Separation of polyphenols by HILIC methods with diode array detection, charged aerosol detection and mass spectrometry: application to grapevine extracts rich in stilbenoids

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Abstract

The characterization of plant extracts is usually accomplished by reverse-phase liquid chromatography, but the development of new complementary approaches, such as HILIC, offers an orthogonal method. In this study, five HILIC stationary phases were evaluated to assess their ability to retain polyphenols. They were selected to cover the main different HILIC mechanisms: bare silica; silica with ethylene bridge; neutral amide; amino; zwitterionic. A total of 31 polyphenol standards were used for the screening, including 9 stilbenes, 8 flavonoids, 6 anthocyanins, and 8 phenolic acids. Three different detections were tested: diode array detector, charged aerosol detector and mass spectrometry.

Results indicated that silica supports were not suitable for retaining polyphenols, with no or low retention observed except for anthocyanins. The effectiveness of stationary phases in retention of phenolics following the order related to increased retention: zwitterionic, amide, and amino.

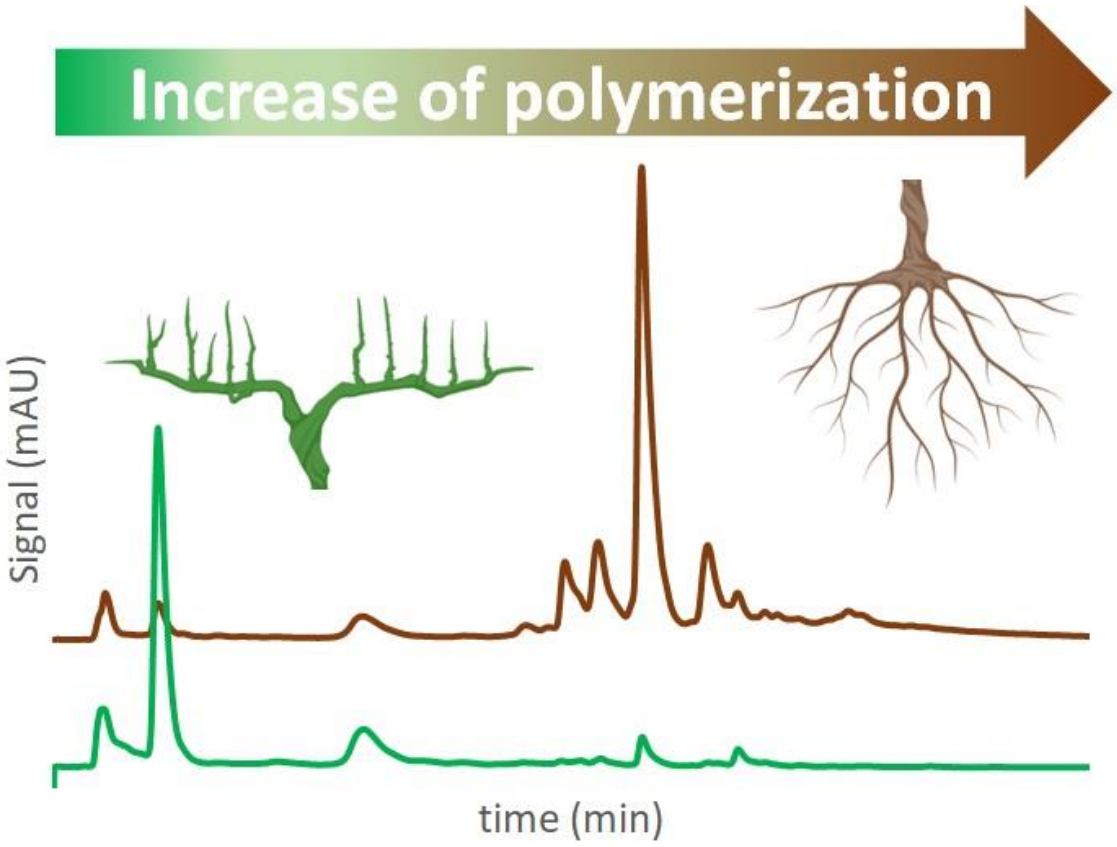
The choice of mobile phase also influenced retention. Mobile phases containing TFA as pH modifier limited retention, while formic acid was found to be more effective for polyphenol retention. Ammonium buffers also improved retention but often compromised peak shape. pH changes mainly impacted ionizable compounds, such as phenolic acids, by increasing their retention when they were ionized.

DAD was wellsuited for detecting polyphenols that possess aromatic rings, though peak wavelengths depend on the structures of the polyphenols. CAD, while less sensitive than DAD and MS, provided an almost similar response for structurally related compounds, even with gradient elution. MS was the preferred detector for quantification when resolution between compounds was challenging, as it is often the case with natural extracts.

The study successfully demonstrated that best HILIC conditions were obtained using an amino stationary phase composed of a polyethylenimine and formic acid-based mobile phase. These conditions were successfully applied to the analysis of stilbenoid-rich extracts from different parts of the vine. The elution order of stilbenoids followed the degree of polymerization. With CAD, the chromatographic profile was more representative of sample composition. It was demonstrated for the first time the interest of a combination of HILIC and CAD for analyzing stilbenes, offering a complementary approach to the classic RP analysis.

Keywords

HILIC, CAD, Stilbene, polyethylenimine, *Vitis vinifera*



HILIC STILBENOIDS

Introduction

Determining the phenolic composition of plant products is a major interest due to the bioactive roles of these compounds in plants and their health benefits for humans [1,2]. Polyphenols play a critical role in the adaptation of plants to their environment [3] and represent a potential alternative to conventional pesticides for plant protection [4]. Additionally, they are active pharmaceuticals as they represent the most abundant antioxidants and micronutrients in our diet, and evidence for their role in neuroprotective, cardioprotective, and chemopreventive actions is increasingly emerging [5,6]. Among them, stilbenoids have attracted a particular attention considering their wide range of biological activities, such as antioxidant and anti-inflammatory properties, as well as their therapeutic potential in cancer and neurodegenerative diseases [7,8]. Stilbenoids are found in many plant species with notable concentrations in grapevine. Shoots, trunks, and roots are recognized for their richness in polyphenols, with more than 150 phenolic compounds reported, primarily stilbenoids [9] and flavonoids [10]. They present different degrees of polymerization depending on the plant part. Utilizing these grapevine by-products for producing bioactive stilbene-rich extracts is of considerable interest [11].

Plant extracts contain complex mixtures of polyphenols with diverse structures and variable number of phenol rings, ranging from monomers to polymers. Many structures have yet to be identified, joined by the lack of commercial standards available for identifying them, their complete characterization is complex and time-consuming, involving several critical steps: sample treatment, extraction, cleaning, and advanced analytical techniques [1,12]. Concerning the last step, more or less sophisticated techniques can be considered. While high-performance reverse liquid chromatography (RP-HPLC) is commonly used, it often fails short to completely characterize phenolic composition. Hydrophilic interaction liquid chromatography (HILIC) mode can afford complementary separation to that obtained with RP-HPLC [13]. It constitutes a powerful technique for separating compounds of biological interest from various matrices, particularly in the fields of -omics sciences, pharmaceutical, environmental, and food analysis [14]. Combining these two modes of retention has been demonstrated to be attractive for two-dimensional analysis of plant extracts [15,16].

One advantage of HILIC is the diversity of stationary phases leading to multimodal mechanisms of retention [11,12]. The main retention mechanism in HILIC involves partitioning of the compounds between a water layer immobilized at the surface of the stationary phase and the organic mobile phase. However, to understand HILIC retention, other secondary interactions, playing important roles, such as ionic interactions, adsorption interactions, and dipole-dipole interactions, must be considered.

HILIC methods have been demonstrated useful for natural products analysis [14] for many different types of compounds, including carbohydrates, phospholipids, amino acids, peptides, and proteins. Due to their aromatic rings and hydroxyl groups, polyphenols are well-suited for HILIC [17–21]. Polyphenols can be divided into flavonoids, such as flavonols, flavanols, flavones, and non-flavonoids (as phenolic acids and stilbenoids). For instance, the challenging separation of proanthocyanidins (condensed tannins), oligomers and polymers of the flavanols, has been successfully obtained using diol stationary phase [15,21–23]. They have been separated according to molecular weight increase, and hence the increase of

polymerization degree. Same degree of polymerization was visualized as multiple peaks indicating the potential of this technique for isomeric discrimination. However, other flavonoids, such as flavonols or dihydrochalcones, and non-flavonoids, such as phenolic acids, were not retained. A study was dedicated to flavonols using a zwitterionic stationary phase [19]. pH value, nature of the organic solvent, salt concentration, and temperature of the mobile phases were investigated. Among these parameters, the use of methanol showed the highest impact for analysis of these molecules helping elution of highly retained molecules. Correlation between the log of retention factor and the number of hydroxyl groups was established which confirmed a HILIC mechanism related to the increase of polarity. Similarly, zwitterionic material has shown also a potential interest for the analysis of isomeric hydroxybenzoic acids [24], where the negatively charged stationary phase played the main role in a mechanism of electrostatic repulsion. HILIC was also successfully utilized for the analysis of anthocyanins using an amide functionalized stationary phase with mobile phase containing 0.4% of TFA [20]. Such acidic mobile phase was essential for ensuring to obtain the flavilium cationic species needed for the improvement of peak shapes. The authors obtained a predictable retention behavior regarding the degree of glycosylation of anthocyanins and their retention times, which was correlated with the increase of the hydrophilic character.

HILIC can be associated with various detectors such as diode array detection (DAD), charged aerosol detector (CAD) or mass spectrometry (MS). DAD is a common detection mode for polyphenols and provides characteristic UV/visible spectra useful for their identification [25]. CAD was less used but promising to achieve the detection and quantification of polyphenols from different agricultural by-products [12]. Its advantage is to provide similar response for related compounds which contributes to quantification even when the standard is missing. MS with different ionization sources has been frequently employed to carry out polyphenol analysis [12]. Electrospray ionization (ESI) negative mode is the most common source due to best sensitivity. The use of MS/MS can be useful for identification and quantification purposes. In HILIC, due to the high percentage content of organic solvent constituting the mobile phases, improved detection can be expected with detectors involving an evaporation step in their process such as CAD [14,26] or ESI/MS [27].

In this study, we explored the potential of HILIC to analyze polyphenols, with a particular focus on stilbenoids, which were to our knowledge not studied in HILIC. We first started with a systematic study of the retention and peak shape of different polyphenols with five mobile phases and five different columns. 31 phenolic molecules were selected representative of different classes: 9 stilbenoids, 8 flavonoids, 6 anthocyanins, 8 phenolic acids (Table S1). We selected columns with different retention mechanisms in order to broadly scan the potential of HILIC. Screening of mobile phases was performed at different pHs, with different ions compatible with various detections. Then, with the most promising combination of mobile and stationary phases, a comparison of different detections was performed for stilbenoids. DAD, CAD and MS were tested for their qualitative and quantitative responses. Finally, we presented an application for bioactive stilbenoid rich extracts obtained from grapevine by-products.

Material and Methods

Chemicals and reagents

Acetonitrile 99.9%, ethanol 96%, formic acid, trifluoroacetic acid, and ammonium formate were purchased from VWR chemicals (France) and were all LC/MS grade. HPLC grade water (minimum resistivity of 18.2 MΩ) was produced in house by ELGA Millipore system (France). Resveratrol-3-*O*-glucuronide (R3G), resveratrol-4'-*O*-glucuronide (R4'G), caffeic acid, gallic acid, gentisic acid, vanillic acid, protocatechuic acid, phloroglucinic, *p*-coumaric, *o*-coumaric, quercetin and quercetin 3-rutinoside were purchased from Sigma-Aldrich (France).
Some stilbenoids had been previously purified from various vine extracts: piceatannol, ε-viniferin, δ-viniferin, pallidol, ampelopsin, myabenol C, α-viniferin, hopeaphenol, isohopeaphenol, vitisin B and A.
The other phenolic standards, quercetin 3-glucoside, and procyanidins: catechin, catechins B2, and C1; stilbenes (*trans*-piceid and *trans*-resveratrol) cyanidin, cyanidin-3-glucoside, malvidin, malvidin-3-glucoside, delphinidin-3-glucoside, were purchased from Extrasynthese (France).

Plant materials

The cultivar / rootstock combinations were *Vitis vinifera* cv. Cabernet sauvignon X 4010 Castel, planted in 1984 in Villenave d'Ornon (region of Bordeaux, France). When the plants were uprooting, they showed no symptoms of wood diseases. The grapevine plants were cut into seven parts as follows: canes, arms, upper trunk, grafting zone, lower trunk, roots, and rootlets. Each sample was ground, dried for two weeks in an oven at 35°C and then stored in a dry environment until extraction [11].

Instrumentation

HPLC-DAD/CAD system

The UltiMate® 3000 HPLC system Dionex (Thermo Scientific, USA) was composed of a pump LPG-3400SD: Quaternary Low Pressure Gradient Pump with integrated solvent degasser; a thermostated auto-sampler WPS-3000SL: thermostated at 5°C and programed at 1 µL-injection volume; a thermostated column compartment with two 6-position, 7-port valves; a Diode Array Detector (DAD) 3000 RS with 2.5 µL and 7 mm flow cell and a Charged Aerosol Detector (CAD) Corona Veo RS (N_2 set at 57.6 PSI and evaporation tube at 35°C); a nitrogen generator Olympia N70-1 (Claind, Italy). The complete system was controlled by 7.2.10 Chromeleon® Chromatography Management Software (Thermo Scientific, USA). Five HILIC columns were chosen as listed in Table 1.

Table 1. Characteristic of the HILIC Columns

Column name	**Dimension (50 × 2.1 mm)**	**HILIC category**	**Chemical**
Kinetex HILIC	1.7 µm, 100 Å	Silica	Silica
Acquity UPLC® BEH HILIC	1.7 µm, 130 Å	Silica	Ethylene Bridged Hybrid Silica
Acquity UPLC® Glycoprotein BEH amide	1.7 µm, 300 Å	Amide	Ethylene Bridged Hybrid Amide
Syncronis™ HILIC	1.7 µm, 100 Å	*Zwitterionic*	*Sulfobetaine*
HILIC Hypersil GOLD™ PEI	1.9 µm, 175 Å	Amino	Polyethyleneimine

UHPLC-HRMS system for the phenolic compound quantification

Chromatographic separation was achieved using a Vanquish Flex system (Thermo Fisher Scientific, Les Ulis, France) consisting in a binary pump, an autosampler and a heated column compartment. MS detection was performed using a Q-Exactive mass spectrometer equipped with a heated electrospray ionization (HESI II) probe (Thermo Fisher Scientific, Les Ulis, France). The mass analyzer was calibrated each week using Pierce® ESI negative and positive ion Calibration Solutions (Thermo Fisher Scientific). The source parameters were sheath gas flow rate 45 arbitrary units (a.u.); auxiliary gas flow rate 15 a.u.; spray voltage 3.7 kV; capillary temperature 320 °C; S lens RF level 100 a.u. and probe heater temperature 250 °C. Full MS scan data were acquired in negative ion mode within the range of *m/z* 70-1050 at a resolution of 70,000 FWHM. The automatic gain control target was set at 3.10^6 ions, with a maximum injection time of 100 ms.

Chromatographic conditions

Column and mobile phase screening

The mobile phases prepared for the screening step contained various pH modifiers (Table 2). The pH values correspond to a measurement performed in water containing the equivalent amount of pH modifier. The gradient conditions for the screening step used binary mobile phases consisted of (A) ACN/H_2O (95:5, v/v) and (B) ACN/H_2O (50:50, v/v) where pH modifiers were added as described in Table 2. The flow rate was set at 0.6 mL.min^{-1}. After an equilibration step of 10 min with 100% of (A) before injection, the gradient was from 100% (A) to 100% (B) in 15 min, with a slope of 3%/min. The mobile phases containing ammonium formate were sonicated 15 min in an ultrasound bath and filtered with 0.22 µm nylon membrane before use. All columns were used at 25°C.

Table 2. Mobile phase composition for the column screening

Mobile phase	Concentration (mM)	pH
TFA 0.085% v/v	11	2.1
Formic acid 1.0% v/v	270	2.1
Ammonium formate buffer	5	3.0
Ammonium formate	5	6.5

Extract analysis

The mobile phase used for the extract analysis with DAD and CAD is an elution gradient composed of mobile phases (A) ACN/aqueous phase with 0.1% formic acid (95:5, v/v) and (B) ACN/ aqueous phase with 0.1% formic acid (70:30, v/v). After an equilibration step of 5 min with 100% of A, the chromatographic conditions started by a 1 min - plateau of (A) and then a gradient from 100% of (A) to 100% (B) in 9 min, follow by a 2 min - plateau at 100% of (B) before the return at 100% of (A) for the next injection. Column oven was set at 25°C. Flow rate was set at 0.6 ml.min^{-1}. A plateau was added before the gradient program to help for method transfer. Percentage range was reduced to focus on stilbene analysis.

The mobile phase used for the extract analysis with MS, is an elution gradient composed of a mobile phases (A) ACN with 0.1% formic acid and (B) water with 0.1% formic acid. After an equilibration step of 6 min with 97% of A, the chromatographic conditions started by a 1.65 min - plateau with 97% of A and then a gradient from 97% of A to 75% of A in 9 min, followed by a 2 min - plateau 75% of A before the return at 97% of A for the next injection. The column oven was set at 25°C. Flow rate was set at 0.6 $mL.min^{-1}$. The difference in the gradient program took into account the difference in dwell volume.

Standard and sample preparation

The volume of injection was 1 µL. All stock standard solutions were in methanol/water (50:50, v/v) at 1 $mg.mL^{-1}$. Then dilutions were performed in acetonitrile. A mixture of ethanol and water (85:15, v/v) was used for the extraction. 50 mg for each sample of the different parts of the grapevine (Fig. 1) were weighed in Eppendorf tubes. 500 µL of the ethanol/water mixture were added. They were sonicated for 15 min in an ultrasonic bath at room temperature and centrifuged at 50,000 g at 7°C for 5 min. For each sample of vine part, the extraction was performed in triplicate.

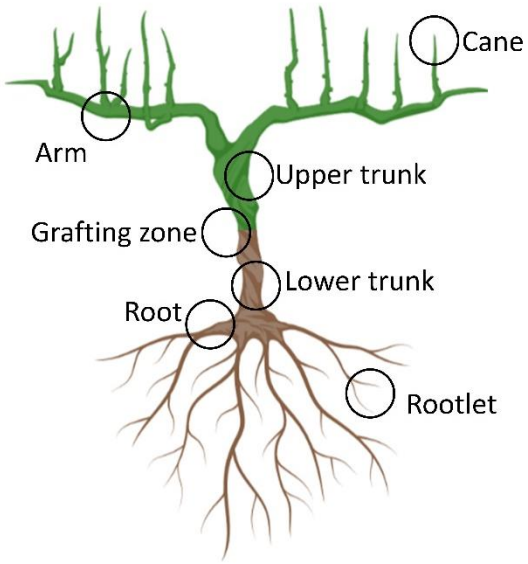


Figure 1. Analyzed grapevine parts.

Results and discussion

Screening of stationary and mobile phases for polyphenol retention

Screening of different stationary and mobile phases was performed with a HPLC-DAD/CAD using a rapid gradient (slope 3%/min) with a large range of percentage, from 5 to 50 % water to ensure the elution of all the compounds. 31 pure phenolic standards were considered: phenolic acids (hydroxybenzoic and hydroxycinnamic acids), stilbenoids and flavonoids (anthocyanins, flavonols, flavanols).

In HILIC, the choice of stationary phase significantly influences selectivity and retention. Therefore, selecting an appropriate stationary phase is crucial for developing an effective HILIC method. The selection of the stationary phase material remains largely a trial-and-error task. The main categories are columns with silica material, which is the typical non-functionalized support, and functionalized HILIC with amide, amino, diol, zwitterionic, etc. [28–30]. In this study, five different HILIC stationary phases (Table 1) were tested, presenting different graftings and covering the main types of HILIC material [31–33]. The selection included: i) a conventional material as an unfunctionalized support composed of bare silica; ii) a modified silica with ethylene bridge (BEH) which modifies the number of polar and anion exchange sites; iii) a neutral material like amide; iv) a cation exchange material with an amino group, and v) a zwitterionic material.

The retention mechanism also depends on the mobile phase conditions. Four mobile phase conditions were tested with pH between 2.0 and 6.5 (Table 2) in the common pH stability range of the used stationary phases.

For each combination, two main parameters were considered: the retention (measured by the retention time (tR) for each polyphenolic compound) and the peak shape (measured as the half-width (w50%) of the peaks). For cases where a compound produced several peaks (this happened especially for the anthocyanins), the time range of the half-width of the peak began from the first peak at its half-height and ended to the half-height of the end of the last peak.

Figure 2A shows an overview of the retention of polyphenol standards obtained with these different conditions. pH had a minimal impact on the retention. The ionization state of most of these compounds was not influenced by the change in pH in the explored domain. However, the surface of the stationary phase such as silica or amino could be modified. The increase in pH seemed to slightly influence the retention with a slight increase in the overall retention. However, pH could not be the only factor to consider but also typology of the acid used. Replacing the formic acid mobile phase at pH 2.1 with TFA at pH 2.1 significantly decreased retention compared to all other mobile phases, probably due to its effect on decreasing the thickness of the water layer [31].

A global classification of the stationary phases could be proposed according to the polyphenol retention. Except for TFA mobile phase, which led to poor global retention, the amino material provided the strongest global retention, followed by the amide stationary phase and then zwitterionic. Silica materials led to the lowest retention, with a very slight advantage of BEH compared to bare silica which is clearly not a candidate in the tested conditions. This ranking may be representative of the thickness of the water-rich layer forms at the stationary phase surface, available for analyte partitioning.

For the shape of the peak (Fig. 2B), the higher the retention, the wider was the peak. So independently of the nature of the compounds, acid conditions provided by the formic acid seemed to offer the best compromise between retention and peak shape.

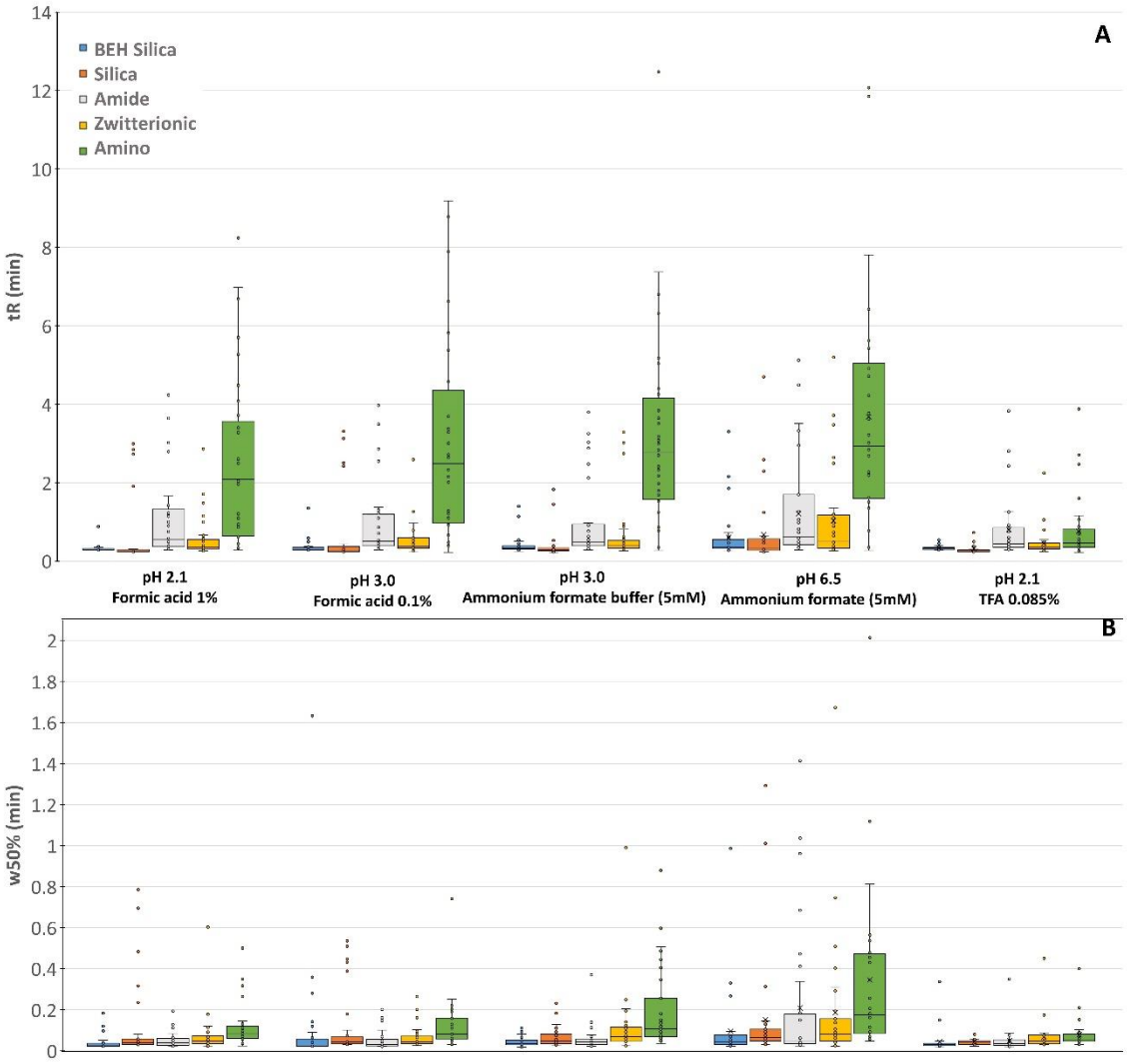


Figure 2. Screening results of stationary and mobile phases on retention time (tR) (A) and 50% peak width represents the peak width at 50% maximum height (w50%) (B) of all polyphenols.

To further explore these results, looking at different types of molecules allowed us to better describe this overall behavior. Figures 3 through 6 illustrate the retention behavior of stilbenoids (Fig. 3), flavanols and flavonols (Fig. 4), anthocyanins (Fig. 5) and phenolic acids (Fig. 6). Since TFA drastically decreased the retention, the corresponding results have not been included, but are reported in Fig S1. Missing data indicated that no elution was observed, due to high retention or significant peak tailing, which hindered peak observation.

Stilbenoid analysis in HILIC

For stilbenoids (Fig. 3A), the retention behavior was similar to the general trends observed, the retention increased with stationary phases following this order: zwitterionic, amide and

amino stationary phases. The amino phase provided the greatest retention compared to amide phase, while the remaining stationary phases offered either limited or no retention. Resveratrol appeared as the least retained compound, while glucuronides with their additional saccharide function and carboxylic acid were the most polar and therefore most retained compounds. Increasing the pH did not affect the retention of most stilbenoids which were not ionizable in the selected pH range of the tested mobile phases, except for glucuronides. R3G and R4'G have near pKa values of 2.79 [34] and 2.78 [35], respectively. At pH 6.5, glucuronides may become ionized, thus the molecules became polar and also could be involved in ion exchange with the amino material or ion pairing with ammonium ions. The addition of a sugar in stilbenoids also resulted in an increase of retention. Retention of piceid (resveratrol 3-glucoside) was found to be higher than resveratrol. ε-Viniferin and δ-viniferin are dimers, more retained than resveratrol. Then trimer α-viniferin eluted after the dimers, followed by tetramer vitisin B. The elution was thus mainly determined by the polymerization degree. With TFA (Fig. S1), even if their retention was slightly higher than other compounds, due to a noticeable increase of polarity such as piceid, TFA still limited the retention. The polar acid function was greatly involved in the retention whatever the nature of the stationary phases.

Peak broadening (Fig. 3B) was generally acceptable (< 0.1 min) except for glucuronides, where the increase of pH was detrimental due to their ionization which induced different retention mechanisms.

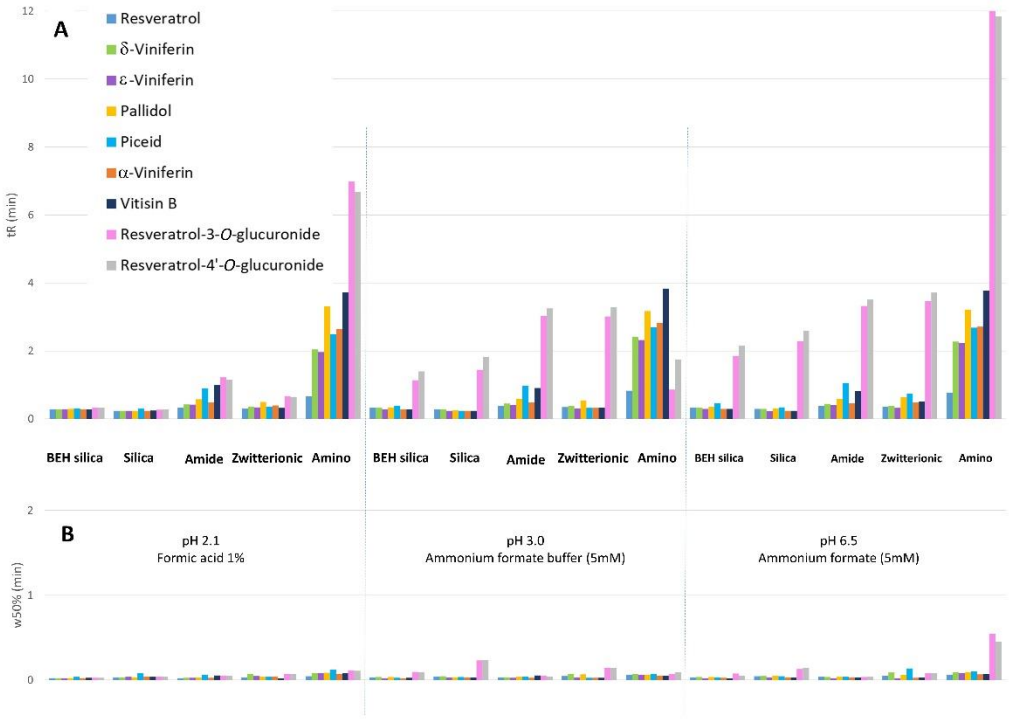


Figure 3. Screening results of the stationary and mobile phases on the retention time (tR) (A) and peak width 50% (w50%) (B) of stilbenes.

Flavonol and flavanol analysis in HILIC

For flavonols (Fig. 4A) and flavanols (Fig. 4C), the retention increased with stationary phases following this order: zwitterionic, amide and amino stationary phases. The use of mobile phases containing TFA always induced less retention but compared to the other compounds tested, retention was observed for amide and amino materials with comparable retention magnitude, the zwitterionic column appeared less retentive (Fig. S1).
For flavonols, myricetin was always more retained than taxifolin due to an additional hydroxyl group, which increased its polarity. The increasing order of elution was quercetin, quercetin-3-glucoside and quercetin-3-rutoside, which follows their increasing polarities. As previously reported, HILIC has been well established for sugar separation, whether as oligomer or polymer [19,36]. Between quercetin and taxifolin, which are isomers, a slight difference in retention appeared with amino material.

The order of elution of flavanols remained the same for all tested mobile phases, correlated with the degree of polymerization (Fig. 4C). Their retention appeared similar with amino support at the different pH values (except with mobile phase containing TFA which slightly decreased the retention). Proanthocyanins eluted depending on their degree of polymerization with this column (order of retention: catechin (monomer) < B2 (dimer) < C1 (trimer)). Their retention was greater than for stilbenoids. Using amide and zwitterionic supports, a huge decrease of retention was obtained at pH 6.5. In the literature, separation of procyanidins was studied mainly with amide [37] and zwitterionic [19] columns, where the correlation between retention and degree of polymerization was also demonstrated.
While tested mobile phases had a weak impact on retention, a huge impact on peak shape was observed (Fig. 4B and 4D). At pH above 2.1, peak broadening occurred, which complicated the measurement of retention times and peak widths, leading to less accurate or missing values. Therefore, raising the pH was clearly not a viable option. This was the first time that an amino column was used for these compounds, resulting in the highest retention but also the broadest peaks under the more favorable mobile phase conditions.

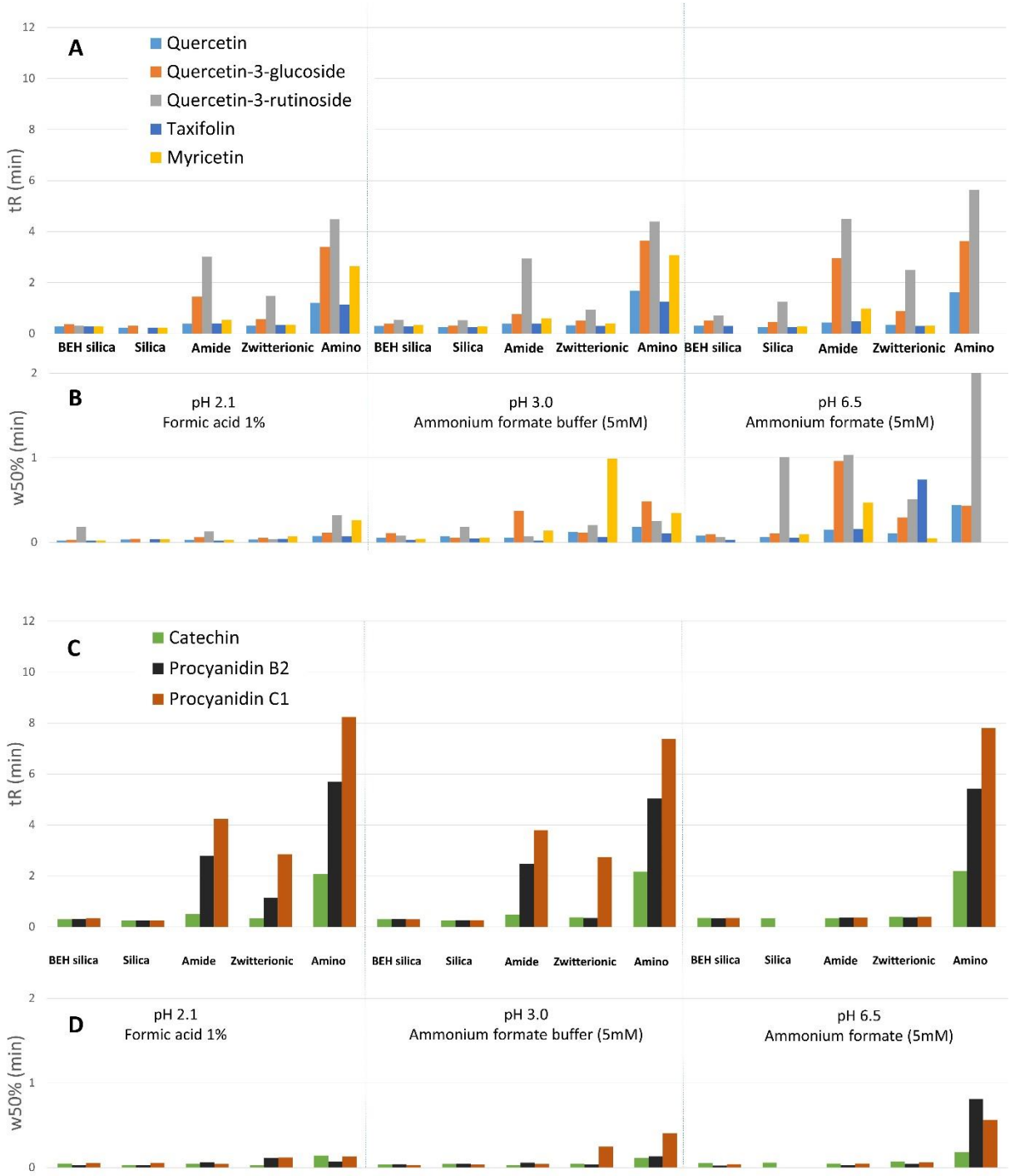


Figure 4. Screening results of the stationary and mobile phases on the retention time (tR) (A) and peak width 50% (w50%) (B) of flavonols; and the retention time (tR) (C) and peak width 50% (w50%) (D) of flavanols.

Anthocyanin analysis in HILIC

Anthocyanins are particularly sensitive to pH change [38]. Among the different chemical forms induced by pH value, the flavylium cation could considerably affect the retention especially

when ion exchange could occur. Therefore, in some cases no measurements were made either due to lack of elution or large peak width (for example taxifolin analyses with amino column and pH 6.5 mobile phase). With silica, due to electrostatic interactions between the positive charges of anthocyanins and the ionized silanols, cation exchange was observed. Additionally, in many cases multiple peaks can be observed, influencing the reliability of a single measurement. Retention was notably higher with bare silica which possesses more silanols than BEH silica (Fig. 5A). Amide and amino materials could retain anthocyanins, particularly with the amino one. The zwitterionic offered weaker retention.

The order of elution of anthocyanins appeared to be, in most conditions, that of malvidin, cyanidin and delphinidin. As expected, the glucoside forms showed higher retention of their respective aglycones for all columns tested, especially with the amino column.

The mobile phase at pH 2.1 provided the highest retention and the amide support appeared the most suitable regarding the retention and peak shape (Fig. 5B). With silica, the retention was significant but broad peaks or multiplicity of peaks were observed. The presence of ammonium ions in the mobile phases induced competitive cation exchange, reducing retention on silica, confirming the retention behavior. The use of TFA was again found ineffective to obtain retention (Fig S1).

The peak widths were difficult to measure (Fig. 5A) due to multiplicity of peaks. There was no combination of mobile and stationary phases offering retention and correct peak shape. When the peak shape seemed correct, the retention was not sufficient.

Our study is in accordance with a previous one in which an amide stationary phase was successfully used for anthocyanins [20]. However, the analysis was performed with more acidic mobile phase (0.4% TFA) as this stationary phase resists a very acidic pH, which led to correct peak shape.

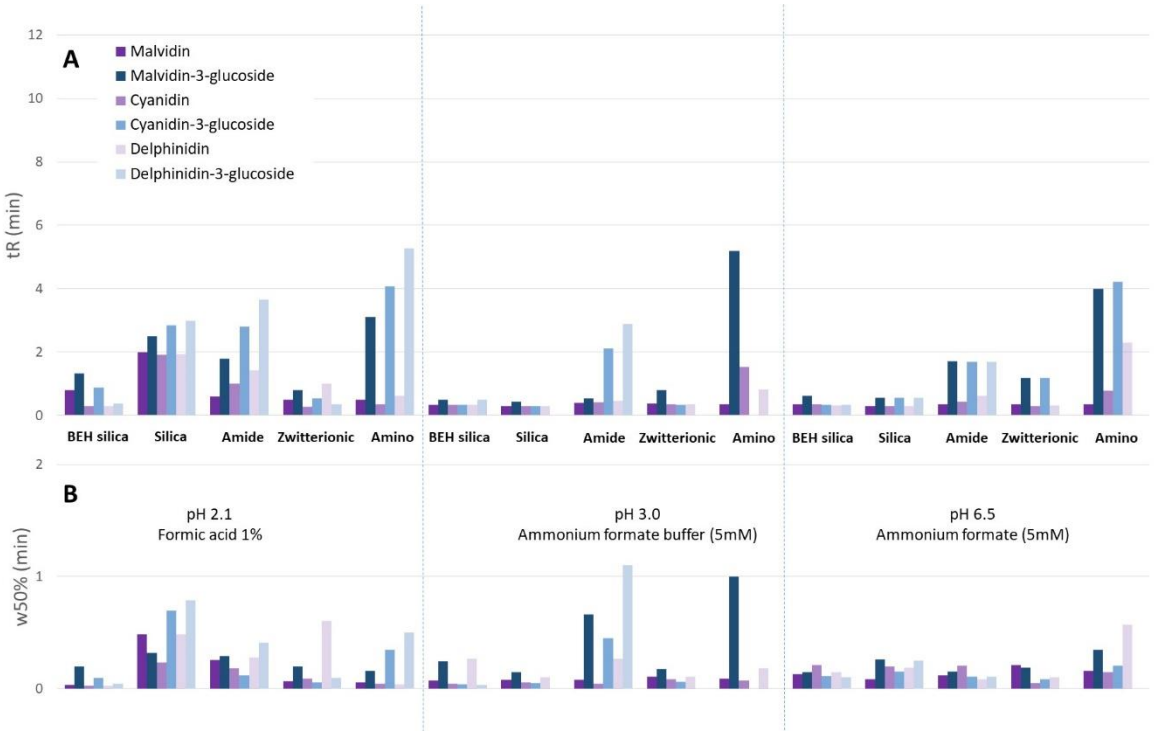


Figure 5. Screening results of the stationary and mobile phases on the retention (tR) (A) and peak width 50% (w50%) (B) of anthocyanins.

Phenolic acid analysis in HILIC

Figure 6A presents the retention of phenolic acids (hydroxybenzoic and hydroxycinnamic acids). Their retention on HILIC columns was difficult. Only the amino column allowed their retention under the various mobile phase conditions tested, except with TFA based mobile phase which prevented retention (Fig S1). It was noteworthy that here the mobile phase conditions at pH 6.5 favored retention regardless of the nature of the stationary phase. This pH could modify the ionic state of these ionogenic analytes allowing the ion exchange mechanism in particular with amino stationary phase. The pKa values of these acids are between 1.7 to 4.6 in water, so the explored pH conditions could lead to their partial ionization, which could affect their polarity. In addition, the presence of ammonium salt may contribute to a thicker water layer, leading to a more efficient partitioning mechanism [24]. Enhanced results may be attributed to the higher ionic strength of mobile phases containing salts compared with simple formic solutions. As previously reported, TFA was found to be ineffective [39]. Interestingly, some acids, such as phloroglucinic and gallic acids, were highly retained due to their number of hydroxyl groups. However, predicting order of elution of these compounds was difficult because of several influencing factors: the number and the position of hydroxyl groups relative to the carboxyl group, and the pKa values of analytes and silanols which depends on acetonitrile percentage in mobile phases [39,40]. Finally, amino column allowed better discrimination of isomers, such as *p*- and *o*-coumaric acids, compared to amide and zwitterionic columns. However, their retention and discrimination were achieved using mobile phases containing ammonium salts.

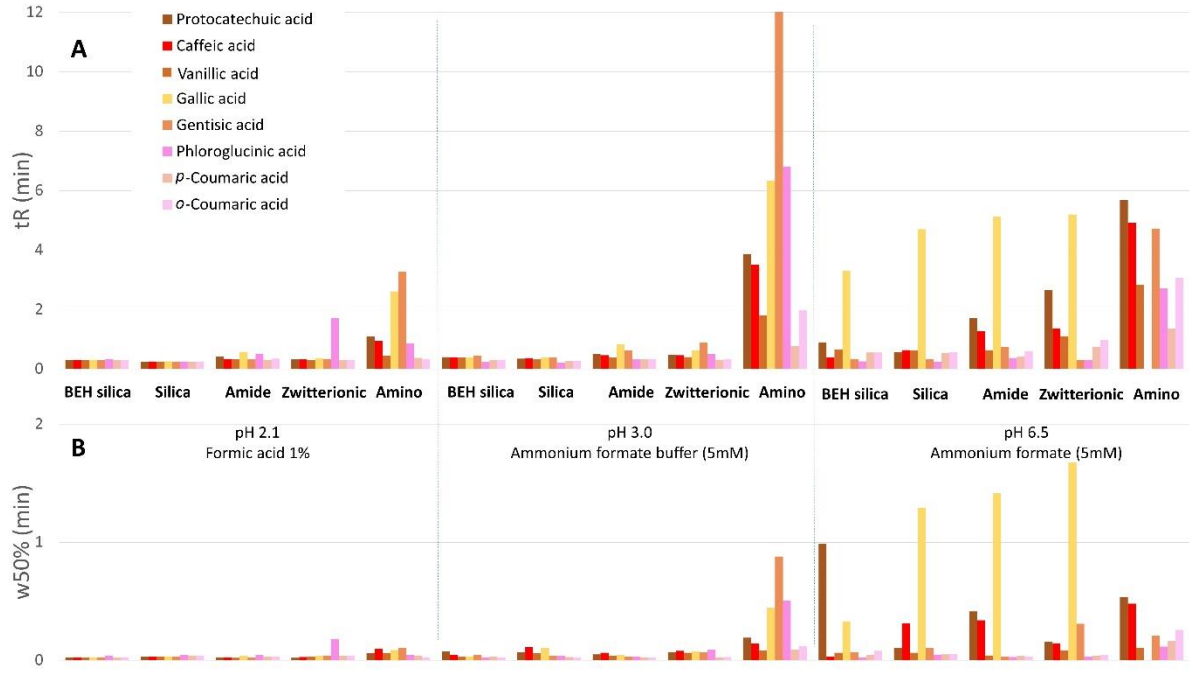


Figure 6. Screening results of the stationary and mobile phases on the retention (tR) (A) and peak width 50% (w50%) (B) of phenolic acids.

The increase of mobile phase pH was accompanied by peak broadening (Figure 6B). For hydroxybenzoic acids, retention was deeply studied with zwitterionic column [24]. The partitioning processes mainly promoted retention, and the presence of salts influenced this retention mechanism by reducing ionic repulsion. As observed here, a mobile phase at pH 6.5 promoted phenol acid retention, even if the silanols could be ionized. However, adsorption processes via hydrogen bonds also can occur with these phenolic acids. The pH and ionic strength of the aqueous-organic phase were important parameters to interpret retention and peak shape. The pKa of acids increased and they became weaker acids. Poor peak shapes may have been caused by silica ionization and interactions with trace metal ions [41]. Maintaining silica in a non-ionized state was challenging.
Overall, for the analysis of polyphenols, the amino and amide stationary phases were more suitable to obtain retention. This classification of the retention potential of the tested materials followed that of previous studies [42]. To achieve retention with correct peak shape, acidic mobile phases were more recommended, avoiding the use of TFA.

Application to stilbenoids

As HILIC columns having not been studied for analysis of stilbenoids in plants, we applied the results of this screening to the analysis of vine extracts. Vineyard maintenance generates significant quantities of waste (wood, vines, roots, etc.) which represent promising source of stilbenoids [43]. Grapevine biomass contain a wide range of stilbenes, mostly monomer in the canes but highly polymerized in the roots [11]. Thus, to help valorize these by-products, HILIC could contribute to their characterization. In addition, these samples, from the aerial part to the roots, represented an interesting molecular model to highlight the HILIC potential to visualize the degree of polymerization of stilbene. Therefore, we used the most retentive stationary phase, the amino, associated with formic acid based mobile phase at pH 2.6 obtained with 0.1% formic acid. Under these conditions, the separation of stilbenoids could be maximized due to their high retention combined with correct peak shape. Figure 7 is a chromatogram of stilbenoid standards which summarizes the distribution of the elution as a function of the degree of polymerization of the stilbenes which increases with increasing retention. HILIC with amino stationary phase could therefore visually highlight the degree of polymerization. In contrast, with RP-HPLC, retention was less correlated with the polymerization degree [11].

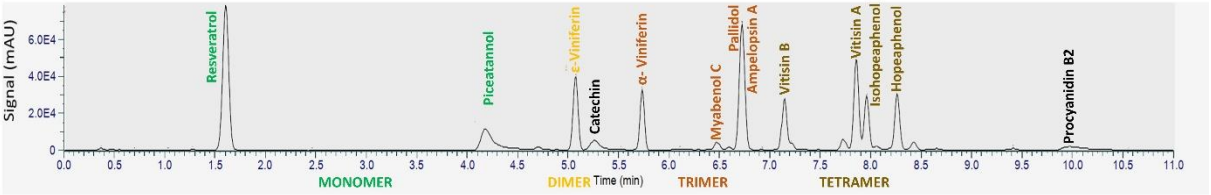


Figure 7. Chromatogram from UV detection (285 nm) obtained with the UHPLC/MS system. Mix of standards at 4.5 µg.mL^{-1}.

Comparison of detection for stilbenoid analysis

To access the composition of these extracts, HILIC was combined with MS, DAD and CAD.
Since MS calibration curves were often not linear, two different concentration ranges were considered to obtain linear calibration curves (Table S2). Their equations varied considerably between compounds based on the ionization capacity of each compound rather than the elution conditions. Table S3 presents the calculated amounts of the main compounds in each extract expressed as mass per sample mass. This confirmed that the tetramer (vitisin A and B, and isohopeaphenol and hopeaphenol) and monomer (resveratrol) were more abundant in the root and rootlets, and the cane, respectively. The results confirmed the considerable stilbenoid variability across the different parts of grapevine (Table S3).
Because MS utilizes expensive equipment, which is not accessible to all laboratories, detections with DAD and CAD were also used in this study which could be used as a first approach in a strategy of extract characterization. In plant analysis, using various detectors could allow for a more comprehensive characterization of the sample.
For polyphenol analysis, UV/Visible detection is ideally suited due to the presence of numerous aromatic rings in these compounds, ensuring an intense and robust detection response. CAD offers simplicity and complements UV/Visible with more universal response for structurally related compounds [44].
Since, in extract samples, full resolution of all compounds is not achieved, and DAD and CAD are not selective as MS, only the response of the three main compounds was taken into account: resveratrol (monomer), ε-viniferin (dimer) and vitisin B (tetramer). Table 3 presents the calibration parameters of the three main stilbenoids to compare CAD and UV detection response.

Table 3. Stilbenoid detection response between CAD and DAD (UV) with standards.

	CAD			**UV**		
	0.003-0.333 mg.mL^{-1}	**R^2**	**S/N** (3 µg.mL^{-1})	**0.003-0.167 mg.mL^{-1}**	**R^2**	**S/N** (3 µg.mL^{-1})
Resveratrol (monomer)	$y = 19.9\ x^{0.86}$	0.9948	36.75	y = 206.66 x - 0.020 (λ 308 nm)	0.9987	211.25
ε-Viniferin (dimer)	$y = 26.2\ x^{0.90}$	0.9973	20.75	y = 101.54 x - 0.027 (λ 324 nm)	0.9992	55.14
Vitisin B (tetramer)	$y = 20.4\ x^{0.88}$	0.9970	30.00	y = 53.79 x - 0.049 (λ 324 nm)	0.9991	49.14

In UV analysis, the selected wavelength was at the maximum absorbance of each compound.
With CAD, calibration curve typically follows a relationship: $y = a\ x^b$, where y is the signal, a and b are response factors, and x is the concentration [45].
The concentration range was slightly larger for DAD than for CAD, showing the slight sensitivity advantage of DAD. With DAD, the slope of the response decreased drastically with the degree of polymerization, being divided fourfold between resveratrol and vitisin B, revealing significant differences in sensibility between compounds. In CAD analysis, the equation

coefficients were rather of the same order of magnitude. CAD is a detection depending on the mobile phase composition [46], since elution was in gradient that explained this slight difference in response. Consequently, CAD could offer a chromatographic profile more representative of the relative amounts of each molecule in the mixture compared to DAD. Concerning the signal-to-noise ratio (S/N), UV/Visible detection can allow higher values than CAD which confirmed the highest peak intensities with UV detection. However, this difference in sensitivity between DAD and CAD decreased with increasing polymerization degree.
In conclusion, the DAD is an essential detector for the analysis of polyphenols; allowing access to their spectrum, thus helping with their identification; and its calibration being linear, quantification is facilitated. The CAD is complementary with its universal response, which allowed for a direct visualization of the composition of the sample without necessarily going through a quantification step, which is very time and sample consuming. MS offers better LOD and selectivity.

Analysis of polyphenols in grapevine by-products

Qualitative and quantitative comparisons were performed between the different detection modes. Figure 8 presents the chromatographic profiles obtained from the different grapevine part extracts recorded with dual detection, DAD and CAD. CAD revealed an additional peak at 6.5 min, which was highly retained compared to stilbenes and not detected by UV/Visible. This compound, which is chemically unrelated to polyphenols and very polar, highlights the utility of CAD to provide complementary information through a more universal detection. As expected, the profiles were differentiated between resveratrol-richer extracts obtained from canes (dark green chromatogram) and tetramer-richer extracts from rootlets (brown chromatogram).

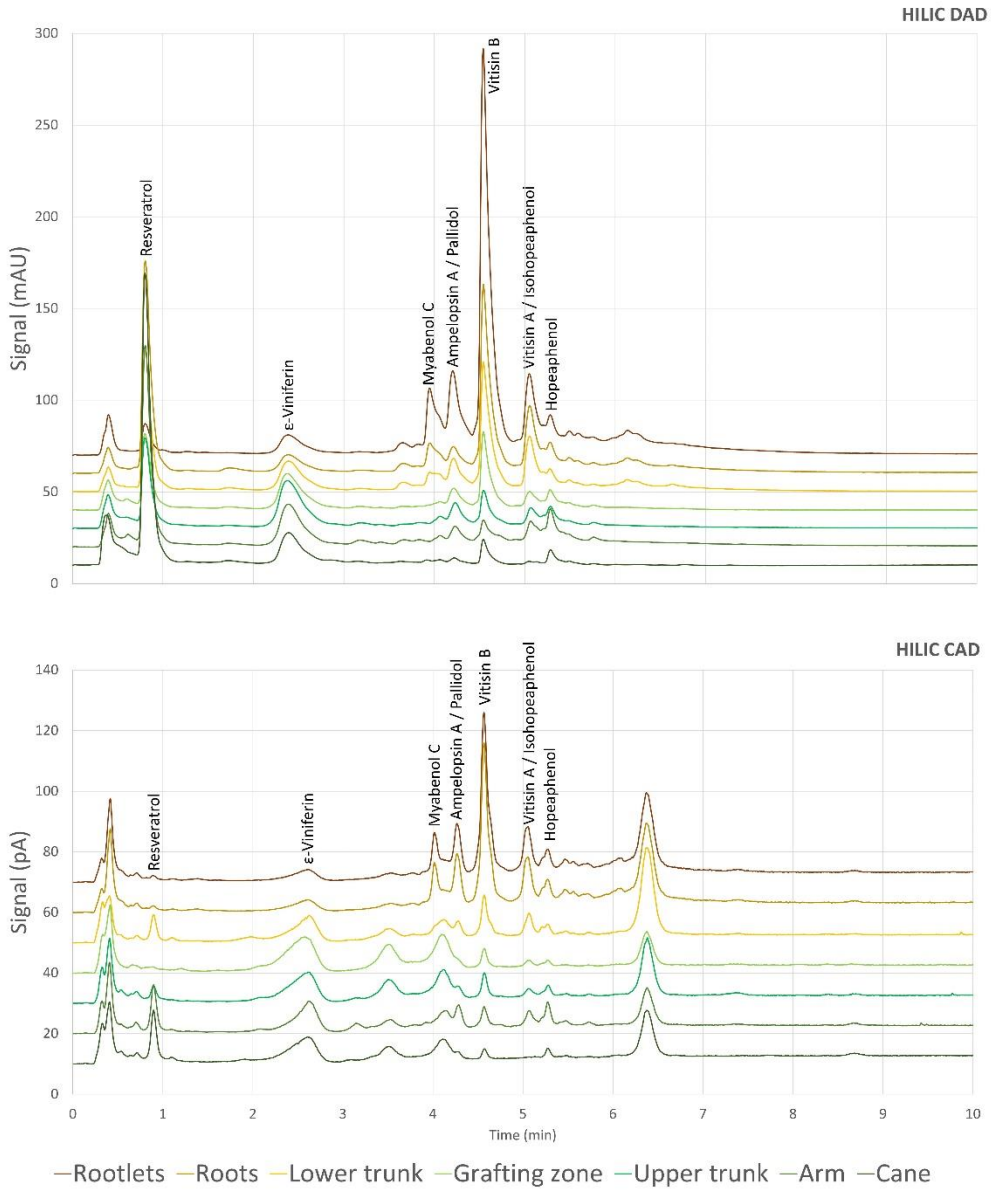


Figure 8. Chromatograms of the different grapevine part extracts analyzed by HILIC/DAD (285 nm) (top) and CAD (bottom). Dark green to brown chromatograms correspond from cane to rootlet extracts.

Quantification of the three main products in the extracts were also performed using DAD and CAD with calibration curves presented in Table 3. These results were compared to those found with MS (Fig. 9). Result for each plant part was obtained from extraction performed in triplicate. So the variability observed could be attributed to plant material which is heterogeneous: as instance, a cane is composed of various tissues with notably, cortex, phloem, xylem and pith that do not have, at all, similar compounds composition [47]. In addition, during the valorization process of this biomass, a grinding step was necessary to facilitate and make the extraction more efficient. However, the size of powder was adapted to Accelerated Solvent Extraction [11], to avoid the clogging of the system. So in our study, we kept this particle size to be able to compared with previous study [11]. Furthermore, the peak area measurement was influenced by the chromatographic resolution and detector

sensitivity. The MS had the advantage of its intrinsic selectivity thanks to being able to filter the chromatogram with the exact m/z, which allowed a much clear form of peaks, and better integration in complex samples. So among the 3 tested detectors, it provided the most reliable quantitative results. Thus, for DAD and CAD, peak identification was performed using retention time and, for DAD, UV spectra gave peak purity. Table S4 provides the retention time precision with RSD less than 4%, confirming the correct identification of peaks.

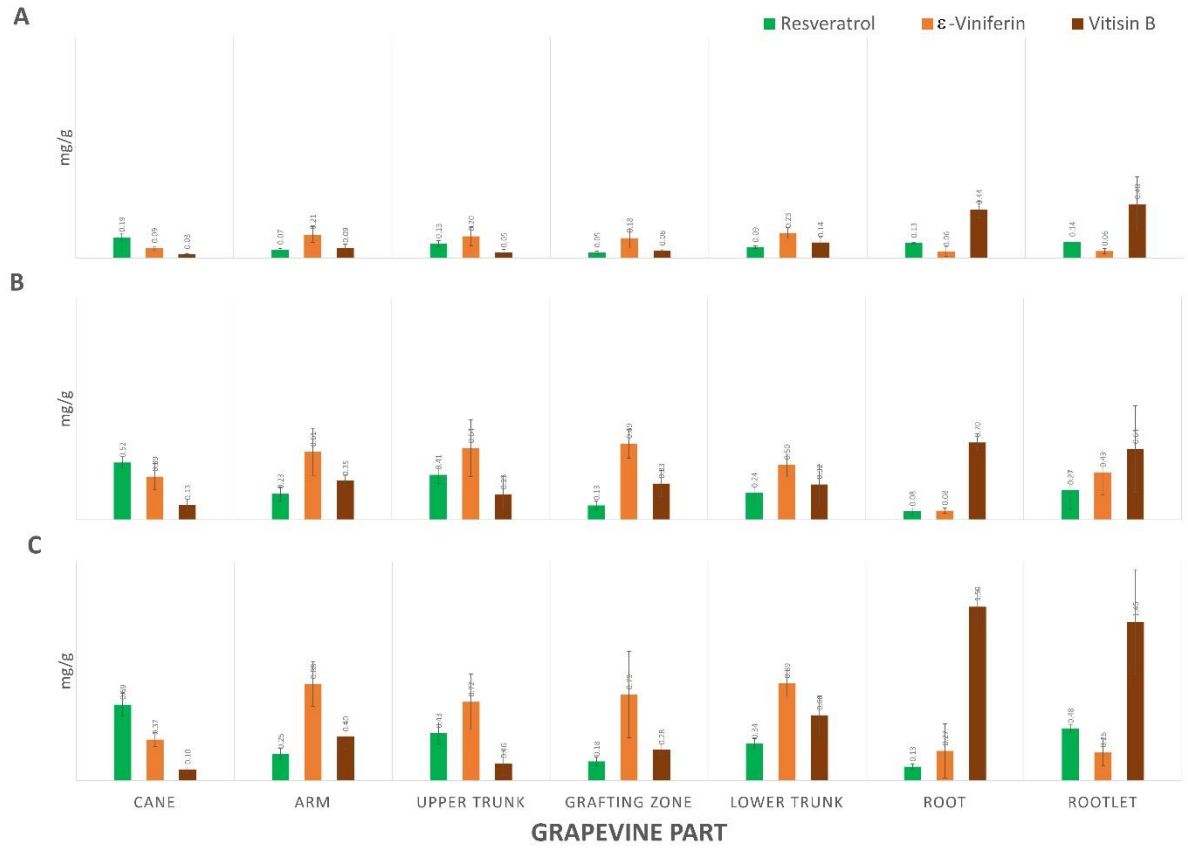


Figure 9. Comparison of quantification results with different detections for the main compounds in grapevine extracts; (A) DAD, (B) CAD and (C) MS.

The results obtained with mass spectrometry showed sample variability (Fig. 9C), which can be very important. Vitisin B, for example, was found in high, though variable, concentrations in the root and rootlets; the same was found to be true for ε-viniferin in many samples. Overall, lower amounts were found with DAD and CAD compared to MS, especially for the tetramer. Vitisin B was eluted in a part of the chromatogram very rich in stilbenoids (Fig. 8), inducing a very approximate and lower measurement of its peak area. The same observation can be made for ε-viniferin, which was co-eluted with catechin. Resveratrol seemed less affected by this problem. We therefore observed that the quantitative profile was more similar between CAD and MS but lower with DAD.

Selectivity study

In order to predict retention in HILIC and also to have a better understanding of the overall chromatographic behavior, selectivity was studied. Correlations between retention and the degree of polymerization (Fig. 10A) or the number of hydroxyl groups (Fig. 10B) were studied. The relationship between retention time and the number of resveratrol degree of polymerization was obtained with the linear regression: y = 1.3562 x - 0.3447 (Fig. 10A), showing a high correlation and the coefficient of determination, R^2 = 0.9578. Other tested models did not improve the correlation (Table S5). When correlation was studied between retention time and the number of hydroxyl groups in the molecule, quadratic or multimodal models showed a better correlation than the linear one (quadratic model: y = -0.061 x^2 + 1.4226 x -3.012 and R^2 = 0.9830 (Fig. 10B). Therefore, a linear relationship using the degree of polymerization was found and could be useful to estimate compounds with different degrees of polymerization.

On the contrary, Figure 10C illustrates the relationship between retention time and calculated log P of stilbenes. The poor correlation (R^2 = 0.0.6570) indicated that using log P was insufficient for retention prediction. Retention behavior in HILIC was not well predicted using global polarity.

With C18 [11,48,49], the order of stilbenoid retention in RP-HPLC was less correlated with the polymerization degree or the OH group number. For example, isohopeaphenol and hopeaphenol (tetramers, nOH = 9) were eluted between resveratrol (monomer, nOH = 3) and ε-viniferin (dimer, nOH = 5).

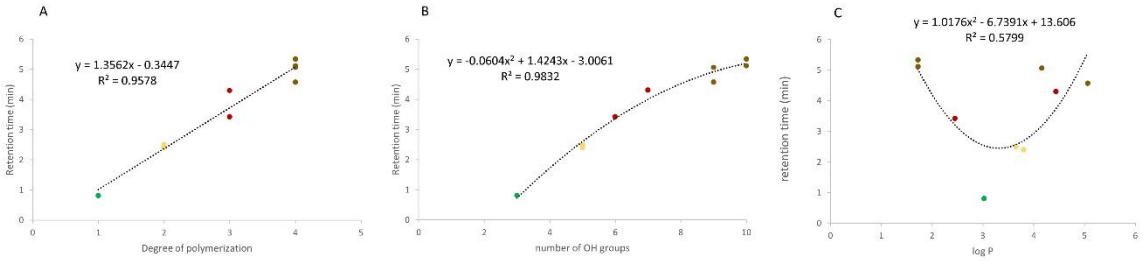


Figure 10. Retention time of stilbenes vs the degree of polymerization (A); the number of hydroxyl groups (B); calculated log P (C) (calculated values [44]): Resveratrol (1/3/3.02); ε-Viniferin (2/5/3.80); δ-Viniferin (2/5/3.65); α-Viniferin (3/6/2.45); Miyabenol C (3/7/4.43); Vitisin A (4/10/4.16); Vitisin B (4/9/5.06); Isohopeaphenol (4/10/1.72); Hopeaphenol (4/10/1.72). Monomer (green); Dimer (yellow); Trimer (red); Tetramer (brown). Chromatographic condition corresponded to gradient elution used for extract analysis.

Conclusion

The global study of several families of polyphenols in HILIC with five columns and five mobile phases showed:
- the amino and amide stationary phases had the best potential in terms of retention and peak quality. Zwitterionic columns have much less retention and silica did not allow to obtain sufficient retention.
- the mobile phase, the study of pH and different pH modifiers, showed that it was generally more judicious to select an acidic pH, avoiding the use of TFA.

Only some glucoside derivatives showed retention with silica stationary phases, however in the studied pH range, no single and symmetric peak could be obtained.

For the first time, to our knowledge, the use of a HILIC approach was developed for the analysis of stilbenoids. It was carried out for the analysis in vine extracts very rich in stilbenoids, in order to demonstrate its interest and complementarity.

As retention of stilbenoids in HILIC was correlated to the degree of stilbene polymerization, chromatographic profiles offered a rapid view of the assessment of stilbene polymerization in the extracts before any quantification.

HILIC uses mobile phases with high-solvent content, especially ACN, which can be advantageous with various detectors. Therefore, DAD, CAD, and MS were compared for qualitative and quantitative results. MS remained the essential detector for the identification and quantification in complex matrices. However, since this instrumentation is very expensive and not very accessible, dual DAD and CAD detection was a relevant alternative as a first method of investigation.

This study demonstrated the complementarity of DAD and CAD for the analysis of stilbenoids in HILIC. Due to the chromophores of polyphenols, they were well detected by DAD. While the sensitivity of DAD was slightly higher than that of CAD, its response was more universal. Thus, direct visualization of the chromatographic profile with CAD is more representative to the sample composition.

For all these reasons, the combination of HILIC with DAD and CAD provided a useful first-line tool in the workflow for the characterization of stilbenoid-rich plants.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

CRediT authorship contribution statement

Karen Gaudin: Writing – original draft, Writing – review & editing, Visualization, Methodology, Investigation, Conceptualization, Supervision, Resources, Validation. **Josep Valls-Fonayet:** Investigation, Validation, Resources, Writing – review & editing, Funding acquisition. **Rémy Cordazzo:** Investigation. **Wiktoria Serafin:** Investigation. **Emma Lafon:** Investigation. **Alexandra Gaubert:** Resources, Writing – review & editing, Funding acquisition. **Stéphanie Cluzet:** Resources, Writing – review & editing, Funding acquisition. **Tristan Richard:** Resources, Writing – review & editing, Funding acquisition.

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Supplementary materials

Separation of polyphenols by HILIC methods with diode array detection, charged aerosol detection and mass spectrometry: application to grapevine extracts rich in stilbenoids

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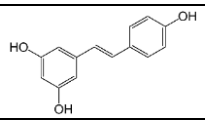
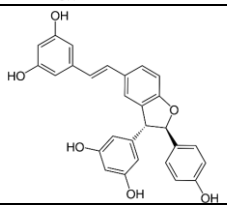
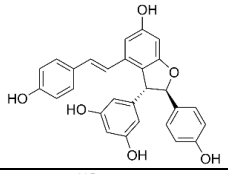
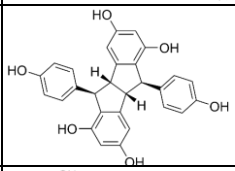
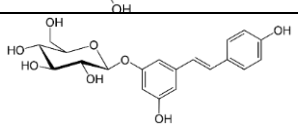
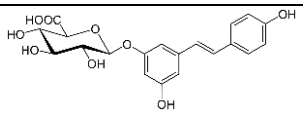
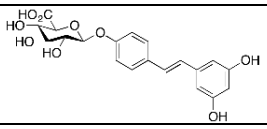
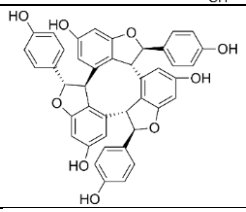
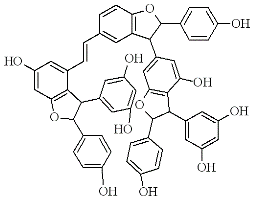
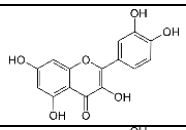
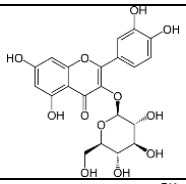
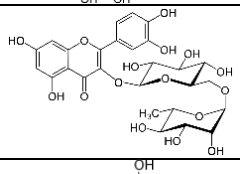
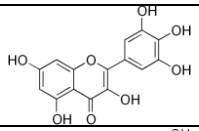
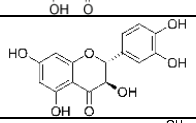
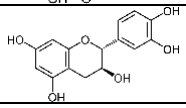
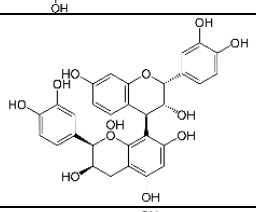
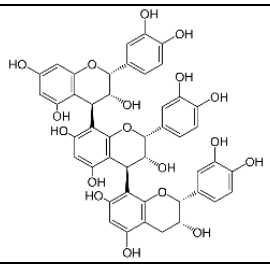
Table S1. List of the studied phenolic molecules.

Polyphenol class	Name	Structure
Stilbenoids	Resveratrol	
	δ-Viniferin	
	ε-Viniferin	
	Pallidol	
	Piceid	
	Resveratrol-3-*O*-glucuronide	
	Resveratrol-4'-*O*-glucuronide	
	α-Viniferin	
	Vitisin B	

Polyphenol class	Name	Structure
Flavonols	Quercetin	
	Quercetin-3-glucoside	
	Quercetin-3-rutinoside	
	Myricetin	
	Taxifolin	
Flavanols	Catechin	
	Procyanidin B2	
	Procyanidin C1	

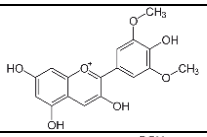
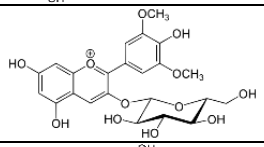
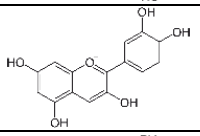
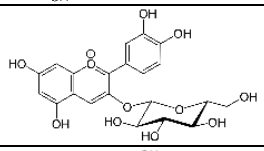
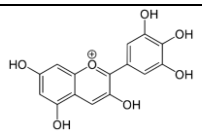
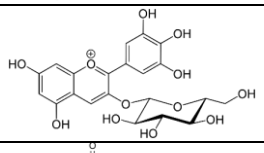
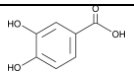
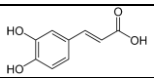
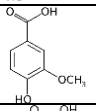
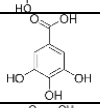
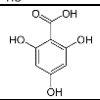
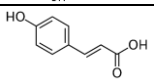
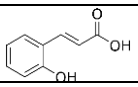
Polyphenol class	Name	Structure
Anthocyanins	Malvidin	
	Malvidin-3-glucoside	
	Cyanidin	
	Cyanidin-3-glucoside	
	Delphinidin	
	Delphinidin-3-glucoside	
Phenolic acids	Protocatechuic acid	
	Caffeic acid	
	Vanillic acid	
	Gallic acid	
	Gentisic acid	
	Phloroglucinic acid	
	p-Coumaric acid	
	o-Coumaric acid	

Table S2. MS calibration curves (Linear model).

Compound	*1.12 - 8.93 µg.mL^{-1}*			*0.07 - 1.12 µg.mL^{-1}*		
	slope	**intercept**	**r^2**	**slope**	**intercept**	**r^2**
trans-Resveratrol	95060	-53842	0.9970			
Piceatannol	77042000	55287600	0.9973	115882000	-1642090	0.9959
ε-Viniferin	4532180	3089910	0.9971	6995180	-52161	0.9943
α-Viniferin	1786240	1227470	0.9989	2825160	-4956	0.9798
Miyabenol C	524005	541838	0.9951	882929	45196	0.9618
Ampelopsin A	10191900	13893900	0.997	20848300	222242	0.9938
Pallidol	1787200	1935960	0.9971	3251440	-4534	0.9935
Vitisin B	1080650	804315	0.9984	1676480	-25639	0.9995
Vitisin A	1335850	1424800	0.9946	2312480	-23135	0.9947
Isohopeaphenol	1357410	1321410	0.9943	2308980	-27921	0.9942
Hopeaphenol	2787980	2649380	0.9963	4668520	21550	0.9885
Catechin	71926900	82360800	0.9944	128605000	2550570	0.9910
Procyanidin B2	6051160	3045010	0.9996	8378350	98407	0.9955
Procyanidin C1	501131	334935	0.9975	832547	-126021	0.9937

Table S3. MS quantification of extracts obtained from various grapevine parts.

	***trans*-Resveratrol**		**Piceatannol**		**ε-Viniferin**		**α-Viniferin**		**Miyabenol C**		**Ampelopsin A**		**Pallidol**		**Vitisin B**		**Vitisin A**		**Isohopeaphenol**		**Hopeaphenol**		**Catechin**		**Procyanidin B2**		**Procyanidin C1**	
	mg.g^{-1}	*SD*	mg.g^{-1}	*SD*	mg.g^{-1}	*SD*	mg.g^{-1}	*SD*	mg.g^{-1}	*SD*	mg.g^{-1}	*SD*	mg.g^{-1}	*SD*	mg.g^{-1}	*SD*	mg.g^{-1}	*SD*	mg.g^{-1}	*SD*	mg.g^{-1}	*SD*	mg.g^{-1}	*SD*	mg.g^{-1}	*SD*	mg.g^{-1}	*SD*
CANE	0.69	*0.10*	0.05	*0.01*	0.37	*0.06*	nd	*nd*	nd	*nd*	0.01	*nd*	0.03	*0.01*	0.10	*0.03*	nd	*nd*	0.01	*nd*	0.10	*0.02*	0.07	*0.01*	0.01	*nd*	0.02	*nd*
ARM	0.25	*0.05*	0.02	*0.01*	0.88	*0.20*	0.02	*nd*	0.11	*0.03*	0.06	*0.02*	0.09	*0.03*	0.40	*0.11*	0.02	*0.01*	0.13	*0.03*	0.13	*0.04*	0.01	*nd*	0.02	*0.01*	nd	*nd*
UPPER TRUNK	0.43	*0.10*	0.02	*0.01*	0.72	*0.25*	0.02	*0.01*	0.08	*0.05*	0.05	*0.02*	0.09	*0.06*	0.16	*0.11*	0.01	*0.01*	0.10	*0.06*	0.11	*0.03*	0.01	*nd*	nd	*nd*	nd	*nd*
GRAFTING ZONE	0.18	*0.04*	0.01	*nd*	0.79	*0.40*	0.01	*0.01*	0.06	*0.02*	0.07	*0.03*	0.06	*0.05*	0.28	*0.05*	0.02	*nd*	0.13	*0.06*	0.07	*0.02*	0.01	*nd*	nd	*nd*	nd	*nd*
LOWER TRUNK	0.34	*0.05*	0.03	*0.01*	0.89	*0.12*	nd	*nd*	0.05	*0.02*	0.14	*0.07*	0.07	*0.02*	0.60	*0.17*	0.06	*0.03*	0.20	*0.06*	0.10	*0.05*	0.02	*nd*	0.01	*nd*	0.01	*nd*
ROOT	0.13	*0.03*	0.03	*nd*	0.27	*0.25*	0.01	*0.01*	0.07	*0.01*	0.38	*0.09*	0.02	*0.01*	1.59	*0.15*	0.12	*0.01*	0.18	*0.02*	0.11	*0.04*	0.01	*nd*	nd	*nd*	nd	*nd*
ROOTLET	0.48	*0.04*	0.05	*nd*	0.26	*0.12*	nd	*nd*	0.07	*0.03*	0.17	*0.14*	0.04	*0.02*	1.45	*0.48*	0.19	*0.12*	0.38	*0.16*	0.17	*0.09*	0.02	*nd*	0.01	*nd*	0.01	*nd*

nd = not determined (value too small)

Table S4. Precision of Retention time (obtained from the injection of extracts)

RSD (n=3)	Resveratrol	ε-Viniferin	Vitisin B
Repeatability	0.55%	1.63%	0.78%
Intermediate precision	1.06%	3.37%	1.97%

Table S5. Precision of Retention time (obtained from the injection of extracts)

Variable	Model	term	estimate	R^2	AIC
log P	Linear $y = b\,x + a$	a	4.151	0.010	38.270
		b	-0.128		
	Quadratic $y = a + b\,x + c\,x^2$	a	13.578	0.578	32.584
		b	-6.725		
		c	1.016		
	Multimodal $y = a + b\,x + c\,\log x$	a	4.811	0.657	30.717
		b	6.898		
		c	-21.149		
Polymerization degree	Linear $y = b\,x + a$	a	-0.345	0.958	9.836
		b	1.356		
	Quadratic $y = a + b\,x + c\,x^2$	a	-1.078	0.963	10.574
		b	2.006		
		c	-0.120		
	Multimodal $y = a + b\,x + c\,\log x$	a	-0.071	0.963	10.636
		b	0.868		
		c	1.173		
Number of OH	Linear $y = b\,x + a$	a	-0.527	0.947	11.892
		b	0.598		
	Quadratic $y = a + b\,x + c\,x^2$	a	-3.012	0.983	3.551
		b	1.426		
		c	-0.061		
	Multimodal $y = a + b\,x + c\,\log x$	a	-3.491	0.979	5.548
		b	-0.018		
		c	3.873		

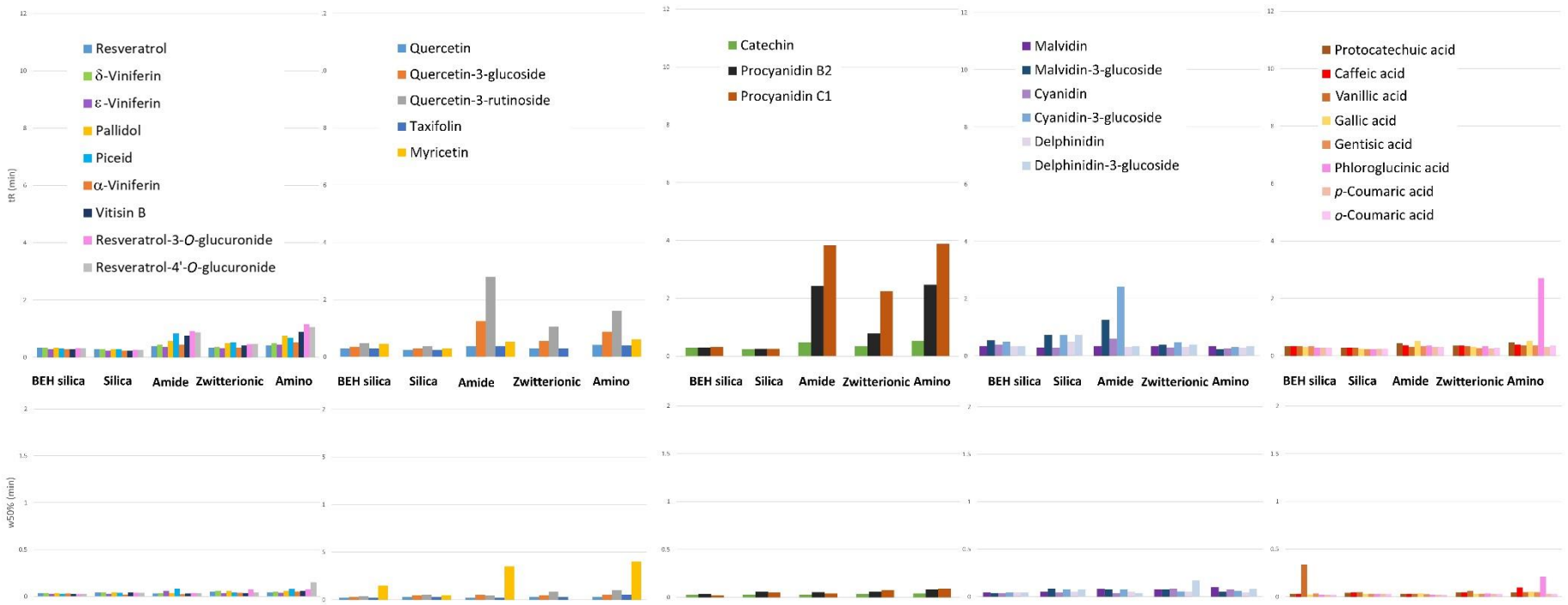


Figure S1. Screening results of the stationary and mobile phases on the retention time (tR) (A) and peak width 50% (w50%) (B) of polyphenols with mobile phase containing TFA 0.085% (pH 2.1).