ROUTINE MEASUREMENT OF FLAVONOID PHENOLS RELATED TO AS-TRINGENCY AND PREMATURE AGING OF WHITE WINES, AND TECH-NICAL CONSEQUENCES

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Summary

The use over many years of a fast and specific colorimetric method for the determination of flavonoid phenols in white wines and musts yielded far-reaching insights into the relationship between these phenols, perceived astringency, and premature oxidative aging. Furthermore, it provided extensive data on the impact of various vinification techniques on their concentration. They are preserved under conditions of reductive must processing and lowered when must oxidation is allowed. The degree of must clarification plays an additional and decisive role in this process. Since, contrary to expectations, flavonoid phenols are already present in the pulp juice of the grapes, gentle grape processing alone is not sufficient to reduce them to a significant extent.

Introduction

Flavonoid phenols, also known as flavonoids for short or simply referred to as catechins, act as tannins and strongly contribute to perceived astringency and bitterness in any kind of wine. In conjunction with oxygen uptake post-fermentation, they are responsible for browning of white wines and strongly accelerate their oxidative aging in respect to both their smell and taste. Thus, they limit their shelf life and play a decisive role in the development of premature oxidative aging (premox). For these reasons, various enological measures are taken to minimize their content from the crushpad onwards. These measures include mechanically gentle grape processing in general and whole-bunch pressing in particular, thorough juice clarification, deliberate juice oxidation by refraining prefermentation SO₂ additions, or the use of fining agents. Concerning the specific aspect of pressing, most press designs and pressing programs aim to minimize the uptake of these phenols. Some wineries only use free-run juice to produce high quality wines on the assumption that it will have little or none of these phenols. However, contrary to popular expectations and according to the results of this study, they are also dissolved in the pulp juice of the intact berries. Hence, gentle grape processing alone is not sufficient to reduce them.

Brief definition of chemical terms

Flavonoid phenols in white grapes and wines comprise essentially the various catechins, also known as flavan-3-ols. These are (+)-catechin, (-)-epicatechin, (-)-epigallocatechin, (-)-epicatechin-3-galate and (-)-epigallocatechin-3-gallate. Catechin and epicatechin prevail in terms of quantity. In contrast to these monomeric forms of catechins, their dimeric and polymeric forms, the so-called procyanidins, are too low to be detected (Carando et al. 1999a) or account for only a minor proportion of all catechins in white wines (Lea et al. 1979, Carando et al. 1999b).

Sensory properties of flavonoids

Flavonoids are predominantly extracted from the seeds and skins during the winemaking process, whilst their counterpart, the nonflavonoids comprising mainly hydroxycinnamates, occur in dissolved form in the pulp juice. Nonflavonoids do not contribute to bitterness or astringency because their contents are close to or below their taste threshold. At best, they contribute to volume and weight on the palate, but they do not play a direct role in 'phenolic taste', color, or aging reactions (Singleton and Noble 1976, Arnold et al. 1980, Vérette et al. 1988, Smith and Waters 2012). In contrast, flavonoid levels describe very precisely the content of astringent phenols that pass from the grapes into the must and that the wine industry aims at minimizing in white wines. As will be shown below, it is an extremely important parameter for assessing the long-time behavior of these wines and their astringency.

Color: White wines without significant amounts of flavonoids are stable in color and not able to produce significant browning under conditions of oxygen uptake during aging even in the absence of free SO₂ (Rossi and Singleton 1966, Simpson 1982, Lee and Jaworski 1988, Fernandéz-Zurbano et al. 1995, 1998). Conversely, flavonoid concentrations strongly correlate with the browning rate and intensity (Schneider 1998, Salacha et al. 2008).

Figure 1 depicts the browning rate of young filtered white wines kept under air without any _{SO2} additions. Browning was measured as the absorbance at 420 nm (A 420). The higher the flavonoid content, the more intensive is the browning achieved. This browning potential is the direct visible evidence of a wine's propensity to undergo drastic chemical changes upon oxygen uptake. Under the same conditions, white wines containing less than 5 mg/L of flavonoids remain stable in color. The underlying reaction of browning is the polymerization of monomeric flavonoids to procyanidins with higher molecular weight.



Taste: Whilst flavonoids are not the only drivers of astringency, bitterness and 'phenolic taste', they are the most important ones. The flavor threshold of catechin in 5 % ethanol was reported to be 5 or 20 mg/L, depending on how this threshold was determined (Dadic and Belleau 1973). Hence, increased flavonoid contents in very young white wines are not necessarily detectable by taste (Arnold and Noble 1979). The reason is that at the very early stage of white wines, they occur predominantly as colorless monomers. Their relatively low taste intensity explains why potential astringency is perceived as less aggressive in young white wines.

When flavonoids undergo polymerization during wine aging, their astringency and color increase at equal concentration. The dimer, consisting of two single molecules, already shows noticeable astringent properties, which further increase as polymerization proceeds towards larger molecule chains (Lea 1978, Lea and Arnold 1978, Lea et al. 1979, Arnold et al. 1980, Delcour et al. 1984, Robichaud and Noble 1990, Noble 1994). Hence, astringency of white wines also depends on their age. Its

sensory perception inevitably presents a snapshot. The increasing astringency of white wines displaying relatively high flavonoid levels is a phenomenon widely known by practitioners. When it occurs, it is usually noticed long before any visible browning can be observed.

Odor: Flavonoid phenols are non-volatile and odorless. Nevertheless, they affect aroma perceived by smell, long-term aroma stability, and white wine shelf life in general when they interact with oxygen. The odor changes occurring thereby are designated as oxidative aging or premox. It is reminiscent of dry herbs, boiled potatoes, preserved mushrooms, boiled vegetables, honey, toasted bread, nuts, etc., sometimes accompanied by a noticeable astringency on the palate. These aroma attributes appear long before any taste and color changes can be observed. They also occur in the presence of free SO₂, but are greatly accelerated when free _{SO2} is no longer present. Any increase in color indicates that profound flavor modifications have already taken place. Hence, the browning potential shown in figure 1 is closely associated with a wine's predisposition to undergo premature oxidative aging.

Ultimately, there is a close relationship between the content of flavonoid phenols and oxidative aging as perceived by smell when white wines are aged under conditions of mild oxygen uptake as it can occur through certain bottle closures. Figure 2 shows this effect on two white varietal wines supplemented with flavonoid phenols extracted from grape seeds and the same amount of a pure flavonoid, (+)-catechin. Similar results can be expected when commercial grape tannins are added to white wines. However, these effects are largely absent if the wine is stored under oxygen exclusion, for example after sealing with bottle closures exhibiting a very low or nil oxygen permeation rate.



When this effect of odorless flavonoids on aroma stability was documented for the first time (Schneider 1989a), it was hypothesized that flavonoids act as a sort of catalyst in the formation of oxidative aging perceived by smell to an extent not known from other grape-derived phenols. It would take another quarter of a century before this phenomenon was substantiated by analytical data: For three chemical markers of oxidative aging – methional, phenylacetaldehyde, and sotolon – an increased formation was shown in the presence of elevated contents of catechin under conditions of nano-oxygenation prevailing when bottles are sealed with oxygen-permeable closures. Oxidation products of catechin and acetaldehyde were hypothesized to be responsible for that formation. Out of 54 white wines bottled with cork closures, those displaying less than 3 mg/L flavonoids showed no noticeable formation of sotolon, methional, and phenylacetaldehyde during prolonged oxidative bottle aging. There was a strong effect of the winery and winemaking techniques (Pons et al. 2015).

It is important to note that 2-aminoacetophenone, which is often listed in the context of white wine aging, does not belong to the aforementioned group of oxidation markers. It is the impact molecule of a distinct kind of aging denominated as 'atypical aging', which in reality is an aroma defect reminiscent of mothballs and furniture varnish usually emerging in rather young wines a few weeks or

months post-fermentation. It is induced by viticultural stress factors as the ultimate cause. Although oxygen is involved in its formation, it is only required in trace amounts that are far from sufficient to trigger oxidative aging (Schneider 2014).

Viticultural factors also are at the origin of the so-called 'petrol flavor', which occurs primarily in aged wines of Riesling regardless of oxygen exposure. These differences underline the importance of sensory discrimination between different types of aging, precursors, and reaction mechanisms.

Typical flavonoid concentrations in white wines

The aforementioned critical concentration limit requires a classification of its magnitude. Thus, surveys on white wines in several countries yielded the following mean concentrations of flavonoids, each expressed as the sum of catechin plus epicatechin:

- 15.1 mg/L on 57 wines from France (Carando et al. 1999b),
- 11.7 mg/L on 29 wines from South Africa (de Villiers et al. 2005)
- 13.6 mg/L on 41 wines from Portugal (Ribeiro de Lima et al. 2006)
- 18.1 mg/L on 16 wines from Greece (Anastasiadi et al. 2009)
- 9.3 mg/L on 37 wines from Austria (Schneider 2009)
- 15.3 mg/L on 14 wines from the Czech Republic (Lampíř et al. 2013)
- A more comprehensive survey (Goldberg et al. 1999) on 664 commercial white wines from all important varieties and growing areas of the world yielded averages of 5.9 mg/L for Chardonnay, 6.1 mg/L for Sauvignon, 4.6 mg/L for Riesling, and 5.2 mg/L for Pinot gris and blanc, with differences related to country, climate, and winemaking techniques.

The entirety of data shows that in many wineries there is much room for improvement regarding shelf life and varietal aroma stability by lowering flavonoid phenols. The only question is how to measure them in routine operations.

Measuring flavonoid phenols

High pressure liquid chromatography (HPLC) has become the golden standard for measuring flavonoids and any other phenols. In commercial winery settings, it is not used because the necessary instrumental equipment is not available and results are very detailed and difficult to interpret from the point of view of real-time decisions.

It has been known since the 1960's that flavonoid phenols represent only a small fraction of the total phenol content of white wines (Kramling and Singleton 1969, Peri and Pompei 1971). Simplifying, it can be said that common white wines display 100-200 mg/L of total phenols and 5-20 mg/L of flavonoid phenols. The differences in total phenols of these wines are primarily due to variable contents of nonflavonoid phenols. Compared with them, the proportion of flavonoid phenols recedes completely into the background. This is shown schematically in figure 3. White wines with an elevated total phenol content can be almost devoid of flavonoids, whilst others with a low total phenol content can display considerable amounts of them. As a result, there is absolutely no correlation between total phenol and flavonoid content in white wines. The classical Folin-Ciocalteu assay used for the determination of total phenols and all methods (Kramling and Singleton 1969, Moutounet 1981) for the quantification of the relatively low levels of flavonoids in white wines derived therefrom lack both specifity and precision. Phenol analyses that work for red wines are not transferable to white wines.



A similar lack of precision has been shown by indirect spectrophotometric measurement in the UV spectrum. Simple measurements of the absorbances at 280 nm and 320 nm and the use of some correction factors have been claimed to allow for assessing total phenols, nonflavonoids, and calculating flavonoids by subtraction (Somers and Pocock 1991). The major drawback of this indirect measurement is that the formula and empirical correction factors it is based on do not apply to all juices and wines. This is clearly demonstrated by negative results obtained on numerous wines.

As an alternative, spectrophotometric methods working in the visible spectrum and using vanillin were developed in the 1970s (Rebelein 1965, Pompei and Peri 1971). Vanillin is a cyclic aldehyde

Figure 4: Basic structural formula of catechins, reaction with DAC.



Pompei and Peri 1971). Vanillin is a cyclic aldehyde that should react relatively specifically with flavonoids. Unfortunately, this method yielded unrealistically inflated values (Schneider 1989b) and was soon abandoned. In a subsequent development, vanillin was replaced by another cyclic aldehyde, namely 4dimethylaminocinnamaldehyde (DAC) (Zironi et al. 1992). This method is based on a condensation reaction of DAC at positions 6 and 8 of the A ring of flavonoids (Figure 4) with formation of a colored product that is measured spectrophotometrically in the visible range at 640 nm. The results are in the range of $\pm 10\%$ of those obtained by HPLC.

Colorimetric measurement of flavonoids with 4-dimethylaminocinnamaldehyde (DAC)

In contrast to more recently developed approaches using voltammetry with disposable carbon electrodes to assess total and easily oxidizable phenols (Ugliano 2016), the flavonoid measurement with DAC directly determines the sensorially relevant phenols. It is easy and rapid to perform in routine analyses, robust, sensitive, specific, and without interferences by non-phenolic wine constituents (Schneider 1995). Of particular interest is the fact that alcohol, sugar, SO₂ and ascorbic acid do not interfere, which allows direct comparison of the values between must and wine. It accurately records all catechins in a single sum value expressed in mg/L catechin. This is comparable to the total acidity, which expresses the sum of all individual acids in g/L of either tartaric or sulfuric acid. The only prerequisite is a simple spectrophotometer operating in the VIS-range and a filter or centrifuge for sample clarification if required. The measuring procedure is as follows:

- Dissolve 100 mg 4-dimethylaminocinnamaldehyde (DAC) in 75 mL methanol 100 % and adjust to 100 mL with HCl 37 %. Fresh reagent should be prepared daily.
- Add 1 mL filtered wine sample to 5 mL reagent, mix, and read absorbance at 640 nm and 10 mm path length when it reaches its maximum after 3 to 5 minutes.

- Obtain the concentration from a calibration curve. To construct the calibration curve, 10 mg (+)catechin are dissolved in 100 ml ethanol (corresponding to 100 mg/L) and diluted to 50 – 25 – 12.5 – 5 – 0 mg/L catechin using ethanol 13 %.
- Results are reported as mg/L catechin equivalents (CE).

Application of the flavonoid measurements to wine

The results of the DAC method closely correlate with perceived astringency of dry white wines with similar matrix properties. The difference threshold is about 3 mg/L catechin. This means that a white wine is perceived as more astringent after the addition of 3 mg/L catechin.

This analytical approach is superior to the sole sensory evaluation of astringency for three reasons:

- It also detects flavonoids that are not yet noticeably astringent during the stage of very young wines and that only come sensorially to the foreground during further aging. If necessary, they can be reduced preventively by fining measures.
- It prevents unnecessary or even strenuous finings against astringent phenols in wines, in which one mistakenly believes that one has detected them without them actually being present. Typical examples of this kind of sensory bias are the mistaking of elevated alcohol or total acidity levels for astringent phenols.
- In the stylistic differentiation of wines within a winery, the variation of total acidity or residual sugar often plays a significant role. This is especially true for varietal wines when several wines of the same variety are produced. However, some wineries obtain further differentiation via modulation of astringency, which can become an additional flavor dimension. The prerequisite for doing so is the use of appropriate vinification methods that provide wines with different flavonoid contents. In this case, flavonoids are measured as routinely as alcohol and total acidity.

Table 1: Sensory assessment of flavonoid phenol concentrations in white wines.	
Concentration (in mg/L catechin)	Interpretation
1	Lowest value ever measured.
3 - 5	White wines with long shelf life.
~ 10	Astringency caused by flavonoid phenols can be perceived.
15 – 25	White wines obtained by consistently reductive grape and juice pro- cessing or by deficient juice clarification.
> 20	Accentuated astringency, which is always perceived as such.

Table 1 gives an overview of the evaluation of flavonoid phenol contents in dry white wines.

The hedonic evaluation of astringency depends on the one that one is used to and on the cultural context.

Results on musts: Impact of grape processing in the absence of SO₂ additions

The flavonoid contents of musts from various V. vinifera cultivars, two countries (Slovenia and Germany) and two vintages (2018 and 2019) were measured and their dependence on vinification conditions was studied. Results:

 Under conditions of standard grape processing (manual harvest, destemming/crushing, no skin contact time, feeding of horizontal pneumatic closed-cage presses by use of a screw, pressing with six press cycles up to 2.0 bar without inert gas protection) but without any pre-fermentation SO₂ additions, free-run juices obtained from the motionless presses yielded an average flavonoid content of 20.1 mg/L. Individual values varied by a remarkable 42 % around this mean. Eight grape varieties (Chardonnay, Riesling, Pinot gris, etc.) of a total of 17 plots were included.

- 2. The setting of the crusher-destemmer had no measurable impact on flavonoid levels. However, this outcome might become different after longer skin-contact periods.
- 3. The values mentioned in point 1 gradually decreased during the holding time in the press drain pan, due to the uptake and enzymatic consumption of oxygen with must oxidation and flavonoid precipitation as a consequence (Schneider 1998). Conversely, they could be stabilized by addition of 50 mg/L SO₂.
- 4. During pressing (2.5 hours, 2.0 bar maximum pressure), the mechanical loading of the must did not lead to an increase in flavonoid levels in the press fractions regardless of the press used. Figure 5 gives an example. This behavior is in contrast to that occurring under conditions of reductive must processing. It can be explained by the effect of must oxidation already occurring in the press and precipitating flavonoids. Oxygen uptake at the beginning of pressing without inert gas protection has been estimated at 4.8-6.4 mg/L O₂ (Cheynier et al. 1993).
- 5. Clarification of the unsulfited juice by flotation with air further contributed to reduce the initial flavonoid content from an average of 20.1 mg/L to a final level of 3 ± 1 mg/L. Flotation with air after SO₂ addition, flotation with nitrogen, and any other form of juice clarification in the presence of free SO₂ maintained the contents at their initial high levels.

Figure 5: Impact of SO₂ addition (70 mg/kg) to grapes on white juice flavonoid levels during pressing. Press system: Horizontal pneumatic closed-cage membrane press with axial feeding.



■ without SO2 (Chardonnay) ■ with SO2 (70 mg/kg) (Sauvignon) ● % juice of total juice volume

Results on musts: Impact of grape processing after SO₂ additions

- 6. When grapes were provided with 70 mg/kg SO₂ before crushing/destemming, pressing as described under point 1 led to an increase in flavonoids in the press fractions as expected. The last pressings displayed the highest contents (Figure 5). The differences between two pneumatic membrane presses from different manufacturers were 10 to 20 %.
- 7. After blending the pressings with the free-run juice, the juice blends obtained displayed flavonoid contents that were only about 10 % higher than in the free-run fractions. This indicates that pressing by means of contemporary membrane presses easily constrains the increase in flavonoids to a moderate level. The increase in pH and potassium as well as the reduction in total acidity are disproportionately greater. Under these conditions, the sometimes practiced separate processing of free-run juice and press fractions loses its significance when it comes to reducing critical phenols.

- 8. Juice clarification by sedimentation or flotation did not affect the initial flavonoid levels as long as free SO₂ was present.
- 9. During alcoholic fermentation, there is an average 25 % depletion of flavonoids regardless of their initial levels and the preceding juice processing. The cause is a partial absorption by yeast cells.

As a result, it can be stated that the pre-fermentation redox regime (with SO₂ vs. without SO₂) exerts a greater influence on must flavonoid contents than crushing, destemming, press settings, and separate processing of the press fractions.

Flavonoid phenols in the pulp juice of unprocessed grapes

The average flavonoid level of 20 mg/L in the free-run juices (point 1) gave rise to amazement, because the phenolic composition of free-run juice obtained without mechanical straining reflects the phenolic composition of the pulp juice, which is not supposed to contain flavonoid phenols as long as the berries' tissue compartmentation is intact. Based on this assumption, the exclusive use of free-run juice and gentle whole-bunch pressing are preferred techniques to obtain juices with low flavonoid levels, in particular for sparkling wine production. However, the average flavonoid levels measured in the free-run juices are considerably higher than those reported in the literature (Goldberg et al. 1999) and in table 1 for standard white wines. The loss of 25 % by absorption on yeast during fermentation cannot explain that difference.

In order to clarify this contradiction and to consistently exclude any mechanical influence of grape processing from the vineyard to after pressing, five healthy grapes were taken from each of 38 plots of different V. vinifera varieties and countries (Slovenia, Portugal, and Germany) in three years (2018, 2019, and 2020). Each grape was lightly pressed by hand according to the gentlest form of whole cluster pressing. SO_2 (100 mg/L) was immediately added to the resulting juices, which were subsequently clarified and analyzed for their flavonoid content. A mean value of 18.45 mg/L flavonoid phenols was found in the pulp juice of the total of 190 grape bunches. This figure does not differ significantly from that of the free-run juices. The mean standard deviation between the grapes of one plot was 24.5 %.

The data obtained do not indicate a clear effect of grape variety or growing region, although such a dependency might exist. However, they show a positive correlation (r=0.63) with Brix numbers. This reflects the property of grapes to synthesize more flavonoid phenols with increasing ripeness or increasing sun exposure.

Consequences for grape and must processing

No information on the flavonoid content of the pulp juice of white grapes can be found in the entire bulk of technical and scientific literature. Nevertheless, it is an established doctrine that flavonoid phenols occur exclusively in the skins and seeds and are extracted from them depending on skin contact time and mechanical load of the fruit. This is the reason for the various efforts to minimize them by mechanically gentle grape processing, whole cluster pressing, separation of the pressings, etc. The data presented here prove for the first time that flavonoid phenols also occur in relevant quantities dissolved in the pulp juice of intact berries. They further show:

- The flavonoids predetermined by the pulp juice are inevitably found in the must, without the slightest mechanical loading or skin-contact of the grapes being required.
- Their amounts are greater than those extracted during the often practiced skin-contact periods of one or two days for aroma enhancement (Figure 6). They are also higher than those released by grape solids during the pressing process (Figure 5). However, this observation only applies to contemporary membrane or other gentle presses and their correct handling.



- Gentle grape processing does not result in white wines with low flavonoid levels. The same applies to whole-cluster pressing or to the sole use of free-run juice.
- A thorough reduction of flavonoids can only be achieved by oxidative must processing without the use of SO₂ ascorbic acid, inert gases or other means to protect must from oxidation, followed by efficient juice clarification. This approach corresponds to passive must oxidation, whereby the flavonoid phenols precipitate and are removed with the must lees (Schneider 2019).
- Passive must oxidation is the result of naturally occurring oxygen uptake and enabling its enzymatic consumption and transfer to phenols in the absence of SO₂ and ascorbic acid. In general, it is sufficient for almost complete removal of flavonoids. Hyperoxidation, developed in the 1970s, involves the active addition of oxygen, is an extreme form of must oxidation, is unnecessary, and can actually lead to the widely feared losses of aroma in some wines (Schneider 1998).
- Juice solids contain flavonoids and additional precursors of a wide range of off-flavors. They release them into the alcohol-containing medium during and after primary fermentation. Hence, the degree of juice clarification is of primary importance in this context. This applies to both reductive and oxidative juice processing, because flavonoids deliberately precipitated by juice oxidation otherwise would dissolve back again and, thus, cancel out the effect of juice oxidation. Thereby, it is irrelevant in which way the juice is clarified. What matters is exclusively the result, measured as residual turbidity and expressed in NTU (nephelometric turbidity units). A residual turbidity in the range of 20 to 80 NTU is recommended (Nicolini et al. 2011); below 20 NTU the frequency of sluggish or stuck fermentations considerably increases. A turbidity meter is helpful and is used successfully in many wineries specializing in fine white wines. There will be no breakthrough in obtaining clean, fruity, and stable varietal aroma as long as producers are not willing or not able to seriously control the results of juice clarification.
- Since the importance of juice clarification and residual turbidity is frequently underestimated, numerous results of must processing trials are neither meaningful nor reproducible. Poor juice clarification also explains the fact that in some experiments on must hyperoxidation no reduction of flavonoids is observed.
- Under conditions of oxidative must processing, oxidizability and flavonoid precipitation depend on the musts' initial hydroxycinnamic acid to glutathione ratio (Cheynier et al. 1990). Therefore, occasional attempts are made to adjust the oxygen supply to the individual must. In the light of the findings on flavonoid removal presented in this report, the naturally occurring oxygen uptake is sufficient, whilst the degree of juice clarification is crucial for residual flavonoids in the wine.
- All measures of reductive vinification, such as the consistent use of SO₂ before fermentation, the use of inert gas inside the press and for flotation, preserve the flavonoid phenols at their original

high level with all the consequences for mouthfeel and shelf life of the resulting wines. The most important factor in modulating flavonoid levels is the decision in favor of reductive or oxidative must processing.

- The more reductive the grape and must processing, the more prone the wine is to premox and oxidative aging in the broadest sense. Accordingly, rigorous protection against oxygen pickup after fermentation and bottling is required.
- In some grape varieties such as Sauvignon, where aroma thiols make a sensorially significant contribution to varietal aroma, oxidative vinification would be detrimental to these thiols. Reductive vinification is therefore necessary to preserve the varietal aroma of such cultivars. The resulting elevated flavonoid phenol content can be reduced by fining. As a guideline, 80 g/hl PVPP reduces the initial content by an average of 75 %.

In summary, elevated levels of flavonoid phenols are a contributing factor to accelerated oxidative aging similar to high oxygen permeability of the bottle closure, low levels of free SO₂, and high storage temperature.

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