Anthocyanins in Berries of Maqui (*Aristotelia chilensis* (Mol.) Stuntz)

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The anthocyanin composition of berries of Maqui [*Aristotelia chilensis* (Mol.) Stuntz] was determined by HPLC with photodiode array and MS detection. Eight pigments corresponding to the 3-glucosides, 3,5-diglucosides, 3-sambubiosides and 3-sambubioside-5-glucosides of delphinidin and cyanidin were identified, the principal anthocyanin being delphinidin 3-sambubioside-5-glucoside (34% of total anthocyanins). The average total anthocyanin content was 137.6 ± 0.4 mg/100 g of fresh fruit (211.9 ± 0.6 mg/100 g of dry fruit). The relative high anthocyanin content and the important presence of polar polyglycosylated derivatives makes the fruits of *A. chilensis* an interesting source of anthocyanin extracts for food and pharmaceutical uses.

Keywords: Quantitative HPLC; anthocyanins, sambubiosides, delphinidin, cyanidin; berries; *Aristotelia chilensis*; Maqui.

INTRODUCTION

Maqui [*Aristotelia chilensis* (Mol.) Stuntz] is a native South America evergreen shrub that grows in dense thickets and can reach 3–5 m in height. It is a dioecious plant that belongs to the family Elaeocarpaceae and produces small edible purple/black berries, about 6 mm in diameter, that are eaten fresh or used for juice, jams or wine-making. The plant prefers slightly acidic, moderately fertile and well-drained soils. It grows rapidly with adequate moisture and readily colonises abandoned, burned or overexploited soils, thus protecting them from erosion. The intense red colour of the aqueous extract of its fruit is due to the presence of anthocyanin pigments causing it to be used as a natural dye. The leaves and fruits are astringent and have been used in Chilean folk medicine as anti-diarrhoeic, anti-inflammatory, anti-haemorrhagic and febrifuge. (Hoffmann *et al.*, 1992).

Previous reports concerning the chemical composition of *A. chilensis* showed the presence of indole and quinoline alkaloids (Silva, 1992; Kan *et al.*, 1997), as well as high levels of polyphenols that have been suggested to be responsible for the high *in vitro* antioxidant activity exhibited by the juice of the fruit (Miranda-Rottmann *et al.*, 2002). Recently, particular attention has been paid to polyphenols and especially the anthocyanins present in the berries, not only for their use as natural colorants, but also for their potential beneficial effects on human health, including suggestions that they be used as dietary supplements in functional food products (Du *et al.*, 2004). Potential effects include anti-oxidant (Heinonen *et al.*, 1998; Prior *et al.*, 1998; Connor *et al.*, 2002; Miranda-Rottmann *et al.*, 2002) and anti-atherogenic activities (Miranda-Rottmann *et al.*, 2002), inhibition of HIV virus (Andersen and Helland, 1995), prevention of visual problems (Morazzoni and Bombardelli, 1996; Sparrow *et al.*, 2003) and activity against some types of human leukaemia and human colon carcinoma (Kamei *et al.*, 1995; Katsube *et al.*, 2003).

The development of techniques such as HPLC coupled to PAD and MS detectors has allowed rapid advances in the identification of the anthocyanin composition of many plants (Giusti *et al.*, 1999; Da Costa *et al.*, 2000; De Pascual-Teresa and Rivas-Gonzalo, 2003). However, the anthocyanin composition of the berries of *A. chilensis* has been scarcely studied. According to our knowledge, only two reports exist concerning the tentative identification of some mono- and di-glucosides of delphinidin, cyanidin, malvidin and petunidin, and these were based on TLC (Mazza and Miniati, 1993) and spectrophotometric characteristics (Díaz *et al.*, 1984) for which no further confirmation has been made. The aim of the present work was to revise and update information about the anthocyanin composition of the berries of *A. chilensis* using modern HPLC-coupled techniques.

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EXPERIMENTAL

Chemicals and standards. Delphinidin-3-glucoside and cyanidin-3-glucoside were purchased from Polyphenols Labs (Sandnes, Norway). D(+)-glucose, D(+)-galactose, L(+)-arabinose, α-L-rhamnose and D(+)-mannose were purchased from Sigma-Aldrich (Steinhein, Germany), and D(+)-xylose was from Panreac Química (Barcelona, Spain). Solvents used were HPLC-grade and purchased from Merck (Darmstadt, Germany). All other chemicals were analytical grade and were supplied by Panreac Química.

Plant material. Wild fruits of *Aristotelia chilensis* (Mol.) Stuntz were picked at maturity in the University Campus of Santiago, Chile. The taxonomic identity was confirmed by comparing with the authenticated herbarium specimen (SQF 17092) at Santiago University herbaria. Fruits were weighed, freeze-dried and stored in dark glass containers until required for analysis.

Extraction of fruit material. The desiccated samples were homogenised in methanol containing 0.1% of concentrated hydrochloric acid, kept overnight at 3–5°C, and then filtered through a Büchner funnel under vacuum. The solid residue was washed exhaustively with methanol, the filtrates obtained were centrifuged (4000 ***g***; 15 min; 2°C) and the solid residue further treated using the same protocol until all of the coloured material was completely extracted. All of the methanolic extracts were combined, a small volume of water was added and the extract concentrated (30°C) by rotary evaporation to remove the methanol. The aqueous extract obtained was washed with *n*-hexane to remove lipids and further purified by CC over a mixed stationary phase composed of 20% Polyclar AT (PVP) and 80% silica gel prepared as previously described (Escribano-Bailón *et al.*, 2002). The extract was carefully added to the column, which was washed with water to remove sugars and acids, and then eluted with methanol containing 0.1% hydrochloric acid in order to obtain the anthocyanins. After adding a little water, the methanol was removed from the eluate in a rotary evaporator and the aqueous extract adjusted with water to a known volume for further analysis.

Fractionation of anthocyanins. The purified berry extract was submitted to semi-preparative HPLC using a Waters (Milford, MA USA) model 600 pump and a Ultracarb ODS20 column (250 × 10 mm i.d.; 5 μm) (Phenomenex, Torrance, CA, USA). The aqueous extract was concentrated at the head of the column and ultrapure (100%) water was passed through the column for 15 min at a flow rate of 3 mL/min. The column was eluted with a mobile phase consisting of 5% acetic acid (solvent A) and methanol (solvent B) initially at 90:10 (A:B), increased to 85:15 over 20 min, followed by isocratic elution at 85:15 for 10 min, increased to 82:18 over 5 min, then to 60:40 18% B over 10 min, and finally to 0:100 over 15 min. The flow rate was 3 mL/min and detection was at 520 nm. Fractions containing anthocyanins were collected in an autosampler, concentrated under vacuum, redissolved in 0.1 M hydrochloric acid and freeze-dried. The purity of the anthocyanins obtained was assessed by HPLC-PAD-MS.

HPLC-PAD-MS analysis. A Hewlett-Packard (Agilent Technologies Inc, Palo Alto, CA, USA) model 1100 system equipped with a quaternary pump, a PAD and a Spherisorb (Waters Corporation, Milford, Massachusetts, USA) ODS2 column (150 × 4.6 mm; 3 μm), thermostated at 35°C, was employed. The mobile phase consisted of 0.1% trifluoroacetic acid (solvent C) and acetonitrile (solvent D), with initial isocratic elution at 90:10 (C:D) for 5 min, increased to 85:15 over 15 min, followed by isocratic elution at 85:15 for 5 min, and finally increased to 65:35 over 15 min. The flow rate was 0.5 mL/min. Eluent flow from the column passed first through the PAD and then to the probe of the MS. Mass spectrometry was performed using a Finnigan LCQ (Thermoquest, San José, CA, USA) equipped with an API source and employing an electrospray ionisation (ESI) interface. Both auxiliary and sheath gases were a mixture of nitrogen and helium. The capillary temperature was 180°C and the capillary voltage 3 V. The MS detector was programmed to perform two consecutive scans: a full scan in the range m/z 120–1500, and an MS^2 scan of the most abundant ion using a relative collision energy of 20%. Spectra were recorded in the positive ion mode.

Quantification of anthocyanins was performed from the peak areas recorded at 520 nm by reference to a calibration curve obtained with a standard of delphinidin-3-glucoside. Berry extracts were analysed in triplicate.

Acid hydrolysis. Isolated anthocyanins were dissolved in 6 M hydrochloric acid and heated to 100°C in screw-cap test tubes for 40 min. Subsequently, the extract was cooled, concentrated under vacuum, and the residue dissolved in 1 mL of water. An aliquot (100 μL) of the solution was analysed by HPLC-PAD for identification of the hydrolysed anthocyanidins. The remaining portion was loaded onto a Waters C18 Sep-Pak cartridge and eluted with water, concentrated under vacuum, and the residue recovered in a minimum volume of water for HPTLC analysis of free sugars.

Analysis of sugars. HPTLC was carried out using silica gel 60 layers (5 × 5 cm; Merck) according to the

Table 1 HPLC retention time (R_t), spectral characteristics, protonated molecular ion and main MS^2 fragments of the anthocyanins detected in berries of *Aristotelia chilensis*

Peak number[a]	R_t (min)	λ_{max} (nm)	Shoulder at 440 nm	Molecular ion $[M^+]$ (m/z)	Fragment ions in MS^2 (m/z)
1	8.2	524	no	759	465, 597, 303
2	9.8	524	no	627	465, 303
3a	11.9	516	no	743	449, 581, 287
3b	12.2	516	no	611	449, 287
4	13.9	524	yes	597	303
5	15.3	524	yes	465	303
6	17.6	516	yes	581	287
7	18.4	516	yes	449	287

[a] Peak numbering as in Fig. 1: for the proposed identities of compounds see Fig. 4.

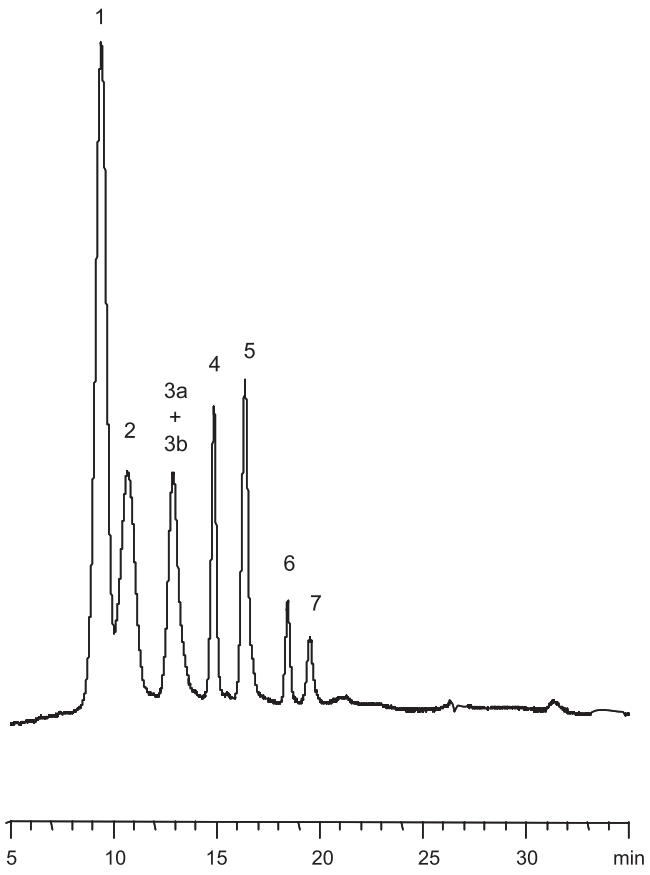


Figure 1 HPLC chromatogram recorded at 520 nm showing the anthocyanin profile of an extract of Maqui fruits (*Aristotelia chilensis*). The proposed identities of compounds associated with the peaks shown are given in Fig. 4. (For chromatographic protocol see Experimental section.)

procedure previously described (Di Paola-Naranjo *et al.*, 2004). The identification of sugars was performed by comparison with standards of D(+)-glucose, D(+)-galactose, L(+)-arabinose, α-L-rhamnose, D(+)-mannose and D(+)-xylose.

RESULTS AND DISCUSSION

Identification of anthocyanins

Figure 1 shows the HPLC profile of the anthocyanins present in an extract of fruits of *Aristotelia chilensis*. Data (retention time, λ_{max} in the visible region, molecular ion and main fragments observed in MS^2) obtained for the anthocyanin peaks in the HPLC-PAD-MS analysis are summarised in Table 1.

Peaks 5 and 7 were readily identified as being associated with delphinidin-3-glucoside and cyanidin-3-glucoside, respectively, by comparison with anthocyanin standards; this was confirmed by UV–vis and MS characteristics (Table 1). Furthermore, the nature of the substituting sugar (glucose) was confirmed by HPTLC after isolation of the compounds and acid hydrolysis.

In determining the structures of the remaining anthocyanins, UV–vis spectra and HPLC retention characteristics were first considered. Compounds associated with peaks 1, 2 and 4, showing λ_{max} at 275, 350 and 524 nm (Fig. 2), similar to that of peak 5, were related to delphinidin derivatives. Similarly, peaks 3 and 6 (λ_{max} at 275 and 516 nm as for peak 7) were assigned as cyanidin-derived anthocyanins. The greater polarity (earlier elution) of the unknown compounds in relation to the corresponding 3-glucosides (peaks 5 and 7) suggested that they were polyglycosylated anthocyanins.

Peak 4 showed a molecular ion at m/z 597 that produced a unique MS^2 fragment at m/z 303 (delphinidin) (Fig. 3). The direct loss of 294 amu (–[162 + 132]) indicated the separation of a disaccharide (hexose + pentose) residue (Giusti *et al.*, 1999; Alcalde-Eon *et al.*, 2004) otherwise fragments corresponding to the sequential loss of the individual sugar residues would have been observed in the MS^2 spectrum. This supposition is also supported by the existence of a shoulder around 440 nm in the visible spectrum of the peak (Fig. 2), which is characteristic of 3-glycosylated

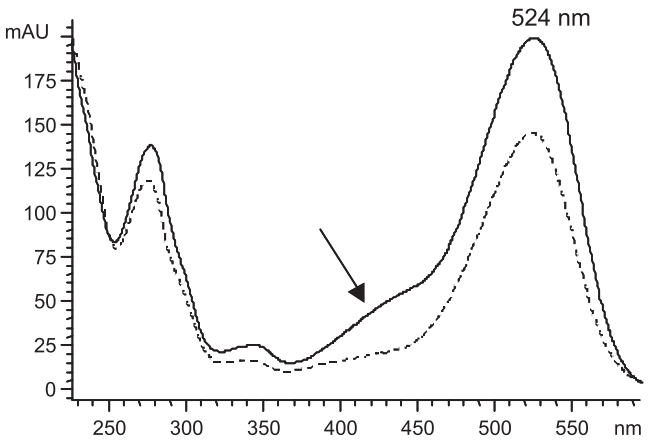


Figure 2 UV–vis spectra of compounds associated with peaks 2 (delphinidin 3,5-diglucoside; dotted line) and 4 (delphinidin 3-sambubioside; solid line) in the chromatogram shown in Fig. 1. The arrow indicates the shoulder around 440 nm that is characteristic of anthocyanidin-3-glycosides.

anthocyanidins (Santos-Buelga *et al.*, 2003). Thus, peak 4 would correspond to a delphinidin-3-diglycoside. In order to identify the sugar residues, the compound was isolated by semi-preparative HPLC and submitted to acid hydrolysis. Analysis by HPTLC revealed glucose and xylose as released sugars. Since sambubiose (i.e. 2-*O*-*β*-D-xylosyl-D-glucose) is the xylosylglucoside most frequently found as an anthocyanin substituent (Mazza and Miniati, 1993), peak 4 was tentatively assigned as delphinidin 3-sambubioside (Fig. 4).

Similar observations to those described for compound 4, regarding fragmentation pattern, absorption spectrum and sugar identification (glucose and xylose) were made for peak 6. This compound showed a molecular ion at m/z 581 that produced a unique MS^2 fragment at m/z 287 (cyanidin), suggesting its tentative identity as cyanidin-3-sambubioside, a major pigment in elderberry (*Sambucus nigra* L.) (see Mazza and Miniati, 1993 and references therein) and other *Sambucus* species (Chiarlo *et al.*, 1978; Johansen *et al.*, 1991; Inami *et al.*, 1996). Cyanidin 3-sambubioside has also been identified in red currant (*Ribes rubrum* L.; Mazza and Miniati, 1993) and fruits of *Viburnum dilatatum* (Kim *et al.*, 2003). Both cyanidin- and delphinidin-3-sambubiosides have been found together in the berries of *Vaccinium padifolium* (Cabrita and Andersen, 1999) and *V. myrtillus* (Du *et al.*, 2004), petals of Roselle (*Hibiscus sabdariffa* L.; Pouget *et al.*, 1990) and purple pods of *Pisum sativum* L. (Mazza and Miniati, 1993). The sambubioside nature of compounds **4** and **6** is consistent with their relative retention times in the HPLC by eluting before the corresponding 3-glucoside derivatives (i.e. delphinidin 3-glucoside, peak 5, and cyanidin 3-glucoside, peak 7) owing to their greater polarity (Santos-Buelga *et al.*, 2003).

A molecular ion at m/z 759 was found for peak 1 that produced MS^2 fragments at m/z 597 (M^+-162, loss of a hexose residue), 465 (M^+-[162+132], loss of hexose + pentose) and 303 (delphinidin) (Fig. 5). Such MS^2 fragments require different substitution positions for each sugar residue on the aglycone. According to the literature (Mazza and Miniati, 1993; Rivas-Gonzalo, 2003), no multi-substituted anthocyanins have been identified that lack a sugar residue at position 3, and the preferred location for a second sugar would be at position 5 rather than at 7 or 4′. The absence of a shoulder around 440 nm in the visible spectrum

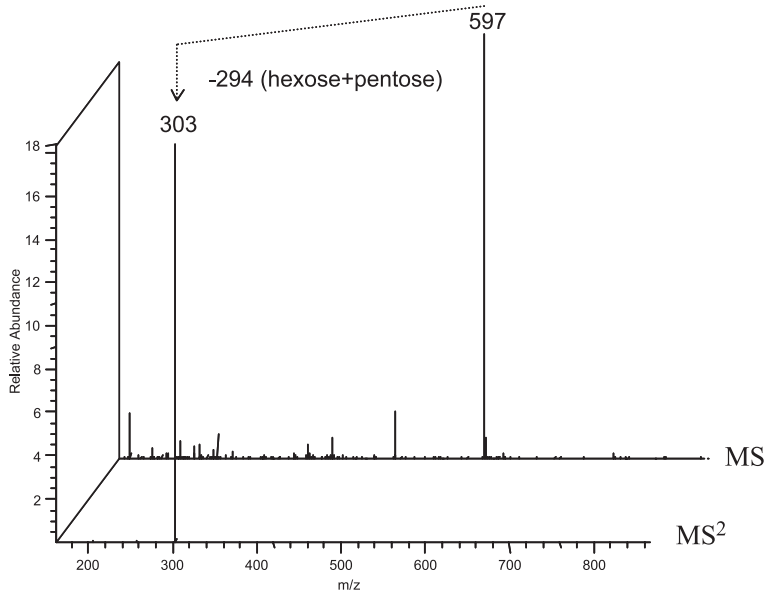
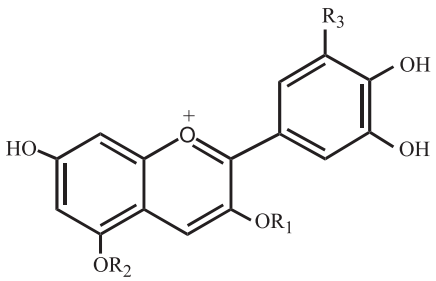


Figure 3 Molecular ion (m/z 597) and MS^2 fragments of compound associated with peak 4 (Fig. 1).



Peak number	R1	R2	R3	Proposed identity
1	Xyl-Glc	Glc	OH	Delphinidin 3-sambubioside-5-glucoside
2	Glc	Glc	OH	Delphinidin 3,5-diglucoside
3a	Xyl-Glc	Glc	H	Cyanidin 3-sambubioside-5-glucoside
3b	Glc	Glc	H	Cyanidin 3,5-diglucoside
4	Xyl-Glc	H	OH	Delphinidin 3-sambubioside
5	Glc	H	OH	Delphinidin 3-glucoside
6	Xyl-Glc	H	H	Cyanidin 3-sambubioside
7	Glc	H	H	Cyanidin 3-glucoside

Figure 4 Suggested identities of the anthocyanins in Maqui fruits. Peak numbers are those shown in the chromatogram of Fig. 1. Glc, glucose; Xyl, xylose.

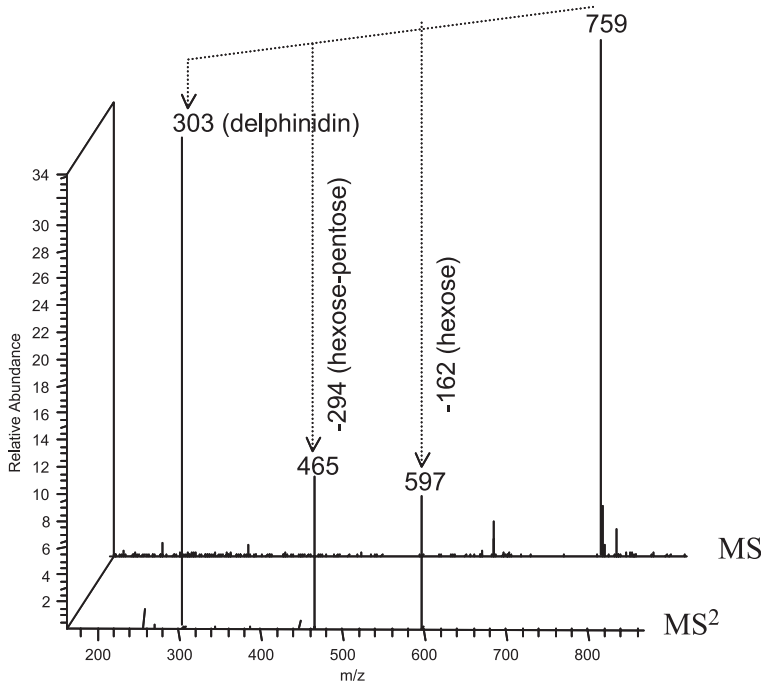


Figure 5 Molecular ion (*m/z* 759) and MS^2 fragments of compound associated with peak 1 (Fig. 1).

(Fig. 2), as observed for anthocyanidin 3,5-diglucosides (Hebrero *et al.*, 1989), would support the existence of substitution at positions 3 and 5 of the aglycone. HPTLC analysis following acid hydrolysis of the isolated anthocyanin revealed glucose and xylose as substituting sugars. Thus, the compound associated with peak 1 was identified as delphinidin 3-sambubioside-5-glucoside. This anthocyanin has been previously

described in purple pods of *P. sativum* L. (Mazza and Miniati, 1993).

Peak 2 showed a molecular ion at *m/z* 627 yielding MS^2 fragments at *m/z* 465 and 303 (delphinidin), resulting from the sequential loss of two hexose residues located at different positions on the anthocyanidin. Glucose was the only sugar released after acid hydrolysis as identified by HPTLC. Following similar reasoning as for the previous anthocyanins, the compound associated with peak 2 was identified as delphinidin 3-glucoside-5-glucoside. This identity was confirmed by comparison of its retention time and spectral characteristics with those of the same compound previously identified in our laboratory in hybrid grapes (Hebrero *et al.*, 1989). The number of sugar residues in compounds **1** and **2** explains their greater polarity (earlier elution) compared with delphinidin 3-glucoside (peak 5). The early elution of peak 2 compared with delphinidin 3-sambubioside (peak 4) is also logical based on the presence of two hexose moieties *versus* one hexose and one pentose.

The tailing character of peak 3 suggested that it was not pure. Even though the UV–vis spectrum did not change across the peak, mass detection revealed the co-elution of two compounds (3a and 3b). The compound associated with peak 3a showed a molecular ion at *m/z* 743 and MS^2 fragments at *m/z* 581 (M^+-162, loss of a hexose residue), 449 ([M^+-[162 + 132], loss of hexose + pentose moieties) and 287 (cyanidin). This fragmentation pattern is similar to that observed for compound **1** and, furthermore, xylose and glucose were detected after acid hydrolysis of the isolated anthocyanin. Thus, peak 3a was identified as being associated with cyanidin 3-sambubioside-5-glucoside. This anthocyanin has been found in *Sambucus* species (Brønnum-Hansen and Hansen, 1983; Mazza and Miniati, 1993; Inami *et al.*, 1996) and purple pods of *P. sativum* L. (Mazza and Miniati, 1993). Furthermore, different acylated derivatives of the compound have been described in various Brassicaceae species, e.g. seedlings of *Sinapis alba* (Takeda *et al.*, 1988), flowers of *Matthiola incana* (Saito *et al.*, 1995) and leaves and stems of *Arabidopsis thaliana* (Bloor and Abrahams, 2002).

Fragmentation of the compound associated with peak 3b (molecular ion at *m/z* 611) yielded fragments at *m/z* 449 [M^+-162] and 287 (cyanidin), and sugar analysis (glucose) was similar to that obtained for peak 2, thus suggesting its identity as cyanidin 3-glucoside-5-glucoside. The identification of the compound associated with peak 3b was confirmed by comparison with standard material and data from our laboratory (Hebrero *et al.*, 1989). The elution of compounds 3a and 3b in relation to cyanidin-3-sambubioside and cyanidin-3-glucoside is coherent with their expected polarity based on the sugar substituents. It is also logical that cyanidin-derived anthocyanins (peaks 3a, 3b, 6 and 7) elute later than their delphinidin-derived counterparts (peaks 1, 2, 4 and 5) showing the same glycosylation pattern.

Among the identified pigments, only delphinidin 3-glucoside and cyanidin 3-glucoside have been previously described in *Aristotelia chilensis* fruits (Díaz *et al.*, 1984). However, the other pigments identified by the these authors were not detected in our extracts, although this could be due to the existence of different Maqui varieties. However, considering that the identifications made in earlier reports were performed using paper chromatography and spectroscopic techniques, and that they have not been confirmed later, we have employed HPLC-PAD-MS techniques to provide more precise information about compound identification.

Content and distribution of anthocyanins in Maqui fruits

The measured concentrations (expressed as delphinidin 3-glucoside equivalents) of the different anthocyanins identified in the fruits of *A. chilensis* are shown in Table 2. Delphinidin derivatives (73%) predominated over those derived from cyanidin (37%), with delphinidin-3-sambubioside-5-glucoside as the major anthocyanin (34% of total anthocyanins). The average total anthocyanin content was 137.6 ± 0.4 mg/100 g of fresh fruit (211.9 ± 0.6 mg/100 g dry weight), which is similar to those concentrations found in other berries considered to be good sources of anthocyanins (Mazza and Miniati, 1993; Clifford, 2000). A prominent feature of the anthocyanin composition of Maqui is that its biosynthetic pathway is largely channelled towards the formation of polyglycosylated derivatives that are highly polar and water-soluble. These characteristics are attractive for extraction and potential use as food colorants, as well as for pharmacological uses.

Table 2 Contents (expressed in equivalents of delphinidin 3-glucoside) and proposed identities of the anthocyanins detected in the berries of *Aristotelia chilensis*

Anthocyanin	Content (mg/100 g)
Delphinidin-3-sambubioside-5-glucoside	46.4 ± 0.1
Delphinidin-3,5-diglucoside	23.7 ± 0.2
Cyanidin-3-sambubioside-5-glucoside Cyanidin-3,5-diglucoside	18.7 ± 0.2
Delphinidin-3-sambubioside	14.2 ± 0.1
Delphinidin-3-glucoside	17.1 ± 0.2
Cyanidin-3-sambubioside	8.9 ± 0.04
Cyanidin-3-glucoside	8.6 ± 0.05
Total anthocyanins	137.6 ± 0.4

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