An Improved Conversion of Hesperidin into Hesperetin Including Purity Determination by Gradient-Elution, High-Pressure Liquid Chromatography

An improved procedure for the conversion of commercial hesperidin into high-purity, crystalline hesperetin is reported. This procedure consists of purifying the crude starting material by insolubles removal and recrystallization, followed by cleaving the saccharides with sulfuric acid–methanol. The use of neat methanol in this transformation facilitates the isolation of a product uncontaminated by resinified sugars. A gradient-elution, reverse-phase, high-pressure liquid chromatography method for rapid and routine analyses of hesperidin and hesperetin is reported. Coinjection provided a means for the identification of the minor impurities isosakuranetin and its 7-β-rutinoside detected in the hesperetin and purified hesperidin, respectively.

Fourteen years ago Horowitz and Gentili reported that citrus peels contained glycosidic flavonoids which could, by way of simple chemical modification, be converted into a new class of sweet compound (1963, 1969). They discovered, for example, that neohesperidin, a bitter flavanone 7-β-neohesperidoside found in the Seville orange, provided an intensely sweet dihydrochalcone (1000× sucrose, molar basis) upon alkaline hydrogenation. Our recently published findings demonstrate that nonglycosidic dihydrochalcones are also intensely sweet. Hesperetin dihydrochalcone derivatives bearing simple 4-*O*-carboxyalkyl (DuBois et al., 1977a) and 4-*O*-sulfoalkyl (DuBois et al., 1977c) substituents have been found to have excellent water solubility and to display taste properties that compare favorably with neohesperidin dihydrochalcone. The simplicity of these analogues suggests the possibility of economic preparation and renders them attractive candidates for potential development as food additives.

A straightforward and generally applicable means for preparation of these simplified sweeteners has been developed (DuBois et al., 1977b). The method involves the regioselective alkylation and direct alkaline hydrogenation of the flavanone hesperetin (**3**). This aglycon, being the basic building block for these sweeteners, must be readily obtainable in quantity and in a high state of purity. Although hesperetin can be prepared by total synthesis (Zemplēn and Bognar, 1942; Boucherie and Hicter, 1963; Honohan et al., 1976), a more economical preparation is the acidic hydrolysis of the naturally occurring rutinoside hesperidin (1), which is the main flavonoid constituent of lemons and oranges.

We report here an improved method for the conversion of commercial hesperidin into hesperetin. The method involves a purification of the rather crude starting material followed by sulfuric acid catalyzed methanolic cleavage of the sugar residues. We also report here gradient-elution, reverse-phase, high-pressure liquid chromatography (HPLC) techniques developed for determining the purity of hesperidin and hesperetin. These techniques complement the isocratic reverse-phase analytical methods recently reported for the analysis of naringin (Fisher and Wheaton, 1976) and neohesperidin dihydrochalcone (Schwarzenbach, 1976; Fisher, 1977).

EXPERIMENTAL SECTION

General. Melting points were taken on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Infrared (IR) spectra were recorded on a Perkin-Elmer Model 137 instrument, whereas ultraviolet (UV) spectra were obtained in methanol with a Cary Model 118 spectrophotometer. Optical rotations were ascertained with a Perkin-Elmer Model 241 polarimeter. Proton nuclear magnetic resonance (^{1}H NMR) spectra were determined with a Varian T-60A spectrometer and are reported as parts per million (δ) relative to tetramethylsilane (internal standard).

Equivalent weights were determined by potentiometric titration (Metrohm Herisau Potentiograph E 576, Brinkmann Instruments, Westbury, N.Y.) with tetrabutylammonium hydroxide in aqueous dimethyl sulfoxide. Thin-layer chromatography (TLC) was carried out on prelayered silica gel 60 F-254 plates (E. Merck A. G., Darmstadt, Germany) of layer thickness 0.25 mm. The plates were developed to 10 cm and visualized with ultraviolet light and iodine. Combustion analyses were performed by the Microanalytical Laboratory, Stanford University, Stanford, Calif.

A gradient-elution, HPLC apparatus was constructed from a U6K universal injector, a Model 660 solvent flow programmer, and two Model 6000 pumps, all from Waters Associates (Milford, Mass.). The detector was a Model SF 770 spectroflow monitor (Schoeffel Instrument Corp., Westwood, N.J.) equipped with a Model GM 770 monochromater operating at 286 nm. A Waters Associates 30 cm × 4 mm i.d. reverse-phase μ Bondapak C-18 column (octadecyltrichlorosilane covalently bonded to 10 μm μ Porasil packing, 9000 plates/meter) was employed.

Purification of Hesperidin (1). A 2-L, three-neck flask, equipped with overhead stirrer and argon inlet, was charged with 100 g of hesperidin (Sunkist Growers Inc., Ontario, Calif., purified grade, lot W3) and 1 L of dimethylformamide (DMF). The dark mixture was stirred rapidly for 30 min, treated with Celite (50 g), and filtered through a 50-g pad of the same material with 200 mL of DMF being used for washing. The clear-yellow DMF solution was added dropwise over 90 min to a continuously boiling mixture of 1 L of H_2O and 25 mL of acetic acid contained in a 3-L three-neck flask equipped with overhead stirrer, reflux condenser, heating mantle, and dropping funnel topped with an Ar inlet. Purified hesperidin began to crystallize from the medium after 300 mL of solution had been added.

The slurry was cooled to 40 °C with continued stirring, filtered, and the residue washed with warm H_2O (1 × 1 I), isopropanol (1 × 0.5 L), and dried in vacuo (50 °C) to afford 62.6 g of fluffy white hesperidin (1): R_f 0.77 (CH_3OH), 0.51 (CH_3OH–$CHCl_3$, 1:1), and 0.57 (*i*-butanol–acetic acid–water, 2:1:1–); mp 260–262 °C (lit. mp 261–263 °C; Pritchett and Merchant, 1946); IR (KBr) 3400, 2950, 1610, and 1580 cm^{-1}; ^{1}H NMR (pyridine-d_5) 1.53 (d, J = 4 Hz, 3, rhamnose CH_3), 2.57–3.43 (m, 2, flavanone C-3 CH_2), 3.73 (s, 3, OCH_3), 3.90–4.66 (m, 10, saccharide O-CH_2-C and C-CH-C), 5.27–5.60 (m, 3, flavanone C-2 CH and saccharide O-CH-O), 6.43 (q, J_{AB} = 2 Hz, 2, A-ring aromatic), and 7.43 (m, 3, B-ring aromatic); UV 285 nm (log ε 4.32) (lit. UV 285 nm; Horowitz and Jurd, 1961).

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Equiv wt calcd: 619. Equiv wt found: 629. Anal. Calcd for $C_{28}H_{34}O_{15}\cdot{}^{1}/_{2}H_2O$: C, 54.29; H, 5.69. Found: C, 54.40; H, 5.76.

Hesperetin (3). A 2-L, three-neck flask, equipped with magnetic stir bar and condenser, was charged with 50.0 g (81.8 mmol) of purified hesperidin, 1 L of dry CH_3OH, and 50 mL of 96% H_2SO_4. The solution was refluxed for 7.5 h and poured into 4 L of ethyl acetate. The mixture was washed with 15% aqueous NaCl (1 × 1 L), H_2O (3 × 1.5 L, final wash colorless), saturated aqueous NaCl, and dried (Na_2SO_4). Evaporation afforded 27.1 g of pale-yellow powder.

The crude product was dissolved in acetone (200 mL) and added dropwise (60 min) to a vigorously stirred (overhead) mixture of 3 L of H_2O and 20 mL of acetic acid maintained at 95–100 °C. The slurry was cooled to 45 °C and the hesperetin filtered and dried in vacuo (24.4 g, 98.7%): R_f 0.34 (ether), 0.57 (ethyl acetate), and 0.37 ($CHCl_3$–CH_3OH, 95:5); mp 220–221 °C (lit. mp 223–225 °C; Arakawa and Nakazaki, 1960); $[\alpha]^{20}_D$ −12.44 (c 2.01, CH_3OH); IR ($CHCl_3$) 1645 and 1600 cm^{-1}; ^1H NMR (acetone-d_6) 2.50–3.38 (m, 2, C-3 CH_2), 3.90 (s, 3,OCH_3), 5.42 (q, 1, C-2 CH), 5.98 (s, 2, A-ring aromatic), and 7.03 (m, 3, B-ring aromatic); UV 288 nm (log ϵ 4.31) (lit. UV 288 nm; Horowitz and Jurd, 1961). Equiv wt calcd: 302. Equiv wt found: 306. Anal. Calcd for $C_{16}H_{14}O_6$: C, 63.57; H, 4.67. Found: C, 63.52; H, 4.67.

(±)-Isosakuranetin (4). The flavanone was prepared from 2,4,6-trihydroxyacetophenone (Aldrich Chemical Co., Milwaukee, Wis.) and *p*-methoxybenzaldehyde (J. T. Baker Chemical Co., Phillipsburg, N.J.) via aldol condensation followed by acid-catalyzed cyclization as previously reported for the synthesis of hesperetin (Honohan et al., 1976): mp 192–193 °C (lit. mp 190–192 °C; Ramakrishnan and Kagan, 1970); ^1H NMR (acetone-d_6) 2.50–3.15 (m, 2, C-3 CH_2), 3.83 (s, 3, OCH_3), 5.42 (q, 1, C-2 CH), 6.00 (s, 2, A-ring aromatic), and 7.21 (AA′BB′ pattern, 2, B-ring aromatic); UV 289 nm (log ϵ 4.25) (lit. UV 289 nm, log ϵ 4.37; Ramakrishnan and Kagan, 1970). Anal. Calcd for $C_{16}H_{14}O_5$: C, 67.13; H, 4.93. Found: C, 67.13; H, 4.96.

HPLC Analysis. Routine hesperidin and hesperetin purity determinations were carried out on 10-μg injected samples with a linear (program 6) gradient elution (15.0 min, 2.0 mL/min) of 10 to 100% spectrophotometric grade methanol in 0.03 M KH_2PO_4 buffer. The buffer was prepared with distilled H_2O and adjusted to pH 4.8 by addition of KOH. HPLC analyses of hesperetin and purified hesperidin samples prepared as described above showed the presence in each of a different minor (~5%), less-polar impurity (identical detector response toward monitored flavanones assumed). Coinjection (routine gradient) of hesperetin samples (retention time 12.7 min) with eriodictyol (12.0 min), naringenin (12.5 min), apigenin (14.0 min), and isosakuranetin (14.2 min) enabled the minor impurity present in the hesperetin to be tentatively identified as isosakuranetin (4). Verification was obtained by coinjection using a nonlinear (program 5) gradient elution (30.0 min, 2.0 mL/min) of 10 to 100% methanol in the buffer (hesperetin, 12.4 min; isosakuranetin, 15.1 min). Attempts to identify the impurity (12.0 min) present in the purified hesperidin (10.6 min) by coinjection with isosakuranetin 7-β-rutinoside and poncirin (isosakuranetin 7-β-neohesperidoside) using the routine gradient elution failed because of the essentially identical retention times of both isosakuranetin glycosides. The impurity was, however, readily identified as the rutinoside (2) by coinjection using an isocratic elution (2.0 mL/min) of 45% methanol in the buffer (hesperidin, 4.8 min; isosakuranetin 7-β-rutinoside, 11.1 min; poncirin, 12.4 min). With the exception of isosakuranetin which was prepared as described above, authentic samples of the potential impurities were supplied by R. M. Horowitz, Fruit and Vegetable Chemistry Laboratory, U.S. Department of Agriculture, Pasadena, Calif.

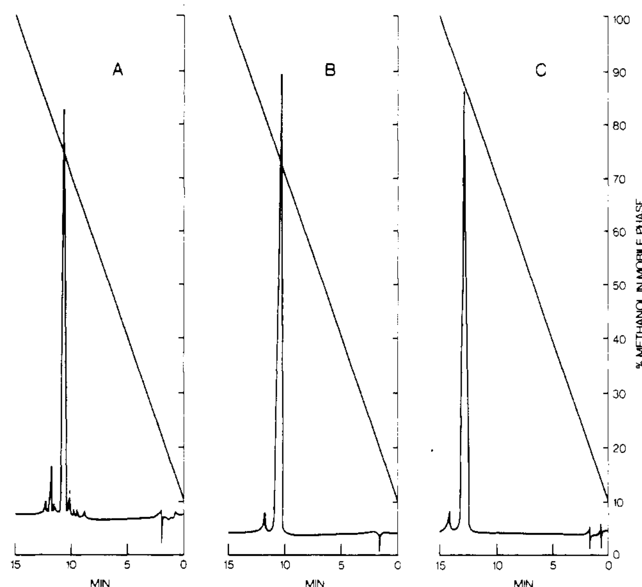
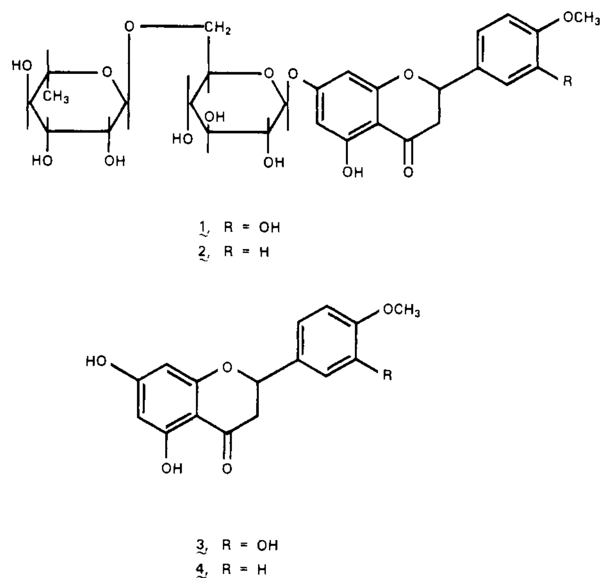


Figure 1. HPLC chromatograms of: (A) unpurified hesperidin; (B) purified hesperidin; (C) purified hesperetin. The chromatograms were recorded using the routine linear gradient (see text) with the diagonal lines representing the increasing concentration (10 to 100%) of methanol in the phosphate buffer.

RESULTS AND DISCUSSION

Hesperidin (1) is obtained by the alkaline extraction of chopped and pulped citrus peels and is isolated, following neutralization, by filtration (Higby, 1947). Hesperidin obtained in this manner is quite impure and our investigations have shown that it cannot be directly converted into high-quality hesperetin. Gradient-elution, reverse-phase HPLC of the commercial material employed in this study detected the presence of several UV-active impurities (Figure 1). These impurities are likely minor phenolic glycosides (Horowitz, 1964) present in the peels. The crude hesperidin also contained 7% volatiles and 9%



of a dark, DMF-insoluble residue. The purity was 75–80%.

The procedure described in the Experimental Section provided purified hesperidin in yields of 80–85% based upon the contents of the original mixture. The product was obtained as a white fluffy solid that was shown by standard methods to be quite pure. Analysis by gradient-elution HPLC (Figure 1) detected the presence of a minor, less polar impurity. This impurity was identified (vide infra) as isosakuranetin 7-β-rutinoside (**2**).

The acidic degradation of hesperidin into hesperetin (**3**), rhamnose, and glucose was first performed nearly 100 years ago (Tiemann and Will, 1881). The original method, which employs ethanolic aqueous sulfuric acid at elevated temperatures, has been utilized by most modern workers (Haley and Bassin, 1951; Sastry and Row, 1960; Looker and Holm, 1960) with little modification. It suffers in that hesperidin is poorly soluble in the medium and therefore extended reaction times or pressure vessels are required. Furthermore, the high temperatures and extended reaction times cause sugar resinification leading to the isolation of a discolored and impure product. Our investigations showed that recrystallization will not effectively remove these dark contaminants. Absorbents, such as activated carbon or acidic alumina, will clean up the product, but not without drastically lowering the yield.

A modification (Wilson, 1955) of the hydrolysis that significantly reduces the reaction time involves first dissolving hesperidin in dilute alkali and then adding this solution to refluxing aqueous hydrochloric acid. This procedure has been further improved by the use of 2-methoxyethanol as cosolvent (Dawson and Otteson, 1975). Hesperetin is still, however, provided containing yellow-brown resinous contaminants.

Of the range of methods investigated for performing this conversion, the utilization of neat methanol as the reaction solvent (a modification of the conditions of Arakawa and Nakazaki, 1960) was found to be vastly superior. Because hesperidin is quite soluble in this medium at reflux, the cleavage could be carried out in less than 8 h for a 5% solution in methanol–96% sulfuric acid (95:5). Little discoloration was afforded because of the lower boiling point of the solvent and the rapid rate of the reaction. Precipitation of an acetone solution of the methanolysis mixture into boiling water provided hesperetin in 99% yield as a slightly off-white, crystalline solid.

Gradient-elution, reverse-phase HPLC (Figure 1) showed the presence of a minor (~5%), less-polar impurity in the hesperetin. Coinjection with a series of citrus flavonoid aglycons (see Experimental Section) identified the impurity as isosakuranetin (**4**). The impurity present in the purified hesperidin was then readily identified as isosakuranetin 7-β-rutinoside (**2**) by coinjection of the hesperidin with **2** and poncirin (isosakuranetin 7-β-neohesperidoside), both potential precursors to the impurity found in the hesperetin.

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Toxaphene Degradation in Estuarine Sediments

Toxaphene in anoxic salt marsh sediments was degraded within a few days to compounds having gas chromatographic retention times shorter than those of standard toxaphene components. This breakdown occurred in sterile as well as unsterile sediments and also in a sand–Fe(II)/Fe(III) system. No breakdown was noticed in a sand system that did not contain the iron redox couple.

Toxaphene is presently the most heavily used chlorinated insecticide in the United States. Domestic consumption averages 26 million kg/year, with a cumulative total of over 500 million kg during the last 30 years (Guyer et al., 1971; vonRumker et al., 1974). The pesticide is made by photochemically chlorinating camphene in the presence

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