New insights about astilbin, a sweet polyphenol: effect of grape variety and stems addition during winemaking

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ABSTRACT

In the usual red winemaking process, grapes are destemmed between the harvest and the filling of the vat. However, in regions like Bourgogne, some winemakers let all or a part of the stems in contact with the juice during vatting. This choice will likely affect the sensory properties of wine, such as its gustatory perception. The present study investigated the effect of adding stems during the winemaking process on the concentration of a sweetening compound, astilbin. The sensory contribution of astilbin in wines was first clarified by measuring its taste detection threshold and comparing it with the concentrations found in various commercial wines. Then, experimental wines resulting from the addition of stems in various proportions were analysed. These practical experiments were carried out in various French wine estates, in Bourgogne, Beaujolais and Bordeaux, over three vintages, allowing the comparison of different grape varieties, namely Pinot noir, Gamay and Merlot. For each experiment, the modality with the addition of stems was compared with a vat of destemmed grapes from the same plot. Samples were taken throughout the winemaking process to be analysed by liquid chromatography coupled with high-resolution mass spectrometry (UHPLC-Exactive, Orbitrap analyser). The results showed that the addition of stems during vatting significantly increased astilbin concentration. Furthermore, this increase varied according to the grape variety. Whereas the astilbin contents were lower in Merlot wines than in Pinot noir or Gamay wines, the ratio between its concentration of wines from the two modalities was higher in Merlot than in Pinot noir and Gamay. The localisation of astilbin in the different components of the bunch, depending on the grape variety, was also investigated to understand this difference better. Thus, a higher abundance of astilbin in stems than in skins was found in the Merlot variety, whereas, for Gamay and Pinot noir, the total quantity of astilbin in a bunch was located in equal proportions in stems and skins. These new results bring new tools to understand better the practice of whole bunch vinification from a chemical perspective.

KEYWORDS: stem, whole bunch vinification, red winemaking, sweetness, astilbin, grape variety, taste detection threshold
INTRODUCTION

From vine growing to bottle ageing, numerous parameters can influence the sensory properties of a wine. The winemaking process of red wines results in a combination of two basic processes: alcoholic fermentation and the partial extraction of soluble constituents from the solid parts of the grapes (Peynaud, 1988). Extraction occurs throughout the contact between the grape juice and the marc, which is composed of seeds, skins and sometimes the stems. The winemaking practices can modulate this extraction and thus influence the sensory properties of the wine. A better understanding of the chemistry involved in these organoleptic modifications can provide new tools for winemakers to monitor them better.

Aromatic and gustatory compounds are responsible for the sensory characteristics of a wine. Recently, the sweet properties of astilbin, a dihydroflavonol rhamnoside, have been highlighted (Cretin, 2016; Fayad et al., 2020). This compound had already been identified in wines and grapes (Landrault et al., 2002; Singleton and Trousdale, 1983; Trousdale and Singleton, 1983), but its taste had not been described. In addition, the presence of three isomers of this compound in red wines, namely neoastilbin, neoisoastilbin and isoastilbin, has been demonstrated (Fayad et al., 2021). These compounds also present sweetening properties, which differ according to their stereochemical configuration and seem to appear over time through the isomerisation of astilbin. Considering their sensory properties, these compounds, and more particularly astilbin, may contribute to the sweetness of dry wines. However, its taste detection threshold in wines has never been established until now, as well as the effect of winemaking practices on this concentration.

Previous work studying the phenolic composition of the stem showed that astilbin was one of the main phenolic compounds after quercetin-3-glucuronide, flavan-3-ol monomers, condensed tannins and phenolic acids (Souquet et al., 2000). More recently, a study was carried out on the localisation of various sweetening compounds in Merlot grapes (Cretin, 2016). It showed that astilbin was present at high concentrations in the stem. Therefore, this study aimed to clarify the contribution of stem addition during winemaking to the content of astilbin in wine.

In a bunch, the stem is the ligneous support to which the berries are attached. In traditional red winemaking, the grapes are generally destemmed after harvesting and before being crushed and placed in the vat. However, some winemakers choose to leave all or part of the stems in contact with the juice during vatting. Generally, whole clusters are added directly to the vats; this process is called whole bunch vinification (Ribéreau-Gayon et al., 2017). In this case, the integrity of the berry is preserved. In other cases, the stems can be partly reincorporated after destemming. Usually, only a percentage of the stems is kept; it can significantly vary according to the origin of the grapes, the vintage conditions and the ripeness of the grapes. In France, this type of winemaking process is traditionally carried out in Bourgogne, Beaujolais and the Rhône Valley, for instance, while in the Bordeaux estates, the grapes are generally destemmed as soon as the harvest arrives in the cellar. The choice of whether or not to keep a part of the stems in the vat is significant and is likely to affect the sensory properties of the resulting wine (Casassa et al., 2021).

At full ripeness, the stems represent between 3 and 7% of the weight of the fresh bunch (Ribéreau-Gayon et al., 2017). They are very rich in water (around 80% of their total composition) and low in sugar (less than 1% of their total composition) (Foulonneau, 2014; Ribéreau-Gayon et al., 2017). The proportion of phenolic compounds represents between 2 and 4% of the total composition of the stems. In addition, in a bunch, the stem can contain up to 20% of the total phenolic compounds despite its low proportion by mass. It also contains acids, nitrogen compounds, mineral compounds and ligneous matter (Foulonneau, 2014; Ribéreau-Gayon et al., 2017). Phenolic compounds are found in their polymeric form. Proanthocyanidins have often been described in stems, with a higher mean degree of polymerisation and a higher percentage of prodelphinidins than in seeds (Pascual et al., 2016).

The influence of incorporating the stems during vinification has already been studied, particularly regarding the aromatic component of the wines. Studies have revealed the presence of volatile compounds with a green aroma, namely methoxypyrazines or methyl salicylate, in wines made from whole bunches compared with wines made from destemmed bunches from Cabernet-Sauvignon (Hashizume and Samuta, 1997; Poitou et al., 2021). In contrast, the work of Spranger et al. (2004) has shown that volatile composition, particularly volatile esters, was not significantly affected by the presence of stems during vinification in Portuguese wines made from Castelão grapes. Regarding the taste component, some studies have focused on bitterness and astringency. Thus, wines vinified with stems were perceived as significantly more astringent and slightly bitter (Casassa et al., 2019, 2021; Pascual et al., 2016), probably linked to the higher concentration of proanthocyanidins (Pascual et al., 2016). The maceration time and the ripeness of the grapes, however difficult it may be to define, can also influence the sensory properties of wine (Llaudy et al., 2008). Nevertheless, most of these studies compared wines made from destemmed bunches with wines in which all the stems had been kept, with no destemming at all.

This study aimed to provide new information on the contribution of astilbin to the sweetness of red wines and investigate the effect of whole bunch vinification on its concentration. For this purpose, the taste detection threshold for astilbin was measured and compared with the concentrations of this compound in various commercial red wines. Then, some experiments were carried out to study the effect of keeping stems during the winemaking process on their content. Finally, the quantitation method was applied to measure astilbin concentration in the various parts of the bunch, namely the stems, skins, seeds and pulp, depending on the grape variety.
MATERIALS AND METHODS

1. Chemicals

Ultrapure water (Milli-Q purification system, Millipore, France), HPLC-grade ethanol and methanol (VWR International, Pessac, France) were used for sample preparation. LC-HRMS analyses was performed with LC-MS grade acetonitrile, formic acid (Fisher Chemical, Illkirch, France) and ultrapure water. Tartaric acid and sodium hydroxide were food-grade quality and were purchased from Sigma-Aldrich (Steinheim, Germany) and Merck (Darmstadt, Germany), respectively. Astilbin was isolated from the wine stem, and its isomers were purified according to the protocol described in our previous work (Fayad et al., 2021).

2. Sensory analyses

The sensory analyses took place in a specific room equipped with individual booths and air-conditioned at 20 °C. Solutions were tasted in INAO normalised glasses. The panel was composed of 20 tasters, 8 men and 12 women, aged from 21 to 40 years. The tasters were informed of the nature and risks of the present study and were asked for their written consent to participate. The analysis was performed in a model wine solution containing 12 % (v/v) alcohol and 4 g/L tartaric acid. The ethanol used was previously bi-distilled. The pH was adjusted at 3.2 by adding sodium hydroxide. The volume of each sample per person was set at 20 mL. The detection threshold of astilbin was determined during two different sessions to avoid saturation for the tasters. Three triangular tests were presented in each session, according to the methodology described in the ISO norms (ISO 4120:2007, 2007). Three increasing concentrations, following the geometric progression of ratio 2, were tasted during the first session: 2.5, 5 and 10 mg/L. The concentrations presented in the second session depended on the results of the first session. If the panellist found the different glass for each concentration, the lowest concentration belonging to a sequence of correct answers and the concentration just below. The geometrical mean of all individual thresholds provided the group threshold.

3. Experiments and sampling

3.1. Wine samples

Astilbin was quantified in 123 commercial red wines. These wines came from several countries: 96 from France, 18 from Slovenia, 8 from Austria and 1 from Germany. The French wines came from several wine regions: 74 from Bordeaux, 16 from Bourgogne (Côte de Nuits) and 6 from Beaujolais (Moulin-à-vent). Among the wines from Bordeaux, a wide range of appellations was studied (Blaye, Graves, Haut-Médoc, Margaux, Médoc, Pauillac, Pessac-Léognan, Pomerol, Saint-Emilion, Saint-Émilion, Saint-Julien). The wines were produced from a diversity of grape varieties. Those from Bourgogne, from Germany and some wines from Slovenia were composed of Pinot noir. The Beaujolais wines were made of Gamay. The wines of Bordeaux, as well as some wines from Slovenia and Austria, were the result of blends of Cabernet-Sauvignon, Merlot, Cabernet Franc and Petit Verdot, with a dominant Cabernet-Sauvignon or Merlot, depending on the wine. The vintages ranged from 1918 to 2020.

3.2. Experiments to study the effect of stems during winemaking

Experiments were set up directly in the cellars of several wineries under the real conditions of a classical winemaking process. They were carried out in two estates in Bordeaux (A and B), one estate in Beaujolais (C) and two estates in Bourgogne (D and E), allowing the comparison of different grape varieties, namely Merlot, Gamay and Pinot noir. These experiments were conducted during the 2019, 2020 and 2021 vintages. Different methods of incorporating the stems were...
implemented depending on the estate. Concerning Estates A and B, the bunches were destemmed, and the desired quantity of stems was then added during the filling of the vats. Properties C, D and E introduced whole bunches among the destemmed bunches directly into the vat. These clusters were added in alternating layers as the clusters entered, as is usually the practice. A pumping-over for homogenisation was carried out at the end of the filling of the vats. For experiments A and B, the percentage of added stems corresponded to the ratio between the weight of bunches whose stems have been added and the total weight of bunches introduced into the vat. For experiments C, D and E, it corresponded to the ratio between the weight of non-destemmed bunches and the weight of total bunches. Thus, different percentages have been studied according to the experiment, ranging from 15 to 50% of the harvest. For Estates C and D, the experiment was duplicated on two different plots (a and b). Codes were assigned for each trial, designating the estate, the vintage and the studied modality. The parameters of each experiment are listed in Table 1.

For each vat containing stems, there was a control vat containing the same bunches but without the stems. Samples were taken throughout the winemaking process, from must to running-off of the vat for experiments A21, B20, C20a, C20b, C21a, C21b, D20a and D20b. Finally, a sampling was carried out at the end of the malolactic fermentation for all the vats.

Other experiments were carried out in the last property (named F) in Bourgogne. This property consisted of 8 hectares located in Côte de Nuits and was composed of 16 plots of land in a single block. The plots were vinified independently of each other, with a proportion of stems varying from 0 to 66% of whole bunches.

Basic oenological analyses (alcohol content and pH) were measured in samples collected for each modality at the end of malolactic fermentation by private service oenological laboratories according to the official methods recommended by the International Organisation of Vine and Wine (OIV).

### 3.3. Bunches and stems

A total of 10 bunches per variety were randomly sampled from 3 different estates. The Merlot clusters were taken from Estate B, Gamay clusters from Estate C and Pinot noir clusters from Estate F. Bunches were harvested at the level of ripeness considered optimal by the winemaker during the 2023 vintage. In addition, single stems from all estates studied were taken during the various experiments. For each plot, between 3 and 8 stems were sampled. Concerning Merlot stems, 2 different plots were sampled in the 2019 and 2020 vintages for Estate A and 1 plot for Estate B in the 2019 vintage. For Gamay, 2 plots from vintage 2020, 6 plots from vintage 2021 were sampled in Estate C and 1 plot from 2020 vintage in Estate E. Finally, concerning Pinot noir stems, 4 plots were sampled in 2021 vintage for Estate D, 3 plots in 2020 vintage for Estate E and 9 plots for 2019, 2020 and 2021 vintages for Estate F. The vegetal material has been conserved at -4°C.

### 4. Quantitation of astilbin in wine and the different components of the bunch

#### 4.1. Liquid Chromatography analysis coupled with High-Resolution Mass Spectrometry

Chromatographic analyses were performed with a Vanquish system (Thermo Fisher Scientific, Les Ulis, France) consisting of a binary pump, an autosampler and a heated column. Our previous article described the quantitative method (Fayad et al., 2021). A C18 column was used, type High Silica Strength (HSST3, 100 mm × 2.1 mm, 1.8 μm) from Waters (Milford, USA). The mobile phase contained water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). The flow rate was set at 400 μL/min, and the injection volume was 5 μL. Eluent B varied as follows: 0 min, 10%; 0.5 min, 10%; 1.5 min, 20%; 3.5 min, 20%; 5.5 min, 25%; 7.5 min, 50%; 8.5 min, 98%; 9.5 min, 98%; 9.6 min, 10%; 11.5 min, 10%. The sample temperature was set at 10°C and 30°C for the column.

Mass detection was performed using an Exacte Orbitrap mass spectrometer equipped with a heated electrospray ionisation (HESI-II) probe (both from Thermo Fisher Scientific). The mass analyser was calibrated each week using Pierce® ESI Negative Ion Calibration Solutions (Thermo Fisher Scientific). The source parameters were set as follows: sheath gas flow rate at 65 arbitrary units (a.u.); auxiliary gas flow rate at 5 a.u.; sweep gas flow rate at 0 a.u.; HESI probe temperature of 320°C; spray voltage -3.5 kV; capillary temperature of 300°C; capillary voltage of -87.5V; tube lens voltage of -130 V; skimmer voltage of -28 V. Mass spectra were acquired in negative mode, from 100 to 1800 m/z at a resolution of 25,000 FWHM. The value of the automatic gain control target was set up at 3.10^6 ions, and the maximum injection time was 200 ms.

Data were processed using the Qual Browser and Quan Browser applications of Xcalibur version 3.0 (Thermo Fisher Scientific). Quantitation was performed by measuring the peak area determined by automatic integration of extracted ion chromatograms (XIC) built in a 3 ppm around the exact mass of the [M-H]+ of the ratio m/z 449.1085, at the retention time 5.06 min.

#### 4.2. Preparation of samples

Samples of wines were diluted by 5 with ultrapure water before being filtered at 0.2 μm to be analysed.

The separation of the constituents of the different bunches, namely the stem, the skins, the seeds and the pulp, was carried out manually. Berries were detached from the stem one by one and peeled to obtain skin before separating seeds from pulp. Each part (skin, seed, pulp and stem) was freeze-dried and macerated in a hydroalcoholic solution for 24 hours. Macerate concentrations were 100 g/L for the pulp, 10 g/L for stems and 40 g/L for skins and seeds. Different hydroalcoholic solutions of maceration were tested with two conditions of pH: without adjustment (namely Ω) and with adjustment to 3.5 by the addition of tartaric acid solution at 4 g/L and then correction to 3.5 with a 0.5 M sodium hydroxide.
solution. For each pH condition, the ethanol percentages were set to 12 % (v/v). Macerates were prepared in triplicate and then diluted by 5 with ultrapure water and 0.2 µm-filtered before injection in LC-HRMS. Each component of each cluster was analysed in triplicate.

Stems sampled without the berries were cleaned, freeze-dried and ground before maceration for 24 hours at 20 g/L in a 50 % (v/v) hydroalcoholic solution.

4.3. Preparation of calibration solution

Calibration solutions were prepared by successive dilutions of the stock solution of astilbin (1 g/L) to give thirteen levels of concentrations: 1 µg/L, 2.5 µg/L, 5 µg/L, 10 µg/L, 25 µg/L, 50 µg/L, 100 µg/L, 250 µg/L, 500 µg/L, 1 mg/L, 2.5 mg/L, 5 mg/L and 10 mg/L. Calibration solution levels were prepared in five-fold diluted and filtered red wine for wine quantification and in ultrapure water for constituents of grape quantification.

4.4. Validation of the quantitation method in different components of the grapes

Concerning wine analysis, the astilbin quantitative method was already validated in a previous study (Fayad et al., 2021). Regarding the grape macerate, the astilbin level was measured with the same LC-HRMS conditions as in wine. The method was validated by studying the same parameters: linearity, sensitivity, intraday repeatability, trueness and specificity.

A calibration curve was established by plotting the areas for each calibration level versus the nominal concentration. Linear regression was performed to obtain the correlation coefficient (R²) to assess linearity. Sensitivity was measured using the approach described by De Paepe et al.. The instrumental detection limit (IDL) was determined by injecting five times the lowest range of calibration solutions (1 µg/L to 25 µg/L). The trueness (recovery ratio) and the precision (% coefficient of variation) were calculated for each level. The lowest concentration, which can be measured with a precision lower than 10 % and trueness higher than 90 %, is defined as the IDL. The concentrations of the macerates were taken into account for the calculation of the limit of detection (LOD). The limit of quantitation (LOQ) was determined as twice the LOD. Trueness was examined by spiking macerate with three different concentrations of astilbin depending on the constituent studied. Thus, the concentrations used for stems were 100 µg/L, 1 mg/L and 10 mg/L; those for skins were 50 µg/L, 500 µg/L and 5 mg/L and those for seeds and pulp were 25 µg/L, 250 µg/L and 2.5 mg/L. The concentration determined using the calibration curve was compared to the actual concentration of the standard and the recovery rate ((determined concentration/actual concentration) × 100) was calculated. Three levels of concentrations (25 µg/L, 250 µg/L and 2.5 mg/L) were also selected to evaluate the intraday repeatability by analysing five replicates of each calibration solution. The coefficient of variation (%) was, thus, calculated. Specificity was determined by assessing retention time repeatability and mass accuracy.

5. Statistical analyses

Statistical analyses were performed with XLSTAT software version 2022.1.2 (Addinsoft, Paris, France). Quantitative data from commercial wine analyses, as well as the data from the different components of the grapes, were examined using the Kruskal-Wallis test with a Dunn comparison. The study of the differences between the wines made with or without stems was carried out using a non-parametric Wilcoxon-Mann-Whitney statistical test for paired samples.

RESULTS AND DISCUSSION

1. Determination of taste detection threshold of astilbin

Previous studies have highlighted the sweet taste of astilbin (Fayad et al., 2021). To reveal its possible contribution to the taste of wine, it was essential to determine its taste detection threshold. This value should be measured in a matrix very close to that of the wine. However, all the wines available in the laboratory contained astilbin. Its taste detection threshold was therefore determined in a model wine solution. To avoid sensory tiredness, the tastings were split into two sessions. The astilbin threshold was calculated at 5.7 mg/L for the panel. Inter-individual variability was very high, with individual thresholds ranging from 0.45 mg/L to 56.5 mg/L. By comparison, the detection threshold of glucose in water was determined at 4.7 g/L (Birch and Munton, 1981). In addition, the taste detection threshold of Quercotriterpenoside I (QTT I), a sweet molecule identified in wines and originating from oak wood, was measured at 590 µg/L in a white wine (Marchal, 2010; Marchal et al., 2011).

The detection threshold of a molecule can also be modified in the presence of other sapid compounds, as in red wine. The sweet perception of astilbin should be studied in the presence of bitter or acid compounds. The gustatory threshold of astilbin in the model wine solution must be compared to the quantitative values to determine the influence of astilbin on the sweetness of dry wines.

2. Quantitation of astilbin in commercial wines. Influence of grape variety

Astilbin concentrations were measured in commercial wines to be compared to their taste detection threshold and to study the influence of grape variety. A quantitative method for astilbin in wine using LC-HRMS has already been developed by Fayad et al. in a previous study (Fayad et al., 2021). This method was used to analyse 123 commercial red wines. Astilbin was found at a mean concentration of 9.2 ± 2.2 mg/L ranging from 0.2 to 56.4 mg/L (Figure 1A). The taste detection threshold for astilbin measured at 5.7 mg/L is, therefore, lower than the average concentration of astilbin found in wines. This means that astilbin can contribute to the taste balance of various wines. The content of this molecule varied considerably depending on the origin of the wines. Therefore, the influence of the grape variety was studied (Figure 1B). Wines made from a blend dominated by Cabernet-Sauvignon or Merlot have been grouped in the same category to compare with wines made from Gamay and...
The average concentration of astilbin found in wines made from a blend of Cabernet-Sauvignon or Merlot was $2.2 \pm 0.2$ mg/L. This concentration was around ten times lower than those found in wines made from Gamay or Pinot noir, which amounted to $19.9 \pm 3.1$ mg/L and $24.6 \pm 4.2$ mg/L, respectively. In addition, statistical tests showed that this difference was significant at the 0.1 % level. These results were similar to the concentrations measured during the last study (Fayad et al., 2021). To compare, another study quantified astilbin in a few Cabernet-Sauvignon wines at between 7 and 9.7 mg/L and in a Merlot wine at around 11.6 mg/L (Landrault et al., 2002). These data are slightly higher than the concentrations found here in Cabernet-Sauvignon and Merlot wines. However, the number of wines analysed was smaller. Other data are also available on Malbec wines from Argentina; astilbin concentrations were measured between 9.1 and 16.3 mg/L (Fanzone et al., 2010) or between 3.6 and 8.8 mg/L (Urvieta et al., 2018). However, the conditions of method development and the parameters used for its validation were poorly described in these studies.

The average concentration of astilbin found in wines made from Gamay and Pinot noir was above its detection threshold. This suggests that astilbin had a stronger effect on sweetness in these wines than in wines made from Cabernet-Sauvignon or Merlot. These results tend to reveal an effect of grape variety on astilbin concentration. Moreover, some Pinot noir and Gamay wines come from the Beaujolais and Bourgogne regions, where whole bunch vinification is generally practised. As mentioned above, astilbin is an abundant compound in the stem. Consequently, another explanation for the high levels observed in some wines could be the presence of stems during vatting, which can increase the release of astilbin. Nevertheless, information on destemming was not available for all wines. Therefore, the effect of the addition of stems during vinification was studied.

### 3. Study of the contribution of stems during the winemaking process

#### 3.1. Overall study

To study the effect of incorporating stems during the winemaking process on astilbin contents, various experiments have been conducted in several wineries, allowing real winemaking conditions. The winemaking method and the percentages of stem addition were chosen at the judgement of the winemaker. Nevertheless, the same methodology was applied to the vats with a certain percentage of stems and the corresponding control vats. Therefore, the presence of stems was the only parameter that changed from one modality to another.

Firstly, the general effect of adding stems on wine composition, whatever the grape variety used, has been studied. Average pH, alcohol content and astilbin levels, measured on samples taken at the end of the winemaking process, were calculated for wines made from destemmed bunches and with stems (Figure 2). Modalities with a certain percentage of stems have been combined and called “with stems”. Wines made from destemmed grapes were referred to as “destemmed” modality.

The average pH was calculated at $3.46 \pm 0.04$ for the destemmed modality and $3.52 \pm 0.04$ for the with stems modality. Concerning the average alcohol content, it was calculated at $13.79 \pm 0.82$ % for the destemmed modality and $13.68 \pm 0.84$ % for the with stems modality. The difference between these modalities was not significant according to the Wilcoxon-Mann-Whitney test for each parameter. However, it is often described that the alcoholic degree is lower in wines vinified with stems than in wines made from totally destemmed clusters (Ribéreau-Gayon et al., 1976; Ribéreau-Gayon et al., 2017). This difference is generally explained by the composition of the stem, which is rich in water and low in sugars, unlike the must, which is very rich in sugars.

![FIGURE 1. A: Box plot of astilbin concentrations in 123 red wines. B: Average astilbin concentrations (mg/L) by grape variety. Error bars indicate 95 % confidence intervals. The letters “a” and “b” indicate significant differences at p-value < 0.001 (***)](image)
The water in the stems may dilute the must, while the ethanol formed during alcoholic fermentation is absorbed by the stems. In addition, some studies described a decrease in acidity in wines made with stems due to the high potassium content of the stems, which can precipitate with tartaric acid (Ribèreau-Gayon et al., 2017). Generally, these observations were made on wines for which all the stems were kept during vinification. The impact of a low proportion of stems (no more than 50% of whole bunches) on these parameters was measured here. Adding between 15 and 50% of stems during vinification did not seem to have any effect on pH or alcoholic strength.

The comparison of average astilbin concentrations according to the winemaking method is also shown in Figure 2. The average value calculated for wines from the modality with stems was 24.3 ± 12.1 mg/L, which was higher than the mean concentration of 19.7 ± 10.9 mg/L found in wines from the destemmed modality, with a significant difference at the 0.1% level according to the Wilcoxon-Mann-Whitney test. The confidence intervals were high for both modalities, which may be linked to the diversity of the wines analysed and, more particularly, their varieties. These results showed that the presence of stems during vinification increased astilbin contents.

In terms of polyphenols, most studies comparing vinification with and without stems have quantified total polyphenols, tannins or anthocyanin content in their experiments (Casassa et al., 2019, 2021; Pascual et al., 2016; Spranger et al., 2004; Wimalasiri et al., 2022). Regarding flavan-3-ols, the concentrations of catechin or epicatechin were higher when 100% of the stems were added, with an increase of around 8 mg/L of catechin for the Castelão wine (Spranger et al., 2004), and around 40 mg/L for Cabernet-Sauvignon wine (Pascual et al., 2016) and Pinot noir wine (Wimalasiri et al., 2022), for example. Quantification of flavones or flavanones has been less described. Concerning quercetin and quercetin-3-glucoside, the concentrations found in the wines of Pinot noir with 100% added stems, or a certain percentage of whole bunches, contained a lower level of total flavonols than the wine without stems, around a few mg/L less (Wimalasiri et al., 2022). The same observation was made in Cabernet-Sauvignon wines, with lower quercetin content in wines with 100% stems (Pascual et al., 2016). It suggests that these compounds can be adsorbed by the stems. None of these studies quantified astilbin. In addition, astilbin was quantified in higher concentrations than quercetin-3-glucoside in Merlot stems (Souquet et al., 2000).

3.2. Evolution of astilbin concentration during the winemaking process

The evolution of astilbin concentration was studied thanks to these experiments. Indeed, samples collected during the winemaking process were used to monitor the extraction of this sweet molecule. Sampling was started when the vats were filled after homogenisation (day 1) and continued until the end of alcoholic fermentation (AF). For some experiments, samples were collected after malolactic fermentation (MLF). Each experiment was carried out without replication. There was, therefore, no biological replicate for a single experiment.

Figure 3 illustrates the variation in the concentration of astilbin in the different experiments. The experiments were carried out on the A and B Bordeaux estates on vinified Merlot wines (Figure 3, A21, B20). Experiment A21 showed an increase in astilbin until the 8th day, when alcoholic fermentation had finished, for the “30% whole bunches” modality with a concentration reaching 8.7 mg/L. In the “destemmed” vat, the maximum astilbin content was measured at 6 mg/L on the 6th day of vinification. The difference calculated between the two modalities was 31% of the maximum content. For experiment B20, the astilbin concentration stabilised at around 5 mg/L for the “destemmed” modality and 8.4 mg/L for the “30% whole bunches” modality around the 11th day of vinification, that is to say, 7 days before the end of alcoholic fermentation. Here, the percentage difference was estimated at 40%.

With regards to the experiments carried out at winery D in Bourgogne, which vinified Pinot noir, astilbin concentrations in the wines coming from plot a (Figure 3, D20a) stabilised from the 8th day, at around 26 mg/L for the “destemmed” vat and around 32 mg/L for the vat with “50% whole bunches.”
FIGURE 3. Astilbin content during the winemaking process. The error bars represent the standard error at 5%. Different stages of vinification are indicated at the end of alcoholic fermentation (End of AF), at the running-off and the end of malolactic fermentation (End MLF). Grape varieties are indicated on the left of the graph. The names of the experiments are shown above each graph in bold.
For the vat with “30 % whole bunches”, astilbin levels reached 31 mg/L at the end of alcoholic fermentation but decreased to 24 mg/L 5 days later, which was slightly lower than the concentration measured in the “destemmed” vat at the same stage. However, the difference between the “destemmed” and “50 % whole bunches” modalities was 19 %. For plot b (Figure 3, D20b), the concentration in the “destemmed” vat stabilised at around 30 mg/L two days before the end of alcoholic fermentation. For the “50 % whole bunches” vat, the content increased to 38.8 mg/L in the sample taken 5 days after alcoholic fermentation. More samples would have been necessary to visualise the stabilisation of astilbin concentration during this experiment. Nevertheless, on the 14th day, the concentration of astilbin in the “destemmed” vat was 26 % lower than in the vat with “50 % whole bunches”. Finally, the latest experiments on Gamay wines carried out at Winery C in the Beaujolais region confirmed the previous observations (Figure 3C). Astilbin levels increased between the 8th and 11th day of vinification, that is, before the end of alcoholic fermentation. Wines from Plot A vinified in 2020 (Figure 3, C20a) and 2021 (Figure 3, C21a) contained higher concentrations of astilbin than those from Plot B in the corresponding modality and vintage. The maximum concentrations found during experiment C21a were 46 mg/L for the “30 % whole bunches” modality, 42 mg/L for the “15 % whole bunches” modality and 38 mg/L for the “destemmed” modality. These concentrations were slightly higher than those measured in the Pinot noir and much higher than those in the Merlot wines. However, the differences between the “destemmed” and “30 % whole bunches” modalities were 17 % and 6 %, respectively, for Plots A and B in the 2021 vintage.

In general, these experiments demonstrated that the addition of stems during winemaking increased the content of astilbin in the wines, regardless of the grape variety. Furthermore, the difference in astilbin content between modalities with and without the addition of stems varied according to the grape variety. Moreover, the majority of the extraction of astilbin occurred during the first part of alcoholic fermentation. This compound was, therefore, released into the aqueous phase when the juice still had low alcohol levels. This is also the case for flavonols such as quercetin and kaempferol, whose levels increase gradually between the 5th and 7th day of fermentation before stabilising (Burns et al., 2001; Koyama et al., 2007). In addition, astilbin concentration in modalities without stems seems to reach its maximum faster than in modalities with stems. This could suggest that astilbin present in the skins was released before that present in the stems. However, this hypothesis needs to be verified by further experimentation.

3.3. Comparison of the difference in astilbin content according to grape variety

The differential effect of the grape variety on the astilbin concentration was measured with and without stem addition. Indeed, the results of the experiments can be distinguished according to the grape variety. Average astilbin concentrations were calculated in wines made from Gamay, Pinot noir and Merlot (Figure 4A). Astilbin concentrations were higher in Gamay and Pinot noir wines than in Merlot wines. These results were in agreement with the observations described above. Indeed, for Gamay, the average concentrations were 27.7 and 31.9 mg/L for the “destemmed” and “with stems” modalities, respectively, and for Pinot noir, the concentrations were 23.9 and 30.3 mg/L for these two modalities. For wines with a majority of Merlot, the average concentration was 5.6 mg/L for the “destemmed” modality and 8.8 mg/L for the “with stems” modality. In comparison with the taste detection threshold, the mean value of astilbin concentration with the addition of stems in Merlot wines exceeded this threshold, which was established at 5.7 mg/L. Finally, the addition of stems provided an average increase of 4.1 mg/L, 6.3 mg/L and 3.2 mg/L of astilbin for wine made from Gamay, Pinot noir and Merlot, respectively.

Nevertheless, it is interesting to note that the difference in astilbin proportion between the two methods (“destemmed” and “with stems”) appeared to be greater for Merlot than for Gamay and Pinot noir. In fact, the presence of stems during vinification contributed an average of 36 % more astilbin for Merlot, compared with 21 % for Pinot noir and 12 % for Gamay (Figure 4B). The statistical test using the Kruskal-Wallis test showed that the difference between the increase ratio of Gamay and Merlot was significant at the 5 % level. This result could be interpreted by a higher proportion of total astilbin in the bunch located in the stems for Merlot than for Pinot noir or Gamay. In the case of Gamay and Pinot noir, astilbin may be more present in the other constituents of the grape, namely the skin, the pulp and the seeds. The localisation of astilbin in the bunch had already been studied for Merlot, with 97 % of the total amount that was present in the stems (Cretin, 2016). However, bunches from other grape varieties were not analysed. The localisation of astilbin in Gamay and Pinot noir bunches was, therefore, necessary to validate this hypothesis.

From a sensory point of view, the contribution of the stems during vinification in these proportions remains to be determined. Another work can be focused on the comparison of the taste of the different modalities by sensory analysis.

4. Development of a quantitation method of astilbin in the bunch constituents

Assessment of astilbin levels in bunches was necessary to understand better the differences observed among grape varieties. The method for quantifying astilbin content in wine, developed by (Fayad et al., 2021), has therefore been adapted to the different grape constituents. Firstly, the extraction conditions have been defined. In this context, two pH conditions (without adjustment (namely Ø) and with adjustment to 3.5) were tested on each constituent. The ethanol level was set at 12 % to stay within wine conditions. The average area of astilbin for each macerate was then calculated (Supplementary Figure S1). The astilbin area varied only slightly according to the extraction method used. Indeed, the error bars between all the conditions overlapped. The difference in extraction between the modalities with and without pH adjustment was not significant.
To compare the extraction of astilbin in each grape component, a common maceration modality must be selected. Therefore, the macerates’ pH was not adjusted for the rest of the study. The analyses carried out on the stems can be divided into two categories. The first category included stems taken from whole bunches, whose berries were also studied. They were macerated directly after freeze-drying in a 12% ethanol solution to enable them to be compared with the other constituents of the bunch. Samples of stems alone were crushed after freeze-drying to obtain a homogeneous powder and thus facilitate comparison with other stems from different modalities. They were macerated in a 50% ethanol solution. The LC-HRMS quantification method was then adapted and validated by studying its linearity, specificity, repeatability, sensitivity and trueness (Supporting Table S1). The linearity range extended from IQL to 10 mg/L. A linear calibration curve was obtained by plotting the peak area as a function of nominal concentration. The value of the correlation coefficient ($R^2$) was calculated at 0.9998, which validated the linearity of the method. The accuracy of mass measurement was evaluated at 0.6 ppm, and the retention time varied by a maximum of 0.04 min. These parameters ensured the specificity of the analysis. The coefficients of variation (%) of five replicates of three calibration solutions were calculated at less than 2.5%, allowing repeatability to be validated. The method described by de (De Paepe et al., 2013) was used to measure the IDL at 2 µg/L. Depending on the concentration of the macerate, the LODs were calculated at 0.25, 0.1, 0.25 and 1 µg/g of freeze-dried extract for the skins, pulp, seeds and stems, respectively. LOQs were therefore estimated at 0.5 µg/g for the skins and seeds, 0.2 µg/g for the pulp and 2 µg/g for the stems. Macerates of each constituent have been spiked with three different concentrations of astilbin stock solution. The recovery ratio ranged from 91.8% to 109.5%, which was in accordance with the specifications (Guidance for Industry, 2018) and therefore demonstrated the trueness. These results allowed the validation of the method for quantifying astilbin in the various bunch components, namely skins, pulp, seeds and stems.

5. Amount of astilbin provided by the different constituents of the bunch. Influence of the grape variety and the vintage.

5.1. General study

The quantification method was used to study the localisation of astilbin in the various components of the bunch. Thus, a total of 10 bunches of Pinot noir, Gamay and Merlot were studied. The different parts of each bunch were macerated in triplicate to ensure the homogeneity of the results. Average astilbin concentrations, expressed in µg/g of dry constituent extract, were calculated for all bunches combined (Figure 5A). Stems were the component with the highest astilbin content, with an average of 463.2 ± 87.8 µg/g of dry extract. They were followed by skins, which contained, on average, 103.9 ± 28.3 µg/g of dry extract. Compared to these two parts, seeds and pulp contained much lower levels, at 2.4 ± 0.6 µg/g and 1 ± 0.3 µg/g, respectively. A study examining the phenolic composition of stems described astilbin as the major favanonol present in the stems (Souquet et al., 2000). In Merlot, it was quantified at 35 µg/g of stems. Another study of phenolic compounds in Tempranillo wine by-products also quantified astilbin (Souza da Costa et al., 2022). In the constituents that underwent freeze-drying before being extracted, astilbin was found at an average of 99.4 µg/g of stems, between 1.33 and 1.52 µg/g of seeds and between 1.49 and 2.64 µg/g of skins. For this study, the seeds and skins were recovered from the pomace after maceration with the wine, so they had already undergone extraction. Overall, the amount of astilbin in stems found in the literature is lower than those measured here. However, the maceration and analysis conditions of these two studies were different.

FIGURE 4. A: Comparison of average astilbin concentrations (mg/L) according to grape variety. B: Percentage difference calculated between modalities destemmed and whole bunch according to grape variety (Gamay $n = 5$, Pinot noir $n = 4$ and Merlot $n = 4$). Error bars indicate 95% confidence intervals. The letters “a”, “ab” and “b” indicate significant differences at $p$-value < 0.05 (*).
FIGURE 5. The mean concentration (n = 30) of astilbin in μg/g dry extract (A) and μg/g fresh extract for each constituent within the cluster (B) was measured in the different components of all grape varieties combined. Error bars indicate a 95% confidence interval. The letters “a”, “b” and “c” indicate significant differences at p-value < 0.001 (***)

FIGURE 6. Mean (n = 10) of the proportions of each component in relation to the weight of the cluster found after separation according to the grape variety. Error bars indicate 95% confidence intervals.
From a global perspective, the stems were, therefore, the component containing the most astilbin, around five times more than the skins in our study. Nevertheless, on the scale of a bunch, the constituents were found in different proportions, and their individual contribution to astilbin content in wine must consequently be weighted by these proportions.

Indeed, after the separation of each cluster, the mass of each component was measured. The average proportions of each component in relation to the overall mass of the bunch were calculated for each grape variety (Figure 6). Pulp was the main component of the bunch, accounting on average for 79.4% for Gamay, 71.6% for Pinot noir and 78.8% for Merlot. It is followed by skins (12% for Gamay, 16.3% for Pinot noir and 12.7% for Merlot), seeds (5.2% for Gamay, 6.9% for Pinot noir and 5.5% for Merlot) and finally, stems (3.4% for Gamay, 5.1% for Pinot noir and 3.1% for Merlot). The proportion of stems was consistent with the literature, which generally measured it at between 3 and 7% of the weight of the ripe bunch (Ribéreau-Gayon et al., 2017). Concerning pulp, skins and seeds, the proportions can be expressed at the berry scale, which means without considering the stem. The proportion of pulp varied from 75.5 to 82.2%, that of skins from 12.5 to 17.2% and that of seeds from 5.3 to 7.3%, depending on the grape variety. These data are within the same range as those found in the literature since, according to (Ribéreau-Gayon et al., 2017), pulp represented between 75 to 85% of the weight of the berry, skins between 8 and 20% and seeds between 0 and 6%. The constituents were also weighed after freeze-drying. The percentage of dehydration was calculated on average at 77%, 57%, 40% and 68% for pulp, skins, seeds and stems, respectively.

For the rest of the study, the percentage of dehydration was used to express astilbin concentrations in µg/g of fresh extract. In addition, the percentage of each component in a bunch according to the grape variety studied was also considered to give a more accurate representation of astilbin concentration (Figure 5B). Ultimately, the amount of astilbin provided by the stems at bunch level (5.6 ± 1.2 µg/g of fresh extract) was close to that provided by the skins (6.3 ± 2 µg/g of fresh extract). However, this proportion may differ depending on the grape variety studied.

5.2. Influence of the grape variety

To study the influence of the grape variety, the total concentrations of astilbin measured in the bunches by grape variety were calculated (Figure 7). Bunches of Pinot noir contained an average of 17.5 ± 7.2 µg/g fresh extract, significantly more than Merlot bunches, with 8.6 ± 2.3 µg/g fresh extract. The average content in Gamay bunches was between the two grape varieties, at 10.3 ± 1.9 µg/g fresh extract. These results are consistent with previous observations showing that Pinot noir wines contained more astilbin than Merlot wines.

To compare with a white grape variety, Landrault et al. (2002) quantified astilbin at levels from about 5 to 30 µg/g of Sauvignon grapes. These analyses were carried out on freeze-dried and crushed botrytised grapes (probably without the stems). Without considering the stems and remaining in concentration per dry extract, astilbin was found at 13.6 µg/g of dry extract for Gamay grapes, 7.5 µg/g for Merlot and 25.9 µg/g for Pinot noir, which remained in the same proportions.

Details of the astilbin concentration provided by each component as a function of the grape variety are shown in Figure 8A. With regards to the pulp and seeds, the difference in astilbin levels between grape varieties was similar, with a higher concentration of astilbin in Gamay and Pinot noir than in Merlot. Nevertheless, these concentrations were much lower (< 0.25 µg/g of fresh extract) than those found in the skins. Indeed, the concentration found in Pinot noir skins (10.5 ± 4.8 µg/g fresh extract) was three times higher than in Merlot skins (3.1 ± 1.4 µg/g fresh extract). The concentration of astilbin found in gamay skins was between these two values, with 5.1 ± 1.1 µg/g fresh extract. In terms of stems, the difference between the concentrations was not significant, with 4.7 ± 1.1 µg/g of fresh extract for Gamay stems, 6.6 ± 3 µg/g for Pinot noir stems, and 5.4 ± 1.6 µg/g
**FIGURE 8.** Astilbin concentration in μg/g of fresh extract (A) and proportion of astilbin (B) measured in different constituents of bunches (n = 10) of different grape varieties. Error bars indicate 95% confidence intervals. The letters “a”, “ab” and “b” indicate significant differences at p-value < 0.05 (*), 0.01 (**) or 0.001 (***) . NS indicates that the difference is not significant with a p-value > 0.05.
for Merlot stems. These results were also represented as a percentage by calculating the proportion of astilbin localised in each component according to grape variety (Figure 8B). Overall, the proportions of astilbin found in the pulp and seeds are less than 2.5 %. The proportion found in Pinot noir skins was 59.4 % and 51.5 % for Gamay, compared with 34.4 % for Merlot skins, with a significant difference between the proportions for Pinot noir and Merlot. The proportion of astilbin located in the Merlot stems was significantly higher (64.6 %) than in Gamay stems (45.1 %) and Pinot noir stems (38.2 %). This means that for a bunch of Gamay and Pinot noir, the proportion of astilbin came half from the stem and half from the skins, whereas for Merlot, two-thirds of the total astilbin in a bunch was found in the stem. Analyses carried out by Cretin (2016) showed that 97 % of astilbin was located in stems for Merlot bunches, but the percentage by mass of each component within a bunch had not been considered.

These results were consistent with the trends observed in the previous section on the effect of winemaking with stems, depending on the grape variety. Indeed, it provides an explanation for the lower difference in astilbin concentrations between destemmed and with-stems winemaking of Pinot noir compared to Merlot and confirms the hypothesis expressed above.

Other measurements were carried out on stems taken from the various estates where the winemaking experiments took place. For each sample, between 3 and 8 stems were taken from different bunches. They were crushed and macerated together to obtain a more homogenous result. In this way, the effect of grape variety on grape stems from several different terroirs can be studied (Figure 9A). The macerates were made with 50 % ethanol (v/v) and on crushed material, which explained the higher concentrations of astilbin than stems macerated in a 12 % ethanol solution, as shown previously. The average astilbin content extracted from Gamay stems was 1.5 ± 0.1 mg/g of dry stems. This was significantly lower than that found in Merlot (2.2 ± 0.2 mg/g). In Pinot noir stems, the average concentration of astilbin was measured at 1.7 ± 0.3 mg/g. These results showed that astilbin was more present in Merlot stems. Therefore, these observations helped explain the greater difference in levels between wines made with stems and those made from destemmed Merlot grapes.

It could be interesting to reproduce these experiments on a larger scale, as there was variability between bunches from the same estate and the same grape variety. Increasing the number of bunches analysed would improve the homogeneity of the sampling.

5.3. Influence of the vintage

The influence of vintage on astilbin contents in wines and stems from the same terroir was also studied. During the 2019, 2020 and 2021 vintages, samples of Pinot noir stems from Estate F were taken from 9 different plots. The average concentration for the 2020 vintage is measured at 2 ± 0.4 mg/g, almost double that of the 2021 vintage, measured at 1 ± 0.1 mg/g (Figure 9B). For the 2019 vintage, the average concentration is 1.3 ± 0.3 mg/g. The Kruskal-Wallis statistical test revealed a significant difference between the 2020 and 2021 vintages. In addition, astilbin levels were measured at the end of vinification in wines from the corresponding plots on this estate (Figure 9C). The average astilbin concentrations of the 2019 and 2020 vintages, 43.8 ± 2 mg/L and 47.7 ± 3.9 mg/L, respectively, are significantly higher than those of the 2021 vintage (27.1 ± 1 mg/L). Thus, depending on the vintage, the concentrations of astilbin extractable from the stems and present in the wines varied. The main factors that differ from one vintage to another for this estate were climatic conditions, which are known to influence the physiology of the vines and the ripeness of the grapes. The effect of the vintage was particularly observed in the variability of the water regime, i.e. the availability of water. Usually, limiting the water availability as veraison approaches increases the oenological potential of the grapes (Ribéreau-Gayon et al., 2017). In addition, water availability is a key parameter in the concentration of phenolic compounds (Matthews and Anderson, 1988; Van Leeuwen and Seguin, 1994).

FIGURE 9. A: Astilbin concentrations in mg/g of dry stems of different grape varieties. B: Astilbin concentrations in mg/g of dry stems (n = 9) from Estate F according to vintage. C: Astilbin concentrations in mg/L in wines (n = 11) from Estate F according to vintage. Error bars indicate 95 % confidence intervals. The letters “a”, “ab” and “b” indicate significant differences at p-value < 0.05 (*), 0.01 (**) or 0.001 (***).
Indeed, it has been demonstrated that significant water stress often leads to a reduction in berry size and, therefore, a high concentration of phenolic compounds (Ojeda et al., 2002). Rainfall for the period between April and the harvest date, which corresponds to the vine’s vegetative cycle, was recorded at the Dijon Longevic station (located 12 km from property F). The accumulation was measured at 256 mm for the 2019 vintage, 193 mm for 2020 and 348 mm for 2021 (data from Météo France). The water stress was, therefore, a priori higher in 2020, which was the vintage in which the concentration of astilbin in the stems and wines was also the highest. The lowest concentration of astilbin was measured for the 2021 vintage, for which cumulative rainfall was higher, which would correspond to lower water stress. These results could raise a first hypothesis related to a link between water availability to the vines and astilbin concentration in the stems. Measurement of carbon isotope composition ($\delta^{13}$C) in wine would be a good indicator for the water status of wine (Gaudillère et al., 2002), and its comparison in a larger range of samples to astilbin concentration would be interesting to investigate this hypothesis further.

**CONCLUSION**

This study was focused on the effect of stems on the content of a sweet compound in wine, astilbin. Firstly, the contribution of astilbin to the sweetness of wine was specified. Its taste detection threshold was established at 5.7 mg/L in the wine model solution. Commercial wines were then analysed, demonstrating that Pinot noir and Gamay wines contained higher concentrations of astilbin than wines made from a blend of Merlot or Cabernet-Sauvignon. Moreover, the concentrations found in wines from Pinot noir and Gamay were above the taste detection threshold, suggesting that astilbin contributed directly to their sweet taste.

The other part of the study was based on some experiments carried out at several wineries to investigate the influence of whole bunch vinification. The addition of stems during vatting significantly increased astilbin concentrations. In addition, the increase in astilbin concentration varied according to grape variety. The ratio between the astilbin concentration of wines made from grapes with added stems and wines made exclusively from destemmed bunches was higher in Merlot than in Pinot noir and Gamay. To understand this observation, the compound was localised in the various constituents of the bunch by applying the quantification method to skins, pulp, seeds and stems macerates. For this purpose, bunches of Merlot, Gamay and Pinot noir were compared. A high abundance of astilbin was found in the stems and skins. However, astilbin was present in greater proportions in the stems than in the skins of Merlot grapes, whereas in Gamay and Pinot noir, it is found in equal proportions in these two constituents. Finally, stems from several terroirs were analysed, showing a higher level of astilbin in Merlot stems than in Pinot noir stems. These results provided an explanation for the less marked difference between wines made from destemmed and with stems grapes in Pinot noir and Gamay than in Merlot.

These various studies showed that the addition of stems during the winemaking process provided a sweetening compound. The influence of whole bunch vinification on the sensory properties of wines, particularly sweetness, needs to be clarified to determine its impact on taste. Indeed, stems can also release bitter and astringent compounds, as well as adding vegetal nuances. Their use must, therefore, be carefully controlled, and knowledge of the sapid and odorous compounds associated with their presence must be further developed.

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