Diffusion of phenolic compounds during a model maceration in winemaking: role of flesh and seeds

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Short Title: Role of pulps and seeds on the diffusion of phenolic compounds during maceration.

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Abstract

Background During red winemaking, diffusion of phenolic compounds from the grape berry cells into the liquid phase occurs simultaneously with the adsorption of the same compounds onto the pulp. In previous studies, we have quantified the proportions of polyphenols diffusing from the skins and then assessed the amounts that can be fixed by the pulp. In this work, we added the impact of seeds, also present during vinification, by carrying out macerations in a model medium with the following berry compartments: skins, seeds, skins + seeds, skins + seeds + pulp.

Results. Interestingly, the seeds alone released a rather high amount of polyphenols. As soon as they were in the presence of cell walls of skin/flesh, and/or anthocyanins, the concentration of seed tannins in the solution dropped dramatically, due to a combined effect of adsorption and/or precipitation and/or chemical reactions. The pulp certainly adsorbed tannins, but they also tend to shift the extraction equilibria and it seems that more tannins could be extracted from skins and seeds when pulp was present. Polyphenol amounts extracted in model systems with skins + seeds+ pulp were close to what was extracted in microvinification.

Conclusion. These model experiments reflect relatively well extraction during microvinification experiments and highlighted the respective impact of the grape berry's different compartments in the wines' final phenolic composition as well as some of the mechanisms involved.

Keywords: simulated maceration, polyphenol diffusion, red winemaking, polysaccharides

1- Introduction

During the red winemaking process, the diffusion of anthocyanins and condensed tannins (or proanthocyanidins (PAs)) is crucial to ensure the expected organoleptic properties of the wine (especially color, bitterness, and astringency). Anthocyanins and tannins in skin cells are mostly located in vacuoles, tannins being also found associated with the cell walls[1,2] . Tannins are also extracted from seeds, although to a lower extent than from skins. Tannin and anthocyanin extraction from the skins/seeds of grapes during winemaking is not total and several studies have shown that there is no direct relationship between their contents in grapes and those found in the corresponding wines [3,4]. The latter depends on the extraction conditions (solvent, temperature, maceration length, …) and the cap-management practices [5] during winemaking, as well as on their ability to cross the barriers represented by cell structure and in particular cell walls[1,6]. Other factors that modulate anthocyanin and tannin concentrations in wines are: (i) their adsorption on insoluble pulp debris (flesh cell walls) [7] and on yeasts [8]; (ii) their interactions with soluble grape compounds (polysaccharides, proteins) extracted during maceration, leading to precipitations [9]; iii) their chemical reactivity [10] . Indeed, once extracted, anthocyanins and tannins undergo several chemical reactions that profoundly change their composition throughout the process [11]. Previous studies have shown that there is no direct relationship between the phenolic composition in grapes and the corresponding wines [3,12,13], whether the phenolic diffusion from grapes was done in a model system or real winemaking conditions. In previous studies [14,15], we highlighted the impact of the grape variety and maturity on the extractability of polyphenols during a model maceration of skins, which was attributed to several differences in the composition of the skin cell walls and phenolic compounds. On the whole, between 15 and 25% of the tannins and between 16 and 45 % of the anthocyanins were extracted from skins (diffusion minus losses).

To understand the impact of interactions between phenolic compounds and flesh on the concentration in wines, they were studied with flesh insoluble material separately [15]. We found that at their usual concentration in wines, more than half of the tannins were adsorbed by flesh water-insoluble material (FWIM) and eliminated from the solution, in agreement with previous works [16]. Although anthocyanins have a very low affinity for FWIMs at 0.5 $g \cdot L^{-1}$ (usual anthocyanin concentration in wines), this adsorption can induce a 15% decrease in their content. An impact of the variety on the interactions was highlighted and was related to differences in the cell wall composition determined by the Comprehensive Microarray Polymer Profiling method.

Our objectives were to study diffusion (mass transfer) and interactions together to understand their respective impact and compare results obtained in model solutions and a real winemaking experiment (microvinification). To this end, new diffusions experiments were performed in model wine-like systems, including seeds and with or without flesh water-insoluble material (FWIM). As in previous works, two varieties of grapes were considered: Carignan and Grenache. These diffusion experiments were then compared with microvinifications to evaluate the relevancy of the model systems.

2- Materials and Methods

Chemicals

Acetonitrile, methanol, ethanol, chloroform, acetic acid, and formic acid were HPLC grade from VWR. Acetone was provided by Fluka. Sodium chloride, tartaric acid, epicatechin, epigallocatechin gallate, lithium chloride, N, N-dimethylformamide, and trifluoroacetic acid were provided by Sigma-Aldrich, sulphuric acid by Roth. Catechin, epicatechin, epicatechin-gallate, epigallocatechin, flavanol dimer B2, flavanol trimer C1, and malvidin-3-*O*-glucoside

chloride were purchased from Extrasynthese (Genay, France). Ultra-pure water was obtained from a Milli-Q Advantage A10 system (Millipore).

Grape sampling

Two *Vitis Vinifera* grape varieties (Carignan Noir, Vitis International Variety Catalogue number 2098 and Grenache, VIVC number 4461), of the 2018 season, were harvested at average potential alcohol of 12 % vol. in the vineyard of the Pech Rouge experimental unit (UE-PR, INRAE, Gruissan, France). The berries were harvested at maturity and sorted in four batches according to their diameter (vol^-, vol^+) and density (deg^-, deg^+) as described before [14]. This sorting results in a good homogeneity within a batch of 30 berries. Only the vol^+deg^+ berries were used in the present work. The total sugar, pH, and total acidity of the corresponding musts are reported in Table 1.

Three sets of 30 berries (triplicate) were and used to separate the different compartments for polyphenol analysis. Skins, seeds, and pulp were immediately frozen in liquid nitrogen and stored at -80 °C before use. The average weight of the berries and each of their compartments was determined (Table 1). Three kilograms of berries were immediately crushed and used for the microvinification (900 g per microvinification, triplicates). Five hundred grams of berries were also immediately frozen at - 80°C for experiments in model solutions.

Macerations in model wine-like conditions

This experiment was designed to follow polyphenol diffusion from skins and seeds during a wine-like maceration experiment and study the impact of their interactions with flesh water-insoluble material (FWIM) on their diffusion and their final composition in the solution. To this end, five different maceration experiments were performed, each being made in triplicate: maceration of A) skins alone, B) seeds alone, C) skins + seeds, D) skins + FWIM, and finally E) skins + seeds + FWIM. Twenty berries of Carignan and Grenache were used for

each experiment. They were manually peeled to separate skins, seeds, and mesocarp. FWIM was prepared from the mesocarp as described in reference[15]. The materials (of 20 grape berries) were immediately immersed in 28 mL of a model aqueous solution containing 3 $g \cdot L^{-1}$ tartaric acid, 50 mM NaCl, and 40 $mg \cdot L^{-1}$ SO_2, at pH 3.5 (adjusted with NaOH 1M). This volume was chosen to obtain a solid/liquid ratio similar to that found in winemaking. Simulated maceration experiments were carried out during 11 days by increasing stepwise the ethanol content from 0 to 15%[14] : 0% ethanol – 24h; 5 % ethanol – 48 h; 10 % ethanol – 48 h; 12% ethanol – 48 h; 14 % ethanol – 48 h; 15 % ethanol – 48 h. Flasks were placed under argon and gently stirred on a stirring plate in dark at 22 °C for 11 days. Samples were taken and centrifuged (15000 xg, 15 min, 15°C) for phenolic analysis at the end of each ethanol increase step. The changes in concentration induced by the addition of ethanol on the one hand, and by the withdrawal of anlaysis solution on the other, were taken into account and calculated.

Winemaking experiment

Fermentation and maceration were performed in low volumes (<1 kg) using "French Press" coffee plungers at 22°C (Supplementary Figure S1). 900 g of berries of each type were crushed. Reactivated Lalvin ICV OKAY yeast (0.2 $g \cdot L^{-1}$) and SO_2 (250 µL of an 8% solution) were added. During the alcoholic fermentation (AF), manual punching down of the pomace cap was carried out daily to homogenize the medium. It is important to note that on such small volumes, the cap of pomace does not form: the solids rise to the surface but do not compact. The decrease in sugar concentration was followed daily, along with polyphenol extraction (total polyphenol index TPI and total red pigment TRP measurements). After 8 days, at the end of the fermentation, the solid parts were manually pressed and the "free-run" and "press" wines were gathered. Classical enology parameters, namely glucose + fructose, total acidity (TA), and pH, were determined according to the CEE-2676/90 official methods of the European Union.

Polyphenol extraction from skins, pulps, and seeds

Frozen skins, pulps, and seeds were ground to a fine powder in liquid nitrogen using a mortar grinder (Pulverisette 2, Fritsch). Extractions were performed on 150 mg of powder with 6 mL of solvent: 750 µL of methanol were added first, followed by 5.25 mL of 60/40/1 (v/v/v) acetone/water/formic acid. Extractions were performed at room temperature on an orbital shaker (Precellys 24, Bertin technologies, program 5000-3*40-20). After centrifugation (3000 *x g*, 5 min, 4°C), 1 mL aliquots of the extracts were dried in a rotary evaporator under vacuum at 35°C for 2h (EZ-2 plus, Genevac SP service). Dried extracts were used for polyphenol analysis.

Polyphenol analysis

Polyphenols diffusion during microvinifications and experiments in model solutions was followed by measuring the total polyphenol Index (TPI) and total red pigments (TRP), determined by UV–visible spectrophotometry (spectrophotometer UV-1800, Shimadzu) at 280 and 520 nm (1 cm path length) after adequate dilution in HCl 1 M. In model maceration experiments and initial skin extracts, free anthocyanins were also analyzed by HPLC using a Waters chromatography system equipped with DAD detection and a C18 reversed-phase column (Atlantis T3, Waters). Anthocyanins were quantified at 520 nm, in equivalent of malvidin-3-*O*-glucoside. PA monomers were analyzed by HPLC-DAD after acid-catalyzed depolymerization in the presence of phloroglucinol, as described by Kennedy *et al*.[17], but with a modification of the elution gradient with mobile phases containing 2% v/v aqueous formic acid (mobile phase A) and acetonitrile containing 2% v/v formic acid (mobile phase B). Eluting peaks were monitored at 280 nm. The elution conditions were 0.4 mL/min; 3% B for 5 min, a linear gradient from 5 to 10% B in 30 min, a linear gradient from 10 to 20% B in 30 min, a

linear gradient from 20 to 25% B in 15 min, a linear gradient from 25 to 80% B in 10 min. The column was then reequilibrated with 3% B for 3 min before the next injection.

The size distribution of polyphenols and the concentration of polymeric tannins (in eq. epicatechin) were also determined by HPSEC in the skin and seed extracts, in the wines, and at the end of the maceration experiments in model conditions, as described previously[15]. Commercial epicatechin, B2 dimer, epigallocatechin gallate, malvidin, and home prepared and characterized tannin fractions were used to determine the retention times of different monomers, oligomers, and polymers (supplementary Figure S2). Quantification was done by integrating the areas under the peak using Agilent software, considering that the polymers correspond to the molecules eluted before 19', the oligomers come out between 19 and 20', and that the peak corresponding to the anthocyanins comes out around 21' (this value was confirmed by the observation of the chromatogram at 520 nm). This quantification, based on the UV response, was done after calibration with catechin and epicatechin.

Statistical analysis

Statistical analysis was performed using Minitab software. The results obtained were assessed by one-way ANOVA analysis followed by a Tukey Test for $p < 0.05$.

3- Results and Discussion

3.1 Analyses of grape polyphenols

The amounts of anthocyanins and tannins quantified in fleshes were negligible compared to the amount of polyphenols found in other grape compartments, even considering their weight in the whole berries. Thus they have not been further considered. Table 1 gives the different compositions of tannins and anthocyanins of Carignan and Grenache seeds and skins determined by HPLC, HPLC after depolymerization, and HPSEC. The two varieties differed

greatly in their anthocyanin content and composition, as discussed previously[14]: Carignan skins were significantly richer in anthocyanins, and proportionally richer in *p*-coumaroylated anthocyanins. Unlike anthocyanins, total tannin contents in skins and seeds did not differ significantly between the two varieties. The only differences observed for skin tannins concerned their proportion of epigallocatechin (EgC) units, higher in the Grenache skin tannins, and the average degree of polymerization in number (DP_n), which was also higher in the Grenache variety. Seed tannins were significantly more galloylated in Grenache than in Carignan, and their DP_n was slightly lower.

HPSEC analyses with DAD detection were also performed on skins and seeds phenolic extracts to determine the size distribution of oligomeric and polymeric polyphenols (Figure 1). Indeed, phloroglucynolysis provides an average degree of polymerization and information on the constitutive units but no information on the size distribution. The total flavanols given by the two methods are of the same order of magnitude with raw materials but differ for two reasons: the depolymerisation reaction is not complete and results for HPSEC are expressed in equivalent of epicatechin. HPSEC chromatograms of skin polyphenols showed three main different peaks corresponding to tannins with different sizes assessed after the calibration with well defined tannins (Supplementary dataS2): one related to polymeric tannins ($DP_n > 5$) at a retention time of 17 minutes, one related to oligomers at a retention time around 19 minutes, and one related to anthocyanins[14] at 20 minutes. The size distributions only slightly differed between Carignan and Grenache. The small peaks observed between 13 and 14 minutes in seeds tannins are due to the larger polymers that are "excluded" because they are too large to enter the pores and thus are the first observed at the end of the column. The anthocyanin peak was wider and shifted for Carignan compared to Grenache, in agreement with the higher content of total anthocyanins in this variety. Quite different chromatograms were found for seed polyphenols. These chromatograms evidenced different populations: catechin/epicatechin,

epicatechin-gallate, oligomeric, polymeric (DP_n>3) tannins, and a much more important exclusion peak. Using tannin fractions of different DP_n extracted from seeds (DP_n between 2 and 24) and skins (DP_n between 3.8 and 39), Kennedy and Taylor[18] observed through HPSEC higher sizes for seed tannins than for skin ones. They attributed this result to more extended conformations of seed tannins, related to galloylation. HPSEC profiles here indicated the presence in the seeds of a majority of high molecular weight tannins, associated with higher contents in low molecular weight tannins than in skins (Figure 1). These higher proportions in low molecular weight tannins explain the lower DP_n found after phloroglucinolysis for seed tannins compared to skin tannins (Table 1).

3.2 Polyphenol diffusion from skins and seeds

Tannins can diffuse from skins and seeds, anthocyanins from skins only. To study the impact of seeds, diffusion experiments were performed with skins and seeds alone and then with skins + seeds. The extraction of polyphenols from skins and seeds during maceration was followed using UV/Vis Spectrometry (TPI and TRP values) (Figure 2). The much higher TPI observed for the extraction from Carignan skins by comparison to Grenache skins is related to a much higher content in anthocyanins[19]. Extraction was rapid (2 days) in our experimental conditions and by comparison to other literature data, especially for seeds[20,21]. This is likely related to the existence of a continuous agitation in the diffusion medium and the rapid increase in the ethanol content (from 0 to 10 % within 3 days). This extraction was observed even at low ethanol concentration and increased with ethanol concentration as reported by Canals *et al.*[20].

The concentrations of polyphenols in the wine-like medium at the end of the maceration experiment and their size distribution are reported in Table 2 and Figures 2E and 2F, respectively. In agreement with our previous works[14], high molecular weight tannins were not extracted from skins and the extraction of polymeric tannins was low (about 21% of the

total skin tannins), this being attributed to their interactions with skin cell walls. HPSEC analysis also highlighted a preferential extraction for seed tannins (Figure 2E and 2F): the highest molecular weight tannins were not extracted, as already observed with skins and in agreement with previous results[21]. This indicates that molecular size is also an important parameter for the extraction of tannins from seeds. However, the distribution profiles largely differed from what was observed with skins: much lower amounts of high molecular weight tannins diffused from skins than from seeds, likely because there are more interactions with the skin cell walls for skin tannins. The percentage of extraction of seed polymeric tannins estimated from the concentrations determined by HPSEC analysis was about 60% and that of oligomers was between 70 and 90 % (Table 2). These extractions led to more than twice higher polymeric tannin concentrations for seeds by comparison to skins at the end of the maceration. These results are in accordance with those of Rousserie et al. [21], who determined for the Merlot grape variety an extraction of more than 70% of the seed tannins at the end of the alcoholic fermentation.

The pholoroglucinolysis results (Table 3) showed that the extracted tannins are of smaller DPn (seeds and especially skins), less galloylated (seeds), and less hydroxylated (skins). This was expected: high DP and high galloylation are the main levers of interactions of polyphenols with polysaccharides and proteins: if they interact more with cell walls they are less extracted. When seeds and skins were mixed (Figure 2 and Table 2), the total polyphenol extraction was much lower than expected: TPI values were much lower than those obtained by summing the values of experiments A and B (Figure 2A and 2B). The presence of seeds did not strongly impact the diffusion/extraction of anthocyanins (red pigments, Figure 2C and D). The decrease observed in TPI or $A_{280\ nm}$ in HPSEC was mainly related to oligomers and polymers,

the most important impact being observed with the highest molecular weight tannins (Figure 2E and F).

The decrease of flavan-3-ols concentration observed when skins and seeds are present together in the diffusion medium may have different origins: 1) high concentrations in the solution that reduces the mass transfer from skins or seeds, according to Fick's law; 2) re-adsorption of seed tannins by skin insoluble material [15] and/or interactions and precipitation with skin soluble polymers (proteins, polysaccharides)[22]; 3) reactions/interactions between polyphenols in solution leading to precipitation[23,24,25]. If the mass transfer may be limited by the tannin concentration in the medium, it does not explain the deficit observed in oligomeric and polymeric tannins, as their concentrations at the end of the maceration when skins and seeds are present together in solution are lower than those observed with seeds only. Thus, the main mechanism leading to tannin deficiency when skins and seeds are present together is likely related to physico-chemical interactions. Tannins in seeds have a higher content of Ec-G. This likely enhances their adsorption/interactions with skin insoluble/soluble materials[13,26–28]. If all the polymeric and oligomeric tannins present in the seeds and skins of 20 berries were extracted, this would lead in our experimental conditions to concentrations in solution of about 8 $g \cdot L^{-1}$, including approximately 4.5 $g \cdot L^{-1}$ of skin tannins. The results of adsorption isotherms with skin cell walls are in agreement with the adsorption of tannins by the skins, even if precipitations can also take part. If everything was extracted with the skins and seeds, we would have concentrations in the order of 7 $g \cdot L^{-1}$ for the polymers and 1 $g \cdot L^{-1}$ for the oligomers. At these concentrations, we are not at the adsorption plateau, as observed by Bindon *et al.*[29], who performed experiments with tannin concentrations up to 8 $g \cdot L^{-1}$. There would therefore still be sites available on the skin cell walls for the seed tannins.

Anthocyanins loss in the presence of seed tannins were only observed with the Carignan variety and anthocyanins interact less with insoluble materials and macromolecules.

Anthocyanins loss, in this case, are therefore more likely caused by chemical reactions[30]. Carignan and Grenache differ by the anthocyanin/tannin ratio, much higher in Carignan, and the percentage of Ec-G of seed tannins is higher than that of skin tannins. These different characteristics may favor reactions between anthocyanins and tannins leading to the formation of less soluble species or non-pigmented derived compounds. It has been recently shown that derived pigments formed by anthocyanin and seeds tannin reactions in the presence of acetaldehyde are much less stable than those formed with skin tannins and tend to aggregate and precipitate [31]. This resulted in a loss of polymeric pigments formed from seed tannins, which increased with tannins molecular mass. Although there was no acetaldehyde in the model solutions used here, other chemical reactions cannot be excluded.

The phloroglucinolysis results (Table 3) did not necessarily show an additional decrease in the average degree of polymerization of extracted tannins from seeds + skins mixtures compared to seeds and skins alone, partly because only the lowest DP were extracted from skins and seeds. However, it should be noted that phloroglucinolysis does not allow for the analysis of all the tannins, because derived tannins may not be depolymerized. The tannins extracted from the mixtures are less galloylated than those extracted from seeds and skins for Carignan and are intermediate for Grenache.

If we consider the amount of tannins extracted from the raw material: between 60 and 90% of seeds tannins were extracted, and between 6 and 11% of skin tannins. Thus, if we calculate what we should theoretically obtain by mixing skins + seeds, we lose about 60% of the Carignan tannins and 80% of the Grenache tannins.

3.3 Impact of FWIM

The presence of FWIM with skins in the diffusion medium (Figure 3) induced a moderate decrease in the total polyphenol concentrations and total red pigments. HPSEC and HPLC analyses (Figure 3E and 3F, Table 2) indicated a decrease in polyphenol polymers and

oligomers of 13 and 27 % for Carignan and Grenache, respectively, mainly related to polymers (18 and 36 % for polymers only), and a decrease in free anthocyanins of 24% and 22%.

With regard to the extraction of polymers from the skins and seedss, whether alone or mixed, there was no significant difference between Carignan and Grenache if we consider the quantity extracted. On the other hand, if we look at the percentage of extracted seed tannins in relation to the initial composition, the percentage extracted was higher in Carignan. When skins and pulp were mixed, significant differences appeared between Carignan and Grenache. The extraction of oligomers showed significant differences between Carignan and Grenache, both in absolute value and in percentage extracted, for all combinations of compartments. The behaviour of anthocyanins was slightly different: their extraction was significantly different in absolute value between Carignan and Grenache, but their extraction percentage is similar. The addition of pulp and seeds did not change this trend: the *percentage* of extracted anthocyanins is not significantly different between Carignan and Grenache.

These decreases can be compared to those expected from adsorption isotherms of skin tannins and anthocyanins on Carignan and Grenache FWIMs[15] . Considering these isotherms, the expected losses were 60-65% for skin tannins and 15-20% for anthocyanins in 15% ethanol and at their concentration in the diffusion medium (1-1.4 $g \cdot L^{-1}$ for tannins and less than 0.5 $g \cdot L^{-1}$ for anthocyanins). Thus, adsorption by FWIMs during maceration experiments is far from inducing tannin losses as large as those expected from adsorption isotherms. Anthocyanin losses are slightly higher than expected from adsorption alone but on the same order of magnitude. It was also less important for Carignan than for Grenache, as expected from previous results[15].

These results indicate first that interactions between FWIMs and extracted polyphenols during maceration shift the solid/liquid equilibrium by decreasing the concentration in solution, resulting in increased diffusion from the skins. This phenomenon is clear for tannins, not for

anthocyanins as there is an impact of tannins on their interactions with FWIMs. The differences observed between Carignan and Grenache, may have two origins:

- the differences in galloylation of skin tannins between the two varieties (twice higher in Grenache than in Carignan), leading to higher adsorption for this variety that counterbalance the 20% higher adsorption capacity evidenced for Carignan with the same polyphenol pool;
- different mass transfer equilibria between polyphenols in skins and polyphenols in the diffusion medium between the two varieties, also related to a different composition of tannins and anthocyanins.

The diffusion/maceration experiments were repeated but this time by mixing skins, seeds, and FWIM (Figure 3 and Table 3). In comparison to the skins+seeds experiment, a decrease in TPI and TRP concentrations was only observed for the Carignan variety (8% in TPI and 16% in TRP). TPI and TRP were slightly higher (16% and 20%, respectively) in the presence of FWIM for Grenache. HPSEC analysis confirmed the results obtained with TPI. In our experimental conditions, the whole results indicated that adsorption by FWIM modulates the polyphenol composition and impacts their final concentrations but less than expected from adsorption results.

The DPm of skin and seed tannins, calculated from phloroglucinolysis (Table 3), which were significantly different when they were in the raw material or diffusing alone, were no longer different when they were in contact with the pulp: it can be assumed that the preferential interaction of the higher DP tannins with the pulp erased the differences and homogenised the size distribution of tannins. Similarly, the percentage of galloylation and trihydroxylation of tannins, which sometimes differed between Carignan and Grenache

depending on the compartment studied, were no longer significantly different once the pulps were added.

The mass balance obtained from phloroglucinolysis did not show an additional loss compared to the skins + seeds system: we went from 38% to 30% of what we should have had if everything had been extracted (Carignan) or even gain a little for Grenache (from 15 to 20%)..

3.4 Comparison of diffusion in model systems and microvinifications

The diffusion of polyphenols from the "skins + seeds + FWIM" model system was compared with that observed in microvinification (Figure 4). Differences in extraction rates were observed, especially within the first days of the maceration, but overall and at the end, the extractions were close in terms of TPI and TRP. These differences in extraction rates can be attributed to differences in the stirring conditions (constant stirring in the model system versus homogenization related to CO_2 release and punching in microvinification) and a more progressive change in the ethanol content during the real fermentation. These two factors, as well as others, have been suggested by Setford et al.[5].

HPSEC analyses (Figure 5) showed close profiles, with slightly higher contents in polymeric tannins in the model maceration experiments than in wines and similar contents in oligomers.

During winemaking, yeasts may be involved in changes in the polyphenol composition through two different mechanisms: 1) chemical reactions involving polyphenols (anthocyanins and tannins) by the release of metabolites such as acetaldehyde or pyruvic acid and also some enzymatic activities (minor); 2) adsorption by cell walls and/or whole cells[8,32]. The impact of chemical reactions related to yeast has not been studied in-depth in this work. When dealing with red wines, adsorption by yeast cell walls only has a minor impact on the total red wine

polyphenolic content [8] and specifically affects high molecular weight polymers. Adsorption by whole yeasts has a higher impact (15-20% of TPI losses, 5% of TRP losses) but occurs within the days after the end of the fermentation, essentially after their death and mainly concerns oligomers and polymers [8,32]. Analysis of wines were performed immediately at the end of fermentaton, so that significant decreases in TPI and TRP related to adsorption are not expected.

4- Conclusion

Macerations in model solutions do not reflect the initial conditions of the winemaking process, but they gave however results close to what is observed during microvinifications and led to several conclusions.

Seed tannins diffuse very well on their own and in our experimental conditions (variety, ethanol content, duration), much more than expected from literature; there may have been a concern in our experiments due to freezing, but the final concentrations usually observed using fresh seeds are of the order of 1-1.5 g/L, which is far from negligible; in terms of selectivity, the highest molecular weight tannins are not extracted.

The percentage of galloylation of tannins extracted from skins+seeds+FWIM mixtures, between 3 and 5%, was much lower than that of seed tannins (between 15 and 20%). However, it was higher than that of skin tannins (between 2 and 4%), which led us to conclude that the tannins found in wine are mostly those of skins. Similarly, the percentage of epigallocatechin units, which come almost exclusively from the tannins of skins, decreased slightly when all the berry compartments were present. Both observations lead to the conclusion that higher levels of skin tannins are found in wine, despite seeds having much higher initial tannin content than the skin. These low contents of seed tannins in wines were attributed to the low and slow extraction of seed tannins requiring longer maceration time[33]. However, this is not what was observed in the present study. Previous results observed from extraction

experiments in a 12 % ethanol solution suggest that extraction rates are highly variable, depending on the vintage and not on the variety[34]. In our experiments, seed tannins were extracted rapidly and easily from seeds, even at low ethanol contents (Figures 2 A and B). Our results, in agreement with those of Rousserie *et al.*[21] suggest then that the low % of Ec-G units in wine can be related to i) selective adsorption of the most galloylated tannins (seed tannins) by skin or flesh insoluble materials; ii) selective precipitation of galloylated tannins with skin soluble polymers or related to chemical reactions. Analyses by phloroglucinolysis of tannins at the end of the wine-like maceration experiments confirm that the percentage of galloylation of tannins decreases when seeds tannins interact with skins and fleshes. Finally, the flesh water-insoluble materials certainly absorb tannins, but they also tend to shift the extraction equilibria and it seems that more tannins can be extracted from skins and seeds when they are added.

The extraction kinetics of anthocyanins and tannins were similar in the case of microvinifications and model solutions containing both skins, seeds and pulp. These expereiments could therefore make it possible to evaluate the potential for polyphenol extraction from a variety on very small quantities of berries, which could for instance be useful in the context of varietal selection.

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References

1 Ortega-Regules A, Romero-Cascales I, Ros-García JM, López-Roca JM, and Gómez-Plaza E. A first approach towards the relationship between grape skin cell-wall composition and anthocyanin extractability. *Anal Chim Acta* **563**:26–32 (2006).

2 Amrani Joutei K, Glories Y, and MERCIER M. Localization of tannins in grape berry skins. *Vitis* **33**:133–138 (1994).

3 Hazak JC, Harbertson JF, Adams DO, Lin CH, and Ro BH. The phenolic components of grape berries in relation to wine composition. *Acta Hortic* **689**:189–196 (2005).

4 Bautista-Ortín AB, Busse-Valverde N, Fernandez-Fernandez JI, Gomez-Plaza E, and Gil-Munoz R. The Extraction Kinetics of Anthocyanins and Proanthocyanidins From Grape To Wine. *J Int Sci Vigne Vin* **50**:91–100 (2016).

5 Setford PC, Jeffery DW, Grbin PR, and Muhlack RA. Factors affecting extraction and evolution of phenolic compounds during red wine maceration and the role of process modelling. *Trends Food Sci Technol* Elsevier Ltd; **69**:106–117 (2017).

6 Hernández-Hierro JM, Quijada-Morín N, Martínez-Lapuente L, Guadalupe Z, Ayestarán B, Rivas-Gonzalo JC, and Escribano-Bailón MT. Relationship between skin cell wall composition and anthocyanin extractability of Vitis vinifera L. cv. Tempranillo at different grape ripeness degree. *Food Chem* Elsevier Ltd; **146**:41–47 (2014).

7 Bindon KA, Smith PA, Holt H, and Kennedy JA. Interaction between grape-derived proanthocyanidins and cell wall material. 2. Implications for vinification. *J Agric Food Chem* **58**:10736–10746 (2010).

8 Mekoue Nguela J, Sieczkowski N, Roi S, and Vernhet A. Sorption of Grape Proanthocyanidins and Wine Polyphenols by Yeasts, Inactivated Yeasts, and Yeast Cell Walls. *J Agric Food Chem* **63**:660–670 (2015).

9 Bindon KA, Carew AL, Mierczynska-Vasilev A, Kassara S, Kerslake F, and Smith PA.

Characterization of macromolecular complexes in red wine: Composition, molecular mass distribution and particle size. *Food Chem* **199**:838–846 (2016).

10 Fulcrand H, Morel-Salmi C, Poncet-Legrand C, Vernhet A, and Cheynier V. Tannins : from reactions to complex supramolecular structures. *Australian Journal of Grape and Wine Research* Adélaïde, Australie; p. 1–9 2006.

11 Hanlin RL, Hrmova M, Harbertson JF, and Downey MO. Review: Condensed tannin and grape cell wall interactions and their impact on tannin extractability into wine. *Aust J Grape Wine Res* **16**:173–188 (2010).

12 Romero-Cascales I, Fernández-Fernández JI, López-Roca JM, and Gómez-Plaza E. The maceration process during winemaking extraction of anthocyanins from grape skins into wine. *Eur Food Res Technol* **221**:163–167 (2005).

13 Fournand D, Vicens A, Sidhoum L, Souquet JM, Moutounet M, and Cheynier V. Accumulation and extractability of grape skin tannins and anthocyanins at different advanced physiological stages. *J Agric Food Chem* **54**:7331–7338 (2006).

14 Abi-Habib E, Poncet- Legrand C, Roi S, Carrillo S, Doco T, and Vernhet A. Impact of grape variety, berry maturity and size on the extractability of skin polyphenols during model wine-like maceration experiments. *J Sci Food Agric* **101**:3257–3269 (2021).

15 Abi-Habib E, Vernhet A, Roi S, Carrillo S, Jørgensen B, Hansen J, Doco T, and Poncet-Legrand C. Impact of the variety on the adsorption of anthocyanins and tannins on grape flesh cell walls. *J Sci Food Agric* (2021).

16 Bindon KA, Kassara S, and Smith PA. Towards a model of grape tannin extraction under wine-like conditions: the role of suspended mesocarp material and anthocyanin concentration. *Aust J Grape Wine Res* **23**:22–32 (2017).

17 Kennedy JA and Jones GP. Analysis of proanthocyanidin cleavage products following acid-catalysis in the presence of excess phloroglucinol. *J Agric Food Chem* **49**:1740–

1746 (2001).

18 Kennedy JA and Taylor AW. Analysis of proanthocyanidins by high-performance gel permeation chromatography. *J Chromatogr A* **995**:99–107 (2003).

19 Boulet JC, Ducasse MA, and Cheynier V. Ultraviolet spectroscopy study of phenolic substances and other major compounds in red wines: relationship between astringency and the concentration of phenolic substances. *Aust J Grape Wine Res* **23**:193–199 (2017).

20 Canals R, Llaudy MC, Valls J, Canals JM, and Zamora F. Influence of ethanol concentration on the extraction of color and phenolic compounds from the skin and seeds of tempranillo grapes at different stages of ripening. *J Agric Food Chem* **53**:4019–4025 (2005).

21 Rousserie P, Lacampagne S, Vanbrabant S, Rabot A, and Geny-Denis L. Influence of berry ripeness on seed tannins extraction in wine. *Food Chem* Elsevier; **315**:126307 (2020).

22 Le Bourvellec C and Renard CMGC. Interactions between polyphenols and macromolecules: quantification methods and mechanisms. *Crit Rev Food Sci Nutr* **52**:213–248 (2012).

23 Poncet-Legrand C, Cartalade D, Putaux J-LL, Cheynier VV, and Vernhet A. Flavan-3-ol aggregation in model ethanolic solutions: Incidence of polyphenol structure, concentration, ethanol content, and ionic strength. *Langmuir* **19**:10563–10572 (2003).

24 Zanchi DD, Vernhet A, Poncet-Legrand C, Cartalade D, Tribet C, Schweins R, and Cabane B. Colloidal dispersions of tannins in water-ethanol solutions. *Langmuir* **23**:9949–9959 (2007).

25 Poncet-Legrand C, Cabane B, Bautista-Ortin A-B, Carrillo S, Fulcrand H, Pérez J, Vernhet A, Pe J, Vernhet A, Poncet-Legrand C, Cabane B, Bautista-Ortin A-B, Carrillo S, Fulcrand H, Pérez J, Vernhet A, Pe J, and Vernhet A. Tannin Oxidation: Intra- versus

Intermolecular Reactions. *Biomacromolecules* **11**:2376–2386 (2010).

26 Le Bourvellec C, Guyot S, and Renard CMGC. Non-covalent interaction between procyanidins and apple cell wall material: Part I. Effect of some environmental parameters. *Biochim Biophys Acta* **1672**:192–202 (2004).

27 Poncet-Legrand C, Gautier C, Cheynier V, and Imberty A. Interactions between Flavan-3-ols and Poly(L-proline) Studied by Isothermal Titration Calorimetry: Effect of the Tannin Structure. *J Agric Food Chem* **55**:9235–9240 (2007).

28 Bautista-Ortín AB, Cano-Lechuga M, Ruiz-García Y, and Gómez-Plaza E. Interactions between grape skin cell wall material and commercial enological tannins. Practical implications. *Food Chem* **152**:558–565 (2014).

29 Bindon KA, Bacic A, and Kennedy JA. Tissue-specific and developmental modifications of grape cell walls influence the adsorption of proanthocyanidins. *J Agric Food Chem* **60**:9249–9260 (2012).

30 Fulcrand H, Duenas M, Salas E, Cheynier V, Dueñas M, Salas E, Cheynier V, Duenas M, Salas E, and Cheynier V. Phenolic reactions during winemaking and aging. *Am J Enol Vitic* **57**:289–297 (2006).

31 Teng B, Hayasaka Y, Smith PA, and Bindon KA. Effect of Grape Seed and Skin Tannin Molecular Mass and Composition on the Rate of Reaction with Anthocyanin and Subsequent Formation of Polymeric Pigments in the Presence of Acetaldehyde. *J Agric Food Chem* **67**:8938–8949 (2019).

32 Vernhet A, Carrillo S, Rattier A, Verbaere A, Cheynier V, and Nguela JM. Fate of Anthocyanins and Proanthocyanidins during the Alcoholic Fermentation of Thermovinified Red Musts by Different Saccharomyces cerevisiae Strains. *J Agric Food Chem* **68**:3615–3625 (2020).

33 Bautista-Ortín AB, Busse-Valverde N, Fernández-Fernández JI, Gómez-Plaza E, and

Gil-Muñoz R. The extraction kinetics of anthocyanins and proanthocyanidins from grape to wine in three different varieties. *J Int des Sci la Vigne du Vin* **50**:91–100 (2016).

34 Zouid I. Etude de l'évolution et de l'extractibilité des composés phénoliques du raisin en milieu hydroalcoolique pendant la maturation. Lien avec les propriétés mécaniques de la baie. Université d'Angers; 2011.

Tables

Table 1. Sugar concentration, acidity, weight, and repartition between the different compartments of Carignan and Grenache berries; High Performance Liquid Chromatography – Diode Array Detector analysis of phenolic compounds in the skins (mg/g of fresh skin), seeds (mg/g of fresh seeds) of the various raw materials. mDP: mean degree of polymerization; EgC: epigallocatechin Ec-G: epicatechin-gallate. Different letters indicate significant differences between varieties for a given parameter (Tukey's test for $p<0.05$). When there were no significant differences, letters were omitted for sake of clarity. High Performance Size Exclusion Chromatography analyses of phenolic compounds (polymer and oligomer peak) in the skins (mg equivalent epicatechin/g fresh skin) and seeds (mg equivalent epicatechin/g fresh seed) of Carignan and Grenache (vol+deg+ berries).

			Car	Gre
Oenological parameters of the berries		Sugars ($g \cdot L^{-1}$)	212 ± 2a	240 ± 2b
		Total acidity (g $H_2SO_4L^{-1}$)	3.72 ± 0.05	3.61 ± 0.13
		pH	3.54 ± 0.14	3.43 ± 0.01
		Berry weight (g)	2.41 ± 0.00	2.41 ± 0.00
		Skin weight (%)	24.5 ± 6.45	18.07 ± 2.07
		Seeds weight (%)	3.06 ± 0.5	3.03 ± 0.5
		FWIM (%)	7.9 ± 0.2a	9.5 ± 0.6b
Phenolic compounds mg/g of fresh skins or seeds by HPLC-DAD. Results in eq. of malvidine glucoside for anthocyanins.	Skins	Total anthocyanins ($mg \cdot g^{-1}$)	5.4±0.2a	2.0±0.1b
		Total flavanols ($mg \cdot g^{-1}$)	10.2±3.2	11.9±0.9
		mDP	18.2±2.3a	23.7±1.5b
		% EgC	35.9±1.3a	41.9±3.0b
		% Ec-G	3.4±0.7	3.3±0.7
	Seeds	Total flavanols ($mg \cdot g^{-1}$)	30.27±6.54	37.80±7.79
		mDP	5.5±0.1a	4.8±0.2b
		% EgC	n.d	n.d
		% Ec-G	14.0±0.2a	19.0±0.5b
Polymeric and oligomeric flavanols by HPSEC-DAD, in mg eq. epicatechin/g	Skins	Polymeric tannins ($mg \cdot g^{-1}$)	8.8±1.2	10.2±0.9
		Oligomeric tannins ($mg \cdot g^{-1}$)	1.8±1.0	1.3±0.1
	Seeds	Polymeric tannins ($mg \cdot g^{-1}$)	58.5±1.2	61.6±12.5
		Oligomeric tannins ($mg \cdot g^{-1}$)	7.2±0.2	9.4±1.7

Table 2: Concentrations in the wine-like solutions at the end of the maceration experiments. Concentrations in flavanol oligomers and polymers are expressed in equivalent epicatechin and were determined from High Performance Size Exclusion Chromatography experiments. Concentrations in anthocyanins (High Performance Liquid Chromatography – Diode Array Detector) are expressed in equivalent malvidin-3-*O*-glucoside. The % of anthocyanins and tannins extracted during maceration experiments were calculated from the mass of the skin and seed used and their contents in polyphenols in mg/g fresh weight. Different letters indicate significant differences (One-way ANOVA) between varieties for a given parameter (Tukey's test for $p<0.05$). When there were no significant differences, letters were omitted for sake of clarity. Capital letters are for the proportion of polyphenols extracted from the raw material.

			Polymers	Oligomers	Anthocyanins
Carignan	Skins	$g \cdot L^{-1}$	0.67 ± 0.08	0.67± 0.11a	0.384 ± 0.027a
		% extracted	*18.1 ± 2.2*	*88.3 ±14.3A*	*16.9 ±1.5*
	Seeds	$g \cdot L^{-1}$	1.83 ± 0.08	0.33 ± 0.01	-
		% extracted	*62.6* ± 2.6*A*	*91.7 ± 3.7 A*	
	Skins + seeds	$g \cdot L^{-1}$	0.84 ± 0.14	0.55 ± 0.08a	0.291 ± 0.036a
		% extracted	*12.7 ± 2.5*	*49.1 ± 8.9A*	*12.8 ± 1.9*
	Skins + FWIM	$g \cdot L^{-1}$	0.55 ± 0.01a	0.61 ±0.04a	0.291 ±0.010a
		% extracted	*14.8 ± 0.3A*	*80.5 ± 4.8A*	*12.8 ± 0.4*
	Skins + seeds + FWIM	$g \cdot L^{-1}$	0.83 ± 0.02	0.66 ±0.02a	0.272 ±0.045a
		% extracted	*12.4 ± 0.3*	*59.0 ± 1.5A*	*12.0 ± 2.0*
Grenache	Skins	$g \cdot L^{-1}$	0.68 ± 0.06	0.29 ±0.01b	0.165 ± 0.007b
		% extracted	*15.8 ± 1.4*	*52.3 ± 1.7B*	*17.6 ± 3*
	Seeds	$g \cdot L^{-1}$	1.62 ± 0.12	0.34 ± 0.05	-
		% extracted	*52.7 ± 3.9B*	*71.7 ±8.8B*	
	Skins + seeds	$g \cdot L^{-1}$	0.90 ± 0.04	0.34 ± 0.01b	0.142 ± 0.017b
		% extracted	*12.3 ± 0.6*	*33.7 ± 1.3B*	*16.8 ± 2.0*
	Skins + FWIM	$g \cdot L^{-1}$	0.46 ± 0.00b	0.25 ±0.01b	0.129 ± 0.013b
		% extracted	*10.7 ± 0.0B*	*45.9 ± 1.4B*	*15.3 ± 1.5*
	Skins + seeds + FWIM	$g \cdot L^{-1}$	0.81 ± 0.13	0.40 ±0.02b	0.105±0.024b
		% extracted	*10.9 ± 1.8*	*39.5 ± 1.5B*	*12.5 ± 2.8*

Table 3: High Performance Liquid Chromatography –Diode Array Detector analysis of tannins after depolymerization in the wine-like solution at the end of the maceration experiment with skins. seeds. skins+ seeds and skins+seeds+FWIM. mDP: mean degree of polymerization; EgC: epigallocatechin Ec-G :epicatechin-gallate. Different letters indicate significant differences (One-way ANOVA, followed by Tukey's test for $p<0.05$) between varieties for a given parameter (for instance DPm of Carignan raw skins compared with DPm of Grenache raw skins). When there were no significant differences, letters were omitted for sake of clarity.

		DPm	% Ec-G	% EgC
Carignan	Raw skins	18.2±2.3a	3.4±0.7	35.9±1.3a
	Raw seeds	5.5±0.1a	14.0±0.2a	0
	maceration - skins	6.4±0.9	7.3±0.4a	25.2±6.0
	maceration - seeds	5.0±0.3a	11.5±1.3	0
	Maceration of skins + seeds	4.9±0.3	5.0±0.8a	9.4±0.3a
	Maceration skins + seeds+ FWIM	4.2±0.4	5.0±1.1	5.3±2.5
Grenache	Raw skin	23.7±1.5b	3.3±0.7	41.9±3.0b
	Raw seed	4.8±0.2b	19.0±0.5b	0
	maceration - skins	7.9±0.5	4.9±0.8b	28.4±1.9
	maceration - seeds	3.8±0.1b	13.3±0.3	0
	Maceration of skins + seeds	4.9±0.5	7.3±0.6b	5.7±1.9b
	Maceration of skins + seeds+ FWIM	5.1±0.9	3.9±1.8	7.1±0.2

Figures

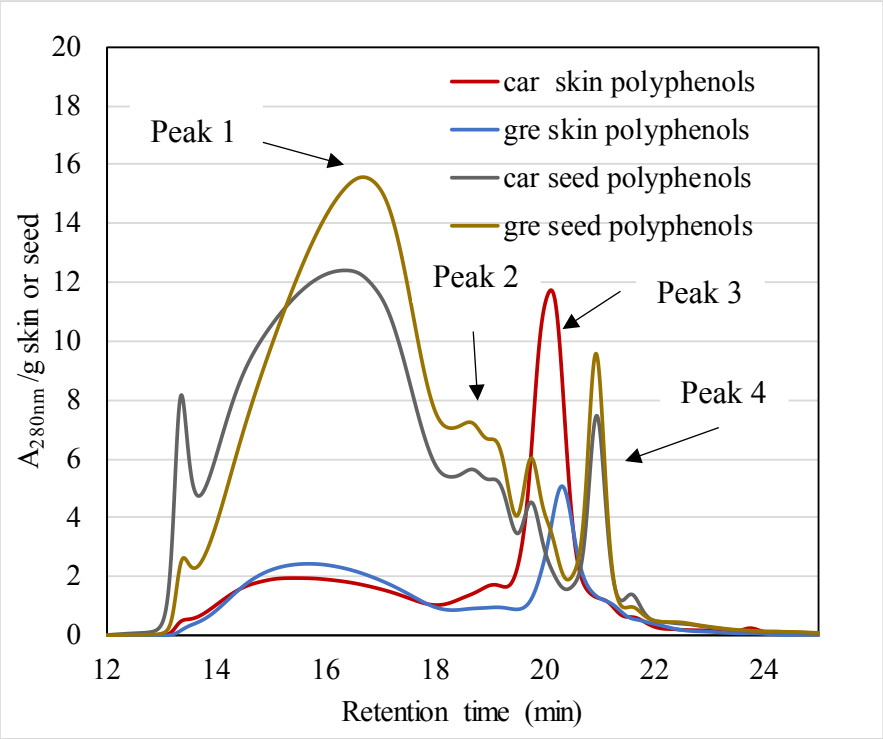

Figure 1. Analysis of the skin and seed polyphenols size distribution of Carignan (car) and Grenache (gre) grape berries by High Performance Size Exclusion Chromatography. Peak 1: polymers; Peak 2: oligomers; Peak 3: monomers (anthocyanins. epicatechin-gallate); Peak 4: monomers (catechin/epicatechin).

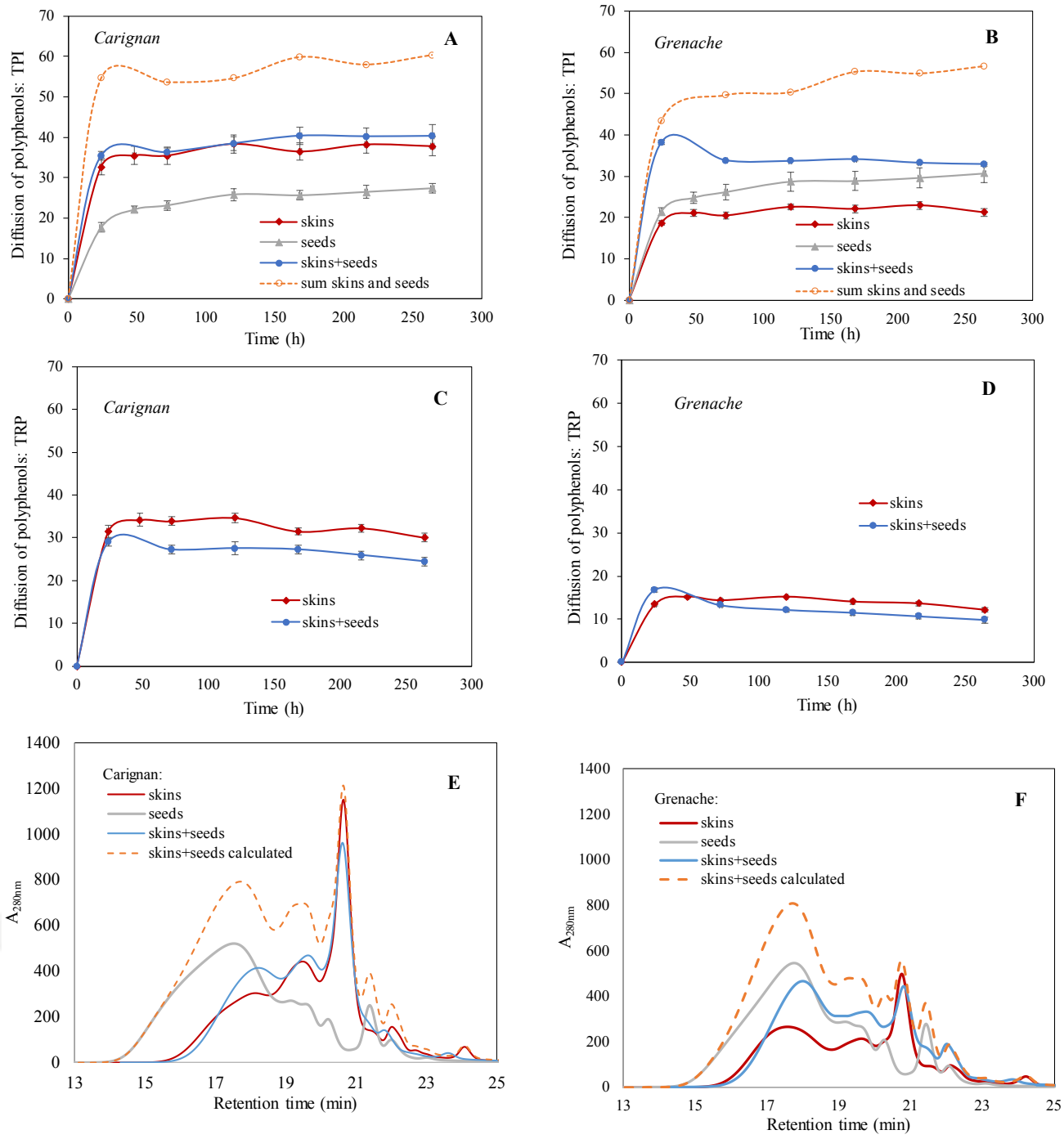

Figure 2. Polyphenols extraction from berries different compartments (skins; seeds; skins+seeds) during wine-like model maceration experiments for Carignan and Grenache. The total polyphenol Index (TPI) and total red pigments (TRP) were determined by UV–visible spectrophotometry at 280 and 520 nm (1 cm path length) after adequate dilution in HCl 1 M and are the absorbany multiplied by the dilution factor. A) car TPI. B) gre TPI. C) car TRP. D) gre TRP. E) and F) High Performance Size Exclusion Chromatography chromatograms at t5 (15%EtOH. 264h): E) carignan. F) Grenache, peaks 1,2,3,4 as in Figure 1.

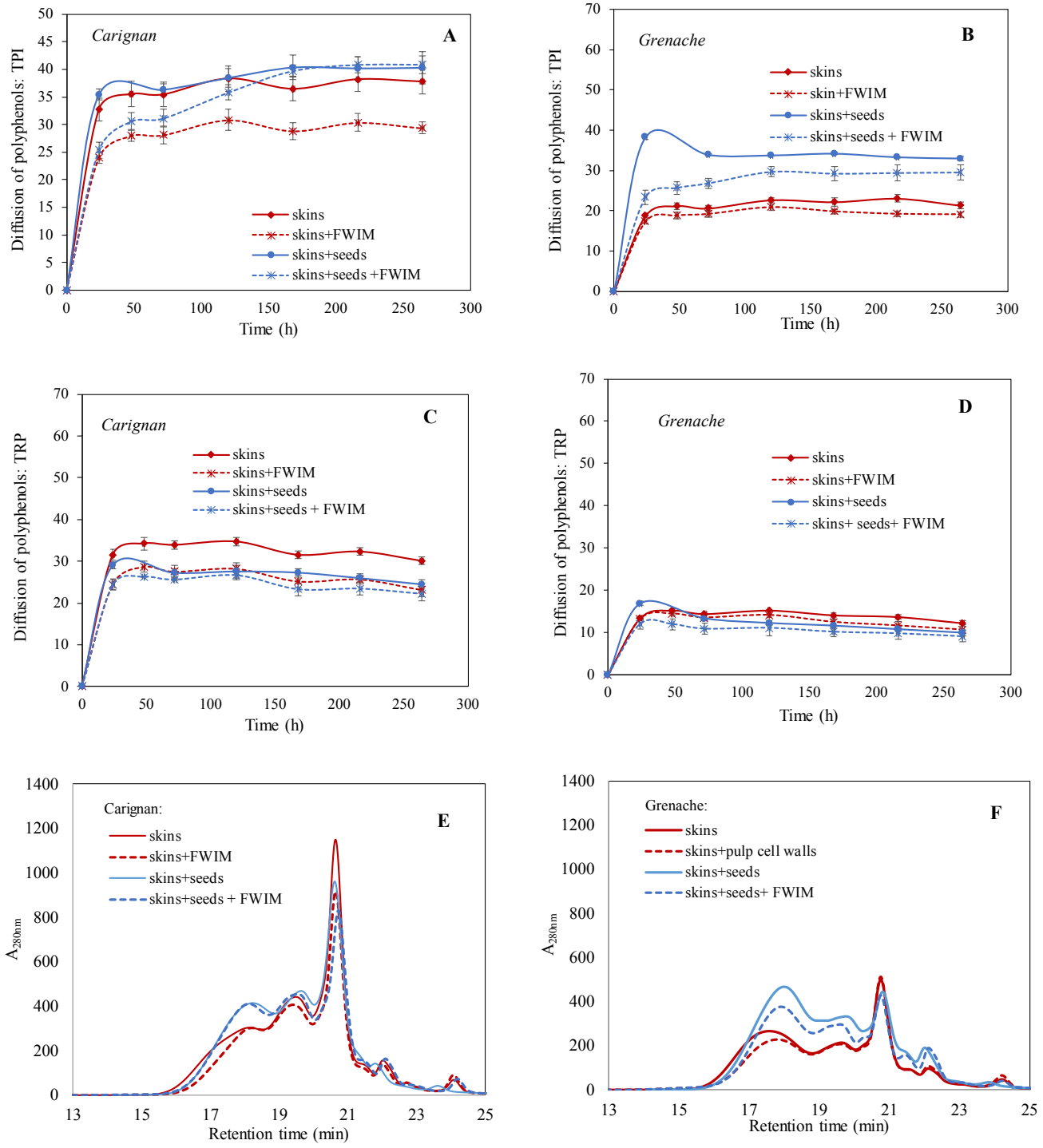

Figure 3. Comparison of polyphenols extraction from the berries different compartments (skin. skins + seeds) with and without Flesh Water Insoluble Material (FWIM) during wine-like model maceration experiments of Carignan and Grenache. The total polyphenol Index (TPI) and total red pigments (TRP) were determined by UV–visible spectrophotometry at 280 and 520 nm (1 cm path length) after adequate dilution in HCl 1 M and are the absorbany multiplied by the dilution factor.A) and B) TPI. C) and D) TRP. E) and F) Size Exclusion Chromatography Chromatograms, peaks 1,2,3,4 as in Figure 1.

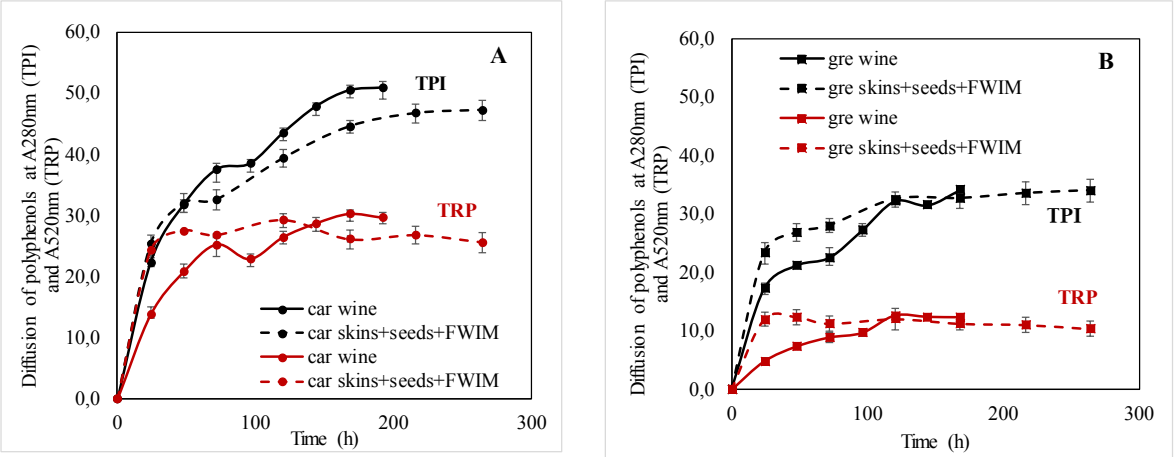

Figure 4. Comparison between the kinetics of phenolic diffusion (TPI and TRP) from whole grape berries in winemaking and wine-like model maceration experiment in A) Carignan and B) Grenache, during the 10 days of experiments.

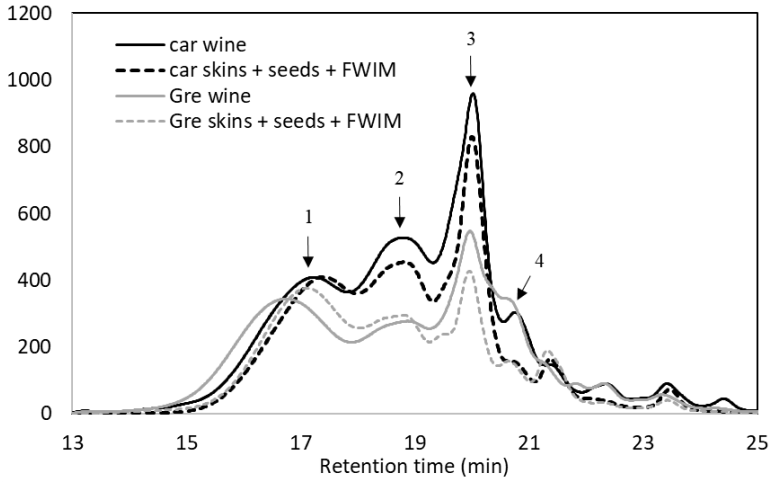

Figure 5. Comparison of the High Performance Size Exclusion Chromatography chromatograms of wines and model maceration experiments (Skins + Seeds + FWIM). Carignan Wine: black line, Carignan model maceration experiment: black dotted line, Grenache model maceration experiment grey dotted line. Peaks 1, 2, 3, and 4 as in Figure 1.