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# Anthocyanins and other phenolic compounds in fruits of red-flesh apples

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Anthocyanins and other phenolics of red-flesh apples, cv. Scugog, were extracted with acidified methanol and purified by Sephadex and by reverse phase high performance chromatography (RP HPLC). Four anthocyanins, three quercetins, two phenolic acids, two flavan 3-ols and one dihydrochalcone glycoside were characterized by HPLC, paper chromatography, spectral characteristics and colour reactions. The major phenolic compound was chlorogenic acid, and the predominant anthocyanin was cyanidin 3-galactoside, with cyanidin 3-glucoside, 3-arabinoside and 3-xyloside present in lower amounts. At 95–100 mg/kg of anthocyanin concentration, 'Scugog' apples are a rich source of the relatively rare cyanidin 3-xyloside.

## INTRODUCTION

Phenolic compounds are important constituents of apples and contribute to the color and flavor of fresh fruit and processed products, such as apple juices (Van Buren, 1970; Timberlake & Bridle 1971; Lea & Timberlake, 1974). In most markets, red skin apples are preferred to others, and within a cultivar, better colored fruits generally earn a higher price. This is not solely a matter of attractive exterior appearance. Consumers generally know from experience that, within a cultivar, brighter colored apples often taste better than green apples.

The pigments causing red coloration in apple skin are mainly anthocyanins, although colorless phenolic compounds also aid in the intensification of color through the copigmentation reaction (Mazza & Brouillard, 1987, 1990). In the skin of red apples the following anthocyanins have been reported: cyanidin 3-galactoside, 3-glucoside, 3-arabinoside, 3-xyloside and 7-arabinoside (Sun & Francis, 1967; Timberlake & Bridle, 1971). The presence of cyanidin 7-arabinoside has, however, not been confirmed. Other phenolic compounds found in apples include: chlorogenic acid, epicatechins, procyanidins, flavonols and dihydrocholcone glycosides (Mosel & Herrmann, 1974; Lea & Timberlake, 1974; Oleszek *et al.*, 1988).

Investigations of anthocyanins and other phenolic compounds in apples have employed fruit with white flesh and red, yellow or green skin. To our knowledge, no study that has used red-flesh apples has been published. Thus, the aim of our present work was to isolate and characterize the anthocyanins and some other phenolics of 'Scugog' apple (*Malus pumila* var. *niedzwetzkyana*) which has red flesh and red skin.

## MATERIALS AND METHODS

### Apples

'Scugog' apples grown at the orchards of Agriculture Canada Research Station, Morden, Manitoba, during the 1988 season were used for this study. Twenty uniform apples with dark red flesh and skin (4 to 5 cm in diameter) were harvested in mid-September from a single tree. The apples were quickly frozen, freeze-dried and stored in the dark at –20°C ± 1° for 6–8 months prior to removing the seeds, grinding and analysis.

### Extraction of phenolic compounds

A 20 g sample was blended with 200 ml of 10 : 1 : 9 ethanol–acetic acid–water (EAW) for 5 min in a Waring blender at full speed. The homogenate was then

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suction-filtered through a Whatman No. 44 filter paper and the residue washed with 50 ml of extraction solvent. The extract was dried under vacuum at 30°C. Just prior to high performance liquid chromatography (HPLC) and paper chromatography (PC), dried pigment extract was redissolved in methanol–acetic acid–water (10:1:9, MAW), and filtered through a 45 μm Millipore filter.

**Standards**

The standard compounds used were as reported by Mazza (1986) and Velioglu and Mazza (1991).

**Purification and HPLC analysis**

Concentrated fruit extract after dissolution in MAW was passed through a Sephadex LH No. 20 column (2 × 40 cm) in 5% aqueous acetic acid to remove the sugars and then in MAW. Three major fractions were collected, taken to dryness and further purified by HPLC.

The equipment used for HPLC consisted of an LKB liquid chromatography system (LKB-Produkter, Bromma, Sweden) equipped with two pumps (LKB Model 2150), a controller (LKB Model 2152), and Rheodyne 7125 injector valve with 20 μl or 100 μl loop, an UltroPac column (250 × 4·6 mm) of Spheri 10-RP18 (10 μm) (Brownlee Labs, Santa Clara, California), a photodiode array detector (LKB Model 2140), interfaced with an IBM personal computer and a Canon A-1210 color printer, and a fraction collector (LKB model 2211 SuperRac).

The following solvent system and elution profiles were used for the separation of phenolics: Solvent A, formic acid–water (5:95 v/v); solvent B, methanol. Elution profile: 0–10 min, 17–22% B (linear gradient) 10–12 min, 22–27% B, 12–33 min, 27–37% B; 33–39 min, 37–55% B. The solvent flow rate was 1·0 ml/min and the column pressure 34–40 bar. All separations were performed at 22 ± 1°C and all solvents were HPLC grade filtered through a 0·45 μm Millipore filter before use. Detection was performed simultaneously at 190–370 nm. The retention times were calculated with an IBM personal computer equipped with a Wavescan Spectral Detector program (LKB 2140–202). The capacity factor ($k'$) was calculated by the following equation (Kirkland, 1971):

$$k' = (t_r - t_0)/t_0 \quad \text{and} \quad \alpha = k'_2/k'_1$$

where $t_r$ = retention time of compound, $t_0$ = the time of zero retention as the time of the nonretained solvent peak, $k'_1$ = capacity factor of compound 1.

The peak area and area percent were calculated with the same IBM computer equipped with Model 2600 chromatography software, version 3.1 (Nelson Analytical, Inc., Cupertiono, California). The UV maxima were determined from the spectrum of each HPLC peak displayed on the IBM personal computer equipped with the Wavescan Spectral Detector program. Most HPLC peaks were collected as separate fractions from the column outlet, concentrated on a rotary evaporator at 30°C and subjected to further analyses.

**Spectral analysis**

Purified anthocyanin pigment was dissolved in methanolic 0·01% HCl; other phenolics were dissolved in methanol. UV and visible spectra were obtained with a Beckman DU-50 spectrophotometer connected to an Epson RX-80 printer and an IBM personal computer equipped with a 'peak pick' program (Beckman Quant 1 Soft-Pak, Beckman Instruments Inc., Scientific Instruments Div., Irvine, CA). Sodium methoxide (NaOMe), aluminum chloride ($AlCl_3$), aluminum chloride + hydrochloric acid ($AlCl_3$ + HCl), sodium acetate (NaOAc) and sodium acetate + boric acid (NaOAc + $H_3BO_3$) shifts were recorded and interpreted as described by Mabry *et al.* (1970) and Markham (1982).

**Paper chromatography**

$R_f$ values of the purified pigments were obtained on Whatman No. 1 paper using the following solvent systems (Mabry *et al.*, 1970; Francis, 1982): BAW—*n*-butanol–glacial acetic acid–water, 4:1:5, upper phase, aged 3 days; Bu–HCl—*n*-butanol-2*N* hydrochloric acid, 1:1, upper phase, paper equilibrated 24 h after spotting and before running, in a tank containing aqueous phase of Bu–HCl mixture; 1% HCl—concentrated hydrochloric acid–water, 3:97; HOAc–HCl—water–glacial acetic acid–12 *N* hydrochloride acid, 82:15:3; TBA—tertiary butanol–glacial acetic acid–water, 3:1:1.

**Hydrolysis of flavonoids**

Purified flavonoids were subjected to acid hydrolysis as described by Velioglu and Mazza (1991). One to 2 mg of flavonoid and methanol in 2 ml of 2N HCl were used. The aglycone and sugar portion of the flavonoid were obtained by heating the pigment–solvent mixture in a water bath at 100°C for 1 h. After cooling, the aglycone was extracted with amyl alcohol and characterized by HPLC. The aqueous solution containing the sugar was dried under vacuum and the sugar was dissolved in a few drops of water and chromatographed on Whatman No. 1 paper with reference standards using solvent 4:1:2·2 *n*-butanol–ethanol–water (BEW), and 4:1:5 *n*-butanol–acetic acid–water (BAW). For anthocyanins, prior to paper chromatography, aqueous solution was washed with 10% di-*n*-octylmethylamine in chloroform as described by Francis (1982). For viewing of sugar paper chromatograms, aniline

hydrogen phthalate reagent was used (Markham, 1982). The position of the sugar in the flavonoid molecule was determined from spectral shifts (Mabry *et al.*, 1970; Markham, 1982) and, by comparing HPLC retention times of apple phenolics with those of authentic compounds, analyzed under the same conditions.

### Total anthocyanins

Total anthocyanins were determined by the pH differential method of Fuleki and Francis (1968) and calculated using the extinction coefficient of cyanidin 3-galactoside in methanol containing 0·01% HCl.

## RESULTS AND DISCUSSION

Column chromatography of 'Scugog' apple phenolics on Sephadex LH No. 20 with MAW gave three major fractions. Analytical HPLC of these fractions showed that the first fraction contained flavan 3-ols and phenolic acids, the second fraction consisted of anthocyanins, and one peak with retention time ($R_t$) of 1524 s, and the third fraction yielded an anthocyanin peak, three flavonols and one peak with $R_t$ of 1740 s (Fig. 1). To obtain individual compounds in a sufficient amount, each Sephadex fraction was run at least five times on a micropreparative HPLC column. The identity of each compound was established by HPLC, paper

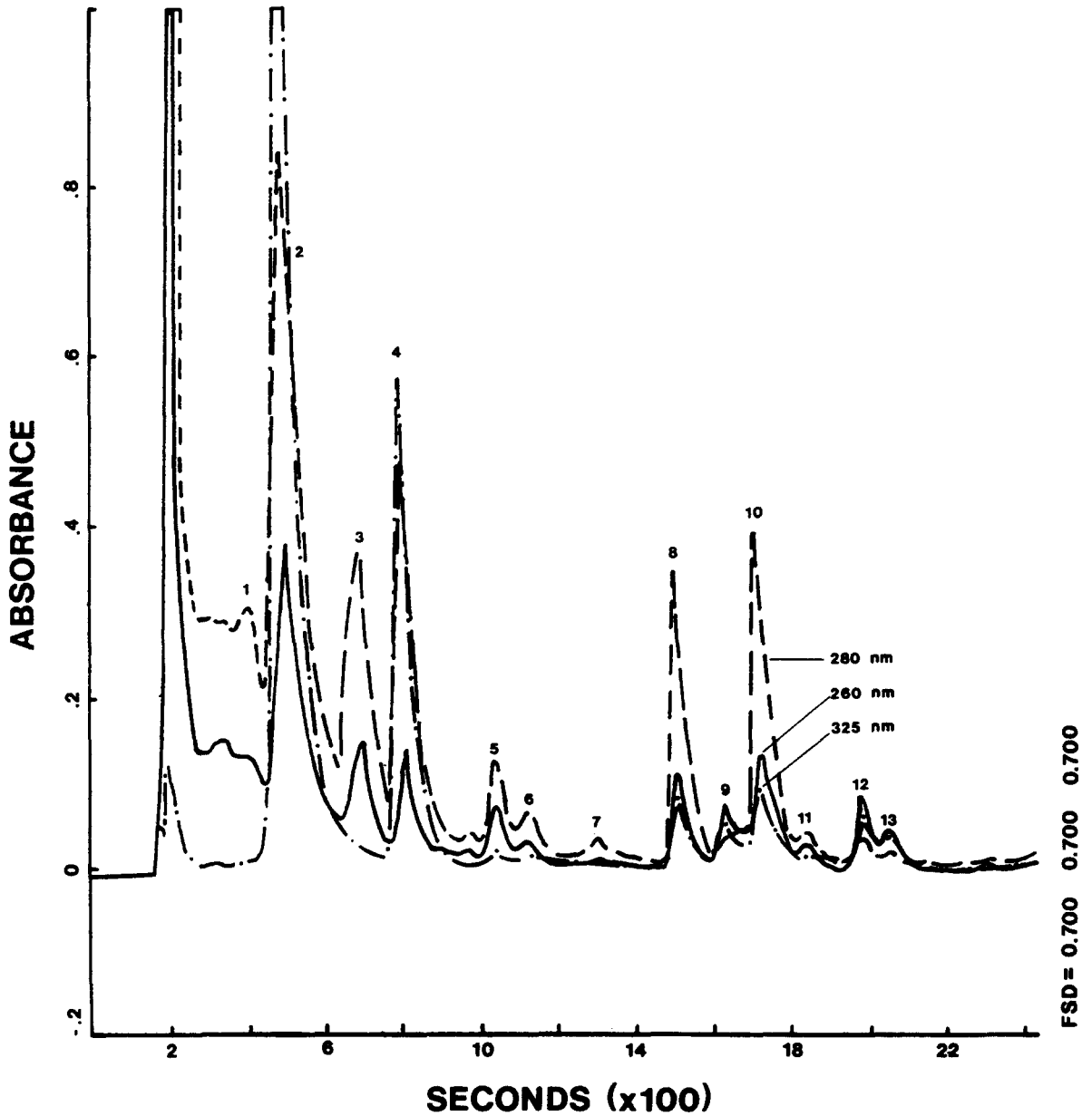


**Fig 1.** Chromatogram of analytical HPLC of Scugog apple phenolics. (1) catechin; (2) chlorogenic acid; (3) a flavan 3-ol; (4) phloretic acid; (5) cyanidin 3-galactoside; (6) cyanidin 3-glucoside; (7) cyanidin 3-arabinoside; (8) unknown; (9) quercetin glucoside; (10) phloretin glucoside; (11) cyanidin 3-xyloside; (12) quercetin arabinoside; (13) quercetin rhamnoside.

**Table 1. Anthocyanin composition of red-flesh apples cv. Scugog**

| Peak[a] | Anthocyanin | $R_f$ (s × 100) | $k'$ | $\alpha$ | Area (%) |
|---|---|---|---|---|---|
| 5 | Cyanidin 3-galactoside | 10·6 | 4·9 | — | 39·1 |
| 6 | Cyanidin 3-glucoside | 11·3 | 5·3 | 1·08 | 27·0 |
| 7 | Cyanidin 3-arabinoside | 13·1 | 6·3 | 1·19 | 23·3 |
| 11 | Cyanidin 3-xyloside | 18·5 | 9·3 | 1·48 | 10·5 |

[a] Refer to peak number in Fig. 1; flow rate = 1 ml/min; $t_0$ = 1·8 s; concentration of total anthocyanins 100 ± 5 mg/kg of fresh fruit.

chromatography, spectral analysis, color reactions and hydrolysis of each peak followed by HPLC, and/or paper chromatography of the aglycone and sugar moieties as described earlier.

Peaks 5, 6, 7 and 9 appeared as magenta-colored spots on chromatograms when seen in visible light, pink spots when seen in ultraviolet light and, after exposure to $NH_3$ vapor, magenta when seen in ultraviolet light. According to Harborne (1958, 1967), these colors and color changes are characteristics of cyanidin derivatives. The $R_f$ values and spectral characteristics for these peaks and for the corresponding aglycones and sugars produced on acid hydrolysis confirmed that peaks 5, 6, 7 and 9 were cyanidin 3-galactoside, 3-glucoside, 3-arabinoside and 3-xyloside, respectively.

Quantitatively, the most abundant anthocyanin of 'Scugog' apples was cyanidin 3-galactoside (Table 1). Sun and Francis (1967), Timberlake and Bridle (1971), and others have reported that the pigment responsible for 80–90% of the red skin color of 'Red Delicious', 'Jonathan', 'Stoker Red', 'Ingrid Marie' and 'Cox's Orange Pippin' apples is cyanidin 3-galactoside. The identity and concentration of minor anthocyanins in these apple cultivars is not definite; however, existing evidence indicates that they are cyanidin 3-glucoside, 3-arabinoside and 3-xyloside, and together they contribute 10–20% of the red skin color of these apples (Timberlake & Bridle, 1971). Thus, the anthocyanin composition of red-flesh 'Scugog' apples is qualitatively the same as that of other apples, but quantitatively the red-flesh apples contain much more cyanidin 3-glucoside, 3-arabinoside and 3-xyloside and less cyanidin 3-galactoside. In spite of the use of a highly sophisticated HPLC system with a diode array detector and computer system, we were unable to detect the presence of acylated anthocyanins as reported by Timberlake and Bridle (1971). At 95–100 mg/kg of anthocyanin content, 'Scugog' apples are, however, a rich source of the relatively rare cyanidin 3-xyloside.

The spectral characteristics, $R_f$ values and HPLC retention times, of peaks 1, 2 and 4 corresponded to (+)-catechin, chlorogenic acid and phloretic acid, respectively. Peak 3 had spectral and chromatographic properties of a flavan 3-ol. However, lack of standards and difficulties with impurities did not allow us to determine whether it was epicatachin, gallocatechin or epigallocatechin.

Peaks 9, 12 and 13 were colorless in visible light, dark purple in ultraviolet light and yellow–green after treatment with $NH_3$. Their MeOH spectra consisted of two absorption maxima in the range 255–266 and 313–357. Acid hydrolysis of purified fractions, followed by HPLC, paper chromatography and spectral analysis of the aglycones gave quercetin, and the sugars were glucose, arabinose and rhamnose, respectively.

Peak 10 was identified as phloretin glucoside (phloridzin) by chromatographic and spectral characteristics as well as by color reactions in diazotized sulfanilic acid and diazotized *p*-nitroaniline (Durkee & Poapst, 1965). The presence of this compound in apples was first reported by Durkee and Poapst (1965) and is known to contribute to the browning reaction of apple ciders (Whitney & Coggins, 1975; Oleszek *et al.*, 1988).

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