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Simple and rapid method for *cis*- and *trans*-resveratrol and piceid isomers determination in wine by high-performance liquid chromatography using Chromolith columns

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Abstract

A simple and rapid HPLC method has been developed for simultaneous determination of the four resveratrol forms (aglycon and glycosidic) in a Grenache wine from Châteauneuf du Pape (Vaucluse). These analyses were achieved by using two commercial monolith HPLC columns and diode array detection. The method provided reliable separations at low pressure with a short analysis time. The limit of detection (LD) and limit of quantification (LQ) were calculated for each standard. The molecules were separated and quantified in a single run without any purification of the sample.

Keywords: Resveratrol; Piceid; Monolithic column; Phytoalexin; HPLC

1. Introduction

The substance, known as resveratrol, is one of a group of compounds (called phytoalexins) that are produced in plants during times of environmental stress such as adverse weather or insect, animal or pathogenic attack [1]. Resveratrol (3,5,4′-trihydroxystillbene) is a naturally occuring polyphenol with two isomers. *trans*- and *cis*-resveratrol have been shown to exist in wine as both the aglycon and the bound glucoside (piceid) (Fig. 1). Stilbenes occur in many plants [2] but grapes and related products are considered the most important dietary sources of these substances [3,4]. It has been established that resveratrol is synthesized and located in the skin but not in the fleshy part of the berries of *Vitis vinifera*. It is not surprising that red wines have greater levels of these four resveratrol derivatives [5–11].

The protective effects offered to humans by moderate consumption of red wine may be related, in part, to the levels of *trans*-resveratrol [12]. However, other natural polyphenolic antioxidant species may contribute to this effect. The potential benefits could be due to resveratrol's ability to inhibit LDL oxidation [13,14], the initial stage of the pathogenesis of arteriosclerosis, and the aggregation of platelets [15,16]. Resveratrol could also have chemo preventive effects against cancer [17,18].

Numerous methods have been described to determine the concentration of the four resveratrol derivatives in wine. The methods employed are varied in their analytical principles. Organic solvent extraction [5,10,19], solid phase extraction [20–26] and direct injection [7,8,11,27] techniques have been used prior to resolution of resveratrol isomers by GC or HPLC. Most GC methods require a derivatization step before column application by flame ionization or mass spectrometry detection [9,28]. Currently, HPLC detection procedures are based upon UV absorption [8,11,29,30], fluorimetry [31–34], electrochemistry [35] and mass spectrometry [36–37].

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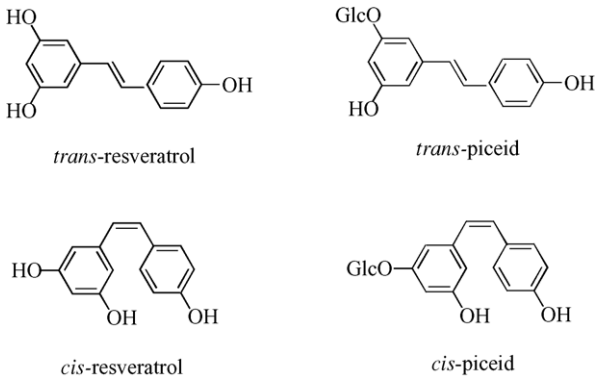

Fig. 1. Chemical structures of resveratrol and piceid isomers.

Additionally, an LC-MS/MS method has been developed [38].

A conventional RP_{18} column with diode array detection has proved to be the most appropriate technique for the separation of *trans*- and *cis*-resveratrol [39] or of the four isomers [40]. However, the majority of these methods require rather long analysis times [27,41–43].

The present paper reports a simple and fast method for the determination of the four resveratrol derivatives in must and wine of the Grenache variety by direct injection using monolithic columns and diode array detection. Monolithic supports, consisting of a single piece of porous material, are now available. Monolithic silica technology attracts the interest of researchers because of their various advantages [44–48].

2. Experimental

2.1. Reagents and standards

Acetonitrile, methanol and water, of HPLC grade, and acetic acid were supplied by VWR International (Darmstadt, Germany). *Trans*-resveratrol was acquired from Sigma (Saint Louis, MO, USA).

2.2. Wine samples

Wines were made by the Viti-RD Company in Villetelle (Hérault, France) from grapes of Grenache, hand harvested in vineyard of Châteauneuf du Pape vineyards (Vaucluse, France) in 2002. After crushing and destemming, the grapes and musts were placed in a stainless steel tank, treated with sulfur dioxide (0.03 g/l) and inoculated with active dry wine yeast (0.10 g/l ICV K1). The alcoholic fermentation was followed by the measure of the density. Both pressed and free-run juices were assembled and fermented by addition of lactic bacterium. After completion of fermentation, the wine was racked, filtered and sulfur dioxide (0.05 g/l) was added prior to bottling and stored at 15 °C in the dark.

2.3. Wine analyses

Wines samples were filtered through a 0.2 μm cellulose regenerated filter (Alltech Associates, Deerfield, IL, USA) and then injected. For each wine, analyses were performed in triplicate immediately after bottle opening.

2.4. HPLC analysis

The analyses were performed using a Waters (Milford, MA, USA) liquid chromatograph equipped with a Model 600 pump and a Model 600 gradient controller, to which were connected a Model 717 auto sampler and a Model 996 photodiode-array detector. The columns were a series of two Chromolith Performance RP-18e (100 mm × 4.6 mm, I.D.) from VWR International. The work is done by means of coupled columns which features a theorical plate number sufficient to separate the four forms of resveratrol. All runs were acquired and processed using Millenium^{32} software (Waters).

The injection volume was 20 μl. The solvent system used was a gradient of (A) water–acetic acid (94:6) (v/v) and (B) water acetonitrile–acetic acid (65:30:5) (v/v/v). The elution gradient is shown in Table 1. Use of monolithic columns permitted mobile phase flow-rate of up to 7 ml/min to be employed in the assays. Increasing the mobile phase flow rate to 7 ml/min significantly increased the speed of the assay and consequently reduced the run time.

Spectrophotometric detection, covering the UV–vis range of 200–310 nm was performed with a resolution of 1.2 nm, and the quantitation was carried out at 285 and 306 nm (where *cis*- and *trans*-isomers have absorbance maximum, respectively).

2.5. Calibration

For the *trans*-resveratrol the calibration curves were drawn from various standard solutions prepared by diluting a stock solution of 200 mg/l of *trans*-resveratrol in methanol, such that they cover a range of concentration between 0.1 mg/l and 10 mg/l. All the solutions were stored at 4 °C and protected from light.

For the calibration of the *cis*-resveratrol, since its commercial standard is not available, working standard solutions of *trans*-resveratrol were taken and left in daylight for a period of 1 h. The exposure to light causes the *trans*-resveratrol to be partially converted into *cis*-resveratrol (80–90% of *trans*-resveratrol was converted to *cis*-resveratrol).

Table 1
Solvent and flow rate gradients

Time (min)	Flow rate (ml/min)	A (%)	B (%)
–	4.00	85	15
10	7.00	70	30
17	7.00	20	80
18	7.00	0	100
20	4.00	85	15

For the irradiation solution, decreased levels of intensity for the *trans*-resveratrol peak was proportional to the area of the new peak corresponding to *cis*-resveratrol.

Since no other peaks were detected under these conditions, the concentrations of *cis*-resveratrol were assigned on the basis of the decrease observed for *trans*-resveratrol following the 1 h period of irradiation.

The quantitation of *trans*-piceid and *cis*-piceid was based on the assumption of identical molar extinction coefficients of *trans*-resveratrol and *cis*-resveratrol at 306 and 285 nm, respectively [49]. For each standard, the limit of detection (LD) and the limit of quantification (LQ) were fixed at 3 and 10 times, respectively, the signal-to-noise ratio (S/N). Baseline noise was measured considering a peak to peak within 3 min selected in three different parts of the chromatogram of the standard solution. The precision of this technique may be expressed by the measure of its repeatability. The repeatability has been estimated by analyzing the concentration of the four compounds in each of the wine samples three times. The precision of the analytical procedure was expressed by the standard deviation (SD).

3. Results and discussion

3.1. Method optimization

Due to different methods employed, with conventional RP_{18} columns, several deviating values have been reported for resveratrol concentrations.

In addition, there are other intrinsic factors responsible for resveratrol variation such as grape variety, environmental factors in the vineyard, and wine-processing techniques. Moreover, these methods often have the disadvantage of excessive analysis run times. Therefore, analysts need methods that provide reliable separations and reduction of analysis time to achieve a routine work.

One approach is to develop high-throughput HPLC methods using monolithic columns. Monolithic silica columns are packed with a single piece of silica gel into a straight rod of highly porous silica with a bimodal pore structure (macroporous and mesoporous structure [50,51]).

Due to their rigid and porous structure, they enable higher solvent flows, shorter assay times, and fast re-equilibration between runs [45].

The method reported in this paper consists of a modification of the Roggero method [6] using a Superspher 100 RP-18e (250 mm × 4 mm, I.D.; 5 μm particle size) column. Due to complex separations of the four forms of resveratrol from wine, coupled monolithic columns are used to produce a column with a theorical plate number significantly higher than conventional columns and with a minimal back pressure. Under the experimental conditions used, *trans*- and *cis*-isomers were well separated and none of the other compounds present in the wine interfered in the determination. A typical chromatogram with the peaks of interest labeled

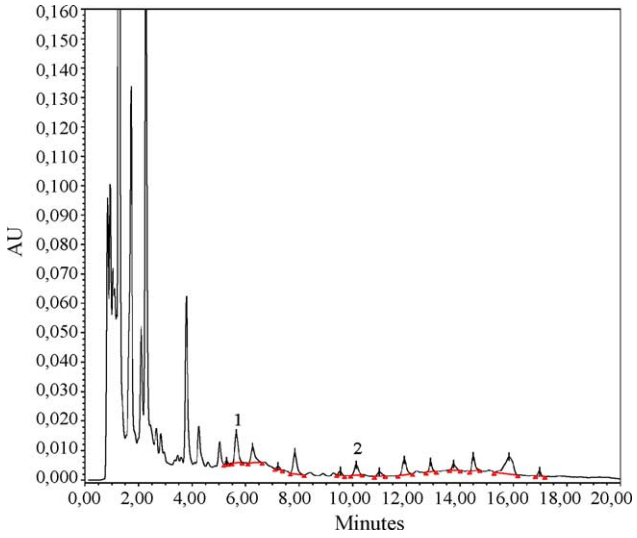

Fig. 2. Chromatogram of a Grenache wine at 306 nm (1: *trans* piceid; 2: *trans* resveratrol).

1–4 is illustrated in Figs. 2 and 3. Peak 2 was identified as *trans*-resveratrol by the following criteria: (a) its retention time and (b) its UV spectrum were identical to the *trans*-resveratrol standard (Fig. 4) and its purity was confirmed by diode array analysis of the spectrum. Peak 4 was identified at *cis*-resveratrol by the following criteria: (a) it co-chromatographed with the new peak which appeared after brief UV irradiation of the pure *trans*-resveratrol standard, (b) its UV spectrum was consistent with that published by Siemman and Creasy [5] for *cis*-resveratrol and its purity was confirmed by diode array analysis of the spectrum. Peaks 3 and 1 were identified as the glucosides of *cis*- and *trans*-resveratrol, respectively, by the following criteria: (a) the UV spectrum of peak 3 was nearly identical with that of

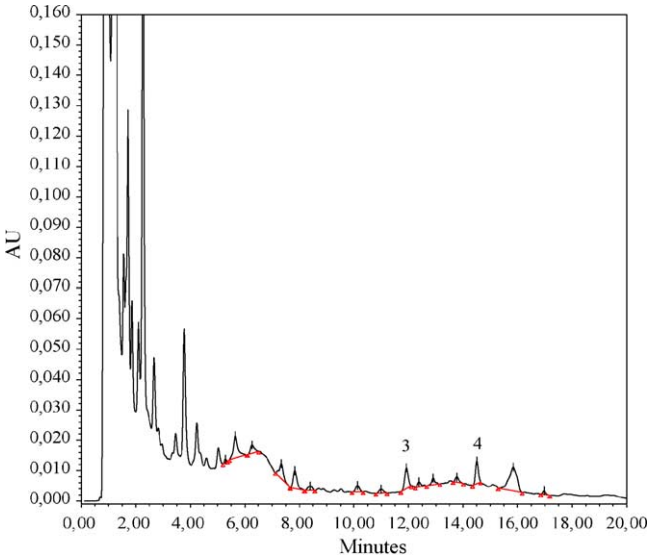

Fig. 3. Chromatogram of a Grenache wine at 285 nm (3: *cis*-piceid; 4: *cis*-resveratrol).

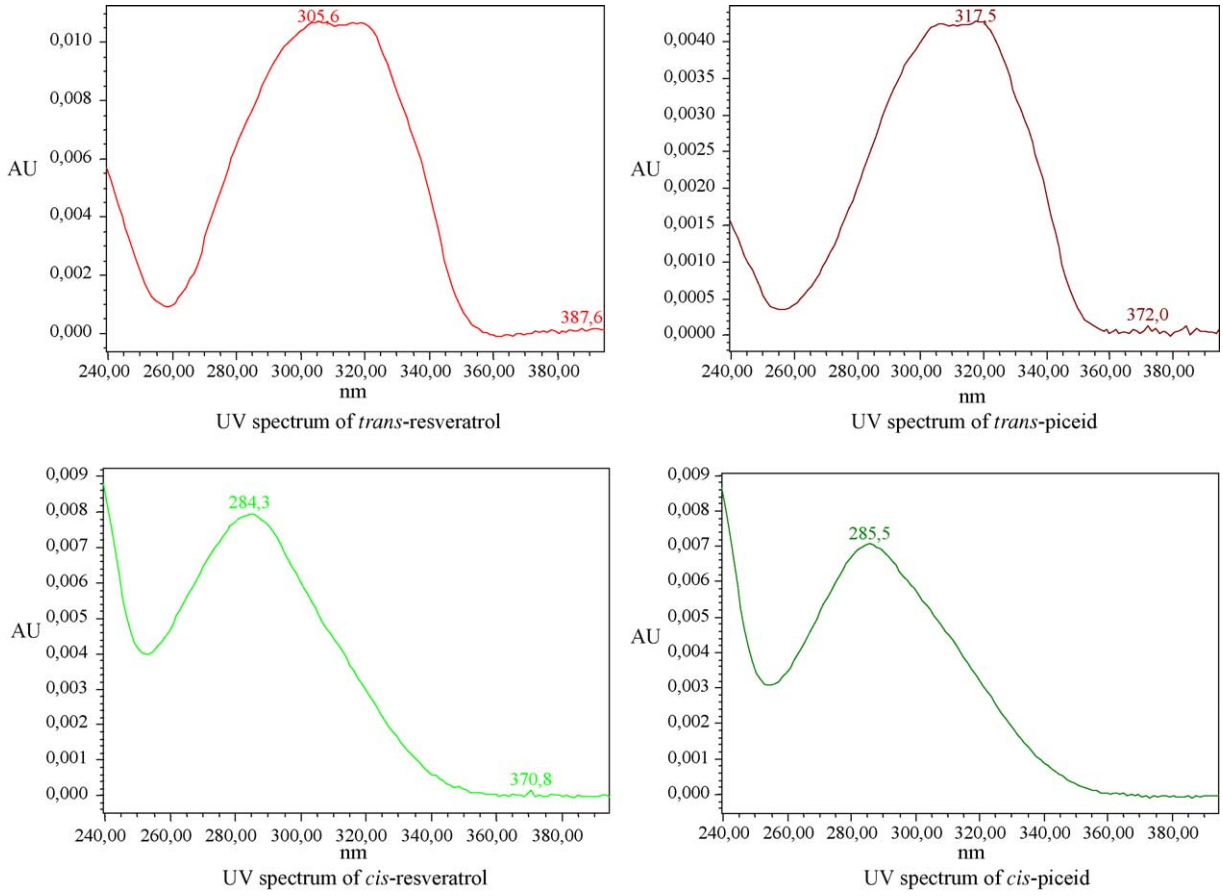

Fig. 4. UV spectrum of *trans*-resveratrol, *trans*-piceid, *cis*-resveratrol and *cis*-piceid.

peak 4 (*cis*-resveratrol), while that of peak 1 provided a close match to the UV spectrum of peak 2 (*trans*-resveratrol) (Fig. 4) [8], (b) by literature data: Waterhouse and Lamuela-Raventos [52] identified β-glucoside of *trans*-resveratrol in grape berries in 1994. The identity of this compound was authenticated from *P. cupsdatum* roots by ^{1}H NMR spectroscopy and treatment with *β*-glucosidase [53].

The four resveratrol derivatives showed lower retention time (*trans*-resveratrol: 10–10.2 min, *trans*-piceid: 5.5–5.7 min, *cis*-resveratrol: 14.4–14.6 min, *cis*-piceid: 11.7–12 min) than these reported in the literature [6] (*trans*-resveratrol: 117–118 min, *trans*-piceid: 101–102 min, *cis*-resveratrol: 125–126 min, *cis*-piceid: 113–114 min). The optimized conditions consisted of a 4 ml/min and a multistage gradient (Table 1), which was fully completed within 20 min, including the washing and re-equilibration time.

Due to the very high porosity of the monolithic column, flow rates as high as 7 ml/min may be achieved with the same quality of resolution. Therefore, a significant reduction of the total time of analysis (85%) could be obtained in comparison with methods employed conventional HPLC columns and direct injection [7,8,11]. At 306 nm the limit of detection and the limit of quantification for *trans*-resveratrol were 0.032 mg/l and 0.10 mg/l, respectively. At 285 nm the limit of detection and the limit of quantification for *cis*-resveratrol were 0.034 mg/l and 0.11 mg/l, respectively.

The calibration curves for both *trans*- and *cis*-resveratrol were prepared as described in Section 2.5 and showed good linearity for both isomers even though the procedure to

Table 2
Calibration parameters of *trans*-resveratrol and *cis*-resveratrol

	λ_{max} (nm)	Calibration curve		LD[a]	LQ[b]
		Equation	r^2		
trans-Resveratrol	306	$y = 0.78 \times 10^5 x - 0.049 \times 10^5$	0.999	0.032	0.10
cis-Resveratrol	285	$y = 0.36 \times 10^5 x - 0.011 \times 10^5$	0.992	0.034	0.11

[a] LD: detection limit (mg/l).
[b] LQ: quantification limit (mg/l).

Table 3
Resveratrol content in Grenache must and wine

	trans-Piceid	*trans*-Resveratrol	*cis*-Piceid	*cis*-Resveratrol	Total resveratrol
Must (t_0)	0.18 ± 0.02	n.d.	0.38 ± 0.03	n.d.	0.33 ± 0.02
End of alcoholic fermentation (t_0 + 6 days)	0.85 ± 0.04	0.29 ± 0.05	1.6 ± 0.06	2.21 ± 0.02	3.94 ± 0.07
End of malolactic separation (t_0 + 21 days)	0.48 ± 0.02	0.56 ± 0.02	1.64 ± 0.07	2.74 ± 0.03	4.53 ± 0.07
Bottling (t_0 + 52 days)	0.52 ± 0.02	0.57 ± 0.03	1.01 ± 0.05	2.10 ± 0.04	3.56 ± 0.06

Results expressed in mg/l and total resveratrol in aglycon. Mean of three replicates ± SD. n.d.: not detected.

achieve *cis*-resveratrol standards involved some errors, which could be avoided if *cis*-resveratrol was available as a commercial standard (Table 2).

3.2. Application

Once the chromatographic conditions for the separation were determined, the procedure was applied to the simultaneously determination of four forms of resveratrol in red Grenache wine from the South-East of France. We studied the evolution of resveratrol during the winemaking process at four distinct stages: in must, at the end of alcoholic fermentation, at the end of malolactic fermentation and at the time of bottling.

As shown in Table 3, the concentration of each isomer of resveratrol is in constant evolution during winemaking process. These variations are largely explained by enological practices, specifically the skin-contact time during maceration which leads to a greater diffusion of resveratrol, the presence of β-glucosidases which release aglycon forms, isomerization of *trans*- into *cis*-, and the likelihood at partial oxidation at the time of bottling.

4. Conclusion

We have developed a new, rapid and sensitive method that allowed the determination of *trans* and *cis* resveratrol and piceid isomers in must and wine.

This method consists of a direct injection of the sample followed by a rapid HPLC quantification step (15 min).

This analytical method was applied to Grenache wine samples of South-East of France and results were shown that each isomer of resveratrol was in constant evolution during the winemaking process.

Analyses were carried out using two monolithic columns. Monolithic support operate at a higher flow rate than conventional reverse phase column with a reduced pressure-drop and shorter washing and re-equilibration times.

We regard the Chromolith column as an useful tool for rapid separation of the four forms of resveratrol.

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