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Chemical characterisation of anthocyanins in tamarillo (*Solanum betaceum* Cav.) and Andes berry (*Rubus glaucus* Benth) fruits

CORALIA OSORIO,[†*] NELSON HURTADO,[†] CORINNA DAWID,[‡] THOMAS HOFMANN, [‡]

FRANCISCO JOSÉ HEREDIA-MIRA,[§] AND ALICIA LUCIA MORALES [†]

Departamento de Química, Universidad Nacional de Colombia, AA 14490, Bogotá, Colombia; Chair of Food Chemistry and Molecular Sensory Science, Technische Universität München, Lise-Meitner-Strasse 34, D-85354 Freising-Weihenstephan, Germany; and Laboratory of Food Colour & Quality, University of Seville, 41012, Sevilla, Spain

Title running header: Anthocyanins from *Solanum betaceum* and *Rubus glaucus*

* Author to whom correspondence should be addressed: C. Osorio. Telephone: +57-1-3165000, ext. 14472. Fax: +57-1-3165220. E-mail: cosorior@unal.edu.co

[†] Departamento de Química, Universidad Nacional de Colombia.

[‡] Technische Universität München

[§] University of Seville

1 **ABSTRACT**

2

3 The anthocyanin composition of tamarillo (*Solanum betaceum* Cav., red variety)
4 and Andes berry (*Rubus glaucus* Benth) was determined by HPLC-PDA and HPLC-
5 ESIMS. From the anthocyanin-rich extracts (AREs), pure compounds (**1**-**7**) were obtained
6 by MLCCC (multilayer countercurrent chromatography) and further preparative HPLC, and
7 their unequivocal structures were obtained by 1D and 2D NMR analyses. The new
8 anthocyanin delphinidin 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside-3'-*O*-β-
9 D-glucopyranoside, as well as the known cyanidin-3-*O*-rutinoside, pelargonidin-3-*O*-
10 rutinoside, and delphinidin-3-*O*-rutinoside were identified as constituents of tamarillo fruit.
11 Although the anthocyanin composition of Andes berry had been reported before in the
12 literature, the unequivocal structure elucidation of the major compound, cyanidin-3-*O*-α-L-
13 rhamnopyranosyl-(1→6)-*O*-β-D-xylopyranosyl-(1→2)-β-D-glucopyranoside, was achieved
14 for the first time.

15

16 *Keywords:* Anthocyanins, *Solanum betaceum*, Tamarillo, *Rubus glaucus* Benth, Andes
17 berry.

18 ## 1. Introduction

19

20 Anthocyanins are the largest group of water-soluble pigments in the plant kingdom.
21 They are responsible for most red and blue colours in fruits and vegetables, and recently,
22 have been used in the food industry as pigments, because of their bright attractive colours,
23 high water solubility, and associated health benefits (Castañeda-Ovando *et al.*, 2009). It is
24 important to note that the studies on the sensory and biofunctional properties of tropical
25 fruits have increased due to their novelty (Sousa De Brito *et al.* 2007; Mahattanatawee *et*
26 *al.*, 2006). These fruits and their derived-products have conquered new markets in
27 subtropical regions because consumer interests have turned to the natural foods.

28 *Solanum betaceum* Cav. syn *Cyphomandra betacea* Sendt. (Solanaceae) is a shrub
29 native to the Andes, specifically in Peru, Ecuador, and Colombia, that belongs to the
30 Solanaceae family. In Colombia, the most common varieties are the traditional yellow-
31 fleshed, and the red one (yellow fleshed with red peel and purple jelly) commonly named as
32 tamarillo (Vasco, Avila, Ruales, Svanberg & Kamal-Eldin¸ 2009*)*. The red-fleshed fruit is
33 the most commercialised variety in Colombia and one of the highly-marketed Colombian
34 tropical fruits in Europe. The fruit is ovoid in shape, a length of 6-8 cm, and a diameter of
35 4-5 cm; when ripe, its peel is dark red, and exhibits a slightly bitter, sour, and astringent
36 taste with a delicate and characteristic aroma. It is generally consumed fresh, or blended
37 together with water and sugar to make juices and desserts. Early studies on phenolics
38 constituents of tamarillo fruit from New Zealand, which were performed by paper
39 chromatography and TLC (thin layer chromatography), reported the presence of 3-
40 rutinosides and 3-glucosides of pelargonidin, cyanidin and delphinidin (Wrolstad &

41 Heatherbell, 1974); whereas pelargonidin 3-glucosyl-glucose, peonidin 3-glucosyl-glucose,
42 and malvidin 3-glucosyl-glucose were identified in tamarillo fruits from Brazil by UV-Vis
43 spectrophotometry and TLC (Bobbio, Bobbio & Rodriguez-Amaya, 1983). Recently,
44 delphinidin 3-rutinoside, cyanidin 3-rutinoside, and pelargonidin 3-glucoside-5-rhamnoside
45 (tentatively) were identified by LC/MS from fruits of Brazil (Vera de Rosso & Mercadante,
46 2007). In fruits from Ecuador, the presence of the anthocyanins, delphinidin glucosyl
47 rutinoside, delphinidin rutinoside, cyanidin rutinoside, and pelargonidin rutinoside; the
48 hydroxycinnamic acids, dicaffeoylquinic acid, caffeoylquinic acid, caffeoyl glucose, and
49 feruloyl glucose; as well as, the esterified carotenoids, lutein and β-cryptoxanthin were
50 reported (Mertz *et al*, 2009; Mertz, Brat, Caris-Veyrat & Gunata, 2010). As can be seen,
51 numerous studies on tamarillo anthocyanins have been performed, but little attention has
52 been paid to the isolation and unequivocal identification by spectroscopic methods.
53 *Rubus glaucus* Benth (Rosaceae), commonly known as Andes berry or "mora de
54 Castilla" (**Figure 1**) is a berry native to South America which is found between 2600 and
55 3100 meters above the sea level and highly consumed in Colombia (annual production
56 surpassing 10000 ton.). The fruit consists of numerous small drupes on a receptacle about
57 1- 2.5 cm long, which are dark-red or purple. It is characterised by an intense aroma and
58 sweet-sour taste; however, this fruit is highly perishable and susceptible during postharvest
59 handling. This fact has motivated the development of processed products with higher shelf-
60 life time as strategy to overcome those problems. The phenolic composition of this fruit and
61 other related ones have been studied by different research groups (Mertz, Cheynier, Günata
62 & Brat, 2007; Vasco, Riihinen, Ruales & Kamal-Eldin, 2009; Cuevas-Rodríguez *et al*.,

63 2010). Thus, high amounts of ellagitannins and proanthocyanidins have been found, as well
64 as hydroxycinnamic acid derivatives, flavonols (mainly quercetin glycosides), and
65 anthocyanins. The main anthocyanins were cyanidin-3-*O*-glucoside (67% of total
66 anthocyanin content), and cyanidin-3-*O*-rutinoside (31% of total anthocyanins). Minor
67 anthocyanins were tentatively reported as cyanidin-3-*O*-malonyl glucoside and a
68 pelargonidin derivative. Garzón, Riedl and Schwartz (2009) reported the anthocyanin
69 content of Andes berry as 45 mg/100 g FW and cyanidin 3-sambubioside, cyanidin 3-
70 glucoside, cyanidin 3-xylorutinoside, cyanidin 3-rutinoside, pelargonidin 3-glucoside, and
71 pelargonidin 3-rutinoside as major constituents. All of the above reported data were
72 obtained only by HPLC-MS analyses, so the unequivocal anthocyanin composition of
73 *Rubus glaucus* Benth is still unknown because the lack of NMR studies on the purified
74 compounds.
75 Tamarillo and Andes berry fruits are a promising source of natural colorants (Osorio
76 *et al.*, 2007), and they have exhibited *in vitro* antioxidant activity (Vasco, Ruales & Kamal-
77 Eldin, 2008; Mertz *et al*, 2009; Hurtado, Morales, González-Miret, Escudero-Gilete &
78 Heredia, 2009) that gives them an interesting added-value. Thus, as part of our ongoing
79 studies on the pigment composition of Colombian fruits (Osorio *et al.,* 2010; Barrios *et al.*,
80 2010; Jaramillo, Dawid, Hofmann, Fujimoto & Osorio, 2011), the anthocyanin composition
81 of the above-mentioned fruits was investigated, by purification and subsequent
82 spectroscopical analyses of these compounds.
83
84 **2. Materials and methods**
85

86 *2.1. General*

87

88 The 1D and 2D NMR spectra were obtained at 303 K using a 400 MHz (DRX) and

89 a 500 MHz Avance III (Bruker, Rheinstetten, Germany) spectrometers in

90 CD_3OD/CF_3COOD (19:1, *v/v*, and 98:2 *v/v* for compound **7**) with solvent peaks as

91 references. The ^{13}C signal and the ^{1}H signal of the CD_3OD were used as secondary

92 references (δ_C 49.3 and δ_H 3.35 ppm). For structural elucidation and NMR signal

93 assignment in 2D NMR experiments, such as COSY-, DEPT-, TOCSY-, ROESY, *g*-

94 HSQC-, and *g*-HMBC-spectroscopy were carried out using the pulse sequences taken from

95 the Bruker software library. Data processing was performed by using XWin-NMR software

96 (version 3.5; Bruker, Rheinstetten, Germany) as well as Mestre-C (Mestrelab Research, A

97 Coruña, Spain). HPLC analyses were performed on an instrument HP 1100 series (Hewlett

98 Packard, Palo Alto, CA, USA) equipped with photodiode array detector (PDA). For

99 preparative purposes, an HPLC apparatus consisting of a Merck-Hitachi L-6000A pump, a

100 Rheodyne injection valve with a 500 μL loop, and a Merck-Hitachi UV-Vis L-4250

101 detector, was used. The CCC system was a multilayer coil countercurrent chromatograph

102 series 521 (P.C. Inc., Potomac, MD, USA). A UV-Vis HP 8452 spectrometer was used for

103 monitoring MLCCC fractions. ESI-MS analyses were performed on a Shimadzu QP-8000α

104 (Shimadzu Corp. Kyoto, Japan). FAB-MS analyses of pure compounds were performed in

105 an AutoSpecQ spectrometer by using positive mode, argon as collision gas and glycerol-

106 sodium iodide as matrix.

107

108 *2.2. Plant material*

109

110 Ripe tamarillo fruits (red variety, peel 100% red, pH 3.5) were collected in Puente
111 Nacional, Santander, Colombia. A voucher specimen was coded as COL 510177 at the
112 Instituto de Ciencias Naturales, Universidad Nacional de Colombia. Pulp, peelings, seeds,
113 and jelly were manually separated. Mora fruits were purchased from different local markets
114 in Chinchiná, Caldas, Colombia, and selected according to their colour ripeness qualities
115 (more than 75% dark-red to wine red), good consistency, and shape.

116

117 *2.3. Chemicals and reagents*

118

119 HPLC grade acetonitrile, and ACS grade *n*-butanol, methanol, and *tert-*
120 butylmethylether (TBME) were purchased from Merck (Darmstadt, Germany). Water for
121 chromatographic separation was purified by means of a Milli-Q water advantage A 10
122 water system (Millipore, Molsheim, France). For LCMS analyses, acetonitrile, water, and
123 formic acid were purchased from Honeywell Burdick and Jackson™ (Muskegon, Michigan,
124 USA). The following compounds were obtained commercially from the sources given in
125 parentheses: hydrochloric acid (Merck, Darmstadt, Germany); CD_3OD and CF_3COOD
126 (Euriso-top, Saarbrücken, Germany); CF_3CO_2H (TFA) (Aldrich Chemical Company,
127 Milwaukee, WI, USA); sodium hydroxide, glucose, and rhamnose (Fluka, Steinheim,
128 Germany); and xylose (Lancaster, Eastgate, England).

129

130 *2.4. Isolation of anthocyanin-rich extracts (AREs)*

131

132 Tamarillo fruits (5 kg) were washed, and after removal of peel and seeds, the jelly
133 was manually separated from the flesh and diluted with water (1:1, *w/w*). Then, portions of
134 250 g were separately applied to an 80 x 4 cm Amberlite XAD-7 resin open column (Rohm
135 and Haas, Darmstadt, Germany). The column was rinsed with water, and the adsorbed
136 pigments were eluted with 1 L of methanol-acetic acid (19:1, v/v), according to the
137 procedure by Degenhardt, Knapp, and Winterhalter (2000). Methanol was removed under
138 vacuum at 35°C and the residue was freeze-dried. The final product was 4.0 g of
139 anthocyanin-rich extract (ARE). Whole Andes berry fruits (945 g) were processed as it was
140 before described to obtain 13.7 g of ARE.

141

142 *2.5. Fractionation of anthocyanin-rich extracts*

143

144 The ARE of tamarillo was separately fractionated by MLCCC (multilayer
145 countercurrent chromatography) in portions of 0.6 g. The solvent system was a mixture of
146 TBME/*n*-butanol/acetonitrile/water (2:2:1:5, *v/v/v/v*, acidified with 0.1% TFA, *v/v*). A
147 single coil (75 x 2.6 mm i.d. PTFE tubing, total volume approx. 410 mL) was used and the
148 revolution speed was set to 800 rpm. The less dense layer was always used as the stationary
149 phase and flow rate of mobile phase was 1.0 mL/min. Eighty fractions (4 mL each one)
150 were collected and pooled based on their UV-Vis absorption as follows: F1 (1-8), F2 (9-
151 24), F3 (25-48), F4 (49-67), F5 (67-80), and F6 (stationary phase). In the same way, 4 g of
152 Andes berry ARE were separately fractionated in portions of 1 g, and ninety fractions of
153 5mL were obtained and pooled as mentioned: F1 (1-11), F2 (12-15), F3 (16-30), F4 (31-

154 36), F5 (37-44), F6 (45-50), F7 (51-85), F8 (86-90), an F9 (stationary phase). All fractions
155 were monitored at 520 nm, the specific wavelength for anthocyanins.
156
157 *2.6. HPLC Analyses*
158
159 Characterization of the anthocyanins in the AREs and MLCCC fractions was done by
160 HPLC using a Zorbax-SB C_{18} 5 μm column (250 x 4.6 mm i.d., Agilent Technologies,
161 Santa Clara, CA, USA), and detection was carried out using a photodiode array detector.
162 Solvent system was a mixture of acetonitrile/formic acid/water (3:10:87, *v/v/v*, solvent A)
163 and acetonitrile/formic acid/water (50:10:40, *v/v/v*, solvent B) and the flow rate was 0.8
164 mL/min. Linear gradient from 6 to 20% B at 0-10 min, 20 to 40% B at 10-20 min, 40 to
165 50% B at 20-30 min, and 50 to 6% B at 30-35 min was used. Prior to injection (volume of
166 100 μL), all samples were filtered through a 0.45 μm Millipore membrane filter.
167 The quantification of anthocyanins was carried out relative to the external standard,
168 delphinidin-3-*O*-β-D-rutinoside (Dp-3-rut, 3-20 mg/mL) and cyanidin-3-*O*-β-D-rutinoside
169 (Cy-3-rut, 3-20 mg/mL) for tamarillo and Andes berry, respectively. Values are means ±
170 four experiments.
171 Pure anthocyanins (**1**-**7**) were obtained by preparative HPLC from MLCCC fractions,
172 on a LUNA C_{18} 5 μm column (250 × 10 mm i.d., Phenomenex®, Torrance, CA, USA).
173 Separations were isocratically made with a 95:5 (*v/v*) mixture of solvents A and B at a flow
174 rate of 4 mL/min. Detection was at 520 nm. In tamarillo, from F2 anthocyanins **1** (22 mg)
175 and **2** (3 mg) were purified; from F3 anthocyanins **3** (93 mg) and **4** (37 mg) were obtained;

176 and from F4 the compound **5** (51 mg) was obtained. In Andes berry, from F2 anthocyanin **7**
177 (73 mg) was obtained; from F4 anthocyanin **4** (76 mg) was isolated; from F6 compound **5**
178 (4 mg) was purified; and from F8 anthocyanin **6** (3 mg) was obtained.

179

180 *2.7. ESI-MS analyses*

181

182 ESI-MS parameters used in the analyses of pure compounds were as follows: spray
183 voltage 4.5 kV, nebulizer gas (N_2) flow rate at 4.5 L/min, probe voltage 4.5 kV, curved
184 desolvation line (CDL) voltage 130 V, CDL temperature 230 °C, deflector voltage 45 and
185 60 V. The instrument was operated in positive ion mode scanning from *m/z* 50 to 800 u.
186 Samples of pure anthocyanins were diluted (1 mg/mL) in a mixture of acetonitrile-formic
187 acid-water (45:10:45) and directly injected into the ESI source at a flow rate of 100
188 µL/min.

189 For the identification of compound **7**, mass and product ion spectra were acquired on an
190 API 4000 Q Trap triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems,
191 Darmstadt, Germany). The isolated fraction was dissolved in a mixture of methanol/water
192 (70:30, v/v) and directly introduced into the mass spectrometer by flow infusion using a
193 syringe pump. The mass spectrometer was activated in full-scan mode under an
194 electrospray ionization (ESI) device running in positive ionization mode with a spray
195 voltage of +5500 V. The MS/MS parameters were optimised for this compound. Data
196 acquisition and instrumental control was completed with the Analyst 1.4.2 software
197 (Applied Biosystems, Darmstadt, Germany).

198

199 *2.8. Sugar analyses*

200

201 For compound **7**, the analysis of glycosidically bound carbohydrates were analysed
202 by means of anion exchange chromatography using an ICS-2500 ion chromatography
203 system (Dionex, Idstein, Germany) consisting of a GS 50 gradient pump, an AS 50
204 autosampler, an AS 50 thermal compartment, and an ED 50 electrochemical detector
205 operating in pulsed amperometric detection mode. The detector was equipped with a gold
206 working electrode operating with a standard carbohydrate quadrupole wave form supplied
207 by manufacturer. Data acquisition and instrumental control was completed with the
208 Chromeleon software (version 6.80, Dionex). Chromatographic separation was performed
209 at 30 °C on a CarboPac PA-20 column (150 x 3 mm, Dionex) connected with a CarboPac
210 PA-20 guard column (30 x 3 mm, Dionex), using an isocratic gradient of sodium hydroxide
211 solution (2.5 mM) for 20 minutes. After each sample, the column was washed with a
212 sodium hydroxide solution (200 mM) and equillibrated with sodium hydroxide solution
213 (2.5 mM) for 10 minutes prior to injection. Moreover chromatography was performed with
214 an injection volume of 10 µL and a flow rate of 0.5 mL/min. For qualitative analysis,
215 glycosidically bound carbohydrates were identified by comparison of retention times and
216 cochromatography of the following reference compounds: glucose, rhamnose, and xylose.
217 For sample preparation an aliquot (1 mg) of the target compound, dissolved in aqueous
218 hydrochloric acid (2 mol/L; 0.5 mL) was placed into a closed glass vial, and then heated at
219 110 °C for 120 min. After cooling to room temperature 150 µL of potassium hydroxide

220 solution (4 N) and 50 mL water was added. Each sample was transformed into autosampler
221 vials for injection into the HPIC system.
222
223 *2.9. Spectroscopic data*
224
225 The structures of compounds **1-7** could be elucidated by means of UV/Vis, LC-
226 MS/MS, LC-TOF-MS, and 1D/2D-NMR experiments.
227
228 **3. Results and discussion**
229
230 *3.1. Identification the anthocyanins in tamarillo fruit*
231
232 The HPLC analysis of the crude jelly extract revealed three major anthocyanins,
233 peaks **3, 4**, and **5** representing *ca*. 97.8% of the total area at λ 520 nm (**Figure 2A**). The
234 minor anthocyanins **1** and **2** were also detected and accountable for 2.2%. Additionally, a
235 major constituent (90.7%) was detected in the ARE of tamarillo peel, whose retention time
236 and spectral characteristics were the same as those of compound **4** from the jelly extract.
237 Both extracts showed *in vitro* antioxidant activity under TEAC assay (Hurtado, Morales,
238 González-Miret, Escudero-Gilete & Heredia, 2009).
239 The use of MLCCC allows diminishing the complexity of the AREs. Polyphenols,
240 such as anthocyanins, are sometimes difficult to separate in classical chromatography; so,
241 counter current chromatography (CCC) uses a biphasic liquid system that allows separating
242 the components of the mixture in preparative scale without loss of material by retention

243 (Ignat, Volf & Popa, 2011). After this procedure the purification of pigments by preparative
244 HPLC was easily carried out.

245 The ESI-MS analyses for isolated anthocyanins showed fragment ions
246 corresponding to three anthocyanidins, delphinidin at *m/z* 303 (**1** and **3**), cyanidin at *m/z*
247 287 (**4**), and pelargonidin at *m/z* 271 (**2** and **5**) (**Table 1**).

248 For compound **1**, a molecular weight of 773 u was confirmed based on the
249 fragments obtained by ESI-MS and FAB-MS. The other ion fragment at *m/z* 303 [M-162-
250 162-146]$^+$ suggests the presence of two hexoses and one pentose as sugar moiety in this
251 compound. The absence absorption at the UV-Vis region between 310 and 355 nm
252 indicates that there are no acylation with aromatic cinnamic acid in this molecule (Giusti &
253 Wrolstad, 2001). The aglycone protons at downfield section on the ^{1}H-NMR spectrum,
254 confirmed the presence of delphinidin as anthocyanidin of compound **1** (**Table 2**). Three
255 anomeric proton signals appear as doublets at δ_H 5.28 (J = 7.7 Hz), 5.05 (J = 7.3 Hz), and
256 4.66 (J = 1.8 Hz) in agreement with the presence of three sugars, as it was suggested from
257 the analysis of ESI-MS spectrum. Although the sugar protons were partially superimposed
258 on each other, the assignment could be completely achieved by the simultaneous analysis of
259 COSY, TOCSY, and HMQC spectra, and were in accordance with two glucosyl and one
260 rhamnosyl units (**Tables 2** and **3**). The ^{1}H-^{1}H coupling constant value indicates a β-
261 configuration for the glucoses and suggests α-configuration for rhamnose. These
262 configurations were confirmed through the heteronuclear HSQC experiment by measuring
263 of $^1J_{CH}$ coupling constants for protons at δ_H 4.66 and 5.28 ppm, which values were 169.8
264 and 164.5 Hz, corresponding to α and β configuration, respectively (Pedersen, Andersen,

265 Dagfinn & Nerdal, 1995). The cross-peaks in the HMBC spectra of **1** at δ 5.28/144.5 ppm
266 and 5.05/146.6 ppm show that one glucosyl moiety is linked to C-3 of the aglycone and the
267 other is attached to C-3' position. Additionally, the cross-peak between the anomeric
268 rhamnosyl proton and C-6" at δ 4.66/67.9 ppm confirms the linkage point between the
269 rhamnosyl moiety and the 3-glucosyl unit. These results were also confirmed by NOE
270 differential experiment. The irradiation of anomeric protons at δ_H 5.28 and 5.05 ppm
271 showed an increment in the signal at δ_H 8.98 (H-4) and 8.09 (H-2') ppm, respectively.
272 Thus, the identity of **1** was found to be delphinidin 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-
273 glucopyranoside-3'-*O*-β-D-glucopyranoside (**Figure 3**). To the best of our knowledge this
274 is the first time that this compound is reported in literature. More than 50 anthocyanins with
275 a glycosyl moiety on the 3'-position of the aglycone have been identified. Most of them
276 have been isolated from species belonging to Orchidaceae, Leguminoseae, Ranunculaceae,
277 and Gentianaceae; however, a few have been found in Compositae, Liliaceae, Rhamnaceae,
278 Nymphaceae, Lobeliaceae, and Commelinaceae (Andersen & Jorheim, 2006).
279 Anthocyanins like compound **1** with a sugar moiety on the 3'-position of the aglycone have
280 not been previously reported in Solanaceae family and this is the first time that this
281 compound is reported as constituent of *Solanum betaceum* Cav.

282 Compound **2** was characterised by the loss of 469 u from the molecular ion *m/z* 740
283 resulting in a fragment ion of pelargonidin aglycone (271). On the basis of the literature
284 (Giusti, Rodríguez-Saona, Griffin, & Wrolstad, 1999), the 469 u was indicative of the
285 anthocyanin disaccharide attached to another moiety of 146 u, which was assigned to a
286 coumaric acid residue, due to the maximum UV-Vis absorption at λ 315 nm. The ^{1}H NMR

287 spectrum of this compound (**Table 2**) showed characteristic signals of a *p*-disubstituted
288 benzene ring at δ_H 6.80 (d, J = 8.3 Hz, 2H) and 7.37 (d, J = 9.5 Hz, 2H), as well as those of
289 an *E*-olefinic bond at δ_H 6.28 (d, J = 15.7 Hz) and 7.59 (d, J = 15.7 Hz), thus confirming the
290 presence of a *p*-coumaroyl residue in this compound. The ^{1}H NMR data of sugar moiety
291 were in agreement with the data of compound **1**, evidencing the presence of a 3-rutinoside
292 moiety. Due to the tiny amount of this compound ^{13}C and 2D-NMR spectra were not
293 acquired, so the attached position of *p*-coumaroyl residue was not able to be defined. Thus,
294 compound **2** was tentatively identified as pelargonidin-*p*-coumaroyl-rutinoside.

295 Based on 1D- and 2D-^{1}H and ^{13}C-NMR data (**Table 3**), the identities of the main
296 compounds were found to be delphinidin-3-*O*-(6''-*O*-α-rhamnopyranosyl)-β-
297 glucopyranoside (**3**), cyanidin-3-*O*-(6''-*O*-α-rhamnopyranosyl)-β-glucopyranoside (**4**), and
298 pelargonidin-3-*O*-(6''-*O*-α-rhamnopyranosyl)-β-glucopyranoside (**5**). ^{1}H-NMR data of
299 these compounds are not shown because the values for aglycon protons were in agreement
300 with those published previously by Andersen and Fossen (2001), and the data of sugar
301 moieties were similar to compound **1**. These results partially agree with those obtained for
302 the tamarillo from Brazil (Vera de Rosso & Mercadante, 2007). They only used HPLC-
303 PDA-MS/MS for the analysis and reported delphinidin 3-rutinoside, cyanidin 3-rutinoside,
304 and pelargonidin-3-glucoside-5-rhamnoside as major tamarillo pigments. In the present
305 work, the structure of the anthocyanin **5** could be unequivocally established.
306
307 *3.2. Identification of anthocyanins in Andes berry fruit*
308

309 The contents of the major anthocyanins in Andes berry fruit are seen in **Table 1**.
310 The HPLC-PDA profile at 520 nm (**Figure 2B**) revealed the presence of two major
311 anthocyanins in Andes berry ARE, cyanidin-3-*O*-(6''-*O*-α-rhamnopyranosyl)-β-
312 glucopyranoside (**4**) and compound **7**.

313 The UV-Vis spectra of the compound **7** showed typical absorption maxima at λ 271
314 and 516 nm. LC-MS analyses evidenced a pseudomolecular ion in the ESI^+ mode $[M]^+$ at
315 *m/z* 727. The fragment ions at *m/z* 287 $[M-132-146-162]^+$, 449 $[M-132-146]^+$, 581 [M-
316 $146]^+$, and 595 $[M-132]^+$ further revealed the presence of one hexose, one methylpentose,
317 and one pentose. Moreover the fragment ion at *m/z* 287 is in agreement with cyanidin being
318 the aglycone. For structure elucidation and NMR signal assignment 2D NMR
319 measurements were performed. The cyanidin moiety was confirmed from the protons of the
320 two 3H ABX systems in the ^{1}H-NMR spectrum. The first typical 3H ABX system could be
321 seen for H-C(6´) at δ 8.29 ppm (dd, 1H, J = 8.6 Hz, 2.4 Hz), H-C(2´) at δ 8.02 ppm (d, 1H,
322 J = 2.3 Hz) and H-C(5´) at δ 7.02 ppm (d, 1H, J = 8.8 Hz) and the second 3H ABX system
323 for H-C(4) at δ 8.87 (brs, 1H), H-C(8) at δ 6.89 (d,1H, J = 2.0 Hz) and H-C(6) at δ 6.66 (d,
324 1H, J = 1.9 Hz). Additionally, the ^{1}H NMR spectrum of compound **7** displayed the three
325 anomeric protons of one hexosyl, one methylpentose, and one pentose moieties, resonated
326 at δ_H 5.45, 4.63, and 4.76 ppm. The anomeric proton of the rhamnosyl moiety showed a
327 coupling constant of 1.6 Hz, which indicates an *α*-configuration, while the glycosyl and
328 xylosyl moieties showed coupling constants of 7.5 and 7.6 Hz, thus indicating *β*-
329 configurations. The identification of glycosidically bound carbohydrates was further
330 confirmed by acid hydrolysis followed by high performance ion chromatography of the

331 corresponding monosaccharides. The sugars of compound **7** were determined to be glucose,

332 rhamnose, and xylose by comparison with authentic compounds. The assignment of the

333 sugar moieties as well as the linkage of the sugar to the aglycone was performed by COSY-

334 , HMQC-, HMBC-, and TOCSY-experiments. On the basis of the heteronuclear data, the

335 structure of compound **7** was identified as cyanidin-3-*O*-(2´´-*O*-*β*-D-xylopyranosyl-6´´-*O*-

336 *α*-L-rhamnopyranosyl-*β*-D-glucopyranoside) previously reported in fruit skin of *Kadsura*

337 *japonica* (Ishikura, 1971), red currant *Ribes rubrum* L. (Goiffon, Mouly & Gaydou, 1999),

338 berries of *Viburnum opulus* L. (Jordheim, Giske, & Andersen, 2007), and black raspberry

339 and raspberry extracts (Tulio et al., 2008), among others. However, to the best of our

340 knowledge this is the first time that is reported as a constituent of *Rubus glaucus* Benth.

341 With an analysis similar to that described above and based on ESI-MS and ^{1}H and

342 ^{13}C NMR data, the identity of anthocyanins **5** and **6** was elucidated as pelargonidin-3-*O*-

343 (6’’-*O*-α-rhamnopyranosyl)-β-glucopyranoside, and cyanidin-3-*O*-*β*-D-glucopyranoside,

344 respectively. The NMR data were in agreement with those before published (Andersen &

345 Fossen, 2001).

346

347 **4. Conclusion**

348

349 The novel delphinidin 3-*O*-*α*-L-rhamnopyranosyl-(1→6)-*β*-D-glucopyranoside-3’-

350 *O*-*β*-D-glucopyranoside was isolated and identified for the first time in tamarillo ARE

351 (*Solanum betaceum* Cav.) as a minor constituent. In addition, the previously reported 3-*O*-

352 rutinosides of cyanidin, delphinidin, and pelargonidin were isolated and their identification

353 was mainly based on 2D-NMR spectroscopy and MS. The unequivocal composition of
354 *Rubus glaucus* Benth ARE was also reported, with cyanidin-3-*O*-(2´´-*O*-*β*-D-
355 xylopyranosyl-6´´-*O*-*α*-L-rhamnopyranosyl-*β*-D-glucopyranoside) and cyanidin-3-*O*-(6''-
356 *O*-α-rhamnopyranosyl)-β-glucopyranoside, being the main constituents. These tropical
357 fruits could be considered as good source of natural pigments with potential antioxidant
358 activity.
359

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361
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364

365 **References**

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Figure Captions

Fig. 1. a) Andes berry (*Rubus glaucus* Benth) and b) tamarillo (*Solanum betaceum* Cav., red variety) fruits.

Fig. 2. HPLC analyses (λ= 520 nm) of a) ARE of tamarillo (*Solanum betaceum* Cav.) fruit, and b) Andes berry (*Rubus glaucus* Benth.) fruit. Peak numbers correspond to the compound numbers in **Table 1.**

Fig. 3. Chemical structure of tamarillo and mora anthocyanins. Glc = glucose, Xyl = xylose.

Figure 1.

A **B**

Figure 2.

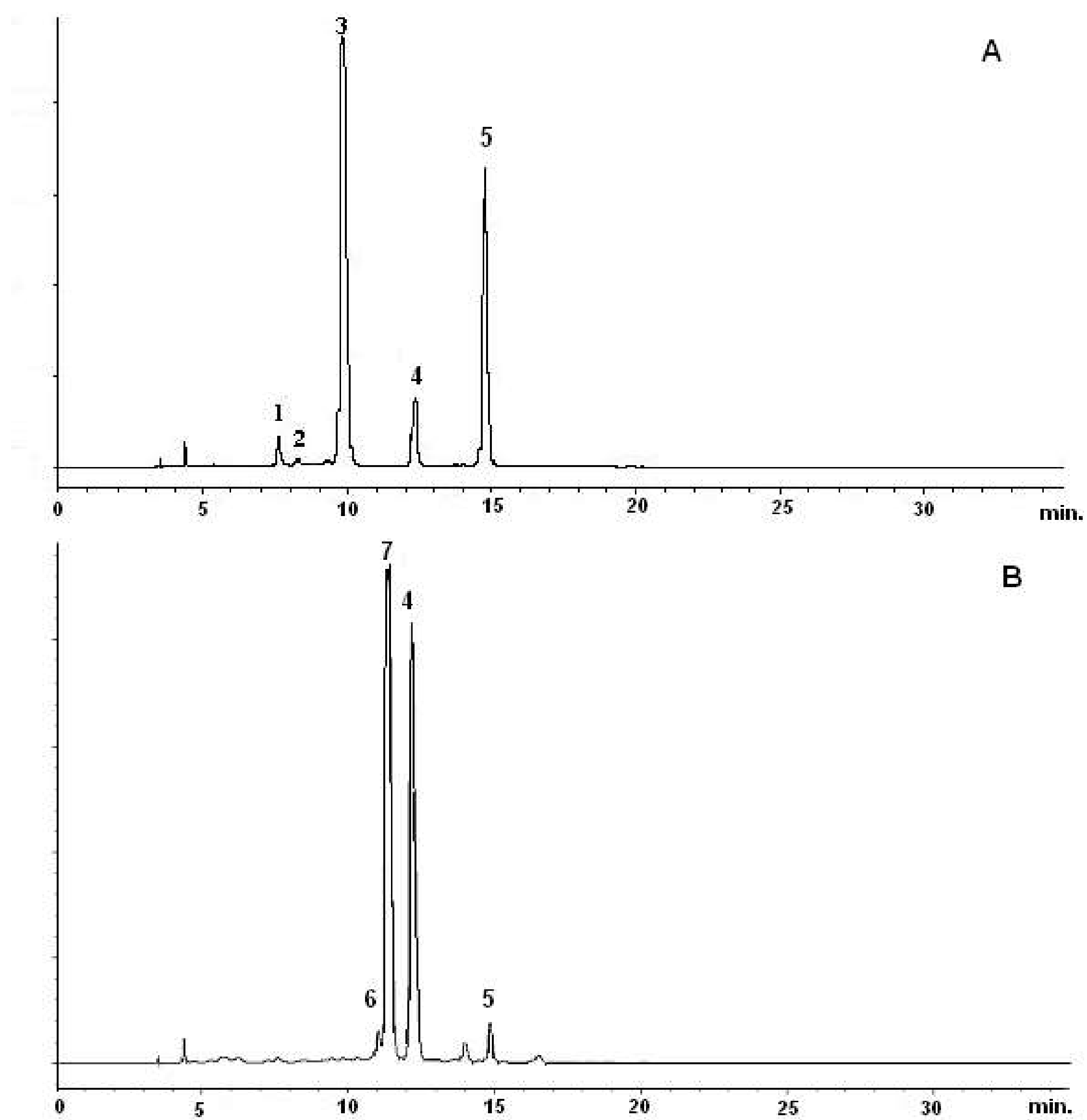
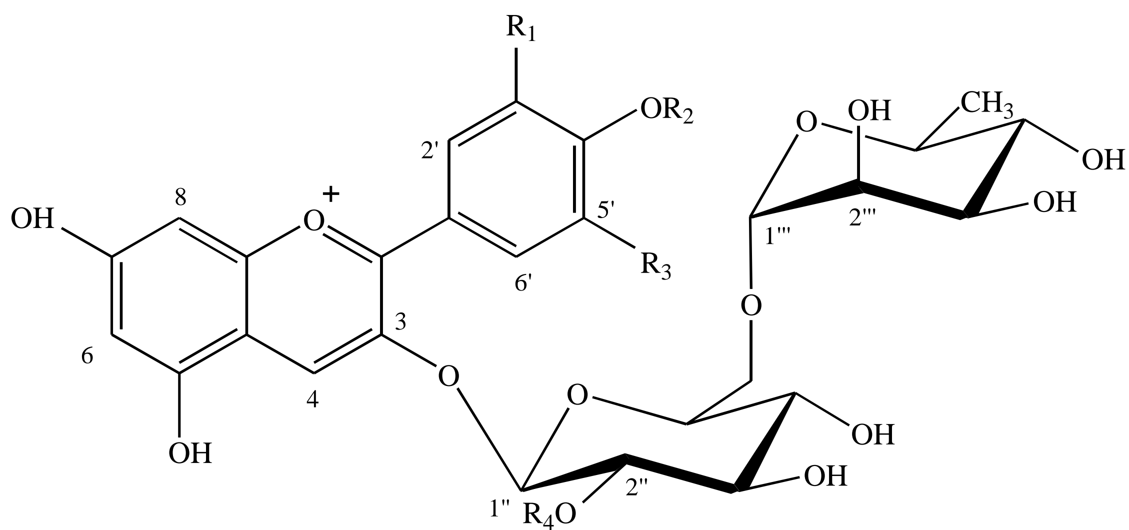


Figure 3.



	R_1	R_2	R_3	R_4
1	OGlc	H	OH	H
3	OH	H	OH	H
4	OH	H	H	H
5	H	H	H	H
7	OH	OH	H	Xyl

Table 1

Chromatographic, spectroscopic, and spectrometric data of the anthocyanins from Tamarillo (*Solanum betaceum* Cav.) and Andes berry (*Rubus glaucus* Benth) fruits.

Compd.[a]	fraction (MLCCC)	t_R (min) (HPLC)[a]	Fragment ions (*m/z*)		λ_{max}(nm)[b]	Amount
			ESI-MS	FAB-MS		
Tamarillo						
1	F2	7.6	773 $[M]^+$, 303 $[M\text{-}162\text{-}162\text{-}146]^+$	795 $[M+Na\text{-}H]^+$, 773 $[M^+]$	514, 241	138.0 ± 17.3[c,d]
2	F2	8.3	741 $[M]^+$, 579 $[M\text{-}162]^+$, 433 $[M\text{-}162\text{-}146]^+$, 271 $[M\ \text{-}162\text{-}162\text{-}146]^+$	763 $[M+Na\text{-}H]^+$, 741 $[M]^+$	496, 413, 315, 255	32.0 ± 0.6[c,d]
3	F3	9.8	611 $[M]^+$, 303 $[M\text{-}162\text{-}146]^+$	633 $[M+Na\text{-}H]^+$, 611 $[M]^+$	518	4934.0 ± 98.7[c,d]
4	F3	12.4	595 $[M]^+$, 287 $[M\text{-}162\text{-}146]^+$	617 $[M+Na\text{-}H]^+$, 595 $[M]^+$	512	438.0 ± 21.9[c,d]
5	F4	14.9	579 $[M]^+$, 271 $[M\text{-}162\text{-}146]^+$	601 $[M+Na\text{-}H]^+$, 579 $[M]^+$	500, 430	2276.0 ± 56.9[c,d]
Andes berry						
6	F8	11.1	449 $[M]^+$, 287 $[M\text{-}162]^+$	-	-	-
7	F2	11.5	727 $[M]^+$, 595 $[M\text{-}132]^{+f}$, 581 $[M\text{-}146]^{+e}$, 449 $[M\text{-}146\text{-}132]^{+f}$, 287 $[M^+\text{-}162\text{-}146\text{-}132]^{+f}$	751 $[M+Na\text{-}H]^+$	516, 271	5063.2[e]

4	F4	12.2	595 $[M]^+$, 287 $[M-162-146]^+$	617 $[M+Na-H]^+$	512, 336, 279	4403.1[e]
5	F6	14.8	579 $[M]^+$, 433 $[M-146]^+$, 271 $[M-162-146]^+$	-	498, 430, 330, 280	440.1[e]

[a] Compound numbers and retention times refer to the numbers given in **Figures 2** and **3**; [b] measured in acidified aqueous solution (5x 10^{-5} M); [c] values are expressed as means ± SE from four measurements; [d] mg Dp-3 rut/100 g ARE; [e] mg Cy-3 rut/100 g ARE; [f] these fragments were obtained by MS/MS; -not determined.

Table 2

^{1}H NMR chemical shifts and [1]H-^{1}H coupling constants for the anthocyanins **1, 2** and **7**. (δin ppm, *J* in Hz).

C	**1**[a]	**2**[a]	**7**[b]
Aglycone			
4	8.98, s	9.14, s	8.87, brs
6	6.67, d 1.9	6.73, d 1.9	6.66, d 1.9
8	6.99, d 1.9	6.98, d 1.9	6.89, d 2.0
2’	8.09, d 2.2	8.65, brd 9.0	8.02, d 2.3
3’	-	7.00, brd 9.0	-
5’	-	7.00, brd 9.0	7.02, d, 8.8
6’	7.98, d 2.2	8.65, brd 9.0	8.29, dd 8.6, 2.4
3-O-glucoside			
1’’	5.28, d 7.7	5.30, d 7.6	5.45, d 7.5
2’’	3.73, dd 9.2, 7.7	3.73, dd 9.1, 7.6	3.97, dd 9.0, 7.8
3’’	3.56, t 9.1	3.56, t 9.5	3.79, t 9.4
4’’	3.43, dd 9.7, 8.7	3.43, dd 9.6, 8.6	3.50, t 9.4
5’’	3.53, m	3.50, m	3.52-3.64, m
6’’ A	4.08, dd 11.1, 1.9	4.06, dd 11.3, 1.9	3.67-3.79, m
6’’ B	3.61, dd 11.3, 6.2	3.61, m	4.02, dd 11.5, 1.6
6’’-O-rhamnosyl			
1’’’	4.66, d 1.8	4.66, d 1.4	4.63, d 1.6
2’’’	3.79, dd 3.6, 1.2	3.80, dd 3.5, 1.2	3.67-3.79, m
3’’’	3.62, dd 9.3, 3.3	3.65, dd 9.0, 3.0	3.52-3.64, m
4’’’	3.34, t 9.6	3.34, t 9.1	3.31, m, overlapped
5’’’	3.55, dd 9.4, 6.7	3.54, m	3.60, dd 9.3, 6.2
6’’’	1.17, d 6.3	1.18, d 6.3	1.14, d 6.5
3’-O-glucoside			
1’’’’	5.05, d 7.3		
2’’’’	3.60, dd 9.6, 6.8		
3’’’’	3.57, t 9.2		
4’’’’	3.43, dd 9.9, 8.3		
5’’’’	3.59, m		
6’’’’ A	4.01, dd, 12.2 2.6		
6’’’’ B	3.78, dd 12.1, 6.0		
2´´-O-xylofuranosyl			

1''''		4.76, d 7.6
2''''		3.20, dd 9.0, 7.5 Hz
3''''		3.32, m, overlapped
4''''		3.38-3.47, m
5''''A		3.74, dd 11.5, 5.5
5''''B		3.05, dd 11.7, 1.1
Coumaroyl		
α	6.28, d 15.7	
β	7.59, d 15.7	
2''''	7.37, d 9.5	
3''''	6.80, d 8.3	
5''''	6.80, d 8.3	
6''''	7.37, d 9.5	

[a] CD_3OD- CF_3COOD (19:1, v/v), 500 MHz, [b] CD_3OD- CF_3COOD (98:2, v/v), 500 MHz.

Table 3

^{13}C NMR chemicals shifts for the anthocyanins **1**, **3**-**5**, **7** (δn ppm).

C	**1**[a]	**3**[a]	**4**[a]	**5**[a]	**7**[b]
Aglycone					
2	162.9	163.8	163.2	163.4	164.3
3	144.5	145.8	144.3	144.2	145.4
4	136.9	135.7	136.5	137.6	135.5
5	157.4	158.0	156.9	157.1	159.1
6	103.4	103.2	103.4	103.5	103.5
7	170.3	170.3	168.4	170.1	170.4
8	95.2	93.8	95.4	95.1	95.2
9	155.8	156.3	156.2	156.5	157.6
10	112.7	113.0	111.8	112.0	113.1
1’	119.5	120.0	120.1	119.8	121.3
2’	113.0	112.8	118.4	135.7	118.6
3’	146.6	147.7	146.2	117.5	147.6
4’	156.4	144.8	154.6	165.7	156.0
5’	145.2	147.7	117.7	117.5	117.4
6’	114.4	112.8	128.6	135.7	128.9
3-O-glucoside					
1’’	103.6	103.5	103.8	104.0	101.3
2’’	76.5	74.6	74.9	74.4	81.7
3’’	77.9	76.6	77.5	74.8	77.4
4’’	71.2	77.6	78.0	77.2	71.0
5’’	74.2	72.4	74.0	73.6	77.9
6’’	67.9	67.8	67.7	67.6	67.7
6’’-O-rhamnosyl					
1’’’	102.4	102.1	102.2	102.1	102.2
2’’’	71.9	69.7	72.6	72.5	71.9
3’’’	72.3	73.9	70.5	73.9	72.5
4’’’	74.7	71.3	71.2	69.7	73.9
5’’’	69.8	67.7	67.7	71.3	69.8
6’’’	17.9	17.8	17.9	17.9	17.9
3’-O-glucoside					
1’’’’	104.5				
2’’’’	72.3				
3’’’’	74.9				
4’’’’	71.2				
5’’’’	74.0				
6’’’’	62.8				
2´´-O-xylofuranosyl					
1´´´´					105.6

2′′′′′	75.8
3′′′′′	78.1
4′′′′′	70.9
5′′′′′	67.3

[a] CD_3OD-CF_3COOD (19:1, v/v), 125 MHz, [b] CD_3OD- CF_3COOD (98:2, v/v), 125 MHz.