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Occurrence and specificity of glucose oxidase (E.C: 1.1.3.4) in botrytized sweet white wine. Comparison with laccase (E.C: 1.10.3.2), considered as the main responsible factor for oxidation in this type of wine

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Summary

Two types of oxidizing enzymes are present in botrytized white grapes and wines: laccase (PPO) and glucose oxidase (GOX). The evolution of these two enzymes is similar both during the over-ripening of grapes and during wine making. Yet, PPO is severely inhibited by the addition of SO_2 following the alcoholic fermentation, and shows a marked instability in both the must and wine environments. GOX, however, remains free and active in solution and helps develop the main characteristics of the wine. In particular, as is to be expected from its activity, GOX oxidizes tartaric acid, ethanol and glycerol, the major components of must and wine, respectively to glyoxylic acid, acetaldehyde and glyceraldehyde. And then, by nucleophilic additions under acidic conditions, these products react with catechins and proanthocyanidins to form several new compounds, some of which appear in a colored form. These reactions can have an impact on the visual quality of the wine.

K e y w o r d s : Botrytized sweet white wine, *Botrytis cinerea*, laccase, glucose oxidase, polyphenols.

Introduction

The effect of polyphenol oxidase on grape ripening and during the production of botrytized sweet white wines or during traditional wine making is well understood and documented (Dubernet and Ribereau-gayon, 1973, Mayer 1978, Ribereau-gayon *et al.* 1979, Salgue *et al.* 1986, Donèche 1991). Laccase (PPO), a fungal polyphenol oxidase from *Botrytis cinerea*, has been widely studied in relation to its effect on the composition and quality of must and wine (Ribereau-gayon *et al.* 1998, Minussi *et al.* 2007, Li *et al.* 2008). In particular, PPO has been shown to be the main enzyme involved in oxidation of red and white wines from rotten grapes. This involves oxidation at all stages: the winemaking process, ageing, and while stored in bottles. This is largely because PPO is an ubiquitous enzyme in the plant world, requiring a polyphenol substrate, and is well adapted to the physicochemical conditions of wine (pH, ethanol, SO_2).

In the wine industry, botrytized grapes are typically considered as belonging to two different groups. The first corresponds to a condition where the grape quality is affected by the formation of gray rot. This is a frequent situation when cloudy/humid conditions occur close to maturity (Donèche 1991, Fregoni *et al.* 1986, Donèche 1989). The second group applies to very specific wine-growing regions, where *Botrytis cinerea* contributes to an over-ripening of the grapes, which can then be used in the production of naturally sweet wines, typically white wines such as Sauternes (Ribereau-gayon *et al.* 1998). The specificity of these wines is the result of two different reactions: a biochemical transformation of the grape berries under the action of *Botrytis cinerea*, and a dehydration/concentration process. Winemakers distinguish three different stages in grapes affected by *Botrytis cinerea*, which are thus harvested at different times: first, the stage of complete botrytization of the grape berry (Stage B), followed by a desiccation process along with the corresponding increase in sugar concentration (BD), and finally, depending on the climatic conditions of the year, several days after the BD stage, we get the richest and best quality grape berries (BD^+).

Botrytis cinerea produces many other enzymes when infecting grapes (Salinas *et al.* 1986, Gunata *et al.* 1989, Movahedi and Heale 1990, Tobias *et al.* 1993, Sasaki and Nagayama 1995). One of these, glucose oxidase (GOX), typically oxidizes polyphenols both in grapes and in wine (Vivas *et al.* 2009). GOX catalyses the conversion of glucose into gluconic acid and releases H_2O_2, which then permitted to oxidize many compounds such as polyphenols. Based on the initial results, a new way of oxidizing botrytized wine through GOX instead of PPO was identified. In this article, we attempt to give a plausible explanation of our preliminary observations.

Material and Methods

R e a g e n t s a n d e n z y m e s : Water was purified using a Milli-Q system (Millipore, Bedford, MA). Ethanol, iron (II) in the form of $FeSO_4$, and SO_2 in the form

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of $NaHSO_3$ were purchased from Prolabo (Fontenay-sous-Bois, France). ß-D-glucose, sodium hydroxide, tartaric acid, glycerol, (+)-catechin, acetaldehyde, glyceraldehyde, glyoxylic acid and methanol were purchased from Aldrich (Saint-Quentin, France). For the laboratory experiments, the following commercial enzymes were used in model solutions: PPO (laccase, E.C: 1.10.3.2) from *Coriolis versicolor* (300 U/g) and GOX (glucose oxidase, EC: 1.1.3.4) from *Aspergillus niger* (148,400 U/g), also purchased from Aldrich. Bound SO_2 was prepared by mixing equimolar amounts of $NaHSO_3$ and acetaldehyde, a mixture ready for use after 2 h at room temperature. Ascorbic acid and bentonite used for must and wine treatment came from Laffort Oenologie (Bordeaux, France).

Measurements in technological conditions: The activities of GOX and PPO in botrytized grapes and in the corresponding wines were estimated both during the over-ripening process and during winemaking in a winery in the Sauternes region (France). For experiments with sulfites, the SO_2 level was measured by the Ripper method (Ribereau-Gayon *et al.* 1977).

Composition of the model solutions: Enzyme activity measurements were recorded for two different solutions: a Must-Like Solution (MLS) and a Wine-Like Solution (WLS). The compositions of these solutions were respectively: for 1 L of MLS, 5 g/L tartaric acid, 180 $g \cdot L^{-1}$ β-D-glucose, 180 $g \cdot L^{-1}$ β-D-fructose, 10 $g \cdot L^{-1}$ glycerol, pH 3.8 (adjusted with 1N NaOH), 1 $g \cdot L^{-1}$ (+)-catechin, 1 $mg \cdot L^{-1}$ $FeSO_4$, with the balance being water; and for 1 L of WLS, 5 $g \cdot L^{-1}$ tartaric acid, 13 % vol. ethanol, 50 $g \cdot L^{-1}$ β-D-glucose, 50 $g \cdot L^{-1}$ β-D-fructose, 20 $g \cdot L^{-1}$ glycerol, pH 3.8 (adjusted with 1N NaOH), 1 $g \cdot L^{-1}$ (+)-catechin, 1 $mg \cdot L^{-1}$ $FeSO_4$, with the balance being water.

Enzyme reactions in the model solutions: All oxidations by enzyme reaction in the laboratory were conducted with MLS and WLS, at 20 °C, to increase the reaction rate, in darkened conditions but with exposure to air. Based on the PPO and GOX activities in must and in wine, a level of 20 U/L was selected. MLS and WLS without any enzymes were used as control samples. Oxidation of (+)-catechin, as shown by the appearance of a characteristic yellow color, was measured at 420 nm using a spectrophotometer. Experiments were repeated 12 times.

LC-DAD-ES: For measurement of residual catechin and characterization of the yellow-brown color developed during the enzyme-induced oxidation of (+)-catechin with GOX, the solution was studied by HPLC and detection was recorded using a DAD and an ESI-MS detector on a Q.Star Elite apparatus (Applied System™) with a Waters separation module system, and using Millenium32 chromatography manager software. Spectra were recorded at 280 nm and 440 nm. The column used was a reverse-phase Interchim C_{18} (10 mm packing, 250 x 4.7 mm i.d.) protected with a guard column of the same material; solvent A, water/formic acid (98:2, v/v); solvent B, acetonitrile/water/formic acid (80:18:2, v/v). The column was used at ambient temperature. The elution program was performed at a constant flow rate of 1 $mL \cdot min^{-1}$, passing from 5 to 30 % of B in 40 min, and then rising to 40 % of B in 10 min, and finally to 100 % of B in 5 min, followed by washing and re-equilibrating the column for 15 min. The injection volume was 20 µL. An electro spray ionization source in negative-ion mode was used. The ion spray voltage chosen was 4.5 kV, with a capillary temperature of 275 °C.

Measurement of GOX and PPO activities, and of gluconic acid: For the GOX assay, Megazyme kits (Bray, Ireland) were used as described in the literature (Vivas *et al.* 2009). For the PPO assay, the syringaldazyne assay described by Dubourdieu *et al.* (1984) was used. Gluconic acid was quantified using an enzyme measuring kit from Boehringer Manheim (Biopharm, Darmstadt).

Standard reaction products of catechin-GOX in MLS and WLS: To identify the products formed by GOX activity, standard compounds were generated using hemisynthesis. As is well documented (Vivas *et al.* 2009), GOX reactions produce several different compounds. These come from oxidation of tartaric acid (to give glyoxylic acid), of ethanol (to give acetaldehyde), and of glycerol (to give glyceraldehyde). A series of solutions containing catechin with acetaldehyde, glyceraldehyde or glyoxylic acid were prepared in 12 % vol. ethanol, pH 2 (1N HCl) in darkened conditions at 25 °C. The catechin/aldehyde or catechin/acid molar ratios were 4/10. After 8 d, the solution was concentrated to a volume of 2 mL, using a rotary evaporator at 30 °C. The main products were purified by semi-preparative HPLC on a C_{18} reverse-phase column. The same procedure as for analytical conditions was used, except for the column (Sunfire 5 µm, 10 x 250 mm), the injection volume (200 µL) and the flow rate (3 $mL \cdot min^{-1}$). All products were characterized using HRMS and ESI^-/MS^2. The corresponding structures are shown in Fig. 1, and the chromatographic and spectrometric characteristics are summarized in Tab. 1.

Results

Evolution of PPO and GOX activity during grapes and wine technology: PPO and GOX activities were tracked during the over-ripening period before harvesting, and during the main winemaking steps. On the vine, the specific ripening process of botrytized grapes followed several stages (Fig. 2). During the botrytization period, both GOX and PPO activities increase to a maximum around the BD stage. Then, depending on the variability of the measurements recorded, the results show that both activities remain more or less constant. During the winemaking phase, the first step consisted of extracting the juice with a press and then, after alcoholic fermentation by *Saccharomyces cerevisiae,* various treatments were applied to the new wine. Tab. 2 compiles GOX and PPO activity values with gluconic acid content and spectrophotometer measurements at 420 nm of the yellow color of must and wine. Pressing caused a continuous extraction of GOX enzymes, consistent with the level of gluconic acid. As regards PPO, activity seems to be stable close to 100 U/mL. Adding sulfites to wine neu-

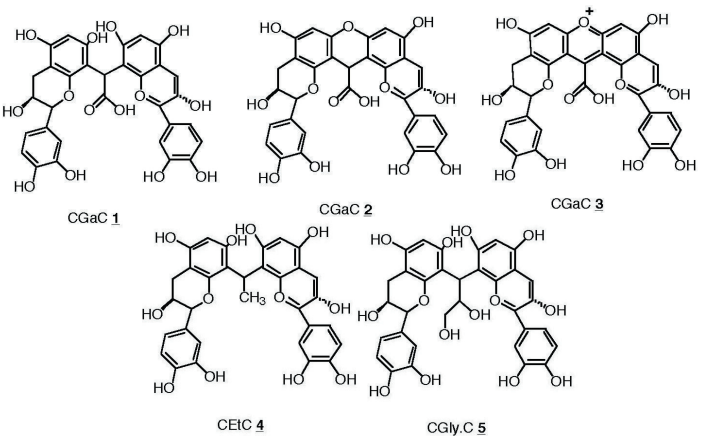

Fig. 1: Structure of some characteristic products derived from nucleophilic additions with catechin in must and wine; these products are used as standards for identifying products resulting from GOX-catechin reactions. CGaC **1**, CGaC **2** and CGaC **3**: Products derived from addition reactions with a tartaric acid oxidation product (glycoxylic acid); CEtC **4**: Product derived from an addition reaction with acetaldehyde produced from ethanol oxidation; CGly.C **5**: Product derived from an addition reaction with glyceraldehyde produced from glycerol oxidation.

Table 1

Chromatic and structural characteristics of certain standard products derived from nucleophilic reactions with catechin, and used to demonstrate the specific effect of GOX in model must and wine solutions

	Rt (min)	λmax, UV, Vis. (nm)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)
CGaC[1] **2a, b, c, d**[2]	17.5, 27.7, 28.5, 32	280	635	617, 345, 289
CGaC[1] **3a, b**[2]	35, 44.8	280	617	327, 289
CGaC[1] **4a, b**[2]	34, 36.7	280, 440	615	289
CEtC[1] **5a, b**[2]	42, 47, 49.5	280	605	317, 289
CglyC[1] **1a, b, c**[2]	7.8, 9.5, 10	280	653	363, 289

[1] C: catechin moiety; Ga:glyoxylic acid linkage; Et: acetaldehyde linkage; Gly: glyceraldehyde linkage.
[2] Numbering of the various peaks on the chromatograms.

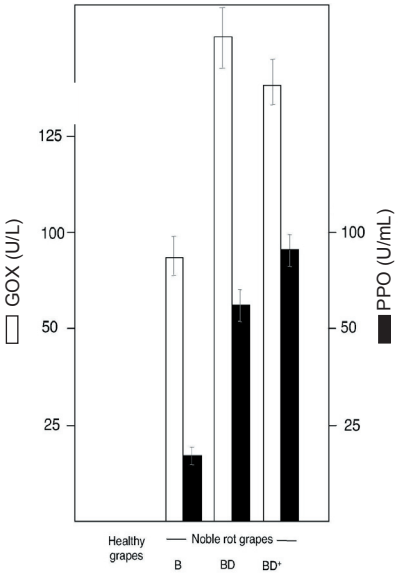

Fig. 2: Evolution of GOX and PPO activities during the botrytization process of 'Semillon' grapes in a Sauternes vineyard (2008). Noble rot stages: B, completely botrytized; BD, botrytized and desiccated; BD^+, highly concentrated, late harvested.

tralized PPO activity, but this was not the case with GOX. The latter shows a significant decrease in activity (78 %), and ascorbic acid or bentonite has a limited impact on residual GOX activity, which implies that the residual GOX remains stable in wine. In addition, the results suggest that sulfites have a determining influence on both enzymes in wine conditions.

Specific effects of sulfites: Sulfites are used during all winemaking and ageing processes. They have a big impact on both GOX and PPO enzymes, but with different intensities (Fig. 3). In winery conditions, a significant inhibiting effect on PPO and GOX activities is noticed with more than 4 $mg \cdot L^{-1}$ of free SO_2 and 40 $mg \cdot L^{-1}$ of bound SO_2. PPO activity is totally suppressed, whereas for GOX, only a third of initial activity is inhibited, with limited effect using SO_2 concentrations in the range of 27-167 $mg \cdot L^{-1}$ of free SO_2 and 217-497 $mg \cdot L^{-1}$ of bound SO_2. The commercial forms of GOX and PPO give similar results in both WLS (Fig. 4) and MLS (data not shown) to those obtained using GOX and PPO from botrytized grapes at the technological maturity stage. The particular impact of free and bound forms of SO_2 was studied under

Table 2

Incidence of certain technological parameters in wine production on the level of PPO and GOX activities, gluconic acid and yellow color at A420 nm for a 2008 vintage from a Sauternes vineyard

Parameters	Enzymes activities		Gluconic acid (g/L)	A420 nm (color unit)
	GOX (U/L)	PPO (U/mL)		
On must				
Pressing[1]:				
0.5 b	108 ± 8.6	90 ± 8	0.36 ± 0.03	0.639
2.0 b	120 ± 10.2	120 ± 11	0.57 ± 0.06	0.497
5.5 b	146 ± 11.4	90 ± 10	0.77 ± 0.06	0.631
6.0 b	194 ± 13.6	95 ± 9	0.74 ± 0.08	0.839
On wine				
Control[2]	32 ± 2.2	0	0.39 ± 0.02	0.193
Ascorbic acid[3]	22 ± 1.5	0	0.38 ± 0.04	0.198
Bentonite[4]	40 ± 3.2	0	0.34 ± 0.03	0.198

[1]Horizontal pneumatic press (Schneider).
[2]After sulfiting (0.25 g/L) ; [3]0.2 g/L ; [4]1 g/L.

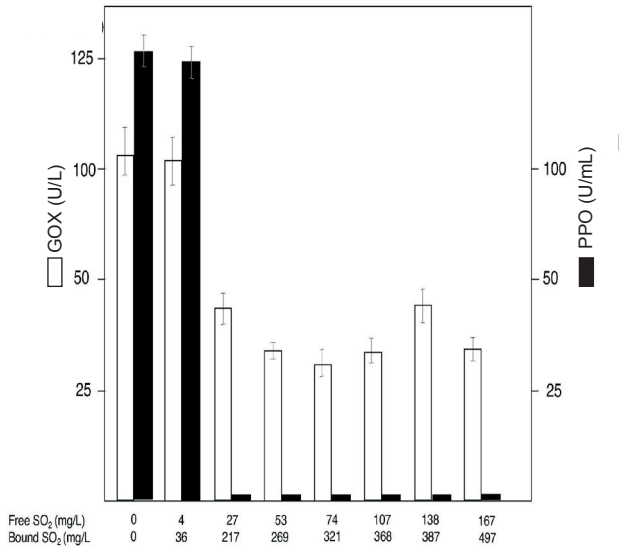

Fig. 3: Effect of free and bound SO_2 on the activities of both GOX and PPO from *Botrytis cinerea* in wines from a Sauternes vineyard (2008). Measurements were recorded directly in the winery. SO_2 was quantified according to the Ripper method after 12 h equilibration. Enzyme activities were also recorded after 12 h.

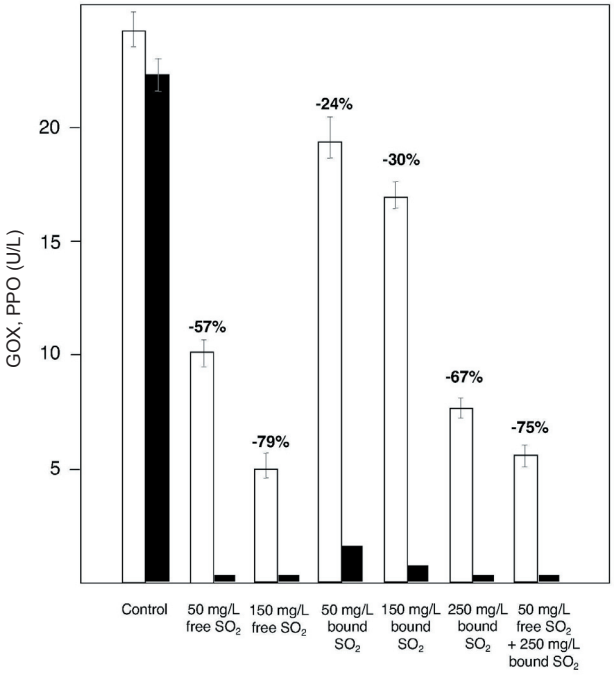

Fig. 4: Laboratory assessment of the effect of SO_2, in both free and bound forms, on GOX and PPO commercial enzymes. Experiments were performed in WLS 12 hrs after adding sulfite solution (at 20 °C, in the dark).

laboratory conditions. Both forms have the same effect on PPO; however, GOX shows different levels of sensitivity to SO_2. Bound SO_2 has less effect than free SO_2 at the same concentration. In sweet white wine formed from botrytized grapes in their technologically mature state, an average mix of 50 mg·L^{-1} of free SO_2 and 250 mg·L^{-1} of bound SO_2 was found to suppress 75 % of GOX activity.

Stability of enzyme activities in must and wine: The key parameter here is the stability of enzyme activities over time, especially during the whole winemaking and ageing processes, which last between 12 and 18 months from fermentation to bottling. GOX and PPO solutions in MLS and WLS were prepared, and regular sampling was carried out for the next 120 d. This consisted of taking a small quantity of each enzyme solution and mixing it with a fresh catechin solution. The oxidizing activity was then estimated by measuring the absorbance at 420 nm of the yellow color, characteristic of the oxidizing products obtained (Fig. 5). To check the relevance of the method, we compared the absorbance intensity and content of residual catechin in the solution by liquid chromatography. A satisfactory negative correlation was observed (Abs. @ 420 nm = 1/[catechin], r: 0.898, standard error: 5.23 %*). The experiments were carried out using commercial enzymes. For PPO, oxidizing capabil-

* Standard error = (r^2-1) x 100

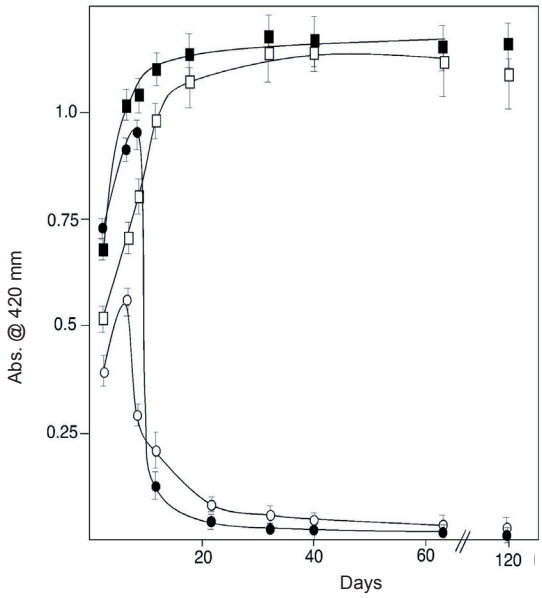

Fig. 5: Stability over time of GOX and PPO enzyme activities. Stability was measured by oxidation of a catechin solution. Tests were conducted in MLS (open symbols) and WLS (closed symbols), for commercial GOX (squares) and PPO (circles) kept at 20 °C in the dark. Over a total period of 120 d, we took regularly a small quantity of each enzyme solution in MLS and WLS, and blended it with sufficient catechin to obtain a concentration of 1 g·L^{-1} (0.003 g catechin in 3 mL of enzyme solution). Oxidation of the solution is represented by absorbance measurements at 420 nm after 48 h at 30 °C. Results are the average of triplicated experiments.

ity increased for the first 10 d, then decreased rapidly, and after 20 d became inactive on the fresh catechin solution. For GOX, the results demonstrate the high level of stability of the oxidizing capability of the enzyme solution in both must and wine.

Modifications induced by GOX in MLS and WLS containing catechins: In order to study the main changes induced by GOX, the evolution of a model solution of catechin, simulating must (MLS) and wine (WLS) conditions, was recorded. In the presence of GOX, the composition of the model solution rapidly became complex: catechin content decreased and several new products were formed (Fig. 6). All these products corresponded to the nucleophilic addition reactions presented in Fig. 1 and Tab. 1. These compounds are the results of a two-step reaction (Vivas *et al.* 2009): first GOX oxidizes glucose to gluconic acid and releases H_2O_2; then H_2O_2, *via* a Fenton reaction, forms a scavenger which oxidizes several compounds, in particular the main must and wine components such as tartaric acid, ethanol and glycerol, to produce respectively glyoxylic acid, acetaldehyde and glyceraldehyde. Then, under acidic conditions, with oxygen and water, different nucleophilic reactions take place to form various compounds. In MLS and WLS, no significant difference in the products formed was noticed, except the presence of acetaldehyde-catechin adducts characteristic of the presence of ethanol in the WLS solution (Fig. 7). Some products showed a yellow color measured at 420 nm, which probably accounts in part for the color

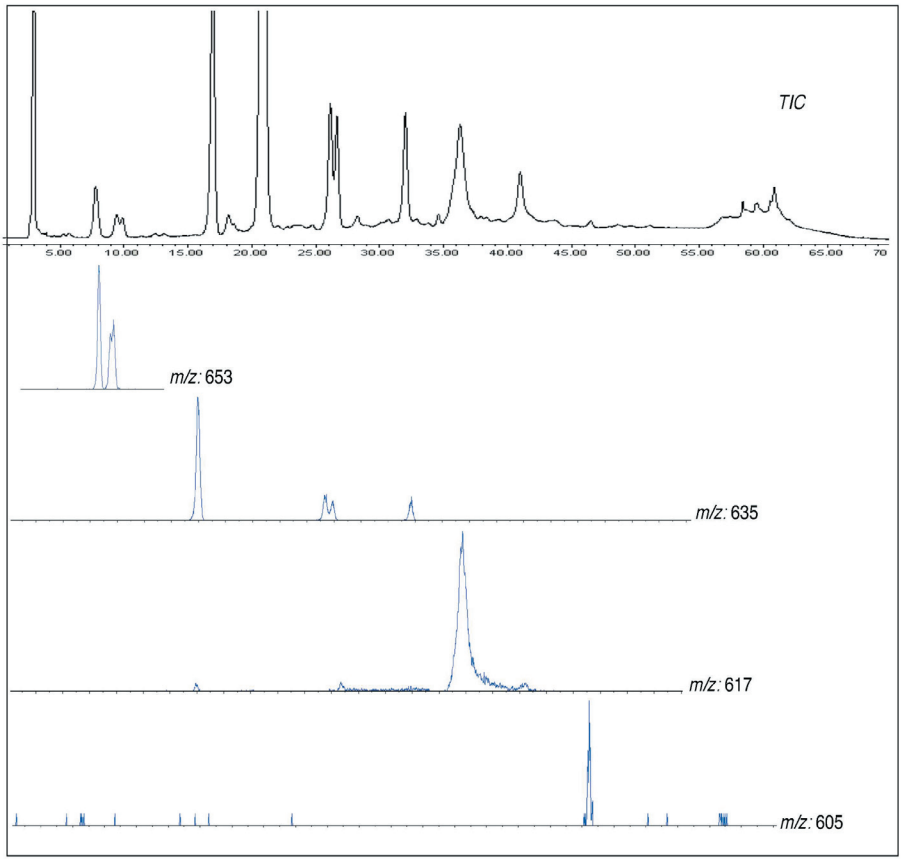

Fig. 6: LS-ES chromatogram of a WLS+Gly medium, after 4 d reaction in the presence of 20 U/L of GOX. The main reaction products were highlighted by fragmentometry (*m/z:* 653, **1a-b**; *m/z:* 635, **2a-d**; *m/z:* 617, **3a-b**; *m/z:* 605, **5a**).

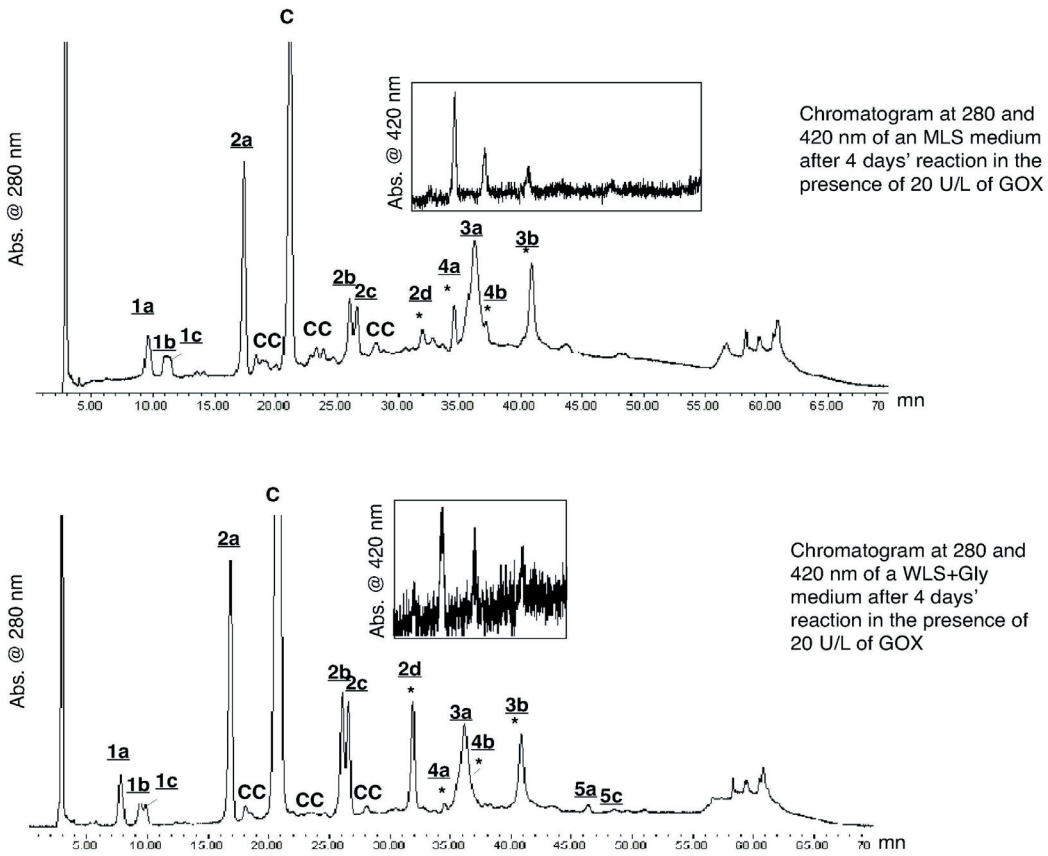

Fig. 7: LS-ES chromatogram of a MLS and WLS + Gly medium after 4 d reaction in the presence of 20 U/L of GOX. The main reaction products were highlighted by fragmentometry (*m/z:* 653, **1a-b**; *m/z:* 635, **2a-d**; *m/z:* 617, **3a-b**; *m/z:* 605, **5a**).

changes over time. These compounds correspond to the xanthylium cations of catechin-glyoxylic-catechin adducts (**4a**, **b**) and show less intense color than the corresponding xanthene form (**3a**, **b**). Comparing the formation of these various products by enzyme reaction (*e.g.* GOX) with formation by chemical oxidation, a significant difference in the kinetics of production was noticed (Fig. 8). It appears that GOX enzymes are necessary in order to create a large amount and variety of nucleophilic addition compounds in a short space of time (Fig. 9).

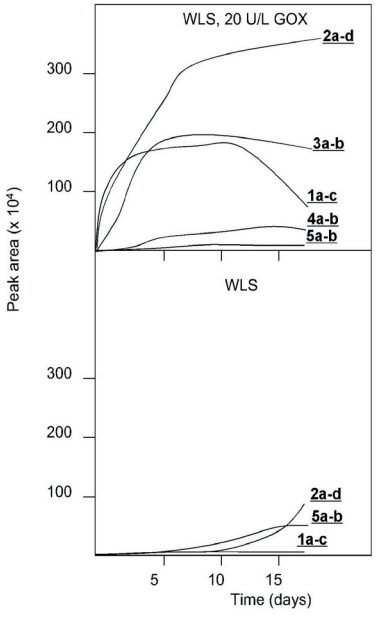

Fig. 8: Evolution over time of the main addition products of glyceraldehyde (**1a-c**), glyoxylic acid (**2a-d**, **3a-b**, **4a-b**), and acetaldehyde (**5a-b**), produced from oxidation of glycerol, tartaric acid, and ethanol, respectively. The first model wine medium contained 20 U/L of GOX and the second one contained none. This gave a comparison of the speeds at which enzyme oxidation and chemical oxidation generated these products.

Discussion and Conclusion

GOX and fungal PPO (laccase) are two oxidizing enzymes involved in the process of *Botrytis cinerea* infection in grapes (Dubernet and Ribereaux-gayon 1973, Li *et al.* 2008). Whereas the role and influence of PPO are well known and documented, very few studies have focused on GOX (Donèche 1987, Donèche 1989). Moreover, there is no research showing a clear relationship between oxidation and GOX activity in grape juice and wine produced from grapes contaminated by *B. cinerea*. Most publications generally accept that laccase is the main oxidizing catalyst in botrytized grapes and in the corresponding wines (Mayer 1978, Ribereau-gayon *et al.* 1998, Duran and Esposito 2000, Gianfreda and Rao 2004). Our recent research work demonstrates that the actions of GOX are complementary to those of PPO. During the infection process of grapes by *B. cinerea,* GOX appears several days after PPO (Donèche, 1987). Then, during the various characteristic stages of over-ripening of grapes affected with noble rot (B, BD, BD^+), both enzymes are present, with a

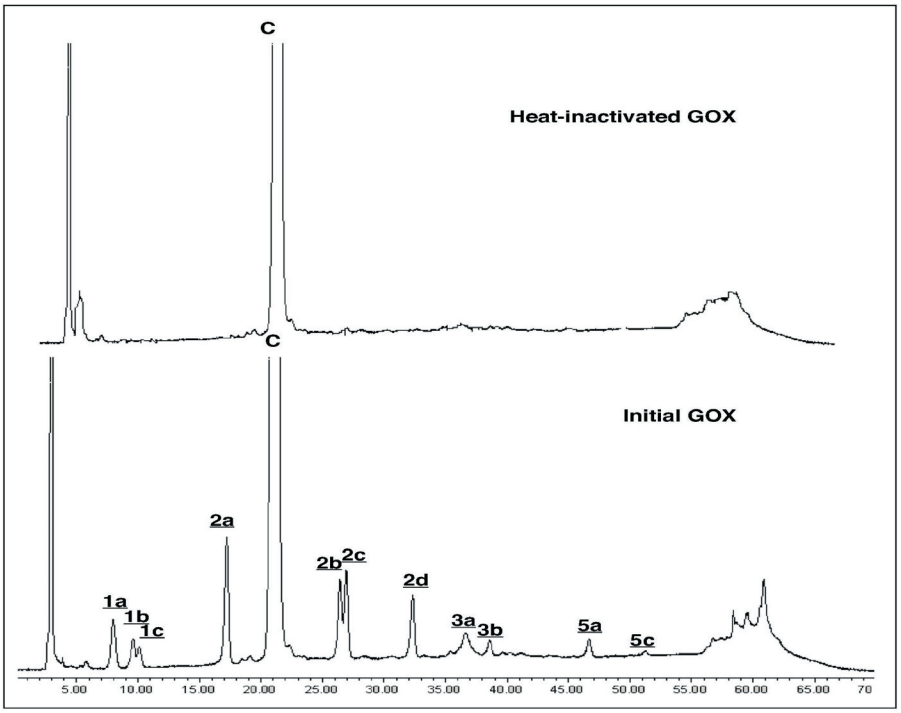

Fig. 9: The effect of inactivating GOX (10 min, 95 °C) on the chromatograms of a WLS + Gly medium after 4 d reaction. The two media contained 20 U/L of GOX.

maximum concentration appearing around the botrytized-desiccated stage (BD). Healthy grapes are free from both enzymes. GOX, as an oxidizing enzyme, is typical of the development of *B. cinerea* on grapes, unlike other oxidizing enzymes, such as peroxidase, which are also present in non-infected grapes (Poux and Ournac, 1972). *B. cinerea* produces many other oxidizing enzymes such as amine oxidase, glycerol oxidase, catalase and peroxidase (Donèche 1987), but most of these are active at pH values which are very different from the pH of must and wine. In addition, due to its general characteristics, GOX is particularly stable in both grape juice and wine (Donèche, 1987).

Over time, in enological conditions, PPO appears as an unstable enzyme. Present and active both in the grape berries and during the pressing process, its activity decreases rapidly upon addition of SO_2 at the end of the alcoholic fermentation. Both free and bound SO_2 have a major inhibitory action on PPO, even at very low concentrations. This observation is confirmed both in winery conditions and in the laboratory. To stabilize sweet white wine made from botrytized grapes, especially to prevent refermentation of residual sugars, higher levels of SO_2 than traditional dry white wines is used (Ribereau-Gayon *et al.* 1998). In addition, the high sugar content of these unique products gives rise to an effective unequalled inhibition in the range of 50-350 $g \cdot L^{-1}$ (Donèche 1991). Furthermore, the results show clearly that, both in must-like and wine-like solutions, PPO loses its oxidizing capability in a fresh catechin solution after a few days. Thus, PPO probably plays a big role in direct oxidation of polyphenols both in the grape and early juice stages. But after sulfiting, and because PPO is naturally unstable, enzyme oxidation of wine is rapidly taken over by other oxidizing enzymes. Of these, GOX is the best candidate.

Apart from the work of Donèche (1989), no publication shows the possible relationship between the metabolism of *B. cinerea* and the accumulation of GOX, as one of the oxidizing enzymes present in botrytized grapes and in the corresponding wines. However, GOX possesses several interesting properties in relation to must and wine. Notably, one would cite the resistance of a residual part of GOX to both free and bound SO_2, and the very stable oxidizing action of GOX over time. These two considerations suggest a long-term influence of GOX on the evolution of botrytized white wine both during ageing in barrels and when bottled. GOX activity has been detected (50-150 U/L) in some old-vintage Sauternes, even after more than 50 years in the bottle (Vivas *et al.* 2009). The fact that GOX has a specific effect on wine composition has already been published (Vivas *et al.* 2009). But whereas PPO acts directly to oxidize polyphenols, GOX achieves this *via* an indirect two-step reaction: first, GOX oxidizes β-D-glucose to gluconic acid and releases H_2O_2; then, H_2O_2 and scavengers resulting from the degradation of H_2O_2 oxidize certain major compounds in must and wine such as tartaric acid, ethanol and glycerol, to produce respectively glyoxylic acid, acetaldehyde and glyceraldehyde. These compounds then react with catechin and proanthocyanidins in a standard nucleophilic addition reaction with oxygen under acidic conditions (Nonier *et al.* 2007) to produce yet further compounds some of which impart a typical yellow color to the must and to the wine (Es-Safi *et al.* 2002) The intensity of the color increases regularly over time and naturally affects the visual quality of white wines (Vivas *et al.* 2008). The same reactions can occur spontaneously by the chemical route, but the kinetics is slower and produces less of the new compounds than the enzyme-induced reactions. These differences between the chemical and the enzyme

reactions need further investigation. In addition, we need to understand the exact role of GOX in the production of colored matter in sweet white wines produced from botrytized grapes.

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