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Flavonoid Glycosides of Spartan Apple Peel

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The flavonoid glycosides of Spartan apples were isolated by column chromatography on polyamide and Sephadex resins and by RP-HPLC. They were characterized by ^1H and ^{13}C NMR as phlorizin and the following glycosides of quercetin: α-L-arabinofuranoside, β-D-galactopyranoside, β-D-glucopyranoside, α-L-rhamnopyranoside, β-D-xylopyranoside. The coupling constants in the ^1H NMR spectra were used to establish anomeric configurations of all glycosides.

A flavonoid glycoside mixture isolated from apples has been reported to have inhibitory properties toward a β-galactosidase of apples and toward softening of fresh apples (Dick et al., 1985). This paper describes the characterization of this mixture. The quercetin glycosides of apple peel were described by identification of the sugar moiety after hydrolysis of the separated glycosides (Siegelman, 1955; Walker, 1964) and by isolation of the individual glycosides by paper and cellulose chromatography (Teuber Wuenscher and Herrmann, 1978). Changes in the quercetin glycoside content of apples during storage have been reported by Donchev (1977). This paper describes procedures for HPLC analysis of the flavonoids in extracts of Spartan apples, procedures for the effective preparative fractionation of the flavonoid glycosides from apples, and their detailed characterization, by NMR methods. The occurrence of phlorizin, long known to be present in other apple tissues (deKoninck, 1835) and in apple juice (Lea and Timberlake, 1974), is now described in apple peel.

MATERIALS AND METHODS

Phlorizin, quercitrin, and rutin were obtained from Sigma Chemical Co. After recrystallization from ethanol–water, each substance gave a single peak on HPLC (see below) and the expected UV spectrum (Mabry et al., 1970). Polyamide 6S was obtained from Riedel-deHaen AG, Hannover. The flavonoid glycoside fraction was prepared from a purified ethyl acetate extract of Spartan apples by chromatography on an acrylic ester resin column (Dick et al., 1985).

High-Performance Liquid Chromatography (HPLC). Apple peel samples for HPLC analysis were prepared by blending 5 g of scraped apple peel in 100 mL of methanol, filtration, concentration to 10.0 mL, and injection of a 10-μL sample. Quantitative extraction of flavonoid glycosides was indicated by the quantitative recovery of rutin from spiked peel samples. Column fraction samples (see later) were injected directly after filtration. A Varian liquid chromatograph was used with detection of absorbance at 270 nm. A Waters Radial-Pak reversed-phase column was used with a solvent program of 25–50% tetrahydrofuran in 0.1% aqueous trifluoroacetic acid over 20 min at a flow rate of 2 mL/min. The labeled peaks of the chromatogram (Figure 1) were identified as (1) quercetin glucoside plus quercetin galactoside (isoquercitrin and hyperin), (2) quercetin xyloside (reynoutrin), (3) quercetin rhamnoside (quercitrin), (4) quercetin arabinoside (avicularin), and (5) phlorizin.

Preparative Column Chromatography. The purified flavonoid glycoside mixture (1.44 g) was fractionated by chromatography on polyamide 6S (240-mL settled bed volume) in a glass column of 3-cm diameter. The sample as a 10% solution in methanol was applied to the column equilibrated with 30% methanol in 0.1% aqueous acetic acid. The column was developed at a flow rate of 3 mL/min with 300 mL of the equilibration solvent followed by a linear gradient of 30–70% methanol in 0.1% aqueous acetic acid of volume 700 mL. This was followed by 700 mL of 70% methanol in 0.1% aqueous acetic acid. Elution of flavonoid glycosides was monitored by absorbance at 260 nm which, however, did not reveal any resolution. Examination of column fractions by HPLC as described gave the profile shown in Figure 2. Phlorizin, HPLC peak 5, was eluted first and resolved from the second component which was a mixture of the quercetin hexosides, the glucoside, galactoside, and rhamnoside, HPLC peaks 1 and 3. The third eluent was the partially resolved pentosides, the xyloside and arabinoside, HPLC peaks 2 and 4. The tail of this peak, labeled 4′ in Figure 2, was quercetin arabinoside. Column fractions of Figure 2 that were pooled were those that contained HPLC peaks 5, 1 and 3, 2 and 4, and 4′. Each was evaporated to dryness. After recrystallization of the residues of peaks 5 and 4′ from ethyl acetate–hexane, 22 mg of phlorizin and 17.5 mg quercetin arabinoside, respectively, were obtained.

The combined fractions containing HPLC peaks 1 and 3 from the polyamide column were evaporated to dryness, dissolved in a minimum volume of methanol and applied to a Sephadex G-10 column (40-mL bed volume), which was developed with a linear gradient of water–20% aqueous methanol at a flow rate of 0.1 mL/min (Redden, 1985). Monitoring by HPLC showed a succession of three

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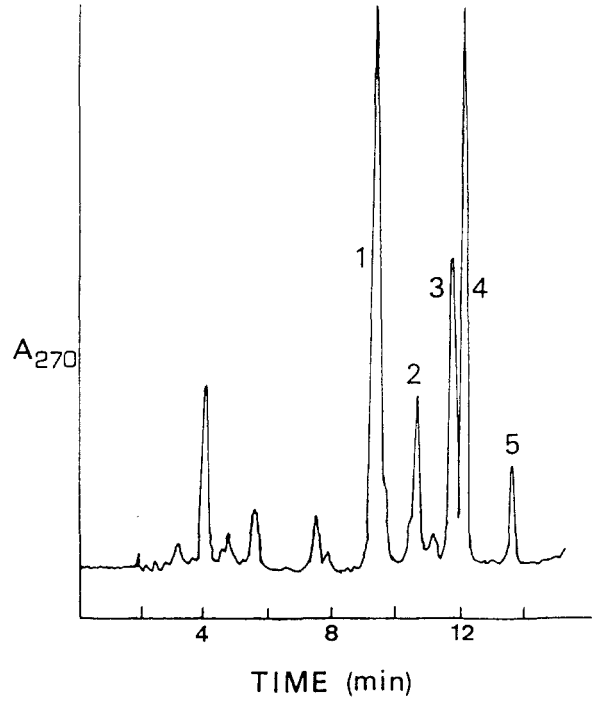

Figure 1. HPLC separation of flavonoid glycosides and phenolic acids of a methanol extract of Spartan apple peel: (1) quercetin glucoside and quercetin galactoside; (2) quercetin xyloside; (3) quercetin rhamnoside; (4) quercetin arabinoside; (5) phlorizin. Conditions: column, radial pak RP (C_{18}); solvent program, 25–50% THF in 0.1% aqueous TFA over 20 min at a flow rate of 2.0 mL/min with detection at 270 nm. Rutin was responsible for the peak at 7.5 min.

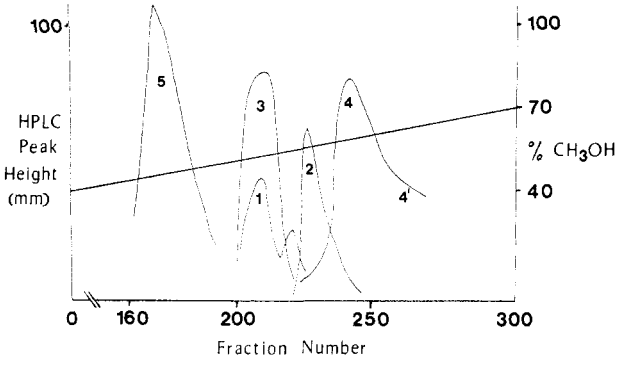

Figure 2. Separation of flavonoid glycoside fraction of Spartan apples on polyamide column of 3-cm diameter and 240-mL volume. Conditions: solvent, 30–70% methanol in 0.1% aqueous acetic acid; effluent monitored by HPLC as in Figure 1. Peaks identified by number refer peaks of Figure 1.

peaks, the first two of which had the appearance of HPLC peak 1 and were labeled 1A and 1B, respectively. The third peak was composed of HPLC peak 3. After evaporation of the solvent and recrystallization from ethyl acetate–hexane, 5.2 mg of 1A, 13.2 mg of 1B, and 11.8 mg of peak 3, quercitrin, were obtained. Chromatography of the fraction containing peaks 2 and 4 from the polyamide column on Sephadex G-10 with gradient elution with methanol–water mixtures did not resolve the two components.

Characterization. Each crystalline product gave a single peak on HPLC and the expected UV–visible spectrum (Mabry et al., 1970). The composition of each crystalline fraction from the column chromatography was established by hydrolysis of 1 mg of each in 1 M H_2SO_4, followed by HPLC of the aglycon (as described earlier) and paper chromatography, using the conditions of Siegelman (1955), of the sugar moeity. ^1H and ^{13}C NMR spectra were recorded at 361.08 and 90.8 MHz on a Nicolet NT-360 NB NMR spectrometer on solutions in dimethyl-d_6 sulfoxide. ^1H NMR chemical shifts are referenced to internal Me_4Si; ^{13}C NMR chemical shifts are referenced to dimethyl-d_6 sulfoxide as 39.56 ppm.

RESULTS AND DISCUSSION

HPLC of the Flavonoid Glycoside Fraction. The flavonoid glycoside fraction obtained from ethyl acetate extracts of Spartan apples by chromatography on acrylic ester resin columns (Dick et al., 1985) may be effectively resolved by reversed-phase HPLC. Figure 1 shows a chromatogram of a methanol extract of Spartan apple peel. The purified flavonoid glycoside fraction gave a profile (not shown) corresponding to retention times between 8 and 15 min in Figure 1 and not containing the other peaks. The flavonoid glycoside peaks were identified in the HPLC chromatogram after preparative isolation (see below) and characterization of individual components and their separate chromatography. The HPLC solvent program developed here was similar to that described by Asen (1982). Quercetin 3-*O*-glucoside (isoquercitrin) and 3-*O*-galactoside (hyperin) are not resolved in this system, but resolution of all of the other major flavonoid glycosides present in apple extracts, including phlorizin and rutin, is realized (Figure 1). Phlorizin has been reported in apple juice (Lea and Timberlake, 1974) but not apple peel. The quercetin glycoside rutin, detected in apples by Siegelman (1955) and Walker (1964), was identified in methanol extracts of apples here (retention time 7.5 min; Figure 1) by comparison with an authentic sample. It is clearly a minor constituent of Spartan apples and was not apparent in the purified glycoside fraction presumably because of its relatively poor solubility in ethyl acetate and water, the extractant solvents.

Preparative Column Chromatography. The preparative resolution of flavonoid glycosides on polyamide and Sephadex columns has been described (Markham, 1975). The separation of the glycoside fraction of Spartan apples on polyamide by elution with an aqueous methanol gradient (Figure 2) was conveniently monitored by absorbance at 260 mn or by HPLC, as shown. Three fractions were obtained in order of increasing methanol concentration in the eluate: phlorizin, a quercetin hexoside mixture, and a quercetin pentoside mixture, respectively. There is some indication from Figure 2 that even greater resolution may be possible, but this was not pursued since our purpose was to characterize each HPLC peak. The great difference in the relative amounts of flavonoid glycosides between Figures 1 and 2 is due to different extraction conditions and the partial purification of the preparative fraction (Dick et al., 1985).

The quercetin hexoside fraction was partially resolved on a Sephadex G-10 column using gradient elution with aqueous methanol (Redden, 1985). Three fractions were obtained in order of increasing methanol concentration: a mixture of quercetin glucoside and galactoside, pure quercetin galactoside, and quercetin rhamnoside. Resolution of the quercetin pentoside fractions on Sephadex was unsuccessful (Redden, 1985).

Characterization of the Flavonoid Glycosides. With one exception, all of the flavonoid glycosides were characterized by comparison of UV–visible and NMR spectra with those of published spectra. In some cases, characterization was made possible by comparison of spectra of mixtures of glycosides to pure compounds and to published spectra. The first glycoside to elute from the polyamide column had UV and ^{13}C NMR spectra identical with those

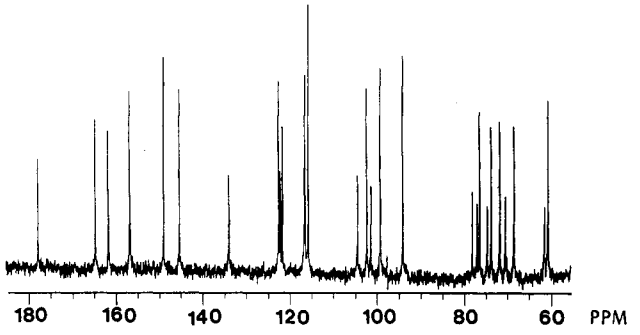

Figure 3. ^{13}C NMR spectrum of fraction 1A of Sephadex G10 chromatography of quercetin hexoside fraction of Spartan apples, a mixture of quercetin 3-*O*-β-D-galactopyranoside, and quercetin 3-*O*-β-D-glucopyranoside.

of an authentic sample of phlorizin. As far as we are aware, the ^1H and ^{13}C NMR spectra of this well-known compound have not been described before: ^1H NMR δ 2.787 (t, 2 H, CH_2Ph, J = 7.2 Hz), 3.1–3.8 (complex m, glucose H and CHC(O)), 4.943 (d, 1 H, anomeric H, J = 7.1 Hz), 4.645, 5.095, 5.198, 5.337 (carbohydrate OH), 5.935, 6.132 (2 d, 2 H, J = 1.90 Hz, aromatic H), 6.646, 7.042 (2 d, 4 H, J = 8.34 Hz, aromatic H), 9.15, 10.6 (2 br s, phenolic OH), 13.54 (s, OH-6); ^{13}C NMR, δ 29.13 (CH_2), 45.08 (CH_2), 60.6, 69.5, 73.3, 76.8, 77.3 (glucose C), 94.4, 96.9, 100.9 (anomeric C), 105.2, 115.1 (2C, CH), 129.3 (2C, CH), 131.6, 155.3, 160.9, 164.6, 165.4, 204.8 (C=O). Phlorizin has previously been described from apple root bark (deKonink, 1835) and from apple juice (Lea and Timberlake, 1974).

The quercetin glycosides were identified by acid hydrolysis followed by paper chromatography of the sugar moiety (Siegelman, 1955) and by HPLC of the aglycon, which showed only quercetin (data not shown). They were characterized by their UV and NMR spectra and by comparison with published spectra. Quercitrin was identified as the α-L-rhamnopyranoside by its ^1H NMR (Markham, 1975) and ^{13}C NMR (Markham et al., 1978; spectrum 65) spectra. The second peak from the Sephadex column was identified as the galactoside hyperin (quercetin 3-*O*-β-D-galactopyranoside) by comparison of its ^{13}C nMR spectrum (Markham and Chari, 1982; spectrum 63). The galactoside was isolated from apples initially by Sando (1955).

The glucoside was characterized from the ^{13}C NMR spectrum of the mixture of the glucoside and galactoside (first peak from the Sephadex column), by subtraction of the galactoside signals, as the β-D-pyranoside isoquercitrin (Markham and Chari, 1982; spectrum 61 and Figure 3). The subtraction technique is well established in NMR spectroscopy (Martin et al., 1980). In the high-field ^1H NMR spectra of these compounds, the signal of the anomeric protons is easily identified. The anomeric configurations can be assigned from their coupling constants, 1.5, 7.7, and 7.3 Hz for the rhamnoside, galactoside and glucoside, respectively. The β-anomeric configuration of the galactoside found here is in contrast to that reported by Teuber Wuenscher and Herrmann (1978) for the corresponding derivative from Golden Delicious apples.

The arabinoside was identified as the quercetin 3-*O*-α-L-arabinofuranoside by comparison of the ^{13}C NMR spectrum of the pure crystalline substance isolated from fraction 4′ (Figure 2) with that of Markham et al. (1978) and Markham and Chari (1982). No evidence was found for the occurrence of other isomers of quercetin arabinoside in apple extracts in this study. Quercetin 3-*O*-β-D-xylopyranoside (reynoutrin; Nakaoki and Morita, 1956) was identified as responsible for HPLC peak 2 from the spectrum of the mixture of the arabinoside and xyloside by subtraction of the arabinoside signals. Although this derivative is well-known (Nakaoki and Morita, 1956; Teuber Wuenscher and Herrmann, 1978), its ^{13}C NMR spectrum has not previously been described: ^{13}C NMR (xylose C) δ 66.1 (C-5), 69.5 (C-4), 73.7 (C-2), 76.1 (C-3), 101.8 (anomeric C), (quercetin C) δ 93.7 (C-8), 98.8 (C-6), 104.0 (C-10), 115.1-116.2 (C-2′, C-5′), 121.2-122 (C-1′, C-6′), 133.2 (C-3), 145.0 (C-3′), 148.7 (C-4′), 156.3–157.0 (C-2, C-9), 161.3 (C-5), 164.5, (C-7), 177.4 (C-4); ^1H NMR δ 5.34 (d, 1 H, J = 7.2 Hz, anomeric H). Xylose carbons were assigned by analogy with those of methyl β-D-xylopyranoside (Gorin and Mazurek, 1975) and quercetin carbons by analogy with quercetin 3-*O*-β-D-glucopyranoside (Markham et al., 1978; Markharm and Chari, 1982). The maximum difference for the quercetin carbons was 0.5 ppm, for C-3.

The flavonoid glycosides described here do not preclude the occurrence of other relatively minor such substances in apples that may be responsible for the small peaks in the HPLC chromatograms in the region of the described compounds. As noted earlier, our extraction procedures do not yield flavonoid disaccharides (e.g., rutin) or oligosaccharides.

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Registry No. Phlorizin, 60-81-1; avicularin, 572-30-5; hyperin, 482-36-0; isoquercitrin, 21637-25-2; quercitrin, 522-12-3; reynoutrin, 549-32-6.

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