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Method to determine resveratrol and pterostilbene in grape berries and wines using high-performance liquid chromatography and highly sensitive fluorimetric detection

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Abstract

Resveratrol and pterostilbene are two hydroxystilbenic phytoalexins synthesized by *Vitaceae*. Produced by leaves and grape berries after biotic or abiotic stress, the determination of their concentration can help to evaluate the disease resistance of different vine varieties. Resveratrol is also found in wines, particularly in red wine. These stilbenes are highly sensitive to light and air oxidation. Extraction must be made under nitrogen and protected from light. HPLC separation of resveratrol and pterostilbene is performed on a reversed phase (C_{18}) with a methanol–formic acid (50 m*M*) gradient. Fluorimetric detection is much more sensitive than UV detection and its specificity allows simple pre-purification of grape berries and direct injection of wines.

1. Introduction

Stilbenes occur naturally in a number of plant families [1]. They can be synthesized by plants after a stress and, in this case, are considered as phytoalexins. Stilbenic phytoalexins have been extensively studied in *Vitaceae* by Langcake and Pryce [2,3]. These authors have described resveratrol (3,5,4′-trihydroxystilbene), pterostilbene (3,5-dimethoxy-4′-hydroxystilbene) and their polymeric forms, the viniferins.

The analysis of resveratrol production by leaves of vitaceae after UV irradiation was considered as a disease resistance index in grape breeding programs [4,5]. More recently resveratrol was identified in wines and considered by Siemann and Creasy [6] as an active ingredient in causing reduction of serum lipids levels in humans. However, this theory is open to discussion in view of the low concentration of resveratrol in wines and the high concentrations of this stilbene necessary to lower lipid levels in the livers of rats with hyperlipemia [7].

Pterostilbene is detected in trace amounts in healthy and immature grape berries [8]. However, its fungitoxicity is much more important than that of resveratrol [9]. Its biosynthesis is not induced by UV irradiation but Langcake *et al.* [2] have detected great amounts of pterostilbene in *Plasmopara viticola* infected leaves of *Vitis vinifera*. This stilbene is probably not synthesized by the same stilbene synthase as resveratrol, as described by Schoeppner and Kindl [10].

Methods to extract and analyse stilbenic phytoalexins in wines and grapes by HPLC have been described by several authors [2,8,11,12]. In

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most cases, extractions required fastidious manipulations and denaturating vacuum rotary evaporation steps. Detection and quantitative determination of stilbenes were made, until now, by UV detection at 295 to 305 nm [2] or at 280 nm after UV irradiation of the sample [6]. GLC analysis of stilbenic phytoalexins has also been described by several authors [4,12,13]. Both methods are not sufficiently sensitive or reliable enough to detect small natural concentrations of stilbenes, as is generally the case, especially for the highly fungicidal pterostilbene [8].

The use of a fluorimetric detector decreases the threshold of measurable concentrations of stilbenes in the nanomole range and is more specific than UV. This specificity requires only two purification steps for grape berries and a direct injection for wines.

This paper demonstrates the possible denaturating effects of rotary evaporation *in vacuo* (40°C) on resveratrol and pterostilbene and proposes a protective method. It describes a fluorimetric detection method that is more sensitive and specific than UV detection.

2. Experimental

2.1. Chemicals

Pure resveratrol (*trans*-3,5,4′-trihydroxystilbene) and pterostilbene (*trans*-3,5 methoxy-4′-hydroxystilbene) were synthesized in our laboratory as described elsewhere [8,9]. Solvents used for extractions were pro analisi grade and HPLC grade for chromatographic analysis (Romil Chemicals, Shepshed, Loughborough, UK). Mixtures of solvents for HPLC were filtered (Acrodisc LC13 PVDF 0.45 μm, Gelman, Ann Arbor, MI, USA) and degassed under a permanent helium stream.

2.2. HPLC analysis

Automatic injector, pump (tertiary gradient system) and UV detector were from Bruker (LC-51, LC-21C, LC-313). The fluorimetric detector was the SFM 25 Model from Kontron. Chromstar software (Bruker) was used to control injector, pump and UV detector and to analyse data provided by the two detectors through a two-way channel peak integration. Analyses were performed on a reversed-phase column (LiChrospher 100-RP 18, 5 μm, 4.5 × 250 mm, Merck) with the following mobile phase: solvent A: methanol–50 m*M* formic acid (20:80, v/v), solvent B: methanol–50 m*M* formic acid (80:20, v/v) and solvent C: pure methanol. Solvents were delivered according to the following programme: linear gradient from 100% A to 100% B in 25 min; 100% B for 5 min; linear gradient from 100% B to 100% C in 1 min; 100% C for 5 min; linear gradient from 100% C to 100% A in 5 min; 100% A for 5 min.

2.3. Fluorimetric and UV detection

Optima in excitation and emission wavelengths were determined with a Kontron fluorimeter (SFM 25) using suitable solutions of pure resveratrol and pterostilbene in a mixture of methanol–50 m*M* formic acid (80:20, v/v). UV absorption was determined with a UV-160 Shimadzu spectrophotometer using the same stilbene solutions. UV optimum absorbances of resveratrol and pterostilbene are at 305.6 and 306.4 nm, respectively, in methanol–50 m*M* formic acid (80:20, v/v). Maximum excitation wavelength is measured at 330 nm and emission at 374 nm for these two stilbenes. Measured parameters were programmed to detect resveratrol and pterostilbene with the HPLC system described above. Solutions which contained different concentrations of these stilbenes (0.1–100 ng per 10 ml injection volume) were obtained by suitable dilutions of a methanolic solution at 0.1 mg ml^{-1}. Standard calibration curves were established by plotting the area of peaks against different concentrations of resveratrol and pterostilbene. Three replicates were made for each concentration. Standard errors and linear correlation were calculated using the GraFit statistical programm (Erithacus Software, Sigma). The lowest detectable concentration for both resveratrol and pterostilbene was 0.1 ng 10

μl^{-1} with the fluorimeter and 5 ng 10 μl^{-1} with the UV detector (306 nm). Linear correlations were excellent from 0.1–100 ng for fluorimetric detection : correlation coefficient $r = 0.9995$ for resveratrol and $r = 0.9975$ for pterostilbene. According to these results the use of a fluorimetric detector enhances by about 50 times the sensitivity of the analysis. In addition, the specificity of the fluorimetric parameter detection, linked to the chemical structure of the stilbenes, decreases the risk of peak confusion and allows for minimal pre-purification of the samples. In order to compare fluorimetric detection to UV detection, a UV detector was connected on-line and signals were computed by the same procedures. In all manipulations, stilbene solutions were protected from light to avoid *cis*-isomerization, decreasing the sensitivity of the detection.

2.4. Sample preparation

Pure resveratrol and pterostilbene were solubilized in methanol at the concentrations of 3 μg ml^{-1} and 3.5 μg ml^{-1}, respectively. An aliquot of 1 ml was evaporated using rotary evaporation (*in vacuo*, 40°C). The residue was solubilized in 1 ml of methanol. Another aliquot of 1 ml was evaporated under a stream of nitrogen (40°C in a water bath). The dried residue was solubilized in 1 ml of methanol. 10 μl of each methanolic solution were separately injected into the analytical HPLC system.

Downy Mildew infected grape berries (var. Chasselas) were harvested at véraison development stage. Eight berries (10.95 g fresh weight) were crushed in 40 ml of methanol using a mechanical homogenizer (VirTis) at maximum speed (25 000 rpm) for 5 min. The resulting suspension was centrifuged (10 000 g, 20 min). 20% of water was added to the supernatant. This solution was pre-purified by solid-phase extraction (Supelclean LC-18 SPE Tubes, 3 ml) in adsorbing impurities on the phase. The eluate was collected, the column was washed with 5 ml of methanol–water (8:2, v/v) and the resulting eluate added to the first one. Eluates were evaporated under a nitrogen stream while the solution was maintained at 40°C in a water bath. The resulting water solution was extracted with diethyl ether three times. The ether fraction was dried with Na_2SO_4, filtered through paper and evaporated to until dry under a nitrogen stream at 40°C as described below. The dried residue was solubilized in 5 ml of methanol and 10 μl of this solution were injected into the HPLC analytical system. Co-chromatographic analysis was performed by adding 20 μl of a 5 μg ml^{-1} stilbene standard solution (resveratrol and pterostilbene) to 200 μl of methanolic grape berry sample solution. 10 μl of this solution was injected.

To evaluate the efficiency of this extraction procedure, 5 ml of a resveratrol and pterostilbene methanolic solution at 5 μg ml^{-1}, obtained by suitable dilution of a stock solution (0.1 mg ml^{-1}), was treated as described for grape berry extraction. 10 μl of this solution was analyzed before the extraction procedure as a check and after the extraction procedure. Three replicates were made and the standard deviation was calculated.

Wine analyses were performed by injecting 5–50 μl of filtered (0.45 μm, Gelman) wine without any purification. Pure resveratrol and pterostilbene were added to samples of wine for co-chromatographic analysis. All extraction procedures were protected from light.

Grape berry and wine analysis were performed using the fluorimetric detector only. Concentrations of resveratrol and pterostilbene in samples were measured using the external standard method. Response factors (amount of standard/peak area) were calculated with data from the standard calibration curve.

3. Results and discussion

Langcake and Pryce [13] described the spectral properties of resveratrol and measured two λ_{max} at 306 nm and 318.7 nm. These values are in complete accordance with our measurements. These authors have observed that under long wavelength UV light (366 nm) stilbenes shown a bright blue fluorescence. Their fluorimetric analysis of the fluorescent compounds extracted from UV-irradiated leaves give an excitation wavelength at 330 nm and an emission wavelength at

374 nm. These values correspond to those described in this paper for resveratrol and pterostilbene.

Stilbenes are unstable in light and sensitive to oxidation in normal air when deposited on thin-layer plates [1]. This sensitivity is probably at the origin of their denaturation observed using rotary evaporation. In this system, when the solvent is evaporated, compounds are uniformly distributed as a thin layer on the wall of the flask and particularly exposed to air oxidation when vacuum is suddenly broken. All solvent evaporation steps must absolutely be done under nitrogen and protected from light.

HPLC analysis of these stilbenes treated by this method show only one peak for each (Fig. 1, peaks 1 and 2). Retention times for resveratrol is 15.6 min and for pterostilbene 26.3 min. Only two negligible minor peaks can be detected (Fig. 1A). The rotary evaporation concentration step (*in vacuo*, max. temp. 40°C and protected from light) induces an important modification of the

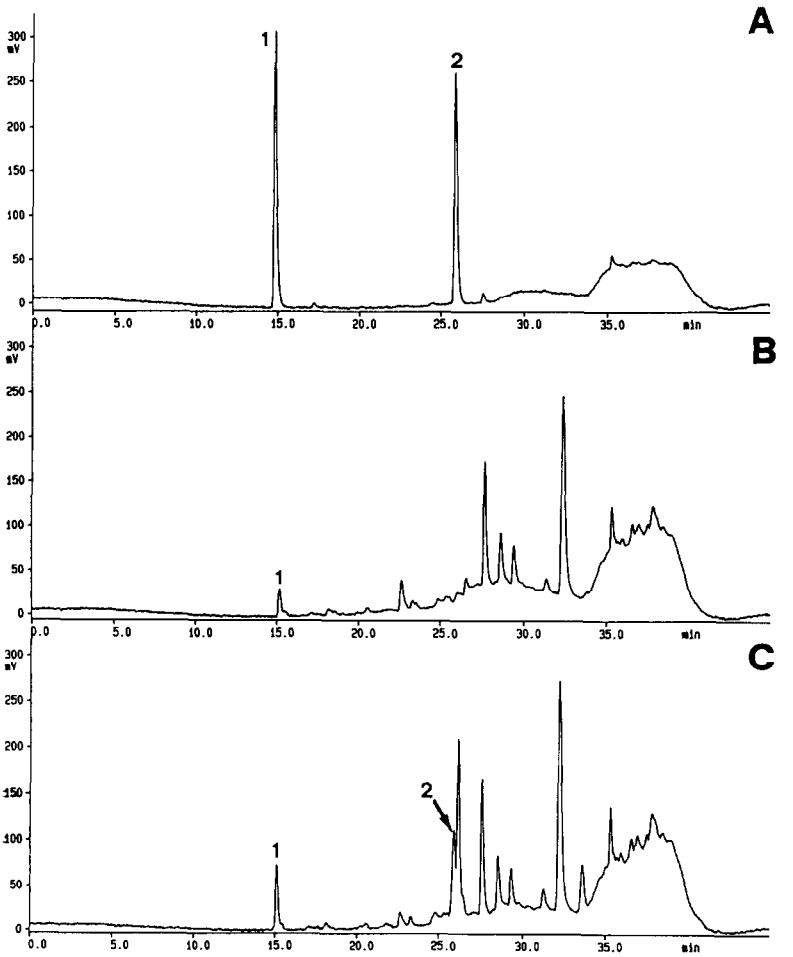

Fig. 1. Demonstration of stilbenes denaturation using rotary evaporation *in vacuo* (40°C). (A) Chromatogram of standard resveratrol (1) (30 ng 10 μl^{-1}; t_R 15.6 min) and pterostilbene (2) (35 ng 10 μl^{-1}; t_R 26.3 min) after concentration of the sample under nitrogen (protective method). Detection: fluorescence, ex. 330 nm, em. 374 nm. (B) Chromatogram of the same concentrations of standard resveratrol (1) after concentration of the sample using rotary evaporation (*in vacuo*; 40°C; protected from light). (C) Chromatogram of the same amounts of standard resveratrol (1) and pterostilbene (2) after concentration using rotary evaporation (*in vacuo*; 40°C; protected from light).

chromatograms. Many secondary peaks appear since major resveratrol and pterostilbene peaks decrease dramatically (Fig. 1B and C). Secondary peaks appear under denaturating extraction conditions for resveratrol at 22.6, 23.3, 27.6, 28.5, 29.3, 31.2, 32.7, and 35.3 min, and four minor peaks between 35.6 and 37.8 min, and for pterostilbene at 26.9 min and 33.6 min. These secondary peaks correspond certainly to oxidation products of these stilbenes as yet not identified.

The recovery concentrations of the extracted standard solutions, determined by HPLC, is 100%. Three replicates give no significant differences between concentration of the check solutions before and after the extraction procedures.

Downy mildew (*Plasmopara viticola*) contaminated grape berries contain 1.21 mg of resveratrol and 35 ng of pterostilbene per gram of fresh weight. Important peaks of other fluorescent compounds appear at retention times situated between those of resveratrol and pterostilbene (Fig. 2A). They were as yet not identified but some of them could correspond to ϵ- and α-viniferine as described elsewhere [2,3]. Co-chromatographic analyses, using standard resveratrol and pterostilbene, are shown in Fig. 2B. Theoretically, 5 ng of each stilbene was added in 10 μl of injected sample. The recovery was 5.6 ng and 5.65 ng, respectively, for resveratrol and pterostilbene.

The specificity of the fluorimetric detection for stilbenes permits direct injection of wines. This procedure avoids any denaturation of the sample. Five swiss wines were analyzed : two white varieties, Chasselas and Chardonnay, and three red varieties: Gamay, Pinot and Gamaret (Gamay × Reichensteiner). In view of the low concentration of resveratrol in white wines, 50 μl of these wines were injected. Due to higher concentrations of resveratrol, only 5 μl of red wines were sufficient to detect this stilbene. Pterostilbene was not detected in wines. Co-chromatographic analyses were realized on a red wine (Gamaret) and a white one (Chasselas). The chromatograms of these wine analyses, with

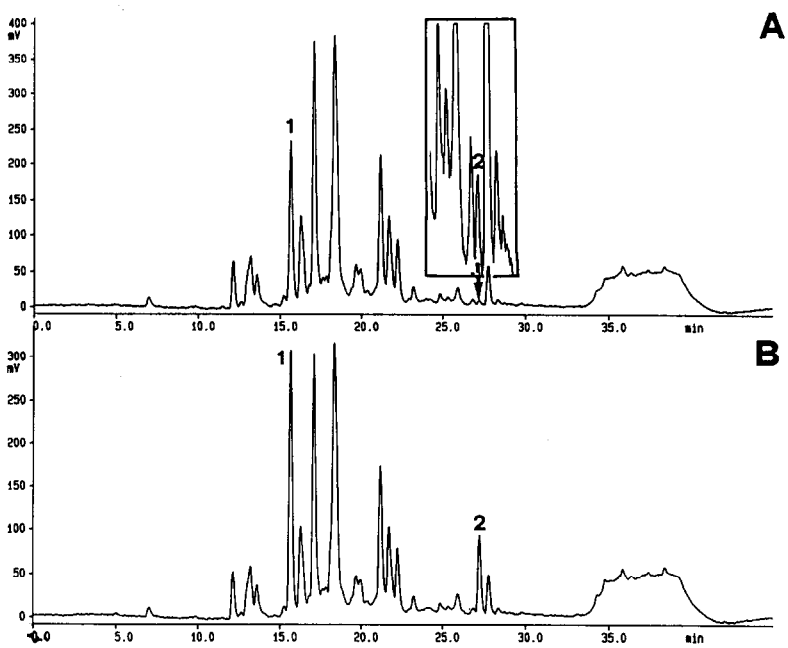

Fig. 2. Chromatograms of Downy Mildew contaminated grape berries (var. Chasselas) extracts using fluorimetric detection (ex. 330 nm, em. 374 nm). (A) Resveratrol: peak 1; pterostilbene: peak 2. In the box: enlarged part of the chromatogram showing the well defined peak of pterostilbene. (B) Co-chromatographic analysis of the same extract after addition of 5 ng each of resveratrol (1) and pterostilbene (2) in 10 μl injected.

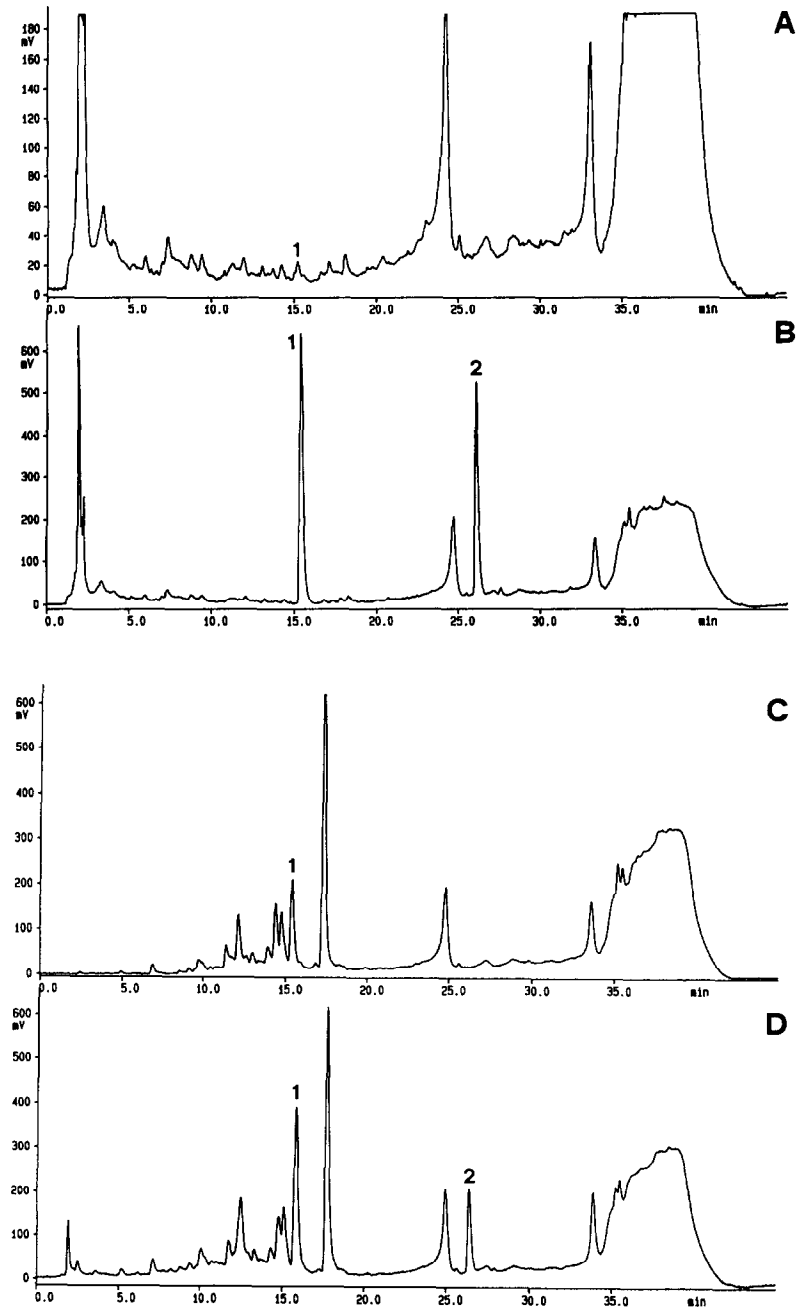

Fig. 3. Chromatograms of wines injected without any purification. Detection: fluorescence, ex. 330 nm, em. 374 nm. (A) and (B) White wine (var. Chasselas) without and with the addition of standard resveratrol (1) and pterostilbene (2), respectively. (C) and (D) Red wine (var. Gamaret) without and with addition of standard resveratrol (1) and pterostilbene (2), respectively.

or without the addition of pure resveratrol and pterostilbene, are shown in Fig. 3A–D. The measured concentrations of resveratrol in the analyzed wines, three replicates drawn from the same bottle for each variety, are presented in Table 1.

Table 1
Amounts of resveratrol in different Swiss wines

Wine variety (1992)		Resveratrol (mmol l^{-1}, mean ± S.D.)
Chardonnay	White	0.14 ± 0.01
Chasselas	White	0.15 ± 0.02
Gamay	Red	6.47 ± 0.54
Pinot	Red	2.95 ± 0.11
Gamaret	Red	2.16 ± 0.25

The use of fluorimetric detection of stilbenes offers much greater sensitivity and selectivity. Many phenolic compounds have a maximum absorbance between 275 to 330 nm while fluorescence parameters are different and specific for each phenolic [14]. This characteristic allows a simple sample preparation and the resulting chromatograms show less unknown peaks, a better resolution and a much greater sensitivity than with UV detection.

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5. References

[1] J.H. Hart, *Ann. Rev. Phytopathol.*, 19 (1981) 437.
[2] P. Langcake, C.A. Cornford and R.J. Pryce, *Phytochemistry*, 18 (1979) 1025.
[3] P. Langcake and R.J. Pryce, *Experientia*, 33, (1977) 151.
[4] M. Barlass, R.M. Miller and T.J. Douglas, *Am. J. Enol. Vitic.*, 38 (1987) 65.
[5] R.M. Pool, L.L. Creasy and A.S. Frackelton, *Vitis*, 20 (1981) 136.
[6] E.H. Siemann and L.L. Creasy, *Am. J. Enol. Vitic.*, 43 (1992) 49.
[7] H. Arichi, Y. Kimura, H. Okuda, K. Baba, M. Kosawa and S. Arichi, *Chem. Pharm. Bull.*, 30 (1982) 1766.
[8] R. Pezet and V. Pont, *Plant Physiol. Biochem. (Paris)*, 26 (1988) 603.
[9] V. Pont and R. Pezet, *J. Phytopathol.*, 130 (1990) 1.
[10] A. Schoeppner and H. Kindl, *FEBS Letters*, 108 (1979) 349.
[11] W. Dercks and L.L. Creasy, *Physiol. Mol. Plant Pathol.*, 34 (1989) 189.
[12] P. Jeandet, R. Bessis and B. Gautheron, *Am. J. Enol. Vitic.*, 42 (1991) 41.
[13] P. Langcake and R.J. Pryce, *Physiol. Plant Pathol.*, 9 (1976) 77.
[14] C.F. Van Sumere, in P.M. Dey and J.B. Harborne (Series editors), *Methods in Plant Biochemistry*, Vol. 1, J.B. Harborne (Editor), Plant Phenolics, Academic Press, London, 1989, Ch. 2, p. 29.