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Thermal and High-Pressure Inactivation Kinetics of Polyphenol Oxidase in Victoria Grape Must

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The inactivation kinetics of polyphenol oxidase (PPO) in freshly prepared grape must under high hydrostatic pressure (100−800 MPa) combined with moderate temperature (20−70 °C) was investigated. Atmospheric pressure conditions in a temperature range of 55−70 °C were also tested. Isothermal inactivation of PPO in grape must could be described by a biphasic model. The values of activation energy and activation volume of stable fraction were estimated as 53.34 kJ mol^{-1} and −18.15 cm^3 mol^{-1} at a reference pressure of 600 MPa and reference temperature of 50 °C, respectively. Pressure and temperature were found to act synergistically, except in the high-temperature−low-pressure region where an antagonistic effect was found. A third-degree polynomial model was successfully applied to describe the temperature/pressure dependence of the inactivation rate constants of the stable PPO fraction in grape must.

KEYWORDS: Grape (*Vitis vinifera ssp. sativa*); polyphenoloxidase; thermal and high-pressure inactivation; thermodynamic model

INTRODUCTION

Biochemical and microbiological preservation of grape musts and wines is a very important aspect in the production process of wine. Polyphenoloxidase (PPO) activity causes undesirable color and turbidity modifications, which can change the stability and organoleptic characteristics of grape juice and musts (*1*). PPO is a copper-containing oxidoreductase that catalyzes two distinct reactions involving phenolic compounds and molecular oxygen, namely, (a) the ortho hydroxylation of monophenols to ortho diphenols or cresolase activity (monophenol monooxigenase, EC 1.14.18.1) and (b) the subsequent oxidation of ortho diphenols to ortho quinones or catecholase activity (diphenol oxygen oxidoreductase, EC 1.10.3.1). The polyphenol oxidation reaction in must is generally controlled using chemical additives such as SO_2. Because sulfites could be detrimental to human health, today many studies are carried out to reduce SO_2 uses in food and beverages (*2*). The strong reduction or the elimination of this additive or of processes (i.e., thermal processing) that can affect the natural composition of must lies within a strategy aimed at an overall improvement of the quality of the final product.

For the last 30 years, many organizations, such as the International Office of Vine and Wine (OIV), have been involved in endeavoring to reduce the use of SO_2 in wines and in other foodstuffs, such as cider, beer, fruit and horticultural products, fruit juices, and syrups. Therefore it is not surprising that the World Health Organization (WHO) has recommended limiting, as much as possible, the use of SO_2 in the treatment of foodstuffs, even to the point of contemplating the possibility of its complete suppression.

On the basis of numerous research studies on this argument, wine figures at the front line (*3*). It is known that the European Community (EC), in its efforts to harmonize the legislation of the member countries, set about reducing, starting in 1987, the maximum content of total SO_2 from 175 to 160 mg/L for red wines and from 225 to 210 mg/L for white and rosé wines. For a sugar content equal to or higher than 5 mg/L, the tolerated maximum dose passes to 210 and 260 mg/L, respectively. According to the actual indications of WHO, the acceptable daily intake (ADI) of SO_2 in human foodstuffs has been cautiously established as 0.7 mg/kg of body weight, but even much lower doses can cause disturbances in subjects lacking the specific enzyme alcohol dehydrogenase (*4*). Therefore it is evident as to how systems or techniques alternative to the addition of SO_2 that are able to inhibit polyphenoloxidase activity are a sought-after goal.

An alternative method for the preservation of musts can be high-pressure treatments. The high-pressure treatment is known to keep quality carriers, such as taste, flavor, and vitamins, intact (*5*). PPO is however very pressure-resistant. In some cases, an

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increase of enzyme activity after pressure treatment has been reported for pear (*6*), onion (*7*), and mushroom (*8*) PPO. Both pressure resistance and thermal resistance of enzymes are seriously dependent on environmental conditions such as pH (*9*) and the presence of sugar, salts, or additives (*10, 11*). The aim of this study was to characterize the pressure and thermal stabilities of PPO in grape must. Hereto, kinetic studies were performed to quantitatively describe the thermal and pressure/heat inactivation of the enzyme. At the same time, the second objective was to develop a mathematical model to describe combined pressure−temperature inactivation of PPO in grape must.

MATERIALS AND METHODS

Materials. White grapes (*Vitis vinifera ssp sativa*, var. victoria, South Africa) were purchased at commercial maturity from a local store. Catechol was obtained from Sigma Aldrich (St. Louis, MO). All others chemicals were of analytical grade.

Methods. Grapes (1 kg) were crushed and filtered through four layers of cheesecloth. The filtrate must was clarified through sedimentation at 4 °C for 24 h and then kept in tubes at −80 °C and used as the PPO enzyme source in the subsequent experiments. The white must had 13.34 °Brix and pH 4.03.

PPO Activity Assay. PPO activity was assayed by a spectrophotometric procedure. The increase in absorbance at 400 nm and 25 °C was recorded automatically for 10 min (Ultrospec 2100 pro, UV−visible spectrophotometer). The sample cuvette contained 1.0 mL of substrate solution (catechol 0.1 M in McIlvaine buffer, pH = 5) and 100 μL of undiluted must. The blank sample contained only 1.0 mL of substrate solution. Enzyme activity was calculated from the linear portion of the curve, which was OD_{400} nm/min. All activity analyses were carried out in duplicate and the relative standard deviations were less than ±1%.

Temperature Inactivation of PPO in Grape Must. Kinetic parameter values (k, E_a) for thermal inactivation of PPO in grape must were determined on the basis of isothermal inactivation experiments. To facilitate the analysis of kinetic results, the applied thermal treatments were designed to be isothermal. Thermal stability of PPO in grape must was investigated at various constant temperatures from 25 to 90 °C using an incubation time of 10 min, whereas detailed thermal inactivation kinetics of PPO in grape must was determined in a temperature range from 55 to 70 °C. The samples were filled in capillaries of 200 μL (Blaubrand, Germany), thermally treated, and cooled in ice water, and the residual activity was measured within 60 min of storage at 0 °C. No reactivation occurred during this storage. The blank (A_0) was defined as the activity of a non-heat-treated enzyme sample.

High-Pressure Inactivation of PPO in Grape Must. The kinetic parameters values for the pressure−temperature inactivation of PPO in grape must were determined on the basis of isobaric/isothermal inactivation experiments and determination of the residual enzyme activity. All pressure experiments were conducted in multivessel high-pressure equipment (HPIU-10.000 serial no. 95/1994, Resato, Roden, The Netherlands) with eight thermostated pressure vessels. The pressure medium is a glycol−oil mixture (TR-15, Greenpoint Oil, Resato, The Netherlands). To enclose the enzyme solution, flexible microtubes of 0.3 mL were used (Elkay, Leuven, Belgium). The microtubes were placed in the pressure vessels, already equilibrated at the inactivation temperature. Pressure was built up slowly (∼100 MPa/min) to minimize temperature increase due to adiabatic heating. After pressure buildup, an equilibrium period of 2 min was taken into account to allow temperature to evolve to its desired value. After the equilibration period, one vessel was decompressed, the sample was immediately cooled in an ice−water bath, and the residual activity was measured, which corresponds to the blank sample (A_0). The other pressure vessels were decompressed after different time intervals. After pressure release, samples were immediately cooled in ice water, and the residual activity was measured within 60 min of storage time in ice water.

Kinetic Data Analysis. Inactivation of enzymes can often be described by first-order kinetics (eqs 1 and 2): or

$$\log A = \log A_0 - \frac{t}{D} \tag{1}$$

$$\ln A = \ln A_0 - kt \tag{2}$$

where A_0 is the initial activity and A the remaining activity at time t (min). For first-order kinetics, the rate constant can be determined by linear regression analysis of log A versus treatment time. Next to characterization in terms of inactivation rate constants (k), first-order inactivations can be described by decimal reduction times, D values (i.e., time needed to reduce the initial activity by one log unit at a constant temperature). Temperature and pressure dependence of the D value can be expressed by z_T and z_P, respectively (eqs 3 and 4). These values represent the temperature or pressure increase needed to obtain a 10-fold reduction of D.

$$\log D = \log D_0 - \frac{T - T_0}{z_T} \tag{3}$$

$$\log D = \log D_0 - \frac{P - P_0}{z_P} \tag{4}$$

When there are several isozymes present that show different behavior toward temperature/pressure (i.e., a labile and stable fraction) and both inactivate according to first-order kinetics, a biphasic kinetic model (eq 5) is obtained. There is a fast inactivation period followed by a decelerated decay. The kinetic parameter values for thermal inactivation in case of biphasic inactivation behavior, can be estimated by nonlinear regression analysis of eq 5

$$A = A_L \exp\left(-k_{L,0} \exp\left(\frac{E_{aL}}{R}\left(\frac{1}{T_0} - \frac{1}{T}\right)\right)t\right) + A_S \exp\left(-k_{S,0} \exp\left(\frac{E_{aS}}{R}\left(\frac{1}{T_0} - \frac{1}{T}\right)\right)t\right) \tag{5}$$

where the subscript L and S refer to labile and stable enzyme fraction, respectively.

Temperature and pressure dependence of inactivation rate constants can be estimated using the Arrhenius (eq 6) or Eyring (eq 7) model:

$$\ln(k) = \ln(k_0) + \left[\frac{E_a}{R_t}\left(\frac{1}{T_0} - \frac{1}{T}\right)\right] \tag{6}$$

$$\ln(k) = \ln(k_0) - \left[\frac{V_a}{R_p T}(P - P_0)\right] \tag{7}$$

where T and P are the absolute temperature (K) and pressure (MPa), respectively, T_0 and P_0 are the reference temperature (K) and pressure (MPa), k_0 is k at T_0 and P_0 (min^{-1}), E_a is the activation energy (kJ/mol), V_a is the activation volume (cm^3/mol), R_t (8.314 J/(mol·K)) and R_p (8.314 cm^3 MPa/(mol·K)) are the universal gas constants.

Mathematical Model To Describe Combined Pressure−Temperature Dependence of PPO in Grape Must Inactivation. The most useful thermodynamic-based model governing the behavior of a system during pressure and temperature changes has been previously described by Hawley (*12*) and later by Morild (*13*). It has often been used to understand the reversible response of a system toward pressure and temperature. Using the Eyring transition-state theory, one can convert this thermodynamic model into a kinetic model, suggesting that enzyme inactivation is accompanied by formation of a metastable/transition activated state (‡), which exists in equilibrium with the native enzyme (*14−16*).

A third-degree polynomial model (derived from the thermodynamic model) was successfully used to describe the heat−pressure inactivation of purified banana and carrot pectin methyl esterase (*17*, *18*). The resulting equation (eq 8) is a linear equation so that a multilinear

regression analysis can be applied for parameter estimation.

$$\ln(k) = a + AX_1 + BX_2 + CX_3 + DX_4 + EX_5 + FX_6 + GX_7 + HX_8 + IX_9 \quad (8)$$

where *a*, *A*, *B*, *C*, *D*, *E*, *F*, *G*, *H*, and *I* are unknown model parameters.

$$a = \ln(k_0) \qquad X_1 = -\frac{(P - P_0)}{T} \qquad X_2 = \frac{(T - T_0)}{T}$$

$$X_3 = -\frac{(P - P_0)^2}{T} \qquad X_4 = \frac{(T - T_0)^2}{T} \qquad X_5 = -\frac{(P - P_0)(T - T_0)}{T}$$

$$X_6 = \frac{(P - P_0)^2(T - T_0)}{T} \qquad X_7 = \frac{(P - P_0)(T - T_0)^2}{T} \qquad X_8 = \frac{(P - P_0)^3}{T}$$

$$X_9 = \frac{(T - T_0)^3}{T}$$

where P_0 (MPa) and T_0 (K) are reference pressure and temperature, respectively, and $k(P,T)$ and $k_0(P_0,T_0)$ are inactivation rate constants (min^{-1}).

By using multivariate tests across the multiple dependent variables, one can select the number of significant variables in the model. The "forward" selection procedure in the multiple linear regression analysis was applied using the statistical software package SAS. The "forward" selection procedure allows one to start with no variables in the model and to calculate the *F** statistic for each independent variable, which will reveal whether the contribution of the several variables is significant to the model. As a measure for the quality of the model fitting to the experimental data, the corrected r^2 and the model standard deviation (SD) were calculated using eqs 9 and 10, respectively.

$$\text{corrected } r^2 = \left[1 - \frac{(m-1)\left(1 - \frac{\text{SSQ}_{\text{regression}}}{\text{SSQ}_{\text{total}}}\right)}{(m-j)}\right] \quad (9)$$

$$SD = \sqrt{\frac{\text{SSQ}_{\text{residual}}}{(m-j)}} \quad (10)$$

Where *m* is the number of observations, *j* is the number of model parameters, SSQ is the sum of squares, and SD is the standard deviation. Besides the corrected r^2 and SD, the Mallow's C_p statistic was computed. C_p is a measure for the total sum of squared errors and defined as (eq 11)

$$C_p = \left(\frac{\text{SSE}_p}{s^2}\right) - (N - 2p) \quad (11)$$

where s^2 is the mean error sum of squares for the full model, SSE_p is the error sum of squares for a model with *p* parameters including the intercept, and *N* is the number of observations. The best situation occurs when C_p is close to *p*.

RESULTS AND DISCUSSIONS

Enzyme Kinetics. The kinetic parameters, Michaelis−Menten constant (K_M) and maximum rate (V_{max}) values, for PPO in grape must were determined by performing PPO activity assays at different catechol concentrations. Activity assays were investigated at 25 °C using catechol solutions of 0.01−1 mol/L in McIlvaine buffer at pH = 5.0. A K_M of 43.5 mM and V_{max} of 219.4 $OD_{400\ nm}$/min for PPO in grape must were obtained using the Michaelis−Menten model. Cash and co-workers (*19*) had reported a K_M of 67 mM for the Concord grape PPO extract with catechol as substrate. Due to problems of cloudiness, it was not possible to evaluate PPO activity at higher substrate concentrations.

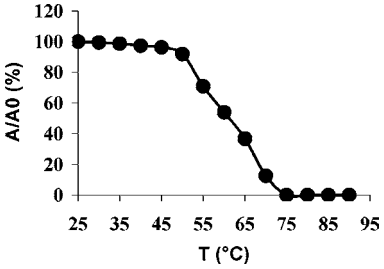

Figure 1. Thermal stability of PPO in grape must. Residual activity was measured after 10-min treatment at different temperatures. Assay conditions were catechol 0.1 mM, pH 5.0, 25 °C.

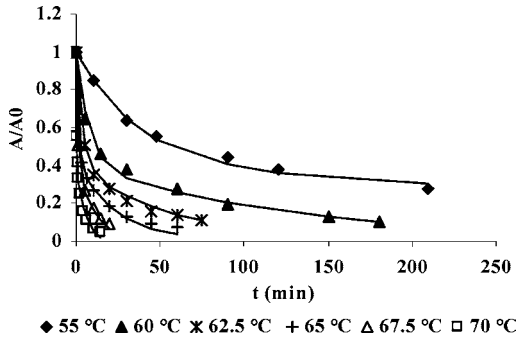

Figure 2. Thermal inactivation curves of PPO in grape must in the temperature range 55–70 °C. Assay conditions were catechol 0.1 M, pH 5.0.

Thermal Inactivation Kinetics of PPO in Grape Must. In **Figure 1**, relative residual PPO activity is plotted as a function of inactivation temperature after a 10-min treatment time. At 60 °C, 50% of PPO activity in grape must was lost after 10 min of treatment, whereas at 65 °C, more than 60% of the PPO activity was lost after 10 min, and at 75 °C, PPO was completely inactivated after 10 min of heating. Lamikanra and co-workers (*20*) reported a loss of about 70% of PPO activity for Welder and Noble grape PPO extract at 60 °C for 30 min, while Wissemann and co-workers (*21*) found a 50% reduction of PPO extract activity after 15 min at 68.1 °C for Ravat and 76.1 °C for Niagara grapes.

Based on the results of thermal stability, a detailed kinetic study of thermal inactivation of PPO in grape must was performed in the range from 55 to 70 °C at atmospheric pressure. **Figure 2** presents the thermal inactivation curves of PPO in grape must in the temperature range from 55 to 70 °C.

The thermal inactivation of PPO in grape must in this temperature range exhibits a biphasic behavior, indicating the presence of a heat-labile and a heat-resistant fraction of PPO, both showing first-order inactivation kinetics. Kinetic inactivation parameters of the stable and labile fractions were estimated in a global approach using nonlinear regression analysis (eq 5) (**Table 1**). Labile and resistant forms of PPO have been shown to occur in a number of other fruits and vegetables including (*22*) palmito (*23, 24*), plantain (*25*), and potatoes (*26*). The inactivation rate constants for the labile and stable PPO fractions at 60 °C were, respectively, 0.135 and 0.008 min^{-1}, while Dalmadi and co-workers (*27*) reported for the labile and stable PPO fractions of strawberry PPO extract at 60 °C, respectively, 1.7855 and 0.1448 min^{-1}. The thermostable PPO fraction amounted to around 50% of the total activity. The activation

Table 1. Estimated Kinetic Parameters of the Stable and Labile Fraction of Thermal Inactivation of PPO in Grape Must

Labile Fraction	
A_L (%)	58.9 ± 1.8[a]
$k_{L,60°C}$ (min^{-1})	0.135 ± 0.01
E_{aL} (kJ/mol)	285.5 ± 6.8
Stable Fraction	
A_S (%)	41.1 ± 1.8
$k_{S,60°C}$ (min^{-1})	0.0078 ± 0.0007
E_{aS} (kJ/mol)	307.2 ± 9.2

[a] Standard error of regression.

energies were estimated as 285.5 ± 6.8 kJ/mol for the labile fraction and 307.2 ± 9.2 kJ/mol for the stable fraction. These activation energy values are in the same range with the ones found for grape PPO from DeChaunac (*28*) and Ravat 51 (*21*) but higher than the one found for Niagara (*21*) grape PPO extract. The larger value of E_a indicates a greater influence of temperature on thermal inactivation (*29*).

Kinetics of PPO in Grape Must Inactivation Due to Combined Pressure and Temperature Treatments. Combined treatments of pressure and temperature for inactivation of PPO in grape must were investigated within the range of 20−70 °C and 100−800 MPa. During the dynamic phase (sample enclosure in pressure vessels, pressure build-up, and equilibration), part of the labile grape PPO fraction was already inactivated. The percentage activity loss during these dynamic conditions depends on the pressure−temperature conditions under study: at milder conditions, such as 500 MPa−55 °C, 39% PPO activity in grape must was lost, whereas at more intense treatment conditions, such as 100 MPa−70 °C or 800 MPa−55 °C, up to 80% of the initial PPO activity in grape must was lost at the start of the isothermal−isobaric treatment conditions, and only inactivation of the stable PPO fraction was observed. Because this stable PPO fraction should be inactivated during processing of grape must to prevent enzymatic browning, for data analysis, only the pressure−temperature inactivation of the stable PPO fraction was taken into account (by discarding all inactivation data with less than 50% activity loss).

From the log linear plots of residual PPO activity stable fraction versus inactivation time at constant pressure, it can be concluded that the pressure−temperature inactivation of grape PPO stable fraction can be adequately described by a first-order model (data not shown). The corresponding first-order inactivation rate constants are given in **Table 2**. At higher temperatures, the inactivation rate accelerated, indicating the synergistic effect of pressure and temperature. However in the high-temperature ($T \geq 60$ °C) and "low"-pressure ($P \leq 200$ MPa) region, an antagonistic effect of pressure and temperature was observed. In this range, a pressure increase at constant temperature resulted in a decrease of the inactivation rate constant. An antagonistic effect of pressure and temperature has already been encountered for enzyme inactivation/protein denaturation, mostly limited to pressures below 300 MPa (*15*, *17*, *18*, *30*−*33*). According to Mozhaev and co-workers (*30*) who studied the behavior of α-chymotrypsin under pressure, pressure stabilization against thermal inactivation/denaturation might be due to counteracting effects of pressure and temperature on the formation or disruption of intramolecular interactions, to their opposing effects on interactions between enzyme/protein and solvent (water), or both. During the initial step of thermal inactivation, proteins can lose a number of essential water molecules, which might lead to structural rearrangements. High pressure may hinder this process owing to its favorable effect on hydratation of both charged and nonpolar groups.

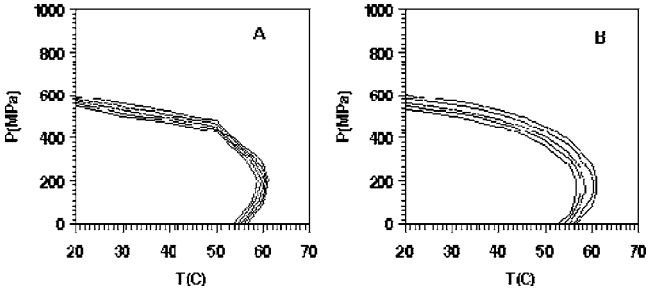

Figure 3. Pressure–temperature kinetic diagram for the *P*/*T* inactivation of the stable fraction of PPO in grape must: (**A**) based on experimental rate constants; (**B**) based on third-order polynomial (eq 12). The inner and the outer lines represent *P*/*T* combinations for which $k = 0.01$ and 0.05 min^{-1}, respectively.

Based on the estimated *k*-values for isothermal−isobaric inactivation of the stable fraction of grape PPO (**Table 2**), a pressure−temperature kinetic diagram (isorate contour) for the stable fraction of PPO in grape must was plotted (**Figure 3A**). The lines represent several combinations of pressure−temperature, resulting in the same inactivation rate constant. The synergistic effect of pressure and temperature in the high-pressure region ($P \geq 200$ MPa) and the antagonistic effect of pressure and temperature in the "low"-pressure region ($P < 200$ MPa) can be deduced from the shape of the contour in this kinetic diagram. Temperature and pressure coefficient models include the Arrhenius and Eyring equations, which, respectively, describe the temperature and pressure dependence of the experimental *k*-values. The Arrhenius relation was valid in the whole pressure domain studied. The inactivation rate constant could be enhanced with increasing temperature at a constant pressure. By plotting the natural logarithm of the inactivation rate constant as a function of the reciprocal absolute temperature for the different pressure levels, we determined the activation energy by linear regression analysis (eq 6). The estimated

Table 2. *k*-Values ($\times 10^{-2}$ min^{-1}) for Combined Pressure–Temperature Inactivation of PPO Stable Fraction in Grape Must[a]

P/T	20	30	40	50	55	60	62.5	65	67.5	70
0.1	ND[b]	ND[b]	ND[b]	ND[b]	0.35 ± 0.04	0.96 ± 0.06	1.69 ± 0.02	3.30 ± 0.35	7.36 ± 0.51	18.03 ± 3.63
100	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]	0.45 ± 0.04	0.89 ± 0.01	1.52 ± 0.07	3.56 ± 0.06	7.13 ± 0.11
200	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]	0.23 ± 0.14	0.48 ± 0.11	0.76 ± 0.04	1.75 ± 0.16	2.76 ± 0.094
300	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]	0.57 ± 0.07	0.89 ± 0.06	1.28 ± 0.03	2.25 ± 0.08	5.29 ± 0.05
400	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]	1.48 ± 0.05	2.07 ± 0.01	3.17 ± 0.08	4.14 ± 0.07	9.09 ± 0.02
500	ND[b]	ND[b]	0.35 ± 0.17	0.64 ± 0.02	2.84 ± 0.14	3.10 ± 0.02	4.09 ± 0.05	5.75 ± 0.40	10.82 ± 0.10	ND[b]
600	0.57 ± 0.03[c]	0.73 ± 0.07	0.94 ± 0.06	2.07 ± 0.07	4.19 ± 0.03	5.06 ± 0.07	7.59 ± 0.03	9.48 ± 0.09	ND[b]	ND[b]
700	1.08 ± 0.08	1.28 ± 0.72	1.57 ± 0.01	3.22 ± 0.04	5.680 ± 0.04	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]
800	1.89 ± 0.01	2.18 ± 1.07	3.45 ± 0.03	5.29 ± 0.09	7.59 ± 0.09	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]

[a] Pressure in MPa; temperature in °C. [b] Not determined. [c] Standard error of regression.

Table 3. z_T and E_a Values for Thermal Inactivation ($P \geq 200$ MPa) of Victoria PPO Stable Fraction in Grape Must at Elevated Pressure

P (MPa)	z_T (°C)	E_a (kJ/mol)
100	8.34 ± 0.45[a]	262.09 ± 2.49
200	9.19 ± 0.24	237.70 ± 3.23
300	10.74 ± 0.41	203.31 ± 8.65
400	13.28 ± 0.79	164.40 ± 13.02
500	18.90 ± 0.26	107.77 ± 10.55
600	35.33 ± 2.71	53.34 ± 6.38
700	50.50 ± 1.26	36.02 ± 8.42
800	57.47 ± 0.09	31.82 ± 4.28

[a] Standard error of regression.

Table 4. z_P and V_a Values for Pressure Inactivation of Victoria PPO Stable Fraction in Grape Must at Different Temperatures

T (°C)	z_P (MPa)	V_a (cm^3/mol)
20	384.61 ± 0.26[a]	−14.61 ± 1.75[a]
30	423.21 ± 0.73	−13.71 ± 0.04
40	315.27 ± 3.91	−19.00 ± 1.52
50	340.64 ± 1.55	−18.15 ± 3.21
55	715.59 ± 0.21	−8.77 ± 0.38
60	292.63 ± 0.61	−21.78 ± 1.51
62.5	327.65 ± 1.22	−19.6 ± 0.57
65	351.60 ± 0.35	−18.40 ± 1.07
67.5	379.08 ± 0.29	−17.19 ± 1.51
70	386.53 ± 2.22	−16.98 ± 0.90

[a] Standard error of regression.

activation energies are presented in **Table 3**. An increase of pressure resulted in a decrease of the activation energy, indicating that the inactivation rate constants are less temperature sensitive at elevated pressure. A linear relation between the activation energy (E_a) and pressure (P) could be established ($r^2 = 0.9692$). Due to the observed antagonistic effect of pressure and temperature, the Eyring equation was not valid for the entire pressure domain. By evaluating pressure dependence, we observe two regions: for pressures exceeding 200 MPa, the inactivation rate constant increased with pressure increase, while below 200 MPa, the inactivation rate constant decreased with pressure increase (ascribed to the antagonistic effect of pressure and temperature). As a result, the estimation of the activation volume values (V_a) was restricted to the higher pressure region (≥200 MPa). By plotting the natural logarithm of the inactivation rate constant as a function of pressure for the different temperature levels, we determined the activation volume by linear regression analysis (eq 7). At temperatures below 60 °C, V_a values were situated between −8 and −19 cm^3 mol^{-1}, and no real trend in the variation with temperature was observed (**Table 4**). When compared to other PPO sources, PPO in grape must at pH 4 seems to be more pressure/temperature sensitive than avocado PPO in phosphate buffer at pH 7 (*15*).

Mathematical Model To Describe Combined Pressure−Temperature Dependence of PPO in Grape Must Inactivation. The relevance to include third-degree terms in the polynomial equation (eq 8) to describe the inactivation rate constant of PPO in grape must as a function of pressure and temperature was statistically evaluated. All the variables significantly contributed to the model, with exception of variable X_7, which was removed from the model, yielding eq 12:

$$\ln(k) = a + AX_1 + BX_2 + CX_3 + DX_4 + EX_5 + FX_6 + GX_8 + HX_9 \quad (12)$$

The isocontour plots based on experimental rate constants and simulated by the third-degree model are visualized in **Figure 3**. The simulated isorate plot based on eq 12 (**Figure 3B**) has a similar shape to the experimental one (**Figure 3A**). In **Figure 3**, the antagonistic effect of pressure and temperature, as previously discussed, can be observed at lower pressure values ($P \leq 200$ MPa) and in the high-temperature domain (≥60 °C). The estimated model parameters of eq 12 are presented in **Table 5**. The model under study resulted in a good accuracy of the estimated model parameters for the inactivation of PPO in grape must. This requirement is, from the experimental point of view, not always possible to accomplish. The Mallow's C_p statistic was also calculated and was found to be equal to the number of variables, including the intercept ($p = 9$). For the model studied, no trend in residuals (differences between experimental and predicted k-values) was noticed as a function of temperature, pressure, experimental k-value, and predicted k-value (data not shown). A parity plot of the natural logarithm of the predicted k-values based on eq 12 compared with the natural logarithm of the experimental k-values was established (**Figure 4**). The divergence from the bisector can be considered as indicator for the inaccuracy of the considered model and its parameters; the more the experimental and the estimated values differ, the less successful the model is. A good correlation between the natural logarithm of the predicted k-values and that of the experimental k-values was found ($r^2 = 0.9535$). Graphically, the third degree model shows good fitting within the temperature−pressure range studied (**Figure 5**).

Table 5. Estimated Model Parameters for PPO Stable Fraction Inactivation in Grape Must Based on Eq 12 at a Reference Pressure of 600 MPa and a Reference Temperature of 323.15 K (50 °C)

parameter	estimated value	RSE (%)
a	−3.88	2.09
A	−2.69	0.54
B	28.04	0.95
C	0.0048	1.01
D	0.7191	0.35
E	0.0316	9.86
F	5.51×10^{-5}	9.56
H	-1.1×10^{-5}	0.66
I	0.00856	0.19
corrected r^2	0.9447	
SD	0.238	
C_p	9	

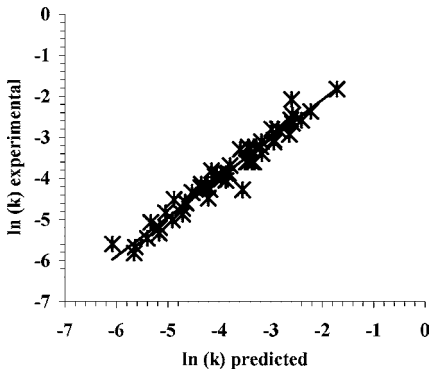

Figure 4. Correlation between the predicted k-values of PPO stable fraction in grape must inactivation determined from experimental isothermal–isobaric inactivation data and simulated using eq 12.

Conclusion. Thermal inactivation of PPO in grape must (pH = 4.03) followed a biphasic model in the temperature range from 55 to 70 °C. The combined temperature and high-pressure inactivation of the stable fraction of PPO in Victoria grape must could be adequately described, whatever the pressure−temper-

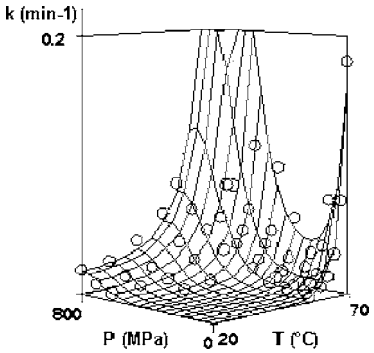

Figure 5. 3D plots for thermal–pressure inactivation of PPO stable fraction in grape must, based on the third-degree polynomial model (eq 12): (○) experimental data points.

ature combinations, by a first-order model in the pressure range of 100−800 MPa and temperature range 20−70 °C. A synergistic effect of pressure and temperature on PPO in Victoria grape must was found, except in the high-temperature−low-pressure region, where an antagonistic effect was noted. A mathematical model was proposed to describe the temperature−pressure behavior of Victoria must PPO inactivation. This insight could be valuable in term of implementing high-pressure technology in grape-based products such as grape juice and wine.

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