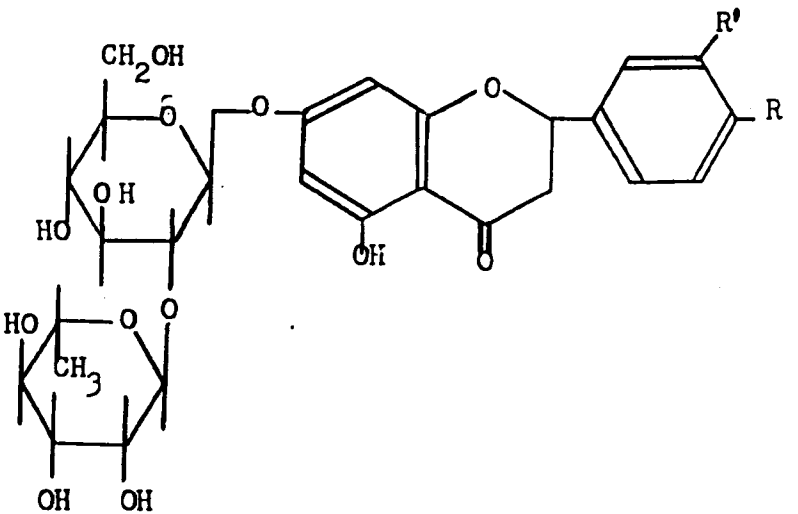
The important correlation between structure and the taste of these flavanone glycosides was made by Horowitz (6) when he proved that the linkage of rhamnose to glucose in the neohesperidosides is 1→2, whereas in the rutinosides the linkage is 1→6. Hence the difference in taste between naringin, neohesperidin, and poncirin, which are extremely bitter, and their tasteless isomers, naringenin-7-β-rutinoside, hesperidin, and isosakuranetin-7-β-rutinoside, can be attributed to the point of attachment of rhamnose to glucose in the sugar moeity.

In subsequent studies on the grapefruit, it was found that small amounts of rutinose, along with a preponderance of neohesperidose, were present in ozonolysis products of unpurified extracts of grapefruit peel flavonoids (15). An obvious implication of this finding was that non-bitter flavanone rutinosides might exist in the grapefruit. No findings of intact flavanone rutinosides in the grapefruit had been reported, however, at the time of initiation of this research (15,16).

A review of the published methods for quantitative determination of naringin revealed that no method had been shown to be specific for

FIGURE 1

BITTER FLAVANONE NEOHESPERIDOSIDES
FOUND IN THE GRAPEFRUIT



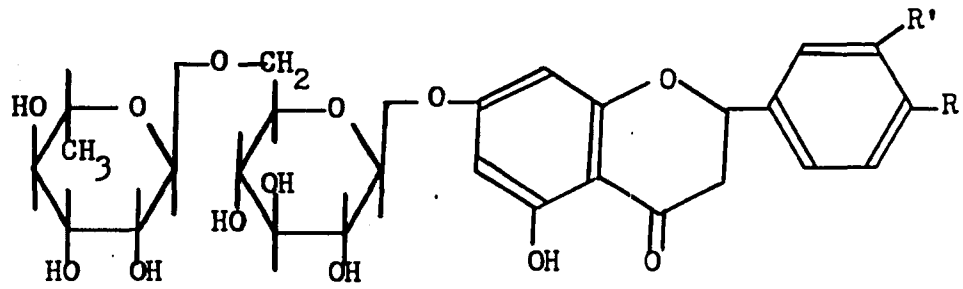
Naringin: R = OH, R' = H

Neohesperidin: R = OCH_3, R' = OH

Poncirin: R = OCH_3, R' = H

FIGURE 2

TASTELESS FLAVANONE RUTINOSIDES FOUND
IN THE ORANGE AND THE GRAPEFRUIT



Naringenin-7-β-Rutinoside: R = OH, R' = H

Hesperidin: R = OCH_3, R' = OH

Isosakuranetin-7-β-Rutinoside: R = OCH_3, R = H

individual flavanone glycosides. At best, the existing methods could be expected only to give values for total flavanone glycoside content.

The discovery of the minor flavanone neohesperidosides in the grapefruit, and the possibility that the grapefruit might contain nonbitter flavanone rutinosides clearly indicated that a re-evaluation of the quantitative methods used to determine naringin was needed. This conclusion was strengthened by the fact that the citrus industry had found that the naringin content of juice, as determined by the available chemical tests, did not correlate with bitterness as determined by taste panels (17).

With the co-operation and support of the U. S. Department of Agriculture, an intensive study was undertaken to develop a new method for the determination of certain individual flavanone glycosides in grapefruit.

In the course of the development of a quantitative method, the important finding was made in this laboratory that "naringin" from Ruby Red grapefruit juice sacs contained significant quantities of tasteless naringenin-7-β-rutinoside (18). Hesperidin and isosakuranetin-7-β-rutinoside, the tasteless isomers of neohesperidin and poncirin, respectively, were also found in the grapefruit. It was thus evident that the quantitative method would need to include procedures for measuring the rutinosides in addition to the neohesperidosides if meaningful information on the biosynthesis and metabolic fate of these compounds in the grapefruit and their role in regard to the taste of grapefruit products were to be obtained.

Although the accuracy and usefulness of some of the older methods

of naringin determination have been discussed elsewhere (19,20), a summary of the available methods will be given here. Poore (21) measured naringin gravimetrically after precipitation with ferric chloride. This direct method of analysis was not reliable since the reagent was not specific for flavonoids. Most other authors subsequently turned to indirect methods of measurement since no suitable separation procedures were available. For example, Harvey and Rygg (22) utilized the red color formed by interaction of naringin with ferric chloride in a colorimetric procedure. Citric acid, however, was found to interfere in color formation.

Hendrickson, Kesterson, and Edwards (17) developed a spectrophotometric method for naringin which measured absorption at 285 mμ under standardized conditions. However, most naturally occurring flavanone glycosides have absorption peaks near 285 mμ, and it is probable that certain other flavonoids absorb in this region also.

Hörhammer and Wagner (20) introduced a colorimetric method based upon the formation of the 2,4-dinitrophenylhydrazone of naringin. The reagent used in this method appears to distinguish between flavanone glycosides and flavonols since Douglass, Morris, and Wender (23) found that flavonols did not react with the reagent under the conditions used. However, it is probable that an ethyl acetate extraction step in the procedure is not quantitative.

The Davis test (24) is the most widely used method for naringin determination because it is convenient and results obtained are no less reliable than those obtained by other previously available methods. This test measures the intensity of the yellow color produced by naringin and other naringenin-7-glycosides in an alkaline-diethylene glycol solution.

While certain other 4'-hydroxy-7-alkoxy or 4'-hydroxy-7-glycosidoxyflavanones may also give this yellow color under Davis test conditions, the hesperetin and isosakuranetin glycosides probably are not measured by this test (25, 26). Although the Davis test appears to be somewhat specific for naringin and naringin-like compounds, naringin and naringenin rutinoside cannot be distinguished from each other, nor can the minor flavanone glycosides be detected by this method.

While none of these methods is specific for naringin, it is possible that they may give a fair estimate of the total flavanone glycoside content. The Davis test has been used extensively in studies of flavanone glycoside content of various parts of the grapefruit at different stages of maturity. Mature grapefruit parts were found to contain flavanone glycosides in the following descending order of concentration: core, albedo (spongy white part of the peel), segment membranes, flavedo (outer pigmented portion of the peel), juice vesicles, and the juice (24,27). In comprehensive studies Hendrickson and Kesterson (28,29) found that the naringin content per whole fruit increased until the diameter of the fruit was about 2 inches. As the fruit matured on the tree for the next 10 months, the flavanone glycoside content decreased in concentration per unit weight, but the total naringin per whole fruit remained essentially constant. This decrease in flavanone glycoside concentration was attributed to dilution.

It should be noted that fruit 0.5 inch in diameter were reported to contain up to 75% flavanone glycoside on a dry weight basis. The percentage gradually decreased until it reached 2 to 3% at maturity (28). In view of the large quantities present, it was suggested that flavanone glycosides may play an important biological role in the developing fruit.

No direct evidence has been reported which supports this suggestion.

It was apparent that the basic problem to be encountered in the development of a quantitative method for determination of individual flavanone glycosides was to find appropriate isolation and separation procedures. The development of the quantitative method was therefore divided into three stages: preliminary isolation of the flavanone glycoside fraction by column chromatography, further isolation and purification of the individual flavanone glycosides by paper or thin-layer chromatography, and final measurement of the individual compounds.

For the column chromatography step, polyamide or ion exchange adsorbents gave the most promise since, using them, flavonoids had been separated directly from aqueous plant extracts (30,31,32). Eventually, in the research reported here, polyvinylpyrrolidone adsorbent was found to give somewhat better separations of components in grapefruit extracts than polyamide or ion exchange adsorbents.

Considerable effort was expended on development of solvent systems to use for paper chromatography since no previously available systems were capable of separating these closely related compounds (33,34,35). The use of non-aqueous solvents for polyamide thin-layer chromatography was suggested by Albach (36). Use of this chromatographic technique revealed that all six known flavanone glycosides of the grapefruit could be separated completely from a mixture. This system was then adapted to a quantitative method.

The method of measurement of the individual flavanone glycosides was adapted from a fluorescence procedure developed earlier in this laboratory (37). The method had to be modified slightly to accommodate

compounds obtained from thin-layer chromatograms.

Chapter II presents a new, specific and sensitive method for individual determination of naringin, naringenin-7-rutinoside, poncirin, isosakuranetin-7-rutinoside, neohesperidin, and hesperidin in grapefruit juice or juice sacs. The procedure involves a preliminary quantitative isolation of the desired flavanone glycosides from juice or juice sacs by polyvinylpyrrolidone column chromatography, further separation and purification of these glycosides by thin-layer chromatography, and quantitative determination by fluorescence analysis of each individual flavanone glycoside after its removal from a thin-layer chromatogram.

Chapter III is devoted to studies on the effect of maturity on the flavanone glycoside concentration in the juice sacs of early and mid-season Ruby Red grapefruit.

Chapter IV gives the results of related studies on the chromatography of flavanone glycosides.

CHAPTER II

A CHROMATOGRAPHIC-FLUOROMETRIC METHOD FOR DETERMINATION OF NARINGIN, NARINGENIN RUTINOSIDE, AND RELATED FLAVANONE GLYCOSIDES IN GRAPEFRUIT JUICE AND JUICE SACS

Detailed Steps of the Analytical Method

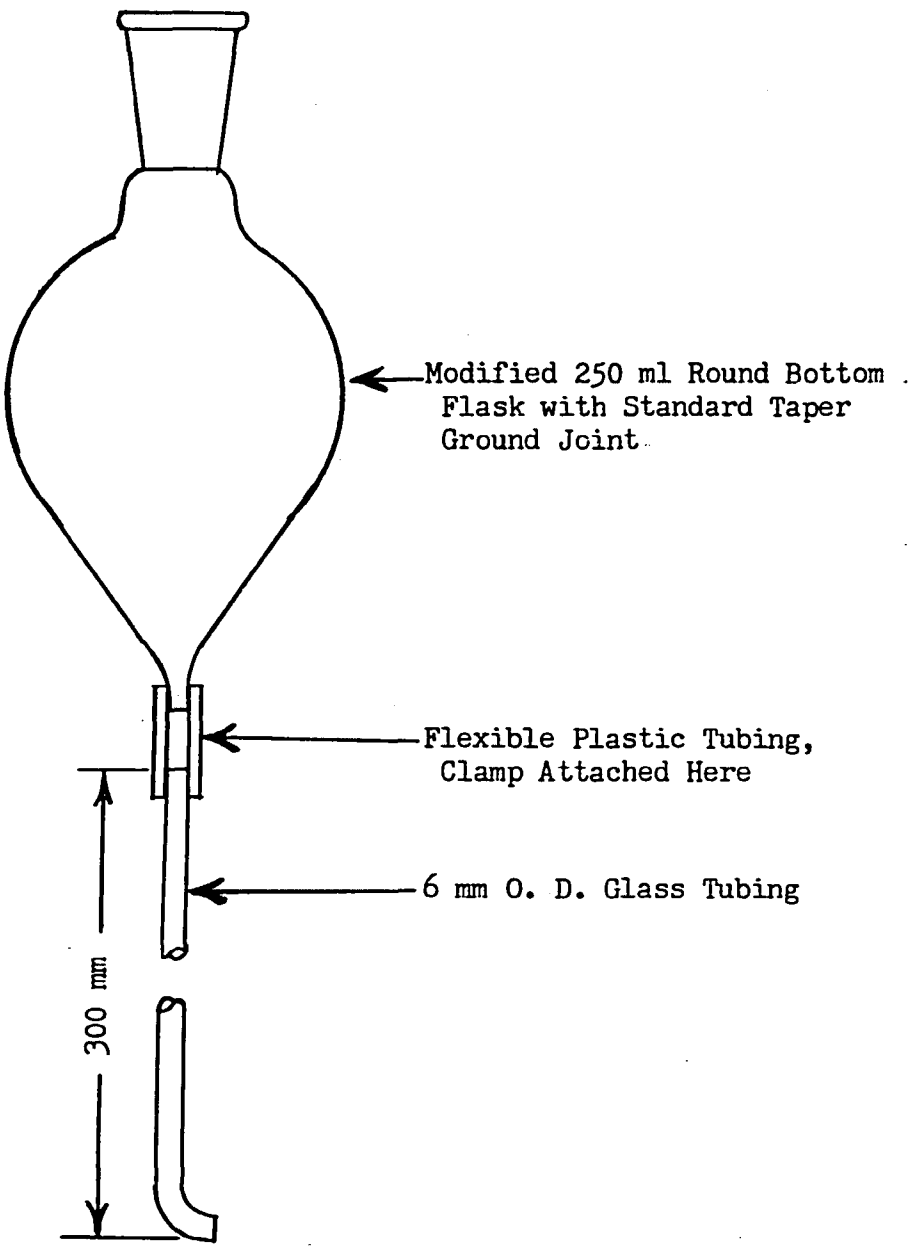
Preparation of Samples for Column Chromatography

Canned grapefruit juice is sampled by mixing 20 ml of juice with an equal volume of hot methanol, stirring the preparation, and allowing it to stand for several minutes until the suspended solids coagulate and settle. The juice-methanol mixture is filtered under reduced pressure through a medium porosity sintered glass filter into a 250 ml special delivery flask (Figure 3). The residue is washed twice with 20 ml hot methanol and 3 times with 20 ml hot 2-propanol. The combined filtrate and washings are then reduced in vacuo to a volume of 5-7 ml at 45°C in the special delivery flask on a rotary evaporator. The resulting aqueous liquid is then applied onto the chromatographic column.

Grapefruit juice sacs (segments with the carpel wall and the seeds removed) are worked up by macerating fresh or partially frozen sacs in a blender. The puree is mixed with an equal volume of hot 2-propanol and the mixture is brought to a boil to denature the enzymes and to coagulate the solids. Filtration and washing are done essentially as described in the above paragraph , except that the solids are macerated a second time before the three final washes are made. A volume of hot solvent equal to that of the original puree is used for each wash. For about 300 to 1000 g

FIGURE 3

FLASK WITH DETACHABLE TUBE FOR CONCENTRATION OF JUICE OR JUICE SACS FILTRATES AND DELIVERY OF THE CONCENTRATES TO A CHROMATOGRAPHIC COLUMN



of fresh sample, the combined filtrate and washings are concentrated to about 300 ml. This is then diluted to 1000 ml in a volumetric flask, by adding sufficient quantities of 2-propanol and water to give a final 3:2 ratio of these solvents. For column chromatography, an aliquot equivalent to 10-18 g wet weight of sacs is transferred from the stock solution to the delivery flask and evaporated to 5-7 ml as described above.

Column Chromatography of Sample Concentrates

Columns are prepared by soaking 10 g of Polyclar AT (General Aniline and Film Corp., Grasselli, N. J.) in 125 ml of distilled water for 1.5 hr, and then packing this slurry, under 5 lb pressure, into a 22 mm I.D. column equipped with a stopcock and 300 ml reservoir. Columns of 12-13 cm height are thus obtained. If any fluorescent material is in the Polyclar, the column is washed with 50% aqueous methanol or with N,N-dimethylformamide-acetic acid-water-methanol solution (1:2:6:4, v/v/v/v). All wash liquid is eluted from the adsorbent with distilled water; 1 mm water is left above the surface of the adsorbent.

Aqueous concentrate of juice or juice sacs (5-7 ml) is added to the column through the delivery flask with tube attached. The delivery flask is rinsed thoroughly with the following sequence of solvents: 3 ml hot distilled water; 3 ml hot 2-propanol; and 3 ml hot water. After each portion has been added to the column, air is forced through the tip of the delivery tube which is positioned near the surface of the liquid. The moving air should be applied until the surface liquid comes to room temperature. A stirring rod is used to mix the upper 1 cm of the Polyclar with the liquid to prevent formation of a gummy layer. After the stirring

rod tip is rinsed with a few drops of methanol, pressure is applied to the column to force the combined concentrate and wash mixture onto the adsorbent. The sides of the column are rinsed twice with individual 5 ml portions of cold distilled water applied through the delivery flask.

Fraction collection is begun after the final rinses are forced onto the column. The column is eluted under 5 lb pressure; the following solvent sequence is used: 125 ml distilled water; 350 ml 25% methanol in water; and 150 ml pure methanol. The fractions obtained and their principal contents are given in Table 1. Complete elution of naringin (fraction 4) from the column is accomplished in about 5 hr. Fractions 2 and 5 are collected to check the completeness of poncirin and naringin elution, respectively, but are not otherwise used in the analysis

Fractions 3 and 4 are reduced to dryness on a rotary evaporator under reduced pressure at 45°C, then transferred in warm methanol to 50 ml beakers, and again evaporated to dryness. Fraction 3 is transferred to a 5 ml volumetric flask and fraction 4 is transferred to a 10 ml volumetric flask. Methanol is the solvent employed.

Thin-Layer Chromatography of Column Fractions

Components of fractions 3 and 4 from the column are further separated by chromatography on thin layers of Polyamide Woelm (Alupharm Chemicals, New Orleans, La.). Plates are prepared from a slurry of one part polyamide in 7.5 parts benzene-methanol (2:3, v/v). A commercial applicator adjusted to give a thickness of 250μ is employed. Since the benzene-methanol solvent may attack plastic, a metal alignment tray is used.

All thin-layer analyses are done in triplicate on 20 x 20 cm plates. Three applications of unknown and three applications of standard are used.

TABLE 1

FRACTIONS OBTAINED IN THE COLUMN CHROMATOGRAPHY PROCEDURE

Fraction	Volume	Eluting Solvent	Should Contain
1	65 ml	H_2O	Sugars and many other compounds.
2	25 ml	H_2O	Mostly blue fluorescing compounds.
3	125 ml	Mostly 25% CH_3OH	All of the isosakuranetin glycosides, some blue fluorescing compounds and some unknown flavonoids.
4	260 ml	25% CH_3OH	All of the naringin and naringenin rutinoside, neohesperidin, and hesperidin, plus some unidentified compounds.
5	150 ml	100% CH_3OH	Unknown compounds.

In naringin and naringenin rutinoside determinations, 10 μl aliquots of fraction 4 concentrate are spotted alternately with 5 μg naringin standards at 2 cm intervals. Spotting is done with Kirk design micro pipettes. Neohesperidin and hesperidin are determined by using 50 μl aliquots of fraction 4 concentrate and 5 μg zones of naringin standard, whereas poncirin and isosakuranetin rutinoside are determined by using 50 μl applications of fraction 3 concentrate and 5 μg zones of poncirin standard. A current of cool air is applied to keep the zones of application less than 7 mm diameter.

Chromatograms are developed at approximately 20-23° C in a nitromethane-methanol solution (5:2, v/v) in an ordinary rectangular thin-layer chromatography jar with a filter paper liner. Development time is 1.5 hr for chromatograms of the naringenin and hesperetin glycosides and 1.0 hr for chromatograms of isosakuranetin glycosides. Use of a paper pad attached to the top of the plate permits continuation of development after the solvent has reached the top of the plate. Better separation of flavanone neohesperidosides from the corresponding rutinosides is thus achieved. Approximate R_f values from a chromatogram developed 40 min are: isosakuranetin rutinoside, 0.67; poncirin, 0.60; hesperidin, 0.50; neohesperidin, 0.43; naringenin rutinoside, 0.37 and naringin, 0.30.

Removal of Zones from the Thin-Layer Chromatograms

The chromatograms are allowed to dry at room temperature for about 10 min after removal from the development jar. Fluorescent zones are located under long wavelength ultraviolet light after exposure of the chromatograms to ammonia vapors or after spraying lightly with 1% $AlCl_3$

in methanol. The zones are outlined by dotting the surface of the adsorbent with a dissecting needle. The area of adsorbent removed for subsequent elution with 10 ml volumes should be twice that removed for 5 ml volumes if both elution volumes are to be compared against the same standard. A blank zone of appropriate dimensions is marked at about the same mobility as the glycoside to be determined.

The outlined zones are removed with a microvacuum cleaner apparatus (Figure 4) similar in design to those of Černý, Joska, and Labler (38) and Millett, Moore, and Saeman (39). A 1 cm diameter circle of glass fiber (cut from a Cambridge filter, Phipps and Bird, Inc., Richmond, Va.) is pushed into one end of the vacuum cleaner until it seats firmly. A vacuum line from a water aspirator is attached to the filter end of the cleaner, and the adsorbent containing the desired sample is swept from the plate into the cleaner chamber. The vacuum line is then disconnected and a small diameter polyethylene tube is inserted into the filter end of the cleaner.

The cleaner is then fitted, with the filter end down, into a 1-hole rubber stopper which seals the top of a specially designed glass vacuum cylinder which is part of the elution assembly shown in Figure 5. The polyethylene tube in the cleaner is placed inside the neck of a volumetric flask contained in the vacuum cylinder. For neohesperidin, hesperidin, and isosakuranetin rutinoside, a 5 ml volumetric flask is used, while a 10 ml flask is used for the naringenin glycosides and poncirin. Pressure in the vacuum cylinder is then reduced to about 350 mm. The intake end of the cleaner is tapped to remove the loose powder, then removed and inverted with the tip held inside the outer

FIGURE 4

MICROVACUUM CLEANER FOR QUANTITATIVE REMOVAL OF SAMPLE ZONES FROM THIN-LAYER CHROMATOGRAMS

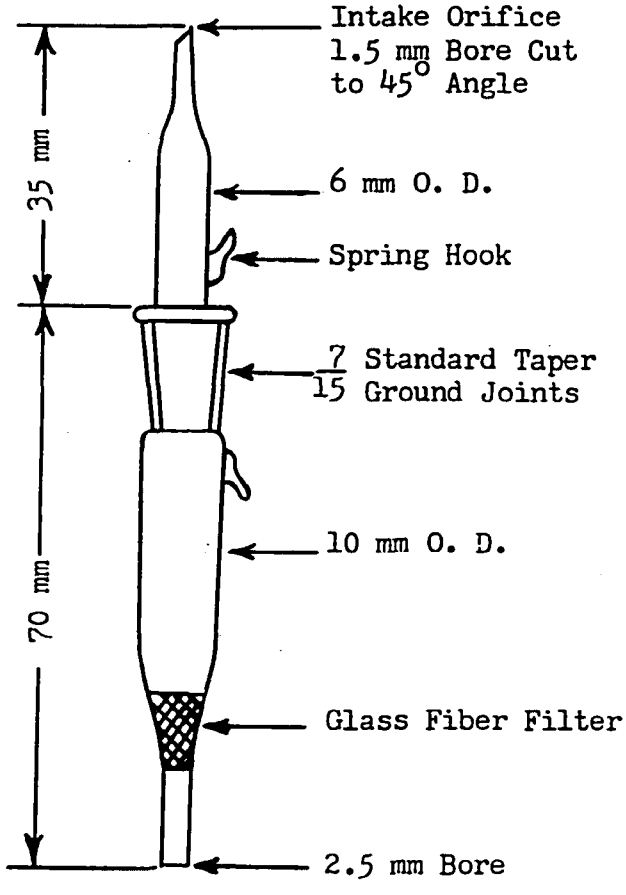
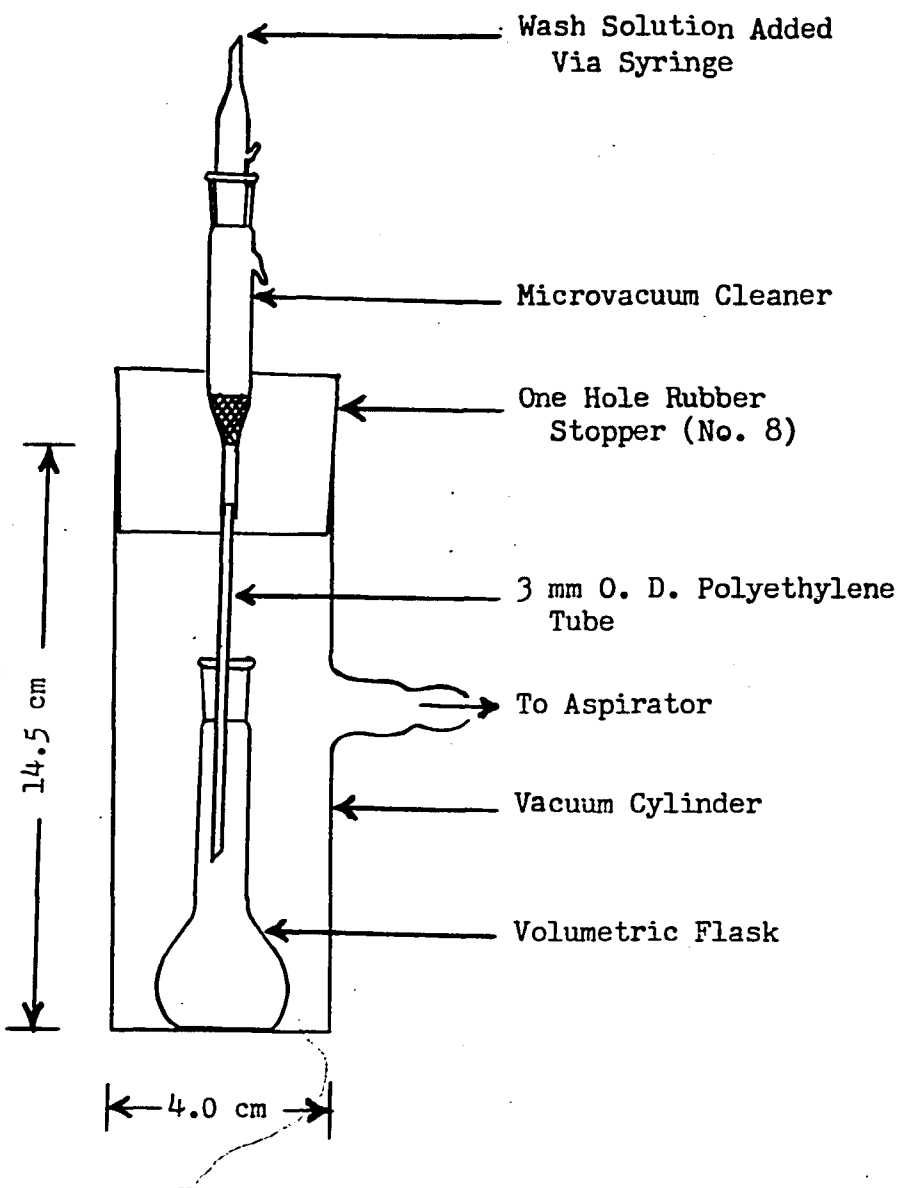


FIGURE 5

ELUTION ASSEMBLY FOR QUANTITATIVE RECOVERY
OF FLAVANONE GLYCOSIDES FROM A
MICROVACUUM CLEANER



joint of the filter end. The intake is rinsed with several milliliters of methanol delivered from a syringe and the flavanone glycoside is eluted from the adsorbent with warm methanol. When the receiving flask is nearly filled with methanol solution, the vacuum is released and the contents are allowed to equilibrate to room temperature. Solutions from chromatograms not sprayed with $AlCl_3$ may be stored until fluorescence determination is convenient, but solutions from sprayed chromatograms should be prepared immediately after elution for fluorescence determination since fluorescence decay begins within 3 hr after formation of the flavanone-$AlCl_3$ complex.

Fluorescence Determination

When readings are to be taken, 50 µl of 1% $AlCl_3$ in methanol is added to each of the nearly filled volumetric flasks which contain either flavanone glycoside solution or the blank, and the solutions are brought to volume with methanol. The solutions are shaken and then allowed to stand about 1 hr to develop maximum fluorescence intensity. For best results, intensities should be read between 1 and 3 hr after addition of $AlCl_3$.

Fluorescence determinations are made with a Turner Fluorometer, Model 110, equipped with a standard cuvette door (G.K. Turner Associates, Palo Alto, Calif.). Pyrex cuvettes (12 x 75 mm) are used in all determinations. An activation wavelength of 325 $\pm$ 2 mµ is isolated from an ultraviolet phosphor lamp (Westinghouse FS4T5, Turner No. 110-855) by an interference filter (Baird Atomic, Inc., 33 University Road, Cambridge, Mass.). A Wratten No. 2A-12 filter, which passes all wavelengths greater than 500 mµ is used as secondary emission filter.

The instrument is corrected with a blank which has been carried through the thin-layer step; then, fluorescence readings are made in pairs concisting of a sample and a standard. In order to minimize error in individual fluorescence dial readings, the average of three readings for each sample is used. Within the concentration range of 0-3.0 μg/ml, the fluorescence intensity is a linear function of flavanone glycoside concentration. Therefore, the average value from the triplicate sample zones can be compared directly with the average value of the triplicate 5 μg standards.

Appropriate corrections in the calculations should be made for fluorescence yield differences between the standard used and the compound to be determined. Based on studies of $AlCl_3$ complexes of purified compounds in methanol solution (1 μg/ml) the following relative fluorescence yields have been used in the calculations; naringin, 1.00; naringenin rutinoside, 1.08; poncirin, 1.24; neohesperidin and hesperidin, 0.44. Sufficient pure isosakuranetin rutinoside was not available for comparison. For the present calculations, it is assumed that it probably has the same fluorescence yield as poncirin.

Studies Relating to the Precision and Accuracy of the Method

The precision of the method was studied by performing the complete analysis on portions representing 16.4 g of juice sacs from a stock solution prepared from a single Texas Ruby Red grapefruit. Table 2 gives results of six successive analyses of naringin, naringenin rutinoside and poncirin.

TABLE 2

ANALYSES FOR FLAVANONE GLYCOSIDES IN SIX IDENTICAL SAMPLES FROM GRAPEFRUIT JUICE SACS

Sample	μg of flavanone glycoside/g of juice sacs (wet weight)		
	Naringin	Naringenin Rutinoside	Poncirin
1	607	276	23.2
2	606	272	24.3
3	597	272	23.7
4	589	266	23.8
5	592	286	25.0
6	598	273	24.0
Mean	598	274	24.0
Standard Deviation (%)	1.2	2.4	2.5

Another study was made to ascertain whether naringin, when added to a whole juice sac concentrate, could be quantitatively detected by the analytical method. Accordingly, 2.12 mg of naringin was added to an aliquot of the Ruby Red juice sac solution, which from the previous studies had been found to contain 9.75 mg naringin, and the enriched solution was then subjected to analysis. The naringin found was 102% of the calculated value.

Application of the Method

To illustrate the use of the method a complete analysis of all six flavanone glycosides was made on a stock solution of juice sac material from 28 Ruby Red Texas grapefruit harvested in late December, 1963. Results from this analysis and a similar analysis of a Texsun brand canned grapefruit juice sample (Code 3008) are presented in Table 3.

Discussion

The quantitative method described here is capable of relatively precise and accurate determination of the individual rutinosides and neohesperidosides of naringenin, isosakuranetin, and hesperetin which are present in grapefruit juice and juice sacs. No previously described method appears to approach the capability of this method, particularly in regard to specificity.

A key step in the present method is the quantitative preliminary isolation of the flavanone glycosides from most of the other components of the exceedingly complex mixture in grapefruit juice and juice sacs. Horowitz has noted that conventional procedures for isolating flavonoids from citrus usually have little quantitative significance since minor,

TABLE 3

ANALYSES FOR FLAVANONE GLYCOSIDES IN JUICE SACS OF TEXAS RUBY RED GRAPEFRUIT AND IN TEXSUN BRAND CANNED GRAPEFRUIT JUICE

Compound	Concentration in Juice Sacs (μg/g, wet weight)	Concentration in juice (μg/ml)
Naringin	546	306
Naringenin rutinoside	210	124
Poncirin	20.6	17.0
Isosakuranetin rutinoside	6.4	5.3
Neohesperidin	24.6	10.5
Hesperidin	13.0	9.9

and even major components are often lost in the process (15). The preliminary isolation procedure, including column chromatography, used in the method described here does not suffer from this shortcoming. Examination of solid residues remaining after preparation of juice or juice sac concentrates for application to columns indicates that all detectable flavanoids present in the original sample are included in the concentrates. Known quantities of pure naringin were quantitatively eluted when subjected to chromatography on Polyclar AT columns, employing the procedure and elution sequences used in the quantitative method, and were accounted for when added to juice sac concentrates carried through the procedure. These results show that no irreversible adsorption occurs on the columns. Other studies showed that no significant quantities of material are lost in the transfers required in the procedure. The elution pattern was found to be identical for two different lots of Polyclar AT.

Although the main function of the column chromatography step is to separate quantitatively the flavanone glycosides which are to be determined from most of the other grapefruit components, this procedure also achieves certain flavonoid separations of importance to the accuracy of the method. With samples investigated, the elution sequence employed resulted in the inclusion in fraction 3 of unknown flavonoid compounds with mobilities on thin-layer chromatograms practically identical to those of naringin and neohesperidin. These compounds are easily separated from the isosakuranetin glycosides of fraction 3 in the subsequent thin-layer chromatography step. However, if they were not separated from fraction 4 by the column chromatography step they would remain mixed with naringin and neohesperidin during thin-layer chromatography.

The result would be slightly high values for the latter two compounds.

The polyamide thin-layer chromatography procedure gives good separation of the desired, individual flavanone glycosides from each other and from the contaminating substances present in fractions 3 and 4. Extensive further thin-layer chromatography of the naringenin glycosides and neohesperidin obtained by such thin-layer chromatography has revealed no detectable impurities in these compounds. It is possible that small quantities of unknown flavonoids may remain mixed with these compounds as well as with the isosakuranetin glycosides and hesperidin on thin-layer chromatograms, but if so, the quantities are probably so low as to be insignificant.

Certain unidentified blue fluorescing impurities do remain mixed with the isosakuranetin glycosides and hesperidin after the thin-layer chromatography step. However, it appears likely that in most cases the selectivity afforded by the specific activating wavelengths employed and the emitted wavelengths measured in the fluorometric analysis virtually eliminates interference by these impurities. Addition of blue fluorescing impurities, isolated from the isosakuranetin glycosides, caused no alteration in the fluorescence intensity produced by these glycosides as determined on the Turner Fluorometer. With but one exception the $AlCl_3$ complexes of the flavanone glycosides obtained in the thin-layer chromatography step of the analyses described herein gave only a single fluorescence peak at 520 mμ on an Aminco Bowman spectrophotofluorometer when activated at 325 mμ. This was true even though considerable impurity was mixed with several of the glycosides. The exception was isosakuranetin rutinoside from Texsun canned grapefruit juice which showed a fluorescence peak at 420 mμ as well as the

major peak at 520 mμ; hence the reported concentration in this instance may be slightly above the true value. In most cases, however, it is probable that the quantitative data obtained will be negligibly affected by such impurities.

Because of the very similar mobilities of the isomeric flavanone rutinoside and neohesperidoside pairs on the polyamide thin-layers, good separation of such pairs of compounds is achieved only if concentrations of individual flavanone glycosides in the applied sample do not exceed 12 μg. The volume of sample spotted is adjusted accordingly.

Virtually no loss of material is encountered when pure naringin standards are eluted from the thin-layer chromatograms in the usual manner. Internal standards have been carried through the thin-layer step, however, to ensure maximum accuracy, since some variations in different lots of Woelm polyamide have been observed.

The precision of the method is relatively good, considering the complexity of the material being sampled. Although precision studies on isosakuranetin rutinoside and the hesperetin glycosides have not been made, reproducibility of results for these compounds should be of the same magnitude as that obtained for poncirin. Limited observations indicate that the major source of precision error is in the thin-layer and fluorescence steps.

In the fluorescence analysis procedure employed, a wavelength of 325 mμ is the optimum activating wavelength for the flavanone glycoside $AlCl_3$ complexes (37). The method was developed however, using an activating wavelength of 313 mμ because, at the time the studies were made, no lamp capable of producing sufficient energy in the 325 mμ

region was available for the Turner fluorometer. The results given in Tables 2 and 3 were obtained with an activation wavelength of 313 mμ which was isolated form a far ultraviolet lamp (General Electric G4T4/1, Turner No. 110-851) by a composite filter consisting of a Corning No. 7-54 filter and a fresh Wratten No. 34-A filter. Studies of the fluorescence of the samples on the Aminco-Bowman spectrophotofluorometer showed that activation at 313 mμ resulted in the same fluoresence spectra as found at an activation of 325 mμ. On the Turner fluorometer, however, the very low intensity 313 mμ line produced by the G4T4/1 lamp necessitated the use of a high sensitivity attachment (Turner No. 110-865). This attachment was not needed when the ultraviolet phosphor lamp was used since this lamp has a relatively high energy yield in the 325 mμ region.

The use of the ultraviolet phosphor lamp (Westinghouse FS45T5) in combination with the 325mμ interference filter has important advantages over the original combination of lamp and filters. Elimination of the unstable Wratten No. 34-A gelatin filter was found to increase considerably the repeatability when reading samples; thus, the precision of the fluorescence analysis was probably improved. In addition a much greater sensitivity was possible. Thus more reliable data can be obtained for hesperidin and isosakuranetin rutinoside, which are present in trace amounts.

This analytical method should be of considerable value in studies in which the quantitative distribution of individual flavanone glycosides in grapefruit is needed; it should find particular application in research studies on the seasonal changes of flavonoids in grapefruit and on the correlation between bitterness of grapefruit and flavanone

glycoside content. Only minor modifications should be required for application of the method to analysis of other parts of grapefruit, and to determination of other flavonoids present in this fruit. The method is somewhat involved for routine analysis, but it should be useful as a criterion for comparing the accuracy of routine methods which may be developed later.

CHAPTER III

STUDIES ON THE EFFECT OF MATURITY ON FLAVANONE GLYCOSIDE CONTENT OF GRAPEFRUIT JUICE SACS

Methods

A study of seasonal changes in flavanone glycoside content was conducted on juice sacs obtained from the fruit of seven Ruby Red grapefruit trees grown at the Texas Agricultural Experiment Station, Weslaco, Texas. These trees were sprayed in June with a 1.0% paraffinic oil emulsion and again in September with a 1.6% emulsion. Sample fruit were harvested on the 28th day of each month, September through January of the 1964-1965 growing season. One fruit was picked each month from the north, south, east, and west sides of each tree; a total of 28 fruit were thus obtained for each month's sample. A somewhat less representative sample consisting of 16 very green fruit picked from the same seven trees was obtained in late July, 1964. Upon completion of picking a given sample, the fruit were shipped immediately to the biochemistry laboratories at the University of Oklahoma where all subsequent work was performed.

When each shipment of fruit arrived at the University of Oklahoma laboratories, the fruit were scrubbed in water and dried. Weights and diameters for individual whole fruit were recorded. The peel (albedo and flavedo)and core were removed by hand from the segments which con-

sisted of the juice sacs, segment walls, and seeds. Segment walls and seeds were carefully removed from the juice sacs of four segments from each fruit. The juice sacs from four segments from each of four fruit were then combined into tared polyethylene bags, weighed, and quick frozen in powdered dry ice. The peels and cores, segment walls, and remaining whole segments were also individually weighed before quick freezing. All grapefruit parts were stored at -10°C until needed for further study.

Juice sac stock solutions were prepared from the combined sacs obtained from one shipment of fruit as outlined in the procedure given in Chapter II.

Soluble solids determinations were carried out on juice sacs stock solutions following a standard procedure for moisture determination (40). An aliquot of stock solution containing about 1 g of solids was mixed with 30 g washed and dried 40-60 mesh sand. The excess liquid was removed by evaporation on a steam bath, then the mixture was brought to constant weight at 63°C under a pressure of about 50 mm Hg.

Insoluble solids were determined by drying at 100°C the solids which remained after preparation of the stock solutions. After 24 hr of drying, the solids came to constant weight. Percent moisture of the juice sacs was calculated directly from the information obtained in the determination of soluble and insoluble solids.

The soluble solids, percent moisture, and other data which describe the monthly samples of grapefruit are presented in Table 4. The average wet weight of juice sacs per fruit was calculated as follows:

$$\text{Avg. wet wt. Juice sacs/fruit} = X + \frac{XZ}{X+Y}$$

X= Avg. wt. juice sacs from 4 segments/fruit
Y= Avg. wet wt. segment walls from 4 segments/fruit
Z= Wet wt. remaining segments/fruit

TABLE 4

DESCRIPTION OF TEXAS RUBY RED GRAPEFRUIT
USED IN FLAVANONE GLYCOSIDE ANALYSES

Harvest Date	Average Diameter Whole Fruit	Average Wet Weight Whole Fruit	Average Wet Weight of Juice Sacs Per Fruit	Moisture of Juice Sacs	Soluble Solids of Juice Sacs
	cm	g	g	%	%
7/28/64	7.0	165.7	97.1	88.4	9.8
9/28/64	8.1	255.7	175.3	88.3	10.3
10/28/64	8.6	294.7	214.3	88.9	10.0
11/28/64	9.5	410.0	274.2	89.6	9.4
12/28/64	9.6	406.5	299.4	89.7	9.4
1/28/65	9.6	432.0	302.9	89.8	9.4

Column chromatography, thin-layer chromatography, and fluorescence analyses for each stock solution were carried out according to the procedures outlined in Chapter II. Naringin, naringenin rutinoside, neohesperidin, hesperidin, poncirin and isosakuranetin rutinoside contents were determined for the six available monthly samples.

The Davis test (24) was applied to these samples to obtain values for comparison with results obtained by the chromatographic-fluorometric method. Diethylene glycol solutions containing aliquots from the stock solutions were allowed to stand 10 min after addition of alkali. Absorbances at 420 mμ were compared in a Beckman Model DU spectrophotometer against a blank which contained the stock solution-diethylene glycol mixture but no added alkali. Alkaline-diethylene glycol solutions containing known quantities of pure naringin were used to construct a standard curve.

The results of the chromatographic-fluorescence analyses for the six individual flavanone glycosides and Davis test are given in Table 5.

Results and Discussion

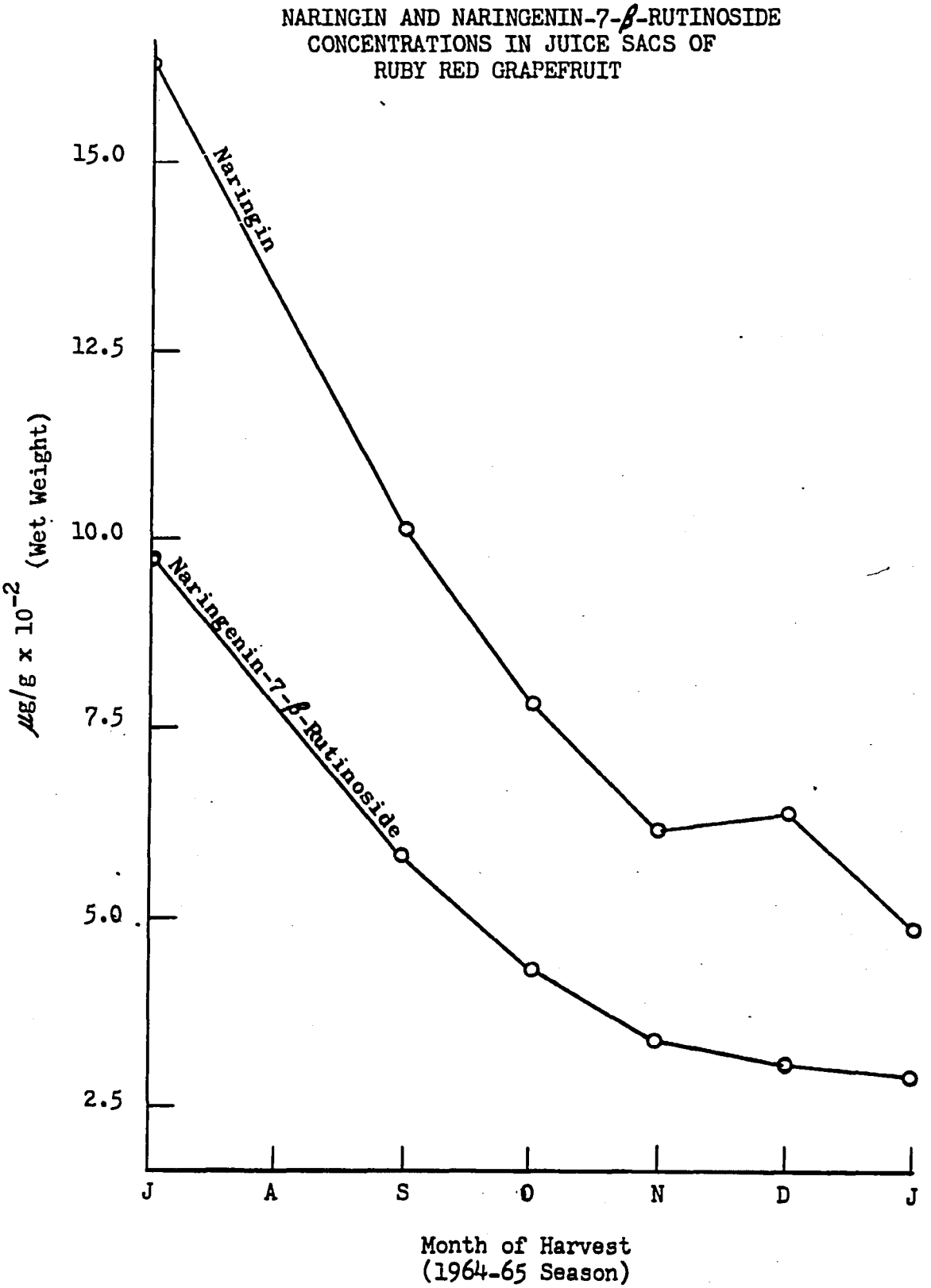
The flavanone glycoside concentrations in juice sacs generally decreased as the fruit matured. The naringin concentration of 1630 μg/g in the July sacs decreased to a low of 494 μg/g in the January sacs while the July naringenin rutinoside content of 976 μg/g decreased to 291 μg/g in the January juice sacs. The naringin and naringenin rutinoside concentrations are plotted versus harvest date in Figure 6. The naringin value for December (642 μg/g) is slightly higher than naringin values in the adjoining months of November (615 μg/g) and January. No corresponding elevation in the December naringenin rutinoside

TABLE 5

MONTHLY VARIATION IN CONCENTRATION OF SIX FLAVANONE GLYCOSIDES IN JUICE SACS OF TEXAS RUBY RED GRAPEFRUIT

Harvest Date	Concentration in Juice Sacs (μg/g Wet Weight)							
	Naringin 1	Naringenin Rutinoside 2	Neohesperidin 3	Hesperidin 4	Poncirin 5	Isosakuranetin Rutinoside 6	Sum of 1 and 2	Davis Value
7/28/64	1630	976	79.5	45.5	59.9	25.1	2606	3900
9/28/64	1010	585	45.4	27.9	42.7	17.5	1595	2100
10/28/64	786	432	35.5	21.0	31.7	12.9	1218	1770
11/28/64	615	336	27.6	15.7	22.4	9.30	951	1270
12/28/64	642	308	31.4	15.9	23.9	8.57	950	1290
1/28/65	494	291	24.2	14.4	18.8	8.80	785	1100

FIGURE 6


NARINGIN AND NARINGENIN-7-β-RUTINOSIDE
CONCENTRATIONS IN JUICE SACS OF
RUBY RED GRAPEFRUIT
Naringin
Naringenin-7-β-Rutinoside
15.0
12.5
10.0
7.5
5.0
2.5
μg/g x 10⁻² (Wet Weight)
J
A
S
O
N
D
J
Month of Harvest
(1964-65 Season)

concentration was observed.

Davis values plotted versus harvest date show a general decrease in flavanone glycoside concentration with advancing maturity of the fruit (Figure 7). The Davis concentration for July of 3900 μg/g falls to a low of 1100 μg/g for the January juice sacs. As in the case of the December naringin content, the December Davis value (1190 μg/g) was slightly higher than values for November (1170 μg/g) and January. It should be noted that the Davis values were about 2.2 times higher than the naringin concentrations and about 1.4 times higher than the combined naringin and naringenin rutinoside concentrations. Thus, while absolute values obtained by the Davis test were high, the decrease in the Davis value was of the same order of magnitude as the decrease in the naringin and naringenin rutinoside concentrations.

The changes in hesperetin and isosakuranetin glycoside concentrations parallel the changes in the naringenin glycoside concentrations although the latter compounds were present in much higher quantity. The neohesperidosides, neohesperidin and poncirin, show a general decrease in concentration as the season progresses as shown in Figure 8. As in the case of naringin, the concentrations of these compounds is somewhat higher for the December sample than they are in months of November and January. The minor rutinosides, hesperidin and isosakuranetin rutinoside, showed no relative increase for December. These results parallel the results obtained for naringenin rutinoside.

The data in Table 6 show that the ratios between the various flavanone glycosides were generally constant for the duration of the study. The main exception is in the high values for the December neo-

FIGURE 7

DAVIS VALUES FOR JUICE SACS
OF RUBY RED GRAPEFRUIT

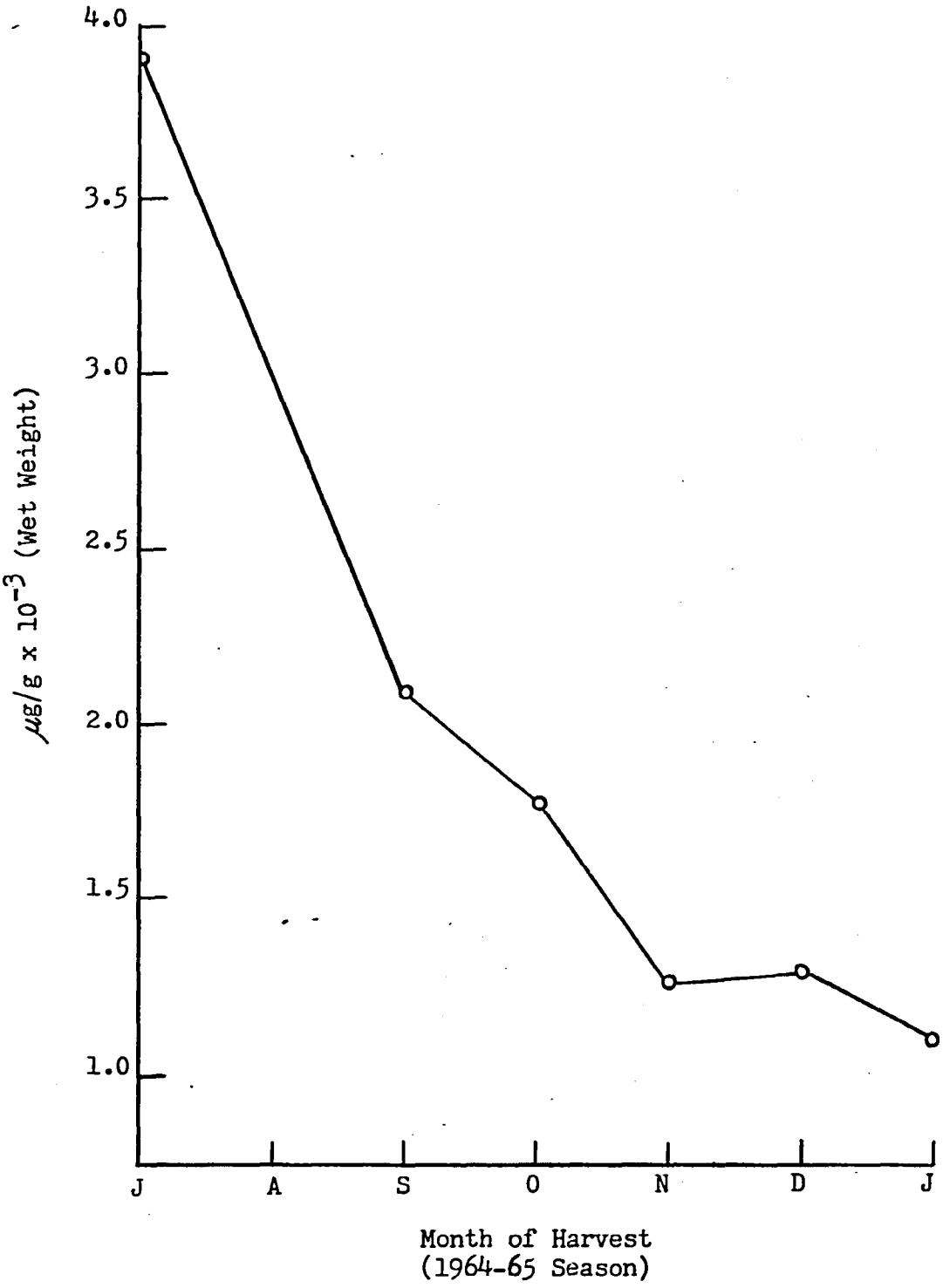
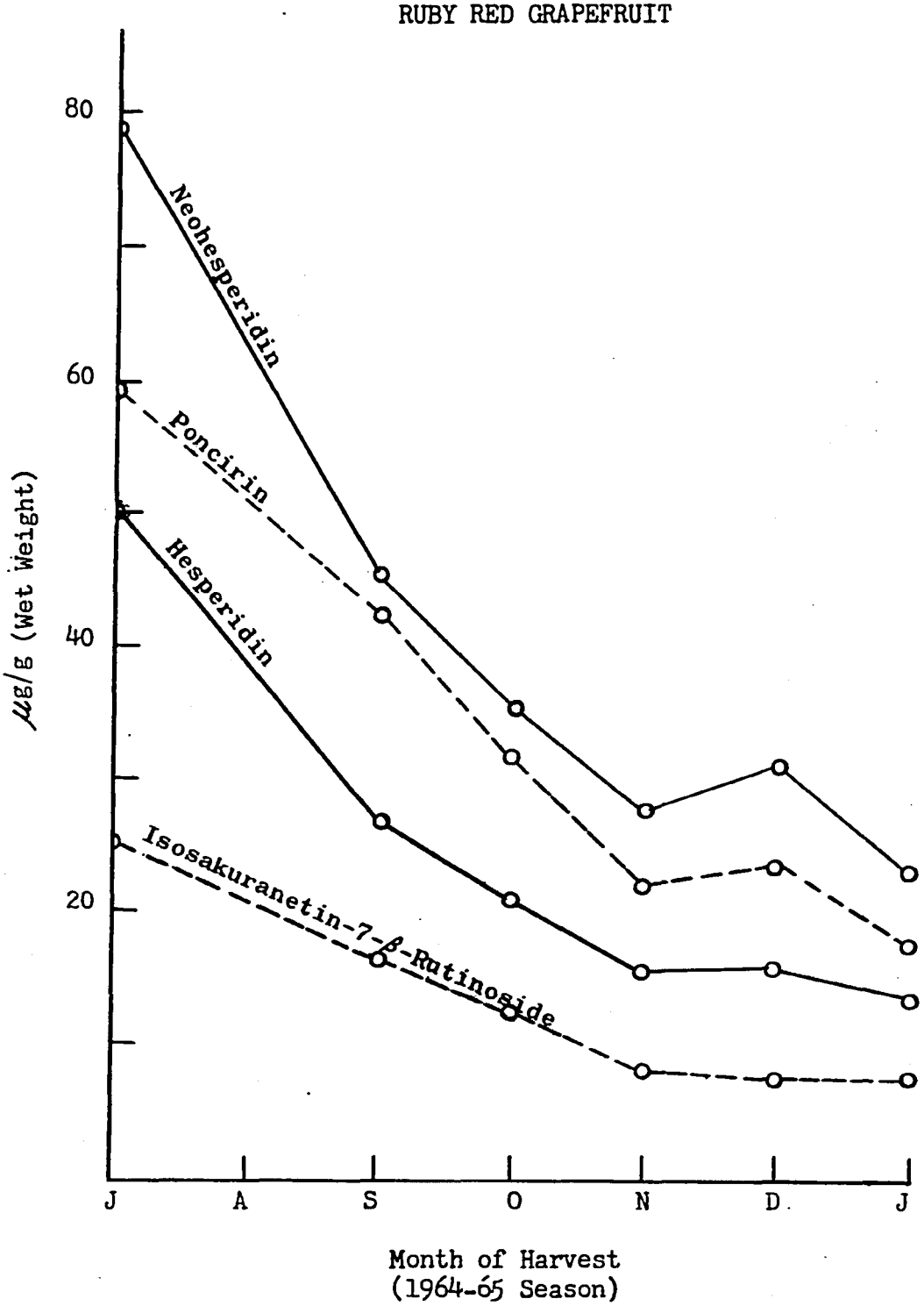


FIGURE 8

CONCENTRATIONS OF FOUR MINOR FLAVANONE GLYCOSIDES IN JUICE SACS OF RUBY RED GRAPEFRUIT



hesperidoside-rutinoside ratios as indicated earlier. The average values for naringin:naringenin rutinoside, and for neohesperidin: hesperidin ratios are very close with values of 1.80:1.00 and 1.74:1.00, respectively. The ratio average of poncirin:isosakuranetin rutinoside, however, is significantly higher with a ratio of 2.42:1.00. These results may prove to be indicative of differences in the metabolism of these compounds in the fruit.

For the first time, the absolute and relative concentrations of these six individual flavanone glycosides in grapefruit juice sacs can be given. In addition to the neohesperidoside-rutinoside ratios, Table 6 also gives data for comparison of the concentration of naringin to each of the five other flavanone glycosides. As mentioned previously, the naringin concentration in juice sacs is 1.80 times the naringenin rutinoside content. Naringin is present in amounts of 21.3 and 37.2 times those of neohesperidin and hesperidin, respectively. Of the neohesperidosides studied, poncirin is present in the lowest quantities, while isosakuranetin rutinoside is present in the lowest quantity of all compounds studied.

These data appear to indicate that neohesperidin and poncirin likely have little influence on total bitterness of the grapefruit studied since they comprise less than 8% of the total measured neohesperidosides. Moreover, Horowitz (6) has found neohesperidin to be about 0.1 as bitter as poncirin and naringin.

In terms of absolute quantities of individual flavanone glycosides in grapefruit juice sacs, it is impossible to compare these data with reports from other workers. As mentioned earlier, the basic reason is that no method capable of measuring individual flavanone glycosides in

TABLE 6

CONCENTRATION RATIOS FOR FLAVANONE GLYCOSIDES
IN JUICE SACS OF TEXAS RUBY RED GRAPEFRUIT

Harvest Date	Naringin / Naringenin Rutinoside	Neohesperidin / Hesperidin	Poncirin / Isosakuranetin Rutinoside	Naringin / Neohesperidin	Naringin / Hesperidin	Naringin / Poncirin	Naringin / Isosakuranetin Rutinoside
7/28/64	1.67	1.73	2.39	20.5	35.9	27.5	65.0
9/28/64	1.73	1.63	2.44	22.3	36.3	23.7	57.8
10/28/64	1.82	1.69	2.38	22.2	37.2	24.8	61.0
11/28/64	1.83	1.77	2.41	22.3	39.2	27.4	66.1
12/28/64	2.09	1.97	2.80	20.5	40.4	26.9	75.0
1/28/65	1.70	1.68	2.14	20.4	34.3	26.3	56.2
Average	1.80	1.74	2.42	21.3	37.2	26.1	63.5

grapefruit has been available previously. Moreover, no data have been published on juice sac preparations. Most published data give figures for juice only, not juice sacs. It would be expected that preparations of juice sacs would have higher flavanone glycoside concentrations than juice since the juice sac membranes are thought to contain larger quantities of flavanone glycosides (24).

The juice of several varieties of grapefruit has been studied by workers who used the Davis test. Studies conducted by Maurer and Burdick (27) on juice of several varieties in the Rio Grande Valley of Texas generally showed a decrease in "naringin" content as the fruit approached maturity in January. It was found, however, that juice from Ruby Red fruit harvested on December 23 gave a Davis value of 0.019% whereas juice from fruit harvested January 27 gave a higher Davis value of 0.039%.

Kesterson and Hendrickson, who studied juice of Florida grapefruit, concluded that there was no decrease in naringin content until after the fruit "had considerably passed peak maturity" (29). Their stated naringin values for juices from fruit harvested on January 1 and February 1, 1952, were 0.021% and 0.018%, respectively.

The Davis values obtained in the present study of Ruby Red grapefruit juice sacs were much higher than the reported values for the juice of Ruby Red fruit. The December value was 0.13% while the January value was 0.11% as indicated in Table 5.

The values obtained for the six flavanone glycosides give no clear cut answers with respect to the effect of maturity on flavanone glycoside concentration in juice sacs. It is possible that the general

decrease in the flavanone glycoside concentration from July to January is a matter of dilution as suggested by Kesterson and Hendrickson (29). This postulation is not clearly confirmed, however, by the data obtained in the present study. The average total naringin content in the juice sacs of a single fruit for the period from July to November is 168 mg, but in December the content climbs to 192 mg per fruit then drops to 150 mg per fruit for January. These data may indicate that a significant change is occurring during the months of December and January. However, these "average" values must be interpreted very cautiously since the total weight of juice sacs per fruit are only approximations.

Horowitz (15) has suggested that in the course of maturing of the grapefruit, a transglycosylation may occur which could result in a decrease of neohesperidoside. Thus, possibly the rutinosides might increase in concentration at the expense of the neohesperidosides. The data presented here do little to confirm or deny this hypothesis; however, it might be noted that the ratio of neohesperidosides to rutinosides for January are somewhat lower than the ratios of the corresponding compounds in the preceding three months.

It is clear that before any definitive statements can be made about the role of the flavanone glycosides in grapefruit metabolism or the taste of grapefruit, much more study is needed. This study was conducted on the juice sacs of fruit picked during the months of July to January. Probably much meaningful information could be obtained by studying the fruit in the very early stages of development prior to July. Also, study of fruit past the peak of commercial maturity (late season fruit) could give important data, particularly if the relation of the flavanone glycosides to loss of bitterness of late season fruit is to be correlated.

CHAPTER IV

RELATED STUDIES ON THE CHROMATOGRAPHY OF FLAVANONE GLYCOSIDES

From the many experiments undertaken in the efforts to devise a feasible method for the quantitative determination of grapefruit flavanone glycosides, several other useful chromatographic techniques and procedures were developed. Although the information recorded in this section was not used directly as part of the quantitative method described in Chapter II, under appropriate circumstances these basic techniques for separation of flavanone glycosides also could be incorporated with or developed into quantitative procedures. In addition, the separation procedures given here should be useful in qualitative studies of flavanone glycosides and of other phenolic compounds occurring in plants.

Ion Exchange Column Chromatography

The use of ion exchange resins in the isolation of flavonoid compounds from aqueous plant extracts was introduced by Wender and coworkers (32,41). The technique consisted of passing large volumes of aqueous plant extracts through columns, which contained Amberlite IRC-50 (H) cation exchange resin (Rohm and Haas Co., Philadelphia, Pa.). The columns, which contained the adsorbed flavonoids, were washed with water until all color-

ed material was removed; then, the flavonoids were removed by elution with alcohol. This method was also used to remove impurities from commercially available flavonoid preparations (32). Recently, the use of Amberlite CG-50, which is a fine mesh chromatographic grade of IRC-50 (H), was reported for the fractionation of various flavonoid and phenolic mixtures (42). These reports indicated that Amberlite CG-50 might be of value for the separation of the grapefruit flavonoids.

Samples, consisting of 20 ml of canned grapefruit juice, were prepared for column chromatography as described in Chapter II. These were chromatographed on 2.5 cm diameter columns packed to various depths with Amberlite CG-50 of different particle sizes. Several different elution rates and elution sequences of water and aqueous methanol or 2-propanol were tried. These experiments revealed that use of resin of particle sizes less than 200 mesh was impractical since columns packed with Amberlite CG-50 Type II (200-400 mesh) became almost completely clogged when concentrated grapefruit juice samples were chromatographed on them.

It was found that a fairly good separation of the components of grapefruit juice could be achieved on a 2.5 cm diameter column packed to a depth of 20-30 cm with Amberlite CG-50 Type I (100-200 mesh), when distilled water was used as the eluting solvent. Resin ranging in particle size from 140-200 mesh obtained by sieving Amberlite CG-50 Type I was also tried, but it seemed to offer little advantage over regular Type I. Sugars, amino acids, and most of the blue fluorescing compounds appeared to be removed in the early fractions from such columns, and later fractions contained naringenin and isosakuranetin glycosides

which were relatively free of contaminants. Relatively large elution volumes were required, however, to obtain the flavanone glycoside fractions. At a flow rate of 2 ml/min under atmospheric pressure, several days of elution were required to remove these compounds from the column. Attempts to increase the flow rate by use of pressure or addition of alcohol to the eluting solvent resulted in decreased resolution.

Ion exchange chromatography was not further developed as a part of a quantitative procedure because of the relatively long time required for elution. However, certain observations and conclusions can be made with respect to the order of elution of individual flavanone glycosides from the column. Naringin and naringenin rutinoside were eluted from the column in fractions preceding the poncirin and isosakuranetin rutinoside fractions. In this respect, the elution pattern is reversed on the ion exchange resin as compared to aqueous elution patterns found with Polyclar AT. Examination of the individual naringenin glycoside fractions on polyamide thin-layers (nitromethane-methanol, 5:2, v/v) revealed that naringenin rutinoside had a somewhat faster flow rate on the ion exchange column than did naringin. From ion exchange studies on the other available neohesperidoside-rutinoside pairs, the rutinoside was found to be less tightly adsorbed than its neohesperidoside isomer.

While these studies indicated that Amberlite CG-50 column chromatography did not have immediate value in this particular method of quantitative analysis of grapefruit flavanone glycosides, they clearly indicate types of separations which may be useful in qualitative or preparative work. These studies show that it may be possible to obtain individual flavanone glycosides of relatively high purity directly from

aqueous citrus extracts. For example, by using water elution, the isosakuranetin glycosides may be obtained free of naringenin glycosides as well as free from most other non-flavonoid material. Also, it is possible that individual neohesperidosides or rutinosides can be resolved to a large extent after a single pass of the aqueous plant extract through the column. Although this technique was tested only on small pilot columns, it appears that this technique could be scaled up for the preparation of relatively large quantities of flavanone glycosides.

Analysis of Grapefruit Flavanone Glycosides by Two Dimensional Polyamide Thin-Layer Chromatography

Preliminary studies were conducted on a simplified procedure for quantitative determination of flavanone glycosides in grapefruit. The lengthy column chromatography step used in the original method given in Chapter II can be eliminated by preliminary isolation of the desired flavanone glycosides from the juice sac stock solution by development of a polyamide thin-layer chromatogram in distilled water. This step, which is the first operation in a two dimensional development procedure, removes most of the interfering material such as the sugars and other highly polar compounds to the solvent front in a manner similar to the action of the Polyclar column. For development of the chromatogram in a second dimension, the nitromethane-methanol solvent (5:2, v/v) is used as in the normal procedure.

To test the accuracy of the two dimensional method, a 50 μl aliquot of juice sacs stock solution, prepared by the usual procedure, was applied directly to one corner of each of three polyamide Woelm thin-layer layer chromatograms. A single 5 μg naringin standard was similarly ap-

plied to each of three additional thin-layer plates. The chromatograms were developed for one hour in distilled water to effect preliminary separation of the flavanone glycosides from the sugars and other potentially interfering substances present in the sample. The plates were then dried thoroughly and chromatographed in the second dimension in nitromethane-methanol for 1.5 hr to separate the individual flavanone glycosides. Naringin and naringenin rutinoside (unknowns and standards) were removed from the thin-layers and subjected to fluorescence analysis by the regular procedures.

The simplified two dimensional thin-layer chromatographic procedure gave the following concentrations for the two isomers in a grapefruit juice sacs stock solution; naringin, 670 μg/g, and naringenin rutinoside, 275 μg/g. When the same stock solution was analyzed by the original chromatographic method, the following values were obtained: naringin, 598 μg/g, and naringenin rutinoside, 274 μg/g.

The higher value of 670 μg/g for naringin obtained by the simplified method as compared to 598 μg/g obtained by the original method was not surprising, since the column chromatography step of the original method had been shown to remove from naringin small quantities of unknown flavonoid compounds which are not likely to be separated from naringin by thin-layer chromatography alone. The values obtained for naringenin rutinoside by the two methods were in excellent agreement. No attempt was made in these studies to determine the known neohesperidosides and rutinosides of hesperetin and isosakuranetin by the simplified method. However, all four compounds were visible on the chromatograms used, and they appeared to be relatively well separated from potentially interfer-

ing substances. Therefore, it seems likely that the simplified method could be employed for their determination, very possibly with good accuracy.

This simplified, two-dimensional chromatography method is less complex and considerably faster than the original column chromatography method for determination of flavanone glycosides in grapefruit. If the decreased accuracy of naringin determination is recognized and can be tolerated, this simplified method may be useful in its present form; also, it has considerable potential for further development into a more accurate as well as rapid method for the determination of flavanone glycosides, and possibly other flavonoids, in grapefruit and other citrus products.

The BzAWN and KFW Solvent Systems

Attention was given in the course of this study to the development of a quantitative procedure based on a separation of individual flavanone glycosides by paper chromatography rather than by thin-layer chromatography. No solvent system listed in the literature was known to separate completely naringin from poncirin. Dunlap (33) had found that poncirin had a slightly higher R_f value than naringin in 1-butanol-acetic acid-water (6:1:2, v/v/v) and a slightly lower R_f value in distilled water, but in a mixed spot, the two compounds were not separated.

Studies with several existing solvent systems commonly used for phenolic acids revealed that benzene-acetic acid-water (125:72:3, v/v/v) gave a fairly clean separation of naringin and poncirin, but poncirin had an R_f value of less than 0.1 and naringin remained at the origin after ascending development on paper. A solvent system, developed in this

laboratory (43), nitromethane-acetic acid-water (4:4:1, v/v/v), was found to separate naringin and poncirin (R_f values of 0.65 and 0.84, respectively), but the zones were elongated and generally unsuited for quantitative work. Combination of these two solvents resulted in a new system which gave good separation of naringin, poncirin, and several other naturally occurring plant phenolic compounds. This system consisted of benzene-acetic acid-water-nitromethane (34:32:5:18, v/v/v/v, called BzAWN). Table 7 shows approximate R_f values in BzAWN for selected flavonoids and other phenolic compounds by ascending chromatography on Schleicher and Schuell No. 589 Red Ribbon paper.

It should be noted that the R_f values given in Table 7 are only approximate since they are composite values obtained from a number of chromatograms run at different times. Nevertheless, they illustrate the excellent separations of naringin, poncirin, naringenin-7-β-glucoside (prunin), and naringenin obtained with the BzAWN system. The BzAWN solvent system moves all the compounds listed away from the origin but does not move any of them into the solvent front. It gives compact, discrete spots of the flavanones; thus, it is possible to obtain fluorescence intensity measurements directly from the chromatogram on a special paper chromatogram door available for the Turner Fluorometer (Turner No. 110-861). It is a fast moving solvent system which permits ascending development of a 30 cm paper in 4-5 hr, and is a relatively volatile solvent system which permits drying of chromatograms in a reasonable time after development. And, it seems to have no adverse effect on either the compounds being chromatographed or on the paper.

A problem common to BzAWN and most classical flavonoid solvent systems

TABLE 7

R_f VALUES OF SELECTED PHENOLIC COMPOUNDS IN THE BzAWN* SOLVENT SYSTEM

Compound	R_f	Compound	R_f
Naringenin	0.89	Kaempferol-3-Rhamno-glucoside	0.31
Poncirin	0.75	Rutin	0.18
Naringenin-7-β-Glucoside	0.63	Caffeic Acid	0.68
Neohesperidin	0.56	Esculin	0.45
Hesperidin	0.55	Scopolin	0.65
Naringin	0.45	Chlorogenic Acid	0.42
Rhoifolin	0.38	Neochlorogenic	0.22

*Benzene-Acetic Acid-Water-Nitromethane (34:32:5:18, v/v/v/v)

is that rutinoside and neohesperidoside derivatives of a given flavanone are not separated. Neither can the solvent give clean separations of rhoifolin and naringin or hesperidin nor neohesperidin from prunin and naringin; however, these compounds when pure give slightly different R_f values in BzAWN. Nevertheless, the BzAWN solvent system has considerable potential for use in procedures involving differentiation of flavanone glycosides in natural products.

A solvent system devised by Runeckles and Woolrich (44) for separation of depsides was found to be very useful also for separation of flavanone glycosides. This solvent system consisting of methyl-iso-butyl ketone-90% formic acid-water (14:3:2, v/v/v, called KFW) was devised for use with paper chromatography, but experimentation with the flavanone glycosides in this laboratory showed that the system gave much better results when used on thin-layer chromatography plates of Avicel SF, a microcrystalline cellulose (FMC Corporation, American Viscose Division, Marcus Hook, Pa.). Whereas BzAWN has no ability to distinguish between neohesperidosides and rutinosides, KFW differentiates between neohesperidoside and rutinoside pairs on both paper and Avicel thin-layer chromatograms. Mixed zones of these pairs can be completely separated on Avicel chromatograms if the plates are developed a second time in the solvent. Table 8 shows the R_f values for certain flavanone glycosides after single and double development on Avicel thin-layer chromatograms and single descending development on Schleicher and Schuell No. 589 Red Ribbon paper.

The R_f values given in Table 8 show that very good separations can be achieved for many of the compounds, particularly naringin, poncirin,

TABLE 8

R_f VALUES OF NARINGENIN AND SELECTED FLAVANONE GLYCOSIDES IN THE KFW* SOLVENT SYSTEM

Compound	R_f Values		
	Avicel Thin-Layers Single Development	Avicel Thin-Layers Double Development	Descending Paper Chromatography
Naringenin	0.97	----	----
Isosakuranetin-7-β-Glucoside	0.82	----	----
Naringenin-7-β-Glucoside (Prunin)	0.62	----	0.53
Hesperetin-7-β-Glucoside	0.57	----	----
Poncirin	0.56	0.68	0.38
Isosakuranetin-7-β-Rutinoside	0.53	0.62	----
Naringin	0.38	0.48	0.26
Neohesperidin	0.35	----	----
Naringenin-7-β-Rutinoside	0.32	0.42	----
Hesperidin	0.29	----	----
Rhoifolin	----	----	0.29

*Methyl-iso-butyl Ketone-90% Formic Acid-Water (14:3:2, v/v/v) (44)

prunin, and naringenin. Naringin, neohesperidin, and hesperidin remain together in mixed spots, but pure samples of these individual compounds give somewhat different R_f values.

Inspection of the R_f values given in Table 7 and 8 show that while neither solvent system can distinguish between all the compounds listed, data compiled from the chromatography of samples in both solvents can give very meaningful information leading to the identity of unknown flavanone glycosides.

CHAPTER V

SUMMARY

Naringin, the bitter 7-β-neohesperidoside of naringenin, had long been considered to be the only major flavanone glycoside present in the grapefruit, Citrus paradisi, until it was discovered that the grapefruit contains significant quantities of naringenin-7-β-rutinoside, a tasteless isomer of naringin, in addition to lesser quantities of several other flavanone glycosides. Since no previously known quantitative method could distinguish between these several flavanone glycosides, research was undertaken to develop a quantitative procedure for analysis of these individual compounds in grapefruit.

A new quantitative method has been developed for the individual determination in grapefruit juice and juice sacs of the bitter flavanone glycosides naringin, neohesperidin, and poncirin, and their tasteless isomers, naringenin-7-β-rutinoside, hesperidin, and isosakuranetin-7-β-rutinoside. This method involves a preliminary quantitative isolation of the desired flavanone glycosides from juice or juice sacs by polyvinylpyrrolidone column chromatography, further separation and purification of these flavanone glycosides by polyamide thin-layer chromatography, and quantitative determination by fluorescence analysis of each individual flavanone glycoside after its removal from a thin-layer chromatogram.

The newly developed chromatographic-fluorometric method was used for the determination of these six flavanone glycosides present in juice sacs of Texas Ruby Red grapefruit harvested monthly from July 28, 1964 to January 28, 1965. These studies revealed that as the fruit became more mature, the concentration in $\mu g/g$ of each of these compounds generally decreased at essentially the same rate. It was noted, however, that the level of the neohesperidosides was slightly elevated in the December sample as compared with the November and January samples. As an example of the range of values observed, the following concentrations in $\mu g/g$ wet weight of juice sacs were obtained for the January sample: naringin, 494; naringenin-7-β-rutinoside, 291; neohesperidin, 24.2; poncirin, 18.8; hesperidin, 14.4; and isosakuranetin-7-β-rutinoside, 8.80. Values obtained on these samples by the Davis test, a classical method for naringin determination, averaged about 2.2 times higher in all samples than the naringin value obtained by the chromatographic-fluorometric method.

From the experiments which were undertaken in order to develop a quantitative method, several other useful techniques were devised for the chromatographic separation of flavanone glycosides. Cation exchange column chromatography was used for the isolation of flavanone glycoside fractions from aqueous grapefruit extracts. A simplified, but somewhat less accurate method, which involves polyamide thin-layer chromatography in two dimensions, was devised for the quantitative determination of certain grapefruit flavanone glycosides. And finally, two solvent systems were developed for the study of flavanone glycosides and related compounds by paper or microcrystalline cellulose thin-layer

chromatography. These solvents are benzene-acetic acid-water-nitromethane (34:32:5:18, v/v/v/v) and methyl-iso-butyl ketone-90% formic acid-water (14:3:2, v/v/v).

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