A Method for the Isolation and Identification of the Flavanone Glycosides of Citrus Fruit Juices

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Flavanone glycoside fractions were isolated from orange, grapefruit, lemon, and lime juices by ethyl acetate extraction and chromatography on Sephadex G-25 fine. The individual flavanone glycosides were separated by thin-layer chromatography on polyamide. The glycosides were identified by thin-layer chromatography, KOH degradation, $KMnO_4$ treatment, acid hydrolysis, and spectral characteristics. Grapefruit juice contained the 7-neohesperidosides and 7-rutinosides of isosakuranetin, hesperetin, and naringenin, while the 7-rutinosides of these same flavanones were found in orange juice. Lemon juice contained the 7-rutinosides of isosakuranetin, hesperetin, naringenin, and eriodictyol, and a glycoside containing rhamnose, glucose, and an unidentified flavanone. Lime juice contained the 7-rutinosides of hesperetin, naringenin, and eriodictyol.

Albach and Redman (1969) have recently reviewed the literature on the flavanone composition of citrus fruit and have presented data concerning the flavanone content of 18 species of citrus and 18 different crosses of citrus species. The flavanone glycosides which have been found in citrus fruit include the 7-rutinosides and 7-neohesperidosides of isosakuranetin (5,7-dihydroxy-4′-methoxyflavanone), hesperetin (5,7,3′-trihydroxy-4′-methoxyflavanone), naringenin (5,7,4′-trihydroxyflavanone), eriodictyol (5,7,3′,4′-tetrahydroxyflavanone), and naringenin-4′-glucoside. Other flavanone compounds which have been reported as present in citrus fruit are naringenin 7-glucoside (Mizelle, 1966), a naringenin 7-rhamnoglucoside which differs from the 7-rutinoside and 7-neohesperidoside of naringenin (Nishiura *et al.*, 1969), 5,6,7,8,3′,4′-hexamethoxyflavanone, 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavanone, eriodictyol glycoside (Albach and Redman, 1969), and possibly a 7-rhamnoglucoside of 5,7-dihydroxy-2′-methoxyflavanone.

The results of Albach and Redman (1969), Harborne (1967), and Nishiura *et al.* (1969) indicate that the flavanone glycoside patterns are useful in the characterization and identification of specific citrus fruits. This would also be expected to be true of the citrus juices, although the patterns and relative levels might be somewhat different due to the solubility of the individual flavanone glycosides in aqueous systems, and possibly to selective degradation during processing. It would, for example, be expected that the amounts of hesperidin (hesperetin 7-rutinoside) in juice would be much lower than in fruit because of its very limited water solubility.

Difficulties have been experienced in the routine isolation of flavanones from citrus and in the separation and detection of individual flavanone glycosides. Albach and Redman (1969) extracted citrus fruit with hot 1-propanol and separated the flavanones by tlc on polyamide. They experienced difficulty in the identification of the less mobile flavanone glycosides because of extraneous materials in the extract. Other procedures (Mizelle, 1966; Nishiura *et al.*, 1969) which have been used for the isolation of these compounds are too lengthy and tedious for use in the routine examination of citrus fruit or juices.

This paper reports on the isolation of the flavanone glycosides from orange, grapefruit, lemon, and lime juices by chromatography on Sephadex, on the thin-layer separation of these compounds on polyamide, and on the identification of some of these compounds by chemical and spectral means.

MATERIALS AND METHODS

Citrus fruit and fruit juices were purchased on the local market. All juices, both commercial and laboratory prepared, were clarified by the AOAC procedure 20.075 (1965).

Naringin (naringenin 7-neohesperidoside), hesperidin (hesperetin 7-rutinoside), neohesperidin (hesperetin 7-neohesperidoside), hesperetin, and naringenin were purchased from chemical suppliers. Eriodictyol was prepared by demethylation of hesperetin, as described by Horowitz and Gentili (1960) and isosakuranetin by methylation of naringin as described by Narasimhachari and Seshadri (1948). Rutinose was obtained by refluxing rutin with 10% acetic acid (Zemplen and Gerecs, 1938).

Isolation of Flavanone Fraction. One hundred-milliliter portions of the clarified juices were extracted twice with 100-ml portions of ether, and the ether extracts discarded. The juices were then extracted four times with 100-ml portions of ethyl acetate. The ethyl acetate extracts were combined and evaporated to dryness on a flash evaporator at 40° C. The residue was dispersed and transferred, using not more than 5 ml water to a 1.5 × 90 cm column of Sephadex G-25 fine in water. The column was eluted with water, discarding the first 50 ml of effluent and then collecting 50 fractions of 5 ml each. The column was washed with a further 250 ml water before reuse. The ultraviolet spectra (200 to 350 mμ) of the individual fractions were determined using a Cary 14 record-

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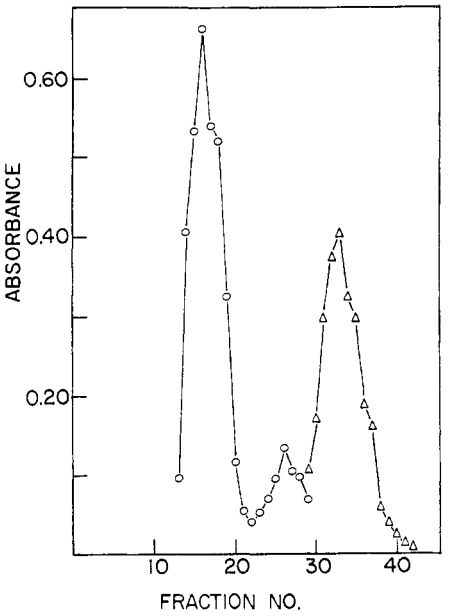

Figure 1. Ultraviolet absorbance of the fractions obtained from 100 ml orange juice by elution with water from Sephadex G-25 fine. All fractions were 5 ml volume and diluted tenfold for measurement (○- -○), 325 mμ; △- -△, 283 mμ)

ing spectrophotometer. The fractions containing flavanones, as indicated by ultraviolet spectra, were combined, evaporated to dryness, and made up to 5 ml with ethanol.

Thin-Layer Chromatography. Thin-layer plates of polyamide were prepared according to the procedure of Fisher *et al.* (1966). Suitable portions of the flavanone fraction were spotted on the plates and developed using nitromethane: methanol:H_2O (5:2:0.025). After drying, the plates were sprayed with 1% $AlCl_3$ in ethanol. The sprayed plates were examined under a long wavelength ultraviolet lamp.

Other Procedures. Acid hydrolysis of flavanone glycosides was accomplished by treating the glycosides with 1 *N* HCl at 100° C for 2 hr. The aglycones were recovered by extraction of the hydrolyzate with ethyl acetate. The acid was extracted from the hydrolyzate with di-*N*-octylmethylamine in chloroform (Puski, 1966), the aqueous solution was evaporated to dryness, the residue dissolved in pyridine, and the sugars separated by paper chromatography using butanol: pyridine:water (10:3:3) (Mes and Kamm, 1969). Sugars were detected on paper chromatograms using alkaline $AgNO_3$ reagent (Smith, 1958). Permanganate oxidation was performed by the method of Chandler and Harper (1961), and KOH degradation as described by Mizelle (1966).

RESULTS AND DISCUSSION

The ultraviolet spectra of the ethyl acetate extracts of citrus juices exhibited peaks at 280 to 285 mμ and secondary peaks at 320 and 325 mμ indicative of flavanones. These peaks were evident at decreasing levels in the first four ethyl acetate extracts of all juices, but could not be detected in later extracts. Thus, it was evident that four extractions were required for the removal of most of the flavanone glycosides from citrus juices. The ultraviolet spectra of the preliminary ether extracts did not indicate the presence of flavanone compounds.

The separation of the flavanone components from the other constituents of the ethyl acetate extracts of citrus juices on a Sephadex column is illustrated in Figure 1 for orange juice. Similar results have been obtained for grapefruit, lemon and

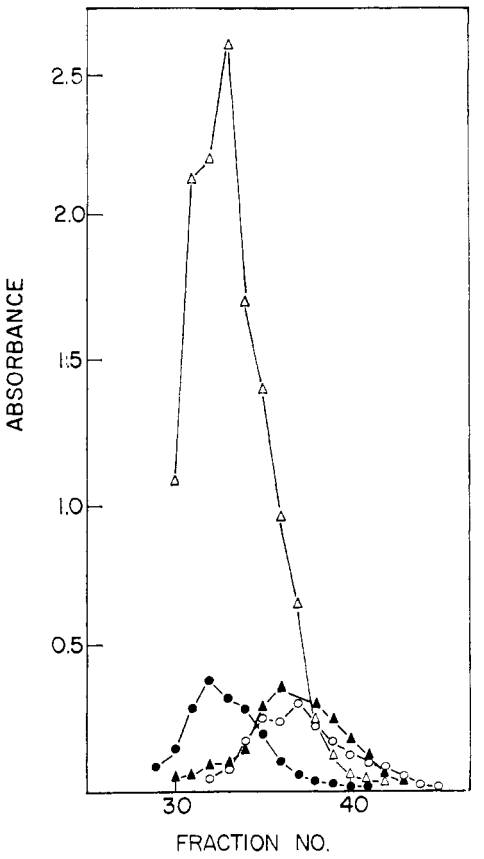

Figure 2. Ultraviolet absorbance at 283 mμ of the fractions containing flavanone glycosides of citrus juices eluted from Sephadex G-25 fine. All fractions were 5 ml volume and diluted tenfold for measurement. ▲- - -▲, lime juice; △- - -△, grapefruit juice; ●- - -●, orange juice; ○- - -○, lemon juice

lime juices. None of the materials obtained from the juices in fractions 1 to 10 from the Sephadex column have been identified. These fractions had no characteristic ultraviolet absorption maxima and are not flavonoid in nature. Fractions 10 to 30 contained materials which exhibited ultraviolet spectra similar to cinnamic acid derivatives with maxima at approximately 295 and 325 mμ. The flavanone glycosides, characterized by absorption maxima at approximately 285 mμ with a shoulder at 325 mμ, appeared in fractions 30 to 45. Flavone and flavonol glycosides, applied separately to this column, were eluted in later fractions. Samples of naringin, naringenin, hesperidin, and hesperetin, applied separately to this column, were completely eluted in fractions 30 to 45. The flavanone glycoside fraction contained only small amounts of impurities which did not interfere with the chromatographic separation or detection of these compounds on polyamide plates.

Figure 2 illustrates the flavanone fractions obtained from the four juices, the fraction in which they appear, and the relative amounts of these compounds in the different juices as measured by absorbance of the individual fractions at 283 mμ. Of interest is the pattern of elution from the Sephadex column of the flavanones from the different juices. The maximum levels of flavanones appeared in fractions 33 or 34 for orange and grapefruit juices, and in fractions 37 to 40 for lime and lemon juices. This feature was noted for all samples of laboratory prepared and commercial juices. This difference in the elution pattern may be due to the differences in the distribution of flavanone glycosides in these juices. This figure also shows that the level of flavanone compounds

was much higher in grapefruit juice than in the other three juices.

Polyamide thin-layer chromatography of the flavanone fractions isolated by Sephadex chromatography showed the distribution of flavanone glycosides in the different citrus juices. These are illustrated in Figure 3. Three compounds were detected in lime juice, five in lemon juice, six in grapefruit juice, and three in orange juice. The flavanone aglycones produced by acid hydrolysis of the flavanone fractions from the four juices were also separated by polyamide thin-layer chromatography. These thin-layer separations are illustrated in Figure 4. Three aglycones were detected in each of orange, grapefruit, and lime juices, and four in lemon juice.

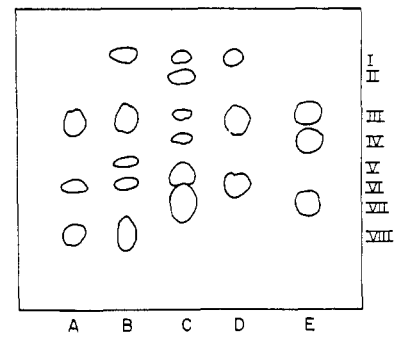

Figure 3. Polyamide thin-layer chromatographic separation of the flavanone glycosides from citrus juices. A, lime juice; B, lemon juice; C, grapefruit juice; D, orange juice; E, standards. I, isosakuranetin 7-rutinoside; II, isosakuranetin 7-neohesperidoside; III, hesperidin; IV, neohesperidin; V, unknown; VI, naringenin 7-rutinoside; VII, narigenin; VIII, eriodictyol 7-rutinoside

ORANGE JUICE

Of the three flavanone glycosides in orange juice, one appeared only in trace quantities, while the other two major components were present at approximately equal levels. One of these major components, on the basis of chromatographic results, was identified as hesperidin (hesperetin 7-rutinoside). The other major component of orange juice moved just ahead of naringin on polyamide plates.

It was isolated in larger quantities from the ethyl acetate extracts of 1000 ml of orange juice by Sephadex chromatography, followed by polyamide column chromatography with elution by nitromethane:methanol (5:2). On acid hydrolysis it yielded naringenin, glucose, and rhamnose. Rutinose was obtained after $KMnO_4$ treatment, and *p*-hydroxybenzoic acid was one of the products of KOH degradation. Ultraviolet and visible spectra in ethanol, in ethanol with AlCl, and in ethanol with NaOH indicated that the flavanone glycoside had a free 5-hydroxyl group, a blocked 7-hydroxyl group and a free 4′-hydroxyl group. The spectra of the aglycone produced by acid hydrolysis indicated free 5- and 7-hydroxyl groups. From these results this flavanone glycoside was identified as naringenin 7-rutinoside. The chromatographic characteristics of the minor flavanone component of orange juice would indicate that it was isosakuranetin 7-rutinoside. Sufficient quantities of this component for further characterization were not isolated.

Polyamide chromatography of the flavanone aglycones produced by acid hydrolysis of the flavanone fraction from orange juice yielded hesperetin, naringenin, and a small amount of isosakuranetin.

GRAPEFRUIT JUICE

Six flavanone compounds were detected in grapefruit juice. The major compound in all samples of grapefruit juice was naringin. Naringenin 7-rutinoside, hesperidin, and neohesperidin were also present in small quantities. Two other fast moving components were detected on the polyamide plates. These presumably were the 7-rutinoside and 7-neohesperidoside of isosakuranetin. The flavanone aglycones produced by acid hydrolysis of the flavanone glycosides were hesperetin, naringenin, and isosakuranetin.

LEMON JUICE

Five flavanone glycosides were detected in lemon juice. Hesperidin was present in moderate amounts and a small amount of naringenin 7-rutinoside was detected. Also present was the same compound which had been detected in orange and grapefruit juice and has been presumed to be isosakuranetin 7-rutinoside. But the major flavanone component of lemon juice was a slow moving one which was isolated in the same manner as was the naringenin 7-rutinoside of orange juice. Acid hydrolysis of this compound yielded eriodictyol, glucose, and rhamnose. Rutinose was obtained by $KMnO_4$ treatment of this compound. Thus it appears that this compound is eriodictyol 7-rutinoside. The fifth component of lemon juice, moving between naringenin 7-rutinoside and neohesperidin on polyamide plates, was present in limited amounts. A small amount was obtained free of other flavanone glycosides during the isolation of eriodictyol 7-rutinoside. It gave a characteristic ultraviolet spectra with a peak at 283 mμ and a shoulder at 325 mμ. Acid hydrolysis of this compound produced glucose, rhamnose, and an unidentified flavanone of lesser mobility than eriodictyol on polyamide plates developed with nitromethane:methanol:H_2O (5.2:0.025). The limited supply of this compound prevented any further attempts at its characterization. The flavanone aglycones produced by acid hydrolysis of the flavanone glycosides were eriodictyol, naringenin, hesperetin, and isosakuranetin. The unidentified flavanone described above was not present in sufficient quantities to be detected in these hydrolyzates.

LIME JUICE

Three flavanone glycosides were present in lime juice. These were hesperidin, eriodictyol 7-rutinoside, and a very

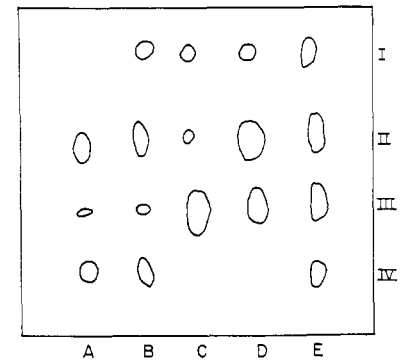

Figure 4. Polyamide thin-layer chromatographic separation of the flavanone aglycones obtained by acid hydrolysis of the flavanone glycosides of citrus juices. A, lime juice; B, lemon juice; C, grapefruit juice; D, orange juice; E, standards. I, isosakuranetin; II, hesperetin; III, naringenin; IV, eriodictyol

small amount of naringenin 7-rutinoside. This may be compared to the results of Albach and Redman (1969), who found only hesperidin in *n*-propanol extracts of lime fruit. The flavanone aglycones produced by acid hydrolysis of the flavanone glycosides were eriodictyol, naringenin, and hesperetin.

With the exception of the presence of appreciable levels of eriodictyol 7-rutinoside in lime juice, the distribution of flavanone glycosides in these citrus juices is similar to that reported by Albach and Redman (1969) for citrus fruit. The flavanone glycosides in these juices indicate that their polyamide chromatographic patterns would be useful for the identification and detection of mixtures of orange, grapefruit, lemon, and lime juices.

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