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► **To cite this version:**

Raul F. Guerrero, Josep Valls-Fonayet, Tristan Richard, Emma Cantos-Villar. A rapid quantification of stilbene content in wine by ultra-high pressure liquid chromatography – Mass spectrometry. Food Control, 2020, 108, pp.1-9. 10.1016/j.foodcont.2019.106821 . hal-02620179

**HAL Id: hal-02620179**

**<https://hal.inrae.fr/hal-02620179v1>**

Submitted on 20 Jul 2022

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1 **A rapid quantification of stilbene content in wine by ultra-high pressure liquid**  
2 **chromatography – mass spectrometry**

3

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20

21 **Abstract**

22 Stilbenes are a family of bioactive phenolic compounds. Wine is one of the main sources of  
23 stilbenes in diet. Very few studies have dealt with a detailed quantitative analysis of stilbenes in  
24 wine. Most methodologies reported until now have been restricted to the analysis of few  
25 stilbenes such as resveratrol and piceid. In this study, a method for the quantification of wine  
26 stilbenes has been developed and validated. The method was simple, fast and sensitive with LOD  
27 between 4-28 µg/L. Matrix effects were assessed, and the methodology was validated in terms of  
28 precision, accuracy, linearity and repetitiveness. The method was able to quantify, in less than 5  
29 minutes, fifteen targeted stilbenes in wines including seven monomers, three dimers, one trimer,  
30 and four tetramers. The methodology was applied to white and red wines. *E*-piceid was the main  
31 stilbene in white wine (mean 155 µg/L). In red wine, *Z*- and *E*-piceid (mean 3.73 and 3.16 mg/L,  
32 respectively) were predominant. Additionally, large amount of other stilbenes including  
33 oligomers such as hopeaphenol (mean 1.55 mg/L) were found in red wines. The developed  
34 methodology could be useful to reveal differences in the contents of stilbenes in wine depending  
35 on variety, season, terroir, treatments, among others and potentially be used as a quality wine  
36 marker.

37

38 **Keywords:** stilbene; viniferin; wine; mass spectrometry.

## 39 **1. Introduction**

40 Wine is a complex evolving matrix in which a large number of compounds with different  
41 chemical nature coexist in a wide concentration ranges. In order to characterize such a complex  
42 mixture, the development of metabolomic approaches based on mass spectrometry has opened  
43 new opportunities to assess wine quality and traceability (Alañón, Pérez-Coello, & Marina,  
44 2015; Arbulu, Sampedro, Gómez-Caballero, Goicolea, & Barrio, 2015). Concerning wine  
45 metabolomics, sometimes referred as Wineomics (Wine-omics, 2008), more than 2000  
46 molecules have been described in wine, including primary wine metabolites such as sugars,  
47 amino acids, biogenic amines, organic acids, fatty acids or minerals, and secondary metabolites  
48 such as phenolics or volatile compounds (Arbulu et al., 2015).

49 Wine polyphenols constitute an heterogeneous family of chemical compounds belonging to  
50 several different chemical structures (Quideau, Deffieux, Douat-Casassus, & Pouységu, 2011).  
51 All these phenolic compounds have attracted a enormous interest because of their organoleptic  
52 properties in wine that included aroma, colour, flavour, bitterness and astringency (Garrido &  
53 Borges, 2013). In addition, their role as bioactive compounds have been widely reported as a key  
54 factor for the protection against cancer, cardiovascular, and neurodegenerative diseases (Quideau  
55 et al., 2011). Because of their chemical complexity, the individual identification and  
56 quantification of all polyphenols remains a challenge (García-Guzmán, Hernández-Artiga,  
57 Palacios-Ponce de León, & Bellido-Milla, 2015). However, recent advances in liquid  
58 chromatography hyphenated with mass spectrometry have allowed a large improvement in the  
59 simultaneous detection and quantification of many polyphenols in wines in the last few years.  
60 Around ninety anthocyanins and anthocyanin derivative pigments such as pyranoanthocyanins  
61 were characterized by liquid chromatography coupled triple quadrupole mass spectrometer (LC-  
62 QqQ-MS) in Sangiovese wines (Arapitsas, Perenzoni, Nicolini, & Mattivi, 2012). Lambert et al

63 developed also a method by LC-QqQ-MS for the selective quantification of up to 152 phenolic  
64 wine compounds, including 100 anthocyanins and derivatives, 15 phenolic acids, 5 flavonols, 11  
65 flavanols and 6 stilbenes (Lambert et al., 2015).

66 Stilbenes are a particular interesting family of non-flavonoid polyphenols in wine, because of  
67 their health related properties (Dvorakova & Landa, 2017; Temsamani et al., 2016; Vang et al.,  
68 2011) and the fact that wine may represent the major source of these compounds in occidental  
69 diets, providing a up to 98% of their intake (Zamora-Ros et al., 2008). Recent studies also stated  
70 that stilbenes could play a role in the preservation of wine (Raposo et al., 2018). It opens the  
71 possibility to identify the stilbene composition and concentration in wines as a quality marker.  
72 Despite a large number of other phenolic compounds are indeed routinely analysed in wines, the  
73 stilbenes analysis is considerably reduced and the number of stilbenes analysed is often limited  
74 to the quantification of *E*-resveratrol, its glucoside *E*-piceid and their corresponding *cis* isomers.  
75 Several reasons can be mentioned to explain this lack of convenient methodologies for the  
76 stilbene's analysis. Firstly, their contents in wines are usually quite low, in a range below of  
77 mg/L (ppm), which requires highly sensitive methods. Secondly, most of these minor  
78 compounds have been recently described. Finally, pure stilbene standards are not yet  
79 commercially available in order to allow a reliable quantification.

80 The development of new methodologies using LC-MS systems can provide a further  
81 improvement in the detection and quantification of wine stilbenes. In relation with identification  
82 methods, the stilbene profile by suspect screening analysis has recently been published. Flamini  
83 and co-authors identified eighteen potential stilbene derivatives to be present in two grape  
84 samples on the basis of accurate mass measurements and isotopic patterns by high resolution  
85 qTOF mass spectrometry (Flamini et al., 2013). Also with a qTOF detector providing high  
86 resolution mass spectra, Moss et al. compiled 41 putative stilbene derivatives potentially present

87 in red wine by screening precursor MS ions as well as characteristic neutral losses in the MS/MS  
88 fragments (Moss et al., 2013). The presence of some of these putative compounds was also  
89 confirmed thanks to the comparison with pure standards such as  $\epsilon$ -viniferin,  $\delta$ -viniferin, or  
90 pallidol. Although qTOF detectors can also be used for quantification purposes, both cited  
91 articles just focused on the qualitative analysis of grape and wine stilbenes. Because of their  
92 efficiency characteristics (sensitivity enhancement and time saving), triple quadrupoles working  
93 in multiple reaction monitoring (MRM) mode are the most often used mass spectrometers for  
94 quantification when standards are available, since they provide a large dynamic range, a great  
95 sensitivity and low LOD and LOQ (Lambert et al., 2015).

96 Despite the advances related to the detection of stilbenes in wine, the matter of their  
97 quantification by throughput methods remains relatively unexplored. Buiarelli et al. developed a  
98 45-minute long LC-QqQ-MS methodology with a C18 column for the direct determination of  
99 resveratrol, piceid and astringin isomers. The method showed a good sensitivity and the  
100 detection limits were around 50 ng/mL (Buiarelli, Coccioli, Jasionowska, Merolle, &  
101 Terracciano, 2007). Erhardt et al described the separation under ten minutes of 38 phenolic  
102 compounds in grapes, including 11 stilbenes (7 monomers : *E*- and *Z*-resveratrol, *E*- and *Z*-  
103 piceid, piceatannol, isorhapontin, astringin; 3 dimers: pallidol,  $\omega$ -viniferin,  $\epsilon$ -viniferin; a  
104 tetramer: isohopeaphenol), with LOD between 0.005 and 0.620 mg/kg grape (Ehrhardt,  
105 Arapitsas, Stefanini, Flick, & Mattivi, 2014). Recently, Hurtado-Gaitan and co-authors have  
106 developed an LC-QqQ-MS method for the analysis of five grapevine stilbenes (4 monomers:  
107 resveratrol, piceid, piceatannol, pterostilbene; and a one dimer:  $\epsilon$ -viniferin) in different complex  
108 matrices including red wine after solid phase extraction. The LOD were in the range of 0.04-0.12  
109 mg/L (Hurtado-Gaitán et al. 2017).

110 In response to the lack of fast and simple methodologies to quantify stilbenes in wine, the aim of  
111 the current work was to develop and validate a fast LC-QqQ-MS methodology to quantify the  
112 fifteen main stilbenes described in grapevine. Among these compounds, six were quantified for  
113 the first time in wine. Finally, the method was validated and applied to real white and red wine  
114 samples.

115

## 116 **2. Materials and methods**

### 117 ***2.1. Stilbene standards and wine samples***

118 *E*-astringin (Carbosynth, UK), *E*-piceid (Sigma Aldrich, Germany), *E*-piceatannol (ChromaDex,  
119 USA), *E*-resveratrol (Sigma Aldrich, USA), *E*-4-hydroxystilbene (Acros organics, Belgium)  
120 were commercially available. Ampelopsin A, hopeaphenol, isohopeaphenol, R2-viniferin,  
121 miyabenol C,  $\epsilon$ -viniferin, R-viniferin,  $\omega$ -Viniferin were isolated from a grapevine raw shoot  
122 following the method described by Biais and coauthors (Biais et al., 2017). The *cis* isomers were  
123 obtained using UV-C irradiation (254 nm) from *trans* isomers (Mattivi, Reniero, & Korhammer,  
124 1995). The white and red wines analyzed in this study were purchased in wine shops (Table 1S,  
125 in supplementary data).

126

### 127 ***2.2. Preparation of wine solutions***

128 Ultrapure water from Milli Q Direct System (Merck Millipore, USA), L-(+)-tartaric acid (Merck,  
129 Germany), ethanol 96% v/v (pharma grade, Panreac, Spain), and sodium hydroxide pure pellets  
130 (pharma grade, Panreac, Spain) were used for wine matrix. For model wine solution (MW),  
131 tartaric acid (4 g) was diluted into 120 mL of ethanol 96% on 1 L volumetric flask. Solution was  
132 flushed with water up to 1 L. pH was adjusted with drops of sodium hydroxide 2 N solution up to  
133 3.6. Standard white wine solution (WW) was composed of a mix of five monovarietal white

134 wines made in the experimental winery IFAPA-Rancho de la Merced: Traminer, Vijiriega, Jaén  
135 blanco, Moscatel and Palomino fino. Into a Schott flask, 500 mL of each wine was poured to  
136 achieve 2.5 L of white wine matrix. Resulting solution showed 3.1 pH, 12.2% alc. vol., and 5.3  
137 total acidity (g/L tartaric acid). Standard red wine solution (RW) was obtained as described for  
138 white using five monovarietal red wines: Pinot noir, Petit verdot, Malbec, Marselan and Tannat.  
139 Resulted red wine matrix showed 3.6 pH, 13.9% alc. vol., and 5.1 total acidity (g/L tartaric acid).  
140 Solutions were centrifuged during 20 min at 4000 rpm and filtrated through PTFE 0.45 µm  
141 filters.

142 Wine samples were diluted in a ratio 1:10 in MW solution. Subsequently, 20 µL of internal  
143 standard solution (*E*-4-hydroxystilbene) was added into 180 µL of sample to achieve a 1.28 ppm  
144 internal standard final concentration.

145

### 146 **2.3. Instrumentation**

147 Ultrapure water from Milli Q Direct System (Merck Millipore, USA), methanol for UHPLC  
148 (Merck, Germany) and formic acid 98-100% (Merck, Germany) were used. Compounds  
149 separation was performed on a Waters Acquity TQD LC/MS/MS System with photodiode array  
150 (PDA) detector equipped with a mass spectrometer Xevo TQD (Waters, USA). The column used  
151 was an Acquity UPLC BEH C18 (2.1 x 100 mm, 1.7 µm, Waters, USA). The mobile phases  
152 consisted of phase A: water 0.1% formic acid; and phase B: methanol 0.1% formic acid. The  
153 6.60 min elution method at flow 0.35 mL/min was 0 min 10% B, 0.20 min 20% B, 1.60 min 40%  
154 B, 3.60 min 70% B, 4.20 min 100% B, 5.20 min 100% B, and recovering initial conditions, 5.60  
155 min 10% B. Wash solvent was water/methanol in a ratio 50/50 and purge solvent was water  
156 0.1% formic acid. Column temperature was kept at 40°C and sample temperature at 10°C.  
157 Injection volume was 10 µL for standards and samples. Mass spectrometer Xevo TQD was



158 driven by software Masslynx v 4.1 (Waters) and set on electrospray negative ion mode (ES<sup>-</sup>).  
159 Mass spectrometer was set on 2.30 kV capillary source voltage, 450°C source desolvation  
160 temperature, 1000 L/h (N<sub>2</sub>) desolvation gas flow and 50 L/h cone gas (Argon) flow. Nitrogen  
161 generator from Peak Scientific (UK), and argon gas bottle (Air Liquide, France) were coupled to  
162 the mass detector for gas supplying. Dwell was automatically adjusted for minimum 12 points  
163 per peak and smoothing was applied on peaks. Smoothing method was on mean, 2 smooth  
164 iterations and 2 smooth widths.

165

#### 166 **2.4. Method development**

167 Model wine (MW), standard white wine (WW) and standard red wine (RW) solutions were used  
168 to validate the method of stilbenes in wine analysis. Firstly, stilbene standards were separately  
169 dissolved in methanol/water in a ratio 50/50 to achieve an approximate concentration of  
170 200 mg/L. Secondly, stilbene solutions were diluted in MW, WW and RW to achieve a  
171 concentration of 10 mg/L of each compound. These solutions were further diluted to achieve a  
172 second stock concentration of 5 mg/L. Finally, 5 mg/L solutions were dissolved in a ration 1/5 to  
173 achieve 1 mg/L solutions. These last solutions were used to evaluate the matrix effect. Solutions  
174 were prepared in triplicates. MW, WW, RW solutions and their dilutions were also injected with  
175 no standard addition.

176 The MW with 10 mg/L of each stilbene (MW-10ppm) solution was further used to prepare  
177 calibration curves. The *E*-4-hydroxystilbene was used as internal standard. This compound was  
178 firstly dissolved in methanol/water in a ratio 50/50 to achieve a 10 mg/L stock solution. The  
179 internal standard was added to each solution to achieve a 1 mg/L final concentration. Five serial  
180 dilutions were prepared from the MW-10ppm solution (5, 1, 0.5, 0.1 and 0.05 mg/L of each  
181 standard). Five serial dilutions were also prepared from 0.05 mg/L solution to achieve 0.02, 0.01,

182 0.005, 0.003 and 0.001 mg/L solutions. Calibration was prepared in duplicates and injected five  
183 times. Area value relation with internal standard area was used as quantification response.  
184 Calibration was calculated considering origin forced inclusion and no weighting function.  
185 Standards were injected to study linearity and accuracy (LOD and LOQ).  
186 For intra- and interday effects, calibration curve with internal standard experiment was re-  
187 injected 5 days after. Vials were kept at 4°C in a fridge. Relative standard deviation (RSD) at day  
188 0 and day 5 were result of 5-times standard injection.

189

### 190 ***2.5. Commercial wine analysis***

191 Wines were diluted in a ratio 1:10 in MW solution. Subsequently, 20 µL of internal standard  
192 solution (*E*-4-hydroxystilbene) was added into 180 µL of sample to achieve a 10 mg/L internal  
193 standard final concentration. All experiments were performed at least in triplicate. Data  
194 presented are means ± standard deviation.

195

### 196 ***2.6. Statistical analysis***

197 Statistical analyses were performed using R scripts in BioStatFlow web application  
198 ([biostatflow.org](http://biostatflow.org), v2.9).

199

## 200 **3. Results and discussion**

### 201 ***3.1. Analysis of individual stilbenes and selection of MRM conditions***

202 Triple quadrupole mass spectrometers (QqQ-MS) are normally programmed in multiple  
203 reactions monitoring (MRM), where several transitions between the parent ion and their  
204 fragment ions are collected. The MRM mode used in LC-QqQ-MS methodology provides the  
205 selectivity required for the analysis by focusing on transitions that are specific to the quantified

206 compounds (Lambert et al., 2015). According to previous reports, the identification using  
207 MS/MS experiments would require the analysis of at least two product ions, the most intense one  
208 being used as a quantifier ion, while the other one is used as a qualifier (Kruve et al., 2015a,  
209 2015b).

210 In order to estimate the operational parameters concerning the optimal detection of the MRM,  
211 both the cone voltage and the collision energy were optimized by direct infusion. A total of  
212 fifteen stilbenes (Figure 1) were selected including seven monomers (*E*- and *Z*-astringin, *E*- and  
213 *Z*-piceid, piceatannol, *E*- and *Z*-resveratrol), three dimers (ampelopsin A,  $\epsilon$ - and  $\omega$ -viniferin), one  
214 trimer (miyabenol C), and four tetramers (hopeaphenol, isohopeaphenol, R2- and R-viniferin).  
215 Each stilbene as well as the internal standard were infused in the detector and the MRM  
216 conditions were optimized in the negative mode. The two most intense fragment ions were  
217 selected to be used in the final LC-QqQ-MS method. All the optimization was performed using  
218 *trans* isomers, and then the transitions were checked to assure their suitability for the  
219 corresponding *cis* isomers. The selected parameters for each compound were those which had a  
220 better response. The results are reported in Table 1. A representative MRM chromatogram of a  
221 model wine spiked with 1 mg/L of each stilbene is presented in Figure 2. Under the described  
222 chromatographic conditions, stilbenes were analysed in less than six min.

223 The stilbene monomers fragmentation pattern was characterized by successive losses of 42 u  
224 fragments (ketene, CH<sub>2</sub>CO). So, for resveratrol, the precursor ion ( $m/z$  227) gives two major  
225 ions:  $m/z$  185 (elimination of one ketene molecule) and 143 (elimination of two ketene  
226 molecules). These ions correspond to previous reports (Buiarelli et al., 2007), the ion at  $m/z$  143  
227 being the most abundant and in consequence it was selected as the quantifier ion. Similarly, for  
228 piceatannol ( $m/z$  243) the fragments at  $m/z$  201 and 159 represent again the loss of ketene  
229 molecules (Wei, Zhao, Li, & Xue, 2016). The glucoside derivatives of these monomers also

230 follow a similar pattern. The qualifier ions for piceid ( $m/z$  389) and astringin ( $m/z$  405)  
231 correspond to the loss of the glucose moiety (162 u), giving the fragment ions at  $m/z$  227 and  
232 243, respectively. This transition is generally the most commonly obtained for stilbene  
233 glucosides (Buiarelli et al., 2007; López-Hernández & Rodríguez-Bernaldo de Quirós, 2016).

234 Concerning dimers,  $\epsilon$ - and  $\omega$ -viniferin, share a parent ion at  $m/z$  453 and a quantifier ion at  
235  $m/z$  359, which corresponds to the loss of the phenol ring (94 u) (Ehrhardt et al., 2014). The  $\omega$ -  
236 viniferin gives also a fragment at  $m/z$  347, consistent with a  $C_7H_6O$  loss (106 u), in accordance  
237 with previous results (Ehrhardt et al., 2014; Moss et al., 2013). Regarding ampelopsin A  
238 ( $m/z$  469), it gives two major ions at  $m/z$  451 (loss of  $H_2O$ ) and 363 (loss of 106 u, 4-  
239 methylenecyclohexan-2,5-dienone). The trimer miyabenol C ( $m/z$  679) quantifier signal at  
240  $m/z$  345 has been previously reported (Vrhovsek et al., 2012). It would correspond to the loss of  
241 two molecules of 4-methylenecyclohexan-2,5-dienone (106 u), a CO group (28 u), and a phenol  
242 (94 u). The corresponding qualifier ion at  $m/z$  451 is formed after the loss of a 4-  
243 methylenecyclohexan-2,5-dienone (106 u), a phenol (94 u) and a CO group (Moss et al., 2013).

244 Four tetramers (ion precursor at  $m/z$  905) were included in the method. The fragment at  $m/z$  359  
245 was observed for all of them, and is the main transition for hopeaphenol and isohopeaphenol.  
246 This ion has been suggested to arise from the symmetrical splitting of the tetramer molecule and  
247 the additional loss of a phenol (Moss et al., 2013). In addition, hopeaphenol and isohopeaphenol  
248 share the main qualifier ion at  $m/z$  451 - which would correspond to the loss of a dimer (Moss et  
249 al., 2013). Meanwhile, R2-viniferin has as a quantifier ion at  $m/z$  811, consistent with the loss of  
250 one phenol group (94 u). The R-viniferin gives a fragment at  $m/z$  799, which consistent to the  
251 loss of a 4-methylenecyclohexan-2,5-dienone (106 u).

252

253 **3.2. Method validation and quality parameters**

254 The validation of the LC-QqQ-MS method for quantification of the fifteen selected stilbenes was  
255 performed by investigating the following quality parameters: linearity, limits of detection (LOD)  
256 and quantification (LOQ), repeatability (intra- and inter-day), and recovery in each wine  
257 matrixes (MW, WW and RW) (Kruve et al., 2015a, 2015b).

258 First at all, the possible matrix effects were investigated. These effects result from co-eluting  
259 matrix compounds that compete for ionization capacity inducing a decrease or increase of the  
260 analyte signal (Choi, Hercules, & Gusev, 2001). Standard white (WW) and red wine (RW)  
261 solutions containing 1 mg/L of each stilbene were analyzed.

262 The same experience was repeated with different dilutions of WW and RW solutions. For the  
263 undiluted wines, the results clearly showed a matrix effect for almost all compounds with a mean  
264 recovery rate of  $50 \pm 30\%$  for the RW solution (Table 2S, supplementary data). The effect is  
265 especially significant for the most polar compounds in the RW solution. The astringin recovery  
266 rate drops to 16% in the undiluted RW solution. Diluting the samples in the model wine solution  
267 increases the recovery rate. In order to reduce matrix effects, a dilution in 1/10 ratio appears to  
268 be the most appropriate for quantifying stilbenes with the minimal ionization suppression. The  
269 mean recovery rates increased to  $101 \pm 9\%$  and  $79 \pm 10\%$  in WW and RW solutions,  
270 respectively.

271 Due to the matrix effects an internal standard was added in the method (Kruve et al., 2015b). An  
272 ideal internal standard should mimic closely the properties of the analyte, differ only slightly  
273 chemically, and have desirable chromatographic properties such as stable isotopes (Wieling,  
274 2002). Such ideal internal standard for the stilbenes analysis is right now unachievable for  
275 practical reasons: first, most stilbenes are simply not commercially available in their natural  
276 form, and secondly the few isotopes available would represent a too high added expensiveness

277 for the analysis. A compromise had to be found and among available stilbene compounds  
278 hydroxystilbene was selected. The main reason was its analogy with the stilbenes selected, its  
279 absence in wine, and its availability as a commercial standard.

280 Quality parameters of the LC-QqQ-MS method were reported in the Tables 2 and 3. The  
281 linearity range of the method was evaluated by serial dilution of a stock solution of the studied  
282 compounds in the model wine solution (range 0.001 to 10 mg/L of each stilbene). The  
283 correlation coefficients ( $R^2$ ) were ranged between 0.981 and 0.999 depending on the analytes.  
284 The regression equations and the linearity ranges of each stilbene are reported in Table 2. One of  
285 the main characteristics of the MRM methodology is the large dynamic range analyte  
286 quantification from few  $\mu\text{g/L}$  to mg/L in our case. The LOD and LOQ values were calculated  
287 using the classical signal-to-noise ratio criterion of 3 and 10, respectively. The values for the  
288 selected stilbenes are given in Table 2. LOQ values were ranged between 15 and 61  $\mu\text{g/L}$ . These  
289 results are coherent with those obtained by other QqQ methodologies (Buiarelli et al., 2007;  
290 Hurtado-Gaitán et al., 2017). For example, Hurtado-Gaitán et al. reported LOQ of 220, 70, 150  
291 and 90  $\mu\text{g/L}$  for resveratrol, piceid, piceatannol and  $\epsilon$ -viniferin, respectively, but with a lower  
292 injection volume (Hurtado-Gaitán et al., 2017).

293 Recovery of each stilbene was calculated in MW, WW and RW solutions containing 1 mg/L of  
294 each stilbene. The data obtained were presented in Table 3. For MW and WW solutions,  
295 recovery values for the stilbenes were within the range of 82 and 120% with a mean recovery  
296 rate of  $105 \pm 12\%$  and  $101 \pm 9\%$ , respectively. In contrast, in the RW solution the recovery  
297 values were lower in the range of 69 and 108% with a mean recovery rate of  $79 \pm 10\%$ . This  
298 decrease of recovery rate in red wines could be due to matrix effects as previously observed.  
299 Recovery values higher than 100% are not unusual in LC-MS and have been previously reported  
300 for stilbenes analysis (Rodríguez-Cabo et al., 2014). Low recovery values for several analytes

301 due to matrix effects in wine have also been previously observed. For example, Lambert et al.  
302 found intensity losses of over 50% in QqQ analysis of phenolic acids when the concentration of  
303 formic acid is not optimized (Lambert et al., 2015).

304 Same experiments were conducted with wine solutions containing 0.5 mg/L of each stilbene. The  
305 obtained recovery rates were similar  $97 \pm 12\%$ ,  $93 \pm 12\%$ , and  $72 \pm 10\%$  for MW, WW and RW  
306 solutions, respectively. Finally, concerning the reproducibility, the relative standard deviation  
307 (RSD%) in term of concentration, was determined in each wine matrix (MW, WW and RW  
308 solution). The results are given in Table 3. The RSD% values were around 10% except for  
309 miyabenol C and R-viniferin.

310

### 311 ***3.3. Evaluation of stilbene pattern in wines***

312 The optimized LC-QqQ-MS method was applied to determine the content of stilbenes in  
313 different commercial mono-varietal white and red wines (Table 1S, supplementary data).  
314 Different cultivars and vintages were selected to observe the wide spectra application of the  
315 method. Stilbene concentration in grape, and therefore in wine, is affected by climate, type of  
316 soil, year, variety, winemaking processes, among others, and therefore wine stilbene  
317 concentration hugely varies (Bavaresco, Mattivi, de Rosso, & Flamini, 2012).

318

#### 319 ***3.3.1. Analysis of white wines***

320 Ten commercial Spanish white wines were analysed from eight different varieties: Albariño  
321 ( $\times 3$ ), Chardonnay, Godello, Moscatel, Riesling, Sauvignon blanc, Verdejo, and Viura. The  
322 content of stilbenes in these white wines is summarized in Table 4.

323 The total stilbene content in white wines was ranged between 0.04 and 0.56 mg/L with a mean  
324 value of 0.23 mg/L. These values are in agreement with literature data (Lamuella-Raventós,

325 Romero-Pérez, Waterhouse, & de la Torre-Boronat, 1995; Ribeiro De Lima et al., 1999). A total  
326 of twelve stilbenes were identified in white wines. But only six compounds were identified  
327 above the limits of quantification (*E*- and *Z*-astringin, *E*- and *Z*-piceid, *E*- and *Z*-resveratrol). The  
328 *E*-piceid (0.11-0.33 mg/L, mean 0.16 mg/L) was the most dominant stilbene in all white wines  
329 followed by a pool of three compounds its isomer *Z*-piceid, and the two isomers of astringin  
330 (11-44 µg/L, mean 21 µg/L). In agreement with others works, piceid seems to be the main  
331 stilbene in white wines (Ribeiro De Lima et al., 1999). The concentrations in astringin and  
332 resveratrol were significantly lower than previously reported in white wines (Lamuela-Raventós  
333 et al., 1995; Ribeiro De Lima et al., 1999), which may be explain due to the huge number of  
334 factor that influence the stilbene concentration. Finally, the presence of stilbene oligomers was  
335 observed for the first time in some white wines including one dimer ( $\epsilon$ -viniferin), one trimer  
336 (miyabenol C), and four tetramers (hopeaphenol, isohopeaphenol, R- and R<sup>2</sup>-viniferin).  
337 However, it was not possible to quantify them because their concentrations were under the limit  
338 of quantification.

339

### 340 **3.3.2. Analysis of red wines**

341 Ten commercial Spanish red wines were investigated from seven different varieties: Cencibel,  
342 Garnacha, Merlot (×2), Monastrell, Syrah, Tempranillo (×3), and Tintilla de Rota. The content of  
343 stilbenes in these red wines is reported in Table 5.

344 In red wines, total stilbene concentration is much more variable than in white wines. Depending  
345 on the wine, stilbene content was ranged from 0.40 mg/L to 35.5 mg/L (mean 13.1 mg/L). The  
346 stilbenes encountered in red wines are mostly glucosylated (Table 3S). Depending of the red  
347 wine, between 40 and 100% (mean 68%) of the stilbene quantified were glucosides. In  
348 agreement with previously described for white wines, piceid (sum *E*- and *Z*-isomers) was the



349 main stilbene in red wines (Total 0.28-15.7 mg/L, mean 6.89 mg/L). The two isomer levels are  
350 comparable (mean 3.16 and 3.73 mg/L for *E*- and *Z*-piceid, respectively). This result is in  
351 agreement with literature data even if their concentrations in some red wines were slightly higher  
352 than those previously reported (Moreno-Labanda et al., 2004). In contrast with white wines, the  
353 second main stilbene in red wines is resveratrol (Total 0.28-9.84 mg/L, mean 3.19 mg/L). As  
354 piceid, the resveratrol concentrations in red wines are strongly contrasted. Levels of *E*- and  
355 *Z*-resveratrol are similar (mean 1.38 and 1.81 mg/L in *E*- and *Z*-resveratrol, respectively). The  
356 astringin isomers are quantified in all red wines. Levels of *E*-astringin (0.06-2.99 mg/L, mean  
357 0.67 mg/L) are significantly higher than that of *Z*-astringin (0.06-0.23 mg/L, mean 0.10 mg/L).  
358 The monomer piceatannol is only quantified in three wines. Even if the results are contrasted, red  
359 wines may contain relative high amount of stilbene oligomers up to 11.2 mg/L representing 13%  
360 of the total stilbene content (Table 3S). The tetramers are the most representative oligomeric  
361 stilbenes. The isohopeaphenol is the main oligomer quantified (0.06-7.47 mg/L, mean 1.55 mg/L),  
362 while its isomer hopeaphenol is only quantified in two wines. In addition, R2-viniferin is  
363 detected for the first time in wine. Red wine R10 (Monastrell, Table 1S) is the only wine which  
364 showed quantifiable amount of miyabenol C,  $\epsilon$ - and  $\omega$ -viniferin (1.41, 0.81 and 0.31 mg/L,  
365 respectively). Monastrell grape has been described as high resveratrol producer (Gatto et al.,  
366 2008), being Monastrell wine reported as a high resveratrol content wine (Moreno-Labanda et  
367 al., 2004).

368 As far as we know, it is the first time that quantitative analysis has been reported for *Z*-astringin,  
369 hopeaphenol, isohopeaphenol, R2-viniferin, miyabenol C and  $\omega$ -viniferin, in wine. Quantitative  
370 data on stilbene monomers *E*- and *Z*-resveratrol, *E*- and *Z*-piceid has usually been reported  
371 (Guerrero et al., 2009; Lamuela-Raventós et al., 1995; Romero-Pérez, Lamuela-Raventós,  
372 Waterhouse, & de la Torre-Boronat, 1996). Vitrac et al., reported also data on astringin,

373  $\delta$ -viniferin and  $\epsilon$ -viniferin in Brazilian wines (Vitrac et al., 2005). Moss et al., were able to detect  
374 piceatannol, astringin,  $\epsilon$ -viniferin,  $\omega$ -viniferin, miyabenol C and hopeaphenol in a red wine  
375 extract (100-folds concentrated) but not to quantify them by direct injection ultra-high-  
376 performance liquid chromatography/electrospray ionization quadrupole time-of-flight mass  
377 spectrometry (Moss et al., 2013).

378 Principal component analysis (PCA) was used to compare white and red wines (Figure 3). The  
379 percentage of total variability explained by PC1 was about 76%. A separation between white and  
380 red whites was observed. The comparison of the scores plot and the loadings plot showed a  
381 tendency to have higher concentration in stilbenes in red wines. In fact, it is widely known that  
382 stilbene concentration in white wines is lower than in red wines because in red winemaking the  
383 must, grape skin and often seeds are in contact during the alcoholic fermentation process (Isabel  
384 Fernandez-Marin et al., 2012). The PCA highlights the great dispersion in stilbene content  
385 between red wines.

386

#### 387 **4. Conclusion**

388 Resveratrol shows a large range of biological effects, including cancer, cardioprotective,  
389 neuroprotective preventions. During the past decades, other natural derivatives of resveratrol  
390 were identified in plant kingdom and more specifically in grapevine. These compounds received  
391 particular attention for their beneficial effects but their content in wine remains relatively  
392 unstudied. The developed method enables the identification and quantification of fifteen  
393 stilbenes well known in wine. The method is fast, does not require sample preparation and  
394 presents a large dynamic range between few  $\mu\text{g/L}$  to few  $\text{mg/L}$ . In addition, this method may  
395 permit increase the number of quantifiable stilbenes as new compounds might be identified.  
396 Concerning their content in wine, twelve stilbenes were quantified in red wines. These wines

397 present a wide dispersion of stilbenes that could be due to numerous factors such as grape  
398 variety, biotic and abiotic stresses or winemaking processes. In further studies, research on the  
399 impact of these and other factors to control the stilbene content especially in red wine may be  
400 affordable due to the development on the described method.

401

## 402 **Funding**

403

## 404 **Notes**

405 The authors declare no competing financial interest.

406

## 407 **Acknowledgments**

408 The authors gratefully thank Actichem (Montauban, France) for providing grapevine products  
409 and to Waters support (MA Cortés and J. Rodriguez) for their contribution in the development of  
410 the method. The work was supported by the Bordeaux Metabolome Facility and MetaboHUB  
411 (ANR-11-INBS-0010 project) and by INIA (RTA2015-00005-C01).

412

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536

537 **Figure Legends**

538

539 **Figure 1.**

540 Stilbene structures.

541

542 **Figure 2.**

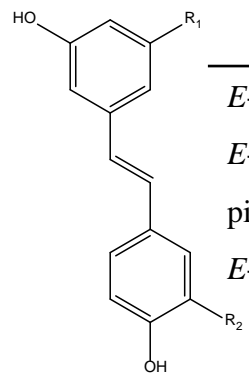
543 Representative MRM chromatograms of a model wine spiked with 1 mg/L of each stilbene. The  
544 transition of quantification is shown for each compound.

545

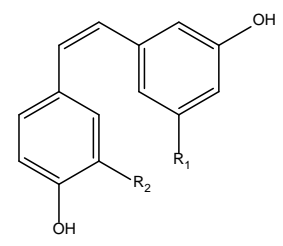
546 **Figure 3.**

547 PCA score and loading plots of the two principal components of white (open circles) and red  
548 wines (full squares).

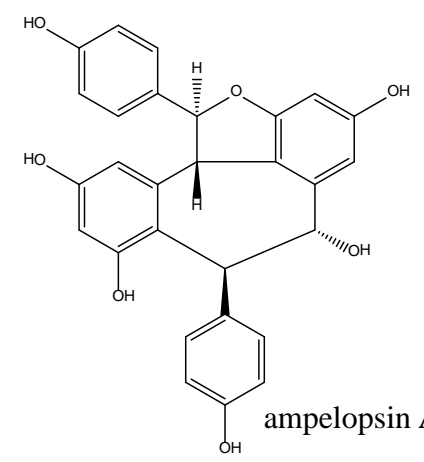
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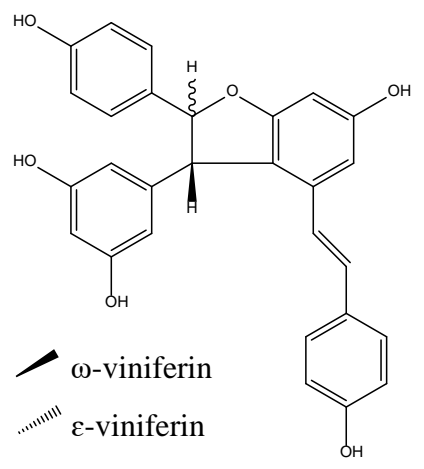
	R <sub>1</sub>	R <sub>2</sub>
<i>E</i> -resveratrol	OH	H
<i>E</i> -piceid	Oglc	H
piceatannol	OH	OH
<i>E</i> -astringin	Oglc	OH



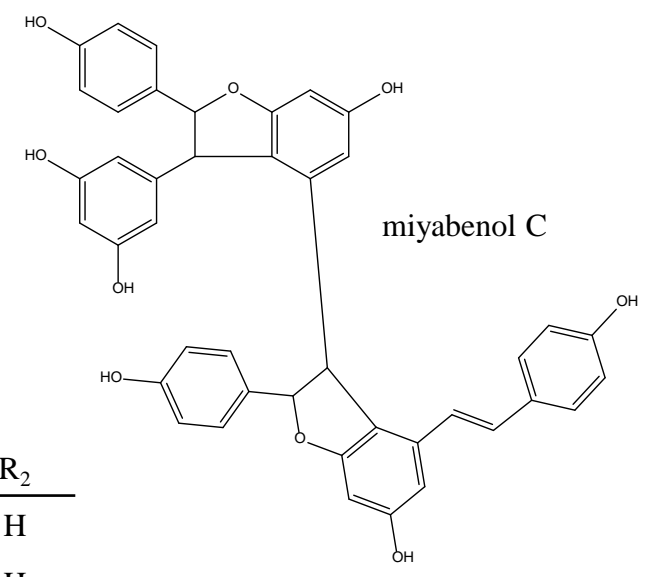
	R <sub>1</sub>	R <sub>2</sub>
<i>Z</i> -resveratrol	OH	H
<i>Z</i> -piceid	Oglc	H
<i>Z</i> -astringin	Oglc	OH



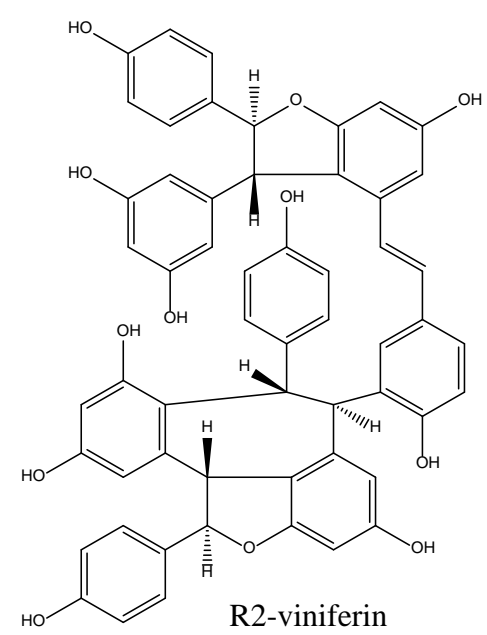
ampelopsin A



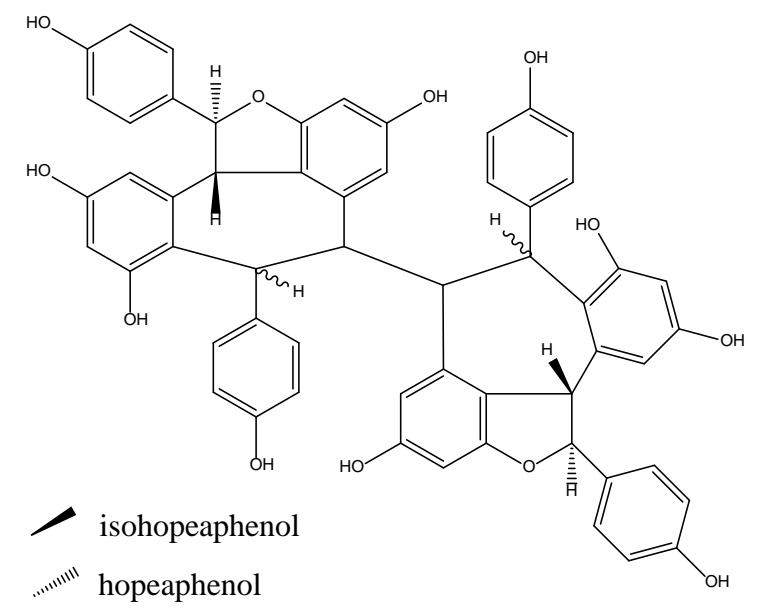
ω-viniferin  
ε-viniferin



miyabenol C



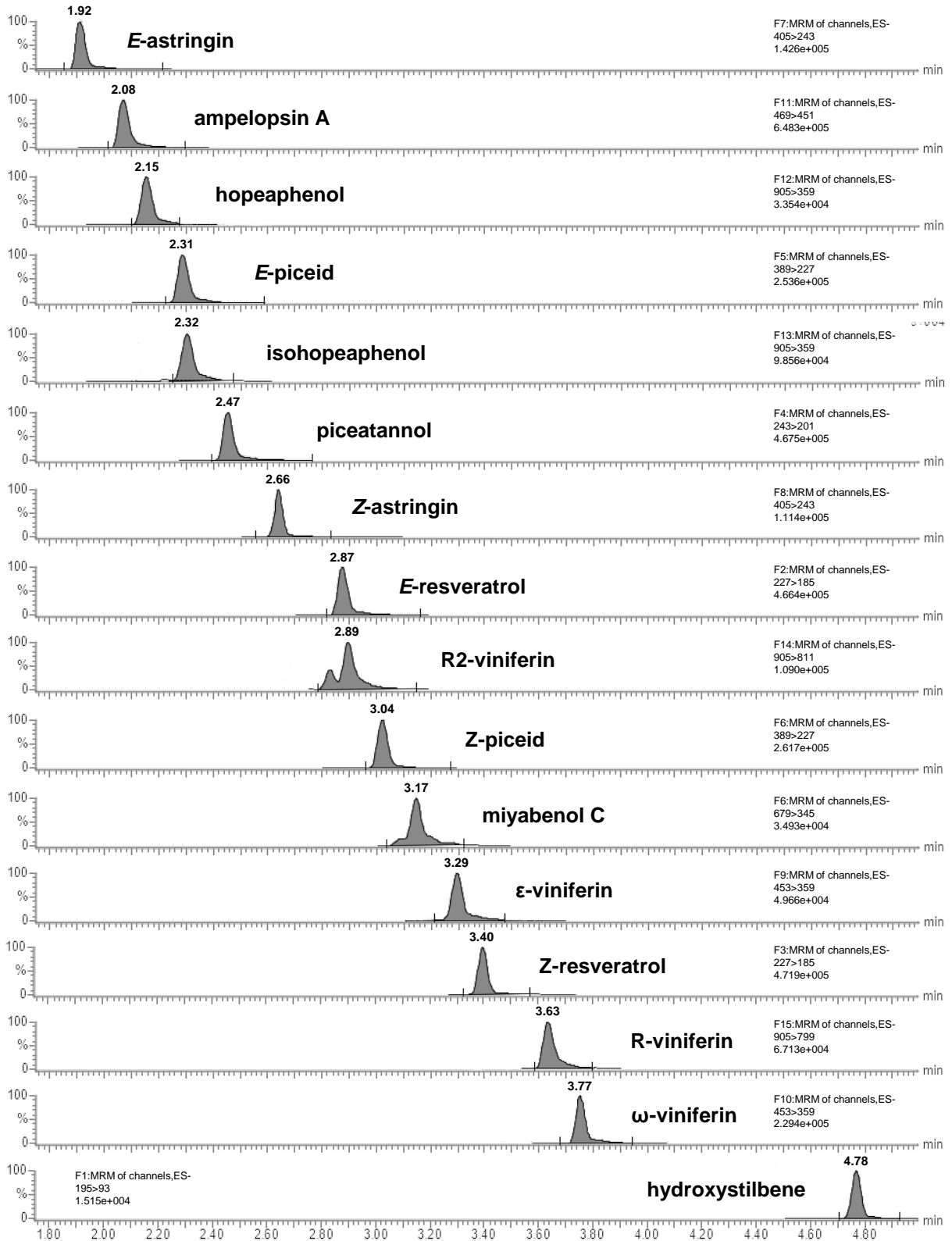
R2-viniferin

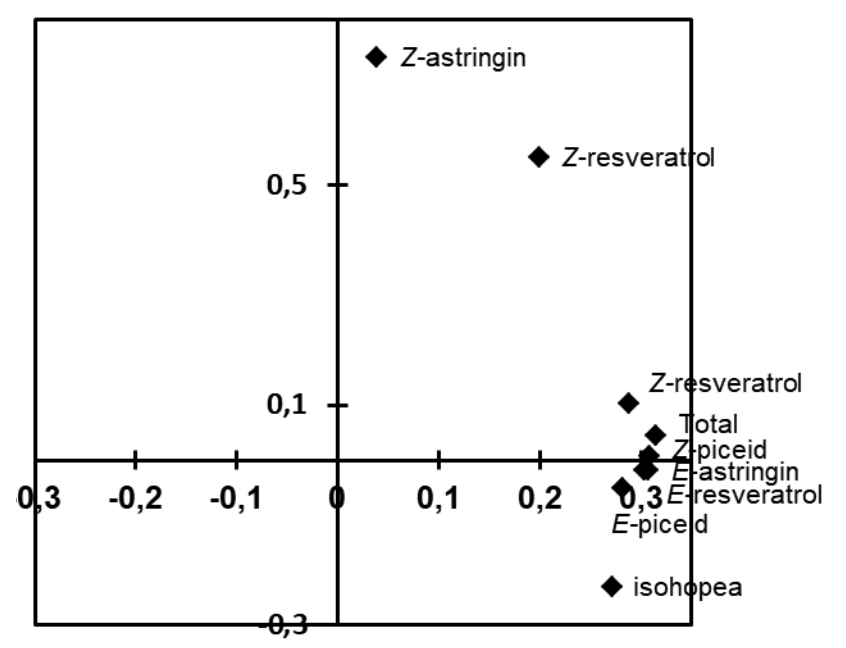
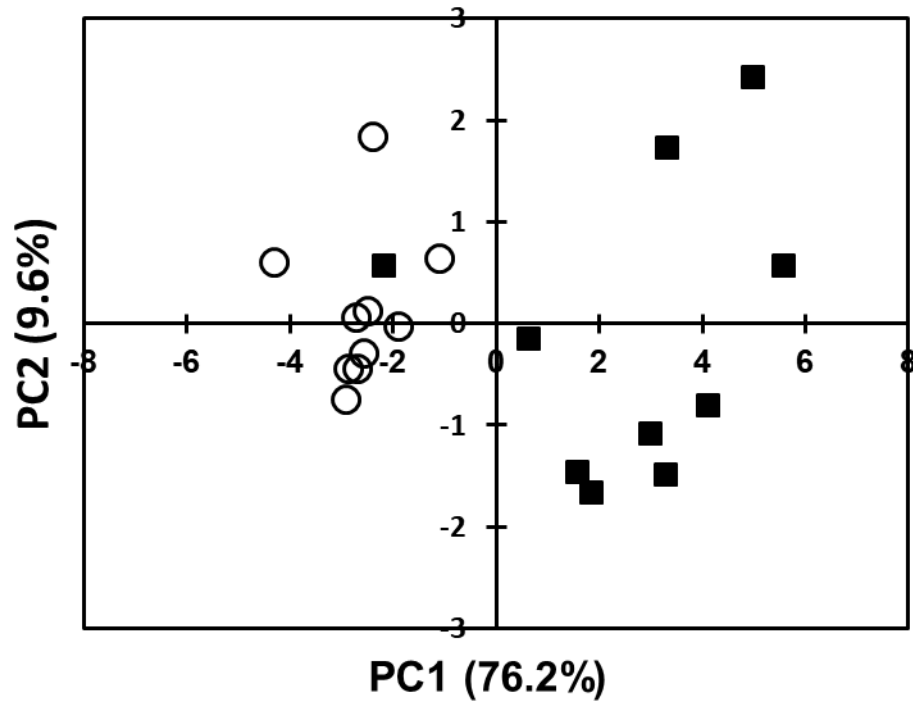


R-viniferin

▲ isohopeaphenol  
▨ hopeaphenol







**Table 1**

Compound name, retention time, and optimized MRM conditions for the analyses of the studied stilbenes by UPLC–MS/MS.

Compound	Rt (min)	Parent Ion ( <i>m/z</i> )	Qualifier				Quantifier			
			Product ion	Dwell (s)	Cone <sup>a</sup> (V)	Coll. <sup>b</sup> (eV)	Product ion	Dwell (s)	Cone (V)	Coll. (eV)
<i>E</i> -astringin	1.92	405	159	0.022	50	50	243	0.022	50	20
ampelopsin A	2.08	469	363	0.021	50	20	451	0.021	50	20
hopeaphenol	2.15	905	451	0.021	65	45	359	0.021	65	45
<i>E</i> -piceid	2.31	389	159	0.021	45	35	227	0.021	45	15
isohopeaphenol	2.32	905	451	0.021	65	45	359	0.021	65	45
<i>E</i> -piceatannol	2.47	243	159	0.021	60	25	201	0.021	60	20
<i>Z</i> -astringin	2.66	405	159	0.016	50	50	243	0.016	50	20
<i>E</i> -resveratrol	2.87	227	143	0.016	50	20	185	0.016	50	30
R2-viniferin	2.89	905	359	0.016	90	45	811	0.016	90	30
<i>Z</i> -piceid	3.04	389	159	0.016	45	35	227	0.016	45	15
miyabenol C	3.14	679	451	0.016	80	25	345	0.016	80	50
$\epsilon$ -viniferin	3.29	453	225	0.016	65	20	359	0.016	65	30
<i>Z</i> -resveratrol	3.40	227	143	0.016	50	30	185	0.016	50	30
R-viniferin	3.63	905	359	0.016	90	40	799	0.016	90	35
$\omega$ -viniferin	3.77	453	347	0.016	70	20	359	0.016	70	20
hydroxystilbene	4.78	195	117	0.097	55	45	93	0.097	55	30

<sup>a</sup>Cone: Cone voltage; <sup>b</sup>Coll.: Collision energy

**Table 2.**

Linearity data, limit of detection (LOD), and limit of quantification (LOQ) of the stilbenes. Z-isomers not included in the table were quantified with the calibration curve obtained for the *E*-isomers.

Compound	Calibration equation	Correlation coefficient (R <sup>2</sup> )	Linearity (mg/L)	LOD (mg/L)	LOQ (mg/L)
<i>E</i> -astringin	$y = 5.75x + 0.212$	0.9959	0.03 – 3.0	0.009	0.030
ampelopsin A	$y = 4.07x - 0.160$	0.9985	0.01 – 5.0	0.005	0.015
hopeaphenol	$y = 1.08x + 0.051$	0.9837	0.02 – 3.0	0.007	0.021
<i>E</i> -piceid	$y = 8.43x + 0.260$	0.9843	0.01 – 7.5	0.004	0.012
isohopeaphenol	$y = 2.38x + 0.087$	0.9811	0.03 – 7.5	0.011	0.033
<i>E</i> -piceatannol	$y = 1.87x + 0.084$	0.9947	0.06 – 4.0	0.018	0.061
<i>E</i> -resveratrol	$y = 3.20x + 0.180$	0.9775	0.03 – 4.0	0.010	0.030
R <sup>2</sup> -viniferin	$y = 0.36x - 0.113$	0.9870	0.06 – 3.0	0.020	0.060
miyabenol C	$y = 0.14x - 0.015$	0.9992	0.08 – 5.0	0.028	0.084
$\epsilon$ -viniferin	$y = 0.32x + 0.030$	0.9936	0.05 – 5.0	0.017	0.051
R-viniferin	$y = 0.99x - 0.262$	0.9940	0.03 – 10.0	0.010	0.030
$\omega$ -viniferin	$y = 1.07x - 0.060$	0.9990	0.03 – 10.0	0.010	0.030

**Table 3.**

Average recovery, intra- and inter-day precision of the concentration of the stilbenes of standard solution (1 mg/L of each compound) in the different wine matrixes (model wine solution, white wine, and red wine).

Compound	Model wine			White wine			Red wine		
	Average recovery (%)	Intraday (RSD %)	Interday (RSD %)	Average recovery (%)	Intraday (RSD %)	Interday (RSD %)	Average recovery (%)	Intraday (RSD %)	Interday (RSD %)
astringin	109	11	7	101	4	7	75	3	5
ampelopsin A	103	10	8	94	9	6	69	6	7
hopeaphenol	107	9	5	105	11	6	80	10	11
piceid	107	9	7	99	8	7	72	6	9
isohopeaphenol	102	9	6	98	10	6	72	8	8
piceatannol	110	9	11	120	12	7	79	7	7
resveratrol	108	11	4	101	11	2	71	6	4
R2-viniferin	82	5	15	116	12	13	108	8	14
miyabenol C	116	15	12	97	14	11	87	21	8
$\epsilon$ -viniferin	130	10	8	99	8	6	74	13	8
R-viniferin	83	24	10	103	19	10	79	14	9
$\omega$ -viniferin	107	8	6	88	11	6	82	7	7

**Table 4.**

Content of stilbenes (in  $\mu\text{g/L}$ ) in commercial white wines. The figure in brackets represents the standard deviation.

Compound	White wines										
	W1	W2	W3	W4	W5	W6	W7	W8	W9	W10	Mean
<i>E</i> -astringin	15 (1)	19 (3)	19 (1)	11 (3)	18 (3)	16 (3)	32 (3)	24 (4)	16 (4)	44 (2)	22 (9)
ampelopsin A	nq	nq	nq	nq	nq	nq	nq	nq	nq	nq	-
hopeaphenol	nd	nq	nd	nd	nd	nd	nd	nd	nd	nq	-
<i>E</i> -piceid	nq	110 (6)	129 (8)	180 (14)	150 (10)	160 (7)	150 (6)	120 (8)	220 (45)	330 (21)	155 (84)
isohopeaphenol	nd	nd	nd	nd	nd	nd	nd	nd	nd	nq	-
piceatannol	nq	nq	nq	nq	nq	nq	nq	nq	nq	nq	-
<i>Z</i> -astringin	21 (5)	9 (2)	11 (3)	12 (15)	15 (7)	11 (2)	16 (3)	48 (2)	14 (3)	20 (4)	18 (11)
<i>E</i> -resveratrol	nd	nq	nq	nq	nq	nq	nq	nq	19 (13)	49 (10)	7 (16)
R2-viniferin	nd	nq	nd	nd	nq	nd	nq	nq	nq	nd	-
<i>Z</i> -piceid	8 (6)	14 (3)	11 (2)	18 (4)	17 (3)	22 (3)	23 (3)	49 (17)	29 (20)	42 (9)	23 (13)
miyabenol C	nq	nd	nq	nq	nd	nq	nq	nd	nq	nq	-
$\epsilon$ -viniferin	nq	nq	nq	nd	nq	nq	nq	nq	nq	nq	-
<i>Z</i> -resveratrol	nd	nd	nq	nq	nq	nq	nq	nq	18 (14)	73 (22)	9 (23)
R-viniferin	nq	nd	nd	nq	nq	nq	nq	nq	nq	nq	-
$\omega$ -viniferin	nq	nq	nq	nq	nq	nq	nq	nq	nq	nq	-
Total	44 (12)	152 (14)	170 (14)	229 (36)	200 (23)	209 (15)	221 (15)	241 (21)	316 (95)	558 (67)	234 (134)

**Table 5.**

Content of stilbenes (in µg/L) in commercial red wines. The figure in brackets represents the standard deviation.

Compound	Red wines										
	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	Mean
<i>E</i> -astringin	57 (6)	233 (18)	443 (29)	212 (12)	340 (13)	530 (30)	540 (23)	549 (29)	825 (110)	2999 (179)	673 (846)
ampelopsin A	nq	nq	nq	nq	nq	nq	nq	nq	nq	nq	-
hopeaphenol	nq	nq	nq	nq	nq	nq	nq	311 (32)	nq	494 (21)	81 (175)
<i>E</i> -piceid	152 (13)	794 (85)	1020 (66)	1070 (70)	1780 (106)	2940 (207)	6570 (530)	3360 (250)	6820 (1060)	7118 (547)	3162 (2714)
isohopeaphenol	nq	490 (41)	353 (22)	nq	2020 (106)	1290 (62)	1150 (65)	2696 (169)	nq	7469 (597)	1547 (2714)
piceatannol	nq	nq	nq	nq	nq	nq	nq	740 (48)	370 (55)	271 (19)	138 (251)
<i>Z</i> -astringin	64 (10)	74 (9)	63 (6)	142 (13)	50 (4)	83 (3)	102 (4)	145 (7)	84 (7)	234 (12)	104 (56)
<i>E</i> -resveratrol	nd	278 (52)	209 (14)	332 (27)	714 (62)	808 (92)	2656 (268)	3755 (38)	3577 (65)	1503 (160)	1383 (1433)
R2-viniferin	nq	nq	nq	nq	nq	nq	nq	930 (39)	nq	3280 (109)	421 (1046)
<i>Z</i> -piceid	125 (12)	999 (109)	1207 (72)	1462 (92)	2024 (142)	2800 (210)	6908 (542)	5457 (399)	8871 (1330)	7469 (597)	3732 (3149)
miyabenol C	nq	nq	nq	nq	nq	nq	nq	nq	nq	1411 (116)	141 (446)
ε-viniferin	nq	nq	nq	nq	nq	nq	nq	nq	nq	805 (47)	81 (255)
<i>Z</i> -resveratrol	nq	302 (52)	nq	232 (17)	537 (46)	1025 (86)	2213 (233)	6085 (538)	3792 (67)	2129 (271)	1813 (2023)
R-viniferin	nq	nq	nq	nq	nd	nq	nq	nq	nq	nq	-
ω-viniferin	nq	nq	nq	nq	nq	nq	nq	nq	nq	305 (12)	31 (96)
Total	399 (41)	3170 (366)	3295 (209)	3450 (231)	7465 (479)	9476 (690)	20139 (1665)	24028 (1665)	24339 (2687)	35487 (2666)	13125 (11975)