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In vivo antioxidant activity of grape, pomace and wine from three red varieties grown in Argentina: Its relationship to phenolic profile

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ABSTRACT

The *in vivo* antioxidant capacity (AC) of natural antioxidants involved in the winemaking of three red grape varieties grown in Argentina and its association with the phenolic composition were studied. Polyphenols from grape, wine and pomace were capable of rescuing yeast cells from oxidative stress, probably by the induction of antioxidant enzymes, such as glutathione reductase (GR) and glutathione peroxidase (GPx). Observed AC was highly correlated with phenolic profiles, as shown by canonical correlation analysis (CCA) and multiple regression analysis (MRA). Grape samples showed the highest activity among sample types, and according to MRA kaempferol-3-glucoside and fertaric acid contributed positively, whereas ethyl gallate contributed negatively to AC of wines and pomaces. With respect to varieties, Syrah was the one with the highest activity, owing to higher contents of anthocyanins, compounds highly related to bioactivity.

Abbreviations: GR, glutathione reductase; GPx, glutathione peroxidase; CCA, canonical correlation analysis; MRA, multiple regression analysis; PCA, principal component analysis; TP, total polyphenols; GAE, gallic acid equivalents; DW, dry weight; LOD, limit of detection; LOQ, limit of quantification; DMSO, dimethyl sulfoxide; YPD, yeast extract peptone dextrose medium

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Chemical compounds: Malvidin-3-glucoside (PubChem CID: 443652); Malvidin-3-coumaroyglucoside (PubChem CID: 71308234); Petunidin-3-coumaroylglucoside (PubChem CID: 44256963); Kaempferol-3-glucoside (PubChem CID: 5282102); Fertaric Acid (PubChem CID: 22298372); Ethyl gallate (PubChem CID: 13250); *trans*-Resveratrol (PubChem CID: 445154).

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1. Introduction

Epidemiologic studies provide convincing evidence that diets rich in plant foods (fruits, vegetables, grains and derivative products from fruits, vegetables and grains) are associated with the prevention or delay of chronic degenerative diseases, such as atherosclerosis, cancer, cardiovascular disease, and type 2 diabetes (Spormann et al., 2008). In the last decades oxidative stress has been proposed to play a fundamental role in these pathologies. The oxidative stress is defined as an imbalance between reactive oxygen species (ROS) production and the inability of the antioxidant biological system to detoxify these free radicals. As a result, this increased level of ROS leads cell to an oxidative stress state, which impacts on a variety of biochemical and physiological processes (Gutteridge & Halliwell, 2000).

In addition to vitamins and minerals, foods obtained from plant kingdom are rich in polyphenols, bioactive compounds capable of reducing the oxidative stress in cells. This biological property is mainly attributed to their behaviour as powerful antioxidants. Among the sources of exogenous natural antioxidants, grapes, grape pomace and wines from red varieties of *Vitis vinifera* L. have received much attention because of their high concentration and great variety of phenolic compounds.

Red grape polyphenols are mainly flavonoid (anthocyanins, flavonols and flavanols) and non-flavonoid compounds (phenolic acids like hydroxycinnamic and hydroxybenzoic acids and stilbenes), all of them are well known for their strong biological action (Monagas, Bartolomé, & Gómez-Cordovés, 2005). These compounds are transferred from the solid parts of the grape into the wine during winemaking operations (crushing, maceration and fermentation). The grape pomace is obtained from the winery industry as a solid waste after alcoholic fermentation, and it is mainly constituted by berry skins and seeds. This residue is characterised by a high phenolic content because of poor extraction during the winemaking process. Since about 80% of the worldwide grape production is used in winemaking and about 25% of the weight of processed grapes remains as pomace, the wine industry produces millions of tons of this residue, which represents an ecological and economical issue of waste management. Particular attention is currently being paid to the exploitation of this winery byproduct because it is considered an alternative and inexpensive source for obtaining natural phenolic compounds with potential application as food antioxidants (Fontana, Antoniolli, & Bottini, 2013).

Chemical antioxidant activity assays (such as FRAP-ferric reducing antioxidant power, TEAC-trolox equivalent antioxidant capacity) are used extensively to evaluate the potential bioactivity of plant foods, yet they do not mimic the complexity of biological systems. The cellular antioxidant activity assay was developed to be a more biologically relevant model to measure antioxidant activity. This approach reflects the cellular physiological conditions and considers the bioavailability and metabolism issues, which influence the net response of the phenolic compounds present in those samples. Among the cell culture models used to support antioxidant research prior to expensive and time-consuming animal studies and human clinical trials, the eukaryotic yeast *Saccharomyces cerevisiae* has been proposed for a rapid screening of AC in wine and food (Baroni, Di Paola-Naranjo, García-Ferreyra, Otaiza, & Wunderlin, 2012; Ignea et al., 2013; López de Lerma, Peinado, & Peinado, 2013; Martorell et al., 2011; Peinado et al., 2013; Stinco et al., 2015). Protective effect of polyphenols against oxidising substances in cells is related to a large number of biological mechanisms, including antioxidant enzyme induction such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and glutathione peroxidase (GPx). These endogenous antioxidants are involved in the metabolism of glutathione (GSH), an important non-enzymatic antioxidant involved in cellular detoxification to maintain redox status (López-Alarcón & Denicola, 2013).

Nowadays, it is known that AC cannot be easily predicted by the content of a specific group of compounds or by measuring a single substance (Baroni et al., 2012). Furthermore, this activity is the result of a synergistic and antagonistic effect of different polyphenols and with other components of the food matrix or of the organism (Rohn, Rawel, & Kroll, 2004). Hence, we need to know the relative contribution of entire phenolic profile to the AC of these exogenous natural antioxidants in order to explain their bioactive behaviour. Accordingly, we propose the use of multiple regression analyses (MRA), a mathematical tool that quantifies the relationship between a dependent variable and twoor more independent variables. Furthermore, MRA allows determining the contribution of each variable to the model; thus, it allows us to identify the key phenolic compounds that contribute to the biological activity observed.

Phenolic profile is strongly affected both quantitatively and qualitatively by the particular grape variety, ripeness, environmental factors and winemaking technological procedure. Although there are a large number of studies that focus on polyphenols and their relationship with winemaking technology, understanding changes in phenolic profile has been always a challenge due to complexity and diversity of these compounds in red wines (Borazan & Bozan, 2013). Moreover, literature is scarce in terms of the association found/shown between these changes and the AC observed. Studies on the possible health benefits of grape and its derivates and on the mechanism underlying these benefits must be accompanied by a comprehensive characterisation of phenol profile.

In the present work we characterised the phenolic profile of grapes (raw material), pomaces (byproducts) and wines (final product) of three *Vitis vinifera* L. red varieties grown in Argentina, and assessed their AC by a cellular model in order to recognise the phenolic compounds associated with the bioactive behaviour of samples by MRA analysis. This work not only describes changes in phenolic profile and *in vivo* AC from grape to wine, but also studies one of the possible mechanisms by which polyphenols would be exerting their effect.

2. Materials and methods

2.1. Samples

Vitis vinifera L. red grapes, grape pomace (skins and seeds) and red young wine, corresponding to three varieties (Syrah, Merlot and Cabernet Sauvignon), were obtained from the "Antonio de

la Torre" cellar in the province of San Juan, Argentina. Three replicates of each sample type and variety were analysed. First, grapes were collected in their optimal ripening stage (22 to 25 g sucrose/100 mL). Pomaces were then collected after alcoholic fermentation and subsequent pressing, while young wines were obtained after stabilisation (4–5 months after primary fermentation) and bottling. All samples were transported to the laboratory at 4–8 °C and protected from light.

2.2. Sample preparation

Extraction of phenolic compounds from whole grapes (previously selected, de-stemmed and washed with distilled water and their remaining water absorbed with blotting paper) and pomaces was carried out as described by Poudel, Tamura, Kataoka, and Mochioka (2008) with minor changes. Briefly, grape and pomace samples were lyophilised and their moisture calculated by weight difference before and after freeze-drying. The moisture percentage ranged from 69 to 72% for grapes, and 49 to 52% for pomaces. After lyophilisation, samples were frozen using liquid nitrogen and grounded until obtaining a fine powder. A portion of 1 g of treated sample was extracted with 15 mL of acidified methanol (0.1% HCl, v/v) in a blender (Ultra-Turrax T18; Ika-Labortechnik, Germany). The homogenate obtained was incubated with agitation for 2 h at 4 °C and then centrifuged at 2058 *g* for 10 min. The supernatant was separated and the solid pellet re-extracted with 5 mL of acidified methanol as previously described. The combined extracts were filtered, fractionated in Eppendorf tubes and stored at –80 °C until analysis. The extraction procedure was carried out in triplicate.

Wines were filtered using Whatman no. 1 filter paper (Whatman, UK), fractionated in 125 mL polyethylene bottles and stored at –80 °C until analysis.

2.3. Determination of total polyphenols

Total polyphenol (TP) content of grapes, pomaces and wines was assayed by the Folin–Ciocalteu method, according to Arnous, Makris, and Kefalas (2001). The absorbance of samples appropriately diluted was read at 750 nm using a UV/VIS Spectrophotometer (Lambda 25, Perkin Elmer, Seer Green, U.K.). TP was calculated using a calibration curve constructed with gallic acid (Riedel-de-Haën, Seelze, Germany). Results of grape and pomace extracts are expressed in mg gallic acid equivalents (GAE) per 100 g of dry weight of sample (DW). Results of wines are reported in mg gallic acid equivalents (GAE) per litre. All samples were analysed in triplicate.

2.4. Determination of phenolic profile

Phenolic compounds were analysed by HPLC-PDA-ESI-MS/MS, using an Agilent Series 1200 LC System (Agilent, Santa Clara, CA, USA), coupled to a PDA detector (Agilent Series 1200) in tandem with an ESI source, connected to a MicroQTOF II (Bruker Daltonics, Billerica, MA, USA) mass spectrometer (MS and MS/MS). The HPLC system was equipped with a binary gradient pump, solvent degasser and autosampler (Agilent Series 1200 L, Santa Clara, CA, USA).

HPLC analyses were performed on a LUNA (Phenomenex, Torrance, CA, USA) C18 column (5 μm, 250 mm x 4.60 mm i.d.), at 35 °C and 0.4 mL min^{-1} flow rate, using 0.5% formic acid (solvent A) and 0.5% formic acid in methanol (solvent B). The gradient programme started with 20% B and changed to 50% B along 3 min, held for 5 min, followed by a second ramp to 70% B along 7 min, held for 5 min, and a third ramp to 80% B along 1 min, and remained in this last condition for 9 min before the next run. The injection volume of properly diluted samples was 40 μL.

UV–Vis analyses were carried out in the range of 200 and 600 nm (PDA). MS spectra were recorded in both negative (for analyses of phenolic acids, stilbenes, flavonols and flavanols) and positive ion modes (for the analysis of anthocyanins) between 80 and 1500 *m/z*. The working conditions for the ESI source were as follows: capillary voltage, 4500 V; nebuliser gas pressure, 4.0 bar; drying gas flow, 8.0 L min^{-1}; and drying gas temperature, 180 °C. Nitrogen and argon were used as nebuliser and collision gases, respectively. The MS detector was programmed to perform an MS/MS scan of the three most abundant ions, using collision energy of 13.0 eV. Data acquisition and processing were performed using Compass (V. 3.1) and Data Analysis (V. 4.0) software, respectively.

Polyphenols present in samples were identified according to their retention times, UV/Vis spectra, MS and MS/MS spectra, in comparison with pure compounds, when available, or with compounds reported in the literature. MS analysis was used for quantification of the polyphenols with external calibration plots, constructed by linear regression from available phenolic standards. Anthocyanin compounds were quantified as malvidin-3-glucoside (Extrasynthese, Genay, France); myricetin, laricitrin and syringetin compounds as myricetin (Sigma-Aldrich, Buenos Aires, Argentina); quercetin and isorhamnetin compounds as quercetin (Fluka, Dorset, U.K.); kaempferol compounds as kaempferol; flavonol glycoside compounds as isoquercetin; flavanol compounds as (+)-catechin (Extrasynthese, Genay, France); hydroxycinnamic acids (HCA) compounds as caffeic acid (Extrasynthese, Genay, France); hydroxybenzoic acid (HBA) compounds as gallic acid and *trans*-resveratrol compounds as *trans*-resveratrol (Sigma-Aldrich, Buenos Aires, Argentina). The limits of detection (LOD) and quantification (LOQ) of the compounds studied were experimentally evaluated considering a signal-to-noise ratio of 3 and 10 respectively. Instrumental LOQ ranged from 0.0013 to 0.0500 mg L^{-1}. All samples were analysed in triplicate.

2.5. Evaluation of antioxidant capacity (AC) using yeast cells

The evaluation of *in vivo* AC of the samples was performed through survival assay in accordance with Baroni et al. (2012). *S. cerevisiae* cells (ATCC36900, American Type Culture Collection) were grown in liquid YPD yeast extract, peptone and dextrose medium, using an orbital shaker at 30 °C and 1.43 g (the flask : medium ratio was 5:1). Yeast cells at the exponential phase (Abs^{600}: 0.5–0.7) were transferred to fresh medium (Abs^{600}: 0.2) and stressed with 2 mM H_2O_2 (enough oxidant concentration to produce 50% of yeast death) for 1 h at 30 °C/1.43 g. To evaluate the *in vivo* antioxidant effect of samples before stress with H_2O_2, cells were treated with the different

samples for 15 min at 30 °C/1.43 g (Belinha et al., 2007). Optimal TP doses from grape and pomace extracts (dried and dissolved in 35% DMSO, Sigma-Aldrich, Buenos Aires, Argentina) and wine were determined in adaptive treatments, exposing cells to increased concentrations of TP from these samples (data not shown). The concentrations chosen were 0.3 μg GAE mL^{-1} and 1.0 μg GAE mL^{-1} for grape and pomace extracts, respectively, and 98.0 μg GAE mL^{-1} for wines. These final concentrations were the lowest, showing the highest rate survival as compared to yeast exposed to H_2O_2 (2 mM) without the addition of sample. These concentrations are in agreement with others previously used (Baroni et al., 2012; Ignea et al., 2013; Martorell et al., 2011; Peinado et al., 2013; Stinco et al., 2015). Two control groups were used: a control plate (yeast exposed to vehicle of phenolic compounds in samples: 35% DMSO in case of grape and pomace extracts, and 12% ethanol in case of wines) and sample control plate (yeast exposed to grape and pomace extracts, and wines alone, without addition of H_2O_2). Cell viability was analysed by plating on solid YPD medium, after proper dilution. Plates were incubated at 30 °C for 72 h. One hundred percent survival was considered the number of colonies observed in the control plate (yeast exposed to vehicle of phenolic compounds in samples). The number of colonies in each plate was between 150 and 200 (Silva et al., 2005). All assays were carried out in triplicate.

2.6. Determination of antioxidant enzymatic defences GPx and GR

For enzymatic assays, the yeast cells were suspended in a lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 50 mM EDTA, pH 7.2), broken using glass beads and alternate cycles of 1 min of agitation and cooling. Cell debris was removed by centrifugation at 14,000 *g* during 15 min and enzyme activities were measured in the supernatants. Determination of GPx activity was based on the oxidation of GSH by GPx, using H_2O_2 as a substrate, coupled to the disappearance of NADPH (Sigma-Aldrich, Buenos Aires, Argentina) by GR (Drotar, Phelps, & Fall, 1985). GR activity was determined by following the decrease in absorbance due to the oxidation of NADPH used in the reduction of oxidised glutathione (Tanaka, Sano, Ishizuka, Kitta, & Kawamura, 1994).

Enzymatic activities were calculated in terms of the protein content for each sample (Bradford, 1976) and are reported in nano katals per milligram of protein (nkat mg $prot^{-1}$), where 1 kat is the conversion of 1 mol of substrate per second. Each enzymatic assay was carried out in triplicate.

2.7. Statistical analysis

Data are expressed as mean ± SD, averaged over at least three independent experiments. The data were analysed using ANOVA test with $p < 0.05$. In all figures, different letters mean statistically significant differences.

Principal component analysis (PCA) was applied to evaluate the relationship between phenolic profile (by families of compounds) and the variety in each sample type.

Canonical correlation analysis (CCA) was used to assess the relationship between the phenolic profile (by families of compounds) and AC (studied by survival rate, GR and GPx activity) of each sample type.

Stepwise multiple regression analysis (MRA) was used to find additional evidence of the relationship between the phenolic profile and AC; thus, we performed different MRA analyses, including only a family of compounds in each test. Additionally, from each of these analyses, the regression (*Beta*) coefficients were analysed to determine the relative contribution of each variable (phenolic compound) to the antioxidant activity (survival rate, GR and GPx activity). We used the statistical package Statistica 7.1 from StatSoft Inc. (2005).

3. Results and discussion

3.1. Phenolic content of grape, pomace and wines

Table 1 shows the content of TP for each sample type of three *V. vinifera L.* red varieties. The TP ranged from 1062 to 1986 mg GAE g^{-1} DW for grapes in accordance with the results obtained by other authors (Lee & Rennaker, 2011; Meyer, Yi, Pearson, Waterhouse, & Frankel, 1997). This content showed significant differences among varieties. Cabernet Sauvignon and Syrah showed the highest and lowest content of TP among grape varieties, respectively. In the case of pomaces, all samples showed important content of TP, indicating that considerable amounts of bioactive compounds could be recovered from these winemaking byproducts for food industries. The TP for pomaces ranged from 985 to 2122 mg GAE g^{-1} DW. In the case of wine samples, content ranged from 1230 to 1475 mg GAE L^{-1}, Merlot and Cabernet Sauvignon being the varieties with the highest and lowest content, respectively. These results are in agreement with those obtained/reported by other authors (Li, Wang, Li, Li, & Wang, 2009).

When the individual phenolic constituents were evaluated, 45 compounds belonging to the family of anthocyanins, flavonols, flavanols, hydroxycinnamic and hydroxybenzoic acids and stilbenes were identified (Tables 1–3 and Supplementary data). Grape samples were characterised by the family of anthocyanins (55% in average among varieties), followed by flavonol glycosides (25% in average among varieties). The most abundant compounds in this sample type were malvidin-3-acetylglucoside, followed by malvidin-3-glucoside and isoquercetin. In the case of pomace samples, anthocyanins and free aglycones of flavonols were the main phenolic families (44% and 24% in average among varieties, respectively), with malvidin-3-coumaroylglucoside and quercetin as the most abundant compounds for these families in the samples. In wines, hydroxybenzoic acids, followed by flavanols and anthocyanins, were the main families (37%, 21% and 17% in average among varieties, respectively), with ethyl gallate, (+)-catechin and malvidin-3-glucoside as the most abundant compounds for these families in the samples. From grape to wine, the main changes in phenolic profile were hydrolysis of glycosidated flavonols, hydroxycinnamoyl tartaric acids (caftaric acid, coutaric acid and fertaric acid) and gallate esters, and also the formation of anthocyanin-derived pigments or pyranoanthocyanin compounds (pigment A, acetyl pigment A and coumaroylvitisin B), as shown in Tables 1–3.

Table 1 – Content of total polyphenols and anthocyanins in *V. vinifera* L. cv. Syrah, Merlot and Cabernet Sauvignon.

	Syrah			Merlot			Cabernet Sauvignon		
	Grape	Pomace	Wine	Grape	Pomace	Wine	Grape	Pomace	Wine
Total polyphenols	1062 ± 104a	1013 ± 63a	1422 ± 31b	1458 ± 145b	2122 ± 214b	1475 ± 58c	1986 ± 187c	985 ± 136a	1230 ± 35a
Dp-3-glc	3.3 ± 0.1b	<LOD	0.70 ± 0.02b	6.9 ± 1.9c	<LOD	0.6 ± 0.1b	1.6 ± 0.4a	<LOD	0.20 ± 0.01a
Cy-3-glc	0.7 ± 0.3a	<LOD	<LOQ	1.8 ± 0.1b	<LOD	<LOQ	0.8 ± 0.1a	<LOD	<LOD
Pt-3-glc	24.1 ± 5.4b	0.9 ± 0.2c	2.40 ± 0.04c	25.0 ± 3.6b	0.4 ± 0.1b	1.6 ± 0.3b	7.4 ± 1.5a	0.10 ± 0.01a	1.00 ± 0.03a
Pn-3-glc	48.4 ± 17.6b	1.0 ± 0.2b	1.60 ± 0.03b	58.9 ± 5.1b	1.7 ± 0.2c	1.9 ± 0.3c	16.7 ± 0.5a	0.80 ± 0.06a	0.40 ± 0.01a
Mv-3-glc	380.5 ± 26.5c	142.2 ± 10.1c	87.4 ± 0.7c	251.5 ± 22.3a	96.8 ± 26.2b	46.6 ± 2.1a	328.9 ± 24.4b	55.8 ± 8.1a	68.6 ± 8.7b
Dp-3-acglc	1.9 ± 0.1a	<LOD	0.10 ± 0.02b	3.4 ± 1.1b	<LOD	0.30 ± 0.03c	1.3 ± 0.2a	<LOD	<LOQa
Cy-3-acglc	0.2 ± 0.1a	<LOD	<LOD	0.6 ± 0.1b	<LOD	<LOD	0.2 ± 0.01a	<LOD	<LOD
Pt-3-acglc	17.7 ± 0.9b	0.9 ± 0.1c	0.60 ± 0.02c	19.9 ± 0.9b	0.4 ± 0.1b	0.5 ± 0.1b	11.6 ± 0.6a	0.03 ± 0.01a	0.20 ± 0.01a
Mv-3-acglc	816.8 ± 58.2c	195.0 ± 16.6c	49.7 ± 2.5c	258.2 ± 26.9a	103.7 ± 23.5b	13.6 ± 3.4a	539.0 ± 25.8b	28.4 ± 2.2a	21.3 ± 1.0b
Pn-3-acglc	72.5 ± 11.1c	1.8 ± 0.7b	2.00 ± 0.02b	47.8 ± 6.1b	3.3 ± 1.6c	2.1 ± 0.3b	32.1 ± 2.4a	0.20 ± 0.01a	0.80 ± 0.02a
Mv-3-cafglc	2.5 ± 1.1b	23.8 ± 4.8b	<LOD	0.5 ± 0.1a	3.4 ± 2.6a	<LOD	0.20 ± 0.03a	2.6 ± 0.2a	<LOD
Dp-3-cmglc	8.0 ± 0.9c	43.9 ± 3.5c	<LOD	3.6 ± 1.1b	7.9 ± 4.8b	<LOD	0.40 ± 0.03a	0.3 ± 0.1a	<LOD
Pt-3-cmglc	17.2 ± 2.2c	72.9 ± 1.2c	<LOQ	7.0 ± 1.4b	24.8 ± 10.7b	<LOQ	1.5 ± 0.6a	1.4 ± 0.5a	<LOD
Mv-3-cmglc	251.7 ± 19.1c	238.9 ± 4.7c	8.90 ± 0.06b	49.6 ± 3.9a	142.8 ± 31.4b	4.2 ± 0.9a	71.1 ± 9.5b	67.5 ± 10.2a	3.8 ± 0.1a
Pn-3-cmglc	63.9 ± 10.4c	42.7 ± 3.5c	1.20 ± 0.02b	23.0 ± 0.8b	24.6 ± 6.9b	1.1 ± 0.2b	13.1 ± 2.7a	1.6 ± 0.6a	0.20 ± 0.01a
Pigment A	<LOD	0.5 ± 0.1b	4.6 ± 0.2c	<LOD	<LOQa	0.5 ± 0.01a	<LOD	12.8 ± 0.5c	3.5 ± 0.1b
Acetyl pig. A	<LOD	0.4 ± 0.1b	1.3 ± 0.09c	<LOD	0.1 ± 0.1a	<LOQa	<LOD	4.2 ± 0.6c	0.80 ± 0.07b
Coumaroylvit. B	<LOD	10.9 ± 2.1b	<LOD	<LOD	0.9 ± 0.5a	<LOD	<LOD	0.7 ± 0.1a	<LOD

Abbreviations: Dp, delphinidin; Cy, cyanidin; Pt, petunidin; Pn, peonidin; Mv, malvidin; glc, glucoside; ac, acetyl; caf, caffeoyl; cm, coumaroyl; Ant, anthocyanin; LOD, limit of detection; LOQ, limit of quantification. Anthocyanin compounds were quantified as malvidin-3-glucoside. Contents are reported in mg kg^{-1} DW for grape and pomace, and mg L-1 for wine. Different letters indicate significant differences ($p < 0.05$) in each sample type among the three varieties.

Table 2 – Content of flavonols and flavanols in *V. vinifera* L. cv. Syrah, Merlot and Cabernet Sauvignon.

	Syrah			Merlot			Cabernet Sauvignon		
	Grape	Pomace	Wine	Grape	Pomace	Wine	Grape	Pomace	Wine
Kaempferol	0.05 ± 0.04c	9.8 ± 0.8a	0.5 ± 0.1c	<LOQa	34.2 ± 5.6c	0.40 ± 0.01b	0.02 ± 0.01b	13.8 ± 0.7b	<LOQ
Myricetin	2.3 ± 0.2b	2.2 ± 0.1a	19.2 ± 2.2c	0.6 ± 0.1a	2.4 ± 0.3a	5.0 ± 0.1a	0.6 ± 0.1a	7.2 ± 0.4b	8.4 ± 1.6b
Laricitrin	0.08 ± 0.01c	0.30 ± 0.03c	<LOQ	<LOQa	0.10 ± 0.01a	<LOQ	0.07 ± 0.01b	0.3 ± 0.1b	<LOQ
Syringetin	0.09 ± 0.01c	0.40 ± 0.02b	<LOQ	0.10 ± 0.01a	0.20 ± 0.02a	<LOQ	0.07 ± 0.01b	0.5 ± 0.03c	<LOQ
Quercetin	0.4 ± 0.2a	93.0 ± 29.5a	56.8 ± 2.3c	2.3 ± 0.4b	251.1 ± 65.6c	39.7 ± 1.3b	3.2 ± 0.4c	163.6 ± 31.9b	31.5 ± 1.5a
Isorhamnetin	0.20 ± 0.07b	16.1 ± 1.7b	28.4 ± 0.2c	0.02 ± 0.01a	12.5 ± 1.5a	10.7 ± 0.2b	0.03 ± 0.01a	20.5 ± 3.3c	10.2 ± 0.6a
Isoquercetin	278.5 ± 89.6b	26.5 ± 1.4c	2.60 ± 0.02a	174.8 ± 18.2a	16.0 ± 0.8a	4.3 ± 0.1c	336.2 ± 26.4c	21.8 ± 1.4b	3.1 ± 0.2b
Myr-3-glc	209.6 ± 30.7b	11.4 ± 0.7c	14.2 ± 0.9c	75.9 ± 8.0a	6.1 ± 0.5b	8.6 ± 0.1b	198.9 ± 29.6b	3.6 ± 0.4a	8.0 ± 0.5a
Myr-3-glcr	1.10 ± 0.05a	1.8 ± 0.1c	0.60 ± 0.05b	1.6 ± 0.4b	0.50 ± 0.08a	0.3 ± 0.1a	4.0 ± 0.1c	0.60 ± 0.07b	0.30 ± 0.01a
Astilbin	2.0 ± 1.0a	7.6 ± 0.2c	6.4 ± 0.3b	2.10 ± 0.05a	2.5 ± 0.3a	2.90 ± 0.01a	4.3 ± 0.4b	3.7 ± 0.3b	6.5 ± 0.6b
Lar-3-glc	4.3 ± 0.4b	6.4 ± 0.2b	8.2 ± 0.02c	2.2 ± 0.1a	2.9 ± 0.2a	2.7 ± 0.01a	5.6 ± 0.3c	2.9 ± 0.3a	3.0 ± 0.1b
Quer-3-glcr	10.9 ± 3.7a	81.4 ± 3.4c	14.5 ± 0.1b	35.2 ± 9.4b	31.9 ± 3.6a	8.3 ± 0.4a	46.8 ± 16.0b	38.3 ± 2.8b	8.2 ± 0.2a
Kp-3-glc	0.10 ± 0.07a	<LOD	<LOD	0.20 ± 0.06a	<LOD	<LOD	0.5 ± 0.2b	<LOD	<LOD
Syr-3-glc	25.4 ± 4.4b	4.9 ± 0.2b	13.2 ± 0.8b	7.2 ± 0.7a	4.2 ± 0.7a	5.3 ± 0.4a	29.9 ± 2.6c	12.0 ± 0.6c	5.3 ± 0.3a
Isorh-3-glc	50.2 ± 20.0c	7.7 ± 0.3c	2.2 ± 0.01	11.4 ± 3.0a	2.0 ± 0.3a	2.2 ± 0.2	34.5 ± 6.7b	2.9 ± 0.3b	2.1 ± 0.2
Procyanidin dimer	13.1 ± 1.3b	10.0 ± 1.1a	20.0 ± 0.3a	<LODa	24.6 ± 3.0b	25.3 ± 0.4b	19.7 ± 1.7c	9.0 ± 1.7a	28.8 ± 1.3c
Procyanidin dimer monogallate	4.0 ± 0.5a	2.6 ± 0.3a	<LOD	6.0 ± 1.3b	13.6 ± 2.0b	<LOD	3.6 ± 0.2a	17.2 ± 1.7c	<LOD
(+)-Catechin	28.7 ± 1.8b	21.8 ± 1.7a	41.9 ± 2.1a	20.8 ± 1.5a	89.7 ± 9.6b	59.4 ± 2.5b	28.4 ± 1.7b	19.6 ± 1.8a	74.9 ± 2.0c
(–)-Epicatechin	74.8 ± 5.0c	27.2 ± 4.0b	40.4 ± 1.2a	68.1 ± 3.2b	112.8 ± 8.2b	55.6 ± 0.8b	57.4 ± 2.9a	17.3 ± 1.7a	65.6 ± 2.1c
Epicatechin gallate	25.4 ± 2.3a	14.7 ± 2.8b	<LOD	75.3 ± 27.9b	45.6 ± 6.7c	<LOD	6.8 ± 0.4a	10.5 ± 1.1a	<LOD

Abbreviations: Myr, myricetin; Lar, laricitrin; Quer, quercetin; Kp, kaempferol; Syr, syringetin; Isorh, isorhamnetin; glc, glucoside; glcr, glucuronide; LOD, limit of detection; LOQ, limit of quantification. Quantification: Myr, Lar and Syr compounds as myricetin; Quer and Isorh compounds as quercetin; Kp compound as kaempferol. Flavonol glycosides compounds as isoquercetin; Flavanols compounds as (+)-catechin. Contents are reported in mg kg^{-1} DW for grape and pomace, and mg L^{-1} for wine. Different letters indicate significant differences ($p < 0.05$) in each sample type among the three varieties.

Table 3 – Content of non-flavonoid phenolic compounds in *V. vinifera* L. cv. Syrah, Merlot and Cabernet Sauvignon.

	Syrah			Merlot			Cabernet Sauvignon		
	Grape	Pomace	Wine	Grape	Pomace	Wine	Grape	Pomace	Wine
Caftaric acid	154.7 ± 15.3c	1.6 ± 0.2b	28.3 ± 1.4a	100.2 ± 4.5a	1.8 ± 0.1c	29.9 ± 1.1b	127.5 ± 21.3b	0.2 ± 0.1a	35.6 ± 0.4c
Coutaric acid	136.0 ± 6.8c	5.4 ± 0.3b	7.9 ± 0.1a	39.0 ± 2.4a	5.1 ± 0.5b	8.9 ± 0.2b	84.9 ± 3.2b	1.4 ± 0.3a	11.0 ± 0.8c
Caffeic acid	<LOD	<LOD	8.4 ± 0.3c	<LOD	<LOD	1.7 ± 0.3a	<LOD	<LOD	3.5 ± 0.5b
Fertaric acid	2.6 ± 1.4	<LOD	<LOQ	2.7 ± 1.3	<LOD	<LOQ	3.6 ± 1.4	<LOD	<LOQ
Gallic acid	<LOD	<LOD	62.8 ± 1.9a	<LOD	<LOD	63.6 ± 2.4a	<LOD	<LOD	75.3 ± 1.5b
Ethyl gallate	<LOD	29.0 ± 4.1a	155.2 ± 8.2a	<LOD	50.5 ± 1.2b	172.0 ± 0.2b	<LOD	53.0 ± 3.0b	197.2 ± 0.2c
trans-Resveratrol	0.08 ± 0.06a	<LOD	0.50 ± 0.01a	7.0 ± 1.5b	<LOD	1.20 ± 0.03c	0.50 ± 0.06a	<LOD	0.60 ± 0.05b

Abbreviations: LOD, limit of detection; LOQ, limit of quantification. Quantification: hydroxycinnamic acids as caffeic acid; hydroxybenzoic acids as gallic acid; trans-resveratrol as trans-resveratrol. Contents are reported in mg kg^{-1} DW for grape and pomace, and mg L^{-1} for wine. Different letters indicate significant differences ($p < 0.05$) in each sample type among the three varieties.

In addition, the phenolic patterns showed important quantitative differences among varieties. In order to evaluate these differences, we applied PCA considering their quantitative phenolic profile by families of compounds. For the three sample types studied, the cumulative percentage of the total variance explained by the first and second principal component was near 100% (Fig. 1). As shown in the biplots, the three varieties studied for all sample types were differently grouped according to their phenolic composition. Syrah variety showed the highest content in anthocyanin compounds in all samples types (Ginjom, D'Arcy, Caffin, & Gidley, 2011), and Merlot showed the highest values for *trans*-resveratrol in grapes and wines, which are in accordance with Atanacković et al. (2012) and Stervbo, Vang, and Bonnesen (2007). Compound characteristics of Cabernet Sauvignon were dependent on sample types; in grapes the phenolic profile was characterised by flavonols, in pomace by HBA and pyranoanthocyanins, and finally in wines by HCA and flavanols. Therefore, the quantitative differences from phenolic profile among varieties are indicative of influence of genotype in the content of these metabolites (Liang et al., 2014).

3.2. *Antioxidant capacity of grape and pomace extracts and wines*

The AC of different samples was evaluated using the *S. cerevisiae* yeast cells as a biological model exposed to oxidative stress induced by H_2O_2 (Baroni et al., 2012). To evaluate the bioactivity of samples in protecting *S. cerevisiae* cells against induced oxidative damage, cell viability was determined with or without the presence of samples as chemoprotector (exogenous antioxidants). As shown in Fig. 2, selected concentrations from samples (see section 2.5) were non-cytotoxic to *S. cerevisiae*. When the oxidative stress was induced, yeast cells showed sensibility to H_2O_2 (2 mM) and only 54% was able to survive the oxidative insult (Fig. 2) (Baroni et al., 2012; Ignea et al., 2013; Stinco et al., 2015). However, pretreatment with different sample types partially suppressed the damage triggered by the oxidant. For example, grape extracts increased between 14% and 20% the survival rate compared to cells exposed to H_2O_2 (Fig. 2a), pomace extracts increased between 8% and 16% (Fig. 2b), and the increment was between 9% and 15% for wines (Fig. 2c). It is important to note that grapes were able to rescue yeast in a higher percentage with the lowest concentration of polyphenols (see section 2.5). In addition to differences observed according to sample types, AC also showed differences among varieties; in this sense, in all sample types, Syrah variety showed the highest AC (Fig. 2).

3.3. *Endogenous antioxidant enzymatic defences of yeast cells*

Previous studies have demonstrated that protective effect of polyphenols is related to their large number of biological mechanisms, including antioxidant enzyme modulation (Martín, González-Burgos, Carretero, & Gómez-Serranillos, 2011; Shin, Yeh, & Yen, 2007; Yeh, Ching, & Yen, 2009). Thus, we evaluated the ability of samples under study to induce the activity of antioxidant enzymes in *S. cerevisiae*. We studied glutathione peroxidase (GPx) and glutathione reductase (GR), enzymes involved in the metabolism of glutathione (GSH), an important biomarker of changes in the redox status of the cell (Gaté, Paul, Nguyen Ba, Tew, & Tapiero, 1999).

When GPx was studied, we saw that its activity was increased when cells were treated only with grape extracts and wines, and decreased in the case of pomace extracts in comparison with control cells (Fig. 3). On the other hand, the treatment of cells with H_2O_2 decreased the activity of this enzyme as compared to that of control cells. However, the co-treatment of cells with sample and the stressing agent increased the enzyme activity in relation to cells exposed to H_2O_2 (Fig. 3). Since GPx is involved in the detoxification of H_2O_2, i.e., conversion into water via oxidation of GSH, our result would be indicating that the activation of this enzyme could be one of the biological mechanisms involved in the AC of samples.

In the case of GR activity, this was not modified when cells were treated with pomace extracts and wines, but it was increased with grape extracts in comparison with control cells (Fig. 3). In this case, as with GPX, the activity was decreased in the presence of H_2O_2 (Fig. 3). However, GR activity was increased during the co-treatment with samples and the stressing agent (Fig. 3). This enzyme replenishes the reduced glutathione (GSH) when oxidised in GSH-dependent redox reactions as in the case of GPx activity; thus, the increment in its activity was expected.

Therefore, the induction of GPx and GR activities would be one of the biological mechanisms that would explain the AC of these samples rich in phenolic compounds, observed through

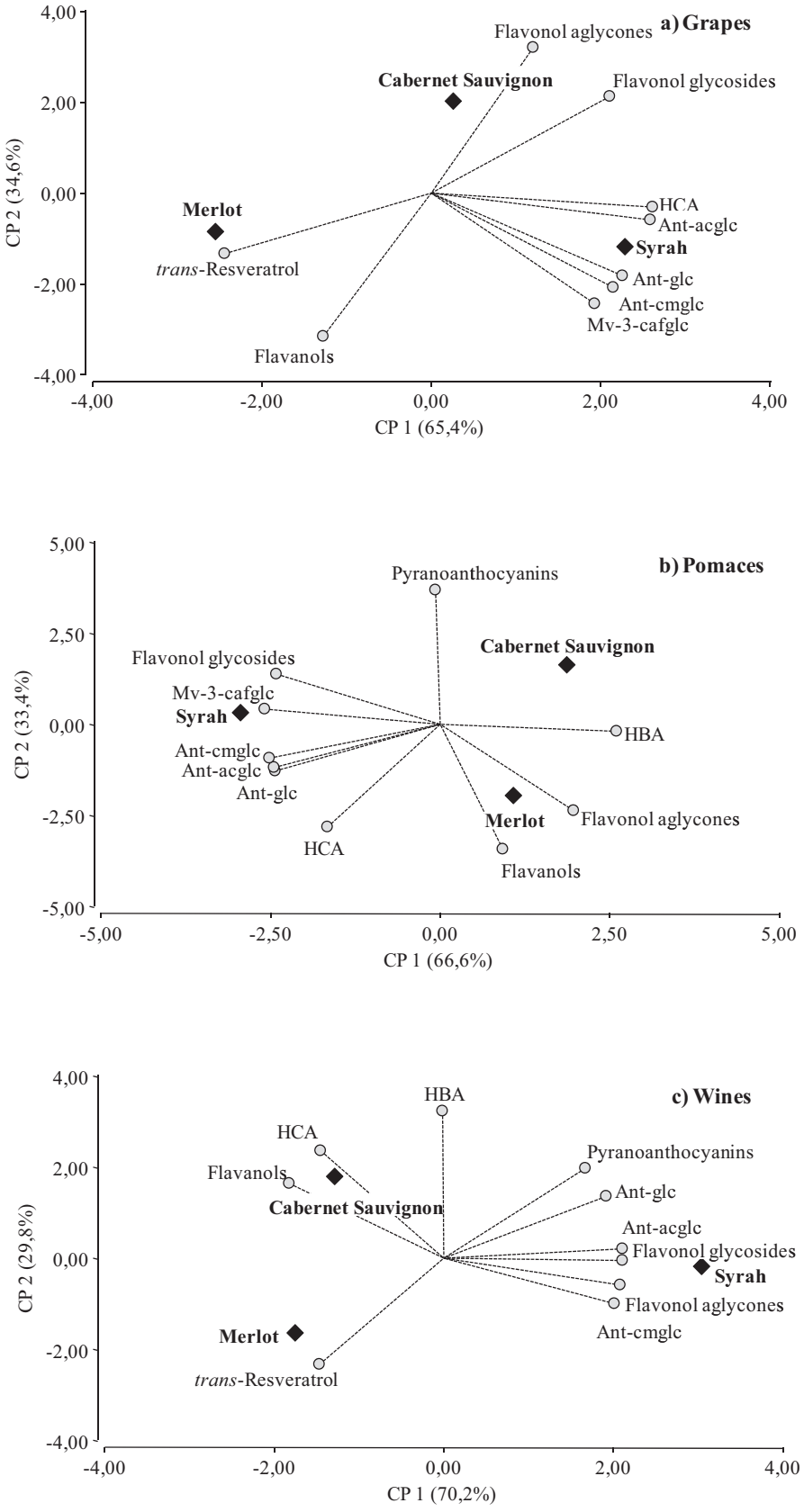

Fig. 1 – Biplot obtained from PCA illustrating the relationship between phenolic profile and *Vitis vinifera* L. cv. Syrah, Merlot and Cabernet Sauvignon in (a) grape, (b) pomace and (c) Wine. Abbreviations: ant, anthocyanins; glc, glucoside; ac, acetyl; caf, caffeoyl; cm, coumaroyl; HCA, hydroxycinnamic acids; HBA, hydroxybenzoic acids.

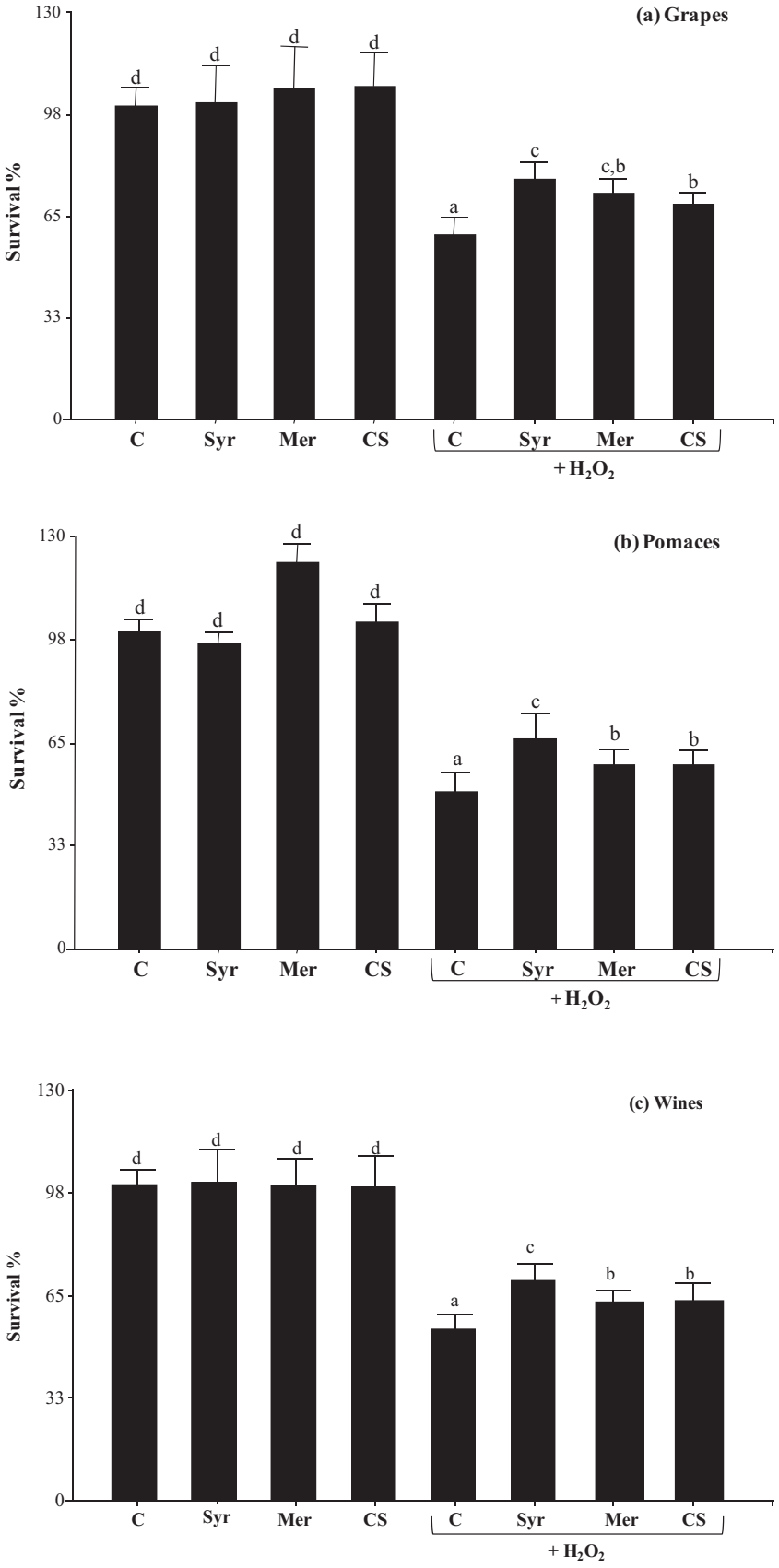

Fig. 2 – Survival rates of *S. cerevisiae* treated and untreated with different sample types and/ or H_2O_2 (mean ± SD). Survival percentage with respect to control cells. Sample types: (a) grape, (b) pomace and (c) wine. Different letters indicate significant differences ($p < 0.01$). C: control cells; Syr: Syrah; Mer: Merlot; CS: Cabernet Sauvignon.

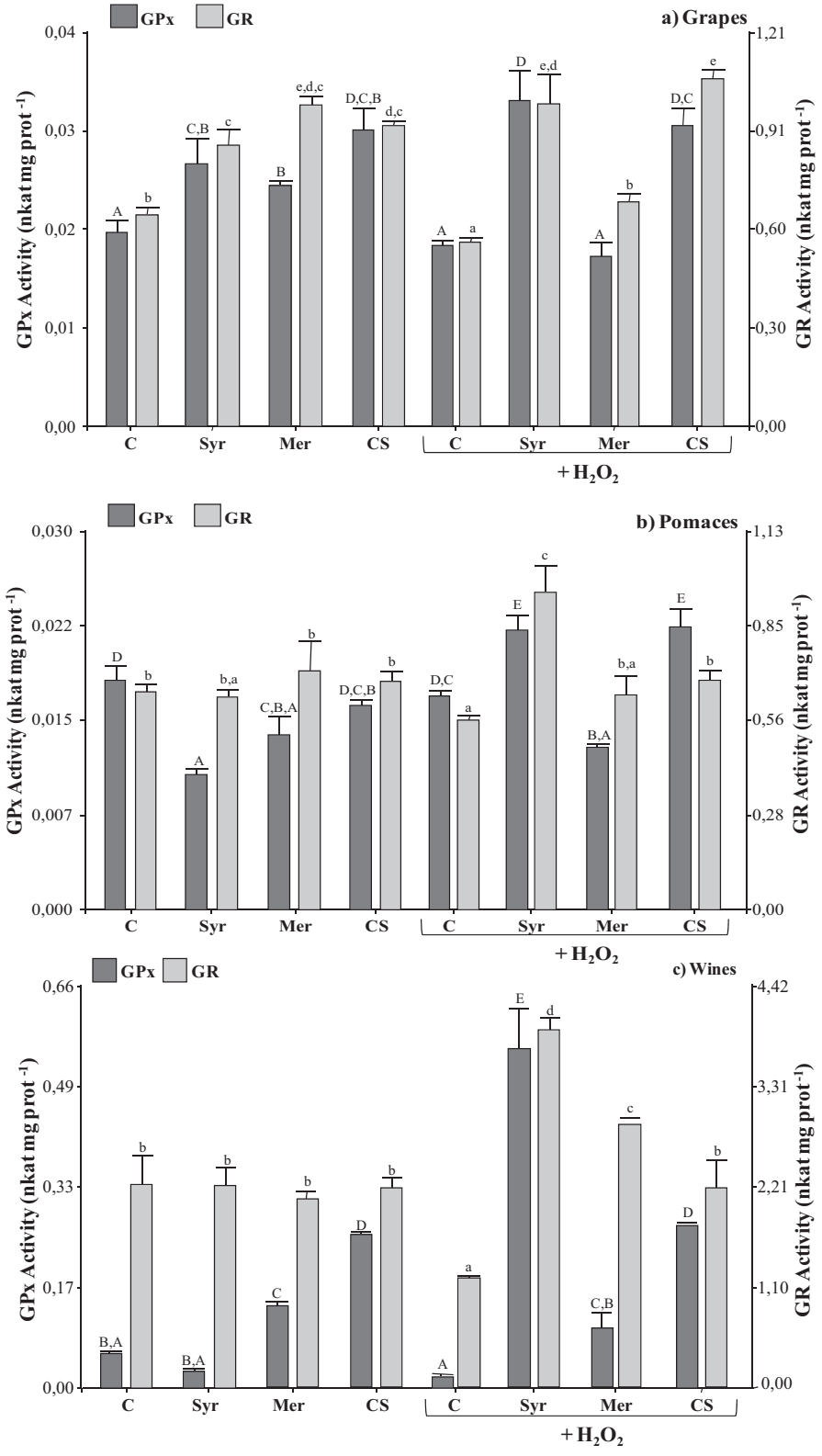

Fig. 3 – Enzymatic activity of *S. cerevisiae* treated and untreated with different sample types and/or H_2O_2 (mean ± SD). GPx: dark grey bars; GR: light grey bars. Sample types: (a) grape, (b) pomace and (c) wine. Different letters indicate significant differences ($p < 0.01$).C: control cells; Syr: Syrah; Mer: Merlot; CS: Cabernet Sauvignon.

the increased survival rate after oxidative stress induced in the yeast cells (Baroni et al., 2012). With respect to differences among varieties, Syrah, in general, showed the highest induction for GPx and GR activities in all sample types (Fig. 3). These results agree with the observation that Syrah was the variety inducing the highest survival rates in *Saccharomyces cerevisiae* (Fig. 2).

3.4. *Relationship between the phenolic profile and antioxidant capacity of grape, pomace and wines*

Observed differences in AC among varieties could be associated to the differences among phenolic compositions, since yeast cells were treated with the same concentration of TP for each sample type (section 2.5). Therefore, in order to evaluate the correlation between the phenolic profile and antioxidant capacity of each sample type, CCA was applied. This analysis showed a significant correlation between the survival rate and GPx and GR activities with the phenolic pattern of grapes, pomaces and wines (r higher than 0.95, $p < 0.001$). The next step was to identify the phenolic compounds that were most important to explain sample bioactivity. In this sense, MRA was applied and the *Beta* coefficient of each compound was analysed in order to study its relative contribution to the survival rate, GPx and GR activities of each sample type. In all cases (grapes, pomaces and wines samples), a high correlation with phenolic profile was observed (for survival rate: r higher than 0.50, $p < 0.05$; for GPx activity: r higher than 0.76, $p < 0.001$; for GR activity: r higher than 0.75, $p < 0.01$).

The analysis of the beta coefficients showed that the compounds with higher contribution to AC were different in each sample type. Fig. 4 shows selected compounds according to sample type, showing the magnitude and sign of *Beta* coefficient.

In this sense, for grapes, malvidin-3-caffeoylglucoside, petunidin-3-coumaroylglucoside, kaempferol and myricetin showed the highest positive contribution to the survival rate, while quercetin-3-glucuronide and *trans*-resveratrol were the most important compounds that contributed negatively to this property (Fig. 4a). For GPx and GR activities, MRA showed that the same compounds were the most important with positive contribution for both enzyme activities: malvidin-3-glucoside, peonidin-3-acetylglucoside, malvidin-3-coumaroylglucoside, kaempferol-3-glucoside, (–)-epicatechin, coutaric acid and fertaric acid, whereas quercetin-3-glucuronide and *trans*-resveratrol were selected as key negative contributors (Fig. 4a). According to Tables 1–3, Syrah grapes had the highest content of the selected compounds with positive contribution to the *in vivo* activity. However, they showed the lowest content of quercetin-3-glucuronide and *trans*-resveratrol. Furthermore, Merlot grapes showed the highest content of *trans*-resveratrol, which probably helps in explaining the lower activity observed (Figs. 2 and 3).

In the case of pomaces, MRA analysis revealed that the same compounds were the most important contributors for the three assays; survival rate, GPx and GR activities (Fig. 4b). Petunidin-3-gucoside, malvidin-3-acetylglucoside, petunidin-3-coumaroylglucoside and coumaroylvitisin B were those with higher positive contribution, while kaempferol, procyanidin dimer monogallate, (+)-catechin and ethyl gallate were highly negatively correlated with *in vivo* antioxidant activity. As shown in Tables 1–3, compounds with higher positive contribution to the AC of pomace were found in higher concentrations in Syrah with respect to Merlot and Cabernet Sauvignon. Conversely, compounds with negative contribution exhibited higher concentrations in Merlot and Cabernet Sauvignon (Tables 1–3). These results are helpful in explaining the higher activity of Syrah pomaces in relation to Merlot and Cabernet Sauvignon.

Finally, when wine samples were studied, *in vivo* assays were significantly correlated with malvidin-3-glucoside, malvidin-3-acetylglucoside, malvidin-3-coumaroylglucoside, acetyl pigment A and isorhamnetin as higher positive contributors, while caftaric acid, ethyl gallate and *trans*-resveratrol were the principal negative contributors. The Syrah variety showed the highest content of malvidin-3-glucoside, malvidin-3-acetylglucoside, malvidin-3-coumaroylglucoside, acetyl pigment A and isorhamnetin, yet Merlot and Cabernet Sauvignon wines showed the highest content in caftaric acid, ethyl gallate and *trans*-resveratrol. These results help explaining the higher activity of Syrah wines.

It should be noted that anthocyanins were described as main contributors to *in vivo* AC in the three types of sample analysed (grape, pomace and wine), and according to Table 1 and Fig. 1 Syrah variety was characterised by the highest content of these compounds (Figs. 2 and 3). Anthocyanin fraction from different berries has already been recognised with strong antioxidant activity in diverse cell lines by other authors (Im et al., 2013; Moze Bornsek et al., 2012). On the other hand, Merlot variety showed the lowest induction of GPx and GR activity in the co-treatment with H_2O_2 (Fig. 3); in some cases, these activities were even equal to or lower than cells exposed to stressing agent (Fig. 3). Merlot grapes and wines showed a phenolic profile characterised by the highest *trans*-resveratrol content among varieties (Table 3 and Fig. 1). According to MRA analysis, the increased concentration of this compound may be related to a decrease in GPx and GR activity (Fig. 4). *Trans*-resveratrol has already been recognised as having a concentration-dependent effect on oxidative stress induced in different human cell lines (Chen, Jiang, Zhao, & Zhang, 2013). In our study, the results observed with respect to modulation of GPx and GR activity could suggest that high concentrations of this compound might have adverse effects, altering the endogenous antioxidant defence system (antioxidant enzymes in this case); therefore, the potential benefit of *trans*-resveratrol should depend on its dosage.

Furthermore, if we consider that the phenolic profile was also conditioned by the winemaking process (Monagas et al., 2005), and that grape extracts showed the highest activity despite using the lowest concentration of polyphenols to treat cells (Fig. 2 and section 2.5), this differential biological activity should be explained by differences in the phenolic profile. According to MRA results, kaempferol-3-glucoside and fertaric acid, quantified compounds in grape extracts but not detected in pomace extracts or wines (Tables 2–3), probably as a result of acid hydrolysis suffered during the winemaking process, were found to be the ones with higher positive contribution to the *in vivo* AC of grapes (Fig. 4a). Both compounds have been recognised by other authors as having high antioxidant capacity (Meyer, Donovan, Pearson, Waterhouse, & Frankel, 1998; Wang, Tang, & Zhang, 2015). On the other hand, by MRA analysis, ethyl gallate was shown to be the compound with higher negative contribution to AC. Ethyl gallate

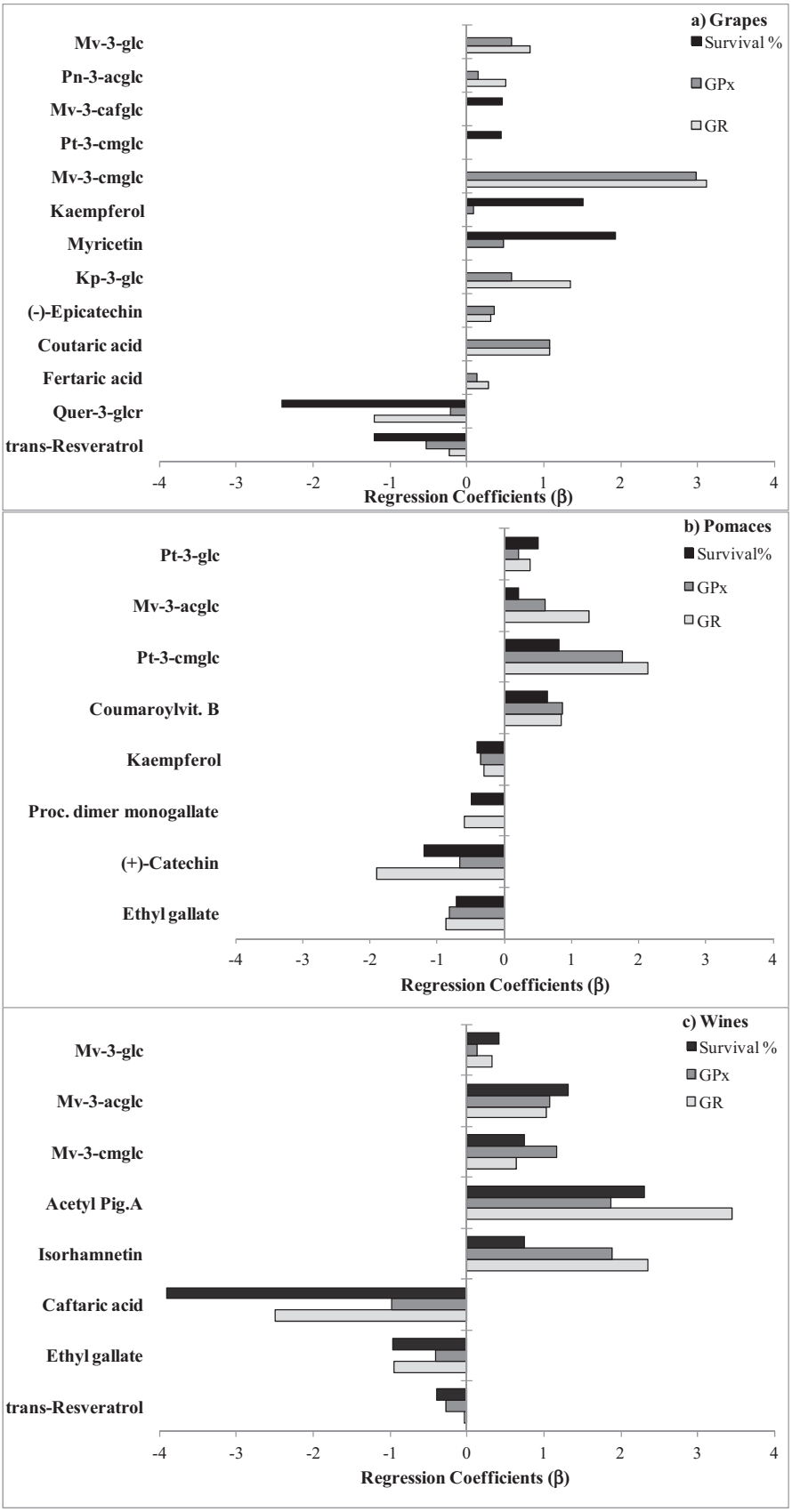

Fig. 4 – The regression (*Beta*) coefficients of key phenolic compounds to explain the antioxidant capacity of samples studied by survival rate, GPx and GR activities. Samples: (a) grape, (b) pomace and (c) wine. Abbreviations: Mv, malvidin; Pn, peonidin; Pt, petunidin; Coumaroylvit, coumaroylvitisin; Pig, pigment; Kp, kaempferol; Quer, quercetin; glc, glucoside; glcr, glucuronide; ac, acetyl; caf, caffeoyl; cm, coumaroyl.

is a compound formed during fermentation process by the esterification of gallic acid with ethanol (Ginjom et al., 2011; Monagas et al., 2005), which is why this compound was quantified in pomace and wine but not detected in grapes, which may contribute to the high activity of grapes (Table 3, Fig. 4b and 4c). Thus, our results could indicate that the changes in the phenolic profile of the grape (raw material) as a consequence of the winemaking process would be affecting the AC of wine (final product) and pomace (byproduct).

4. Conclusion

The results presented in this study underline differences in phenolic profile and *in vivo* antioxidant activity of products involved in the winemaking process: grape as a raw material, wine as a final product and pomace as a byproduct. In addition, we observed differences in both parameters according to the variety studied. Results from MRA analysis showed that Syrah had the highest AC, as a consequence of their phenolic profile characterised by the highest content in anthocyanin compounds, while the content of *trans*-resveratrol in Merlot grapes and wine probably contributes to its lower activity. Furthermore, changes in the phenolic profile from grape to wine, as a consequence of the winemaking process, affected the AC. In this sense, grapes were characterised by higher content of kaempferol-3-glucoside and fertaric acid that may contribute positively to its higher *in vivo* AC. On the other hand, ethyl gallate contributed negatively to the AC of wines and pomaces. The MRA model obtained successfully described the impact of changes in phenolic profile by the grape variety and by the winemaking process on *in vivo* antioxidant activity.

Based on our results, the comparable AC of grape pomace and wine is worth mentioning. Therefore, the recovery of this waste from the winemaking process as a potent natural antioxidant and its use in the development of multifunctional ingredients constitute a major challenge, while helping to reduce pollution and adding value to raw material.

Finally, results presented in this work show that some phenolic compounds played a critical role in the antioxidant activity of samples, thus providing important references for the improvement of Argentinean wine quality.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi: 10.1016/j.jff.2015.10.034.

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