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ARTICLE

Polyphenol Oxidase Activity from Three Sicilian Artichoke [*Cynara cardunculus* L. Var. *scolymus* L. (Fiori)] Cultivars: Studies and Technological Application on Minimally Processed Production

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Several papers helped with the development of more methods to control browning, or study thermal polyphenol oxidase (PPO) inactivation, but did not provide any solutions to technological process problems and food process improvement. Artichokes [*Cynara cardunculus* L. var. *scolymus* L. (Fiori)] are susceptible to browning; this alteration could affect and reduce the suitability for its use, fresh or processed. Within this study, the catecholase and cresolase activities of PPO from three different Sicilian artichokes cultivar were characterized with regard to substrate specificity and enzyme kinetics, optimum pH and temperature, temperature and pH stability, and inhibitor test; all of the results were used for technological purposes, particularly to optimize minimally processed productions (ready-to-eat and cook-chilled artichokes).

KEYWORDS: Polyphenol oxidase; enzymatic browning; *Cynara cardunculus* L. var. *scolymus* L. (Fiori); inhibition

INTRODUCTION

The artichoke, *Cynara cardunculus* L. var. *scolymus* L. (Fiori), is a member of the Asteraceae family; it is cultivated in the Mediterranean regions and produces edible flower heads with a high nutritional value (*1*−*5*). In Italy, the production of artichoke flower heads is over 419800 tons/year (*6*), and it is largely used in the fresh market.

The artichoke (*Cynara scolymus* L.) is known for its nutritional properties. The chemical components of artichoke leaves and globe have been studied extensively, and many authors have found different polyphenolic compounds with antioxidant activity (*7*, *8*). In many studies, including in vitro, in vivo, and human trials, pharmacological activities, including choleretic (*9*), lipid-lowering, and antiatherogenic (*10*, *11*), hepatoprotective (*12*), and inhibition of cholesterol biosynthesis (*13*), were well documented.

Moreover, this vegetable contains an enzyme called polyphenol oxidase (PPO; EC 1.10.3.1), which is responsible for browning in plants. Browning is the main process responsible of quality loss during postharvest handling, storage (*14*−*16*) and a limiting factor to processing artichoke.

In the presence of oxygen, this copper enzyme catalyzes the hydroxylation of monophenols to *o*-diphenols (cresolase activity) and the oxidation of *o*-diphenols to their corresponding *o*-quinones (catecholase activity) (*17*). Management of the browning process represents the major challenge to food scientists. The control of browning in artichoke hinges upon an understanding of several parameters: its pathway, the polyphenol oxidase properties, their substrates and inhibitors, and the chemical, biological, and physical factors that affect each of these parameters. Several authors studied and characterized PPO from artichokes: Espin et al. (*17*) isolated PPO from artichoke, avoiding the use of severe methods, and tested and confirmed the reaction mechanism of the monophenolase activity previously proposed for other PPOs; Ziyan and Pekyardimci (*18*) extracted and characterized PPO from artichoke skin and flesh; Lattanzio et al. (*19*) studied browning phenomena in stored artichoke heads trying to understand the enzymatic or chemical nature. None of these studies, although they helped in the development of methods to control the browning process or studied PPO thermal inactivation, provided solutions to problems with the technological process or suggested improvements to the food process.

The aim of this work was to provide an innovative approach on PPO study and characterization. All of the biochemical information (particularly, total enzymatic activity, biochemical seasonal changes, and thermal chemical inactivation parameters) could help the development of specific technological purposes for food, particularly to optimize the minimally processed productions (ready-to-eat and cook-chilled artichokes). Because enzymes have been successfully used as chemical markers to evaluate raw material quality and heat treatment in other products, we hypothesize that PPO may be employed as a potential intrinsic index for cultivar selection and thermal pasteurization validation in minimally processed artichokes.

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MATERIALS AND METHODS

Plant Material. Artichokes, *C. cardunculus* var. *scolynus* L. (Violetto di Sicilia, Violetto di Provenza, and Tema 2000 cultivars) were grown in the experimental fields (Ramacca, Catania, Italy; Menfi, Agrigento, Italy) of the University of Catania. Soil preparation, fertilization, irrigation, plant protection, and other growing practices were those commonly applied by Italian farmers. The artichokes heads were harvested by hand for two consecutive seasons (winter and spring 2007) and stored at a temperature of 10.0 ± 0.5 °C with 85% relative humidity for 24 h until analysis. Artichoke heads (capitules) were harvested at marketing stage regardless of size. During the fall−winter harvest period (from October to February), harvesting was once a week. In the spring (from March to May) harvesting was done every 3−4 days. All artichoke heads were weighed without stalk. In this work, only marketable heads were considered.

Violetto di Sicilia, Violetto di Provenza, and Tema 2000 cultivars were selected and studied in this experiment because of their wide use and their rapid browning after cutting.

Sample Preparation. The heads were gently washed with drinking water, and excess water was removed with a manual salad spinner; afterward, external leaves were removed down to the heart, and the top was sliced off. The obtained heads were ground by an Ultra-Turrax T25 homogenizer (Janke & Kunkel, Staufen, Germany) set at speed 3 for 60 s.

Determination of Total Phenolics. Total polyphenol analysis was carried out according to the method of Singleton and Rossi (*20*). The results were expressed as milligrams of chlorogenic acid per 100.0 g of fresh weight of plant material.

PPO Extraction and Assay. PPO extraction was carried out according to the method of Espin (*17*). One hundred and fifty grams of ground artichoke was homogenized at 0 °C in 100 mL of 0.1 M phosphate buffer (pH 6.5). The homogenate was filtered, and the filtrate was centrifuged at 15000*g* for 30 min at 4 °C. The obtained supernatant was used as crude extract and was ultrafiltered with 50 kDa cutoff (Millipore, Bedford, MA). PPO partially purified was stored at 20 °C, and its assay was carried out according to the method of Mazzocco (*21*) and Espin (*17*) by using cathecol as substrate. Enzymatic activity was expressed as enzymatic units (mmol min^{-1} g^{-1}) per gram of vegetal material.

Different phenols were tested as substrates to find the best one: chlorogenic acid (*22*), 4-methylcatechol, 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxyhydrocinnamic acid, and caffeic acid.

Substrate Specificity and Enzyme Kinetics. Six different commercial grade substrates (3,4-dihydroxyphenylacetic acid, 3,4-dihydroxyhydrocinnamic acid, 4-methylcatechol, caffeic acid, catechol, chlorogenic acid_ were used to study the Michaelis−Menten constant (K_m) and maximum reaction velocity (V_{max}) at five different concentrations and at standard conditions. Data were plotted as $1/V$ and $1/S$ concentration (*23*).

Optimum pH and Temperature. PPO activity, as a function of pH, was determined under standard assay conditions (20 °C using catechol as substrate) using various buffers: 0.1 M sodium acetate (pH 5.0−5.6), 0.1 M citrate phosphate buffer (pH 2.7, 4.0), and 0.1 M phosphate buffer (pH 6.5, 7.0, 8.0). PPO activity, as a function of temperature, was determined under standard assay conditions (pH 6.5, using catechol as substrates) using temperatures from 4 to 90 °C.

Stability to Temperature. PPO extracted was incubated at 75 °C at pH 4.0 (canned vegetable pH) and 100 °C at pH 4.0 and 5.8 (artichoke pH) for different times (minutes), and, after cooling, the residual enzyme activity was calculated. Thermal stability of PPO was determined by heating the enzyme solution at various temperatures between 4 and 90 °C for several minutes at pH 6.5.

***D*, *z*, and E_a (*24*).** The kinetic data *D* and *z* on the inactivation of PPO at 75 (pH 4.0) and 100 °C (pH 5.8; 4.0) were analyzed using a conventional first-order model described by Bigelow's primary and secondary relationships:

$$\log A_t = (\log A_0 - t)/D$$

A_t is the mean residual of enzyme activity after t minutes of thermal treatment, A_0 is the mean initial enzyme activity, and D is the decimal reduction time definite as the temperature treatment time needed for 90% inactivation of initial activity at a given temperature; D values allowed application of Bigelow's secondary relationship, calculating the values of z (decimal reduction temperature)

$$\log D_0/D_t = (T_t - T_0)/z$$

with D_0 being the initial decimal reduction time, D_t the decimal reduction time at time t, T_0 (°C) the initial temperature, and T_t (°C) the temperature at time t. The temperature dependence of the D values is given by the z value. The z value equals the temperature increase necessary to obtain a 10-fold decrease of the D value.

Activation energy (E_a) was determinated, according to the Arrhenius equation, by measuring the maximal initial rate at different temperatures and plotting the logarithmic value of V_{max} versus $1/T$ (*22*).

Stability to pH. The stability to pH of the enzyme was examined by incubating the extract at different pH values, 4.0 (canned vegetable pH) and 6.0 (natural artichoke pH), at 25 °C for 0, 10, 30, and 120 min and calculating the residual activity.

Inhibitor Tests. To analyze the inhibition of PPO activity by ascorbic acid, citric acid, and tartaric acid, a constant aliquot of each inhibitor was (respectively) added to the reaction solution. PPO activity was determined using the methods described previously with five different substrate concentrations. The relative enzymatic activity was expressed considering the activity without inhibitor as 100.

Color Analysis. The CIELAB coordinates (L^*) of artichokes cut on the surface were directly read with a colorimeter NR-3000 (Nippon Denshoku Industries Co. Ltd., Tokyo, Japan). For each artichoke, measurements were taken at five different points, immediately after cutting at 25 °C.

Statistical Analysis. All determinations were done in triplicate, unless noted otherwise. Analysis of variance (ANOVA) of the data was evaluated by the Statistical Analysis System (SAS ver. 9.0). Least significant difference (LSD) was employed to determine the statistical significance of the differences between the means ($p \leq 0.05$).

Table 1. Total Polyphenols in Two Different Seasons of Harvest (Winter and Spring)

cultivar	harvest	total polyphenols (mg of chlorogenic acid/g of FW)	PPO activity units/g of FW (mmol g^{-1} min^{-1})
Violetto di Sicilia	Dec 18, 2004 (winter)	1.33	20.06
Violetto di Provenza		1.42	13.05
Tema 2000		1.79	27.04
Violetto di Sicilia	April 4, 2005 (spring)	8.88	13.64
Violetto di Provenza		5.19	14.44
Tema 2000		10.19	19.32

RESULTS AND DISCUSSION

Determination of Total Phenols, Browning, and Total PPO Activity. Comparison of total polyphenol contents between the two harvest seasons (winter and spring) led to significant differences ($p < 0.05$) being higher in the spring harvest, according to published literature (*25*) (data shown in **Table 1**). Polyphenols increase improved the nutritional quality and biochemical stability of the artichokes due to their antioxidant activity.

Similarly, harvest season affected PPO activity; a decrease in activity was noted in the spring harvest except for cultivar Violetto di Provenza, which showed a quite stable and low level of enzymatic activity in both seasons. Tema 2000, although it showed a drop in activity during spring harvest, maintained the highest level of PPO activity. Browning varied among different cultivars. All L^* values showed a very significant decrease after cutting. Particularly, Tema 2000 presented the highest drop, whereas Violetto di Provenza showed the lowest (**Figure 1**).

To better understand the relationship between PPO, polyphenol content, and browning, the correlations between these

parameters were calculated. A good correlation was found between PPO activity and total phenols (0.98), between browning and PPO activity (0.91), and between browning and total polyphenols (0.97) as described by other authors in different vegetal matrices (*25*).

Substrate Specificity and Enzyme Kinetics. PPO activity in partially purified extracts was examined with regard to its monophenolase and diphenol oxidase activities. The result suggested that this enzyme was an *o*-diphenol oxidase, and no cresolase activity has been found. K_m and V_{max} values calculated with different substrates at various concentrations are shown in **Table 2**.

Cultivar Tema 2000 showed the highest PPO activity for each substrate analyzed, whereas cultivar Violetto di Provenza showed the lowest; this result led us to consider this cultivar as the most suitable for minimal processing production. Chlorogenic acid resulted in the highest level of enzyme activity in cultivars Tema 2000 and Violetto di Sicilia, whereas 4-methylcatechol resulted in the highest level of enzyme activity in cultivar Violetto di Provenza. Maximum activity toward chlorogenic acid could be explained by its natural presence in artichoke tissue as the main polyphenolic compound.

Optimum pH. As shown in **Figure 2** the pH optima for artichoke PPO were found to have a very broad range between 4.0 and 7.0, with some differences among cultivars. Outside these values, a significant drop in the activity was found in each cultivar as other authors have described (*26*)

Optimum Temperature. The effect of temperature between 4 and 90 °C on PPO activity from different cultivars is shown in **Figure 3**. The optimum temperature for each cultivar was 50 °C with a gradual decrease with temperatures changes. An anomalous, but common, trend for each cultivar has been noted in the temperature range between 4 and 16 °C, due probably to the presence of isoenzymes, but this hypothesis needs further investigation.

Stability to Temperature. It was found that the enzyme was stable at 4 °C for 240 min, but was unstable at 75 and 100 °C in each cultivar as other authors have described (*18*).

To better understand the thermal technological properties of cultivars, *D* and *z* parameters were calculated at 100 °C. Data are shown in **Table 3**. The *D* value was affected by PPO stability and content. Because different cultivars showed different PPO levels, it was necessary to include the contribution of PPO activity on *D* definition. Cultivar Violetto di Sicilia showed the lowest absolute *D* value, and for this reason it was selected as reference to

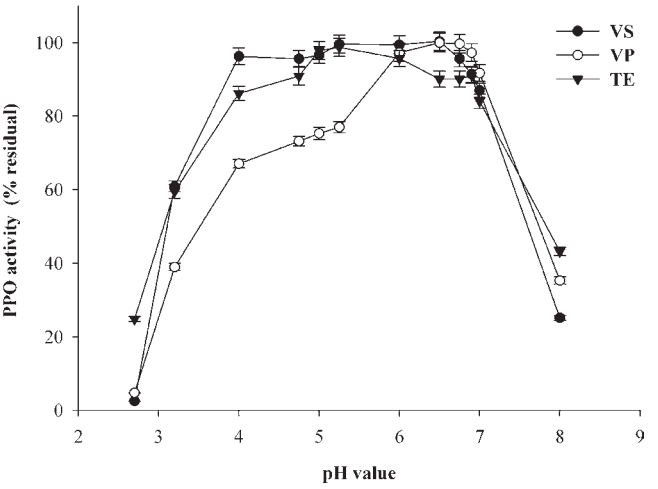

Figure 2. Optimal pH for cultivars Violetto di Sicilia, Violetto di Provenza, and Tema 2000.

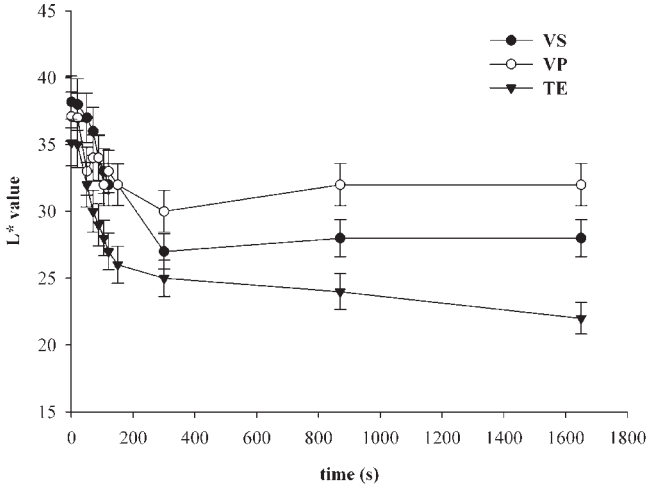

Figure 1. Browning expressed as *L** parameter in Violetto di Sicilia, Violetto di Provenza, and Tema 2000.

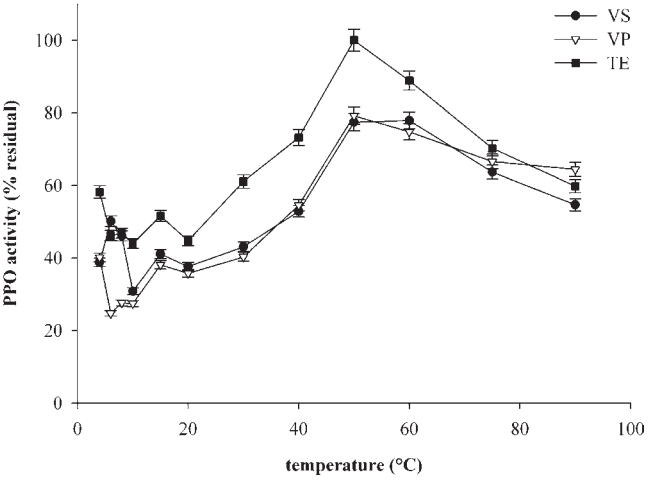

Figure 3. Optimal temperature for cultivars Violetto di Sicilia, Violetto di Provenza, and Thema 2000.

Table 2. K_m and V_{max} Calculated with Different Substrates at Various Concentrations in Violetto di Sicilia, Violetto di Provenza, and Tema 2000

	Violetto di Sicilia			Violetto di Provenza			Tema 2000		
substrate	V_m	K_m	V_m/K_m	V_m	K_m	V_m/K_m	V_m	K_m	V_m/K_m
catechol	4.79	1.35	3.56	3.04	1.81	1.67	6.17	2.38	2.59
4-methylcatechol	8.79	2.75	3.19	20.89	10.2	2.05	22.69	4.23	5.37
3,4-dihydroxyphenylacetic acid	9.4	3.8	2.47	5.07	5.01	1.01	9.43	1.03	9.19
3,4-dihydroxyhydrocinnamic acid	10.13	1.38	7.37	6.76	0.82	8.22	24.08	2.29	10.52
caffeic acid	4.89	1.5	3.25	3.1	2.99	1.04	12.35	1.48	8.32
chlorogenic acid	17.91	2.5	7.17	7.39	2.84	2.61	31.66	2.5	12.66

Table 3. *D* and *z* Parameters Calculated at 100 and 75 °C at pH 4.00 and 5.8 in Cultivars Violetto di Sicilia (VS), Violetto di Provenza (VP), and Tema 2000 (TE)

	heat treatment at 100 °C						heat treatment at 75 °C		
	pH 5.8			pH 4.0			pH 4.0		
parameter	VS	VP	TE	VS	VP	TE	VS	VP	TE
PPO activity (mmol $min^{-1}g^{-1}$)	2.79	1.33	3.94	2.78	0.89	3.40	2.78	0.89	3.40
D (min)	41.00	43.80	58.10	0.25	0.32	0.26	11.25	9.22	11.22
z	53.60	56.00	60.50	15.17	17.19	15.36	15.17	17.19	15.36
E_{disatt} (kJ mol^{-1})	45.14	43.21	40.04	144.66	127.67	142.93			
real *D*[a]	41.00	20.85	82.26	0.25	0.16	0.29	11.23	4.64	12.19

[a] All relative values were calculated using cultivar Violetto di Sicilia as reference.

calculate all relative values according to the following formula:

$$D_{relative} = (D_{absolute} \times PPO_{cultivar})/PPO_{Violetto\ di\ Sicilia}$$

Violetto di Provenza showed the lowest *D* and *z* parameters; this result suggested that it could be the most suitable cultivar for the blanching treatment.

Nevertheless, all *D* values obtained at 100 °C probably affected the nutritional content of artichokes; with regard to the PPO, in fact, a heat treatment at 100 °C from 20 to 80 min decreased enzyme activity (62.5%). To decrease nutritional loss, a heat treatment at 75 °C in a low pH (4.0) solution was tested according to hurdle technology. This treatment successfully decreased the *D* value (from 41 to 13 min) and enzyme activity (10%).

Stability to pH. The stability of PPO at different pH values is shown in **Figure 4**. At pH 6.0 it is possible to note that all cultivars were stable with quite an increase of activity from 6 to 8% around 30 min, but at 120 min the activity decreased by about 30%. Probably this phenomenon is due to the fact that the enzyme's conformation will be more favorable at this pH condition, which in turn is very similar to the artichoke's pH.

At pH 3.0 PPO the activity decreased quickly and strongly (about 60% after 120 min of treatment), whereas at pH 4.0 it was quite stable in each cultivar (about 20% after 120 min of treatment).

Inhibition of PPO. Inhibition study experiments were carried out to determine the inhibitory effect of three different organic acids on PPO activity from different cultivars (**Table 4**). Citric acid and tartaric acid treatments had a similar inhibitory effect on Violetto di Sicilia and Violetto di Provenza; in particular, tartaric acid was the most successful in reducing the activity at low concentrations (about 70% reduction), whereas citric acid resulted in 45% activity reduction. The same inhibitors at low concentration did not affect or reduce PPO activity from Tema 2000 until 0.2 M concentration.

The inhibitory action of ascorbic acid on cultivars Violetto di Sicilia and Tema 2000 was noted from low (0.04 mM) to high concentrations, whereas this effect on cultivar Violetto di Provenza was completely different. In fact, it was restricted to a small range of concentrations (around 0.4 mM). For lower concentrations it did not have any effect, whereas for higher concentrations it stimulated PPO activity.

With regard to artichokes as raw material for minimal processing, it could be concluded that Violetto di Provenza seems to be the most suitable cultivar for that purpose due to its low PPO activity with no seasonal variability; Violetto di Sicilia showed the lowest PPO activity with strong seasonal variability, whereas Tema 2000 showed the highest PPO activity throughout the year. The characterization of the PPO extracted showed the optimal conditions of catalysis and inhibition by organic acids and ascorbic acid. This information could be extremely useful in the production of artichoke heads ready to eat. Ascorbic acid was found to be the most suitable organic acid to increase quality and shelf life because it was successful at low concentration and because it did not affect sensory parameters and at the same time increased the vegetal nutritional value with low additional cost (vitamin C price around 20 euros/kg).

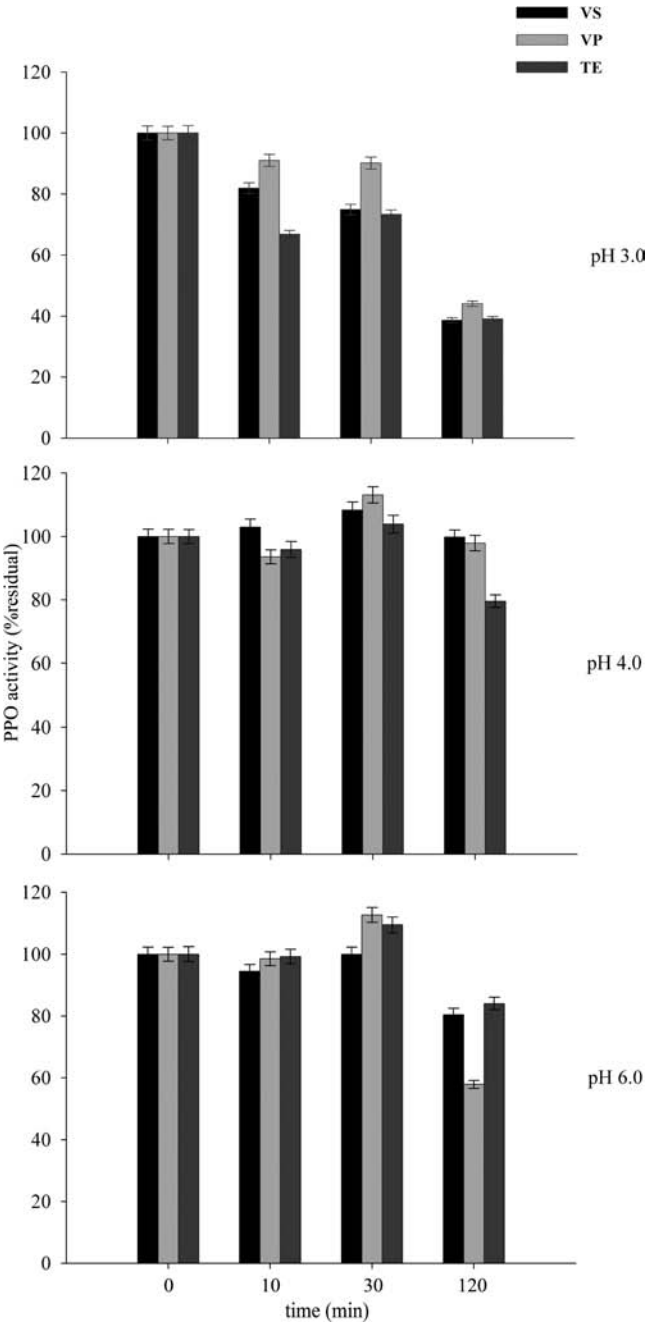

Figure 4. PPO stability to pH in cultivars Violetto di Sicilia, Violetto di Provenza, and Thema 2000.

Table 4. Inhibitor Effect on PPO Activity

		PPO activity decrease (%)		
	mM	Violetto di Sicilia	Violetto di Provenza	Tema 2000
citric acid	0	100	100	100
	40	48	89	92
	100	44	63	94
	160	39	61	95
	200	33	59	22
	400	37	60	18
tartaric acid	0	100	100	100
	40	49	61	94
	100	28	34	95
	160	14	33	93
	200	2	28	83
	400	2	18	29
ascorbic acid	0.000	100	100	100
	0.004	62	58	37
	0.040	47	47	34
	0.400	27	29	11
	2.000	11	68	8

Related to chilled-cooked artichokes the study of *D* and *z* parameters showed that Violetto di Sicilia seems to be the most suitable cultivar to be processed due to the heat instability of its PPO enzyme that minimized thermal nutritional and quality losses.

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