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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.

All compounds were identified based on PDA spectra characteristics, elution order, hydrolysis behavior, and mass spectra data.

2.8. Anthocyanin quantification by HPLC–PDA analysis

Quantification of anthocyanins was performed by HPLC–PDA in the whole extract without SPE purification. Solvents, gradients, injection volume, and flown rate were the same as described in Section 2.7. Chromatograms were obtained at 520 nm for quantification purposes. Calibration curves in three concentration ranges (from 0.5 to 4.5 ppm; from 10 to 90 ppm; and from 100 to 250 ppm) were performed in different days ($n = 3$) to quantify each anthocyanin as mg of cyanidin 3-*O*-rutinoside equivalents per 100 g of fresh sample weight. Linearity, detection, and quantification limits were evaluated as described by ICH (2005).

2.9. Acid and alkaline hydrolysis of non-anthocyanin phenolic compounds

Acid hydrolysis of purified fraction of phenolic compounds (Section 2.2) was performed as reported by Ayaz, Hayirlioglu-Ayaz, Gruz, Novak, and Strnad (2005). After sonication for 10 min, 1 mL of sample was mixed with 5 mL of 6 M HCl and sealed under a N_2 atmosphere. After 1 h in boiling water bath, the reaction mixture was immediately cooled (ice bath/15 min).

For alkaline hydrolysis, 1 mL of 4 M NaOH was mixed with 1 mL of phenolic compounds purified fraction (Section 2.2). The mixture was sealed under a N_2 atmosphere and saponified for 4 h in the darkness. The reaction was stopped with 6 M HCl.

Fractions after acid and alkaline hydrolyses were load in C-18 SPE cartridge previously conditioned with water. Retained compounds were washed with 4 volumes of HPLC–MS water and eluted with pure methanol. Fractions in methanol were concentrated under nitrogen, recovered with water until remained 20% of methanol in the final solution, and filtered through a 0.22 μm GE Magna nylon membrane filter prior to injection.

2.10. Preparation of phenolic standards

Standard solution, containing 12 target compounds (gallic acid; protocatechuic acid; 4-hydroxybenzoic acid; catechin; chlorogenic acid; vanillic acid; caffeic acid; syringic acid; epigallocatechin gallate; *p*-coumaric acid; quercetin-3-*O*-rutinoside; quercetin), was prepared mixing 100 μL of each stock solution at 1000 ppm in methanol. The resulting pool of standards (1300 μL) was mixed with water (5200 μL) to reach in a concentration of 20% of methanol in water in the final solution.

2.11. Non-anthocyanin phenolics tentative identification by HPLC–PDA–ESI/MS analysis

Purified fractions, alkaline, and acid hydrolysates were analyzed using the HPLC equipment previously described in Section 2.2. The same column, guard column, and filters as described in Section 2.7 were used for this analysis.

A solution of 10% of methanol in acidified water (0.1% of formic acid) was used as mobile phase A and pure acetonitrile as mobile phase B. Separation was obtained using a isocratic condition (2% mobile phase B) during the initial 15 min, from 15 to 65 min, an linear gradient from 2% to 40% of mobile phase B, 40% was kept for more 3 min. At the end of the gradient, the column was washed increasing to 100% B, 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 100 μL. Spectral data were collected from 250 to 700 nm and chromatograms obtained in 280 nm for hydroxybenzoates and flava-3-ols (proanthocyanidins), 320 nm for hydroxycinnamates, and 360 nm for flavonol derivatives.

A 0.2 mL flow rate was diverted to the mass spectrometer. Mass spectrometric analysis was performed under negative ion mode, and other settings were the same reported in Section 2.7. Total ion chromatography (TIC) was performed with a mass range from 100 to 2000 m/z.

A tentative identification and/or classification were proposed using information from UV–visible spectra, comparison with standards, elution order, acid/alkaline hydrolysis behavior, and mass spectra data.

2.12. Statistical analysis

Statistica software (Statsoft® version 7.0) was used to perform statistical analysis. Analysis of variance (ANOVA) was used to evaluate the main effects of a three factor experimental design (year, sampling date, and ripening stage) for each anthocyanin compound. Tukey test with 95% of confidence interval was used for mean comparisons of three replications ($n = 3$). Linear regression was performed on calibration curves of cyaniding-3-glucoside to evaluate linearity.

3. Results and discussion

3.1. Tentative identification of major anthocyanin compounds

Chromatography and spectral characteristics (Table 1) were used for anthocyanin identification. Additionally, acid hydrolysis products from Ceylon gooseberry extracts, spectral characteristics and retention time comparisons with in-house berry extracts anthocyanins were used as reference materials.

Fig. 1 shows the chromatogram obtained for *Dovyalis hebecarpa* extracts in which there are 7 tentatively identified anthocyanins from 10 detected peaks. The two major anthocyanins represented more than 80% of the total area (Table 1) and were tentatively identified as delphinidin-3-rutinoside (peak 2) and cyanidin-3-rutinoside (peak 4). Identification of major peaks was based on their molecular mass, the molecular mass of the daughter ions, elution order, and by comparison with anthocyanin chromatographic and spectral behavior obtained from in-house prepared berry extracts as detailed described below.

The MS fragmentation data analysis clearly showed that the two early peaks in PDA chromatogram (peaks 1 and 2) produced fragments that could correspond to the aglycon structure of delphinidin. Similarly, fragments that could correspond to cyaniding aglycon structure were detected for peaks 3 and 4, and petunidin, peonidin, and malvidin aglycones (peaks 5, 6, 8) for the three last peaks in the chromatogram a 520 nm. Moreover, the elution order in C-18 column is in agreement with the structural and chemical characteristics of these compounds. Thus, as expected, hydroxylation at B-ring structure of anthocyanins should decrease retention time in reverse phase chromatography as metoxylation should increase. It is in agreement with acid hydrolysis results obtained for Ceylon gooseberry extracts in which it was possible to perceive longer retention as metoxylation of B-ring structure part appears (petunidin) and shorter retention time for hydroxylated structures (delphinidin and cyanindin). Thus, acid-hydrolyzed extracts showed clear peaks in the chromatogram at 520 nm for delphinidin (16.4 min, m/z 303), cyanidin (20.97 min, m/z 287), and petunidin (22.6 min, m/z 317) confirming the presence of these aglycon structures in non-hydrolyzed extracts. Other two minor peaks were detected (25.02 and 26.95 min), however due to the low concentration no clear PDA and MS spectra was obtained (See Supporting material for anthocyanidin chromatographic profile).

Table 1
Chromatographic and spectral data from HPLC–PDA–MS analysis of anthocyanins in Ceylon gooseberry samples.

Peak number	HPLC–PDA		MS		Tentative identification		Relative percentage	
	R_t (min)*	λ_{max} (nm)	MS^+	MS^2	Aglycone	Anthocyanidin	Pulp*	Skin*
1	11.71 ± 0.05	275, 523	465	303	Delphinidin	Delphinidin-3-glucoside	9.77 ± 0.73	12.15 ± 0.37
2	12.71 ± 0.06	275, 524	611	303	Delphinidin	Delphinidin-3-rutinoside	60.44 ± 2.86	63.14 ± 2.75
3	14.01 ± 0.05	279, 516	449	287	Cyanidin	Cyanidin-3-glucoside	4.51 ± 1.02	4.46 ± 0.55
4	15.30 ± 0.05	279, 516	595	287	Cyanidin	Cyanidin-3-rutinoside	20.91 ± 1.94	17.04 ± 2.31
5	16.85 ± 0.04	275, 525	625	317	Petunidin	Petunidin-3-rutinoside	3.19 ± 0.56	1.94 ± 0.54
6	19.33 ± 0.04	279, 522	609	301	Peonidin	Peonidin-3-rutinoside	0.28 ± 0.05	0.21 ± 0.09
7	19.82 ± 0.03	274, 521	633, 493	303	Delphinidin	Non identified	0.15 ± 0.07	0.28 ± 0.01
8	20.41 ± 0.06	272, 527	639	331	Malvidin	Malvidin-3-rutinoside	0.48 ± 0.20	0.14 ± 0.06
9	22.49 ± 0.03	277, 517	477	287	Cyanidin	Non identified	0.15 ± 0.05	0.14 ± 0.04
10	26.66 ± 0.03	270, 512	475	303	Dephinidin	Non identified	ND	0.27 ± 0.05

* Results are mean ± standard deviation ($n = 4$). ND: non detected.

Peak 1, identified as delphinidin-3-hexoside (Fig. 1) had a molecular ion at m/z 465 and a detected fragment at m/z 303 which could correspond to a loss of a glucoside moiety (162u) due to the abundance of this hexose linked to anthocyanins in nature. Same fragmentation behavior was found for peak 3, however with a detected fragment at m/z 287 that probably correspond to cyaniding aglycone. The mass spectra of delphinidin-3-rutinoside, cyanidin-3-rutinoside, petunidin-3-rutinoside, peonidin-3-rutinoside, and malvidin-3-rutinoside showed peaks at the molecular weight of each compound (Table 1), and a loss of m/z 308 fragment. UV–visible spectra data of these peaks showed no absorption band at 310–320 nm range for peaks 1–7 and, which could indicate the absence of acylations with aromatic organic acids in the molecular structure of these anthocyanins. Moreover, even with different proportion between peaks after alkaline hydrolysis all rutinoside derivatives were detected, a strong evidence of the absence of acylation at these peaks. Additionally, 3-rutinoside derivatives are expected at longer retention times than 3-hexosides due to a specific structural characteristic of rhamanose that increases non-polarity of the molecule (Giusti & Jing, 2008).

Aiming to confirm the identification, all observed retention times obtained for anthocyanins extracted from Ceylon gooseberry were compared to the chromatographic profile of anthocyanins from cherry, Concord grape juice, and strawberry anthocyanins that were considered as reference materials. Berry anthocyanins from these in-house extracts were identified based on elution order, UV–visible spectra, fragmentation pattern on mass spectroscopy, comparison with previous reports from literature, and the extensive familiarity of our laboratory with these samples (Fig. 2). Anthocyanin profiles from all of them were in agreement with previous reports (Wang, Race, & Shrikhande, 2003; Wu & Prior, 2005). Thus, cherry extract presented three major anthocyanins from 5 tentatively identified compounds, named as cyanidin-3-rutinoside, cyanidin-3-hexoside, and peonidin-3-rutinoside with retention times that match with anthocyanins in *D. hebecarpa* chromatograms.

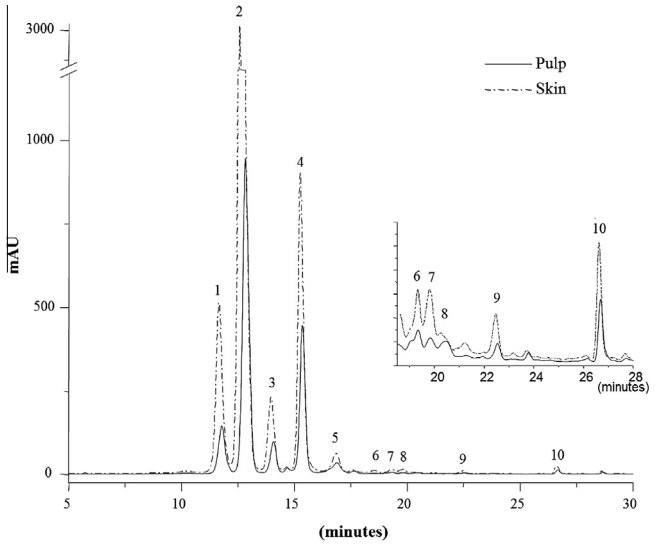

Fig. 1. Chromatographic profile of anthocyanin fraction from Ceylon gooseberry (*Dovyalis hebecarpa*) skin and flesh fruit part at 520 nm. Number in peaks are (1) delphinidin-3-*O*-hexoside; (2) delphinidin-3-*O*-rutinoside; (3) cyanidin-3-*O*-hexoside; (4) cyanidin-3-*O*-rutinoside; (5) petunidin-3-*O*-rutinoside; (6) peonidin-3-*O*-rutinoside; (7) non-identified; (8) malvidin-3-*O*-rutinoside; (9 and 10) non-identified compounds. See Supporting material for MS and PDA spectra of each peak.

Anthocyanin composition of Concord grape juice showed their typical anthocyanin profiles, as reported in previously published data (Wang et al., 2003) with two glycosided anthocyanins as major compounds (delphinidin-3-*O*-hexoside and cyaniding-3-*O*-hexoside, peaks 3 and 5, Fig. 2). Acylated compounds were detected after 20 min being delphinidin-3-coumaroylhexoside the major one. Comparing the elution order of anthocyanins in Ceylon gooseberry samples and in Concord grape juice it was possible to perceive that delphinidin-3-*O*-hexosides (peak 1) and cyaniding-3-*O*-hexosides (peak 3) in both samples had similar retention times. Moreover, malvidin-3-*O*-hexoside and petunidin-3-*O*-hexoside were detected in Concord grape juice in shorter retention times than that observed for rutinoside derivatives of these anthocyanidins in *D. hebecarpa* samples (peaks 5 and 8). These evidences were used as additional information to indicate a tentatively identification for peaks 1, 3, 5 and 8 in Ceylon gooseberry samples (Table 1 and Fig. 1).

Ten different anthocyanins were detected in strawberry freeze-dried fruits, but only cyanidin-3-hexoside, pelargonidin-3-*O*-hexoside, and pelargonidin-3-*O*-rutinoside were major peaks representing 1.79%, 91.55%, and 4.61% of total area. Chromatographic profile and identification of major anthocyanin in strawberry matched with previous data (Cerezo, Cuevas, Winterhalter, Garcia-Parrilla, & Troncoso, 2010; Fredericks et al., 2013). The retention time for cyanidin-3-*O*-hexoside in chromatograms of strawberry samples matched with peak 3 in Ceylon gooseberry chromatogram (Fig. 2). There were no detected peaks in *D. hebecarpa* with the same retention times as pelargonidin-3-hexoside and pelargonidin-3-rutinoside detected in strawberry extract. It could indicate the absence of these compounds or the presence in concentrations that were not able to be detected by the method.

Previously, it was reported a similar chromatographic profile for a hybrid of *D. hebecarpa* with *D. abssinica*. This hybrid of species has a yellow pulp and a red skin, in which anthocyanins showed the same elution order and major compounds as observed for the *D. hebecarpa* specie (de Rosso & Mercadante, 2007). In this work,

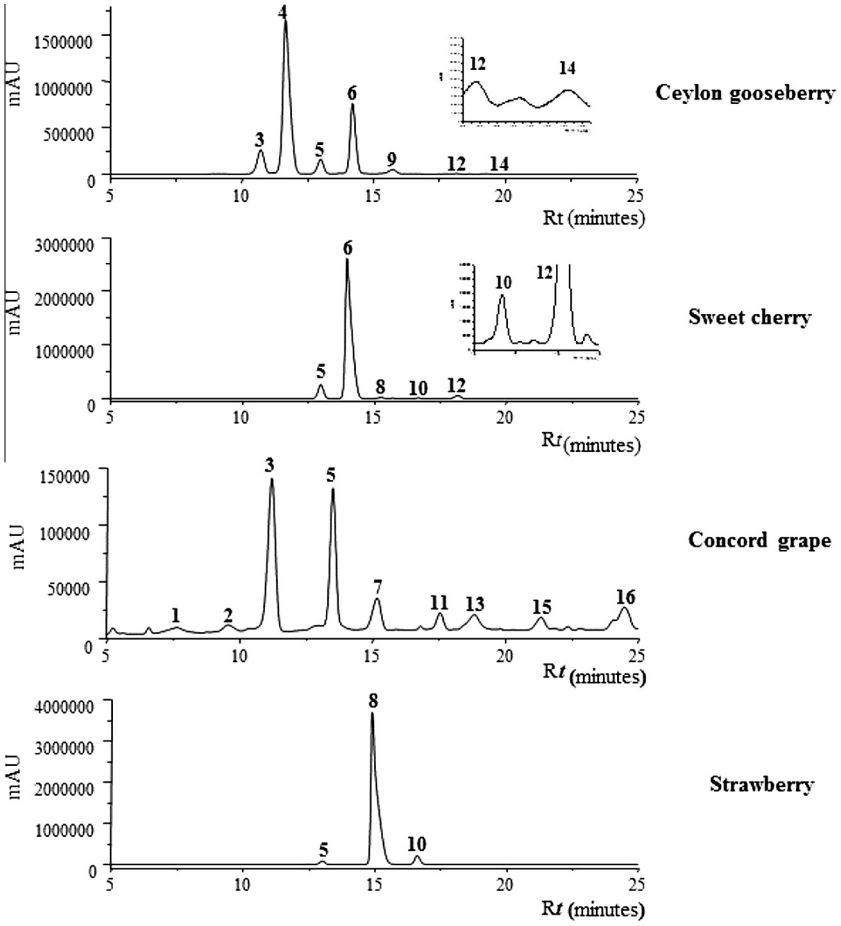

Fig. 2. Chromatographic profile comparison of anthocyanins from Ceylon gooseberry with in-house berry extracts. (1) Delphinidin-3-pentoside; (2) cyanidin-3,5-dihexoside; (3) delphinidin-3-hexoside; (4) delphinidin-3-rutinoside; (5) cyanidin-3-hexoside; (6) cyanidin-3-rutinoside; (7) petunidin-3-hexoside; (8) pelargonidin-3-hexoside; (9) petunidin-3-rutinoside; (10) pelargonidin-3-rutinoside; (11) peonidin-3-hexoside; (12) peonidin-3-rutinoside; (13) malvidin-3-hexoside; (14) malvidin-3-rutinoside; (15) delphinidin-3-(6″-acetoyl)hexoside; (16) delphinidin-3-(6″coumaroyl)-5-dihexoside.

the MS^n fragmentation reactions allowed the detection of fragments from disaccharide linkage between the sugar moieties, glucose and rhamanose. It has happen due to the free molecule rotation, increasing gas accessibility which resulted in this fragmentation pattern. In our work, fragmentation was obtained during ionization process (in source fragmentation) which does not have enough energy to linkage the molecule at this site. Nevertheless, in the delphinidin-3-rutinoside mass spectra, it was possible to perceive a small peak in m/z 463 that could correspond to 1,6-glucosyl linkage and loss of deoxyhexose moiety (146u).

Peaks 7, 9, and 10 (representing less than 0.28% of the total peak area in skin and pulp samples, Table 1) were not identified since MS spectra did not show a logical fragmentation pattern or PDA spectra did not match with aglycon identification. However, MS fragments and PDA information obtained for peak 7 strongly indicate a presence of a delphinidin derivative since the λ maximum value was similar to values obtained for delphinidin-3-hexoside and delphinidin-3-rutinoside. Moreover, one possible structure to this compound could be delphinidin-3-(6″-coumaroyl)hexoside, in which ion fragment 633 m/z is a sodium adduct of this compound. Nevertheless, the characteristic absorbance peak in the 310–340 nm range (Rodriguez-saona et al., 1998) was not detected in PDA spectra characteristic from acylation.

Ceylon gooseberry anthocyanin profile was very similar to black currant pigments (Frøytlog, Slimestad, & Andersen, 1998) with 3-rutinoside derivatives of delphinidin (30.6%) and cyanidin (43.6%) as the major pigments followed by delphinidin-3-glucoside (12.9%) and cyanidin-3-glucoside (9.3%). Peonidin-3-rutinoside (1.5%) and malvidin-3-rutinoside (2.1%) were detected as minor pigments, similarly to *D. hebecarpa* with malvidin-3-rutinoside as one of minor compounds detected. Another publication for black currants confirmed previous data and identified more 5 minor anthocyanins that correspond to less than 2% of total area (Borges, Degeneve, Mullen, & Crozier, 2010).

3.2. Qualitative and quantitative comparison of anthocyanins in Ceylon gooseberry flesh and skin fruit parts over two seasons

Anthocyanins in flesh *versus* skin fruit parts of *D. hebecarpa* were evaluated for qualitative and quantitative differences over two yearly harvests (in autumn and winter) (Table 1).

For quantification purposes using PDA detector, anthocyanins were quantified as cyanidin-3-glucoside equivalent using three different concentrations ranges in calibration curves. The highest range (from 100 to 250 ppm of cyanidin-3-glucoside) was used to quantify delphinidin-3-rutinoside in skin samples. An intermediate range (from 10 to 80 ppm of cyaniding-3-glucoside) was used for peaks 1, 3, 4, and 5 in skin and 1, 2, and 4 in pulp samples. Finally, the lowest range (from 0.5 to 4 ppm of cyaniding-3-glucoside) was used to quantify compounds in lower concentrations (peaks 6 and 8 in skin samples and peaks 3 and 5 in pulp samples). As recommended (ICH, 2005), statistical treatment of data from calibration curves was performed to evaluate the linearity. Regression results showed that model is linear without lack of

Table 2
Content of anthocyanins from flesh and skin of Ceylon gooseberry samples.

Peak #	Tentatively identification anthocyanins	R_t (min)	Concentration (mg/100 g FW)				ACY/total concentration proportion			
			Flesh		Skin		Flesh		Skin	
			April	July	April	July	April	July	April	July
1	Delphinidin-3-*O*-glucoside	11.71 ± 0.05	7.26 ± 0.41	8.46 ± 0.65	82.45 ± 4.23	68.96 ± 5.95	0.12	0.11	0.13	0.13
2	Delphinidin-3-*O*-rutinoside	12.71 ± 0.06	35.39 ± 1.31	48.17 ± 4.11*	403.00 ± 22.24	324.08 ± 27.65*	0.58	0.62	0.62	0.61
3	Cyanidin-3-*O*-glucoside	14.01 ± 0.05	4.35 ± 0.33	4.57 ± 0.23	31.66 ± 1.47	24.14 ± 9.34	0.07	0.06	0.05	0.05
4	Cyanidin-3-*O*-rutinoside	15.30 ± 0.05	14.19 ± 0.07	16.82 ± 1.01*	102.63 ± 6.53	93.76 ± 7.61	0.23	0.22	0.16	0.18
5	Petunidin-3-*O*-rutinoside	16.85 ± 0.04	n.q.	n.q.	16.27 ± 1.00	14.97 ± 0.68	n.q.	n.q.	0.03	0.03
6	Peonidin-3-*O*-rutinoside	19.33 ± 0.04	n.q.	n.q.	4.82 ± 0.29	4.66 ± 0.16	n.q.	n.q.	0.01	0.01
8	Malvidin-3-*O*-rutinoside	20.41 ± 0.06	n.q.	n.q.	4.72 ± 0.48	4.40 ± 0.37	n.q.	n.q.	0.01	0.01
	Sum of total concentration FW		61.19	78.02	645.55	534.97				

Results are mean ± SEM (n = 6) expressed as cyanidin-3-rutinoside equivalent. The signal * means that values are different from values of April samples (Tukey test, $p \leqslant 0.05$). ACY: anthocyanin, FW: fresh weight. DM: dry matter by freeze-drying. n.q.: not quantified. See Supporting material for PDA and MS spectra of each peak, and calibration curve for cyanidin-3-rutinoside.

fitness in all evaluated ranges (see Supplementary material for calibration curve data). Moreover, using the calibration curve data (ICH, 2005) values of 1.4 and 4.3 mg/100 g of sample were the detection and quantification limits adopted in this work, respectively.

A similar chromatographic profile for anthocyanins extracted from skin and pulp samples were obtained. Only minor peaks (peak 10) was not detected in extracts from pulp samples which could be present in concentration under the method detection limit (Table 1).

Quantification results showed that all anthocyanin were present in higher concentrations in skin than in pulp (Table 2). Consequently, the concentration sum of all compounds resulted in a total that was higher in skins (534.97 and 645.55 mg as cyanidin-3-rutinoside equivalent/100 g of FW) than in pulp samples (61.19 and 78.02 mg as cyanidin-3-*O*-rutinoside equivalent/100 g of FW).

Comparison of anthocyanin content determined in Ceylon gooseberry fruits with levels of other sources from literature should consider sample treatment. The mainly reason is because these phytochemicals are frequently analyzed in entire fruits in literature. In the present work, skins were analyzed separated because this fruit is commonly consumed after peel removal. Thus, pulp fruit part showed higher concentration of anthocyanin than entire raspberries (39.7 mg/100 g of FW), red currant (14.7 mg/100 g of FW), and cranberries (32.5 mg/100 g of FW) and lower than entire blackcurrants (247.9 mg/100 g of FW) and blueberries (215.9 mg/100 g of FW) all of them determined by HPLC–PDA in equivalents of cyaniding-3-glucoside and detected at 520 nm (Borges et al., 2010). However, entire fruit could present similar values to reported anthocyanin content in entire blueberries and blackcurrants because skin fruit part showed 6–10 times higher anthocyanin levels than pulp of Ceylon gooseberry.

Anthocyanin levels determined for skin samples of *D. hebecarpa* were higher than values reported for Gamay grape skins (64.3–84.4 mg of cyaniding-3-glucoside equivalent/100 g FW) (Pace et al., 2014), similar to Legacy highbush blueberry skin (580 mg of cyaniding-3-glucoside equivalent/100 g FW), and lower than skins from Bluegold and Brigitta highbush blueberry varieties (715 and 733.5 of mg cyaniding-3-glucoside equivalent/100 g FW).

Anthocyanin concentration reported for different cultivars of cherries in equivalents of cyandin-3-rutinoside were similar to Ceylon gooseberry pulp content specially for the darkest variety of the species *Prunus avium* (65.08–73.73 mg/100 g FM) and *Prunus cerasus* (59.75–68.71 mg/100 g FW) (Cao et al., 2015).

Samples analyzed over 2 years did not show significant differences in the anthocyanin profile and the total content of anthocyanin. Notwithstanding, ANOVA results revealed a significant season effect (for results of autumn *versus* winter samples) on cyanidin-3-*O*-rutinoside and delphinidin-3-*O*-rutinoside for pulp samples, and only in delphinidin-3-*O*-rutinoside for skin samples. In skin samples the highest values were obtained in April (autumn) and for flesh fruit part in July (winter) which could be a result of plant defense mechanism to different non-ideal climate conditions.

As reported before, *D. hebecarpa* requires moisture and temperatures higher than 20 °C for perfect fruit development (Morton, 1987). The climate information available for this region reports that June and July are months of a dry weather and lower average temperatures recognized as a dry winter season. It could submit plant to a situation of water stress and chilling accumulation increasing levels of anthocyanin in pulp fruit part. Moreover, in skins anthocyanins can act as a barrier against UV radiation (Dieter Treutter, 2006) and it could explain the higher concentration of these compounds after earlier autumn (March and April). Since this season is recognized by higher averages of day and night temperatures than in winter. In agreement with this hypothesis, a previous study with different black currants cultivars cultivated under different latitudes and weather conditions clearly showed a positive correlation of delphinidin-3-glucoside and delphinidin-3-rutinoside levels with temperature and radiation (Zheng et al., 2012).

Profile composition previously reported for hybrid of *D. hebecarpa* × *D. abssinica* had 9 identified compounds, in which 7 of them were the same identified in this work. Delphinidin-3-(6″-acetyl)-glucoside and pelargonidin-3-rutinoside were identified in this hybrid after malvidin-3-rutinoside. These anthocyanins had the same elution order than two non-identified compounds in *D. hebecarpa* samples, but with different PDA and MS spectral information. Individual compound concentrations for each anthocyanin were from 5 to 20 times greater in *D. hebecarpa* than in the previously reported hybrid fruit (De Rosso & Mercadante, 2007).

3.3. Other phenolics in Ceylon gooseberry flesh and skin

Previously to purification using solid-phase extraction, analyses of skin and flesh crude extracts showed that anthocyanins were the major phenolic class in this specie corresponding alone to over 68.5% and 85.93% of total peak area in flesh and skin samples, respectively. Thus, Fig. 3A and B showed the chromatographic profile of polyphenols obtained after purification and concentration of extracts from pulp and skin samples of *D. hebecarpa*, respectively.

Both fruit parts showed a complex composition of phenolic compounds with 30 compounds in flesh and 29 in skin samples (Table 3 and Fig. 3). The UV–visible maximum absorption bands of each compound was compared with authentic standards and

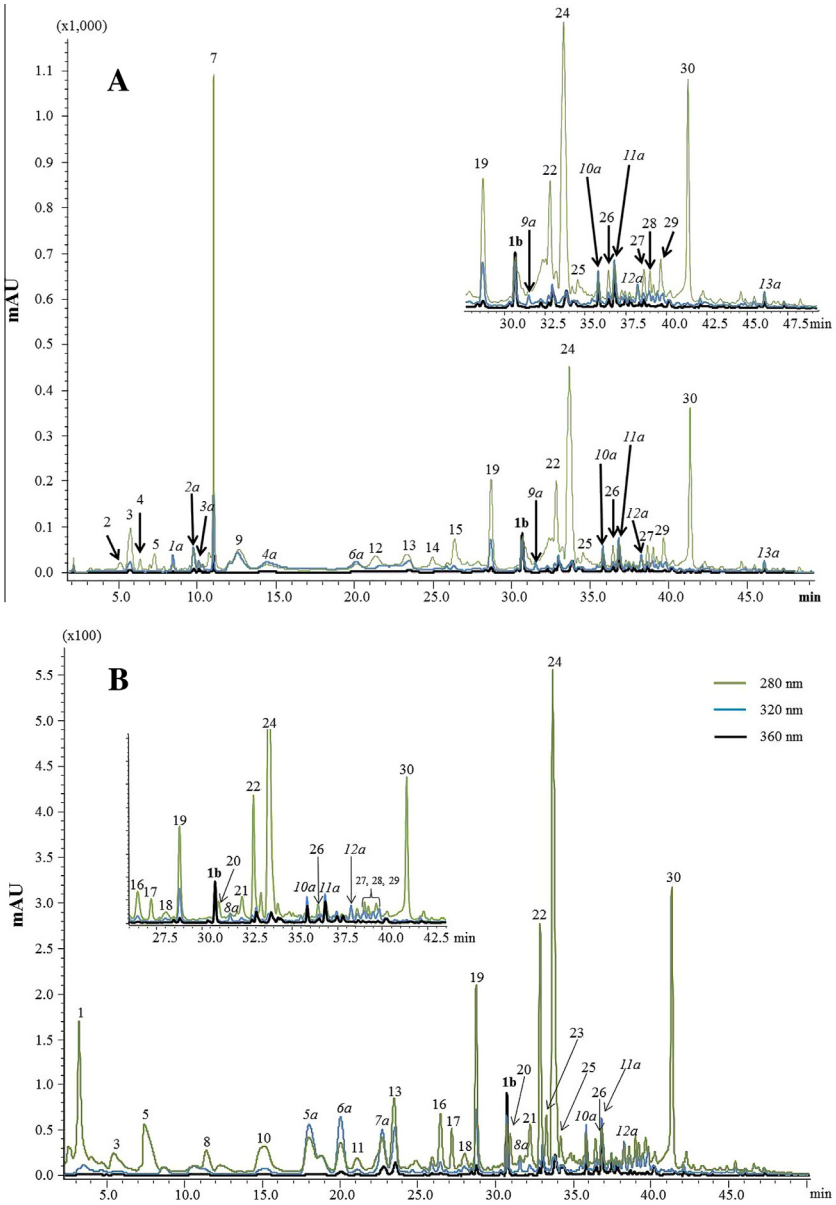

Fig. 3. Chromatographic profile of phenolic compounds from Ceylon gooseberry pulp (A) and skin (B) fruit part. Figures correspond to the purified fraction phenolic compounds (without anthocyanins) previously to hydrolysis (whole extract). Peak numbers were assigned as in Table 3.

used to classify phenolics into classes (Table 3) as previously proposed (Määttä, Kamal-Eldin, & Törrönen, 2003; Määttä-Riihinen, Kamal-Eldin, & Törrönen, 2004). The MS spectral information and the mild in-source fragmentation obtained for negative ions were used to obtain more information about the molecular masses of conjugates and the structural complexity of each compound. Since phenolics from Ceylon gooseberry have not been previously reported, comparison with previous literature was not possible. However, whenever possible, previous reports on characterization of other berries were used for comparison with Ceylon gooseberry profile aiming to support the tentative identification of each peak. Finally, acid or alkaline hydrolysis was performed attempting to obtain aglycons and non-acylated compounds from structure linkage of native forms (Xu & Howard, 2012).

Thirty different hydroxybenzoic acid derivatives were detected in skin and pulp samples of Ceylon gooseberry in which most of them were tentatively identified as galloyl esters (peaks 1–3, 5, 6, 8–11, 13, 15–18, 20, 27, and 29). The spectral features used to classify these compounds were previously described by Määttä-Riihinen and coworkers in their research with *Ribes* species (Määttä et al., 2003) and with *Rubus* species (Määttä-Riihinen et al., 2004). Thus, soluble galloyl esters will show no changes in the spectrum shape but due to the esterification to a polyol core these compounds will show a significant bathochromic shift (10–15 nm) in the maximum absorption band (280–285 nm) when compared to of its aglycon structure, the gallic acid (270 nm). These features are show in Table 3.

Peaks 12, 22, 23, and 28 were assigned as flavan-3-ols derivatives or proanthocyanidins due to spectral similarity with epigallocatechin-gallate (Table 3), the available standard for this phenolic class. Moreover, 271 nm was previously reported as the major absorbance band for flavan-3-ols (Treutter, 1998). A recent work with four Brazilian berries reported proanthocyanidins as minor compounds being present as epicatechin-epicatechin in only one of these fruits (Da Silva, Rodrigues, Mercadante, & de Rosso, 2014).

The mild fragmentation and negative ions obtained in this study did not allow an unequivocal identification of each compounds detected in *D. hebecarpa* extracts. However, for some peaks it

Table 3
Chromatographic and spectral data from HPLC–PDA–MS analysis of phenolic compounds in Ceylon gooseberry pulp and skin samples.

Peak*	R_t (min)	λ_{max}	Base peak MS (m/z)	Other fragments MS (m/z)	Tentative identification	Pulp	Skin
280 nm – Flavan-3-ols and hydroxybenzoic acid derivatives compounds							
1	3.23	225, 284	347	337, 399	Galloyl ester		X
	4.48	214, 270	169		Gallic acid (Std)		
2	5.09	283	375		Galloyl ester	X	
3	5.47	284	481	331 (monogalloyl hexoside), 579, 517, 319 (M-162)	Di-galloyl hexoside	X	X
4	6.35	252, 290	329	400, 375	Vanillic acid hexoside	X	
5	7.27	278	375		Galloyl ester	X	
6	7.45	216, 285	381	153	Galloyl ester		X
	8.47	215, 259, 293	153		Protocatechuic acid (Std)		
7	11.03	212, 268	485	439.05, 465.05, 232.95, 375.05, 331(monogalloyl hexoside), 233	Unidentified	X	
8	11.45	215, 275	ND		Galloyl ester		X
9	12.65	289	485.2	475.05, 337	Galloyl ester	X	
	15.08	254	137		4-Hydroxybenzoic acid (Std)		
10	15.17	226, 284	659		Galloyl ester		X
	16.38	278	289		Catechin (Std)		
11	21.11	286	ND		Galloyl ester		X
	21.24	217, 260, 292	353		Vanillic acid (Std)		
12	21.35	217, 272	477		Flavan-3-ol	X	
13	23.32	289	319		Galloyl ester	X	X
	24.31	213, 273	197		Syringic acid (Std)		
14	24.94	218, 266	431	421	4-Hydroxybenzoic acid derivative	X	
	25.12	211, 273	457		EGCG (Std)		
15	26.36	278	431.15	329, 421	Galloyl ester	X	
16	26.47	211, 279	329	659	Galloyl ester		X
17	27.22	280	437		Galloyl ester		X
18	28.03	214, 285	ND		Galloyl ester		X
19	28.68	289	481.05	579, 963	HHDP hexoside	X	X
20	30.95	215, 280	ND		Galloyl ester		X
21	32.23	215, 286	577, 613		Dimer B2 (Flavanol)		X
22	32.82	224, 273	359.05	421	Flavan-3-ol	X	X
23	33.28	215, 271	ND		Flavan-3-ol		X
24	33.66	228, 274	451.05	461, 471, 441	HHDP-trigalloyl lactonized	X	X
25	34.53	214, 281	ND		Galloyl ester	X	X
26	36.43	219, 281, 325sh	479.05		Unindentified	X	X
27	38.63	226, 286	421.1	479	Galloyl ester	X	X
28	38.99	220, 276	421		Flavan-3-ol	X	X
29	39.66	225, 281	591.2	489.10, 387.15	Galloyl ester	X	X
30	41.34	222, 283	543.15	589.15, 579.25, 1087.55	HHDP-monolactonized tergalloyl group	X	X
320 nm – Hydroxycinnamic acid derivative compounds							
1a	8.46	231, 313	315	231, 413, 631	p-Coumaric derivative	X	
2a	9.77	287sh, 319	323	233, 353, 519	Ferulic and/or cafeic acid derivative	X	
3a	10.10	220, 299sh, 326,	323,315		Ferulic and/or cafeic acid derivative	X	
4a	14.45	311	ND		p-Coumaric derivative	X	
5a	18.03	225, 309	337		p-Coumaric derivative		X
6a	20.12	212, 313	325 (164 + 162 + 45)		p-Coumaric sugar ester	X	X
	20.80	218, 324	353	707	Chlorogenic acid (Std)		
	22.34	217, 233sh, 322	179		Caffeic acid (Std)		
7a	22.74	215, 292sh, 322,	485	475, 367	Ferulic or cafeic acid derivative		X
	28.84	225, 308	163		p-Coumaric acid (Std)		
8a	31.58	216, 291	ND		Unidentified		X
9a	32.98	214, 270, 331	ND		Unidentified	X	
10a	35.82	267, 333	577		Unidentified	X	X
11a	36.82	267, 331	431		Unidentified	X	X
12a	38.25	217.2, 310.7	713		p-Coumaric derivative	X	X
13a	46.08	220.6, 314.8	577		p-Coumaric derivative	X	
360 nm – Flavonols							
1b	30.67	264, 292, 353,	479		Myricetin hexoside	X	X
	33.14	254, 354	609		Quercetin-3-*O*-rutinose (Std)		
	43.26	254, 364	301		Quercetin (Std)		

* Numbers were assigned in accordance with Fig. 3. Std: standard. ND: not detected.

was possible to identify the molecular weight and some fragments that in agreement with UV–visible spectrum data and literature information were used to suggest a possible identification. Thus, peak 3 showed the negative ion at m/z 481 which could correspond to a sum of two de-protonated gallic acid molecules (169 amu) with a hexose moiety (162 amu) less one water molecule (18). Furthermore, at the same retention time another fragment at m/z 319 could correspond to the loss of one hexose moiety (M-162 = 319). Additionally, the UV–visible spectra showed maximum bands similar to gallotannins at 275–285 nm (Määttä-Riihinen et al., 2004) helping to assume that peak 3 could be a molecule of di-galloyl hexoside.

Peak 4 was assigned as vanillic acid hexoside with a molecular weight of 329 which correspond to a negative charged vanillic acid (167) plus a hexose moiety (162). Besides, a previously published data showed UV–visible spectral maxima values at 260–264 nm and 290–296 nm and a molecular weight of 330 for vaniloylhexose and for vanillic acid hexoside in *Ribes* species (Määttä et al., 2003)

and in the Brazilian berry Araça (Da Silva et al., 2014), respectively. These data are in agreement with spectral characteristics observed for peak 4 (Table 3). Moreover, peak 3 and 4 showed the same elution order than reported elution for di-galloyl hexoside and vanillic acid hexoside in Araça samples (Da Silva et al., 2014).

Gallotannins are precursors of ellagitannins which are formed by C–C oxidative coupling of two adjacents galloyl groups forming HHDP which will be linked to a polyol core (Talcott & Krenek, 2012). Due to this reaction a hypochromic effect will be observed maximum absorption at 275–285 nm of galloyl esters and gallotannins as well (Määttä-Riihinen et al., 2004). As PDA spectra reported by Määttä-Riihinen et al. (2004), ellagitannins will present a great absorption at 220–230 nm, a less intense absorption at 275–285 nm that depend of the number of HHDP and galloyl molecules esterified in the structure, and a the absence of the characteristic band at 360 nm of ellagic acid. Based on this evidences, peaks 24 and 30 were assigned as ellagitannins and the MS negative ions are matching with reported fragments for HHDP-trigalloyl lactonized and HHDP-monolactonized tergalloyl group found in Uvaia (Da Silva et al., 2014), another Brazilian berry from southwest region, and in flower buds of Syzygium aromaticum Merr. & Perry (Bao et al., 2012), respectively. The two fruit sources (Araça e Uvaia) and the flower bud of Syzygium aromaticum Merr. & Perry belongs to *Myrtaceae* family from *Magnoliopsida* class as well as *D. hebecarpa*. This evidence could support some similar phenolic compounds between these plant materials.

Peaks with greater absorbance at 320 nm than at 280 or 360 nm were tentatively classified as hydroxycinnamic acid derivatives and UV–visible spectral characteristics used to classify as p-coumaric acid derivatives and caffeic and/or ferulic acid derivatives as proposed by Määttä-Riihinen et al. (2004). This classification is supported by the presence of peaks with the same retention time than cafeic and p-coumaric acid standards in samples of Ceylon gooseberry that were treated by alkaline hydrolysis, in some cases with the same MS and UV–visible spectra than this standards (see Supporting material for chromatograms of hydrolyzed samples). MS spectral information did not additional provide structural elucidation with identification of possible linkage with sugar or between phenolic acids.

Only one peak (peak 8) showed greater absorbance at 360 nm than at 280 or 320 nm and it was assigned Myricetin-hexoside. These compound showed two major absorption bands at 264 and 353 nm in UV spectra which are typical to UV absorption bands for flavonols (Tomás-Barberán & Ferreres, 2012). This maximum absorption bands are reported as corresponding to the B-ring or cinnamoyl-nucleus (Band I from 325 to 400 nm) and A-ring or benzoyl-nucleus (Band II from 240 to 295 nm). Moreover, peak 8 showed a fragment at m/z 479 that correspond to the molecular weight of Myricetin linked to a hexose moiety. Finally, this flavonol was detected and identified as Myricetin hexoside in other berries (Lago-Vanzela, Da-Silva, Gomes, García-Romero, & Hermosín-Gutiérrez, 2011; Määttä et al., 2003) with similar spectral characteristics than peak 1b (Fig. 3) from Ceylon gooseberry polyphenol chromatographic profile.

Peak 7 is the major phenolic compound in chromatogram of pulp samples with UV spectra maximum band that could suggest a 4-hydroxycinnamic acid derivate with major absorption near to 262 nm (Määttä et al., 2003). However, MS spectra showed many fragments with similar intensity to the major negative ion (base peak, 485, Table 3) which could indicate co-elution. Furthermore, there is a ion at m/z 331 that could correspond to galloyl hexoside molecule present as a result of in-source fragmentation or as co-elution.

In summary, even no clear structure elucidation was obtained for minor phenolic compounds in Ceylon gooseberry pulp and skin, it was possible to classify major phenolic compounds into classes as flavonol, hydroxycinnamic acid, hydroxybenzoic acid, and flavanols derivatives (Table 2). Except for peak 7 in pulp samples chromatogram which is the major compound only in this fruit part, peaks numbered as 19, 22, 24, and 30 were the major phenolic compounds found in both fruit parts, in pulp and in skin samples of Ceylon gooseberry (Fig. 3). The tentative identification proposed in this work has assigned these peaks as HDDP-hexoside, a flavonol derivative, and two ellagitannins (HHDP-trigalloyl lactonized and HHDP-monolactonized tergalloyl group) corresponding to 47.9% and 45.4% of total phenolic compounds than not anthocyanins in pulp and skin samples, respectively.

Anthocyanins were the major polyphenols in this specie correspond alone to more than 50% and 60% of phenolic compounds in Ceylon gooseberry pulp and skin, respectively. A total of 10 different anthocyanins were detected and 7 of them were tentatively identified, corresponding to 99.6% of the total content.

D. hebecarpa was a rich source of glycosylated anthocyanins with delphinidin-3-rutinoside and cyanidin-3-rutinoside as major compounds. Moreover, higher concentration of anthocyanins was detected in skin (2054.91 and 1616.96 mg cy-3-glu eq/100 g of freeze-dried samples in autumn and winter, respectively) with higher proportions of glycosylated delphinidin compounds than in flesh samples.

Anthocyanins quantification showed a possible effect of season variation in a different manner for flesh and skin samples, mainly in delphinidin-3-rutinoside level. Thus, after autumn season, an increased level of this compound was found in skin samples, and after months of dry and lower weather temperatures, in flesh.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.12.041.

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