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Modeling of Urea Degradation in White and Rosé Wines by Acid Urease

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The specific activity of a whole cell acid urease preparation was tested in five wines manufactured in the Apulia region of Italy in the 2003 vintage at both short and long treatment times, thus confirming the validity of the pseudo-first-order kinetic model to describe urea removal in real wines. The ratio between the experimental pseudo-first-order kinetic rate constant (k_{Ie}) for any real wine tested and that (k_I) referred to a model wine solution having the same composition and pH reduced from about 0.21 to 0.02 as the overall content of phenolic compounds (P) increased from 109 to 853 g m^{-3} of gallic acid equivalent (GAE). The specific inhibitory effect of such compounds was explained by accounting for the equilibrium constant (K_P) of the reaction of polyphenols with acid urease, which was found to be about 21 g of GAE m^{-3} for the real wines tested, whereas it ranged from about 16 to 45 g of GAE m^{-3} when the model wine solution was enriched with tannins extracted from grape seeds or skins, respectively. A sequential experimental procedure consisting of accelerated acid urease tests at high doses of enzyme followed by accelerated ethyl carbamate tests on the resulting acid urease treated wine was recommended to assess preliminarily the technoeconomic feasibility of the acid urease hydrolytic process for the wine of concern.

KEYWORDS: Acid urease; real and model wines; phenolics; pseudo-first-order kinetic rate constant; inhibitory effect; urea degradation kinetics

INTRODUCTION

Ethyl carbamate (urethane, EC) is a naturally occurring component in all fermented foods and beverages, being spontaneously produced by the reaction between urea and ethanol (*1*). Because EC has shown a potential carcinogenic activity when administered in high doses in animal tests (*2*, *3*), there is a great deal of interest in reducing EC levels in food products.

Several preventive actions, such as control of fertilization techniques, adjustment of nutrient contents in grape musts, use of suitable yeast and lactic acid bacteria strains, acid urease application, and control of storage conditions, have been recommended by the U.S. Food and Drug Administration to reduce EC levels in wine (*4*).

Although the feasibility of acid urease application for the removal of urea from several type wines has been extensively demonstrated (*5*−*10*), the efficiency of such a treatment was found to vary with the type of wine, content of inhibiting factors, and usage conditions. More specifically, the most effective inhibitors of urease present in wines were found to be, in order of importance, fluoride, malate, ethanol, and phenolic compounds (*11*−*13*).

Actually, Famuyiwa and Ough (*12*) attributed the greater content of fluoride in California wines than in European and Japanese wines to the extensive use of cryolite (AlF_6Na) as an insecticide in California vineyards. Because no Italian vineyard is presently treated with cryolite, no documentation on the fluoride content in the Italian wines is presently available, and the maximum acceptable limit for fluoride in wines is 1 g m^{-3} in accordance with the *Compendium of International Methods of Analysis* (*14*). Further evidence was independently reported by Rodríguez Gómez et al. (*15*), who found that the average (0.15 ± 0.07 g m^{-3}) and maximum (0.50 g m^{-3}) values of the fluoride content in bottled wines of the different types and areas of the Canary Islands, as determined by direct potentiometry with a fluoride specific electrode, were by far lower than the maximum allowed by the International Office of the Vineyard and the Wine (*14*).

For fluoride concentrations of $<$0.5 g m^{-3}, the inhibitory effect of fluoride on acid urease activity was regarded as negligible as observed by Kodoma (*13*). Thus, in previous work (*16*), the

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Table 1. Mean Values and Standard Deviations of the Main Wine Analyses for the Five Italian Wines Investigated in This Work and Experimental Pseudo-First-Order Kinetic Rate Constants (k_{Ie}) with Their Corresponding Coefficients of Determinations (r^2) Together with the Specific Acid Urease Activity (π_A) and Pseudo-First-Order Kinetic Rate Constant (k_I) Calculated via Equations 8–14 and Referred to Model Wine Solutions Having the Same Composition and pH as the Real Wines Tested

parameter	sample A	B	C	D	E	unit
ethanol	12.30 ± 0.02	12.13 ± 0.10	14.38 ± 0.12	17.00 ± 0.15	11.80 ± 0.02	% v/v
pH	3.40 ± 0.04	3.53 ± 0.06	3.89 ± 0.05	3.86 ± 0.05	3.65 ± 0.04	
total acidity	4.85 ± 0.35	4.90 ± 0.28	6.18 ± 0.38	5.90 ± 0.35	5.00 ± 0.27	kg[a] m^{-3}
volatile acidity	0.37 ± 0.08	0.34 ± 0.05	0.36 ± 0.05	0.50 ± 0.09	0.36 ± 0.06	kg[b] m^{-3}
overall SO_2	94 ± 3	90 ± 2	57 ± 2	110 ± 3	86 ± 2	g m^{-3}
$K_2S_2O_5$	163 ± 5	156 ± 3	99 ± 3	191 ± 5	149 ± 3	g m^{-3}
urea	15 ± 3	17 ± 5	20 ± 5	118 ± 20	45 ± 8	mmol m^{-3}
ammonium	5.0 ± 0.4	14.0 ± 1.1	6.5 ± 0.8	19.2 ± 1.9	6.7 ± 1.5	g m^{-3}
ethyl carbamate	1.2 ± 0.1	1.4 ± 0.1	4.2 ± 0.3	4.4 ± 0.4	1.5 ± 0.1	mg m^{-3}
TPI	7.5 ± 0.3	7.1 ± 0.2	25.2 ± 0.4	18.7 ± 0.3	11.1 ± 0.2	
total phenolics	109 ± 5	112 ± 7	853 ± 24	289 ± 20	254 ± 15	g m^{-3}
tartaric acid	3.7 ± 0.3	3.7 ± 0.1	3.8 ± 0.4	4.8 ± 0.4	3.4 ± 0.1	kg m^{-3}
L-malic acid	1.1 ± 0.3	0.8 ± 0.1	0.42 ± 0.04	0.28 ± 0.06	0.03 ± 0.01	kg m^{-3}
L-lactic acid	0.4 ± 0.1	0.7 ± 0.1	2.5 ± 0.3	1.65 ± 0.03	4.5 ± 0.5	kg m^{-3}
citric acid	0.23 ± 0.04	0.30 ± 0.00	0.05 ± 0.01	nd[c]	nd	kg m^{-3}
$k_{Ie} \times 10^5$	2.1 ± 0.1	1.7 ± 0.1	0.44 ± 0.01	1.20 ± 0.09	1.7 ± 0.2	$m^3\ g^{-1}\ min^{-1}$
r^2	0.98	0.96	0.995	0.97	0.94	
π_A	0.102	0.117	0.209	0.180	0.188	units mg^{-1}
$k_I \times 10^5$	10.16	11.68	20.91	18.02	18.84	$dm^3\ mg^{-1}\ min^{-1}$
k_{Ie}/k_I	0.207	0.145	0.021	0.066	0.092	

[a] As tartaric acid equivalent. [b] As acetic acid equivalent. [c] Not detectable by the analytical method used.

effects and interactions of the concentrations of malic (M) and lactic (L) acids and potassium metabisulfite (K), ethanol volumetric fraction (y_E), and pH on the specific activity (π_A) of a commercial preparation of whole cell acid urease in model wine solutions were assessed by performing a central composite design. The experimental responses were then fitted by using a second-order polynomial reduced to its canonical form to identify the only statistically significant principal axes (*16*).

Among the wine components tested, malic acid was found to be the greatest inhibitor. Actually, as the M level or pH of the model wine solutions was increased from 1.25 to 3.75 kg m^{-3} or from 3.25 to 3.75, respectively, the variation in the specific enzyme activity (π_A) was about similar, but of opposite sign, thus leading correspondingly to a decrease or an increase in π_A (*16*). In accordance with Trioli and Ough (*11*), this was attributed to the fact that the greater the pH of the wine model solution, the smaller the fraction of undissociated malic acid became.

For urea concentrations of <1 mol m^{-3} the ammonium formation rate was assumed to be of pseudo-first-order with respect to urea (*16*). However, when the experimental pseudo-first-order kinetic rate constants (k_I) of the real wines assayed by Kodama (*13*) and Trioli and Ough (*11*) were compared to those estimated by means of the empirical model set up previously (*16*), it was found that the latter were largely overestimated with respect to the former.

The main aim of this work was to assess the validity of the pseudo-first-order kinetic model to describe urea removal in five wines manufactured in the Apulia region of Italy in the 2003 vintage at both short and long treatment times. To this end, the experimental pseudo-first-order kinetic rate constant values were compared to those observed in model wines enriched with different amounts of phenolic compounds as extracted from grape skins or seeds to develop an empiric modeling capable of evaluating the contribution of the main inhibitory components present in wines and thus estimating the technoeconomic feasibility of such a detoxification process.

MATERIALS AND METHODS

Raw Materials. The commercial preparation Enzeco Acid Urease (Enzyme Development Corp., New York) from *Lactobacillus fermentum* was used. It consisted of a partially soluble powder to be stored at 4 °C. Its claimed specific activity was 3.3−4.0 units mg^{-1}, where 1 unit corresponds to the amount of powder that liberates 1 μmol min^{-1} of ammonia from urea at 37 °C, once it is dissolved in a 0.1 kmol m^{-3} sodium acetate buffer (pH 4) enriched with urea (83.33 mol m^{-3}) and ethanol (12.5% v/v).

Five Italian wines, produced from grapes of the vintage of 2003 in the Apulia region of Italy, were filtered and then submitted to acid urease tests about 9 months after their alcoholic fermentation. Four of these were white wines, namely, two table white wines labeled A and B and two types of the typical Italian liqueur wine Moscato di Trani, labeled C and D, whereas the fifth sample was a rosé wine labeled E.

Two extracts of grape skins (Grap'tan S) and grape seeds (Grap'tan PC), manufactured by Ferco Oenologie (Saint Montan, France), were used as sources of phenolic compounds for the model wine solution representing the central point of the composite design previously described (*16*). This model solution was prepared by dissolving constant amounts of urea (1 mol m^{-3}), tartaric (TA = 5 kg m^{-3}), malic (M = 2.5 kg m^{-3}) and lactic (L = 1.75 kg m^{-3}) acids, potassium metabisulfite (K = 0.2 kg m^{-3}), and ethanol (y_E = 13% v/v) in deionized water and then adjusting the resulting pH to 3.50. All reagents were of analytical grade.

Wine Analyses. All samples were stored in 0.75 dm^3 bottles at 4 °C before testing. Their ethanol volumetric fraction, pH, and titrable and volatile acidities, as well as total SO_2, urea, and phenolic compounds, were determined by using the OIV analytical methods (*17*). The total phenolic index (TPI) of each sample was calculated as the absorbance measured at 280 nm times the corresponding dilution factor (*18*). The overall content of phenolic compounds (P) was colorimetrically assessed at 700 nm using the Folin−Ciocalteu reagent and expressed as gallic acid equivalent (GAE) by referring to a calibration curve valid for a gallic acid concentration range of 0−30 g m^{-3} (*19*). By using the same analytical method, the total phenolics of the grape skin and seed extracts were found to be 0.476 ± 0.02 and 0.622 ± 0.01 g of GAE g^{-1} of extract, respectively.

All spectrophotometric measurements were carried out using a Lambda25 spectrophotometer (Perkin-Elmer Inc., Wellesley, MA) and quartz cells with a 1-cm path length.

The acidic wine composition, that is, the concentrations of tartaric, L-malic, L-lactic, and citric acids, was determined by high-pressure liquid chromatography (HPLC-DAD) (*20*) using an apparatus provided by Dionex Corp. (Sunnyvale, CA), consisting of a P680A pump coupled to a PDA-100 diode array detector and controlled by Chromeleon software. The column was a Nova-Pak C_{18}, 250 × 4 mm, 4 μm, protected by a guard column packed with the same material (Waters Corp., Milford, MA). Both columns were thermostatically controlled at 30 °C. The chromatographic conditions, as well as peak detection, identification, and quantification, were carried out as described by Cane (*20*).

All analytical data are shown in **Table 1**.

Acid Urease Treatment of Wine Samples. To assess the time course of the hydrolytic process, each wine sample was spiked with 1 mol m^{-3} of urea, conditioned at 20.0 ± 0.2 °C, and then poured into 50 cm^3 rubber-capped flasks, each containing 0.0119 g of the enzyme preparation (E_0 = 238 g m^{-3}). These were immersed in a water bath placed over a magnetic multistirrer (model Multistirrer 15, Velp Scientifica, Milan, Italy) to maintain the reaction temperature constant at 20.0 ± 0.2 °C by means of a thermostat model F3 (Haake, Karlsruhe, Germany). Several samples (50 mm^3) were withdrawn from any flask for as long as 10−30 h and were diluted with 960 mm^3 of deionized water at 4 °C and stored at −18 °C before being assayed for ammonium and urea by using the enzymatic kit from Boehringer Mannheim GmbH (Mannheim, Germany). The absence of any interference between the assay and substances present in the wine sample was checked by using an internal standard as a control.

A few validation tests were performed on wine sample D by adding 236 g m^{-3} of acid urease and different amounts of urea in the range of 0.833 and 1.667 mol m^{-3}.

Thirty-day-long acid urease tests were finally carried out. Each wine sample, as such (test TE) or spiked with 0.417 mol m^{-3} of urea (test UE), was further enriched with 25 g m^{-3} of acid urease and stored in 0.265 dm^3 dark glass bottles. After the samples, as well as the control wine (test T), had been maintained at 18.0 ± 0.5 °C for 30 days, they were assayed for the residual urea and ammonia contents. Any test was carried out in triplicate.

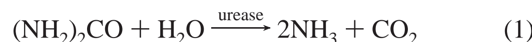
Acid Urease Treatment of Model Wine Solutions. To assess the effect of the phenolic compounds, the grape seed and skin extracts were added to the model wine solution in the ranges of 0−1.2 and 0−0.3 kg m^{-3}, respectively. Kinetic data were determined by using the same procedure mentioned above.

Potential Ethyl Carbamate Analysis. The 250 cm^3 wine samples resulting from the above acid urease treatment were heated at 70 ± 1 °C in a thermostatic oven for 48 h to accelerate ethyl carbamate formation. The so-called *potential ethyl carbamate* was assessed by GC-MS (*21*). Wine (50 cm^3) was integrated with 2 cm^3 of a hydroalcoholic solution (80% v/v in ethanol) containing 5 g m^{-3} of methyl carbamate (Sigma-Aldrich, Milan, Italy) as internal standard and 25 cm^3 of dichloromethane before being submitted to ultrasonic extraction for 10 min. The extract was separated by centrifugation (4000 rpm for 10 min), whereas the aqueous phase was further incorporated with 25 cm^3 of dichloromethane, ultrasonically extracted, and centrifuged. Both supernatants were mixed, dried using anhydrous Na_2SO_4, and reduced to a volume of 1 cm^3 using a rotary evaporator at 40 °C without vacuum.

A Hewlett-Packard 5890 gas chromatograph interfaced with a 5972 mass selective detector was used for the detection and quantification of ethyl carbamate. The concentrate (1 mm^3) was fed into a HP-624 capillary column (30 m × 0.32 mm × 0.25 μm), using a splitless injection mode for 1 min. The carrier gas was ultrapure helium with a flow rate of 1 cm^3 min^{-1}. After injection, the column temperature program used was as follows: 40 °C hold for 1 min, 70 °C min^{-1} ramp to 60 °C, 60 °C hold for 3 min, 2 °C min^{-1} ramp to 110 °C, 20 °C min^{-1} ramp to 250 °C, and finally 250 °C hold for 5 min. Both temperatures of the transfer line and injector were kept at 250 °C. The primary electron ionization (EI) mass spectra and spectra of analytes, methyl carbamate, and ethyl carbamate (Sigma-Aldrich) were recorded in the full-scan mode (with the ion mass-to-charge ratio, *m/z*, ranging from 35 to 100) to determine the retention times and characteristic mass fragments. For qualitative and quantitative analyses the responses for *m/z* 46.05, 62.05, 89.0, and 75.0 ions were taken into account. For quantification, peak area ratios of the analytes to the internal standard were calculated as a function of the concentration of the substances. The calibration curve was constructed by using six standards containing 5, 10, 20, 50, 100, and 200 mg m^{-3} of ethyl carbamate (*21*).

RESULTS AND DISCUSSION

Acid Urease Kinetics. The hydrolysis of urea to ammonia and carbon dioxide by acid urease (EC 3.5.1.5)



$$(NH_2)_2CO + H_2O \xrightarrow{\text{urease}} 2NH_3 + CO_2 \quad (1)$$

is generally described by means of a modified Michaelis−Menten reaction rate expression, which incorporates pH-dependent kinetics, substrate inhibition, and noncompetitive product inhibition by NH_4^+ (*22*). More specifically, the Michaelis−Menten constant (K_M) was reported to be practically independent of pH (*23*), its value for the urease from *Lactobacillus fermentum* being equal to 3 ± 2 mol m^{-3} at pH 3 and 20 °C (*16*).

In previous work (*16*), the ammonium formation rate (r_A) was found to vary quite linearly with the urea concentration (S) up to $S \approx K_M$, thus allowing its kinetic model to be reduced to the pseudo-first-order one

$$r_A \cong k_I E_0 S \quad (2)$$

where k_I is the pseudo-first-order kinetic rate constant and E_0 the enzyme level.

By referring to eq 1, the un-steady-state urea (S) and ammonium (A) mass balances in the well-mixed liquid phase can be written as

$$\frac{dS}{dt} = -r_S \quad (3)$$

$$r_A = \frac{dA}{dt} = -2\frac{dS}{dt} \quad (4)$$

to be integrated with the initial conditions

$$S = S_0 \text{ and } A = A_0; \qquad \text{for } t = 0 \quad (5)$$

where r_S is the urea removal rate.

By replacing eq 2 into eq 4 and integrating both eqs 3 and 4 with the initial conditions (*5*), it was possible to determine the time dependence of A and S as

$$S = S_0 \exp\left(-\frac{1}{2}k_I E_0 t\right) \quad (6)$$

$$A = A_0 + 2S_0\left[1 - \exp\left(-\frac{1}{2}k_I E_0 t\right)\right] \quad (7)$$

where k_I is to be expressed in m^3 g^{-1} min^{-1}, E_0 in g m^{-3}, and A and S in mol m^{-3}.

In previous work (*16*), the specific enzyme activity $\pi_A (= r_{A0}/E_0)$ was determined in several model wine solutions at different levels of pH, ethanol volumetric fractions (y_E), and concentrations of malic (M) and lactic (L) acids and potassium metabisulfite (K) and fitted by means of the following second-order canonical regression:

$$\pi_A = 0.029 + 0.0088X_1^2 + 0.0104X_2^2 + 0.0125X_3^2 + 0.0034X_4^2 \quad (8)$$

In eq 8

$$X_1 = 0.742(x_1 - 1.045) - 0.028(x_2 - 0.517) + 0.442(x_3 - 0.747) - 0.413(x_4 - 0.677) - 0.290(x_5 + 0.964) \quad (9)$$

$$X_2 = -0.452(x_1 - 1.045) - 0.332(x_2 - 0.517) + 0.264(x_3 - 0.747) + 0.042(x_4 - 0.677) - 0.783(x_5 + 0.964) \quad (10)$$

$$X_3 = 0.474(x_1 - 1.045) - 0.012(x_2 - 0.517) - 0.498(x_3 - 0.747) + 0.603(x_4 - 0.677) - 0.404(x_5 + 0.964) \quad (11)$$

$$X_4 = 0.120(x_1 - 1.045) - 0.931(x_2 - 0.517) - 0.209(x_3 - 0.747) - 0.119(x_4 - 0.677) + 0.248(x_5 + 0.964) \quad (12)$$

and

$$x_1 = (M - 2.5)/1.25$$
$$x_2 = (L - 1.750)/0.875$$
$$x_3 = (K - 0.20)/0.05 \quad (13)$$
$$x_4 = (y_E - 13.0)/1.5$$
$$x_5 = (\mathrm{pH} - 3.50)/0.25$$

where X_i and x_i are the generic principal axis and coded input variable, respectively.

Thus, use of eq 2 allowed k_I to be estimated as

$$k_I \cong \frac{\pi_A}{S_0} \quad (14)$$

where the initial urea concentration (S_0) used throughout the original experimental design was about 1 mol m^{-3}.

Acid Urease Activity in Real Wines. The same operating conditions (S_0 = 1 mol m^{-3}, E_0 = 238 g m^{-3}) previously selected to study the enzyme activity in several model wine solutions (*16*) were used to determine the time course of urea degradation in five Italian wines, the main chemicophysical characteristics of which are shown in **Table 1**.

Figure 1 shows a semilogarithmic plot of the current urea concentration (S) against time (t) for all wines assayed.

By virtue of eq 6, it was possible to relate the natural logarithm of S and time by using the least-squares method, thus determining the average value and standard deviation of the experimental pseudo-first-order kinetic rate constant (k_{Ie}) for all wines examined (**Table 1**).

Because the coefficients of determination (r^2) ranged from 0.94 to 0.995, the pseudo-first-order kinetic model was regarded as appropriate to describe the evolution of this hydrolytic process not only in model wine solutions (*16*) but also in real wines, as shown by the continuous lines plotted in **Figure 1**. This concurred with previous findings by other authors (*5*, *9*, *13*).

To confirm further the validity of the above kinetic model, two additional series of trials were carried out.

The first series was aimed at assessing the independence of k_I from the initial concentration of urea (S_0) in wine sample D, as spiked with 0.833 or 1.667 mol m^{-3} of urea when using an initial enzyme content of 236 g m^{-3}.

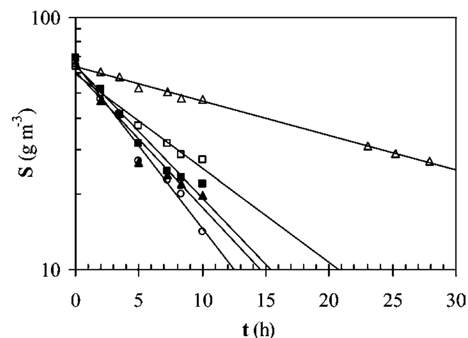


Figure 1. Time course of urea degradation for the five Italian wines listed in **Table 1** (○, A; ■, B; △, C; □, D; ▲, E) enriched with 1 mol of urea m^{-3} and treated with 238 g m^{-3} of acid urease. The continuous lines represent the mean squares regression lines.

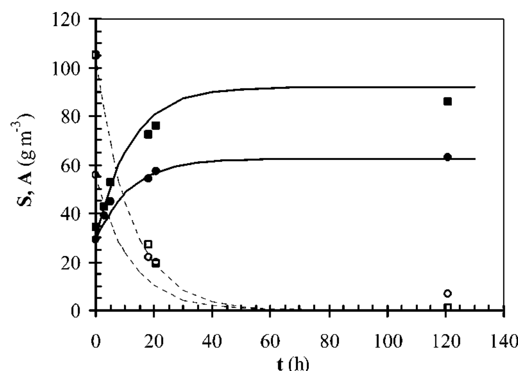


Figure 2. Time courses of urea (open symbols) and ammonium (solid symbols) concentrations for Moscato di Trani wine D (**Table 1**) enriched with 1.667 (□, ■) or 0.833 (○, ●) mol of urea m^{-3} and treated with 236 g m^{-3} of acid urease. The continuous lines were calculated using eqs 6 and 7 together with the corresponding experimental pseudo-first-order kinetic rate constants (k_{Ie}) listed in **Table 1**.

As shown in **Figure 2**, the experimental time courses of urea degradation and ammonium formation in all of these tests were quite accurately reconstructed by using eqs 6 and 7 in conjunction with the same value of k_{Ie} (= 1.2 × 10^{-5} m^3 g^{-1} min^{-1}) previously estimated for S_0 = 1 mol m^{-3} (**Table 1**).

The second series of experiments was directed to test the process performance in all five wines examined under the operating conditions recommended by the enzyme manufacturer (i.e., E_0 = 25 g m^{-3} of acid urease, equivalent to ca. 100 units dm^{-3} of wine), this enzymatic dose being smaller than the maximum one allowed by the EU regulation 1622/00 (75 mg of acid urease per liter of wine treated or 375 units per liter) (*6*).

All of these treatments were prolonged up to 30 days to account for the effects of all of the inhibitors present in real systems.

As shown in **Table 2**, in four of the five wines the acid urease degraded urea to ammonium up to residual levels smaller than those detectable by the analytical method used here, even in those fortified with 0.417 mol m^{-3} of urea. Only in the case of wine sample C, in either test TE or test UE, was the acid urease preparation incapable of degrading all of the urea initially present, probably because of the highest initial content of phenolic compounds (853 g m^{-3}).

By resorting to previous acid urease tests, use of the pseudo-first-order kinetic model together with the experimental k_{Ie} values listed in **Table 1** allowed the residual concentrations of A and S to be estimated using eqs 6 and 7, as shown in **Table**

Table 2. Experimental Concentrations of Ethyl Carbamate (EC), Urea (*S*), and Ammonium (*A*) in the Five Italian Wines under Study as Such (T) or Treated with 25 g m^{-3} of Acid Urease either with the Natural Urea Content (TE) or with 0.417 mol m^{-3} of Urea Added (UE) As Compared with Those Calculated via Equations 6 and 7 in Conjunction with the Corresponding Experimental Pseudo-First-Order Kinetic Rate Constants (k_{Ie}) Listed in **Table 1**

sample	parameter	T	TE		UE		unit
			exptl	calcd	exptl	calcd	
A	EC	10.2 ± 1.5	6.6 ± 1.7	-	12.7 ± 1.4		mg m^{-3}
	S	0.9 ± 0.2	nd[a]	1.0×10^{-5}	nd	3.0×10^{-4}	g m^{-3}
	A	5.0 ± 0.4	5.4 ± 0.5	5.5	19.1 ± 1.8	20.6	g m^{-3}
B	EC	14.8 ± 2.3	6.9 ± 1.4		9.3 ± 0.9		mg m^{-3}
	S	1.0 ± 0.3	nd	1.1×10^{-4}	nd	2.7×10^{-3}	g m^{-3}
	A	14.0 ± 1.1	14.4 ± 1.5	14.6	28.2 ± 3.1	29.6	g m^{-3}
C	EC	55.5 ± 4.5	31.8 ± 3.7		182 ± 18		mg m^{-3}
	S	1.2 ± 0.3	1.1 ± 0.3	0.11	4.7 ± 1.7	2.4	g m^{-3}
	A	6.5 ± 0.8	6.6 ± 0.7	7.2	16.7 ± 1.9	20.8	g m^{-3}
D	EC	243 ± 24	81.1 ± 9.1		83.4 ± 1.7		mg m^{-3}
	S	7.1 ± 1.2	nd	1.1×10^{-2}	nd	5.0×10^{-2}	g m^{-3}
	A	19.2 ± 1.9	22.6 ± 2.5	23.5	39.5 ± 4.2	38.5	g m^{-3}
E	EC	27.0 ± 2.5	14.6 ± 1.4		28.9 ± 2.4		mg m^{-3}
	S	2.7 ± 0.5	nd	2.2×10^{-4}	nd	2.3×10^{-3}	g m^{-3}
	A	6.7 ± 1.5	8.2 ± 2.2	8.4	22.0 ± 2.6	23.4	g m^{-3}

[a] Not detectable by the analytical method used.

2. Despite a small variation in the reaction temperature used in both tests, quite good agreement was noted between the experimental and calculated *A* and *S* values, except for wine C. In this case, the experimental data shown in **Table 2** appeared to be more or less stoichiometrically incongruent with eq 1, the moles of ammonium formed in tests TE or UE being about 3.3 or 1.6 times the moles of urea consumed, respectively.

In spite of the above discrepancy, the experimental k_{Ie} values estimated in short-run acid urease tests appeared to be able to describe the hydrolytic degradation of urea independently of the initial concentrations of enzyme and urea, confirming further the accuracy of the first-order kinetic model mentioned above.

Finally, the efficiency of the enzymatic treatment was assessed by submitting all wine samples to accelerated ethyl carbamate tests (**Table 2**).

When the wines containing natural levels of urea were treated with 25 g m^{-3} of acid urease for 30 days (test TE), the potential ethyl carbamate decreased with respect to its initial value (test T), thus showing the efficiency of the hydrolytic treatment. In the case of wines amended with 0.417 mol m^{-3} of urea, the same enzymatic treatment was generally able to restore the potential ethyl carbamate content of nontreated wines, except for wine sample C.

Among the wines submitted to the control test T, wine sample C containing a residual urea of about 1.2 g m^{-3} yielded quite an important amount of potential ethyl carbamate (56 mg m^{-3}), about 5 or 4 times greater than that observed in white wine A (ca. 10 mg m^{-3}) or B (about 15 mg m^{-3}), respectively, both of these containing an analogous initial concentration of urea (15−17 mmol m^{-3}). In accordance with the chemical equilibrium law this was likely due to the higher ethanolic fraction of wine C (14.4% v/v). In fact, by referring to the raw wines stored at 4 °C, the initial EC content of the most alcoholic wines, C and D, was about 4 mg m^{-3}, whereas that of the other samples ranged from 1.2 to 1.5 mg m^{-3} (**Table 1**).

The higher levels of ethanol and urea present in wine D are due to the alcohol enrichment process described in the production regulatory of the typical Italian liqueur wine Moscato di Trani, this process inducing yeast cell lyses and thus release of the endogenous urea in the end product.

The quite great concentrations of potential EC (ca. 80 mg m^{-3}) detected in wine D, once submitted to both tests TE and UE (**Table 2**), are highly likely ascribable to the reaction between ethanol (its volumetric fraction being as high as 17% v/v) and residual urea and/or other precursors such as citrulline and arginine (not determined). In fact, at the end of the accelerated test at 70 °C for 48 h in both tests TE and UE, the urea concentration was not detectable, being <17 mmol m^{-3}. Moreover, the wine samples were free of yeast cells so as to avoid further urea extraction. Thus, in the case of ethanol contents >14% v/v and minimum amounts of urea of the order of 17 mmol m^{-3}, wine storage at high temperatures may result in significant EC levels, even greater than the level (60 mg m^{-3}) presently established as a voluntary target for ethyl carbamate in fortified wines by the U.S. wine industry (*6*).

Effect of Phenolic Compounds on Acid Urease Activity. To clarify the specific inhibitory effect of phenolic compounds on the k_{Ie} values pertaining to the five Italian wines mentioned above, use of eqs 8−14 allowed a preliminary estimation of their pseudo-first-order kinetic rate constants (k_I) as due to their peculiar chemicophysical characteristics (that is, *M*, *L*, *K*, *E*, and pH) only, disregarding any other inhibitory effect, as reported in **Table 1**.

By referring the experimental pseudo-first-order kinetic rate constants (k_{Ie}) to those (k_I) concerning model wine solutions having the same composition and pH of the real wines tested, it was possible to observe that the relative activity of acid urease reduced from about 0.21 to 0.02 as the overall content of phenolic compounds (*P*) increased from 109 to 853 g m^{-3} of GAE (**Figure 3**).

By assuming that acid urease activity is strongly inhibited by such compounds behaving as competitive inhibitors by linking to the enzyme active site to form a stable enzyme−inhibitor complex (EP), it was possible to express the effective kinetic rate constant (k_{Ie}) as

$$k_{Ie} = \frac{k_I}{1 + \frac{P}{K_P}} \qquad (15)$$

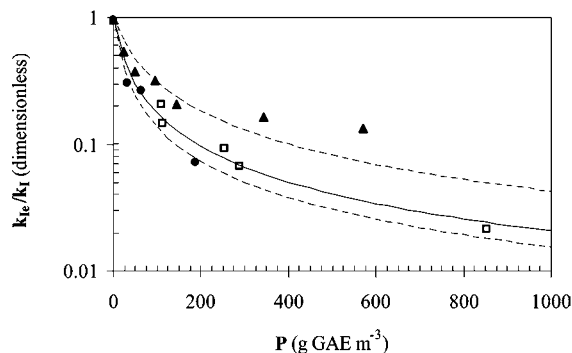


Figure 3. Effect of phenolic compounds on the ratio between the experimental pseudo-first-order kinetic rate constant (k_{Ie}), pertaining either to the real wines listed in **Table 1** (□) or to the model wines enriched with grape skin (▲) or seed (●) tannins, and calculated constant (k_I) as referred to model wines devoid of any phenolic compound, but with the same composition and pH.

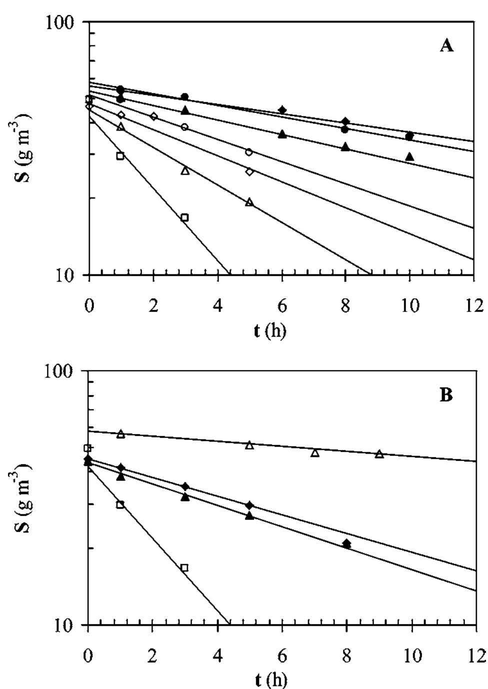


Figure 4. Time course of urea degradation in model wine solutions enriched with different amounts of grape skin [(**A**) ◆, 1200 g m^{-3}; ●, 720 g m^{-3}; ▲, 300 g m^{-3}; ○, 200 g m^{-3}; ◇, 100 g m^{-3}; △, 50 g m^{-3}; □, 0 g m^{-3}] or seed [(**B**) △, 300 g m^{-3}; ◆, 100 g m^{-3}; ▲, 50 g m^{-3}; □, 0 g m^{-3}] extract, treated with 238 g of acid urease m^{-3}. The continuous lines represent the mean squares regression lines.

where K_P is the equilibrium constant of the reaction between the enzyme (E) and inhibitor (P).

By fitting the ratio k_{Ie}/k_I as a function of P via a nonlinear estimation method, it was possible to minimize to 14.5% the mean percentage error among the experimental and calculated ratios by setting K_P to 21.1 ± 0.5 g of GAE m^{-3} (see continuous line in **Figure 3**).

To verify whether the observed correlation between k_{Ie} and P was due to the inhibitory effect of the phenolic compounds, the model wine solution corresponding to the composite design previously performed (*16*) was enriched with two different tannin sources extracted from grape skins or seeds in the ranges of 0−1.2 and 0−0.3 kg m^{-3}, respectively.

Figure 4 shows a semilogarithmic plot of the current urea concentration (S) against time (t) for the model wine solution enriched with both phenolic sources.

By using eq 6 and fitting the natural logarithm of S against t via the least-squares method, it was possible to determine the average value and standard deviation of the experimental pseudo-first-order kinetic rate constant (k_{Ie}) as a function of P.

By resorting to the competitive inhibition model previously applied to describe the phenolic compound inhibition, it was possible to determine the inhibition constant for both tannin sources as

$$K_P = 44.7 \text{ g of GAE m}^{-3} \quad \text{for grape skin extract}$$

$$K_P = 15.7 \text{ g of GAE m}^{-3} \quad \text{for grape seed extract}$$

The broken lines in **Figure 3** show a quite satisfactory agreement between the experimental and calculated kinetic constant rate ratios in both tests, the corresponding mean percentage errors being about 23.8 and 12.3%, respectively.

This result clearly confirms the greater inhibitory effect of phenolic compounds extracted from grape seeds than those extracted from grape skins. This agreed with previous findings by Trioli and Ough (*11*), who observed a greater reduction in the activity of the acid urease activity when the model wine samples were integrated with grape seed tannins with respect to those enriched with catechin in the range of 0−880 g of GAE m^{-3}. However, it is difficult to explain why the phenolic content of the wines assayed tended to inhibit the acid urease activity in just a little milder way ($K_P \approx 21$ g of GAE m^{-3}) than grape seed tannins ($K_P \approx 16$ g of GAE m^{-3}).

Thus, the main results of this study can be summarized as follows:

(1) The kinetics of acid urease in model or real wines can be safely regarded as being of pseudo-first-order with respect to urea for $S \leq 1$ mol m^{-3}. Thus, by virtue of eqs 6 and 7 this hydrolytic process can be described by means of a single independent parameter, that is, the pseudo-first-order kinetic rate constant (k_I).

(2) The specific acid urease activity of about 4 units mg^{-1}, as generally claimed by the manufacturers, is limited to a specified buffer solution enriched with ethanol (12.5% v/v) and urea (83.33 mol m^{-3}), the urea level being about 1000 times greater than that generally encountered in real wines (*7*).

(3) The pseudo-first-order kinetic rate constant (k_{Ie}) for a given wine to be treated may be roughly estimated by resorting to the empirical model developed here, that is, by using eqs 8−15 and referring to model wine solutions enriched with grape seed tannins to ensure the same composition and pH of the real wine of concern. In this way, by using eq 6 it is possible to obtain a preliminary estimation of the processing time (τ) needed to reduce the initial urea content (S_0) by a given factor, once the wine has been theoretically amended with the enzyme dose recommended (<75 g m^{-3}) to avoid any unpleasant taste in the wine after treatment (*6*).

(4) To check for such estimates (k_{Ie}, τ), it is suggested that the wine samples under study, as such or enriched with 1 mol m^{-3} of urea, be submitted to accelerated acid urease tests at 20 °C using a high dose of enzyme ($E_0 \approx 250$ g m^{-3}) to shorten the process to only a few days.

(5) Once the effective pseudo-first-order kinetic rate constant has been determined, it will be possible to check for the preliminary estimates of τ and k_{Im}, as well as for the hydrolytic process efficiency, by submitting simultaneously samples of the filtered raw and urea-unspiked, acid urease treated wines to accelerated ethyl carbamate tests.

In conclusion, the short-cut experimental procedure outlined here appears to be useful to assess preliminarily not only the contribution of the inhibitory components present in the wine of concern but also the technoeconomic feasibility of such an acid urease based detoxification process (*16*).

NOTATION

A concentration of ammonium ions (mol m^{-3})
E_0 initial enzyme concentration (g m^{-3})
K concentration of potassium metabisulphite (kg m^{-3})
K_P phenolic compound inhibition constant (g GAE m^{-3})
K_M Michaelis−Menten constant (mol m^{-3})
k_I pseudo-first-order kinetic rate constant for ammonia (m^3 g^{-1} min^{-1})
L concentration of lactic acid (kg m^{-3})
M concentration of malic acid (kg m^{-3})
m/z ion mass-to-charge ratio
P phenolic compound concentration (g of GAE m^{-3})
r^2 coefficient of determination (dimensionless)
r_A ammonium formation rate (mol m^{-3} min^{-1})
r_S urea degradation rate (mol m^{-3} min^{-1})
S concentration of urea (mol m^{-3})
t reaction time (min)
TA concentration of tartaric acid (kg m^{-3})
X_i generic principal axis of eq 8
x_i generic coded independent variables, as defined by eq 13
y_E ethanol volumetric fraction (% v/v)

Greek Symbols

π_A specific enzyme activity ($= r_A/E_0$, units mg^{-1})
τ processing time needed to reduce urea concentration by a given factor (day)

Subscripts

0 initial condition
e experimental

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