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Levels of Stilbene Oligomers and Astilbin in French Varietal Wines and in Grapes during Noble Rot Development

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Phenolics from grapes and wines can play a role against oxidation and development of atherosclerosis. Stilbenes have been shown to have cancer chemopreventive activity and to protect lipoproteins from oxidative damage. A method for the direct determination of stilbene oligomers (viniferin and pallidol) as well as astilbin in different types of wine using high-performance liquid chromatography with UV detection is described. In a survey of 21 commercial wines from the south of France, levels of pallidol and viniferin are reported for the first time in different types of wines. Viniferin was found to be present only in red and botrytized sweet white wines with levels between 0.1 and 1.63 mg/L; pallidol was not found in dry and sweet white wines but only in wines made by maceration with stems, with levels between 0.38 and 2.22 mg/L. Highest levels of astilbin were found in Egiodola (15.13 mg/L), Merlot (11.61 mg/L), and Cabernet Sauvignon (8.24 mg/L) for red wines and in Sauvignon (5.04 mg/L) for white varietal wines. Astilbin levels are highest for recent vintages, but pallidol is not found in older vintages. During noble rot development in Sauvignon or Sémillon grapes from the Sauternes area, levels of *trans*-astringin, *trans*-resveratrol, *trans*-piceid, and pallidol are quite low (<0.5 mg/kg of grapes). Viniferin and astilbin levels become optimum at 2 and 30 mg/kg, respectively, during spot grape and speckle grape stages.

KEYWORDS: Wine; phenolics; stilbenes; grapes; pallidol; viniferin; astilbin; noble rot

INTRODUCTION

Phenolic compounds play an important role in plant life. Stilbenes occur naturally in a number of plant families and can act as resistant agents to pathogens (bacteria, viruses, yeasts, and insects) (*1*). Resveratrol can be synthesized by plants in response to microbial infection or stress. However, it is also produced by plants after chemical treatments, such as herbicide or fungicide application (*2*), or after UV exposure (*3*). Grapes and related products are considered to be the most important dietary sources of these substances (*4*).

Wine consumption has been inversely correlated to coronary heart disease in many epidemiological studies, such as Renaud's French paradox study (*5*). A proposed mechanism for this correlation involves the antioxidant activity of the flavonoids and stilbenes, some of the phenolic compounds found in wine. Moreover, *trans*-resveratrol seems to have a variety of biological activities. It inhibits the oxidation of human low-density lipoproteins (*6*, *7*) and platelet aggregation (*8*), which may explain the decrease in coronary heart disease observed among wine drinkers. Recently, we reported the occurrence of original stilbenes such as astringin in wines (*9*) and concentrations of stilbenes (*trans*-resveratrol, *trans*-astringin, and *trans*-piceid) in Portuguese and French wines (*10*). Although the nature and concentrations of stilbenes are various and depend on multiple factors including grape cultivar, fungal pressure, wine-making procedures, and climate, they can reach higher concentrations (up to 20 mg/L) often with a dominance of the resveratrol glycosides (*11*, *12*). Moreover, wine polyphenols have been reported to have anticarcinogenic properties, delaying tumor onset in transgenic mice (*13*). *trans*-Resveratrol has also been shown to have cancer chemopreventive activity in assays on three major stages of carcinogenesis (*14*, *15*). *trans*-Astringin and, to a greater extent, its aglycon are active as antileukemic agents (*16*, *17*), and *trans*-piceatannol is a known inhibitor of protein−tyrosine kinases, which are positive regulators of cell proliferation (*18*, *19*). Recently it was found that resveratrol decreases tumor growth in a rat tumor model. Resveratrol administration to AH-130 rats caused a very significant decrease (25%) in tumor cell content by causing apoptosis in the tumor

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cell population (*20*). Resveratrol was also shown to reduce viability and the DNA synthesis capability of cultured promyeloctic leukemia (HL-60) cells (*21*).

The possible effects of resveratrol on the mitochondrial respiratory chain in rat brains was investigated by Zini et al. (*22*). It was demonstrated by these authors that resveratrol inhibits the mitochondrial respiratory chain through complexes II and III and was able to scavenge the superoxide anion. Resveratrol can decrease complex III activity by competition with coenzyme Q. This complex is the site where reactive oxygen substances (ROS) are generated. By decreasing the activity of complex III, resveratrol can not only oppose the production of ROS but also scavenge them.

Activities of new types of oligostilbenes need to be tested and quantified in several models and biological matrices to determine their potential effects. For example, two new stilbene dimer glucosides, resveratrol (*E*)-dehydrodimer 11-*O*-*β*-D-glucopyranoside and resveratrol (*E*)-dehydrodimer 11′-*O*-*β*-D-glucopyranoside, were isolated together with the known resveratrol (*E*)-dehydrodimer and pallidol from *Vitis vinifera* cell cultures (*23*). Recently, seven novel stilbene derivatives have been isolated from a Riesling wine (*24*). The newly identified compounds included the monostilbene 2,4,6-trihydroxyphenanthrene-2-*O*-glucoside, as well as two isomeric resveratrol-2-*C*-glucosides. In addition, four dimeric stilbenes, that is, *cis*- and *trans*-*ϵ*-viniferin diglucoside as well as pallidoldiglucoside, have also been obtained for the first time from Riesling wine. A real interest in the research of new stilbenes in grapes and wines is necessary to increase our knowledge of their nature and occurrence.

In the present study we report the determination of stilbene oligomers in wines and grapes. We report viniferin, pallidol, and astilbin (a flavanonol) concentrations for a range of 21 commercial red and white wines from France. The contents of stilbene oligomers as well as *trans*-resveratrol, *trans*-piceid, *trans*-astringin, and astilbin were also determined in grapes during the five stages of development of noble rot in two grape varieties from the Sauternes area using an HPLC method coupled with UV detection.

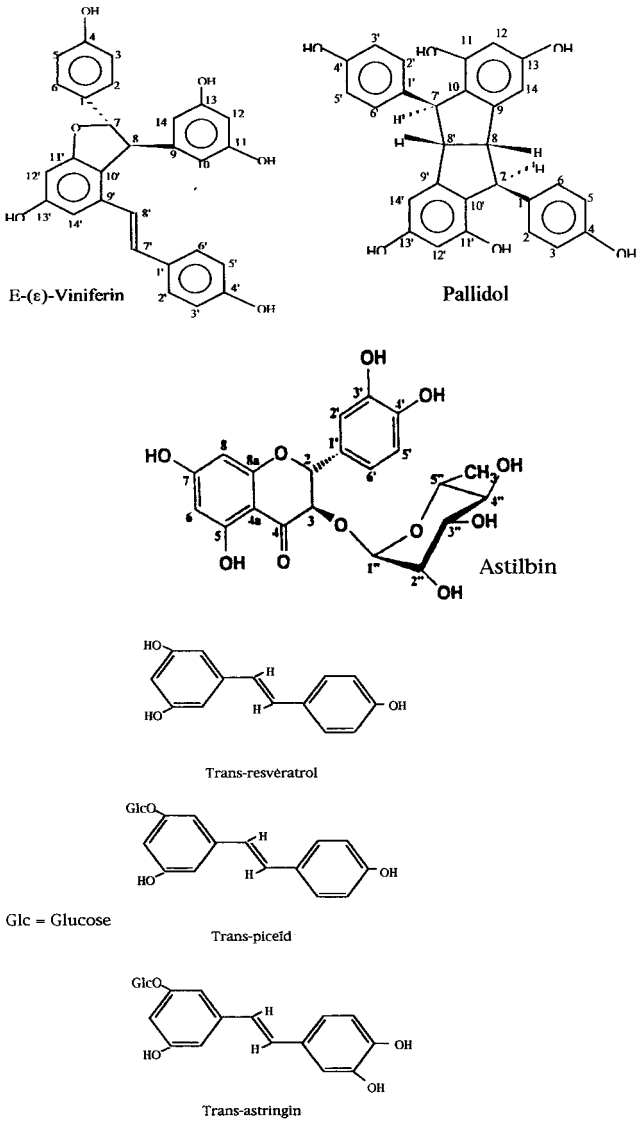


Figure 1. Structures of stilbene monomers and oligomers and astilbin (a flavanonol) analyzed in wines and grapes.

MATERIALS AND METHODS

Materials. *trans*-Resveratrol was purchased from Sigma (St. Quentin Fallavier, France). *trans*-Piceid and *trans*-astringin were obtained from cell suspension cultures as previously described (*25*, *26*). Pallidol, (*E*)-*ϵ*-viniferin, and astilbin were obtained from Merlot stalks.

Methanol and acetonitrile (HPLC grade) were purchased from Carlo Erba (Val de Reuil, France) and Merck (Nogent sur Marne, France).

Structures of the studied compounds are given in **Figure 1**.

Isolation and Identification of Pallidol, Astilbin, and Viniferin. Final purifications were achieved by semipreparative HPLC with a 4 × 250 mm Ultrasep RP18 reversed phase column (4 *μ*m) (Bischoff) at room temperature using the following solvents: A, H_2O adjusted to pH 2.4 with TFA; B, 20% A with 80% CH_3CN, with gradient system as described earlier (*26*) and detection at 280 nm. ^{1}H NMR spectra were recorded at 303 K in the Fourier transform mode at 500.13 MHz on a Bruker AMX 500 spectrometer equipped with a broad band 20-mm probe, using a spectral width of 20 ppm and TMS as internal standard. Chemical shifts were expressed as parts per million relative to the CD_3OD (3.30 ppm) resonance. Mass spectra were recorded on a VG Autospec-Q in the FAB^+ mode.

The ^{1}H NMR spectrum of the first compound studied showed signals corresponding to pallidol, the symmetrical dimer of resveratrol (**Figure 1**): four aliphatic protons at *δ* 3.75 and 4.50 (two large singlets) and two AA′BB′ systems characterized by two pairs of proton doublets at *δ* 6.68 and 6.95 (J = 8.5 Hz), corresponding to protons of the B and D rings, and by two pairs of proton doublets at *δ* 6.13 and 6.55 (J = 2 Hz), corresponding to the A and C rings. These NMR data are identical to those published in 1986 (*27*) for pallidol, and its identity was confirmed by HPLC comparison with an authentic standard isolated from grape cell suspension cultures (*23*).

The ^{1}H NMR spectrum for the second compound studied showed a signal corresponding to a rhamnose residue at a typical shift of *δ* 1.18 (J = 6.2 Hz). The large singlet at *δ* 4.06 (J = 1.5 Hz) indicated an α configuration of the anomeric proton of the sugar (H-1″). Identification as astilbin (dihydroquercetin 3-*O*-α-rhamnoside) (**Figure 1**) was confirmed by FAB^+MS data (*m*/*z* 451) and by comparison of the ^{1}H NMR shifts and coupling constants with literature data (*28*, *29*).

Viniferin has been already isolated and identified (*30*, *31*).

Wine Samples. We analyzed 21 samples of different French varietal wines: 6 red (Merlot, Cabernet Sauvignon, Egiodola, and Trempanillo); 14 white [8 sweet (Sémillon) and 6 dry (Chardonnay, Viognier, and Sauvignon); and 1 rosé from Syrah variety in commercial bottles from France. The wine samples analyzed were from all viticultural areas of southern France and of different vintage years, that is, 1986−2000 (**Table 1**). All wines analyzed are frequently consumed in France.

Grapes Samples. Grapes samples (1 kg each) were collected by clusters: 50 clusters were harvested in each vineyard (with 10−15 berries by clusters) at the five stages of noble rot development in the Sauternes area from two vineyards with Sémillon and Sauvignon varieties. Clusters were selected at random throughout the vineyard. The different stages based on botrytis infection for harvesting the grapes

Table 1. Levels of Stilbene Oligomeric Compounds and Astilbin for French Varietal Wines

wine	type	vintage	color	astilbin (mg/L)	viniferin (mg/L)	pallidol (mg/L)
Cabernet Sauvignon	red dry	1998	red	7	1.63	ND[a]
Cabernet Sauvignon	red dry	1999	red	7.95	0.1	ND
Cabernet Sauvignon	red dry	1999	red	9.77	1.35	2.22
Merlot	red dry	1998	red	11.61	0.53	ND
Tempranillo	red dry	1998	red	1.19	0.13	ND
Egiodola	red dry	1999	red	15.13	1.12	1.33
rosé from Syrah	rosé dry	1999	rosé	3.84	ND	0.38
Chardonnay "red"[b]	white dry	1999	white	12.98	ND	0.30
Chardonnay	white dry	1999	white	3.3	ND	ND
Chardonnay	white dry	1999	white	4.33	ND	ND
Sauvignon	white dry	1999	white	0.77	ND	ND
Sauvignon	white dry	2000	white	9.30	ND	ND
Viognier	white dry	1999	white	2.99	ND	ND
Sémillon	botrytized sweet white	1986	white	0.84	0.11	ND
Sémillon	botrytized sweet white	1991	white	1.66	0.10	ND
Sémillon	botrytized sweet white	1993	white	2.07	0.14	ND
Sémillon	botrytized sweet white	1994	white	1.90	0.17	ND
Sémillon	botrytized sweet white	1995	white	1.77	0.08	ND
Sémillon	botrytized sweet white	1997	white	5.86	0.13	ND
Sémillon	botrytized sweet white	1998	white	1.84	0.11	ND
Sémillon	botrytized sweet white	1999	white	3.45	0.12	ND
average				5.21	0.27	0.20

[a] Not detected. [b] Natural phenolic enriched by special wine-making.

are as follows: 1, healthy grapes; 2, speckle grapes; 3, spot grapes; 4, full rotten grapes; 5, roast rotten grapes. Grape samples were immediately frozen at −30 °C prior to analysis. The harvest time period was from September 4 to November 4 (September 4, stage 1; September 16, stage 2; October 6, stage 3; October 15, stage 4; and November 4, stage 5).

Sample Preparation for HPLC Analysis. Stilbene extraction was carried out with 20 mL of wine by adding three times 20 mL of EtOAc. After solvent evaporation, 500 μL of methanol was added to the residue. The solution was filtered on a 0.45 μm filter and directly injected into the HPLC system. Stilbene extraction was carried out on 5−10 g of lyophilized grapes. A freeze-dryer Virtis Sentry from the Virtis Co. was used to lyophilize grapes. Freeze-dried grapes were obtained by adding 100 g of mixed fresh grapes to 70 mL of distilled water and 70 mL of CH_3CN (HPLC grade). This solution was placed in the Virtis freeze-dryer for 24 h. The same procedure as was used for wine analysis was then applied.

HPLC Analysis. Separation and quantification of stilbenes (pallidol and viniferin) and astilbin in wines and grapes were carried out by HPLC. A Hewlett-Packard model 1090 with three low-pressure pumps and a diode array UV−visible detector coupled to an HP Chem Station was used for solvent delivery system and detection. A Nucleosil 100C18 column, 4.0 × 250 mm (Hewlett-Packard), thermostated at 30 °C was used as the stationary phase with a flow rate of 0.5 mL/min.

The solvents used for the separation were as follows: solvent A, methanol; solvent B, bidistilled water. The gradient solvent system was as follows: 5 min, 2% A, 98% B; 12 min, 7% A, 93% B; 18 min, 11% A, 89% B; 28 min, 15% A, 85% B; 38 min, 30% A, 70% B; 48 min, 40% A, 60% B; 55 min, 50% A, 50% B; 75 min, 50% A, 50% B; 85 min, 70% A, 30% B; 100 min, 70% A, 30% B. Twenty-five microliters of extract was injected into the HPLC system after filtration on a 0.45 μm Millipore membrane. After each analysis, the column was re-equilibrated with phase A for 10 min. Detection was at 280, 286, and 321 nm for pallidol, astilbin, and viniferin, respectively. Measurements were carried out in duplicate. An HPLC chromatogram and spectra at 280, 286, 321 nm of stilbene (pallidol and viniferin) and an astilbin standard solution with the retention time for each compound are given in **Figure 2**. Analytical characteristics of stilbenes and astilbin determinations are given in **Table 2**. *trans*-Resveratrol, *trans*-piceid, and *trans*-astringin were separated and quantified in grape samples according to the HPLC procedure described (*10*).

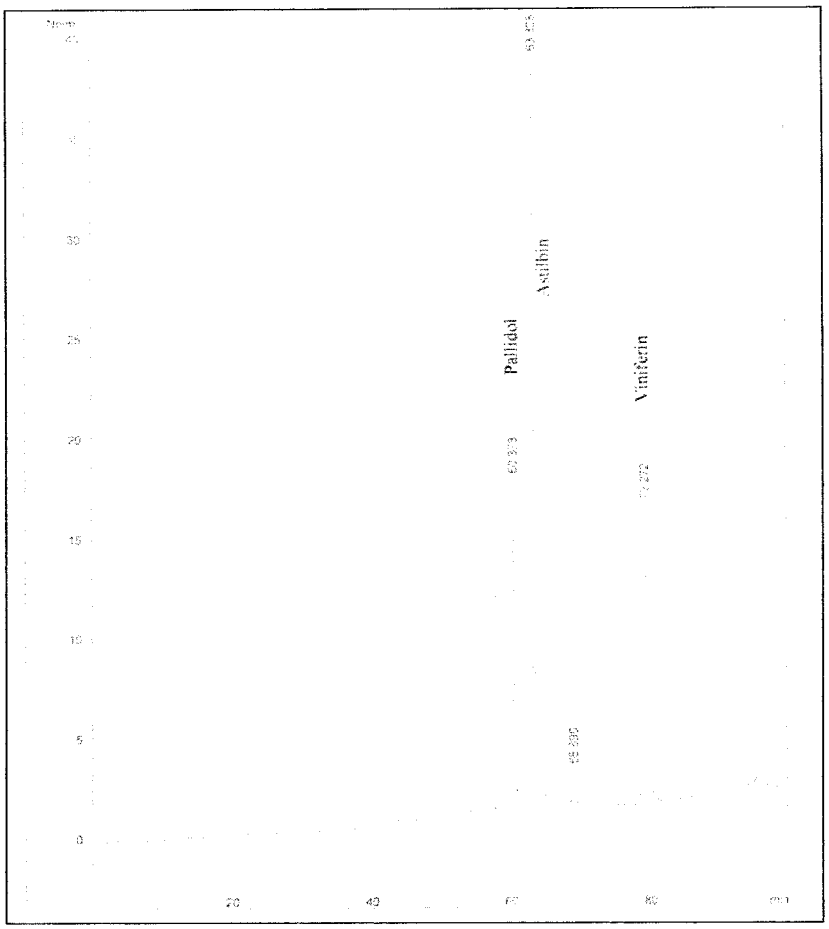
Table 2. Analytical Characteristics for the Determination of Stilbenes (Pallidol and Viniferin) and Astilbin in Wine Samples by HPLC-UV

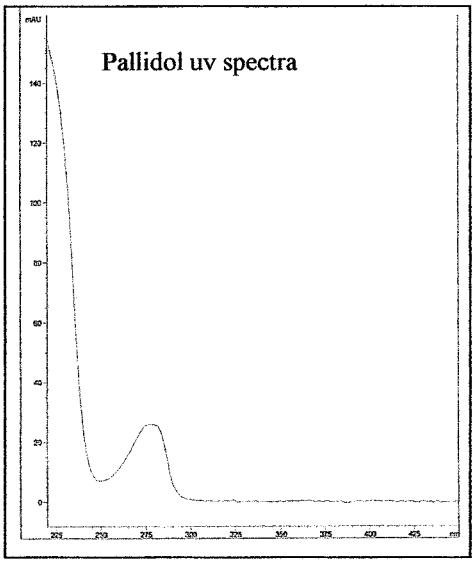
stilbene	detection limit[a] (ng)	calibration range[b] mg/L	calibration range[b] *r*	accuracy[c] recovery (%)	precision[d] RSD (%)
pallidol	8	0.0–50	0.9994	99.3 ± 3.9	1.6–3.3
viniferin	8	0.0–50	0.9995	99.4 ± 3.5	1.2–3.0
astilbin	5	0.0–50	0.9993	99.1 ± 3.8	1.7–2.9

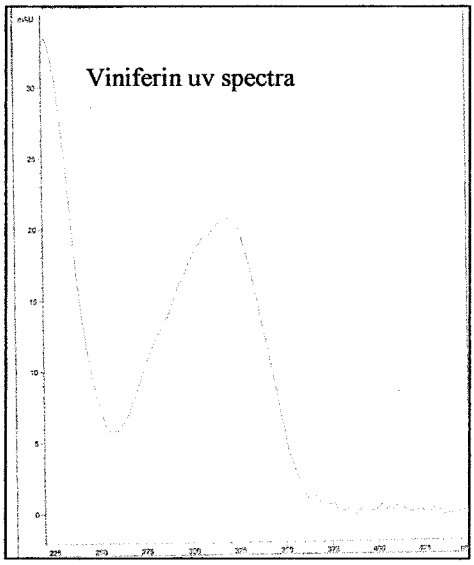
[a] Detection limit calculated according to IUPAC rules (25 μL). [b] Calibration range in mg/L and coefficient of correlation (*r*) obtained for five points. [c] Mean value ± SD of determinations in two different samples. [d] Relative standard deviation (%) of six determinations in five different samples.

RESULTS AND DISCUSSION

Chardonnay wine enriched in phenolics by a special wine-making technique was obtained by crushing the grapes with must, seeds, and skins fermentation; the wine-making procedure was thus the same as for a red wine, including a maceration step (6 days) with an increase of temperature to 28 °C. The concentrations of viniferin determined by the HPLC method varied from 0.1 to 1.63 mg/L for the red wines and from 0.08 to 0.17 mg/L for the botrytized sweet white wines (**Table 1**). To our knowledge this is the first time that viniferin has been found in botrytized sweet white wine. Viniferin was not detected in traditional white wine, the Chardonnay enriched in phenolics by the special wine-making technique, or in rosé wine. This interesting finding could be attributed to the fact that botrytized sweet white wines are produced with grapes via a procedure in which noble rot is part of the step maturation process. The fungal development of *Botrytis cinerea* could induce defense reactions of the vine plant to synthesize stilbene oligomers such as viniferin. Pallidol results are different (**Table 1**): the levels varied between 0.38 and 2.22 mg/L, but this compound was not found in sweet and traditional white wines. It was found only in some red and rosé wines and in a Chardonnay wine enriched in phenolics by the special wine-making technique. In fact, all wines containing pallidol were made with a maceration step incorporating the stalks, which could influence pallidol levels







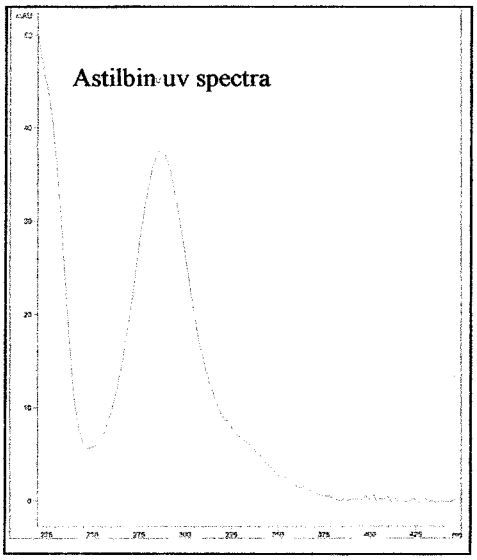


Figure 2. HPLC chromatogram and spectra at 280, 286, and 321 nm of stilbenes (pallidol and viniferin) and astilbin standard solution.

in wine. Astilbin was found in all wine types (**Table 1**), and the levels varied between 1.19 and 15.13 mg/L for red wines, between 0.77 and 9.3 mg/L for dry white wines, and between 0.84 and 5.86 mg/L for botrytized sweet white wines. Average levels of astilbin are quite similar between rosé and white dry wines, but the level of this compound in the Chardonnay wine enriched in phenolics by the special wine-making technique is greater than the average red wine value.

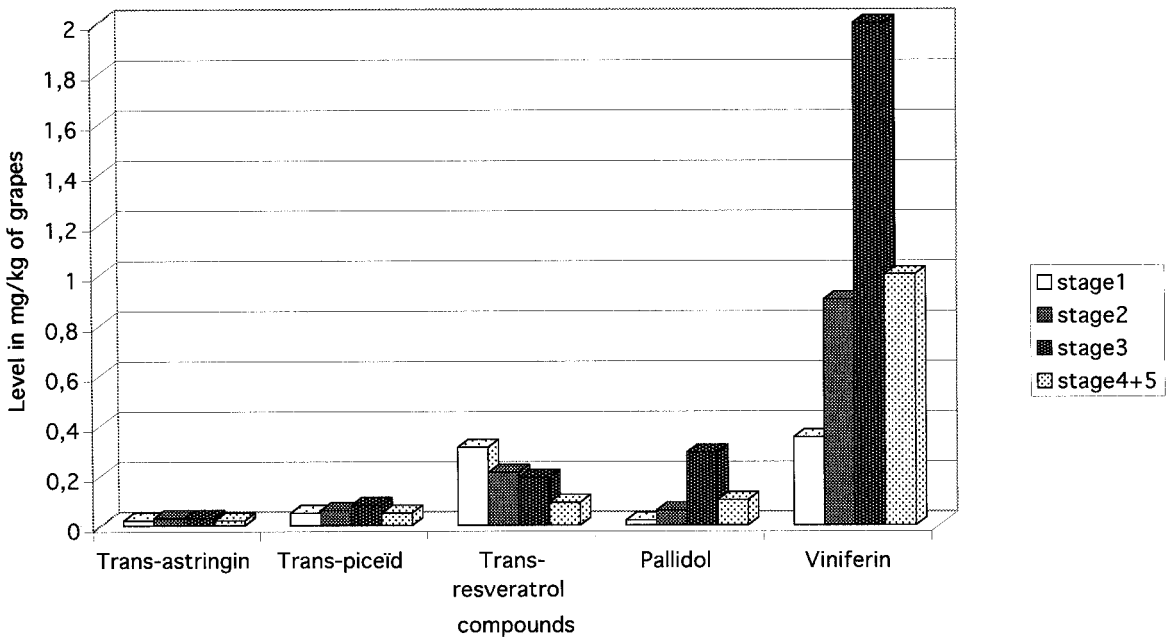


Figure 3. Levels of stilbenes (monomers and oligomers) in Sauvignon grapes during noble rot stages.

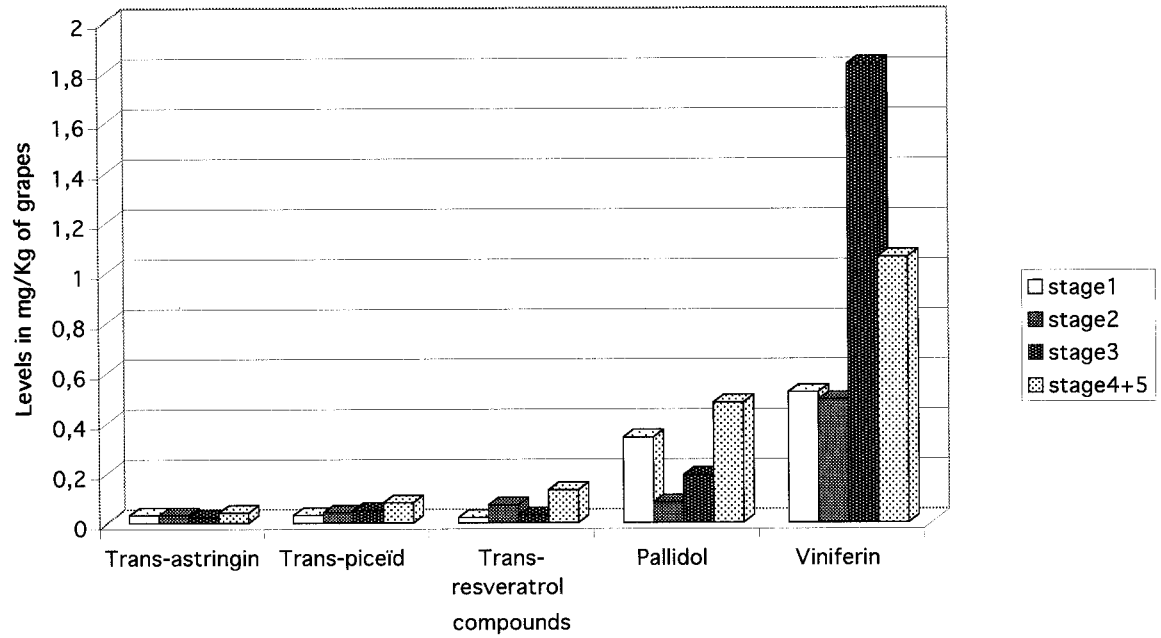


Figure 4. Levels of stilbene compounds (monomers and oligomers) in Sémillon grapes during noble rot stages.

For average levels of stilbenes as a function of grape variety, the highest levels of astilbin were found in Egiodola (15.13 mg/L), Merlot (11.61 mg/L), and Cabernet Sauvignon (8.24 mg/L) for the red varieties and in Sauvignon 5.04 mg/L for the white wines. For viniferin, the highest levels are found in Egiodola (1.12 mg/L) and Cabernet Sauvignon (1.03 mg/L). For white varieties, only Sémillon contains viniferin (0.10 mg/L). Pallidol was not found in white varieties but only in Egiodola (1.33 mg/L) and Cabernet Sauvignon (0.74 mg/L). Stilbenes and astilbin levels classified according to vintage indicated that levels are greater in the more recent vintages for astilbin. No pattern was observed for viniferin or pallidol levels as a function of vintages. Pallidol was absent from the 1986 to 1998 vintages; this compound was found only in the 1999 vintage.

In **Figures 3** and **4** we can observe the evolution of stilbene monomers and oligomers for Sauvignon and Sémillon grapes from the Sauternes appellation area. For Sémillon grapes, the levels of *trans*-astringin, *trans*-piceid, *trans*-resveratrol, and pallidol increase, reaching an optimum at stages 4 and 5 (full rot grapes and roast rot grapes). In the case of Sauvignon grapes, the levels of *trans*-resveratrol appear to decrease during all stages of noble rot development, but levels of *trans*-astringin, *trans*-piceid, and pallidol are highest for the third stage (spot grapes). However, for both varieties, the levels of *trans*-astringin, *trans*-resveratrol, and *trans*-piceid are quite low (<0.13 mg/kg for each compound), which is in agreement with the levels reported in Merlot and Chardonnay (*32*), reaching a maximum of 0.5 mg/kg for pallidol, during the different stages of noble rot development on grapes. Our findings are also in agreement with results reported (*33*) on the ability of pathogenic *B. cinerea* strains to biotransform resveratrol into a variety of unidentified oxidized metabolites as a means of reducing the antifungal effects of resveratrol, thus facilitating invasion of the fungus into host-plant tissues. In this work, studies utilizing growing

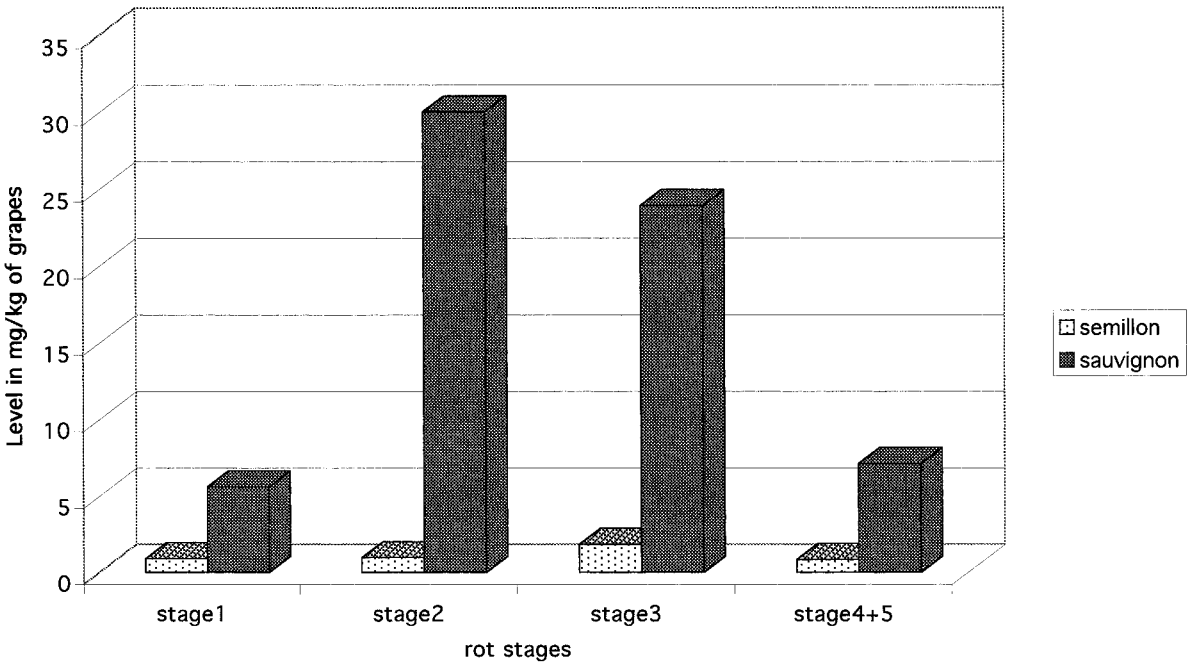


Figure 5. Astilbin levels in Sauvignon and Sémillon grapes during noble rot stages.

incubations of *B. cinerea* ATCC 11542 with resveratrol resulted in the production of three new restrytisols A−C and three known oxidized resveratrol dimers (resveratrol *trans*-dehydrodimer, leachinol F, and pallidol).

For viniferin, the highest level is obtained during stage 3 of noble rot development, for both Sémillon or Sauvignon grapes with levels close to 2 mg/kg. Stages 4 and 5 seem to affect the level in this molecule for both varieties in that the levels are reduced almost to 50%. During the last stages of noble rot development viniferin could be used by the plant to act against *Botrytis* development or could be combined with other phenolic compounds to protect some vegetal cell structures. Grapes and wines containing viniferin could be of health interest because the inhibitory effect of (+)-α-viniferin was reported to be ∼3−4-fold that of resveratrol on cyclooxygenase activity of prostaglandin H2 synthase partially purified from sheep seminal vesicles and (+)-α-viniferin was found to exhibit a dose-dependent inhibition on cyclooxygenase activity (*34*).

Astilbin levels are given in **Figure 5** for Sauvignon and Sémillon varieties. Levels of astilbin during noble rot development appear almost stable for Sémillon but increase considerably for Sauvignon: between +300 and +400% to reach concentrations between 25 and 30 mg/kg of grapes for stages 2 and 3, falling to 7.11 mg/kg at the fifth stage. Like viniferin, astilbin could be used by the plant to act against botrytis development. Recently a hepatoprotective effect of astilbin greater than that of vitamin E was demonstrated and astilbin was capable of restoring lipoperoxides and tissue prostanoids to basal values in a study using rat models (*35*). The ability to increase the levels of astilbin in grapes or wine could be of great interest.

Concentrations of pallidol and viniferin (two silbene oligomers) are reported for the first time in different type of wines. Viniferin is present only in red and botrytized sweet white wines with levels between 0.1 and 1.63 mg/L; pallidol is found in dry and botrytized sweet white wines, but only in wines made by maceration with stalks where levels are between 0.38 and 2.22 mg/L. Highest levels of astilbin were found in Egiodola (15.13 mg/L), Merlot (11.61 mg/L), and Cabernet Sauvignon (8.24 mg/L) for red wine varieties and in Sauvignon (5.04 mg/L) for white varietal wines. Astilbin levels are highest for recent vintages, but pallidol is not found in older vintages. During noble rot development in Sauvignon or Sémillon grapes, levels of *trans*-astringin, *trans*-resveratrol, *trans*-piceid, and pallidol are quite low (<0.5 mg/kg of grapes). Viniferin and astilbin reach optimum levels during spot grape and speckle grape steps.

It would be important in the future to investigate other stilbene compounds in grapes and wines. However, it will also be important in the future to obtain data on the bioavailability of stilbene compound monomers and dimers in the plasma after absorption of different wine types.

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