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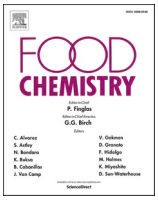

Focus on the relationships between the cell wall composition in the extraction of anthocyanins and tannins from grape berries

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ABSTRACT

Concentrations of anthocyanins and tannins after extraction from berries in wines and from skin macerations in model solutions have been studied for two grape varieties, two maturation levels and two vintages berries. Characterization of the cell wall polysaccharides has also been performed, the classical method based on the analysis of the neutral sugars after depolymerization being completed by a comprehensive microarray polymer profiling (CoMPP). Extraction was lower in model solutions than in wines, with the same ranking: non acylated anthocyanins> tannins > p-coumaroylated anthocyanins. The polysaccharidic composition suggested a role of homogalacturonans, rhamnogalacturonans and extensins in the extraction process. A global explanation of the interactions between anthocyanins, tannins and polysaccharides is proposed.

1. Introduction

Polyphenols are secondary metabolites of the grape berries that play a major role in the wine sensory properties. The two most important families of polyphenols are anthocyanins and condensed tannins, i.e. oligomers and polymers of flavan-3-ols, since they contribute to the color and to the astringency of red wines, respectively. Extraction of anthocyanins and tannins is a natural process which begins when the grape berries are crushed and continue throughout maceration until pressing. However, the extraction is far from complete and no clear relationship has been established between the berry composition and the wine concentration in polyphenols (Harbertson, Kennedy, & Adams, 2002). Specific processes have been developed to increase the extraction rate, e.g. using enzymes, heating, vacuum (Sacchi, Bisson, & Adams, 2005; Morel-Salmi, Souquet, Bes, & Cheynier, 2006). Nevertheless, a large part of the anthocyanins and tannins remain trapped in the pomace. Varietal differences in the extraction rates have been related to berry size, i.e. skin to juice ratio, skin composition and/or cell wall structure (Cheynier et al., 2006; Bindon, Kassara, & Smith, 2017; Abi-Habib et al., 2021) but the underlying mechanisms are still poorly understood. Thus more knowledge about the factors that influence polyphenol extraction from grape during wine-making is necessary.

The major grape anthocyanins are 3-glucosides of five aglycones: malvidin, cyanidin, delphinidin, peonidin, petunidin. These anthocyanins can be acylated with acetic, caffeic and p-coumaric acids. In most grape varieties, the two main families of anthocyanins are the non acylated anthocyanins, and the p-coumaroylated anthocyanins. Tannins are flavan-3-ol oligomers and polymers issued from four major

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monomeric units: catechin, epicatechin, epigallocatechin and epicatechin gallate. Seed tannins do not contain epigallocatechin units while skin tannins are poorer in epicatechin gallate units (Cheynier et al., 2006).

Grape berries consist in three parts: the skin, the pulp or mesocarp and the seeds. Anthocyanins are located in the skins, except in the teinturier cultivars, whereas tannins are located mainly in the seeds and the skins. Within a cell, anthocyanins and tannins accumulate in the vacuole (Amrani-Joutei, Glories, & Mercier, 1994; Kennedy, Hayasaka, Vidal, Waters, & Jones, 2001). Thus, the release of anthocyanins and tannins depends on the integrity of the vacuole membrane, the tonoplast, and of the cell walls which have a key role as they appear to limit the extraction of polyphenols by binding them (Amrani-Joutei et al., 1994).

The structure of berry cell walls has been detailled elsewhere (Hanlin, Hrmova, Harbertson, & Downey, 2010; Gao, Zietsman, Vivier, & Moore, 2019). Primary cell walls are composed of linear cellulose microfibrils linked together by hemicelluloses such as xyloglucans, through hydrogen and certainly covalent bonds (Hanlin et al., 2010). The 3-dimensional network of cellulose-hemicellulose is filled with pectins composed of linear domains such as homogalacturonans and branched domains such as rhamnogalacturonans. Pectins are linked together by extensins which are glycoproteins. Galacturonic acids of homogalacturonans are highly methylated. However, de-esterification which occurs during maturation (Garrido-Banuelos et al., 2019; Gao, Fangel, Willats, Vivier, & Moore, 2021) releases COO^- functions which allow Ca^{2+} bridges between homogalacturonans (Hanlin et al., 2010). As a consequence, the texture becomes less rigid and evolves towards a gel. Secondary cell walls are thicker than primary cell walls and contain lignin and more cellulose. They have been evidenced in seeds. Skins may also contain lignin (Yang, Wang, Tan, Fu, & Sun, 2019) but in so low concentrations that their presence in skins has long been considered as unlikely (Hanlin et al., 2010). Moreover, seeds remain intact while skins are disrupted during vinification. Thus, extraction of polyphenols is expected to be more difficult from seeds than from skins.

When cell walls are disrupted, the extraction of polyphenols from the vacuole is very fast (Hanlin et al., 2010). But simultaneously, two phenomena occur: (1) binding on the cell walls, due to hydrogen bonding and hydrophobic interactions, and (2) chemical modifications. Both can contribute to the observed losses in anthocyanins and tannins, i.e. the difference between the quantities measured in berries and the quantities measured in wines, lees, and pomaces (Abi-Habib et al., 2021).

Binding between polyphenols and polysaccharides has been extensively studied. Proanthocyanidins, also called tannins, have drawn most of the attention. Both the binding capacity of the cell walls and the extractability of polyphenols increase during maturation (Gao et al., 2019). These binding properties would be due to linear pectins or homogalacturonans that represent around 40% of the cell wall composition, and to branched pectins or rhamnogalacturonans (Gao et al., 2019). Highest affinities were observed between highly methylated linear pectins (Gao et al., 2019; Watrelot & Norton, 2020; Liu, Renard, Rolland-Sabate, & Le Bourvellec, 2021) and tannins with high degrees of polymerization (Watrelot, Le Bourvellec, Imberty, & Renard, 2013; Watrelot & Norton, 2020). On the other hand, tannins with DP 9 had no affinity with pectins showing a low degree of methylation (Watrelot et al., 2013). Arabinans, which are one of the main side chains of branched pectins, present a higher binding affinity with highly polymerized tannins (Fernandes et al., 2020). The same authors suggest that low branched rhamnogalacturonans would allow stacking of other polyphenols, increasing the overall binding. On the whole, tannins can be bound up to 70% on cell wall polysaccharides (Hanlin et al., 2010; Abi-Habib et al., 2022). Both homogalacturonans and rhamnogalacturonans are concerned, e.g. in apple 62% of tannins were bound on homogalacturonans and 38% on RGI (Fernandes et al., 2020). Globally, anthocyanins are less bound than tannins (Abi-Habib et al., 2022). However, important differences exist between non acylated and p-coumaroylated anthocyanins. Coumaroylated anthocyanins have a higher affinity for grape cell walls than non acylated anthocyanins (Goncalves, Rocha, & Coimbra, 2012; Abi-Habib et al., 2022). Cyanidin-3-glucoside, a non acylated anthocyanin, had a higher affinity with less methylated pectins (Fernandes et al., 2020).

Enzymes, through their impact on cell wall structure and composition, play a role in such mechanism. Both pectinases (pectine-methyl esterase or PME, pectine lyase and polygalacturonase), cellulases and xylanases, when added to cell walls and tannins in solution, decreased the binding of polyphenols, the lowest binding being observed with polygalacturonase and pectine-lyase (Osete-Alcaraz et al., 2021). However, in berries, pectinases, and particularily PMEs, are the most important enzymes, whereas cellulases are missing (Nunan, Davies, Robinson, & Fincher, 2001; Barnavon et al., 2001). By decreasing the methylation rate of the pectins, PMEs contribute to weaker bonds between pectins and tannins. Enzymes still contribute to the degradation of the cell walls even after crushing of berries and binding of polyphenols, releases of soluble polysaccharides have been observed mainly with pectinases and cellulases (Osete-Alcaraz et al., 2021).

Extractions of anthocyanins and tannins from the skins, which implies unbinding, occur mainly during alcoholic fermentation. It is triggered by temperature and ethanol increase (Medina-Plaza et al., 2019), a gradient of concentration between skins or pulps and juice (Bindon et al., 2017), and depends on the chemical structure of the cell walls. Anthocyanins increase the quantities of extracted tannins (Kilmister, Mazza, Baker, Faulkner, & Downey, 2014; Bindon et al., 2017), but the mechanism involved remains unclear. Chemical modifications also contribute to the explaination of losses of anthocyanins and tannins. During wine making, anthocyanins may be degraded into lower molecular weight compounds, converted to pyranoanthocyanins or involved in reactions with tannins, leading to the formation of adducts, e.g. pigmented tannin-anthocyanin and tannin-ethyl-anthocyanin or colorless anthocyanin-tannin (Cheynier et al., 2006). The incorporation of anthocyanins within tannins begins very early, as soon as anthocyanins and tannins are released from the vacuole (Campbell, Grosnickel, Kennedy, & Waterhouse, 2021). Similarly, tannins can react with other polyphenols, e.g. other tannins, anthocyanins, phenolic acids, especially under oxidative conditions. These derived polyphenols are only partly quantified by depolymerization methods such as thiolysis or phloroglucinolysis, and therefore are considered as losses. Over 50% losses have been observed for anthocyanins and tannins after extraction from skins in model solutions (Abi-Habib et al., 2022), but little is known about the respective parts of irreversible binding vs. other losses induced by the chemical modifications.

The composition of polymers such as polyphenols and polysaccharides is usually determined by chemical analysis of their constitutive units after a depolymerization step. The different polysaccharide families can be quantified accurately, but without more information on their reactivity or physico-chemical properties. The comprehensive microarray polymer profiling (CoMPP) technique is a method using cell wall probes, e.g. antibodies, to follow cell wall derived polymers, mainly polysaccharides. This method is based on the reaction between an antibody and an epitope, a structural part of a polysaccharide specific of each antibody. The signal reflects the epitope abundance which is accessible to the antibody. Compared to the chemical composition, it provides an enriched series of datasets to assess the conditions of the cell walls, depending on ripening, variety, etc. Recent studies, using the technique of CoMPP together with classical analytical methods, confirmed the development pattern of berry cell wall changes, during grape ripening, at the polymer level (Gao et al., 2021; Abi-Habib et al., 2022).

2. Hypothesis

The objective of the present work was to explore the relationships between polyphenol extraction and berry cell wall composition,

determined by compositional analysis of sugars and CoMPPs, during maceration of grape skins in wine-like model systems and during wine-making. This was performed on two cultivars, Grenache and Carignan, that show large differences in terms of anthocyanin-to-tannin ratios and of anthocyanin and tannin extraction rates, over two vintages. Further variability was achieved by sorting the berries by size and/or sugar level. The different modalities of the experimental design such as grape variety or level of maturation were considered as factors of variability, and processed together.

The novelty is the complementarity between two extraction processes, model solution and wine, combined with a characterization as complete as possible of the anthocyanins, tannins and cell wall polysaccharides including CoMPPs. The underlying hypothesis was that our results, combined with the bibliography, would lead to propose a general frame explaining what occurs in cell walls as soon as anthocyanins and tannins are released.

3. Material and methods

3.1. Sampling

Two *Vitis vinifera* red grape varieties, Grenache noir and Carignan, were harvested from vineyards of the INRAE experimental domain of Pech Rouge, Gruissan, France at an average potential alcohol of 12% vol., in 2018 and 2019. The berries were sorted as described earlier (Abi-Habib et al., 2021) then split into homogeneous groups. Two criteria were selected. The first one was the volume of the berries, low or high, noted V- or V+, respectively. The threshold was a diameter of 1.4 cm. The second one was the sugar content, yielding low or high alcoholic degrees, noted D- or D+, respectively. In 2018 the two criteria were applied, so four groups were obtained: V-D-, V-D+, V + D- and V + D. These first results showed no major influence of the berries volumes on their composition, so the protocol was simplified in 2019 with only two groups: D- and D+.

Grape skins were separated from 30 berries with a scalpel and immediately used for diffusion experiments. Skins, seeds and pulps were separated from other samples (30 berries) with a scalpel and immediately frozen in liquid nitrogen then stored at −80°C for later analysis of their composition. Sample preparation and analysis were performed in triplicate.

3.2. Analytical methods

The experimental design for each sampling is detailed in supplementary Fig. 4. Five types of materials were obtained, namely polysaccharide and polyphenol extracts from berries (skins, seeds and/or pulps), plus model solutions and wines after microvinification. The polysaccharide extracts were named hereafter alcohol insoluble cell wall solids (AISs) as previously used (Abi-Habib et al., 2021), a synonym of alcohol insoluble residues (AIR)(Gao et al., 2021).

- Material 1: AISs for CoMPP. The AISs of Carignan and Grenache were isolated using the following procedure, as the optimal one for CoMPP technology (Nguema-Ona et al., 2012; Moore et al., 2014). The frozen ground flesh (10 g) was incubated in 100% v/v absolute ethanol at 80 °C for 15 mn to deactivate endogeneous enzymes. After centrifugation, the pellets were washed sequentially by a series of solvents (ethanol, methanol, chloroform, acetone) using a stirring plate. Thereafter, the pellets were suspended in deionized water and freeze-dried to yield dry powder of flesh cell wall materials, which were used for structural composition analysis (CoMPP).
- Material 2: AISs for neutral sugars composition. The procedure was different in 2018 and 2019. In 2018 AISs were extracted at 40 °C with two cycles of 96% ethanol, then one cycle of 70% ethanol, in an ultrasound bath, using the procedure described previously (Abi-Habib et al., 2021). In 2019 AISs were extracted at 100 °C with two cycles of ethanol 80%, using a Thermo ASE350 accelerated solvent extractor (Lahaye et al., 2021).
- Material 3: Polyphenolic extracts. The extraction procedure was adapted from Mane et al., 2007. Frozen skins and seeds from 30 berries (3 replicates) were ground to a fine powder in liquid nitrogen with a Mortar Grinder Pulverisette 2 (Fritsch, Idar-Oberstein, Germany). The powder (150 mg) was added first with methanol (750 µL), then extracted with 5.25 mL of 60/40/1 (v/v/v) acetone–water-formic acid at room temperature. The mixture was crushed with Precellys (Bertin Technologies, Montigny-leBretonneux, France) during three cycles (3 x 40 s each). Both extracts were pooled. After centrifugation with a Heraeus Multifuge X3R Centrifuge (Thermo-Fischer Scientific, Waltham, USA) (21320 g, 5 min, 4 °C), aliquots (1 mL) of the supernatant were dried under reduced pressure at 35 °C, using a Genevac (SP Scientific, Warminster, PA, USA).
- Material 4: Extractions from skins in model solutions. Three replicates of fresh skins from 30 berries were weighted and immediately immersed in 42 mL of a model solution containing 3 g/L tartaric acid, 50 mM NaCl and 40 mg/L SO_2, at pH 3.5 adjusted with NaOH. This volume of 42 mL was chosen from the data obtained by grape sampling (average weight of berries, skins and microvinifications of 900 g of berries). These averages slightly differed depending on the berry size, and the volume varied between 28–31 mL (V-) and 38–44 mL (V+). The chosen value of 42 mL for all samples corresponded to the V + modalities and the highest liquid-to-solid ratios. Simulated maceration experiments were carried out by increasing stepwise the ethanol content from 0 to 15% as described in Abi-Habib et al., 2021. All experiments were performed in triplicate. Flasks were placed under argon and gently stirred in the dark at 22 °C. Polyphenol diffusion was followed during 11 days by measuring the total polyphenolic index (TPI) and total red pigments (TP) daily, as well as the HPLC-DAD and HPSEC profiles of samples taken and centrifuged (15000 g, 15 mn, 15 °C) at the end of each ethanol increase step. TP and TPI were measured as absorbance at 520 nm and 280 nm after a 100-fold dilution in 1 M hydrochloric acid, in a 10 mm cuvette. To account from differences in initial fresh skin mass, depending on the modality, results were divided by the initial mass and reduced to 1 g fresh skin for all modalities.
- Material 5: Vinification of whole berries Fermentations were carried out in triplicate at 21 °C in a thermostatically controlled chamber, in low volume tanks (<1 kg) using French press coffee plungers with similar and standard conditions. Berries (900 g) were crushed and placed in the plunger. Lalvin ICV OK yeast (20 g/hl) and SO_2 (250 µL of a 8% solution) were added simultaneously. Cap management was carried out daily during ahcoholic fermentation by submerging the pomace with the plunger.The fermentations, which lasted 7–8 days, were considered finished with densities below 995 g/L. At the end of the alcoholic fermentation, some classical parameters of the wine such as alcoholic degree or total acidity were determined. TP and TPI were measured at the beginning (T0) and at the end (T3) of alcoholic fermentation. An UV–vis spectrophotometer was used with three replicates at each time of estimation.

The following analytical methods were applied to the previous materials.

- Comprehensive microarray polymer profiling (CoMPP) extracts from material 1. Samples were sequentially extracted first with 50 mM CDTA (cyclo-hexane-diamino-tetra-acetic acid pH 7.5) and then with NaOH-4 M (Moller et al., 2007) to obtain the pectin and hemicellulose rich fraction, CDTA fractions and NaOH fractions, respectively. After centrifugation, the extracts from each fraction were printed onto a nitrocellulose membrane and then probed with a series of 27 monoclonal antibodies (mAbs) plus a carbohydrate-binding module (CBM). The raw data were normalized, and the relative abundance of different polymer epitopes were displayed on a scale of 0–100 with a

cut-off value of 5 on the raw data (Moller et al., 2007). The values were the means of the three biological repetitions and four dilutions. By convention, the units were noted hereafter au/mgAIS, to recall that the arbitrary units of the CoMPPs reffer to given weights of AISs.

- Determination of neutral sugar compositions from material 2. The neutral glycosyl-residue composition of the AISs was determined according to the Saeman procedure, by gas chromatography after polysaccharide prehydrolysis (H_2SO_4, 72%, 30 mn, 30 °C) then hydrolysis after dilute by Milli-Q water (2 h, 100 °C) and neutral sugar conversion in their alditol acetate derivatives (Hoebler, Barry, David, & Delort-Laval, 1989; Lahaye et al., 2021).
- Polyphenols by MRM from material 3 and material 5. Anthocyanins, flavan-3-ol monomers, dimers and trimers were determined by UHPLC-QqQ-MS in the MRM mode, using a method adapted from those previously described (Pinasseau et al., 2017), with an Acquity UPLC system hyphenated to a triple quadrupole (QqQ) TQD mass spectrometer equipped with a reversed-phase Acquity HSS T3 1.8 µm 1.0 × 100 mm column (Waters, Saint Quentin en Yvelines, France). Tannin composition was determined by analysis of the flavan-3-ol constitutive units released after phloroglucinolysis using UHPLC-QqQ-MS in the MRM mode as described earlier (Pinasseau et al., 2016).
- Polyphenols by HPLC from material 3, material 4 and material 5. Dried extracts were redissolved in a wine-like solution (ethanol 12%, 3 g/L tartaric acid, 50 mmol/L NaCl, 40 mg/L SO_2, pH 3.5) for HPLC-DAD analysis of anthocyanins in model solutions (Abi-Habib et al., 2021), results expressed in equivalent of malvidin-3-glucoside. The size distributions and concentrations of polymeric tannins, expressed in equivalent of epicatechin, were determined by HPSEC in dimethylformamide with 1% acetic acid (v/v), 5% water (v/v), 0.15 M lithium chloride, at 60 °C and 1 mL/mn, injected in an Agilent HPLC 1260 Infinity II system equipped with a diode array detector (DAD) and Phenogel columns connected in series (Abi-Habib et al., 2021). The columns were calibrated with regards to the polyphenol elution times using commercial standards (catechin, epicatechin, dimer B2 and trimer C1) along with tannin fractions of different degrees of polymerization, results expressed in equivalent of epicatechin.

3.3. Processings

A few unit conversions were mandatory for a quantitative comparison of anthocyanins and tannins analyzed in berry, model solution, wine. New units with their calculation workflow have been reported in supplementary Table 4. Then, extraction rates from berries to model solution or from berries to wines were calculated.

Data analysis was based on standardized principal component analysis (PCA) and on correlation coefficients. Models were also built with supervised multivariate methods, but the results, not concluding, were not presented.

4. Results

All data, raw or after unit conversion, were merged in a single dataset T0 of dimensions (12 × 294), 12 observations and 294 variables. The separation of the berries in two classes, D + and D-, intended to obtain two different levels of maturity. From supplementary Table 5 extracted from T0, this goal has been achieved. For each variety and each year, the wines from the D + modalities presented a higher alcoholic content, same or lower total acidity, more color and polyphenols, than the D-modality. From T0, two datasets were extracted, T1 and T2, each having a different purpose. T1 of dimensions (12 × 18) was focused on the comparison of the quantities of anthocyanins and tannins in berries, in model solutions and in wines. All results were expressed in mg/L, calculated according to supplementary Table 4. T2 of dimensions (12 × 91) was dedicated to the explanation of the extraction of anthocyanins and tannins in model solutions and wines, using variables concerning the CoMPP results from skins (19) and pulps (30) in arbitrary units, au/mg AIS, the acidic sugars from skins and in pulps (2) in mg/g AIS, the neutral sugars of the polysaccharides from skins(7) and pulp (7), in %, anthocyanins and tannins in skins, seeds, berries (14), in µg/g berry, anthocyanins and tannins in model solutions (4), in µg/g berry, anthocyanins and tannins in wines (8), in µg/g berry.

The results are presented in three parts: (1) an overview of anthocyanins and tannins in berries, model solutions and wines, with selection of variables of interest, based on T1; (2) the polysaccharidic composition of the cell walls, based on T2; and (3) an explanation of the variables of interest using explanatory variables issued from berry analysis, based on T2.

Correlations are widely used. But correlation does not imply causation. The causation, the berry composition, was determined by the experimental design (year, variety, maturation) with consequences on any of its element, skins, pulps or seeds. This explains why, for example, correlations between pulp CoMPPs on one hand, and polyphenols extractions in model solutions performed without pulps on the other hand, were presented and discussed.

4.1. Anthocyanins and tannins in berries, model solutions and wines

A standardized PCA summarized the T1 table, with scores and correlation circle represented in supplementary figure S5. Scores 1 and 2 confirmed the differences between Carignan and Grenache, showing also an effect of the year. However, such interpretation has already been developped in previous papers (Abi-Habib et al., 2021; Abi-Habib et al., 2022) and will not be detailled any more. This part is dedicated to the selection of variables of interest, and to a focus on the extraction of anthocyanins and tannins in model solutions and in wines.

4.1.1. Selection of variables of interest (anthocyanins, tannins)

Many variables are relative to the quantification of anthocyanins or tannins. We wish to select the most interesting ones, for further processsings in part 3. Anthocyanins were all well correlated to the first dimension in the correlation circle in supplementary figure S5. Nevertheless, from our previous results and the bibliography concerning the difficulties of extracting p-coumaroylated anthocyanins, both the non acylated and the p-coumaroylated anthocyanins were selected. Concerning tannins, the variables related to MRM analysis were opposite on PC1 with the variables related to HPSEC. MRM would be more accurate on anthocyanins and native tannins, but HPSEC would be more robust on evolved tannins which may be present in large amount in model solutions and wines. Finally, four variables of interest were selected to represent anthocyanins and tannins: the non acylated anthocyanins, the p-coumaroylated anthocyanins, the total anthocyanins, plus the total tannins which is the sum of the monomeric, oligomeric and polymeric tannins. All four were quantified in model solutions and in wines with their corresponding analytical method, MRM or HPLC-DAD for anthocyanins, HPSEC for polymeric polyphenols.

4.1.2. Extractions of non acylated anthocyanins, p-coumaroylated anthocyanins and tannins in model solutions and wines

Correlations between non acylated and p-coumaroylated anthocyanins were illustrated in Fig. 1 for berries, model solutions and wines. Mean values were also gathered in Table 1. Non acylated and p-coumaroylated anthocyanins represented most of the anthocyanins in grape berries of Carignan and Grenache. Their values showed differences between varieties, years and maturities. The main effect was the variety, i.e. Carignan berries were richer than Grenache berries, Fig. 1-a. Lower variations were observed for the year and the maturity, p-coumaroylated and non acylated anthocyanins having opposite behaviours. The p-coumaroylated anthocyanins were mainly impacted by the year, with higher quantities in 2019 than in 2018, whereas non acylated anthocyanins increased with the maturity, but not with the year, Fig. 1-a. When comparing to their concentrations in model solutions, Fig. 1-b, -b,

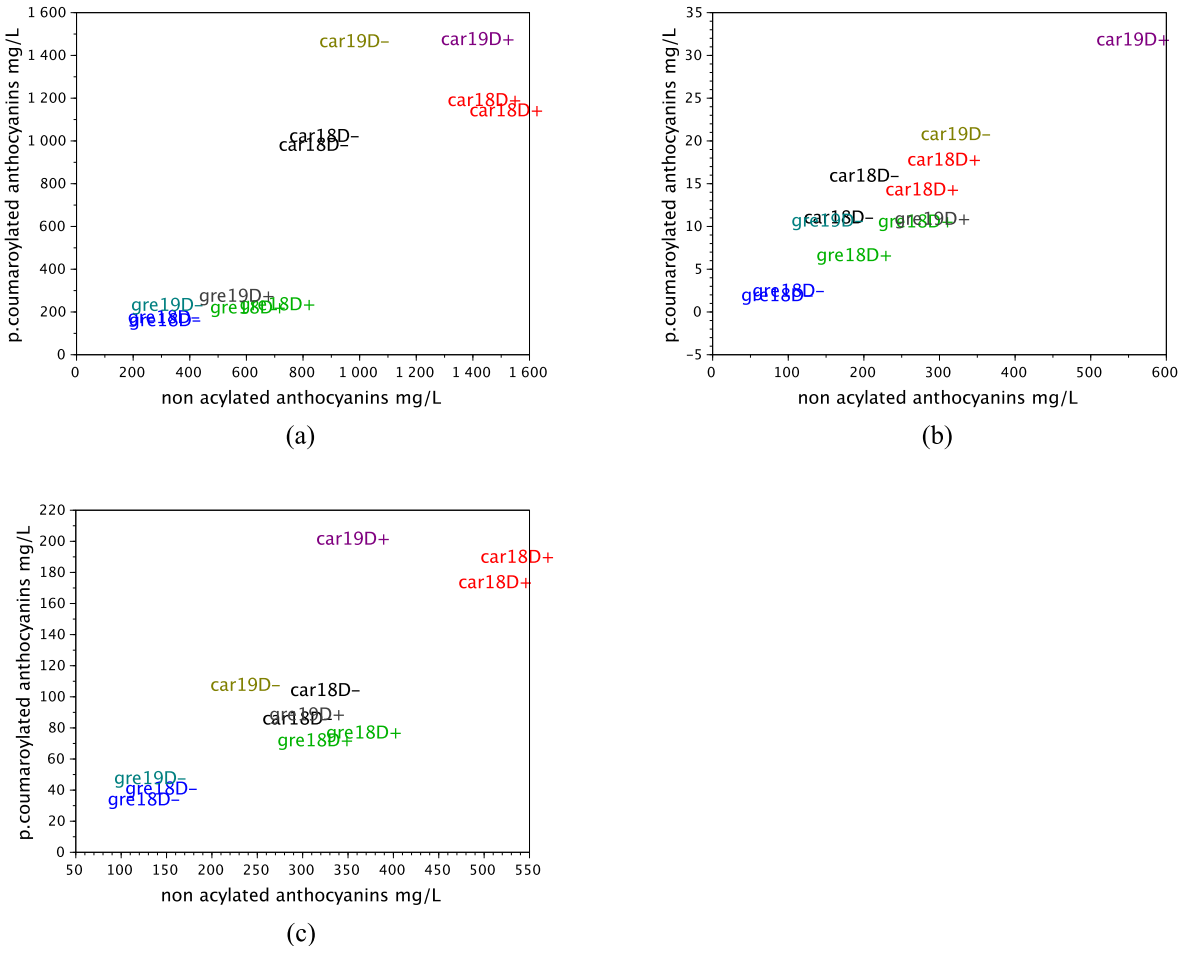

Fig. 1. Non acylated vs. p-coumaroylated anthocyanins in berry skins (a), in model solution after extraction from skins (b) and in wines (c). The 2 repetitions observed for 2018 correspond to the V + and V- modalities.

Table 1
Averages of the extraction of anthocyanins and tannins in model solution and in wine for the Carignan and Grenache varieties, 2018 and 2019 vintages. m = MRM, s = SEC, d = DAD

	Berries	Model sol.	Wine	Extraction		
	mg/L	mg/L	mg/L	model sol.	wine	difference
non acylated anthocyanins	692 m	193 d	262 m	28%	38%	10%
p.coumaroyl anthocyanins	652 m	11 d	93 m	2%	14%	12%
total anthocyanins	1397 d	210 d	344 d	15%	25%	9%
	1345 m		377 m		28%	
tannins, total	8022 s		1345 s		17%	
	6236 m		547 m			
- from skins	3906 s 3325 m	726 s		19%		
- from seeds	4117 s 2911 m					

between a quarter and half of the non acylated, but only a few percent of the p-coumaroylated anthocyanins, had been extracted. Thus, there was a real problem of extractibility of the p-coumaroylated anthocyanins in model solutions, previously reported on the same dataset for 2018 (Abi-Habib et al., 2021) and latter explained by a higher adsorption with lower reversibility (Abi-Habib et al., 2022). It should also be noted that the values align along a straight line, with a ratio p-coumaroylated/ non acylated around 1/20 and a correlation coefficient of 0.94. Anthocyanins were more extracted in wines than in model solutions, Fig. 1-c and Table 1. The increase was sharp for the p-coumaroylated anthocyanins, with extraction rates of 2% in model solutions compared to 14% in wines.

The relationships between tannins and anthocyanins are illustrated by Table 1 and Fig. 2 for berries, model solutions and wines. Tannin and anthocyanin concentrations in berries can separate clearly the two varieties, Carignan and Grenache, and the two years, 2018 and 2019, Fig. 2-a,b. From Table 1, extraction rates for tannins were in-between non acetylated and p-coumaroylated anthocyanins. Extractions of anthocyanins were higher in wines than in model solutions. This means that an additional mechanism concerning wines but not model solutions improved the anthocyanin extraction. On the other hand, extractions of tannins were comparable in wines and in model solutions, 17 vs. 19%. Wines tannins originated from skins and seeds, model solution tannins from skins only. So, extraction of skin tannins was higher in model solution than in wine.

Nevertheless, even with different extraction rates, non acylated and p-coumaroylated anthocyanins both exhibited an alignment with tannins which is visible in model solutions and is more clearly observed in wines, Fig. 2-c,d,e,f. These results suggest that non acylated anthocyanin, p-coumaroylated anthocyanin and tannin extractions were tied together, all three depending from the same underlying factors. The

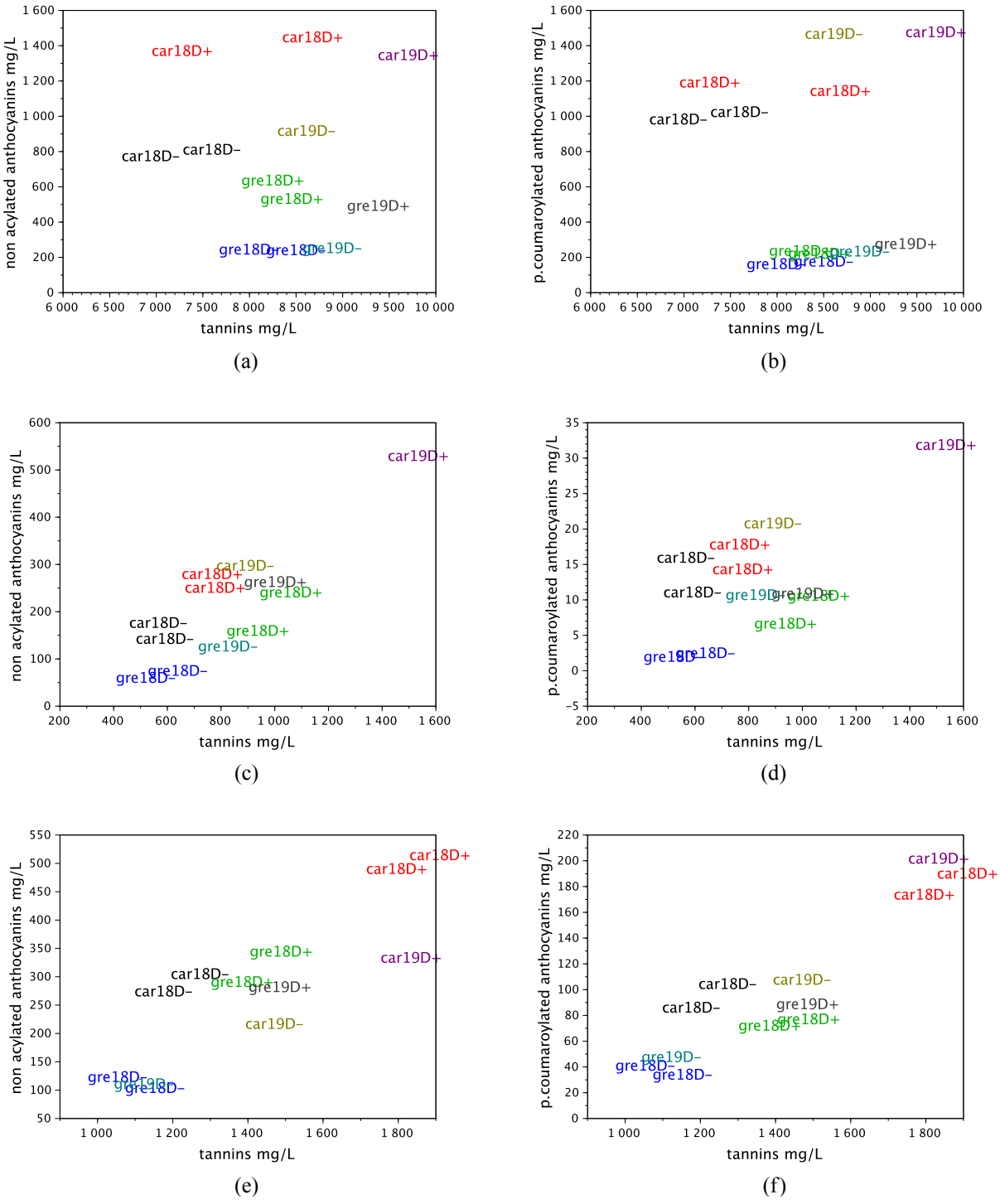

Fig. 2. Tannins vs. non acylated anthocyanins (a, c, e) or p-coumaroylated anthocyanins (b, d, f) measured on berries (a,b), model solutions (c, d) and wines (e, f).

extracted quantities in % decreased from non acylated anthocyanins to tannins then to p-coumaroylated anthocyanins and from wine to model solution.

4.2. *The polysaccharidic composition of the cell walls*

The polysaccharides of the cell walls have been characterized by two methods: (1) the neutral sugar composition obtained after acidic hydrolysis; (2) the comprehensive microarray polymer profiling (CoMPP) of cell wall polysaccharides. Results were provided by the T2 dataset.

4.2.1. *Neutral sugar composition of cell wall polysaccharides*

Polysaccharides were analyzed in skins and pulps on the basis of their main 7 neutral sugars. Each was expressed as a percentage of the total neutral sugars, in order to reveal possible differences of composition between the two varieties and/or the two years. Supplementary figure S6 summarizes the data. Slight differences of compositions were observed between Carignan and Grenache in 2018. However, these differences were dramatically increased in 2019, Carignan pulps and skins presenting a lower % of glucose and a higher % of arabinose, and Grenache pulps and skins a lower % of rhamnose and a higher % of mannose. Compared to the effects of variety and year, maturation had a

minor effect on the neutral sugar composition.

4.2.2. Comprehensive microarray polymer profiling (CoMPP) on cell wall polysaccharides

The CoMPP results presented a great variability. Table 2 reports each antibody which presented a non null signal on at least one sample in each of the 4 analytical situations: CDTA skins or pulps and NaOH skins or pulps. On the whole, almost all of the families of antibodies yielded signals. An important exception was the cellulose, with no signal at all.

The main families of pectins and hemicelluloses yielded signals, but with different behaviours. Homogalacturonans and AGPs were found mostly in CDTA extracts, from skins or pulp. Rhamnogalacturonans were found mostly in pulps. Finally, extensins were found in pulp and skin-NaOH extracts and in pulp-CDTA extracts, but not in skin-CDTA extracts.

4.3. Explanation of the variables of interest using the explanatory variables

The T2 dataset was used to explain the variables of interest previously selected: total, non acylated and p-coumaroylated anthocyanins measured by MRM, and total tannins measured by HPSEC, in model solutions and in wines, leading to a total of 8 variables of interest. Correlation coefficients were computed between these variables of interest and the variables in T2 which concerned the berry composition (the explanatory variables). Table 3 reports the correlation coefficients with the highest absolute values. The threshold of 0.65 was a breakpoint in the series of ordered R absolute values. The presentation of the results will concern successively the anthocyanins, the tannins, the CoMPP and the neutral sugars, all measured on extracts issued from berries.

Table 2
List of the monoclonal antibodies (mAbs) and the carbohydrate binding module (CBM). The sign + indicates that signals have been observed. Otherwise, signals were 0 for all samples.

Name	Function	skins CDTA	skins NaOH	pulps CDTA	pulps NaOH
	Homogalacturonans				
LM19	HG unesterified	+		+	
JIM5	HG partially methyl esterified + unesterified	+		+	
LM18	HG partially methyl esterified + unesterified	+		+	
JIM7	HG methyl esterified	+		+	
LM20	HG methyl esterified	+		+	
2F4	HG Ca^{2+} crosslinked				
PAM1	HG blockwise de-esterified				
LM8	xylogalacturonan				
	Rhamnogalacturonans				
INRA-RU1	backbone of rhamnogalacturonan I			+	+
INRA-RU2	backbone of rhamnogalacturonan I	+	+	+	+
LM5	(1→4) β-D galactan			+	
LM6	(1→5) α-L arabinan	+	+	+	+
LM13	linear (1→5) α-L arabinan			+	+
	Arabinogalactan −proteins				
JIM8	AGP	+		+	
JIM13	AGP	+		+	+
LM14	AGP			+	
LM2	AGP β-linked GlcA				
	Extensins				
LM1	extensin		+	+	+
JIM11	extensin		+	+	+
JIM20	extensin		+	+	+
	Hemicelluloses				
LM10	(1→4) β-D xylan				
LM11	(1→4) β-D xylan/arabinoxylan				+
BS400-2	(1→3) β-D glucan		+		+
LM15	xyloglucan		+		+
LM24	galactosylated xyloglucan		+		
LM25	xyloglucan/unsubstit. β-D glucan		+	+	+
LM21	(1→4) β-D galacto glucomannan		+		+
	Celluloses				
CBM3a	cellulose				

4.3.1. Correlations between the variables of interest and the berries anthocyanins and tannins

According to the principle of diffusion, the concentrations of anthocyanins observed in the model solutions and the wines should be correlated to the concentrations in the berries. It was verified by the non acylated and the p-coumaroylated anthocyanins in the model solution, and by the non acylated anthocyanins in wines. Results were different for the p-coumaroylated anthocyanins in wines, more correlated to the skins tannins ($R = 0.85$) than to the skin non acylated anthocyanins ($R = 0.69$) or to the skin p-coumaroylated anthocyanins ($R<0.65$). Same observation can be drawn for tannins. Extracted tannins in model solution and wine present high and similar correlations with berry anthocyanins and berry tannins, confirming the importance of anthocyanins in the extraction of tannins.

4.3.2. Correlations between the variables of interest and the comprehensive microarray polymer profiling (CoMPP)

Significant correlations with anthocyanins and tannins from model solutions or wines were observed for the four families: homogalacturonans (HG), rhamnogalacturonans (RG), extensins and hemicelluloses. Homogalacturonans were represented by mAbs LM18 and LM19 in the CDTA fraction, designed for unesterified homogalacturonans (LM19) and for partially methyl esterified or unesterified homogalacturonans (LM18) (Verhertbruggen, Marcus, Haeger, Ordaz-Ortiz, & Knox, 2009). It is noteworthy that the sign of the correlations was positive for the skins and negative for the pulps in model solutions, whereas model solutions did not contain pulps. Similarily, positive correlations were found between skin rhamnogalacturonans in the CDTA extracts and polyphenols. In model solutions, skin mAbs LM6 (1→6 α-L-arabinan) and INRA-RU2 (backbone of rhamnogalacturonan I) correlated with anthocyanins and tannins, skin mAb CDTA-JIM13 (AGPs) with tannins. Correlations were also observed between mAbs LM6, INRA-RU1, INRA-RU2, LM13 and p-coumaroylated anthocyanins. In wines, skin CDTA LM6 correlated with p-coumaroylated anthocyanins, pulp NaOH LM13 with anthocyanins. The only negative correlations were observed between pulp mAb LM5 (1→4 β-D-galactan) and non acylated anthocyanins. LM5 targets galactan ends. This suggests that a lower number of galactan chains would favor the extraction of non acylated anthocyanins.

Extensins were involved in the extraction process with mAbs LM1, JIM11 and JIM20 and concerned mainly the anthocyanins. But their correlation depended on the conditions: negative values on pulp, NaOH extraction, for anthocyanins in model solution or anthocyanins and tannins in wines (JIM11, JIM20); positive values with anthocyanins on pulp for CDTA extraction in model solutions (LM1, JIM20); positive values with non acylated anthocyanins on skins for NaOH extraction in wines (JIM11, JIM20). As for homogalacturonans, extensins presented a different behaviour in skins and in pulps.

Hemicelluloses were also involved in the extraction process. In model solutions, tannins correlated positively to pulp NaOH mAb LM11 (1→4 β-D-xylan/arabinoxylan) but negatively to pulp CDTA mAb LM25 (xyloglucan/unsubstituted β-D glucan). In wines, non acylated and total anthocyanins correlated negatively to pulp mAbs LM21(1→4 β-D-galactoglucomanan) and BS400-2 (1→3 β-D-glucan), both extracted with NaOH. Skin mAbs did not provide correlations with anthocyanins or tannins from model solutions or wines.

4.3.3. Correlations between the variables of interest and the neutral sugars issued from the polysaccharides

Neutral sugars were correlated to the extraction of anthocyanins,

Table 3
Highest correlation coefficients between the explanatory variables from the berries and the variables of interest, anthocyanins and tannins. The threshold was set to 0.65 in absolute value.

Explanatory variables	Variables of interest							
	Model solution				Wine			
	Anthocyanins			**Tann.**	**Anthocyanins**			**Tann.**
	all DAD	non acyl. DAD	p.coum. DAD	SEC	all MRM	non acyl. MRM	p.coum. MRM	SEC
Anthocyanins								
skin total $_{SEC}$	0.77	0.76	0.83		0.92	0.81	0.70	0.90
skin total $_{MRM}$	0.77	0.76	0.86		0.83	0.72	0.65	0.83
skin non acylated$_{MRM}$	0.77	0.76	0.79		0.93	0.84	0.69	0.92
skin p.coum.$_{MRM}$	0.73	0.72	0.86		0.70			0.71
Tannins								
skin total$_{SEC}$	0.82	0.82	0.79	0.73	0.73		0.85	0.82
skin oligo.$_{SEC}$	0.79	0.78	0.79		0.94	0.79	0.81	0.93
skin polym. DP> 3$_{SEC}$	0.69	0.69		0.74			0.72	
berry total$_{SEC}$		0.65		0.73			0.73	
Homogalactur.								
skin CDTA-LM19	0.87	0.88	0.73	0.91	0.73		0.79	0.76
skin CDTA-LM18	0.71	0.73		0.86			0.70	
pulp CDTA-LM19	−0.69	−0.67	−0.85					
pulp CDTA-LM18			−0.72					
Rhamnogalactur.								
pulp CDTA-LM6			0.77					
skin CDTA-LM6	0.84	0.84	0.80	0.84			0.71	
pulp CDTA-INRA-RU1			0.66					
pulp CDTA-INRA-RU2			0.66					
skin CDTA-INRA-RU2	0.74	0.74	0.71	0.82				
pulp CDTA-LM5					−0.66	−0.70		
pulp CDTA-LM13			0.82					
pulp NaOH-LM13					0.69			
skin CDTA-JIM13				0.71				
Extensins								
pulp CDTA-LM1	0.70	0.69	0.84					
pulp CDTA-JIM20	0.70	0.68	0.85					
pulp NaOH-JIM11	−0.69	−0.68	−0.68		−0.83	−0.82		−0.72
pulp NaOH-JIM20					−0.73	−0.75		
skin NaOH-JIM11						0.67		
skin NaOH-JIM20						0.70		
Hemicelluloses								
pulp NaOH-LM11				0.75				
pulp CDTA-LM25				−0.77				
pulp NaOH-LM21					−0.72	−0.68		
pulp NaOH-BS400-2					−0.67	−0.67		
Neutral sugars								
skin arabinose %	0.84	0.84	0.87	0.75			0.70	
pulp arabinose %	0.74	0.74	0.71	0.75				
skin mannose %	−0.89	−0.88	−0.90	−0.78			−0.72	−0.65
skin glucose %	−0.71	−0.70	−0.74	−0.68				
pulp glucose %	−0.86	−0.85	−0.91	−0.72			−0.68	
pulp xylose %			0.68					
skin xylose %			0.65					

total, non acylated, p-coumaroylated, and tannins in model solutions, but only to the extraction of p-coumaroylated anthocyanins in wines. Pulps and skins were concerned. Arabinose and xylose correlated positively, whereas mannose and glucose correlated negatively. This supports the previous observations on hemicellulose CoMPPs, the opposition between mAb LM11 on the one hand, mAbs LM21, LM25 and BS400-2 on the other hand. Polysaccharides targetted by mAb LM11 (xylan/arabinoxylan) contain mainly arabinose and xylose, whereas polysaccharides targetted by mAbs LM21, LM25 and BS400-2 (galactoglucomanan, xyloglucan/unsubstitued β-D glucan and β-D glucan respectively) contain mainly glucose and mannose.

To illustrate the importance of polysaccharides in the extraction of anthocyanins and tannins, and the effects of grape varieties and vintages, four parameters covering the diversities of antibodies and neutral sugars were selected: mAb LM19 (unesterified homogalacturonans) CDTA skin extract, mAb JIM11 (extensins) NaOH pulp extract, skin arabinose and skin mannose in %. Their plots against the wine anthocyanins and tannins are represented in Fig. 3. The four CoMPP figures presented the same shapes, with a clear increase for mAb LM19 and a clear decrease for mAb JIM11 which tended to 0 for the highest extractions of anthocyanins and tannins. Neutral sugars presented a different behaviour. A slight increase for arabinose and a decrease for mannose can be observed during the maturation. But the final values remained fixed by the initial values, due to the grape variety and to the year, they were just slightly affected by the maturation, as already stated.

5. Discussion

Our results confirmed for wines the difficulty to extract p-coumaroylated anthocyanins previously reported in model solutions (Abi-Habib et al., 2022). In both situations the concentrations of p-coumaroylated anthocyanins and tannins were correlated, suggesting a same underlying mechanism. The main difference was a higher release of p-coumaroylated anthocyanins in wines than in model solutions, almost negligeable. In model solutions, p-coumaroylated anthocyanins were shown to be preferentially found in the precipitates analyzed at the end of the diffusion experiments (Abi-Habib et al., 2022). A possible explanation is

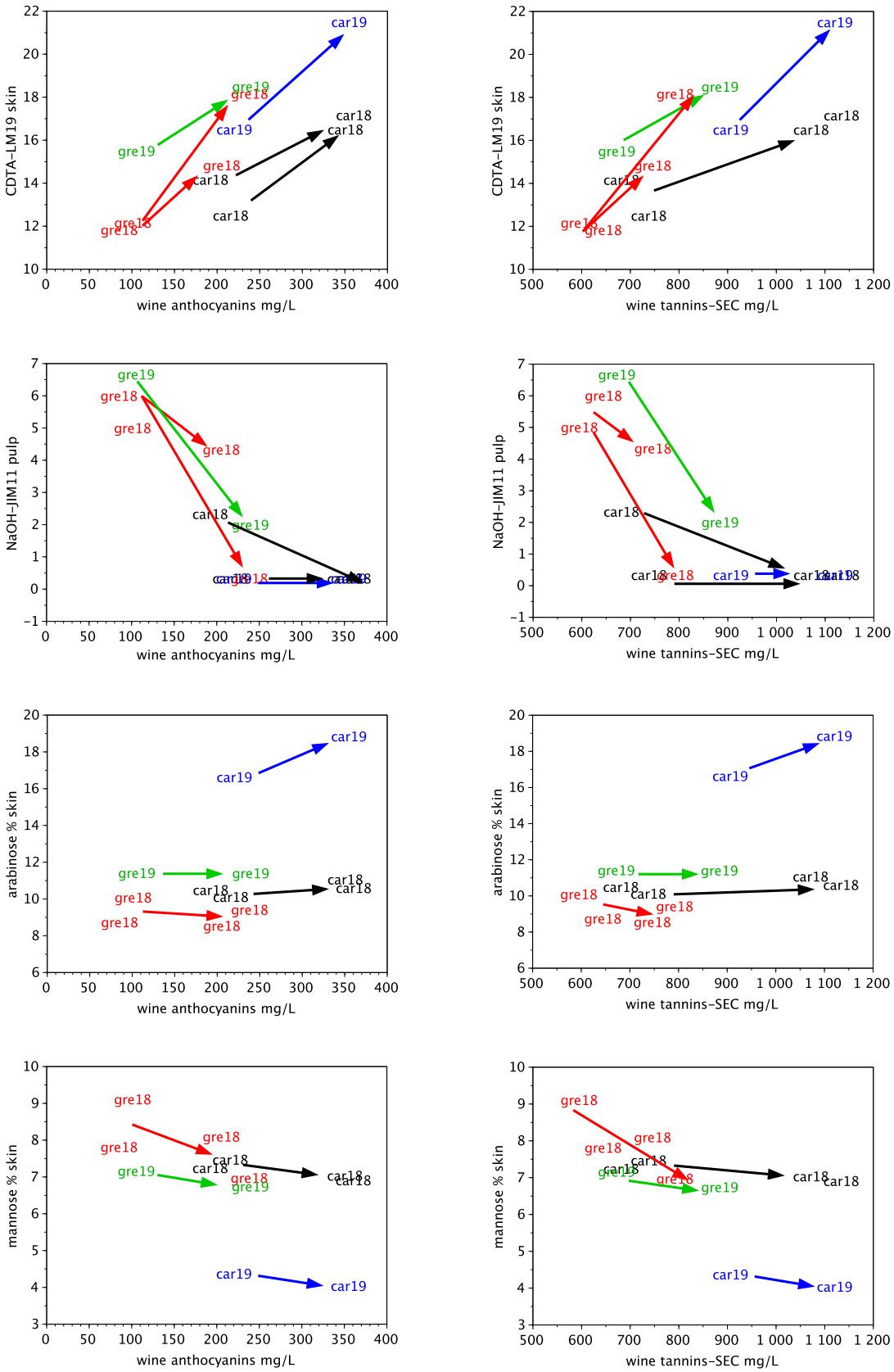

Fig. 3. mAb LM19 HG (unesterified) in CDTA-skin extracts, mAb JIM11 (extensin) in pulp NaOH extract, skin arabinose % and skin mannose % vs. wine total anthocyanins MRM (left) and wine total tannins HPSEC (right). The arrows represent the maturation, from low degree (D-) to high degree (D+). The 2018 V- and V+modalities were represented by the same arrow when close.

that wines present a different composition, due to yeasts, seeds and pulps, which contribute to prevent this precipitation.

Another result was the impact of cell walls on the polyphenol extractions (Amrani-Joutei et al., 1994; Hanlin et al., 2010). The contributions of the different families of polysaccharides were precised. Higher extractions of polyphenols were associated to (1) CDTA homogalacturonans vs. anthocyanins and tannins, high levels in skins for wines and model solutions, low levels in pulps for model solutions, (2) CDTA rhamnogalacturonans vs. anthocyanins and tannins, high levels in skins and pulps, except for LM5, low levels in pulps, (3) extensins vs. anthocyanins, high levels when extracted in pulps with CDTA (model solutions) or in skins with NaOH (wines), low levels when extracted in pulps with NaOH (wines and model solutions), and (4) hemicelluloses vs. anthocyanins in wines, tannins in model solutions, rich in xylans but poor in glucans and galactomannans.

Several correlations can be surprising. A typical example is given by the correlations between the compositions of berry pulps and polyphenol extractions in model solutions, performed without the pulps. In fact, the real causes are due to the variability of the experimental design, which had an impact on the skins and pulps (and seeds). The correlations involving the pulps would betray a composition and a state of degradation also present in the skins, and influencing the polyphenol extractions.

A different behaviour was observed between skins and pulps for homogalacturonans and extensins. Globally, extractions would be associated to skins rich in easily extractible low-methylated homogalacturonans and extensins difficult to extract, and with pulps poor in easily extractible low-methylated homogalacturonans but rich in easily extractible extensins. This description suggests a high level of degradation of the pulp, and a previous action of the PME in the skins.

Neutral sugar compositions completed the CoMPPs results. Higher extractions of anthocyanins and tannins were associated to higher % of arabinose and lower % of mannose in the skins. This conclusion echoes a previous report of a lower anthocyanin extractibility associated to a lower % of arabinans and a higher % of mannose in skins of Monastrell when compared to skins of Syrah, Cabernet-Sauvignon or Merlot (Ortega-Regules, Ros-Garcia, Bautista-Ortin, Lopez-Roca, & Gomez-Plaza, 2008). Arabinose and mannose remained roughly stable during the maturation, in accordance with previous results (Garrido-Banuelos et al., 2019). Mannose is found in glucomannans and galacto-glucomannans, which belong to the hemicellulose families. These hemicelluloses are present in lower quantities than xyloglucans, yet they greatly contribute to the strength and the 3-dimentional structure of the cell walls (Melton, Smith, Ibrahim, & Schroder, 2009). An example is provided by CoMPP mAb LM21 (galacto-glucomannan), correlated to a decrease of the extractibility of anthocyanins in wine, see Table 3. Less glucomannans and galacto-glucomannans, yielding lower levels of mannose, may lead to less rigid cell walls, being more accessible to tannins. Arabinose is found in arabinans and arabino-galactans, which form the branched chains of RGI polysaccharides. As homogalacturonans and rhamnogalacturonans, arabinans have the capacity to bind tannins (Fernandes et al., 2020).

Without proofs of causalities, it is difficult to draw explanations between correlated variables. So, more questions were asked than answered. Why are soluble (CDTA) low methylated skin homogalacturonans and skin rhamnogalacturonans, plus non soluble (NaOH) extensins, positively correlated to the extractions of anthocyanins and/or tannins? Why opposite correlations could be observed between skins and pulps? The question of the extraction of anthocyanins and tannins is far from simple. Moreover, the dominant hypothesis in the litterature is that binding of polyphenols on the cell walls is always detrimental for their extraction. This has been shown for the highly polymerized tannins bond on the highly methylated pectins. But isn't there another possible explanation, where binding would be benefical for the polyphenol extraction? This theory is presented hereafter.

It is acknowledged that tannins bind to the cell walls (Hanlin et al., 2010), and that anthocyanins ease the tannin extraction (Kilmister et al., 2014; Bindon et al., 2017). The proposed mechanism would rely on two steps: (1) a competition between anthocyanins and tannins for the binding sites, mainly homogalacturonans and low branched arabinans, (2) a stacking of the remaining polyphenols on the ones already binded to the cell walls. Non acylated anthocyanins would bind preferabily to the lower methylated and more hydrophilic and negatively charged pectins (Ortega-Regules et al., 2008), whereas p-coumaroylated anthocyanins and highly polymerized tannins would bind preferabily to the highly methylated and most hydrophobic pectins of the cell walls (Gao et al., 2019; Watrelot & Norton, 2020). In other words, the binding of p-coumaroylated anthocyanins would prevent the binding of tannins on highly methylated pectins, thus favoring their extractibility. It would also explain the correlations between tannins and p-coumaroylated anthocyanins in wines and model solutions, and why p-coumaroylated anthocyanins are so difficult to unbind. Then, when all binding sites have been saturated, some of the remaining free polyphenols would stack on the polyphenols already bond to the cell walls, as already suggested (Renard, Baron, Guyot, & Drilleau, 2001; Fernandes et al., 2020). The release processus would depend mainly on ethanol and temperature conditions, enzymes having a limited action for releasing bound tannins (Osete-Alcaraz et al., 2021). It would be the reverse of the previous steps: unstacking then unbinding, following previous observations on apples (Renard et al., 2001). Non acylated anthocyanins and low polymerized tannins would unbind more easily than p-coumaroylated anthocyanins and highly polymerized tannins because the former present weaker interactions with cell walls than the latter. It can also be postulated that some pectins would be released under temperature and ethanol conditions, enhanced by cellulase and pectinase activities (Osete-Alcaraz et al., 2021), explaining the positive correlations of low methylated pectins and rhamnogalacturonans. Stability over time would be achieved when soluble oligosaccharides and polysaccharides have wrapped and therefore have formed a protecting layer around polyphenols. This theory was suggested by previous results about wine astringency (Boulet et al., 2016). Para coumaroylated anthocyanins were reported to be more reactive than non acylated anthocyanins with salivary enzymes (Paissoni et al., 2018). Let us extend this property to some of the berry proteins. Para coumaroylated anthocyanins would be better protected in the case of higher levels of extractible pectins, e.g. advanced maturations. But in model solutions, without pulps, there would be a deficiency in soluble pectins when compared to wines, leading to a lack of protection and therefore more precipitations of the p-coumaroylated anthocyanins.

6. Conclusion

CoMPPS and neutral sugars confirmed the importance of the cell wall compositions in the extraction of anthocyanins and tannins. The main binding sites were homogalacturonans and rhamnogalacturonans in the skin cell walls. Polyphenols accessibility to theses binding sites depended on extensins and some hemicelluloses. Moreover, CoMPPs and neutral sugars can describe the keypoints for polyphenols extractions from grapes. For example, the % of arabinan and mannose in the composition of cell wall polysaccharides would characterize the grape variety and the vintage. And a selection of antibodies from the CoMPPs analysis, e.g. mAb JIM11 NaOH pulp extract, which decreases sharply, could be an indicator of the maturity of the cell walls, reached when the signal tends to 0.

Predicting the extraction of anthocyanins and tannins in wines from the grapes remains a challenge. To put forward the importance of the cell walls composition in this process, the *phenolic maturity* concept, focused on polyphenolic extractions in model solutions (Bautista-Ortin, Lopez-Roca, & Gomez-Plaza, 2006; Nel, 2018), could be updated by a *cell wall maturity* concept taking into account polysaccharides and polyphenols in the berry composition. This characterization would help to predict the ability of grapes to give wines more or less rich in

polyphenols. It could be a good support for grape vine selection.

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CRediT authorship contribution statement

Jean-Claude Boulet: Software, Writing - original draft, Visualization. **Elissa Abi-Habib:** Methodology, Validation, Investigation, Resources. **Stéphanie Carrillo:** Investigation, Resources. **Stéphanie Roi:** Investigation, Resources. **Fréderic Veran:** Investigation, Resources. **Arnaud Verbaere:** Investigation, Resources. **Emmanuelle Meudec:** Investigation, Resources, Data curation. **Anais Rattier:** Investigation, Resources. **Marie-Agnès Ducasse:** Investigation, Resources. **Bodil Jørgensen:** Validation, Investigation, Resources. **Jeanett Hansen:** Validation, Investigation, Resources. **Sophie Le Gall:** Investigation, Resources, Writing - review & editing. **Céline Poncet-Legrand:** Conceptualization, Methodology, Data curation, Writing - review & editing, Supervision, Funding acquisition, Project administration. **Véronique Cheynier:** Conceptualization, Methodology, Writing - review & editing, Writing - review & editing, Supervision. **Thierry Doco:** Conceptualization, Methodology, Data curation, Writing - review & editing, Supervision. **Aude Vernhet:** Conceptualization, Methodology, Validation, Data curation, Writing - review & editing, Supervision, Funding acquisition, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, athttps://doi.org/10.1016/j.foodchem.2022.135023.

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