Anal. Chem. **1997,** *69,* 5172−5177

Technical Notes

HPLC Analysis of Grapevine Phytoalexins Coupling Photodiode Array Detection and Fluorometry

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A reversed-phase HPLC method useful for the analysis of the grapevine phytoalexins resveratrol, its β-D-glucoside, ϵ-viniferin, and pterostilbene in leaf extracts was developed by coupling diode array detection and fluorometry. Phytoalexins were extracted from UV-irradiated grapevine leaves with methanol/water (80:20) and prepurified on C18 solid phase extraction cartridges. Separation by HPLC was achieved using a C18 column and a gradient elution with acetonitrile and water (from 10 to 85% acetonitrile). Analyses of grapevine leaf extracts were performed by injecting the equivalent of 1 mg of leaf fresh weight. Recovery was nearly 100% for the three stilbenes resveratrol, pterostilbene, and ϵ-viniferin (ranging from 100.2 to 104.8%), and replicate analyses gave coefficients of variation of 0.6−2.5%. Identification of each phytoalexin was accomplished by line spectral comparisons with known standards, and ϵ-viniferin was further characterized by MS and GC/MS. Simultaneously, stilbenes were detected by fluorometry, allowing specific identification of these compounds. This procedure provided excellent separation and enabled quantitation of all grapevine phytoalexins present in the extracts. The method can easily be extended to the analysis of wine or biological fluids.

Phytoalexins are biologically active compounds that are produced by plants in response to fungal infection or abiotic stresses such as heavy metal ions or UV light.[1] In grapevines, such a response includes the synthesis of a simple stilbene, resveratrol (*trans*-3,5,4′-trihydroxystilbene),[2] and its glucoside,[3] together with the biosynthetically related compounds ϵ-viniferin and pterostilbene (*trans*-3,5-dimethoxy-4′-hydroxystilbene)[4,5] (Figure 1). Stilbenes have provoked an intense interest due to their antifungal properties, and their presence has been shown to be closely related to grape disease resistance, namely to gray mold caused by *Botrytis cinerea*.[6−8] Moreover, resveratrol is one of the constituents of wine which is thought to confer protection against artherosclerosis, coronary heart diseases,[9] and cancer.[10] In light of these findings, recent papers have described analytical methods to assay resveratrol in wines[11−20] or in grapevines.[15,21−23] In contrast, there is only one work describing a method suitable for the simultaneous analysis of the other stilbenic phytoalexins of grapes,[24] but the latter method did not allow for the identification of the compounds analyzed, i.e., resveratrol, pterostilbene, and

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S0003-2700(97)00582-9 CCC: $14.00

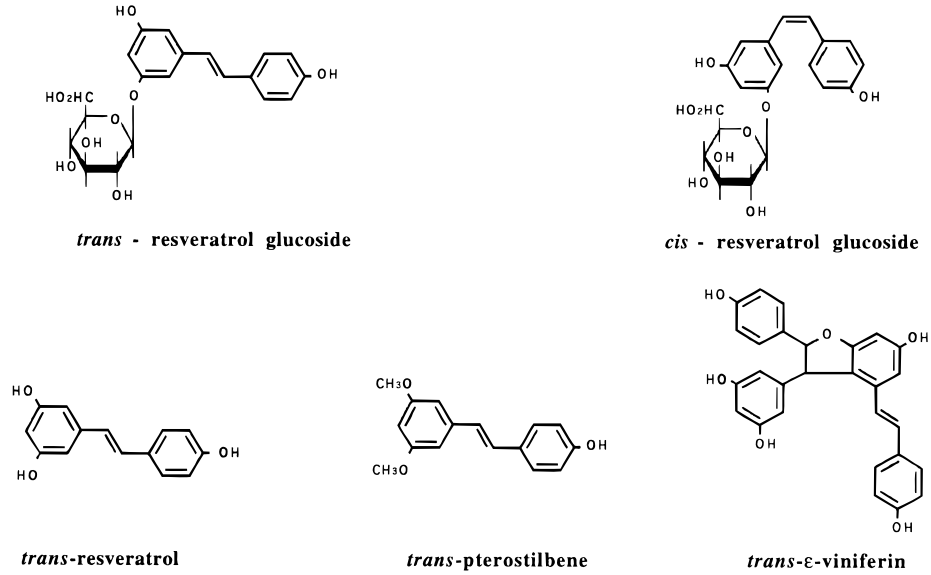


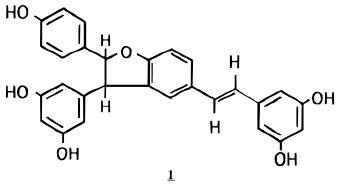
Figure 1. Chemical structures of the stilbene phytoalexins measured by this method.

ϵ-viniferin. Moreover, the resveratrol glucosides were not included in this analysis.

We now describe an HPLC technique coupling diode array detection and fluorometry that is useful for the analysis of the cis and trans isomers of resveratrol and its β-D-glucoside, ϵ-viniferin, and pterostilbene in grapevine leaf extracts. Identification of each phytoalexin was accomplished by line spectral comparisons with known standards, and ϵ-viniferin was further characterized by MS and GC/MS. Specific detection of all stilbenes was also obtained using fluorometric analysis.

MATERIAL AND METHODS

Standards. A chemically pure standard of *trans*-resveratrol was synthesized by a Wittig condensation between a phosphorus ylide and a silylated hydroxybenzaldehyde, as previously described.[22,25] *trans*-Pterostilbene was obtained from Drs. R. Pezet and V. Pont (Swiss Federal Agricultural Station of Changins). *trans*-Piceid, the 3-β-D-glucoside of resveratrol,[3,16,26] was isolated from the dried roots of *Polygonum cuspidatum* as described by Waterhouse and Lamuela-Raventos.[3] The purity of the glucoside obtained was compared to a standard furnished by Professor D. M. Goldberg (University of Toronto, Canada). A resveratrol dehydrodimer analogous to ϵ-viniferin was obtained by dimerization of *trans*-resveratrol with horseradish peroxidase−hydrogen peroxide utilizing a modification of the method of Langcake and Pryce[27] or Calderon et al.[28] *trans*-Resveratrol (6 mg, 26.3 μM) was treated with 2 mL of H_2O_2 and 0.1 mg of horseradish peroxidase (type IX, Catalog No. P-1139, 275 units/mg solid, purchased from Sigma, St. Louis, MO) in 100 mL of citrate buffer and 8% dioxane (to ensure solubility of resveratrol) at pH 6.0. The mixture was stirred for 15 min in the dark and then extracted twice with ethyl acetate. The dehydrodimer **1** obtained under these conditions was found to be an isomeric form of the grapevine dehydrodimer ϵ-viniferin.[27] Compound **1** was purified by preparative TLC on reversed-phase C18 material (RP 18 F254S, Merck, Darmstadt, Germany) in 7:3 (v/v) MeOH/water (R_f = 0.53), leading to 3 mg (6.61 μM) of **1** (50% yield). Its UV spectrum



showed a *trans*-stilbene chromophore similar to *trans*-resveratrol, but in contrast the spectrum showed no base shift of the long-wavelength chromophore in the presence of diluted NaOH, indicating the *trans*-stilbene lacking a phenolic group in the 2- or 4-position of the stilbene moiety.[27] Moreover, the mass spectrum of this compound (EI, M^+ at *m/e* 454, relative intensity = 100) and that of its trimethylsilyl ether (GC/MS, M^+ at *m/e* 814, relative intensity = 100) suggested that it consists of a pentaphenol with a dehydrodimeric structure.

The structure of **1** was also confirmed by ^{1}H-NMR (500 MHz, acetone-d_6).[27] Cis isomers of each stilbene were obtained by UV irradiation of diluted solutions (5 μg/mL) of the corresponding *trans*-stilbene. The standards were placed in a UV cuvette and irradiated for 10 min at 366 nm.

Plant Material. Two *Vitis* species differing in susceptibility to gray mold were used in this study: *Vitis vinifera* L. cv Pinot Noir (susceptible species) and *Vitis rupestris* L. cv Rupestris du Lot (tolerant species). Only leaves (from in vitro grown plantlets or from plants growing in the fields) have been assayed for phytoalexin production. Stilbene synthesis was induced in grape leaves by UV irradiation as previously described.[22]

Sample Preparation. UV-irradiated leaves were ground in a mortar with sand and 30 mL of 8:2 (v/v) MeOH/water. After centrifugation at 10000*g* for 15 min, the supernatant was prepurified on a Sep-Pak C18 cartridge (Waters, Milford, MA). After elution with 8:2 (v/v) MeOH/water (30 mL), the eluate was evaporated to dryness (<40 °C). Leaf extracts were then redissolved in 10 mL of methanol/g fresh weight and filtered. For HPLC analysis, 10 μL of each sample (i.e., 1 mg fresh weight) was injected. During sample preparation, extracts were constantly

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protected from light to avoid photochemical isomerization of *trans*-resveratrol to the cis form.

To evaluate the efficiency of this extraction procedure, 100 μg portions of each of the three stilbenes were added to 1 g of uninduced fresh material and treated as described above. This was done in triplicate. The recovery concentrations of the extracted standards determined by HPLC were 103.6 ± 4.6% for resveratrol, 104.8 ± 5.3% for pterostilbene, and 100.2 ± 4.5% for the dehydrodimer **1**. The precision of the method was evaluated by performing six replicate analyses of three different grape leaf extracts. The coefficients of variation ranged from 0.6 to 2.5% for all stilbenes.

HPLC Analysis. Analyses were performed on a Lichrocart Merck C18 (Merck-Clevenot Corp., Darmstadt, Germany) reversed-phase column (250 mm × 4 mm, 5 μm) preceded by a guard column of Lichrospher 100 RP-18 (4 mm × 4 mm, 5 μm, Merck). A Waters system comprising a Model W 600 system controller, a Model W 717 sample injector, a Model W 996 photodiode array detector, and a Model W 474 fluorometer was used. For fluorometric detection, maximum excitation wavelength was measured at 330 nm and emission at 374 nm.[2,15] Stilbenes were eluted from the HPLC C18 column with a gradient comprising acetonitrile (solvent A) and water (solvent B). Solvents were delivered according the following program: linear gradient elution from 10% A and 90% B to 85% A and 15% B within 18 min; 85% A and 15% B for 5 min; linear gradient elution from 85% A and 15% B to 10% A and 90% B within 7 min. This was followed by a 5 min equilibrium period with 10% A and 90% B prior to injection of the next sample. The flow rate was 1 mL/min.

Identification and Quantification of Stilbenes. Identification of resveratrol, its β-D-glucoside, pterostilbene, and the dehydrodimer ϵ-viniferin in extracts 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. Further identification of resveratrol glucosides was achieved by enzymatic hydrolysis (see below). MS and GC/MS were also used for the characterization of ϵ-viniferin (see below). Quantification of *trans*-resveratrol, *trans*-pterostilbene, and *trans*-ϵ-viniferin was performed using the fluorometer only since these three stilbenes were found to have the same fluorometric characteristics (λ_{ex} = 330 nm and λ_{em} = 374 nm). Standard calibration curves were established using peak area vs different amounts of each stilbene (i.e., 2, 10, 20, 50, 100, and 200 ng). We used the calibration curve obtained for the dehydrodimer **1** to quantify ϵ-viniferin in the extracts. Three replicates were made for each concentration. Mean coefficients of variation (CV) and linear correlations were calculated using the Waters Millenium system. Linear correlations for fluorometric detection were excellent (r^2 = 0.999 for *trans*-resveratrol, r^2 = 0.9995 for *trans*-pterostilbene, and r^2 = 0.9975 for the *trans*-dehydrodimer **1**). The trans form of the resveratrol glucoside was quantified using calibration curves of *trans*-resveratrol, since both compounds have the same fluorometric characteristics. The cis forms of stilbenes are normally not present in grape leaf extracts.[2,19,22] Quantification of *cis*-stilbenes was thus not necessary, except for the cis form of the resveratrol glucoside, which was always present in the extracts. *cis*-Resveratrol glucoside was quantified by UV at 285 nm by using the calibration curve obtained for *cis*-resveratrol (r^2 = 0.995). The *cis*-glucoside has UV properties similar to those of *cis*-resveratrol. Concentrations of resveratrol, its glucosides, pterostilbene, and ϵ-viniferin in grape leaf extracts were measured using the external standard method. Response factors (i.e., amounts of standard/peak area) were determined with data from the calibration curves. The detection limit was measured as the concentration corresponding to the lowest signal which differs significantly ($p < 0.01$) from the baseline. This value was ~0.1 μg/g fresh weight for the four stilbenes and was sufficient due to the high levels of each compound found in the extracts.

Enzymatic Hydrolyses of Resveratrol Glucosides. Enzymatic hydrolyses of resveratrol glucosides with β-D-glucosidase (Catalog No. 49290, 6.76 units/mg protein, Fluka, Buchs, Switzerland) or α-D-glucosidase (Catalog No. G-6136, 25 units/mg protein, Sigma, St. Louis, MO) were conducted as follows: methanolic extracts of grape leaves (see above) were diluted with deionized water such that the concentration of alcohol does not exceed 10% and adjusted to pH 6.0 with 0.1 N NaOH. Enzymes were added in a concentration of 1 mg/mL of diluted samples. The mixtures were then incubated at 30 °C for 17 h (overnight). Samples were concentrated and dissolved in methanol and filtered before HPLC analysis.

MS and GC/MS Analyses. MS analysis of the resveratrol dehydrodimers (ϵ-viniferin and its isomeric form) was carried out using a mass spectrometer (Kratos, Concept IS, Great Britain) with direct introduction of the samples. GC/MS analysis of both dimers was done in the form of their trimethylsilyl derivatives[18,22] using a quadrupole mass spectrometer (Nermag R 10-10C). In both cases, ionization was obtained by electron impact (electron energy, 70 eV, and filament current, 0.19 mA).[29] Samples were injected into a Chrompack gas chromatograph (Chrompack Corp., The Netherlands) equipped with a de Ros injector. Analysis was performed on a capillary column SE-30 (25 m × 0.32 mm) operating at 300 °C. The pressure of the carrier gas (nitrogen) was maintained at 50 kPa at a flow rate of 1 mL/min. Under these conditions, the retention times of both resveratrol dehydrodimers were 17.0 min.

RESULTS AND DISCUSSION

Analysis of Standards. Stilbenes are highly fluorescent compounds that are very easily detected by fluorometry. Figure 2A shows the HPLC profile corresponding to the four standards using fluorometric detection. Under our chromatographic conditions, retention times were 12.63, 15.30, 17.38, and 21.28 min for the trans form of the resveratrol glucoside, *trans*-resveratrol, the *trans*-dehydrodimer **1**, and *trans*-pterostilbene, respectively. Simultaneously, these four compounds were analyzed by a photodiode array detector and characterized by their UV spectra from 250 to 400 nm. All compounds showed two bands corresponding to a high absorbance from 308 to 336 nm (band I) and from 281 to 313 nm (band II), bands which are characteristic of the *trans*-stilbenes[30] (Figure 3A,B). UV maximum absorbances of *trans*-resveratrol and *trans*-pterostilbene were both at 307.8 nm, values which are consistent with those previously published.[2,5,18,31] Unlike resveratrol and pterostilbene, the synthetic *trans*-dehydrodimer of resveratrol showed a maximum absorbance at 224 nm (data not shown). This compound also contains a *trans*-stilbene moiety

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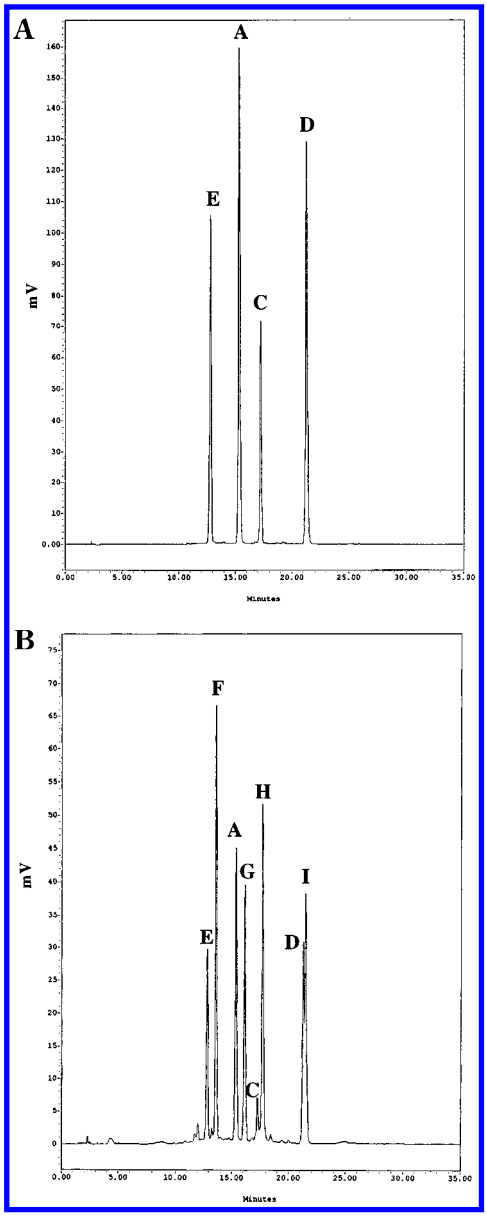

Figure 2. HPLC analysis of standards of phytoalexins (detection by fluorometry). (A) Peak A, *trans*-resveratrol; peak C, *trans*-resveratrol dehydrodimer **1**; peak D, *trans*-pterostilbene; peak E, *trans*-resveratrol glucoside. (B) HPLC profile obtained after partial photochemical isomerization of the standards. Peaks A, C, D, and E were the same as in part A; peak F, *cis*-resveratrol glucoside; peak G, *cis*-resveratrol; peak H, *cis*-resveratrol dehydrodimer; peak I, *cis*-pterostilbene.

with a long-wavelength chromophore similar to that of resveratrol ($\lambda_{max} = 312.5$ nm). The UV spectrum of the resveratrol glucoside is very close to that of resveratrol ($\lambda_{max} = 320$ nm). UV maximum absorbances of the cis forms were, for the four stilbenes (Figure 3C,D), at 285 nm. UV spectra of *cis*-stilbenes are characterized by the lack of band I (see above). Light-induced isomerization of the *trans*-stilbene moiety in the dehydrodimer **1** leads to the cis isomer.

Analysis of Grape Leaf Extracts. Figure 4 shows the HPLC profiles corresponding to the injection of UV-induced leaf extracts of *V. vinifera* (A) and *V. rupestris* (B). Five major peaks appeared within the extracts of *V. rupestris* (peaks A, C, D, E, and F), and only two major and two minor peaks appeared within the extracts of *V. vinifera* (peaks A, C, E, and F). Peak A was identified as *trans*-resveratrol by the following criteria: (1) its retention time was identical to that of synthetic *trans*-resveratrol, and it cochromatographed with pure resveratrol; and (2) its UV spectrum was identical to that of *trans*-resveratrol, and its purity was confirmed as >99% by diode array detection of the spectrum. Peak D was identified as *trans*-pterostilbene using the same criteria as those used for resveratrol. The two peaks (noted E and F) found in grape leaf extracts (Figure 4B,C) were identified as the β-D-glucosides of *cis*- and *trans*-resveratrol, respectively, by the following criteria: (1) treatment of samples with β-D-glucosidase resulted in the disappearance of these peaks with a concomittant increase of peak A (corresponding to the *trans*-resveratrol peak) and the occurrence of a new compound (noted peak G), whose UV spectrum and retention time were identical with those of *cis*-resveratrol (Figure 3C); (2) treatment of samples by an α-D-glucosidase had no effects on these peaks; (3) UV spectra of the two peaks were very close to those of *cis*- and *trans*-resveratrol, respectively, and their purities were confirmed as >99% by diode array detection of the spectrum; and (4) the two compounds cochromatographed with authentic *cis*- and *trans*-piceid. The occurrence of the cis form of the glucoside of resveratrol is surprising since extracts have constantly been protected from light during sample preparation. Thus, the presence of *cis*-resveratrol glucoside in the extracts is likely not attributable to the photochemical isomerization of the trans form (see below).

Peak C was identified as being the resveratrol dehydrodimer, ϵ-viniferin, by the following criteria: (1) it cochromatographed with the synthetic resveratrol dehydrodimer **1**; (2) its UV spectrum was identical to that of the pure dehydrodimer **1**; and (3) photochemical isomerization of peak C gives a new peak corresponding to a *cis*-stilbene, which cochromatographed with the cis form of **1**. Further identification of C was achieved by mass spectrometry. In this way, small quantities of C were purified from grapevine leaf extracts (*V. rupestris*) by preparative TLC using reversed-phase material RP 18 in 7:3 (v/v) MeOH/water. Observation of the TLC plates under long-wavelength UV light revealed the presence of four fluorescent compounds, three of which were identified as resveratrol ($R_f = 0.66$), its glucoside ($R_f = 0.78$), and pterostilbene ($R_f = 0.30$), respectively. The fourth compound cochromatographed with the synthetic dehydrodimer **1** ($R_f = 0.53$). After TLC separation and extraction, HPLC analysis confirmed the correspondence between peak C and the compound collected from TLC plates. Compound C was then analyzed by MS and GC/MS (after derivatization with BSTFA) (see Materials and Methods). The mass spectrum of the free phenol (EI, M^+ at *m/e* 454, relative intensity = 100) (Figure 5) and that of the trimethylsilyl ether obtained by GC/MS (M^+ at *m/e* 814, relative intensity = 100) confirm the dimeric and pentaphenolic nature of this compound. With diluted NaOH, its UV spectrum showed a base shift of the long-wavelength chromophore, indicating the presence of a *trans*-stilbene unit with a free *p*-hydroxy group.[27] It can thus be assumed that peak C corresponds to the resveratrol dehydrodimer, ϵ-viniferin. However, further characterization by NMR was not possible, due to the small quantities available.

The concentrations of stilbenes in grape leaf extracts are presented in Table 1. It can be seen that the phytoalexin production potential (determined by the amounts of free resveratrol, its glucosides, and ϵ-viniferin) is higher for the tolerant species, *V. rupestris*, than for the susceptible one, *V. vinifera*. Pterostilbene can also be synthesized in relatively large amounts

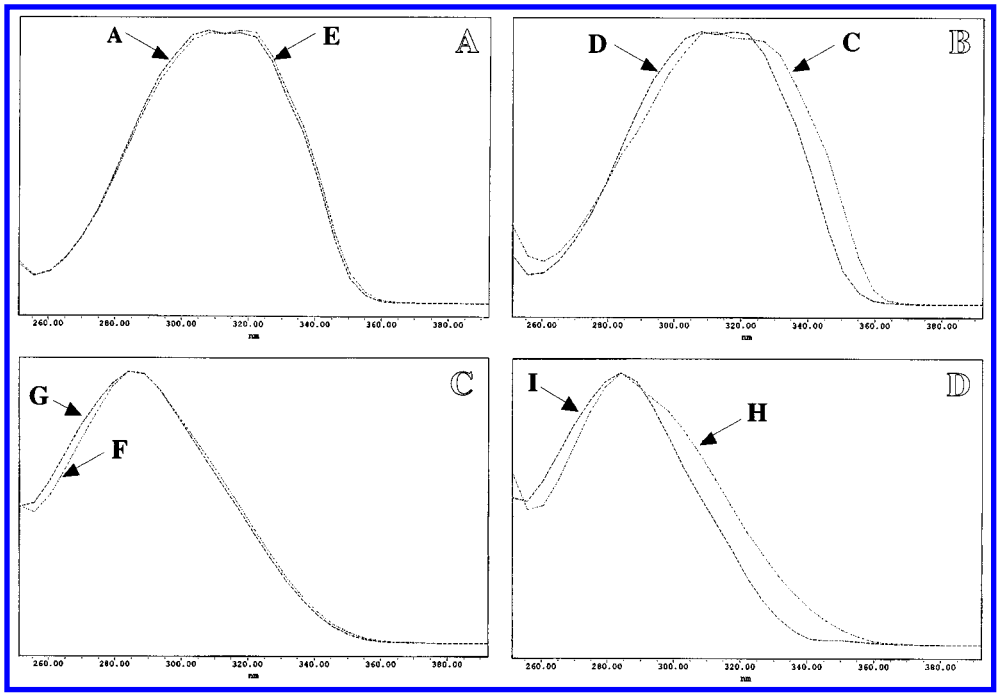

Figure 3. Spectra of *trans*- and *cis*-stilbenes obtained by diode array detection. (A) Spectra of *trans*-resveratrol (peak A) and its *trans*-glucoside (peak E). (B) Spectra of the *trans*-dehydrodimer **1** (peak C) and *trans*-pterostilbene (peak D). (C) Spectra of *cis*-resveratrol (peak G) and its *cis*-glucoside (peak F). (D) Spectra of the *cis*-dehydrodimer **1** (peak H) and *cis*-pterostilbene (peak I).

Table 1. Production of Phytoalexins in *Vitis* spp. As Induced by UV Irradiation[a]

	V. vinifera	*V. rupestris*
trans-resveratrol[b]	102 ± 25	216 ± 42
trans-resveratrol glucoside	8 ± 2	80 ± 20
cis-resveratrol glucoside	3 ± 1	48 ± 12
trans-ϵ-viniferin	25 ± 6	70 ± 18
trans-pterostilbene	0.2−0.8	14 ± 7

[a] All leaves were placed in Petri dishes on moist filter paper and then irradiated on their abaxial surfaces for 10 min with UV radiation (0.36 J cm^{-2}). After 24 h in darkness, phytoalexins were extracted as described in the Materials and Methods section. The values represent average phytoalexin production of six leaves of each species. [b] Expressed in micrograms per gram fresh weight. Quantification of all stilbenes was done by using the fluorometer, except for the *cis*-resveratrol glucoside which was quantified in UV at 285 nm (see Materials and Methods section).

in the leaves of *V. rupestris*, but this was not consistently observed in our experiments. Pterostilbene is a very biologically active compound which is usually found in low quantities in grapevines.[5,32]

The fact that extracts of the variety Pinot Noir contained very low concentrations of resveratrol glucosides confirmed other previously published results,[33] which showed that Pinot Noir wines generally have a low resveratrol glucoside content. In contrast, in *V. rupestris* leaf extracts, the sum of the glucosides (cis and trans) can exceed the free resveratrol content. The existence of free and bound forms of resveratrol in grapevine leaf extracts leads one to formulate interesting hypotheses regarding the metabolism of this stilbene within the plant. *trans*-Resveratrol is likely synthesized in the free form by the action of stilbene synthase and then rapidly glycosylated by a glycosyl transferase to *trans*-

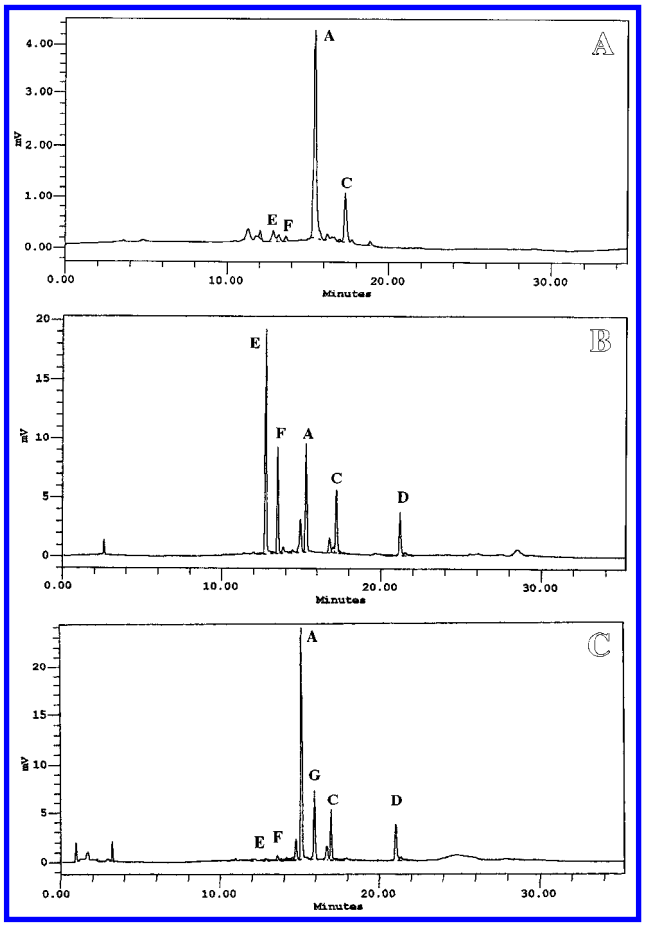

Figure 4. HPLC chromatograms of *Vitis* spp. extracts. (A) *V. vinifera* extract. (B) *V. rupestris* extract. (C) The same extract as in part B but obtained after treatment with β-D-glucosidase for 17 h, showing disappearance of peaks E and F, increase in peak A, and occurrence of a new peak (peak G). Peak A, *trans*-resveratrol; peak C, *trans*-ϵ-viniferin; peak D, *trans*-pterostilbene; peak E, *trans*-resveratrol glucoside; peak F, *cis*-resveratrol glucoside; peak G, *cis*-resveratrol.

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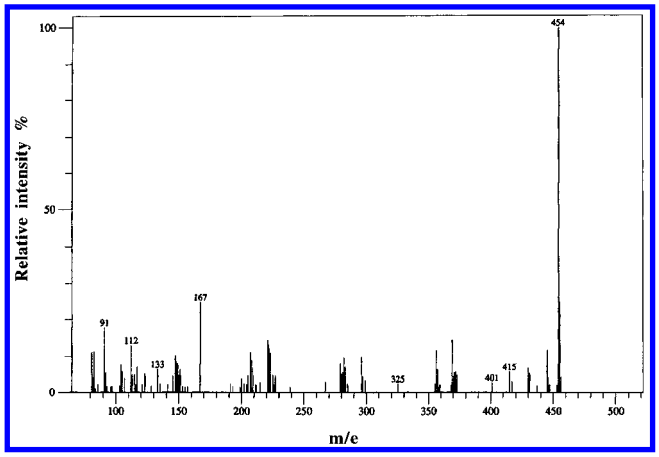

Figure 5. Mass spectrum (electron impact, direct introduction) of compound C obtained after purification by TLC.

resveratrol glucoside; after this, isomerization to the *cis*-glycosidic form is possible by a *cis*-isomerase (which has not yet been characterized to date). The occurrence of free resveratrol in the extracts is linked to the presence of endogenous β-D-glucosidases, which probably remain active during the extraction process.[34] Interestingly, we observed a direct negative correlation between the amounts of free resveratrol and those of glycosylated resveratrol in the plant extracts.

In this paper, we have described a method suitable for the analysis of the four major stilbene phytoalexins of grapevines, resveratrol, its β-D-glucoside, pterostilbene, and ϵ-viniferin. Given the high level of peak resolution, both the cis and the trans isomers of these compounds could be detected. Use of fluorometric detection together with diode array detection permits the unambigous characterization of stilbene compounds, and this is due, first, to the specificity of the fluorometric parameter detection linked to the particular structure of stilbenes and second, to the characteristic absorbance of the stilbene skeleton between 260 and 350 nm.[30] Simultaneous determination of pterostilbene and ϵ-viniferin together with that of resveratrol and its glucosides allows an excellent assessment of the grapevine phytoalexin response. In fact, although there are a number of works on the relationship between resveratrol production and grape disease resistance,[6,8,35,36,37] the biological role of the other stilbenes in the defense mechanisms of grapevines is poorly understood, largely due to the lack of data concerning their chromatographic determination. Moreover, study of pterostilbene and ϵ-viniferin is also of great interest since they are both considered to be more fungitoxic than resveratrol itself.[6,38] This analytical method thus allows the determination of the major phytoalexins in grapevine leaves with the aim of clarifying their role in the resistance of this species against fungal attack.

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ACKNOWLEDGMENT

We thank Professor D. M. Goldberg (University of Toronto, Canada) and Mr. G. Soleas (Andres Wines Limited, Grimsby, Canada) for the generous gift of a purified sample of resveratrol glucoside. We are also indebted to Drs. R. Pezet and V. Pont (Swiss Federal Agricultural Station of Changins) for providing us a pure sample of pterostilbene.

Received for review June 5, 1997. Accepted September 25, 1997.[⊗]

AC970582B

[⊗] Abstract published in *Advance ACS Abstracts*, November 1, 1997.