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Low molecular-weight phenols in Tannat wines made by alternative winemaking procedures ☆

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ABSTRACT

Low molecular weight phenols of Tannat red wines produced by Traditional Maceration (TM), Prefermentative Cold Maceration (PCM), Maceration Enzyme (ENZ) and grape-Seed Tannins additions (ST), were performed and discussed. Alternatives to TM increased wine phenolic contents but unequally, ST increased mainly smaller flavans-3-ol, PCM anthocyanins and ENZ proanthocyanidins (up to 2250 mg/L). However low molecular weight flavan-3-ols remained below 9 mg/L in all wines, showing that there is not necessarily a correspondence between wine richness in total tannins and flavan-3-ols contents at low molecular weight. PCM wines had particularly high concentrations of tyrosol and tryptophol, yeast metabolism derived compounds. The use of grape-seed enological tannins did not increase grape seed derived phenolic compounds such as gallic acid. Caftaric acid was found in concentrations much higher than those reported in other grape varieties. Wine phenolic content and composition was considerably affected by the winemaking procedures tested.

1. Introduction

Red-wine is a complex matrix of phenolic compounds in solution. They are important for wine quality because of their influence in chemical, sensorial and nutraceutical wine properties (Cheynier, Dueñas-Patron, Souquet, Sarni-Manchado, & Fulcrand, 2006). For instance, anthocyanins are responsible for the colour of young red wines, and by combining with tannins and others metabolites, for the colour of wines throw ageing (Fulcrand, Dueñas, Salas, & Cheynier, 2006). Flavan-3-ols and their polymers are also responsible for bitter flavour and astringency tactile sensation (Cheynier et al., 2006). Others phenol compounds, even when present at lower concentrations, play important roles in wine as copigments, such as flavonols (Boulton, 2001), in oxidation process or pigments stability such as cinnamic acids (Boulton, 2001; Cheynier et al., 2006), or are particularly bioactive compounds, like stilbenes (De Nisco et al., 2013).

Because these molecules are located only, or mainly, in the solid part of grapes, their concentrations in red-wines depend mostly on maceration. In traditional red winemaking this process develops mainly simultaneously with alcoholic fermentation.

In accordance with the cited importance of phenolic compounds for red-wine quality, alternative winemaking procedures to traditional one have been developed in order to improve the phenolic extraction. Some of them imply the modification of duration and conditions of maceration, as when a phase of low temperature maceration (less than 15 °C), precedes the fermentative maceration. In that case what is aimed at is to increase water-soluble compounds such as anthocyanins above mostly hydro-alcoholic polyphenols such as tannins (Cheynier et al., 2006). Others options, imply the deliberated addition of phenols to must or wine, for example, when "enological tannins" (commercial preparations containing tannins) are employed. Wine phenolic concentrations, are also intended to be increased through the use of commercial enzymatic preparations, mainly known as "maceration-enzymes".

While main phenolic compounds present in wine can be estimated through spectrophotometric analyses, quantifications of total phenols and anthocyanins, proanthocyanidins, flavan-3-ols reactive to vanillin etc., HPLC-DAD analysis of wine or wine extracts has proved efficient to separate and identify several individual phenols. Both approximations have allowed grape-varietal characterisation (Boido et al., 2011; González-Neves, Ferrer, &

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Gil, 2012a), nutraceutical wine valorization (De Nisco et al., 2013), and to enhance the understanding of how different enological practices affect wine composition (Alcalde-Eon et al., 2014; Busse-Valverde et al., 2010; González-Neves et al., 2013; Soto-Vázquez, Río Segade, & Orriols-Fernández, 2010).

The aim of this work was to study how alternative winemaking procedures to the traditional one, may modify the phenolic composition of young red wines (cv. Tannat). Additionally we present innovative information on the phenolic composition of Tannat wines. The alternatives to traditional maceration tested were: pre-fermentative cold-maceration (PCM), maceration with addition of maceration-enzymes (ENZ) and finally, maceration with grape-seed enological tannin addition (ST).

2. Material and methods

2.1. Grapes

The experiment was carried out during the 2011 vintage. Tannat grapes were grown in the south of Uruguay and were harvested according to their sugar contents, total acidity and pH which were respectively 240 g/L, 4.1 g/L and 3.29. Berry weight at harvest was 1.52 g. For the precedent analytical determinations, duplicate samples of grapes were collected in each vineyard and analysed according to the methods proposed by O.I.V (2013). The analyses were carried out using an Atago N1 refractometer (Atago, Japan) and a Hanna HI8521 pH metre (Hanna Instruments, Italy). Additionally, duplicate samples of 250 berries were collected at harvest for the analysis of phenolic potential of the grapes (Table 1) according to Glories and Augustin (1993). Briefly, each sample was processed in a home-mixer (Phillips HR-2855, Eindhoven, Netherlands) and 50 g of the homogenate was put it in each of two 400 mL glass container, adding in one of them 50 mL of a buffer solution pH 3.2 and in the other 50 mL of a solution pH 1.0. From solution addition, macerations developed for 4 h, mixing by hand every hour to re-homogenate the macerated. Then the macerated was filtered using cotton as filter. The filtrated extract was centrifuged 3 min a 3000 rpm, and supernatant was used for the spectrophotometric analyses, determining the total potential in anthocyanins (total anthocyanins measured in the solution derived from the extract macerated to pH1), extractable anthocyanins (total anthocyanins measured in the solution derived from extract macerated to pH 3.2) and phenolic richness (absorbance measured at 280 nm in the solution derived from the extract macerated to pH 3.2). Using the previous determinations, indexes that estimate the tannic contents of the skins (dpell), seeds (dTpep), their relative proportions (dpell% and Mp%) and the cellular maturity index (EA%) were calculated as described by González-Neves et al. (2012a). A Shimadzu UV-1240 Mini spectrophotometer (Shimadzu, Japan) was used for the required measurements. At harvest, the clusters were transported to the winery in plastic boxes (20 kg each).

2.2. Winemaking

Two batches of grapes (70 kg each) were used in each winemaking procedure. Grapes were destemmed and crushed with an Alfa 60 R crusher (Italcom, Italy), and the barrelling was done in stainless steel tanks (100 L capacity each). Traditional Maceration (TM), the addition of maceration enzymes (ENZ) or enological Seed Tannins (ST), and cold pre-fermentative maceration before traditional maceration (CPM) were the winemaking techniques compared. The TM was employed as the control treatment. Potassium metabisulphite (50 mg SO_2/100 kg of grapes) was added and musts were inoculated with dry active yeast (20 g/HL *Saccharomyces cerevisiae* Natuferm 804; OenoBioTech, France). The sulphur dioxide additions were done immediately after the crushing of the grapes. The yeast inoculations were added immediately in the TM, ST and ENZ musts, whereas they were added after the pre-fermentative process in CPM.

The TM wines were made by classical fermentation on skins for 8 days, according to the polyphenolic potential of the grapes. The wines were pumped over twice daily, involving approximately one and a half the total volume of must a day, followed by punching down the cap, along with the skin contact. The temperature of fermentation was between 23 and 26 °C. The CPM wines were elaborated with skin contact at low temperature (10–15 °C) for 5 days prior to fermentation, cooling the must with frozen water containers. After that, a classical fermentation with skin contact was carried out for 5 days at the same conditions that in the control wines. The ENZ and ST wines were produced with the addition of 2.5 g/100 kg of grapes, of commercial maceration enzymes (Rapidase Ex Colour; DSM, Netherlands), and 200 mg/kg of commercial enological grape-seed tannins respectively, following the destemming and crushing of the grapes. Classical fermentation on the skins was carried out, according to the control wines. At devatting, free-run juice was obtained and the marcs were pressed with a stainless steel manual press. In all cases, free-run juices and press juices were mixed. The wines were kept in the stainless steel tanks, where the fermentations were completed, until racking. At the end of alcoholic fermentation, sulphur dioxide additions (50 mg SO_2/L) were made to inhibit malolactic fermentation. Finally, the wines were kept in glass containers with a capacity of 10 L and closed with cork stoppers until analyses.

2.3. Wine analyses

The wines were analysed 9 months after vinification except for HPLC analysis of anthocyanins that was performed 4 months after vinification. Two replications of the analyses were performed in all cases. The base composition (level of alcohol, total acidity, volatile acidity, residual sugars, total sulphur dioxide, and pH) was analysed with infrared analyser Winescan TM AutoSampler 79000 (Foss, USA) and the software Foss Integrator version 154 (Foss, Denmark). Total polyphenols, flavanols reactive to vanillin and proanthocyanidin contents were analysed according to the spectrophotometric methods described by Paronetto (1977). Total anthocyanins measured by spectrophotometry were analysed according to Ribéreau-Gayon and Stonestreet (1965). The CIELAB parameters brightness (L^*), chromaticity (C^*), red–greenness (a^*) and yellow–blueness (b^*) were determined, using the D65 illuminant and a 10° observer according to Ayala, Echávarri, and Negueruela (1997).

The wines were centrifuged for 3 min at 3000 rpm before spectrophotometric analyses. The measurements were carried out

Table 1
Phenolic characterization of Tannat grapes at harvest.

A 280 nm (AU)	ApH 1 (g/L)	ApH 3.2 (g/L)	EA%	dpell (g/L)	dTpep (g/L)	dpell%	Mp%
75.5	1583	832	47.5	33.3	42.2	44.1	56.0

A 280, phenolic richness; ApH 1, total potential in anthocyanins; ApH 3.2 extractable anthocyanins, EA% anthocyanin extractability index; dpell tanin component on the skins; dTpep, tannin components of the seeds; dpell% relative percentage of tannins on the skins, Mp% relative percentage of tannins on the seeds.

Table 2
Main phenolic families determinate spectrophotometrically in Tannat wines produced by different winemaking procedures.[a]

	Total phenols [b]	Anthocyanins	Flavan-3-ols reactive to vanillin	Proanthocyanidins
TM	1227 ± 92 c	281 ± 14 b	690 ± 105 c	1653 ± 173 b
PCM	1417 ± 33 b	347 ± 18 a	871 ± 94 bc	1976 ± 69 ab
ENZ	1586 ± 85 a	312 ± 22 ab	1184 ± 300 ab	2257 ± 180 a
ST	1380 ± 30 b	298 ± 24 b	1435 ± 271 a	1802 ± 208 b

[a] TM, traditional maceration; PCM prefermentative cold maceration; ENZ vinification with pectolytic enzymes addition at barrelling, ST grape-seed enological tannin addition at barrelling.
[b] Different letters in the same column indicate differences significatives Tukey 0.05.

using a Cole Parmer S2100-UV+(Cole Parmer, USA) UV–VIS spectrophotometer, employing glass cells with a 1 mm path length for colour determinations and 1 cm path length for the others.

2.3.1. HPLC analysis of anthocyanins

Anthocyanins were analysed by HPLC-DAD, according to Revilla, Pérez-Magariño, González-SanJosé, and Beltrán (1999). Briefly, after filtration through a 0.45 μm pore size membrane, the samples were injected directly into a chromatographic system equipped with two pumps Waters 510 and 515, a Rheodyne 7725i injector and a photodiode detector Waters 2996 (Waters Corp., USA). The system was controlled with Millennium 32 Software (Waters Corp., USA). A Luna C18 reverse phase column, 5 μm, 150 x 4.6 mm (Phenomenex, USA) was used as the stationary phase, with a mobile phase flow rate of 0.8 mL/min. The solvent A was an aqueous solution (10%) of formic acid, and solvent B was an aqueous solution of methanol (45%) and formic acid (10%). A gradient was established from 35 to 95% B for 20 min, from 95 to 100% B for 5 min, isocratic 100% B for 5 min. Two replications of the analyses were performed in all the cases. The identification of the compounds was carried out taking into account the spectrum of each and the retention time of each peak. Previously, the identification was confirmed (González-Neves et al., 2007) using a chromatographic system with a mass spectrophotometer as a reference.

The separation carried out by HPLC allow the quantification of the non-acylated glucosides of delphinidin, cyanidin, malvidin, petunidin and peonidin, the acetylated glucosides of the same anthocyanidins and the coumarylic glucosides of delphinidin, cyanidin, malvidin and petunidin.

The concentration of each pigment was calculated using a calibration curve with malvidin glucoside chloride (Extrasynthese, France) and the results are expressed in mg/L of malvidin-3-O-glucoside. The total anthocyanin contents of the wines were calculated considering the sum of all the anthocyanins quantified.

2.3.2. HPLC analysis of non-anthocyanic low molecular weight phenols

An aliquot of 50 mL from each wine was extracted three times with 20 mL of ethyl ether and three times with 20 mL of ethyl acetate. The organic fractions were combined, drying with anhydrous Na_2SO_4, and then evaporated to dryness under vacuum at 30 °C. The residue was dissolved in 2 mL of methanol/water (1:1, v/v), filtered through a 0.45 μm pore size membrane, and then, 30 μL was injected into the HPLC-DAD system. Analyses were performed using a chromatographic system equipped with a photodiode array detector model G1315B, quaternary pump model G1311A, and auto sampler model G1329A (Agilent Technologies, Palo Alto, CA). For separation of the compounds a reverse phase Nova-Pack C18 column (300 mm × 3.9 mm i.d., 4 μm) was used. Gradient elution was employed, with Solvent A being water/acetic acid (98:2, v/v) and Solvent B being water/acetonitrile/acetic acid (78:20:2, v/v/v). The gradient profile was 0–55 min, 100–20% A and 0–80% B; 55–57 min, 20–10% A and 80–90% B; 57–90 min, 10% A and 90% B isocratic, followed by washing (methanol) and re-equilibration of the column. The flow rate was 1.0 mL/min from 0 to 55 min and 1.2 mL/min from 55 to 90 min. Detection was performed by scanning from 210 to 360 nm with an acquisition speed of 1s as has been described previously (Peña-Neira, Hernández, García-Vallejo, Estrella, & Suarez, 2000).

The identification of the compounds was carried out taking into account the spectrum and retention time of each peak and the relation among them. Previously, the identification was confirmed by HPLC-DAD/ESI-MS (Fanzone, Zamora, Jofré, Assof, & Peña-Neira, 2011).

Quantitative determinations were made using the external standard method with commercial standards. The calibration curves were achieved by injection of standard solutions under the same conditions used for the samples analysed, over the range of concentrations observed. The compounds for which no standards were available were quantified with the curves of quercetin (flavonol glycosides, syringetin-3-glucoside), myricetine (myricetine glycosides), trans-resveratrol (trans-resveratrol glucoside, cis-resveratrol-glucoside), caffeic acid (hydroxycinnamic acid esters), and (+)-catechin (procyanidins). All of the solvents were of HPLC grade and purchased from Merck (Darmstadt, Germany). All of the analyses were performed in duplicate.

2.4. Statistical analyses

Analyses of variance and media separation by Tukey at 5% were performed using statistical software INFOSTAT (Di Rienzo J.A., Balzarini M.G., Tablada M., & C.W. INFOSTAT., 2011).

3. Results and discussion

3.1. Spectrophotometric analyses

Table 2 shows that the three alternative winemaking produced wines having more total phenol contents than TM ones, being those elaborated with ENZ the ones with the highest concentrations. A more comprehensive analysis of the influence of each winemaking procedure on wine composition can be achieved by observing results per phenolic type. The ENZ wines had the highest content of proanthocyanidins, while PCM wines of anthocyanins and ST ones of flavans-3-ols reactive to vanillin, even when that compounds were also significantly higher in ENZ wines. These results can be related to the modifications implied by the alternative procedures tested, compared to traditional maceration. Anthocyanin compounds can be easily extracted in water solutions, considering their solubility properties and tissue localisation (Cheynier et al., 2006). The condition of vinification implied by the PCM allowed the highest accumulation of anthocyanins in wines. Considering that the conventional maceration time was shorter than in the others vinification alternatives, the time of maceration prior to fermentation (absence of ethanol) must have thus allowed anthocyanins to accumulate into must as has been found in similar studies (Busse-Valverde, Gómez-Plaza, López-Roca, Gil-Muñoz, & Bautista-Ortín, 2011; Koyama, Goto-Yamamoto, & Hashizume, 2007). Nevertheless other works report wines produced by

prefermentative cold maceration with less anthocyanin concentrations than control ones (González-Neves, Gil, Favre, & Ferrer, 2012b). Anyhow, wines elaborated with PCM also had more tannins contents than TM wines. That result supports Busse-Valverde et al. (2010), Busse-Valverde, Gómez-Plaza, López-Roca, Gil-Muñoz, and Bautista-Ortín (2011), Hernández-Jiménez, Kennedy, Bautista-Ortín, and Gómez-Plaza (2012) findings, that in absence of ethanol significant contents of skin and even seed tannins may be obtained. Hernández-Jiménez et al. (2012) found that after 6 days of maceration in model solutions, seed proanthocyanidin extraction rate was independent of the ethanol content in the extraction medium (from 0 to 15% v/v) expressing that this fact should be taking into consideration when managing techniques such as prefermentative cold maceration. Because of those reports and the results of previous experiences of our group (González-Neves et al., 2013) a vinification protocol for the PCM with a fermentative maceration time shorter than in TM was decided (Section 2.2). Eventually the total maceration time appear to be a more important factor determining tannin wine content than the fermentative maceration one. On the other hand when the whole maceration time is the same than in the traditional vinifications a decrease in tannin concentration has been obtained (Koyama et al., 2007) as would be expected. Defining an optimised total time of maceration and the relative time of maceration at low temperatures, could be amongst the key factors to reach the objectives aimed at with the implementation of this vinification technique.

In the ENZ experiment, even when those enzymes tested were commercially recommended to enhance pigment extraction, a higher increase in flavanols wine contents than in anthocyanins was obtained. Maceration enzymes contain a mix of enzymes that mainly target middle lamella and grape cell-wall pectin's and polysaccharides (Bautista-Ortín, Fernández-Fernández, López-Roca, & Gómez-Plaza, 2007), and these tissues contain tannins or are barriers for their extraction (Amrani, Ouazzani Chahdi, Bouya, Saucier, & Glories, 2003). That would explain our findings and those reported in other works (Busse-Valverde et al., 2010; Ducasse et al., 2010; González-Neves et al., 2013). Recently, Busse-Valverde et al. (2011) have found that maceration enzymes also facilitate seed phenolic extraction. Nevertheless, in our work ENZ wines presented higher contents of skin-derived compounds (anthocyans and flavonols) than TM wines while not of seeds compounds, like gallic acid, suggesting that their effect was mainly exerted on grape skin.

The highest contents of phenols reactive to vanillin were measured in ST wines (Table 2). Since vanillin reacts only with free flavan-3-ols, or with the terminal units of the proanthocyanidins (Hagerman, 2002), and ST wines had less proanthocyanidins contents than PCM and ENZ wines, and no statistical differences respect TM wines, the results could reflect a smaller degree of polymerisation in the flavan-3-ol of ST wines. As may be observed in Busse-Valverde et al. (2010) results, flavan-3-ols of grape seeds are characterised for a low degree of polymerisation while Moutounet et al. (2004) found that the degree of polymerisation of grape enological tannins was inferior to that in grapes. Bautista-Ortín, Cano-Lechuga, Ruiz-García, and Gómez-Plaza (2014) have proved using different pronanthocyanidinic enological tannins, that cell wall material have great affinity for tannins particularly for those of a higher molecular mass. Having enriched the ST musts in tannins, it is thus also expected that the less polymerised would be those reaching the final wines.

3.2. HPLC analysis of anthocyanins

Despite the treatment considered, malvidinin forms of the anthocyanins were the most abundant class of anthocyanin (Table 3), followed by petunidin and delphinidin forms, according to the anthocyanic profile defined for Tannat red young wines previously (González-Neves et al., 2012a) and with the fact that even when the technique of vinification may modify the wine anthocyanin profile, the grape variety effect prevails (González-Neves et al., 2012b). The HPLC results of total free anthocyanins shows that PCM wines had the highest concentrations followed by ENZ, ST and TM wines, supporting the discussion developed in Section 3.1 around the total anthocyanins quantified spectrophotometrically. Interestingly, the alternatives of vinification differed not only in the magnitude of the increase of anthocyanin concentration compared to TM, but also considering individual anthocyanin concentrations. The wines produced with PCM had the highest contents of malvidin anthocyanic forms, which are the most stable ones (Cheynier, Souquet, Kontek, & Moutounet, 1994), which could have a positive impact in maintaining long-term wine colour. On the other hand with the ENZ alternative, the delphinidinic forms where increased the most, and being those anthocyanins the least stable forms (Cheynier et al., 1994), their enological significance could be less relevant. In ST wines none of the anthocyaninc forms increased statistically, but all were present in higher concentration than in MT wines. The antioxidants properties cited for commercial tannins (Celotti, Battistutta, Comuzzo, Poinsaut, & Zironi, 1999) could be the explanation for these findings where delphinidinic forms increased in the highest proportion. Alcalde-Eon et al. (2014) also found higher contents of anthocyanins in wines with tannins additions respect to control wines, point out that tannin addition may protect anthocyanins from degradation.

3.3. Analysis of non-anthocyanic low molecular weight phenols

In Table 4 the non-anthocyanic low molecular weight phenols compounds (NA-LMWP) identified are listed. Compound numbers are correlative with chromatogram pic numbers in Fig. 1. For compounds which quantification was no possible due to their very low concentration (out of range of the calibration curve) the value was substituted by a "tr" (traces). Fig. 2 illustrates the total contents by treatment showing the contribution of each family to that total.

A total of 35 NA-LMWP compounds in all Tannat wines produced for the four winemaking procedures tested were identified, of which 28 could be quantified. They totalled between 66 mg/L in TM wines up to more than 86 mg/L in PCM ones.

The results show that among the NA-LMWP, the concentrations of non-flavonoid compounds were higher than those of flavonoids (accounting for 78.2% in ENZ to 81.6% in TM wines) in all wines. Even when referred to the non-anthocyanic low molecular weight fraction, the findings were unexpected based on the results reported for red-wines by several works (Boido et al., 2011; Fanzone, Peña-Neira, Jofré, Assof, & Zamora, 2010; Monagas, Suarez, Gómez-Cordovés, & Bartolomé, 2005).

Within non-flavonoid compounds, phenolic alcohols and related compounds (49.47% in TM to 56.40% in PCM wines), hydroxycinnamic acids and their derivatives (24.66% in ENZ to 26.74% in TA wines) and hydroxybenzoic acids (17.54 in PCM to 23.83% in TM wines) were the most abundant compounds. Stilbenes were present in much lower proportions (approximately 1.1% in all treatments).

Several compounds were detected in lower concentrations than those reported by other authors using similar analytical methods (Fanzone et al., 2010 in Malbec, Monagas et al., 2005 in Tempranillo, Graciano, Cabernet Sauvignon and Merlot) or other methodologies (Boido et al., 2011 in Tannat). However it is well documented that wine phenolic concentrations are very variable, depending among other factors on grape cultivar (González-Neves et al., 2012a; Monagas et al., 2005), climate during grape development and maturation (González-Neves et al., 2012a), winemaking conditions (Bautista-Ortín et al., 2007), and wine age, because they are in

Table 3
Concentrations of anthocyanins analysed by HPLC in Tannat wines produced by different winemaking procedures.[a]

	TM[d]	PCM	ENZ	ST
Cyanidin[b]	0.90 ± 0.04 b	1.11 ± 0.04 a	1.02 ± 0.15 ab	0.98 ± 0.07 ab
Delphinidin	11.77 ± 1.52 b	16.89 ± 2.12 ab	18.02 ± 4.24 a	14.94 ± 1.56 ab
Petunidin	23.37 ± 2.56 b	33.83 ± 3.88 a	31.29 ± 5.64 ab	27.09 ± 2.44 ab
Peonidin	6.69 ± 0.40 b	8.11 ± 0.57 a	8.33 ± 0.70 a	7.37 ± 0.51 ab
Malvidin	142.99 ± 9.02 b	194.62 ± 24.62 a	168.16 ± 24.65 ab	157.02 ± 10.79 ab
Total free anthocyanins[c]	185.72 ± 13.54 b	254.55 ± 31.23 a	226.83 ± 35.37 ab	207.41 ± 15.37 ab

[a] TM, traditional maceration; PCM prefermentative cold maceration; ENZ vinification with pectolytic enzymes addition at barrelling, ST grape-seed enological tannin addition at barrelling.
[b] The value of each anthocyanidin consider the sum of the quantified non-acylated glucoside, acetylated glucoside, coumarylic glucoside and caferylic glucoside.
[c] The total free anthocyanin content of the wines was calculated considering the sum of all the free anthocyanins quantified.
[d] Different letters in the same row indicate differences significatives Tukey 0.05.

constant reactions in the wine (Fulcrand et al., 2006). Respect to this former point, whereas in the previous cited works that have analysed NA-LMWP in wines, analyses were made at devatting, we did it at 9 month. Peña-Neira et al. (2000) report NA-LMWP concentrations even lower, than those that we found, for aged Spanish red-wines.

In all wines the concentrations of cinnamic acids found were much higher than those reported for wines derived from other grape varieties: Fanzone et al. (2010) for Malbec, Monagas et al. (2005) for Tempranillo, Graciano, Cabernet and Merlot, Peña-Neira et al. (2000) in wines from different Spanish regions. The concentrations of trans-caftaric acid (Table 4 pick 3) mainly explained the cited differences. At the same time while former cited authors present benzoic acids having higher concentration than cinnamic acids, in our case this was just the opposite, as in previous reports for Tannat wines (Boido et al., 2011) where high concentrations of caftaric acid are also reported. If this is a distinctive characteristic of wines derived from Tannat grape variety, this should deserve further investigation.

Peña-Neira et al. (2000) express that contents of caftaric acid in wines are mostly related to grape variety and that concentrations of tartaric esters of cinnamic acids are not related to the vinification method. We obtained high concentrations of caftaric acid in all wines, although it was even higher in the wines elaborated with the three alternatives to TM.

It was found in all wines very low concentrations of low molecular weight phenols (LMWP) belonging to flavanoĺs phenolic family, even when they presented high contents of proanthocyanidins (up to more than 2250 mg/L in ENZ wines). Vrhovsek, Vanzo, and Nemanic (2002) found that the coefficient between wine concentrations of proanthocyanidins of rather low molecular weight (less than 5 units), and those of more polymerised fractions, was between 0.39 and 0.46 in 5 month Blaufränkisch wines, indicating a preponderance of larger polymers. It would be expected to get coefficients even lower, if just monomers and dimers are taken into account. Nevertheless these findings markedly differ from those presented by other authors (Fanzone et al., 2010; Monagas et al., 2005) who used the analytical technique applied in our study. Boido et al. (2011) using others analytical procedures for NA-LMWP determination, in very phenols rich Tannat wines, found flavanols being the most abundant NA-LMWP compounds, reporting much higher concentrations than those reported for us. Then, it could not be associated to a varietal characteristic. In addition, in the cited works, (+)-catechin and (−)-epicatechin were the flavanols present in the highest concentration. In our research, we measured very low quantities of (+)-catechin, and (−)-epicatechin was not detected.

It should be considered, that while we present results of nine-month wines, most works present outcomes of analyses made at devatting. Peña-Neira et al. (2000) in aged red wines, reported catechin concentrations in the range of our findings. Recently, Chiara, Pacella, Jourdes, and Teissedre (2011) found a correlation between mDP and wine age that suggest that the mDP decreases through time, but nine-month wines are still very young as to interpretive results in terms of aging. Bautista-Ortín et al. (2007) report a significant decrease in flavan-3-ols of low molecular weight after 8 month of bottling, showing that time elapsed may be among the causes of the lower contents reported in our work. Inter-flavan bond formation and cleavage determine the size distribution of tannin in wines (Cheynier et al., 2006), further investigation being needed for a better understanding of the process (Fulcrand et al., 2006). Finally, our results show that there is no necessarily a correspondence between wine richness in total flavan-3-ols and LMWP flavan-3-ols.

Within the hydroxi-benzoic fraction, five acids and three derivatives were identified in all wines. In this family the most abundant compound was gallic acid as reported by other authors (Boido et al., 2011; Fanzone et al., 2010; Peña-Neira et al., 2000) follow by syringic acids (pick N° 14 Fig. 1) in agreement with other works (Fanzone et al., 2010; Monagas et al., 2005). Nevertheless in a recent work Boido et al. (2011) report that syringic acid was not found in Tannat grapes and wines.

The benzoic acid contents in wines were not greatly affected by the alternative of vinification used (Fig. 2). It is well documented that grape-seeds are an important source of gallic acid (Boido et al., 2011; Koyama et al., 2007). Busse-Valverde et al. (2011) demonstrated that maceration enzymes may facilitate seed phenolic extraction, and Hernández-Jiménez et al. (2012) that prefermentative cold maceration also may increase seed derivatives. So higher contents of gallic acid should be expected in wines elaborated with those treatements, but this was not confirmed in our studies. Soto-Vázquez et al. (2010) report significantly higher contents of gallic acid when maceration enzymes and enological tannin where used together during vinification, not evidencing if the cause was the enzymatic addition or the tannin one. But, Bautista-Ortín et al. (2007) testing separately these two enological additives obtained increased concentrations of gallic acid only in the wines derived from the tannin treatment. Alcalde-Eon et al. (2014) report that enological tannins increased levels of gallic acid just in one of the 2 years of essays, expressing that the effect of the enological tannins depend on vintage, that is, on grape compositional differences.

Seven cinnamic acids and derivatives were identified. Tartaric esters were present in much higher concentrations than free acids in all wines. *Trans* and *cis* p-coumaric acids were detected in so small concentration that they could not be quantified. Trans-caftaric acid was the compound of the family present in highest concentration (representing between 74% and 78% of the cinnamic acids and derivatives quantified). In all wines produced by alternative procedures to TM, its concentration was higher, but just in ST wines those differences were statistically significant. That increase as product of the grape seed-tannin addition is not expected,

Table 4
Non-anthocyanin low molecular weight phenols [Mean (mg/L) ± sd] in Tannat wines produced by different winemaking procedures.[a]

		Vinification alternative			
Peak	Compound	TM[b]	PCM	ENZ	ST
	Non-flavonoid Phenols				
	Hydroxybenzoic acids and derivatives				
1	Gallic acid	5.13 ± 0.48 a	4.80 ± 2.21 a	5.67 ± 0.70 a	5.04 ± 0.68 a
2	Protocatechuic acid	1.25 ± 0.04 ab	1.11 ±0.15 b	1.21 ± 0.07 ab	1.35 ± 0.12 a
11	Vanillic acid	1.78 ± 0.11 a	1.50 ± 0. 11 b	1.58 ± 0.09 ab	1.53 ± 0.12 b
14	Syringic acid	2.22 ± 0.14 a	2.01 ± 0.25 ab	1.86 ± 0.08 b	1.85 ± 0.14 b
4	Methyl gallate	0.47 ± 0.07 b	0.58 ± 0.03 ab	0.57 ± 0.06 ab	0.68 ± 0.09 a
17	Ethyl gallate	1.14 ± 0.05 a	1.10 ± 0.08 a	1.10 ± 0.05 a	1.10 ± 0.10 a
27	Ellagic acid	1.00 ± 0.04 ab	1.11 ± 0.05 a	0.93 ± 0.09 b	0.86 ± 0.09 b
	Total hydroxybenzoic acids and derivatives	**12.99 ± 0.46 a**	**12.22 ± 0.40 a**	**12.92 ± 0.87 a**	**12.40 ± 0.91 a**
	hydroxycinnamic acids and derivatives				
3	Trans-caftaric acid	10.85 ± 1.57 b	13.50 ± 2.09 ab	11.47 ± 1.18 ab	14.49 ± 1.40 a
6	Trans-coutaric acid	1.41 ± 0.56 a	1.81 ± 0.51 a	1.82 ± 0.12 a	2.29 ± 0.50 a
5	Cis-coutaric acid	0.28 ± 0.10 a	0.48 ± 0.17 a	0.35 ± 0.09 a	0.51 ± 0.11 a
12	Trans-caffeic acid	0.86 ± 0.14 b	1.04 ± 0.07 a	1.01 ± 0.03 ab	0.85 ± 0.03 b
18	Trans-p-coumaric acid	tr	tr	tr	tr
16	Cis-p-coumaric acid	tr	tr	tr	tr
13	Hexose-ester-trans-p-coumaric acid	0.51 ± 0.17 a	0.57 ± 0.12 a	0.76 ± 0.02 a	0.68 ± 0.25 a
	Total hydroxycinnamic acids and derivatives	**13.91 ± 2.20 b**	**17.41 ± 2.74 ab**	**15.40 ± 1.38 ab**	**18.82 ± 2.13 a**
	Total phenolic acids and derivatives	**26.90 ± 2.61 a**	**29.63 ± 3.12 a**	**28.33 ± 2.24 a**	**31.22 ± 2.97 a**
	Stilbenes				
15	Stilbene	tr	tr	tr	tr
25	Trans-resveratrol-glucoside	0.26 ± 0.01 a	0.31 ± 0.06 a	0.30 ± 0.04 a	0.28 ± 0.04 a
32	Cis-resveratrol-glucoside	0.38 ± 0.03 a	0.44 ± 0.08 a	0.43 ± 0.08 a	0.48 ± 0.04 a
	Total stilbenes	**0.64 ± 0.03 b**	**0.75 ± 0.02 a**	**0.73 ± 0.06 a**	**0.76 ± 0.05 a**
	Phenolic alcohols and related compounds				
26	Tryptophol	0.64 ± 0.06 b	1.45 ± 0.08 a	1.02 ± 0.05 ab	1.04 ± 0.43 ab
7	Tyrosol	26.32 ± 3.88 b	37.85 ± 5.02 a	32.37 ± 3.94 ab	37.35 ± 0.75 a
	Total phenolic alcohols and related compounds	**26.96 ± 3.94 b**	**39.30 ± 5.00 a**	**33.39 ± 3.93 ab**	**38.39 ± 0.82 a**
	Total non-flavonoid phenols	**54.50 ± 5.28 b**	**69.68 ± 8.08 a**	**62.45 ± 5.48 ab**	**70.38 ± 3.49 a**
	Flavonoid phenols				
	Flavan-3-ols				
10	Catechin	0.80 ± 0.16 b	1.49 ± 0.64 ab	1.55 ± 0.28 a	0.57 ± 0.05 c
19	Procyanidin-dimer	1.03 ± 0.36 b	1.56 ± 0.16 ab	1.52 ± 0.31 ab	2.03 ± 0.40 a
8	Procyanidin-dimer-B3	1.48 ± 0.42 a	1.65 ± 0.47 a	1.74 ± 0.51 a	1.99 ± 0.45 a
9	Procyanidin-dimer-B1	0.61 ± 0.02 c	1.99 ± 0.14 a	1.61 ± 0.79 ab	0.87 ± 0.15 bc
20	Procyanidin-dimer-C1	1.49 ± 0.05 b	2.05 ± 0.29 a	2.18 ± 0.31 a	2.28 ± 0.17 a
	Total flavan-3-ols	**5.41 ± 0.98 b**	**8.74 ± 1.01 a**	**8.61 ± 1.57 a**	**7.74 ± 0.87 ab**
	Flavonols				
21	Flavona	tr	tr	tr	tr
35	Quercetin	1.60 ± 0.13 a	1.95 ± 0.09 a	1.50 ± 0.19 a	1.91 ± 0.43 a
29	Quercetin-3-glucuronide	2.15 ± 0.24 b	2.53 ± 0.20 b	3.00 ± 0.09 a	2.51 ± 0.29 b
30	Quercetin-3-galactoside	0.62 ± 0.12 b	0.67 ± 0.07 b	1.08 ± 0.13 a	0.74 ± 0.03 b
31	Quercetin-3-glucoside	0.36 ± 0.09 a	0.45 ± 0.07 a	0.37 ± 0.06 a	0.41 ± 0.09 a
33	Naringetine	tr	tr	tr	tr
34	Syringetine-3-glucoside	0.49 ± 0.03 ab	0.58 ± 0.03 a	0.45 ± 0.07 b	0.40 ± 0.07 b
22	Myricetin-3-glucuronide	tr	tr	tr	tr
23	Myricetin-3-galactoside	tr	tr	tr	tr
24	Myricetin-3-glucoside	1.12 ± 0.25 b	1.38 ± 0.06 ab	1.58 ± 0.19 a	1.58 ± 0.19 a
28	Di-OH-Kaempferol	0.50 ± 0.08 c	0.64 ± 0.08 bc	0.80 ± 0.08 a	0.65 ± 0.02 b
	Total flavonols	**6.85 ± 0.41 b**	**8.20 ± 0.36 a**	**8.79 ± 0.31 a**	**8.19 ± 1.06 a**
	Total flavonoids	**12.26 ± 1.33 b**	**16.94 ± 1.05 a**	**17.40 ± 1.31 a**	**15.94 ± 0.29 a**
	Total LMWP	**66.76 ± 6.41 b**	**86.63 ± 8.30 a**	**79.85 ± 6.77 ab**	**86.31 ± 3.57 a**

[a] TM, traditional maceration; PCM prefermentative cold maceration; ENZ vinification with pectolytic enzymes addition at barrelling, ST grape-seed enological tannin addition at barrelling.
[b] Different letters within the same row indicate differences significatives Tukey 0.05. tr, compounds detected but not quantifiable.

because it is not a grape seed compound (Boido et al., 2011). Nevertheless Obreque-Slíer, Peña-Neira, López-Solís, Ramírez-Escudero, and Zamora-Marín (2009) show that enological tannin commercial label is not always accurate about the vegetable or tissue origin of the declared tannin. Additionally, as was commented in Section 3.2, commercial tannins are cited as having antioxidants properties (Celotti et al., 1999), and it is well documented that caftaric acid is the main oxidisable wine compound (Cejudo-Bastante, Castro-Vázques, Hermosín-Gutiérrez, Pérez-Coello, 2011). Finally our findings could have been the result of a commercial formulation including material from other origin than grape seed (such as grape skin) or the consequence of oxidative protection of must and wine native compound by the adding tannins.

Flavan-3-ol LMWP family compounds contents were statistically higher in all wines elaborated with alternatives to TM, especially in PCM. The less polimerizated flavanols are the easiest extracted at the beginning of maceration (Koyama et al., 2007), then the time of prefermentative maceration would let their accumulation. In ENZ wines even when concentrations were higher than in those elaborated with TM, the increase was much less evident that the measured in proanthocyanidins (Section 3.1, Table 2). Results are consistent with previous findings that report maceration enzymes enhancing particularly the extraction of tannin fraction associated to the skin cell-wall (Amrani et al., 2003), which are those in grapes of highest molecular weight (Cheynier et al., 2006).

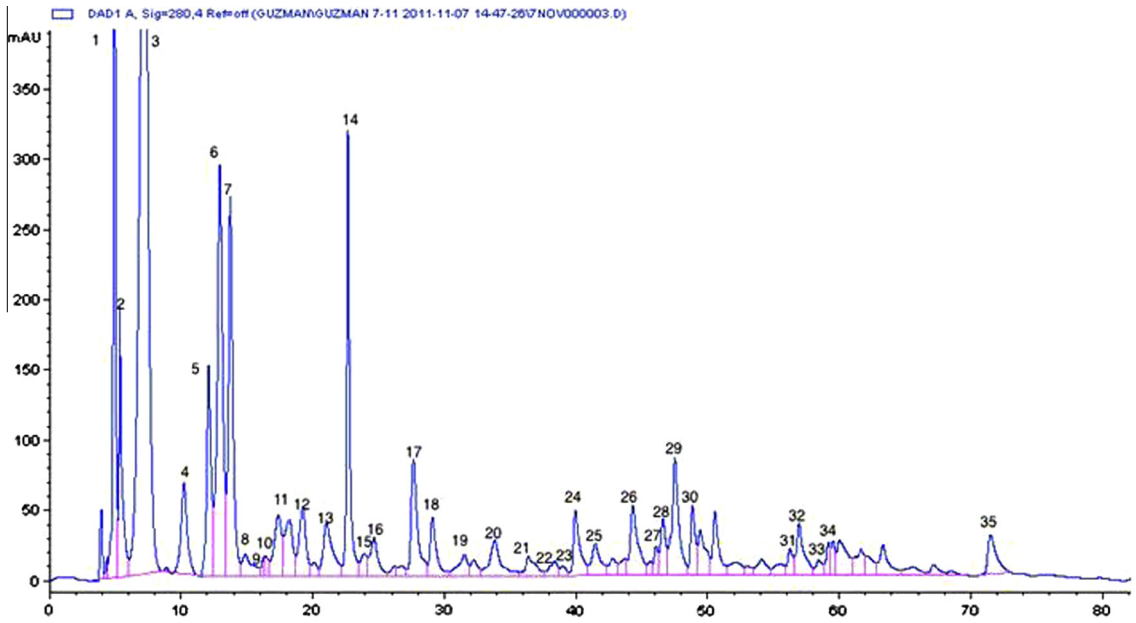

Fig. 1. Chromatogram at 280 nm of non-anthocyanin phenolic compounds of diethyl ether/ethyl acetate extract from a wine of the maceration enzyme essay. Peak assignment is given in Table 4. 1. Gallic acid; 2. protocatechuic acid; 3. trans-caftaric acid; 4. methyl gallate; 5. cis-coutaric acid; 6. trans-coutaric acid; 7. tyrosol; 8. procyanidin B3; 9. procyanidin B1; 10. catechin; 11. vanillic acid; 12. trans-caffeic acid; 13. hexose ester trans-p-coumaric acid; 14. syringic acid; 15. stilbene; 16. cis-p-coumaric acid; 17. ethyl gallate; 18. trans-p—coumaric acid; 19. procyanidin-dimer; 20. procyanidin-trimer; 21. flavona; 22. myricetin-3-glucuronide; 23. myricetin-3-galactoside; 24. myricetin-3-glucoside; 25. tans-resveratrol-glucoside; 26. tryptophol; 27. ellagic acid; 28. Di-OH-kaempferol; 29. quercetin-3-glucuronide; 30. quercetin-3-galactoside; 31. quercetin-3-glucoside; 32. cis-resveratrol-glucoside; 33. naringetine; 34. syringetine-3-glucoside; 35. quercetin.

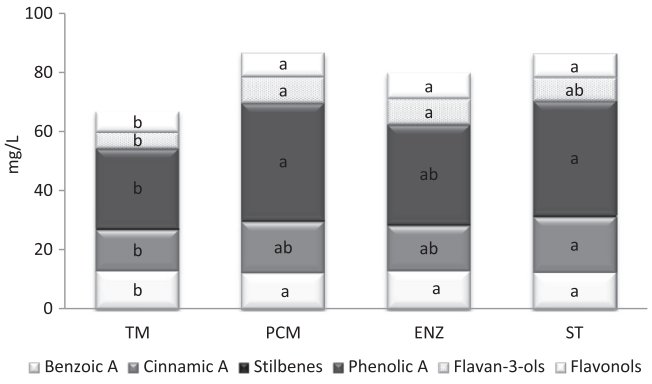

Fig. 2. Wine phenolic families determined by HPLC after liquid/liquid extraction by winemaking procedure. TM, traditional maceration; PCM prefermentative cold maceration; ENZ vinification with maceration enzymes addition at barrelling, ST grape-seed enological tannin addition at barrelling. Different letters within the same column indicate differences significatives Tukey 0.05.

Is there a great background of evidence about grape seeds being an important source of flavan-3-ols (Boido et al., 2011; Koyama et al., 2007). Bautista-Ortín et al. (2007) report increases of wine flavan-3-ols of low molecular weight when proanthocyanidin enological tannins were used. Unexpectedly, in our work the addition of enological tannins of grape-seed to the must, except for procyanidin-dimer (Table 4, pick 19), did not increase in a great extent the wine contents of these compounds, even more, these wines presented the lowest catechin concentrations. Bautista-Ortín et al. (2014) express that when adding at barreling, enological tannins may be absorbed in great extent by grape skin cell wall material, even when those authors found polymeric proanthocyanidinds being the most absorbed.

Among flavonols, eleven compounds were detected and seven quantified. In red wines the first importance adjudicated to these compounds has been their activity as copigments (Boulton, 2001). In the present study, quercetine-3-glucuronide was the main flavonol compound quantified in all wines followed by the aglycone quercetine and myricetin-3-glucoside. Boido et al. (2011) report in Tannat wines quercetin and myricetin being the principal flavonols found in wines, but do not report glucuronides. All wines elaborated with the alternatives to TM presented higher flavonol contents than those of TM. These results may also have a nutraceutical interest, because flavonols such as quercetine, have been suggested to have important activity, even more than resveratrol, in prevention of some specific kind of illnesses (Song et al., 2014). The highest flavonol concentrations were measured in ENZ wines, where the greatest contents of quercetine-3-galactoside, quercetine-3-glucuronide and di-OH-kaempferol were confirmed. Because flavonols are located in the grape skins, cited results are consistent with the improved extraction of skin components cited for maceration enzymes (Romero-Cascales, Ros-García, López-Roca, & Gómez-Plaza, 2012). Interestingly our result suggest that macerating enzymes increases skin derivated components (anthocyanins, flavonols) while not from seeds (like gallic acid), even when studies like Busse-Valverde et al. (2011) have shown that macerating enzymes also may increase seed phenolic extraction. The extended time of maceration in aqueous solutions in PCM winemaking should explain the higher flavonol contents found in PCM wines compared to TM ones. PCM was the only treatment where all quantified flavonols presented higher concentrations than in TM wines. They also presented the highest contents of syringetine-3-glucoside.

In ST wines, higher flavonol contents than in TM ones were also found. That cannot be expected to be a direct result of a seed enological tannin addition, because flavonols are just present in skins tissues. As mentioned above for cinnamic acids, the observed could be the result of oxidative protection of them and formulations containing more grape derivatives than seed ones.

Finally our results demonstrated that the winemaking procedure may change flavonol wine contents although, Monagas et al. (2005) have expressed that the flavonol profile depends on grape variety.

The stilbenes quantified were represented by trans-resveratrol glucoside and cis-resveratrol glucoside, being the later in highest concentration in all wines, differently from what was reported in others works (De Nisco et al., 2013). Winemaking alternatives to traditional one, increased wine stilbene levels in similar magnitude, but concentrations remained below the 1 mg L^{-1}. In ST wines, higher concentrations of these compounds can be explained by the facts discussed for cinnamic acids and flavonols, because they are not components of grape seeds.

Significant differences were observed regarding contents of phenolic alcohols and related compounds (tyrosol and tryptophol respectively), PCM wines showing the highest concentrations. An increase in yeast diversity in first steps of PCM has been demonstrated (Feuillat, 1997). Since tyrosol and tryptophol presence in wines derive from the yeast metabolism of tyrosine and tryptophan (Monagas et al., 2005), more extraction of those amino acids and more yeast diversity could explain the observed in PCM wines.

3.4. Wines colour study

Table 5 shows the results of CIELAB wine colour. The wines produced with the vinification alternatives to TM had better colour quality. That means wines with fewer values of lightness, more saturated and reddish. Nevertheless, just in ENZ wines the differences were statistical respect to TM wines. Wines others than TM had the highest concentrations of anthocyanins (Table 2 and 3), the molecules responsible of young red wine colour (Cheynier et al., 2006) and of compounds like flavonols (Table 4), cited by Boulton (2001) as important cofactors capable to exert a dramatic influence on colour even at very low cofactor to anthocyanins ratios. Even when PCM wines had the highest contents of anthocyanins, ENZ wines had higher anthocyanin contents but in addition, the highest flavonols concentrations, particularly of quercetine-3-glucuronide, quercetine-3-galactoside and Di-OH-Kaempferol, cited by Boulton (2001) among the strongest known cofactors. Other authors also have reported increases of wines colour when macerating enzymes are used, adjudging the result to an increase in anthocyanins extractions (Romero-Cascales et al., 2012). Nevertheless in other works wines with more chromacity and reddish colour have been obtained using maceration enzymes despite less anthocyanin contents than control wines (González-Neves et al., 2013). Soto-Vázquez, Río Segade and Orriols-Fernández (2010) reports improvement of colour and copigmentation in wines with enzyme and tannin addition, but only the level of kaempferol was higher than in control wines. These compositional results could contribute to explain the colour differences reported among wines produced with the different winemaking alternatives. However it must be taken into consideration that wine colour depends on multiple factors (Boulton, 2001; Cheynier et al., 2006). Anyhow, as our results suggest, it would be worthy to take into consideration for wine colour analysis, particularly when extraction phenomena are under study, factors like specific flavonol contents.

Table 5
CIELAB colour in Tannat wines produced by different winemaking procedures.[a]

	TM[c]	PCM	ENZ	ST
L^{*}[b]	51.68 ± 3.23 a	50.33 ± 0.51 a	48.03 ± 1.24 a	50.00 ± 1.86 a
C^{*}	49.35 ± 2.93 b	51.51 ± 0.64 ab	53.25 ± 0.67 a	52.22 ± 1.78 ab
a^{*}	49.06 ± 2.95 b	51.19 ± 0.64 ab	53.03 ± 0.65 a	51.96 ± 1.79 ab
b^{*}	5.29 ± 0.75 a	5.66 ± 1.12 a	4.55 ± 1.51 a	4.91 ± 1.95 a

[a] TM, traditional maceration; PCM prefermentative cold maceration; ENZ vinification with pectolytic enzymes addition at barrelling, ST grape-seed enological tannin addition at barrelling.
[b] (L^{*}) lightness, (C^{*}) chromaticity, (a^{*}) red–greenness and (b^{*}) yellow–blueness.
[c] Different letters within the same row indicate differences significatives to Tukey 0.05.

4. Conclusions

Results provide information of putative enological and nutraceutical importance.

The alternatives to traditional winemaking evaluated increased and modified red-wine phenolic composition. While in PCM and ENZ that would be the result of a better exploitation of grape composition (without use of additives in PCM), in ST this would be the consequence of the tannin formulation added and its potential wine phenolic compounds oxidative protection effect.

PCM produced wines with the highest contents of some hidrosoluble phenolic compounds such as anthocyanins, but flavan-3-ols content was also increased. Additionally yeast metabolism derived compounds concentrations; tyrosol and triptophol, were significantly higher in PCM than in TM wines, possibly due to the activity of native yeast strains in the prefermentative phase.

ENZ produced the wines with the highest total phenolic content, but that was mainly due to the increase in proanthocyanidin concentrations, even when more anthocyanins than in TM wines were obtained. The extraction of NA-LMWP, was also increased. Particularly significant higher contents of flavan-3-ols, flavonols and stilbenes than in TM wines were confirmed, whereas contents of phenolic acids, also present in grape pulp, did not increase.

ST produced wines much more concentrated in flavans-3-ols reactive to vanillin than the other wines. The use of grape-seed enological tannins did not increase some phenolic compounds usually described as components of grape seeds, for example catechin or gallic acid. Instead, we obtained significant increase of phenolic compounds not derived from seed, such as flavonols and caftaric acid.

Tannat phenolic characterisation was improved. Syringic acid was reported for the first time in Tannat wines while caftaric acid was found in much higher concentrations than those cited for most grape varieties.

The three alternatives evaluated produced wines with higher concentrations of recognised nutraceutical compounds such as flavonols and stilbenes. This should be taken into account when increasing these wine properties is aimed at.

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