A High-Pressure Liquid Chromatographic Method for the Resolution and Quantitation of Naringin and Naringenin Rutinoside in Grapefruit Juice

Naringin and naringenin rutinoside were resolved from filtered grapefruit juice by high-pressure liquid chromatography (HPLC) using a micro C-18 column and eluting with a water–acetonitrile system. These flavonoids were detected at 280 nm.

Naringin, the 7β-neohesperidoside of naringenin (Horowitz, 1964), is the principal flavanone glycoside, as well as the primary bitter component in grapefruit juice. Its isomer, 7β-rutinoside of naringenin, also present in grapefruit, is tasteless. Other compounds present in grapefruit, such as limonin (Maier and Dreyer, 1965) and poncirin (Horowitz, 1964), are secondary contributors to bitterness.

Because bitterness is frequently cited as the principal deterrent to the profitable marketing of grapefruit products, several procedures have been reported for the determination of naringin. The Davis test (Davis, 1947), when applied to grapefruit juice, suffers from the disadvantage of not being able to differentiate between naringin and its tasteless isomer. Therefore, it is not a reliable measure of naringin bitterness. The chromatographic–fluorometric method reported by Hagen et al. (1965), the thin-layer chromatographic–colorimetric method reported by Fisher et al. (1966), and a shorter thin-layer chromatographic method reported by Tatum and Berry (1973) are somewhat involved for routine determination of naringin.

The importance of naringin in grapefruit juice bitterness required the development of an improved method for the quantitation of naringin. The simultaneous quantitation of naringenin rutinoside would allow a determination of the "bitter to tasteless" ratio.

The object of this work was to design such an assay. The following procedure was developed and used in this laboratory.

MATERIALS AND METHODS

Apparatus. A Model ALC 202 high-pressure liquid chromatograph (HPLC) with a Model 6000 A pump and U6K injector (Waters Associates, Milford, Mass.) was used. The recorder was a Texas Instrument Servo/Riter II 2-pen. A Schoeffel uv–visible liquid chromatography analyzer Model SF 770 (Schoeffel Instrument Corp., Westwood, N.J.) was the detector. A Spectra-Physics integrator (minigrator, Spectra-Physics, Santa Clara, Calif.) was used.

A Model BB Burrell wrist action shaker (Burrell Corp., Pittsburgh, Pa.) was used. A Waters Associates sample clarification kit with 1.2 or 0.45 μm Millipore aqueous filter system was used.

Column. A Waters Associates 30 cm × 4 mm i.d. reverse phase μ Bondapak C-18 column (octadecyltrichlorosilane chemically bonded to <10 μm porasil packing) was used.

Reagents. The eluting system was water–acetonitrile (80:20, v/v). Both solvents were degassed.

Thin-Layer Chromatography. System A was Analtech polyamide microlayer 10 × 10 cm sheets with nitromethane–methyl alcohol (5:2). Visualization was accomplished by spraying the sheets with 1% aluminum chloride in ethyl alcohol and viewing under 360-nm light. System B was Quantum Industries silica gel LQD plates with ethyl acetate–isopropyl alcohol–water (65:23.5:11.5). A freshly prepared solution of 0.5 ml of anisaldehyde in 50 ml of glacial acetic acid with 1.0 ml of concentrated sulfuric acid was the spray reagent. The plates were heated at 100 °C for 10 min. System C was the same as B except that visualization was as in system A. System D employed the same plates, spray reagent, and heat treatment as B. Methyl ethyl ketone–glacial acetic acid–methanol (3:1:1) was the developing solvent. Observing the plates under uv light (360 nm) intensified the spots in systems B and D.

Sample Preparation. Fresh hand-squeezed, processed single-strength grapefruit juice or reconstituted concentrate was filtered.

High-Pressure Liquid Chromatographic (HPLC) Resolution and Quantitation of Naringin and Naringenin Rutinoside. An aliquot (10 to 50 μl) of the above filtered juice was injected onto the column with a flow rate of 2.0 ml/min. Detection was accomplished at 280 nm with 1.0 absorbance unit full scale. Integration was conducted at an attenuation of 1.0, peak width setting of 24, and slope sensitivity of 80. The recorder chart speed was 12 in./h.

The quantity of both flavonoid glycosides in unknown samples was determined from a linear regression equation. This equation was obtained from standard samples of naringin over the range of 1 to 60 μg. These samples were eluted and detected under the above conditions.

Identification of Naringin and Naringenin Rutinoside. The eluates corresponding to the peak areas labeled naringin and naringenin rutinoside in Figure 1 were collected. Both gave positive reactions with the flavanone reagent (Horowitz, 1957). Both displayed all the characteristic uv spectra of 7-substituted naringenin (Horowitz and Jurd, 1961; Jurd, 1962). The electrophoretic behavior of the eluates corresponding to naringenin 7-rutinoside and naringin (naringenin 7-neohesperidoside) in Figure 1 was consistent with that reported by Gentili and Horowitz (1965). When cochromatographed with authentic standards, using system A, both eluates exhibited the separation and R_f values reported by Hagen et al. (1965) for these two flavanone isomers. Acid hydrolysis (Mizelle et al., 1965; Coffin, 1971) of both eluate residues followed by thin-layer chromatography with reference flavanone aglycones in systems A and C showed naringenin as the aglycone portion. The sugars obtained by hydrolysis of both eluates were identified as rhamnose and glucose by cochromatography with standard sugars in systems B and D. Additional confirmation was obtained by HPLC retention volumes and peak enrichment experiments with authentic samples using the conditions reported above.

Percent Recovery and Precision. The reliability of the procedure was determined by a series of recovery experiments in which a base sample of grapefruit juice was fortified with known amounts of naringin. The native naringin in the base sample was previously determined by this liquid chromatographic procedure. Individual samples were fortified with sufficient naringin to provide a concentration of 250 to 1000 ppm of naringin in 50-ppm increments.

The repeatability of the method was determined by analyzing five aliquots from a grapefruit juice sample.

RESULTS AND DISCUSSION

This procedure constitutes an improvement in simplicity, speed, and accuracy over existing methods. The average time for six complete analyses was about 3.0 h. A

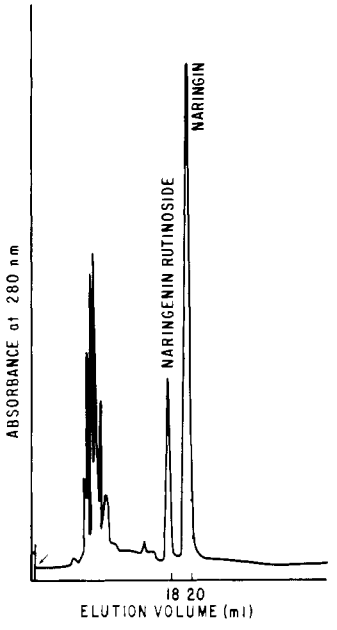

Figure 1. Separation of naringenin rutinoside and naringin in grapefruit juice. For experimental details, see text.

comparison of the Davis method with this procedure showed that the Davis test gave consistently higher naringin values. The Davis values were approximately twice those found for naringin by this HPLC method. The Davis values have always been rather nebulous. The test is not specific. The values reflect the detection of several compounds; however, the Davis values have been customarily reported as naringin. The naringenin rutinoside may be quantitated at the same time as naringin. Both compounds have identical uv characteristics.

If both naringin and limonin bitterness (Fisher, 1975) are to be determined on the same grapefruit sample, the sample is first extracted with chloroform to remove the limonin. The samples are filtered to remove particulate material which may clog the column. Naringenin rutinoside and naringin were eluted isocratically after approximately 18 and 20 ml, respectively (Figure 1).

The number of theoretical plates for the column, using naringin as the reference peak, was 2600, equivalent to a plate height of 0.12 mm. The column capacity factor, k', was 5.1. The amount of native naringin in the base sample was 210 ppm. The recoveries of naringin from the fortified samples were all within ±10% of the total naringin. The range of naringin found in the five repeatability experiments was 390–400 ppm, with a mean of 394 and a standard deviation of ±3.9.

A plot of peak areas vs. micrograms of naringin showed linearity over the range of 1 to 60 μg of naringin ($r = 0.996$). The naringin used contained 2 mol of water, mp 170–175 °C (Merck Index, 1968).

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James F. Fisher*
T. A. Wheaton

Florida Department of Citrus
University of Florida, IFAS
Agricultural Research & Education Center
Lake Alfred, Florida 33850

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