Resveratrol: Isomeric Molar Absorptivities and Stability

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Resveratrol has attracted interest as a wine constituent that may reduce heart disease. Published data on the molar absorptivity and chemical stability of *cis*- and *trans*-resveratrol have varied greatly. Accurate values for UV absorbance for *trans*-resveratrol [UV λ_{max} (EtOH) nm (ϵ) 308 (30 000)] and *cis*-resveratrol [UV λ_{max} (EtOH) nm (ϵ) 288 (12 600)] were determined and are used to improve chromatographic quantitation methods. Trials conducted under a variety of commonly encountered laboratory conditions show that *trans*-resveratrol is stable for months, except in high-pH buffers, when protected from light. *cis*-Resveratrol was stable only near pH neutrality when completely protected from light.

Keywords: *Antioxidant; phenolic; wine; grape; stilbene; isomer; UV spectroscopy; absorptivity*

INTRODUCTION

The non-flavonoid phytoalexin *trans*-resveratrol *(trans*-3,5,4′-trihydroxystilbene) and its various derivatives are widely distributed in gymnosperms and dicotyledons (Gorham, 1980) including groundnuts (Ingham, 1976; Schoeppner *et al*., 1984). *trans*-Resveratrol was discovered in the grape vine, *Vitis vinifera*, and was observed to occur there as a response to fungal infection or injury in 1976 (Langcake and Pryce, 1976). Later studies revealed *trans*-resveratrol in grape skins (Creasy and Coffee, 1988; Jeandet *et al*., 1991, 1995a; Roggero and Garcia-Parilla, 1995) and more recently in wines (Siemann and Creasy, 1992; Lamuela-Raventós and Waterhouse, 1993; Jeandet *et al*., 1993, 1995b,c; Mattivi, 1993; Roggero and Archie, 1994; McMurtrey *et al*., 1994; Pezet *et al*., 1994; Goldberg *et al*., 1994, 1995a; Vrhovsek *et al*., 1995). *trans*-Resveratrol has been suggested as a cause for reduced CHD mortality in wine drinkers and numerous studies describe potential mechanisms by which *trans*-resveratrol could reduce heart disease. *trans*-Resveratrol has been shown to inhibit platelet aggregation (Chung *et al*., 1992; Kimura *et al*., 1985; Pace-Asciak *et al*., 1995; Bertelli *et al*., 1995), protect the liver from lipid peroxidation (Kimura *et al*., 1983), and inhibit low-density lipoprotein oxidation (Frankel *et al*., 1993). These studies of health effects were, in part, stimulated by previous reports of the physiological activity of resveratrol-containing polygonaceous plants such as *Polygonum cuspidatum* used in China and Japan. These plants were used as herbal folk remedies, such as Kojo-kon, for the treatments of various fungal, bacterial, inflammatory, and lipid atherosclerosis ailments (Nonomura *et al*., 1963; Kimura *et al*., 1983, 1985).

Resveratrol occurs in the *cis* and *trans* isomeric forms (Figures 1 and 2). In Vitaceae fungal infection or UV light stimulates the production of stilbene synthase and catalyzes the reaction of 4-hydroxycinnamoyl-CoA and malonyl-CoA to produce *trans*-resveratrol (Fritzemeier and Kindl, 1981). In the grape berry, *trans*-resveratrol production is stimulated by UV light exposure, fungal infection, or injury (Creasy and Coffee, 1988; Jeandet *et al.*, 1991, 1995a). *cis*-Resveratrol has not been reported in *Vitis vinifera*; however, it has been shown to be present in wines (Jeandet *et al*., 1993, 1995b; Mattivi *et al*., 1995; Lamuela-Raventós and Waterhouse, 1995; Goldberg *et al*., 1995b,c; Soleas *et al*., 1995). Physiological activity of *cis*-resveratrol has only been described once, and was shown to inhibit kinase activity, a factor related to cancer (Jayatilake *et al*., 1993).

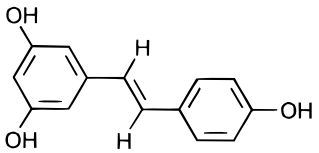

Figure 1. *trans*-Resveratrol.

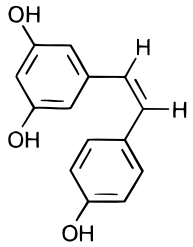

Figure 2. *cis*-Resveratrol.

There has been no specific information on the properties of *cis*-resveratrol, and its light sensitivity has made it difficult to purify. There is a large disparity in the reported molar absorptivities and maximum absorbance wavelengths for each of the two isomers. This has created difficulties in the quantitative reporting of individual resveratrol isomer levels. One study quantitatively expressed the *cis* isomer in *trans*-resveratrol equivalents (Lamuela-Raventós and Waterhouse, 1995). In other studies, a known amount of *trans*-resveratrol was irradiated to induce partial isomeric conversion to *cis*-resveratrol, and that isomerization was assumed to result in a mixture of only *cis*- and *trans*-resveratrol (Goldberg *et al*., 1995b; Jeandet *et al*., 1995b).

This study determines the molar absorptivities of both compounds at two wavelengths to facilitate chromatographic quantitation at either compound's absorbance maximum. In addition, stability trials under a variety of conditions were also conducted to test some commonly encountered laboratory conditions and their effect on the isomerization or degradation of resveratrol.

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MATERIALS AND METHODS

trans-Resveratrol was purchased from Sigma Chemical Co. (St. Louis, MO). *cis*-Resveratrol was obtained by irradiating a 8.94 mmol/L stock 50% ethanol solution of *trans*-resveratrol for 3 h. *trans*-Resveratrol was UV irradiated through a 2.0 mm thick borosilicate glass liquid scintillation vial placed next to a Mineralite lamp Model UVGL-25 (Ultraviolet Products, San Gabriel, CA) operating with an intensity of 180 μW/cm^2 at 366 nm and, for one comparison, at 254 nm with an intensity of 750 μW/cm^2.

To test resveratrol stability in various buffers at differing pH values, a 50 mM phosphoric acid buffer was made using 85% phosphoric acid. Sulfuric acid was used to lower the pH, and ammonium hydroxide was used to raise buffer pH. The four buffers used were 1.0, 3.5, 7.0, and 10.0 pH units. Resveratrol solutions were mixed in a 1:1 ratio with the buffers for a final concentration of 276 μmol/L and 50% EtOH.

trans-Resveratrol evaporation was conducted using rotary evaporation *in vacuo* at 37 °C in EtOH and the various buffers. Copper contamination ($CuSO_4$) at the 3.2 and 7.9 μmol/L copper (Cu^{2+}) levels and with rotary evaporation was also tested in triplicate.

UV spectra were obtained with a Hewlett-Packard (HP) (Palo Alto, CA) 8452A diode array spectrophotometer controlled by HP 89532A UV−vis software (DOS) Rev. A.00.00 through a 10 mm quartz cuvette. To reduce moisture contamination of the standards and solvents, all containers were rinsed with acetone and dried with nitrogen gas.

cis and *trans* isomer separation and collection was performed on the 8.94 mmol/L solution and the semipreparatory system described below. Each fraction of *cis*-resveratrol was purified with this method at least twice. Long-term storage of standards in 100% EtOH was done at −5.0 °C in sealed, light-protected borosilicate glass containers.

Semipreparative HPLC was performed with a Waters 510 pump (Milford, MA), a Rheodyne U6K injector with a 5 mL fixed loop, and an HP Model 1050 UV−visible detector set at 306 nm. The column was a Waters RCM Prep Nova-Pak HR C_{18}, 6 μm, 25 mm × 100 mm, preceded by a 25 mm × 10 mm Guard-Pak guard column of the same specifications. Elution was carried out at 20 °C using 55% aqueous methanol mobile phase at 5 mL/min flow rate.

Analytical HPLC Procedure. An HP 1090 series II with a diode array UV−visible detector was controlled by chromatography and quantitation software Chemstation (DOS) Rev. 02.00. Detector light path length was 0.6 cm. All resveratrol samples were sterile filtered through 4 mm poly(tetrafluoroethylene) (PTFE) 0.45 μm pore size filters before HPLC injection.

Analyses were performed using an HP ODS Hypersil, 5 μm, 2.1 mm × 100 mm stationary phase column with a 2.1 mm × 20 mm precolumn with the same phase. The mobile phase flux was 0.5 mL/min. The eluents were (A) deionized, sterile filtered H_2O and (B) methanol. Injection volume was 5.0 μL. The column was thermostatically controlled to maintain a temperature of 40.0 °C.

The separation was conducted using a linear gradient of 100% solvent A to 100% solvent B in 15 min, and then the column was flushed for 5 min with solvent A to re-establish the initial conditions before another sample was injected.

The eluent was monitored at 306 and 286 nm, the UV optimum absorbancies of *trans*- and *cis*-resveratrol, respectively, and the UV spectra of the eluent peaks were obtained.

The results of the analyses are expressed in units of moles per liter or percent *cis*-resveratrol of the overall area of *cis*- and *trans*-resveratrol at the specified wavelength corrected for molar absorptivity ratio difference. Expression as a percent *cis*-resveratrol of the total chromatographed peak areas (100% *cis*- and *trans*-resveratrol) simplifies matters and negates solvent evaporation effects.

RESULTS AND DISCUSSION

The analytical HPLC method was optimized for simplicity and quick, efficient separation of *trans*- and *cis*-resveratrol in simple mixtures. This method is not adequate for analysis of resveratrol isomers in wines, which was not addressed here. Parallel analyses, however, were carried out using a resveratrol wine analysis method developed by Lamuela-Raventós and Waterhouse (1995), and the results were equivalent. Since all samples were filtered before HPLC injection, possible filter adsorption of resveratrol was tested. No filter adsorption loss was observed for either isomer when done in triplicate. *trans*-Resveratrol eluted first with a retention time of 7.3 min, followed by *cis*-resveratrol at 8.0 min. A typical chromatogram of the isomers is illustrated in Figure 3. Peak A at retention time 7.3 min was confirmed to be *trans*-resveratrol since its retention time and UV spectrum were identical to those of pure *trans*-resveratrol. Peak B at retention time 8.0 min was formed after brief UV irradiation of the pure *trans*-resveratrol standard. Comparison of its ^{1}H NMR spectrum was consistent with that of Jayatilake *et al.* (1993). In addition, ultraviolet spectra for each isomer (Figure 4) were consistent with those published by Goldberg *et al.* (1995b), who also performed GC−MS identification, and our λ_{max} values were similar to those of Jayatilake *et al.* (1993), although the molar absorptivity values described by Jayatilake *et al.* (1993) were much lower than those graphically reported by Goldberg *et al.* (1995b) or the data herein (Table 1). The UV spectrum of *cis*-resveratrol was not, however, consistent with that of Siemann and Creasy (1992), who reported a UV λ_{max} of 230 nm. Thus, it appears that the spectrum reported by Siemann and Creasy (1992) was not of *cis*-resveratrol but of some other product of UV irradiation, as described by Roggero and Garcia-Parrilla (1995).

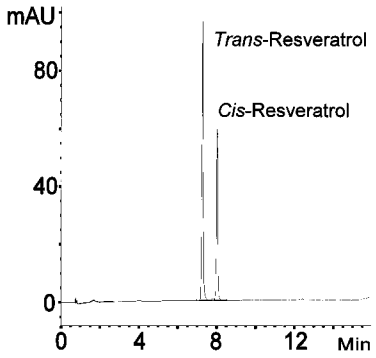

Figure 3. Typical chromatogram of *cis*- and *trans*-resveratrol at 286 nm.

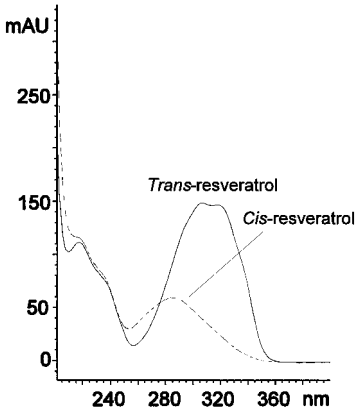

Figure 4. UV spectra of chromatogram in Figure 3.

UV-Induced Isomerization. A solution containing 418 μmol/L of pure *trans*-resveratrol was irradiated for 120 min at 366 nm and monitored for isomerization and *cis*-resveratrol formation every 10 min. Partial conversion to *cis*-resveratrol was maximum at about 100 min

Table 1. *trans*- and *cis*-Resveratrol UV λ_{max} (EtOH)

		trans-		*cis*-	
author	year	λ_{max} (nm)	ϵ	λ_{max} (nm)	ϵ
Hathway and Seakins	1959	305[a]	28200[b]		
Ingham	1976	307	ND[c]	285	ND
Langcake and Pryce	1976	306	26700		
Siemann and Creasy	1992	306	26800	261	22800
		320	25900		
Jayatilake *et al.*	1993	305	10400[b]	230	1600
		320	2200	290	1400
		306[a]	16200		
this work		308	30000	286	12500[d]
		320	29000	288	12600
		306	31800[e]	286	13100[e]

[a] Piceid (*trans*-resveratrol glucoside). [b] Originally expressed as log ϵ). [c] ND = not determined. [d] = not λ_{max}. [e] UV λ_{max} (10% EtOH) nm (ϵ).

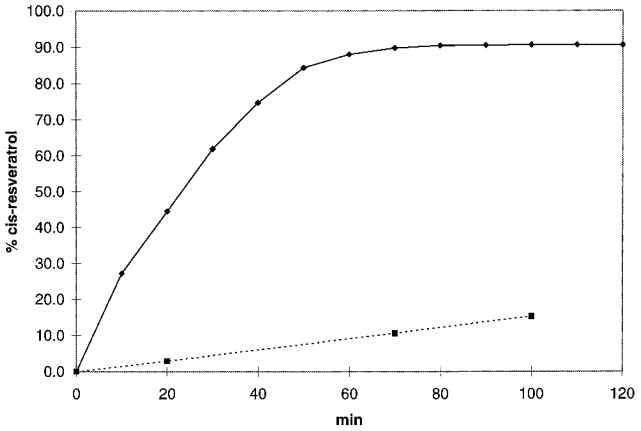

Figure 5. *cis*-Resveratrol conversion from a 418 μmol/L solution of 100% *trans*-resveratrol irradiated at 366 nm at 180 μW/cm^2 and at 254 nm at 750 μW/cm^2 (—, 366 nm; - - -, 254 nm).

of irradiation. *trans*-Resveratrol was converted to 90.6% *cis*-resveratrol. For comparison, another 418 μmol/L solution of 100% *trans*-resveratrol solution was irradiated at 254 nm for 60 min (Figure 5), although the borosilicate container would dramatically attenuate the low wavelength radiation. Even after 10 h of irradiation at 254 nm, the *trans* isomer had converted to ≤63% *cis*-resveratrol. No other products appeared in the chromatograms after irradiation at either wavelength, and 100% of the peak areas accounted for all of the initial *trans*-resveratrol. The isomeric ratios in the irradiated solutions were determined using the molar absorptivity ratios from chromatographic integration data at both 286 and 306 nm. The results were the same at both wavelengths. The data confirmed that under these conditions there was a quantitative transformation of the *trans* isomer to the *cis* isomer, as described by Goldberg *et al.* (1995b). Another solution of 8.94 mmol/L *trans*-resveratrol was irradiated at 366 nm for 3 h, and the resulting ≥80% *cis*-resveratrol mixture was separated to obtain the *cis*-resveratrol.

Calibration. Calibration curves for *trans*-resveratrol were obtained with ≥0.999 (r^2) linear correlation of integration response with concentration. The curves did not vary by more than 5% over 3 months, limiting the need for recalibration, though it was performed multiple times during this time period. Standards were stored at −5 °C in 100% EtOH in the dark and protected from stray light in sealed, light-proof containers. Other sets of standards were stored in light-proof, Parafilm-sealed containers in 50% aqueous ethanol, unrefrigerated. These standards also appeared to be stable, though

Table 2. *trans*- and *cis*-Resveratrol Molar Absorptivities (EtOH)

	absorbances				
concn (μmol/L)	286 nm	288 nm	306 nm	308 nm	320 nm
	trans-Resveratrol				
5.52	0.0746	0.0846	0.1493	0.1514	0.1517
11.04	0.2227	0.2374	0.3396	0.3416	0.3336
33.13	0.6850	0.7405	1.0481	1.0561	1.0190
55.61	1.0186	1.1107	1.6236	1.6284	1.5789
slope (ϵ)	18987	20668	29859	29959	28972
r^2 [a]	0.993	0.994	0.998	0.997	0.998
CV[b]	18.0	15.8	6.7	6.5	5.1
	cis-Resveratrol (Corrected Absorbances)[c]				
5.26	0.0726	0.0726	0.0574	0.0552	
10.56	0.1473	0.1466	0.1052	0.0997	
31.60	0.3987	0.3985	0.2790	0.2621	
52.76	0.6732	0.6728	0.4675	0.4385	
slope (ϵ)	12536	12626	8715	8163	
r^2	1.000	1.000	0.999	0.999	
CV	5.2	5.1	10.3	11.4	
trans/cis	1.515	1.637	3.426	3.670	

[a] r^2, square of Pearson's correlation coefficient. [b] CV, coefficient of variance (%). [c] See text.

solvent evaporation was noticeable through the Parafilm seal as an increase in standard HPLC area, but no new peaks were observed. Standard solutions that were unprotected from light and exposed to laboratory fluorescent lighting isomerized to about 80% *cis*-resveratrol over 30 days.

cis-Resveratrol was extremely light sensitive, and the preparation of standards, while executed in near total darkness, allowed enough light to cause slight isomerization. Purified fractions of ≥98% purity yielded standards of ≥95% purity after handling. *cis*-Resveratrol stored in solution in the dark at ambient temperatures in 50% EtOH remained stable for at least 35 days over the range of 5.3−52.8 μmol/L.

Detection Limits and Precision. The detection limit was measured as the concentration corresponding to the lowest signal measurable above baseline with a signal-to-noise ratio of 3:1 when done in triplicate with four standards. The limits of detection for *trans*-resveratrol were 1.2 and 0.6 μmol/L at 306 and 286 nm, respectively. The limits of detection for *cis*-resveratrol were 0.9 and 0.6 μmol/L at 306 and 286 nm, respectively. Instrument precision was evaluated by performing 10 replicate injections of a 22.1 μmol/L *trans*-resveratrol standard. The coefficients of variation (CV) were 1.4% and 1.7% at 306 and 286 nm, respectively, indicating very high reproducibility.

Molar Absorptivities. Absorptivity was determined by measuring absorbance at four concentrations and determining the slope of the line that best fit these data (Table 2). Absorptivity was UV λ_{max} (EtOH) nm (ϵ) 288 (12 600) for *cis*-resveratrol, CV = 5.1%, over the range of 5.26−52.76 μmol/L. Actual concentrations were corrected for the 95% purity based on the area of the *trans*-resveratrol peak and subtracting its absorption contribution. This gave an excellent 0.99 correlation coefficient across the standards. Absorptivity in 10% ethanol was greater, UV λ_{max} (10% EtOH) nm (ϵ) 286 (13 100), possibly due to altered hydrogen bond interactions with the water present. The coefficient of variation equaled 0.4%, performed in triplicate at only one concentration.

trans-Resveratrol absorptivity was done identically and appeared to be 100% pure by HPLC. Absorptivity was again based on the slope of four measurements

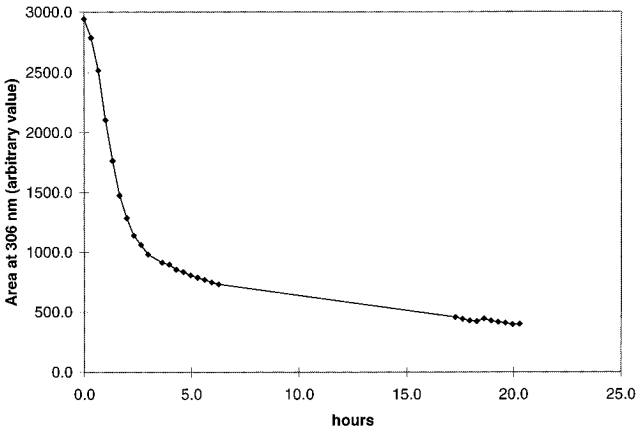

Figure 6. *trans*-Resveratrol, 276 μmol/L, in pH 10.0 buffer.

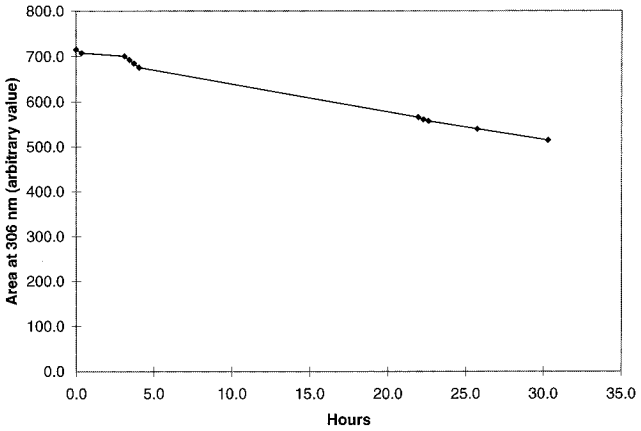

Figure 7. *cis*-Resveratrol, 276 μmol/L, in pH 10.0 buffer.

equaling UV λ_{max} (EtOH) nm (ϵ) 308 (30 000) with a correlation coefficient $\geq$0.99 and CV = 6.5%. This absorptivity value is higher than previously reported, likely due to rigorous protection from light. The molar absorptivity in 10% ethanol was greater, UV λ_{max} (10% EtOH) nm (ϵ) 306 (31 800), again possibly due to hydrogen bond interactions with the water. The coefficient of variation equaled 0.3%, and it, too, was performed in triplicate at one concentration.

The molar absorptivities of both *cis*- and *trans*-resveratrol were determined at four to five wavelengths (Table 2). The higher variability of the nonmaxima values is likely due to the higher variability in the wavelength-dependent change in absorbance at these values.

Stability. When protected from light, *trans*-resveratrol was stable for at least 42 h (CV $\leq$ 1.0%) and for at least 28 days with a CV $\leq$ 4.7% in buffers pH 1−7. The initial half-life for *trans*-resveratrol in pH 10.0 buffer was 1.6 h (Figure 6).

A 418 μmol/L *trans*-resveratrol solution was irradiated with Mineralite (366 nm) for 2 h, becoming 90.6% *cis*-resveratrol, and it was then set on the counter exposed to laboratory fluorescent lighting conditions. The percent of the *cis* isomer dropped to 86.1% over 60 days. New samples in the various buffers were exposed to the 366 nm UV irradiation for 5 min; the equilibrium proportion at 30+ days under laboratory lighting averaged about 86% except at pH 1.0, which had about 33.7% of the *cis* isomer, and at pH 10.0, which had no *cis*-resveratrol and very little *trans*-resveratrol.

Under identical buffer conditions, *cis*-resveratrol degraded at pH 10.0 with a half-life of 63.7 h (Figure 7). *cis*-Resveratrol was stable at pH 7.0 (CV = 0.7%), and less stable at pH 3.5 (CV = 2.6%); however, at pH 1.0

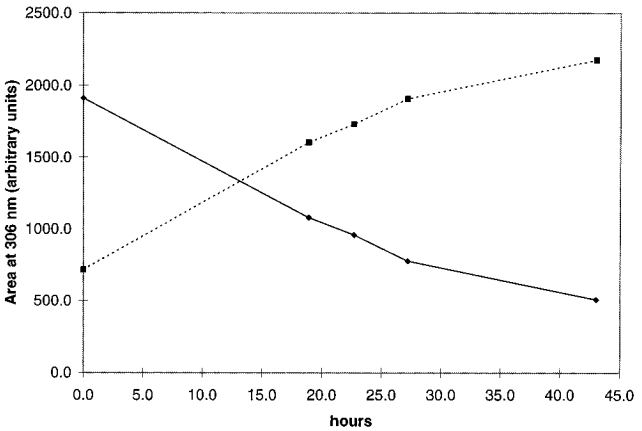

Figure 8. *cis*-Resveratrol, 276 μmol/L, in pH 1.0 buffer (- - -, *trans*-resveratrol; —, *cis*-resveratrol).

isomerization to *trans*-resveratrol was efficient—after 22.8 h ($t_{1/2}$), 50% of the *cis* form had isomerized to the *trans* isomer (Figure 8). As noted above, the *cis* isomer is sensitive to light exposure; and one 95% pure 79 μmol/L *cis* isomer fraction in ethanol was placed on the laboratory counter in borosilicate glass exposed to the overhead fluorescent lighting and isomerized to 91% *cis*-resveratrol over 30 days. Thus, under the laboratory fluorescent lighting used here, the equilibrium mixture was between 86% and 91% *cis*-resveratrol as determined over a time span of up to 2 months at various concentrations. The exact equilibrium mixture is likely dependent on the specific spectrum of light reaching the sample.

Sample dry-down operations with rotary evaporation were checked for resveratrol stability, and contrary to Pezet *et al.* (1994) and McMurtrey *et al.* (1994), no degradation of *trans*-resveratrol was observed after evaporation of alcohol, confirmed by Jeandet even when heated at 40 °C (unpublished results) or in buffers at various pH values except for the pH 10.0 buffer. Triplicate 1 mL unbuffered solutions of 55.6 μmol/L *trans*-resveratrol in 10% EtOH were rotary evaporated at 37 °C and redisolved in 1 mL of 10% EtOH. The recoveries of *trans*-resveratrol in the reconstituted solutions were each 82%, though no new products were observed by HPLC analysis after this procedure. When 3.2 and 7.9 μmol/L Cu^{2+} were added and evaporated with the resveratrol, no degradation or oxidation was observed, and the coefficients of variation at both wavelengths averaged 1.2% and 3.2% for each copper level, respectively. When performed without copper, the CV averaged 1.6% at both wavelengths. Evaporation with the buffers also showed no degradation. Analysis of an 87.6 μmol/L standard solution of *trans*-resveratrol in pH 7.0 buffer gave an average CV of 0.8% before evaporation and 5.7% after evaporation at both wavelengths. At pH 3.5 the CV at both wavelengths averaged 6.6% before evaporation and 3.3% after evaporation. Again, no new peaks were seen in the chromatograms. On two occasions fractions obtained by preparative HPLC decomposed to many new compounds. Numerous attempts to reproduce this occurrence failed. Glassware contamination is speculated to be the cause. Storage of dry *cis*-resveratrol was problematic. After one 20 mg sample was dried, it isomerized 60% into *trans*-resveratrol over 3 months when stored in the dark at −5 °C; the cause for this reaction was not clear.

Conclusions. Molar absorptivities over a range of commonly reported wavelengths were accurately determined for both *cis*- and *trans*-resveratrol. *trans*-Resveratrol should be handled with caution, but can be

handled in the laboratory without stringent lighting precautions, whereas *cis*-resveratrol cannot. Laboratory lighting conditions favor an equilibrium of about 91% *cis*-resveratrol. Low pH causes *cis*-resveratrol isomerization to the *trans* isomer, a sterically more stable form. Some literature reports of *cis*-resveratrol are inaccurate and were addressed here. There is a need for a quantitative kinetic study of resveratrol isomerization under well-defined light fluxes and in the presence of acid.

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