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Color, Ellagitannins, Anthocyanins, and Antioxidant Activity of Andean Blackberry (*Rubus glaucus* Benth.) Wines

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ABSTRACT: Twenty-eight blackberry (*Rubus glaucus* Benth.) wines elaborated under different processing conditions were analyzed for total phenolics, ellagitannins, anthocyanins, color, and antioxidant activity. Ellagitannins were the main phenolic compounds and the most determinant factor in the antioxidant capacity of wines (r = 0.980). The major anthocyanins were cyanidin 3-rutinoside (64 ± 6%) and cyanidin 3-glucoside (19 ± 4%), followed by several minor compounds (17 ± 4%). Two of them were native blackberry anthocyanins, namely, cyanidin 3-rutinoside-5-glucoside and cyanidin 3-xylorutinoside. The remaining seven compounds were anthocyanin-related pigments generated during and after the alcoholic fermentation, identified as A-type and B-type vitisins and hydroxyphenylpyranoanthocyanins. The presence of fruit solids in contact with the liquid fraction during fermentation and the ratio of water to fruit employed in the preparation of the musts had a great impact on the content of ellagitannins, total phenolics, and the antioxidant activity of wines and a minor impact on their color and anthocyanin composition.

KEYWORDS: *Rubus glaucus, fruit wines, anthocyanins, ellagitannins, antioxidant activity*

INTRODUCTION

Grape wine is the most abundant fruit-fermented product in the world and probably the food product with the highest accumulated scientific knowledge. Nevertheless, worldwide, particularly in climatic zones where the production of *Vitis vinifera* grapes is not possible, such as at tropical and high latitudes, there is little production of wines elaborated with other fruits. Among these, berry fruits seem to be the most suitable raw material for wine production,[1−14] probably due to their high content of anthocyanins and other phenolic compounds. Polyphenols are determinant factors in several wine properties such as color, astringency, and bitterness. The chromatic characteristics of wine and their evolution over a wine's shelf life depend on the absolute and relative concentration of anthocyanins in the fruit, the wine production method, and the multiple chemical reactions taking place during fermentation and aging such as the reactions between anthocyanins and various wine compounds, including other phenolics. These reactions are responsible for the generation of new, more stable pigments related to the natural evolution of the red wine color from red to orange nuances.[15]

On the other hand, the high polyphenolic content of berry fruits makes them an important source of dietary antioxidants that reduce the risks of various chronic diseases, including cardiovascular disease and cancer. Over the past decade, many studies have focused on phenolic composition and in vitro antioxidant capacity of many fruits,[16,17] including blackberries.[18−26] The main class of phenolics in blackberries (*Rubus* sp.) are ellagitannins, which are also present in a few fruits such as raspberries (*Rubus idaeus* L.), strawberries (*Fragaria* × *ananassa* D.), pomegranate (*Punica granatum* L.), and muscadine grapes (*Vitis rotundifolia*). Ellagitannin content is usually estimated by acid hydrolysis or methanolysis, followed by HPLC of hydrolytic products, mainly ellagic acid.[27−29] Over the past years, new methods have been developed to achieve a more accurate and direct quantification of ellagitannins.[22,30,31]

The Andean blackberry (*R. glaucus* Benth.) is native to the high-altitude areas of Central and South America, mainly Colombia and Ecuador. The berry is appreciated for its attractive dark-red color, juiciness, and flavor, in comparison to most cultivated blackberries.[32] It is consumed fresh or processed into products such as frozen pulp, juice, jam, and, to a minor extent, wines. The phenolic composition, mainly with regard to ellagitannins[33,34] and anthocyanins,[33−35] and the antioxidant activity[35−37] of *R. glaucus* berries and derived products[38] have been addressed only in recent years. Other Andean blackberry studies have focused on flavor compounds.[39−41] As far as we are aware, no studies about *R. glaucus* wines have been published previously in a peer-reviewed journal.

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The aim of the work was to characterize the color, antioxidant activity, and phenolic composition of Andean blackberry wines from Ecuador. The work was focused on the major phenolic compounds of *R. glaucus* berries, that is, ellagitannins and anthocyanins. First, the results obtained with regard to the hydrolysis of ellagitannins in the blackberry wines are discussed, comparing them with the results obtained with the same method in raspberry extracts.[28] The second deals with the identification by means of HPLC-DAD-ESI-MS/MS analysis of the main individual anthocyanins and anthocyanin-derived compounds found in the wines. Afterward, the characteristics of wines concerning the color, antioxidant activity, and contents in total phenolics, anthocyanins, and ellagitannins are described and compared with previous findings in other berry and red grape wines. Finally, the paper is completed with a discussion about the effects on wine characteristics of some technological factors, such as the yeast strain, the presence/absence of fruit solids during fermentation, and the proportion of fruit in the initial juices.

MATERIALS AND METHODS

Chemicals. All of the chromatographic solvents were of HPLC grade. Methanol, acetonitrile, formic acid, hydrochloric acid, gallic acid, Folin−Ciocalteu reagent, sodium carbonate, and sodium metabisulfite were purchased from Panreac (Spain). 2,2-Diphenyl-1-picrylhydrazyl (DPPH), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were obtained from Sigma-Aldrich (Germany). Cyanidin 3-glucoside (≥96%), cyanidin 3-rutinoside (≥96%), and ellagic acid (≥90%) were obtained from Extrasynthese (Genay, France).

Wines. The basic raw materials for the elaboration of wines were *R. glaucus* blackberries, cane sugar, and water. Potassium bisulfite ($K_2S_2O_5$) was used to sulfite musts and wines. The wines were elaborated in the Technical University of Ambato (Ambato, province of Tungurahua, Ecuador) on a laboratory scale in plastic containers of 25 L.

Two different studies were carried out. The experimental factors and their levels are summarized in Table 1. In study 1, 16 wines were fermented using four different yeast strains (factor Y) in either the presence or absence of fruit solids during fermentation (factor S). Bread instantaneous dry active yeast was included in the study due to the fact that it is the cheapest, most readily available, and most used commercial yeast in the region. In study 2, 12 wines were obtained from musts prepared by mixing fruit and water in three different proportions (factor W). The practice of diluting fruit with water is common in the region and is applied to diminish the viscosity and acidity of the juices and also for economic reasons.

Table 1. Experimental Designs

Study 1:[a] Factor Y (Yeast Strain)
Y0, LEVAPAN (Ecuador), *S. cerevisiae* var. *cerevisiae*
Y1, UVAFERM CM (Lallemand), *S. cerevisiae* var. *cerevisiae*
Y2, Lalvin EC1118 (Lallemand), *S. cerevisiae* var. *oviformis*
Y3, Lalvin QA 23 (Lallemand), *S. cerevisiae* var. *oviformis*
Study 1: Factor S (Fruit Solids in Fermentation)
S0, without solids
S1, with solids
no. of trials: 4 × 2 × 2 repetitions = 16 wines
Study 2:[b] Factor W (Ratio Water/Fruit in Musts)
W0, 4 L water/kg fruit
W1, 3 L water/kg fruit
W2, 2 L water/kg fruit
no. of trials: 3 × 4 repetitions = 12 wines

[a]All of the wines of study 1 were obtained from musts prepared with 2 L of water per kilogram of fruit. [b]All of the wines of study 2 were fermented in the presence of fruit solids and with the same yeast strain (Lalvin QA 23).

The vinification procedure was as follows. *R. glaucus* blackberries were purchased in June 2009 (study 1) and June 2010 (study 2) at the "Alborada" Women's Association, located in Santa Rosa, at 3000 m altitude, close to the city of Ambato. The basic characteristics of the fruit were soluble solids content, 7.3 ± 1.3 °Brix; total acidity, 2.20 ± 0.30 g malic acid/100 g fresh fruit; and pH, 3.0 ± 0.1. Fruit was mixed with water, sulfited (100 mg $K_2S_2O_5$/L), and crushed. At this point, but only in half of the 16 trials of study 1, fruit solids were removed manually through a sieve (850 μm). In all cases, the next steps were the enrichment of musts with cane sugar to achieve a solids content of 21 °Brix, followed by the inoculation of the yeasts (0.3 g dry yeast/L). During fermentation, the temperature in the 28 trials fluctuated between 18 and 24 °C. Sugar attenuation finished after 11−20 days in the case of wine yeast strain fermentations and after 17−23 days in the case of bread yeast strain fermentations. Subsequently, the solids were removed and each wine was transferred clean into a new container and sulfited (75 mg $K_2S_2O_5$/L). The wines were maintained at 18 ± 1 °C for 2 months. In half that time, the wines were racked and sulfited again (75 mg $K_2S_2O_5$/L). After this clarification phase, the general characteristics of the wines were as follows: soluble solids, 7.0 ± 0.2 °Brix; total acidity, 0.97 ± 0.04 g malic acid/100 mL; pH, 3.0 ± 0.1; and alcohol content, 12.1 ± 1.0% (vol). Samples of all 28 wines were sent to Spain and kept refrigerated (4 °C) and under nitrogen until analyses, which included color measurements, total phenolic content, antioxidant activity, ellagitannins, and anthocyanins by means of HPLC-DAD analysis. These analyses were carried out in a period of 1 month from the arrival of the samples. All determinations were made in triplicate. In addition, 8 months later a HPLC-DAD-ESI-MS/MS analysis was applied with the aim of achieving a better identification of the native anthocyanins and the anthocyanin-derived pigments observed in the blackberry wines.

Finally, a small frozen sample of *R. glaucus* berries was also sent to Spain. A fraction (0.2 kg) was employed to elaborate another wine (trial 29) by mixing and crushing it with 0.6 L of water, adding sugar until 21 °Brix, and inoculating the yeast (Lalvin QA23, 0.3 g/L). The aim of this additional assay was to know the anthocyanin composition of the raw material and to have an approximate idea of its change during the fermentation (11 days). This was done by HPLC-DAD analysis.

Color Measurements. These measurements were made in a double-beam spectrophotometer (Zuzi TU 1901, Spain). The absorbances at 420, 520, and 620 nm were used to calculate the color intensity ($CI = A_{420} + A_{520} + A_{620}$); the contributions to the CI of the yellow ($100A_{420}/CI$), red ($100A_{520}/CI$), and blue ($100A_{620}/CI$) components;[42] and the hue (A_{420}/A_{520}) of the wines.

Wine color (WC), anthocyanin color (AC), color due to pigments that are resistant to SO_2 bleaching (CDR_{SO2}), and chemical age (CAW) were obtained according to the method described by Somers and Evans.[43] All spectrophotometric measurements were made at 520 nm. WC is the absorbance of wine read in a 1 mm cuvette and corrected to a 10 mm path length by multiplying by 10. CDR_{SO2} is the residual absorbance in a 10 mm cuvette of a wine containing 0.3% sodium metabisulfite. AC is the color at wine pH mainly due to monomeric anthocyanins, calculated as $AC = WC - CDR_{SO2}$. CAW is the proportion of wine color assignable to pigments that are resistant to SO_2 bleaching ($CAW = CDR_{SO2} \times 100/WC$).

Total Phenolic Content (TPC). The TPC was determined according to the Folin−Ciocalteu method.[44] In a 100 mL volumetric flask, 1 mL of diluted wine, 50 mL of deionized water, 5 mL of the Folin−Ciocalteu reagent, and 20 mL of a 20% (w/v) sodium carbonate solution were added in that order. The volumetric flask was filled to its volume with water. After 30 min, the absorbance of the samples was measured at 750 nm in a double-beam spectrophotometer (Cintra 20, Germany). The results are expressed as milligrams per liter of gallic acid equivalents (concentration range = 100−600 mg/L, R^2 = 0.999).

Antioxidant Activity. The DPPH assay was used to evaluate the radical-scavenging activity of wines by means of the technique described by Rivero-Pérez et al.[45] Sixty microliters of wine (previously diluted with methanol, FD = 20) was mixed with 2940 μL of a 60 μM DPPH methanolic solution in a polystyrene cuvette. The absorbance at 515 nm was measured using the kinetic mode on a double-beam spectrophotometer (Cintra 20) initially ("time zero") and every 30 s until the reaction reached the steady state plateau. The difference between the absorbance at time zero and at 60 min was employed to quantify the antioxidant activity, expressed as millimoles of Trolox equivalents per liter of wine, by means of the corresponding standard curve (concentration range = 0.1−1 mM Trolox, R^2 = 0.999).

Ellagitannins. The acid hydrolysis of wine ellagitannins and the subsequent analysis by HPLC were performed under similar conditions to those described for *Rubus* fruits by Vrhovsek et al.[28] The samples were submitted to hydrolysis at 85 °C in 4 M HCl (20 mL of sample with 16.6 mL of 37% HCl and 14.4 mL of methanol). To optimize the acid hydrolysis time, preliminary tests were carried out at 1, 2, 4, 6, and 20 h. After hydrolysis, the sample was deposited in 50 mL of methanol. An aliquot (5 mL) was partially neutralized with 3 mL of 4 M NaOH and diluted to 10 mL with methanol. Subsequently, the sample was filtered with 0.45 μm pore size, 13 mm PVDF syringe-tip filters (TeknoKroma, Spain) and transferred into LC vials. Each sample was submitted to hydrolysis twice.

HPLC analysis was carried out in a Waters 2695 Separations Module liquid chromatograph, coupled to a Waters 996 PDA detector. The separation was performed using a LiChroCART Purospher Star RP-18e column (250 × 2 mm i.d., 5 μm, Merck, Germany) and a Purospher precolumn (4 × 4 mm, 5 μm). The solvents were 1% formic acid in water (A) and acetonitrile (B). The gradient was from 0 to 5% B in 10 min and from 5 to 30% B in 30 min. The column was washed with 100% B for 2 min and equilibrated for 5 min prior to each analysis. The flow rate was 0.3 mL/min, and the oven temperature was 40 °C. The injection volume was 20 μL. Spectra were monitored between 240 and 450 nm. Ellagic acid and the four additional hydrolytic compounds that showed a UV−vis spectrum similar to that of ellagic acid (see Results and Discussion) were quantified at 254 nm by means of the calibration curve obtained with an ellagic acid standard (concentration range = 10−150 mg/L, R^2 = 0.996). The sum of their contents was used to estimate the global richness in ellagitannins, after subtraction of the free ellagic acid concentration obtained before the acid hydrolysis.

Anthocyanins. Method 1: HPLC-DAD Analysis. HPLC-DAD analysis of anthocyanins was performed with the same equipment and column described for ellagitannins. A modified version of the method described by Vasco et al.[34] was employed. The solvents were 5% formic acid in water (solvent A) and acetonitrile (solvent B). The gradient conditions were from 5 to 10% B in 5 min, 10% B for 5 min, from 10 to 42% B in 20 min, and a return to the initial conditions in 5 min. The flow rate was 0.2 mL/min, and the oven temperature was 25 °C. Samples were directly injected (20 μL) after filtration with 0.45 μm pore size, 13 mm, PVDF syringe-tip filters. Spectra were recorded between 250 and 550 nm. Cyanidin 3-glucoside and cyanidin 3-rutinoside were identified by comparing their retention times with those of the corresponding pure standards. Both compounds were quantified from the areas recorded at 520 nm and expressed as milligrams per liter (cyanidin 3-glucoside, 1−110 mg/L, R^2 = 0.9997; cyanindin 3-rutinoside, 1−200 mg/L, R^2 = 0.9999). The calibration curve of cyanidin 3-glucoside was also used to estimate the content of several additional minor anthocyanins and anthocyanin-derived pigments (see Results and Discussion).

Anthocyanins. Method 2: HPLC-DAD-ESI-MS/MS Identification. HPLC identification of Andean blackberry wine anthocyanins was performed using an Agilent 1200 series system equipped with DAD (Agilent, Germany) and coupled to an AB Sciex 3200 Q TRAP (Applied Biosystems) electrospray ionization mass spectrometry system (ESI-MS/MS). The chromatographic system was managed by the Agilent Chem Station (version B.01.03) data-processing station. The mass spectral data were processed with Analyst MDS software (Applied Biosystems, version 1.5). The wine samples were filtered (0.20 μm, polyester membrane, Chromafil PET 20/25, Machery-Nagel, Düren, Germany) and directly injected (50 μL) into a Zorbax Eclipse XDB-C18 reversed-phase column (4.6 × 250 mm; 5 μm particle; Agilent, Germany) thermostated at 40 °C. The solvents were water/acetonitrile/formic acid (87:3:10, v/v/v, solvent A; 40:50:10, v/v/v, solvent B), and the flow rate was 0.63 mL/min. The linear gradient for solvent B was as follows: 0 min, 6%; 15 min, 30%; 30 min, 50%; 35 min, 60%; 38 min, 60%; 46 min, 6%. For identification, the ESI-MS/MS in positive ionization mode was operated using a combination of +EMS (enhanced mass spectrum; MS conditions) and +EPI (enhanced product ion; MS/MS conditions) experiments, setting the following parameters: scan, 100−1500 Da (250 Da/s); declustering potential, 65 V; entrance potential, 10 V; collision energy, 10 (arbitrary units); curtain gas, 15 psi; collision gas, medium; ion spray voltage, 4000 V; temperature, 450 °C; ion source gas 1, 70 (arbitrary units); ion source gas 2, 50 (arbitrary units); and Q3 barrier, 12 V. Standards of cyanidin 3-glucoside and cyanidin 3-rutinoside and previously reported LC-DAD-MS/MS data for anthocyanins and pyranoanthocyanins were used to confirm peak assignations.[46,47]

Statistical Methods. Two-factor and simple analysis of variance (ANOVA) and Fisher's least significant differences tests (p = 0.05) were used to assess significant differences among the wines. Pearson's correlations were applied to study the relationship among wine parameters. All statistical methods were performed using Statgraphics 5.1. (Statistical Graphics Corp., Rockville, MD, USA).

RESULTS AND DISCUSSION

Hydrolysis of Ellagitannins. Following the acid hydrolysis of the blackberry wine samples, six hydrolytic products were observed (Figure 1). Three of them were clearly the most

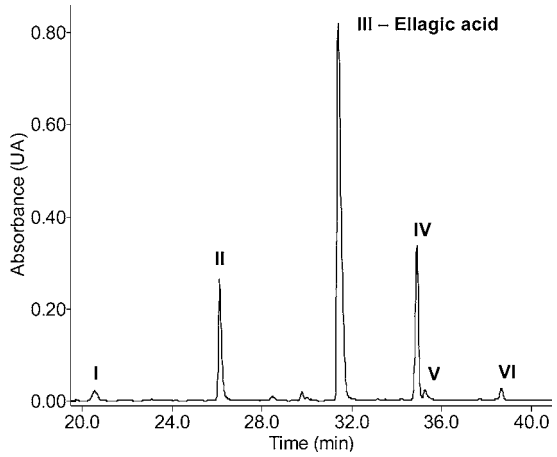

Figure 1. HPLC-DAD chromatogram at 254 nm of a blackberry wine after hydrolysis. Compound **III**: ellagic acid. Peaks I, II, IV, V, and VI, unknown hydrolytic products.

abundant: ellagic acid, identified by comparison with a pure standard (RT = 30.9 min, λ_{max} = 254 and 365 nm), and compounds **IV** and **II**. They accounted for 60 ± 1, 18 ± 1, and 15 ± 1% of the sum of the areas at 254 nm of the six detected peaks, respectively. The remaining 7% belonged to three minor peaks, compounds **I**, **V**, and **VI**. We used the same hydrolytic and chromatographic methods that Vrhovsek et al.[28] applied to raspberry extracts, which reported the presence of ellagic acid (RT = 30.8 min) and three additional hydrolytic products: methyl gallate, methyl sanguisorboate, and an unidentified compound called derivative 1. The retention time and UV−vis spectra of compounds **I**, **IV**, and **II** were very close to those of these products, respectively. Compound **I** (RT = 22.2 min) showed a different DAD spectrum (λ_{max} = 272) from that of

ellagic acid and similar to that reported for methyl gallate (RT = 21.8 min, λ_{max} = 274). Compound **IV** (RT = 34.4 min, λ_{max} = 372 nm), as methyl sanguisorboate did (RT = 34.6 min λ_{max} = 371 nm), showed a bathochromic shift to a longer wavelength compared with the spectrum of ellagic acid. This was also observed for compound **II** (RT = 25.7 min, λ_{max} = 371) and derivative 1 (RT = 25.1 min, λ_{max} = 369). It should be noted that the relative abundance of compound **II** in the blackberry wines was much higher than that of derivative 1 in the raspberry extracts. Finally, the DAD spectra of small peaks for compounds **V** (RT = 34.8 min, λ_{max} = 364) and **VI** (RT = 38.2 min, λ_{max} = 373) resembled those of ellagic acid and compound **IV**, respectively.

Figure 2 shows the evolution of the releases of the above-mentioned compounds during the hydrolysis of a blackberry

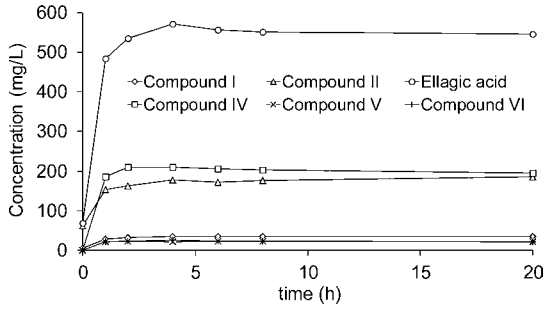

Figure 2. Evolution of the release of hydrolytic products during hydrolysis of ellagitannins in 4 M HCl at 85 °C.

wine with 4 M HCl at 85 °C. The maximum amount of ellagic acid was obtained after 4 h. At longer hydrolysis times, this concentration was slightly but significantly ($p < 0.05$) lower. In the case of compounds **I** and **II**, the rates of release became constant after 4 and 6 h of hydrolysis, respectively. Compounds **IV**, **V**, and **VI** showed similar tendencies as ellagic acid. Their rates of release after 20 h were 88−95% of those obtained after 4 h.

These decreases could have been due to condensation reactions within degradation products present in the hydrolysate that probably occurred because the hydrolytic medium was not strictly methanolic.[27] Our first purpose was to comply with this premise, but it was not possible because a very strong precipitation took place while the wine dry residue obtained after vacuum evaporation at 30 °C in methanol was redissolved. Therefore, we decided to mix the wine with methanol and 37% HCl directly, as described under Materials and Methods.

Finally, 4 h was considered to be the optimum hydrolysis time, which is 2 h less than that reported by Vrhovsek et al.[28] The repeatability of the hydrolysis procedure was evaluated. Five aliquots of one blackberry wine were separately hydrolyzed for 4 h and analyzed by HPLC-DAD. The relative standard deviations for ellagic acid and compounds**II**, **IV**, **V**, and **VI** were 2.3, 3.1, 3.5, 3.0, and 2.9%, respectively. Compound **I** was not considered for quantification purposes because its UV−vis spectrum was very different from that of ellagic acid.

Anthocyanin Composition. Figure 3, panels a and b show the HPLC-DAD chromatogram (method 1) obtained at 520 nm for the blackberry juice at the fermentation starting point and for a blackberry wine, respectively. Two main peaks (2 and 4) can be observed, which were identified after comparison with the corresponding pure standards, cyanidin 3-glucoside and cyanidin 3-rutinoside, respectively. Cyanidin 3-glucoside has been identified as the main anthocyanin in different blackberry species.[8,20−25] Nevertheless, in this work the most abundant pigment was cyanidin 3-rutinoside, which agrees with most of the previous references on *R. glaucus*. Percentages of 45% for cyanidin 3-rutinoside and 40% for cyanidin 3-glucoside have been reported in a sample from Colombia,[36] whereas in blackberries from Ecuador, these values were 62 and 38%,[33] respectively. The last values are very similar to those observed in the raw material employed in trial 29 (Figure 4). During the 11days of fermentation, the absolute and relative contents of both anthocyanins greatly changed. Although its concentration also declined, cyanidin 3-rutinoside proved to be much more stable than cyanidin 3-glucoside (Figure 5). This is consistent with the tendencies previously observed during winemaking in the anthocyanin composition of raspberries [8] and Evergreen blackberries.[9]

Besides these two cyanidin glycosides, only four anthocyanins have been previously identified in the Andean blackberry: cyanidin 3-sambubioside, cyanidin 3-xylorutinoside, pelargonidin 3-glucoside,[36] and pelargonidin 3-rutinoside.[34,36] Vasco et al.[34] also reported the presence of cyanidin and a cyanidin glycoside that might be the cyanidin 3-malonylglucoside. In trial 29, we detected four additional small peaks (Figure 3a, peaks A−D), which accounted for around 4% of the anthocyanin content in the juice and 6% after 11 days of fermentation (Figure 4). In wines 1−28, which were elaborated in Ecuador and analyzed approximately 2 months after the end of fermentation, the number and the relative importance of the additional peaks were notably higher than in the juice and wine of trial 29. Up to 16 additional peaks were detected, 7 of them well-defined (Figure 3b, peaks A′−G′), which accounted for between 9 and 26% of the total anthocyanin content, 17% on average (Figure 4). These compounds probably had several origins. Some anthocyanins came from fruit, but it is certain that new pigments could have been generated during the fermentation and maturation of wines. In recent years, great advances have been made in the elucidation of the chemical nature and the mechanisms of formation of these anthocyanin derivatives in red grape wines. Within them, the most important are probably the pyranoanthocyanins, a large group of compounds that have absorbance maxima at lower wavelengths than native anthocyanins. The presence and progressive predominance of these pigments with respect to monomeric anthocyanins would explain the color change observed during aging in red grape wines from purple-red to orange hues.[15]

The DAD spectra of the small peaks denoted in Figure 3b were as follows. Peaks A′ and E′ showed maximum wavelengths at 518−520 nm, typical of cyanidin-based anthocyanins. Peaks B′ and C′ showed a very particular DAD spectra, with a maximum wavelength hypsochromically shifted (λ_{max} = 485 nm) in comparison to that of monomeric anthocyanins. The shape and visible absorption maximum shown in the UV−vis spectra of the latter compounds closely resembled those of B-type vitisin-like pyranoanthocyanins.[15,47] Finally peaks D′, F′, and G′ showed maximum wavelengths between 502 and 509 nm (red-orange color).

Figure 3c shows the chromatographic profile (absorbance at 520 nm) of the occurring anthocyanic pigments in the blackberry wines, as obtained by means of method 2. DAD detection was coupled with mass spectrometry, with the aim of achieving a better identification of peaks (Table 2). Chromatograms of Figures 3b (method 1) and 3c were different, first,

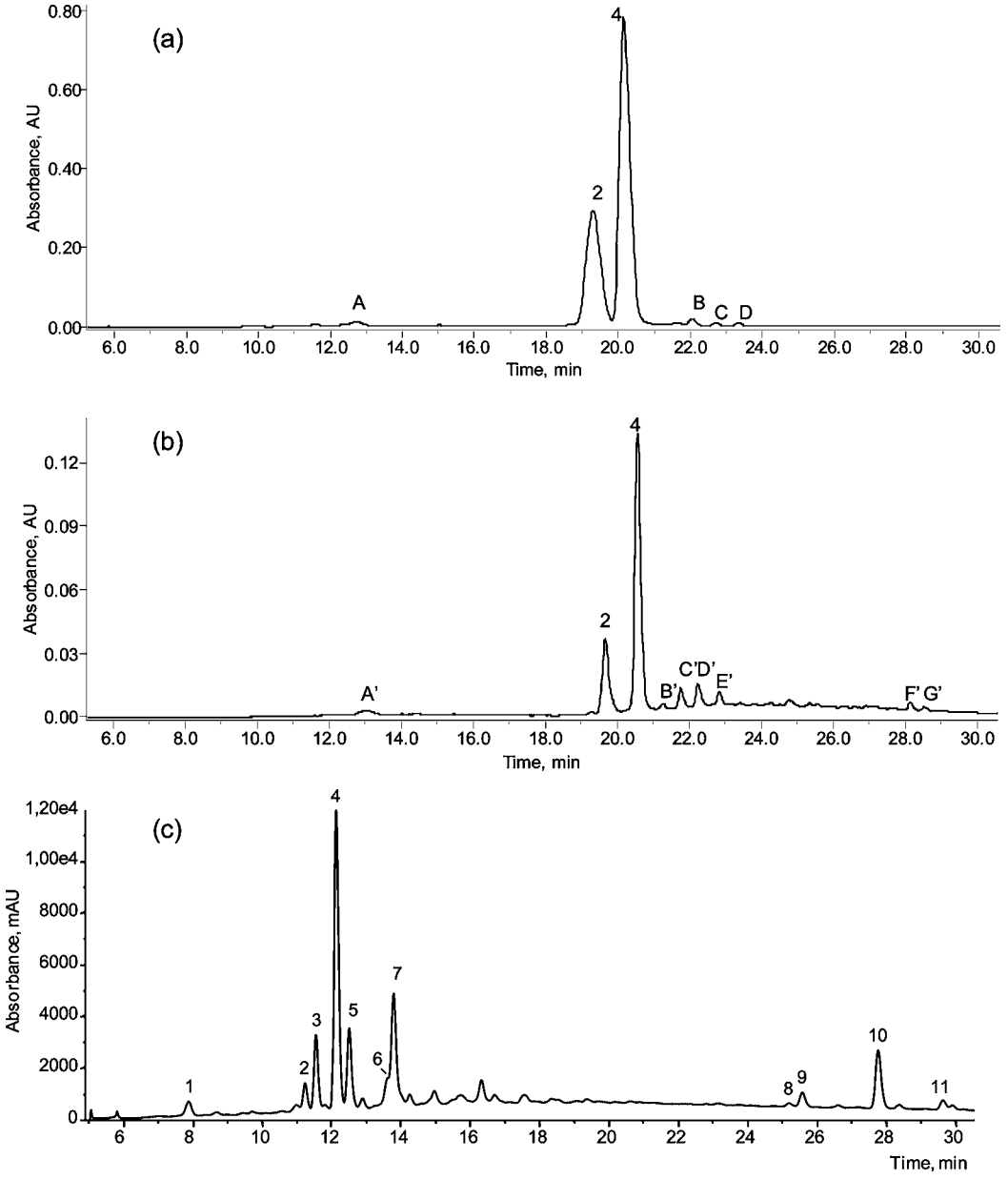

Figure 3. Chromatograms at 520 nm of blackberry juice (a) and wines (b, c). Chromatograms a and b were obtained with method 1 (HPLC-DAD), and chromatogram c was obtained with method 2 (HPLC-DAD-ESI-MS/MS).

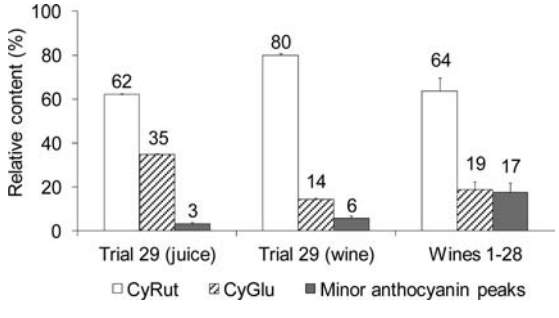

Figure 4. Relative contents of cyanidin 3-rutinoside, cyanidin 3-glucoside, and minor anthocyanin compounds in the blackberry juice and wines.

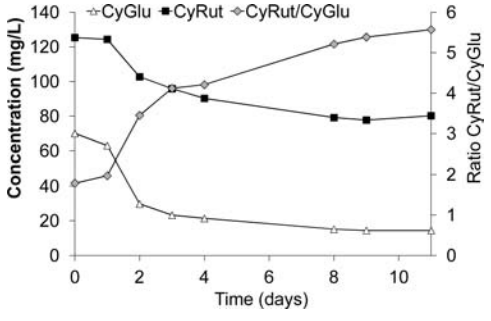

Figure 5. Evolution of the contents of cyanidin 3-rutinoside and cyanidin 3-glucoside and their ratio during fermentation of trial 29.

because the columns and chromatographic conditions were not the same, and, second, because method 2 was applied around 8 months later than method 1. Although during this time wines were stored under refrigeration (4 °C) and nitrogen, we must assume that some evolution of their color and anthocyanin composition took place.

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Table 2. Anthocyanins and Anthocyanin-Derived Pigments Identified in the Blackberry Wines by Using HPLC-DAD-ESI-MS/MS, Respective Standards, and Previous Identification Data[a]

peak	RT (min)	λ_{max} (vis)	parent ion (M^+)	product ions (MS/MS)	tentative identification
			Cyanidin Glycosides		
1	7.9	514	757	595, 449, 287	cyanidin 3-rutinoside-5-glucoside
2	11.3	514	449	287	cyanidin 3-glucoside (std)
3	11.6	518	727	581, 287	cyanidin 3-xylorutinoside
4	12.1	518	595	449, 287	cyanidin 3-rutinoside (std)
			Vitisin-like Pyranoanthocyanins		
5	12.5	508	663	517, 355	10-carboxypyranocyanidin 3-rutinoside
6	13.6	488	751	619, 473, 311	10*H*-pyranocyanidin 3-xulorutinoside
7	13.8	488	619	473, 311	10*H*-pyranocyanidin 3-rutinoside
			Hydroxyphenyl-like Pyranoanthocyanins		
8	25.2	510	727	nd	10-catechylpyranocyanidin 3-rutinoside
9	25.6	502	843	403	10-hydroxyphenyl 3-xylorutinoside
10	27.8	502	711	403	10-hydroxyphenyl 3-rutinoside
11	29.6	508	741	433	10-guaiacyl 3-rutinoside

[a]Abbreviations: nd, not detected; std, standard. Retention times (RT) refer to Figure 3c.

Native blackberry anthocyanins and anthocyanin-related pigments formed during and after alcoholic fermentation were found. All of the identified pigments were based on cyanidin, although pelargonidin-based anthocyanins have been sometimes reported as minor compounds in Andean blackberry (*R. glaucus* Benth.),[34,36,38] but other studies did not found them.[29]

Native cyanidin-based anthocyanins identified were tentatively assigned as cyanidin 3-rutinoside-5-glucoside (peak 1 in Figure 3c), cyanidin 3-glucoside (peak 2), cyanidin 3-xylorutinoside (peak 3), and cyanidin 3-rutinoside (peak 4), which was the main occurring anthocyanin, followed by cyanidin 3-xylorutinoside. Assignations were largely based on UV−vis and MS/MS data (Table 2) together with the matching of chromatographic and spectral data in the cases of cyanidin 3-glucoside and cyanidin 3-rutinoside with those of true standards. All of the aforementioned compounds presented a visible absorbance maximum at 514−518 nm typical of cyanidin-based anthocyanins.[46] The MS/MS spectrum of cyanidin 3-rutinoside-5-glucoside showed the same product ions (m/z values of 449 and 287) in similar relative intensities to that of 3-rutinoside, together with a new signal at m/z 595 that can be attributable to the loss of a hexose moiety. On the basis of literature data,[48] it was assumed that the aforementioned result indicated that a glucose moiety was linked to the C-5 position of the cyanidin aglycon, whereas a 6″-rhamnosyl glucoside (rutinoside) was the substituent at C-3 position. As far as we know 3,5-diglycosides have been never described to occur in Andean blackberry. In the case of cyanidin 3-xylorutinoside (very likely 3-(2″-xylosyl)rutinoside), its assignation was based on the consideration that the 2″-glycosylated glucose moiety (fragment weight of 294 amu), resulting from the previous release of the 6″-rhamnosyl unit (product ion at m/z 581), is usually lost as an entire fragment under ESI-MS/MS conditions.[46] Cyanidin 3-xylorutinoside has been previously detected in Andean blackberry,[36,38] and its occurrence in black raspberry has been unequivocally demonstrated by NMR spectroscopy.[49] Comparison of the anthocyanin profiles obtained by methods 1 and 2 suggests that peaks A and A′ of Figure 3, panels a and b, respectively, could correspond to cyanidin 3-rutinoside-5-glucoside (peak 1 of Figure 3c). With regard to cyanidin 3-xylorutinoside, which in Figure 3c eluted between cyanidin 3-glucoside and cyanidin 3-rutinoside, it was not observed in Figure 3a,b, perhaps because it coeluted with one of the previously cited compounds.

The native blackberry anthocyanins are expected to be transformed into anthocyanin-related pigments during and after the alcoholic fermentation, as it is well documented for the winemaking process using grapes. Pyranoanthocyanins are a class of anthocyanin-derived pigments formed by the reaction of anthocyanins with several low molecular weight compounds having a polarizable double bond.[47] On the one hand, some yeast metabolites such as pyruvic acid and acetaldehyde lead to the formation of the so-called A-type and B-type vitisins, respectively, during the alcoholic fermentation. On the other hand, hydroxycinnamic acids give rise to hydroxyphenylpyranoanthocyanins during and after the alcoholic fermentation.

With regard to vitisin-like pyranoanthocyanins, one A-type vitisin (peak 5 in Figure 3c) and two B-type vitisins (peaks 6 and 7) derived from the main anthocyanins present in Andean blackberry wine were tentatively identified on the basis of the characteristic UV−vis spectra of such anthocyanin-derived pigments[47] and the expected molecular ions and ESI-MS/MS fragmentation patterns (Table 2). The compound eluting under peak 5 showed a visible maximum absorbance at 508 nm (red-orange color) and was tentatively assigned as 10-carboxypyranocyanidin 3-rutinoside as it showed the same fragmentation pattern as cyanidin 3-rutinoside (product ions corresponding to the subsequent losses of rhamnosyl and glucosyl moieties). This A-type vitisin derived from the main anthocyanin found in Andean blackberry wine, and it was not possible to clearly identify (MS/MS spectra) any other A-type vitisin derived from the other anthocyanins. In contrast, two B-type vitisins (peaks 6 and 7 in Figure 3c) were detected, and they derived from the two main anthocyanins. As observed for A-type vitisin, the B-type vitisins showed the same fragmentation patterns as their respective precursor anthocyanins, and they were tentatively assigned as 10*H*-pyranocyanidin 3-xylorutinoside (peak 6) and 10*H*-pyranocyanidin 3-rutinoside (peak 7). Moreover, these B-type vitisins showed a visible maximum absorbance at 488 nm (orange color) according to the expected characteristic UV−vis spectra of such compounds.[47]

The hydroxyphenyl-like pyranoanthocyanins found in Andean blackberry wine derived from the reaction of *p*-coumaric, caffeic, and ferulic acids with the main anthocyanin, cyanidin 3-rutinoside (peaks 8, 10, and 11 in Figure 3c), together with the derivative from *p*-coumaric acid with cyanidin 3-xylorutinoside (peak 9). All of these compounds showed the characteristic UV−vis spectra of hydroxyphenylpyranoanthocyanins[47] following the number of substituent in the E-ring linked to the C-10 position (Table 2; 502 nm for monosubstituted and 508−510 nm for the disubstituted ones). The MS and MS/MS confirmed the suggested structures, although, in these cases, only one product ion corresponding to the pyranoanthocyanidin aglycon was observed in the MS/MS spectra.

On the basis of the results obtained by mass spectrometry, the quantification of the minor anthocyanin compounds detected by means of method 1 was performed as follows. We have assumed that peaks B′ and C′ of Figure 3b, with a maximum wavelength at 485 nm, were visitin-like pyroanthocyanins, and we have quantified them jointly. The assignation for the five remaining minor peaks (A′, D′−G′) was not so obvious, so we decided to quantify all of them together.

Characterization of the Blackberry Wines. The characteristics of blackberry wines related to color, anthocyanins, ellagitannins, total phenolics, and antioxidant activity are summarized in Table 3.

Color intensity (CI) and wine color (WC) were similar to or even higher than those reported in blackberry wines,[5,9,14] except in the case of wines produced from fruit juice subjected to a high-temperature−short-time pasteurization treatment prior to fermentation.[9] Among the three components of color intensity, red was clearly the most important, followed by yellow. On average, these two contributions accounted for 95% of the CI. Therefore, the blue component seems to have less importance in blackberry wines in comparison to red grape wines, in which it normally achieves values of 10−12%.

The blackberry wines were analyzed 2 months after the end of fermentation. Therefore, they must be classified as young wines. This agrees with the facts that their hue was still low (0.42−0.63) and that the main component of WC was the monomeric anthocyanin color (AC). The color due to pigments resistant to SO_2 bleaching (CDR_{SO2}) was responsible, on average, for 26.6% of WC. This was lower than the chemical age (CAW) observed in Evergreen blackberry wines by Rommel et al.,[9] but higher than those previously reported by the same authors in red raspberry wines.[8] This could be related to their different anthocyanin profiles. The major anthocyanin in red raspberries was cyanidin 3-sophoroside, whereas that in Evergreen blackberries was cyanidin 3-glucoside (72%) followed by cyanidin 3-rutinoside (7%), just the opposite of what we detected in the *R. glaucus* wines. The authors observed that cyanidin 3-glucoside was more labile during winemaking than cyanidin 3-sophoroside, which was consistent with the fact that their blackberry wines had CDR_{SO2} values of 2.4−2.8 times higher than the red raspberry wines had.[8,9] They also reported that after fermentation, the relative content of cyanidin 3-rutinoside in their blackberry wines was increased significantly because of its greater stability in comparison to cyanidin 3-glucoside,[8] as we also observed in trial 29 (Figure 5). This would explain why our *R. glaucus* wines showed lower CDR_{SO2} values than the Evergreen blackberry wines did.

The TPC of the wines (601−1624 mg GAE/L) was situated in the lower side of the range observed in other red berry fruit wines[1,4−6,10−14] in which TPC values from 600[11] to 4044 mg GAE/L[5] have been reported. With regard specifically to blackberry wines, different authors showed average values of 1232, 1548, and 2212 mg GAE/L in commercial wines of Turkey,[14] Croatia,[1] and Illinois (USA),[4] respectively. The content in polyphenols of a wine depends mainly on the richness of the raw material and the winemaking procedure. Bearing in mind that the blackberry wines were obtained from juices in which fruit was diluted approximately 3−5 times with water, the TPC results could be considered consistent with most previous studies on blackberry fruits and juices.[16,25,26] Nevertheless, higher amounts have been found in several papers.[19,33] It is obvious that during winemaking, only a partial extraction of the raw material phenolic content could be achieved, particularly with regard to the less extractable compounds, that is, the ellagitannins. The TPC of the wines was strongly correlated with ellagitannins (r = 0.924). As expected,[33−35] ellagitannins were the major phenolic compounds found in the blackberry wines (265−1445 mg EAE/L). Direct comparison with the literature was not possible, because no previous references about ellagitannin content in wines produced with blackberries or other *Rubus* sp. fruits were found.

The total anthocyanin content of the wines (35−121 mg/L) was very similar to that previously observed (13−192 mg/L) in blackberry wines,[4−6,9] but lower than those previously observed in fresh *R. glaucus* berries: 450−510 mg/kg FW.[34,36] There are many examples in the literature showing how the free anthocyanin content of any anthocyanin-rich raw material, including blackberries,[9,23,38,41] declines during fermentation[1,3,8,9] or other kinds of transformation processes[23,41] and

Table 3. Statistical Summary of Analytical Parameters[a] of the Blackberry Wines

variable	mean ± SD	min−max	RSD (%)
color intensity	5.9 ± 1.7	2.7−8.4	29
yellow (%)	32.5 ± 2.1	28.7−36.3	6
red (%)	63.0 ± 2.9	57.5−68.3	5
blue (%)	4.5 ± 1.3	2.7−6.9	28
hue	0.52 ± 0.06	0.42−0.63	11
wine color	3.7 ± 1.1	1.7−5.1	29
anthocyanin color	2.7 ± 0.8	1.4−4.0	29
CDR_{SO2}	1.0 ± 0.5	0.3−2.1	48
chemical age (%)	26.6 ± 8.1	15.1−45.0	30
CyGlu (mg/L)	13.0 ± 4.2	7.0−23.9	33
CyRut (mg/L)	45.4 ± 17.5	19.4−87.2	38
vitisins (mg/L)	3.4 ± 0.8	1.4−5.0	25
A-others (mg/L)	8.0 ± 1.4	5.5−11.0	17
A-total (mg/L)	70.0 ± 21.1	35.3−121.0	30
ellagic acid (mg/L)	12.6 ± 4.5	4.8−21.6	36
ellagitannins (mg/L)	778 ± 330	265−1445	43
TPC (mg/L)	1048 ± 276	601−1624	26
AA (mM Trolox)	8.7 ± 2.8	3.8−14.2	32

[a] CDR_{SO2}, color due to pigments resistant to SO_2 bleaching; CyGlu, cyanidin 3-glucoside; CyRut, cyanidin 3-rutinoside; vitisins, sum of peaks B′ and C′ in Figure 3b, with a maximum wavelength at 485 nm; A-others, sum of the rest of minor peaks in Figure 3b (A′, D′−G′); A-total, sum of all peaks in Figure 3b; TPC, total polyphenol content; AA, antioxidant activity.

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Table 4. ANOVA Results for Color Parameters and Anthocyanins[a] (Means and Standard Errors)[b]

	study 1, two-way ANOVA[c]						study 2, one-way ANOVA		
	factor Y, yeast strain				factor S, fruit solids in fermentation		factor W, water/fruit ratio in the musts		
variable	Y0, LEVAPAN	Y1, UVAFERM CM	Y2, Lalvin EC1118	Y3, Lalvin QA23	S0, without	S1, with	W0, 4 L/kg	W1, 3 L/kg	W2, 2 L/kg
color intensity	6.3 ± 0.5 a	7.2 ± 0.3 a	7.4 ± 0.7 a	6.4 ± 0.5 a	6.4 ± 0.4 a	7.3 ± 0.3 a	2.8 ± 0.1 a	4.7 ± 0.3 b	6.2 ± 0.3 c
yellow (%)	30.7 ± 0.4 a	30.4 ± 0.8 a	31.9 ± 0.2 b	30.7 ± 0.3 a	30.5 ± 0.4 a	31.4 ± 0.3 b	33.4 ± 0.2 a	34.9 ± 0.3 b	35.3 ± 0.3 b
red (%)	65.9 ± 0.6 b	65.2 ± 1.3 b	62.2 ± 0.7 a	65.5 ± 0.6 b	65.7 ± 0.6 b	63.7 ± 0.8 a	63.5 ± 0.3 b	59.8 ± 0.6 a	59.0 ± 0.5 a
blue (%)	3.4 ± 0.3 a	4.4 ± 0.6 a	5.8 ± 0.6 a	3.8 ± 0.3 b	3.8 ± 0.3 a	4.9 ± 0.5 b	3.1 ± 0.2 a	5.4 ± 0.3 b	5.7 ± 0.2 b
hue	0.47 ± 0.01 a	0.47 ± 0.02 a	0.51 ± 0.01 b	0.47 ± 0.01 a	0.47 ± 0.01 a	0.49 ± 0.01 b	0.53 ± 0.01 a	0.58 ± 0.01 b	0.60 ± 0.01 b
wine color	4.2 ± 0.3 a	4.7 ± 0.2 a	4.6 ± 0.4 a	4.2 ± 0.3 a	4.2 ± 0.3 a	4.6 ± 0.1 a	1.8 ± 0.1 a	2.8 ± 0.2 b	3.7 ± 0.2 c
anthocyanin color	3.3 ± 0.3 a	3.4 ± 0.2 a	2.9 ± 0.2 a	3.3 ± 0.3 a	3.2 ± 0.2 a	3.3 ± 0.1 a	1.5 ± 0.1 a	2.0 ± 0.2 b	2.4 ± 0.1 c
CDR_{SO2}	0.9 ± 0.1 a	1.3 ± 0.2 ab	1.7 ± 0.3 b	0.9 ± 0.1 a	1.0 ± 0.1 a	1.4 ± 0.2 b	0.3 ± 0.7 a	0.8 ± 0.1 b	1.3 ± 0.1 c
chemical age (%)	20.7 ± 1.4 a	28.4 ± 3.3 b	36.0 ± 3.5 c	20.5 ± 2.6 a	23.7 ± 2.0 a	29.1 ± 3.5 b	17.2 ± 0.1 a	28.8 ± 2.4 b	34.4 ± 1.7 b
CyGlu (mg/L)	16.8 ± 1.3 b	11.5 ± 0.8 a	8.9 ± 0.3 a	19.4 ± 3.3 b	13.5 ± 1.4 a	14.7 ± 2.0 a	10.3 ± 1.7 a	10.7 ± 2.1 a	11.8 ± 0.7 a
CyRut (mg/L)	50.5 ± 5.1 a	56.4 ± 9.5 a	36.3 ± 3.7 a	64.2 ± 12.1 a	54.8 ± 6.6 a	49.0 ± 6.6 a	43.8 ± 5.2 a	31.4 ± 6.8 a	30.8 ± 1.7 a
vitisins (mg/L)	3.8 ± 0.3 a	3.8 ± 0.2 a	4.2 ± 0.4 a	3.0 ± 0.6 a	3.6 ± 0.2 a	3.8 ± 0.4 a	1.9 ± 0.4 a	2.8 ± 0.3 a	3.6 ± 0.1 b
A-others (mg/L)	10.9 ± 0.4 a	11.4 ± 0.9 a	8.4 ± 0.5 a	8.8 ± 0.9 a	10.1 ± 0.7 a	9.6 ± 0.7 a	11.6 ± 1.2 a	13.4 ± 1.0 a	14.1 ± 0.2 a
A-total (mg/L)	82.0 ± 6.9 a	83.0 ± 10.4 a	57.9 ± 4.2 a	95.4 ± 13.6 a	82.0 ± 7.7 a	77.1 ± 8.3 a	67.6 ± 8.2 a	58.3 ± 10.0 a	60.3 ± 2.1 a

[a] CDR_{SO2}, color due to pigments resistant to SO_2 bleaching; CyGlu, cyanidin 3-glucoside; CyRut, cyanidin 3-rutinoside; vitisins, sum of peaks B′ and C′ in Figure 3b, with a maximum wavelength at 485 nm; A-others, sum of the rest of minor peaks in Figure 3b (A′,D′−G′); A-total, sum of all peaks in Figure 3b. [b] Different letters indicate significant statistical differences among the means for each factor ($p < 0.05$). [c] No significant interaction Y × S was observed for any variable.

during subsequent storage.[1,8,23,38] Figure 5 shows how in trial 29 the concentrations of the two major anthocyanins drastically decreased during fermentation. After 11 days, the contents on cyanidin 3-glucoside and cyanidin 3-rutinoside fell to 23 and 65% of the contents existing in the initial juice, respectively.

With regard to the antioxidant activity (AA) of wines, values from 3.8 to 14.2 mM Trolox were obtained (Table 3). AA was highly correlated with TPC ($r = 0.945$), as has been observed many times in a wide range of phenolic-rich raw materials and products, including fruits[16,20,25,26,34] and wines made with fruits.[1,4−7,10−14] A direct comparison of the AA data with most previous papers on the antioxidant capacities of berry wines was not possible due to differences in the analytical method[1,4−7,10,11] or in the way that results were expressed.[1,13,14] By means of the DPPH method, Schmitzer et al.,[12] in elderberry wines, and Fernández-Pachón et al.,[50] in red grape wines, reported radical-scavenging capacities of 6.3−9.95 and 4.7−17.4 mM Trolox, respectively. On the other hand, the TPC values were 1544−2004 mg GAE/L for elderberry wines[12] and 1313−2389 mg GAE/L for the red grape ones,[50] whereas in the Andean blackberry wines values from 601 to 1624 mg GAE/L were obtained. The AA/TPC ratios calculated for each different type of wines were, on average, 4.5 ± 0.6, 4.9 ± 1.5, and 8.4 ± 1.0 mmol Trolox/g GAE, respectively. These data suggest that in relative terms, the blackberry wines were more effective in vitro antioxidants than the elderberry and red grape wines, which is in agreement with previous findings comparing the superoxide anion scavenging capacity[7] and the ferric reducing antioxidant power[6] of blackberry and red grape wines. This fact could be attributed to the different natures of the major phenolic compounds of the wines.[6] In red grape wines AA was particularly associated with the flavanols and anthocyanins,[50] whereas in the elderberry wines anthocyanins were the most abundant phenolic compounds.[12] In the Andean blackberry wines, AA seemed to be strongly related to the content of ellagitannins ($r = 0.980$). No significant correlation was obtained between AA and anthocyanins. The lower impact in the antioxidant capacity of the anthocyanins in comparison with the ellagitannins has been previously reported in tropical highland blackberries (*Rubus adenotricus*)[25] and in raspberries (*R. idaeus*), in which anthocyanins were responsible for 11% of the AA; this percentage reached 58% for the sum of the ellagitannins lambertianin C and sanguiin H-6.[17] In the same way, in different small fruits of the genera *Vaccinium*, *Rubus*, and *Ribes*, AA correlated less with the anthocyanin content than with TPC.[16] TPC provides a global evaluation of the phenolic richness of the material in which it is measured. In red berries the anthocyanins represent a part of such richness, sometimes not the most important. By contrast, other authors have shown that anthocyanins contribute appreciably to the antioxidant activity of blackberry extracts.[20,21,24] Nevertheless, it must be noted that in the latter studies, the ellagitannins were not considered.

Influence of Technological Factors. From the three experimental variables evaluated in this work, the yeast strain factor showed the lowest influence on the analytical parameters (Table 4). With regard to the color variables yellow, red, blue, hue, and CAW, Lalvin EC 1118 wines had a slightly more evolved color than the wines elaborated with the other three yeast strains. This seemed to be consistent with the fact that Lalvin EC 1118 wines had low anthocyanin contents, although statistical differences with respect to the other wines were not observed in all cases. The inclusion of fruit solids during fermentation also produced a slight acceleration of the color

evolution, but this effect was not clearly appreciated when wines of each yeast strain were analyzed separately. On the other hand, this factor had a great impact on the content of ellagitannins and ,consequently, on the TPC and AA of the wines (Figure 6a). On average, the wines fermented in the

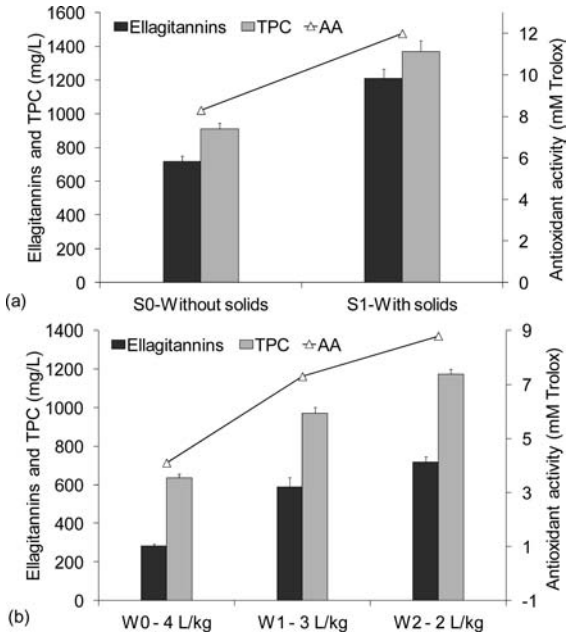

Figure 6. Ellagitannins, total phenolic content (TPC), and antioxidant activity (AA) of blackberry wines elaborated with/without the presence of fruit solids during fermentation (a) and from juices prepared with different proportions of water and fruit (b). Statistically significant differences were observed for the three variables and for both factors ($p < 0.01$).

presence of fruit solids showed 71, 50, and 46% higher values in these variables, respectively, than those obtained from filtered juices. By contrast, this factor did not affect the anthocyanin content of the wines. In blackberries, anthocyanins are located in the flesh, whereas ellagitannins are distributed around all parts of the fruits, the seeds being largely their main source.[18,30] During the preparation of the juices, crushing the raw material caused an intense disruption of the fruit drupelets. In addition, the process took place in a highly diluted medium (2−4 L of water/kg of fruit). This might have caused a massive and rapid extraction of anthocyanins in all of the treatments in such a way that subsequent maceration in those treatments fermented with fruit solids did not provide any further increase in their anthocyanin concentration. Figure 5 seems to confirm this assumption. On the other hand, the integrity of the fruit seeds was not affected by crushing, and they therefore should have retained most of their ellagitannins content. This might explain the important subsequent increment in ellagitannins, TPC, and AA observed in those wines in which a permanent contact of solids (seeds) with the liquid existed. Comparable results were obtained in black raspberry (*Rubus coreanus*) wines from Korea:[5] the presence of fruit pulp during fermentation led to increases in the TPC and the wine's radical-scavenging activity of 7.5 and 4.4%, respectively, whereas the inclusion of both pulp and seeds soared these values by up to 46.6 and 25.5%, respectively.

As could be expected, when the proportion of fruit in the musts was increased, significant increments for TPC, ellagitannins, and AA were observed in wines (Figure 6b). In addition, with growing amounts of fruit, the wines showed progressively more intense (CI, WC, AC) and more stable (CDR_{SO2}, CAW) color, with more hue, yellow, and blue and less red nuances (Table 4). With regard to the anthocyanin composition, the single significant differences were obtained for vitisins (Table 4), in a way consistent with that discussed for color parameters. In this sense, it must be noted that a strong correlation ($r = 0.944$) was observed between hue and the content of the vitisin-type compound corresponding to peak B′ in Figure 3b. In addition, the latter is negatively correlated with cyanidin 3-rutinoside ($r = -0.717$), which in turn is positively correlated with the red nuance ($r = 0.805$). Therefore, these results would indicate the occurrence of a relationship between the progressive decline and transformation of cyanidin 3-rutinoside on new and more stable pigments, such as the vitisins, and the evolution of wine color from red to yellow nuances.

In summary, *R. glaucus* berries have proven to be an advisible raw material to produce red wines with a relevant in vitro antioxidant activity comparable to that of red grape wines. The optimum winemaking conditions to obtain a blackberry wine deeply colored and rich in health-promoting compounds should include the preparation of juices with the lowest water-to-fruit ratio compatible with the appropriate technological and sensory restrictions and the presence of the fruit solids during fermentation. The high relative contents of cyanidin 3-rutinoside in *R. glaucus* in comparison with other blackberry species, in which the more labile cyanidin 3-glucoside usually prevails, could be a suitable characteristic for obtaining wines or other derivative products with a long shelf life color. Further research is needed to confirm these suggestions. The characterization of the composition of the blackberry wines with regard to flavonols, flavanols, and proanthocyanidins, among other phenolic compounds, might be also interesting.

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ABBREVIATIONS USED

AA, antioxidant activity; AC, anthocyanin color; A-total, sum of all anthocyanins; CDR_{SO2}, color due to pigments resistant to SO_2; CAW, chemical age index; CI, color intensity; CyGlu, cyanidin 3-glucoside; CyRut, cyanidin 3-rutinoside; DPPH, 2,2-diphenyl-1-picrylhydrazyl; DW, dry weight; EA, free ellagic acid; EAE, ellagic acid equivalent; FW, fresh weight; GAE, gallic acid equivalent; RT, retention time; SD, standard deviation;

RSD, relative standard deviation; TPC, total polyphenol content; WC, wine color.

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