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Abstract

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

Keywords: stilbene; viniferin; wine; mass spectrometry.

1. Introduction

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

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

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

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

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

2. Materials and methods

2.1. Stilbene standards and wine samples

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

2.2. Preparation of wine solutions

Ultrapure water from Milli Q Direct System (Merck Millipore, USA), L-(+)-tartaric acid (Merck, Germany), ethanol 96% v/v (pharma grade, Panreac, Spain), and sodium hydroxide pure pellets (pharma grade, Panreac, Spain) were used for wine matrix. For model wine solution (MW), tartaric acid (4 g) was diluted into 120 mL of ethanol 96% on 1 L volumetric flask. Solution was flushed with water up to 1 L. pH was adjusted with drops of sodium hydroxide 2 N solution up to 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 40ºC and sample temperature at 10ºC.
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^-).
159 Mass spectrometer was set on 2.30 kV capillary source voltage, 450ºC source desolvation
160 temperature, 1000 L/h (N_2) 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,

0.005, 0.003 and 0.001 mg/L solutions. Calibration was prepared in duplicates and injected five times. Area value relation with internal standard area was used as quantification response. Calibration was calculated considering origin forced inclusion and no weighting function. Standards were injected to study linearity and accuracy (LOD and LOQ).

For intra- and interday effects, calibration curve with internal standard experiment was re-injected 5 days after. Vials were kept at 4ºC in a fridge. Relative standard deviation (RSD) at day 0 and day 5 were result of 5-times standard injection.

2.5. Commercial wine analysis

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

2.6. Statistical analysis

Statistical analyses were performed using R scripts in BioStatFlow web application (biostatflow.org, v2.9).

3. **Results and discussion**

3.1. Analysis of individual stilbenes and selection of MRM conditions

Triple quadrupole mass spectrometers (QqQ-MS) are normally programmed in multiple reactions monitoring (MRM), where several transitions between the parent ion and their fragment ions are collected. The MRM mode used in LC-QqQ-MS methodology provides the 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, ε- and ω-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_2CO). 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

follow a similar pattern. The qualifier ions for piceid (*m/z* 389) and astringin (*m/z* 405) correspond to the loss of the glucose moiety (162 u), giving the fragment ions at *m/z* 227 and 243, respectively. This transition is generally the most commonly obtained for stilbene glucosides (Buiarelli et al., 2007; López-Hernández & Rodríguez-Bernaldo de Quirós, 2016). Concerning dimers, ε- and ω-viniferin, share a parent ion at *m/z* 453 and a quantifier ion at *m/z* 359, which corresponds to the loss of the phenol ring (94 u) (Ehrhardt et al., 2014). The ω-viniferin gives also a fragment at *m/z* 347, consistent with a C_7H_6O loss (106 u), in accordance with previous results (Ehrhardt et al., 2014; Moss et al., 2013). Regarding ampelopsin A (*m/z* 469), it gives two major ions at *m/z* 451 (loss of H_2O) and 363 (loss of 106 u, 4-methylenecyclohexan-2,5-dienone). The trimer miyabenol C (*m/*z 679) quantifier signal at *m/z* 345 has been previously reported (Vrhovsek et al., 2012). It would correspond to the loss of two molecules of 4-methylenecyclohexan-2,5-dienone (106 u), a CO group (28 u), and a phenol (94 u). The corresponding qualifier ion at *m/z* 451 is formed after the loss of a 4-methylenecyclohexan-2,5-dienone (106 u), a phenol (94 u) and a CO group (Moss et al., 2013). Four tetramers (ion precursor at *m/z* 905) were included in the method. The fragment at *m/z* 359 was observed for all of them, and is the main transition for hopeaphenol and isohopeaphenol. This ion has been suggested to arise from the symmetrical splitting of the tetramer molecule and the additional loss of a phenol (Moss et al., 2013). In addition, hopeaphenol and isohopeaphenol share the main qualifier ion at *m/z* 451 - which would correspond to the loss of a dimer (Moss et al., 2013). Meanwhile, R2-viniferin has as a quantifier ion at *m/z* 811, consistent with the loss of one phenol group (94 u). The R-viniferin gives a fragment at *m/z* 799, which consistent to the loss of a 4-methylencyclohexan-2,5-dienone (106 u).

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 µg/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 µg/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-Gaitan et al. reported LOQ of 220, 70, 150
291 and 90 µg/L for resveratrol, piceid, piceatannol and ε-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 ± 12% and 101 ± 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 ± 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 ± 12%, 93 ± 12%, and 72 ± 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 (×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 (Lamuela-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 (ε-viniferin), one trimer
336 (miyabenol C), and four tetramers (hopehaphenol, isohopeaphenol, R- and R2-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

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

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

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

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

4. Conclusion

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

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

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536

Figure Legends

Figure 1.

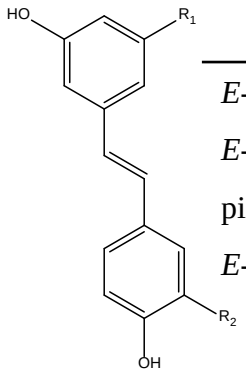
Stilbene structures.

Figure 2.

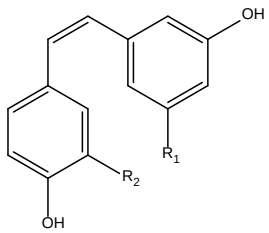
Representative MRM chromatograms of a model wine spiked with 1 mg/L of each stilbene. The transition of quantification is shown for each compound.

Figure 3.

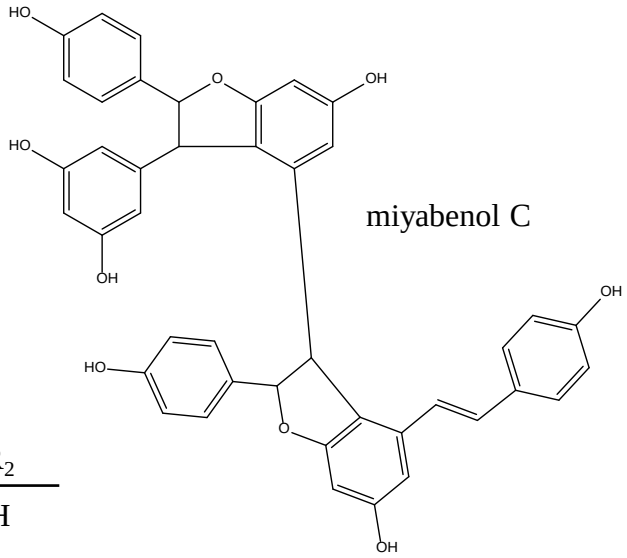
PCA score and loading plots of the two principal components of white (open circles) and red wines (full squares).



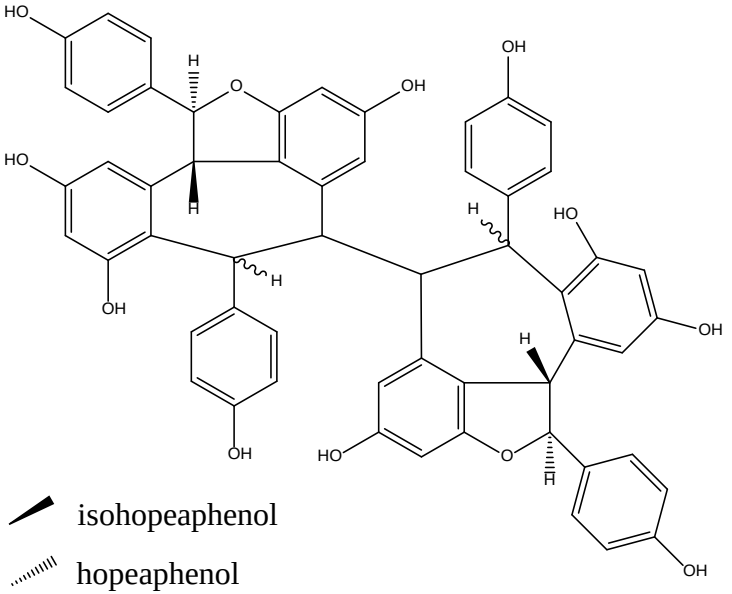
	R_1	R_2
E-resveratrol	OH	H
E-piceid	Oglc	H
piceatannol	OH	OH
E-astringin	Oglc	OH



	R_1	R_2
Z-resveratrol	OH	H
Z-piceid	Oglc	H
Z-astringin	Oglc	OH

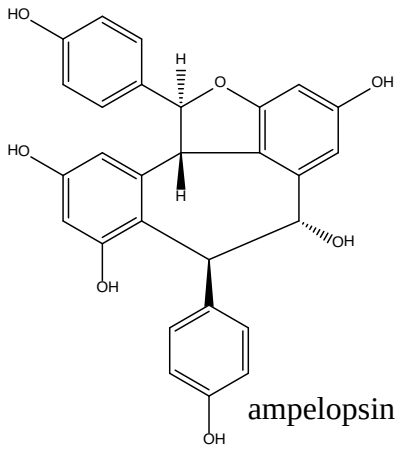


miyabenol C

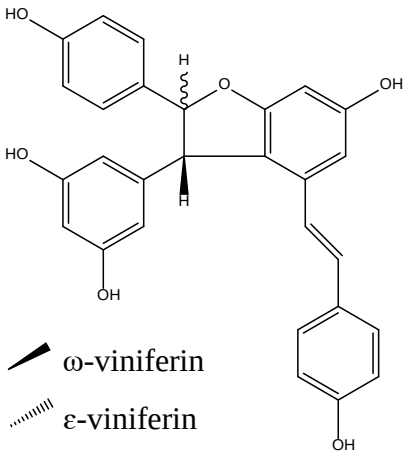


isohopeaphenol

hopeaphenol

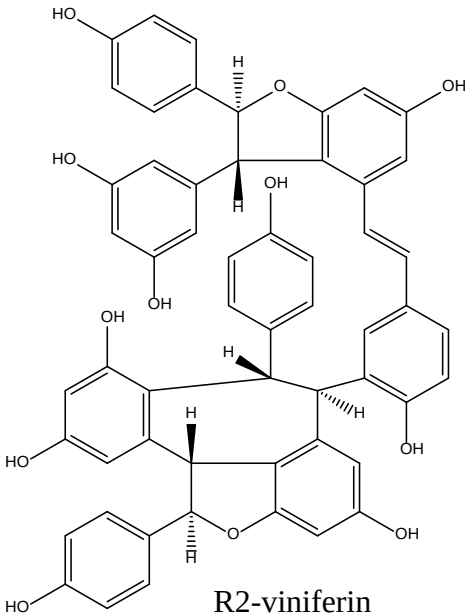


ampelopsin A

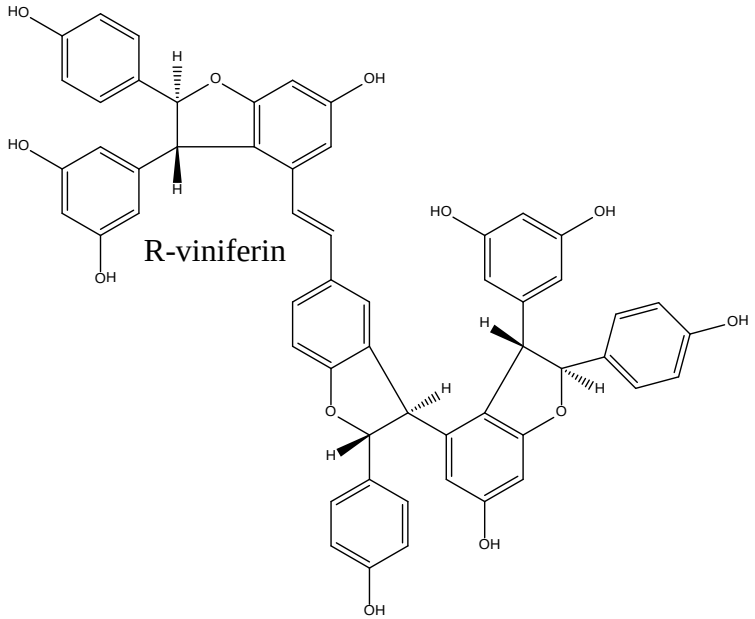


ω-viniferin

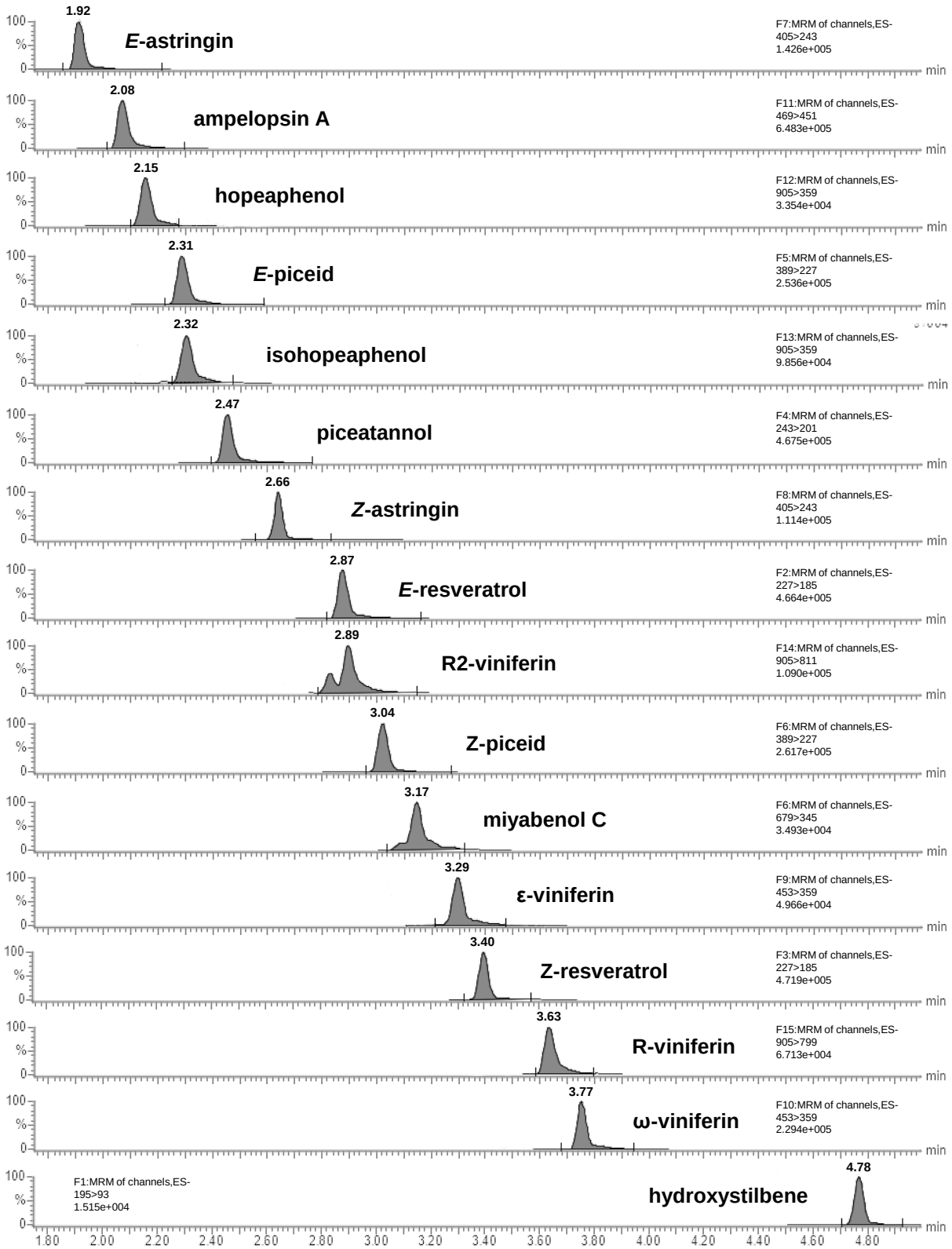
ε-viniferin

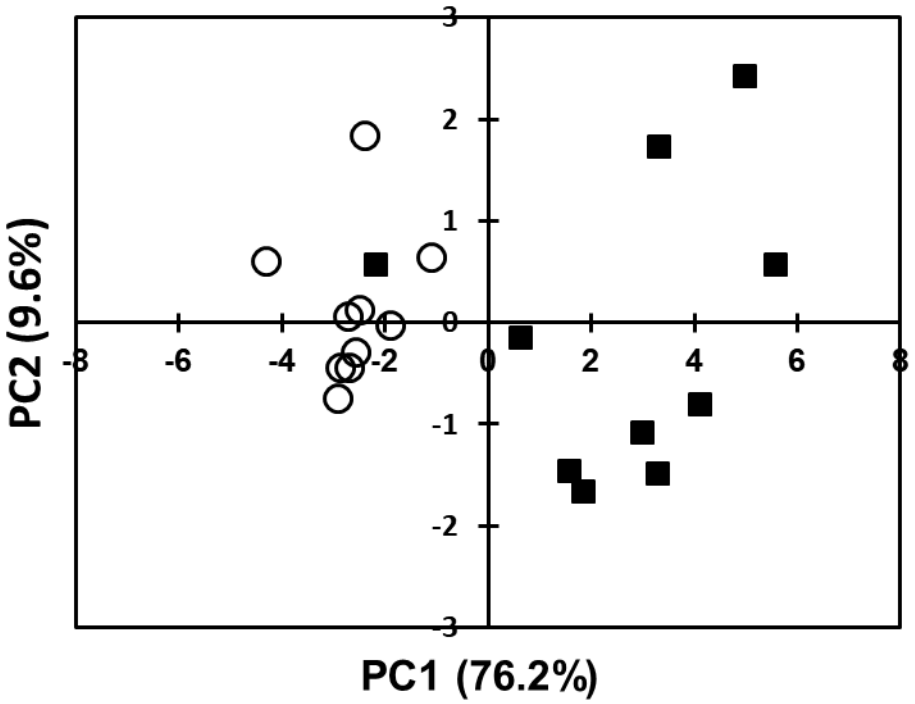


R2-viniferin



R-viniferin


1.92
E-astringin
F7:MRM of channels,ES-
405>243
1.426e+005
2.08
ampelopsin A
F11:MRM of channels,ES-
469>451
6.483e+005
2.15
hopeaphenol
F12:MRM of channels,ES-
905>359
3.354e+004
2.31
E-piceid
F5:MRM of channels,ES-
389>227
2.536e+005
2.32
isohopeaphenol
F13:MRM of channels,ES-
905>359
9.856e+004
2.47
piceatannol
F4:MRM of channels,ES-
243>201
4.675e+005
2.66
Z-astringin
F8:MRM of channels,ES-
405>243
1.114e+005
2.87
E-resveratrol
F2:MRM of channels,ES-
227>185
4.664e+005
2.89
R2-viniferin
F14:MRM of channels,ES-
905>811
1.090e+005
3.04
Z-piceid
F6:MRM of channels,ES-
389>227
2.617e+005
3.17
miyabenol C
F6:MRM of channels,ES-
679>345
3.493e+004
3.29
ε-viniferin
F9:MRM of channels,ES-
453>359
4.966e+004
3.40
Z-resveratrol
F3:MRM of channels,ES-
227>185
4.719e+005
3.63
R-viniferin
F15:MRM of channels,ES-
905>799
6.713e+004
3.77
ω-viniferin
F10:MRM of channels,ES-
453>359
2.294e+005
F1:MRM of channels,ES-
195>93
1.515e+004
4.78
hydroxystilbene
100
%
0
min
1.80
2.00
2.20
2.40
2.60
2.80
3.00
3.20
3.40
3.60
3.80
4.00
4.20
4.40
4.60
4.80


PC2 (9.6%)
PC1 (76.2%)
3
2
1
0
-1
-2
-3
-8
-6
-4
-2
0
2
4
6
8

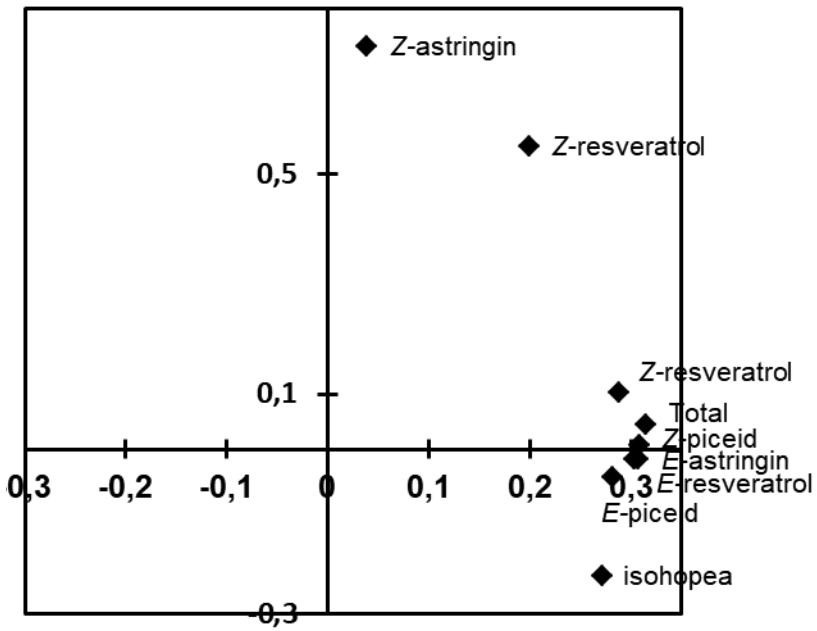

Z-astringin
Z-resveratrol
0,5
0,1
-0,3
0,3
-0,2
-0,1
0
0,1
0,2
0,3
Z-resveratrol
Total
Z-piceid
E-astringin
E-resveratrol
E-piceid
isohopea

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 (*m/z*)	Qualifier				Quantifier			
			Product ion	Dwell (s)	Cone[a] (V)	Coll.[b] (eV)	Product ion	Dwell (s)	Cone (V)	Coll. (eV)
E-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
E-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
E-piceatannol	2.47	243	159	0.021	60	25	201	0.021	60	20
Z-astringin	2.66	405	159	0.016	50	50	243	0.016	50	20
E-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
Z-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
ε-viniferin	3.29	453	225	0.016	65	20	359	0.016	65	30
Z-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
ω-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

[a]Cone: Cone voltage; [b]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^2)**	**Linearity (mg/L)**	**LOD (mg/L)**	**LOQ (mg/L)**
E-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
E-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
E-piceatannol	y = 1.87x + 0.084	0.9947	0.06 – 4.0	0.018	0.061
E-resveratrol	y = 3.20x + 0.180	0.9775	0.03 – 4.0	0.010	0.030
R2-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
ε-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
ω-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
ε-viniferin	130	10	8	99	8	6	74	13	8
R-viniferin	83	24	10	103	19	10	79	14	9
ω-viniferin	107	8	6	88	11	6	82	7	7

Table 4.

Content of stilbenes (in μg/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**
E-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	-
E-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	-
Z-astringin	21 (5)	9 (2)	11 (3)	12 (15)	15 (7)	11 (2)	16 (3)	48 (2)	14 (3)	20 (4)	18 (11)
E-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	-
Z-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	-
ε-viniferin	nq	nq	nq	nd	nq	nq	nq	nq	nq	nq	-
Z-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	-
ω-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**
E-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)
E-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)
Z-astringin	64 (10)	74 (9)	63 (6)	142 (13)	50 (4)	83 (3)	102 (4)	145 (7)	84 (7)	234 (12)	104 (56)
E-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)
Z-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)
Z-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)