# Method for the Quantitative Extraction of Resveratrol and Piceid Isomers in Grape Berry Skins. Effect of Powdery Mildew on the Stilbene Content

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A simple method for the quantitative extraction of resveratrol and its glycosides from grape berry skins has been developed. Optimal conditions for the extraction were investigated. Type of solvent, time, and temperature assayed influenced resveratrol and piceid yield. Adequate extraction was attained with ethanol/water (80:20 v/v) maintained at 60 °C for 30 min. Recovery (>96%) and reproducibility (6.83−15.13%) were satisfactory. After extraction, resveratrol and piceid isomers were quantified by high-performance liquid chromatography coupled to a ultraviolet−visible diode array detector. The amounts detected in 13 samples of 7 different varieties analyzed were, on average, 92.33 μg/g of dry skin for *cis*-piceid, 42.19 μg/g of dry skin for *trans*-piceid, and 24.06 μg/g of dry skin for *trans*-resveratrol. *cis*-Resveratrol was not detected in any sample. In grape berries infected by powdery mildew the contentw of these compounds were considerably increased and the degree of infection was positively related to their stilbene content.

**Keywords:** *Resveratrol; piceid isomers; stilbene; grape; extraction method; powdery mildew*

## INTRODUCTION

Resveratrol is a phytoalexin present in wines (*1*−*5*), grape juices (*6*−*8*), grapes (*9*, *10*), and peanuts and peanut products (*11*, *12*).

Resveratrol exhibits chemopreventive and antitumor activities (*13*−*16*) and inhibits reactions that increase the risk of coronary heart diseases (*17*, *18*).

In vines, the synthesis of resveratrol is initiated in response to ultraviolet (UV) irradiations (*19*−*21*) or stress, especially interaction of the plant with pathogens such as *Botrytis cinerea* (*22*−*25*) and *Plasmopara viticola* (*22*, *26*−*28*).

Resveratrol in vines is produced by leaves and berries; in the latter case, resveratrol is synthesized by berry skin and to a lesser extent by seeds (*20*, *26*, *29*, *30*).

Different authors have measured the content of *trans*-resveratrol in grape berry skins (*6*, *9*, *10*, *20*, *29*) and its glycoside, *trans*-piceid (*9*), including samples affected by *Botrytis cinerea* (*29*) or *Plasmopara viticola* (*31*). In most cases, the methods employed required consecutive extractions or sample derivatization for the analysis of *trans*-resveratrol. Table 1 summarizes previous conditions used in the literature to extract resveratrol and piceid isomers from different grapevine tissues.

This work was undertaken to establish a simple method for the extraction of *trans*-piceid, *cis*-piceid, and *trans*-resveratrol from grape berry skins. The presence of *cis*-piceid has been previously described in grape leaf extracts (*32*) and in grape juices (*2*, *8*, *40*), and these authors suggested that *cis*-piceid was present in the solid parts of grape berries. However, to our knowledge, a method for the extraction of *cis*-piceid from grape berry skins has not been previously described.

With this method we have determined the concentration of these compounds in some white and red grape berries, and we have examined the changes in resveratrol and piceid production in response to increasing *Uncinula necator* infection in the vineyard.

## MATERIALS AND METHODS

**Samples.** *Healthy Grapes.* The resveratrol and piceid concentrations of healthy grape berries (*Vitis vinifera*) were determined in 13 samples, 6 were from white grape berries and 7 were from red varieties. The varieties of white grapes included were Chardonnay, Xarel·lo, Macabeo, and Parellada; the red ones were Merlot, Cariñena, and Cabernet Sauvignon. All of the samples were hand-harvested from different Penedès and Costers del Segre regions (Spain). Values of °Brix were measured in all grape samples.

*Grapes Infected by Powdery Mildew.* The grape berries selected were from the Cariñena red variety, susceptible to oidium *(Uncinula necator)* attacks. The clusters were made from two different vineyards from the Penedès region (Spain), and three samples were selected as a function of their infection degree: apparently noninfected clusters (they had no visible signs of infection), slightly infected clusters (5−10% of their berries had visible signs of infection), and highly infected clusters (25−75% of their berries had visible signs of infection). All of the samples were hand-harvested at maturity.

The samples were protected from light to avoid light-induced isomerization during sample treatment. One hundred berries of each sample were randomly taken from different places on 10 clusters. Their skins were removed by hand and immediately frozen, freeze-dried, and stored at −20 °C until extraction.

**Extraction Method.** *Procedure.* Prior to analysis, 0.2 g of freeze-dried grape skins was homogenized for 30 s in a blender with 25 mL of ethanol/water (80:20 v/v) and maintained at 60 °C for 30 min, with gentle stirring. The extract was filtered

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**Table 1. Extraction Conditions Described in the Literature To Extract Resveratrol and Piceid Isomers from Grapevine Tissues**

| extraction solvent | sample | time and temp conditions | compounds extracted[a] | reference |
|---|---|---|---|---|
| methanol 70% | grape berry skin | nd[b] | *trans*-resveratrol | Creasy and Coffee (*20*) |
| methanol 90% (in ethanol) | grape berry skin | nd | *trans*-piceid and *trans*-resveratrol | Waterhouse and Lamuela-Raventós (*9*) |
| methanol 80% | grape berry skin | nd | *trans*-resveratrol | Soleas et al. (*6*) |
| ethyl acetate | grape berry skin | nd | *trans*-resveratrol | Okuda and Yokotsuka (*10*) |
| methanol 95% | grape berry skin and seeds | overnight, room temp | *trans*-resveratrol | Jeandet et al. (*29*) |
| methanol | grape berries | 5 min | *trans*-resveratrol | Pezet et al. (*31*) |
| methanol | grape berries | 48 h, 3 °C | *trans*-resveratrol | Ector et al. (*30*) |
| methanol 95% | grape berries | 20 min | *trans*-resveratrol | Bavaresco et al. (*25*) |
| methanol 70% | grapevine leaves | nd | trans-resveratrol | Langcake and Pryce (*26*) |
| methanol 70% | grapevine leaves | nd | *trans*-resveratrol | Dercks and Creasy (*28*) |
| methanol 80% | grapevine leaves | 15 min | *trans*-piceid, *cis*-piceid, and *trans*-resveratrol | Jeandet et al. (*31*) |
| methanol 85% | grapevine leaves | nd | *trans*-piceid and *trans*-resveratrol | Douillet-Breuil et al. (*39*) |
| methanol 50% | grapevine pomace | 2 min | *trans*-resveratrol | Soleas et al. (*3*) |

[a] Only resveratrol or piceid isomers have been included. [b] nd, not described.

through a stainless steel colander and concentrated to 10 mL by rotary evaporation (in vacuo) at room temperature. The concentrated extracts were filtered through Whatman inorganic Anopore membrane filters (Anotop 10 plus, 0.2 $\mu$m) and injected into the high-performance liquid chromatograph (HPLC) without further processing.

*Optimization.* The following extraction conditions were optimized: temperature, time, and extraction solvent. Samples were extracted with ethanol/water (80:20 v/v) at temperatures that varied from 25 to 80 °C, for 15−120 min, and the extraction profiles obtained were evaluated.

After optimal conditions of temperature and time had been established, five extraction solvents were tested: ethanol/water (80:20 v/v), ethanol 100%, ethyl acetate/methanol (50:50 v/v), acetone/water (75:25 v/v), and acetone 100%. The amounts extracted for each one of these solvents are expressed in micrograms per gram of dry grape berry skin.

*Validation.* To validate the extraction method with ethanol 80%, at 60 °C for 30 min, we have evaluated recovery and precision. Recovery was calculated by comparing *trans*-resveratrol values in grape skins with those in the same sample spiked with known amounts of standard compound, on three different days. *trans*-Resveratrol standard was added at 20, 40, and 100% of the original concentration on grape skins, before the sample was homogenized in the extraction solvent.

To evaluate precision (or degree of reproducibility), the same sample was extracted on eight different days. The precision of the extraction, for each compound, was expressed as the coefficient of variation.

**HPLC Analysis of Resveratrol and Piceid Isomers.** *trans*-Resveratrol (*trans*-3,5,4′-trihydroxystilbene) standard was purchased from Sigma Chemical Co. (St. Louis, MO). *trans*-Piceid (*trans*-resveratrol 3-*O*-$\beta$-D-glucoside) standard was extracted from the roots of *Polygonum cuspidatum*, as described by Waterhouse and Lamuela-Raventós (*9*). *cis*-Resveratrol (*cis*-3,5,4′-trihydroxystilbene) and *cis*-piceid (*cis*-resveratrol 3-*O*-$\beta$-D-glucoside) standards were obtained by sunlight exposure of the *trans* isomers. The compounds were characterized as *cis*-isomers by their UV−visible spectra using a diode array detector (DAD) and by comparison with literature data (*32*, *33*).

The samples were injected in duplicate onto a Hewlett-Packard (HP) 1050 instrument equipped with an HP autoinjector, ChemStation, and an HP 1050 diode array UV−visible detector. The volume injected was 100 $\mu$L. The system was equipped with a Tracer Nucleosil column $C_{18}$ 120 (25 × 0.4 cm), 5 $\mu$m particle size, with a precolumn of the same material; the column temperature was maintained at 40 °C.

The HPLC conditions were described previously (*8*). In summary, the elution profile was as follows: 0 min, 83.5% A, 16.5% B; 13 min, 82.0% A, 18.0% B; 15 min, 82.0% A, 18.0% B; 17 min, 77.0% A, 23.0% B; 21 min, 75.0% A, 25.0% B; 27 min, 68.5% A, 31.5% B; 30 min, 0% A, 100% B, where solvent A was glacial acetic acid in water (52.6:900 v/v) and solvent B was 20% phase A and 80% acetonitrile.

Identification of piceid isomers and *trans*-resveratrol was carried out by comparison of the retention time of each standard and that within the extracts. They were also characterized by their UV spectra from 250 to 400 nm using the photodiode array detector and by line spectral comparisons with the standards.

UV maximum absorbances of *trans*-forms were at 306 nm and at 285 nm for *cis*-piceid.

The UV spectra of the resveratrol glucosides are very close to that of resveratrol aglycons, and the quantitation of *trans*-piceid and *cis*-piceid was based on the assumption of identical molar extinction coefficient of *trans*-resveratrol and *cis*-resveratrol at 306 and 285 nm, respectively. The standard curves of *trans*-resveratrol and *cis*-resveratrol for the quantitation of *trans* isomers and *cis*-piceid were performed as described previously (*4*).

The values of selectivity, precision, limit of detection (0.003 mg/L), and limit of quantitation (0.01 mg/L) determined with a *trans*-resveratrol standard were similar to those obtained previously (*8*).

## RESULTS AND DISCUSSION

**Extraction of Resveratrol and Piceid Isomers from Grape Berries.** *Optimization of Temperature and Time.* We have performed extractions at 25, 40, 60, and 80 °C to determine the effect of temperature in the extraction process. In each one of the temperature conditions tested we have quantified piceid and resveratrol isomers at different times of extraction (15, 30, 45, 60, and 120 min), on duplicate samples. After revision of previous extraction conditions described in the literature (see Table 1), the extraction solvent employed was ethanol/water (80:20 v/v). As methanol and ethanol have the same properties to extract both hydrophilic and hydrophobic compounds and methanol is included in ICH guidelines as a solvent to be avoided in medicinal products (*34*), we have selected ethanol as a less toxic solvent.

Table 2 shows the maximum amount of stilbenes extracted in grape berry skin at each temperature assayed. By comparison of the concentrations reached at 25, 40, 60, and 80 °C, the highest extraction for *trans*-resveratrol and piceid isomers was observed at 60 °C for 30 min. *cis*-Resveratrol was not detected in any

**Table 2. Maximum Amounts of Resveratrol and Piceid Isomers in Grape Berry Skin Extracted in Ethanol 80% at Each Temperature**

| temp (°C) | time (min) | μg/g of dry skin *trans*-resveratrol | *trans*-piceid[a] | *cis*-piceid[b] | total amount |
|---|---|---|---|---|---|
| 25 | 120 | 97.80 | 82.65 | 41.17 | 221.62 |
| 40 | 120 | 121.70 | 151.89 | 70.91 | 344.50 |
| 60 | 30 | 246.35 | 157.73 | 85.23 | 489.31 |
| 80 | 30 | 283.13 | 142.62 | 38.33 | 464.08 |

[a] Quantified as *trans*-resveratrol. [b] Quantified as *cis*-resveratrol; *cis*-resveratrol was not detected.

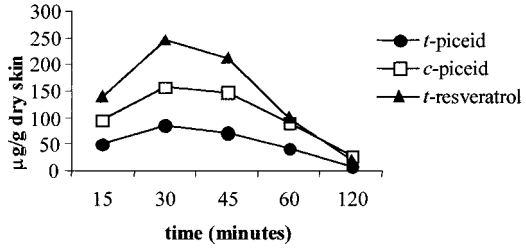


**Figure 1.** Extraction profile of stilbenes versus time from grape skin sample at 60 °C using ethanol/water (80:20 v/v) as extraction solvent. The values are means of two different extractions.

condition assayed. At 25 °C, the maximum of extraction was reached at 120 min; however, only 40 and 50% of the extraction obtained for the three compounds at 60 °C for 30 min was obtained. The maximum of extraction at 40 °C was also at 120 min, and it was very high for *trans*-piceid (95%) and *cis*-piceid (80%) but very low for *trans*-resveratrol (50%), compared with the amounts extracted at 60 °C for 30 min.

Prior to stipulating the extraction conditions of temperature and time at 60 °C and 30 min, respectively, we have confirmed that a higher increase in temperature was not related to a higher increase in the extraction. For that reason, we carried out the extraction at 80 °C (on duplicate samples), and piceid and resveratrol isomers were quantified at 15 and 30 min. In these conditions the maximum of the extraction is reached at 30 min (Table 2). In comparison with 60 °C and 30 min conditions, the extraction for *trans*-resveratrol was higher (115%), was slightly lower for *trans*-piceid (90%), and was very much lower for *cis*-piceid (45%).

In Figure 1 the evolution of the three compounds at 60 °C with time is shown; the maximum was reached at 30 min, and then there was a depletion of all of them, probably due to their degradation after 45 min of extraction.

Finally, we have tested in duplicate the stability of a *trans*-resveratrol standard solution (5 mg/L, exposed for 5 min to sunlight) in ethanol 80% at 60 °C for 30 min, and no significant degradation was observed for *trans*- and *cis*-resveratrol (between 2 and 4%, respectively).

*Optimization of Extraction Solvent.* To ensure the extraction solvent selected was the best extractor, we assayed different polarity solvents to evaluate their effect in the extraction at 60 °C during 30 min. The solvents employed were ethanol/water 80:20 (v/v), ethanol 100%, ethyl acetate/methanol 50:50 (v/v), acetone/water 75:25 (v/v), and acetone 100%. The results obtained are shown in Figure 2. As we can see, the highest extraction for all compounds was obtained with ethanol/water 80:20 (v/v), and the lowest was obtained with acetone 100%.

*Validation.* Recovery of *trans*-resveratrol spiked to the sample at 20, 40, and 100% of the original concentration on grape skins was very high for the three concentrations: 96.2% for the lowest, 103.4% for the medium, and 97.8% for the highest, respectively.

The precision of the extraction, expressed as the coefficient of variation, was 15.13% for *trans*-resveratrol, 7.95% for *trans*-piceid, and 6.83% for *cis*-piceid, the means obtained for each compound being 18.13 μg/g of dry skin for *trans*-resveratrol, 64.31 μg/g of dry skin for *trans*-piceid, and 307.18 μg/g of dry skin for *cis*-piceid. These precision values are satisfactory according to Huber (*35*).

The methods published previously to extract resveratrol and piceid isomers from grapevine tissues (Table 1) required different treatments of extracts before the chromatographic analysis. Furthermore, in the majority of them only *trans*-resveratrol was studied. With the extraction method described in this paper, *trans*-resveratrol, *trans*-piceid, and *cis*-piceid can be quantified from grape berry skins in a simple and reproducible manner.

**Resveratrol and Piceid Isomers in Healthy Grapes.** Table 3 shows the amounts of *trans*-piceid, *cis*-piceid, and *trans*-resveratrol found in skins of grape berries from different varieties. To our knowledge, this is the first time that the *cis*-piceid concentration has been studied in grape berry skin.

The samples analyzed were from white grape berries from four different varieties (Parellada, Macabeo, Chardonnay, and Xarel·lo) and five red grape berries from three different varieties (Cariñena, Cabernet Sauvignon, and Merlot). In Figure 3 a chromatogram of a grape berry skin extract is shown.

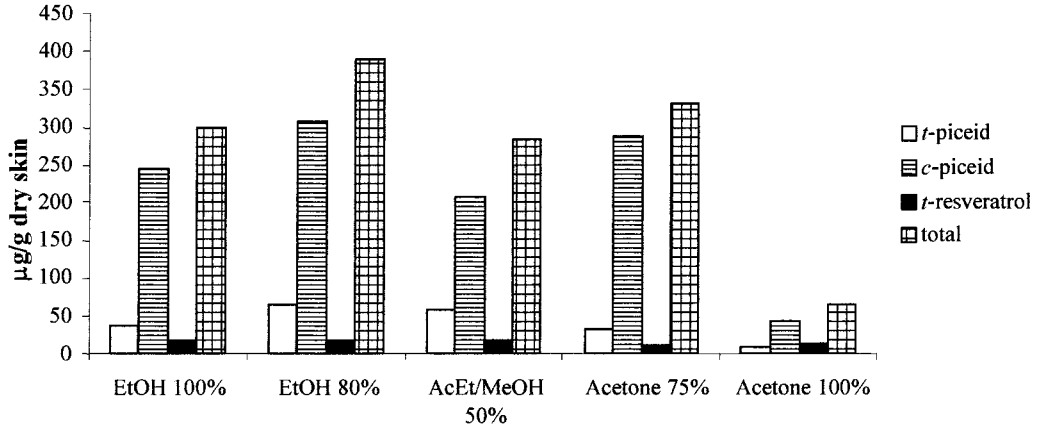


**Figure 2.** Grape skin extraction with different solvents. The extraction was performed at 60 °C for 30 min in triplicate.

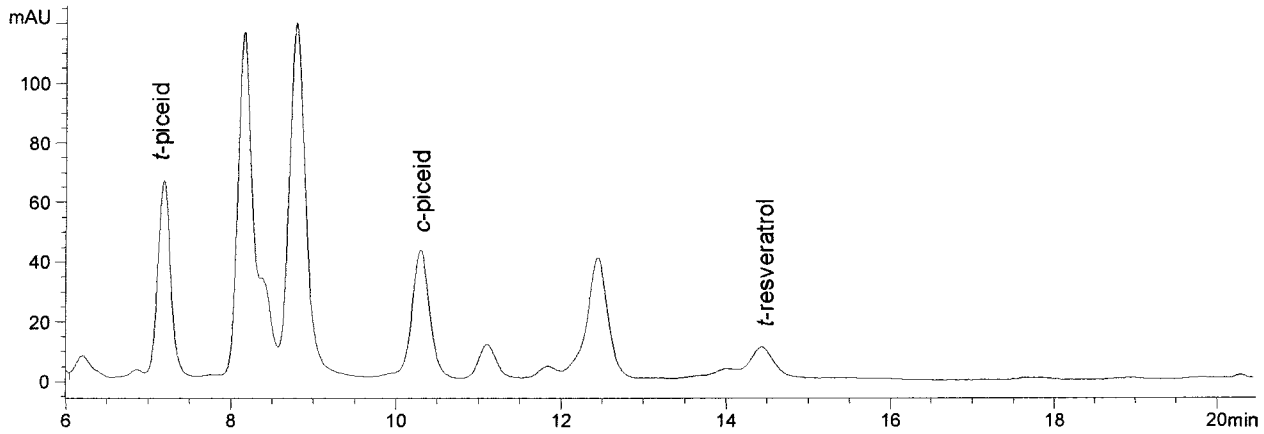


**Figure 3.** Chromatogram obtained at 306 nm corresponding to a grape skin extract.

**Table 3. Content of Resveratrol and Piceid Isomers in Grape Berry Skins Extracted in Ethanol 80% at 60 °C during 30 min**

| | | μg/g of dry skin | | | |
|---|---|---|---|---|---|
| grape variety | °Brix | *trans*-resveratrol | *trans*-piceid[a] | *cis*-piceid[b] | total amount |
| white | | | | | |
| Parellada | 16 | 12.54 | 9.92 | 37.80 | 60.26 |
| Parellada | 15 | 11.04 | 8.08 | 43.27 | 62.39 |
| Parellada | 19 | 16.59 | nq[c] | 5.50 | 22.09 |
| Macabeo | 19 | 47.60 | 5.04 | 11.82 | 64.46 |
| Chardonnay | 18 | 26.25 | 12.13 | 39.68 | 78.22 |
| Xarel·lo | 18 | 18.13 | 64.31 | 307.18 | 389.61 |
| red | | | | | |
| Cariñena | 19 | 18.32 | 24.03 | 11.14 | 53.49 |
| Cariñena | 21 | 21.35 | 38.43 | 20.89 | 80.66 |
| Cariñena | 17 | 17.28 | 20.89 | 12.39 | 50.55 |
| Cabernet Sauvignon | 20 | 26.65 | 11.12 | 51.31 | 89.09 |
| Cabernet Sauvignon | 17 | 19.35 | 5.49 | 9.80 | 34.64 |
| Cabernet Sauvignon | 21 | 39.38 | 6.44 | 4.00 | 49.83 |
| Merlot | 16 | 38.26 | 342.66 | 645.47 | 1026.39 |

[a] Quantified as *trans*-resveratrol. [b] Quantified as *cis*-resveratrol. [c] nq, not quantified; *cis*-resveratrol was not detected.

*cis*-Resveratrol was not detected in any sample, in accordance with the data published previously by Langcake and Pryce (*26*), Jeandet et al. (*29*), and Soleas et al. (*6*).

*cis*-Piceid was the major compound in 6 of 11 samples analyzed (4 white and 2 red grapes). The amount of *cis*-piceid found was between 4 and 645.47 μg/g (average = 92.33 μg/g), the amount for *trans*-piceid was between not quantified and 342.66 μg/g (average = 42.19 μg/g), and the amount for *trans*-resveratrol was between 11.04 and 39.38 μg/g (average = 24.06 μg/g). The greatest content of total resveratrol was found in Merlot sample (1026.39 μg/g). The content of resveratrol in this grape variety was much higher than in the others, as has previously been observed in wines (*1*), followed by one white grape berry, Xarel·lo (389.61 μg/g) (*5*).

In accordance with data published by Okuda and Yokotsuka (*10*) and Soleas et al. (*3*), we have found similar amounts of *trans*-resveratrol in red and white grape berries (average = 28.39 and 22.02 μg/g, respectively).

**Resveratrol and Piceid Isomers in Mildew-Infected Grapes.** Powdery mildew, caused by the fungus *Uncinula necator*, is the most enduring and persistent disease on grapes, especially among California *Vitis vinifera* vineyards. The degree of susceptibility to powdery mildew varies from variety to variety, and Cariñena variety is one of the most seriously affected (*36*).

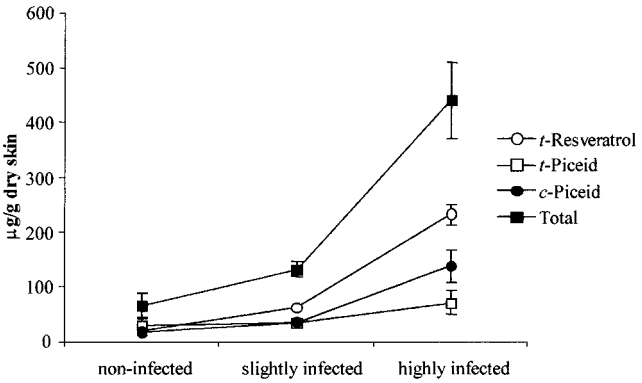


**Figure 4.** Evolution of stilbene content versus the increasing *U. necator* infection (powdery mildew) in the vineyard. The values are expressed as means of two different vineyards with their error bars. Extraction was performed with ethanol 80% at 60 °C for 30 min.

Figure 4 shows the evolution of resveratrol and piceid contents versus the infection degree of Cariñena grape berries from two different vineyards. As we can see in the error bars, the evolutions of *trans*-resveratrol and piceid isomers were similar in both vineyards. Resveratrol and piceid concentrations increased when the infection degree was higher. In grape berries not apparently infected by powdery mildew the amounts of the three compounds were similar. In highly infected grape berries, *trans*-resveratrol was the compound with a great increase (~12-fold higher than in healthy grapes) followed by *cis*-piceid (~8-fold higher than in healthy grapes). However, the content of *trans*-piceid is only ~2.5-fold higher than in healthy grapes.

*Botrytis cinerea* infection is the most studied fungus−grapevine interaction (*22*−*24*). These authors observed that the fungal pressure in the vineyards increased the synthesis of *trans*-resveratrol. However, in the case of grapes highly infected by *Botrytis* the concentration of resveratrol is lower than in healthy grapes, probably due to their degradation promoted by exocellular enzymes of this fungus, for example, a laccase-like stilbene oxidase (*22*, *37 38*).

In *Plasmopara viticola*−grapevine interaction, this fungus cannot detoxify resveratrol and was not able to suppress phytoalexin production in *Vitis* spp. (*28*). The results obtained in *Uncinula necator*−grapevine interaction are in accordance with this last observation: a higher infection degree is correlated with a higher concentration of *trans*-resveratrol in grape berry skins. These results could suggest that, in a manner similar

to that in *Plasmopara* infection, *U. necator* cannot degrade resveratrol.

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