Isolation, Characterization, and Evolution in Red Wine Vinification of Resveratrol Monomers

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Isolation from red wine and characterization of *trans*- and *cis*-resveratrol, *trans*-resveratrol β-D-glucopyranoside, and *cis*-resveratrol β-D-glucopyranoside are described. Extraction from grape skin and chemical changes of the four compounds during a conventional red wine vinification were monitored, showing the close relationship among the contents of the compounds. Factors influencing the model, such as the ethanol concentration, the hydrolytic cleavage of glucose moieties in glucosides and *trans/cis* isomerization, are discussed on the basis of the experimental data.

Keywords: *Vitis vinifera; stilbenes; trans-resveratrol; cis-resveratrol; trans-resveratrol β-D-glucopyranoside; cis-resveratrol β-D-glucopyranoside; NMR*

INTRODUCTION

Resveratrol (3,5,4′-trihydroxystilbene) and its oligomers are natural compounds, found in various families of plants (Gorham, 1980; Sotheeswaran and Pasupaty, 1993; Casabuono and Pomilio, 1994; Powell et al., 1994). Grapes and related products are probably the most important foodstuff containing these compounds. Hydroxylated stilbenes have been investigated both for their biological role in plant defense against pathogens and for their pharmacological properties. The genus *Vitis* is characterized by the presence of a wide number of such compounds in berries, leaves, and roots (Langcake and Pryce, 1977; Pryce and Langcake, 1977; Langcake et al., 1979; Pezet and Pont, 1988; Creasy and Coffee, 1988; Jeandet et al., 1991; Mattivi and Reniero, 1992; Korhammer et al., 1995).

Recently Siemann and Creasy (1992) hypothesized a link between the study reporting decreased lipid deposition in rats' liver from *trans*-resveratrol (**1**) and clinical studies showing reduced serum lipid levels in humans from drinking wine. One of the physiological mechanisms is based on the proved inhibition of LDL oxidation by phenolic substances (Frankel et al., 1993a). The possible benefit of wine on human health due to the presence of **1** is under debate because, in many cases, the concentration was assessed to be very low, so that it would be impossible to have a sufficient ingestion of the active principle with a normal consumption of wine (Frankel et al., 1993b).

More recently, further studies reported the presence of **1** in wine in higher concentrations than previously reported and the presence of the *cis* isomer (**2**) (Jeandet et al., 1993; Mattivi, 1993a,b; Roggero and Archier, 1994). The higher content measured in experimental wines with respect to marketed ones showed the possibility of increasing the resveratrol levels by means of a proper choice of vinification techniques (Mattivi and Nicolini, 1993).

The presence in red wines, at up to 14.5 mg/L, of one of the *trans*-resveratrol glycosides—which was supposed to be the *trans*-resveratrol β-D-glucopyranoside (**3**) named piceid—was reported (Roggero and Archier, 1994). Piceid was isolated and structurally characterized in the roots of the medicinal plant *Polygonum cuspidatum* and was used for confirming its presence in grape skin (Waterhouse, 1994).

In this paper we report the isolation and characterization by NMR of **3** from wine. Furthermore, the presence of *cis*-resveratrol β-D-glucopyranoside (**4**) and its characterization by ^1H and ^{13}C NMR are described. As far as we know, **4** has never been reported as a natural product but has been obtained by photochemical isomerization of **3** (Jayatilake et al., 1993). The contents of **1**–**4** during conventional red wine vinification are also studied, and the relationships between free and bound forms are discussed, taking into account the role of some main factors, such as extraction from grape skin, hydrolysis of glucosides, and *trans/cis* isomerization.

MATERIALS AND METHODS

As we observed that glucosides **3** and **4** show low susceptibility to oxidation, we performed their purification in the presence of air, thus improving the removal of other highly oxidizable coeluting compounds (such as oligomeric proanthocyanidins and tannin–anthocyanin pigments). Their isolation from wine was carried out with a double extraction (EtOAc, 14 L) on about 5 L of a Slovenian red wine of the *Blaufränkisch* variety, chosen for its high content of both isomers. The acidic compounds were removed by washing three times with 3% $NaHCO_3$ aqueous solution. After drying, the solution was concentrated to low volume and passed through semipreparative HPLC (LiChrospher 100 RP-18 column, 10 μm particle size, column dimensions 25 × 1 cm, λ = 350 nm), eluting it with a H_2O–MeCN mixture (70:30, isocratic, flow rate 1.6 mL/min). A fraction of 5.6 mg of **4** was obtained (15–16 min), whereas the fraction including **3** (12–13.5 min) contained a high amount of tyrosol and other minor compounds. The fraction was thus dissolved into a H_2O–EtOH (1:1) solution saturated with NaCl, and tyrosol was extracted with pentane–CH_2Cl_2 (2:1). The H_2O–EtOH solution was then evaporated to remove ethanol, loaded on a C_{18} Sep-Pak cartridge (1 g), washed with H_2O, and finally eluted with EtOAc. In this way a 4.1 mg fraction of **3** was obtained.

^1H-NMR spectra were recorded at 500 MHz, and ^{13}C-NMR spectra at 125 MHz, in acetone-d_6. The resonances of the methyl group of acetone-d_6 were used as reference for δ values: ^1H δ = 2.04; ^{13}C δ = 29.8. FAB-MS spectra were obtained in positive mode using a glycerol matrix. UV measurements were performed in ethanol.

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Table 1. ^{1}H-NMR Parameters (δ Values; J in Hertz) of Compounds 1–4

	1	2	3	4
H-2′	7.41	7.12	7.41	7.13
H-3′	6.83	6.69	6.82	6.72
H-5′	6.83	6.69	6.82	6.72
H-6′	7.41	7.12	7.41	7.13
$J_{2',3'}$	8.70	8.60	8.60	8.50
$J_{5',6'}$	8.70	8.60	8.60	8.50
H-α	6.87	6.32	6.89	6.34
H-β	7.00	6.42	7.07	6.45
$J_{\alpha,\beta}$	16.40	12.40	16.30	12.20
H-2	6.53	6.27	6.79	6.49
H-4	6.26	6.21	6.47	6.41
H-6	6.53	6.27	6.66	6.43
$J_{2,4}$	2.00	2.20	2.10	1.90
$J_{4,6}$	2.00	2.20	bs[a]	1.90
$J_{2,6}$	2.00	2.20	1.60	1.90
H-1 Glc[b]			4.93	4.76
H-2 Glc			3.44	3.38
H-3 Glc			3.51	3.45
H-4 Glc			3.43	3.44
H-5 Glc			3.52	3.35
H-6 Glc			3.72	3.68
H-6′Glc			3.91	3.78
$J_{1,2}$			7.70	7.60
$J_{2,3}$			aa[c]	9.00
$J_{3,4}$			aa	aa
$J_{4,5}$			9.10	aa
$J_{5,6}$			5.80	4.80
$J_{5,6'}$			2.50	2.40
$J_{6,6'}$			11.20	11.80

[a] bs, broad signal. [b] Glc, β-D-glucopyranose. [c] aa, according to axial–axial coupling.

The identity and the purity of the isolated compounds were also confirmed by assessing each product of hydrolysis. Quantitative enzymatic hydrolysis of **3** and **4** (0.5 mg) was carried out in a citrate buffer (citric acid–sodium citrate, 0.11 M, to pH 5.0 with NaOH), at 45 °C with almond β-glucosidase (Sigma; activity 5.5 units/mg of solid). After reaction (24 h), the solution was loaded on an activated C_{18} Sep-Pak cartridge (1 g). Sugars were recovered in the percolate (plus 5 mL of water for washing the column) and analyzed by GC after silylation of the *O*-methyloxime derivatives (Versini et al., 1984). A subsequent elution of the cartridge with 5 mL of EtOAc gave the aglycons, which were analyzed by HPLC (Mattivi, 1993a). Compound **1** is commercially available (Sigma), while **2** was obtained through UV-induced photoisomerization of **1** (100 mg/L in EtOH, λ = 254 nm, 24 h) and purified by means of preparative HPLC. Pure **1** and **2** were also isolated from wine under the same preparative conditions used for **4**. Care has to be taken in using pure **2** as a standard, because it undergoes partial rearrangement to **1**. For this reason the hydrolysis of **4** led to the formation of a mixture containing about 85% **2** and 15% **1**.

The extraction and transformation of compounds **1–4** during alcoholic fermentation and prolonged skin maceration were investigated throughout a conventional red wine vinification of the *Lambrusco a foglia frastagliata* variety. Samples of free juice were collected daily; finally the total wine (free + press run after dejuicing) was sampled (Mattivi and Nicolini, 1993). Fermentation of the samples (250 mL) was stopped by adding $NaHSO_3$ (200 mg/L) and cooling at 4 °C. The analyses were performed the same day of sampling. The wine had a final total stilbenes content of 57.2 μmol/L (72.1 for the free run juice), thus allowing us to perform the quantitative analysis of each compound easily.

RESULTS AND DISCUSSION

Glucosides Characterization. The structures were elucidated by 1D- and 2D-NMR experiments (1D- and 2D-^{1}H,^{1}H-COSY, 2D-^{1}H^{13}C-COSY, 1D-NOE difference). ^{1}H- and ^{13}C-NMR data for compounds **1–4** are reported in Tables 1 and 2, respectively. By comparison with the literature (Jayatilake et al., 1993; Mannila et al., 1993) and with our results on pure compounds, the aglycons were confirmed to be *cis*- and *trans*-resveratrol. By comparing the NMR data with those from literature (Bock and Thøgersen, 1982; Jayatilake et al., 1993) and with our experimental data, we concluded the carbohydrate unit was β-D-glucopyranose. Because of the overlapping signals, we also performed 1D-relayed coherence transfer experiments using Gaussian pulses for selective excitation (Kessler et al., 1986, 1991). The ABC-spin system indicates that the glucose moiety is bound to the stilbenic benzene ring bearing two hydroxyl groups. NOEs from H-α to H-2 and H-6 and from H-1 Glc to H-2 and H-4 indicate the position of the substitution to the benzene ring (Figure 2).

Table 2. ^{13}C-NMR Spectral Data of Compounds 1–4 (δ Values)

C	1	2	3	4
1	140.9	140.6	140.9	140.5
2	105.7	107.9	106.2	109.1
3	159.6	159.3	160.3[a]	160.0[a]
4	102.7	102.4	103.9	103.8
5	159.6	159.3	159.4	159.1[a]
6	105.7	107.9	108.2	110.5
α	126.9	129.0	126.5	128.9
β	129.1	130.5	129.9	131.0
1′	130.0	129.4	130.0	129.3
2′	128.8	131.1	128.9	131.0
3′	116.4	115.8	116.4	115.9
4′	158.2	157.5	158.2[a]	157.6[a]
5′	140.9	115.8	116.4	115.9
6′	105.7	131.1	128.9	131.0
1 Glc[b]			102.1	102.1
2 Glc			74.8	74.7
3 Glc			78.1	77.9
4 Glc			71.5	71.2
5 Glc			77.8	77.5
6 Glc			62.8	62.6

[a] Exchangeable. [b] Glc, β-D-glucopyranose.

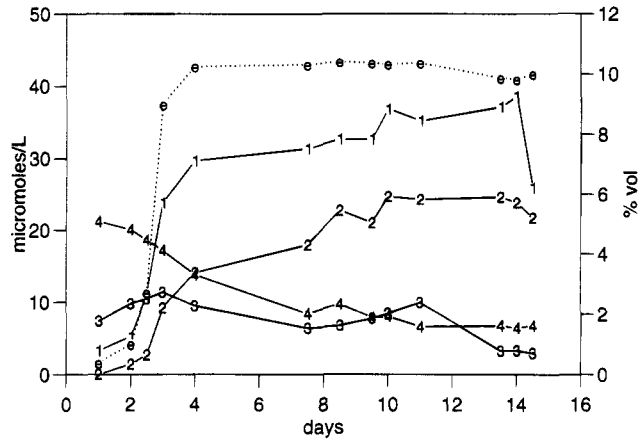

Figure 1. Evolution of compounds **1–4** and of ethanol (**e**) in the free run juice during a conventional red wine vinification. The last points indicate the content in wine (press run juice added).

FAB-MS spectra showed a $M^+ + 1$ peak at m/z 391 ($C_{20}H_{22}O_8$ requires 390).

The UV spectra in ethanol are identical to those of the relevant aglycons (Ingham, 1976).

Enzymatic hydrolysis of resveratrol glucosides led to a quantitative formation of free *trans*- and *cis*-resveratrol and glucose.

HPLC Analysis. The HPLC method developed for the analysis of *trans*-resveratrol (Mattivi, 1993a) was used for the quantification of compounds **1–4**.

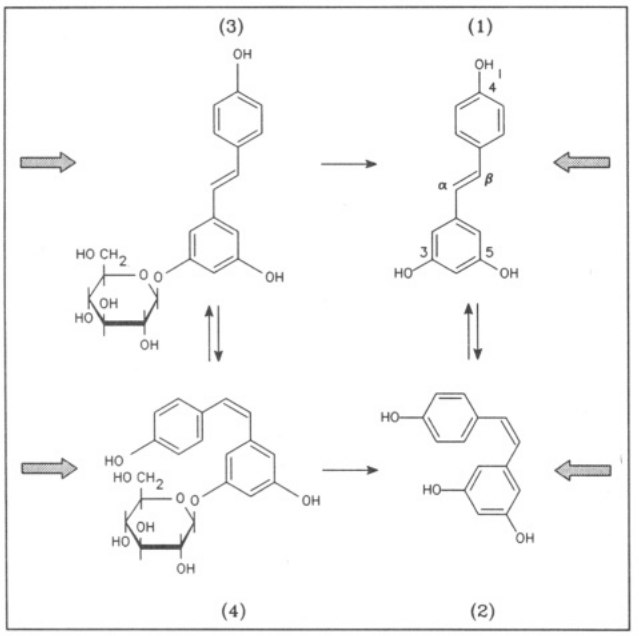

Figure 2. Diagram of the principal factors influencing stilbenes concentration during red wine vinification (shaded arrow, extraction from solid parts of grape; single arrow, hydrolysis; double arrow, isomerization).

The preparative stage on a C_{18} cartridge allows the recovery of all the compounds. The UV spectral profiles of the glucosides are identical to those of the relevant aglycons, and the molar extinction coefficients are also very similar. The most suitable analytical wavelengths are 310 nm for *trans* and 282 nm for *cis* isomers. For this study, we chose to use the chromatograms at 310 nm as this was used previously in a study of the kinetic of extraction of *trans*-resveratrol (Mattivi and Nicolini, 1993), estimating the *trans* and *cis* isomers by assuming log ϵ values of 4.453 and 3.865, respectively (experimental values in absolute ethanol for free compounds).

Evolution during Vinification. At the beginning of the fermentation (Figure 1) **2** is absent while **1** is present (3.2 μmol/L). Their amounts increase in relation to the maximal production of ethanol; after that, a slight increase is observed. After dejuicing, the content decreases by adding the press run to the free run juice. The final concentration is higher in the *trans* than in the *cis* isomer.

Glucosides are extracted from the grape skin in the early stage of vinification before the extraction of the relevant aglycons, **3** being in lower concentration (7.4 μmol/L) than **4** (21.2 μmol/L).

The concentration of **4** decreases by a factor of 3 during vinification, with the maximum drop concomitant with the maximum yeast activity (ethanol production).

Compound **3** shows a slight growth up to the third day of the vinification (11.4 μmol/L); after that, it shows a slow decrease to a final content of 2.9 μmol/L.

An explanation of these results should consider the influence of three main factors: (1) direct extraction from the grape skins (glucosides extracted at a larger extent in earlier stages and free stilbenes at a later point, enhanced by the presence of ethanol); (2) hydrolysis of the glucosides, which leads to the formation of aglycons; (3) *trans/cis* isomerization. A diagram of the influence of these factors on the concentrations of **1**–**4** during vinification is shown in Figure 2.

The pattern of evolution of glucosides is consistent with the simultaneous extraction from the skins and hydrolysis to aglycons. The hydrolysis could be either acid catalyzed or enzymatic. The former can take place in a slightly acidic medium such as must or wine, yet it is usually a very slow reaction; the latter could explain better the experimental data. It is well-known that both grape and yeasts have β-glucosidase activities (Biron et al., 1988; Lecas et al., 1991; Darriet et al., 1988). The exogenous β-glucosidase of yeast cell membrane usually reaches its utmost activity during a short time (2–3 days) around the exponential growth of yeast population; after that, it undergoes a rapid decrease because of its weak stability at acidic pH (Gunata et al., 1992; Sponholz, 1992). The formation of the aglycons is thus explainable in the presence of both a direct extraction of free compounds from the grape skins and the hydrolysis of glucosides. The observed disappearance of 14.5 μmol/L of **4** during vinification and the formation of 21.7 μmol/L of **2** suggest that hydrolysis is the main source. The contemporaneous reaction of *trans/cis* isomerization can also play a role, especially with regard to the aglycons, which are less stable than the relevant glucosides. The high amount of **1** with respect to the relevant glucoside suggests that its direct extraction from the solid parts of the grape is more important—with respect to the hydrolysis—than for **2**.

At the beginning of the fermentation, the glucosides are the main stilbenes, the *cis* isomer being the most important. In the final wine a higher content of aglycons was found, especially in the case of the *trans* isomer, but the *cis* isomer was also present at comparable levels. According to our experience and to the data reported in a study on 106 samples (Mattivi et al., 1994), Italian red wines—as the one in this study—usually show a content of glucosides lower than the relevant free compounds.

Conclusions. The characterization and quantification of compounds **1**–**4** confirm that red wine is a potential foodstuff source of stilbenes. The recognition of **4**, present in the early stage of the vinification, and its dropping during the fermentation, allow a better understanding of the origin of **2.**

The strong connections among compounds **1**–**4** indicate that a quantification of all four compounds is necessary to obtain an exhaustive estimate of the resveratrol monomers in red wine.

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