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THE OCCURRENCE OF PICEID, A STILBENE GLUCOSIDE, IN GRAPE BERRIES

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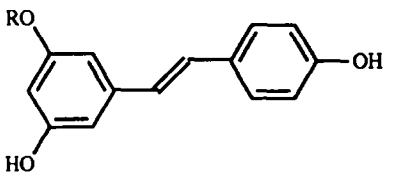
Key Word Index—piceid; polydatin; grapes; *Vitis* Vitaceae resveratrol; glucoside; stilbene glucoside.

Abstract—Piceid, the 3-β-glucoside of resveratrol, was observed in berries of two of the three varieties tested. These results suggest that the source and fate of this glucoside could be related to the biosynthesis of resveratrol and its production or loss in response to plant stress. Also, the levels of piceid in wine could affect the physiologically available amounts of resveratrol to consumers of wine.

INTRODUCTION

Resveratrol (**1**) is a well-established secondary metabolite that is found in *Vitis* species. It was first noted in grape leaves in 1976 by Langcake and Pryce [1] and later in berries [2, 3]. It has also been found in many other species, including peanut and pine [4]. Resveratrol production is related to fungal infection; most studies have focused on *Botrytis cinerea*. Initial studies examined the relationship between *Botrytis* infection and the production of **1** [5], whereas others compared the vine's ability to produce **1** and related stilbenes as a function of external factors [6, 7]. Additionally, some investigations addressed factors affecting the accumulation of **1** [8–10], as well as the induction and molecular analysis of the enzyme responsible for production of **1** [11, 12] including site-directed mutagenesis [13]. Perhaps most importantly, the gene for stilbene synthase was cloned into tobacco and the tobacco produced **1** in response to *B. cinerea* infection [14].

There is additional interest in **1** from another perspective, as it has been implicated as being responsible for the reduced heart disease rate amongst wine drinkers [15]. There have been a large number of very recent studies describing procedures and results for the analysis of wine for **1** [15–20]. This interest is based on various biological tests of **1** and piceid (**2**) which suggest they have a potential to reduce cardiac heart disease. These tests were initiated because *Polygonum cuspidatum* root, which contains high piceid levels, is used in Asia as a treatment for a number of diseases including atherosclerosis [21]. Resveratrol and/or piceid have been found to have *in vitro* effects in: inhibiting the copper-catalysed oxidation of LDL (low density lipoprotein, a serum cholesterol fraction) [22], inhibiting platelet clotting [23], arachidonate metabolism [24] and reducing liver injury from peroxidized oil [25].



	R
1	H
2	Glc

Preliminary studies on wine showed that when fermenting musts were treated with glucosidase-containing enzyme mixtures, increased levels of **1** were detected in the finished wine [26]. This suggested the presence of **2** in the must, and hence in the grapes used to make the wine. To establish whether this was the case or not, five grape berry samples were examined for the presence of **2**.

RESULTS AND DISCUSSION

The structure of **2** was established by Nonomura *et al.* as 3,4',5-trihydroxystilbene-3-β-mono-D-glucoside [27]. Authentic **2** was obtained by extracting *P. cuspidatum* and purifying the major UV absorbing component by preparative HPLC. The identity of the chromatographically purified material was authenticated in three different ways. First, the purified material was analysed by ^{1}H NMR spectroscopy and the spectrum correlated with literature data for **2** [28]. Second, a sample of this material was treated with β-glucosidase, and **1** was formed as evidenced by HPLC analysis. Third, the UV absorption spectrum of **2** was identical to literature values [29].

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Table 1. HPLC analysis of berry samples*

Grapes: Species, authority, 'variety', UC Davis selection number	Brix†	Piceid **2** ($\mu g\,g^{-1}$ dry wt skins)	Resveratrol **1** ($\mu g\,g^{-1}$ dry wt skins)
Vitis x labruscana Bail. 'Concord', UCD-05	19.3	n.d.	9.3
V. vinifera L. 'Syrah', UCD-01	23.0	2.8	11.3
V. vinifera 'Pinot noir', UCD-31	18.6	187	n.d.
V. vinifera 'Pinot noir', UCD-19	19.0	7.1	44.8
V. vinifera 'Pinot noir', UCD-29	20.0	66.3	78.5

*n.d. ≡ not detected.

†Degree of ripeness determined by measuring berry juice density equivalent to g sucrose $100\,g^{-1}$ solution.

Table 2. Recovery levels from first extract to HPLC analysis

Compound	Amount added ($\mu g\,g^{-1}$)	Amount found ($\mu g\,g^{-1}$)	Recovery (%)
Piceid	2.89	2.11	73
Resveratrol	4.95	1.83	37

The five grape samples were extracted using a procedure adapted from the *P. cuspidatum* extraction and berry extraction of resveratrol. Only the berry skins were extracted, as **1** is reported to be formed only in the skins [3]. A very general procedure was used which extracts the berries with methanol–ethanol followed by evaporation and partitioning of the resultant oil between aqueous and organic solvents. Because we utilize a concentration step and eliminated the grape pulp from the original extraction, our limit of detection was quite low for piceid ($0.08\ \mu g\,g^{-1}$ dry wt skins, approximately $0.001\ \mu g\,g^{-1}$ fr. wt whole grapes).

The levels of **1** observed here were comparable to previous reports on ripe grapes, in the range of $1\ \mu g\,g^{-1}$ fresh wt [3]. It appears (Table 1) that there is no proportionality between the levels of **1** and **2**. Most interesting was the sample of *Pinot noir*, UC selection 31 which had the highest levels of **2**, but no measurable amount of **1**.

One comment regarding the low recovery, especially of **1**, is in order (Table 2). Our procedure measured the levels of both **1** and **2** from the same sample, by separating the two components into organic and aqueous phases at one point. Efforts to improve the efficiency of recovery of one component would have reduced the recovery of the other. We chose to instead get some data on both components from a single sample, with an emphasis on the recovery of **2**. Higher extraction efficiencies would yield data with a more precise measure of berry levels, but the efficiency of our procedure leaves no doubt about the existence of piceid in these samples.

This initial report establishes that piceid is present in significant amounts in grape berry skins. Future studies will be able to address the origin and fate of this material in the berry, variety and species differences, and the presence of this material in other parts of the plant. In addition, the levels of **2** in wine should be studied to determine whether or not piceid could significantly contribute to the physiologically available pool to humans of resveratrol in wine.

EXPERIMENTAL

Standards. Piceid (polydatin) **2** was obtained by the extraction of *P. cuspidatum* Sieb. et Zucc. (grown from authentic cuttings obtained at the University of California, Berkeley Botanical Garden) as follows. Crude extracts were obtained by blending 8.62 g dry wt of lyophilized roots with 400 ml MeOH. The extract was centrifuged and the supernatant was washed with petroleum ether (2×50 ml). The remaining MeOH solubles were evapd to a syrup that was dissolved in 50 ml H_2O to which 50 ml EtOAc was added. The resulting aq. phase was adsorbed on to a 60 ml C-18 cartridge; this was eluted first with H_2O, with **2** subsequently eluted with MeOH. The MeOH solubles were then evapd *in vacuo*, with the resulting solids redissolved with 10 ml H_2O, 1 ml of which was injected on to a prep. C-18 column (300 mm $\times$ 7 mm, 5 μm particle size). The mobile phase was 84% 0.2 M orthophosphoric acid adjusted with ammonium hydroxide to pH 1.5 and 16% of acetonitrile with a flow rate of $4.2\ ml\,min^{-1}$, with detection (10 m cell) at 325 nm. The major component was collected, evapd to dryness, redissolved in H_2O and desalted using a C-18 cartridge as described above to yield 55 mg of **2**.

Purified **2** had the same UV spectrum as piceid, **2**, with peaks at 306 and 321 nm [29]. A ^1H NMR spectrum taken in D_2O was essentially identical to the lit. spectrum, previously taken in acetone-d_6 [28]. In addition, 2 mg '**2**' was dissolved in a pH = 6 aq. soln with $2\ mg\,100\,ml^{-1}$ β-glucosidase from almonds, $5.5\ U\,mg^{-1}$ [EC3.2.1.21] (Sigma). HPLC analysis of the soln after 14 hr at 21° showed largely **1** and a small amount of **2**. The standard of **1** was purchased from Sigma.

Extraction of berry skins. The skins were removed from berries and lyophilized. From *ca* 500 g fr. wt grape berries, 5 g of dry skins of each sample was extracted by blending in 100 ml of an alcoholic solution of MeOH–EtOH (90:10). The alcoholic extract was washed twice with 50 ml petroleum ether and evapd to a syrup. The syrup was dissolved in 30 ml of salt (NaCl) satd H_2O

and 30 ml of EtOAc was then added. The 2 phases were sepd. The aq. phase was adsorbed on to a C-18 cartridge as above, washed with H_2O and **2** was eluted with MeOH. The MeOH extract was evapd and the final residue was dissolved in H_2O and analysed by HPLC for **2**. The organic phase was evapd and the solids redissolved in 4 ml 1:1 H_2O–acetonitrile for HPLC analysis of **1**.

Analytical HPLC. An HPLC system with a diode array UV-visible detector was used with a reversed phase C-18 column, 4 × 250 mm, 5 μm particle size. The solvents used for the sepn were: A, 0.2 M orthophosphoric acid adjusted with ammonium hydroxide to pH 1.5; B, 20% A with 80% acetonitrile. Analysis of **2** was carried out using 20% B and 80% A, while **1** was analysed using 30% B and 70% A, in each case using a flow rate of 1 ml min^{-1}. The eluate was detected at 306 nm, and the UV spectrum of the peaks of **1** and **2** were compared to standard spectra to verify identity. In addition, 5 UV spectra at the maxima and leading and trailing sides of the peaks of interest were rescaled and overlaid in order to detect impurities in the peak.

The quantification of both compounds was carried out using external standards. The recovery of the extraction was calcd by adding both standards to half of one alcoholic berry extract and then comparing the incremental HPLC response to a calibrated response. The calibrated response curve was created by adding known amounts of piceid to a MeOH soln over the equivalent range of 80–0.008 $\mu g\,g^{-1}$ dry wt grape skins. This was treated as the MeOH extract of grapes above. The response was linear with a correlation coefficient of 0.999, with a limit of detection at 0.08 $\mu g\,g^{-1}$ dry wt grape skins. The response was linear with a correlation coefficient of 0.997. Levels lower than 4 $\mu g\,g^{-1}$ were not tested.

Note added in proof

A recent report describes the presence of a resveratrol glycoside in wines: Roggero, J. P. and Archier, P. (1994) *Sci. Aliments* **14**, 99.

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