Original article
Effect of a cold-active pectinolytic system on colour development of Malbec red wines elaborated at low temperature

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Summary   The effect of a new cold-active pectinolytic system on colour of Malbec wines was studied under the following winemaking conditions: (i) fermentation at low temperature (20 °C) and (ii) prefermentative cold maceration (PCM) (5 °C–7 days) followed by traditional fermentation (28 °C). The pectinolytic system was mainly composed of polymethylgalacturonase and pectin lyase activities, detected under similar conditions to those in winemaking (pH 3.6–20 °C). The results show that the enzyme system significantly accelerated colour extraction by reducing the maceration time necessary for vinification at low temperature and shortening the PCM stage. Enzyme-treated wines exhibited better chromatic parameters than their controls at devatting and after 6 months of storage. The cold-active enzyme compensated the decrease in colour extraction due to the low maceration temperature, achieving high-quality wines with chromatic characteristics similar to those of traditional wines.

Introduction

Polyphenols are one of the most important quality parameters of red wines. Among these compounds, anthocyanins are the pigments responsible for the colour of red grapes and young red wines, while tannins exhibit high antioxidant capacity and they are responsible for astringency and bitterness (Cheynier *et al.*, 2006). Polyphenols are also considered bioactive compounds because of their nutraceutical effects on human health, including anticarcinogenic and antioxidant properties (Basli *et al.*, 2012).

Grape polyphenols are mainly located in the skins and seeds, and they diffuse into the wine during the maceration process. However, grape skin cell walls constitute a barrier against diffusion of these compounds. The permeability to polyphenols can be increased by partial hydrolysis of their structural polysaccharides (pectins, hemicelluloses and cellulose), a process that can be facilitated by maceration enzymes (Romero-Cascales *et al.*, 2012). Particularly, pectinolytic enzymes are able to break down pectic polymers of plant cell walls. These enzymes comprise two main types: methy-

lesterases, which remove methoxyl groups from pectin, and depolymerases. The depolymerases include hydrolases and lyases, which cleave the bonds between galacturonate units, the former by hydrolysis of the α-(1,4) glycosidic bonds and the latter by β-elimination. The use of exogenous pectinolytic enzymes in vinification may increase colour extraction during the maceration stage. The enzymes used in enology are complex mixtures of pectinases (mainly consisting of polygalacturonase, pectinesterase and pectin lyase activities), cellulases, hemicellulases, and acid proteases. At present, commercial pectinases are obtained from the genus *Aspergillus* and, besides desirable activities, they generally contain high levels of pectinesterase and β-glucosidase activities (Romero-Cascales *et al.*, 2008; Alimardani-Theuil *et al.*, 2011) that can negatively affect the wine quality.

Traditional red winemaking is characterised by the simultaneous development of alcoholic fermentation and maceration. New winemaking techniques have been proposed considering the selective effect of maceration conditions on the extraction of grape components and the reactions in which they participate. Among these methods, prefermentation cold maceration (PCM) is employed prior to fermentation to encourage extraction in aqueous medium at low temperature (5–15 °C), which results in preferential solubility of

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water-soluble compounds and encourages selective extraction of anthocyanins and tannins of low molecular weight (Sacchi *et al.*, 2005; Alvarez *et al.*, 2006). Additionally, it results in a better-structured product, one that is richer in phenolic and aromatic compounds, thus preserving a strong relationship with the production area (Alvarez *et al.*, 2006). To another hand, fermentations conducted at low temperatures (15–20 °C) can increase the production and retention of volatile compounds, thus improving the aromatic profile of wines (Molina *et al.*, 2007). Vinification at low temperature is traditionally applied in white winemaking, but it is currently gaining ground in red winemaking. However, low maceration temperatures can diminish colour extraction in red wines, an effect that can be counteracted using cold-active pectinolytic enzymes as they ensure good colour extraction at low temperature.

Malbec (*Vitis vinifera* L.) is considered to be the emblematic cultivar of Argentina. Recent studies carried out in Mendoza, Argentina, have shown the polyphenolic richness of Malbec grapes and their potential to produce quality wines (Fanzone *et al.*, 2010). This middle-maturing grape variety of French origin is well adapted to the soil and dry climate of this region and produces wines with a very deep colour and high tannin concentration, a fruity aroma and a particular plum-like flavour.

Recently, Martín and Morata de Ambrosini (2013) reported on a cold-active pectinolytic system obtained from a psychrotolerant *Bacillus* sp., which increased colour extraction in short macerations with red grape skins at low temperature. The aim of the present work was to evaluate the effect of this cold-active enzymatic system on colour development of Malbec wines elaborated at low temperatures, to propose a technology that will achieve to highlight the richness in pigments and polyphenols of regional wines.

Materials and methods

Production of the cold-active enzymatic system

The pectinolytic enzymatic system was obtained from *Bacillus* sp. CH15 bacterial strain, which was previously isolated from grape berries from San Rafael, an enological area in the province of Mendoza, Argentina (Cabeza *et al.*, 2011), and studied for secretion of cold-active acidic pectinolytic enzymes (Martín & Morata de Ambrosini, 2013). The bacterial strain was grown overnight at 30 °C under agitation (100 rpm) in pectin medium according to Kobayashi *et al.* (2001) but with minor modifications. Concentrations were (g L^{-1}) pectin from citrus peel (Sigma Chem. Co., St. Louis, MO, USA) 5.0, soy peptone 5.0, yeast extract 5.0, K_2HPO_4 1.0, $MgSO_4$ $7H_2O$ 0.2, $MnSO_4·6H_2O$ 0.05, $CaCl_2·2H_2O$ 0.05 and 50 mM acetate buffer (pH 5.0). All chemical reagents were of analytical grade.

After cells were removed by centrifugation (14 000 *g*; 15 min at 4 °C), the supernatant was dialysed overnight at 4 °C using 0.1 M NaCl and 0.2 g L^{-1} $CaCl_2$ solution in 50 mM acetate buffer (pH 5.0). Then, the supernatant was concentrated fivefold by rotary evaporation under reduced pressure (40 min at 35 °C and 85 KPa). The corresponding concentrate was called CH15 pectinolytic preparation (CH15-PP) and was used as maceration enzyme for colour extraction.

Enzyme activity assays

Polymethylgalacturonase, polygalacturonase, cellulase and xylanase comprised the total enzymatic activity present in the CH15-PP that contributed to the release of reducing sugars from the corresponding substrate. These polysaccharide hydrolases were analysed by measuring the increase in reducing end groups with 3,5-dinitrosalicylic acid reagent (DNS; Miller, 1959) using high methoxyl citrus pectin, polygalacturonic acid, birchwood xylan and microgranular cellulose from Sigma Chem. Co as substrates (0.25% of each substrate in 50 mM acetate buffer, pH 3.6). The enzyme/substrate ratio was 1/10 (v/v), and the reaction temperature was 20 °C.

Pectin lyase and pectate lyase activities were determined spectrophotometrically at 235 nm by monitoring the formation of unsaturated products from pectin and polygalacturonic acid, respectively (Collmer *et al.*, 1988). The reaction conditions were the same as those used for the hydrolase analyses, and molar extinction coefficients of unsaturated pectin and polygalacturonic acid were 5500 and 4600 L mol^{-1} cm^{-1}, respectively.

Pectinesterase activity was measured by titration of the carboxylic groups released from 0.5% high methoxyl citrus pectin in 0.1 M NaCl (adjusted to pH 4.5 with 0.5 M NaOH) at 20 °C (Moyo *et al.*, 2003).

β-glucosidase activity was determined by quantification of glucose released from a 0.5% D-(+)-cellobiose (Fluka, Buchs, Switzerland) solution using an enzymatic kit (Ghose, 1987). The reaction conditions were identical to those used for the hydrolase assays.

In all cases, one unit of enzyme activity (U) was defined as 1 μmol of galacturonic acid released per min under the given assay conditions (da Silva *et al.*, 2005), except for β-glucosidase activity, where one unit is the enzymatic activity that releases 2 μmol of glucose per min from cellobiose under the assayed conditions (Yang *et al.*, 2008).

Extracellular protease and amylase activities were determined by inoculation of the bacterial strain on skim milk agar or gelatin agar plates for protease activity and on starch agar plates for amylase activity,

according to Charoenchai *et al.* (1997), at pH 4.5. Agar cultures were examined for clear zones surrounding bacterial growth after incubation at 28 °C for 3–5 days and flooding of the plates with 50 g L^{-1} of acetic acid (protease activity) or Lugol's solution (amylase activity). Additionally, gelatin agar cultures were examined for liquefaction of the medium after incubation for 3 days.

Protein content was determined according to Bradford (1976), using bovine serum albumin as a standard for the reaction.

Grape samples and microvinification assay

Malbec grape must (*Vitis vinifera*) was obtained from a winery in San Rafael, southern Mendoza, Argentina [250 g L^{-1} reducing sugar; 3.6 g L^{-1} total acidity, expressed as tartaric acid; 180 mg L^{-1} yeast assimilable nitrogen (YAN); pH 4.22]. Microfermentations were carried out in 1-L Erlenmeyer flasks containing 800 mL of fresh must supplemented with sodium metabisulfite (80 mg SO_2 L^{-1}). Total acidity was corrected to 5.5 g L^{-1} with tartaric acid, and selected yeasts (strain IOC-2007 from the Institut Œnologique de Champagne, Épernay, France) were added to the flasks at a concentration of 10 g of dry yeast per 100 kg of must. Two different winemaking techniques were applied: (i) fermentation at low temperature (20 °C) (LTF) and (ii) PCM (5 °C–7 days) followed by traditional fermentation (28 °C) (PCM + FT). Two wines were elaborated with each technique, one supplemented with the CH15-PP (6.096 U L^{-1} must) and the other one not (control). All fermentations were carried out in triplicate at a controlled temperature using cold chambers or stoves. Additionally, wines elaborated with traditional maceration (28 °C) and without enzymes were used as general controls. Progress of the alcoholic fermentation was monitored daily by measuring weight loss using flasks with stoppers containing a Müller valve that allowed only CO_2 to escape from the system. Constant weight for two consecutive days indicated the end of the fermentation.

During the period of skin contact, fermentations were stirred for 30 min at 90 rpm twice a day. At the end of the fermentations, wines were drained and only free run juice was collected and kept in 330-mL glass bottles at 18 °C.

Wine chemical characteristics

Enological parameters such as ethanol percentage (v/v), total and volatile acidity (g L^{-1}), pH, residual sugar (g L^{-1}) and total and free SO_2 (mg L^{-1}) were determined according to the OIV official methods (O.I.V., 1990).

Wine chromatic characteristics

Wines were analysed during the maceration process and after 6 months of storage. Samples were centrifuged before analyses. Absorbance was measured using an UV/Visible spectrophotometer (Metrolab 1700; Capital Federal, Buenos Aires, Argentina), with 0.1-cm pathlength glass cells for colour intensity and tint, and with 1-cm pathlength cells for total phenols and anthocyanins, after adjusting the pH of the wine samples to 3.6.

The colour intensity (CI) was calculated as the sum of the absorbances measured at 420, 520 and 620 nm, and the tint as the ratio between 420 and 520 nm, according to Glories (1984) and Sudraud (1958), respectively. Total polyphenolic index (TPI) was calculated according to Ribéreau-Gayon *et al.* (1998), after measuring the absorbance at 280 nm of a 100 times diluted wine sample using deionised water. Total anthocyanins were determined according to the method by Puissant-León (Cayla *et al.*, 2002), which consists in measuring the absorbance at 520 nm after incubation of a wine sample for 30 min in 0.1 N HCl (1:40 ratio, v/v).

To calculate the CIELAB parameters, the whole visible spectrum (380–780 nm) was recorded, and the illuminant *D*65 and 10º observer were used in the calculus. *L** (lightness), *C** (chroma), *H** (hue angle), *a**(red colour intensity), and *b**(yellow colour intensity) parameters were obtained using MSCV® software (Negueruela, 2005). The CIELAB differences (*Δ*Er,s*) were calculated using the following equation:

$$\Delta \text{Er}, s^* = \left[\left(L_A^* - L_B^* \right)^2 + \left(a_A^* - a_B^* \right)^2 + \left(b_A^* - b_B^* \right)^2 \right]^{1/2},$$

where the subscripts A and B correspond to different samples.

Monomeric, polymeric and copigmented anthocyanins were determined using the colorimetric effects of SO_2 and acetaldehyde on anthocyanins, according to the method proposed by Levengood and Boulton (2004). Samples were analysed spectrophotometrically by measuring absorbance at 520 nm with 0.1-cm pathlength glass cells. Readings were corrected by multiplying them with a factor 10. Colour caused by monomeric anthocyanins (MA), polymeric pigments (PP) and copigmented anthocyanins (CA) was determined through the following equations:

$$\text{MA} = A_{20} - A_{SO_2}$$

$$\text{PP} = A_{SO_2}$$

$$\text{CA} = A_{\text{acet}} - A_{20}$$

Statistical analysis

Vinifications and analyses were performed in triplicate, and the data are presented as mean $\pm$ standard deviation. Analysis of variance (ANOVA) and Fisher's LSD test ($P < 0.05$) were applied to all experimental data, using STATGRAPHICS Plus 5.1 (Manugistics, Rockville, MD, USA).

Results and discussion

Enzymatic activities present in the CH15-PP

The cold-active pectinolytic system CH15-PP was produced by *Bacillus* sp.CH15. The strain was isolated and selected as previously described by Martín and Morata de Ambrosini (2013) and deposited in the Strain Bank of Autochthonous Microorganisms at the Biotechnology Laboratory of the Faculty of Industrial Applied Sciences, National University of Cuyo, Argentina. Martín and Morata de Ambrosini (2013) found that the enzymatic system demonstrated high pectinolytic activity at a low temperature range (10–20 °C) and at wine pH, and it increased the pigment content during a short maceration of red grape skins. The present study was conducted to evaluate the effect of this enzyme under actual vinification conditions.

Table 1 shows the enzymatic activities, expressed as specific activity (units per gram of total protein), present in the CH15-PP and determined under conditions resembling those in winemaking (20 °C and pH 3.6).

Table 1 Specific enzymatic activities present in the CH15-PP determined at 20 °C and pH 3.6 (resembling winemaking conditions)

Enzyme	Specific activity (U g^{-1}protein)*
Polymethylgalacturonase (PMG)	3775 $\pm$ 46
Polygalacturonase (PG)	875 $\pm$ 22
Pectin lyase (PNL)	2716 $\pm$ 57
Pectate lyase (PAL)	ND
Pectinesterase (PE)	ND
Cellulase	491 $\pm$ 11
Xylanase	583 $\pm$ 16
β-glucosidase	ND
Protease	(+)
Amylase	(+)

Values are means $\pm$ standard deviation ($n = 3$). (+) indicates substrate degradation by enzymes secreted by the bacterial strain on skim milk agar and gelatin agar plates (protease activity) and starch agar plates (amylase activity).
ND, not detected.
*U: one unit is the enzymatic activity that catalyses the release of 1 μmol of product per minute under the reaction conditions.

Pectin degradation requires the combined action of several pectinolytic enzymes. The main pectinolytic activities observed in the CH15-PP were polymethylgalacturonase (PMG), polygalacturonase (PG) and pectin lyase (PNL). A commercial preparation frequently used in winemaking, Endozym® Ice (Pascal–Biotech, Paris, France), was used as control enzyme and showed an activity of 3228 $\pm$ 85, 2700 $\pm$ 85 and 5400 $\pm$ 56 U g^{-1} for PMG, PG, and PNL, respectively. This means that the bacterial strain preparation, CH15-PP, showed a higher PMG activity than the control, while its PG and PNL activities were about 32% and 50% of the control enzyme. Pectate lyase (PAL) and pectinesterase (PE) activities were not detected in the CH15-PP under the conditions assayed. Romero-Cascales *et al.* (2008) found PAL activity in six commercial enzymatic winemaking preparations, but only at low levels. PE activity should be very low or absent in winemaking preparations to avoid excessive methanol production.

Additionally, the enzymatic system showed cellulase and xylanase activities under the conditions assayed. These activities help degrade polysaccharides present in the grape skin cell wall. They have been attributed to increase colour and liberate tannins bound to the cell wall (Amrani Joutei *et al.*, 2003).

β-glucosidase activity can negatively affect wine colour, as it is able to degrade anthocyanins (Romero-Cascales *et al.*, 2008), but this activity was not detected in the CH15-PP.

Other enzymes such as proteases and amylases were also present in the studied enzyme. Particularly proteases catalyse the degradation of proteins from the grape skin cell membrane and may help increase extraction of polyphenols located inside vacuoles.

Microvinification

Two winemaking techniques were applied, and the effect of the CH15-PP enzyme system was studied and compared with a traditional winemaking.

Wine fermentation kinetics are shown in Fig. 1. Two wines obtained with alternative techniques to the traditional vinification were elaborated with skin contact during 20 days. TF control wines reached 12% ethanol (v/v) after 11 days of maceration. The fermentation rate is defined as the amount of alcohol or CO_2 produced per unit of time. It is known that this parameter increases with increasing temperature, thus shortening the fermentation time. As a result, it took significantly more time to obtain high alcohol levels in wines produced with techniques at low temperature. However, the fastest fermentation rate was found in wines produced by PCM followed by TF, especially wines produced with the enzyme (0.271 and 0.320 g of ethanol h^{-1} for PCM + TF control and PCM + TF enzyme wines, respectively) (measured
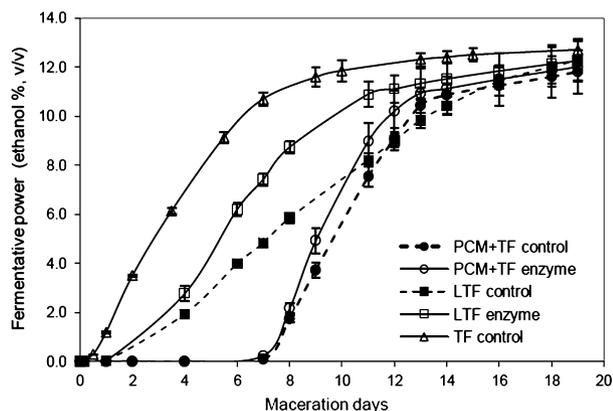
Figure 1 Fermentation kinetics of the elaborated wines. (●) Prefermentation cold maceration (PCM) followed by traditional fermentation without enzyme (PCM + TF control); (○) PCM followed by traditional fermentation with CH15-PP enzyme (PCM + TF enzyme); (■) fermentation at low temperature without enzyme (LTF control); (□) fermentation at low temperature with CH15-PP enzyme (FLT enzyme); (△) traditional fermentation without enzyme (TF control).

after PCM stage), whereas TF control wines showed a rate of 0.195 g of ethanol h^{-1}. This could indicate that the PCM process like the enzymatic system increased the availability of grape fermentable sugars as a consequence of a further degradation of skin cell wall components, accelerating the fermentation process.

On the other hand, after 4 days of maceration, fermentation kinetics of LTF enzyme wines was similar to those of TF control wines. After 11 days of maceration, LTF enzyme wines reached an ethanol percentage of about 11, a behaviour not observed in LTF control wines, which showed a significantly lower fermentation rate. Consequently, the CH15-PP enzyme used in vinification at low temperature significantly reduced the total maceration time.

The chemical composition of the wines at the end of the alcoholic fermentation was also determined (data not shown). Significant differences in ethanol content were found between TF control wines and wines made with alternative techniques, especially those supplemented with the enzyme, which showed the highest ethanol levels (13.10%, 12.70% and 12.10% ethanol, for PCM + TF enzyme, LTF enzyme, and TF control wine, respectively). Total acidity in enzyme-treated wines was higher than in wines without the enzyme, but no significant difference in pH could be observed between any of the samples. Volatile acidity was only lower in enzyme-treated PCM + TF wines, while TF control wines showed the largest levels of total and free SO$_2$. All parameters were below the maximum values permitted.

Effect of the enzyme on colour development during winemaking

Figures 2 and 3 show the colour development of wines elaborated measured through classical and CIELAB parameters. The two winemaking techniques assayed showed differences in colour extraction. In PCM + TF wines, the effect of the enzyme on all chromatic parameters was evidenced during the first 24 h of the PCM stage. After this time, colour intensity (CI), total polyphenolic index (TPI) and the chroma parameter (C^*) were significantly higher in enzyme-treated wines than in control wines without enzyme, and consequently, the tint and lightness (L^*) parameters significantly decreased. Thus, enzyme musts became darker and with a higher polyphenol content than control musts, which was maintained throughout the PCM stage. Other authors have also found the most profound colorimetric changes during the first 2 or 3 days of the PCM stage (Gómez-Míguez *et al.*, 2007; De Santis & Frangipane, 2010). A fast colour extraction, particularly extraction of anthocyanins, is suitable for the future stability of the colour. Different time–temperature combinations in PCM processes have been reported, ranging between 2–8 days and 3–15 °C (Alvarez *et al.*, 2006; De Santis & Frangipane, 2010). However, there are no reports yet regarding the influence of cold-active pectinolytic enzymes on colour extraction in red wines. According to our results, use of the CH15-PP would shorten the PCM stage to only 24 h, at 5 °C.

In PCM + TF vinification, significant differences during fermentation were observed between enzyme-treated wine and its control regarding CI, tint and TPI, but C^* and L^* parameters were not significantly different. Maximum values for CI and C^* were found near the third day of maceration for both vinification methods. Free anthocyanins are the main responsible compounds for the red colour at this stage, and they significantly contribute to these two parameters: CI and C^*. Usually, anthocyanins reach a maximum around the third or fourth day of maceration, after which they drop, probably partly because of oxidation and polymerisation reactions, and also to adherence of anthocyanins to yeasts and solid grape parts (Fulcrand *et al.*, 2006). After that, during the devatting stage, significant differences between enzyme-treated wine and its control were only observed for CI and tint.

On the other hand, in LTF wines, the CI and C^* parameters were significantly higher in enzyme-treated wines than in their respective controls, with a peak close to day 7 of the maceration stage. Then, a slight decrease in these parameters was observed, probably because of the reasons mentioned above. Tint significantly decreased throughout the fermentation period in wines with the enzyme, indicating a higher extraction of red than yellow colour compounds. However,
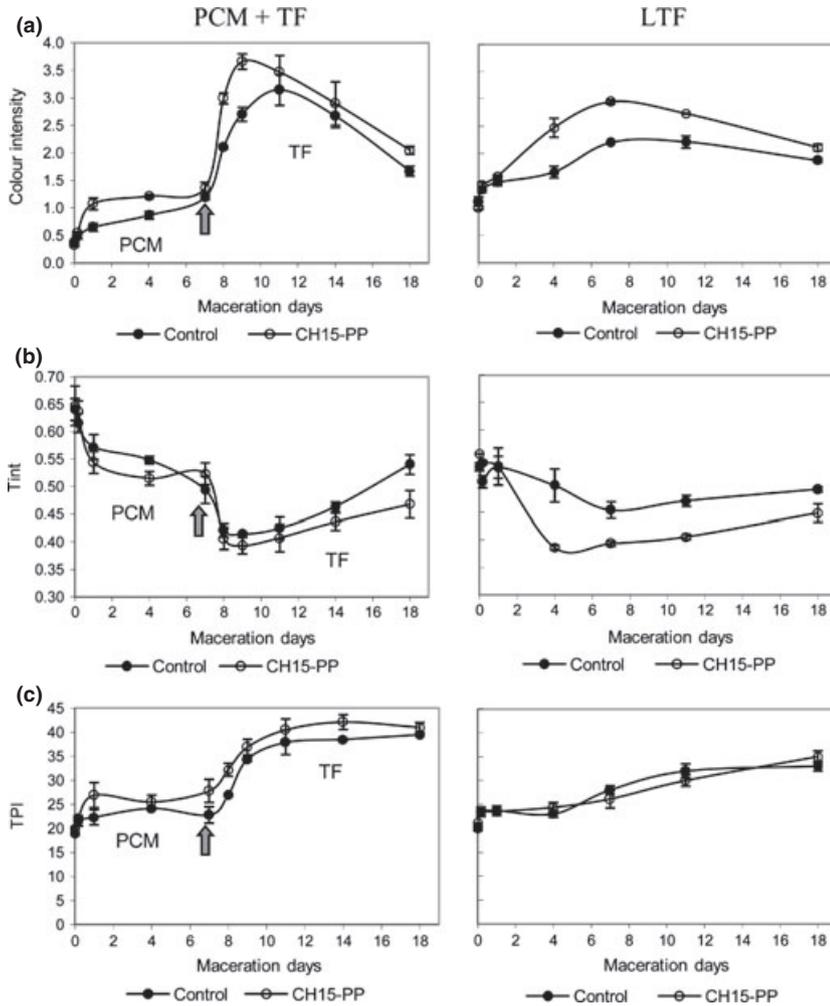
Figure 2 Colour development of wines elaborated with CH15-PP enzyme (empty marker) and without enzyme (filled marker), during (i) prefermentation cold maceration (PCM) followed by traditional fermentation (TF) (left figures), and (ii) fermentation at low temperature (LTF) (right figures). Colour was measured using classical parameters. (a) Colour intensity, (b) tint, (c) total polyphenolic index (TPI). The arrow indicates the moment PCM finished, and yeast was inoculated.

no significant difference was observed in TPI and L^* between enzyme-treated wines and their controls. These results differ from those previously obtained (Martín & Morata de Ambrosini, 2013), when during a short maceration assay with grape skin the enzyme increased both the CI and TPI compared with natural extraction. In LTF, it seems that the enzyme produced a high extraction of certain phenolic compounds, particularly anthocyanins, the main compounds for the red colour, whereas the extraction was not sufficient to produce a significant effect in the case of TPI. Other authors found that small must volumes corresponding to winemaking at a low scale increased fixation of polyphenols onto grape skins and seeds (González-Neves *et al.*, 2010). So winemaking at a larger scale should be considered when confirming these results.

A comparison between the chromatic characteristics of the elaborated wines at the end of the alcoholic fermentation and after 6 months of storage was made (Table 2 and 3).

At devatting, TF control wines and the two wines made with alternative winemaking supplemented with the CH15-PP enzyme showed similar chromatic parameters. Only tint, *h**and TPI values in TF control wines were significantly higher than those in the other wines, which were less reddish and had a more purple tone, although they showed the highest total polyphenol content. CIELAB colour differences between the TF control wine and enzyme-treated wines, $\Delta Er, s^* = \left(\left(L_A^* - L_B^* \right)^2 + \left(a_A^* - a_B^* \right)^2 + \left(b_A^* - b_B^* \right)^2 \right)^{1/2}$, were calculated, but values were in no case higher than 2.7 units (data not shown). A value of 2.7 CIELAB units is the threshold for the human eye to distinguish between the colour of wines, when trained tasters use standardised winetasting glasses (Martínez *et al.*, 2001). Consequently, any difference between these wines was not visually detectable. However, CIELAB colour differences were also calculated between wines treated with the enzyme and their respective controls (without
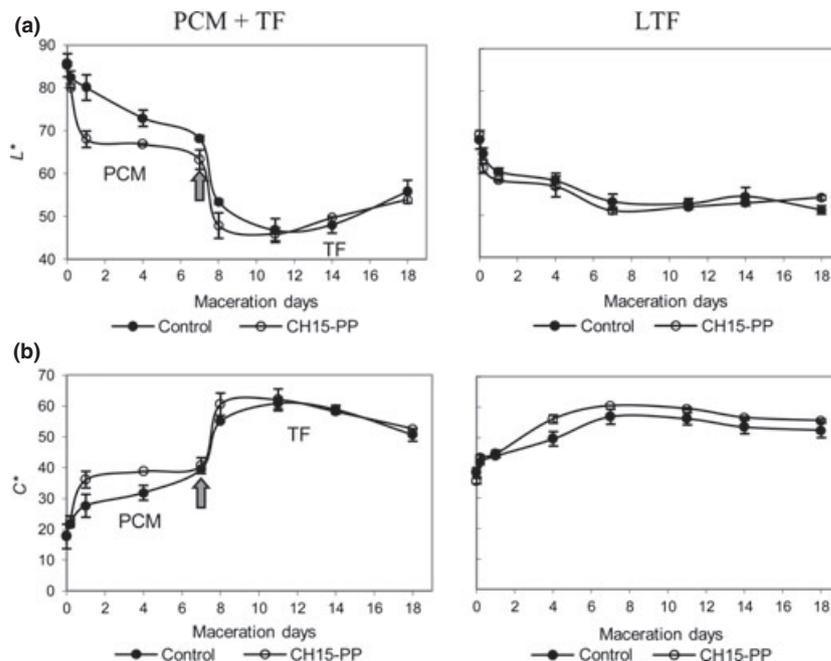
Figure 3 Colour development of wines elaborated with CH15-PP enzyme (empty marker) and without enzyme (filled marker), during (i) prefermentation cold maceration (PCM) followed by traditional fermentation (TF) (left figures) and (ii) fermentation at low temperature (LTF) (right figures). Colour was measured by CIELAB parameters. (a) Lightness (L^*), (b) chroma (C^*). The arrow indicates the moment PCM finished, and yeast was inoculated.

Table 2 Chromatic parameters of the wines assayed at the end of each alcoholic fermentation

	PCM + TF			LTF			TF
	Without enzyme	With CH15-PP enzyme	Sig.	Without enzyme	With CH15-PP enzyme	Sig.	TF control wine
CI	1.67 ± 0.089a	2.05 ± 0.071bc	*	1.87 ± 0.035b	2.11 ± 0.063c	*	2.05 ± 0.024bc
Tint	0.54 ± 0.18c	0.45 ± 0.03a	*	0.49 ± 0.01ab	0.45 ± 0.01a	*	0.52 ± 0.01bc
TPI	35.5 ± 0.05c	37.3 ± 2.97bc	ns	34.1 ± 2.83a	33.0 ± 0.10ab	ns	40.3 ± 0.07c
L^*	55.8 ± 2.69a	53.9 ± 4.3a	ns	51.35 ± 2.3a	54.3 ± 0.6a	*	47.8 ± 1.9a
C^*	50.6 ± 2.1a	54.6 ± 2.5b	ns	52.3 ± 2.4a	55.5 ± 0.4b	*	55.8 ± 0.1b
h^*	(−2.40) ± 0.57b	(−1.55) ± 0.10b	ns	(−0.35) ± 0.07a	(−1.11) ± 0.14b	*	2.51 ± 0.08c
ΔE^*	–	2.90	–	–	3.73	–	–
Free anthocyanins	3.78 ± 0.46a	4.45 ± 0.21a	*	4.49 ± 0.35a	4.19 ± 0.20a	ns	4.43 ± 0.85a
Copigmented anthocyanins	1.28 ± 0.47a	1.88 ± 0.62a	ns	2.00 ± 0.20a	2.01 ± 0.41a	ns	1.76 ± 0.30a
Polymeric pigments	0.248 ± 0.003b	0.245 ± 0.021b	ns	0.164 ± 0.014a	0.161 ± 0.030a	ns	0.408 ± 0.021c
Total anthocyanins (mg L^{-1})	449.3 ± 13a	482.2 ± 5.0a	*	470.7 ± 22a	501.6 ± 2.1a	*	449.6 ± 40a

PCM, prefermentation cold maceration; TF, traditional fermentation; LTF, fermentation at low temperature; CI, colour index; TPI, total polyphenolic index; L^*, lightness; C^*, chroma; h^*, hue angle; Sig., significant difference between the means within the same treatment, according to the ANOVA test; ns, no significant difference; *, significant difference.

ΔE^* is CIELAB colour difference $\left(\Delta E^*_{n,s} = \left[(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2 \right]^{1/2} \right)$. Values >2.7 CIELAB units indicate differences between two wines perceived by the human eye (Martínez *et al.*, 2001). ΔE^* values were calculated within each treatment.

The values are the average of three vinification ± standard deviation. Different letters in the same row indicate significant differences ($P < 0.05$) between the five wines.

enzyme), with 2.9 and 3.73 CIELAB units for PCM + TF and LTF wines, respectively. From these results, it may be deduced that the cold-active enzyme compensated the colour extraction limited by the effect of low maceration temperatures.

About pigments derived from anthocyanins, only polymeric pigments were significantly higher in the TF control wines. Moreover, significant differences between enzyme-treated wines and their respective controls (without enzyme) were found in total anthocyanins for both vinification techniques, as well as in free anthocyanins only for CPM + TF wines, reaching similar levels to those of the traditional wines.

Nevertheless, after 6 months of storage in bottles, significant differences were found in chromatic parameters between TF control wines and enzyme-treated

Table 3 Chromatic parameters of the wines assayed after 6 months of storage

	PCM + TF			LTF			TF
	Without enzyme	With CH15-PP enzyme	Sig.	Without enzyme	With CH15-PP enzyme	Sig.	TF control wine
CI	1.21 ± 0.11a	1.63 ± 0.015b	*	1.58 ± 0.058b	1.75 ± 0.010c	*	1.68 ± 0.040b
Tint	0.63 ± 0.04bc	0.62 ± 0.07bc	ns	0.57 ± 0.16ab	0.55 ± 0.01a	ns	0.68 ± 0.07c
TPI	25.1 ± 0.55a	28.1 ± 0.71b	ns	25.0 ± 0.25a	23.8 ± 0.21a	ns	28.2 ± 1.20b
L^*	58.6 ± 2.5b	50.5 ± 2.5a	*	55.6 ± 1.5b	52.3 ± 0.5a	*	50.2 ± 0.5a
C^*	28.8 ± 1.2a	37.0 ± 1.5c	*	30.5 ± 0.8a	38.6 ± 2.2c	*	35.5 ± 2.5b
h^*	11.5 ± 0.15b	8.5 ± 0.01a	*	10.0 ± 0.25b	7.5 ± 0.0a	*	12.5 ± 0.0c
ΔE^*	–	4.25	–	–	4.48	–	–
Free anthocyanins	0.95 ± 0.02a	1.20 ± 0.01b	*	1.25 ± 0.14b	1.15 ± 0.10b	ns	1.54 ± 0.20b
Copigmented anthocyanins	2.48 ± 0.07a	3.31 ± 0.03b	*	3.36 ± 0.26b	3.75 ± 0.01c	*	3.70 ± 0.05c
Polymeric pigments	0.481 ± 0.010a	0.578 ± 0.059a	*	0.600 ± 0.007a	0.570 ± 0.010a	ns	0.567 ± 0.010a
Total anthocyanins (mg L^{-1})	113.3 ± 3.3a	153.9 ± 11b	*	148.4 ± 3.0b	160.2 ± 2.5b	*	180.1 ± 6.5b

PCM, preformentation cold maceration; TF, traditional fermentation; LTF, fermentation at low temperature; CI, colour index; TPI, total polyphenolic index; L^*, lightness; C^*, chroma; h^*, hue angle; Sig., significant difference between the means within the same treatment, according to the ANOVA test; ns, no significant difference; *, significant difference.

ΔE^* is CIELAB colour difference $\left(\Delta E_{n,s}^* = \left[(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2 \right]^{1/2} \right)$. Values >2.7 CIELAB units indicate differences between two wines perceived by the human eye (Martínez *et al.*, 2001). ΔE^* values were calculated within each treatment.

The values are the average of three vinification ± standard deviation. Different letters in the same row indicate significant differences ($P < 0.05$) between the five wines.

LTF wines. The latter wines achieved a significantly higher CI and C^*, while tint, h^* and TPI were significantly lower. This resulted in darker wines with a more purple tone, but with less total polyphenol content, while enzyme-treated PCM + TF wines showed chromatic parameters like those of TF control wines. Similar results were obtained by González-Neves *et al.* (2010), who found that after 12 months of storage, wines produced with diverse winemaking techniques presented no differences in total pigments, compared with traditional wines.

CIELAB colour differences showed a similar behaviour to that after devatting. Enzyme-treated PCM + TV and LTF wines showed values higher than 2.7 units (4.25 and 4.48 CIELAB units, respectively), compared with their respective controls (without enzyme).

In general, after 6 months of storage, all wines demonstrated a higher content of pigments derived from anthocyanins, at the expense of a decrease in free and total anthocyanins. It is known that ageing favours the formation of pigments derived from anthocyanins, particularly polymeric pigments that increase the colour stability of red wines (Fulcrand *et al.*, 2006). At this moment, only significant differences in pigments derived from anthocyanins were observed in enzyme-treated wines and their respective controls, thus promoting the colour stability of the wines supplemented with enzyme, except in polymeric pigments for LTF wines.

It can be inferred from these results that the use of the new cold-active CH15-PP enzyme in the winemaking techniques assayed compensated the colour extraction, which is decreased due to the effect of the low maceration temperature. The enzyme system probably also favoured a higher colour stability of aged wines. Vinification at low temperatures is advantageous because it allows a more controlled process and preserves flavours. The assay conditions studied could render high-quality wines, with chromatic characteristics similar to those of traditional wines. These results are a first approach to this study, so supplementary studies on a larger scale of wines with a longer ageing process and evaluation of their aromatic profiles are currently being conducted to confirm these conclusions.

Acknowledgments

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Graphical Abstract

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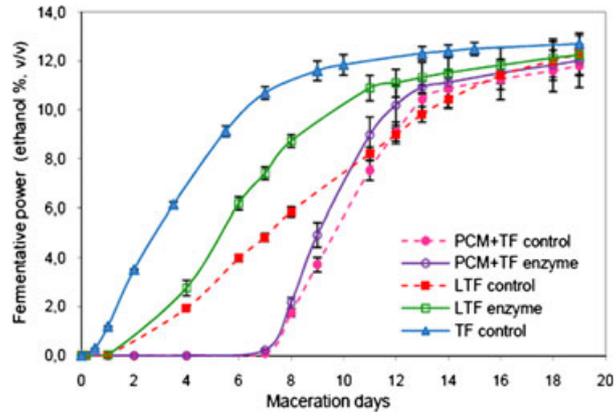
The enzyme system significantly accelerates colour extraction by reducing the maceration time necessary for vinification at low temperature and shortening the PCM stage, achieving high-quality wines with chromatic characteristics similar to those of traditional wines..