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Abstract

Astilbin (2R, 3R) was recently reported to contribute to wine sweetness. As its aglycon contains two stereogenic centers, three other stereoisomers may be present: neoisoastilbin (2S, 3R), isoastilbin (2R, 3S), and neoastilbin (2S, 3S). This work aimed at assaying their presence for the first time in wines as well as their taste properties. The isomers were synthesized from astilbin and purified by semi-preparative HPLC. With the four stereoisomers, a sweet taste was perceived whose intensity varied with the configuration. Their content was assayed by developing a UHPLC-Q-Exactive method. The method was applied to screen astilbin and isomers in various wines, especially in different vintages from the same estate. While young wines contained higher concentrations of astilbin than the old ones, the concentrations of the other isomers, mainly neoastilbin, were higher in the old wines, suggesting their formation over time.

Keywords: Sweetness**,** method validation, taste, isomers, MS/MS, Q-Exactive

Highlights

1. First identification of neoastilbin, neoisoastilbin and isoastilbin, three stereoisomers of astilbin in wine.
2. Evaluation of sweet perception for all stereoisomers.
3. Development of an LC-HRMS method for quantifying astilbin isomers in wine.
4. Application of the method to analyze wines up to one century old
5. Unlike astilbin, neoastilbin levels were higher in old wines than in young ones.

1. Introduction

Wine is a complex matrix containing thousands of compounds, many of them remain unidentified. Some of them have organoleptic properties (Ribéreau-Gayon et al., 2012). They are likely to contribute to the different flavors of wine and especially the soft component, which plays a major role in the taste balance of dry wines by reducing their acidity and their bitterness (Peynaud, 1980). While these taste balances are intimately linked to the composition of the grapes, they are modulated during winemaking by the selective extraction of the berry constituents, and they evolve during aging in both barrel and bottle (Marchal et al., 2013). Indeed, natural sweet compounds released by oak wood (Gammacurta et al., 2019; Marchal et al., 2011b) or yeast lees (Marchal et al., 2011a) have been identified by taste-guided purification. Recently, such an approach allowed the isolation of two compounds from grapes that might contribute to the sweetness of dry wines: *epi*-DPA-G and astilbin (Crétin, 2016; Cretin et al., 2019).

Astilbin, or (2R,3R)-3,3′,4′,5,7-pentahydroxyflavanon-3-α-L-rhamnopyranoside, is a dihydroflavonol rhamnoside found in many plants and plant-derived products, such as *Rhizoma Smilax glabra* (Zheng et al., 2018), *Engelhardtia chrysolepis* (Igarashi et al., 1996), *Rhizoma Smilax Chinae* (Zhang et al., 2012), grape and wine (Crétin, 2016; K. Trousdale and L. Singleton, 1983; Landrault et al., 2002). It exerts a variety of biological activities such as anti-bacterial (Wang et al., 2019), antioxidative (Zhang et al., 2012) and regulation of fat metabolism (Chen et al., 2001). The aglycon of astilbin is dihydroquercetin, also named taxifolin, and it contains two stereogenic centers: carbons C-2 and C-3. Depending on the configuration of these carbons, astilbin (2*R*, 3*R*) has three other stereoisomers, i.e. neoisoastilbin (2*S*, 3*R*), isoastilbin (2*R*, 3*S*), and neoastilbin (2*S*, 3*S*), as shown in **Figure 1** (Gaffield et al., 1975).

Several authors have studied the stability of astilbin in order to predict the duration of its physiological effects in foods and beverages. In 1960, Tominaga suggested the existence of *cis* and *trans* isomers of astilbin involving C-2 and C-3 of the heterocyclic ring (Tominaga, 1960). The interconversion between isomers has been described in various works (Gaffield et al., 1975; Zheng et al., 2018). Based on studies on dihydroquercetin (Elsinghorst et al., 2011), the putative mechanism of this isomerization involves the formation of a quinone methide that can either recyclize to give neoisoastilbin or epimerize *via* a hydroxychalcone to provide isoastilbin and neoastilbin after recyclization (Zhang et al., 2013).

Astilbin was identified in wine for the first time by Trousdale and Singleton (K. Trousdale and L. Singleton, 1983) within a concentration range of 0.10-2 mg/L. Later on, its presence was also reported in red wine, in sweet wines made with botrytized grapes, and in Champagne (Chamkha et al., 2003; Landrault et al., 2002; Vitrac et al., 2001). The sweet taste of astilbin was described only recently (Crétin, 2016) and an LC-HRMS method has been developed to quantify it in dry wines (Fayad et al., 2020). However, the presence of astilbin isomers has never been reported in wine. In a study on Malbec wine from Argentina, Fanzone *et al.* mentioned the presence of an astilbin derivative on the basis of UV data, but no structure was proposed (Fanzone et al., 2010). Yet the sweet properties of these isomers have already been suggested (Kasai et al., 1988), which highlights their potential value.

The present work investigated the presence of astilbin isomers in red wines. First, neoisoastilbin, isoastilbin, and neoastilbin were synthesized from astilbin and their sensory properties were assessed. Their presence was sought in commercial red wines by LC-HRMS targeted screening. This method was validated to quantitate astilbin and its isomers in a repeatable and sensitive manner. The method was then applied to screen astilbin and its isomers in various commercial wines, especially in different vintages from the same estate, to analyze their evolution over time.

2. Materials and methods

2.1. Chemicals and commercial wines

Astilbin (LC-MS purity ≥ 95 %), was isolated from vine stems by centrifugal partition chromatography and semi-preparative high performance liquid chromatography (HPLC) according to the procedure described by Cretin (2016) (Crétin, 2016). Ultrapure water (Milli-Q purification system, Millipore, France) and HPLC-grade methanol (VWR International, Pessac, France) were used for sample preparation. Butan-1-ol and acetonitrile used for the purification of isomers were supplied by VWR International (Pessac, France). LC-MS-grade acetonitrile, water and formic acid used for mass spectrometry analysis were purchased from Fisher Chemical (Illkirch, France). Samples of 63 commercial red wines were used for isomer identification and quantitation. The wines were from various regions (39 from Bordeaux, 16 from Burgundy, 6 from Beaujolais, 1 from Roussillon and 1 from Germany) with vintages varying from 1918 to 2017. Among them, two series of different vintages from the same winery were analyzed: 16 Clos des Lambrays from 1918 to 2017 (CDL1918 – CDL2017) and 20 Pessac-Léognan between 1998 and 2017 (PL1998 – PL2017).

2.2. Astilbin isomerization

An aliquot of 340 mg of astilbin was dissolved in 300 mL of hydro-ethanolic solution (12 % v/v EtOH in ultrapure water) and pH was adjusted to 5 with formic acid. This value had been chosen after preliminary tests at various pHs. The mixture was heated at 60 °C for 7 days. After five liquid-liquid extractions with 50 mL of butanol saturated with water, the combined organic layers were evaporated to dryness, suspended in water and freeze-dried to obtain 323 mg of pale orange powder.

2.3. Purification by semi-preparative liquid chromatography

Semi-preparative HPLC analyses were performed using a Waters Prep 150 LC including a 2545 Quaternary Gradient Module, a 2489 UV/ Visible detector, and a 2424 ELSD detector (Waters, Guyancourt, France). An Atlantis T3 OBD prep column (19 × 250 mm, 5 µm, Waters, Guyancourt, France) was used. The mobile phase was a mixture of ultrapure water containing 0.1 % of formic acid (Eluent A) and acetonitrile with 0.1 % of formic acid (Eluent B). The flow rate was set to 20 mL/min. The gradient was 0 min, 10 % (B); 2.46 min, 10 % (B); 4.91 min, 20 % (B) 14.73 min, 20 % (B); 24.56 min, 25 % (B); 34.38 min, 50 % (B); 39.29, 98 % (B); 44.20 min , 98 % (B); 44.70, 10 % (B).

Aliquots (around 40 mg) of powder were dissolved in 200 µL of methanol and in 200 µL of ultrapure water, 0.45 µm-filtered and successively introduced manually into the system. A total of 320 mg were injected. UV detection was carried out at 254 and 280 nm and chromatographic peaks were collected manually in tubes just after the detector. For each tube, 100 µL was taken, diluted 10-fold with ultrapure water before being injected in LC-HRMS to check the purity of the obtained compounds. Samples obtained were pooled, evaporated *in vacuo* to remove acetonitrile, and freeze-dried to obtain white powders.

Thus, 59 mg of astilbin, 29 mg of neoastilbin, 10.80 mg of isoastilbin and 25.40 mg of neoisoastilbin were obtained. Their relative stereochemistry was determined by ROESY NMR experiments on a Bruker Avance 600 NMR spectrometer (^{1}H at 600 MHz) equipped with a 5-mm TXI probe. The specific optical rotations were measured with a JASCO P-2000 polarimeter with a sodium emission wavelength (λ = 589 nm).

Neoastilbin: white amorphous powder; $[\alpha]^{25}_{D}$ -107 (*c* 0.01, MeOH); ^{1}H NMR (CD_3OD, 600 MHz), see **Table S1 (supplementary data)**; HRMS *m/z* 449.1078 $[M-H]^-$($C_{21}H_{21}O_{11}^-$) (-1.1 ppm)

Isoastilbin: white amorphous powder; $[\alpha]^{25}_{D}$ -129 (*c* 0.01, MeOH); ^{1}H NMR (CD_3OD, 600 MHz), see **Table S1 (supplementary data)**; HRMS *m/z* 449.1076 $[M-H]^-$($C_{21}H_{21}O_{11}^-$) (-1.3 ppm)

Neoisoastilbin: white amorphous powder; $[\alpha]^{25}_{D}$ +51,2 (*c* 0.01, MeOH); ^{1}H NMR (CD_3OD, 600 MHz), see **Table S1 (supplementary data)**; HRMS *m/z* 449.1078 $[M-H]^-$($C_{21}H_{21}O_{11}^-$) (-1.1 ppm)

2.4. Sensory analysis

The sensory analysis took place in a specific room air-conditioned at 20 °C and equipped with individual booths. The compounds were dissolved at 5 mg/L in a non-oaked white wine (Bordeaux, 2013, 100 % Sauvignon blanc, 13 % vol. alc.) with a low astilbin level (<0.5 mg/L). Samples were tasted in clear INAO wine glasses by five experts in winetasting (four women, one man, aged from 24 to 54 years old). The tasters were informed of the nature and risks of the present study and were asked for their written consent to participate. They were asked to describe the gustatory perception of each compound using the vocabulary of winetasting. Sweetness and acidity intensity were evaluated on a scale from 0 (not detectable) to 5 (strongly detectable) and compared to a blank solution. Even though the compounds were observed in wines, the panelists were advised not to swallow but to spit out the samples after tasting.

2.5. Sample preparation

Stock solutions of astilbin, isoastilbin, neoastilbin and neoisoastilbin were prepared in methanol at 1 mg/mL and stored at 4 °C. Working solutions were obtained by diluting the stock solutions to the corresponding concentration. Each sample of wine was diluted to 1/3 in pure water and 0.45 µm-filtered before injection in LC-HRMS.

2.6. Liquid chromatography – High Resolution Mass Spectrometry (LC-HRMS)

Chromatographic separation was achieved using a Vanquish Flex system (Thermo Fisher Scientific, Les Ulis, France) consisting in a binary pump, an autosampler and a heated column compartment.

Three C18 columns were tested: Hypersil Gold (2.1 mm x 100 mm, 1.9 µm) from Thermo Fisher Scientific, High Silica Strength (HSST3; 100 mm x 2.1 mm, 1.8 µm) and Bridged Ethylsiloxane/silica Hybrid (BEH; 100 mm x 2.1 mm, 1.7 µm) both from Waters. The flow rate was set at 600 µL/min for Hypersil Gold and 400 µL/min for HSST3 and BEH. The injection volume was 5 µL and the eluents were (A) 0.1 % formic acid in water and (B) 0.1 % formic acid in acetonitrile. For the optimized gradient, eluent B varied as follows: 0 min, 10 %; 1 min, 20 %; 3 min, 20 %; 5 min, 25 %; 7 min, 50 %; 8 min, 98 %; 10 min, 98 %; 10,1 min, 10 %; 12 min, 10 %. The column and sample temperatures were 25 °C and 10 °C, respectively.

MS detection was performed using a Q-Exactive mass spectrometer equipped with a heated electrospray ionization (HESI II) probe (both from Thermo Fisher Scientific, Les Ulis, France). The mass analyzer was calibrated each week using Pierce® ESI Negative and Positive Ion Calibration Solutions (Thermo Fisher Scientific). The source parameters were optimized by direct injection of an astilbin solution (5 mg/L) as follows: sheath gas flow rate 65 arbitrary units (a.u.); auxiliary gas flow rate 5 a.u.; sweep gas flow rate 0 a.u.; spray voltage 2.7 kV; capillary temperature 300 °C; S lens RF level 55 a.u. and aux gas heater temperature 300 °C. Full MS scan data were acquired in negative ion mode within the range of *m/z* 150–600 at a resolution of 70,000 FWHM. The automatic gain control target was set at 3.10^6 ions, with a maximum injection time of 200 ms.

To identify the astilbin isomers present in red wine, product ion spectra were recorded using targeted SIM / data-dependent acquisition mode (t-SIM / dd-MS^2) at a resolution of 17,500 FWHM with *m/z* 449.1 ion in the inclusion list.

185 For quantitation of isomers, peak areas were determined by automatic integration of extracted ion
186 chromatograms (XIC) built in a 3 ppm window around the exact mass of the $[M-H]^-$ ion. All data
187 were processed using the Qualbrowser and Quanbrowser applications of Xcalibur version 2.1
188 (Thermo Fisher Scientific).

189 ***2.7. Validation of analytical method***

190 The method was validated for linearity, accuracy, sensitivity, and recovery. A commercial red
191 wine (Bordeaux 2018, 13.8 % alc. vol.) was chosen to validate the method. This sample contained
192 astilbin at a concentration of 3.30 mg/L as obtained in the previous method (Fayad et al., 2020).
193 Calibration curves were designed by plotting neoastilbin, astilbin, neoisoastilbin and isoastilbin
194 areas (*yi*) against the nominal concentration of each calibration standard (*xi*). These calibration
195 standards were prepared by spiking the red wine with standards to give thirteen levels of
196 concentrations; 0.002, 0.005, 0.01, 0.02, 0.04, 0.07, 0.15, 0.30, 0.60, 1.25, 2.50, 5, 10 and 20
197 mg/L. Linear regression was performed and the correlation coefficient (r^2), slope (a) and intercept
198 (b) were determined. The intra- and inter-assay accuracy and precision were evaluated for each
199 compound in terms of relative standard deviation (RSD) on retention time (tr) and peak area (A)
200 with five replicates (n=5) at eight different levels on a single assay and five assays on three non-
201 consecutive days.
202 LOD and LOQ were defined as the concentrations of the compounds that produced a signal-to-
203 noise ratio (S/N) of 3 and 10, respectively. The recovery was analyzed by spiking the red wine
204 with three different concentrations of neoastilbin, astilbin, neoisoastilbin and isoastilbin (100
205 µg/L, 500 µg/L and 1 mg/L; n=3). The concentration determined by means of the calibration
206 model was compared to the real concentration of the standard by calculating the recovery rate
207 ((determined concentration/real concentration) × 100).

3. Results and discussion

3.1. Synthesis and Sensory Characterization of Astilbin Stereoisomers

In a recent study, the analysis of a red wine by LC-HRMS revealed different signals in the extracted ion chromatogram (XIC) corresponding to *m/z* ions characteristic of the empirical formula of astilbin (Fayad et al., 2020). These results might suggest the presence of astilbin isomers in wine. Previous studies reported the isomerization of astilbin and the mechanism of this reaction has been clearly established for taxifolin using quantum chemistry calculation and circular dichroism (Elsinghorst et al., 2011). The same mechanism was proposed for the rhamnosyl derivatives (Zhang et al., 2013). The interconversion between astilbin (2R, 3R) and its stereoisomers involved a ring opening leading to a quinone methide. This compound can lead to neoisoastilbin (2S, 3R) by recyclization. The quinone methide can also epimerize by the formation of an α-hydroxychalcone to give isoastilbin (2R, 3S) and neoastilbin (2S, 3S) by recyclization. Preliminary tests guided the choice of a pH suited for isomerization and avoiding hydrolysis of the glycoside moiety. Mild acidic conditions (pH 5) were subsequently chosen to stay close to the composition of wine. From a solution of pure astilbin, a mixture of four main compounds was obtained after 7 days at 60 °C. LC-HRMS confirmed that these compounds had the same *m/z* ions. After extraction with butan-1-ol, the reaction mixture was submitted to semi-preparative HPLC to purify the four isomers. Only the fractions with a high level of purity (> 95 %) were kept. ROESY NMR correlations (**Figure S-1 to Figure S-4**) and comparison of optical rotations with literature data allowed the identification of astilbin ($[\alpha]^{25}_{D}$ -8), neoisoastilbin ($[\alpha]^{25}_{D}$ +51.2), isoastilbin ($[\alpha]^{25}_{D}$ -129) and neoastilbin ($[\alpha]^{25}_{D}$ -107).^{1}H NMR assignments for astilbin, neoastilbin, neoisoastilbin and isoastilbin are listed in **Table S-1**.

230 The sensory properties of the four stereoisomers purified were then investigated. Five experts in
231 winetasting evaluated the taste characteristics of a white non-oaked wine spiked individually with
232 the compounds. They rated the intensity of sweetness, bitterness, and sourness on a scale from 0 to
233 5 (**Table 1**). The non-spiked wine used as a reference was evaluated as 1/5 for bitterness and
234 sweetness, and 5/5 for acidity. An increase in sweetness was perceived for the modalities added
235 with astilbin and isoastilbin (3/5 both). The taste of neoastilbin and neoisoastilbin was evaluated
236 as sweeter (4/5 both). For all compounds, a decrease in acidity was also observed (3/5 for astilbin
237 and neoisoastilbin, 4/5 for isoastilbin and neoastilbin). No impact on bitterness was detected.
238 These results highlighted the sweetness of the four isomers, which confirmed and supplemented
239 previous studies. Indeed, Kasai et al. (1988) had extracted astilbin and its isomers from
240 *Engelhardtia chrysolepis* leaves. Only neoastilbin was reported as sweet but the tasting conditions
241 were not described (Kasai et al., 1988). Recently, Cretin identified astilbin as a sweet compound
242 in wine (Crétin, 2016).
243 The sweetness intensity of the isomers seemed to be influenced by their stereochemistry.
244 Interestingly, for the sweetest compounds, neoisoastilbin and neoastilbin, the stereogenic center
245 C2 had an *S* absolute configuration. Such effects of stereochemistry on taste properties have
246 already been described. For instance, naringin, which is present in grapefruit, has a different
247 bitterness depending on its majority form (2R or 2S) (Gaffield et al., 1975). For wine compounds,
248 lyoniresinol, which is extracted from oak wood and is the dextrorotatory form, develops a strong
249 bitterness, whereas its enantiomer is tasteless (Cretin et al., 2015).

250 ***3.2. Identification of astilbin isomers in red wine by LC-HRMS targeted screening***

251 Neoastilbin, astilbin, neoisoastilbin and isoastilbin are considered as marker constituents of plants
252 such as *Smilax Glabrae* (Chen et al., 2007, 2014; Zhang et al., 2019). To separate and quantify

253 these compounds, Chen et al. (Chen et al., 2007) developed an HPLC method to assay *Rhizoma*
254 *Smilacis Glabrae* samples from different locations in China. Later on, Li et al., (2012) developed
255 an LC-MS method to separate astilbin and its isomers by the interpretation of their retention time
256 and MS/MS data and by comparing these with the data provided by the literature under the same
257 LC-MS conditions. These methods have made significant contributions to the separation of
258 astilbin and its isomers. However, they are time-consuming.
259 Recently, a method was developed by LC-MS to quantitate astilbin and *epi*-DPA-G in dry red
260 wines (Fayad et al., 2020). A Hypersil C18 column was used with an elution gradient of water and
261 acetonitrile both acidified with 0.1 % formic acid. This method was rapid (less than 10 min),
262 sensitive (LOQ ≤ 20 µg/L), repeatable (RSD ≤ 3 %) and with a good recovery (≥ 89 %) (Fayad et
263 al., 2020). However, the separation of the purified isomers was not sufficient, particularly for
264 isoastilbin and neoisoastilbin.
265 To overcome this issue, the gradient elution was optimized by modifying the composition of the
266 eluents at the retention time of neoisoastilbin and isoastilbin (between 3 and 5 min). The best
267 conditions were obtained by increasing the percentage of the acetonitrile from 3 min to 7 min very
268 slowly. Therefore, instead of passing from 25 % (B) at 3 min to 90 % (B) at 7.5 min, the gradient
269 was delayed to 20 % (B) at 3 min to 50 % (B) at 7 min. Using this gradient, neoisoastilbin and
270 isoastilbin were better separated but the resolution obtained was less than 1. To increase this
271 resolution, HSST3 and BEH columns were also tested with a flow rate of 400 µL/min. The BEH
272 presented similar results to that of Hypersil, while the resolution with HSST3 was much better (R_S
273 = 1.2). This column was therefore chosen for the detection of astilbin isomers in red wines.
274 Negative ionization mode was chosen for mass spectrometry, since flavonoids have been shown to
275 exhibit stronger signal responses (Huang and Liaw, 2017). The ionization parameters were
276 optimized for astilbin detection by determining the most intense and characteristic product ions

277 and to ensure optimal transmission of ions to the mass analyzer. This optimization was carried out
278 by varying the nebulizing and drying gas flow rates, the spray voltage, the transfer capillary and
279 the vaporizer temperatures, resulting in a significant increase in signal intensity.
280 This improved LC-HRMS method was used to search for the presence of astilbin isomers in red
281 wine. Due to its mass accuracy measurement, Orbitrap mass spectrometry is well suited for
282 targeted screening of natural extracts containing a high diversity of compounds (Marchal et al.,
283 2015). For each sample of wine analyzed, extracted ion chromatograms (XIC) was built in a 5-
284 ppm window around *m/z* 449.10681, which corresponded to the theoretical *m/z* of the
285 deprotonated $[M-H]^-$ ion of astilbin. An example of such XIC obtained for CDL1946 is presented
286 in **Figure 2**. In most samples, five main peaks were observed in the XIC. Among them, astilbin
287 was detected at 4.39 min. Considering the mass measurement accuracy, the additional peaks at
288 2.77, 4.14, 5.09, and 5.30 min, suggested the presence of astilbin isomers. To assign these peaks,
289 the pure standards of astilbin stereoisomers were injected using the same method. The retention
290 times of neoastilbin, neoisoastilbin and isoastilbin were 4.14, 5.09, and 5.30 min, respectively.
291 Spiking wine samples with these standards led to a perfect co-elution and an increase in peak
292 areas. To confirm this hypothesis, MS^2 spectra were recorded for the five peaks. For signals at
293 4.14, 5.09, and 5.30 min, these spectra were similar to that of astilbin and showed main fragment
294 ions at *m/z* 303 and 285. The ions at *m/z* 303 differed from the deprotonated $[M-H]^-$ ion by 146.0
295 corresponding to the loss of the rhamnosyl moiety and were characteristic of the
296 taxifolin/epitaxifolin aglycons, with dehydrated species at *m/z* 285. These results confirmed the
297 presence of neoastilbin, isoastilbin and neoisoastilbin in red wine.
298 For the peak at 2.77 min, the MS^2 spectra of $[M-H]^-$ ion showed different fragments at *m/z* 287
299 and 269. These product ions corresponded to the loss of a hexosyl moiety (162.0) and a further
300 dehydration (loss of 18 Da), suggesting that this compound was not a stereoisomer of astilbin. The

301 ion at *m/z* 287 was associated with a $C_{15}H_{12}O_6$ moiety that might correspond to eriodictyol or
302 dihydrokaempferol (also named aromandendrin). Baderschneider and Winterhalter identified
303 dihydrokaempferol-3-*O*-glucoside in white Riesling wines (Baderschneider and Winterhalter,
304 2001). Moreover, dihydrokaempferol and its 3-*O*-rhamnoside (Singleton and Trousdale, 1983) as
305 well as kaempferol-3-*O*-glucoside (Cheynier and Rigaud, 1986) had already been described in
306 grapes and wine. These previous works suggested that the compound detected at 2.77 min might
307 be dihydrokaempferol-3-*O*-glucoside. However, another study mentioned the presence of
308 eriodictyol-glucoside in the skins of Sercial grapes (Perestrelo et al., 2012). Even though the
309 description of the identification process in that paper lacked clarity and although the presence of
310 eriodictyol in wine is not well documented, this hypothesis cannot been excluded. Analysis of
311 pure standards or the isolation of the compound eluted at 2.77 min would be necessary to identify
312 its chemical structure unambiguously.
313 Regardless of this unknown isomer, the LC-HRMS targeted screening allowed the identification
314 of neoastilbin, neoisoastilbin and isoastilbin in red wine. To our knowledge, the presence of these
315 three stereoisomers of astilbin has never been reported in wine.

316 ***3.3. Validation of quantification method to assay astilbin stereoisomers in red wine***

317 The method developed for targeted screening was also used for absolute quantitation of astilbin
318 isomers. A commercial red wine was used to build the calibration curves in order to avoid strong
319 matrix effects. The quantitation method was validated by evaluating linearity, repeatability,
320 sensitivity and recovery. Validation was performed in accordance with the regulatory guidelines
321 stipulating that a method used for the quantitative measurement of analytes should be reliable and
322 reproducible for the intended use (Peris-Vicente et al., 2015).

To study linearity, eight calibration samples of astilbin, neoastilbin, neoisoastilbin and isoastilbin were prepared in a red wine covering a range from 0.002 to 20 mg/L, in accordance with the astilbin concentrations previously measured (Fayad et al., 2020). The wine used for method validation contained astilbin at a concentration of 3.3 mg/L that had been used to build the calibration curve. The other stereoisomers were below the LOQ. **Table S-2 in supporting information** summarizes the correlation coefficient (r^2) of each isomer and the corresponding equation. For the four compounds, the calibration curves were satisfactorily linear with $r^2 \geq 0.9993$. Each back-calculated standard concentration was within the acceptance limits (CV ≤ 15 %).

Good sensitivity was obtained with LOD values of 21, 5, 7 and 20 µg/L for neoastilbin, astilbin, neoisoastilbin and isoastilbin, respectively (**Table S-2**). Precision was evaluated by performing intra- and inter-day repeatability (RSD) studies. RSD on retention time and area (RSD_{tr} and RSD_A) evaluated for the different compounds were ≤ 6.2 % and inter-day RSD were ≤ 7.2 %, indicating the stability of this proposed method.

To complete the validation, the recovery of each compound was evaluated by spiking three different red wines at three concentrations (100 µg/L, 400 µg/L and 4 mg/L) of neoastilbin, astilbin, neoisoastilbin and isoastilbin. The recovery values ranged from 81.3 to 101 %, which met the requirements of the guidelines and validated the accuracy of the method. These results indicated that the method was satisfactory for the analysis of astilbin and its isomers in red wine.

3.4. Quantitation of astilbin stereoisomers in commercial red wines

The validated LC-HRMS method was used to assay astilbin and its stereoisomers in 63 commercial wines from different regions and different vintages. As shown in **Table 2**, astilbin and neoisoastilbin were quantified in all wines, isoastilbin was below LOQ in two wines and

346 neoastilbin was not detectable or quantifiable in 12 wines. In all wines, astilbin was the most
347 abundant stereoisomer.
348 **Figure 3** shows the distribution of astilbin, neoastilbin, isoastilbin and neoisoastilbin
349 concentrations in 63 wines. The average concentration of astilbin was 9.10 mg/L with a minimum
350 value of 0.60 mg/L and a maximum value of 41.10 mg/L. Regarding isomers, the mean values of
351 neoastilbin, neoisoastilbin and isoastilbin were 1.08, 0.70 and 1.03 mg/L respectively. The
352 maximum concentrations of neoastilbin, neoisoastilbin and isoastilbin were 5.94, 2.73 and 4.45
353 mg/L respectively, found in CDL1946. All isomers were shown to increase the sweetness
354 perception of a wine at 5 mg/L, so the quantitative results demonstrated the sensory potential of
355 these flavanonols for some wines. Indeed, the concentrations of astilbin and its stereoisomers
356 varied considerably according to the origin of the wines. Wines from Beaujolais (BJ01 to BJ06)
357 contained high values of astilbin (from 15.51 mg/L to 23.67 mg/L). High concentrations of astilbin
358 and its stereosiomers were also found in wines from Burgundy and Ahr, whereas wines from
359 Bordeaux and Roussillon contained lower amounts. Apart from these regional differences, the
360 wines also differed in their grape variety: Gamay for Beaujolais, Pinot noir for Burgundy and Ahr,
361 Cabernet-Sauvignon, Merlot and Cabernet franc for Bordeaux, Mourvedre and Grenache noir for
362 Roussillon. One hypothesis explaining the differences between regions might be the grape
363 composition, some varieties being richer in astilbin than others, as already shown for Egiodola,
364 Merlot or Cabernet-Sauvignon (Landrault et al., 2002). However, previous works established the
365 abundance of astilbin in grape stems (Crétin, 2016; Souquet et al., 2000) and winemaking in
366 whole bunches is traditionally practiced in Beaujolais and Burgundy. For instance, this was the
367 case for wines from Clos des Lambrays analyzed here. Therefore, another explanation of the high
368 levels observed in some wines could be the presence of stems during vatting, which may have
369 increased the release of astilbin and isomers. Information on destemming was not available for all

370 wines, but no stems were present during the making of Beaujolais wines BJ01 to BJ05. For these
371 reasons, it seemed that the variations in astilbin isomer concentrations might result from various
372 factors such as grape variety and winemaking practices. Future studies will aim to clarify the
373 relative contribution of these parameters.
374 Interestingly, in the set of samples analyzed in this work, there were two series of vintages from
375 the same winery. Even if weather conditions and, to a lesser extent, winemaking techniques may
376 differ from one vintage to another, such series could be useful for comparing the concentrations of
377 astilbin stereoisomers in old or recent vintages. First, a series of samples of a well-known red
378 wine from Burgundy, Clos des Lambrays (CDL), covered 16 vintages over one century. A
379 previous study using the same wines revealed significant concentrations of astilbin, even in old
380 wines. The method developed in the present work allowed the quantitation of the other
381 stereoisomers. **Figures 4** and **S5** tend to suggest that young wines contained higher concentrations
382 of astilbin than old ones, while the concentrations of the isomers, mainly neoastilbin, were higher
383 in old wines. The difference in concentrations between astilbin and neoastilbin appeared to
384 decrease over time. For instance, in CDL2017, the concentrations of astilbin and neoastilbin were
385 40.90 mg/L and 0.15 mg/L, respectively, whereas in CDL1918 they were 8.00 mg/L and 5.84
386 mg/L. By plotting the vintage and the concentration, inverse correlations were observed for
387 neoastilbin ($r^2 = 0.62$) and astilbin ($r^2 = 0.31$) (**Figure 4**). These results suggest that neoastilbin
388 was formed over time, maybe through isomerization of astilbin. The levels of isoastilbin and
389 neoisoastilbin, albeit slightly higher in old wines, seemed less affected by the age of the wine.
390 A second series of wines from Pessac-Leognan (PL) allowed the comparison of astilbin
391 concentrations in 20 samples from a more limited range of vintages between 1998 and 2017. The
392 overall concentrations were lower than in CDL but the same trend was observed, with astilbin
393 varying from 3.50 mg/L in PL2017 to 1.25 mg/L in PL1998 and neoastilbin from 0.09 mg/L to

394 0.77 mg/L. **Figure S-5** (in supporting information) shows similar correlations to those observed in
395 CDL, which might indicate an increase in neoastilbin (r^2 = 0.65) and a decrease in astilbin (r^2 =
396 0.49) over time.
397 These results highlight the same trends and suggest that astilbin could be a native compound that
398 is present in grape, while the other stereoisomers are mainly obtained by isomerization.
399 Interestingly, astilbin and neoastilbin were the most abundant isomers in old wines. They have a
400 2,3-*trans* configuration and are therefore more stable. Kiehlmann et al. showed that 2,3-*trans*-
401 dihydroquercetin can epimerize in hot aqueous or alcoholic solution to give approximately 10 %
402 of *cis* isomer (Kiehlmann and Li, 1995). As wine is an acidic hydro-alcoholic solution, we
403 hypothesize that astilbin is first released during winemaking and then evolves slightly toward
404 thermodynamic equilibrium by the formation of neoastilbin. To confirm this hypothesis, future
405 work will study the presence of astilbin isomers in grape as well as their evolution during
406 winemaking and bottle aging.
407 From a sensory point of view, these findings are promising since neoastilbin and neoisoastilbin
408 have been shown to develop more sweetness than astilbin. The isomerization occurring over time
409 could be related to the usual gain of sweetness observed in old wines. This assumption could be
410 confirmed by determining the gustatory detection thresholds of these isomers and comparing the
411 quantitative data obtained in wines.

412 **4. Conclusion**

413 This study reports the first identification of astilbin stereoisomers in wine. Isoastilbin, neoastilbin
414 and neoisoastilbin were synthesized to allow the study of their sensory properties in wine. Their
415 addition to a wine modified the taste balance by increasing the perceived sweetness, whose

intensity varied according to the stereochemistry. Neoastilbin and neoisoastilbin were the most active compounds.

Thanks to the development and validation of an LC-HRMS analytical method, astilbin stereoisomers were identified and quantified for the first time in 63 commercial wines from different regions and different vintages. Astilbin was the predominant isomer in all the wines with an average concentration of 9.10 mg/L, while the other isomers were quantified at concentrations of the order of mg/L. Analysis of a series of vintages from two wineries revealed higher levels of astilbin, and especially neoastilbin, in old wines than in young ones. On the contrary, astilbin was generally more abundant in young wines. These results suggest that the isomerization of astilbin occurs during bottle ageing and leads mainly to the formation of neoastilbin, which is a trans isomer and might be thermodynamically more stable than isoastilbin and neoisoastilbin. Interestingly, neoastilbin and neoisoastilbin are sweeter than astilbin, so the isomerization of astilbin might be related to the gain in sweetness often observed in old wines.

Beyond providing new knowledge on the molecular origin of the sweet taste of dry wine, this study offers promising perspectives. Further studies are required to determine the impact of grape variety and winemaking practices on the presence of astilbin and its isomers. The determination of the gustatory detection thresholds of all isomers will be an asset to evaluate the influence of astilbin isomerization during aging on the taste balance of old wines.

Acknowledgements

The authors declare that there are no conflicts of interest.

Syntia Fayad's grant was supported by Région Nouvelle Aquitaine and CIVB whereas Marie Le Scanff's was funded by Chaire Denis Dubourdieu – Fondation Bordeaux Université. The authors are also grateful to Château Haut Bailly, B. Champy, and E. Guittard for providing samples and Biolaffort for material support. We thank Dr. Ray Cooke for proofreading the manuscript.

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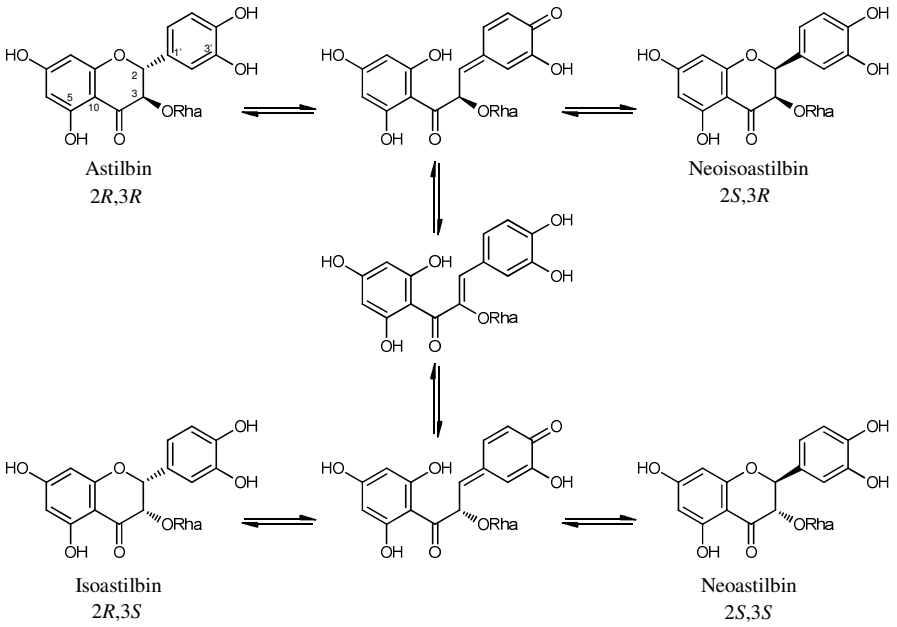

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552 **Figure 1.** Interconversion of astilbin and its isomers neoisoastilbin, isoastilbin and neoisoastilbin.

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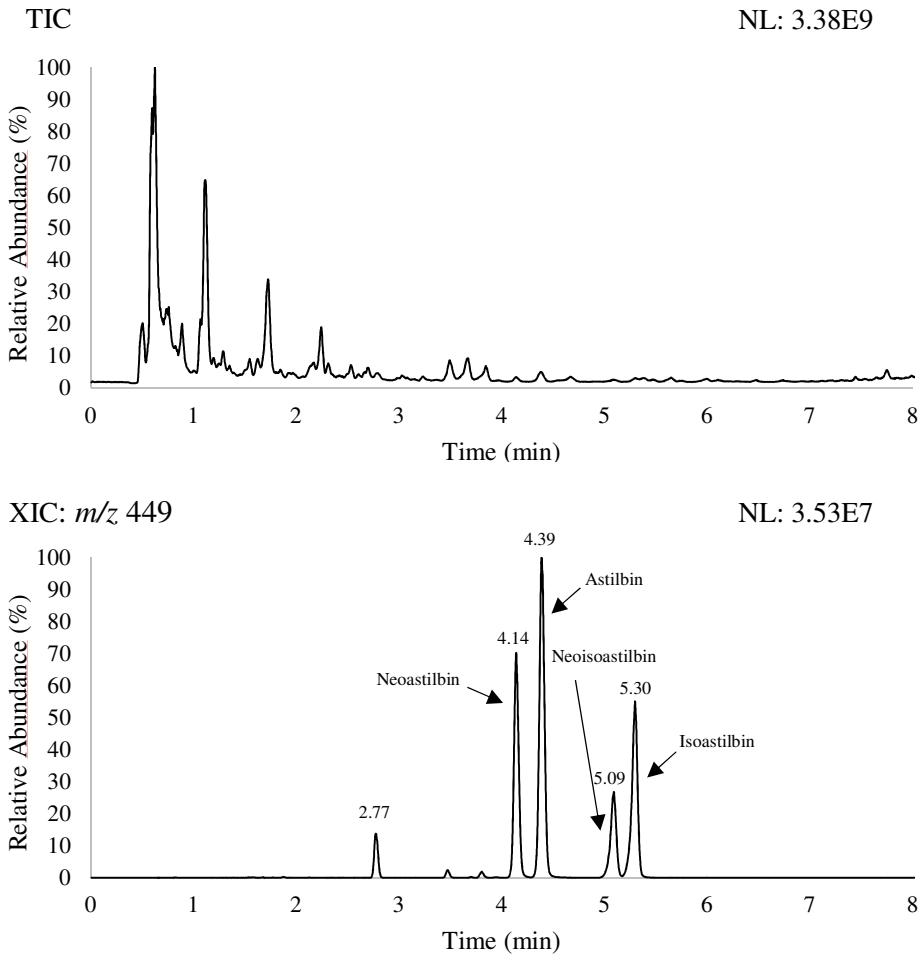

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555 **Figure 2.** Chromatograms TIC (top) and XIC (bottom) in negative ionization mode corresponding

556 to ion *m/z* 449 in Clos des Lambrays, vintage 1946 (CDL1946).

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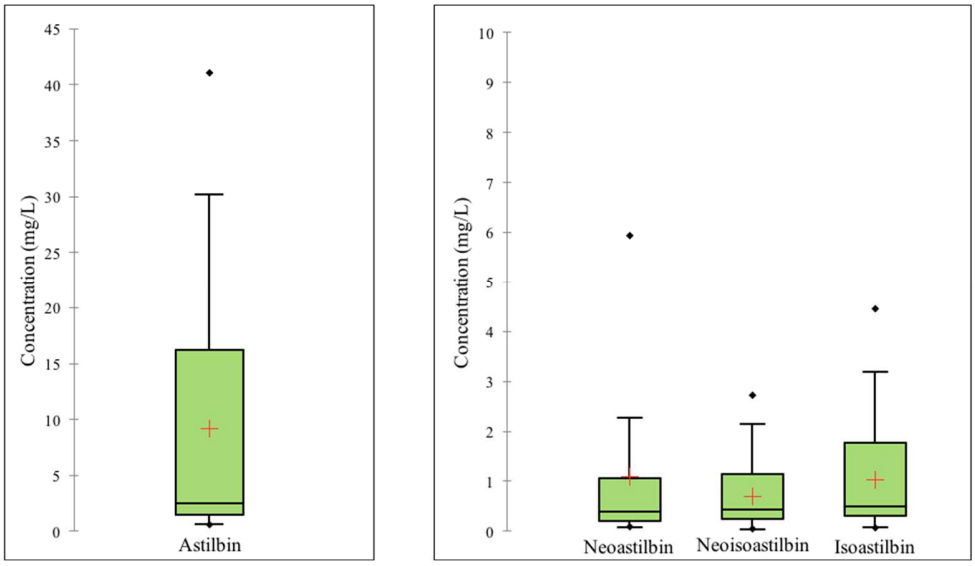
561 

562 **Figure 3.** Box plot of astilbin, neoastilbin, neoisoastilbin and isoastilbin concentrations in 63 red
563 wines.

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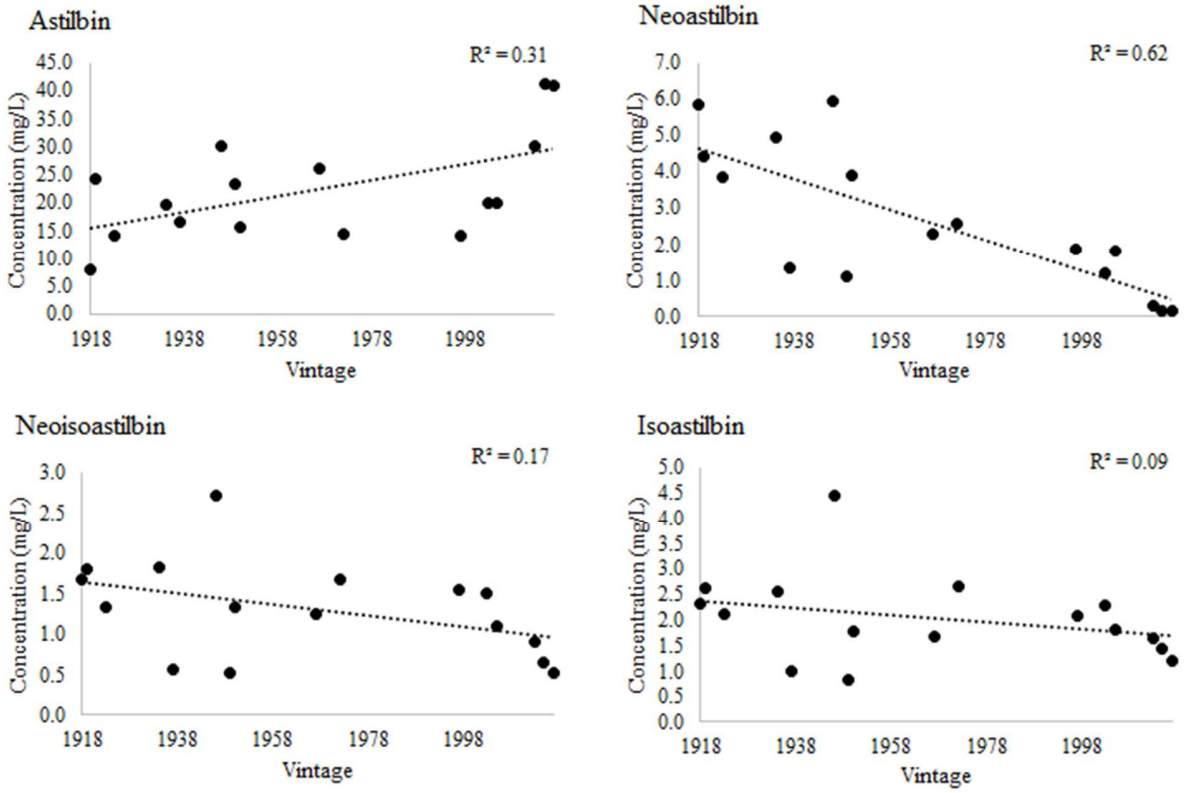

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566 **Figure 4.** Relationship between concentration of compounds and aging of Clos des Lambrays
567 (CDL) wines.

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570 **Table 1.** Gustatory description of isolated compounds.

Compounds	Taste in white wine		
	Sweet	**Acid**	**Bitter**
Astilbin	3/5	3/5	1/5
Neoastilbin	4/5	4/5	1/5
Neoisoastilbin	4/5	3/5	1/5
Isoastilbin	3/5	4/5	1/5

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574 **Table 2.** Quantification of astilbin, neoastilbin, neoisoastilbin and isoastilbin in several vintages of
575 red wine. Concentration values were measured in mg/L.

Num	Region	Appellation	Grape Variety [1]	Vintage	Astilbin	Neoastilbin	Neoisoastilbin	Isoastilbin
BD01	Bordeaux	Blaye	Blend / Merlot	2016	2.93	<LOD	0.10	0.15
BD02	Bordeaux	Graves	Blend / Cabernet Sauvignon	2011	2.53	0.09	0.30	0.36
BD03	Bordeaux	Haut-Médoc	Blend / Merlot	2012	1.64	0.10	0.26	0.22
BD04	Bordeaux	Haut-Médoc	Blend / Merlot	2015	1.45	<LOD	0.11	0.10
BD05	Bordeaux	Margaux	Blend / Cabernet Sauvignon	2012	0.63	<LOQ	0.09	<LOQ
BD06	Bordeaux	Médoc	Blend / Cabernet Sauvignon	2013	3.30	<LOD	0.25	0.30
BD07	Bordeaux	Pauillac	Blend / Cabernet Sauvignon	2010	0.75	<LOD	0.10	0.07
BD08	Bordeaux	Pauillac	Blend / Cabernet Sauvignon	2012	1.28	<LOQ	0.19	0.17
BD09	Bordeaux	Pomerol	Blend / Merlot	2014	4.87	0.16	0.55	0.65
BD10	Bordeaux	Saint-Emilion	Blend / Merlot	2012	2.23	0.09	0.29	0.32
BD11	Bordeaux	Saint-Estèphe	Blend / Cabernet Sauvignon	2012	1.38	0.09	0.23	0.19
BD12	Bordeaux	Saint-Julien	Blend / Cabernet Sauvignon	2002	2.26	0.47	0.50	0.53
BD13	Bordeaux	Saint-Julien	Blend / Cabernet Sauvignon	2003	0.82	0.23	0.21	0.18
BD14	Bordeaux	Saint-Julien	Blend / Cabernet Sauvignon	2012	2.44	<LOQ	0.23	0.29
BD15	Bordeaux	Saint-Julien	Blend / Cabernet Sauvignon	2012	1.18	<LOD	0.10	0.11
BD16	Bordeaux	Saint-Julien	Blend / Cabernet Sauvignon	2012	2.02	<LOQ	0.23	0.25
BD17	Bordeaux	Saint-Julien	Blend / Cabernet Sauvignon	2012	2.30	<LOQ	0.22	0.28
BD18	Bordeaux	Saint-Julien	Blend / Cabernet Sauvignon	2014	3.46	0.13	0.38	0.44

BD19	Bordeaux	Saint-Julien	Blend / Cabernet Sauvignon	2014	4.20	<LOQ	0.34	0.46
PL1998	Bordeaux	Pessac-Léognan	Blend / Cabernet Sauvignon	1998	1.25	0.77	0.44	0.42
PL1999	Bordeaux	Pessac-Léognan	Blend / Cabernet Sauvignon	1999	0.86	0.35	0.25	0.26
PL2000	Bordeaux	Pessac-Léognan	Blend / Cabernet Sauvignon	2000	1.33	0.99	0.50	0.51
PL2001	Bordeaux	Pessac-Léognan	Blend / Cabernet Sauvignon	2001	1.35	0.90	0.49	0.50
PL2002	Bordeaux	Pessac-Léognan	Blend / Cabernet Sauvignon	2002	1.75	0.60	0.50	0.54
PL2003	Bordeaux	Pessac-Léognan	Blend / Cabernet Sauvignon	2003	1.48	0.76	0.51	0.50
PL2004	Bordeaux	Pessac-Léognan	Blend / Cabernet Sauvignon	2004	0.76	0.40	0.27	0.26
PL2005	Bordeaux	Pessac-Léognan	Blend / Cabernet Sauvignon	2005	0.78	0.20	0.20	0.23
PL2006	Bordeaux	Pessac-Léognan	Blend / Cabernet Sauvignon	2006	1.06	0.33	0.30	0.33
PL2007	Bordeaux	Pessac-Léognan	Blend / Cabernet Sauvignon	2007	1.83	0.40	0.43	0.51
PL2008	Bordeaux	Pessac-Léognan	Blend / Cabernet Sauvignon	2008	1.34	0.33	0.35	0.37
PL2009	Bordeaux	Pessac-Léognan	Blend / Cabernet Sauvignon	2009	1.44	0.44	0.41	0.43
PL2010	Bordeaux	Pessac-Léognan	Blend / Cabernet Sauvignon	2010	1.90	0.28	0.37	0.47
PL2011	Bordeaux	Pessac-Léognan	Blend / Cabernet Sauvignon	2011	1.95	0.19	0.30	0.41
PL2012	Bordeaux	Pessac-Léognan	Blend / Cabernet Sauvignon	2012	1.75	0.20	0.31	0.39
PL2013	Bordeaux	Pessac-Léognan	Blend / Cabernet Sauvignon	2013	3.07	0.21	0.42	0.53

PL2014	Bordeaux	Pessac-Léognan	Blend / Cabernet Sauvignon	2014	4.39	0.25	0.53	0.70
PL2015	Bordeaux	Pessac-Léognan	Blend / Cabernet Sauvignon	2015	2.11	0.13	0.27	0.35
PL2016	Bordeaux	Pessac-Léognan	Blend / Cabernet Sauvignon	2016	1.96	0.09	0.20	0.32
PL2017	Bordeaux	Pessac-Léognan	Blend / Cabernet Sauvignon	2017	3.50	0.09	0.26	0.40
CDL1918	Burgundy	Clos des Lambrays	Pinot Noir	1918	8.00	5.84	1.67	2.31
CDL1919	Burgundy	Clos des Lambrays	Pinot Noir	1919	24.20	4.42	1.81	2.63
CDL1923	Burgundy	Clos des Lambrays	Pinot Noir	1923	14.10	3.84	1.33	2.10
CDL1934	Burgundy	Clos des Lambrays	Pinot Noir	1934	19.50	4.92	1.83	2.56
CDL1937	Burgundy	Clos des Lambrays	Pinot Noir	1937	16.50	1.31	0.56	1.00
CDL1946	Burgundy	Clos des Lambrays	Pinot Noir	1946	30.10	5.94	2.73	4.45
CDL1949	Burgundy	Clos des Lambrays	Pinot Noir	1949	23.30	1.10	0.52	0.83
CDL1950	Burgundy	Clos des Lambrays	Pinot Noir	1950	15.50	3.89	1.33	1.76
CDL1967	Burgundy	Clos des Lambrays	Pinot Noir	1967	26.10	2.27	1.24	1.67
CDL1972	Burgundy	Clos des Lambrays	Pinot Noir	1972	14.50	2.56	1.66	2.64
CDL1997	Burgundy	Clos des Lambrays	Pinot Noir	1997	14.20	1.84	1.54	2.08
CDL2003	Burgundy	Clos des Lambrays	Pinot Noir	2003	19.80	1.18	1.50	2.28
CDL2005	Burgundy	Clos des Lambrays	Pinot Noir	2005	20.00	1.82	1.09	1.80
CDL2013	Burgundy	Clos des Lambrays	Pinot Noir	2013	30.20	0.30	0.91	1.63
CDL2015	Burgundy	Clos des Lambrays	Pinot Noir	2015	41.10	0.16	0.66	1.45
CDL2017	Burgundy	Clos des Lambrays	Pinot Noir	2017	40.90	0.15	0.51	1.20
BJ01	Beaujolais	Moulin-à-vent	Gamay	2010	15.91	1.03	2.10	2.71
BJ02	Beaujolais	Moulin-à-vent	Gamay	2012	25.05	0.75	2.15	3.19
BJ03	Beaujolais	Moulin-à-vent	Gamay	2015	20.25	0.44	1.22	2.18
BJ04	Beaujolais	Moulin-à-vent	Gamay	2015	23.67	0.69	1.71	2.78
BJ05	Beaujolais	Moulin-à-vent	Gamay	2015	19.18	0.71	1.54	2.44
BJ06	Beaujolais	Moulin-à-vent	Gamay	2017	15.51	0.40	1.07	1.72

GE01	Germany/ Ahr	Walporzheim	Pinot Noir	2016	20.01	0.40	1.41	2.16
RO01	Roussillon	Collioure	Blend / Mourvèdre	2016	0.97	<LOD	0.04	<LOQ

LOQ: limit of quantification; LOD: limit of detection

1: Majority grape variety is mentioned when it concerns a blend.

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