Direct HPLC Analysis of *cis*- and *trans*-Resveratrol and Piceid Isomers in Spanish Red *Vitis vinifera* Wines

Keywords: *Resveratrol; piceid; stilbenes; isomers; red wine; HPLC*

INTRODUCTION

The phytoalexin *trans*-3,5,4′-trihydoxystilbene (resveratrol) was first reported in the skins of grapes (Creasy and Coffee, 1988; Jeandet *et al.*, 1991) and later in wines (Siemann and Creasy, 1992; Lamuela-Raventós and Waterhouse, 1993; Mattivi, 1993; Jeandet *et al.*, 1993; Roggero and Archie, 1994; McMurtrey *et al.*, 1994; Pezet *et al.*, 1994; Goldberg *et al.*, 1995). There has been a great interest in the presence of *trans*-resveratrol in wine for its protective effect against heart diseases since it inhibits platelet aggregation (Kimura *et al.*, 1985), it inhibits the oxidation of low-density lipoproteins oxidation (Frankel *et al.*, 1993), it reduces the levels of triacylglycerol, and it protects the liver from lipid peroxidation (Shan *et al.*, 1990). The levels described in red U.S. wines are below 1 mg/L (Siemann and Creasy, 1992; Lamuela-Raventós and Waterhouse, 1993) and much higher in Italian and French wines (Jeandet *et al.*, 1993; Mattivi, 1993; Roggero and Archie, 1994). Recent surveys report similar levels that are not quite so different (McMurtrey *et al.*, 1994; Goldberg *et al.*, 1995).

The physiological effects attributable to this compound in wines could be affected by the levels of other resveratrol derivatives. The 3-β-glycoside of *trans*-resveratrol, piceid, was noted in the skin of grapes (Waterhouse and Lamuela-Raventós, 1994). Roggero and Archie (1994) reported the presence of an undefined resveratrol glycoside in wines based on its UV spectral properties, which were similar to those of resveratrol. Piceid is the major polyphenol found in the root of *Polygonum cuspidatum*. The powder of this root is used in China and Japan as a treatment for atherosclerosis and for other therapeutic purposes (Kimura *et al.*, 1983, 1985; Shan *et al.*, 1990). Here the evidence for piceid in red wines is established by the use of a piceid standard extracted from *P. cuspidatum*.

HPLC using UV detection (Siemann and Creasy, 1992; Lamuela-Raventós and Waterhouse, 1993; Mattivi, 1993) and GC/MS (Jeandet *et al.*, 1993; Goldberg *et al.*, 1994) have been the methods most employed to measure *trans*-resveratrol of wines. These methods required prior sample treatment, before the injection. Direct HPLC anlysis of wines for *trans*-resveratrol was accomplished by McMurtrey *et al.* (1994) with an electrochemical detector and by Pezet *et al.* (1994) with a fluorometric detector. These methods were very sensitive to this compound and decreased the time needed for analysis.

The *trans* isomers are transformed to the *cis* forms under UV light (Siemann and Creasy, 1992). Except in the case of kinase inhibition, a factor related to anticancer activity (Jayatilake *et al.*, 1993), the physiological activity of the *cis* forms has not been studied previously, so it is important to distinguish it from the *trans* isomer and to quantify each separately. Also, in the studies of *P. cuspidatum* listed above, the activity of the resveratrol glycoside is observed to be quite different from that of the aglycon, but the human digestive tract is known to have glycosidase activity (Hackett, 1986), so it is possible that the glucoside of resveratrol could release the aglycon on ingestion.

To carry out an initial survey of resveratrol derivative concentrations in Spanish wines, we developed a new simple procedure that separates and quantitates the four compounds, *trans*- and *cis*-resveratrol and *trans*- and *cis*-piceid, in red wines.

MATERIALS AND METHODS

Standards. *trans*-Resveratrol was purchased from Sigma; piceid extract was obtained from *P. cuspidatum* as previously described (Waterhouse and Lamuela-Raventós, 1994). The *cis* forms of the aglycon and the glycoside were obtained by sunlight exposure of the *trans* isomers.

Samples. Eighteen monovarietal red wines from different Spanish appellations were analyzed. As described in Table 1, the varieties tested were Cabernet Sauvignon, Merlot, Pinot noir, Tempranillo, and Grenache. Some were samples purchased from local Barcelona markets, and some samples were obtained from national wineries.

The samples were analyzed by direct HPLC injection, after filtration through Whatman inorganic Anopore membrane filters (Anodisc, 0.2 μm); two replicates were performed for each sample. The wines were protected from light to avoid light-induced isomerization during sample handling.

HPLC Analysis. The HPLC system used for the analysis was a Hewlett-Packard (HP) 1050 gradient liquid chromatograph with a diode array UV–visible detector HP 1040M coupled to a Chem Station HP 79995A. A Nucleosil (Tracer) reversed-phase column, C_{18} 120 (25 × 0.4 cm), 5 μm particle size, with a precolumn of the same material, was used for the stationary phase, at 40 °C. Injection was by means of a Rheodyne injection valve (Model 7125) with a 100 μL fixed loop.

Two solvents were used for the separation: solvent A, glacial acetic acid in water, pH 2.40; solvent B, 20% phase A with 80% acetonitrile, with a flow rate of 1.5 mL/min.

The elution profile was as follows: 0 min, 82% A, 18% B; 10 min, 82% A, 18% B; 17 min, 77% A, 23% B; 21 min, 75.5% A, 24.5% B; 27 min, 68.5% A, 31.5% B; 30 min, 0% A, 100% B.

The eluate was monitored at two different wavelengths, 285 and 306 nm, where *cis* and *trans* isomers have absorbance maxima, respectively. After each analysis, the column was re-equilibrated with phase A for 5 min. Results of the analyses are expressed in milligrams per liter of *trans*-resveratrol equivalents based on absorbance of the *trans* and *cis* isomers at 306 and 285 nm, respectively, assuming equal absorptivities at those wavelengths.

RESULTS AND DISCUSSION

Method. Since HPLC analysis requires sample filtration, a number of filters were compared, including ones made of nylon, PVDF, and polysulfone. However, these retained more than 60% of *trans*-resveratrol. The filter selected, Anodisc 0.2 μm, did not retain any of this compound.

The HPLC conditions described are critical for the separation of the four stilbenes analyzed (see Figure 1). The pH of solvent A and the elution program were very important, since a pH higher than 2.40 did not allow the separation of *cis*-resveratrol from the next peak at 21.9 min. The separation of the *trans* forms of resveratrol and piceid from the adjacent peaks depended on the percentage of solvent B. Both parameters were optimized and yielded a complete resolution of the four

Table 1. Concentration of Resveratrol and Piceid Isomers in Spanish Red Wines

wine variety	vintage	apellation	*t*-piceid[a] (mg/L)	*c*-piceid[a] (mg/L)	piceid *t/c* ratio	*t*-resveratrol (mg/L)	*c*-resveratrol[a] (mg/L)	resveratrol *t/c* ratio	total *t/c* ratio	total (mg/L)
Pinot noir	1990	Penedès	0.97	0.57	1.70	8.00	2.13	3.76	3.32	11.67
Pinot noir	1990	Costers del Segre	3.96	0.79	5.01	2.26	0.11	20.55	6.91	7.12
Merlot	1989	Costers del Segre	4.01	0.68	5.90	4.64	0.67	6.93	6.41	10.00
Merlot	1992	Navarra	1.54	0.81	1.90	0.98	0.36	2.72	2.15	3.69
Merlot	1992	Penedès	2.36	1.20	1.97	7.74	2.48	3.12	2.74	13.78
Merlot	1993	Penedès	4.01	1.98	2.03	2.59	0.68	3.81	2.48	9.26
Carernet Sauvignon	1991	Penedès	0.96	0.40	2.40	0.95	0.20	4.75	3.18	2.51
Cabernet Sauvignon	1992	Navarra	1.00	0.52	1.92	1.32	0.38	3.47	2.58	3.22
Cabernet Sauvignon	1993	Penedès	1.11	0.35	3.17	1.55	0.20	7.75	4.84	3.21
Cabernet Sauvignon	1993	Penedès	1.23	0.55	2.24	1.86	0.40	4.65	3.25	4.04
Tempranillo	1990	Penedès	1.26	0.54	2.33	1.87	0.35	5.34	3.52	4.02
Tempranillo	1992	Ribera de Duero	1.06	0.95	1.12	2.04	0.46	4.43	2.20	4.51
Tempranillo	1992	Navarra	0.74	0.54	1.37	0.60	0.15	4.00	1.94	2.03
Tempranillo	1993	Penedès	1.19	0.61	1.95	1.28	0.19	6.74	3.09	3.27
Tempranillo	1993	Penedès	1.51	0.87	1.74	1.13	0.26	4.35	2.34	3.77
Tempranillo	1993	Penedès	1.05	0.62	1.69	1.05	0.25	4.20	2.41	2.97
Grenache	1992	Priorat	2.66	0.82	3.24	2.83	0.41	6.90	4.46	6.72
Grenache	1993	Priorat	2.60	0.95	2.74	2.04	0.46	4.43	3.29	6.05
Pinot noir av			2.46	0.68	1.54	5.13	1.12	4.58	4.21	9.39
Merlot av			2.98	1.17	2.55	3.99	1.05	3.80	3.14	9.19
Cabernet Sauvignon av			1.07	0.45	2.38	1.42	0.29	4.90	3.36	3.23
Tempranillo av			1.13	0.69	1.64	1.33	0.28	4.75	2.54	3.43
Grenache av			2.63	0.88	2.99	2.43	0.43	5.65	3.86	6.37
total av			1.85	0.76	2.43	2.48	0.56	4.43	3.28	5.65

[a] Amount expressed as *trans*-resveratrol.

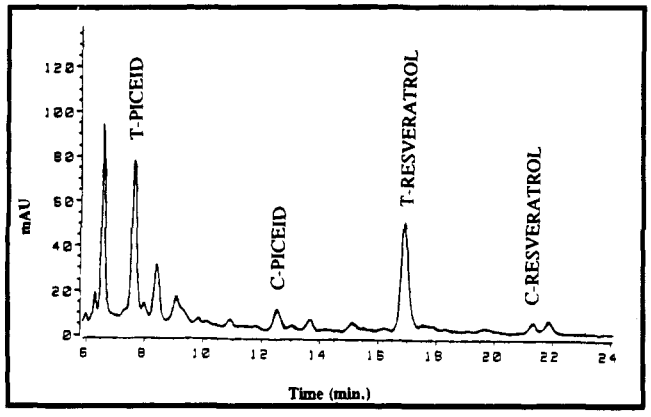

Figure 1. Chromatogram of a red wine at 306 nm.

compounds, each isomeric pair of which had nearly identical UV spectra at all points.

The validation of the analytical method was established according to *The United States Pharmacopeia* (USP XXII).

To evaluate precision, the same sample was injected on six different days. The precision or the degree of reproducibility is expressed as the coefficient of variation (CV). The four compounds studied, *cis* and *trans* isomers of resveratrol and piceid, had very low CV: 0.94% for *cis*-resveratrol, 1.56% for *trans*-resveratrol, 0.74% for *cis*-piceid, and 0.37% for *trans*-piceid.

The limit of detection (LD) and limit of quantitation (LQ) were calculated by measuring the magnitude of analytical background response by running six blanks, using the maximum sensitivity allowed by the system and calculating the standard deviation of this response. LD was estimated by multiplying the standard deviation (SD) by a factor of 3; the LQ was defined as 10 times the SD. At 306 nm the LD for *trans*-resveratrol was 0.003 mg/L and the LQ was 0.01 mg/L. The LQ was subsequently validated by the analysis of six standards prepared at a concentration at 0.01 mg/L.

Wines. Eighteen varietal Spanish red wines from different appellations and vintages were analyzed to identify the presence of the four stilbenes (Table 1).

These results corroborate other studies which show that, compared to the other varieties, wines made from Pinot noir grapes have a high level of *trans*-resveratrol, averaging 5.13 mg/L compared to 3.99 mg/L for Merlot, 1.42 mg/L for Cabernet Sauvignon, 1.33 mg/L for Tempranillo, and 2.43 mg/L for Grenache. Analyses of California wines show higher levels of *trans*-resveratrol in Pinot noir (Lamuela-Raventos and Waterhouse, 1993; McMurtrey *et al.*, 1994). The same pattern was observed in a comparison of French commercial wines (Roggero and Archie, 1994) as well as by a global survey (Goldberg *et al.*, 1995). It would appear that either the production of the *trans* isomer is apparently genetically controlled in some way or Pinot noir is consistently cultivated under conditions that enhance the production of resveratrol. However, the total amounts of resveratrol derivatives were very similar in Merlot (9.19 mg/L) and Pinot noir (9.39 mg/L) wines.

The levels of *trans*-resveratrol found in these wines were higher than those described for U.S. wines (Siemann and Creasy, 1992; Lamuela-Raventós and Waterhouse, 1993) but closer to the values obtained in Italian wines (Mattivi, 1993), where the high levels observed were attributed to the different environmental and cultural conditions. *trans*-Resveratrol is produced by grape berries in response to fungal infection and UV irradiation (Creasy and Coffee, 1988; Jeandet *et al.*, 1991). Thus, it is is possible that sun exposure of grapes may be a factor in increased resveratrol levels. However, it should be noted that each of the above reports was carried out using different procedures: Goldberg *et al.* (1995) using gas chromatography analysis and McMurtrey *et al.* (1994) applying direct HPLC analysis found smaller differences between European and American wines.

In some wines, for example, the Pinot noir from Costers del Segre, the amount of piceid is greater than resveratrol, and this is reflected in the *trans* and *cis* forms. In the study of piceid in grapes, there were some instances where piceid predominated and some where resveratrol predominated (Waterhouse and Lamuela-Raventós, 1994).

The *cis* isomer of resveratrol has not been previously reported as a natural product in other studies of *Vitis vinifera* grapevines (Langcake and Pryce, 1976; Jeandet *et al.*, 1991). It is possible that the *cis* isomer was present but was not detected, since other natural products contain both *cis* and *trans* isomers (Ingham, 1976). In wines, the *cis* isomers of both stilbenes could arise from light exposure of must or wine during the wine-making process or possibly from light exposure of wine bottles during storage.

The ratios of *trans* to *cis* of both piceid and resveratrol were always greater than 1, and sometimes as high as 20. This corroborates but does not confirm the supposition that *V. vinifera* produces only the *trans* isomer.

The presence of these four constituents in red wines means wine consumers ingest considerably more resveratrol than previously thought. Moderate consumers (200 mL/day) of Spanish red wines would ingest between 0.676 and 2.75 mg/day of *trans*-resveratrol and its derivatives. However, as Waterhouse and Frankel (1993) noted, the importance of this material will depend on its absorption and metabolism, as well as the level necessary to elicit an effect *in vivo*.

Conclusions. This rapid direct injection HPLC method for quantitation of both the *cis* and *trans* isomers of resveratrol and piceid in red wine facilitates analysis of the large number of samples needed for statistical comparison of treatments or varieties. The production level of stilbenes appears to depend on the grape variety, so their biosynthesis may be under some genetic control. The relatively high levels of *trans*-resveratrol in this sample of Spanish wines indicate that environmental factors can also affect resveratrol production. The generally high ratio of *trans* to *cis* isomers supports the supposition that resveratrol is produced as a *trans* isomer and the *cis* isomer is a contaminant derived by isomerization of the *trans* isomer. The data on the levels of all the available resveratrol derivatives in wine suggest that the amount available for its physiological effects may be greater than previously thought.

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