Identification of Some Phenolic Compounds in Apples

Wieslaw Oleszek,[1] Chang Y. Lee,* Antoni W. Jaworski, and Keith R. Price

Five quercetin and two phloretin glycosides were isolated from apple skins. By chemical and spectral methods they were identified as quercetin 3-*O*-galactoside (hyperin), 3-*O*-glucoside (isoquercitrin), 3-*O*-xyloside (reynoutrin), 3-*O*-arabinoside (avicularin), and 3-*O*-rhamnoside (quercitrin), phloretin glucoside (phlorizin), and phloretin xyloglucoside. The glycosides of phloretin were also found in apple flesh.

The polyphenolic compounds of apples have been studied fairly well because they contribute to the color and flavor of apple juices (Lea and Timberlake, 1974; Timberlake and Bridle, 1971). Recently, the presence of quercetin 3-glycosides in apple peels has attracted considerable interest due to their possible role in suppressing firmness loss in apples during cold storage (Lidster et al., 1986). It was suggested that these compounds inhibit β-galactosidase activity, the enzyme that may be involved in the regulation of apple texture (Lidster and McRae, 1985; Dick et al., 1985). In addition, dihydrochalcone glycosides, such as phlorizin and phloretin xyloglucoside, are known to be related to the browning reactions in apple ciders (Durkee and Poapst, 1965; Whiting and Coggins, 1975). However, it was shown that their oxidation products by polyphenol oxydase (PPO) resulted in the yellow color, suggesting that they are not the enzyme substrates (Durkee and Poapst, 1965). On the other hand, their chemical, enzymatic, or microbial degradation produced phenolic acids that then brown readily (Barnes and Williams, 1961; Holowczak et al., 1960). Earlier study on these quercetin glycosides was very much limited to the qualitative analysis based on thin-layer chromatography (TLC) or column chromatography. The main reason was that the separation and identification of quercetin glycosides by the conventional chromatographic methods were very difficult due to the similarity of their molecular weight and polarity (Bernardi et al., 1984). Since a high-performance liquid chromatograph (HPLC) equipped with a diode array detector was able to isolate many phenolic compounds in some fruits (Jaworski and Lee, 1987; CoSeteng and Lee, 1987), pure phenolic standards are required for the quantitative analysis. Thus, the aim of our present work was to isolate, identify, and purify some flavanoid glycosides in apple using HPLC and mass spectrometer.

MATERIALS AND METHODS

Apples. Rhode Island Greening apples grown at the New York State Agricultural Experiment Station orchards during the 1986 season were used for this study. A 22-kg portion of apples was peeled and cored mechanically. The peels and sliced fleshes were immediately immersed in the potassium metabisulfite ($K_2S_2O_5$, 1000 ppm) water solution to prevent browning, then quickly drained, frozen at −23 °C, and freeze-dried. Yields in dry weight were 300 g of skin and 2.22 kg of flesh.

Extraction. The dried skin (300 g) was blended with 3 × 3 L of 85% methanol in a Waring blender for 5 min each. The mixture was filtered, and the filtrate was concentrated in vacuo to the final volume of 1 L. The extract was washed 3× with hexane in a separatory funnel to remove lipids, carotenoids, and chlorophylls, adjusted to pH 7.0 with 15% NaOH, and then applied to the C_{18} column (6 cm × 10 cm, 55–105 μm, Waters Associates, 50 g) to remove sugars and phenolic acids. The column was first washed with 1 L of distilled water (1 mL/min), and then the phenolics were eluted with 1 L of methanol. Methanol was removed in vacuo, and the final concentrate of viscous brown material was 7.2 g.

Isolation of Glycosides by Preparative HPLC. The concentrated extract was dissolved in 50 mL of distilled water and loaded to the preparative C_{18} column (cartridge 5 × 30 cm, Waters 1000 PREP-PAK with pump, Waters Model 590, 80-mL version). The column was eluted with 8% acetonitrile in 5% acetic acid (10 mL/min) to elute catechins and procyanidins (fraction 1), and then the mobile phase was switched to 20% acetonitrile in 5% acetic acid (flow rate 10 mL/min) to yield fractions 2–4 (Figure 1).

Separation of Glycosides by Micropreparative HPLC. The glycosides of the fractions 2 and 3 were separated by micropreparative HPLC column (Waters Associates C_{18}, particle size 5 μm, 8 mm × 10 cm) with isocratic 10% acetonitrile in 5% acetic acid and a flow rate of 2 mL/min. One-hundred microliter samples containing approximately 5–8 mg of glycosides were repeatedly injected.

Analysis of Glycosides by Analytical HPLC. The purity of the compounds separated by micropreparative HPLC, their R_t's, and general phenolic composition in apple extracts were measured with an analytical HPLC unit (Hewlett-Packard, Model 1090M, equipped with a diode array detector and 95580A series 300 computer) using an 8 mm × 10 cm C_{18} Radial-PAK column (Waters) according to the method of Jaworski and Lee (1987).

Analysis of Hydrolysis Products. Individual glycoside was hydrolyzed in 2 N HCl in 50% methanol for 3 h at 80 °C. Sugars produced were separated on cellulose TLC plates with benzene–butanol–pyridine water (1:5:3:3) as a solvent system and stained with silver nitrate. Aglycons were identified with an analytical HPLC as described above by comparing retention time (R_t) with those of commercial standards (Sigma).

Spectral Analyses of Glycosides. The spectra and batochromic shifts were recorded with a spectrophotometer (Hewlett-Packard 8452A) equipped with a diode array detector and series 300 computer according to the method of Mabry et al. (1970).

Fast Atom Bombardment Mass Spectrometery (FAB-MS). FAB mass spectra (negative ion mode) were recorded on Kratos MS/50TC spectrometer. The samples

Department of Food Science & Technology, New York State Agricultural Experiment Station, Cornell University, Geneva, New York 14456 (W.O., C.Y.L., A.W.J.), and AFRC Institute of Food Research, Norwich, England (K.R.P.).

[1] On leave from the Institute of Soil Science and Plant Cultivation, 24-100 Pulawy, Poland.

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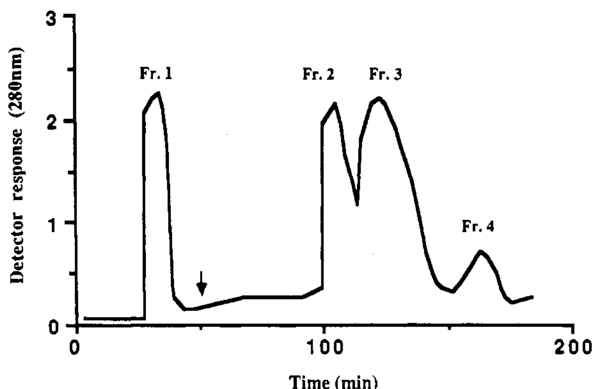

Figure 1. Preparative reversed-phase HPLC separation of apple skin phenolics. The HPLC conditions are described in Materials and Methods. Arrow shows the time when mobile phase was switched from 8 to 20% acetonitrile in 5% acetic acid.

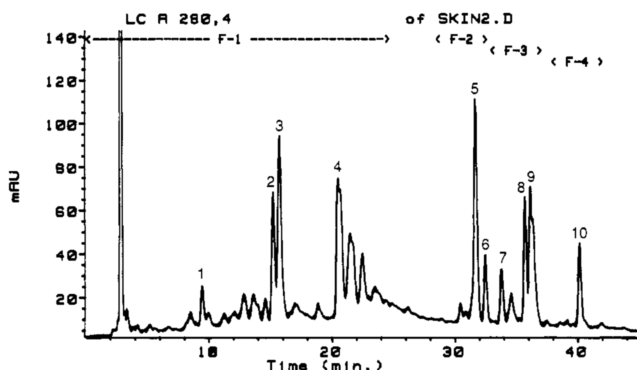

Figure 2. Chromatogram of analytical HPLC of apple skin phenolics: (1) catechin; (2) chlorogenic acid; (3) procyanidin B2; (4) epicatechin and procyanidin C1; (5) quercetin galactoside; (6) quercetin glucoside; (7) quercetin xyloside; (8) quercetin arabinoside; (9) quercetin rhamnoside and phloretin xyloglucoside; (10) phloridzin. Fractions 1–4 correspond to those in Figure 1.

were dissolved in glycerol and bombarded with a 9-kV (nominal) beam of xeon atom produced by an Ion Tech 11 NF atom gun. The spectra were recorded on a UV galvanometer recorder.

RESULTS

Preparative HPLC was able to separate phenolics into four major fractions (Figure 1). Analytical HPLC of these fractions showed that fraction 1 contained mainly catechin, epicatechin, and their dimers and polymers (R_t 0–25 min); fraction 2 consisted of two peaks that had R_t's 31.7 and 32.5 min; fraction 3 revealed three peaks with R_t's 33.8–36.1 min; and fraction 4 yielded only one peak with R_t 40.2 min (Figure 2). To obtain individual glycoside in a sufficient amount, fractions 2 and 3 were run at least 30 times on a micropreparative column. Identities of each compound were as follows:

Compound F-2-1: 80 mg; R_t 31.7 min; UV, λ_{max}, nm (MeOH) 260, 364, ($AlCl_3$) 272, 406, ($AlCl_3$–HCl) 272, 404, (NaOAc) 272, 378, (NaOAc–H_3BO_3) 264, 388; FAB-MS, m/z 463 $(M - H)^-$. Acid hydrolysis yielded quercetin and galactose (TLC, HPLC).

Compound F-2-2: 21 mg; R_t 32.5 min; UV, λ_{max}, nm (MeOH) 260, 360, ($AlCl_3$) 272, 406, ($AlCl_3$–HCl) 270, 404, (NaOAc) 268, 370, (NaOAc–H_3BO_3) 266, 394; FAB-MS, m/z 463 $(M - H)^-$, 325, 311, 301 $(M - Glu - H)^-$. Acid hydrolysis revealed quercetin and glucose.

Compound F-3-1: 35 mg; R_t 33.8 min; UV, λ_{max}, nm (MeOH) 258, 360, ($AlCl_3$) 272, 302 sh, 366 sh, 410, ($AlCl_3$–HCl) 270, 302 sh, 366, 402, (NaOAc) 268, 368, (NaOAc–H_3BO_3) 264, 384; FAB-MS, m/z 433 $(M - H)^-$. Acid hydrolysis yielded quercetin and xylose.

Compound F-3-2: 30 mg; R_t 35.7; UV, λ_{max}, nm (MeOH) 260, 356, ($AlCl_3$) 274, 302 sh, 362 sh, 404; ($AlCl_3$–HCl) 272, 302 sh, 360 sh, 404; (NaOAc) 266, 364, (NaOAc–H_3BO_3) 264, 378; FAB-MS, m/z 433 $(M - H)^-$, 301 $(M - Ara - H)^-$, 285. Acid hydrolysis furnished quercetin and arabinose.

Compound F-3-3: 12 mg; R_t 36.1; UV, λ_{max}, nm (MeOH) 286; FAB-MS, m/z 567 $(M - H)^-$, 435 $(M - Xyl - H)^-$, 409, 405, 395, 325, 311, 297, 273 $(M - Xyl - Glu - H)^-$. Acid hydrolysis yielded phloretin, xylose, and glucose.

Compound F-3-4: 12 mg; R_t 36.2; UV, λ_{max}, nm (MeOH) 256, 350, ($AlCl_3$) 272, 420, ($AlCl_3$–HCl) 270, 356, 398, (NaOAc) 268, 366, (NaOac–H_3BO_3) 262, 368; FAB-MS, m/z 447 $(M - H)^-$, 301 $(M - Rha - H)^-$, 285. Hydrolysis afforded quercetin and rhamnose.

Compound F-4: 50 mg; R_t 40.2 min; UV, λ_{max}, nm (MeOH) 286; FAB-MS, m/z 435 $(M - H)^-$. Acid hydrolysis yielded glucose and phloretin.

DISCUSSION

The 85% methanol extracts of the RI Greening apple peels contained several phenolic glycosides in addition to catechins and procyanidins. They were successfully separated from the other phenolic compounds by reversed-phase C_{18} preparative high-performance liquid chromatography (Figure 1). However, the separation into individual glycosides by a preparative HPLC was not successful since most of these compounds possess very close molecular weights and polarities. They were successfully separated and collected by a micropreparative HPLC in repetitive runs in 10% acetonitrile in 5% acetic acid isocratic solution system. Five of the isolated compounds (F-2-1, F-2-2, F-3-1, F-3-2, F-3-4) were identified by chemical and spectral methods as quercetin galactoside, glucoside, xyloside, arabinoside, and rhamnoside (R_t 31.7, 32.5, 33.8, 35.7, 36.2 min, respectively; Figure 2). The site of the chemical bond of sugars to aglycon was established by bathochromic shifts method (Mabry et al., 1970). All five compounds showed bathochromic shifts of band I (in MeOH) to band Ia (in $AlCl_3$–HCl) ranging from 40 to 48 nm, a characteristic feature of 5-hydroxy 3-O-substituted flavonols. The bathochromic shifts of band II (6–12 nm) with AcONa also indicated the presence of unsubstituted hydroxy groups at C-7 positions (Mabry et al., 1970). Moreover, the shifts of band I in the presence of NaOAc–H_3BO_3 ranging from 14 to 34 nm indicated the presence of the unsubstituted B-ring *o*-dihydroxyl groups. Thus, by the spectral features it was concluded that all the isolated flavonoids were 3-*O*-glycosides of quercetin. The five flavonoids reported here have been recently identified in Spartan apples skin by Dick et al. (1987). However, they were unable to separate quercetin galactoside and quercetin glucoside. The solvent systems used in the present research were more effective for separation of flavonoids, and those two glycosides were successfully isolated. The HPLC analysis time presented here was longer than that of Dick et al. (1987); however, it provided good resolution that made possible quantification of quercetin and phloretin glycosides and flavans simultaneously.

In addition, two other glycosides were separated and identified. Compound F-4, which was easily separated from the others by preparative HPLC, yielded phloretin and glucose when hydrolyzed. Its UV spectrum, retention time on analytical HPLC, and molecular weight as established by FAB-MS were identical with those of commercially available phlorizin, whose presence in apples was reported (Lea and Timberlake, 1974). The second glycoside (F-3-3) migrated together with quercetin arabinoside (F-3-2) in the isocratic solvent system (10% acetonitrile in 5% acetic acid) of micropreparative HPLC. Therefore,

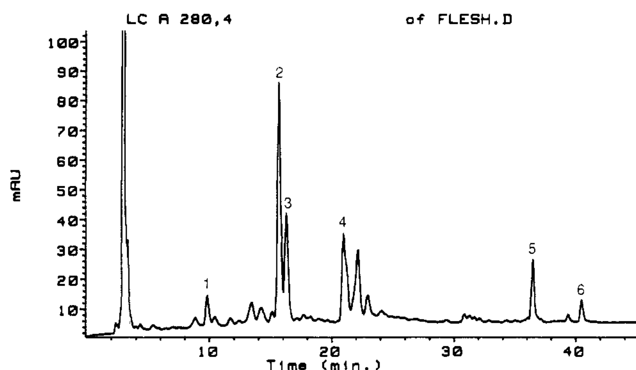

Figure 3. Chromatogram of analytical HPLC of apple flesh phenolics: (1) catechin; (2) chlorogenic acid; (3) procyanidin B2; (4) epicatechin and procyanidin C1; (5) phloretin xyloglucoside; (6) phloridzin.

these two compounds were successfuly separated by a liquid–liquid extration procedure by dissolving the mixture in distilled water and then extracting the quercetin arabinoside (F-3-2) with ethyl acetate, while the F-3-3 compound remained in the water solution. This compound was extracted with 1-butanol, and the butanol extract yielded a compound with a molecular weight of 567 and a UV spectrum similar to that of phlorizin. Acid hydrolysis produced phloretin, xylose, and glucose. FAB-MS in the negative-ion mode produced a spectrum containing main ions at m/z 567, 435, and 273, corresponding to the $(M - H)^-$ ion and sequential losses of xylose (132 μm) and glucose (162 μm). The fragment m/z 273 is consistent with the presence of phloretin (M_r 274). Thus, compound F-3-3 was proven to be phloretin xyloglucoside, with xylose being a terminal sugar. The site of sugar attachment to the aglycon moiety as well as the mode of the sugar linkage was not established in this research. The presence of this compound in the apples has been reported by Whiting and Coggins (1975).

As shown in Figures 2 and 3, apple skin and apple flesh differ distinctly in the phenolic glycoside composition. The apple flesh contains mainly two dihydrochalcone glycosides whereas apple skin possesses both dihydrochalcone and quercetin glycosides. The presence of phloretin xyloglucoside in apple skin was not reported before since this compound merges with quercetin arabinoside and/or quercetin rhamnoside in HPLC and consequently could not be recognized. A new generation of HPLC equipped with a diode array detector and the computer system, like the one used in the this study, makes it possible to detect the presence of this compound in apple skin extracts.

Registry No. Hyperin, 482-36-0; isoquercitrin, 482-35-9; reynoutrin, 549-32-6; avicularin, 572-30-5; quercitrin, 522-12-3; phlorizin, 60-81-1; phloretin xyloglucoside, 113532-28-8.

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