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degree of

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BY

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Norman, Oklahoma

1966

STUDIES OF FLAVANONE GLYCOSIDES

FROM THE GRAPEFRUIT

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STUDIES OF FLAVANONE GLYCOSIDES FROM THE GRAPEFRUIT

CHAPTER I

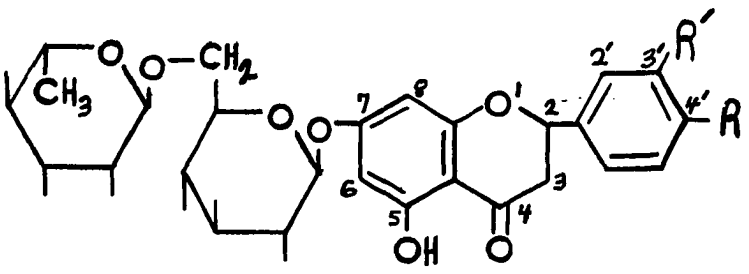
INTRODUCTION

Flavonoid compounds occur widely in higher plants and are particularly prevalent as flavanone glycosides in the fruits of the genus Citrus. The first isolation of a flavonoid compound from citrus fruit was recorded in 1828 by Lebreton (1) who obtained from a tincture of macerated oranges a crystalline substance which he called hesperidin. Subsequent investigation over many years finally established that hesperidin is a flavanone glycoside, the aglycone being 2S-hesperetin (3',5,7-trihydroxy-4'-methoxyflavanone) (2,3,4), and that it is the principal flavonoid of the sweet orange, Citrus sinensis. The sugar moiety of hesperidin was shown to be a diglycoside consisting of L-rhamnose and D-glucose, with the glucose attached to the 7-position of the aglycone through a β-glycosidic linkage (5,6); its structure was found to be identical with that of rutinose (7), the disaccharide component of rutin. The structure of rutinose, determined by Zemplén and Gerecs (7)

to be 6-O-β-L-rhamnopyranosyl-D-glucose, was revised by Gorin and Perlin (8) to 6-O-α-L-rhamnopyranosyl-D-glucose since the trialdehyde obtained by oxidizing rutinose with periodate had the α-configuration. The complete structure of hesperidin, then, is that of the 7β-rutinoside of hesperetin (I, Figure 1).

In 1936, Kolle and Gloppe (9) isolated from the Seville orange, C. aurantium, a glycoside that was isomeric with hesperidin which they called neohesperidin. Neohesperidin occurs along with hesperidin in the Seville orange and upon hydrolysis it gives, as does hesperidin, L-rhamnose, D-glucose, and hesperetin as products. The attachment of the disaccharide to the aglycone in neohesperidin was shown by Horowitz (10) to be the same as that in hesperidin, indicating the isomeric difference must reside in the linkage of rhamnose to glucose. As early as 1938, however, Zemplén and Tettamanti (11) had shown that the disaccharide of neohesperidin, which they named neohesperidose, differs from rutinose and suggested that its structure was probably 4-O-L-rhamnosyl-D-glucose. This suggestion was left unchallenged until 1961 when Horowitz and Gentili (12, 13) made an important and significant advance in the chemistry of the flavanone glycosides by proving that the postulation of the 1 to 4 rhamnose to glucose linkage in neohesperidose was incorrect. These workers established, by alkaline degradation and by methylation and hydrolysis studies, that

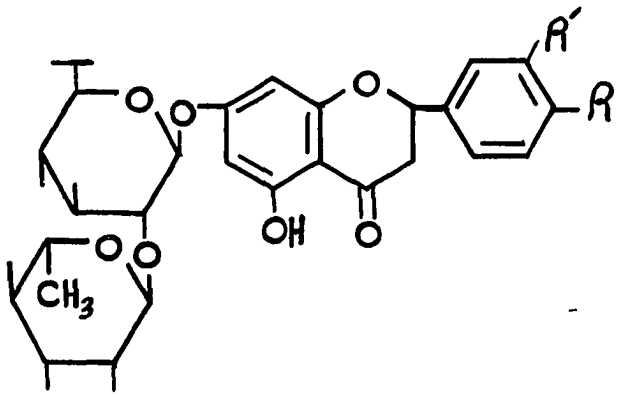
FIGURE 1



I. Hesperidin; R = OCH_3, R' = OH

II. Naringenin-7β-Rutinoside;
R = OH, R' = H

III. Isosakuranetin-7β-Rutinoside;
R = OCH_3, R' = H



IV. Neohesperidin; R = OCH_3, R' = OH

V. Naringin; R = OH, R' = H

VI. Poncirin; R = OCH_3, R' = H

the structure of neohesperidose was actually 2-O-α-L-rhamnopyranosyl-D-glucose. Therefore, neohesperidin was shown to be the 7β-neohesperidoside of hesperetin (IV, Figure 1).

During the elucidation of the structure of neohesperidose, Horowitz and Gentili demonstrated that the rhamnoglucoside moiety of at least two other flavanones isolated from citrus species was also neohesperidose. Naringin, first isolated from the flowers of the shaddock, C. grandis, by DeVry in 1847 (14), and poncirin (15), from Poncirus trifoliata, both gave identical glycosides after alkaline degradation and identical methylated sugar derivatives after methylation and hydrolysis (13). These products were, in turn, identical with those obtained from neohesperidin when the latter was treated under similar conditions. Thus, naringin was established as the 7β-neohesperidoside of naringenin (4',5,7-trihydroxyflavanone) (V, Figure 1) and poncirin as the 7β-neohesperidoside of isosakuranetin (4'-methoxy-5,7-dihydroxyflavanone) (VI, Figure 1). The peel of the navel and Valencia oranges, C. sinensis, have been shown by Gentili and Horowitz (16) to contain the rutinoside isomers of naringin and poncirin, i.e., naringenin-7β-rutinoside (II, Figure 1) and isosakuranetin-7β-rutinoside (III, Figure 1).

Based on a tabulation of various glycosides isolated from Citrus, Horowitz (17) suggested that citrus fruits

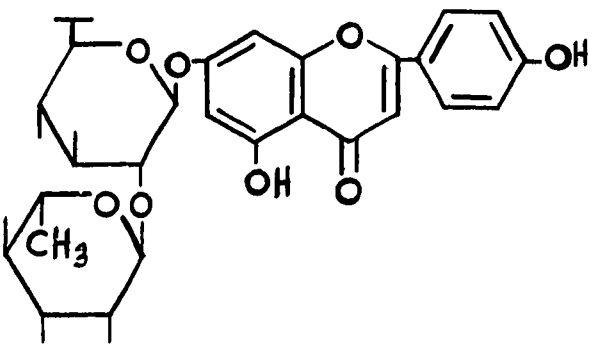
can be divided into two broad categories: (a) those containing mainly rutinosyl glycosides; and (b) those containing mainly neohesperidosyl glycosides. This generalization was supported by the information obtained after ozonolysis of crude extracts from various citrus fruits and identification of the resulting sugars by paper chromatography (10). All species examined yielded glucose, however, only two, lemons, (C. limon), and Valencia oranges, yielded large amounts of rutinose and apparently no neohesperidose. Two others, green Seville oranges and green Ponderosa lemons (C. limon f. ponderosa), yielded chiefly neohesperidose together with a trace of another disaccharide which was probably rutinose. At the initiation of the research described in this dissertation, the Seville orange was the only species of citrus fruit in which both a neohesperidoside (neohesperidin) and a rutinoside (hesperidin) had been isolated intact (9).

In addition to the Seville orange and the Ponderosa lemon, the grapefruit, C. paradisi, also appeared to contain both neohesperidosides and rutinosides. Ozonolysis of grapefruit extract yielded a preponderance of neohesperidose but a small amount of rutinose was also present (10). Dunlap and Wender (18) isolated from the grapefruit a naringenin rhamnoglucoside which was shown not to be naringin, thus indicating the possibility that the grapefruit may contain naringenin-7β-rutinoside. Naringin has long been

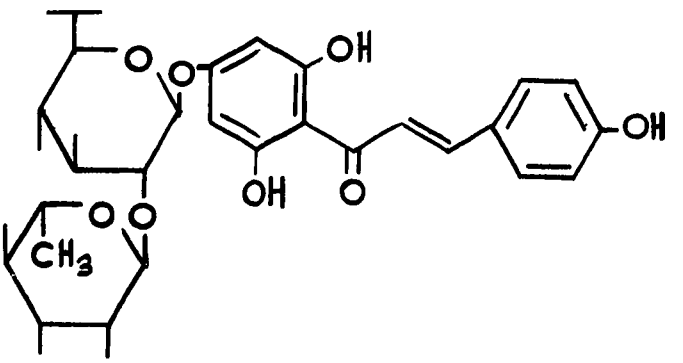
known to be the principal flavanone glycoside of the grapefruit. Recently, neohesperidin (18) and poncirin (12) have also been found to occur in small amounts in the grapefruit.

Horowitz (10, 13) has noted that an outstanding feature of the neohesperidoside-rutinoside isomerism is the strikingly different taste phenomena it produces. The neohesperidosides of naringenin, hesperetin, and isosakuranetin are extremely bitter compounds while the rutinosides of the same aglycones are completely tasteless. The degree of bitterness of naringin and poncirin is comparable to that of quinine and is detectable to the extent of 1 part in 50,000 (19). Neohesperidin is reported to be approximately one-tenth as bitter as these (13). In studies on the relationships between taste and bitterness of some phenolic glycosides, Horowitz (10) found that the presence of rutinose in a glycoside is apparently sufficient to render the compound tasteless since all rutinose derivatives tested so far have no detectable taste. The presence of neohesperidose in a flavonoid compound, however, does not guarantee its bitterness. Rhoifolin, the 7β-neohesperidoside of apigenin (4',5,7-trihydroxyflavone) (VII, Figure 2) is tasteless and naringin chalcone (VIII, Figure 2) is intensely sweet (10). In fact, the presence of neohesperidose in a flavanone is not necessary for this response since prunin, the 7β-glucoside of naringenin (IX, Figure 2), elicits a bitter taste sensation which is slightly greater

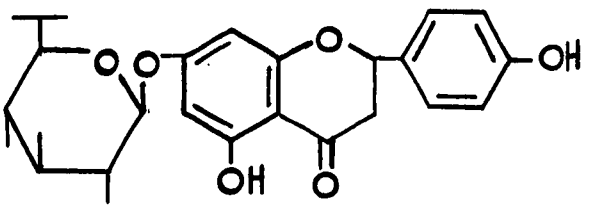
FIGURE 2



VII. Rhoifolin



VIII. Naringin Chalcone



IX. Prunin

than that of neohesperidin. Neohesperidose itself is almost imperceptibly sweet (10).

Certain citrus fruits tend to be bitter, especially when they are green, but as the fruit ripens. bitterness may decline or disappear entirely. These observations invite speculation as to the mode of the loss of bitterness. It has been generally observed (19, 20, 21, 22) that the absolute flavonoid content per fruit increases during the growth of citrus fruits up to a certain equatorial diameter (about 2 in. for certain varieties of grapefruit and oranges) and then becomes approximately constant; the increase in size and weight of the growing fruit then results in a decrease of the percentage of flavonoids with maturity. This dilution is a probable factor responsible for the subsequent decrease in bitterness during maturation of the grapefruit.

A different means of debittering has been postulated (10) for the Seville orange. For example, Kolle and Gloppe (9), as mentioned previously, have shown that bitter neohesperidin and tasteless hesperidin are both present in unripe Seville oranges while no neohesperidin is to be found in the ripe fruit. This suggests the possibility that bitter flavanone neohesperidosides, initially present in high concentration, gradually undergo a transglycosylation reaction that changes them to the corresponding tasteless flavanone rutinoside. There is,

however, no direct experimental evidence to support this debittering mechanism.

The bitterness which naringin and other neohesperidosides impart to grapefruit products has a substantial effect on consumer acceptance of these products and this is one of the reasons why the flavonoid glycosides of the grapefruit are of considerable interest. It is believed that bitterness is the major factor contributing to decreasing consumption of grapefruit products, particularly in early season grapefruit. Steps taken to eliminate or greatly reduce this bitterness and to insure grapefruit products of reliable quality would enhance their market value and thus be of considerable economic importance. Possible approaches might involve new or improved methods of processing grapefruit products, measures taken to prevent the original accumulation of bitter components in the very young fruit, or treatment of grapefruit products during processing to transform the bitter components to tasteless ones. An example of the latter technique is enzymic debittering suggested by Griffiths and Lime (23).

Before new methods or improvements on existing methods could be made, it was obvious that more information was necessary concerning the flavonoids of the grapefruit. The work of Dunlap and Wender (18) and of Horowitz and Gentili (10) showed that many problems involving the flavanone glycoside content of the fruit were

still unresolved. Furthermore, the citrus industry had found that the naringin content of grapefruit juice, as determined by the available chemical tests, often did not correlate with bitterness as determined by taste panels (24), further indicating the complex and confused situation of these components in the grapefruit.

With financial aid from the U. S. Department of Agriculture, research was undertaken at the University of Oklahoma to gain further information concerning the flavanone glycoside content of the grapefruit. It was hoped that knowledge disclosed by these qualitative investigations would aid in elucidating the chemistry of the flavanone glycosides and subsequently in establishing the nature of their precursors and degradation products. Such information could possibly serve eventually as a basis for the development of improved processing methods to provide grapefruit products of reduced bitterness. The research presented in this dissertation was concerned primarily with the separation and identification of pure, individual flavanone glycosides of grapefruit.

Chapter II describes the isolation and identification of the tasteless 7β-rutinosides of naringenin, hesperetin (hesperidin), and isosakuranetin from the segments of mid-season grapefruit and of the bitter monoglucoside, prunin, from the same source.

Chapter III is devoted to isolation and identification

studies of two naringenin rhamnodiglucosides from late-season grapefruit segments.

CHAPTER II

ISOLATION AND IDENTIFICATION OF SOME FLAVANONE RUTINOSIDES AND PRŪNIN FROM THE GRAPEFRUIT

Preparation of Mid-Season Grapefruit Segment Concentrate

The peel and core were carefully removed from the segments (juice sacs, carpel walls, and seed) of 29 lb of Ruby Red grapefruit obtained near Weslaco, Texas, in late December, 1963. The segments were quick frozen in powdered dry ice and stored at -20°F until they were used. The frozen segments were chopped and sliced with a knife to break the segment walls, and then dropped into boiling methanol. The resulting slurry was filtered through a bleached muslin cloth. The solids remaining on the cloth were squeezed to break intact juice sacs and remove as much liquid as possible, and then were transferred to a Waring Blendor and further macerated with a small amount of methanol. The slurry obtained was transferred to 60 x 180 mm Soxhlet extraction thimbles, and the liquid which passed through the thimbles was added to the original filtrate. The solids remaining in the thimbles were

extracted three times successively for 24 hr each time, with methanol, and the extracts were added to the combined filtrates. The combined filtrates and extracts were concentrated to a total volume of about 2 liters at low temperature by means of a cyclone evaporator, and finally reduced in a rotary evaporator to obtain a segment concentrate of about 1-liter volume.

Preliminary Isolation of Flavanoids from Grapefruit Segments

Several workers had indicated the usefulness of polyamide adsorbents for the isolation of flavanoids and phenolics from plant materials (25, 26, 27, 28, 29). The general procedure involved application of the plant material to an aqueous polyamide column and elution with water, followed by aqueous alcohol of increasing alcohol concentration. Preliminary experiments in this laboratory (using 2.5 x 20 cm pilot columns of various polyamides) indicated the applicability of this method to the grapefruit segment concentrate.

Approximately 500 ml of the segment concentrate was chromatographed on each of two 8-cm diameter columns packed to a depth of 73 cm with Ultramidpulver polyamide (Badische Anilin- und Soda-Fabrik AG, Ludwigshafen am Rhein, Germany). The columns were packed under 5 lb pressure from aqueous slurries of 1175 gm polyamide which had been soaked in water for 1.5 hr prior to packing. The columns were each

washed with about 20 liters of solution containing dimethylformamide-acetic acid-water-methanol (1:2:6:4, v/v/v/v, called DMF), followed by several liters of distilled water. Samples were applied under 5 lb pressure, with stirring of the top 8-10 cm of the column to prevent formation of a flow-reducing plug of oily material from the concentrate at the top of the column. Reapplication of pressure reduced the sample zone to 6 cm. Washed Ultramid-pulver was then gently poured onto the sample zone to a depth of 5 cm to prevent channeling and disruption of the sample zone as more eluting solvent was poured on. Elution was accomplished with distilled water under atmospheric pressure, with 1-liter fractions being collected. Flow rates were approximately 150 ml per hr. Fractions obtained were taken to dryness or near dryness on a flash evaporator and the residues were taken up in two 25 ml volumes of methanol. The resulting fractions were examined by spotting 25 μl of each on paper (Schleicher and Schuell - 589 Red Ribbon) and thin-layer (Adsorbosil-1, silica gel, Applied Science Laboratories, Inc., State College, Pa.) chromatograms and developing the chromatograms in benzene-acetic acid-water-nitromethane (34:32:5:18, v/v/v/v, called BzAWN). For visual observation of flavonoid material, the chromatograms were sprayed with 1% methanolic $AlCl_3$ solution and observed under 3660 Å ultraviolet (U.V.) light. The chromatograms indicated that fractions 18 through 34 from

these columns were rich in flavonoid compounds. These fractions were combined and rechromatographed on a 4.5-cm diameter column packed to a depth of 18 cm with Polyclar AT, a polyvinylpyrrolidone obtained from General Aniline and Film Corp., Grasselli, N. J. The column, designated MS-C, was prepared and washed in the same manner as the Ultra-midpulver column described above. The sample was reduced in volume, adsorbed onto 10 gm of washed Polyclar AT and this was charged to the column as an aqueous slurry. Elution was accomplished using successively the following percentages of methanol in water: 10%-2250 ml, 25%-700 ml, 50%-500 ml, and 100%-1000 ml. Volumes of fractions collected were: 1 through 25, 50 ml each; 26 through 43, 100 ml each; 44, 500 ml, and 45, 1000 ml. These were taken to dryness and taken up in methanol as described before. Thin-layer chromatography revealed that fractions 17 through 38 contained a considerable quantity of material that appeared to be pure naringin by most chromatographic criteria, but which was resolved into at least two compounds on thin layer chromatograms of Woelm polyamide (Alupharm Chemicals, New Orleans, La.) developed in a solvent system consisting of nitromethane-methanol (5:2, v/v, called NM). These fractions also contained substances which were chromatographically similar to neohesperidin and poncirin, but appeared to contain only low quantities of substances which were not flavonoids. Therefore, they were combined, taken to a

sirup on the flash evaporator, and the sirup was mixed well with approximately 3 parts of isopropyl alcohol. The solvent was stripped off, resulting in the deposition of 9.8 gm of a yellow solid material. This was designated MS-C3. Fractions 39 through 43 were combined and designated MS-C4, but no further work was done on MS-C4 since it appeared to contain only naringin. Both fractions 44 and 45 contained a component which was chromatographically identical to standard prunin. These fractions were therefore combined, concentrated, and designated MS-C5. Further work on this sample is described on page 40.

The use of nitromethane-methanol mixtures and other nonaqueous solvents on polyamide thin-layer chromatograms was suggested by Albach (30), a suggestion which subsequently proved to be invaluable in this research. Experiments in this laboratory showed that a mixture containing standards of the neohesperidosides and rutinosides of naringenin, hesperetin, and isosakuranetin could be cleanly resolved into its six components by TLC on Woelm polyamide, using NM as developing solvent. An immediate implication was that such TLC systems could be translated to column chromatographic systems for preparative work. Subsequent pilot column chromatographic experiments on samples of the flavonoid rich MS-C3 fraction showed that resolution of its components was possible using Polyclar AT with benzene-methanol mixtures as eluting solvents.

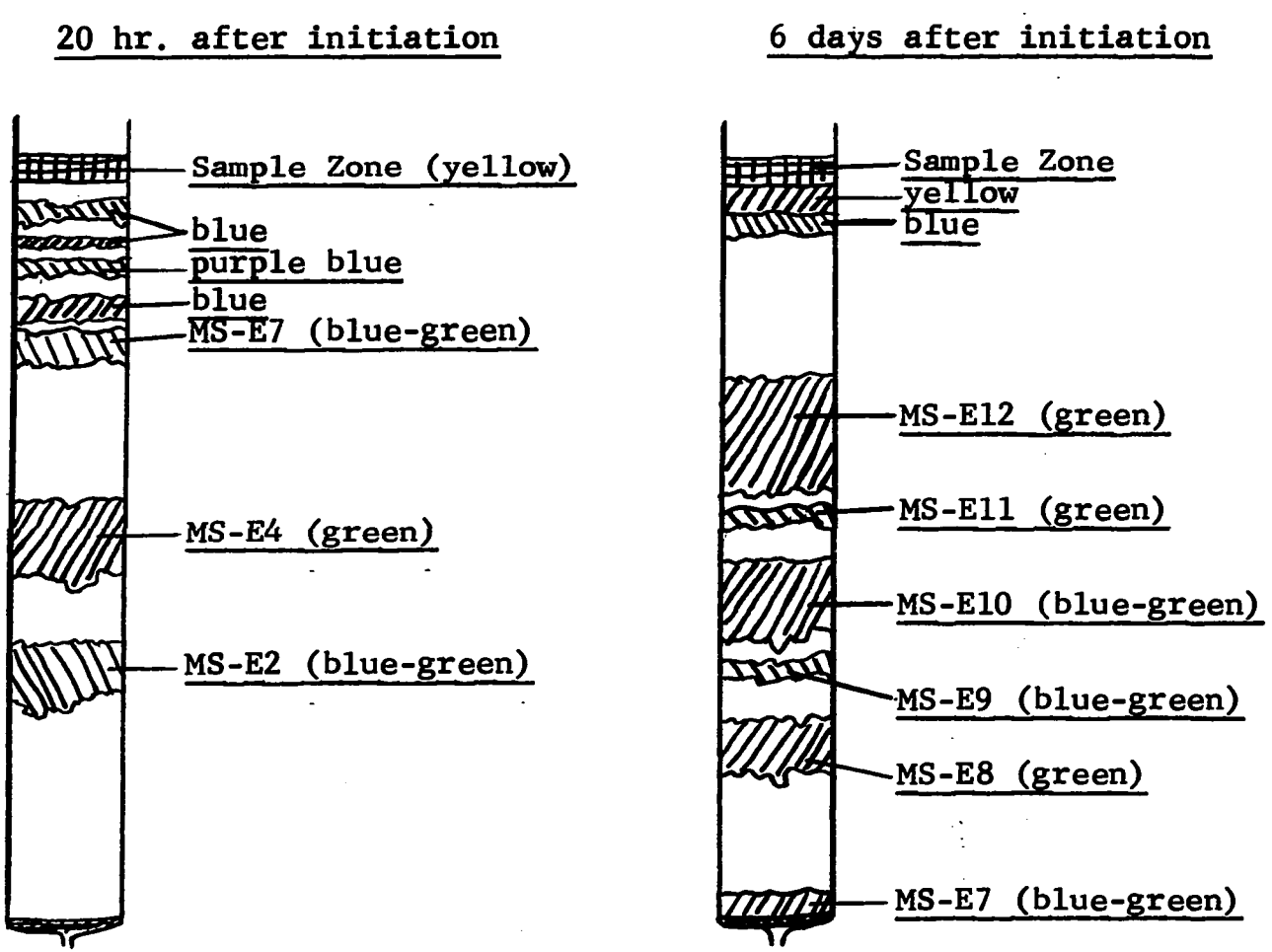
Separation and Purification of Individual Flavanone Glycosides--Column Chromatography of MS-C3

A column 7.5 cm in diameter was packed, under atmospheric pressure, to a depth of 65 cm from a slurry of 500 gm Polyclar AT in 4 liters of 75% benzene-25% methanol, and was thoroughly washed with the benzene-methanol solution. The column was labeled MS-E. Three grams of MS-C3 were charged to this column by dissolving the sample in 50 ml methanol and then adding, just prior to application to the column, 150 ml benzene to give the desired solvent ratio. The top 2 cm of the column adsorbent were stirred thoroughly with the sample solution to insure against irregularities in the leading edge of the sample zone due to sample application. When the level of the sample solution reached the top of the adsorbent, the inside of the column was rinsed with 50 ml of the benzene-methanol solvent. When the top of the adsorbent was again at the point of incipient dryness, 25% methanol in benzene was added as collection of eluant fractions was begun for the first time. Elution of compounds from the column was accomplished with varying percentages of methanol in benzene according to the following sequence: 25% methanol, 35 liters; 30% methanol, 2 liters; 35% methanol, 2 liters; 40% methanol, 8 liters; and 50% methanol, 22 liters. Flow rate was approximately 325 ml per hour. Eight zones which fluoresced either blue-green or green in 3660 Å U.V. light, as shown in Figure 3

Figure 3

Polyclar AT Column Chromatography of Flavanone Glycosides from the Grapefruit

Fluorescence in 3660 Å U.V. Light



were individually collected from the column. The fractions containing these zones, designated according to the order in which they moved off the column as fractions E2, -4, -7, -8, -9, -10, -11, and -12 (Figure 3), were reduced to dryness on a rotary evaporator. Each residue was reprecipitated by dissolving it in 1 part methanol, adding 3 parts benzene, and stripping off the solvent. Yellow solids were deposited in all the samples except E11, which could not be taken to dryness at 45-50°C on the flash evaporator.

Extensive thin-layer chromatography of samples prepared from these fractions indicated each to consist of a single individual flavanone glycoside. Furthermore, the close correlation between the R_f values of E2, E7, and E10 and those of isosakuranetin-7β-rutinoside, hesperidin, and naringenin-7β-rutinoside, respectively, as tabulated in Table 1, strongly indicated the presence of all three rutinosides in the grapefruit.

In an attempt to ensure the final removal of any Polyclar AT adsorbent or other extraneous nonflavonoid material which might be present, the individual flavanone glycosides were subjected to rechromatography on columns of silicic acid. E12 and E10 were purified on 7.5 x 50 cm silicic acid columns packed under 5 lbs pressure. Samples were dissolved in 50 ml methanol and silicic acid was poured in until a pasty mass resulted. Benzene (450 ml)

TABLE 1

R_f VALUES OF ISOLATED GLYCOSIDES AND STANDARDS ON THIN-LAYER CHROMATOGRAMS

Compound	Adsorbent / Solvent[2]	Woelm Polyamide A	Woelm Polyamide B	Avicel SF[1] C	Avicel SF[1] D	Avicel SF[1] E	Avicel SF[1] F
E12, E11, and Naringin		0.30	0.60	0.82	0.38	0.60	0.26
E10, E9, and Naringenin Rutinoside		0.37	0.62	0.79	0.32	0.56	0.21
E8 and Neohesperidin		0.43	0.61	0.68	0.35	0.57	0.39
E7 and Hesperidin		0.50	0.63	0.66	0.29	0.52	0.36
E4 and Poncirin		0.60	0.62	0.55	0.56	0.72	0.68
E2 and Isosakuranetin Rutinoside		0.67	0.63	0.52	0.53	0.67	0.64

1. Avicel SF--Microcrystalline cellulose (FMC Corporation, American Viscose Division, Marcus Hook, Pa.).
2. Solvent A. nitromethane-methanol (5:2, v/v, called NM).
   B. methanol-water (1:1, v/v, called MW).
   C. benzene-ethyl acetate-formic acid-water (18:42:12:5, v/v/v/v, called BzEFW).
   D. methyl isobutyl ketone-formic acid-water (14:3:2, v/v/v, called KFW).
   E. n-butanol-acetic acid-water (6:1:2; v/v/v, called BAW-1).
   F. benzene-acetic acid-water-nitromethane (34:32:5:18, v/v/v/v, called BzAWN).

was added, the sample was mixed thoroughly and charged to the column. Elution was effected using 10% methanol-90% benzene under atmospheric pressure. The flavanone glycoside zone, followed by its yellow brown fluorescence in 3660 Å U.V. light, pulled away from some yellow-green fluorescing material which remained at the top and was collected as it moved off the column. The fraction containing the zone was taken to dryness; the residue was picked up in 50 ml methanol and to this was slowly added 150 ml benzene. The solvent was stripped off, resulting in 605 mg of white amorphous precipitate.

The purification of E10 was accomplished in a similar manner and gave 530 mg of a white precipitate. The other six components were purified using the same general procedure except for the employment of different column sizes. For E9 and E11, 4.5 x 20 cm columns of silicic acid were used; for E4, a 3 x 20 cm column, and for E2, E7, and E8, the column dimensions were 2.5 x 15 cm. Preparation of the sample charge was adjusted accordingly. The weights of the purified components obtained were: E2 = 35 mg; E4 = 100 mg; E7 = 49 mg; E8 = 63 mg; E9 = 60 mg; E11 = 260 mg.

Studies on the Individual Flavanone Glycosides

Naringin and Naringenin-7β-Rutinoside

Fraction E12, which fluoresced green on the nonaqueous

Polyclar column, contained a greater quantity of material than any other fraction obtained from this column. It appeared to consist of a single, chromatographically pure compound that was extremely bitter in taste and was indicated by thin-layer chromatography to be naringin. Fraction E10, which fluoresced blue-green on the column, appeared practically identical with E12 on a number of paper and thin-layer chromatograms. It was, however, tasteless, and its R_f values, as seen in Table 1, when chromatographed on thin layers of Avicel in benzene-ethyl acetate-formic acid-water (18:42:12:5, v/v/v/v, called BzEFW), methyl isobutyl ketone-formic acid-water (14:3:2, v/v/v, called KFW), and n-butanol-acetic acid-water (6:1:2, v/v/v, called BAW-1) solvent systems were slightly but consistently shorter than those of E12 and of authentic cochromatographed standard naringin. When chromatographed on thin layers of Woelm polyamide in NM, E10 with an R_f of 0.37 was clearly differentiated from E12 and standard naringin, both of which had an R_f of 0.30. On all chromatograms, the R_f values of E10 agreed exactly with those of cochromatographed naringenin-7-rhamnoglucoside isolated from the sweet orange, which is believed to be identical with the naringenin-7β-rutinoside recently reported by Gentili and Horowitz (16) to be present in this fruit.

Hydrolysis. The products obtained after hydrolysis of E12 and E10 were identical. Two milligrams of each

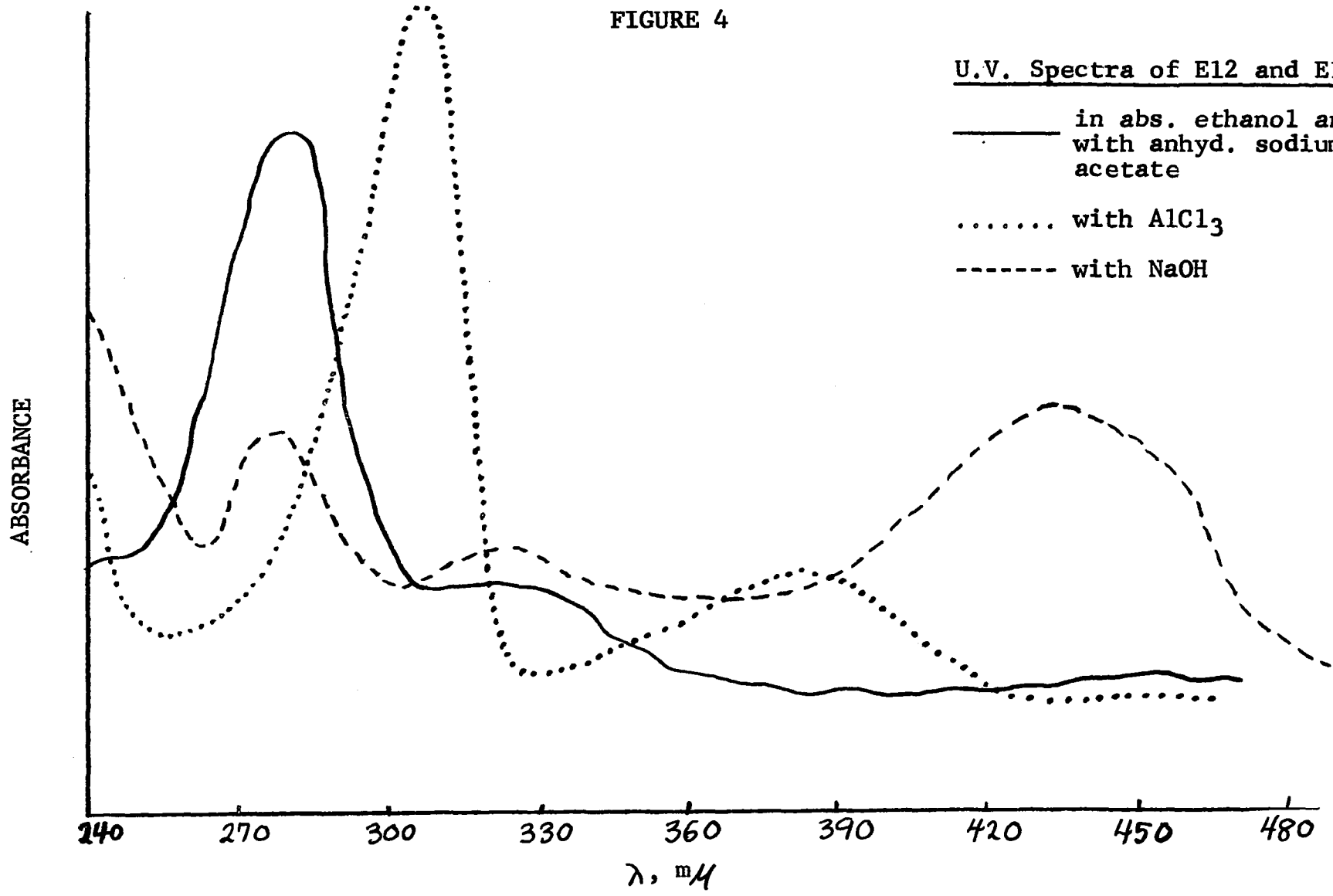
compound were hydrolyzed by refluxing for 3 hr with 2 ml of 3% hydrochloric acid. The cooled hydrolysate was poured into a 20 ml glass stoppered test tube and the 5 ml hydrolysis flask was rinsed successively with two 1 ml volumes of water and 4 ml of ethyl acetate. These rinsings were added to the hydrolysate in the test tube. The ethyl acetate was removed with a pipette and the extraction was repeated 4 more times. The combined ethyl acetate extracts were taken to dryness and the residue was dissolved in 3 ml of methanol. Cochromatography of the aglycones obtained with reference flavanone aglycones on thin layers of Adsorbosil 1 silica gel in benzene-acetic acid-water (125:72:3, v/v/v, called BzAW-1), benzene-nitromethane-water (3:2:5, v/v/v, upper layer, called BNW), and chloroform-acetic acid-water (2:1:1, v/v/v, lower layer, called CAW) showed that both E10 and E12 were glycosides of naringenin. The R_f values of the aglycones of E10, E12, and standard naringenin in the three solvent systems were 0.85, 0.38, and 0.59, respectively.

The aqueous layer remaining after extraction of each hydrolysate with ethyl acetate was deionized by passing the solution through a 2.5 x 7 cm ion-exchange column of Amberlite MB-1 (with a small amount of MB-3 at the bottom). The deionized eluate was taken to dryness and the residue was picked up in 1 ml water. The sugars thus obtained by hydrolysis of both E10 and E12 were shown to be rhamnose

and glucose by cochromatography with reference sugars on thin layers of Adsorbosil 1 in n-propyl alcohol-ethyl acetate-water (7:1:2, v/v/v, called PEW-1) and Avicel SF in PEW-1 and n-butyl alcohol-pyridine-benzene-water (5:3:1:3, v/v/v/v, called BPBW). Sugar spots were developed by spraying the chromatograms with aniline-oxalic acid reagent (31) and heating the sprayed chromatograms in the oven at 105°C for 20 minutes. R_f values obtained on the thin layers were similar to those obtained with the same solvents on paper chromatograms (32), but spots obtained were generally smaller and more compact on the thin layers, thus providing better differentiation of compounds with similar mobilities.

Ultraviolet Spectra. Information concerning the mode of attachment of the sugar residue to the aglycone in E10 and E12 was obtained by ultraviolet spectral studies. Absolute ethyl alcohol solutions of each compound (2 x 10^{-2} mg/ml) produced identical flavanone glycoside ultraviolet absorption spectra, with λ max of 283 and 330 mμ (Figure 4). Sodium acetate is a sufficiently strong base to ionize the 7-hydroxy group of a flavanone if this hydroxy is unsubstituted. Addition of the anhydrous reagent to an alcoholic solution of either compound, however, failed to produce a shift in the λ max at 283, showing that neither compound possessed a free 7-hydroxy group (33, 34). The λ max at 283 was shifted to 304 mμ in

FIGURE 4


U.V. Spectra of E12 and E10
in abs. ethanol and with anhyd. sodium acetate
..... with AlCl3
------- with NaOH
ABSORBANCE
240
270
300
330
360
390
420
450
480
λ, mμ

alcoholic aluminum chloride in both cases, indicating chelation of the $AlCl_3$ between the 5-hydroxy and 4-carboxyl groups and, therefore, the presence in both compounds of a free 5-hydroxy group (33, 34). Relatively rapid formation of the chalcone, as indicated by the appearance of a broad peak at 430 mμ, in dilute alcoholic sodium hydroxide, showed the simultaneous presence of a blocked 7-hydroxy and a free 4'-hydroxy group in both E10 and E12 (34). These results clearly indicated that the glycosidic linkage in both compounds must be at the 7-hydroxy of naringenin.

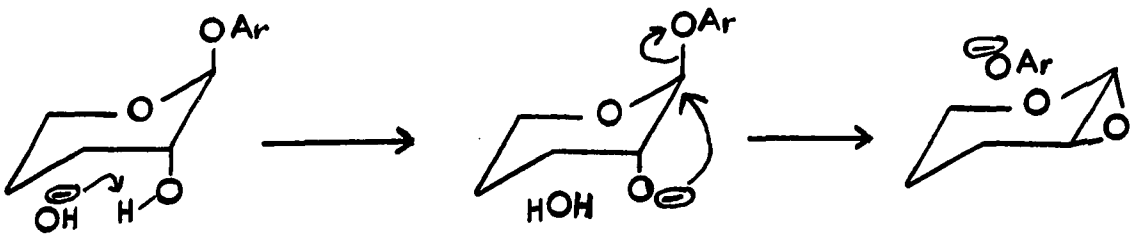
Enzyme Studies. The selective rhamnosidase isolated from naringinase C-100 by Dunlap, Hagen, and Wender (35) provided a means of demonstrating that the disaccharides of E10 and E12 were linked through glucose to the aglycone. Enzymic hydrolysis of E10 and E12 with the rhamnosidase yielded a compound in each case which appeared identical with authentic cochromatographed prunin. Ten milliliters each of solutions consisting of 2.0×10^{-3}% E10 or E12, 1.0% citric acid, and sufficient NaOH to give a pH of 4.0 were incubated with rhamnosidase for 6 hr at 50°C. Controls consisted of substrate-buffer solutions without the enzyme. Each solution was extracted immediately after incubation with two 10 ml volumes of ethyl acetate, the combined extracts were taken to dryness, and the residue was dissolved in 250 μl methanol. Samples thus obtained from the enzymic hydrolysates and controls were chromatographed on

thin layers of Avicel SF in BAW-1 and BzAWN solvent systems and the developed chromatograms were observed under 3660 Å U.V. light after spraying the chromatograms with 1% methanolic $AlCl_3$. Prunin, in the hydrolysates, was clearly differentiated from the original rhamnoglucosides E10 and E12 which remained unhydrolyzed in the control samples. These results confirmed that E10 and E12 were naringenin-7-glycosides, and indicated that both compounds were indeed naringenin-7β-rhamnoglucosides which could differ only in the mode of attachment of rhamnose to glucose in the sugar moiety.

Paper Electrophoresis. Further confirmation of the structure of E10 and -12 was gained by paper electrophoresis. The electrophoretic mobility of E10 was found to be identical with that of naringenin-7-rhamnoglucoside from the sweet orange, but significantly less than that of E12, which corresponded to authentic naringin, during paper electrophoresis in 0.1 M sodium tetraborate buffer of pH 2.5 (16). These results were consistent with those reported by Gentili and Horowitz (16), who state that flavanone-7β-rutinosides can be readily distinguished from the corresponding neohesperidosides by paper electrophoresis because of the relative lower migration rates of the rutinosides.

Alkaline Degradation. Horowitz and Gentili have shown that the degradation of flavanone-7β-neohesperidosides

on heating with 20-25% aqueous potassium hydroxide proceeds by a significantly different pathway than similar degradation of flavanone-7β-rutinosides (12, 13). The neohesperidosides yield phloracetophenone-4'-neohesperidoside as a degradation product, while the rutinosides yield mostly deglycosylated products, including phloroglucinol, as shown in Figure 5. The rationalization for this behavior is that in aromatic glycosides strong alkali ionizes the trans C-2 hydroxyl group resulting in displacement of the trans C-1 aryloxy group and produces the 1,2-anhydro compound which is further degraded (36, 37). If the C-2 group is substituted, this

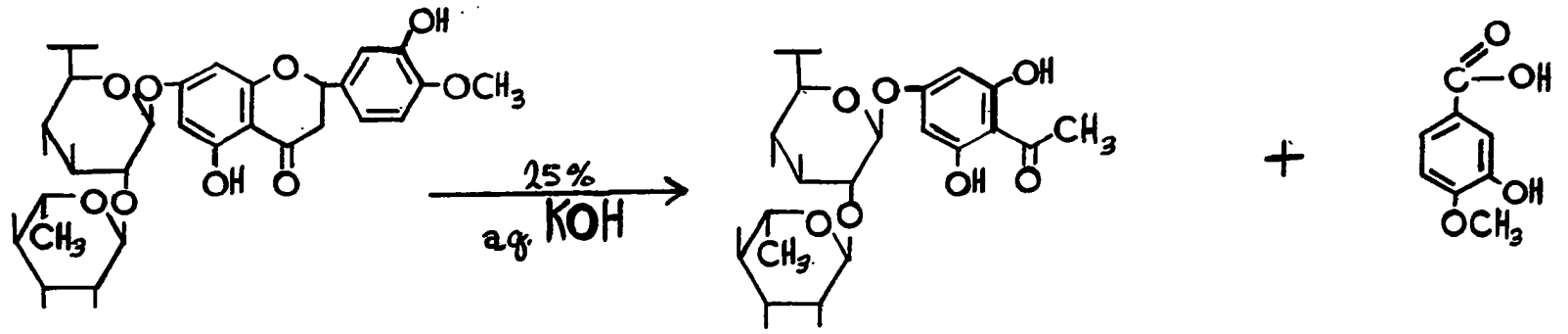


displacement cannot occur and the glycosidic linkage remains intact. Studies in this laboratory employing authentic hesperidin and neohesperidin indicated that this degradation could be adapted successfully to a micro scale, with identification of the products by thin-layer chromatography. Thus this procedure could serve as a useful tool in confirming the structure of small quantities of flavanone glycosides. Two milligrams each of E10 and E12 were, therefore, placed in a 25 ml conical flask equipped with a side arm and the flask was flushed with nitrogen. Four milliliters of 25% aqueous KOH was delivered

FIGURE 5

Alkaline Degradation of a Neohesperidoside (1) and a Rutinoside (2).

(1)

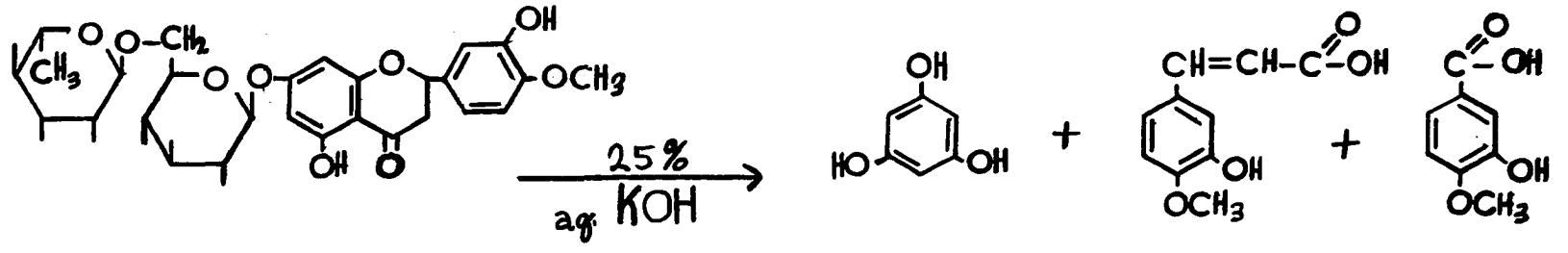


Neohesperidin

Phloracetophenone-4'-Neohesperidoside

Isovanillic Acid

(2)



Hesperidin

Phloroglucinol

Isoferulic Acid

Isovanillic Acid

to the flask and the solution was refluxed under N_2 (flow rate = 40 ml/min) for 3.5 hr. The pH of the cooled solution was adjusted to ca. 3 with 25% HCl, transferred to a separatory funnel, diluted to 25 ml, and extracted with three 25-ml volumes of ethyl acetate. The combined ethyl acetate extracts were taken to dryness and the residue was dissolved in 1 ml methanol. The samples were chromatographed with standard compounds on Avicel SF thin layers, in KFW, BzEFW, CAW, BzAW (6:7:3, v/v/v, upper layer, called BzAW-2), and 2% acetic acid solvents. To detect any phloracetophenone-4'-neohesperidoside, each plate was dried, sprayed with 1% $AlCl_3$ in methanol, and observed under 3660 Å U.V. Phenolic acids were detected by observation under 3660 Å U.V. after exposing the plate to NH_3 vapors and by the characteristic color reaction after spraying with diazotized sulfanilic acid reagent (38). The products obtained by alkaline degradation of E10 were thus found to include phloroglucinol, p-hydroxybenzoic acid, and p-hydroxycinnamic acid but not phloracetophenone glycoside, while E12 yielded mainly phloracetophenone-4'-neohesperidoside and p-hydroxybenzoic acid.

The evidence described above confirmed that fraction E12 was indeed naringenin-7β-neohesperidoside (naringin) and indicated that E10 was naringenin-7β-rutinoside.

Aglycone Oxidation. Further proof that the disaccharide of E10 was rutinose was achieved by oxidation of

the aglycone portion of the molecule by the permanganate method of Chandler (39) which released an intact disaccharide. The sugar was shown to have R_f values identical with those of cochromatographed authentic rutinose on thin layers of Adsorbosil 1 in PEW-1 and in n-propyl alcohol-ethyl acetate-water (32:57:13, v/v/v, called PEW-2) solvents and on thin layers of Avicel SF in PEW-1 and -2, BPBW, and n-butyl alcohol-acetic acid-water (4:1:5, v/v/v, upper phase, called BAW-2).

Gas Chromatography of Methylated Sugars.[1] Final confirmation that the rhamnose to glucose linkage in E10 was 1 to 6, and not 1 to 2, 1 to 3, or 1 to 4, was accomplished by completely methylating the flavanone glycosides (40), methanolyzing the permethyl ethers produced, and identifying the methyl glucosides obtained by gas chromatography (41, 42). The procedures employed by Albach (43) for methylation, methanolysis, and preparation of samples for gas chromatographic analysis were modified to accommodate 10 mg of glycoside.

In a typical run, 10 mg of flavanone glycoside were dissolved in 1 ml of anhydrous N,N-dimethylformamide in a 10 ml Erlenmeyer flask. Methyl iodide (0.06 ml) and freshly precipitated silver oxide (0.06 g) were added, the flask was stoppered, and the contents were stirred for 24

[1]The work described in this section was done by Dr. William J. Dunlap, Research Chemist for the University of Oklahoma Research Institute.

hr. Chloroform (5 ml) was then added to the solution, and the silver iodide and silver oxide were removed by filtration. The filtrate was evaporated on a rotary evaporator, and the residual sirup was dissolved in 5% hydrogen chloride in methanol. The solution was refluxed for 24 hr, cooled, neutralized with sodium isopropoxide, and the solvent was removed by flash evaporation. The residue was suspended in chloroform, and the sodium chloride formed in the neutralization step was removed by filtration. The chloroform was again removed by flash evaporation and the residue was dissolved in 0.5 ml fresh chloroform. This solution was subjected to gas chromatographic analysis on an F&M Model 810 gas chromatograph equipped with dual flame ionization detectors. Standard methylated sugars for use in the gas chromatographic analysis were prepared by methylation of various standard sugars and glycosides. The starting materials and the methylated sugar standards obtained were: methyl-α-D-glucopyranoside, methyl-2,3,4,6-tetra-O-methyl-α-D-glucopyranoside; maltose, methyl-2,3,4,6-tetra-O-methyl-β-D-glucopyranoside and α- and β-methyl glycosides of 2,3,6-tri-O-methyl-D-glucopyranoside; melibiose, α- and β-methyl glycosides of 2,3,4-tri-O-methyl-D-glucopyranoside; and naringin, α- and β-methyl glycosides of 3,4,6-tri-O-methyl-D-glucopyranoside.

Table 2 presents the relative retention times obtained for the standards and the tri-O-methyl-D-glucopyranosides

TABLE 2

RELATIVE RETENTION TIMES FOR METHYLATED GLUCOSES AND PRODUCTS FROM COMPLETE METHYLATION AND METHANOLYSIS OF FLAVANONE GLYCOSIDES

Compound	Relative Retention Times	
	Butanediol Succinate Column	Carbowax 20M Column
2,3,4,6-α-D-glucopyranoside	1.00	1.00
" -β-"- "	1.49	1.39
2,3,4-β- " "	2.65	2.70
3,4,6-β- "- "	3.06	3.07
2,3,6-β- "- "	3.44	3.58
2,3,4-α- "- "	3.75	3.82
3.4.6-α- "- "	3.64	3.81
2.3.6-α- "- "	4.69	4.84
Isosakuranetin-7β-Rutinoside (E2)	2.68,3.79	2.68,3.80
Hesperetin-7β-Rutinoside (E7)	2.66,3.72	2.68.3.80
Naringenin-7β-Rutinoside (E10)	2.70.3.80	2.67,3.80

Conditions: Butanediol Succinate Column--6 ft x 1/4 in, 5% on Gas Chrom Q (80-100 mesh); 172°; 60 ml/min He.

Carbowax 20M Column--6 ft x 1/4 in, 6% on Gas Chrom Q (80-100 mesh); 158°; 65 ml/min He.

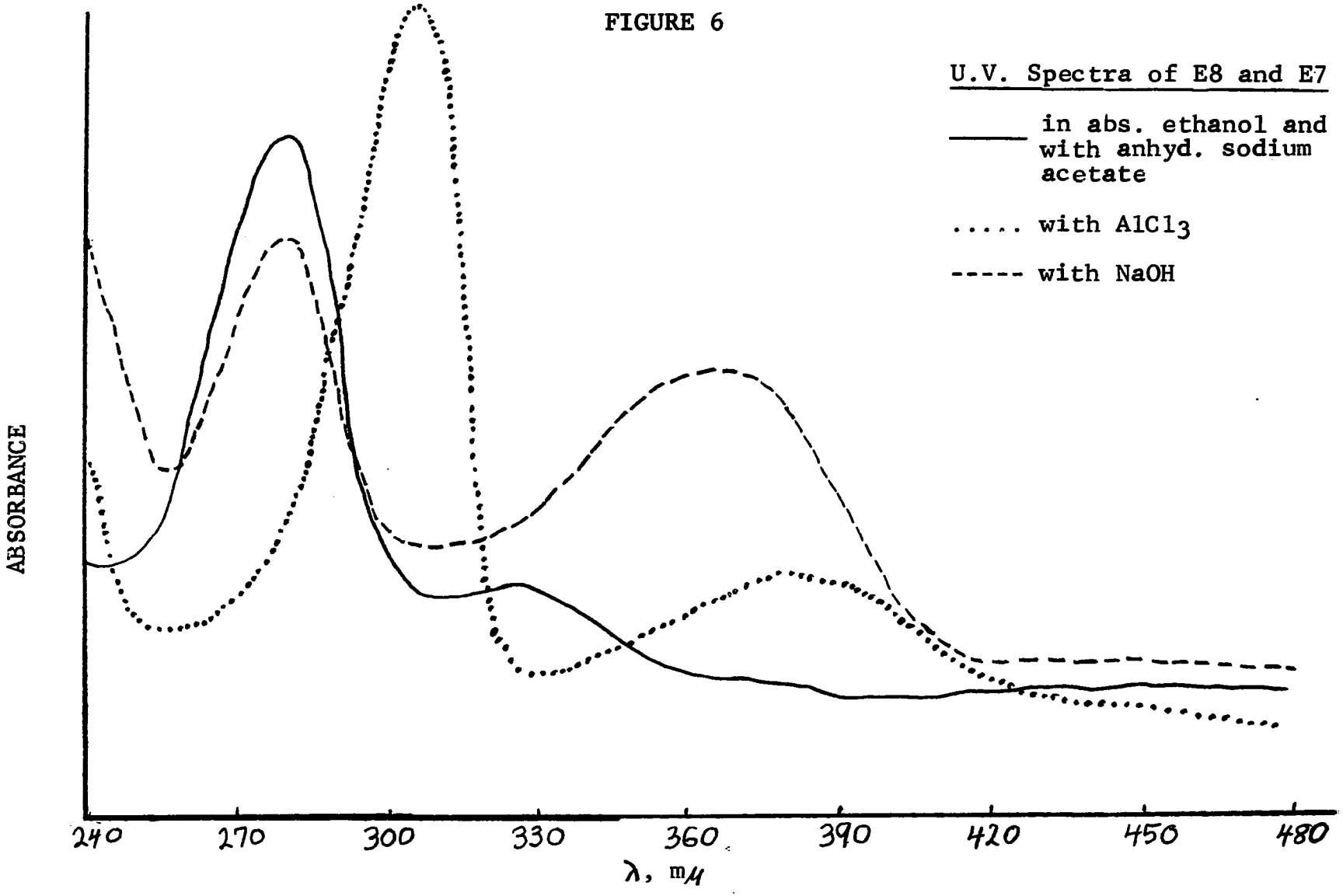
produced from E10. These data indicate that the methyl glucosides resulting from permethylation and methanolysis of E10 are the α- and β-methyl glycosides of 2,3,4,-tri-O-methyl-D-glucopyranoside and hence the rhamnose to glucose linkage in E10 must be 1 to 6.

The methods employed to identify naringin and naringenin-7β-rutinoside were also used to identify the remaining isolated flavanone glycosides and these will not be described again in detail. Any significant or important changes made in procedures or material used are mentioned.

Neohesperidin and Hesperidin

Fraction E8, which fluoresced green on the Polyclar AT column, and fraction E7, which fluoresced blue-green, were found to correspond in R_f values with authentic cochromatographed neohesperidin and hesperidin, respectively (Table 1), when subjected to thin-layer chromatography. The compounds were clearly differentiated by chromatography on thin layers of Woelm polyamide, using NM solvent (neohesperidin and E8, R_f 0.43; hesperidin and E7, R_f 0.50). Fraction E8 possessed a very bitter taste, while E7 was tasteless. Ultraviolet spectra (Figure 6) of both showed the usual flavanone glycoside peaks with λmax at 281 and 326 mμ in absolute ethanol. No shift was observed in these maxima upon treatment with anhydrous sodium acetate indicating in each case a substituted 7-hydroxy

FIGURE 6


U.V. Spectra of E8 and E7
in abs. ethanol and with anhyd. sodium acetate
..... with AlCl3
----- with NaOH
ABSORBANCE
240
270
300
330
360
390
420
450
480
λ, mμ

group; addition of alcoholic $AlCl_3$ produced a shift of the λmax at 281 to 305 mμ indicating the presence of a free 5-hydroxy group in both. Both E8 and E7 were converted to chalcones by treatment with dilute alcoholic sodium hydroxide. However, chalcone formation was slow, requiring approximately 30 minutes, and the characteristic λmax appeared at 365 mμ (in contrast to λmax of 430 mμ for naringin chalcone) indicating that both the 7-hydroxy and 4'-hydroxy groups were closed (33, 34).

Acid hydrolysis and identification of the products by thin-layer chromatography showed both E8 and E7 to consist of hesperetin, glucose, and rhamnose. Upon enzymatic hydrolysis with rhamnosidase, E7 and E8 yielded identical glycosides which corresponded exactly on thin-layer chromatograms with cochromatographed hesperetin-7β-glucoside obtained by partial hydrolysis of authentic hesperidin. The combined spectral and hydrolysis studies thus indicated E7 and E8 to be hesperetin-7β-rhamnoglucosides.

E7 was found to have an electrophoretic mobility on paper which was identical to that of hesperidin, but less than that of E8, which corresponded to neohesperidin. Upon alkaline degradation, E8 yielded phloracetophenone-4'-neohesperidoside and isovanillic acid, while E7 yielded phloroglucinol, isoferulic acid, and isovanillic acid, but no phloracetophenone glycoside.

On the basis of this evidence, E8 was confirmed as hesperetin-7β-neohesperidoside (neohesperidin) and E7 was indicated to be hesperetin-7β-rutinoside (hesperidin). Further study of E7 showed that it yielded, upon permanganate oxidation, a disaccharide with chromatographic properties identical with those of authentic rutinose, and upon permethylation and methanolysis, α- and β-methyl-2,3,4-tri-O-methyl-D-glucopyranosides (Table 2). Hence the identity of E7 was confirmed as hesperidin.

Poncirin and Isosakuranetin-7β-Rutinoside

Fractions E4, which fluoresced green on the Polyclar AT column and E2, which fluoresced blue-green, were found to correspond in R_f values on thin-layer chromatograms to authentic cochromatographed poncirin and isosakuranetin-7β-rhamnoglucoside from the sweet orange (Table 1). The R_f values of E4 and -2 on Woelm polyamide with nitromethane-methanol (5:2) solvent, which gave the best separation of these compounds which could be obtained, were 0.60 and 0.67 respectively. Ultraviolet spectral, acid and enzymic hydrolysis, paper electrophoresis, degradation,[2] oxidation, and methylation studies, as previously described, confirmed

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[2]To identify the p-methoxycinnamic and anisic acids produced by degradation of E2 and -4, it was necessary to use the solvent systems: n-butanol-ethanol-ammonium carbonate buffer (40:11:19, v/v/v, upper layer) and n-butanol-nitromethane-ammonium carbonate buffer (40:11:19, v/v/v, upper layer) as recommended by Dunlap and Wender (44). The buffer contains 72 g $(NH_4)_2CO_3$ and 25.5 g NH_3 per liter H_2O.

E4 to be isosakuranetin-7β-neohesperidoside (poncirin) and E2 to be isosakuranetin-7β-rutinoside.

Fractions E9 and E11

Fraction E9 was a blue-green fluorescing zone which moved off the Polyclar AT column just prior to naringenin-7β-rutinoside, E10, while E11 was a green fluorescing zone moving off the column after the rutinoside but just prior to naringin, E12 (Figure 3). Except for differing mobilities on the nonaqueous Polyclar AT column, E9 and E11 appeared identical with E10 and E12, respectively, in all studies conducted on the previously identified six flavanone glycosides. Fraction E9 was tasteless, while E11 was very bitter. The R_f values of E9 and -11 were identical, respectively, to those of cochromatographed E10 and -12 in every paper and thin-layer chromatography system tried, including Woelm polyamide with nitromethane-methanol solvents. The migration rates during paper electrophoresis of E9 and -11 were likewise identical with those of E10 and E12. Acid hydrolysis, ultraviolet spectral studies, and enzymic hydrolysis with rhamnosidase showed that E9 and E11 were naringenin-7-rhamnoglucosides. It was postulated that E9 and -11 are diastereoisomers of E10 and -12 respectively, differing from these compounds only in the configuration of the carbon atom number 2. If this were true, there would possibly be a detectable difference in the optical

rotations of the corresponding diastereomeric pairs. The specific rotations of the four compounds were therefore determined in methanol solution. Also included in this study were samples of E9, naringenin rutinoside, E11, and naringin purified from late-season grapefruit segments by methods similar to those used for their isolation from the mid-season grapefruit segments. The results are presented below:

	$[\alpha]_D^{25^\circ}$	
Compound	Mid-Season	Late Season
Naringin	-88°	-96.5°
E11	-94°	-96.5°
Naringenin Rutinoside	-99°	-103°
E9	-99°	-104°

The results were disconcerting for two reasons: different rotations were obtained for the corresponding compounds from mid- and late-season fruit; and there seemed to be no significant difference in rotations of the compounds which were thought to be diastereomers, except possibly in the case of naringin and E11 from mid-season fruit. Several explanations may be advanced to account for the erratic results obtained. Naringin and E11 can be re-crystallized from water and dried at 138°C to give the dihydrate. No suitable solvent could be found to re-crystallize naringenin rutinoside and E9; therefore, these

had to be weighed as the white amorphous precipitate. Thus there was doubt concerning the actual quantity of compound present in solutions. The adsorption of water by the sample during weighing and changes in concentration of the solution through evaporation of some of the volatile methanol solvent may be other possible sources of error. Horowitz has reported the specific rotation of naringin in water as -84.4°C (13); however, attempts to prepare an aqueous solution of naringin that was sufficiently concentrated for measurement of the rotation with the equipment available were unsuccessful. The possibility also exists that the practically identical rotations obtained for the compounds thought to be diastereomers may indicate that racemization occurred during purification of the compounds after their separation on the nonaqueous Polyclar AT column.

Fraction MS-C5

Identification of Prunin

The thin-layer chromatography system: Woelm polyamide with nitromethane-methanol (5:2), which was used to monitor the fractions from column MS-C, does not separate standard neohesperidin (R_f = 0.43) from standard prunin (R_f = 0.42). Fractions MS-C3 and MS-C5 from column MS-C both had components which migrated to the 0.42-0.43 zone; however, they appeared to be different compounds since MS-C4 showed no $AlCl_3$

positive material at R_f = 0.42-0.43. Moreover, when chromatographed in methanol-water (1:1, v/v, called MW) solvent on Woelm polyamide, a system which clearly resolves standard neohesperidin (R_f = 0.61) from standard prunin (R_f = 0.44), a component was detected which cochromatographed with prunin but none was detected corresponding to neohesperidin. MS-C5 was, therefore, subjected to column chromatography in an attempt to isolate sufficient quantities of the material, presumably prunin, for identification.

Column Chromatography. Fraction MS-C5 was taken to dryness on a flash evaporator using isopropyl alcohol to remove the water. The residue (205 mg) was dissolved in 6 ml methanol, the methanol solution was adsorbed onto 1.5 g washed Polyclar AT, and this was allowed to dry. The dry Polyclar AT containing the sample was slurried with a small amount of solvent (benzene-methanol, 3:1) and delivered to a 2.5 x 24 cm Polyclar AT column, labeled MS-H, prepared from a slurry of 15 g of the polyamide in 100 ml of the solvent. Elution was accomplished using benzene-methanol (3:1) under atmospheric pressure. Individual zones, as observed under 3660 Å U.V. light, were collected as they moved off the column. Thin-layer chromatograms indicated fraction MS-H8 (300 ml), which began eluting after 1775 ml solvent had passed through the column, contained the compound of interest. It was further purified by chromatography on a 2.5 x 10 cm silicic acid

column with benzene-methanol (3:1) as solvent, yielding 27.9 mg of pure compound.

Characterization Studies. MS-H8 cochromatographed with authentic prunin on thin layers of Woelm polyamide with NM and MW solvent systems as mentioned previously, and also on Avicel SF in BAW-1 (R_f = 0.42, 0.44, and 0.84, respectively). The compound possessed a bitter taste and upon hydrolysis yielded naringenin and glucose. Ultraviolet adsorption spectra in ethanol and in the presence of various reagents were similar to those of naringin indicating that the glucose was attached to the aglycone at the 7-hydroxy position. On the basis of this evidence, MS-H8 is almost certainly prunin.

Discussion

The probable presence of rutinosides in grapefruit was indicated previously by the identification of rutinose among the sugars liberated by ozonolysis of crude extracts of grapefruit peel flavonoids (10), but this is the first reported isolation of an intact flavanone rutinoside from grapefruit. An impure fraction apparently consisting mostly of naringenin rhamnoglucoside which was not naringin was previously isolated by Dunlap and Wender (18) from grapefruit in this laboratory, but thin-layer chromatography indicates that this fraction does not contain naringenin-7β-rutinoside.

The identification in grapefruit of the rutinosides of naringenin, hesperetin, and isosakuranetin should be of importance to the grapefruit industry. The poor correlation often obtained between bitterness of grapefruit and flavonoid content may be partially explained by the discovery of these tasteless rutinosides, particularly naringenin-7β-rutinoside, in the fruit, since analytical procedures which have been employed for flavonoid determination in citrus fruit, such as the Davis method (45), are unable to distinguish between the rutinosides and the bitter neohesperidosides.

Knowledge of the occurrence of the rutinosides along with the neohesperidosides should be a significant contribution to the elucidation of the metabolism of these compounds in the fruit and aid in formulating theories as to the biosynthesis and transformation of citrus flavonoids in general. It has been postulated that the decrease in bitterness of grapefruit which often occurs with maturity may be the result of a transglycosylation reaction which changes the bitter flavanone neohesperidosides to tasteless rutinosides (10, 17). Recent quantitative studies (46) in this laboratory on the seasonal variations in grapefruit of individual neohesperidosides and rutinosides, however, did not give any evidence for a transglycosylation mechanism. The percentage of flavonoids in fruit in the early stages of development is very high. Kesterson and Hendrickson

in Florida (19, 20) reported up to 75% flavonoids on a dry weight basis in grapefruit one-half inch in diameter; the absolute flavonoid content was reported to increase until a certain equatorial diameter (ca. 2 in.) was reached and then to become approximately constant. In the seasonal variation study, Hagen, Dunlap and Wender (46) confirmed that the original high flavonoid concentration was decreased during growth of the grapefruit; however, it was also revealed that the ratio of neohesperidosides to rutinosides remained essentially constant throughout maturation, indicating that transglycosylation was not the reason for the reduced bitterness of the late season fruit. This constant ratio does not eliminate the possibility of a transglycosylation reaction occurring, since the constant seasonal ratio of these compounds could be the reflection of steady-state equilibrium concentrations involving interconversion of neohesperidosides and rutinosides. Whether such a dynamic situation exists, or whether the neohesperidoside and rutinoside content of the fruit is fixed at an early stage of development and not further changed must await other studies on the precursors and biosynthesis of the grapefruit flavonoids. An obvious possibility is the use of isotopic tracers to determine the presence or absence of a transglycosylation reaction.

The isolation and identification of prunin from grapefruit has apparently not been reported before.

Prunin, like the flavanone neohesperidosides, is also a bitter compound (10) and therefore may be of importance to the grapefruit industry if its concentration in the fruit is sufficient to impart a significant bitterness to grapefruit products. Since naringin and naringenin rutinoside occur in relatively large quantities in the grapefruit, it may be argued that the prunin isolated was actually an artifact produced by the partial hydrolysis of small quantities of the naringenin rhamnoglucosides during sample processing. However, two-dimensional thin-layer chromatography of Ruby Red grapefruit juice sacs concentrates prepared under relatively mild conditions by Hagen, Dunlap, and Wender (46) for the quantitative determination of grapefruit flavanone glycosides, clearly showed prunin to be present, indicating the artifactual nature of prunin to be unlikely. Any biosynthetic scheme postulated for the flavanone glycosides of the grapefruit should include, or be able to account for, prunin as a natural metabolite.

The polyamide and polyvinylpyrrolidone chromatography procedures described were extremely useful for the study of the closely related flavonoid glycosides of the grapefruit and appear superior to any other method available for preliminary separation of the flavonoids from crude concentrates and for subsequent separation of the very similar flavanone glycosides obtained in preliminary

isolation procedures. Polyamides, in general, function by hydrogen bonding between the phenolic groups and the amide groups in the interior of the particle as well as on the surface. A column therefore has a capacity a hundred times greater than one based on simple adsorption (47). Practically all previous uses (25, 26, 27, 28, 29) of polyamides for the fractionation of plant materials on columns employed essentially aqueous solvents. The success with the nonaqueous Polyclar AT columns in resolving the neohesperidosides from the closely related rutinosides should prompt the consideration of nonaqueous polyamide column chromatography as a method for preparative separation of other closely related plant phenolics.

CHAPTER III

ISOLATION AND IDENTIFICATION STUDIES ON TWO FLAVANONE TRIGLYCOSIDES OF THE GRAPEFRUIT

During the course of the work on the isolation of the neohesperidosides and rutinosides from the mid-season grapefruit segment concentrate, two additional compounds, judged to be flavanones because of the fluorescence of their $AlCl_3$-complex, were encountered in the fractions eluted early from the aqueous Ultramidpulver columns. Thin layer chromatograms of fractions from pilot column studies of this concentrate had previously indicated the presence of these compounds and comparable components were subsequently shown to occur also in concentrates of late-season grapefruit segments. Due to the potential importance and interest of additional flavanones in the grapefruit, work was undertaken to isolate sufficient quantities of these compounds for identification. Late-season grapefruit segments were chosen for this effort.

Preliminary Isolation

Twenty-five pounds of fruit, harvested in early April,

1964, from the same trees from which the mid-season fruit was obtained, provided the segments used in this study. After the core and peel were removed, the segments were immediately worked up in the manner described for the mid-season segments. The resulting 1 liter of concentrate from segments of late-season grapefruit was chromatographed on an 8-cm diameter column packed to a depth of 64 cm with Polyclar AT. The column was packed under 5 lb pressure from an aqueous slurry of 600 g of the polyamide (which had previously been soaked in water for 1.5 hr), washed with about 20 1 of DMF solution, and rinsed with distilled water. The sample was applied to the column under 5 lb pressure. However, channeling often occurred during sample application and it was frequently necessary to repair the damage by stirring the upper portion of the column. Channeling was finally arrested by applying a protective layer of Polyclar AT to the top of the column. Elution was begun with distilled water under 5 lb pressure with 1 liter fractions being taken. Fractions 7, 8, and 9, indicated by thin-layer chromatography to contain the compounds of interest, were combined, labeled LS-C, and the compounds were precipitated with isopropyl alcohol to yield 1.66 g of light brown powder. Thin-layer chromatography of a sample from LS-C showed the pair of new flavanone-like compounds to have R_f values of 0.20 and 0.25 in NM on Woelm polyamide, values which were lower

than that of naringin (R_f = 0.30). This thin layer chromatography system separates the standard flavanone glycosides in pairs according to the aglycone and the pairs are further resolved according to the sugar moiety. From a mixed spot, naringin (R_f = 0.30), naringenin rutinoside (0.37), neohesperidin (0.43), hesperidin (0.50), poncirin (0.60), and isosakuranetin rutinoside (0.67), will separate from origin to solvent front in the order just given. In each pair the rutinoside is the faster migrating compound and the neohesperidoside the slower migrating compound. The similarity in the pattern of resolution between the two flavanone-like components of LS-C suggested that these compounds might also constitute a rutinoside-neohesperidoside pair of the same aglycone.

Separation and Purification of LS-C Components

LS-C was subjected to chromatography on a 7.5 x 50 cm Polyclar AT column as described for fraction MS-C3. The column was eluted with 22 liters of benzene-methanol (3:1), then the methanol concentration was increased 5% for every 4 liters of solvent. Fraction LS-C14 (4 liters), which began coming off the column after 45 liters of solvent had passed through, contained the faster migrating component and fraction LS-C15 (4 liters) contained the slower migrating component. Extraneous $AlCl_3$ positive material was removed from LS-C14 by rechromatography on a 2.5 x 20

cm Polyclar AT column with benzene-methanol (7:3) as solvent, and it was further purified on a 1.2 x 8 cm silicic acid column with benzene-methanol (3:1) solvent. A yield of 39.7 mg of white amorphous solid was obtained. Fraction LS-C15 was separated from other contaminating flavonoids by successive rechromatography on a 3 x 15 cm Polyclar AT column with benzene-methanol (3:1) as solvent, and then on a 1.5 x 10 cm column packed with Woelm polyamide (column grade) and eluted with nitromethane containing from 5 to 15% methanol, to yield 8.2 mg of a light yellow amorphous solid. Due to the small amount of LS-C15 obtained from the last column, it was not further purified. Thin-layer chromatography showed both LS-C15 and LS-C14 to consist of single, individual flavanone glycosides and to contain no other detectable components.

Identification Studies on LS-C15 and -C14

Preliminary Characterization

The appearance of LS-C15 and -C14 on thin layer chromatograms sprayed with methanolic $AlCl_3$ indicated that they were probably flavanone glycosides, but they did not correspond chromatographically with any flavanone glycoside standards available. The R_f values of these compounds and of naringin on two adsorbents with four different solvent systems are given below:

	Woelm Polyamide		Avicel SF		
	Nitromethane-methanol (2:1)	Methanol-water (1:1)	BAW-1	BzEFW	KFW
LS-C14	0.48	0.85	0.29	0.08	0.02
LS-C15	0.46	0.81	0.31	0.08	0.03
Naringin	0.50	0.59	0.70	0.82	0.30

Hydrolysis. Acid hydrolysis of LS-C15 and LS-C14 and identification of the products by thin-layer chromatography showed both to contain naringenin, rhamnose, and glucose. Visual comparison on the thin-layer chromatograms of sugars obtained from either LS-C15 or -C14 with standards indicated that the glucose to rhamnose ratio was greater than one.

Ultraviolet Spectra. LS-C14 and LS-C15 produced identical ultraviolet adsorption spectra, with λmax at 283 and 330 mμ. Addition of anhydrous sodium acetate to an alcoholic solution of either compound failed to produce a shift in the λmax at 283 mμ, showing that neither compound possessed a free 7-hydroxy group; the λ max at 282 mμ was shifted to 305 mμ in alcoholic $AlCl_3$, indicating the presence in both compounds of an unsubstituted 5-hydroxy group (33, 34). Prolonged standing (30 min.) of either compound in dilute alcoholic NaOH resulted in chalcone formation which produced a λmax at 358 mμ. The slow formation of the chalcone and the appearance of its λ max at 358 instead of 430 mμ indicated that in both

compounds the 4'-hydroxy group was substituted (34).

The ultraviolet adsorption spectra which showed that both the 7- and 4'-hydroxy groups of naringenin are substituted in LS-C15 and -C14 and the possibility that they may contain more than two moles of monosaccharides per mole of aglycone made it evident that the ratio of naringenin:glucose:rhamnose in the compound must be determined. Existing methods for the determination of the aglycone to sugar ratio in milligram amounts of glycosides did not seem applicable for these particular compounds, therefore, a method was developed to determine the naringenin:glucose:rhamnose ratio in LS-C15 and -C14.

Ratio Determination Method

Hydrolysis. Approximately 1 mg of glycoside was weighed accurately and placed in a 5 ml 19/22 ℸ conical flask. Five milliliters of 3% HCl was added, the solution was refluxed for 1 hr and cooled to room temperature.

Polyclar AT Column. Polyclar AT (0.25 g) was slurried in 4 ml of methanol-water (1:1). After standing for 30 min., the slurry was poured onto a small glass wool pad at the bottom of a 1.2 x 15 cm column equipped with a 1 mm bore stopcock. The resulting 1.5 cm column of adsorbent was washed successively with 30 ml of H_2O, 60 ml of methanol, and again with 30 ml of H_2O. Five millimeters of H_2O was left over the adsorbent.

Ion-Exchange Column. Amberlite IR-45 (OH form) Analytical Grade (3.0 g) was soaked overnight in H_2O and washed into a 1.2 x 15 cm column equipped with a coarse fritted glass disc and a 1 mm bore stopcock. The resin was backwashed and allowed to settle. Two centimeters of water was left above the resulting 5 cm column of resin.

Elution. The hydrolysate was poured into an 18 cm stemmed delivery (thistle) tube equipped with a stopcock just below the bulb and dripped onto the Polyclar AT column, which retains the aglycone and allows the sugars to pass through. The eluate from the Polyclar was, in turn, dripped directly onto the ion-exchange resin to neutralize the acid. The hydrolysis flask and delivery tube were rinsed successively 3 times with 5 ml volumes of water which were delivered to the polyamide column. After each rinse had passed through, the column was eluted with an additional 30 ml of water. The 50 ml of water eluate from the Polyclar column was dripped directly onto the ion-exchange column, maintaining a height of approximately 2 cm water over the resin at all times. To insure removal of all sugar from the resin, it was eluted with an additional 25 ml water, the total 75 ml water eluate was caught in a 100 ml round bottom 24/40 ℈ flask and taken to dryness by flash evaporation. The sugars were dissolved in three 5 ml volumes of water, transferred to a 25 ml conical

19/22 T flask, and the contents were taken to dryness by flash evaporation. The sugars were then dissolved in 500 µl water delivered with a Kirk micropipette.

After the 50 ml of water eluate had passed through the Polyclar, the column was removed from above the ion-exchange resin and the aglycone was stripped from the Polyclar with 75 ml of methanol, the first three 5 ml volumes being used to rinse the hydrolysis flask and delivery tube. The eluate was caught in a 100 ml round bottom 24/40 T flask and taken to dryness by flash evaporation. The aglycone was dissolved in two 10 ml volumes of methanol, transferred to a 25 ml volumetric flask and the contents were brought up to volume with methanol.

Glucose and Rhamnose Determination. Two 10 µl samples of the sugar solution were applied, using Kirk micropipettes, to an Adsorbosil 1 thin layer (250 microns) plate activated for 1.5 hr at 105°C, 5 cm from the bottom edge, along with glucose and rhamnose standards of 1, 2.5, 5.0, 7.5, and 10 µg amounts. The chromatogram was developed in n-propyl alcohol-ethyl acetate-water (7:1:2, v/v/v) to a distance of 10 cm beyond sample application, dried 3 hr in an air current, sprayed with 10 ml of aniline-oxalic acid reagent (31), heated at 105° for 20 min., and allowed to cool. Chromatograms were prepared in quadruplicate to reduce errors due to individual plate variation. Density of the brown (glucose) and yellow-brown (rhamnose)

colors of the reaction products was determined on a Photovolt TLC Desnitometer Model 530 (Photovolt Corporation, New York), using a Wratten 47B filter to enhance response. A standard curve was obtained by plotting the average values of the four values obtained for each standard sample. The response was linear from 1 to 10 μg for both rhamnose and glucose.

Naringenin Determination. Three 250 μl samples of the naringenin solution were transferred to 10 ml volumetric flasks, 50 μl of 1% methanolic $AlCl_3$ was added 1 hr prior to the determination to develop full fluorescence intensity, and each solution was brought to volume with methanol. Standard solutions were prepared containing 1, 2.5, 5, 7.5, and 10 μg of naringenin per 10 ml of methanol. Fluorescence determinations were made with a Turner Fluorometer, Model 110 (G. K. Turner Associates, Palo Alto, Calif.), using Pyrex cuvettes (12 x 75 mm) in a standard cuvette door. An activation wavelength of 325 $\pm$ 2 mμ was 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 510 mμ was used as secondary emission filter. The amount of naringenin was determined from the standard curve which was linear from 1 to 10 μg.

To assess the accuracy of the method just described,

three samples of standard naringin dihydrate were subjected to analysis for the naringenin:glucose:rhamnose ratio. Inspection of recovery values for the results obtained, as presented in Table 3, indicated the method was acceptable for the ratio determination. When analysed by this method, both LS-C15 and LS-C14 were shown to have a naringenin:glucose:rhamnose ratio of 1:2:1 (Table 3).

Further Studies on LS-C15 and LS-C14

Glucosidase Studies. Information as to the mode of attachment of the sugars to the aglycone was obtained by enzymic hydrolysis with the β-glucosidase of Dunlap, Hagen, and Wender (35). Solutions consisting of 0.5 mg of either LS-C15 or LS-C14, in 1% citric acid buffer at pH 4 were incubated with the enzyme at 50° for 12 hr. Control solutions contained all but the enzyme. After incubation, the solutions containing LS-C15 and LS-C14 were extracted with five 5 ml volumes of ethyl acetate, the combined extracts were taken to dryness and redissolved in 10 ml of absolute ethanol.

The ultraviolet spectra of the products obtained from both LS-C15 and LS-C14 showed the usual λmax at 282 mμ and 330 mμ. No shifts in these maxima were observed upon addition of anhydrous sodium acetate showing that both still possessed a substituted 7-hydroxy group. Treatment of either product with sodium hydroxide to form

TABLE 3

TABULATION OF DATA OBTAINED IN AGLYCONE-SUGAR RATIO DETERMINATION ON LS-C15, LS-C14, AND STANDARD NARINGIN DIHYDRATE

Compound	Sample Weight (μg)	Naringenin (μg) M.W. = 272.27			Glucose (μg) M.W. = 180.16			Rhamnose (μg) M.W. = 164.16			Ratio
		fd.	Calc.	%Rec.	fd.	Calc.	%Rec.	fd.	Calc.	%Rec.	N:G:R
Naringin 1 (dihydrate)	1027	460	454	101	300	300	100	295	273	108	1.01:1:1.07
" 2	1058	440	467	94	260	308	85	260	281	93	1.12:1:1.1
" 3	1001	435	446	98	272	292	93	270	269	100	1.06:1:1.08
LS-C15	1048	255	-	-	333	-	-	169	-	-	1:1.95:1.09
LS-C14	1015	260	-	-	330	-	-	161	-	-	1:1.92:1.03

the chalcone resulted in the appearance of a broad λmax at 430 mμ instead of at 358 mμ, which was observed before enzymatic incubation, therefore, the 4'-hydroxy group of the naringenin was now unsubstituted in both LS-C15 and LS-C14.

The remaining ethanol solutions were concentrated and subjected to thin-layer chromatography on Woelm polyamide in NM and MW, and on Avicel SF in BzEFW, KFW, and BAW-1. The product obtained from the β-glucosidase hydrolysis of LS-C15 was shown to be naringin and the product obtained from LS-C14 was shown to be naringenin rutinoside by cochromatography with standard naringin and naringenin rutinoside, respectively.

The aqueous solutions remaining after ethyl acetate extraction of the enzyme incubation were deionized by passage through a 1.2 x 5 cm Amberlite MB-1 ion-exchange column. Chromatography of the water soluble product on silicic acid in PEW-1 and -2 and on Avicel in PEW-1, -2, BPBW, and BAW-2 verified that the only sugar cleaved from both LS-C15 and -C14 was glucose.

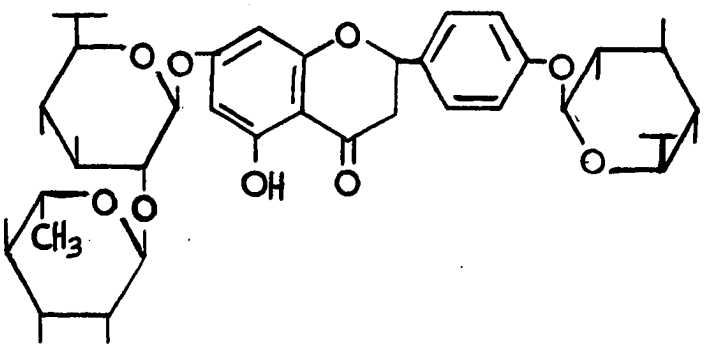
Partitioning of the control solutions of the enzymic incubation with ethyl acetate did not result in the extraction of any glycoside, showing that both LS-C15 and -C14 were quite water soluble. The control solutions, therefore, were deionized by passage through MB-1 ion-exchange columns and then concentrated. Thin-layer

chromatography showed both LS-C15 and -C14 to be unchanged in the controls.

In accordance with the evidence presented above, the structure 4'-β-D-glucopyranosyl-7-β-neohesperidosylnaringenin was postulated for LS-C15 (X, Figure 7) and 4'-β-D-glucopyranosyl-7-β-rutinosylnaringenin for LS-C14 (XI, Figure 7). Insufficient amounts of LS-C15 were available for a definitive taste evaluation but tests showed LS-C14 to be tasteless.

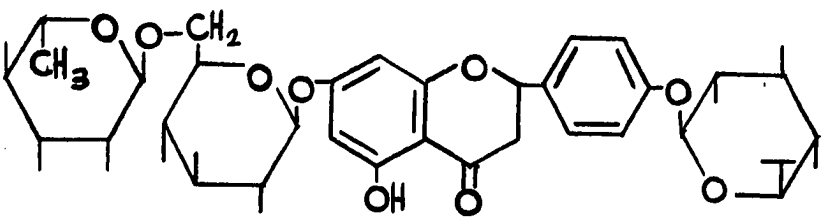
Rhamnosidase Studies. In an attempt to verify further the proposed structures, LS-C15 and -C14 were incubated with rhamnosidase (35) to show that the action by this enzyme would cleave rhamnose and yield the same glycoside naringenin-4', 7-diglucoside, from both LS-C15 and -C14. The particular preparation of rhamnosidase used for this experiment, however, was found to be contaminated with glucosidase and thin-layer chromatograms of the ethyl acetate extracts and aqueous layers of the incubation solutions showed that several products were present. From the LS-C15 incubation, spots corresponding to LS-C15, naringin, prunin, and naringenin were obtained, along with two others which were postulated to be naringenin-4'-glucoside and naringenin-4', 7-diglucoside on the basis of their migration in various TLC systems given below (prunin is included for reference):

FIGURE 7



X. LS-C15

4'-β-D-glucopyranosyl-7-β-neohesperidosylnaringenin



XI. LS-C14

4'-β-D-glucopyranosyl-7-β-rutinosylnaringenin

	Woelm Polyamide		Avicel SF	
	NM	MW	KFW	BAW-1
Prunin	0.43	0.50	0.56	0.78
Naringenin-4'-glucoside	0.36	0.37	0.56*	0.78*
Naringenin-4', 7-diglucoside	0.32	0.65	0.06	0.39

*Assumed to be at same position as prunin.

The same components were detected from the LS-C14 incubation except that naringenin rutinoside was seen instead of naringin. The compound believed to be naringenin-4', 7-diglucoside, like LS-C14 and LS-C15, was not extractable with ethyl acetate.

Search for Possible Additional Components. Reference to Table 3 shows that the amounts of naringenin, glucose, and rhamnose obtained for each triglycoside account for only 67% of the total weight of LS-C15 and only 74% of LS-C14. This weight discrepancy indicated the possibility that the molecule contained another component or components in addition to the naringenin, glucose and rhamnose. Samples of LS-C15, LS-C14 and their acid hydrolysates were subjected to chromatography on Avicel thin layers in 15% acetic acid, BPBW, BAW-1, and BzAW-1 and sprayed with diazotized sulfanilic acid reagent without detection of any additional constituents. Thin layer chromatograms of the samples on Adsorbosil 1 in BAW-1, PEW-1, and -2 charred with 10% sulfuric acid failed to reveal any extraneous material. An Avicel plate of the hydrolyzed samples,

developed in BAW-1 and sprayed with methanolic $AlCl_3$ failed to detect any intermediate glycoside, indicating complete hydrolysis. Another ratio determination on a sample of LS-C14 which had been dried at 138°C for 12 hr *in vacuo* resulted in a recovery of 77%, only a 3% increase from the original 74% recovery.

Discussion

No suitable solvent could be found for recrystallization of the triglycosides and the gross contamination is probably a consequence of the method by which the compounds were precipitated from the benzene-methanol solution. This is a hazard that may be expected when working with such small amounts of material. It is unlikely that these glycosides contain any constituent other than naringenin, glucose, and rhamnose since the extensive search by thin-layer chromatography of the hydrolysates should have revealed any component that could account for approximately one-fourth of the weight. It is also unlikely that the products of the β-glucosidase action would cochromatograph exactly with the standard compounds in the various TLC systems if other components remained bonded to the cleaved glucose, naringin, or naringenin rutinoside. The likelihood of the weight discrepancy being due to solvent entrapment was reduced when a dried sample of LS-C14 showed no significant increase in total weight recovered. A likely

possibility is that LS-C15 and LS-C14 are only approximately 67 and 74% pure, respectively. The IR spectrum of LS-C14 shows adsorption at 1740 cm^{-1} probably due to an ester carbonyl; however, the adsorption is weak and should be at least as strong as the carbon 4 carbonyl at 1640 cm^{-1} if it were actually part of the molecule.

The naringenin rhamnodiglucosides are of considerable interest because of their possible importance in the metabolism of naringin and naringenin-7β-rutinoside in the grapefruit. The latter compounds are relatively insoluble in water and the attachment of an extra glucose moiety at the 4'-hydroxy of naringenin may provide a means of solubilizing the flavanone since both the triglycosides are quite water soluble.

This is the first report of the isolation of any such naringenin derivatives from the grapefruit or any other source. However, subsequent experiments by the author (unpublished data) have produced evidence which suggests that the neohesperidoside derivative (LS-C15) occurs in the shaddock and the isomeric rutinoside derivative (LS-C14) occurs in the sweet orange.

In retrospect, one can rationalize the behavior of the triglycosides on the aqueous Polyclar AT column and on thin layer chromatograms of Woelm Polyamide developed in NM. Due to their high water solubility, the observed quick elution from the aqueous column should be expected.

The low R_f values observed in the thin-layer chromatography system are due not to greater hydrogen bonding with the polyamide, but to the lack of solubility of the compounds in the relatively nonpolar solvent. Future isolation procedures of these compounds from other sources should exploit this solubility in water since most other flavonoid material could be removed by a simple ethyl acetate extraction.

With only minor modifications, the method developed for the aglycone-sugar ratio determination of the triglycosides should be applicable to any glycoside whose aglycone will bond to the Polyclar AT long enough for the sugars to be eluted. The densitometric method for the determination of the sugars should find wide applicability where only micro amounts of plant or animal material are available for analysis.

CHAPTER IV

SUMMARY

The flavanone rhamnosylglucosides of citrus fruits are of two principal types: rutinosides, which are tasteless; and neohesperidosides, which are distinguished by their extremely bitter taste. Rutinosides are derivatives of the disaccharide rutinose, 6-O-α-L-rhamnopyranosyl-D-glucose, while neohesperidosides are derived from the isomeric disaccharide neohesperidose, 2-O-α-L-rhamnopyranosyl-D-glucose. In general, a single species of citrus fruit contains either rutinoside or neohesperidoside glycosides. Naringin, poncirin, and neohesperidin, which are the bitter flavanone neohesperidosides of naringenin, isosakuranetin, and hesperetin, respectively, have previously been shown to occur in the grapefruit and are of considerable interest to the grapefruit industry due to their effect on the taste of grapefruit products and consequently on consumer acceptance. Previous studies on the flavonoids of grapefruit have indicated the probable presence of other closely related flavanone glycosides in this fruit. The present research was undertaken to

to investigate further the qualitative aspects of the flavanone glycoside content of the grapefruit.

Pilot column chromatographic studies of a concentrate of segments from mid-season grapefruit demonstrated the usefulness of polyamide adsorbents for the preliminary separation of the flavonoids from the concentrate and for the further purification of individual compounds from mixtures of very similar flavanone glycosides. Subsequent preparative column chromatography of the segment concentrate on Ultramidpulver and Polyclar AT polyamides with water and water-methanol as eluting solvents afforded a flavonoid-rich fraction which was shown by thin-layer chromatography to contain several components, which by R_f value and fluorescence of their $AlCl_3$-complex, appeared to be flavanone glycosides. By further column chromatographic resolution of the flavonoid-rich fraction on Polyclar AT with benzene-methanol as eluting solvent, isolation of the tasteless 7-rutinosides of naringenin, hesperetin (hesperidin), and isosakuranetin in addition to the bitter isomeric neohesperidosides previously known to be present in the grapefruit was achieved. Acid and enzymic hydrolysis, degradation, oxidation, methylation, and U.V. spectral studies were employed to establish the structures of these compounds. Except for the Seville orange, which has been shown to contain hesperidin and neohesperidin, the grapefruit is apparently the only citrus species from

which both an intact rutinoside and neohesperidoside have been isolated.

Prunin, the bitter 7β-glucoside of naringenin, was also isolated from the same concentrate of mid-season grapefruit segments and characterized by the methods used to identify the flavanone rhamnoglucosides.

Similar aqueous and non-aqueous polyamide column chromatography of a concentrate of segments from late-season grapefruit resulted in the isolation of two water soluble naringenin rhamnoglucosides which did not correspond chromatographically to any available flavonoid glycosides. A method developed for the determination of aglycone-sugar ratio in flavanone glycosides showed both compounds to have a naringenin-glucose-rhamnose ratio of 1:2:1. Enzymic hydrolysis of these compounds with β-glucosidase yielded glucose and naringin from one and glucose plus naringenin-7β-rutinoside from the other. It is proposed that these compounds are 4'-β-D-glucopyranosyl-7-β-neohesperidosylnaringenin and 4'-β-D-glucopyranosyl-7-β-rutinosylnaringenin. This work constitutes the first report of such naringenin triglycosides.

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