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# A High-Performance Liquid Chromatographic Method for the Quantitation of Hesperidin in Orange Juice

Hesperidin was resolved from filtered orange juice by high-performance liquid chromatography (LC) using a micro C-18 column and eluting with a water–acetonitrile system. Detection was accomplished at 285 nm. This procedure improves on present hesperidin analysis by shortening analysis time and enhancing analytical confidence.

Hesperidin is the 7$\beta$-rutinoside of 2*S*-hesperetin (Horowitz, 1964). This compound is the predominant flavonoid glycoside in Florida orange juice. Hesperidin is tasteless and does not contribute to juice quality; however, its concentration in juice may be used as a measure of extraction pressure and related juice quality. Hesperidin has been reported to exert an apparent regulatory action on erythrocyte concentration and tissue perfusion in humans (Robbins, 1975).

Methods for the isolation and measurement of hesperidin have been reviewed by Kefford and Chandler (1970). The object of this work was to see if the technique of LC could improve on the present analyses for hesperidin. The following procedure was developed and used in this laboratory.

## MATERIALS AND METHODS.

**Apparatus.** A Model ALC 202 high-performance liquid chromatograph with a Model 6000 A pump and U6K injector (Water 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 Perkin-Elmer Model 457 infrared spectrophotometer equipped with a Wilks micro sampling system Model 45A and KRS–5, 2-mm crystal was used to help identify hesperidin. Peak areas were determined with a Spectra-Physics integrator (minigrator, Spectra-Physics, Santa Clara, Calif). A Waters Associates sample clarification kit with 1.2 or 0.45 Millipore aqueous filter system was used.

**Column.** A Waters Associates 30-cm × 4-mm i.d. reverse-phase $\mu$ Bondapak C-18 column (octadecyltrichlorosilane chemically bonded to <10 $\mu$ Porasil packing) was used.

**Reagents.** The eluting system was water–acetonitrile (80:20, v/v). The system was degassed with an ultrasonic bath. The crude hesperidin was obtained from Sigma (St. Louis, Mo.).

**Sample Preparation.** Fresh hand-squeezed, processed single-strength orange juice or reconstituted concentrate was filtered through glass wool. The resulting filtrate was refiltered through the Millipore clarification system.

**High-Performance Liquid Chromatographic Resolution and Quantitation of Hesperidin.** An aliquot (10–50 $\mu$L) of the above filtered juice was injected onto the column with a flow rate of 1.5 mL/min. Detection was accomplished at 285 nm with 0.1 absorbance unit full scale. Integration was conducted at an attenuation of 1.0, peak width setting of 35 and slope sensitivity of 150. The recorder chart speed was 12 in./h.

The quantity of hesperidin in unknown samples was determined from a linear regression equation. This equation was obtained from ten standard samples of hesperidin in dimethylformamide over the range of 1.0 to 10.0 $\mu$g. These samples were eluted isocratically and detected under the above conditions.

**Percent Recovery and Precision.** The reliability of the procedure was determined by fortifying five identical base samples of orange juice with sufficient hesperidin to provide concentrations of 100 to 600 ppm. The native hesperidin in the base sample was previously determined by this LC procedure.

The precision of the method was determined by analyzing five aliquots from an orange juice sample containing hesperidin.

**Identification of Hesperidin.** Recycle of the peak labeled hesperidin in Figure 1 showed this peak to be composed of only one constituent after seven passes through the column. The eluate corresponding to this peak area was collected and concentrated by freeze-drying. The identity of this fraction as hesperidin was based on the flavanone test (Horowitz, 1957), characteristic UV spectra (Jurd, 1962), and comparison of its infrared spectrum with

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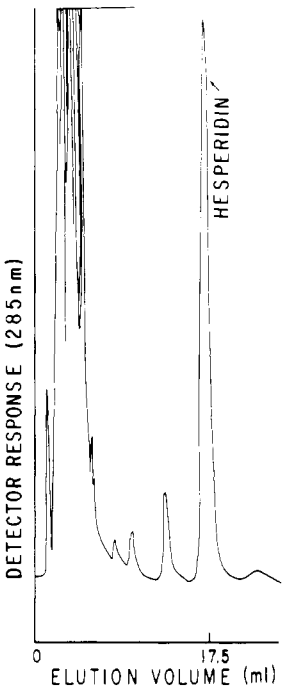


**Figure 1.** Separation of hesperidin in orange juice. For experimental details see text.

authentic hesperidin. The identity was also supported by peak enrichment. The hesperidin used in all the above standardization and identification work was purified by three crystallizations from dimethylformamide–water and drying in vacuo at 110 °C, mp 259–261 °C.

## RESULTS AND DISCUSSION

This procedure can be carried out directly on filtered orange juice without extractions. The determination is quantitative, strictly objective, simpler, and faster than prevalent hesperidin determinations. The time required for a complete analysis was 25 min. The fresh hand-squeezed, processed single-strength orange juice or reconstituted concentrate all gave the same general chromatographic pattern (Figure 1). The samples were filtered to remove particulate material which may clog the system. The hesperidin was eluted isocratically after approximately 17.5 mL (11.5 min) (Figure 1). The number of theoretical plates for the column, using hesperidin as the reference peak, was 2675, equivalent to a plate height of 0.11 mm. The column capacity factor, $k'$, was 5.0.

The base sample of fresh Hamlin orange juice used in the reliability test was collected under conditions designed to minimize its hesperidin content. The amount of native hesperidin in this base sample was 50 ppm. The recoveries of hesperidin from the five fortified base samples were all within ±8% of the hesperidin added. In order to fortify the base orange juice samples with hesperidin, it was necessary to add the appropriate aliquot of a solution of hesperidin dissolved in dimethylformamide to each base sample. The ultrasonic bath was helpful in effecting dissolution. The range of hesperidin found in the five repeatability experiments was 210 to 225 ppm, with a mean of 216 and a standard deviation of ±5.6 ppm.

Table I. Hesperidin[a] in Orange Juice Obtained at Different Extractor Pressures

| juice | soft squeeze, ppm[b] | medium squeeze, ppm[b] | hard squeeze, ppm[b] |
|---|---|---|---|
| Valencia | 98 | 107 | 120 |
| Hamlin | 54 | 73 | 100 |

[a] Calculated from the linear regression equation. [b] Average of five samples.

A plot of peak areas vs. μg of hesperidin showed linearity over the range of 1.0 to 10.0 μg ($r$ = 0.993).

An example of the use of this procedure is seen in Table I which shows the increasing amount of hesperidin found in Valencia and Hamlin orange juice obtained with increasing extractor pressure.

In the author's opinion, this procedure improves on previous hesperidin analyses in time of sample preparation and detection, as well as precision and reliability.

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# Gas Chromatographic Determination of Diquat Residues in Potato Tubers

A gas chromatographic method is described for the determination of diquat residues in potato tubers by reduction of diquat with sodium borohydride to a volatile diamine derivative. It is demonstrated that reduction with sodium borohydride effectively displaces diquat from both soil and potato tubers without prior acid hydrolysis. After extractive cleanup the diquat derivative could be detected at levels down to 0.01 ppm in potatoes with a nitrogen–phosphorus specific detector. Recoveries of diquat from potatoes at fortification levels of 0.05 to 1.00 ppm averaged 87.4 ± 4.1%.

The herbicide diquat (1,1′-ethylene-2,2′-bipyridylium dibromide) is an extremely effective chemical dessicant, widely used for destruction of the potato foliage prior to harvest. This treatment occasionally causes damage to the

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