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Anthocyanin and other phenolic compounds in Ceylon gooseberry (*Dovyalis hebecarpa*) fruits

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ABSTRACT

Ceylon gooseberry is a deep-purple exotic berry that is being produced in Brazil with great market potential. This work aimed to determine major phenolic compounds in this specie by HPLC–PDA–ESI/MS. Samples were collected in two different seasons. Pulp and skin were analyzed separately. Non-acylated rutinoside derivatives of delphinidin (~60–63%) and cyanidin (~17–21%) were major anthocyanins tentatively identified. All anthocyanins had higher concentration in skin than in pulp (64–82 and 646–534 mg of cyaniding-3-glucoside equivalents/100 g skin and pulp, respectively). Moreover, anthocyanin profile changed between sampling dates ($p < 0.05$). Mainly for delphinidin-3-rutinoside which could be a result of season variation. In this specie, non-anthocyanin polyphenols represent less than 35% of total extracted polyphenols. The tentative identification proposed a flavonol and three ellagitannins as major compounds of the non-anthocyanin phenolics fraction. Finally, anthocyanin is the major phenolic class in this fruit and its composition and content are significantly affected by season.

1. Introduction

Phenolic compounds are non-essential secondary metabolites formed in normal metabolism of plant tissues playing an important role in fruit development and survival. Most of them can act as strong antioxidants (Dai & Mumper, 2010) and, as recently discussed, it is capable to interact with enzymes and cell mediators in the prevention of chronic diseases development and in the maintenance of a healthy status (Del Rio et al., 2013; He & Giusti, 2010). Accordingly, there is an increased interest in chemical elucidation and quantification of these compounds in commonly consumed fruits and vegetables (Aaby, Mazur, Nes, & Skrede, 2012) or in exotic plant materials with an unknown polyphenolic profile (Agawa et al., 2011; Longo & Giuseppe, 2005; Mertz et al., 2009).

Berry fruits are recognized as rich sources of these beneficial compounds. Among them, anthocyanins are the major phenolic class being responsible for the red-to-purple color and high acceptance of these fruits. Moreover, significant amounts of tannins and phenolic acids are also reported as frequent phenolic compounds in berries (Seeram, 2008).

The knowledge of fruit phenolic composition, concentration, and its content oscillation due to environmental condition is crucial to determine good phytochemical sources, for quality cropping purposes, for supporting future studies on biological properties, and in the development of industrial applications.

Ceylon gooseberry is a deep red-to-purple berry originally from Sri Lanka (Asia) (Morton, 1987) that is being produced with satisfactory yields in the southwest regions of Brazil. Flesh and Skin are rich sources of phenolic compounds and anthocyanin with high antioxidant activity (Bochi, 2013). However, up to date there are no reports about phenolic profile composition of these compounds. Moreover, these compounds are enrolled in plant resistance to environmental conditions (Harborne, 2000) and it was hypothesized that fruit could have different composition among tissues and between two harvesting periods.

Thus, the purpose of this study was to characterize anthocyanin and other major phenolic compounds in Ceylon gooseberry flesh and skin. Furthermore, samples were monitored over two years in fruits that were harvested in autumn and in winter aiming to evaluate possible oscillations due to climate changes on anthocyanin content.

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2. Materials and methods

2.1. Reagents and materials

Optima LCMS grade acetonitrile, methanol, water, formic acid (88%), ACS grade acetone, sodium hydroxide, hydrochloric acid, as well as 0.22 μm GE Magna nylon membrane filter were obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.). Catechin (98%) was purchased from Sigma Aldrich. Sep-Pak® C-18 cartridges (6 cc, 500 mg) were obtained from Waters corporation (Milford, MA, U.S.A.). Standards of caffeic acid (99.7%), chlorogenic acid (99.25%), ellagic acid (99.9%), gallic acid (98%), p-coumaric acid (98%), protocatechuic acid (99.9%), rutin (95%), syringic acid (99.5%), and vanillic acid (97.9%) were obtained from MP Biochemicals (Santa Ana, CA, U.S.A.), (+)-catechin hydrate (98%), epigallocatechin gallate (97%), quercetin dihydrated (98%), and cyaniding-3-glucoside (Kuromanin, 97%) from Sigma–Aldrich (St. Louis, MO, U.S.A.), and 4-hydroxybenzoic (99%) from Acros Organics (Geel, Belgium).

2.2. Equipment

Samples were analyzed using an HPLC (high-performance liquid chromatography, Shimadzu; Columbia, MD, U.S.A.) equipped with LC-20AD pumps, SIL-20AC auto sampler, and a CTA-20A Column Oven coupled to a LCMS-2010, SPD-M20A Photodiode Array (Shimadzu), and Mass Spectrometer (Shimadzu) detectors. LCMS Solution Software (Version 3, Shimadzu) were used for data analyses. Mass spectrometry was conducted on a quadrupole ion-tunnel mass analyzer (QoQ system, Q-array – Octapole – Quadrupole mass analyser, Shimadzu) equipped with electrospray ionization (ESI) interface (Shimadzu). A Terroni Freeze-dryer, model LS-3000E (São Paulo, Brazil) and analytical grinder with refrigeration system (model Q298A, Qhimis, São Paulo, Brazil) were used for sample preparation.

2.3. Sample preparation

Ripened (12.6 ± 1.8 °Brix, 3.5 ± 0.8 g of citric acid/100 g sample, transversal and longitudinal diameters of 20.81 × 22.07 mm) berries were obtained from a producer region located between Pinhalzinho and Bragança Paulista cities (at 22°48′06.8″S 46°33′28.1″W, São Paulo, Brazil) in 2009 and 2010. Fruits were washed with water and allowed to dry before frozen at −20 °C. Manual skin removal by hand was made in frozen fruits to minimize enzymatic degradation and juice loss. Frozen flesh fruit parts were crushed using a food processor (Philips' Walita Master food processor, model RI3142) and placed into trays to return to freezing conditions. Frozen skins and flesh were immersed in nitrogen and immediately freeze-dried until the pressure was reduced to stable values lower than 22 μHg. Freeze-dried samples were ground to obtain a visually homogenous fine powder.

2.4. Extraction

It was performed using a previously optimized method developed for Ceylon gooseberry samples (Bochi et al., 2014). Flesh and skin freeze-dried powdered samples were added to the extraction solvent media in a proportion of 1:120 (w/v). The extraction solvent was composed of 0.35% v/v of formic acid solution in 20% v/v of acetone in distillated water. After 20 min under mixing using a magnetic stirrer at 1500 rpm, the homogenate was filtered, the residue discarded, and the slurry was concentrated in a rotary evaporator (35 °C ± 2 °C) for acetone removal. The final extract was made up to a known volume with 0.35% (v/v) formic acid solution in distillated water.

2.5. SPE-C18 purification procedure

Previous to identification analysis, crude extracts were semi-purified to obtain one fraction mainly with anthocyanins and another with other phenolic compounds. Purification was performed as previously described (Rodriguez-saona & Wrolstad, 2001) with some modifications. Water-based crude extracts with 0.35% (v/v) of formic acid (4 ml) were loaded into solid phase extraction (SPE) C-18 cartridges (Waters Corporation, Milford, MA, USA), previously activated with methanol and conditioned with acidified water (0.35% v/v formic acid). Polar compounds were washed out with two volumes of formic acid aqueous solution (0.35% v/v). Less polar phenolic compounds were eluted using two volumes of ethyl acetate and lastly anthocyanins were recovered with acidified methanol (0.35% formic acid). The ethyl acetate fraction was dried under nitrogen and made up to a known volume (2 ml) with 20% methanol in water. After methanol removal in rotary evaporator (38 ± 2 °C), the anthocyanin fraction was made up to a known volume (2 ml) with acidic water (0.35% formic acid v/v). All fractions were directly analyzed as purified fractions without hydrolysis. Additionally, as described in items 2.6 and 2.9, a portion of each fraction was used for acid and alkaline hydrolysis for additional structural information.

2.6. Acid hydrolysis of anthocyanins

Purified anthocyanin fractions obtained in 2.5 were hydrolyzed with HCl 3 N (1:5 v/v) for 45 min at 100 °C in a screw-cap test tube, and then cooled in an ice bath (Rodriguez-saona, Giusti, & Wrolstad, 1998). The hydrolysate was loaded into a C-18 SPE cartridge previously conditioned with water. Polar compounds were washed with 4 volumes of HPLC–MS water and anthocyanins were then eluted with pure acidified methanol (0.35% formic acid). The methanol was removed in a rotary evaporator (38 ± 2 °C) and the remaining fraction made up to a known volume with acidic water (0.35% formic acid v/v).

2.7. Anthocyanin identification by HPLC–PDA–ESI/MS analysis

Whole extract, purified fractions, and acid hydrolysates were analyzed using the HPLC equipment previously described in Section 2.2. A reverse phase Symmetry C-18 column (4.6 × 150 mm; 3.5-μm particle size; Waters Corp. Mass. U.S.A.) was connected to a guard column (4.6 × 22 mm. Symmetry 2 micro; Waters Corp. Mass. U.S.A.) for the analysis. Solvents and samples were filtered through a 0.22 μm GE Magna nylon membrane filter (Fisher Scientific).

Mobile phases consisting of 3% (v/v) formic acid in water (solvent A) and 100% acetonitrile (solvent B) were used for anthocyanin analysis. Separation was achieved using a linear gradient from 5% to 20% B, in 30 min, 20% of B was kept until 32 min. At the end of the gradient, the column was washed increasing B to 100%, keeping it for 5 min, and equilibrated to initial conditions for 5 min.

The flow rate was 0.8 mL/min and the injection volume was 50 μL. Spectral data were collected from 250 to 700 nm. Flow rate of 0.2 mL/min was diverted to the mass spectrometer.

Mass spectrometric analysis was performed under positive ion mode. It was used the following settings: nebulizing gas flow, 1.5 L/min; interface bias, ±4.50 kV; block temperature, 200 °C; focus lens, −2.5 V; entrance lens, −50 V; pre-rod bias, −3.6 V; main-rod bias, −3.5 V; detector voltage, 1.5 kV; scan speed, 2000 amu/s. Full scan for total ion chromatography (TIC) was performed with a mass range from 100 to 1000 m/z and selective ion monitoring (SIM) was used to search for the molecular ions of the common anthocyanidins throughout the analysis.