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# Combining Laccase-Mediated Dimerization of Resveratrol and Centrifugal Partition Chromatography: Optimization of E -Labruscol Production and Identification of New Resveratrol Dimers

Emmanuel Sursin, Amandine Flourat, Z. Akissi, Agathe Martinez, Nicolas Borie, Cedric Peyrot, Eric Courot, Jean-Marc Nuzillard, Jean-Hugues Renault, Laurence Voutquenne-Nazabadioko, et al.

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Combining laccase-mediated dimerization of  
resveratrol and Centrifugal Partition  
Chromatography: Optimisation of *E*-Labruscol  
production and identification of new resveratrol  
dimers.

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KEYWORDS Biocatalysis, Centrifugal Partition Chromatography NMR spectroscopy, Resveratrol, Labruscol

ABSTRACT Resveratrol dimers are of great interest for pharmaceutical and cosmetic applications. Nevertheless, the yield of their bio-production is limited by both the competition between the possible radical-radical coupling pathways and complex isolation procedures. Alternative organic synthesis methods do not afford higher yields. Although enzymatic routes can provide dimers in one step from resveratrol, bio-catalysis optimisation is required to improve yields and orient radical-radical coupling selectivity toward a specific resveratrol dimer, *E*-labruscol herein. After a rapid study of the relative importance of the bio-catalysis parameters, a design of experiments was implemented to produce *E*-labruscol in high yield by laccase-mediated dimerization of resveratrol. *E*-labruscol and  $\delta$ -viniferin were identified and isolated by flash chromatography as major products in 21% and 52% yields, respectively. As an alternative to purification on silica gel, an efficient separation of the aforementioned compounds was achieved by centrifugal partition chromatography (CPC). This technology provided  $\delta$ -viniferin in 63.1% yield (90% purity) and labruscol isomers in 20.4% yield with a purity of 95% after a CPC polishing step, but it also revealed the presence of *E*-labruscol diastereomers, leachianol F and

leachianol G, as major reaction products, as well as less abundant products: pallidol, Z-labruscol,  $\epsilon$ -viniferin and two new resveratrol dimers named iso- $\delta$ -viniferin and iso- $\epsilon$ -viniferin.

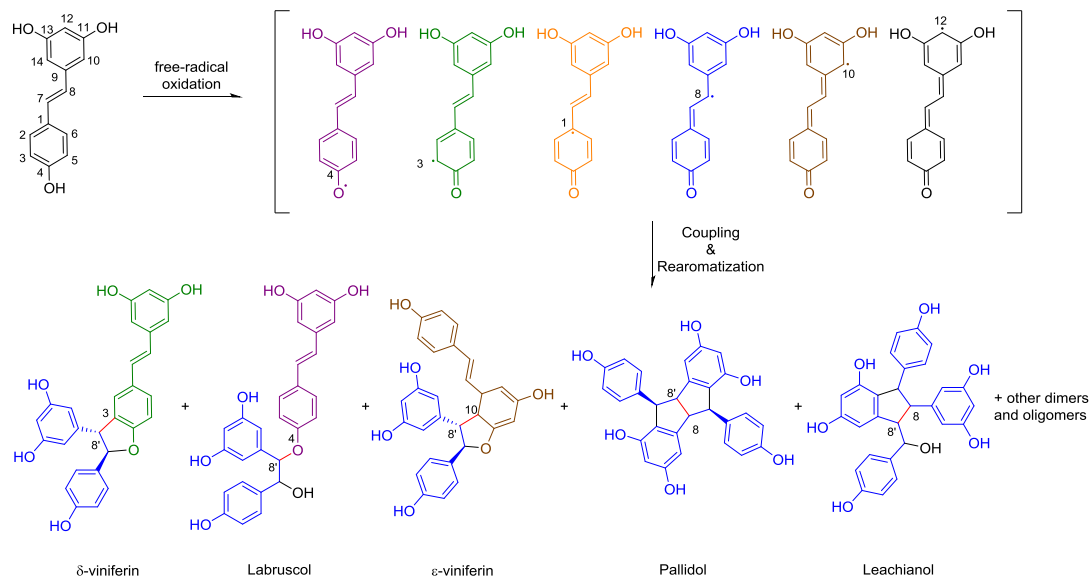
## INTRODUCTION

Resveratrol and its dimers, such as viniferins, are well-known plant specialized metabolites.<sup>1,2</sup> These phyto stilbenes have attracted much attention due to their antioxidant,<sup>3,4</sup> anticarcinogenic,<sup>1,5-8</sup> antiaging<sup>9</sup> and anti-inflammatory activities.<sup>10</sup> Although their extraction from vine shoots used to be industry-scale processes, thorough and rather expensive purification procedures were required due to their low concentration in plants, especially for dimers such as viniferins.<sup>11</sup> As an alternative, the bio-production of resveratrol and/or its dimers was first achieved in engineered micro-organisms (*e.g.*, *E. coli*, *S. cerevisiae*) but concentration in fermentation broth remained often under the 1 g.L<sup>-1</sup> limit. The highest yield value reported in the review by Thapa *et al.* was 2.3 g.L<sup>-1</sup> of resveratrol but it required expensive starting material such as *p*-coumaric acid and cerulenin.<sup>11</sup> Bio-production of resveratrol and dimers such as  $\delta$ -viniferin, pallidol and  $\epsilon$ -viniferin (Scheme 1) has proven to be more efficient from cell suspension of *Vitis labrusca* L. and elicitation with methyl jasmonate and cyclodextrins resulting in a concentration up to 4.2 g.L<sup>-1</sup> in a 20 L bioreactor for resveratrol, simply using sucrose as carbon source.<sup>12</sup> A new dimer of resveratrol, *E*-labruscol, was also isolated from this culture.<sup>13</sup> *E*-Labruscol showed IC<sub>50</sub> values of about 20  $\mu$ M against two human skin malignant melanoma cancer cell lines (HT-144 and SKMEL-28) and a healthy human dermal fibroblast HDF line.<sup>14</sup> However, as 10 L of culture medium was required to produce only 41 mg of *E*-labruscol,<sup>13</sup> the search for a new approach had to be considered in complement of grapevine cell culture optimization. In a recent study, *E*-Labruscol diastereomers were isolated in small amounts (7.7

and 1.1 mg) from biotransformation of a mix of pterostilbene and resveratrol using a secretome of *Botrytis cinerea*.<sup>15</sup> Their activities against breast cancer cell were evaluated.<sup>15</sup>

Reaction of resveratrol with an oxidase such as a peroxidase or a laccase resulted in its oligomerization.<sup>16–20</sup> *In planta*, such processes leads to a wide diversity of oligomers through different radical-radical coupling patterns.<sup>21</sup> Although  $\delta$ -viniferin is often the major product of the relatively complex mixture of oligomers obtained by enzymatic dimerization of resveratrol (Scheme 1), Ponzoni *et al.* have reported the presence of a very small amount of labruscol ( $\approx 5\%$ ) during the laccase-mediated dimerization of resveratrol.<sup>17</sup> In a previous study, dedicated to laccase dimerization of sinapyl alcohol, we have demonstrated that the initial selectivity of the reaction toward the 8-*O*-4 dimer can be reversed and favour the production of 8-8 dimer by fine-tuning the experimental biocatalysis conditions.<sup>22</sup> Increasing yields of *E*-labruscol by optimizing the conditions of the laccase-mediated biocatalytic dimerization of resveratrol thus appeared to be a realistic goal. A design of experiment (DoE) approach was carried out to determine the optimal settings of the enzymatic reaction parameters (*i.e.*, resveratrol concentration, enzyme/substrate ratio, media composition, addition rate of the enzymatic solution) for *E*-labruscol production. Purification of the resulting stilbenes was then achieved by a combination centrifugal partition chromatography (CPC)<sup>13</sup> and preparative HPLC. CPC is a solid support-free liquid-liquid chromatographic technique based on the partitioning of solutes between at least two immiscible liquid phases. A CPC column consists on a series of interconnected cells engraved circumferentially on stacked disks rotating around a single axis. The liquid stationary phase is maintained inside the column by a constant centrifugal force field while the other non-miscible liquid phase is pumped through it. This technique is very attractive in terms of selectivity, sample loading capacity and scaling-up ability together with avoiding irreversible adsorption on

chromatographic solid-support, the latter aspect justifying its importance in the field of natural product purification.<sup>10</sup> Moreover, the chemical profiling of the produced dimers was achieved by combining a <sup>13</sup>C NMR based dereplication workflow for the identification of the already known compounds and a classic *de novo* structure elucidation approach for the original dimers.



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resveratrol dimerization and corresponding dimers.

## MAIN METHODS

### General reaction procedure

Resveratrol (114 to 456 mg) was dissolved in the organic solvent (EtOAc or acetone) then the additional volume of distilled water was added to reach 25 mL. The reaction medium was stirred at 700 rpm and brought to the targetted temperature. Using a syringe pump, 7 mL of laccase solution at the desired concentration (0.43 to 17.1 U.mL<sup>-1</sup>) was added at the predetermined rate (7 to 50 mL.h<sup>-1</sup>). The reaction was stopped by adding 1 M HCl solution (1 mL). The layers were

separated when the reaction mixture was biphasic and when it was homogeneous, acetone was evaporated under reduced pressure. The aqueous layer was extracted twice with ethyl acetate (2x10 mL). The organic layers were combined, washed with brine (10 mL), dried over anhydrous MgSO<sub>4</sub>, filtered and concentrated.

### **Scaled-up reaction procedure**

Resveratrol (2.0 g, 8.76 mmol) was dissolved in acetone (305 mL) then distilled water (130 mL) was added. The reaction medium was vigorously stirred and heated at 45 °C. Using a syringe pump, 28 mL of laccase solution (18.8 U.mL<sup>-1</sup>, 60 U.mmol<sup>-1</sup>) was added over 4 hours. Two hours after the end of the addition, only a small amount of residual resveratrol was detected by TLC, the reaction was stopped by adding 1 M HCl solution (5 mL). Acetone was distilled under reduced pressure. The aqueous layer was extracted twice with ethyl acetate (2x100 mL). The organic layers were combined, washed with brine (100 mL), dried over anhydrous MgSO<sub>4</sub>, filtered and concentrated.

### **CPC fractionation**

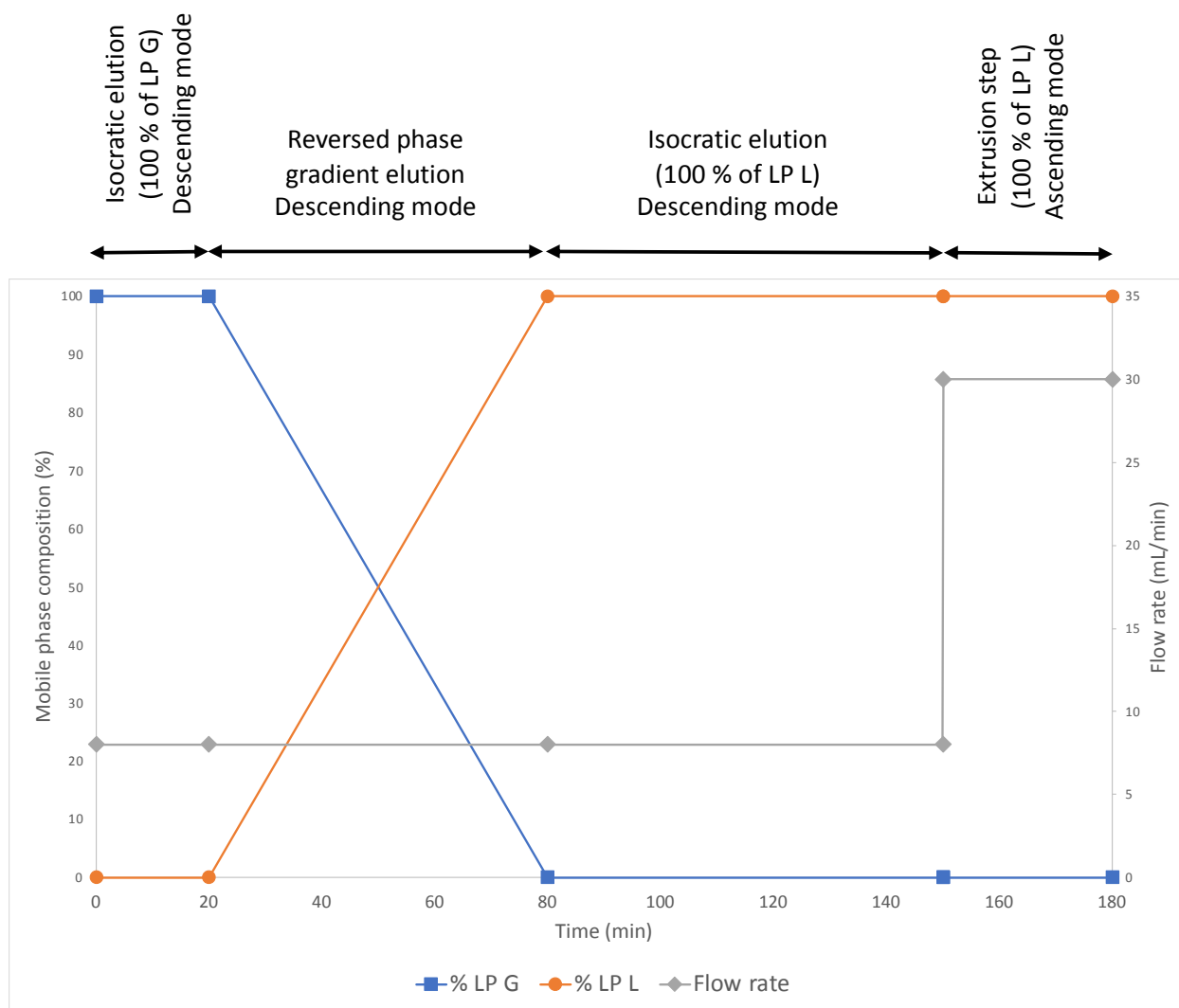
Centrifugal partition chromatography (CPC) was carried out on a lab-scale FCPC<sup>®</sup> A200+PF 5125 column of 270 mL capacity (Rousselet Robatel Kromaton, Annonay, France) containing twenty-one circular partition disks, engraved with a total of 840 oval partition twin-cells (~10 mL per twin-cell) and connected to an integrated and automated peripheral PF 5125K including the injection valve, the gradient pump, the UV/Vis detector, the fraction collector and the control unit.

The crude extract (2 g) obtained from the enzyme catalysis was fractionated using a gradient elution method in the descending mode in a single run of 150 min. Three biphasic solvent

systems were prepared independently by mixing *n*-heptane, EtOAc, MeOH and water in the proportions 2/3/2/3 (v/v) for system L, 2/5/2/5 (v/v) for system J and 1/4/1/4 (v/v) for system G. The L, J and G letters for naming the biphasic solvent systems correspond to the notations used to describe the systems of the HemWat solvent scale.<sup>23</sup> These three systems were selected to gradually decrease the polarity of the phase mobile from system G to system L while ensuring the biphasic character of the global system with MeOH and water as major constituents in the lower phase. The sample solution was prepared by directly dissolving the crude stilbene mixture (2 g) in 12 mL of a mixture of System J upper and System G lower phases (75/25, v/v). The CPC column was filled with the upper organic phase of System J at 600 rpm. After loading the sample solution into the column through a 20 mL sample loop, the rotation speed was adjusted to 1400 rpm and the flow rate was set at 8 mL.min<sup>-1</sup>.

The elution was carried out, as shown in Figure 1, in descending mode by pumping 100% of the lower phase of system G for 20 min (first isocratic section), then this phase was reduced from 100% to 0% in 60 min (gradient section). The lower phase of system L was held at 100% for 70 min (second isocratic section). Finally, the column was extruded by switching from descending mode to ascending mode, while continuing to pump 100% of the lower phase of system L at 30 mL.min<sup>-1</sup>.





**Figure 1.** CPC fractionation: description of the reversed phase gradient elution mode procedure.

LP G: lower aqueous phase of system G; LP L: lower aqueous phase of system L.

The experiments were carried out at room temperature. 8 mL fractions were collected every minute and spotted onto Merck TLC plates coated with silica gel 60 F254 and developed with chloroform/methanol/acetic acid (83:17:3, v/v/v). After detection at UV254 and UV366, the plates were sprayed with vanillin-sulfuric acid and heated at 100 °C for 5 min. Fractions were

then pooled based on their TLC profile similarities and solvents evaporated under vacuum. A total of 17 fractions were obtained.

### **Chemical profiling of CPC fractions**

Identification of compounds was performed in collected fractions using a dereplication procedure based on  $^{13}\text{C}$  nuclear magnetic resonance (NMR) <sup>14,24</sup>

About 15 mg of each fraction were dissolved in 600  $\mu\text{L}$  DMSO-*d*<sub>6</sub> and analyzed by nuclear magnetic resonance (NMR) spectroscopy at 298 K on a Bruker Avance AVIII-600 spectrometer.  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were acquired at 600.15 MHz and 150.91 MHz, respectively. Additional heteronuclear single quantum coherence (HSQC), heteronuclear multiple bond correlation (HMBC), and homonuclear correlation spectroscopy (COSY) 2D-NMR experiments were performed on fractions containing putatively identified compounds using standard Bruker microprograms. After spectra processing using the TOPSPIN 4.0.5 software (Bruker), the absolute intensities of all  $^{13}\text{C}$  NMR signals detected in all spectra were collected by automatic peak picking. Then the  $^{13}\text{C}$  NMR spectral width (from 0 to 240 ppm) was divided into chemical shift buckets of 0.2 ppm and the absolute intensity of the NMR peaks detected in all spectra was associated to the corresponding bucket. The empty chemical shift buckets throughout the fraction series are removed from the list of buckets that provide useful information. The remaining chemical shift buckets are then used to build a two-dimensional matrix, where each row corresponds to a chemical shift bucket of, in this case, 0.2 ppm, and each column corresponds to a CPC fraction. Each cell in the table contains the spectral intensity value corresponding to its chemical shift and the reference of its CPC fraction. Hierarchical Clustering Analysis (HCA) was performed on the rows (*i.e.* chemical shift buckets) for data visualization of signals

corresponding to major compounds. The resulting clusters of  $^{13}\text{C}$  NMR chemical shifts were visualized as dendrograms on a heat map (Figure 5). The  $^{13}\text{C}$  NMR chemical shifts regrouped with the HCA were submitted as search keys to a local database containing the structures and predicted NMR chemical shifts (ACD/NMR Workbook Suite 2012 software, ACD/Labs, Ontario, Canada) to identify the corresponding chemical structures. Finally, each proposal given by the database search was confirmed by interpretation of 1D and 2D NMR data ( $^1\text{H}$  NMR, HSQC, HMBC, COSY).

## RESULTS AND DISCUSSION

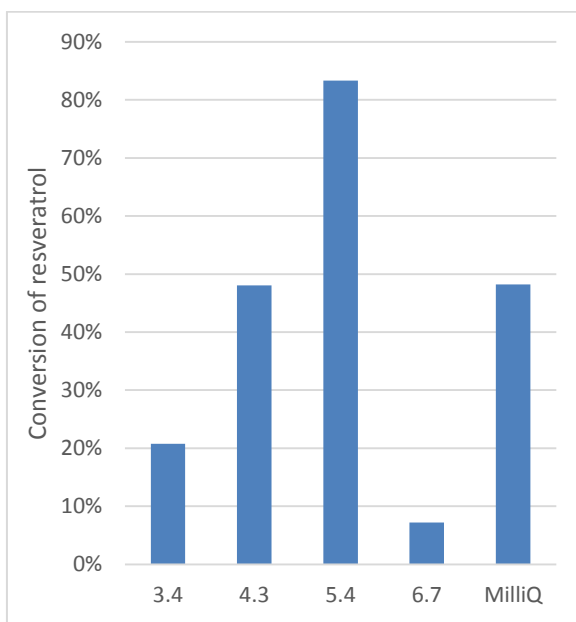
Controlling the yield of enzymatic reactions is a multifactorial issue as several parameters such as temperature, stirring, nature of the medium (*i.e.*, pH, presence of co-solvent), concentration of substrates or products can directly affect enzyme activity.<sup>25</sup> In addition, in the present case, dimerization proceeds through radical-radical coupling, whose selectivity is also influenced by the amount of radical species in solution, by medium dilution, and by reaction duration. The reduction of the number of parameters to consider in the perspective of a global yield optimization was studied by means of a preliminary “one variable at a time” (OVAT) approach.

### ***E*-Labruscol production**

#### **One variable at a time (OVAT)**

Solvent volume (25 mL) and stirring speed (700 rpm) were identically set for all reactions to ensure the same level of oxygenation as laccases are oxygen-dependent enzymes.<sup>25</sup> Resveratrol conversion was monitored to validate the enzymatic activity of laccase as well as the resveratrol availability in solution, the latter being only sparingly soluble in water ( $0.3 \text{ mg}\cdot\text{L}^{-1}$ ).<sup>26</sup> Laccase

having already proven to be active in biphasic media,<sup>17,27</sup> a first set of experiments was conducted in ethyl acetate (EtOAc) and highly pure water (MilliQ grade) or buffer solutions (EtOAc:Aqueous phase 3:1 v:v). Considering that laccase activity is high between pH 3 and 7,<sup>25</sup> the influence of pH on resveratrol conversion was monitored by HPLC within this range for 1.5 h (Figure 2). Operating at pH 3.4 and 6.7 has proven unfavourable. These results are in accordance with the maximum activity of laccase from *Trametes versicolor* being generally described at mid-acidic conditions.<sup>25</sup> Interesting resveratrol conversion was observed with MilliQ water:EtOAc (1:3) as reaction medium. Indeed, even though measuring pH is not easy in pure MilliQ water,<sup>28</sup> its pH is considered mid-acidic due to dissolution of atmospheric carbon dioxide. Biphasic media containing AcOEt led to an emulsion from which extraction was difficult to achieve. An assay was performed in a monophasic solvent mixture composed of acetone and MilliQ water in a 2:1 volume ratio and resulted in more than 95% of resveratrol conversion.



**Figure 2.** Influence of pH for buffering solutions and MilliQ water (uncontrolled pH) 25% in ethyl acetate.

Considering the aforementioned data, the monophasic acetone-MilliQ water medium was selected to further investigate the laccase-mediated dimerization of resveratrol.

### Design of experiments

A parametric optimization of *E*-labruscol yield by a DoE approach was then carried out. The variables were the acetone/water and the enzyme/substrate ratios, the initial resveratrol concentration, the addition rate of the enzymatic solution through a syringe pump, and the temperature (Table 1). A design matrix was generated using Modde 12.0 software according to fractional face-centered cubic design resulting in 29 experimental points that included 26 factor points and three replications at the middle point to evaluate the reproducibility.<sup>29</sup>

**Table 1.** Independent parameters used for the CCF design

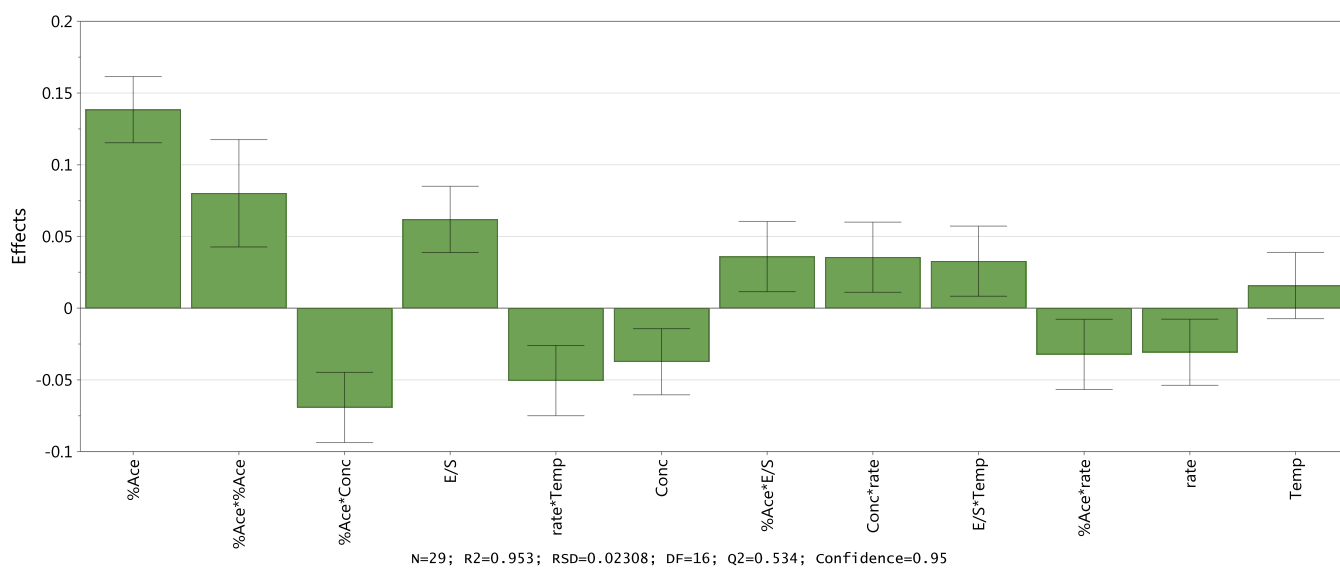
Parameters	Name	Abbr.	Units	Levels			Precision
				-1	0	1	
X <sub>1</sub>	%Acetone	%Ace	%	20	45	70	1.25
X <sub>2</sub>	Enzyme/substrate ratio	E/S	U/mmol	6	33	60	0.45
X <sub>3</sub>	Concentration	Conc	mmol/L	20	50	80	1.5
X <sub>4</sub>	Enzyme addition rate	rate	mL/h	7	28.5	50	1.08
X <sub>5</sub>	Temperature	Temp	°C	25	35	45	0.5

Aliquots of the medium were taken at three different times (1.5 h, 3 h and 4.5 h). The shortest reaction time (1.5 h) was sufficient to reach the maximum yield of *E*-labruscol in all cases, so

that the following discussion relies on the sole basis of results obtained after 1.5 h of reaction. Aliquots of the medium were taken at three different times (1.5 h, 3 h and 4.5 h).

After having performed the 29 experiments, a polynomial model for HPLC yield of *E*-labruscol (Y) was obtained with a satisfying R<sup>2</sup> (0.95) and acceptable fit between prediction and experimental data (Q<sup>2</sup> = 0.53). Equation of the model is given below on unscaled form to provide direct application with real parameters values (Equation 1). Scaled and centered coefficients are given in Figure 3. Data demonstrates that increasing percentage of acetone has a positive impact on *E*-labruscol production. In consequence, this parameter was fixed at its maximum (70 %) to generate the 4D contour plot given in Figure 4.

$$Y = - 7.009 \cdot 10^{-2} - 7.165 \cdot 10^{-4}X_1 - 2.177 \cdot 10^{-3}X_2 + 6.710 \cdot 10^{-4}X_3 + 3.369 \cdot 10^{-3}X_4 + 2.135 \cdot 10^{-3}X_5 + 6.410 \cdot 10^{-5}X_1^2 + 2.667 \cdot 10^{-5}X_1X_2 - 4.617 \cdot 10^{-5}X_1X_3 - 3.000 \cdot 10^{-5}X_1X_4 + 6.065 \cdot 10^{-5}X_2X_5 + 2.752 \cdot 10^{-5}X_3X_4 - 1.174 \cdot 10^{-4}X_4X_5 \text{ (Equation 1)}$$



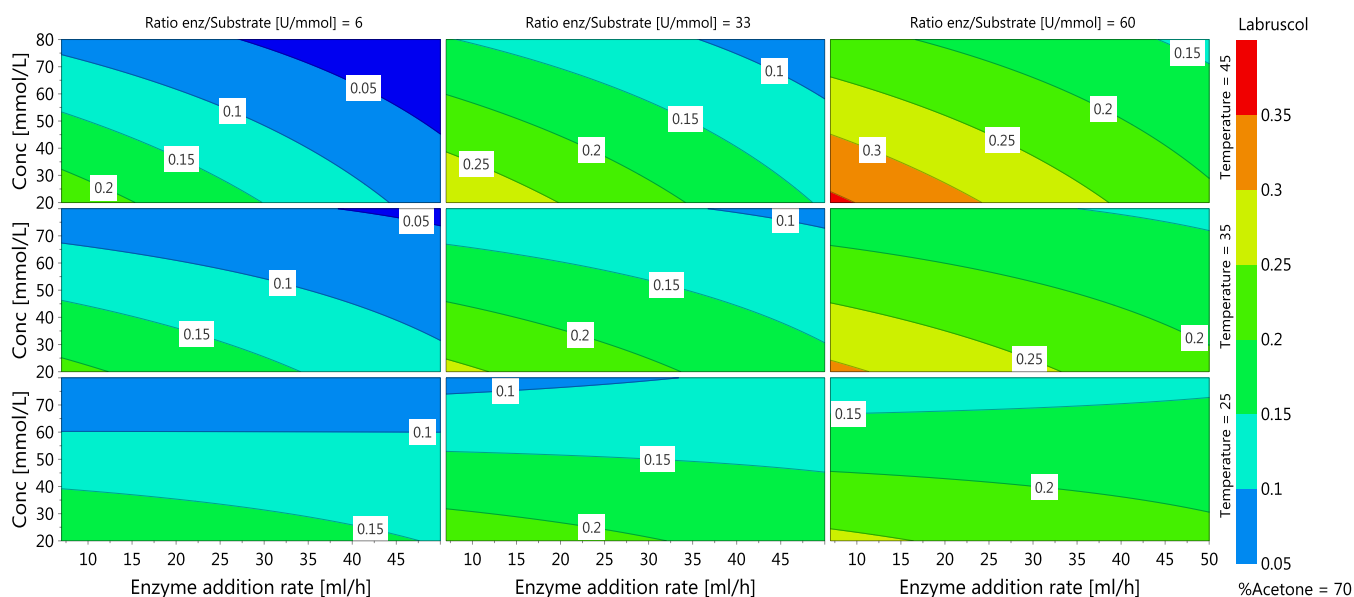
**Figure 3.** Model coefficients [centered and scaled (SC)] ranked in decrescent order on effect, at 95% confidence interval. %Ace ( $x_1$ ), E/S ( $x_2$ ), Conc ( $x_3$ ), rate ( $x_4$ ), Temp ( $x_5$ ).

Figures 3 and 4 provide a deep insight into the linear, quadratic and square effects of the



parameter variations. Figure 4, only the percentage of acetone have a significant impact on the

response of the model. Temperature did not significantly influence *E*-labruscol yield by itself but



exhibited synergic effect regarding the addition rate of the enzyme and the enzyme/substrate ratio. As one can observe on contour plots, the hot spot is located in the bottom left area. Even though this position is not ideal, further decreasing the concentration is not appropriate to achieve production at gram scale as it would require large medium volumes. In the same way, decreasing addition rate of the enzyme would result in very long reaction times. Finally, increasing enzyme/substrate ratio is costly and temperature cannot be increased without risking laccase denaturation. Thus, the observed hot spot corresponds to minimum concentration of substrate ( $20 \text{ mmol.L}^{-1}$ ) and rate addition of the enzyme ( $7 \text{ mL.h}^{-1}$ ) of the maximum enzyme/substrate ratio ( $60 \text{ U/mmol}$ ) and temperature ( $45 \text{ }^{\circ}\text{C}$ ) box. In these conditions, the software predicted a *E*-labruscol HPLC yield of  $36.0 \pm 4.1\%$ . In order to confirm this prediction, an experiment was performed in 7:3 acetone:water medium at initially  $20 \text{ mmol.L}^{-1}$  of resveratrol with the addition of 7 mL of enzymatic solution over 1 h (final enzyme/substrate ratio of  $60 \text{ U.mmol}^{-1}$ ) and at  $45 \text{ }^{\circ}\text{C}$ . After 1.5 h an aliquot was taken and analyzed by HPLC, revealing a yield of *E*-labruscol of 37.2%, in full agreement with the predictive model.

**Figure 4.** 4D contour plots of *E*-labruscol yield at 70% of cosolvent

### **Scale-up**

In order to produce *E*-labruscol in sufficient amount for biological assays, a scaled-up synthesis was attempted.

The amount of resveratrol was increased from 114 mg in 25 mL of reaction medium to 2 g in 300 mL of acetone and 130 mL of milliQ water, resulting in a 17.5 scale-up factor. Laccase solution (28 mL, 60 U.mmol<sup>-1</sup>) was added over 4 h, then the reaction was pursued for 6 h, leading to a resveratrol conversion of 95%.  $\delta$ -viniferin (1.14 g) was isolated from the reaction medium by normal phase flash chromatography (1:1 cyclohexane:EtOAc). A second purification step by reversed phase flash chromatography was performed yielding 412 mg (21% yield) of pure *E*-labruscol and 201 mg of laechianol F-G. For *E*-labruscol, the reduced isolated yield of 21% compared to the HPLC yield of 37% can be explained by losses during the two successive flash purifications. Indeed, small amount of desired product could not be recovered from the normal phase purification. Moreover, only highly pure fractions were collected after the reverse phase purification discarding some fractions containing *E*-labruscol as well as minor impurities. The TLC analysis of the reaction medium revealing a high number of synthesized compounds, a <sup>13</sup>C NMR dereplication workflow, starting by a CPC fractionation step, was carried out<sup>26</sup> in order to further characterize the compounds present in the extracted reaction mixture.

### **CPC fractionation and Structure elucidation**

### **CPC fractionation and dereplication**

The crude mixture (2 g) obtained through the DoE optimized biocatalytic reaction (*i.e.*, 20 mmol.L<sup>-1</sup> of resveratrol (2.0 g) in 7:3 acetone/water (438 mL), 45 °C, 7 mL.h<sup>-1</sup> enzymatic solution addition (28 mL), total reaction duration 6 h) was fractionated by an original gradient centrifugal partition chromatography (CPC) procedure involving a descending reversed phase elution mode using three quaternary biphasic solvent systems composed of *n*-heptane, EtOAc, methanol and water. This solvent combination was firstly tested based on previous results obtained for the purification of stilbenes including labruscol by CPC.<sup>14,24</sup>

Gradient elution in CPC is an efficient technique for complex mixture fractionation with a reduced experimental time compared to the isocratic elution mode. Nevertheless, its applications in solid support free liquid–liquid chromatography remains scarcely used, mainly due to possible modifications of the stationary phase composition during the mobile phase evolution. The mobile and the stationary phases were herein based on three solvent systems corresponding to the systems L (Hept/EtOAc/MeOH/W, 2/3/2/3 v/v/v/v), J (Hept/EtOAc/MeOH/W, 2/5/2/5 v/v/v/v) and G (Hept/EtOAc/MeOH/W, 1/4/1/4 v/v/v/v) in the HEMWat solvent scale.<sup>23</sup> The aqueous phase of the more polar system (system G) was used as the initial mobile phase, the aqueous phase of the less polar system (system L) was used as the final mobile phase, while the organic phase of the intermediary system (system J) was used as stationary phase.

This original reversed phase gradient elution in CPC enabled a good solubility of the reaction mixture and a selective elution of the different synthesized resveratrol dimers while keeping the unreacted resveratrol inside the CPC column. Generally, the gradient elution mode in CPC implies the use of type 2 ternary biphasic systems, *i.e.*, with converging tie lines, in order to minimize composition modifications of the stationary phase during the separation, both stationary and mobile phases being in a thermodynamic equilibrium. The implementation of an

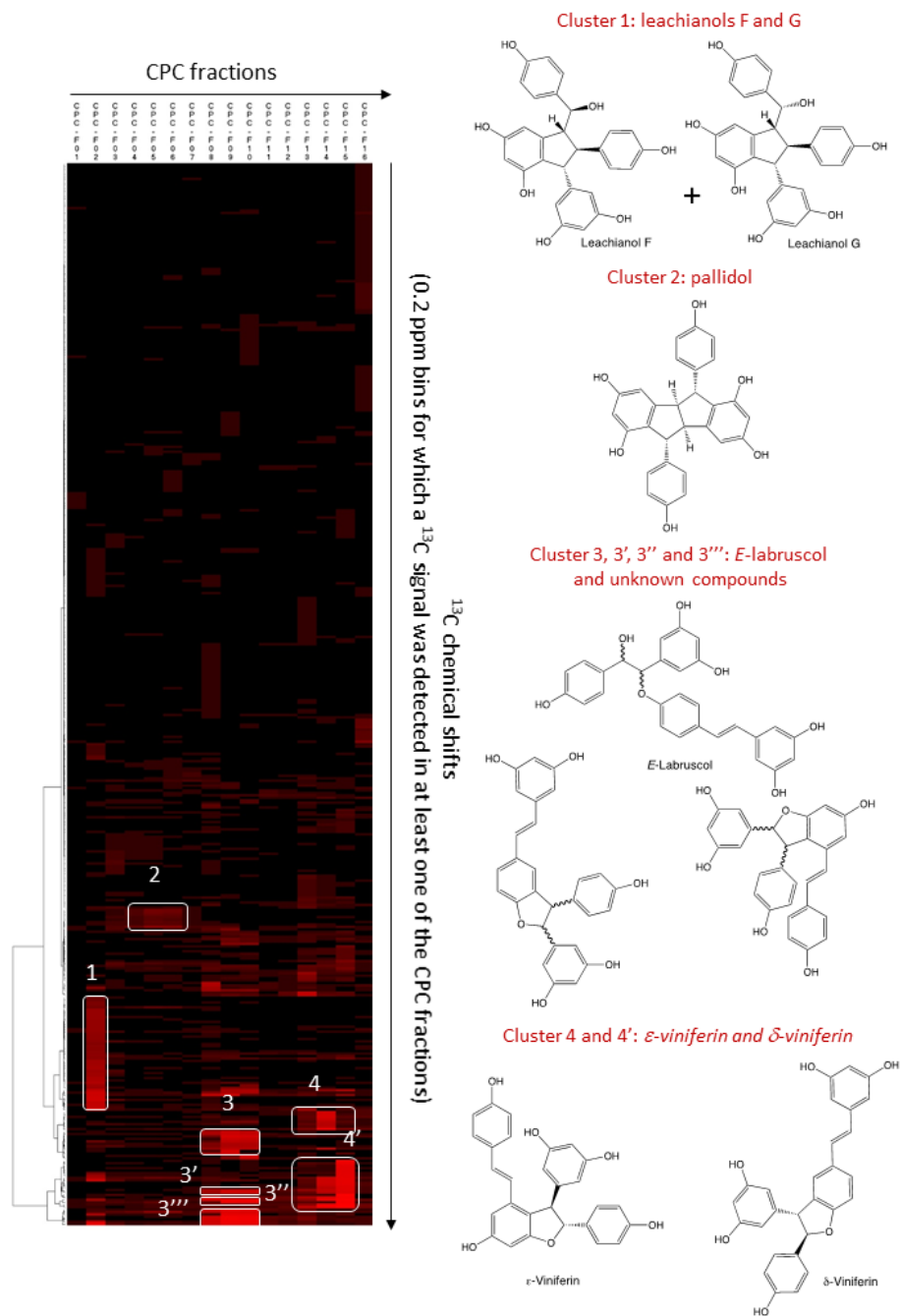
elution gradient within the HemWat solvent scale requires some care in order to minimize the modification of the stationary phase composition, which progressively re-equilibrates during the CPC run. Table 2 shows the advantage of taking the organic phase of the intermediate system (system J) as stationary phase to decrease this phenomenon and thus the inherent hydrodynamic disturbances leading to continuous stationary phase loss during the process.

**Table 2.** Upper phase compositions measured by  $^1\text{H}$  NMR spectroscopy of the three used biphasic systems

<b>Solvent systems</b>	<b>n-heptane (% v/v)</b>	<b>Ethyl acetate (% v/v)</b>	<b>Methanol (% v/v)</b>	<b>Water v/v)</b>
<b>System L</b>	47.73	48.10	3.04	1.12
<b>System J</b>	33.09	62.42	2.61	1.87
<b>System G</b>	22.65	72.84	2.1	2.2

After combining the collected fractions on the basis of TLC profile similarities, 16 adjacent fractions (F1, F2 [...] F16) containing simplified mixtures or even pure compounds were obtained (HPTLC fractogram is shown in Figure S3). The major stilbenes were annotated by using a  $^{13}\text{C}$ -NMR based dereplication workflow as explained in the material and methods section<sup>30,31</sup>. The resulting heat map after hierarchical clustering analysis on the  $^{13}\text{C}$  chemical shifts highlights four clusters (Figure 5). The chemical shifts corresponding to each cluster were submitted as search keys to an in-house developed database that contains more than 5000 compounds to date and associates natural product structures to the predicted  $^1\text{H}$  and  $^{13}\text{C}$  NMR chemical shifts calculated by the ACD/Labs predictor. As a result, seven stilbenes were identified and their structures confirmed through 1D and 2D NMR analysis.

Fraction 2 (120.9 mg, elution time from 13 to 33 min, cluster 1) corresponded to a mixture of leachianols F and G. Pallidol (27.2 mg) was mainly obtained from fractions 5- 6 eluted between 44 and 56 min (cluster 2). The main compounds from fraction 8 to fraction 10 (517 mg representing 25.8% of the crude injected sample, elution time from 58 min to 96 min, (clusters 3, 3', 3'',3''')) where the two diastereomers of *E*-labruscol, which were present at a purity greater than 80%. The same fraction contained three other <sup>13</sup>C chemical shift clusters which could not be annotated by the dereplication workflow used, probably because they correspond to compounds not present in the database and being therefore potentially new. Fraction 14 and 15 (1262.4 mg, from 100 min to 134 min, (clusters 4 and 4')), collected during the extrusion step of the CPC run contained  $\delta$ -viniferin as the main product with a purity of 90%, and a small amount of  $\epsilon$ -viniferin and unreacted resveratrol. The structures of  $\delta$ -viniferin, *E*-labruscol diastereomers, leachianol F and leachianol G,<sup>32</sup> *i.e.*, the major reaction products, as well as minor products such as pallidol,<sup>33</sup>  $\epsilon$ -viniferin were confirmed through 1D and 2D NMR analysis.

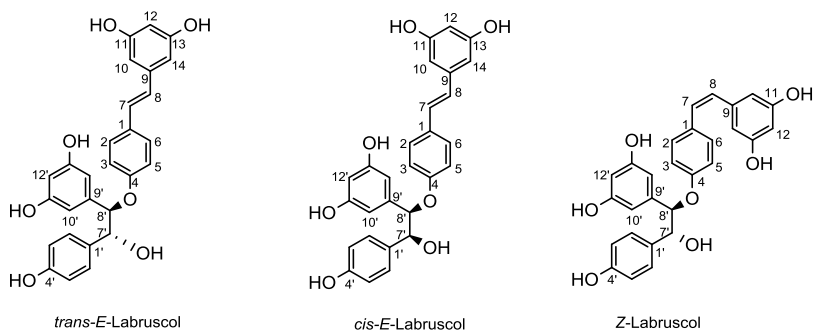


**Figure 5.** Heatmap of peak intensities of  $^{13}\text{C}$  NMR (rows) and fractions (columns) obtained from the crude mixture CPC fractionation. This representation allows the visualization of the carbon skeleton of the major compounds. The 4 clusters represent the 8 major compounds identified by the  $^{13}\text{C}$ -NMR based dereplication procedure.

Purity greater than 90% was targeted for the products of interest, particularly for *E*-labruscol isomers. Labruscol-containing fractions (517 mg) were pooled and submitted to an additional CPC purification step again in a gradient elution mode using the same three biphasic solvent systems G, J and L, but in the ascending mode. The latter was chosen as there was no issue related to the presence of residual resveratrol. The organic phase of the less polar system (system L) was used as the initial mobile phase, the organic phase of the more polar system (system G) was used as the final mobile phase, while the aqueous phase of the intermediary system (system J) was used as stationary phase. This CPC purification step (see Figure S3 for the HPTLC fractogram) allowed the recovery of 408 mg of *E*-labruscol isomers with an average purity of 95.1%, estimated by CLHP-DAD based on the peak area measured at 286 nm.

### Structure elucidation

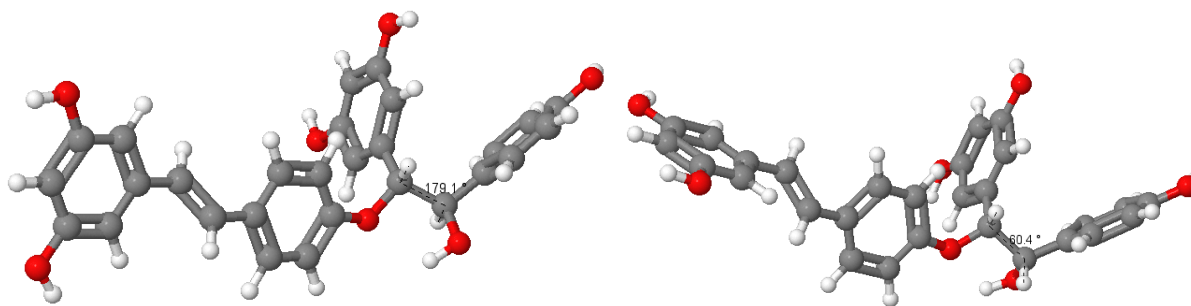
The structural elucidation of the diastereomers of *E*-labruscol, *Z*-labruscol (Figure 6, S5-10 and Table S1-3) and of the two new resveratrol dimers (Figure 8, S11-12 and Tables S4-5), has been unambiguously obtained by 1D and 2D NMR analyses, including  $^1\text{H}$ ,  $^{13}\text{C}$ , heteronuclear single quantum correlation (HSQC), heteronuclear multiple bond correlation (HMBC), correlation spectroscopy (COSY) and, when necessary, rotating-frame nuclear Overhauser effect correlation spectroscopy (ROESY).<sup>29</sup>





**Figure 6.** Structures of isolated labruscol isomers

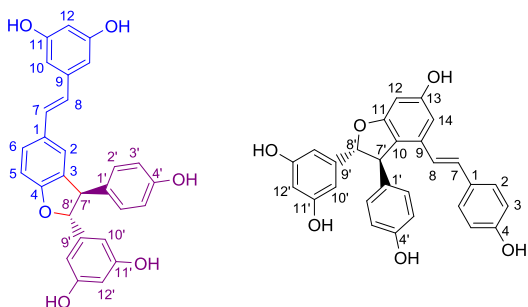
Molecular modelling shows that the diastereomers of *E*-labruscol have some rotation freedom around the C7'-C8' bond. Models of these structures are presented in Figure 7. The value of the H7'-C7'-C8'-H-8' dihedral angle in the lowest energy conformation is 180° for the (7'R, 8'R or 7'S, 8'S) isomer and 60° for the (7'S, 8'R or 7'R, 8'S) isomer. The  $^3J(\text{H}7'-\text{H}8')$  coupling constant values are 7.4 Hz and 5.4 Hz for the first and the second eluted compounds, respectively. The assignment of the highest coupling constant value (7.4 Hz) to the (7'R, 8'R or 7'S, 8'S) isomer, the first one eluted on the C-18 flash chromatographic phase, led to name it *trans-E*-labruscol. This molecule was identical to the single *E*-labruscol isomer isolated from *Vitis labrusca* cell culture.<sup>13</sup> The isomer with the lowest coupling constant value (5.4 Hz), the second that eluted, was therefore named *cis-E*-labruscol of (7'S, 8'R or 7'R, 8'S) configuration. NMR analysis of the initial mixture revealed a 4:1 ratio in favour of the *trans* isomer.



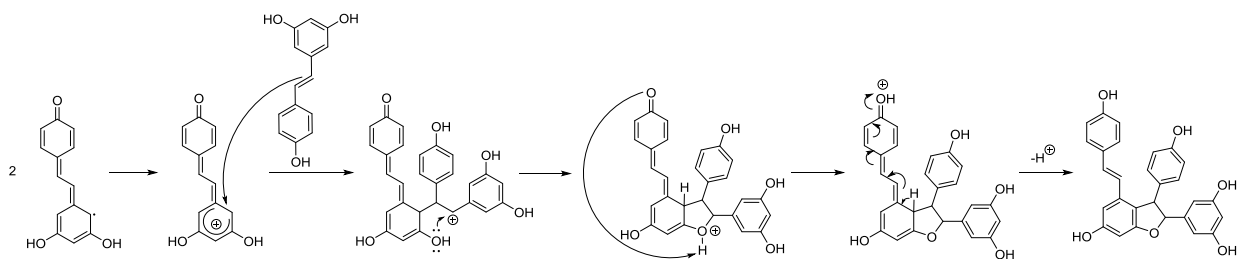
**Figure 7.** 3D structure of minimal energy conformation of *trans-E*-labruscol (left) and *cis-E*-labruscol (right)

The novel resveratrol dimers were called iso- $\delta$ -viniferin and iso- $\epsilon$ -viniferin, respectively (Figure 8). In the NMR spectrum, HMBC correlations were observed between protons H-2 and H-5 and carbons C-3 and C-4 and between H-8' and C-3 suggesting the structure of iso- $\delta$ -

viniferin. The HMBC correlations observed between protons H-12, H-14 and H-8' and carbon C-11 confirmed the structure of iso- $\epsilon$ -viniferin. The molecular formula of  $C_{28}H_{23}O_6$  was determined by high-resolution electrospray ionization mass spectrometry (HR-ESI-MS) analysis, revealing the molecular ions  $[M-H]^-$  at  $m/z$  455.1499 in the negative ionization mode. Whereas formation of the iso- $\delta$ -viniferin was easily understandable from the *O*-4 and 8 radical forms of resveratrol (Scheme 1), 11-*O*-8' or 10-7' linkages in iso- $\epsilon$ -viniferin cannot be explained by simple radical-radical coupling. Dismutation of radical species to afford cationic forms constitutes a possible mechanism for the formation of the iso- $\epsilon$ -viniferin (Scheme 2).<sup>34</sup>



**Figure 8.** Structure of the new iso- $\delta$ -viniferin and iso- $\epsilon$ -viniferin



**Scheme 2.** Hypothetical mechanism for the formation of iso- $\epsilon$ -viniferin

## CONCLUSIONS

The laccase-mediated synthesis of labruscol from resveratrol was optimized and its yield multiplied 7-fold (determined by HPLC) compared to previously published works. The fine tuning of reaction conditions (temperature, solvent composition, concentration, amount and addition rate of enzyme) favoured the dimerization of resveratrol into labruscol. However, the selectivity was not perfect and a purification step was necessary. Successive normal and reverse phase flash chromatography allowed to recover only 21% of pure labruscol (>98% purity) and 52% of  $\delta$ -viniferin. Alternatively, purification by CPC isolated these two compounds in high yield and purity (20.4 and 63.1%, respectively). CPC fractions were submitted to a  $^{13}\text{C}$  NMR based dereplication workflow for the identification of the already known compounds. The structures of the unknown compounds were assigned by usual *de novo* elucidation by NMR, after their isolation by a supplementary purification step by liquid chromatography. Thus, four novel structures were determined, those of *cis-E*--labruscol, *Z*-labruscol, iso- $\delta$ -viniferin and iso- $\epsilon$ -viniferin

## ASSOCIATED CONTENT

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acssuschemeng.3c01997>. Its include full material and methods as well as  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra. In addition, raw data (NMR FID, CSV files relative to laccase activity determination and the DoE, pdf files relative to HRMS and flash chromatography purifications) have been deposited in a repository available at <https://doi.org/10.57745/HBRMRX>

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## Author Contributions

‡These authors contributed equally.

A.L.F. C.P. and F.A. conceptualized the project. E.S. and A.L.F. performed the enzymatic synthesis, flash purification and structure elucidation of trans- and cis-labruscol. Z.L.E.A. performed the CPC purifications and structure elucidation steps, J.H.R. supervised the CPC purification steps, L.V. supervised the de novo structure elucidation of new compounds, N.B. co-supervised the CPC purification steps, A.M. recorded and interpreted the NMR data at 600 MHz, J.M.N. performed the molecular modeling of *E*-labruscol isomers, A.L.F (lead), Z.L.E.A., L.V., J.M.N. and J.H.R. wrote the original draft. E.C. acquired funding, manages the project. E.C. and F.A. reviewed and edited this article.

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## TABLE OF CONTENT



## SYNOPSIS

Challenging synthesis and purification of the valuable dimer of resveratrol *E*-Labruscol were optimized. Additionally, 4 new resveratrol dimers were identified.