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Characterization and winemaking application of a novel pectin-degrading enzyme complex from *Aspergillus sojae* ATCC 20235

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Summary

A novel pectin-degrading enzyme complex produced by *Aspergillus sojae* ATCC 20235 (PC-AS) using low-cost substrates was characterised in terms of its enzyme activities relevant in winemaking. This novel PC-AS was applied at the maceration/fermentation stage during the elaboration of 'Tempranillo' red wines to study its effect on colour development and the phenolic and amino acid wine composition. PC-AS polygalacturonase activity was the major enzyme activity detected and quantified under winemaking conditions (pH 3.5, 20 °C) and proved being stable and active in the presence of sulfur dioxide. Xylanase activity, albeit in lesser amounts, was also present in PC-AS, and neither pectinesterase, which produces methanol, nor β-glucosidase, which is detrimental to wine colour, were detected in PC-AS. This pectin-degrading complex promoted a faster colour extraction since maximum colour intensity of the enzyme treated wines was reached earlier compared to their controls. After 6 months of storage under winery conditions, wines elaborated with PC-AS presented higher concentrations of caffeic acid, coumaric acid and aspartic acid ($p < 0.05$), suggesting an improved extraction of grape cell components. In conclusion, the application of PC-AS yielded results that showed that it can be used in red winemaking to shorten the maceration time needed to reach high CI values and to improve the extraction of some phenolics and other compounds that enhance the quality of the final product.

Key words: *Aspergillus sojae*; red wine; pectinases; colour; phenolics.

Abbreviations: PC-AS: pectin-degrading enzyme complex produced by *Aspergillus sojae* ATCC 20235; ATCC: American type culture collection; GRAS: generally regarded as safe; GABA: gamma-amino butyric acid; PGA: polygalacturonic acid; CPB: citric acid – phosphate buffer; DNS: 3,5-dinitrosalicylic acid reagent; O.D.: optical density; MBS: potassium metabisulfite; HPLC: high pressure liquid chromatography; OIV: Organisation Internationale de la Vigne et du Vin; UV/Vis: ultraviolet/visible; CI: colour intensity; ANOVA: analysis of variance; PCA: principal component analysis; *A. sojae*: *Aspergillus sojae*; *V. vinifera*: *Vitis vinifera*; C: control wines with no enzyme addition; L: wines with addition of 0.030 g·L^{-1} of a commercial pectinase; P1: wines with addition of PC-AS 0.015 g·L^{-1}; P2: wines with addition of PC-AS 0.030 g·L^{-1}.

Introduction

The main stages for winemaking are: pre-fermentation processing, fermentation, post-fermentation processing and ageing (Moreno-Arribas and Polo 2009). Pectin-degrading enzymes can be used during winemaking to improve: juice extraction, clarification, filtration, colloidal stability (by preventing haze from forming later in processing), aroma extraction (Styger *et al.* 2011), and in the case of red grapes, to improve colour extraction (Santos-Buelga and De Freitas 2009).

The pre-fermentation processing of grape berries includes destemming and crushing, which are the first mandatory procedures for grape juice extraction, and simultaneously maceration starts as grape skins are disrupted. At this stage, commercial pectolytic enzyme preparations can be used to degrade the polysaccharides of cell walls and middle lamellae, and thus to facilitate juice release and liberation of polyphenols (pigments, tannins) and aroma molecular precursors that are located in the grape skin cells (Busse-Valverde *et al.* 2011). A current model for grape berry cell walls includes three main types: an hemicellulose-rich cell wall (corresponding to peripheral skin cells), a pectin-rich cell wall of skin cells containing highly branched heteropolysaccharides, and a pectin-rich cell wall of pulp cells (Gao *et al.* 2016). The berry pulp zone is easily released and crushed, whereas the peripheral zone of skin cells is the most difficult to extract (Conde *et al.* 2007) and needs either extra pressure to release its components, or the addition of pectolytic preparations, which in turn improve grape varietal characteristics and give character to the must and wine. These commercial pectolytic preparations are complex cocktails of several enzymes that degrade grape tissues as well as polysaccharide colloids of the produced musts (Ducasse *et al.* 2011). They act on the grape skin pecto-cellulosic cell walls by partially cleaving their structural polysaccharides (Zietsman *et al.* 2015a). They may also potentiate wine aroma expression when they contain specific glycosidases that hydrolyze the linkage of volatile terpenes

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and isoprenoids to sugar residues and release the odorous molecules that were glycosylated inside the grape berry cells (Ugliano 2009). Thus, a plethora of enzyme activities can participate in the disruption of grape berry tissues and their synergies contribute to degrade cell wall polysaccharides (Zietsman *et al.* 2015b).

In the case of red wine making, during alcoholic fermentation red grape skins are allowed to be present, and polyphenols (anthocyanins and tannins) responsible for the red colour and sensorial properties of bitterness, astringency and mouth-filling (Brossaud *et al*. 2001), are further extracted as the ethanol produced during the fermentation facilitates their release from skin cells. In red wine fermentations the use of enzyme preparations to enhance wine colour and aroma extraction should include an optimum combination of specific enzymes (Gao *et al.* 2016) and it is important to note that among the secondary activities present in commercial pectolytic preparations, cinnamoyl esterase activities should be absent because they release free phenolic acids (Figueiredo-González *et al.* 2014) that can become precursors of volatile phenols that confer off-odours described as "barnyard" or "horsey" odours to red wines. In addition, the pectin methyl-esterase activity should be negligible because no methanol should be generated when the pectolytic preparation is added to the crushed grapes.

Pectolytic preparations include several activities, among which the following can be included: a) endo- and exo- polygalacturonases (EC 3.2.1.15 and EC 3.2.1.67) that catalyze hydrolysis of α-1→4 glycosidic endo- linkages and linkages of the non-reducing end of pectin chains respectively; b) pectin lyases **(**EC 4.2.2.10**)** that catalyze the eliminative cleavage of α-1→4 glycosidic linkages of pectins; and c) pectinesterases **(**EC 3.1.1.11**)** that catalyze the hydrolysis of the ester linkage between the methoxyl or acetyl groups and the carboxylic group of galacturonic acid residues in the pectin chain (Jayani *et al.* 2005). Fungi are known to be some of the best producers of pectolytic enzymes, which they secrete to infect plants (De Lorenzo and Ferrari 2002). The produced enzymes degrade the plant cell wall and middle lamella facilitating the fungus penetration and infection of the plant. For industrial purposes the filamentous fungus *Aspergillus sojae* ATCC 20235 has been used in the production of the Japanese food named koji (Nunokawa 1986), and its production of pectolytic enzymes in solid state and submerged fermentations has been studied in the last few years (Tari *et al.* 2007, Mata-Gómez *et al.* 2015, Fratebianchi *et al.* 2017) showing high production using low cost carbon and nitrogen sources such as sugar beet pulp, corn steep liquor, apricot pomace or orange peel. Moreover, this strain possesses the safety GRAS (Generally Regarded As Safe) status, which means that its metabolites are not toxic for humans, and it is a natural and non-genetically-modified organism. The objective of this research was to produce and characterize the novel pectin-degrading enzyme complex (named with the acronym PC-AS from here onwards) produced by *Aspergillus sojae* ATCC 20235 under submerged fermentation, and to further investigate its potential use in the process of red wine making. The significance of this study lays in providing novel information for enzyme and wine industries and a deeper insight on additives used for red grape juice and wine production that increase the extraction of grape components that improve certain aspects related to sensorial properties such as colour.

Material and Methods

Reagents: 3,4′,5,7-Tetrahydroxy-3′,5′-dimethoxyflavylium chloride (malvidin chloride), catechin, quercetin and p-coumaric acid were purchased from Extra synthèse (Lyon, France). Gallic acid from Scharlau (Australia) and caffeic acid from Fluka (Sigma, St Louis, MO, USA). All chromatographic solvents were of HPLC grade. Acetonitrile and methanol from J. T. Baker, Avantor Performance Materials (Center Valley, PA, U.S.A), diethyl ether from Biopack (Zárate, Bs.As., Argentina), ethyl acetate and formic acid from Anedra, Research AG (Tigre, Bs.As., Argentina).

Aspartic acid, serine, threonine, isoleucine and leucine were from Anedra. Glycine, GABA, proline, agmatine, ornithine, spermidine, putrescine, l-2-aminoadipic acid, sodium azide and diethyl ethoxy-methylene malonate were purchased from Sigma (St Louis, MO, USA). Glutamic acid, alanine and histamine were from Fluka. Valine and methionine were from Merck. Trytophan was from Biopack and lysine from Gibco (NY, USA).

Polygalacturonic acid (PGA), pectin from apples, high methoxyl citrus pectin, birchwood xylan and p-nitrophenyl-β-D-glucopyranoside were purchased from Sigma. All other reagents used were of analytical grade.

Microorganism and spore production: The *A. sojae* ATCC 20235 strain belonged to the American Type of Culture Collection. It was propagated on YME plates, containing malt extract (10 $g \cdot L^{-1}$), yeast extract (4 $g \cdot L^{-1}$), glucose (4 $g \cdot L^{-1}$) and agar (20 $g \cdot L^{-1}$), and incubated at 30 °C until sporulation. Stock cultures were preserved in 20 % glycerol and stored at -80 °C.

Molasses agar slants were used to obtain the spore suspensions for the seed inoculums (Gögus *et al.* 2006). Spores were harvested from the slants with 0.2 $g \cdot L^{-1}$ Tween 80-water after incubation for 1 week at 30 °C, and counted in a Neubauer Chamber (Marienfeld, Germany) to determine the spore concentration.

Culture conditions and enzyme complex production: The pectinase complex was produced by submerged fermentation of *A. sojae* in an aqueous solution of grinded orange peel (40 $g \cdot L^{-1}$) and ammonium sulfate (2.75 $g \cdot L^{-1}$).The initial pH of the culture medium was adjusted to 3.3 before inoculation with 1.4×10^5 spores$\cdot mL^{-1}$. Batch cultures of 200 mL performed in 1000 mL Erlenmeyer flasks were incubated at 30 °C and 200 rpm in an orbital shaker for 6 d, time after which the entire content of each flask was withdrawn for processing. The fungal biomass and other solid particles were removed from the liquid medium by centrifugation (5900 × g, 20 min) followed by filtration through cheesecloth. The filtered supernatant was further clarified by refiltering through 0.45 μm cellulose nitrate membrane filters (Sartorius, Goettingen, Germany), and then concentrated and washed with five volumes of 12.5 mM cit-

ric acid – 6.25 mM Na_2HPO_4 buffer (pH 5.0) by diafiltration using a 10,000 molecular weight cut-off polyethersulfone laboratory crossflow cassette (Sartorius). The resulting concentrate was lyophilized to obtain a dry preparation, which was shelf-stored at room temperature until used.

Enzyme assays: Polygalacturonase, xylanase and endo-glucanase activities were determined by measuring the release of reducing end groups from different substrates, as described below. Polygalacturonase activity was measured with PGA and pectin from apple as substrates (Cavalitto *et al*. 1999), xylanase activity was determined using birchwood xylan as substrate (Bailey *et al.* 1992), whereas for endo-glucanase activity determination carboxymethylcellulose was used (Ghose 1987). Substrates were prepared as 2 $g \cdot L^{-1}$ solutions in 50 mM citric acid – 25 mM Na_2HPO_4 buffer (CPB), and the enzyme/substrate ratio used in the reaction mixtures was 1/10 (v/v). Total cellulase activity was determined with the filter paper assay according to Ghose (1987), using Whatman No. 1 filter paper in CPB. The reducing end sugars derived from these carbohydrolase activities were measured using the 3,5-dinitrosalicylic acid reagent (DNS; Sigma) (Miller 1959). One unit of enzyme activity was defined as the amount of enzyme that releases 1 µmol of reducing groups from its substrate per minute.

Pectin lyase activity was determined spectrophotometrically by optical density (O.D.) at 235 nm by measuring the initial rate of formation of unsaturated reaction products from 5 $g \cdot L^{-1}$ high methoxyl citrus pectin in CPB (Edstrom and Phaff 1964). Pectinesterase activity was measured by continuous titration of the carboxylic groups released from an unbuffered 0.5 % high methoxyl citrus pectin solution (Moyo *et al*. 2003). Beta-glucosidase activity was assayed following the procedure given in Vita *et al*. (2009) by measuring O.D. at 405 nm under alkaline conditions, due to the release of p-nitrophenol from p-nitrophenyl-β-D-glucopyranoside.

Enzyme activities were measured at pH 3.5 and 20 °C in all cases, so as to resemble conditions normally occurring during winemaking.

Effect of sulfur dioxide on polygalacturonase activity and stability: The influence of sulfur dioxide on PC-AS polygalacturonase activity was tested by performing the corresponding activity assay in the presence of different concentrations of sulfur dioxide, ranging from 12.5 to 200 $mg \cdot L^{-1}$. Potassium metabisulfite (MBS; Enartis, Novara, Italy) was used to obtain the desired concentration.

The effect of sulfur dioxide on polygalacturonase stability was studied by incubating PC-AS at 20 °C for 6 d in the presence of 200 $mg \cdot L^{-1}$ sulfur dioxide. Aliquots were withdrawn at regular time intervals and polygalacturonase activity was measured under standard assay conditions.

Wine production: Red wines were produced from *Vitis vinifera* L. 'Tempranillo' red grapes from local vineyards of the northern Spanish region of La Rioja. Grapes were manually destemmed, crushed, and the resulting must (reducing sugar concentration 212 $g \cdot L^{-1}$, pH 3.60) was supplemented with 80 $mg \cdot L^{-1}$ MBS. Fermentations were carried out at 18-20 °C following traditional winemaking techniques in 3.8 L glass vats containing 3 L of fresh must. Four different wines were elaborated with respect to the addition of pectinases, as follows: control wines with no enzyme addition (C), with addition of PC-AS 0.015 $g \cdot L^{-1}$ (wines P1) and 0.030 $g \cdot L^{-1}$ (wines P2), and with addition of 0.030 $g \cdot L^{-1}$ of the commercial pectinase Lallzyme C-Max (Lallemand, Barcelona, Spain) for oenological use (wines L). Experiments were performed in triplicates. Pectinases were added to the must-containing vats immediately before inoculation with 30 $g \cdot L^{-1}$ of pre-hydrated selected yeasts (Lalvin 71B, Lallemand). Progress of the alcoholic fermentation was monitored daily by density measurement of the fermenting wines, and considered finished when the reducing sugar concentration decreased under 3 $g \cdot L^{-1}$ (5 d). A post-fermentative maceration period of wine in contact with pomace and lees was extended for four additional days. At the end of this period wines were drawn off from solids and the free-running wine was collected. The collected wine was kept at 5 °C for static clarification for 30 d. Clarified wines were submitted to chemical and chromatic analyses and finally bottled and stored at 15 °C under winery conditions for 6 months. Chromatographic analyses by HPLC were performed at the end of storage and the potential generation of off-odors was also evaluated by sensorial analysis with a panel of three expert analysts.

Chemical analyses: Determinations of ethanol percentage, total and volatile acidity, pH, total and free sulfur dioxide and residual sugars were performed according to OIV official procedures (OIV 2014).

Chromatographic analyses

Anthocyanins: Analysis of anthocyanins was carried out following the official method OIV-MA-AS315-11 of the International Organisation of Vine and Wine by direct analysis of red wine samples by reverse phase HPLC (OIV 2014). The five most important non acylated anthocyanins and four major acylated anthocyanins were identified on the basis of retention times and detection at 518 nm, using malvidin as the external standard.

Non-anthocyanins: Non-anthocyanin analyses were carried out using the method by Ginjom *et al*. (2011). According to this method 5 mL of wine samples were dealcoholized by vacuum and subsequently acidified. Non-anthocyanin phenolic compounds were extracted with diethyl ether (3 x 5 mL) from the acidified samples, and with ethyl acetate (3 x 10 mL). These organic fractions were pooled and dried under nitrogen gas, and then stored at 20 °C until analyzed by reverse phase HPLC. Identification of these compounds was based on retention times, UV/Vis spectra, using external standards. Quantification was made at the wavelengths that show more sensitivity to each phenolic group (Ginjom *et al*. 2011): 280 nm for hydroxybenzoic acids and flavonols, using gallic acid and catechin as external standards; 320 nm for hydroxycinnamic acids, using caffeic acid and coumaric acid as external standards; and 360 nm for flavanols with quercetin as standard.

Amino acids: Amino acids and biogenic amines were analyzed by reverse phase HPLC after derivatization with the reagent diethyl ethoxy-methylene malonate fol-

lowing the method of Gómez-Alonso *et al*. (2007) with a slight modification in the injection volume, which was set to 20 µL. Fifteen amino acids and four biogenic amines were detected on their aminoenone derivative form. They were identified according to their retention times, using the external standards indicated in Reagents section. Analyses were performed in an HPLC Waters system (Milford, MA, USA) equipped with a Waters 1525 binary pump, a Waters 717 plus autosampler and a Waters 2996 photodiode array detector. The column was a reverse-phase XBridge column Waters C18 (5 µm packing, 250 mm x 4.6 mm).

Chromatic analyzes: Colour intensity (CI) was analysed during the fermentation and the post-fermentative maceration period. Samples were clarified by centrifugation (6500 × g, 10 min) before the analyses. Sample CI values were determined by spectrophotometry using 0.1 cm pathlength glass cuvettes, and calculated as the sum of O.D. at 420, 520 and 620 nm (OIV 2014).

Statistical analysis: Analysis of variance (ANOVA) was applied as data showed normal distribution and homogeneous variances. Statistical significance between mean values was discriminated according to Fisher's LSD tests. IBM-SPSS Statistics 19.0 software for Windows (IBM-SPSS Inc., Chicago, IL, USA) was used for data processing.

Results and Discussion

Characterization of the enzyme activities present in PC-AS: Pectin-degrading preparations used in oenology contain a number of enzyme activities, which play an essential role in the winemaking process and can therefore impact on wine quality. We determined the activities of seven major degrading enzymes in PC-AS and results are displayed in Tab. 1. It is shown that xylanase and polygalacturonase activities represented the total enzyme activity of PC-AS, and the polygalacturonase was the major activity. In commercial enzyme preparations polygalacturonase activity represents in some cases 70-80 % (Ceci and Lozano 1998). The average polygalacturonase activity towards PGA of twelve commercial preparations used for colour extraction determined at 40 °C by Guérin *et al*. (2009) was 142 $U \cdot mg^{-1}$ protein, which means that the polygalacturonase activity of our PC-AS was around 2.5 times higher than that reported by these authors. This high activity might compensate the absence of pectin lyase activity in PC-AS which, although in less proportions than polygalacturonase, is often found in commercial enzyme preparations. The polygalacturonase substrate specificity, reflected in the difference in activity observed when PGA is replaced by pectin as the reaction substrate, is consistent with the substrate specificity shown by several reported polygalacturonases (Contreras-Esquivel and Voget 2004, Pedrolli and Carmona 2010). Additionally, no pectinesterase activity was detected in PC-AS under the assayed conditions, which is very important since this enzyme catalyses the release of methanol from pectic substances to the wine, and methanol is highly toxic for humans, including blindness among other severe toxic effects (Robins *et al.* 1981).

Table 1

Enzyme activities analyzed in PC-AS

Enzyme activity (substrate)	Units·mg^{-1} protein	Units·g^{-1} dry preparation
Polygalacturonase (PGA)	357 ± 17	1046 ± 51
Polygalacturonase (pectin)	74.5 ± 10.9	219 ± 32
Pectin lyase	nd	nd
Pectinesterase	nd	nd
Xylanase	1.07 ± 0.15	3.14 ± 0.45
Total cellulase	nd	nd
Endo-glucanase	nd	nd
β-glucosidase	nd	nd

Values are means calculated for three determinations ± standard deviation. Activities were determined at pH 3.5 and 20 °C, resembling conditions normally occurring during winemaking.
nd: not detected; PGA: polygalacturonic acid.

The effectiveness of pectinases in winemaking processes can be affected by the levels of sulfur dioxide (Takanayagi *et al.* 2001), which is normally added to the must and wine as an antimicrobial and antioxidant agent. The polygalacturonase activity present in PC-AS proved to be unaffected by concentrations of sulfur dioxide up to 200 $mg \cdot L^{-1}$, which is the legally permitted limit for red wines according to European legislations, and remained fully stable upon incubation for 6 d in the presence of this additive (data not shown).

Cellulases comprise a heterogeneous group of enzymes including endo-glucanases, exo-glucanases and cellobiases, which along with hemicellulases (xylanases, galactanases), act synergistically to achieve efficient degradation of the cell wall. Besides its contribution to clarification processes by removing haze-forming glucans from grape must and wine, these enzymes have been attributed to increase colour and allow the release of cell wall-bound tannins (Joutei *et al.* 2003). PC-AS did not show activity towards either cellulose or cellulose-derived substrates, and it proved to be positive for xylanase activity. Zietsman *et al.* (2015a) reported a synergistic effect of xylanase and polygalacturonase activities in degrading grape tissues during maceration in a model system of grape skin cell preparations. Consequently, the presence of both activities polygalacturonase and xylanase in our PC-AS is a distinctive and relevant trait of this enzyme complex.

β-glucosidase activity can be deleterious to wine quality as it may hydrolyze β-glucosidic linkages of anthocyanins, which provides stability to these pigments responsible for the colour of red wines (Wightman and Wrolstad 1996). This activity was absent in PC-AS.

Wine production and colour development during fermentation: In order to assess whether PC-AS can be applied to improve of colour extraction, CI was monitored during maceration/fermentation of the four different wines, *i.e.*, those where PC-AS or a commercial enzyme preparation was added (P1, P2 and L), and those without enzyme addition (C). Significant differences in CI were found at the second day of maceration/fermentation between the P2 and L wines compared with control wines (Tab. 2). Even though the CI of enzyme-treated wines was higher than control wines virtually throughout the entire process, these differences decreased when wines were kept in contact with grape

Table 2

Colour intensity of the produced red wines during fermentation (day 0-5) and the post-fermentative maceration period (day 6-9)

Day	Treatment	Colour Intensity (CI)
0	C	3.84 a ± 0.99
	P1	4.24 a ± 0.55
	P2	4.42 a ± 0.73
	L	4.48 a ± 0.59
2	C	6.22 b ± 0.46
	P1	7.87 bc ± 1.47
	P2	8.26 cd ± 1.36
	L	8.11 cd ± 1.36
4	C	9.18 cdef ± 1.04
	P1	10.21 ef ± 0.89
	P2	10.54 ef ± 0.68
	L	10.87 f ± 1.07
6	C	9.13 cde ± 0.79
	P1	9.61 def ± 0.67
	P2	10.09 ef ± 0.34
	L	10.16 ef ± 0.74
9	C	9.31 cdef ± 0.89
	P1	8.91 cde ± 0.72
	P2	9.29 cdef ± 0.48
	L	9.55 cdef ± 0.17

C: control wines with no enzyme addition; P1, wines with 0.015 g·L^{-1} PC-AS; P2, wines with 0.030 g·L^{-1} PC-AS; L, wines with 0.030 g·L^{-1} Lallzyme c-max. Values are means of three vinifications ± standard deviations. Different letters within the same column indicate significant differences according to the LSD test ($p < 0.02$).

pomace and lees during the post-fermentative maceration period that was extended for four additional days (day 6 to 9) before wines were drawn off from solids. Ethanol content in combination with the extended maceration can account for extraction of polyphenols and colour (Casassa *et al*. 2013) and explains the progressive increase of CI values of our control C wines during the post-fermentative maceration. Our enzyme-treated wines (P1, P2 and L) reached their corresponding highest CI values at the end of fermentation and the extended maceration did not improve colour extraction. Similar results were also obtained by Bautista-Ortín *et al*. (2005), who found the greatest differences in CI between enzyme-treated wines and control wines at the third day after addition, time after which differences progressively declined and became not always significant. The trends in CI along the maceration/fermentation were rather similar in our enzyme-treated wines, showing a substantial increase at a first stage followed by slight drop which is evidenced as the process comes to an end. Monomeric anthocyanins are the main responsible polyphenols for the increase of CI witnessed during maceration/fermentation, pigments which normally decline along the process, partly due to hydrolysis, oxidation and polymerization reactions into which they are involved (Fulcrand *et al*. 2006); anthocyanins also precipitate with the lees since they adhere to the yeasts and grape solid parts (Salmon *et al.* 2002). Therefore, our results show that the enzyme treated wines reached their highest CI values at the end of fermentation and no extended maceration period would have been required, whereas for control wines maximum CI values were reached at the end of the extended maceration period, implying that the enzymes present both in PC-AS and in the commercial preparation promoted a faster anthocyanin extraction compared to maceration with no added enzymes.

Tab. 3 presents the general composition of the elaborated wines in terms of the oenological parameters. The residual sugar level of wines P2 supplemented with 0.030 g·L^{-1} PC-AS was significantly lower compared to the other elaborated wines, and almost half the value compared to control wines elaborated with no enzyme addition. Other oenological parameters determined were similar in all wines with the exception of the ethanol levels, which were slightly higher in wines P2. All parameters were normal and within the maximum permitted values.

At the end of the elaboration and storage period, sensorial analysis revealed that wines did not show any aroma deviation. Hydroxycinnamic acid derivatives are present in red grape skins and they can be hydrolyzed by cinnamoyl esterase activities, as mentioned above, which release the free phenolic acids. These are substrates for the cinammate decarboxylase of some yeasts, which transforms the free

Table 3

Oenological parameters of the elaborated red wines at the moment of bottling

	C	P1	P2	L
Residual sugar (g·L^{-1})	2.37 a ± 0.12	2.37 a ± 0.05	1.23 c ± 0.05	2.09 b ± 0.07
pH	3.86 ab ± 0.01	3.86 ab ± 0.00	3.89 b ± 0.02	3.83 a ± 0.01
Total acidity[a] (g·L^{-1})	4.6 a ± 0.1	4.5 a ± 0.1	4.5 a ± 0.2	4.7 a ± 0.1
Volatile acidity[b] (g·L^{-1})	0.07 a ± 0.02	0.06 a ± 0.01	0.05 a ± 0.01	0.07 a ± 0.00
Alcohol Degree (% at 20 °C)	12.4 a ± 0.06	12.5 a ± 0.10	12.7 a ± 0.06	12.4 a ± 0.10
Free SO_2 (mg·L^{-1})	< 30.0	< 30.0	< 30.0	< 30.0
Total SO_2 (mg·L^{-1})	< 60.0	< 60.0	< 60.0	< 60.0
L-malic acid (g·L^{-1})	1.48 a ± 0.09	1.63 a ± 0.06	1.69 a ± 0.11	1.67 a ± 0.10
L-lactic acid (g·L^{-1})	0.22 a ± 0.06	0.29 a ± 0.07	0.30 a ± 0.03	0.23 a ± 0.08

C, control wines with no enzyme addition; P1, wines with 0.015 g/L PC-AS; P2, wines with 0.030 g·L^{-1} PC-AS; L, wines with 0.030 g·L^{-1} Lallzyme c-max. [a] Expressed as tartaric acid; [b] Expressed as acetic acid. Values are means of three vinifications ± standard deviations. Different letters indicate significant differences according to the LSD test ($p < 0.02$).

phenolic acids into volatile phenols that confer off-odours (Figueiredo-González *et al.* 2014), and which were not present in any of the wines of our study as judged by a panel of expert sensorial analysts, indicating aroma stability of our elaborated wines.

Phenolic and amino acid content of wines: In order to determine the efficiency of the extraction of components from grape skins to the wine, anthocyanins and non-anthocyanin phenolics were analyzed in the elaborated wines. Their identification was based on chromatographic retention times (Ginjom *et al*. 2011) and comparison with the reference standards. Anthocyanins are responsible for the colour, one of the main attributes of wine quality, and other wine organoleptic characteristics such as bitterness and astringency. Nine anthocyanins were identified and quantified by reverse phase HPLC (Fig. 1 and Tab. 4). Malvidin-3-glucoside was the main anthocyanin, in agreement with the majority of studies on *V. vinifera* wines ('Cabernet Sauvignon', 'Shiraz', 'Merlot', 'Graciano', 'Tempranillo') (Ginjom *et al.* 2011). Other derivates of malvidin - coumaroylglucoside and acetylglucoside derivates- were also present in our wines, as previously reported (Ginjom *et al.* 2011). The second anthocyanin in abundance was cyanidin-3-glucoside, followed by the glucoside, acetylglucoside and coumaroylglucoside derivates of other anthocyanidins (delphinidin, peonidin and petunidin) which are commonly found in red wines (Flamini *et al.* 2013).

Regarding the quantified non-anthocyanins in our wines (Tab. 4), the major compound was gallic acid, which appears from the hydrolysis of gallate esters of tannins present in the grape seeds, and it has been reported to be the benzoic acid readily visible by chromatographic analysis after ageing of red wines (Waterhouse 2002). Among the hydroxycinnamic acids, the major acids caffeic and coumaric acids were present in our wines as expected, since they and their derived esters are ubiquitous in fruits and all plants tissues

Table 4

Contents ($mg \cdot L^{-1}$) of anthocyanins, non-anthocyanins, amino acids and biogenic amines in the red wines studied

	C	L	P2	P1
Anthocyanins				
Delphinidin-3-glucoside	2.04 a ± 0.11	1.98 a ± 0.08	2.03 a ± 0.08	1.97 a ± 0.07
Cianidin-3-glucoside	2.62 a ± 0.18	2.45 a ± 0.09	2.58 a ± 0.12	2.55 a ± 0.12
Petunidin-3-glucoside	1.68 a ± 0.01	1.66 a ± 0.01	1.68 a ± 0.01	1.69 a ± 0.02
Peonidin-3-glucoside	1.98 a ± 0.26	1.73 a ± 0.02	1.80 a ± 0.02	1.80 a ± 0.07
Malvidin-3-glucoside	13.74 ab ± 1.49	11.90 a ± 0.37	13.49 ab ± 0.67	14.14 b ± 1.39
Peonidin-3-acetylglucoside	1.75 a ± 0.01	1.76 a ± 0.04	1.74 a ± 0.03	1.73 a ± 0.01
Malvidin-3-acetylglucoside	2.18 a ± 0.05	2.22 a ± 0.04	2.15 a ± 0.10	1.88 a ± 0.40
Peonidin-3-coumaroylglucoside	2.01 ab ± 0.01	1.94 a ± 0.04	1.98 ab ± 0.06	2.05 b ± 0.06
Malvidin-3-coumaroylglucoside	2.05 ab ± 0.09	1.92 a ± 0.03	1.99 ab ± 0.02	2.08 b ± 0.11
Non-anthocyanins				
Gallic acid	7.69 a ± 4.07	12.12 a ± 6.04	11.21 a ± 5.40	9.40 a ± 1.59
Cathequine	4.66 a ± 1.41	3.28 a ± 0.88	4.03 a ± 0.37	3.38 a ±0.55
Caffeic acid	5.00 a ± 1.24	5.70 ab ± 0.78	8.85 b ± 2.71	5.73 ab ± 1.42
Coumaric acid	4.67 a ± 0.56	4.22 a ± 0.79	6.38 b ± 0.91	5.52 ab ± 0.20
Quercetin	1.93 a ± 0.03	1.94 a ± 0.01	2.06 a ± 0.16	1.96 a ± 0.01
Amino acids				
Aspartic acid	12.69 a ± 0.50	8.92 a ± 0.98	18.41 b ± 5.59	8.56 a ± 2.13
Glutamic acid	40.13 a ± 0.31	34.05 a ± 0.16	32.14 a ± 10.75	36.48 a ± 1.26
Serine	2.02 ab ± 0.29	ND	2.06 b ± 0.42	1.58 a ± 0.06
Glycine	15.26 ab ± 0.20	13.97 a ± 1.14	18.35 b ± 3.65	14.28 a ± 0.26
Threonine	6.35 a ± 0.14	5.02 a ± 0.67	6.68 a ± 0.54	9.20 a ± 5.91
Proline	316.21 a ± 18.51	276.16 a ± 1.50	494.85 b ± 179.24	265.24 a ± 49.44
Valine	12.10 a ± 1.18	11.60 a ± 0.47	12.84 a ± 1.57	11.13 a ± 1.29
Tryptophan	7.13 ab ± 0.25	6.17 a ± 0.10	8.33 b ± 1.41	7.37 ab ± 1.13
Leucine	3.09 ab ± 0.04	2.46 a ± 0.12	3.70 b ± 0.75	2.53 a ± 0.35
Ornitine	18.34 a ± 1.76	13.72 a ± 4.36	19.66 a ± 0.67	17.27 a ± 0.19
Lyisine	15.41 ab ± 0.27	14.08 a ± 0.20	17.35 b ± 2.36	14.10 a ± 0.61
Biogenic amines				
GABA	33.89 ab ± 1.91	29.01 a ± 1.35	36.80 b ± 4.94	31.81 ab ± 2.39
Putrescine	3.72 a ± 0.66	7.20 a ± 5.99	4.93 a ± 0.09	2.84 a ± 0.25
Spermidine	5.49 a ± 0.37	5.77 a ± 0.68	8.03 a ± 0.46	5.43 a ± 0.26

C: control wines with no enzyme addition; P1, wines with 0.015 $g \cdot L^{-1}$ PC-AS; P2, wines with 0.030 $g \cdot L^{-1}$ PC-AS; L, wines with 0.030 $g \cdot L^{-1}$ Lallzyme c-max. Values are mean values (three vinifications) ± standard deviation. Different letters (a, b) indicate significant differences ($p < 0.05$) for each analysis.

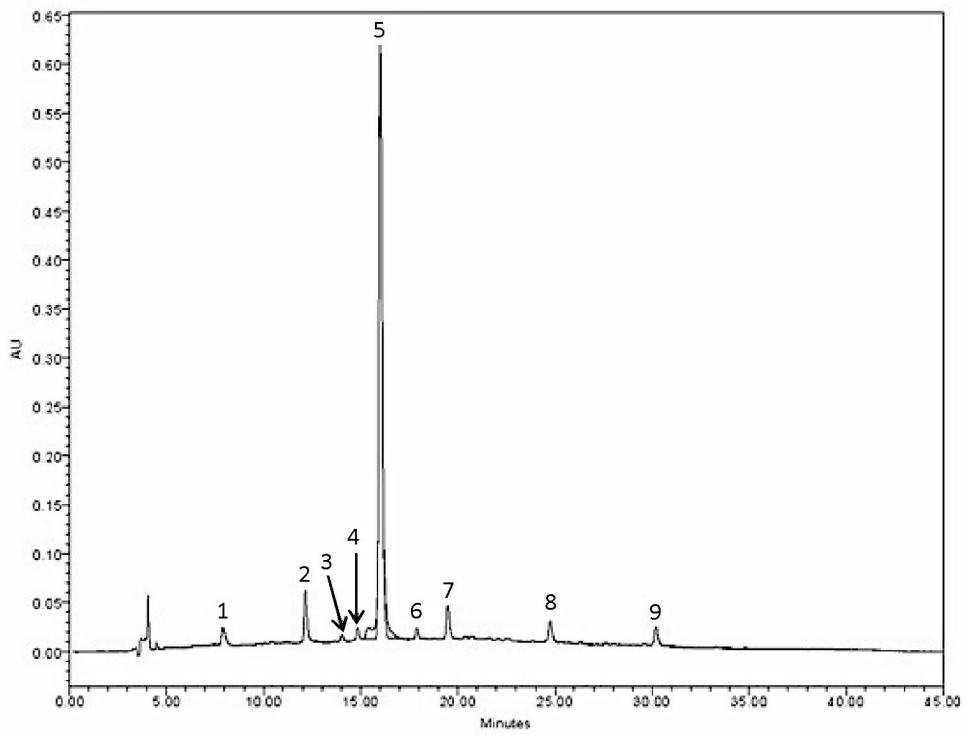

Fig. 1: HPLC chromatogram of anthocyanins in the red wines determined at 520 nm: 1 delphinidin-3-glucoside; 2 cyanidin-3-glucoside; 3 petunidin-3-glucoside; 4 peonidin-3-glucoside; 5 malvidin-3-glucoside; 6 peonidin-3-acetylglucoside; 7 malvidin-3-acetylglucoside; 8 peonidin-3-coumarylglucoside; 9 malvidin-3-coumaroylglucoside.

(Waterhouse 2002). Tab. 4 shows that the concentrations of caffeic and coumaric acids for wines P2 (supplemented with 0.030 g·L^{-1} PC-AS) were significantly higher than those of the control wines without enzyme addition. The obtained values of 8.85 mg·L^{-1} caffeic acid and 6.38 mg·L^{-1} coumaric acid were in the range of concentrations reported for European red wines (0.30-30.8 mg·L^{-1} caffeic acid; 0.30-14.19 mg·L^{-1} coumaric acid) and Brazilian red wines (3.59-10.45 mg·L^{-1} caffeic acid; 0.30-7.83 mg·L^{-1} coumaric acid) (Dias *et al.* 2016) without spoilage off-odours. In the case of our P2 wines, the value of 6.38 mg·L^{-1} coumaric acid, compared to 4.67 mg·L^{-1} of control wines, indicated that the PC-AS slightly facilitated the release of the coumaric acid naturally occurring in grape skins. The other two non-anthocyanin flavonoids quantified in our wines were free quercetin and catechin, whose concentrations were below the levels found for the three former phenolic acids (Tab. 4). Our results are in agreement with previous reports which demonstrated that commercial pectolytic enzyme preparations facilitate the release of phenolic compounds from grape skin cell walls by degrading the pectin fraction of the cell wall (Romero-Cascales *et al.* 2012).

The amino acid content of our elaborated wines was also analyzed to determine the extraction efficiency with respect to grape cell disruption, both skin and pulp cells. Fig. 2 shows the HPLC chromatogram of the identified aminoenone derivatives of amino acids and biogenic amines at 280 nm. The most abundant amino acid was proline, whose

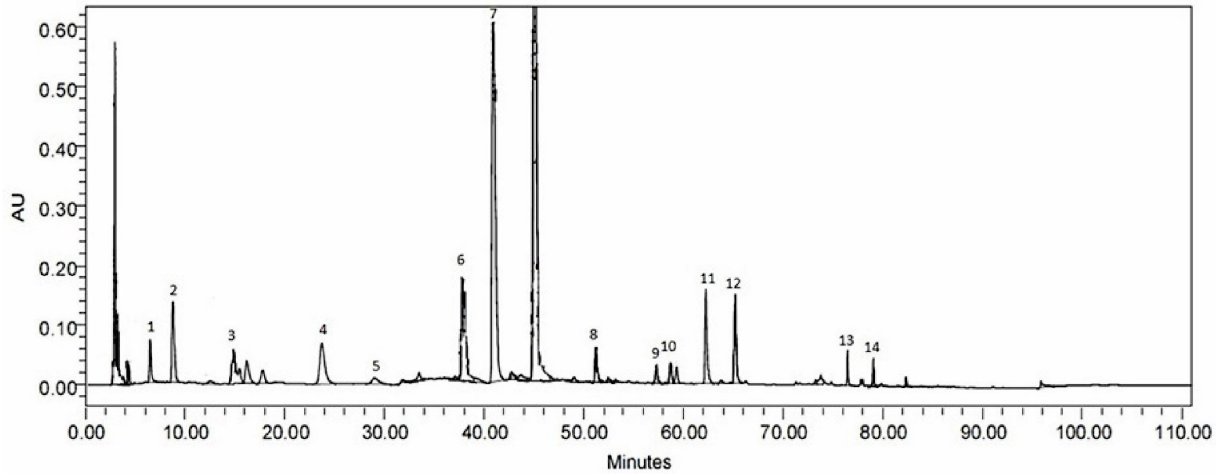

Fig. 2: HPLC chromatogram of amino acids and biogenic amines in the red wines: 1 aspartic acid; 2 glutamic acid; 3 aminoadipic acid; 4 glycine; 5 threonine; 6 GABA; 7 proline; 8 valine; 9 tryptophan; 10 leucine; 11 ornithine; 12 lysine; 13 putrescine and 14 spermidine.

mean concentration ranged from 265 to 495 mg·L^{-1} (Tab. 4), which reflects the fact that the fermenting *Saccharomyces cerevisiae* yeasts are not able to utilise proline (Pinu *et al*. 2014). The next in abundance were glutamic acid (Tab. 4) with mean values reaching up to 40.13 mg·L^{-1}, and GABA with 36.8 mg·L^{-1}. The other amino acids showed mean values under 18.4 mg·L^{-1}, with the exception of ornithine, whose concentrations were in the range 13.72-19.66 mg·L^{-1}, and of wines P2 treated with 0.030 g·L^{-1} of PC-AS, that showed significantly higher values of aspartic acid (18.41 mg·L^{-1}) compared to the other elaborated wines. Similarly, Tab. 4 shows that caffeic and coumaric acid concentrations in wines P2 reached higher values than in control wines without pectinase treatment, and that the coloured anthocyanins malvidin derivatives and peonidin-3 coumaroylglucoside reached also significantly higher concentrations in wines P1 treated with 0.015 g·L^{-1} of PC-AS than in wines L treated with 0.030 g·L^{-1} of the commercial pectinase. These results indicate that pectolytic enzymes help to release not only skin cell associated molecules, but also compounds contained in the inner part of the cells by degrading the pectins of cell walls and middle lamella during maceration of grape berries. In order to highlight these differences among wines, principal component analysis (PCA) was applied to all the data obtained from the HPLC analysis (Fig. 3). Wines P2 were grouped separately and positioned on the right extreme of the X-axis in the PCA, where function 1 accounted for 95.9 % of the total variance; caffeic acid and aspartic acid concentrations characterize the right side of this axis. Thus, PCA showed that wines P2 treated with 0.030 g·L^{-1} of PC-AS were significantly different from the other elaborated wines and showed higher extraction of the grape cell components: caffeic acid and aspartic acid.

Conclusions

The application during red wine making of a β-glucosidase-free, pectinesterase-free pectin-degrading enzyme complex produced by *A. sojae* (PC-AS) promoted a faster colour extraction compared to wines elaborated without enzyme during maceration/fermentation of *V. vinifera* L. 'Tempranillo' red grapes.

This PC-AS was shown to be rich in polygalacturonase activity, stable and active under winemaking conditions, and it is naturally produced by a GRAS fungal strain known for being used for food production. Results obtained with our enzyme complex in terms of colour extraction were similar to those attained with a commercial pectinase complex of oenological use. HPLC analysis of the phenolic and amino acid composition of the elaborated wines revealed a higher caffeic acid and aspartic acid content in wines produced with the higher dose of PC-AS, consistent with an improved extraction of the grape cell components by the catalytic activities present in this enzyme preparation. Thus, this study proves the usefulness of a pectin-degrading enzyme complex that can be easily isolated from the broth of *A. sojae* cultures performed with low cost substrates, and it poses this pectin-degrading PC-AS complex as a potential alternative pectinase source for oenological use.

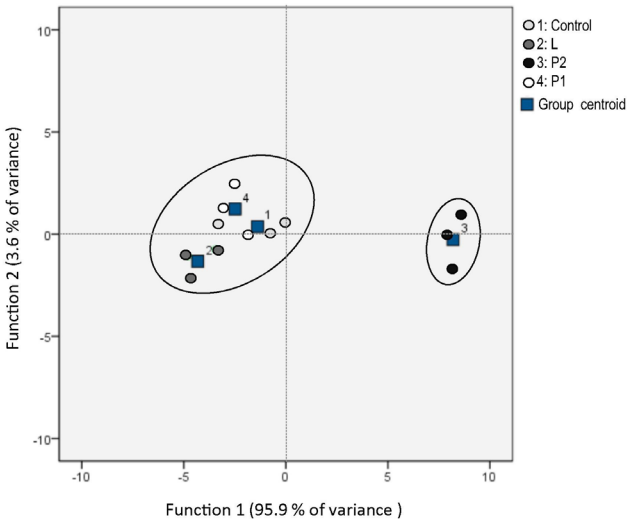

Fig. 3: Principal Component Analysis (PCA) of the analytical composition of the elaborated red wines determined by HPLC.

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