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ORIGINAL PAPER

Determination of stilbene derivatives in Burgundy red wines by ultra-high-pressure liquid chromatography

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Abstract The polyphenols, for example stilbenes and flavonoids, are an important family of compounds present in grapes and wines. Several studies have shown that stilbenes are antioxidants and cancer-preventing agents. For the first time, eight natural stilbenes *(trans*-resveratrol, *trans*-piceid, *cis*-piceid, *trans*-astringin, *trans*-piceatannol, (+)-*trans*-ε-viniferin, pallidol, and hopeaphenol), isolated and purified from *Vitis vinifera*, were simultaneously analysed by ultra-high-pressure liquid chromatography coupled with photodiode-array detection. Separation of the stilbenes by UHPLC was optimized with the assistance of "Quality-by-Design" commercial software. Four different reversed-phase columns packed with 1.5–1.7-μm particles were tested and compared for their retention behaviour and separation efficiency. On the basis of the performance characteristics determined, the VisionHT C18 HL column was selected for the stilbenes studied, because resolution of the critical pair was 1.5 with a peak width of 2–4 s. The optimized method resulted in highly repeatable retention times (RSD 0.03–0.07%), peak areas (RSD 3–6%), and linear ranges were between 0.005 and 50 mg L^{-1} for most of the compounds. All stilbenes, except *trans*-astringin, *trans*-piceatannol, and pallidol were identified and quantified in Burgundy red wines at different concentrations after direct injection of the wines.

Keywords Stilbenes · Wine · UHPLC · Chromatographic columns · Quality-by-Design

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Introduction

The stilbenes belong to an important group of polyphenolic secondary metabolites present in various families of plants, among which grapes are certainly the most widespread natural source over the world. Associated products, and in particular wine, are regarded as the most important dietary sources of these compounds [1, 2]. Stilbene derivatives have a variety of distinct chemical structures based on the 1,2-diphenylethylene skeleton, which vary, for instance, according to steric configuration, the number of hydroxyl groups, glycosylation, or oligomerization (Fig. 1). Their

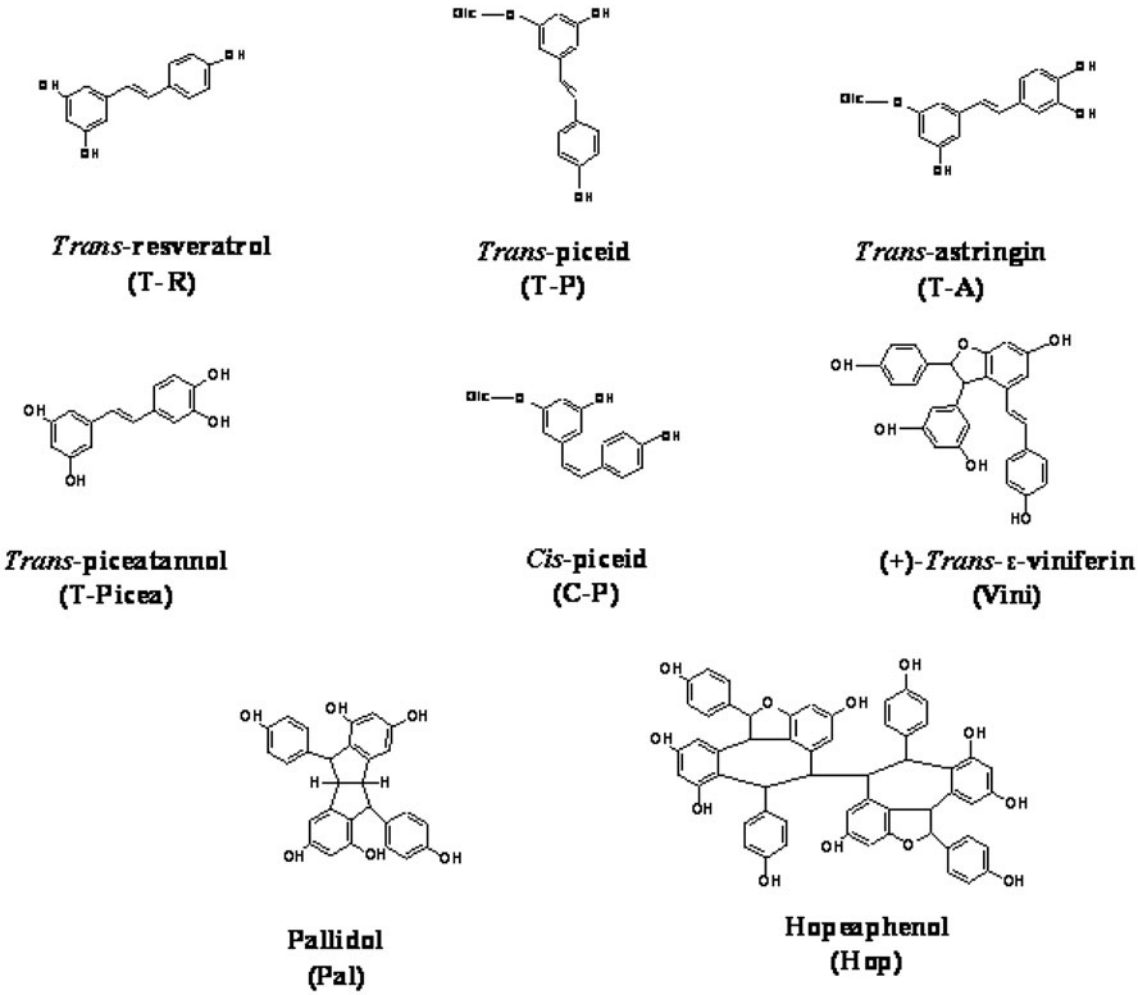


Fig. 1 Chemical structures of resveratrol and selected derivatives

presence in grapes is considered to result from the accumulation of phytoalexins on exposure to pathogens or environmental stresses such as UV exposure or water deficiency [3]. Among the different stilbenes, resveratrol has undoubtedly been the most studied compound since its identification in wine and its association with various health benefits. However, all of the stilbene derivatives show biological or therapeutic properties [4], including antioxidant and antifungal activity [3, 5–7], and chemoprevention of cancer, cardiovascular disease, aging, and neurodegeneration [8, 9].

Several analytical studies have attempted to extract, purify, or quantify these species in vines and wine [10, 11]. High-performance liquid chromatography (HPLC) has been used to analyse phenolics and stilbenes in wines [12, 13]. Coupled with UV detection, HPLC has been used for separation and the analysis of *trans* and *cis*-resveratrol (T-R and C-R), *trans* and *cis*-piceid (T-P and C-P), *trans*-astringin (T-A), δ-viniferin (δ-Vini), and ε-viniferin (ε-Vini) in grapes and wine, which had previously been fractionated [14–16]. Because of their fluorescent properties, stilbene derivatives (T-A, T-P, and C-P, T-R and C-R, and Pallidol "Pal") can be detected in red and white wines without sample preparation [17, 18]. Recently, HPLC coupled with mass spectrometry (MS) with negative electrospray ionisation has enabled detection of stilbene derivatives in directly injected wines with high sensitivity [19]. Alternative methods, for example gas chromatography or capillary electrophoresis, have also been used for separation of stilbenes [20, 21]. However, they only enabled detection of T-R.

Although HPLC is established as an easy and efficient method for stilbene separation, it still requires long experimental runs, which means large volumes of solvent and low-throughput analysis. Recently, ultra-high-pressure liquid chromatography (UHPLC) has introduced a new era in liquid chromatography [22]. This advanced method, which relies on the use of high back pressures on columns packed with <2 μm beads, enables very fast analysis with improved resolution and sensitivity. Various studies already report UHPLC analyses of phenolics or pesticides [22, 23], but so far it has not been used for the simultaneous

detection of stilbene derivatives. Beyond the substantial interest connected with their potential health benefits, high throughput analysis of stilbenes in wines is likely to provide new directions for authentication and new insights into understanding of the mechanisms of metabolite expression in grapes as a function of environmental conditions. Several authors have, indeed, shown that the resveratrol content of wines is affected by vintage effects and/or viticultural practices [3, 24].

In this study, we report the UHPLC separation of eight stilbene derivatives (Fig. 1) extracted from the stem of *Vitis vinifera*, and their quantification in Pinot noir wines from four distinct appellations in Burgundy.

Experimental

Reagents

Acetonitrile, methanol, water, and formic acid (liquid chromatography grade) were purchased from Biosolve (Valkenswaard, The Netherlands). Chemically pure standards of the stilbenes *trans*-resveratrol (T-R), *trans*-piceid (T-P), *cis*-piceid (C-P), *trans*-astringin (T-A), *trans*-piceatannol (T-Picea), (+)-*trans*-ε-viniferin (Vini), pallidol (Pal), and hopeaphenol (Hop) were purified from grape stems as described elsewhere [10]. The purity of the standard materials was measured by ^{1}H nuclear magnetic resonance (NMR) spectroscopy. Stock solutions (1000 mg L^{-1}) were prepared in methanol and kept at −20 °C in the dark. Working solutions were prepared by dilution with water.

Wine samples

Four red Burgundy wines (pinot noir, 2008 vintage) were analysed. They were obtained from the same wine producer, who followed the same winemaking process for all the wines. Therefore, the four wines differ only in the geographic location of grapes from which they were made: Clos “Les Avaux” and Clos “Les Couchereaux” are from the *Côte de Beaune*, and “Clos Saint Jacques” and “Clos Vougeot” are from the *Côte de Nuit*.

Quality-by-Design (QbD) software

DryLab 2000 plus software (Molnar-Institut, Berlin, Germany) was used to optimise temperature and elution time for separation of the eight stilbenes. UHPLC experiments were carried out at two column temperatures, 30 and 60 °C, combined with two chromatographic gradient times, 5 and 15 min. The conditions and results from these four experiments were imported to the Drylab software which helped to determine the optimum experimental conditions.

UHPLC analysis

An Acquity UPLC system (Waters, Milford, MA, USA) equipped with a model 2996 PDA detector was used for the analysis. Four columns (Table 1) were tested for the measurements: BEH C18 was purchased from Waters (Eschborn, Germany) and VisionHT C18, VisionHT C18-HL, and VisionHT C18-P from Alltech Grom (Rottenburg–Hailfingen, Germany). Under the optimised conditions, the column oven was thermostated at 60 °C and the sample system at 27 °C. The sample (10 μL) was injected via full-loop injection. We used water–acetonitrile–formic acid 100:10:0.1 (*v*/*v*) as solvent A, and acetonitrile as solvent B. The optimised system consisted in a stepwise gradient as follows: 0% B (0–1.73 min), 16% B (1.73–2.73 min), 42% B (2.73–4.12 min). This was followed by a 0.88-min equilibration period with the initial conditions before injection of the next sample. The flow rate was set to 0.8 mL min^{-1} for the BEH C18 column and 0.5 mL min^{-1} for the VisionHT C18, VisionHT C18-P and VisionHT C18-HL columns, which resulted in a maximum system pressure of 950 bar. Detection was performed at 286 nm and 305 nm, and the chromatographic characteristics were calculated by use of Waters Empower software.

NMR spectroscopy

^{1}H NMR spectra of approx. 300 μg of the stilbenes in 125 μL d6-acetone (99.95% ^{2}H; Merck, Darmstadt) were acquired in sealed 2.5-mm tubes (Bruker Match System) at 303 K with a Bruker DMX 500 NMR spectrometer (Rheinstetten, Germany), operating at a proton resonance frequency of 500.13 MHz. A 5 mm dual observe cryogenic ^{13}C/^{1}H probe head equipped with an actively shielded z-gradient coil [90-deg (^{1}H and ^{13}C):10 μs] was employed. ^{1}H NMR spectra were acquired with 90-degree excitation

Table 1 Characteristics of the columns tested

Columns	BEH C18	VisionHT C18	VisionHT C18-HL	VisionHT C18-P
Dimension (mm)	2.1×150	2.0×150	2.0×150	2.0×150
solid phase	Hybrid	Silica	Silica	Silica
Particle size (μm)	1.7	1.5	1.5	1.5
Pore size (A°)	130	100	120	100
Surface area (m2/g)	185	200	220	200
Maximum allowed pressure (Bar)	1000	830	830	830
pH range	1–12	1–10	1–10	1–10
Carbon load	18%	6%	10%	5%
End capping	Yes	Yes	Yes	No

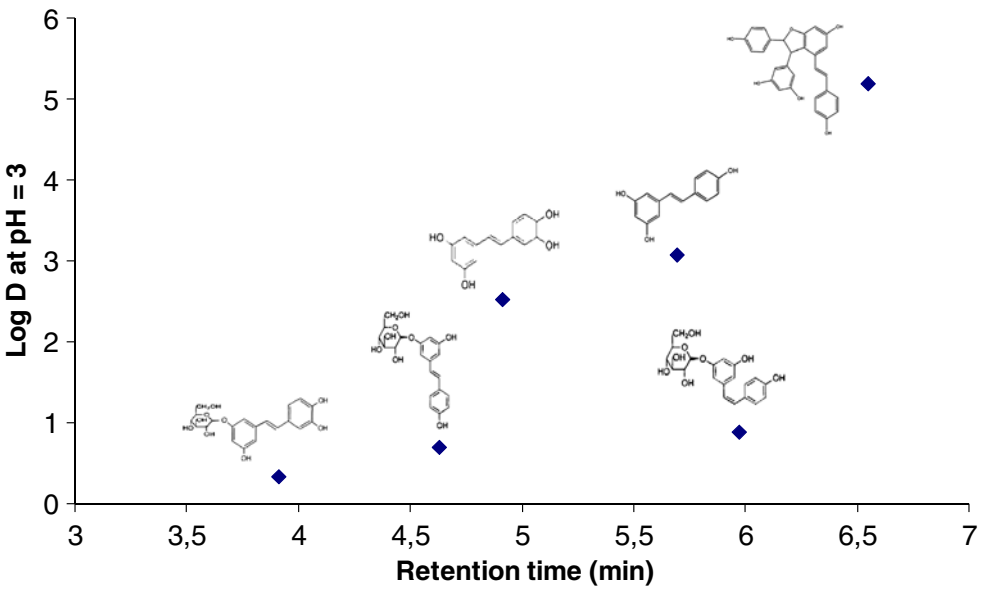


Fig. 2 Diagram showing the order of separation of the eight stilbenes on the basis of their measured retention times on the BEH C18 column, with their log *D* values at pH 3

pulses (acquisition time 5 s, relaxation delay 15 s, exponential line broadening 0.3 Hz; reference d6-acetone: 2.04 ppm). The authenticity of the samples was confirmed by analysis of spectra acquired with selected standard pulse sequences (cosygpmfqf, dipsi2etgpsi19, hsqcetgpsi, hmbcacgplpgf) with sufficient digital resolution to clearly indicate any significant impurity in the targeted stilbenes.

Results and discussion

Separation of stilbenes

The eight stilbenes were first analysed using the BEH C18 column with a linear solvent strength (LSS) gradient. When the methanol concentration was increased from 0 to 60% in acidified water (0.1% formic acid) within 7 min, only six analytes out of eight were separated. The retention behaviour of the separable stilbenes was dependent not only on their polarity but also on the degree of substitution of the phenolic rings. As can be seen from Fig. 2, the log *D* values at pH 3 (0.33–8.42) indicate that these stilbenes are hydrophobic compounds [25]. Nevertheless, the stilbenes with glucosidic moieties (T-P, C-P, and T-A) had low log*D* (0.69, 0.89, and 0.33, respectively) and were of low polarity. However, C-P was eluted at 5.97 min. This indicates that the *cis* isomer has a higher molecule dipole moment capable of interacting more strongly with the carbon material of the column by means of dispersion interactions.

In order to be able to perform the separation of the eight studied stilbenes, the previously described LSS gradient was improved. Because the two stilbenes that could not be initially detected (Pal and Hop) are actually more hydrophobic

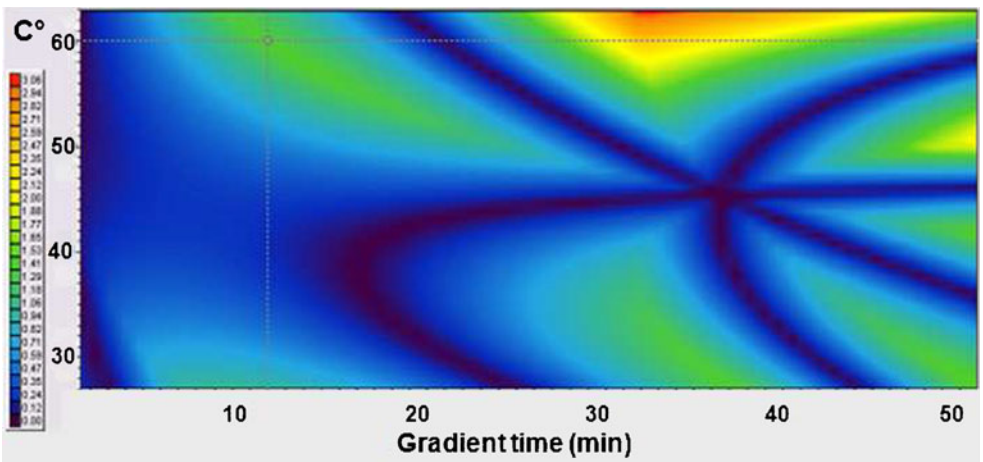


Fig. 3 The QbD map obtained after importing the chromatographic data from the four realised measurements for the conditions (50 min, 60 °C and below)

than the six others, methanol was replaced by acetonitrile. Concentration of the two stilbenes to 10 mg L^{-1} finally led to their elution within 7 min. However, to overcome the co-elution of Pal and C-P, a 15-min gradient had to be used to detect all eight stilbenes.

In order to achieve optimum separation, with sharp, symmetrical chromatographic peaks, and to reduce analysis time, the semi-empirical Drylab software was used to describe the efficiency of the column through the Quality-by-Design (QbD) process. The chromatographic characteristics (retention time, peak area) obtained for the four UHPLC measurements were imported into the software and the average resolution was visualised in a map (Fig. 3).

Colours on the map are accompanied with a left scale, which reflects the resolution of the critical pair in the chromatogram. Above 1.2–1.5, the peaks will be separated to baseline. Our map showed that the optimum separation (green zone, value of 1.41) should occur at a gradient time of 12 min and a column temperature of 60 °C. Use of these designed conditions enabled the separate detection of the stilbenes within 6 min, as shown in Fig. 4a. The stepwise gradient thus obtained and described in the "Experimental" section, was tested for the BEH C18 column for validation with real experimental conditions. The chromatogram obtained with this column is presented in Fig. 4b. Although slight differences were observed between the predicted and measured retention times, as also shown for other applications [26, 27], good agreement was obtained between the theoretical chromatogram from the DryLab software and the experimental one (Fig. 4a, b).

Choice of column

In addition to the BEH C18 column, three other columns (VisionHT C18-HL, VisionHT C18, and VisionHT C18-P) were tested for separation of the eight stilbenes. The optimised method obtained from the QbD software was applied to all four columns to study the different retention of the stilbenes on the different reversed stationary phases (for phase characteristics refer to Table 1). Here, the flow rate had to be reduced to 0.5 mL min^{-1} in order to remain below the maximum allowed pressure. The chromatograms obtained are presented in Fig. 4 and compared with the predicted chromatogram. Baseline separation for all the targeted stilbenes was achieved with these columns with the exception of VisionHT C18-P, as expected. Indeed, because the C-loading of this column was low and it was not endcapped, some of the stilbenes were not eluted in this run. To select the best column, chromatographic characteristics (optimum resolution, retention time, and separation efficiency of the stationary phase) were calculated for the eight stilbenes and are presented in Fig. 5.

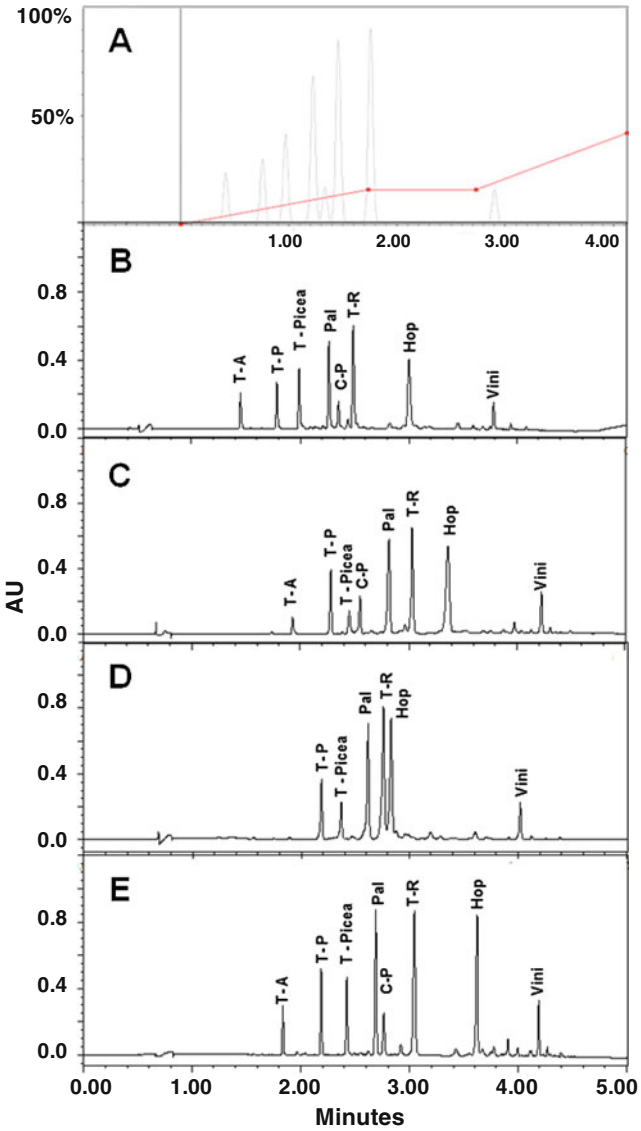


Fig. 4 Comparison of the theoretical chromatogram predicted by the DryLab software (**A**) and those obtained under real conditions with four different columns (**B**, BEH C18; **C**, VisionHT C18; **D**, VisionHT C18-P; and **E**, VisionHT C18-HL). Abbreviations: T-A, *trans*-astringin; T-P, *trans*-piceid; T-Picea, *trans*-piceatannol; Pal, pallidol; C-P, *cis*-piceid; T-R, *trans*-resveratrol; Hop, hopeaphenol; Vini, (+)-*trans*-ε-viniferin

Another chromatographic characteristic, the apparent retention factor (k^*) was taken into consideration to study the elution of the baseline-separated analytes. Figure 5b compares k^* values of the eight stilbenes for the three tested columns. We noticed significantly higher values for some of the stilbenes with the BEH C18 column; this is possibly because of the hybrid nature of the stationary phase.

Although peaks were narrow (2–6 s) as shown in Fig. 5a, significant differences between peak-widths were observed. In case of the three tested columns Pal and Hop had slightly broader peaks for all columns, which indicates

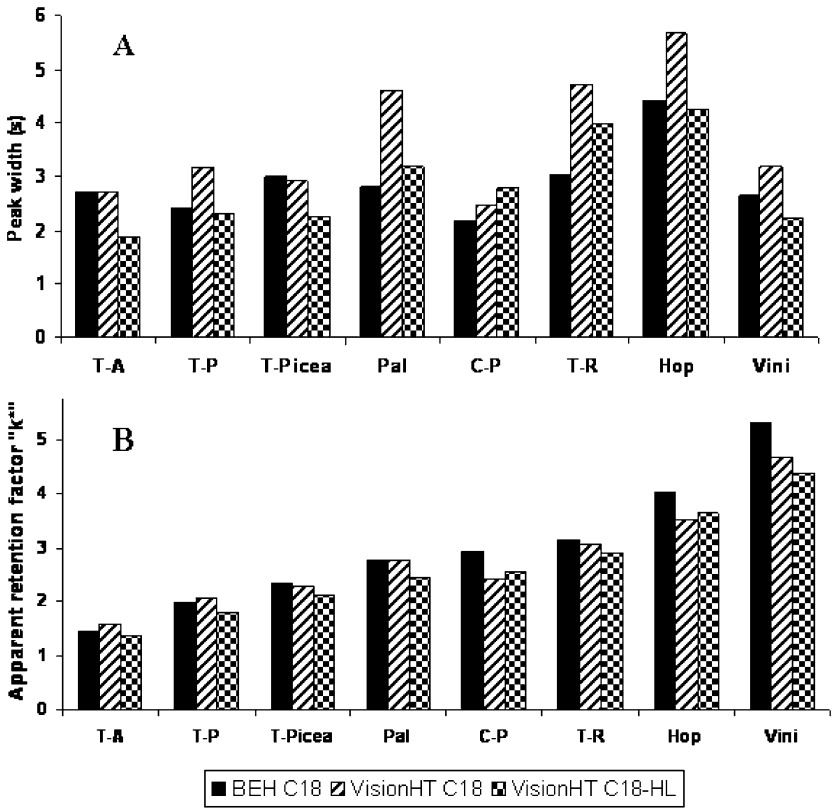


Fig. 5 Comparison of the peak-widths (**A**) and the apparent retention factor "*k**" (**B**) of the eight stilbenes for the three tested columns. Abbreviations: T-A, *trans*-astringin; T-P, *trans*-piceid; T-Picea, *trans*-piceatannol; Pal, pallidol; C-P, *cis*-piceid; T-R, *trans*-resveratrol; Hop, hopeaphenol; Vini, (+)-*trans*-ε-viniferin

the presence of partial shape selectivity [28]. The highest peak widths were obtained with the VisionHT C18 column. Moreover, a strong tailing effect was observed with this column, possibly because the lower C-loading resulted in the presence of additional interactions between the stationary phase and the analytes. Smaller values of peak widths were noticed for the BEH C18 and VisionHT C18-HL columns, and differences between peak-widths of the separated stilbenes with these two columns also seemed to be smaller (Fig. 5a). From Fig. 5a, we conclude that under our experimental conditions the VisionHT C18 is the best column for efficient separation of these stilbenes.

Both of the columns finally selected before the validation step (BEH C18 and VisionHT C18-HL) were used to analyse four red wines (pinot noir grape variety) from Burgundy to study the matrix effect of the wine on the retention of the stilbenes, as shown in Table 2. As deduced from Table 2 slight differences between retention times of standards and those in wines are noticed with the BEH C18 column (decrease of approx. 0.01 min). In contrast, remarkable agreement and good repeatability were observed for the VisionHT C18-HL column. Moreover, the VisionHT C18-HL column enabled the observation of Hop in the Avaux wine, whereas it was not reliably detectable with the BEH C18 column. Therefore, on the basis of these comparisons, the VisionHT C18-HL column was selected as the best for high-efficiency separation of stilbenes from wines.

Determination of performance characteristics

The optimised method for the eight stilbenes was validated with the VisionHT C18-HL column in which no variance of retention times was observed. Identification data, for example retention times, peak widths, resolution, and quantification data (linear range, limit of detection "LOD", limit of quantification "LOQ", etc.), are gathered in Table 3. Each compound was detected at its UV absorption maximum, i.e. 286 nm for Pal, C-P, and Hop, and 305 nm for T-A, T-P, T-Picea, T-R, and Vini, and the UV–visible spectra were always investigated to ascertain the purity of the analyte peaks.

Measurements were repeated three times for each concentration and the relative standard deviations (RSDs) of retention times were between 3 and 6%. The peak widths obtained were independent of the concentrations and did not vary within repeated measurements. Linear ranges were dependent on the analytes; the lowest concentration was 0.005 mg L^{-1} for five compounds (Pal, Vini, and Hop were detectable from 0.01, 0.01, and 0.05 mg L^{-1}, respectively) and the upper limit was 100 mg L^{-1} for Pal, C-P, and Hop. On the basis of these linear ranges, the calibration curve for

Table 2 Investigation of the matrix effect. Comparison of retention times of the analytes in standard solutions and in different wines using the BEH C18 and VisionHT C18-HL columns

	Retention time [min]									
	Applying BEHC18 column					Applying VisionHT C18-HL column				
	standard	Avaux	Vougeot	Saint Jacques	Couchereaux	standard	Avaux	Vougeot	Saint Jacques	Couchereaux
T-A	1.456					1.836				
T-P	1.788	1.778	1.777		1.791	2.188	2.187	2.186		2.186
T-Picea	1.993					2.422				
Pal	2.266					2.688				
C-P	2.355	2.347	2.348	2.353	2.353	2.761	2.762	2.761	2.763	2.762
T-R	2.489	2.479	2.479	2.487	2.486	3.041	3.043	3.042	3.046	3.044
Hop	3.003		2.945	2.950	2.950	3.620	3.615	3.615	3.617	3.616
Vini	3.790	3.775	3.780	3.781	3.782	4.193	4.196	4.197	4.197	4.197

T-A, *trans*-astringin; T-P, *trans*-piceid; T-Picea, *trans*-piceatannol; Pal, pallidol; C-P, *cis*-piceid; T-R, *trans*-resveratrol; Hop, hopeaphenol; Vini, (+)-*trans*-ε-viniferin

each compound was determined and used for their quantification in the red wines studied. As deduced from Table 3, excellent determination coefficients (R^2) were calculated for these curves. The precision of the optimised method was tested for all the analytes studied; the accuracy ranged between 85% and 100% in the low ppm range.

The purity of the analytes was evaluated and we noticed impurities in the obtained chromatograms (Fig. 4). Thus ^{1}H NMR spectra were recorded in order to determine the purity of these stilbenes (Electronic Supplementary Material Table S1). These purities were taken into consideration to calculate stilbene concentrations in the wines studied. The purity of the stilbenes was derived by integration of the 10–6 ppm region of ^{1}H NMR spectra in order to quantify the ratio of $H_{ar\text{-}C}$ protons to the overall proton signals (Electronic Supplementary Material Fig. S1).

Wine analysis

Figure 6 shows chromatograms obtained from the four wines with the VisionHT C18-HL column and with detection at 286 and 305 nm for comparison. For each wine, several peaks were detected, which can be distinguished as a function of the absorption wavelength (Fig. 6), in agreement with the intrinsic complexity of the wine matrix. Moreover, and most interestingly, we clearly observed that for a given wavelength, relative peak intensities of the analytes vary with wines, demonstrating that wines made from grapes from vineyards very close to each other can have very distinct stilbene profiles. Figure 7 indeed shows that five stilbene derivatives (T-P, T-R, C-P, Vini, and Hop) of the eight considered in this study could be quantified in all four wines. T-R and C-P were present in

Table 3 Characteristics selected for validation of the optimised method with the VisionHT C18-HL column

	T-A	T-P	T-Picea	Pal	C-P	T-R	Hop	Vini
wavelength [nm]	305	305	305	286	286	305	286	305
retention time [min]	1.836	2.188	2.422	2.688	2.761	3.041	3.62	4.193
TR within day RSD [%]	0.1	0.07	0.19	0.08	0.07	0.4	0.13	0.04
Resolution	9.6	5.62	5.8	1.48	4.67	7.9	9.97	
peak width [s]	2	2.4	2.6	2.9	3	4.2	4.6	2.3
working range [mg/L]	0.005-50	0.005-50	0.005-10	0.01-100	0.005-100	0.005-50	0.05-100	0.01-50
R^2	0.999	0.997	0.997	0.999	0.999	0.991	0.999	0.995
RSD of peak area at 1 mg/L [%]	3.79	3.06	4.59	6.56	5.48	5.47	5.37	3.83
LOD [mg/L]	0.005	0.005	0.005	0.01	0.005	0.005	0.05	0.01
Accuracy (%)	84	100	99	92	87	77	78	80

T-A, *trans*-astringin; T-P, *trans*-piceid; T-Picea, *trans*-piceatannol; Pal, pallidol; C-P, *cis*-piceid; T-R, *trans*-resveratrol; Hop, hopeaphenol; Vini, (+)-*trans*-ε-viniferin

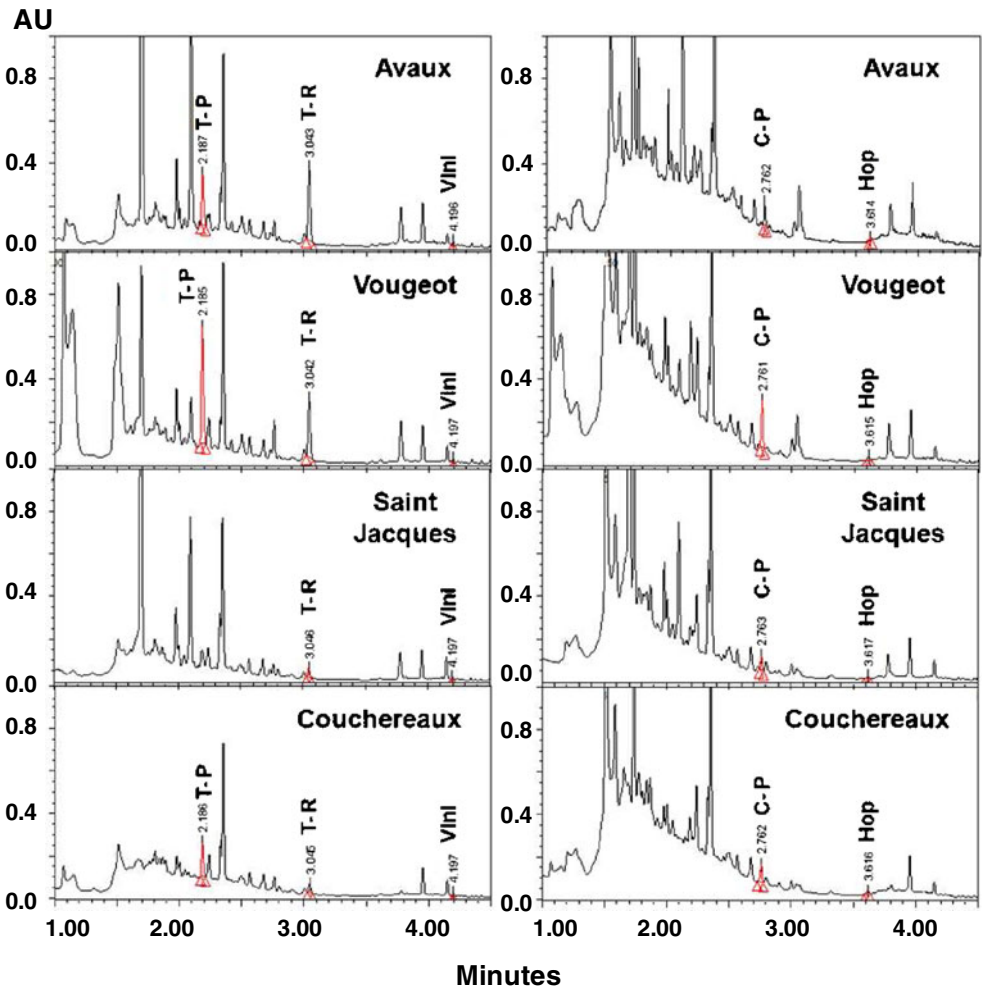


Fig. 6 Wine chromatograms obtained with the VisionHT C18-HL column, with detection at 286 nm (*left*) and 305 nm (*right*), the absorption maxima of the compounds. Abbreviations: T-P, *trans*-piceid; C-P, *cis*-piceid; T-R, *trans*-resveratrol; Hop, hopeaphenol; Vini, (+)-*trans*-ε-viniferin

all of the wines with concentrations ranging from 0.53 mg L^{-1} to 5.08 mg L^{-1} for T-R with the highest concentration in the *Clos des Avaux* wine, and from 2.40 mg L^{-1} to 8.87 mg L^{-1} for C-P with the highest concentration in the *Clos Vougeot* wine. For this same wine, piceids clearly seem to be the major forms with the *trans* isomer peaking at a concentration of up to 14.27 mg L^{-1}, whereas the latter is absent from the *Clos Saint Jacques* wine and the *cis* isomer concentration is nearly a third of that of the *Clos Vougeot* wine. Similarly, it is worth of mentioning that the *Clos des Avaux* and the *Clos des Couchereaux* wines originating from very close vineyards (distance less than 500 m) contain approximately similar concentrations of the piceids, vini, and Hop. They can, nevertheless, be clearly distinguished on the basis of their T-R content which is approximately six times higher in the former than in the latter (Fig. 7). As a whole, the different concentrations measured here agree with results already reported for wines from several geographical origins, for which levels of T-P were between 5 and 25 mg L^{-1}, and levels of C-P were lower than 10 mg L^{-1} [23], whereas concentrations for T-R can vary between 1 and 34 mg L^{-1} [29]. Although three stilbene derivatives (T-A, T-Picea, and Pal) could not be detected in these red wines, to the best of our knowledge, Vini and Hop were determined in Burgundy wines for the first time. Application of this method to real wines indicated that simultaneous quantification of stilbene derivatives enabled pinpointing of stress-related metabologeographic expressions [30] in wines at a spatial resolution as low as the *climat* scale of Burgundy

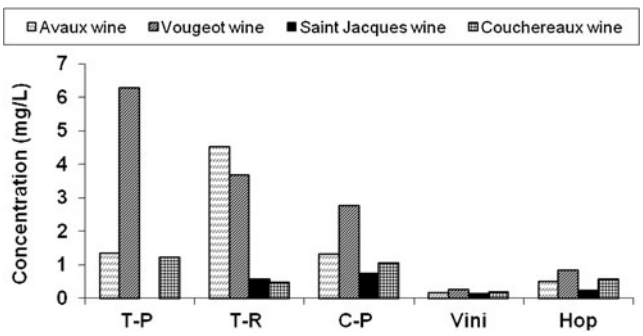


Fig. 7 Concentration of the identified stilbenes in wines (mg L^{-1}). Abbreviations: T-P, *trans*-piceid; C-P, *cis*-piceid; T-R, *trans*-resveratrol; Hop, hopeaphenol; Vini, (+)-*trans*-ε-viniferin

appellations, thus emphasising possible *terroir* effects resulting from interplays between several environmental factors such as soil microbiology, UV exposure, or vine status [31].

Conclusion

In this work, a useful and fast method for separation of stilbenes was optimised by use of Quality-by-Design software, validated by means of a targeted UHPLC approach, and used for the direct analysis of wines after selection of the VisionHT C18-HL column as the best stationary phase. It could be concluded that UHPLC is an attractive alternative to traditional methods for direct determination of stilbenes in wines. The results obtained indicate that this method is very sensitive (5 ppb without preconcentration) and repeatable (4–6%) and has no matrix effect. The validated method enabled investigation of possible *terroir* effects resulting from interplays between several environmental factors, for example soil microbiology, UV exposure, or vine status.

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