ANTHOCYANINS THAT CONFER CHARACTERISTIC COLOR TO RED COPIHUE FLOWERS (*LAPAGERIA ROSEA*)

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ABSTRACT

The Copihue (*Lapageria rosea*), also known as the Chilean bellflower, is the national flower of Chile and is the only species in the genus *Lapageria*. The copihue's tepals are commonly red, with white or pink being less common. The red color of the copihue has been glorified in legends, poems and popular songs. The present work studies the pigments that confer red copihues their characteristic color. The principal types of cyanidin present in red copihue's tepals are cyanidin-3-*O*-rhamnosylglucoside, followed by cyanidin-3-*O*-glucoside, and while only the latter is detected in pink tepals and neither one are detected in white flowers.

Based on the obtained results by HPLC-ESI-MSⁿ and HPLC-DAD, it is concluded that rhamnosyl- and glucosyl-derivatives of cyanidin, which present respectively an absorption maximum at 518 and 516 nm, confer the characteristic red color to red copihues. Furthermore, glycosylated cyanidin derivatives, pigments derived from other anthocyanidins, were not detected in red copihue flowers even when they are present in other red flowering plants.

Keywords: flower, copihue, *Lapageria rosea*, anthocyanins, cyanidin-3-*O*-rutinoside, cyanidin-3-*O*-glucoside.

INTRODUCTION

The copihue (*Lapageria rosea*), also known as the Chilean Bellflower and the Chilean Glory Flower, is the national flower of Chile (Fig. 1). It grows in forests in southern Chile, forming part of the Valdivian flora. It is the only species in the genus *Lapageria*, and is an evergreen climbing plant reaching up to 10 m high among shrubs and trees¹. The flowers are between 5 to 10 cm long, with six red, pink or white tepals (Fig. 1). It was first described by Ruiz y Pavón in 1802, and was named *Lapageria rosea* in honor of Joséphine Beauharnais de Lapagerie, the first wife of emperor Napoleon I². In the scientific literature, studies have been reported on pollination³, culture^{5,6} and genetics⁷, but since Lawrence et al 1938⁸ there isn't any publication about the pigments which confer the characteristic red color to the national flower of Chile, which has been glorified in legends, poems and popular songs^{9,10,11}. Currently, one of the techniques used for anthocyanins identification in fruits and plants has been HPLC-MS¹²⁻¹⁴.

The present work studies the pigments present in the red copihue's tepals by HPLC-ESI-MSⁿ and HPLC-DAD, comparing their anthocyanin profiles with those of the less common pink and white flowering copihue tepals.

Figure 1: White, pink and red copihue flowers

EXPERIMENTAL

Samples of copihue tepals.

Samples of copihue flowers were collected at the end of the flowering season because this plant is a protected species. Red copihue tepals were collected at two places in the Araucanía Region in Southern Chile between May and July 2007. Red copihue tepals were collected on the "El Encanto Farm", Gorbea (39° 06' 17", 72° 41' 46" W), and tepals of red, pink and white flowering copihue varieties were obtained at the Llamas del Sur Farm

in Huichahue, Padre Las Casas. The samples were kept at -4 °C until their extraction for analysis.

Reference compounds and chemicals

Anthocyanin reference compounds were purchased from Extrasynthese (Lyon, France) as part of a cyanidin derivatives kit, which contained respectively as chlorides cyanidin, kuromanin (cyanidin-3-*O*-glucoside), ideain (cyanidin-3-*O*-galactoside chloride), keracyanin (cyanidin-3-*O*-rutinoside). Additionally, 3-*O*-glucosides derived from delphinidin, peonidin, petunidin, malvidin, as well as other anthocyanins were also obtained from the Polyphenols Laboratories AS (Sandness, Norway), Extrasynthese (Lyon, France), and PhytoLab (Vestenbergsgreuth, Germany), and were submitted to HPLC analysis as described below. All solvents were HPLC grade and water was of Milli-Q quality.

Sample Extraction Procedure

A total of 10 g of tepals were ground in 3% (v/v) formic acid in methanol. The homogenized mixture was centrifuged at 3000 rpm for 10 min, collecting the supernatant. The extracts were filtered through a GV Durapore filter (0.22 mm pore size, 13 mm diameter, Millipore, USA).

HPLC with diode array detection (HPLC-DAD) analysis

The HPLC-DAD analysis protocol based on the International Organization of Vine and Wine (OIV) resolution OENO 22/2003, with minor modifications, was described recently by von Baer et al¹⁵. The chromatographic system consisted of a quaternary LC-10ADVP pump, a FCV-10ALVP elution unit, a DGU-14 degassing unit, a SIL-10ADVP autoinjector, a CTO-10AVP column oven, a SPD-M10AVP diode array UV/VIS detector, a SCL-10AVP controller and a CLASS-VP data system, all from Shimadzu Corporation, Japan. Zorbax Eclipse XDB (C-18), 4,6 µm, 250 x 4,6 mm. i.d. column (Agilent, USA) were set up at 40 °C and a flow rate of 0.8 mL min⁻¹. Sample volume was 50 µL and detection at 518 nm. Formic acid (p.a. 98-100%), Milli-Q water and HPLC grade acetonitrile were used for gradient elution. Solvent A was water/formic acid/acetone nitrile 87:10:3 (v/v/v); solvent B was water/formic acid/acetone nitrile 40:10:50 (v/v/v). The gradient starts linear at 6% B up to 30% B at 15 minutes, 50% B at 30 minutes, 60% B at 35 minutes and then down to 6% B at 41 minutes. This gradient is followed by a post-run time of 5 minutes at initial conditions.

HPLC with Electrospray Ionization Multiple Mass Spectrometry (HPLC-ESI-MSⁿ).

HPLC identification of copihue tepals pigments were performed on an Agilent 1100 Series system (Agilent, Germany), equipped with DAD (G1315B) and LC/MSD Trap VL (G2445C VL) electrospray ionization mass spectrometry (ESI-MSⁿ) system, and coupled to an Agilent Chem Station (version B.01.03)

data-processing station. The mass spectra data were processed with the Agilent LC/MS Trap software (version 5.3). The copihue tepals extracts, after filtration (0.20 mm, polyester membrane, Chromafil PET 20/25, Macherey-Nagel, Düren, Germany) were injected (50 mL) on a reversed-phase column Zorbax Eclipse XDB-C18 (4.6 x 250 mm; 5 mm particle; Agilent, Germany), thermostated at 40 °C. MS parameters: positive ion mode, dry gas: N₂, 11 L/min, dry temperature: 325 °C, nebulizer: 60 psi, capillary: -2500 V, capillary exit offset: 70 V, end plate offset: -500 V, skimmer 1: 20 V, skimmer 2: 10 V, scan range: 50-1200 *m/z*, chromatographic conditions as below.

Quantitative determination of anthocyanins in copihue tepals

Copihue tepals were extracted and analyzed by HPLC as described in section 2.4. For quantitative determination, a calibration curve was made with cyanidin-3-*O*-glucoside standard from which were quantified all the cyanidin derivatives taking under consideration the gravimetric factor involved. Working standard solutions spanning the concentration range from 1.0 – 80.0 mgL⁻¹ of cyanidin-3-*O*-glucoside were prepared by appropriate dilution of stock standard solutions in mobile phase A. The limits of detection (LOD) and quantification (LOQ) were obtained as three and ten times the noise signal from a chromatogram of a low concentration standard.

RESULTS AND DISCUSSION

Anthocyanin profiles of red *L. rosea* tepals.

As shown in Fig. 2, two red pigments were detected in the extract of red copihue tepals by HPLC- DAD. Their retention times correspond to cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside. No other relevant concentrations of any other anthocyanins, including delphinidin-3-*O*-glucoside, peonidin-3-*O*-glucoside or malvidin-3-*O*-glucoside, which are usually found in red wine, were detected¹⁵. Cyanidin-3-*O*-galactoside, which under these conditions elutes at 10.4 min, was neither detected in the extract.

Figure 2: HPLC-DAD chromatograms of anthocyanins in red copihue tepals

A: red copihue tepal extract; B and C: Anthocyanin standards. Peak labels: (1) cyanidin-3-*O*-glucoside; (2) cyanidin-3-*O*-rutinoside.

Figure 3: HPLC-DAD spectra of (A) cyanidin-3-*O*-glucoside and (B) cyanidin-3-*O*-rutinoside. Continuous lines correspond to the copihue tepal extract and dashed lines to reference anthocyanins. The structures of the commercial standards are drawn below.

In Fig. 3, the UV-VIS spectra from HPLC-DAD chromatography of both anthocyanidins from copihue (continuous line) and for the reference pigments are compared, showing respectively a good fit with absorption maxima at 516 nm for cyanidin-3-*O*-glucoside and at 518 nm for cyanidin-3-*O*-rutinoside, both in mobile phase.

On the other hand, HPLC-MSⁿ analysis confirms that in red copihue tepals, the predominant anthocyanins are glycosylated derivatives of cyanidin, which shows a characteristic signal at 287 (*m/z*) after losing the sugar moiety by fragmentation. Cyanidin-3-glycoside elutes first at 12.3 minutes followed by cyanidin-3-*O*-rutinoside at 13.3 minutes (Fig. 4). The loss of a fragment of *m/z* 162 indicates that the cyanidin might be conjugated with either galactose or glucose, since they have the same molecular weight, but the galactose derivative has a shorter retention time (10.4 min) and is absent in copihue tepals in the HPLC-DAD analysis. For the diglycoside form of cyanidin, the loss of a *m/z* 146 fragment indicates that it is conjugated with a rhamnose and the *m/z* 162 fragment is most likely to be a glucose. This result is consistent with the fragmentation patterns reported for these anthocyanins by Tian *et al*¹². In the HPLC-MSⁿ there is also a third minor peak, which might correspond to cyanidin-3-(6''-formyl) glycoside, showing a loss of *m/z* 190 in copihue's tepals, although this could be an artifact because extraction was made with methanol acidified with formic acid and this minor peak was not detected in fresh extracts by HPLC with DAD detection. In spite of this artifact, the use of weakly acid media (formic or acetic acid) is best to avoid the hydrolysis of glycosylated anthocyanins during the extraction, and at this pH the extract is more stable than in the alkaline region¹⁶.

Figure 4: HPLC-MSⁿ of anthocyanins of red copihue tepal extract. A: Chromatogram UV-VIS at 518 nm (Peak labels are the same as in figure 2). Product-ion analysis of B: m/z 448.9 (cyanidin-3-*O*-glucoside); and C: m/z 595.0 (cyanidin-3-*O*-rutinoside).

Anthocyanin content in copihue tepals

The two mayor peaks identified were quantified by calibration with cyanidin-3-glucoside standard, while considering the differing molecular weights for the cyanidin-3-*O*-rutinoside quantification. Both analytes have the same chromophore (cyanidin) and have a similar molar absorptivity as calculated from absorption spectra of both reference compounds (Fig. 3).

The relevant elements of the regression equation and other characteristic parameters of the method show a good linearity response for cyanidin-3-glucoside between 1.0 – 80.0 mg L⁻¹. From the calibration curve, regression equation $y \text{ (mAU)} = 153430 \times (\text{mg L}^{-1}) - 52142$ ($r = 0.999$) was obtained with a $S_{y/x}$ of 49701. The LOD of 0.01 mg L⁻¹ and a LOQ of 0.03 mg L⁻¹.

In Table 1 summarizes the anthocyanin concentrations found in copihue tepals. Whereas neither cyanidin-3-glucoside nor cyanidin-3-rutinoside was detected in white copihues, only cyanidin-3-glucoside was detected in those of pink copihues. In the sample of red copihue tepals, both cyanidin glycosides were present at quantifiable levels, where the concentration of cyanidin-3-*O*-rutinoside was almost 4-times greater than that of cyanidin-3-*O*-glucoside.

Table 1: Concentration of anthocyanins in the copihue tepal extracts.

Tepal color	Cyanidin-3- <i>O</i> -glucoside		Cyanidin-3- <i>O</i> -rutinoside	
	mg L ⁻¹ in extract	µg g ⁻¹ in tepals (fresh weight)	mg L ⁻¹ in extract	µg g ⁻¹ in tepals (fresh weight)
White	N.D.	N.D.	N.D.	N.D.
Pink	1,90±0,02	2,1± 0,1	N.D.	N.D.
Red	16,8±0,3	18,8±0,3	75,5±0,9	84,4±0,9

In the literature, the presence of cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside has been reported in flowers of various plant species. Norbæk *et al.* reported them together with other anthocyanes in tepals of 28 Chilean species of *Alstroemeria* and in 183 interspecific hybrids¹⁷, whereas this author

reported the presence of cyanidin-3-*O*-rutinoside, but together with cyanidin-3-*O*-rutinoside-7-*O*-glucoside¹⁸, in *Lilium* flowers. This substance was not detected in copihue tepals, but was reported recently to be present in flowers of *Gladiolus* cultivars, where it is found together with other anthocyanidins that are absent in copihue¹⁹.

Other ornamental flower species, such as *Camellia* cultivar ‘Dalicha’ (*Camellia reticulata*), present red colored petal due to the presence of five cyanidin derivatives, but conjugated with xylose derivatives in position 3, some of which were esterified with organic acids²⁰. Bright red flowers contain higher proportions of cyanidin and its derivatives^{21,22,14}. Anthocyanins that contain cyanidin and pelargonidin were isolated from the ornamental flowers of an Ugandan *Hippeastrum* cultivar. The most reddish petals contained the highest relative proportion of cyanidin derivatives²³.

The absence of other colored anthocyanins in the analyzed copihue samples, and the fact that the blue or violet color in copihues only appears in some rare varieties⁵ could be explained looking to the biosynthetic pathway of anthocyanins in flowers of ornamental plants²⁴. As illustrated in Fig.5, this pathway can be divided mainly in 3 lines, where each starts with a common precursor, naringenin, a flavanone that is substrate for the flavanone-3-hydroxylase (F3H), that then forms dihydrokaempferol, which under the action of flavanone-3',5'-hydroxylase (F3'5'H) and flavanone-3'-hydroxylase (F3'H) forms dihydromyricetin and dihydroquercetin, respectively. These three dihydroflavonols with the enzymes dihydroflavonol reductase (DFR), anthocyanidin synthetase (ANS) and glucosyl transferase (GT) produce the corresponding anthocyanins of cyanidin, pelargonidin and delphinidin. When increasing the hydroxyl substitutions on the B-ring or the aromatic acyl groups attached to it, the color of the anthocyanin tends to blue²⁵. So pelargonidin, with only one –OH group tends to orange, cyanidin with two hydroxyl groups present a red color, delphinidin with three –OH tends to a violet color. Successive acylations in the B-ring will produce petunidin and malvidin with a deeper hue of blue. Also the presence of copigments can alter petal color^{26, 27}.

Figure 5: Biosynthetic pathway of anthocyanins in ornamental plants (Adapted from Tanaka *et al.*²⁷)

D: Detected in red and pink copihue tepals ND: Not detected in copihue tepals

The manipulation of the enzymes DFR, F3'H and F3'5'H has been reported as key for the expression of different colors in flowers²⁸, and also the competition of the different pathways for a common substrate also should be considered²⁹. For example in case of marginal picotee petals of *Petunia*, in the uncolored sections of the corolla's margins presented higher levels of flavonol and flavonol synthase transcripts, while the transcript levels involved in the anthocyanidin biosynthesis were the same in the colored part as in the unpigmented, so the lack of colored anthocyanins is due to a decrease of dihydroflavonols in the anthocyanin pathway which are derived to the formation of flavonols³⁰.

If the red copihue presents a higher concentration of cyanidin-3-*O*-rutinoside than cyanidin-3-glucoside, it would be expected to maintain that proportion in the pink copihue, but only cyanidin-3-*O*-glucoside is present in tepals. This result could indicate that in the pink copihue variety the rhamnosyl transferase required to produce the cyanidin-3-*O*-rutinoside²⁴ is lacking, so that tepals in this case have only a slight pink instead of red as happens in the red

variety, where the latter anthocyanin is the main contributor to red color, due to its higher concentration.

CONCLUSIONS

Two cyanidin glycosides, cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside, were detected in red copihue tepals, whereas only cyanidin-3-*O*-glucoside, which is only present in minor proportions in the red tepals, was found in the pink ones. The biogenetic pathway of anthocyanins, in which the latter anthocyanidin is the precursor for the rutinoside derivate, could explain this result. In white copihue tepals, neither anthocyanidin was detected. It is concluded that in red copihue tepals, cyanidin-3-*O*-rutinoside followed by cyanidin-3-*O*-glucoside are principally responsible for the bright red color of the Chilean national flower so priced in legends, poems and popular songs and by the inhabitants of the country.

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