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Purification and identification of Capulin (*Prunus serotina* Ehrh) anthocyanins

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Abstract

Capulin (*Prunus serotina* Ehrh) anthocyanins were extracted from liquid nitrogen powdered epidermal tissue using acetone, the aqueous acetone extract partitioned with chloroform and purified in a C-18 solid-phase cartridge. pH-differential and bisulfite bleaching methods were used to determine monomeric anthocyanin content and polymeric color. Pigments were identified by spectral analyses, HPLC and mass spectroscopy. Information from HPLC profiles, saponification and acid hydrolysis of the capulin anthocyanins showed that the two major pigments were cyanidin-3-glucoside (34%) and cyanidin-3-rutinoside (63%), with no acylating groups. A third yellow/orange pigment (λ_{max} 480 nm, MW = 632) possibly containing rutinose was detected by MS.

1. Introduction

Capulin (*Prunus serotina* Ehrh) is the name given to a tree and its fruits growing wildly or under cultivated conditions in the Mexican highlands and other regions of the American continent. In USA, the fruit is known as 'black' or 'wild' cherry, 'cerezo' in Guatemala, 'cerezo criollo' in Venezuela and 'cerezo de los Andes' in Colombia (Chavez, 1990). Capulin corresponds to a polymorphic species composed of a large number of varieties and subspecies (Venero, 1966). Taxonomy: Division Atophyta, Class Dicotiledonae, Order Rosales, Family Rosaceae, Subfamily Prunoidea and Genus *Prunus* (Heywood, 1982). The proposed complete scientific name is *Prunus serotina* Ehrh subsp. *capuli* (Cav.) McVaugh (INI, 1994). *Prunus capuli* is a similar species found in Ecuador (McVaugh, 1951).

The *Prunus serotina* tree and its fruit have been used for food and medicinal purposes since pre-hispanic times (INI, 1994). The fruit is consumed fresh, dried or as marmalade, and the roasted seeds are eaten as a snack (Martínez, 1959). A problem with the fruit is that it cannot be harvested all at once since its maturity varies throughout the two-month harvesting season. The fruit, similar to a sweet cherry, is small (1 cm diam.) with a dark-red skin and green translucent flesh. The abundance of this fruit may be a potential source of anthocyanin pigments for use by the food industry. Cyanidin (cyd) is the most ubiquitous anthocyanidin in the *Prunus* genus, peonidin is rare and delphinidin completely absent. Glycosidic substitution is relatively simple and the sugars present are glucose (glc), rutinose (rut) and sophorose. There is no clear evidence of acylation in the edible fruit (Wrolstad, 1993; Timberlake, 1981).

Anthocyanins are flavonoid phenolic compounds that occur in some plant tissues as glycosides (mono-, di- or tri-glycosides) with or without organic acids (chlorogenic, ferulic, caffeic, etc.) esterified to one or more of the sugar moieties. Acylated anthocyanins may participate in co-pigmentation interactions in which the anthocyanin and acid rings form sandwiched structures. The resulting complexes resist hydration and the deleterious effect of high pH. A hyperchromic shift also results that intensifies the expressed color (Combe, 1996). The interest in identifying sources of anthocyanin pigments by the food, pharmaceutical and cosmetics industries is based on consumer demand for natural instead of synthetic colorants, the possible ban by the FDA of FD&C Red #3 and #40, and because of the

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nutraceutical properties (antioxidant) reported for flavonoids (LaBell, 1993; I-San Lin, 1994).

The anthocyanin pigments in *Prunus serotina* have not been identified. The objective of this study was to identify the anthocyanin pigments in the skin of capulin fruit.

2. Materials and methods

2.1. Plant material

Capulin fruits hand-harvested in July 1996 in Huejotzingo, Puebla, Mexico (19°10′02″ N and 98°23′27″ W) were washed, placed in polyethylene bags and stored at −40°C until used.

2.2. Pigment extraction

The extraction was done following the procedure described by Giusti & Wrolstad (1996). Hand-peeled skin from partially thawed fruit was frozen in liquid nitrogen and powdered using a stainless-steel Waring Blendor. Powdered samples (50 g) were blended with 70 mL acetone and stored overnight at 5°C. The samples were filtered and the filter cake residue re-extracted with 70% acetone until a clear solution was obtained. Filtrates were combined, shaken in a separatory funnel with 2 volumes of chloroform and stored overnight at 5°C. The aqueous portion was collected and dried in a Büchi rotavapor at 40°C for 5–10 min, until all residual acetone was evaporated. The aqueous extract was placed in vials, flushed with nitrogen and frozen until used.

2.3. Monomeric anthocyanin content and polymeric color

Monomeric anthocyanin content and polymeric color were determined using the pH differential and bisulfite bleaching methods, respectively (Wrolstad, 1993). A Shimadzu 300 UV spectrophotometer and 1 cm pathlength disposable cells were used for spectral measurements at 420, 510 and 700 nm, respectively. Pigment content was calculated as cyanidin-3-glucoside (cyd-3-glc), using an extinction coefficient of 29,600 L cm^{-1} mg^{-1} and molecular weight of 448.8.

2.4. Determination of pH and refractive index

pH measurements were made using a Corning 340 pH-meter (Corning, NY, USA) calibrated with pH 4 and 7 buffers. Refractive index was measured with an Auto-Abbe 10500 refractometer (Reichert-Jung, Leica, NY, USA).

2.5. Anthocyanin purification

The aqueous extract was passed through a 20 mL capacity/5 g sorbent weight C-18 mini column (high-load C-18 tube, Alltech Assoc., IL) previously activated with methanol followed by 0.01% aqueous HCl (aq-HCl). Anthocyanins and other phenolics were adsorbed on the mini-column; sugar, acids and other water-soluble compounds were eluted with 2 volumes of aq-HCl. Anthocyanins were subsequently eluted with methanol containing 0.01% HCl. The methanolic extract was concentrated using a Büchi rotavapor at 35°C and pigments were dissolved in deionized water containing 0.01% HCl.

2.6. Alkaline and acid hydrolysis of anthocyanins

Purified pigments (2 mL) of capulin and grape (Concord grape Welch's concentrate, Welch's, NY, USA) were saponified in screw-cap test-tubes with 10% aqueous KOH (10 mL) for 8 min at room temperature in the dark (Hong & Wrolstad, 1990). The solution was neutralized using 2 N HCl, and the hydrolysate purified using a C-18 Sep-Pak cartridge (Waters Assoc., Milford, MA).

Purified saponified pigment (1 mL) was mixed with 15 mL of 2 N HCl in a screw-cap test tube, flushed with nitrogen and capped. The pigment was hydrolyzed for 45 min at 100°C and cooled in an ice bath. The hydrolysate was purified using a C-18 Sep-Pak cartridge (Hong & Wrolstad, 1986, 1990). Alkaline and acid hydrolyses were done in duplicate.

2.7. High performance liquid chromatography (HPLC)

Apparatus: The analytical system used was Perkin-Elmer Series 400 liquid chromatograph equipped with a Hewlett-Packard 1040A photodiode array detector and a Hewlett-Packard 9000 computer system, was used with simultaneous detection at 280, 310, and 520 nm, respectively. The spectra (detection wavelengths from 250 to 600 nm) were recorded for all peaks.

Column and mobile phase: The analytical system used was a PolyLC ODS C-18 column (5 micron), 250 × 4.6 i.d. (PolyLC, MD), fitted with a ODS-10, 4 cm × 4.6 mm i.d. Micro Guard column (Bio-Rad Lab.) was used. Solvent A: 100% HPLC grade acetonitrile, B: 1% phosphoric acid, 10% acetic acid, 5% acetonitrile (v : v : v) in water. Flow rate: 1 mL min^{-1}.

Analyses conditions: For anthocyanins a linear gradient from 0% to 12% A in 13 min and to 20% A in 15 min was used, and for saponified anthocyanins and anthocyanidins a linear gradient from 0% to 30% A in 30 min was used.

2.8. Mass spectroscopy of capulin anthocyanins

Low-resolution MS was done using electrospray MS and MS-MS (Giusti & Wrolstad, 1996). The instrument was a Perkin-Elmer SCIEX API III + Mass Spectrometer, equipped with an ion spray source (ISV = 4700, orifice voltage of 80) and loop injection. Purified capulin anthocyanin extracts (20 μL) were injected directly into the system.

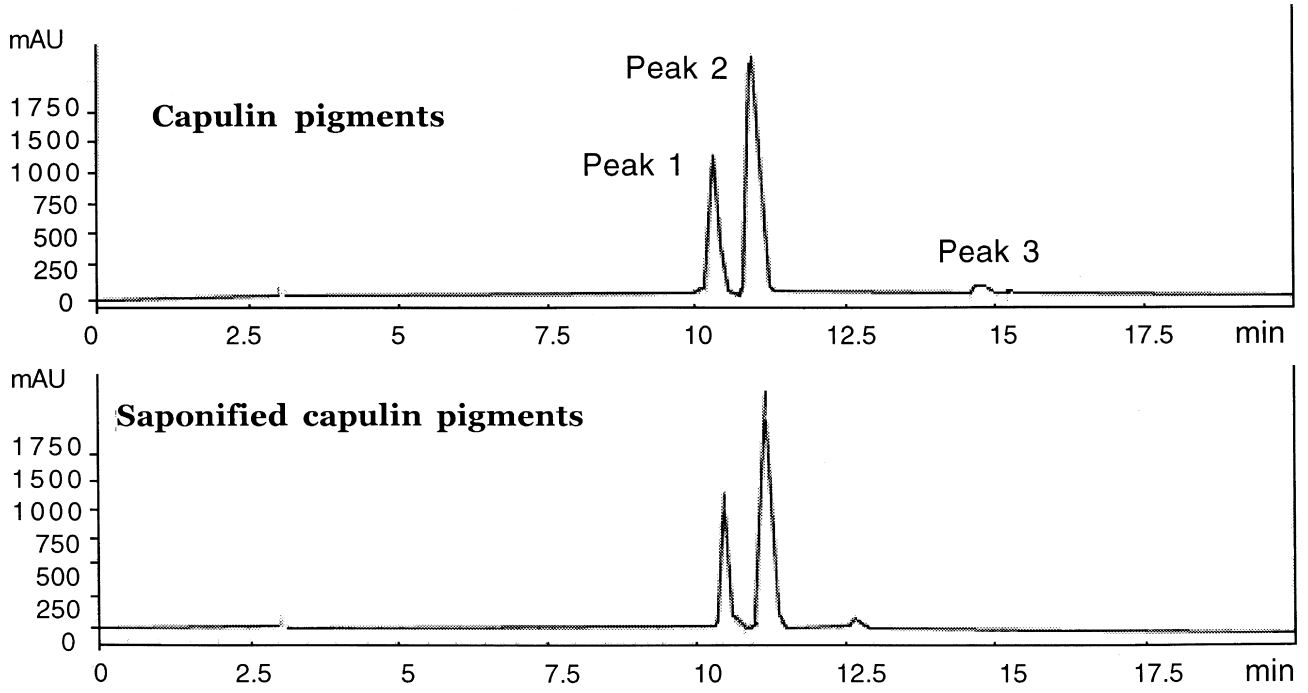

Fig. 1. HPLC separation of purified (top) and saponified (bottom) capulin anthocyanins. PolyLC ODS C-18 250 × 4.6 mm i.d. column. Solvent A: 100% acetonitrile; B: 1% phosphoric acid, 10% acetic acid, 5% acetonitrile and 84% water. Top: linear gradient from 0% to 12% A in 13 min and to 20% A in 15 min; Bottom: linear gradient from 0% to 30% A in 30 min. Flow rate: 1 mL min^{-1}.

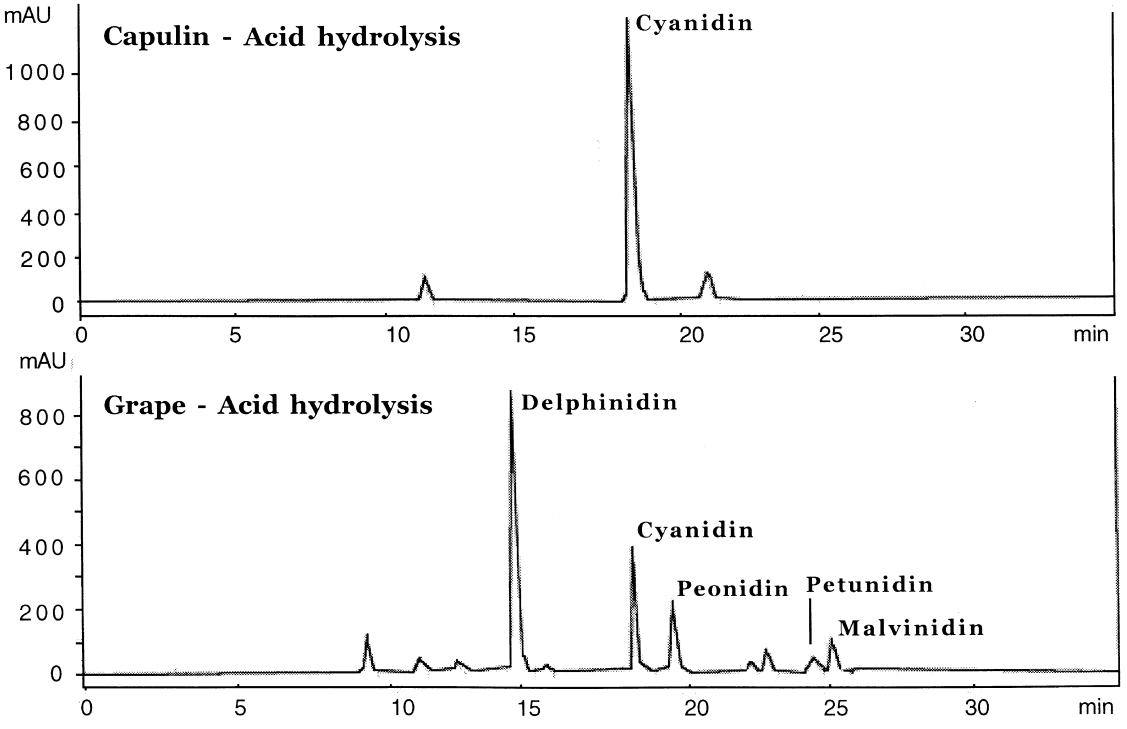

Fig. 2. HPLC separation of acid-hydrolyzed capulin (top) and grape (bottom) anthocyanins. Same HPLC conditions as for Fig. 1.

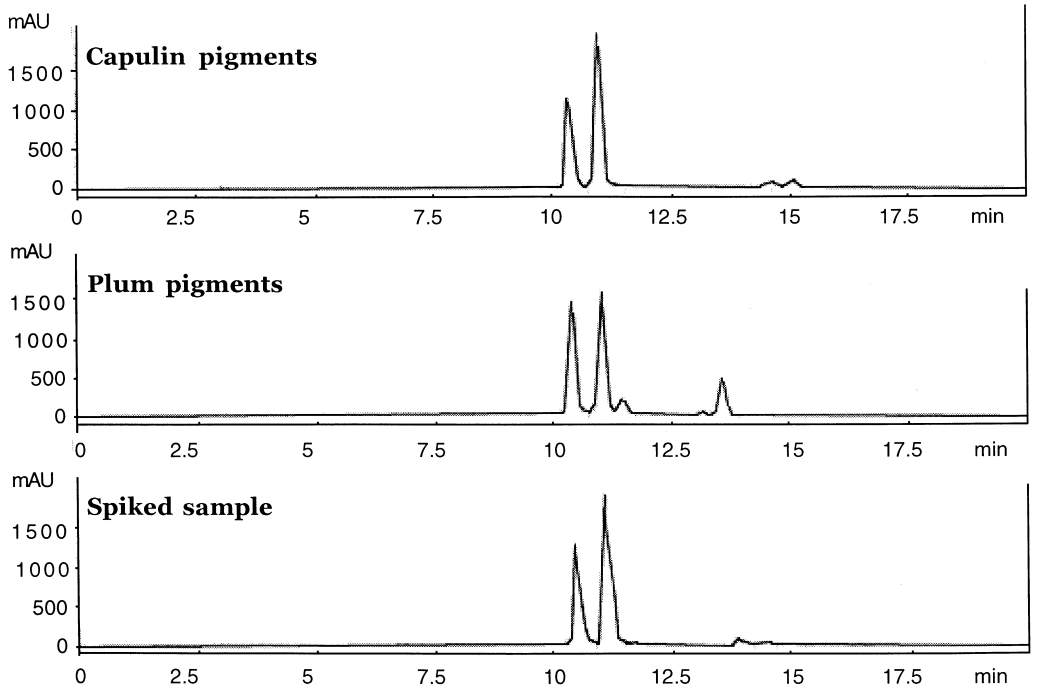

Fig. 3. HPLC separation of purified anthocyanin pigments from capulin (top), plum (middle) and of a capulin sample spiked with plum (bottom). Same HPLC conditions as for Fig. 1.

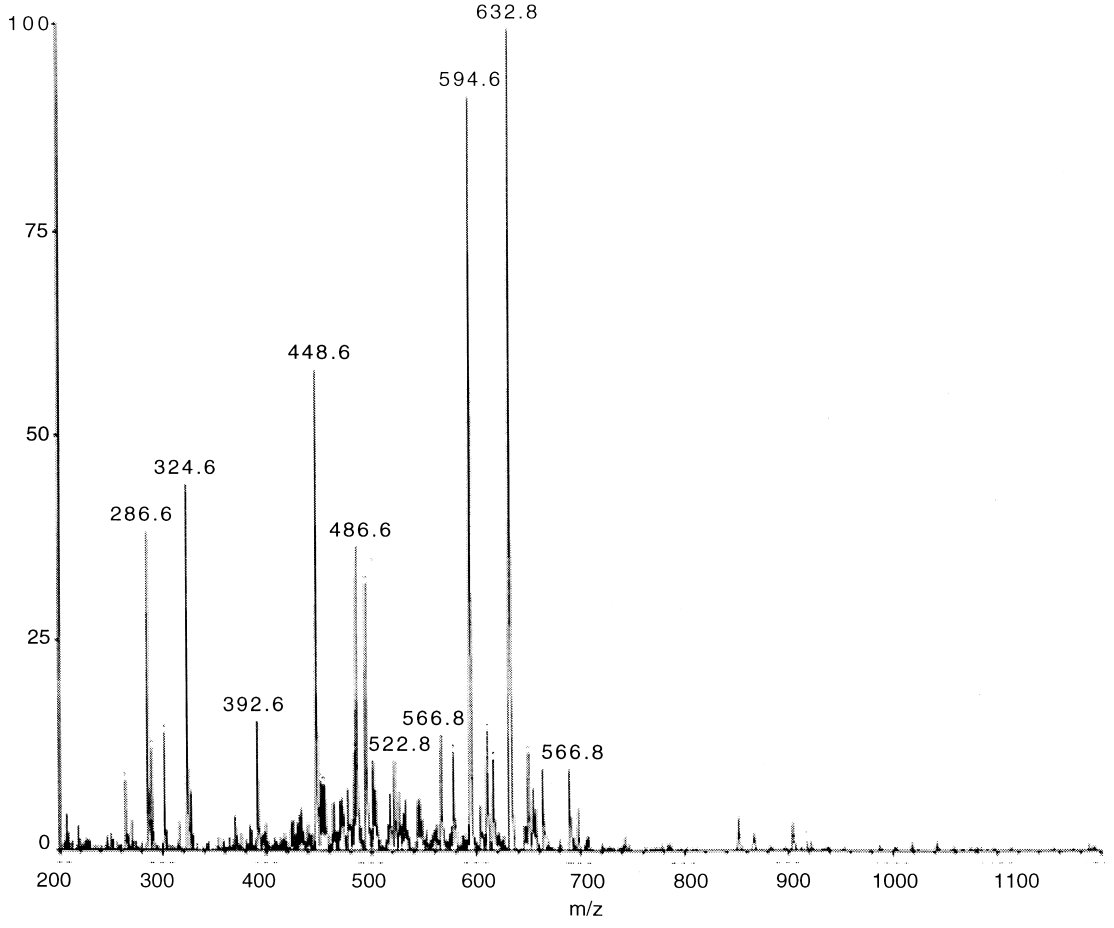

Fig. 4. MS of capulin anthocyanins. The molecular weights obtained correspond to cyd-3-rut (595) and cyd-3-glc (449) and a third unidentified pigment (633) possibly containing rutinose.

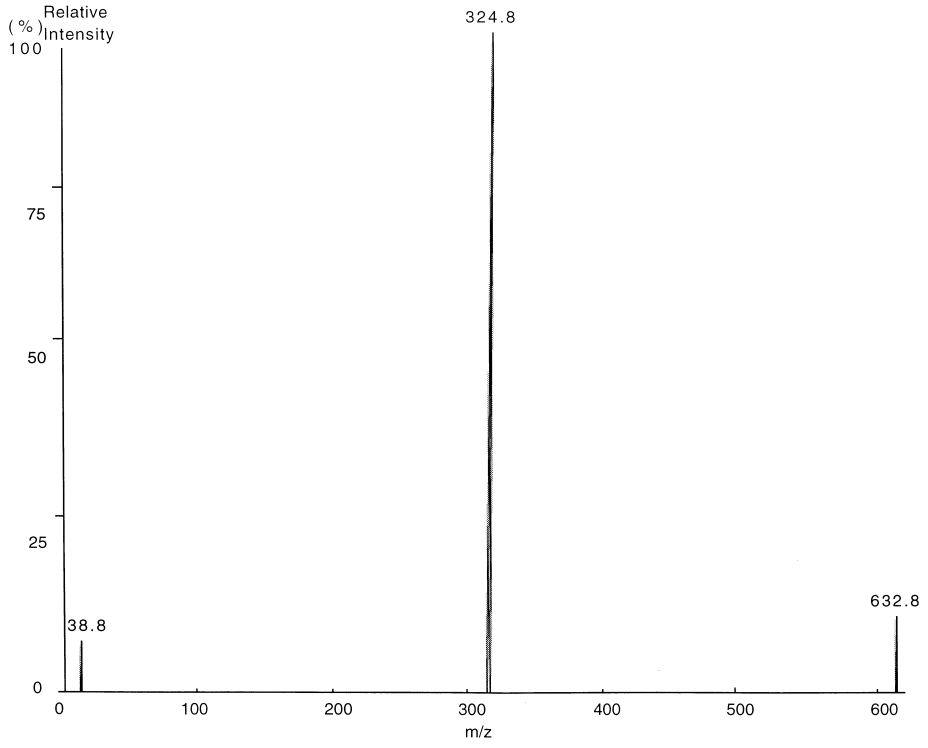

Fig. 5. MS–MS of the unknown pigment (peak 3, Fig. 1). The molecular weights obtained correspond to a 325 MW fraction associated to a moiety with a similar MW as rutinose.

3. Results and discussion

3.1. Monomeric anthocyanin content and polymeric color

The anthocyanins are present only in the skin of the fruit and the pigment content in the ripe fruit, determined on an cyd-3-glc basis, was 272 mg/100 g skin (31.7 mg/100 g fresh fruit). The anthocyanin concentration in the skin was higher than that found in red radish (39.3–185 mg/100 g skin) (Giusti, Rodríguez-Saona, Baggett, Reed, Durst & Wrolstad, 1998), and blueberries (110–190 mg/100 g fresh fruit) (Gao & Mazza, 1994). The purified aqueous portion obtained had a mean pH of 4.2, a soluble solid content of 17.2°Brix and 22.6% polymeric color.

Table 1
λ_{max}acyl (340 nm) and λ_{max}Acy (520 nm) and λ_{max}acyl/ λ_{max} Acy ratio for capulin anthocyanin peaks 1 and 2 (see Fig. 1)

	Peak 1	Peak 2
λ_{max}acyl	185 mAU	160 mAU
λ_{max}Acy	1850 mAU	1800 mAU
λ_{max}acyl/λ_{max}acy	0.10	0.09

3.2. HPLC separation of capulin anthocyanins

Anthocyanins from capulin were separated by HPLC (Fig. 1). Two anthocyanins (peaks 1 and 2) represented ca. 98%, while one minor peak (peak 3) represented ca. 2% of the total area at 520 nm. According to Harborne (1958) the spectra of anthocyanin peaks can provide information about the presence of acylating groups and reported that the ratio of absorbance at the acyl maximum (340 nm) to the absorbance at the anthocyanin (Acy) maximum wavelength (520 nm), λ_{max}acyl/ λ_{max}Acy, is a measure of the molar relation of the cinnamic acid to the anthocyanidin. In acidified methanolic solution, a ratio of 48% to 71% is indicative of a 1/1 molar ratio, while a ratio of 83% to 107% is characteristic of a 2/1 molar ratio of cinnamic acid to anthocyanin. The λ_{max}acyl/λ_{max}Acy ratios found for the capulin anthocyanins were 9% and 10% for peaks 1 and 2 respectively (Table 1). These ratios suggest that the two major anthocyanins found in capulin epidermal tissue were not acylated. Peak 3 showed an unusual spectrum, with a λ_{max} of 480 nm.

Saponification of capulin anthocyanins confirmed that the two major pigments found did not contain acylating groups (Fig. 1) and the retention times of the saponified anthocyanins coincided with those obtained with the purified capulin pigments.

Acid hydrolysis of the saponified capulin anthocyanins resulted in one major anthocyanidin (Fig. 2). The retention time and spectra of the hydrolyzed pigment matched those of cyanidin from hydrolyzed grape anthocyanins (Fig. 2). One minor peak was also observed, however, the retention time did not match those of any of the common anthocyanidins present in grape (Fig. 2) or red radish (pelargonidin) (Giusti & Wrolstad, 1996).

The anthocyanin pigment profiles obtained from capulin were compared to those of pigments isolated from other *Prunus* species, plum concentrate. The pattern of both was very similar (Fig. 3). Wrolstad (1993) reported that the major anthocyanin present in plum were cyd-3-rut (41%) and cyd-3-glc (31%). The capulin sample was spiked with plum concentrate and the results showed an overlap of the two major pigments suggesting that the anthocyanin present in capulin are cyd-3-glc (34%) and cyd-3-rut (64%). Other fruits reported to contain cyd-3-glc and cyd-3-rut are rhubarb, red and black raspberries, boysenberry, loganberry, blackberry and red and black currant (Wrolstad & Heatherbell, 1968; Nybom, 1968; Barrit & Torre, 1973, 1975).

3.3. Mass spectroscopy

Results from MS showed molecular weights of 595 and 449 corresponding to cyd-3-rut and cyd-3-glc, respectively (Fig. 4).

The MS also detected the presence of a third pigment with a MW of 633 that did not match the mass of any anthocyanin reported in *Prunus* species. This pigment may correspond to peak 3, with a λ_{max} of 480 nm and possibly containing a rutinoside fraction (Fig. 5).

4. Conclusions

Anthocyanin content of capulin, determined on a cyd-3-glc basis was 2.72 mg g^{-1} skin, while polymeric color was 22.6%. The major pigments in capulin, identified by spectral analyses, HPLC and MS, were cyd-3-glc and cyd-3-rut. An additional yellow/orange pigment (λ_{max}480 nm) was detected by MS (MW 632) with rutinose possibly attached to it.

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