Research Article

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Chiral analysis of *E*-ε-viniferin enantiomers, towards a new chemotaxonomic marker of the vine

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Abstract

BACKGROUND: The accurate characterization of grapevine cultivars (*Vitis vinifera*) is crucial for grape growers, winemakers, wine sellers, consumers and authorities, considering that mistakes could involve significant damage to the wine economic system. To avoid any misunderstanding, morphological, molecular and chemical tools are developed to positively identify grape varieties.

RESULTS: *E*-ε-viniferin is a stilbene dimer mainly present in the woody part of grapevine and present as a mixture of two enantiomers: (*7aR*, *8aR*)-(−)-*E*-ε-viniferin (1) and (*7aS*, *8aS*)-(+)-*E*-ε-viniferin (2). In addition to phenotypic and genotypic approaches, a chemotaxonomic method using *E*-ε-viniferin enantiomers as chemical markers of grapevine cultivars was investigated. The isolation and purification of *E*-ε-viniferin enantiomers by preparative high-performance liquid chromatography (HPLC) and chiral HPLC from 14 red and eight white grapevine cane cultivars enabled us to determine the proportion of each enantiomer and therefore to calculate the enantiomeric excess for each variety. The relative abundance of each *E*-ε-viniferin enantiomer permitted us to distinguish grape varieties, as well as to establish cultivar relationships and patterns through statistical analysis.

CONCLUSION: This pioneering work highlighting the enantiomeric excess of *E*-ε-viniferin as a chemical marker of grapevine paves the way for further studies to understand what mechanisms are involved in the production of these enantiomers in grapevine.

Keywords: *Vitis vinifera*; grapevine cane; chirality; stilbenes; viniferin

INTRODUCTION

The cultivation of vines (Vitaceae) associated with the production of wine is an ancestral practice dating back thousands of years, and today is carried out all over the world. Among the Vitaceae family, *Vitis* is the genus that has attracted most attention because of its economic and qualitative importance.[1] More specifically, *Vitis vinifera* is the major cultivated vine and wine-producing grape.[2] More than 6000 grape varieties have been reported in the world but only 100 cultivars are used for winemaking.[3] Regarding economic and regulatory issues, the identification of different grapevine varieties is important to grape growers, winemakers, wine sellers, consumers and authorities. In fact, an error can involve significant economic repercussions for all the actors throughout the wine production chain.

Ampelography is the science of phenotype identification and classification of grapevines. From the Greek 'ampelos' meaning vine and 'graphe' meaning description, ampelography is a traditional vine characterization based on morphological identification. The distinction was performed by comparing shape, color, size or texture of grapevine leaf and berries.[4] Mainly used in the 20th century, ampelography can, however, be relatively subjective depending on the observer. Moreover, some cultivars are morphologically very similar and difficult to differentiate by visual comparison, allowing slight mistakes.

In the early 1990s, grapevine cultivar identification was revolutionized by DNA fingerprinting. This genotypic technology was based on the study of microsatellite DNA, which is a small repeat unit (less than four nucleotides) that generates repeating regions (between 20- and 30-fold) in the genome.[5] The microsatellite profiles allow us to distinguish different cultivars but also to establish parent linkage through their reproducibility and their codominant

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nature.[6] The DNA fingerprint, which reflects the genotype and is independent of the phenotype, provides more objective descriptors for cultivar identification.

More recently, chemotaxonomy was used to differentiate red grape cultivars. Anthocyanins, which are chemical compounds of the flavonoid group (polyphenols) and are responsible for the color of grape cultivars, were used owing to their relative abundance in grape berry skin.[7] Anthocyanin profiles were relatively stable during ripening and vintage, allowing the discrimination of 50 grape cultivars from a Spanish germplasm.[8] Due to the development of sensitive analytical tools, it is now possible to discriminate non-*V. vinifera* species as well as interspecies hybrids to *V. vinifera* cultivars owing to the analysis of diglucosylated anthocyanins.[7] Although anthocyanins are chemical markers of the vine, it is important to mention that they only allow the differentiation of red cultivars.

Stilbenes are another group of naturally occurring polyphenolic compounds derived from secondary plant metabolism. Over the past decades, more than 1000 compounds belonging to this phenolic class have been discovered. Structurally, stilbenes possess a 1,2-diphenylethylene nucleus generating a C_6–C_2–C_6 skeleton.[9] Within the common stilbenes resveratrol is the basic monomeric unit, which can lead to oxidative coupling reactions and the formation of oligomeric forms. Concerning oligomers, we can find dimers, trimers and tetramers or even larger structures such as hexamers and octamers.[3] Notwithstanding the wide chemical diversity, the production of these secondary metabolites is limited to a dozen families in the plant kingdom, such as the Pinaceae, Polygonaceae, Gnetaceae and Vitaceae.[2]

Among the Vitaceae, the oxidative condensation of *E*-resveratrol gives rise to a relatively limited group of oligomers named viniferins.[10] One of the major viniferins in grapevine is *E*-ε-viniferin, a dehydrodimer of resveratrol.[11] It was first described by Langcake and Pryce in 1977 in grapevine leaves as a response to a fungal infection and ultraviolet (UV) radiation.[12] Since the early 2010s, several authors have reported that *E*-ε-viniferin was the main compound in vine woody parts, especially in grapevine cane, with a content reaching 1–6 g kg^{-1} of cane.[13,14] It is important to emphasize that *E*-ε-viniferin is a mixture of the two enantiomers: (*7aS*, *8aS*)-(+)-ε-viniferin and (*7aR*, *8aR*)-(−)-ε-viniferin. In fact, through oxidative condensation reactions of resveratrol, the neoformed dimeric *E*-ε-viniferin possesses two stereogenic centers in C-7a and C-8a of the benzofurane ring, thereby generating chiral compounds.[15]

Currently, the enantiomeric excess, i.e. the absolute value of the difference between the quantities of two enantiomers of *E*-ε-viniferin, in several grapevine canes has never been studied. The enantiomeric excess is a historical and current chemical parameter used as a reference for deciphering an enantiomeric composition. It is largely applied in pharmacopeia methods,[16,17] and several authors working on polyphenol/stilbene enantiomers have used it.[18-21] Due to the variation in genotype and phenotype of the various cultivars, these values can vary substantially. For this goal, the present work investigated the enantiomeric excess of *E*-ε-viniferin in 22 commonly cultivated *V. vinifera* cultivars. A statistical approach was carried out to discriminate the various grape varieties according to their *E*-ε-viniferin enantiomeric excess. The *E*-ε-viniferin enantiomeric excess as chemical markers of the grapevine is discussed.

MATERIALS AND METHODS

Chemicals and standards

Absolute ethanol purchased from VWR (Fontenay-sous-bois, France) and purified water using an Elga water purification system (High Wycombe, UK) were employed for hydro-alcoholic extraction. Liquid chromatography–mass spectrometry (LC-MS)-grade acetonitrile from VWR and formic acid from Fisher Scientific (Loughborough, UK) were used for analysis by ultra-high-performance liquid chromatography–diode array detection–mass spectrometry (UHPLC-DAD-MS). HPLC-grade acetonitrile and trifluoroacetic acid (TFA) from Sigma-Aldrich (St Louis, MO, USA) were purchased for purification of *E*-ε-viniferin by preparative HPLC. Hexane and isopropanol from VWR were used for chiral analysis by HPLC-DAD.

Standards of *E*-ε-viniferin were isolated and purified in our laboratory from *V. vinifera* cane by preparative HPLC. The identity and purity of the dimeric stilbene were analyzed by UHPLC-DAD-MS and nuclear magnetic resonance and compared to data from the laboratory.[11,14] The purity of *E*-ε-viniferin was estimated to be ≥95% by UHPLC-UV-DAD.

Plant material

The 22 *V. vinifera* grape varieties of interest (14 red cultivars – Cabernet franc N, Cabernet-Sauvignon N, Carignan N, Cot N (syn. Malbec), Gamay N, Merlot N, Mourvèdre N (syn. Monastrell), Pinot noir N, Prunelard N, Sangiovese N, Syrah N (syn. Shyraz), Tannat N, Tempranillo N, Touriga nacional N – and eight white cultivars – Chardonnay B, Chenin B, Colombard B, Muscadelle B, Riesling B, Sauvignon B, Semillon B, Ugni blanc B – belong to the 'VitAdapt' experimental plot of INRAE, Villenave d'Ornon, France.[22]

They were all planted in 2009 and were cultivated on a gravelly soil, representative of this region of Bordeaux (Pessac-Léognan appellation). They were also similarly grafted on a rootstock and were all managed by conventional methods. In this sense, there was no variable influence of grapevine age, soil conditions, rootstock origin, agronomic factors or environment changes that could impact stilbene composition. Within the 'VitAdapt' parcel, each cultivar was cultivated on ten different vine stocks and all cultivars were repetitively planted in five blocks. In our study, we collected grapevine cane of the 22 grape varieties mentioned above from two different blocks as biological replicate.

For each grape variety in the two different blocks, 15 grapevine canes were randomly harvested from ten different vine stocks in January 2020. The grapevine canes were cut into pieces, then dried in an oven (35 °C) for 2 weeks and finally finely ground into powder (4 mm) with a cutting mill (Fritsch, Idar-Oberstein, Germany). Samples were stored in a dry place until extraction.

Extraction

Pressurized liquid extraction was conducted on 5 g of grapevine cane powder using an accelerated solvent extraction apparatus (Dionex Corporation, Sunnyvale, CA, USA). A 34 mL cartridge was used with a cellulose filter. Extraction was performed with the following parameters: temperature, 60 °C; heat time, 5 min; static time, 5 min; rinse volume, 60%; purge, 100 s; pressure, 100 bars; extraction solvent, ethanol/water (85/15; v/v); two independent cycles. Organic solvent was removed using a rotary evaporator and then residual aqueous solvent was lyophilized in order to obtain extract powder (recovery percentage from 4.28% to 8.14% depending on grape cultivar; mean of 6.67%). Two sets of extractions were carried out on separate days for each *V. vinifera* cultivar from each block.

Quantification by UHPLC-DAD-MS

Each grapevine cane extract was analyzed on an Agilent 1290 Series UHPLC apparatus (Agilent Technologies, Santa Clara, CA, USA) equipped with an autosampler module, a binary pump with

a degasser, a column heater/selector and a UV–visible diode array detector. The column was a 100 mm × 2.1 mm i.d., 1.8 µm, Zorbax SB-C18 with a 2.1 mm × 5 mm i.d. guard column from the same manufacturer. Solvents A (water acidified with 0.1% of formic acid) and B (acetonitrile acidified with 0.1% of formic acid) were eluted at a flow rate of 0.4 mL min^{-1} with the following gradient: 17% B (0–0.4 min), 17–30% B (0.4–4.4 min), 30–38% B (4.4–7.4 min), 38–50% B (7.4–9.0 min), 50–100% B (9.0–10.0 min). The UHPLC apparatus was coupled to an Esquire 6000 ion trap mass spectrometer (Bruker-Daltonics, Billerica, MA, USA) using an electrospray ionization source. The mass spectrometry parameters were as follows: negative mode (range of *m/z* 100–1200); nitrogen drying gas, 10 L min^{-1}; nebulizer pressure, 40 psi; temperature, 365 °C; capillary voltage, 3100 V; capillary exit voltage, −118.3 V; skimmer voltage, −40 V; trap drive, 58.1.

Extracts were dissolved in an methanol/water mixture (50/50, v/v) at 1 mg mL^{-1}, filtered on 0.45 µm polytetrafluoroethylene (PTFE) and injection of 1 µL was performed. Each extract was analyzed independently in triplicate.

Calibration and equation curves were performed by injecting pure *E*-ε-viniferin in quintuplicate at several concentrations (1, 5, 10, 50, 100 µg mL^{-1}). The maximum wavelength of absorption of *E*-ε-viniferin was 320 nm. The linearity of the standard responses was carried out by plotting the peak area *versus* the *E*-ε-viniferin concentration. The limit of detection (LOD) and the limit of quantification (LOQ) were evaluated according to Feinberg's processing.[23] All quantitation data were processed using Bruker Data Analysis 3.2 software.

E-ε-viniferin isolation by preparative HPLC

The preparative HPLC apparatus was a Gilson PLC 2050 equipped with a 4.5 mm pump head, a 5 mL injection loop, a UV–visible detector (dual-wavelength spectrophotometer) and a fraction collector. The separation was performed on a Phenomenex Kinetex XB-C18 column (150 mm × 21.2 mm). The solvent system consisted of solvent A (water acidified with 0.025% TFA) and B (acetonitrile acidified with 0.025% TFA) with elution at a flow rate of 20 mL min^{-1} with the following gradient: 15% B (0–5.0 min); 15–35% B (5.0–25.0 min); 35–100% B (25.0–26.0 min).

Grapevine cane extracts were solubilized at 50 mg mL^{-1} in methanol/water (50/50, v/v) and filtered on PTFE 0.45 µm. *E*-ε-Viniferin of each grapevine cane from the different cultivars was automatically collected after UV detection with an adapted threshold. Organic solvent was removed using a rotary evaporator and then residual aqueous solvent was lyophilized, allowing us to obtain different powders of *E*-ε-viniferin.

Chiral analysis by HPLC-DAD

Chiral HPLC analyses were performed on an Agilent Technologies 1100 Series apparatus fitted to an autosampler, a Prostar Pump model 210 and a Prostar UV–visible diode array detector model 335. A Phenomenex Lux Amylose-1 (150 × 4.6 mm) column was used for the chiral separation. The solvent system was composed by hexane acidified with 0.5% TFA as solvent A and isopropanol acidified with 0.5% TFA as solvent B. Runs were conducted at a flow rate of 1 mL min^{-1} using an isocratic mode at 20% B (0–40 min). *E*-ε-Viniferin powder isolated from each cultivar was solubilized at 1 mg mL^{-1} in methanol. The enantiomeric excess (ee) of *E*-ε-viniferin in grapevine canes, expressed as a percentage, was calculated as follows:

$$\text{ee}\,(\%) = |\%\text{enantiomer}\,\mathbf{1} - \%\text{enantiomer}\,\mathbf{2}\,|$$

where % enantiomer **1** + % enantiomer **2** = 100%.

Statistical analysis

Two independent extractions on 22 grapevine cane cultivars in two different blocks were performed. Each grapevine cane extract was analyzed independently in triplicate. Data are shown as means ± standard deviation (SD). A one-way analysis of ANOVA followed by Tukey's multiple-comparison post hoc tests was performed to differentiate the *E*-ε-viniferin content in the different grapevine cultivars. GraphPad Prism software version 9 (La Jolla, CA, USA) was used. In order to discriminate the several grape varieties according to the proportion of each enantiomer as well as enantiomeric excess, multivariate statistical analysis was carried out using R x64 3.6.3 software with the Multivariate Analysis FactomineR package.[24] Principal component analysis (PCA) was conducted to visualize the acquired data and observe the discrete trend between grapevine cane cultivars.

RESULTS AND DISCUSSION

Quantification of *E*-ε-viniferin in grapevine cane

E-ε-Viniferin was quantified by UHPLC-DAD-MS in the various grapevine cane cultivars using pure standard. Analyses were conducted at the maximum absorption (λ_{max}) of the targeted compounds, i.e. at 320 nm. The UHPLC quantification method was validated according to Feinberg's method with regard to precision, accuracy, sensitivity and linearity.[23] A correlation coefficient superior to 0.99 was obtained highlighting a sharp linearity between peak areas and concentrations. All parameters of *E*-ε-viniferin, such as retention time, UV data, mass spectrometry data, and calibration curve, are presented in Table 1.

The quantitation of *E*-ε-viniferin in grapevine cane of the different grape varieties is presented in Fig. 1. Focusing on the *E*-ε-viniferin content in the 14 red cultivars, the highest concentrations were found in blocks 1 and 2, respectively, with the Portuguese cultivar Touriga nacional N (3.2 and 2.6 g kg^{-1} dry weight (DW)), followed by Prunelard N (2.4 and 2.3 g kg^{-1} DW) and Pinot noir N (2.4 and 2.0 g kg^{-1} DW). Conversely, the lowest contents were observed with the cultivars Tempranillo N (0.5 and

Table 1. Retention time, UV data, mass spectrometric data (pseudomolecular ion and produced ions, negative mode), calibration curve,[a] limit of detection (LOD), limit of quantification (LOQ), and determination coefficients (R^2) of *E*-ε-viniferin

	t_R (min)	λ_{max} (nm)	$[M-H]^-$ (*m/z*)	MS/MS fragments	slope *a*	Intercept *b*	LOD (µg mL^{-1})	LOQ (µg mL^{-1})	R^2
E-ε-viniferin	7.2	320	453	435, 411, 369, 359, 347, 265	6.0433	0.3763	0.13	0.29	0.9999

[a] The equation curve is $y = ax + b$, where y is the area under the peak, x is the concentration of *E*-ε-viniferin (µg mL^{-1}), a is the slope and b is the intercept.

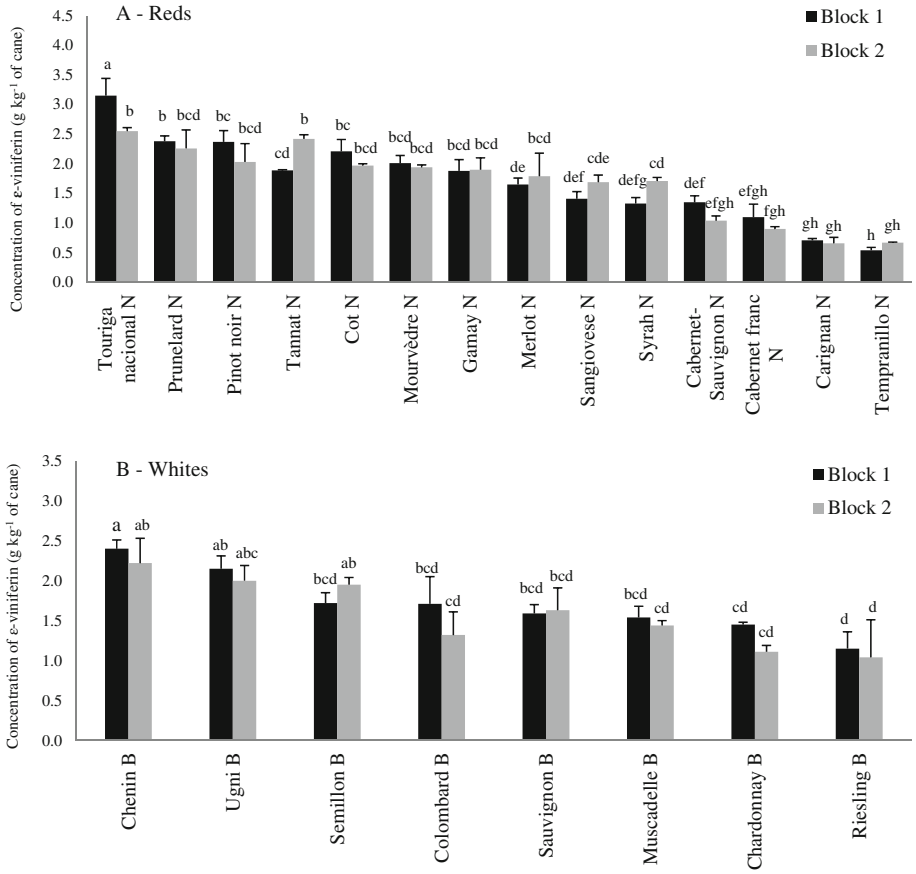

Figure 1. *E*-ε-Viniferin content (g kg^{-1} DW) in grapevine canes of *V. vinifera* cultivars from block 1 (black bars) and block 2 (grey bars): (A) red cultivars (upper part); (B) white cultivars (lower part). Different letters indicate significant difference between *E*-ε-viniferin concentrations according to one-way ANOVA, followed by Tukey's multiple comparison post hoc tests.

0.7 g kg^{-1} DW) and Carignan N (0.7 and 0.7 g kg^{-1} DW) (Fig. 1(A)). With regard to the eight white cultivars, the cultivars Chenin B (2.4 and 2.2 g kg^{-1} DW) and Ugni blanc B (2.2 and 2.0 g kg^{-1} DW) were the richest in *E*-ε-viniferin in blocks 1 and 2, respectively, while the cultivars Chardonnay B (1.5 and 1.1 g kg^{-1} DW) and Riesling B (1.2 and 1.0 g kg^{-1} DW) were the poorest in blocks 1 and 2, respectively (Fig. 1(B)). The average of *E*-ε-viniferin content for all the cultivars was estimated as 1.7 g kg^{-1} DW. Several studies have already investigated the stilbene content in grapevine cane and our results on *E*-ε-viniferin concentration were in agreement with those of other authors. Lambert *et al.* conducted experiments in 16 grape varieties of *V. vinifera* showing that *E*-ε-viniferin was one of the most abundant stilbenes in all cultivars.[14] The lowest *E*-ε-viniferin value was at 0.9 g kg^{-1} DW in Carignan N and the highest at 3.7 g kg^{-1} DW in Pinot noir N, which was in accordance with our findings. Similarly, Guerrero *et al.* (2016) worked on 22 grape cultivars, showing *E*-ε-viniferin concentrations between 0.9 g kg^{-1} DW (Palomino fino cultivar) and 2.8 g kg^{-1} DW (Gewürztraminer cultivar).[24] Furthermore, Pawlus *et al.* (2013) investigated stilbene content in grapevine cane from 16 wild-type *Vitis* in which the *E*-ε-viniferin content ranged between 0.7 and 5.7 g kg^{-1} DW.[13]

Regarding the comparison of *E*-ε-viniferin content between the two parcels, it appeared globally that no notable difference of concentration occurred for each grapevine cultivar. Indeed, the *E*-ε-viniferin content in blocks 1 and 2 was similar in most of cultivars such as in Cabernet franc N (1.1 and 0.9 g kg^{-1} DW, respectively), in Merlot N (1.7 and 1.8 g kg^{-1} DW, respectively), or in Sangiovese N (1.4 and 1.7 g kg^{-1} DW, respectively) (Fig. 1). The only differences occurred with the Touriga nacional N and Tannat N cultivars, with significant differences between the two blocks.

Overall, the *E*-ε-viniferin content varied among various *V. vinifera* cultivars, whereas it was relatively stable for the same grape variety located in two different plots. These findings suggest a key role of the genotype to determine the concentration of *E*-ε-viniferin in vegetative parts of the vine.

Distribution of *E*-ε-viniferin enantiomers in grapevine cane

E-ε-Viniferin is a mixture of two enantiomers that were unambiguously characterized in our previous work.[25] The determination of *7aS*, *8aS* configuration of the chiral centers corresponded to the dextrorotatory form (i.e. positive rotatory power), whereas the *7aR*, *8aR* configuration related to the levorotatory form (i.e. negative rotatory power). The two enantiomers (*7aR*, *8aR*)-(−)-*E*-ε-viniferin (**1**) and (*7aS*, *8aS*)-(+)-*E*-ε-viniferin (**2**) are presented in Fig. 2.

The proportions of each enantiomer as well as the enantiomeric excess were calculated in the various grapevine cultivars owing to accurate isolation of *E*-ε-viniferin followed by a chiral analysis by HPLC-DAD. Based on an isocratic separation, the first peak, with a retention time of 9.0 min, matched the (*7aR*, *8aR*)-(−)-*E*-ε-viniferin and the second one, with a retention time at 16.3 min, corresponded to the (*7aS*, *8aS*)-(+)-*E*-ε-viniferin (Fig. 3). The

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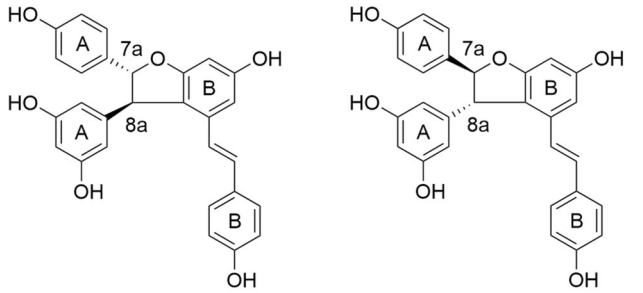

Figure 2. Structure of *E*-*ε*-viniferin enantiomers **1** and **2**.

chromatographic elution monitored at 320 nm allowed us to determine the peak areas and therefore the relative abundance of each enantiomer and the enantiomeric excess for the 22 *V. vinifera* cultivars. The enantiomeric excess is an absolute value highlighting the degree to which one sample contains one enantiomer in greater quantity than the other. In this sense, a racemic mixture has an enantiomeric excess of 0%, while a single completely pure enantiomer has an enantiomeric excess of 100%.

Among the red cultivars, the grape varieties Cabernet-Sauvignon N and Cabernet franc N were strongly rich in enantiomer **2**, the (*7aS*, *8aS*)-(+)-*E*-*ε*-viniferin, with mean values at 94.8% and 92.4%, showing an enantiomeric excess of 89.6% and 84.8%, respectively (Table 2). The Carignan N cultivar was also mainly composed of the enantiomer **2** form, with a mean proportion at 68.7% and an enantiomeric excess of 37.4%. Conversely, several red cultivars such as Cot N, Syrah N, Tannat N, Merlot N and Prunelard N were predominantly constituted of enantiomer **1**, (*7aR*, *8aR*)-(−)-*E*-*ε*-viniferin, with mean values at 71.0%, 69.2%, 68.8%, 63.8% and 55.7%, generating an enantiomeric excess between 42.0% and 11.4%. A racemic mixture, i.e. an equal proportion of the levorotatory and dextrorotatory enantiomers of a chiral compound, was observed for the grape varieties Touriga nacional N, Sangiovese, Gamay N, Mourvèdre N and Pinot noir N, with enantiomeric excess values of between 1.6% and 7.8% (Table 2).

Regarding the white cultivars, the grape varieties Muscadelle B and Chardonnay B showed the highest proportion of enantiomer **1**, with mean values at 67.5% and 62.0%, respectively, and therefore an enantiomeric excess of 35.0% and 24.0% (Table 2). Other studied white cultivars such as Semillon B, Sauvignon B, Riesling B, Colombard B, Chenin blanc B and Ugni blanc B presented globally a racemic mixture. It could be noted that the cultivar Ugni blanc B was the only one that exhibited a slightly major proportion of enantiomer **2** (54.6%).

This was the first time that the enantiomeric excess of *E*-*ε*-viniferin had been determined in several grapevine canes. Some studies have focused on the chiral separation of viniferins, especially *E*-*δ*-viniferin and *E*-*ε*-viniferin, which were synthetically obtained using an enzymatic- or metal-catalyzed oxidative coupling approach.[26,27] In plant extracts, the enantiomeric excess of some naturally occurring compounds have been reported. With regard to polyphenols, the enantiomers of the flavonoid catechin were isolated with 35% enantiomeric excess in *Arbutus unedo* L. (strawberry tree),[28] while several norlignan, neolignan and lignan enantiomers were characterized in *Acorus tatarinowii* S. with variable enantiomeric excess values oscillating between 0% and 75%.[29] As each enantiomer often has different biological activities, as one possesses beneficial therapeutic effect and the other has no or negative impact, the chiral analysis of natural compounds in plant extracts is essential to ensure their pharmaceutical properties.

Our study also showed that the percentage of each enantiomer in the various *V. vinifera* cultivars was similar between the two blocks (Table 2). For instance, enantiomer **1** was present in Cot N cultivar at 69.5% and 72.4% in blocks 1 and 2, respectively, in Merlot N 62.9% and 64.7% in blocks 1 and 2, respectively, and in Syrah N at 71.6% and 66.7% in blocks 1 and 2, respectively. Similarly, in a non-exhaustive way, enantiomer **2** was evaluated in blocks 1 and 2 in Cabernet franc N at 93.2% and 91.7%, respectively, in Cabernet-Sauvignon N at 96.0% and 93.6%, respectively, and in Tempranillo N at 60.6% and 55.8%, respectively. Despite the substantial variation of enantiomeric excess between the different cultivars, the proportions of enantiomers from a grape variety were close. To support these claims, in our previous work dealing with the structural elucidation of viniferin stereoisomers, the enantiomeric excess of *E*-*ε*-viniferin was determined in the red cultivars Cabernet-Sauvignon N and Merlot N from a vineyard in the region of Bordeaux, in which values obtained were 98.34% (mainly enantiomer **2**) and 23.32% (mainly enantiomer **1**), respectively.[25] The similar enantiomeric excess values from a grapevine cultivar such as Cabernet-Sauvignon N or Merlot N belonging to different vineyard and different years underlined the influence of the genetic material specific to each grape variety in the production of *E*-*ε*-viniferin and more particularly in the differences of enantiomeric ratio observed. In order to achieve a robust varietal classification, more studies are needed in order to demonstrate whether the intervarietal differences observed are more prevalent than the intravarietal differences due to different edaphoclimatic conditions.

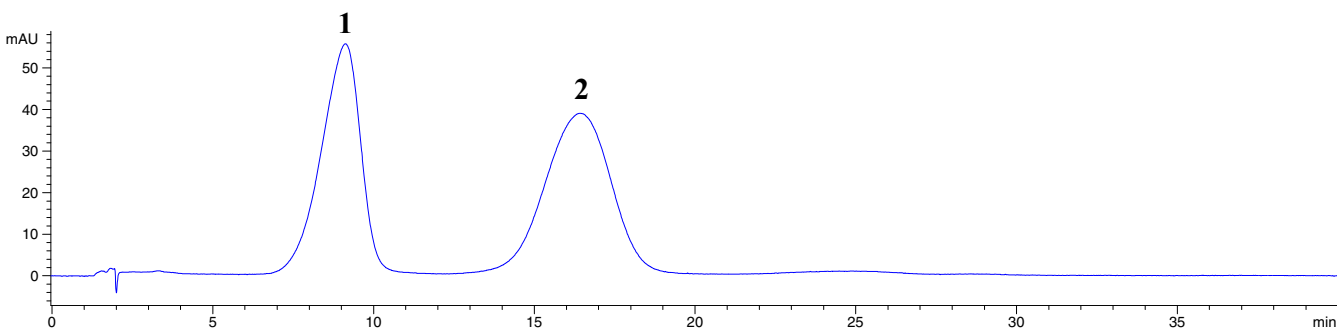

Figure 3. HPLC-UV chromatogram at 320 nm of the chiral separation of the *E*-*ε*-viniferin enantiomers **1** and **2**.

Table 2. Proportions (%) and enantiomeric excess (%) of *E*-ε-viniferin enantiomers in grapevine cane of red and white *V. vinifera* cultivars from two different blocks (1 and 2)

	Enantiomer **1** (%)	Mean	Enantiomer **2** (%)	Mean	Enantiomeric excess (%)
Red cultivars					
Cabernet franc N-1	6.8	7.6	93.2	92.4	84.8
Cabernet franc N-2	8.3		91.7		
Cabernet-Sauvignon N-1	4.0	5.2	96.0	94.8	89.6
Cabernet-Sauvignon N-2	6.4		93.6		
Carignan N-1	28.8	31.3	71.2	68.7	37.4
Carignan N-2	33.8		66.2		
Cot N-1	69.5	71.0	30.5	29.0	42.0
Cot N-2	72.4		27.6		
Gamay N-1	46.4	49.0	53.6	51.0	2.0
Gamay N-2	51.6		48.4		
Merlot N-1	62.9	63.8	37.1	36.2	27.6
Merlot N-2	64.7		35.3		
Mourvèdre N-1	52.9	50.8	47.1	49.2	1.6
Mourvèdre N-2	48.6		51.4		
Pinot noir N-1	53.3	51.8	46.7	48.2	3.6
Pinot noir N-2	50.3		49.7		
Prunelard N-1	57.4	55.7	42.6	44.3	11.4
Prunelard N-2	54.0		46.0		
Sangiovese N-1	46.8	48.3	53.2	51.7	3.4
Sangiovese N-2	49.8		50.2		
Syrah N-1	71.6	69.2	28.4	30.8	38.4
Syrah N-2	66.7		33.3		
Tannat N-1	73.5	68.8	26.5	31.2	37.6
Tannat N-2	64.0		36.0		
Tempranillo N-1	39.4	41.8	60.6	58.2	16.4
Tempranillo N-2	44.2		55.8		
Touriga Nacional N-1	46.9	46.1	53.1	53.9	7.8
Touriga Nacional N-2	45.3		54.7		
White cultivars					
Chardonnay B-1	61.5	62.0	38.5	38.0	24.0
Chardonnay B-2	62.5		37.5		
Chenin B-1	55.1	56.2	44.9	43.8	12.4
Chenin B-2	57.3		42.7		
Colombard B-1	53.0	54.6	47.0	45.4	9.2
Colombard B-2	56.1		43.9		
Muscadelle B-1	63.3	67.5	36.7	32.5	35.0
Muscadelle B-2	71.6		28.4		
Riesling B-1	50.7	53.3	49.3	46.7	6.6
Riesling B-2	55.9		44.1		
Sauvignon B-1	46.3	50.0	53.7	50.0	0.0
Sauvignon B-2	53.7		46.3		
Semillon B-1	49.0	49.1	51.0	50.9	1.8
Semillon B-2	49.2		50.8		
Ugni blanc B-1	41.0	45.4	59.0	54.6	9.2
Ugni blanc B-2	49.8		50.2		

Overall, this pioneering work paves the way for further studies to understand the mechanisms involved in the production of these enantiomers in grapevine. A hypothesis could be the presence of a chiral intermediate involved in the oxidative condensation reactions, resulting in the formation of *E*-ε-viniferin enantiomers. This key role of intermediate could be played by the dirigent proteins, which are proteins imparting stereoselectivity on the phenoxy radical-coupling reaction.[30] They were discovered by Lewis and coauthors in 1997, who characterized a protein in *Forsythia intermedia* that controls the monolignol coniferyl alcohol radical coupling, catalyzed by peroxidases, to afford the (+)-pinoresinol.[31] The dirigent proteins were principally described in the scientific literature for lignan biosynthesis, where two chiral centers are introduced during the formation of a covalent bond between C-8 and C-8′.[31,32] However, Borges and coworkers showed the presence of dirigent proteins in *V. vinifera* and suggested that an alternative lignan biosynthetic pathway exists or that these proteins could be involved in reactions other than

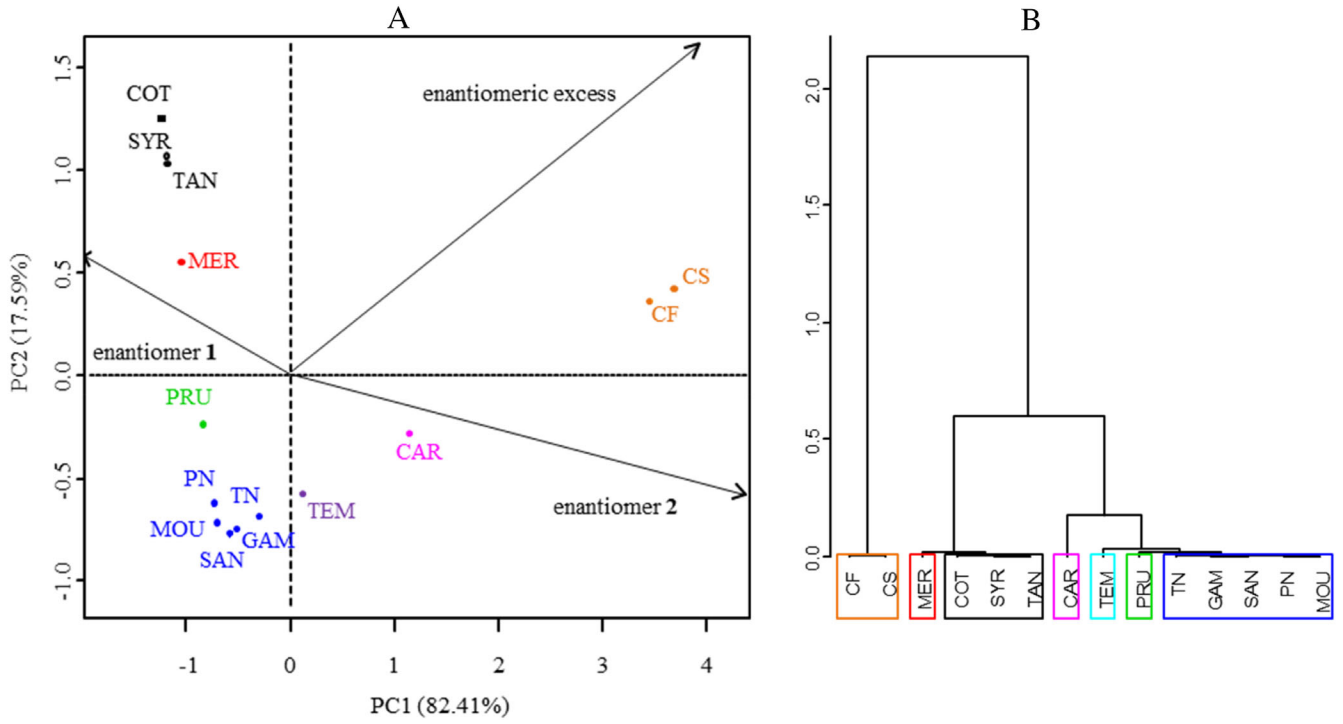

Figure 4. (A) Principal component analysis, showing the distribution of the 14 red *Vitis* cultivars, colored by cluster, according to the enantiomer proportions and enantiomeric excess variables. (B) Hierarchical clustering on principal components, illustrating the proximity of the different red *V. vinifera* cultivars studied. CF, Cabernet franc N; CS, Cabernet-Sauvignon N; CAR, Carignan N; COT, Cot N; GAM, Gamay N; MER, Merlot N; MOU, Mourvèdre N; PN, Pinot noir N; PRU, Prunelard N; SAN, Sangiovese; SYR, Syrah N; TAN, Tannat N; TEM, Tempranillo N; TN, Touriga nacional N.

the dimerization of coniferyl alcohol.[33] In this sense, dirigent proteins could be expressed differentially according to cultivar genotype and may possibly be involved in the oxidative condensation reactions of resveratrol in grapevine.

Enantiomeric excess of *E*-ε-viniferin as a chemotaxonomic marker of the vine

To appreciate the similarities and differences between the 14 red cultivars genotypes studied, a principal component analysis (PCA) was carried out considering the balance of each enantiomer as well as the enantiomeric excess (Fig. 4(A)). The first dimension (PC1), accounting for 82.41% of the total variance, was associated with high content of enantiomer **2** as well as high enantiomeric excess values. The cultivars Cabernet franc N and Cabernet-Sauvignon N were clustered together on the positive side of the PC1. The cultivar Carignan N, and to a lesser extent the cultivar Tempranillo N, were also individually represented on the positive side of the PC1. The second dimension (PC2), explaining 17.59% of the total variance, was associated with the high concentration of enantiomer **1**. The grape varieties Cot N, Syrah N and Tannat N were grouped together on the positive side of PC2 as well as the cultivar Merlot N, which was individually classified. On the negative side of both dimensions, corresponding to a racemic mixture of *E*-ε-viniferin enantiomers, the rest of the cultivars Pinot noir N, Gamay N, Touriga nacional N, Mourvèdre N, and Sangiovese N were clustered together and separated from the cultivar Prunelard N, also represented on both negative sides (Fig. 4(A)). Additionally, a hierarchical clustering on principal components (HCPC) was carried out in order to visualize the closeness of the different red *V. vinifera* cultivars (Fig. 4(B)). This classification confirmed the proximity of Cabernet-Sauvignon N and Cabernet franc N. Based on a parentage study, the cultivar Cabernet-Sauvignon N was reported as the progeny of Cabernet franc N and therefore sharing a genetic inheritance.[34] Moreover, it has been shown that the cultivars Merlot N and Cot N have a parent in common – *V. vinifera* cv. Magdeleine Noire des Charentes – which would explain their richness in enantiomer **2** as well as their hierarchical proximity.[34] Similarly, Gamay N's ancestor was investigated, and it was Statistically supported that the cultivar Pinot noir N was a parent.[35] These two grape varieties were part of the same cluster, marked by a racemic mixture and an enantiomeric excess close to zero.

Besides the red cultivars, a PCA was conducted on the white cultivars (Fig. 5). The first dimension, which explained 95.44% of the total variance, was associated with both high content of enantiomer **1** and enantiomeric excess values. Conversely, the second dimension, accounting for 4.56% of the total variance, corresponded to a high content of enantiomer **2** (Fig. 5(A)). The cultivars Muscadelle B and Chardonnay B were represented on the positive side of the PC1, meaning a notable content of enantiomer **1**. Conversely, the cultivar Ugni blanc B was represented on the positive side of the PC2, exerting an unbalanced enantiomeric ratio towards enantiomer **2**. Other cultivars, such as Semillon B, Sauvignon B, Riesling B and Colombard B, were found on the negative side of both dimensions, corresponding to a racemic mixture of *E*-ε-viniferin enantiomers (Fig. 5(A)). HCPC on white cultivars was also performed, consolidating a notable proximity between the cultivars Muscadelle B and Chardonnay B (Fig. 5(B)). A parental relationship study has reported that Muscadelle B and Chardonnay B possess a parent in common – *V. vinifera* cv. Gouais blanc B – which would explain the closeness of these cultivars.[36] Similarly, another work has demonstrated that the cultivar Colombard

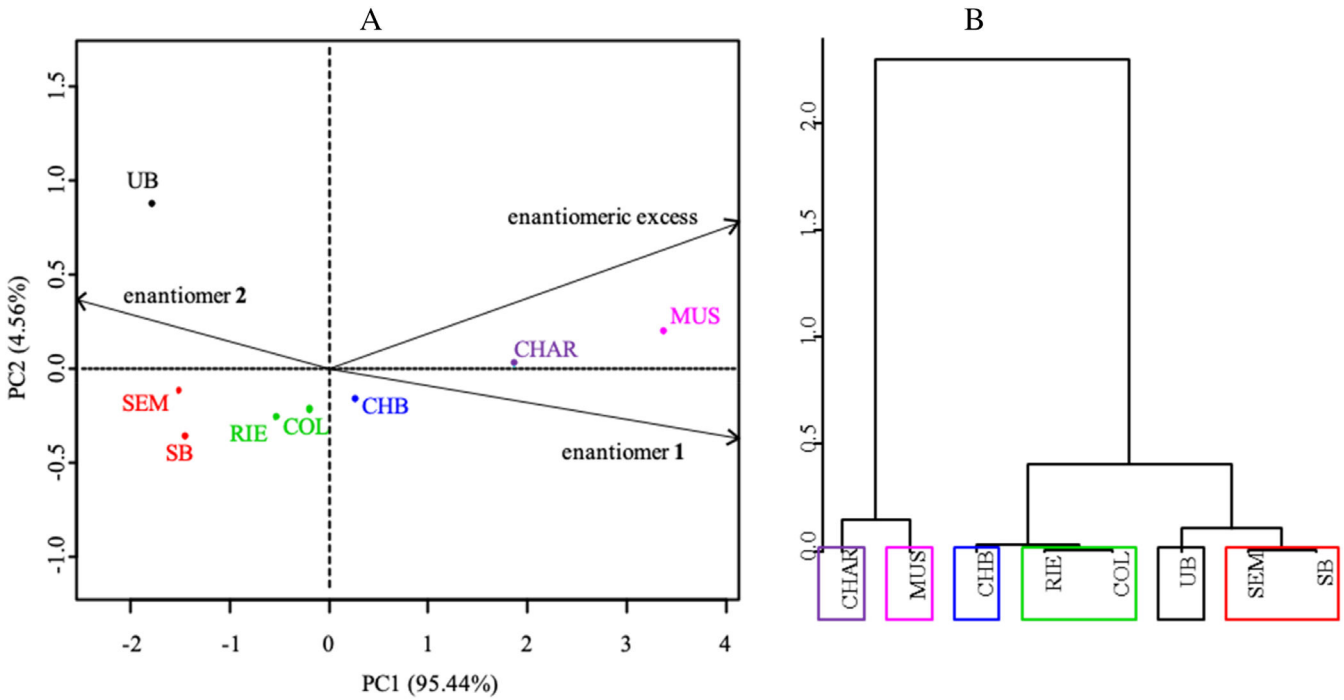

Figure 5. (A) Principal component analysis, showing the distribution of the eight white *Vitis* cultivars, colored by cluster, according to the enantiomer proportions and enantiomeric excess variables. (B) Hierarchical clustering on principal components, illustrating the proximity of the different white *V. vinifera* cultivars studied. CHAR, Chardonnay B; CHB, Chenin blanc B; COL, Colombard B; MUS, Muscadelle B; RIE, Riesling B; SB, Sauvignon B; SEM, Semillon B; UB, Ugni blanc B.

B was the progeny of the cultivars Chenin blanc B and Gouais blanc B.[37] These findings are in accordance with the observed HCPC in which Colombard B and Chenin blanc B were closely grouped (Fig. 5(B)). A relationship between the enantiomeric excess of *E*-ε-viniferin and the genetic patrimony of *V. vinifera* grape varieties was strongly suggested.

CONCLUSIONS

To summarize, the present study provides a suitable method to distinguish *V. vinifera* cultivars through the analysis of the enantiomeric excess of *E*-ε-viniferin in grapevine cane. A small amount (only few grams) of the vegetative part is needed for the extraction and chiral analysis. The relative abundance of the two enantiomers (*7aR*, *8aR*)-(−)-*E*-ε-viniferin (**1**) and (*7aS*, *8aS*)-(+)-*E*-ε-viniferin (**2**) allowed us to evaluate the enantiomeric excess, which depends directly on the cultivar. In addition, to be able to distinguish grape varieties, the use of *E*-ε-viniferin enantiomers allowed us to establish cultivar relationships and pattern through statistical analysis. The enantiomeric excess of *E*-ε-viniferin could thus be considered as a chemical marker of grapevine, and the chemotaxonomic method described in the present work could be complementary to phenotypic or genotypic approaches. Further investigation should be undertaken to understand the mechanism involved in the biosynthesis of each enantiomer as well as to elucidate the reason for *E*-ε-viniferin enantiomeric excess variation in the different grapevine cultivars.

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CONFLICT OF INTEREST

The authors declare no competing financial interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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