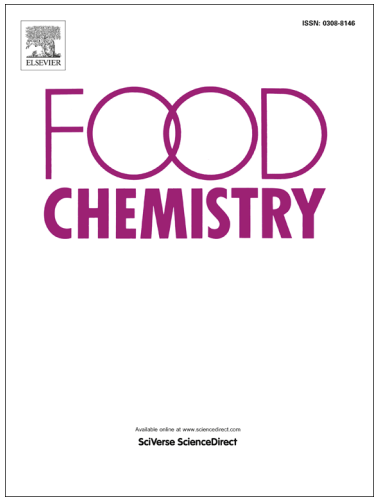

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Recovery of phenolic antioxidants from Syrah grape pomace through the optimization of an enzymatic extraction process

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Abstract

Phenolic compounds are highly valuable products that remain trapped in grape pomace, an abundant winery by-product. Therefore, efficient extraction procedures of these compounds represent a route for grape pomace valorisation. Here we performed a screening of the factors affecting the aqueous enzymatic extraction of phenolic compounds from Syrah grape pomace, including the following independent variables: temperature, pH, pectinase, cellulase and tannase; and a subsequent optimization through response surface methodology. At the optimal region, the enzymatic treatment enhanced the extraction yield of phenolics by up to 66 % and its antioxidant capacity by up to 80 %, reducing the incubation time and enzyme doses in respect to previous studies. We found that tannase raises the antioxidant capacity of the extract by the liberation of gallic acid, while cellulose favours the liberation of p-coumaric acid and malvidin-3-*O*-glucoside. We also tested the procedure in different grape pomace varieties, verifying its wide applicability.

Keywords: Phenolics; Antioxidants; Extraction; Grape pomace; Tannase; Cellulase

Chemical compounds studied in this article: Gallic acid (PubChem CID: 370), (+)-Catechin (PubChem CID: 9064); p-Coumaric acid (PubChem CID: 637542); Syringic acid (PubChem CID: 10742); Malvidin-3-*O*-glucoside (PubChem CID: 443652); Resveratrol (PubChem CID: 445154).

1. Introduction

Winemaking generates millions of tons of grape pomace waste per year worldwide, which represents a substantial waste management problem due to its high pollution load (Devesa-Rey et al., 2011). Phenolic compounds, which are majorly responsible for the organoleptic and healthy properties of wines, still remain in great proportion in the grape pomace (Makris, Boskou, & Andrikopoulos, 2007). The main actual uses of grape pomace include tartaric acid extraction, ethanol production, distillation processes, as fertilizer and as an additive in animal feeding. Nonetheless, the applicability in these two last cases is limited, since high levels of phenolics inhibit germination and polymeric polyphenols reduce digestibility (Fontana, Antoniolli, & Bottini, 2013).

Therefore, extraction of phenolics from grape pomace serves a double purpose: reducing the pollution load of the waste and recovery of highly valuable natural products (Sri Harsha, Gardana, Simonetti, Spigno, & Lavelli, 2013). Grapes and their derived products are rich in phenolic compounds that can be classified in the following three main groups: phenolic acids (benzoic and hydroxycinnamics acids), simple flavonoids (catechins, flavonols, and anthocyanins) and tannins and proanthocyanidins (Fontana et al., 2013). A substantial amount of possible applications of these compounds in the food, cosmetic and pharmaceutical industries can be found in the literature, deriving from their beneficial effects on human health (Lachman et al., 2013; Pandey & Rizvi, 2009; Tournour et al., 2015). Grapes have been associated with a lowered risk of chronic diseases in numerous epidemiological studies, including the prevention of cancer and cardiovascular diseases (Yu & Ahmedna, 2013). *In vitro* and *in vivo* studies have demonstrated that grape phenolics are involved in a broad spectrum of biological activities, such as antioxidant activity, inhibition of cancer cell proliferation, anti-inflammatory, and anti-cholesterol properties, which potentially explains the associated beneficial effects. These beneficial effects are sometimes

controversial and depend also on phenolics bioavailability (J. Yang & Xiao, 2013). In addition, they can be employed as natural colorants, as a preservative in foods, and in the development of functional food (Dos Santos et al., 2017; García-Lomillo & González-SanJosé, 2017).

The traditional extraction method of phenolics from grape pomace is the solid-liquid extraction, which employs an organic solvent. This method presents various drawbacks, including safety hazards, low product quality because of remaining solvent traces and environmental risk (Puri, Sharma, & Barrow, 2012; B. Yang, Jiang, Shi, Chen, & Ashraf, 2011). On the other hand, aqueous extraction assisted by enzymes is an alternative greener strategy (Puri et al., 2012). This approach relies on the enzymes' ability to degrade or disrupt cell wall complex materials, favouring the release of trapped compounds.

The cell walls of grape skins are highly complex and dynamic, being composed of polysaccharides (cellulose, xyloglucan, arabinan, galactan, xylan, etc.), acidic pectin substances, lignin, structural proteins) and are stabilized by ionic and covalent linkages (Ortega-Regules, Romero-Cascales, Ros-García, López-Roca, & Gómez-Plaza, 2006; Pinelo, Arnous, & Meyer, 2006). Phenols in grapes include those bound to cell-wall, which are bound to polysaccharides by hydrophobic interactions and hydrogen bonds, and those confined in the vacuoles of plant cells or associated with the cell nucleus (Pinelo et al., 2006). Therefore, it is necessary to use several enzymes to degrade this complex matrix and release the phenols trapped in it, such as cellulase, pectinase, tannase, etc.

Pectinases constitute a group of enzymes that catalyse the degradation of pectic polymers in plant cell walls (Garg et al., 2016), cellulases act in the depolymerization of cellulose to fermentable sugars (Sharma, Tewari, Rana, Soni, & Soni, 2016), and tannase catalyses the hydrolysis of ester and depside bonds in hydrolysable tannins producing glucose and gallic acid

(Belur & Mugeraya, 2011). Therefore, a selective enrichment on different phenols in the extract is expected according to each enzyme action.

At present, a few studies have demonstrated the feasibility of the application of pectinases, cellulases, and tannase for phenolics extraction from grape pomace in aqueous media (Arnous & Meyer, 2010; Chamorro, Viveros, Alvarez, Vega, & Brenes, 2012; Kammerer, Claus, Schieber, & Carle, 2005). The action of pectinases and cellulases in grape pomace was tested together by Kammerer *et al.* (Kammerer et al., 2005), who found that cellulases action was evident only in conjunction with pectinase. Chamorro *et al.* tested pectinases, cellulases, and tannase individually. Though changes were found in the extract composition, a significant increase in the total phenolics recovery was only found in the case of tannase (Chamorro et al., 2012). Fernández *et al.* found improvements in total phenolics extraction yield from grape seeds and skins with the three enzymatic activities individually, but could not reach a better effect by a sequential treatment with these enzymes (Fernández, Vega, & Aspé, 2015). A more recent work by Martins *et al.* tested the activity of these enzymes but employing a subsequent methanol extraction, which may mask the enzymatic effect on total phenolic recovery (Martins, Roberto, Blumberg, Chen, & Macedo, 2016). They also found changes in the extract composition, but only a significant increase in the total phenolics and antioxidant capacity recovery in the case of tannase.

In the aforementioned reports from literature, pH and temperature recommended as optimal by the manufacturer were employed, i.e, no variation of these parameters were explored to test their influence on the extraction yield. In addition, excessive enzyme doses and/or prolonged incubation times were chosen. Therefore, these previously published studies have not evaluated the effect of the different involved factor on the enzymatic extraction. The aim of the present study was to test the factors significantly affecting the aqueous enzymatic extractions of phenolics and antioxidant capacity and to perform an optimization by response surface

methodology to make this extraction a more realistic method for its industrial application. At the same time, the model describing the response surface methodology allowed us to describe the effect of the factors on the extraction yield. We have also evaluated the effect of the optimized simultaneous treatment and individual enzymes in the extract composition.

Syrah variety was chosen as grape pomace model for the screening and optimization because it combines the following characteristics: 1) it is one of the most grown grape worldwide, distributed in different countries and regions (Kym & Aryal, 2013); 2) it has a great content of tannins (Ky & Teissedre, 2015), and therefore an effect of tannase enzyme is expected, and 3) it is recognized as one of the varieties with the highest polyphenol and antioxidant content (Ky, Lorrain, Kolbas, Crozier, & Teissedre, 2014). In addition, the efficiency of the optimized treatment was also tested in other worldwide relevant red grape pomace varieties, such as Cabernet Sauvignon, Malbec and Pinot Noir, and a variety of regional interest, Marselan.

2. Materials and Methods

2.1. Materials

Grape pomace of red wine grapes (*Vitis vinifera L.)* of Syrah, Cabernet Sauvignon, Malbec Pinot-Noir and Marselan varieties were obtained from Argentinian wineries (Victoria, Entre Ríos Province and Tunuyán, Mendoza Province).

The following enzymes were employed: pectinase (EC 3.2.1.15) and cellulase (EC 3.2.1.4) from *Aspergillus niger* (Sigma-Aldrich, St. Louis, Missouri, USA); and tannase (EC 3.1.1.20) from *A. oryzae* (Kikkoman, Minato-ku, Tokyo, Japan). Folin-Ciocalteu´s phenols reagent, ABTS (2,2′-azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt), and gallic acid, p-coumaric acid, syringic acid, malvidin-3-*O*-glucoside, resveratrol and (+)-catechin standards were from Sigma-Aldrich (St. Louis, Missouri, USA).

2.2. Grape pomace preparation

Grape pomace (GP), consisting in residual grape seeds, pulp, and skins, was collected after winemaking fermentation in wineries, and stored frozen at -20 °C until processing in the laboratory. GP was dried in a drying oven (San-Jor, SL60SDB, San Andrés, Buenos Aires, Argentina) to reach < 6 % humidity and milled in coffee grinders to a particle size of 0.25 – 2.38 mm (sieved by mesh No.8 and No.60) before extraction. When refereeing to "g of GP", we refer to the dried and milled GP.

2.3. Determination of enzymatic activity

The amount of enzyme units per g of commercial power was determined in enzymes solutions according to the reference method employed by each supplier. In the case of tannase, one unit (U) is defined as the amount of enzyme which hydrolyses 1 µmol of the ester bond in tannic acid per min at 30°C and pH 5.50 under the conditions described by Iibuchi *et al.* (Iibuchi, Minoda, & Yamada, 1967). In the case of cellulase, one U is defined as the amount of enzyme which liberates 1 µmole of glucose from cellulose in 1 h at pH 5.00 at 37 °C. In the case of pectinase, 1 U corresponds to the amount of enzyme which liberates 1 µmol galacturonic acid from polygalacturonic acid per min at pH 4.00 and 50 °C. The amount of glucose and galacturonic acid liberated in each reaction was determined by the dinitrosalicylic acid (DNS) method (Miller, 1959). Afterwards, the desired enzyme units in each experiment were added by weight.

2.4. Extraction of phenolics from grape pomace

Extraction of phenolics from grape pomace was performed in 50 mM acetate buffer at a solid: solvent ratio of 1:10 w/v, pH was that indicated by the experimental design in the screening procedure (Supplementary Table 1) and 5.00 in the case of the Box–Behnken design. The enzymes were added after being manually weighted. Samples were incubated in continuous agitation (125 rpm) in an orbital shaker at a controlled temperature (Infors HT incubation shaker, Ecotron model,

Bottmingen, Switzerland). At the chosen time, samples were ice-cooled to stop the reaction and centrifuged at 10,397 x *g* for 15 min (Sigma 3-18 KH Laboratory centrifuge, Rotor N° 19776–H, St. Louis, Missouri, USA). The supernatant was then filtered through a 0.2 µm syringe filter (Sartorius, Minisart RC 4, Goettingen, Germany) and analysed immediately after filtration.

2.5. Factors screening for the extraction of grape pomace

A fractional factorial design was performed in order to assess the factors involved in the aqueous enzymatic extraction of phenolics (William G. Cochran & Gertrude M. Cox, 1992). The following six factors were included: pectinase, tannase, cellulase, pH, temperature, and incubation time, resulting in 16 runs.

Upper and lower limits for pH and temperature were delimited in accordance to manufacturer recommendations (Table 1). Upper and lower limits for incubation time were delimited by a preliminary test, in which extraction yields were tested at 6 and 24 h. Since no differences were observed between 6 and 24 h incubation, we reduced the range to 2 and 6 h (Table 1).

One of our objectives was to minimize enzyme doses; therefore a low amount of enzyme was employed as the upper limit in comparison to previous similar studies (Table 1).

Experiments for this screening procedure and subsequent optimization were performed with GP of Syrah variety. Design matrix and experimental results for Total phenolics (TP) and Trolox equivalent antioxidant capacity (TEAC) as responses are shown in Supplementary Table 1. Factorial regression was performed with TP and TEAC as the response variables.

2.6. Response surface design and optimization of the responses

Response surface methodology (RSM) was employed to optimize the enzymatic extraction (Myers, Montgomery, & Anderson-Cook, 2016). A Box–Behnken design was performed with the

following three factors selected as significant according to the screening data analysis: temperature, tannase, and cellulase. The design consisted of 15 runs, including three central points. The upper limit of enzyme units was increased to 200 U / g of GP; the temperature interval was 25-45 °C. The incubation time was fixed at 2 h and pH at 5.00. Design matrix and experimental results for TP and TEAC as the response variables are shown in Supplementary Table 2. The experimental data were subjected to a full quadratic regression fitting, including a stepwise method which removes the least significant term for each step.

Optimization of the treatment was performed in order to maximize TP and TEAC simultaneously. The exploration of the optimal region was done by applying the desirability function method. The predicted results for TP and TEAC at the optimized factor levels were contrasted with experimental data by performing three replicates of the extraction at these conditions (188 U of cellulase / g of GP, 198 U of tannase / g of GP, at a temperature of 45 °C, pH 5.00, 2 h incubation, and all the other conditions as described in section 2.3). A control experiment was performed under the same conditions but avoiding enzymes addition.

2.7. Determination of total phenolics as gallic acid equivalents

We employed the Folin-Ciocalteu method for total phenolics estimation (Singleton & Rossi, 1965). In general, a dilution 1/5 of the extracts was performed for the assay in order to fall in the linear range of the response. Determinations were made in triplicates and results were expressed in g of gallic acid equivalents (GAE) / 100 g of GP.

2.8. Determination of antioxidant activity

The improved Trolox Equivalent Antioxidant Capacity (TEAC) assay was used (Re et al., 1999). In general, a dilution 1/5 of the extracts was performed for the assay in order to fall in the linear range of the response. Determinations were made in triplicates and results were expressed as mmol Trolox equivalents (TE) / 100 g of GP.

2.9. Phenolic content analysis by HPLC-DAD

The analysis was performed employing a Dionex Ultimate 3000 SD HPLC system with a Diode Array Detector (Thermo Fisher Scientific, Waltham, MA, USA). Typically 20 µl of a filtered sample or a convenient dilution was injected on a Hypersil Gold C18 3 µm; 2.1 mm x 100 mm column (Thermo Fisher Scientific, Waltham, MA, USA). Mobile Phase consisted of a mixture of deionized water (solvent A) and acetonitrile (solvent B), both acidified by acetic acid 0.5 %; at a flow rate of 0.25 ml/min. The separation method was as follow: 2.5 min at 10 % B, gradient from 10 % B to 50 % B in 6.5 min; gradient to 80 % in 2 min; 4 min at 80 % B; and then back to 10 % B. Chromatograms were recorded at 260, 280, 320 and 520 nm. Quantification was performed by comparison with a calibration curve performed with each standard for the following compounds: gallic acid, p-coumaric acid, syringic acid, malvidin-3-*O*-glucoside, resveratrol and (+)-catechin; at the most representative wavelength in each case. Results were expressed as g / 100 g GP.

2.10. Phenolics extraction at the optimal region from different red grape pomace varieties

Grape pomaces of the following varieties were employed: Marselan, Cabernet Sauvignon, Malbec, and Pinot Noir. Extractions of phenolics from grape pomace were performed as described in Section 2.4, under the optimized conditions for the Syrah variety: pH 5.00, 2 h incubation, 188 U of cellulase / g of GP, 198 U of tannase / g of GP, at a temperature of 45 °C.

A control experiment was performed under the same conditions but avoiding enzymes addition. Relative increments on TP or TEAC extraction yields were calculated as follow:

$$\frac{Treatment\ extraction\ yield}{Control\ extraction\ yield} \times 100 - 100$$

2.11. Statistical data analysis

Experimental design, data analysis, and optimization were performed with the Minitab 17.1.0 software. The statistical significance of regression models was evaluated by the Fisher´s test

(F-test) analysis of variance (ANOVA), considering a p-value < 0.05. Factors with a p-value < 0.05 were considered to have significant effects on the response value.

For means comparisons, the experiments were performed in triplicates, and the mean values were analysed statistically by ANOVA followed by the Tukey's post-hoc test. The significance of the results was established at p-value < 0.05.

3. Results and Discussion

3.1. Screening of the factors affecting the enzymatic extraction of phenolics by a fractional factorial design

The intervals tested for the factors pH and temperature and enzymes units for pectinase, tannase, and cellulase are shown in Table 1. A fractional factorial design was performed including the six mentioned factors, for which 16 runs were performed. For each run, TP and TEAC were measured (Supplementary Table 1).

Factorial regression was performed with TP as the response variable (Supplementary Table 3). The linear component of the model was found to be significant (p-value = 0.016). Two-way interactions instead were globally not significant, (p-value=0.173). Significant effects (p-value < 0.05) were found only in the cases of temperature, tannase, and cellulase factors. Similar results were obtained with TEAC as the response variable (Supplementary Table 4).

Pectinase did not have a significant effect on TP or TEAC content of the extracts (p-value > 0.05). It is worth mentioning that some studies have reported evidence of pectinase action in aqueous extraction, but with no increase in total phenolics extraction yield (Chamorro et al., 2012; Landbo & Meyer, 2001). In contrast, other studies have reported increments in the extraction yields of the individual (Kammerer et al., 2005) and total phenolics (Fernández et al., 2015). These discrepant results may be related to the pectinase preparation used, and also the grape variety

selected. On the other hand, we found a significant effect of cellulase on TP extraction yield, which was not evident in other studies (Chamorro et al., 2012; Martins et al., 2016), probably because we have extended the temperature range for its effect assessment (Table 2).

In the case of the factors pH and incubation time, we found no significant effect in the tested intervals (p-value > 0.05). For this reason, the screening analysis allowed us to minimize the incubation time to 2 h, and to employ an intermediate pH (5.00) for cellulase and tannase treatment. This proposed simultaneous treatment differs from the sequential treatment reported by Fernández *et al.* (Fernández et al., 2015), in which changes in pH were performed to work at optimal pH for each enzyme. Nonetheless, that procedure did not succeed in adding a positive effect by the second enzymatic treatment, probably because synergistic effects were avoided by doing a sequential instead of simultaneous treatment. For this reason, we propose here to perform the treatment simultaneously with cellulase and tannase at an adequate pH.

3.2. Response surface design and optimization for simultaneous treatment of grape pomace with cellulase and tannase

A Box-Behnken design (BBD) was performed with the three selected factors — temperature, tannase and cellulase—, according to the intervals shown in Table 1. For each run, TP and TEAC were measured (Supplementary Table 2).

The results of the regression and ANOVA on the model of the BBD for TP as the response variable are shown in Supplementary Table 5. This regression model was statistically significant (p-value < 0.0001). The goodness of the fit was corroborated by the lack-of-fit test and the adjusted determination coefficient (Supplementary Table 5). The regression equation was as follow:

$$TP = 0.706 + 0.001018\ cellulase + 0.000847\ tannase - 0.0240\ T + 0.000468\ T*T - 0.000004\ cellulase*tannase$$

The model includes linear terms for the three factors, a quadratic positive term for temperature and a small negative two-way interaction term for cellulase and tannase. Therefore, it can be interpreted that the employment of simultaneous high doses of cellulase and tannase give rise to saturation on TP extraction yield.

The results of the regression and ANOVA on the model of the BBD for TEAC as the response variable are shown in Supplementary Table 6. This regression model was statistically significant (p-value < 0.0001). The goodness of the fit was corroborated by the lack-of-fit test and the adjusted determination coefficient (Supplementary Table 5). The regression equation was as follow:

TEAC = 14.85 + 0.01774 cellulase + 0.01360 tannase - 0.822 T

- 0.000070 cellulase*cellulase - 0.000044 tannase*tannase

+ 0.01274 T*T

The model includes linear and quadratic terms for the three factors.

The graphical representation of the models and desirability function are shown in Figure 1. It can be concluded that while TP increases mostly linearly proportional with each enzyme dose, TEAC reaches saturation with high enzyme doses. TEAC does not necessarily have to be directly proportional to TP; instead, it would depend on the antioxidant activity of each phenolic on the extract and their relative abundance. Therefore, the behaviour found could be related to the relative abundance of each phenolic compound in the extract at each enzyme concentration. In addition, other phenomena cannot be discarded, such as instability of the antioxidants at high enzymes concentrations. This is a relevant fact since most of the studies performed at present have employed an excessive amount of enzymes (Table 1) and these results indicate that excessive enzymes doses could have additional drawbacks.

In the case of temperature factor, TP shows a quadratic growth, while TEAC shows a more complex behaviour. It is known that the antioxidant properties of phenolics are sensitive to temperature (Larrauri, Sánchez-Moreno, & Saura-Calixto, 1998). Therefore TEAC behaviour can be interpreted as a result of a compromise between an increment in the extraction of TP with temperature and the loss of antioxidant capacity.

Employing the final response surface models, the factors values for maximization of TP and TEAC simultaneously were predicted to be as follow: 188 U of cellulase / g of GP and 198 U of tannase / g of GP and at 45 °C. The extraction performed at these conditions retrieved experimental results which were in good agreement with those predicted by the model (Table 2).

This treatment significantly increases TP ($p < 0.001$) and TEAC ($p < 0.0001$) in respect to a control extraction performed at identical conditions, but without enzymes addition (Table 3). Moreover, the relative increases of TP and TEAC in respect to the control extraction are greater than the ones obtained in a comparable previous study (Chamorro et al., 2012) (Table 3). Enzymes amounts employed by Chamorro *et al.* were in general excessive, and they employed also an excessive incubation time. In conclusion, better relative increments of extraction yields were obtained by our simultaneous treatment by exploring the optimal values for the involved variables.

We have also compared the obtained extraction yield with a typical hydroalcoholic extraction (Ethanol/ water 1:1; T: 50°C; incubation time: 6 h) performed with the same GP and solid/solvent ratio as the aqueous extraction. This hydroalcoholic extraction gave rise to 2.5 GAE/ 100 g of GP and 19.6 mmol TE / 100 g of GP. Both parameters are approximately three times greater than those obtained with the aqueous extraction assisted by the simultaneous enzymatic treatment, probably due to the greater solubility of some polyphenols in organic solvent. A combination of both methodologies, as has been proposed by Martins *et al.* and Binaschi *et al*.

(Binaschi, Duserm Garrido, Cirelli, & Spigno, 2018; Martins et al., 2016) could result in better extraction yields and minimization of the employed organic solvent.

3.3. Analysis of the cellulase and tannase individual effects on TP and TEAC recovery and on the phenolics composition of the extracts.

In order to explore the contribution of each enzyme on TP and TEAC recovery and on the phenolics composition of the extracts, we also performed a treatment with cellulase or tannase (all the other conditions being preserved in respect to the simultaneous treatment). The phenolics gallic acid, p-coumaric acid and (+)-catechin were quantified by HPLC-DAD in these extracts, and in those corresponding to the control and the optimized combined treatment (Table 4).

We found that each enzyme has a significant effect on TP and TEAC recovery individually in respect to the control. Though increments in TP and TEAC recovery are observed in the simultaneous treatment in respect to the individual treatments, their effect is not additive. These experimental results are in agreement with the saturation effects at high enzymes doses described by the regression models of TP and TEAC responses.

We found a significant effect of tannase on the release of gallic acid ($p < 0.001$), which can be correlated to its effect on TEAC. These results provide evidence of the tannase effect, which reduces the complexity of tannins and liberates the small and potent antioxidant gallic acid (Shahrzad, Aoyagi, Winter, Koyama, & Bitsch, 2001).There is evidence that polymeric and complex phenols are poorly absorbed; while monomeric phenols are absorbed in the small intestine (De Pascual-Teresa, Moreno, & García-Viguera, 2010).Therefore, the action of tannase may also increase bioavailability of the liberated phenols.

On the other hand, the effect of cellulase on the extraction of gallic acid is not significant (Table 4), while we found a significant effect of cellulase on the liberation of p-coumaric acid ($p < 0.001$), which could in part account for its effect on TEAC. The p-coumaric acid content in plants

can be divided into soluble and insoluble fractions. The insoluble fraction is attached by ester bonds to lignocellulose. The soluble fraction can be found in its free form or bound to small molecules through ester linkages and is stored in vacuoles (Mattila & Kumpulainen, 2002; Robbins, 2003). The degradation of cell wall components by cellulase is probably favouring the release of this soluble fraction of p-coumaric acid.

We also found that tannase significantly increases the release of syringic acid, while cellulase has no effect when employed alone. In combination with tannase, an additive effect is observed, that increases syringic acid extraction in respect to the tannase treatment alone.

In the simultaneous enzymatic treatment, a concomitant enrichment of the extracts in the phenolics acids gallic acid, p-coumaric acid and syringic acid is achieved.

In respect to catechin, though we would expect it to increase in the case of tannase treatment due to its liberation from gallocatechin, we found no significant changes in any of the treatments. This would imply that the liberation of gallic acid arises from a wider group of hydrolysable and condensed tannins.

The anthocyanin malvidin-3-*O*-glucoside was detected in all the extracts. A significant increase was observed only in the case of cellulase treatment but not in the simultaneous treatment. Kammerer *et al.* have reported previously a negative effect of some enzymes preparation in anthocyanins recovery, due to side enzyme activities that produce glycoside hydrolysis or de-esterification of acylated anthocyanins (Kammerer et al., 2005). This fact should be taken into consideration when enrichment in anthocyanins in a requirement for the aqueous extract.

3.4. Testing the optimized conditions in diverse red grape pomace varieties

We applied the extraction conditions obtained with Syrah variety for simultaneous optimization of TP and TEAC, to varieties of Cabernet Sauvignon, Malbec, Pinot-Noir and Marselan

GP, and measured TP and TEAC (Supplementary Table 7). We found significant increments in the extraction yield, between 20 and 40 % for TP and 9 and 70 % for antioxidant capacity by the enzymatic treatment (Figure 2). Though they are lower than the increment found in the case of Syrah GP (66 % for TP and 80 % for TEAC), they still represent substantial increments in comparison to previously reported enzymatic treatment, which were in general between 10 and 30 % for TP and 4-30 % for antioxidant capacity (Table 3) (Chamorro et al., 2012; Fernández et al., 2015; Martins et al., 2016).

In the case of Pinot-Noir, which has the lower relative increment in the extraction for TP and TEAC (Figure 2), the aqueous extraction control gave the larger absolutes values for these parameters (Supplementary Table 7). It is probable that phenolic compounds are less tightly trapped in this GP variety, giving rise to a greater extraction yield and a less evident enzymes action. Previous studies have only employed one or two varieties to test the enzymatic treatments. Binaschi *et al.* employed two grape pomace varieties with different particle size, and found differences in the enzymatic effect on the phenolics extraction, suggesting an influence of these two factors (Binaschi et al., 2018). Here we provide evidence of the relevance of taking into consideration GP variety when testing extraction procedures and their application.

4. Conclusions

In the present study, the phenolics extraction yield from red grape pomace assisted by enzymes was optimized by response surface methodology with the Syrah variety. The model obtained allowed us to describe for the first time the behavior of total phenolics and antioxidant capacity responses in respect to tannase and cellulase enzymes doses and temperature. Extraction was improved by up to 66 % and the antioxidant capacity recovery by up to 80 % by optimizing the simultaneous enzymatic treatment.

We also showed the individual effect of each enzyme in the extract composition: while tannase enriches the phenolic extract in gallic and syringic acids; cellulase enriches it in p-coumaric acid and malvidin-3-O-glucoside. We can relate these results to enzymes actions: tannase degrades hydrolysable tannin, liberating mainly gallic acid, while cellulase hydrolyses cellulosic fibres where phenolics can remain trapped, liberating other compounds. The enzymes combination gives rise to additive or negative effects depending on the phenolic compound. This fact should be taken into consideration when selective enrichment of particular compounds is required.

The practical goals of our approach include adequate conditions for a productive simultaneous treatment with cellulase and tannase to extract phenolic compounds with antioxidant activity, minimization of enzyme doses and incubation time in respect to previous reported treatments, and demonstrated applicability for different grape pomace varieties of worldwide and regional relevance.

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The authors declare no conflict of interest associated with this manuscript.

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Tables

Table 1. Independent variables values employed in previous studies and recommended by the enzymes manufacturers; and delimitation of the ranges for screening and optimization designs.

	Enzyme Units (U) / g of GP					
	Pectinase (P)	Cellulase (C)	Tannase (T)	Incubation time (h)	Temperature (°C)	pH
(Fernández *et al.*, 2015)[a]	190	1500	750	3	25/37/30 (P/C/T)	4.00/5.00/5.70 (P/C/T)
(Chamorro *et al.*, 2012)[b]	6.75-13.5	158-315	2000-4000	24	35	5.5
Manufacturer specifications[c]					50/37/40 (P/C/T)	4.00/5.00/5.50 (P/C/T)
Ranges for screening[d]	0-100	0-100	0-100	2-6	25-45	4.00-5.50
Ranges for optimization[d]	-	0-200	0-200	2	25-45	5.50

[a] In this study enzymatic treatment was performed sequentially, under the conditions indicated for each enzyme.

[b] In this study enzymatic treatment was performed simultaneously under the indicated conditions.

[c] This are the optimal Temperature and pH as indicated by the enzyme manufacturers.

[d]. These are the ranges employed in the present study for each experimental design.

Table 2. Validation of the method at the optimal region by comparison of experimental data with the model predicted values for TP and TEAC.

Maximized response	Factors			TP (g GAE / 100 g of GP)		TEAC (TE mmol / 100 g of GP)	
	Cellulase (U / g of GP)	Tannase (U / g of GP)	T (°C)	Predicted	Experimental	Predicted	Experimental
TP and TEAC	188	198	45	0.78	0.81 ± 0.04	5.52	5.58 ± 0.05

Results are shown as the mean ± standard error; number of replicates = 3.

Table 3. Comparison of the enzymatic treatment in the optimal region as obtained in this study and other published study.

	Enzyme Units/ g of GP [a]						Total polyphenols (g of GAE/ 100 g GP)						
	Pectinase	Cellulase	Tannase	t (h)	T (°C)	pH	Control	Pectinase	Cellulase	Tannase	Simultaneous treatment	Pooled SEM	Relative increment [b] (%)
(Chamorro *et al.*, 2012)	6.75-13.5	158-315	2000-4000	24	35	5.50	0.11	0.11-0.12	0.10-0.11	0.11-0.14 **	0.11-0.14 ** (P + C + T)	0.01	0-30
Optimal region (This study)	-	188	198	2	45	5.00	0.49	-	0.76 **	0.74 **	0.81 *** (C + T)	0.02	66

	TEAC (TE mmol / 100 g of GP)						
	Control	Pectinase	Cellulase	Tannase	Simultaneous treatment	Pooled SEM	Relative increment (%)
(Chamorro *et al.*, 2012)	1.65	1.78-1.85 ***	1.71-1.74	1.82-1.98 ***	2.09-2.17 *** (P + C + T)	0.4	30
Optimal region (This study)	3.1	-	4.5 ***	4.6 ****	5.6 **** (C + T)	0.1	80

[a] Ranges of enzymes doses are shown, along with their corresponding results.
[b] Relative increments correspond to simultaneous enzymatic treatment in respect to the control experiment of each study, which consist in the extraction at identical conditions but avoiding enzymes addition.
[d] Data was extracted from a bar graph.
Significant differences are indicated in respect to the control experiments of each study:
*p < 0.05
** p < 0.01
*** p < 0.001
**** p < 0.0001

Table 4. Cellulase and tannase effects on TP, TEAC and phenolics composition of the extracts.

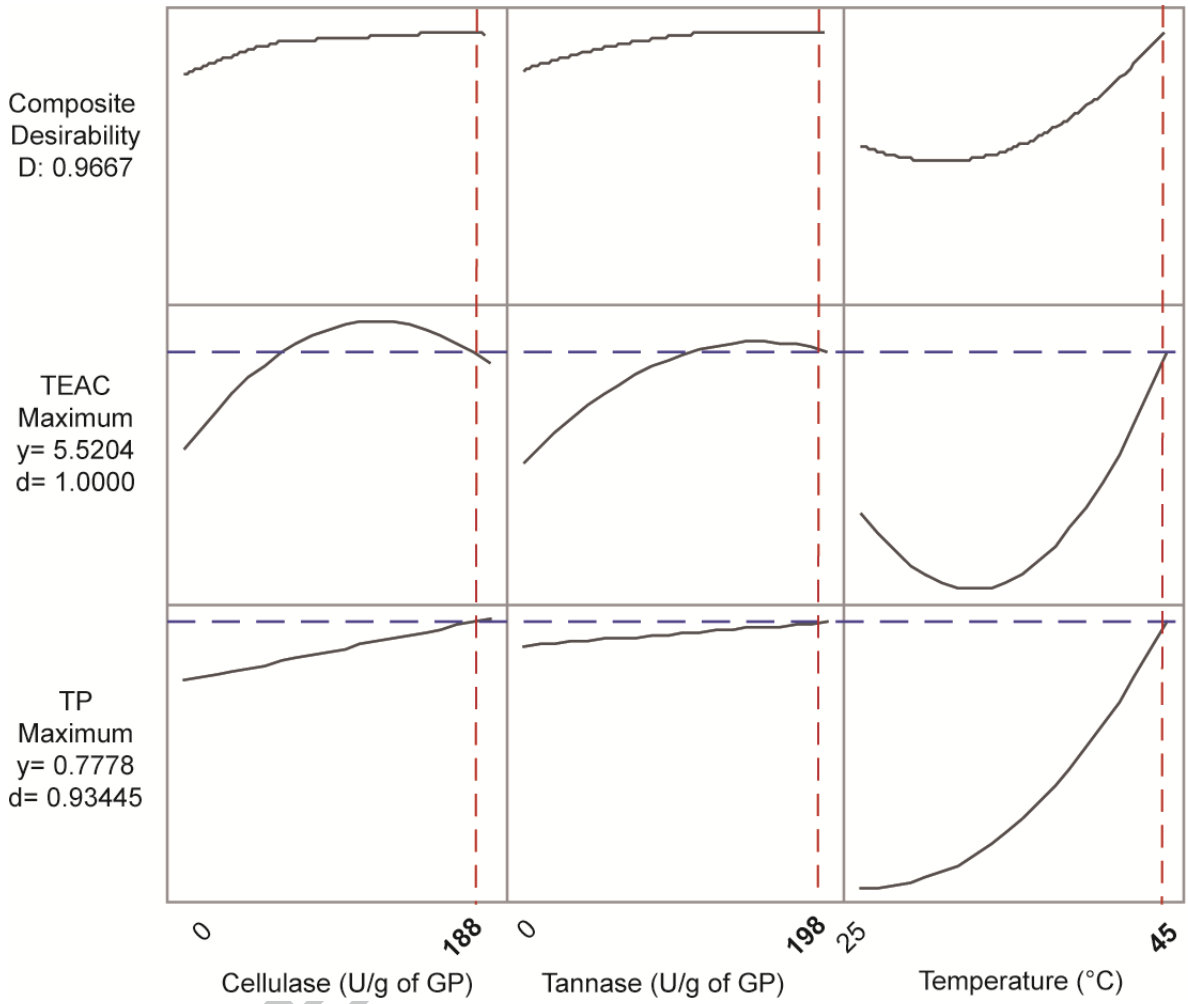
	Treatment				
	Control	C 188 U	T 198 U	C 188 U + T 198 U	Pooled SEM
TP (g GAE / 100 g GP)	0.49	0.76 ***	0.74 ***	0.81 ***	0.02
TEAC (mmol TE / 100 g GP)	3.1	4.5 ***	4.6 ****	5.6 ****	0.1
Individual Phenolics (g / 100 g GP)					
gallic acid	0.03	0.08 ns	0.16 **	0.16 **	0.02
p-coumaric acid	0.002	0.014 **	0.009 *	0.016 ***	0.002
Syringic acid	0.13	0.15 *	0.13 ns	0.17 ***	0.01
(+)-catechin	0.018	0.022 ns	0.028 ns	0.026 ns	0.004
Resveratrol	nd	nd	nd	nd	
Malvidin-3-*O*-glucoside	0.005	0.011***	0.007 ns	0.004 ns	0.001

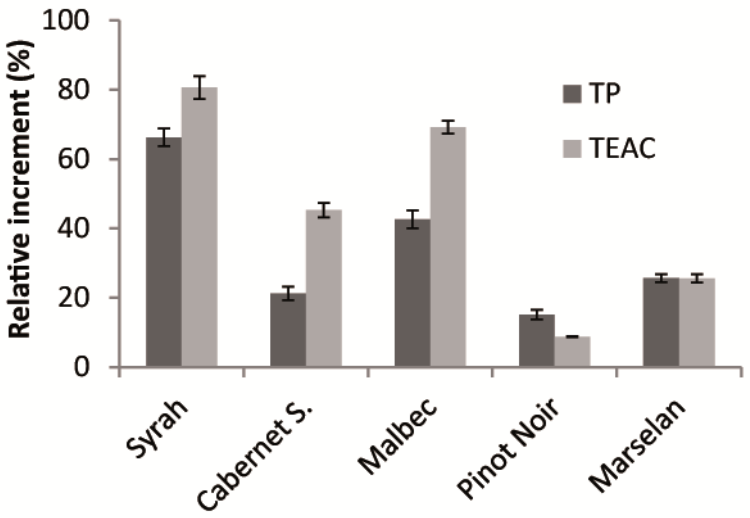
SEM, standard error of the means; number of replicates = 3.
Significant differences are indicated in respect to the control experiment:
ns, not significant
* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$
**** $p < 0.0001$
nd, not detected (< 100 µg / 100 g of GP)

7. Figures Captions

Figure 1: Response surface model for total phenolics (TP) and antioxidant capacity (TEAC) extraction from grape pomace (GP) vs tannase, cellulase, and temperature. TP (g GAE / 100 g of GP) and TEAC (TE mmol / 100 g of GP) responses were modelled against tannase, cellulase, and temperature factors in order to estimate the values of these variables for optimization of the simultaneous enzymatic treatment.

Figure 2: Increments in the extraction yield of total phenolics (TP) and antioxidant capacity (TEAC) from grape pomace (GP) of five different Argentinian varieties by the optimized enzymatic treatment. Relative increments in TP and TEAC were calculated in respect to a control performed at identical conditions avoiding enzymes addition. The conditions employed correspond to those found for concomitant maximization of TP and TEAC response with the Syrah variety.





Highlights

Tannase and cellulase in conjunction enhance grape pomace extraction of phenolics.

Optimization provides practical enzyme doses, time and temperature for the extraction.

Gallic acid liberation by tannase raises grape pomace extracts antioxidant capacity.

Cellulase favours the liberation of p-coumaric acid and malvidin-3-*O*-glucoside.

Cellulase and tannase simultaneously employed favour the liberation of phenolic acids.