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Characterization of polyphenols and evaluation of antioxidant capacity in grape pomace of the cv. Malbec

Andrea Antoniolli, Ariel R. Fontana *, Patricia Piccoli, Rubén Bottini

Laboratorio de Bioquímica Vegetal, Instituto de Biología Agrícola de Mendoza (IBAM), Facultad de Ciencias Agrarias, CONICET – Universidad Nacional de Cuyo, Almirante Brown 500, M5528AHB Chacras de Coria, Argentina

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ABSTRACT

Low molecular weight polyphenols (LMW-PPs) and anthocyanins, along with the antioxidant capacity, were assessed in grape pomace extract (GPE) of red grape (*Vitis vinifera* L.) cv. Malbec. Twenty-six phenolics (13 LMW-PPs and 13 anthocyanins) were characterized and quantified by HPLC-MWD and UPLC–ESI-MS. The maximum concentrations of LMW-PPs corresponded to the flavanols (+)-catechin and (−)-epicatechin, whereas malvidin-3-glucoside was the most abundant anthocyanin. Piceatannol, a stilbene analogue to resveratrol with higher antioxidant activity, was firstly identified and quantified in GPE of the cv. Malbec. The antioxidant activity for Malbec GPE determined by oxygen radical absorbance capacity (ORAC) assay was 2756 μmol TE g^{-1} GPE. Therefore, the data reported sustain the use of winemaking by-products as a cheap source of phenolic compounds suitable for biotechnological applications, as a strategy for sustainable oenology.

1. Introduction

Grape is the world's largest fruit crop with an annual production of more than 67 million tons of berries. About 80% of the worldwide grape production is used in winemaking (Fontana, Antoniolli, & Bottini, 2013; Kammerer, Claus, Carle, & Schieber, 2004), and thus this industry is relevant for several countries. Argentina has 228,575 ha of vineyards which is around 3% of the global grape area. Malbec is the main cv. produced in Argentina representing 31% of red grape area, and is considered the emblematic wine cv. for the country (Díaz, Ventura, & Galceran, 2005; Fanzone et al., 2012). Grape pomace (GP) is obtained from winemaking process as the residue remaining after fermentation, mainly constituted by skins and seeds of berries (Fontana et al., 2013). During white grape winemaking, seeds and skins are removed before fermentation, in red grape winemaking, seeds and skins are removed after a maceration period in contact with fermenting must. However, GP still contains high levels of polyphenols because of a partial extraction during maceration (Kammerer et al., 2004). Since about 20% of the weight of processed grapes remain as GP, the wine industry produces millions of tons of left-overs that represent an ecological and economical waste management issue (Fontana et al., 2013).

The recovery of phenolics from GP has attracted increasing attention in the past years, and industries are finding high value and sustainable alternative to their residues. This is because GP is a potential source of phytochemicals that may be recovered as functional compounds for the pharmaceutical, cosmetic, and food industries, and used also, as biopesticides (Fontana et al., 2013). Thus, the phenolic and antioxidant characterization of the winemaking industry by-products is the first step to promote such applications. GP may be an alternative natural source of antioxidants that are considered safer in comparison with synthetic compounds, which are widely used in the food industry although having undesirable toxicological effects (Iglesias, Pazos, Lois, & Medina, 2010). On the contrary, it is known that polyphenols have health-promoting effects and anti-aging properties (Fontana et al., 2013) since they capture free radicals and others reactive oxygen species (ROS) involved in conditions ranging from inflammatory-immune injury to myocardial infarction and cancer (Middleton, Kandaswami, & Theoharides, 2000). Researchers have observed the action of polyphenols in controlling or preventing risk factors related to metabolic syndrome and several chronic diseases in aging humans (Galleano et al., 2012; Prasain, Carlson, & Wyss, 2010). These biological properties of polyphenols are attributed mainly to their powerful antioxidant, metal chelating and antiradical activities (Wu et al., 2010; Šeruga, Novak, & Jakobek, 2011).

* Corresponding author. Tel.: +54 0261 4135010x1228.
E-mail addresses: afontana@mendoza-conicet.gob.ar, fontana_ariel@yahoo.com.ar (A.R. Fontana).

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Polyphenols constitute one of the most numerous and widely distributed groups of natural products in the plant kingdom. That includes not only an ample variety of molecules with a polyphenol structure (i.e., several hydroxyl groups on aromatic rings) but also molecules with one phenol ring, such as phenolic acids and phenolic alcohols (Fontana et al., 2013). Polyphenols contained in grapes and wine can in general be classified into two main groups: non-flavonoids (hydroxybenzoic and hydroxycinnamic acids and their derivatives, stilbenes and phenolic alcohols) and flavonoids (anthocyanins, flavanols, flavonols and dihydroflavonols) (Fanzone et al., 2012). Many polyphenols have been identified in GP, where the most abundant are anthocyanins, flavanols, flavonols, hydroxybenzoic and hydroxycinnamic acids, and stilbenes (Barcia et al., 2014; Kammerer et al., 2004).

The phytochemicals characteristics of GP are associated with bioactive properties, and therefore it is of utmost relevance to determine their composition. Despite comprehensive studies on the polyphenols profile of GP, quantitative data have been mostly expressed as total phenolic contents (TPC) and often correlated with the antioxidant activity of GPE. Although these procedures give a broad-spectrum information for preliminary characterization of GP extracts (Fontana et al., 2013), such approaches might be complemented by chromatographic techniques for the identification and quantification of individual polyphenols. The determination of compounds has particular interest to recognize possible relation between the content of polyphenols and the antioxidant properties of GPE. These data may provide valuable information for the characterization of samples and also increase the economic value of the product.

The objective of this work was the characterization of phenolic compounds and the *in vitro* antioxidant activity of GPE obtained during the winemaking process of grapes cv. Malbec, the most representative red grape variety cultivated in Argentina. To our knowledge, this is the first report on the phenolic composition of Malbec GPE. The data obtained are discussed with new insights for the winemaking industry to upgrade the value of Malbec wine residues as potential source of natural antioxidants in diverse biotechnological applications.

2. Materials and methods

2.1. Chemicals

Hydrochloric acid, ethanol and Folin–Ciocalteu reagent were purchased from Merck (São Paulo, Brazil). Trolox reagent (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), $NaH_2PO_4{\cdot}2H_2O$, $Na_2HPO_4{\cdot}12H_2O$, fluorescein and 2,2′-azobis-2-methylpropionamidine dihydrochloride (AAPH) were purchased from Sigma–Aldrich (Steinheim, Germany). Trolox standards solutions at different concentrations (0, 3.125, 6.25, 12.5, 25 and 50 μmol L^{-1}) were prepared with 75 mmol L^{-1} potassium phosphate buffer pH 7.0 (NaH_2PO_4–Na_2HPO_4). Fluorescein was prepared as a 20 nmol L^{-1} solution in 75 mmol L^{-1} potassium phosphate buffer pH 7.0. The AAPH reagent was prepared at 140 mmol L^{-1} in 75 mmol L^{-1} potassium phosphate buffer pH 7.0.

Standards of gallic acid (99%), 3-hydroxytyrosol (⩾99.5%), (−)-gallocatechin (⩾98%), caftaric acid (⩾97%), (−)-epigallocatechin (⩾95%), (+)-catechin (⩾99%), (−)-epicatechin (⩾95%), (−)-epigallocatechin gallate (⩾95%), caffeic acid (99%), syringic acid (⩾95%), coumaric acid (99%), ferulic acid (⩾99%), polydatin (⩾95%), piceatannol (99%), *trans*-resveratrol (⩾99%), quercetin hydrate (95%), cinnamic acid (99%), quercetin 3-β-D-glucoside (⩾90%), kaempferol-3-glucoside (⩾99%) and malvidin-3-O-glucoside chloride (⩾95%) were purchased from Sigma–Aldrich. The standard of 2-(4-hydroxyphenyl) ethanol (tyrosol) (⩾99.5%) was obtained from Fluka (Buchs, Switzerland). Stock solutions of the above mentioned compounds were prepared in methanol at concentration levels of 1000 μg mL^{-1}. Calibration standards were dissolved in the initial mobile phase of each method (LMW-PPs or anthocyanins, respectively). HPLC-grade Acetonitrile (MeCN) and formic acid (FA) were acquired from Mallinckrodt Baker (Inc. Phillipsburg, NJ, USA). Primary-secondary amine (PSA) and octadecylsilane (C_{18}) were both obtained from Waters (Milford, MA, USA). Reagent grade NaCl, anhydrous Na_2CO_3, anhydrous $MgSO_4$ and anhydrous $CaCl_2$ were purchased from Sigma–Aldrich. Ultrapure water was obtained from a Milli-Q system (Millipore, Billerica, MA, USA).

2.2. Sampling

This study was performed with GP obtained from *Vitis vinifera* L. cv. Malbec, provided by Catena Institute of Wine from the Adriana vineyard located in Gualtallary, Mendoza, Argentina, and harvested in 2013. The vinification procedure was conducted with mechanical daily pumping over and contact of the skins and seeds with the juice for 11 days. After that, must was pressed, fresh GP samples were collected and placed in ice cooled boxes during transportation to the laboratory, and then, stored at −20 °C until processing.

2.3. Polyphenol extraction

Solid–liquid extraction was applied to obtain a rich extract in phenolics. Conditions (time, solvent-to-solid ratio and temperature) were chosen from literature (Amendola, De Faveri, & Spigno, 2010; Bucić-Kojić, Planinić, Tomas, Jakobek, & Šeruga, 2009; Spigno, Tramelli, & De Faveri, 2007; Vatai, Skerget, & Knez, 2009). Briefly, GP samples were grinded in a laboratory mixer with an aliquot of the extraction solvent (ethanol:water, 50:50 v/v) at a 25:1 solvent-to-sample (DW) ratio. The extraction was carried out during 120 min under continuous stirring at 60 °C. The liquid was filtered through a filter paper and concentrated in a rotary evaporator at 40 °C. The concentrate extracts were freeze-dried for 96 h at 0.12 bar and −45 °C and then, placed in sealed tubes and kept at −20 °C in dry atmosphere and darkness prior analysis. Dry matter content of GP was determined by drying at 105 °C to constant mass in triplicate. Yields were expressed in percent and calculated as grams of freeze-dried extract per 100 g of dried GP. Extraction trials were carried out in triplicate.

2.4. Sample preparation

Anthocyanins were directly analyzed by dissolving an aliquot of 5 mg freeze-dried extract in the initial mobile phase of HPLC method for anthocyanins. LMW-PPs were extracted according to a previously reported method (Fontana & Bottini, 2014). It should be pointed out that freeze-drying is the ideal pre-treatment to better preserve the polyphenolic fraction, although it is practically and economically not feasible for the real industrial exploitation of GP. In such a case, either an immediate extraction of the fresh GP or a drying pre-treatment are more likely applied; for the later, it comes that most probably extraction yields may be a little different as compared with the process used here. Briefly, 50 mg of freeze-dried extract were dissolved in water, made up to 5 mL and extracted with 2.5 mL acidified MeCN. For phase separation 1.5 g NaCl and 4 g $MgSO_4$ were added, shaken 1 min and centrifuged 10 min at 3000 rpm. Then, 1 mL aliquot of the upper MeCN phase was transferred to a 2 mL clean tube containing $CaCl_2$, PSA and C_{18}, vortexed and centrifuged. Finally, an aliquot of extract was evaporated to dryness, the residue was reconstituted in the initial mobile phase for LMW-PPs and then, analyzed by high

performance liquid chromatography-multiple wavelength detector (HPLC-MWD).

2.5. Total phenolic content (TPC)

The TPC was spectrophotometrically measured with an UV–vis spectrophotometer Cary-50 (Varian Inc., Mulgrave, Australia) from 5 mg freeze-dried extract dissolved in ethanol 50% (v/v) aqueous solution. Following (Spigno et al., 2007), TPC was determined by two different methods: the Folin–Ciocalteu assay (FC) and the direct reading of the absorbance at 280 nm (Ribéreau-Gayon, Glories, Maujean, & Dubourdieu, 2006). In this case the sample was diluted 1:100 v/v and the absorption at 280 nm was measured. The TPC was calculated from a calibration curve made with standard solutions of gallic acid (three replicates) in the range between 5.4 and 31.5 mg L^{-1} (R^2 = 0.999), and expressed as gallic acid equivalents (GAE 280, mg g^{-1}). For the FC method an aliquot of 0.25 mL of dissolved sample, 12.5 mL of distilled water, 1.25 mL of Folin–Ciocalteu reagent and 5 mL of 20% Na_2CO_3 solution were mixed into a 25 mL flask and added with distilled water to final volume. Prior to the measurement of the absorbance at 765 nm, the mixture was homogenized and incubated 30 min in darkness and 25 °C. TPC was expressed as GAE mg g^{-1} freeze-dried GPE by using a calibration curve with gallic acid as standard (three replicates) in a range between 0 and 200 mg L^{-1} (R^2 = 0.999).

2.6. Antioxidant activity

The ORAC of GPE was determined according to Berli, Alonso, Bressan-Smith, and Bottini (2012). Re-suspended GPE solutions, as described in TPC determination, were diluted 1:750 v/v in 75 mmol L^{-1} potassium phosphate buffer (pH 7.0). Fifty microliters aliquots of diluted samples and trolox standards (0, 3.125, 6.25, 12.5, 25 and 50 μmol L^{-1}) were added to a 96-well plate. Then, 100 μL of fluorescein solution were added and the mixture was incubated at 37 °C 7 min before the addition of 50 μL of 140 mmol L^{-1} peroxyl radical generator AAPH. Fluorescence was monitored by using 485 nm excitation and 538 nm emissions at 1 min intervals for 60 min on a microplate fluorometer (Fluoroskan Ascent FL, Thermo Fisher Scientific Inc, Wilmington, DE). The area under the curve of the fluorescence decay during 60 min was calculated and the ORAC was expressed as μmol of trolox equivalents per gram of GPE (μmol TE g^{-1}).

2.7. Low molecular weight polyphenols

HPLC separations/quantifications were carried out with a Dionex Ultimate 3000 HPLC-MWD system (Dionex Softron GmbH, Thermo Fisher Scientific Inc., Germering, Germany) and a reversed-phase Kinetex C_{18} column (3.0 mm × 100 mm, 2.6 μm) Phenomenex (Torrance, CA, USA). Ultrapure water with 0.1% FA (A) and MeCN (B) were used as mobile phases. Analytes were separated using the following gradient: 0–2.7 min, 5% B; 2.7–11 min, 30% B; 11–14 min, 95% B; 14–15.5 min, 95% B; 15.5–17 min, 5% B: 17–20, 5% B. The mobile phase flow was 0.45 mL min^{-1} from 0 to 2.5 min and 15.5 min to 20 min; while the flow was 0.8 mL min^{-1} from 2.7 min to 14 min. Temperature column was 40 °C and the injection volume 5 μL. The identification and quantification of polyphenols in the GPE were based on the comparison of the retention times (t_R) and maximum absorbance values of detected peaks in samples with those obtained by injection of pure standards. Samples were quantified by using an external calibration with pure standards. Linear ranges between 0.5 and 20 μg mL^{-1} were obtained with the exception of (+)-catechin and quercetin (2.5–50 μg mL^{-1}), and quercetin-3-glucoside and kaempferol-3-glucoside (0.5–10 μg mL^{-1}) with coefficient of determination (R^2) higher than 0.999 for all the studied LMW-PPs.

2.8. Anthocyanins

For HPLC-MWD analysis of anthocyanins, separations were carried out in a reversed-phase Symmetry C_{18} column (4.6 mm × 250 mm, 5 μm) Waters (Milford, MA, USA). The chromatographic analysis of anthocyanins were carried out according to the method of Kammerer et al. with some modifications (Kammerer et al., 2004). The mobile phase consisted of ultrapure water/FA/MeCN (87:10:3, v/v/v; eluent A) and ultrapure water/FA/MeCN (40:10:50, v/v/v; eluent B) using the following gradient: 0 min, 10% B; 0–10 min, 25% B; 10–15 min, 31% B; 15–20 min, 40% B; 20–30 min, 50% B; 30–35 min, 100% B; 35–40 min, 10% B; 40–47 min, 10% B. The mobile phase flow was 0.8 mL min^{-1}, column temperature 35 °C, and injection volume 10 μL. Quantifications were carried out by area measurements at 520 nm, and the anthocyanin content was expressed as malvidin-3-glucoside, using an external standard calibration curve (1–250 μg mL^{-1}, R^2 = 0.9984). With the aim to confirm the anthocyanins compounds detected in Malbec GPE analyzed with LC-MWD, a liquid chromatography/mass spectrometry (UPLC–MS) system was used. In this way, an Aqcuity UPLC system, (Waters, Milford, MA, USA) was employed to confirm anthocyanins structure. It was equipped with a sprayer needle where ions were generated by electrospray ionization (ESI) in positive ionization mode. Single quadrupole mass spectrometry detection was applied and the chromatographic conditions were the same described for HPLC-MWD analysis.

3. Results and discussion

3.1. Extraction yield and TPC

Considering the extraction solvent applied in solid–liquid extraction from GP, the published results are not conclusive about an ideal solvent, and different mixtures have been proposed (Amendola et al., 2010; Bucić-Kojić et al., 2009; Fontana et al., 2013; Spigno & De Faveri, 2007). In the present study GPE were obtained by using non-toxic, cheap, and available method of extraction with the aim to achieve a GPE suitable for different industrial applications. Among the most commonly solvents used in antioxidants extraction, ethanol is the best option because it is the natural solvent of these compounds in the wine-making process (Spigno & De Faveri, 2007).

The total extraction yields obtained from Malbec GP samples in the present research are shown in Table 1. In previous studies with red GP (Spigno et al., 2007) obtained values of 9 to 12%, while an average of 7.5% were attained by (Amendola et al., 2010) although with a different cultivar (Barbera). Rockenbach et al. (2011) reported yields of *ca.* 25 mg per 100 g of GP in extractions with

Table 1
Extraction yields parameters, total phenolic content and antioxidant activity.

Total extract yield DW%[a]	16.1 ± 3.1
TPC GAE FC[b]	196.2 ± 22.7
TPC GAE 280[c]	165.7 ± 30.2
Yield DW[d]	31.6 ± 3.7
ORAC[e]	2756.0 ± 109.1

[a] g freeze-dried extract/100 g GP (DW).
[b] mg GAE g^{-1} GPE.
[c] mg GAE g^{-1} GPE.
[d] mg GAE g^{-1} GP (DW).
[e] μmol TE g^{-1} GPE.

Table 2
Levels of LMW-PPs in freeze-dried Malbec GPE. Average contents (μg g^{-1} GPE) with their standard deviations, $n = 3$ replicates.

Analyte	Concentration
Hydroxybenzoic acids	
Gallic acid	252.8 ± 18.5
Syringic acid	1731.7 ± 156.3
Total	1984.5
Hydroxycinnamic acids	
Caftaric acid	n.d.
Cafeic acid	16.0 ± 2.6
p-Coumaric acid	64.6 ± 5.3
Ferulic acid	24.1 ± 1.1
Total	104.6
Stilbene	
Polydatin	12.3 ± 2.7
Piceatannol	38.8 ± 5.4
Trans-resveratrol	36.0 ± 4.9
Total	87.0
Flavanols	
(+)-Catechin	3387.5 ± 374.7
(−)-Epicatechin	1763.4 ± 221.8
(−)-Gallocatechin	n.d.
(−)-Epigallocatechin	n.d.
(−)-Epigallocatechin gallate	n.d.
Total	5150.8
Flavonols	
Quercetin-3-glucoside	112.2 ± 12.1
Kaempferol-3-glucoside	n.d.
Quercetin	557.3 ± 83.9
Total	669.5
Other compounds	
OH-tyrosol	n.d.
Tyrosol	34.0 ± 2.7
Total	39.1
Total LMW-PPs	8035.5

n.d., not detected.

acidified methanol for the cvs. Cabernet Sauvignon, Merlot and Bordeaux, and of around 15 g per 100 g of GP for cv. Isabel. In general, other authors do not express total yield in terms of the amount of dried extract obtained.

The production of secondary metabolites by vine plants depends not only on genetic characteristics but also on growth environmental conditions. The content of the polyphenols in Malbec grapes may vary between different cultivars, being influenced by locations, harvest time, and the growth environment (Berli et al., 2008, 2012).

The obtained TPC of GPE determined by FC and absorbance 280 methods were lower than the reported by other authors by using similar extraction solvent (ethanol–water 50%) and methodology. Spigno et al. (2007) informed TPC levels of 441 mg GAE FC g^{-1} GPE and 227 mg GAE 280 g^{-1} GPE for cv. Barbera and Amendola et al. (2010) found 269.0 mg GAE FC g^{-1} and 261.1 mg GAE 280 g^{-1}). While GPE of cv. Norton extracted with 80% ethanol at 1:10 ratio (m/v) produced 475 mg GAE 280 g^{-1} GPE (Hogan, Canning, Sun, Sun, & Zhou, 2010). In commercial grape seeds extracts, TPC varied from 78.5 to 563 mg GAE FC g^{-1} GSE (Monagas et al., 2005).

The polyphenols yield relative to dry GP was an average of 31.6 mg GAE FC g^{-1} DW (Table 1). Using similar conditions to the present work (Spigno et al., 2007) obtained higher polyphenols yield in cv. Barbera GP resulting in 42.5 mg GAE g^{-1} DW, while Vatai et al. (2009) reported lower yields (17.3 mg GAE g^{-1}) from red GP cv. Refošk, and Bucić-Kojić et al. (2009) achieved higher values from red grape seeds cv. Frankovka (129.59 mg GAE g^{-1}). Rockenbach et al. (2011) with the extraction system mentioned above, reported yields in Cabernet Sauvignon of 74.7, in Bordeaux 63.3, in Merlot 46.2, and in Isabel 32.62 mg GAE g^{-1}. González-Centeno et al. (2013) determined total phenolic yields ranged from 31 to 47 mg GAE g^{-1} from white GPs, which was submitted to solid/liquid consecutive extractions with acetone/water (80:20, v/v) and with MeOH/water (60:40, v/v) as solvent systems.

3.2. Antioxidant activity

The interest in the measurement and assessment of antioxidant capacity is increasing due to the importance of ROS in aging and pathogenesis of many diseases in which ROS are involved (Prior et al., 2003). As well, it represents a useful strategy to evaluate extraction methods and provide a preliminary characterization of samples before chromatographic analysis or biological assays. There is disparity in the types of tests with different bases that are applied to evaluate the antioxidant activity of extracts. Also, differences in the expression of results and use of reference antioxidants have been reported (Fontana et al., 2013). The antioxidant activity measured by ORAC assay has been related with chain-breaking antioxidants against peroxyl radical, interrupting the radical chain reaction (propagation and branching) (Ou, Hampsch-Woodill, & Prior, 2001). In this way, ORAC method is based on the inhibition of the peroxyl-radical-induced oxidation initiated by thermal decomposition of AAPH, where fluorescein is protected from oxidation by the peroxyl radical. Then, the antioxidant activity is quantified by the areas under the curves of relative fluorescence intensity. There are few reports about the use of ORAC method to determine the antioxidant capacity in extracts derived from GP. However, this technique has been used to other kind of samples such as grape cane extract (Karacabey & Mazza, 2010), de-alcoholized wines (Bogianchini, Cerezo, Gomis, López, & García-Parrilla, 2011) and extracts of Brazilian blueberries (Pertuzatti et al., 2014) among others. The ORAC technique is the only one that combines both time and degree of inhibition into a single magnitude (Fontana et al., 2013; Prior et al., 2003).

The antioxidant activity data for the Malbec GPE obtained in the current research through the ORAC assay was 2756 ± 109 μmol TE g^{-1} GPE. Yilmaz and Toledo (2003, 2006) reported the antioxidant capacity of grape seed extract (GSE) and skin extracts obtained from winemaking and juice industry of grapes cv. Chardonay, Merlot and Muscadine by the ORAC method. Their results showed that grape seed powder has higher ORAC values as compared with skin extracts (between 303 to 638 and 70 to 103 μmol TE g^{-1} DW raw material, respectively). They also proposed that the high antioxidant capacities of GSE would most likely be due to the presence of polymeric procyanidins, in addition to the monomers. Monagas et al. (2005) used ORAC to quantify the antioxidant capacity of commercial dietary GSE, obtaining values among 2860 to 26,200 μmol g^{-1} GSE within their extracts. Ky, Lorrain, Kolbas, Crozier, and Teissedre (2014) found an ORAC value between 202 and 561 μmol TE g^{-1} DW raw material when studied grape seeds from six red wine cultivars. Furthermore, a freeze-dried GPE obtained by enzymatic extraction has an average ORAC value of 4239 μmol TE g^{-1} (Rodriguez-Rodriguez et al., 2012) and spray-dried GPE extracted using microwave assisted method by Pérez-Serradilla and Luque de Castro (2011) resulted in 3930 μmol TE g^{-1} of spray-dried GPE.

As can be observed the variability in the results could be related to different factors involved in extraction of GP, winemaking processes, as well as the genetic and environmental conditions of grape varietals.

3.3. Identification and quantification of polyphenols

The identified and quantified LMW-PPs (non-anthocyanins) in Malbec GPE were gallic acid, tyrosol, (+)-catechin, (−)-epicatechin, caffeic acid, syringic acid, coumaric acid, ferulic acid, polydatin,

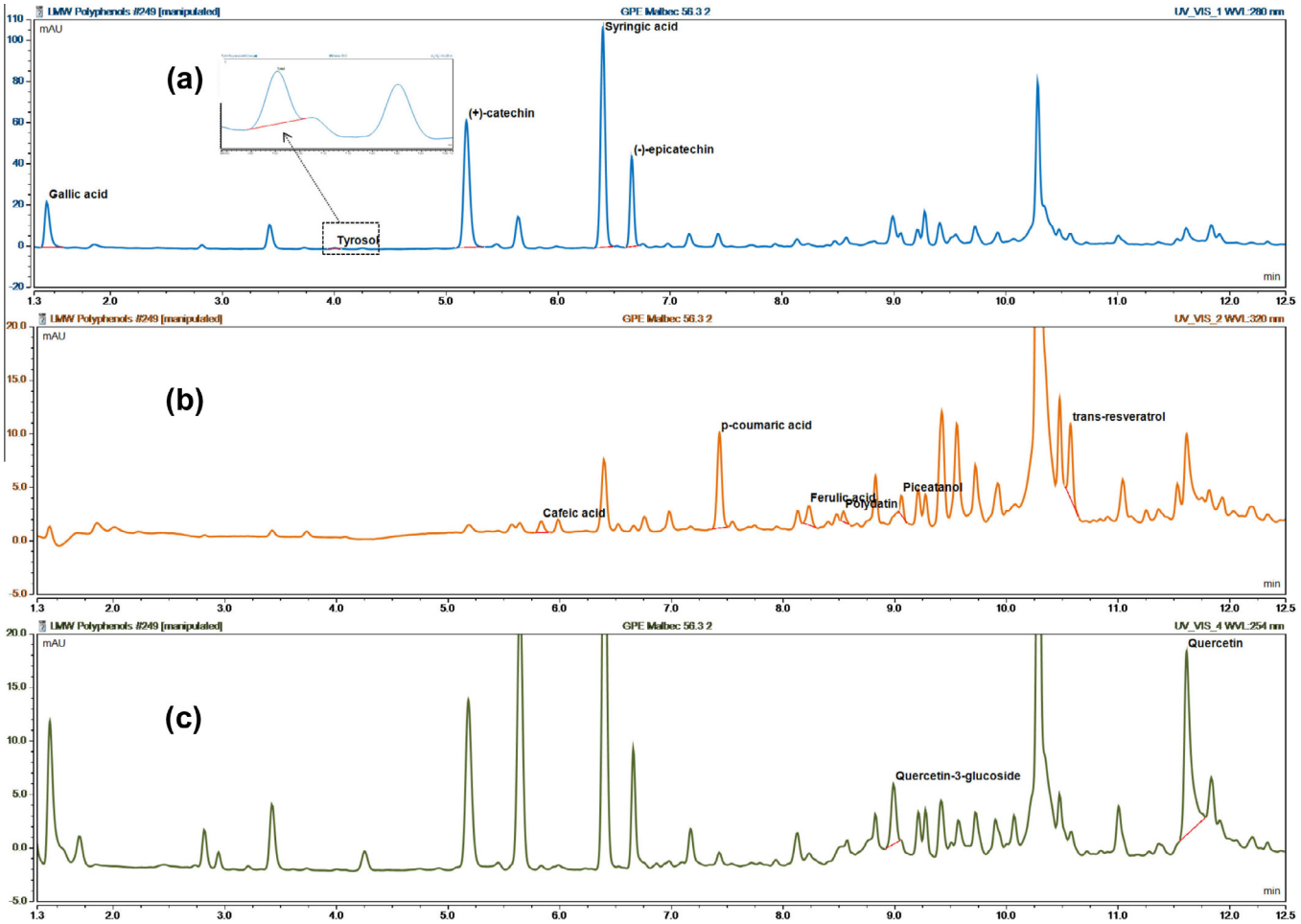

Fig. 1. Extracted chromatograms of each detection wavelength for LMW-PPs in freeze-dried Malbec GPE analyzed by HPLC-MWD. (a) 280 nm, (b) 320 nm and (c) 254 nm.

Table 3
Anthocyanins quantified in freeze-dried Malbec GPE. Average contents (μg g^{-1} GPE) with their standard deviations, $n = 3$ replicates.

Anthocyanins	μg g^{-1} of freeze dried GPE
Delphinidin 3-O-glucoside	4581 ± 412
Cyanidin 3-O-glucoside	870 ± 104
Petunidin 3-O-glucoside	6880 ± 481
Peonidin 3-O-glucoside	2460 ± 248
Malvidin 3-O-glucoside	26,658 ± 1866
Total glucosylated	41,449
Delphinidin 3-O-acetylglucoside	1043 ± 115
Petunidin 3-O-acetylglucoside	1424 ± 152
Peonidin 3-O-acetylglucoside	1902 ± 215
Malvidin 3-O-acetylglucoside	4021 ± 322
Total acetylated	8391
Cyanidin 3-O-p-coumaroylglucoside	1886 ± 151
Petunidin 3-O-p-coumaroylglucoside	2481 ± 199
Peonidin 3-O-p-coumaroylglucoside	1854 ± 204
Malvidin 3-O-p-coumaroylglucoside	12,864 ± 772
Total coumaroylated	19,085
Total anthocyanins	68,924

piceatannol, *trans*-resveratrol, quercetin-3-glucoside and quercetin grouped into non-flavonoids (hydroxybenzoic and hydroxycinnamic acids, and stilbenes), flavonoids (flavanols and flavonols) and other compounds. The studied analytes were successfully separated and identified by comparing their elution times and spectra with pure standards. Table 2 presents the concentration of individual LMW-PPs, while Fig. 1 shows the chromatogram of LMW-PPs detected and quantified. The flavonoids (+)-catechin and (−)-epicatechin as well as syringic acid were the most abundant compounds in the studied GPE samples with concentrations between 1731 and 3387 μg g^{-1}, followed by gallic acid, quercetin and quercetin-3-glucoside. The stilbene compounds showed concentration ranged between 12 and 39 μg g^{-1} for polydatin and piceatannol, respectively. The most interesting result, from both qualitative and quantitative point of views, was the identification of piceatannol in GPE from Malbec grapes. Piceatannol is a tetrahydroxy stilbene, and similarly to *trans*-resveratrol, the most important sources in the human diet are grapes and wine. Contrary to resveratrol, scientific papers providing detailed information concerning the concentration of piceatannol in wine (or derivatives) are limited. Different reports showed that piceatannol content in grapes is about 4-times lower than that of resveratrol. However, its concentration in some kinds of red wine may be up to 2-times higher than *trans*-resveratrol (Piotrowska, Kucinska, & Murias, 2012). Compared to *trans*-resveratrol, piceatannol has reported higher *in vitro* antioxidant activity showing its importance. Considering the probable synergic effects with other stilbenes (and polyphenols in general), the quantification of piceatannol could add novel information for supporting the use of GPE as a complementary nutritional/pharmacological additive. In this work, the concentration of *trans*-resveratrol and piceatannol were similar, adding a potential of Malbec GP as a source of stilbenes. Other compound quantified in GPE for first time was the phenylethanoid

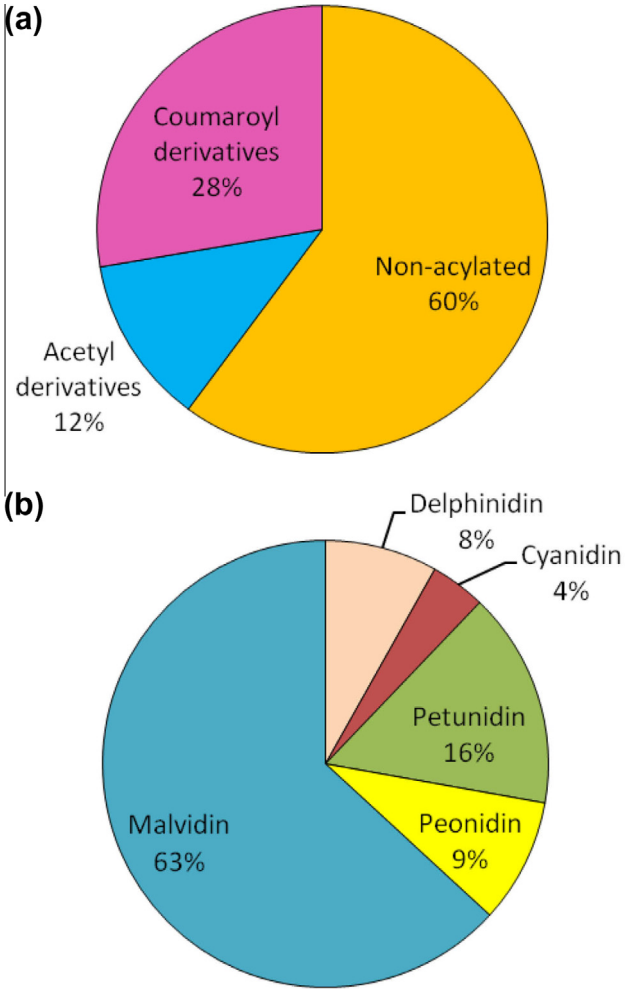

Fig. 2. (a) Distribution of anthocyanins based on type of derivative (non acylated, acylated and coumarylated); (b) anthocyanins distribution by type of anthocyanidinin.

tyrosol, which also has important antioxidant properties (Cañuelo et al., 2012).

There are only few works related to phenolic characterization in the cv. Malbec and they are focused on the analysis of wine (Fanzone, Peña-Neira, Jofre, Assof, & Zamora, 2010; Fanzone et al., 2012). Unfortunately; there is not any statement in Malbec GP to establish an appraisal.

The obtained results for Malbec GPE showed similarities in terms of the identified LMW-PPs and their relative contents with those obtained in the mentioned papers of Malbec wine. The majority of compounds were quantified by Fanzone et al. (2010, 2012) with the exception of piceatannol. A difference between Malbec wines and GPE was observed with respect to the concentration of syringic and gallic acids. In GPE, opposed to wine, syringic acid was considerable more abundant than gallic acid. A similar behavior was observed for quercetin and quercetin-3-glucoside.

Comparison of the chromatographic profiles and quantitative data of Malbec GP with others reports in a similar matrix could provide some evidences for the variety of compounds present in the extract. The concentration of *trans*-resveratrol found in this work is in agreement with those reported by Kammerer et al. (2004) for GP of Cabernet Mitos (123 μg g^{-1} dried GP). However, the majority of works on GP characterization of red grape varieties reported minor concentrations of *trans*-resveratrol, between 6 and 64 μg g^{-1} dried GP (Careri, Corradini, Elviri, Nicoletti, & Zagnoni, 2003; Casazza, Aliakbarian, De Faveri, Fiori, & Perego, 2012; Hogan et al., 2010; Rockenbach et al., 2011). For the flavonoids (+)-catechin and (−)-epicatechin the concentrations obtained in this work were lower than the reported for GPE of Pinot noir (between 5700 and 8970 μg g^{-1} GPE) (Cheng, Bekhit, McConnell, Mros, & Zhao, 2012). The same authors reported similar concentrations for Pinot meunir GPE (2130–3610 μg g^{-1}) (Cheng et al., 2012) and lower than the reported in this study for Merlot, Cabernet sauvignon and Canaiolo seeds (1276–1400 μg g^{-1}). These results indicate that while the range of phenolics content in GP from different studies may be comparable, the qualitative content of the individual phenolics may be different and dependent on grape variety, environmental conditions and winemaking procedures.

3.4. Anthocyanin composition

The anthocyanin transfer from grape to wine is rather limited and values lower than 40% has been suggested (Kammerer, Gajdos Kljusuric, Carle, & Schieber, 2005). Consequently, a high amount of grape anthocyanins remains in GP being an interesting source of natural food colorants for food industry. For Malbec GPE, 13 glycosylated and acylated (acetyl and p-coumaroyl derivatives) anthocyanins, were identified by HPLC-MWD and confirmed by HPLC-MS. Table 3 summarizes the individual anthocyanin and concentrations in Malbec GPE. As expected, malvidin 3-O-glucoside was the predominant compound, mostly followed by malvidin 3-O-p-coumaroylglucoside.

Concentrations of monoglucosylated anthocyanins in Malbec GPE ranged from 870 to 26,658 μg g^{-1} GPE for cyanidin 3-O-glucoside and malvidin 3-O-glucoside, respectively. The concentrations for acetylated anthocyanins found in the studied GPE ranged from 1043 to 4021 μg g^{-1} for delphinidin, 3-O-acetylglucoside and malvidin 3-O-acetylglucoside, respectively. In the case of coumaroyl derivatives, the concentrations ranged from 1854 to 12,864 μg g^{-1} for petunidin 3-O-p-coumaroylglucoside and malvidin 3-O-p-coumaroylglucoside, respectively.

As expected, malvidin derivatives were the predominant compounds. Fig. 2a shows that the non-acylated glucosides were the most abundant group of pigments in Malbec GPE (60%) compared with the acylated forms. Fig. 2b shows that the amount of malvidin-3-glucoside was highest among all of the anthocyanins. The second most abundant anthocyanidin was petunidin, followed by peonidin. The achieved results are in agreement in terms of the identified anthocyanins and their relative concentrations with those obtained (Fanzone et al., 2010, 2012) for Malbec wines. A difference between Malbec wines and GPE was observed respect to the pattern of acetylglucosides and coumaroylglucosides. In GPE, opposed to wines, coumaroylglucosides were more abundant than acetylglucosides. It could be related to the different compounds extractability during winemaking and GP extraction.

With regard to previous works in GP, a comparable but not identical phenolic profile was observed. Kammerer et al. (2004) stated the anthocyanin content for the red variety Cabernet mitos, noticing a similar pattern distribution to Malbec GP in this work. Both works showed a higher concentration of coumaroylglucosides than of acetylglucosides derivatives. In terms of total concentration of anthocyanins, Kammerer et al. (2004) reported 50,616 and 131,868 μg g^{-1} DW of anthocyanins for grape skins of GP collected in two consecutive years, showing the effect of different environmental conditions and winemaking procedures on the final GP obtained.

4. Conclusions

Qualitative and quantitative characterization of phenolic compounds of Malbec GPE allowed the identification of 26 compounds

(13 LMW-PPs and 13 anthocyanins) representing the first report for Malbec GP. As far as we know, the stilbene piceatannol reported in Malbec grapes and GPs adds originality to the most cultivated grape in Argentina. Also, the new data reported in terms of antioxidant capacity and phenolic composition of Malbec GPE provides information for winemaking industry to use by-products of this cultivar as a cheap source of phenolic compounds suitable for biotechnological applications. This evaluation may be included as an innovative strategy for sustainable oenology.

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