



ORIGINAL RESEARCH ARTICLES

Insights into multiscale chemical characterisation for the understanding of berry maturation

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Article number: 8286



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Associate editor:
Fernando Zamora



Received:
30 August 2024

Accepted:
27 November 2024

Published:
30 January 2025

ABSTRACT

Among secondary metabolites, phenolic and volatile compounds are major contributors to wine quality. Their quantities in wines will depend on several factors, their concentrations in grapes but also their extraction rates, along with a number of other factors. Extraction is a complicated process which involves interactions with other compounds; *e.g.*, polysaccharides and proteins. The aim of this work was to evaluate the potential of merging different analytical methods for identifying different compounds at different scales. Known methods in wine analysis, such as phloroglucinolysis, were complemented with methods usually applied to other food products and rarely or never on grapes or wines, such as solid-state NMR and NMR water mobility by ¹H MRI. The feasibility of merging these methods was validated on this first trial. While the results did not allow us to answer questions related to the study, they raised other interesting questions to be addressed in future experiments.

KEYWORDS: wine, phenolic compounds, polysaccharides, volatiles, composition, wax, ¹³C solid-state NMR, water mobility



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INTRODUCTION

Red wine quality is determined to a large extent by its aroma and phenolic composition, depending on grape berry composition and the extraction and reactions of these compounds and their precursors during the winemaking process. Grapes contain a few odorant molecules, such as monoterpenols, pyrazine (Flanzy, 1998) and rotundone (Wood *et al.*, 2008), whose extraction is often limited; for example, the extraction rate of rotundone (pepper aroma) is around 10 % (Geffroy, 2020). In addition, most of the aroma potential of grapes (99 %) is due to non-volatile and non-odorant molecules, such as carotenoids, glycosylated precursors, thiol and dimethyl sulfur (DMS) precursors (Genovese *et al.*, 2013a). After extraction, non-volatile and non-odorant molecules, bound with glycosides, cysteine derivatives, phenolic acids and fatty acids, are transformed into free odorant volatile molecules during the winemaking process. Whether odorous or not, some volatile molecules can be transformed into other odorous molecules due to their instability (*e.g.*, terpenols, terpene diols and C13 norisoprenoids; Paolini *et al.*, 2018). Aroma compounds and their precursors can be found in the pulp, but most of them are located in the skin (Pineau *et al.*, 2011).

In addition to the abovementioned compounds, grapes contain phenolic compounds. Two major families of phenolic compounds are essential for red wine quality: i) anthocyanins, which are red grape pigments, and ii) flavanols, which include monomers, oligomers and polymers; *i.e.*, proanthocyanidins, usually referred to as condensed tannins. The latter have an impact on mouthfeel and ageing potential, contributing to astringency and bitterness, and they react with anthocyanins to form derived pigments, thus taking part in colour changes and colour stabilisation. Anthocyanins are found in berry skins, and the majority of flavanols is found in skins and seeds, with low amounts in berry pulp. Consequently, red winemaking involves the extraction of both anthocyanins and flavanols from the solid parts of the berry, which determines their concentrations in wine. As in the case with aromas, total extraction of anthocyanins and tannins does not take place, and therefore their concentrations in wine do not necessarily reflect berry composition (Abi-Habib *et al.*, 2021). Indeed, large differences in extraction rates of phenolic compounds have been observed during winemaking (Harbertson *et al.*, 2002). For example, experiments involving skin maceration in model solutions have shown that anthocyanins, and to a lesser extent tannins, are more easily extracted from Grenache (GRE) than from Carignan (CAR) (Abi-Habib *et al.*, 2021). This is thought to be related to differences in the composition of berry cell walls, likely reflecting structural differences (Bindon *et al.*, 2017). The adsorption of proanthocyanidins onto cell wall material has been reported (Bindon *et al.*, 2017), and has been found to be higher in CAR than in GRE, explaining varietal differences in the extraction rates of tannins (Abi-Habib *et al.*, 2021). A certain amount of adsorption of anthocyanins—especially of coumaroylated anthocyanins—onto pulp cell walls was also observed in the latter study, which is

consistent with their lower rate of extraction from skins (Abi-Habib *et al.*, 2021). Thus the extraction process appears to be complex, since it involves several chemical families and their interactions, which highly depend on variety and maturation level. In a recent review (Boulet *et al.*, 2024), we proposed a general mechanism to explain the extraction of polyphenols as a consequence of their interactions with skin and pulp polysaccharides; for example, pulp and skin pectins may precipitate and spare phenolic compounds from precipitation. Another issue after polyphenolic extraction is their stabilisation, which could also be triggered by polysaccharides from the skin and the yeast. In other words, the understanding of phenolic extraction may be tied to a better characterisation of the skins. Such knowledge would be very valuable in the context of new variety selection: early quality predictions based on berry composition could enhance selection efficiency by adding quality parameters to currently used parameters, which are linked to agronomic properties, such as resistance to diseases.

Berry skin cell walls are consequently drawing more attention by the oenological research community. Recently, comprehensive microarray polymer profiling (CoMPPs) was carried out in order to quantify berry skin polysaccharides (Gao *et al.*, 2021; Abi-Habib *et al.*, 2021). Our goal was to evaluate other complementary approaches at different scales (molecular, tissue) in order to obtain further information on the chemical composition of berries. In particular, we evaluated the potential of two NMR-based methods currently used on other food products, but not on grapes and wines. Maturation is characterised by the demethylation of insoluble pectins (Barnavon *et al.*, 2001), which results in higher cell wall porosity (Bindon *et al.*, 2014). Water mobility may therefore be modified during maturation, which could be assessed via proton magnetic resonance imaging (¹H MRI), which has been demonstrated to be a suitable tool for studying the state of water in biological systems. As well as being non-invasive, ¹H MRI enables the generation of several image contrasts from which biological markers can be derived; for example, proton density and transverse magnetic relaxation images have been used to study the internal structure of the grape berry (Andaur *et al.*, 2004; Ciampa *et al.*, 2019), and three-dimensional diffusion images (*i.e.*, diffusion tensor imaging), have been used to extract further microstructural (*i.e.*, beyond the resolution limit of an MRI spectrometer) information from a grape berry (Dean *et al.*, 2014). Meanwhile, ¹³C solid-state nuclear magnetic resonance (NMR) could provide an overview of the chemical composition of the skin.

MATERIALS AND METHODS

Two *Vitis vinifera* grape varieties, Carignan and Grenache (hereafter referred to as CAR and GRE) were studied. Berries were harvested at an average potential alcohol of 12% vol. from the vineyard of the Pech Rouge experimental unit (INRAE, Gruissan, France) in 2019. They were sorted according to their natural heterogeneity in terms of density (degree of maturity: D), yielding two batches (D- and D+) per variety, as described in previous work (Abi-Habib *et al.*, 2021).

Triplicate samples of each batch were collected, processed and analysed. In particular, grape skins were separated from the berries with a scalpel and immediately used for diffusion experiments in wine-like model solutions, as also described in the previous study (Abi-Habib *et al.*, 2021). Sample preparations are detailed below for each analysis. Wines were made in triplicate under standard conditions in a room thermostatically controlled at 21 °C and using French press coffee makers (1 L volume, Bodum, Neuilly-sur-Seine, France). After mechanical crushing, berries (900 g) were put in the Bodum and inoculated with Lalvin ICV OK yeast (20 g/hl). SO₂ (250 µL of a 8 % solution) was then added. Cap management was carried out during alcoholic fermentation by plunging the pomace with the plunger daily until the end of fermentation; i.e., when the density remained stable with values below 995 g/L. SO₂ (40 mg/L) was then added to prevent malolactic fermentation and oxidation occurring.

At different scales and times of the process, analyses were performed on whole grape berries, extracts from berry skin, seed and pulp, and then on the wine.

1. Basic berry analysis

A batch of berries was weighed and their skins, pulps and seeds were separated using a scalpel and their proportions calculated. Another batch was crushed for quantification of sugars, pH and total acidity in the must applying the OIV methods of analysis.

2. Analysis of berry water mobility

Water mobility and distribution in the grape berries was analysed using high resolution magnetic resonance imaging (MRI) transversal relaxation (T2) mapping. Three to five samples for each of the four experimental batches (GRE D-, GRE D+, CAR D- and CAR D+) were analysed. Each berry was first weighed before being put in a 30 mm-diameter glass tube sealed with a screw cap with septum, and flushed with nitrogen gas. MRI experiments were carried out at room temperature (22 °C) using a Bruker Ascend 400WB (9.4 T) instrument (Bruker, Ettlingen, Germany) equipped with a microimaging accessory. A 32 mm-diameter birdcage radiofrequency coil was used for both excitation and signal reception. To enable T2 map generation, 30 echo (echo time, TE, of 5 to 30 ms 5 ms step) images were acquired using a Multi Slice Multi Echo (MSME) pulse sequence. The remaining acquisition parameters were: a 19.2 mm x 19.2 mm field of view (FOV), and an acquisition matrix (192 x 192) resulting in the in-plane resolution of 100 µm. Depending on the berry size, 34 to 40 slices (0.5 mm-thick) were obtained from the entire berry. The repetition time (TR) of 10 s was used with two averages, resulting in a total acquisition time of 64 min. Three datasets were obtained per sample.

To carry out image processing and T2 mapping, T2 relaxation maps were reconstructed from each of the obtained 30 echo images using an in-house Matlab (MathWorks, USA) implementation of the NNLS–Non-Negative Least Square algorithm (Lawson and Hanson, 1985). Images were first segmented slice per slice using a home-made Matlab programme based on a region growing algorithm that enabled

interactive thresholding. For each slice, two masks were then obtained: namely, of the noise and the seedless berry; i.e., central, cavity. The noise image was then used to estimate the mean noise level. The berry T2 maps were constructed by fitting the signal decay (signal intensity versus TE) pixel-wise to a sum of exponentials. Only pixel signals higher than three times the mean noise were included in the echo decay. The NNLS algorithm was fed with a parsimonious decomposition basis of 40 T2 logarithmically spaced from 10 ms to 1000 ms. A parsimonious T2 basis was demonstrated to be as efficient as the use of a smoothing factor in the minimisation of the curvature of the T2 distribution (Bonny *et al.*, 2020).

3. Skin cuticle and wax analysis

The cuticle composition of grapevine skins was studied using gas chromatography coupled with flame ionisation and mass detector (Girard *et al.*, 2012). Cuticular waxes were twice extracted from isolated cuticles of grape berry skins in 4 mL of methylene chloride. The resulting solution of cuticular waxes was spiked with cholestane as internal standard. Dewaxed cutins were trans-esterified by treatment with 14 % boron trifluoride in methanol for 16 h at 70 °C including 10 µg of heptadecanoic acid as internal standard. Extraction was performed with CH₂Cl₂ (2 x 2 mL), then the extract was dried over anhydrous sodium sulfate and under an N₂ stream. Prior to gas chromatography analysis, wax and cutin samples were silylated with 100 µL of bis-(trimethylsilyl) trifluoroacetamide containing 1 % trimethylchlorosilane at 70 °C for 60 min. The qualitative composition was studied with a capillary GC-MS (Agilent 122-5532UI) equipped with a DB5ms column (30 m x 0.32 mm, 0.1 µm [J&W]) and a mass spectrometric detector (70eV, m/z 50-700). For the cutin analyses, GC was carried out in an oven temperature programmed as follows: 1 min at 90 °C, 40 °C/min up to 200 °C, 1 min at 200 °C and 3 °C/min up to 320 °C. For the wax analyses, GC was carried out in an oven temperature programmed as follows: 2 min at 50 °C, 40 °C/min up to 220 °C, 2 min at 200 °C, 3 °C/min up to 320 °C and 30 min at 320 °C. The quantitative composition was studied using GC-FID under the same GC conditions, but H₂ was used as carrier gas at 2 mL/min.

4. Analysis of skin cell wall polysaccharides

Alcohol insoluble residues (AIRs) were prepared using Dionex ASE 350 (Thermo Scientific) at 100 °C with 80 % ethanol from grape skins previously separated from the berries, as described above. Polysaccharides were compared for their sugar composition and their NMR spectra. The sugar composition of the cell wall polysaccharides was analysed after sulfuric acid hydrolysis and gas chromatography analysis (Boulet *et al.*, 2023). Solid state ¹H/¹³C CP/MAS NMR experiments were performed on a Bruker Avance III 400 MHz spectrometer operating at a ¹³C frequency of 100.62 MHz. A double resonance H/X CP/MAS 4 mm probe was used. AIR samples were spun at a rate of 12 kHz at room temperature. The cross-polarisation pulse sequence parameters were 2.5 µs proton 90° pulse, 2 ms contact time and 10 s recycle time. Typically, an accumulation of 2048 scans was used. The carbonyl signal of glycine (176.03 ppm)

was used as an external standard for the chemical shift of the ^{13}C solid-state NMR spectra. Before carrying out a Fourier transform, all data were processed with an exponential multiplication parameter of line broadening of 20 Hz.

5. Analysis of skin volatile compounds

Extractions were performed in model solutions (hereafter *model solution*) by increasing the ethanol content stepwise (Abi-Habib *et al.*, 2021). Berries (hereafter *berries*) were sampled before and after extraction. Each batch of frozen grapes of each variety was thawed overnight at 4 °C. After manual deseeding, pulps and skins from 50 berries were crushed with an Ultra-Turrax T25 for 1 min at 15000 rpm in 12 % ethanol. Seeds were reintroduced to their must samples and were homogenised for 2 h at 25 °C, then centrifuged for 8 min at 4000 rpm. 10 ml of supernatants were aliquoted into 20 mL screw-cap vials and stored at -20 °C before processing.

In order to determine the aroma potential of the glycosidic precursors, volatiles of the bound fraction were released after acid hydrolysis, as described by Pedroza *et al.* (2010). Ten milliliters of centrifuged berry extracts and model solutions were hydrolysed by addition of 2 M citric acid solution to pH 2.5 for 2 h at 70 °C. NaCl (3 g) and the internal standard (100L of hexanal at d_{12} at 109 ng/ μL in ultrapure water) were added and mixed for 30 min. The solution was then stirred with a PDMS-coated stir bar (1 mm film thickness, 10 mm length, Twister, Gerstel, Mulheim and der Ruhr, Germany) at 500 rpm for 2 h at 70 °C. The stir bars were removed from the samples, rinsed with distilled water, dried on an absorbent sheet of paper towel and finally transferred into thermal desorption tubes for the GC/MS analysis.

Volatile compounds were desorbed from the stir bar in the thermal desorption unit (TDU, Gerstel, Mulheim and der Ruhr, Germany) of a 7890A gas chromatography apparatus (Agilent Technologies, Palo Alto, CA, USA) under the following conditions: initial temperature at 30 °C, raised to 280 °C at 100 °C/min, final temperature at 280 °C for 5 min. The desorbed analytes were cryo-focused at -100 °C using liquid nitrogen, in a Cool Injection System injector (CIS 4, Gerstel, Germany). The compounds were then transferred to the GC-MS instrument with the programme temperature as follows: final temperature of 280°C (held for 5 min) at 12 °C/s. Helium was used as the carrier gas to separate volatile compounds, at a velocity rate of 40 cm/s, in a Agilent DB-HeavyWAX (30 m x 0.25 mm *i.d.*, 0.5 μm film thickness; J&W Scientific) column. The chromatographic separation conditions were set at 40 °C to 250 °C at a rate of 4 °C/min. The transfer line temperature was 250 °C. Mass spectrometry analysis were performed on a 5975C mass selective detector (MSD, Agilent Technologies, Palo Alto, CA, USA) in electron impact mode (EI) at 70 eV, at a rate of 4 scans/s, covering a m/z range from 29 to 350 Da, with a source temperature at 230 °C and a detector temperature at 150 °C. Three replicates of each berry extract and model solution were analysed. Molecules were identified by comparison of the experimental linear retention index and the obtained spectra using several databases: US National Institute of Standards and Technology

(NIST08), WILEY11N17 and the in-house INRAMass. Data treatment was made with MSD ChemStation software. Total ion chromatogram peak area for each identify component was calculated and divided by the peak area of the internal standard to obtain a relative quantification. The obtained values were used for statistical data analysis using R 4.3.1 with the stats and pheatmap packages.

First, the whole data was visualised in a PCA in order to identify potential outliers to be removed. After removing the outliers, the three replicates were averaged to keep only one intensity per (variety x type x alcohol x hydrolyse) in order to represent the logged intensities on a heatmap. Then, to observe potential significance, ANOVA with fixed effects with four factors: type (berry or model solution), variety (CAR or GRE), alcohol (D- or D+) and hydrolyse (H or NH) was conducted for each volatile organic compound (VOC) on the whole data, including potential outliers. P-values of these ANOVAs were corrected with Bonferroni for each effect. When the alcohol effect was significant for the ANOVA, additional Wilcoxon tests were computed.

6. Analysis of skin, seed and wine phenolic compounds

Berry and wine phenolic compounds, including anthocyanins and flavanol monomers, dimers and trimers, were determined by UHPLC-MS/MS in the MRM mode, using an Acquity UPLC system coupled to a triple quadrupole (QqQ) TQD mass spectrometer with an electrospray ionisation (ESI) source, equipped with a reversed-phase Acquity HSS T3 1.8 μm 1.0 x 100 mm column (Waters, Saint-Quentin-Yvelines, France), as described in a previous study (Lambert *et al.*, 2015). Constitutive units of flavanol derivatives released after acid-catalysed depolymerisation in the presence of phloroglucinol were analysed by UHPLC-QqQ-MS in the MRM mode (Pinasseau *et al.*, 2016). Total flavanol units released by phloroglucinolysis (FlaT) were calculated and average degree of polymerisation (mDP) was evaluated as the molar ratio of the sum of all flavanol units to the sum of terminal units. Phenolic compounds from skins, wines and model solutions were also analysed by high performance size exclusion chromatography (HPSEC) in dimethylformamide containing 1 % acetic acid (v/v), 5 % water (v/v), and 0.15 M lithium chloride, at 60 °C with a 1 mL/min flow rate, using an Agilent HPLC 1260 Infinity II system equipped with a diode array detector (DAD) and Phenogel columns connected in series, as described previously (Abi-Habib *et al.*, 2021).

7. Classical wine analysis

Classical oenology parameters (pH, alcoholic degree, glucose-to-fructose ratio and volatile acidity) were determined at the end of fermentation applying the OIV methods of analysis. Total polyphenol index (TPI) and total pigments (TP) were determined by measuring absorbance values at 280 nm and 520 nm after dilution in 1 % HCl before and after fermentation using a Shimadzu UV-1800 spectrophotometer.

TABLE 1. Average composition of fresh berries and musts. Pulp weights were calculated from skin and seed weights.

	Unit	GRE D-	GRE D+	CAR D-	CAR D+
berry weight	g	1.65	1.86	1.82	1.79
skin weight	g/g berry	0.20	0.17	0.16	0.20
seed weight	g/g berry	0.04	0.04	0.03	0.03
pulp weight	g/g berry	0.76	0.79	0.81	0.77
must sugars	g/L	184	238	190	223
must pH	-	3.15	3.33	3.19	3.28
must total acidity	g/L H ₂ SO ₄	3.60	3.09	5.20	4.42

TABLE 2. NMR water mobility.

	Unit	GRE D-	GRE D+	CAR D-	CAR D+
berry, short T2	%	90	88	91	89
berry, short T2	mS	44.7	34.1	53.2	49.7
berry, long T2	mS	771	748	829	828

RESULTS

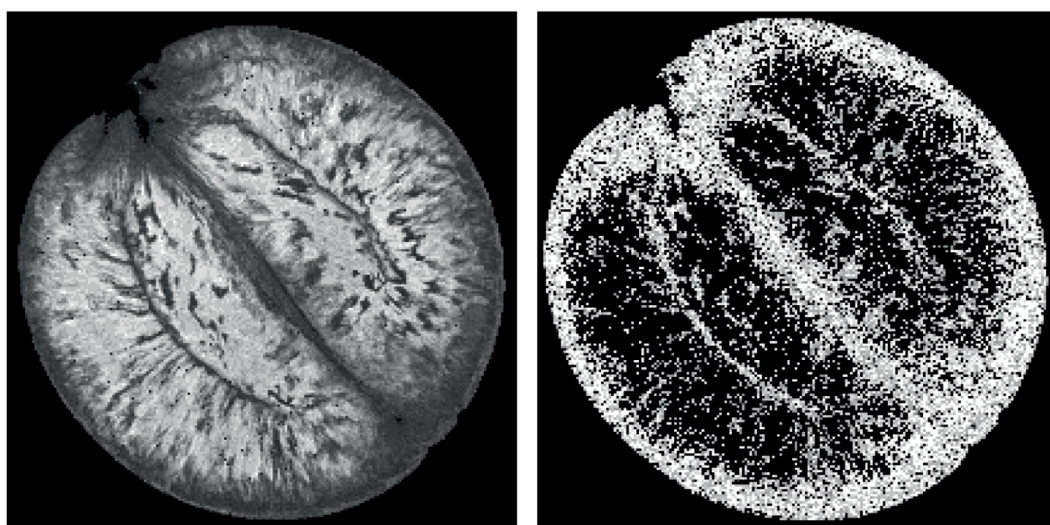
The results associated with the different methods described in the materials and methods section are each reported in turn below.

1. Berry characteristics

The main effect of maturation is an increase in sugars and pH and a decrease in total acidity (Table 1), as is classically observed. GRE berries of the lowest maturity level were significantly smaller than the other three groups, while there was no significant impact of maturity on CAR berry weight. As was expected, sugar contents, pH values and total acidity levels confirmed that the D + grapes were riper than the D- grapes, with significantly higher sugar levels, higher pH values and lower acidity in D+ than in D-. However, larger differences were observed between the GRE samples, CAR D- being significantly riper than GRE D-, and GRE D+ significantly riper than CAR D+.

2. Berry water mobility

T2 water relaxation values are characteristic of the state of water within the samples. More mobile or free water is characterised by a longer T2 in tissue while confined/structured or water bound to macromolecules is characterised by a shorter T2. An increase in short T2 values, or a decrease in long T2 values, is associated with less free and more highly bound water. Despite their heterogeneity in terms of their fresh weight (from 1.39 g to 3.09 g), all the analysed berries had a similar total water content of around 75 %. The results of the NNLS inversions show that more than 95 % of the pixel decay curves resulted in two T2 components; *i.e.*, in two water fractions in the berries. An example of the T2 maps resulting from GRE D- is shown in Figure 1: there is a major water fraction (90 %) with a short T2 of 20 ms to 90 ms and a mean of 55 ms, and a minor (10 %) water fraction with a long T2 with a mean value of 800 ms. The spatial distribution of these water fractions is displayed in the T2 maps. The major water fraction can be seen to be mainly concentrated in the

**FIGURE 1.** Representative T2 maps for a GRE D- berry derived from pixel-wise NNLS inversion of the signal decays. Short T2 on the left and long T2 on the right.

pulp, whereas the minor (long T2) water fraction is mainly located at the periphery just below the skin film and in the vascular bundles within the berries. All the berry results were averaged and are reported in Table 2. For each grape variety, an increase in the maturation degree was accompanied with a decrease in short T2: -22 % and -7.5 % from D- to D+ for GRE and CAR respectively.

When dealing with proton mobility in biological systems, T2 images can be considered as maps of the distribution of mobile protons (mainly from water and/or fat) contrasted with T2 relaxation. Therefore, the observed decrease in short T2 relaxation time with degree of maturity of the two grape varieties may be due to the decrease in mobility of the main water fraction resulting from the increased sugar concentration. In the same way, the contrasting T2 images allow the spatial distribution of sugar concentration in the berry to be inferred. As shown in Table 2, the short water fraction in GRE D-, and therefore a high sugar concentration, is mainly located in the pulp, while the high mobility of the sub-skin film can indicate a lower sugar concentration in the

vicinity of the skin. It should be noted that no conclusion could be drawn regarding the skin itself, because the pixel resolution of the NMR images (100 μm) was too large when compared to skin thickness, and border effects may also have occurred. Therefore, skin analysis would need to be improved by, for example, stacking several skin layers.

3. Skin cuticle and wax composition

The wax and cutin compositions are reported in supplementary material (Table S1). The amount of wax extracted from the skins of the GRE and CAR varieties were significantly ($p < 0.05$) different for both experimental batches (D+ and D-). In addition, for each grape variety, a significant difference was observed between D+ and D-. Wax composition was also analysed. Like that of many flesh fruits, the cuticle of grapevine berries is rich in both aliphatic waxes and triterpenoids (Yang *et al.*, 2021; Lara *et al.*, 2015). In the present study, the wax composition was comparable in the two grape varieties. Nevertheless, terpenic compounds seem to be the most variable between D+ and D- of each variety. These results are

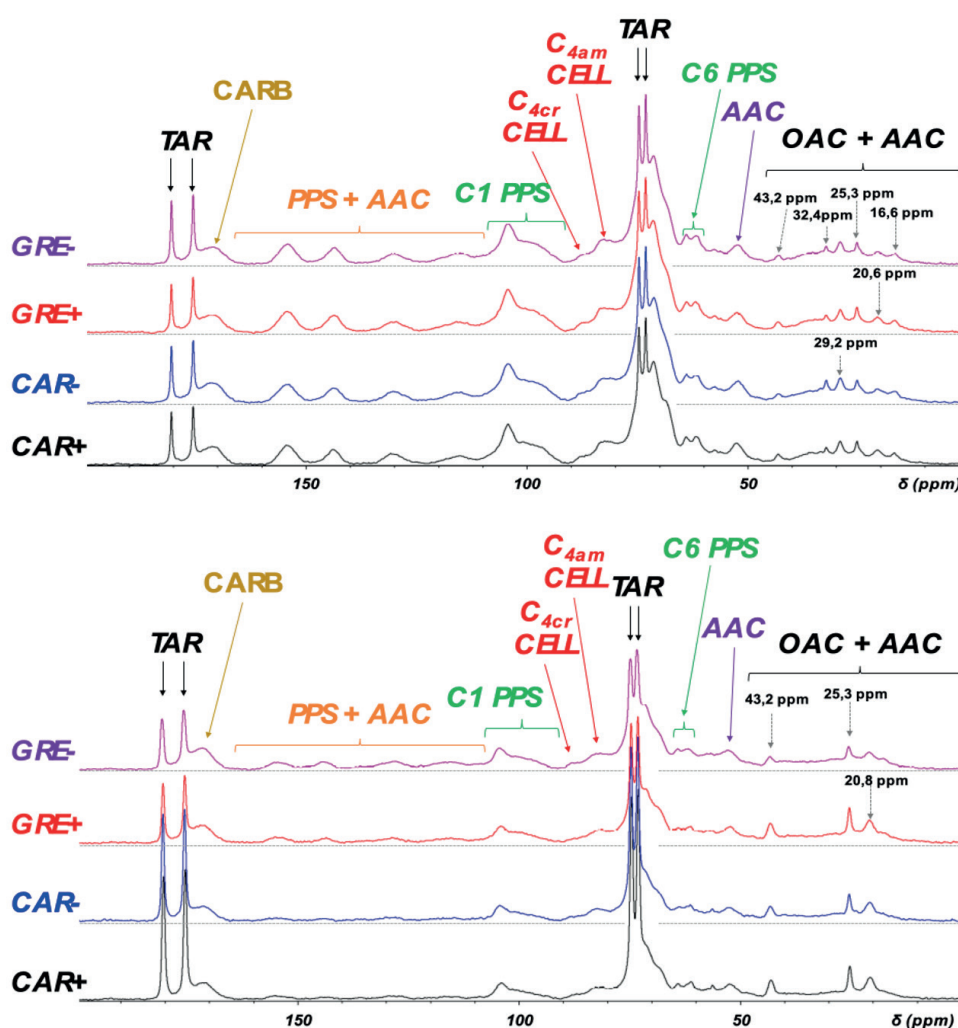


FIGURE 2. Annotated ^{13}C CPMAS spectra of skin (top) and pulp (bottom) alcohol insoluble residues. CARB=carbonyl COOH/COOR; PPS+AAC=polyphenols and/or aromatic rings of amino-acids; C1PPS = C1 of polysaccharides; $\text{C}_{4\text{cr}}\text{CELL} = \text{C}_{4\text{cr}}$ of cellulose; $\text{C}_{4\text{am}}\text{CELL} = \text{C}_{4\text{am}}$ of cellulose; TAR = tartaric acid, C6PS = C6 of polysaccharides; AAC = C α of amino-acids; OAC+AAC = $-\text{CH}_3$ organic acids, side-chains of amino-acids.

consistent with a previous study (Pensec *et al.*, 2014), suggesting that these modifications could impact either the mechanical properties of the berry or its susceptibility to pathogens at the maturation stage. The composition of the polymeric scaffold of the cuticle (cutin) was also studied. For both varieties of grape vine berry the cutin polyester is rich in C18 monomers, in particular in 9,10 epoxy18-hydroxyoctadecanoic acid. These data are in line with those of pioneer work on cutin composition obtained from other varieties via different depolymerisation methods (Baker & Holloway, 1970; Walton & Kolattukudy, 1972). In the present study, the cutin composition of both grape varieties seemed only slightly affected by degree of maturation.

4. Quantification of skin cell wall polysaccharide composition

The results of the ¹³C solid-state NMR and neutral sugar composition analyses are reported in supplementary material (Table S2). No differences were observed in terms of polysaccharides, amino and organic acids between GRE and CAR or D- and D+. The only differences that were found were within-variety (GRE and CAR) for tartaric acid and pulp phenolics. The presence of phenolic compounds and tartaric acid in the quantities found was surprising, as the AIR preparation comprised a precipitation of polysaccharides in 80 % ethanol, and thus most of the polyphenols and tartaric acid should have been removed with the supernatant. This was certainly the case for malic acid, whose concentration was of the same magnitude as tartaric acid. Tartaric acid may be involved in complexes with AIR polysaccharides, but malic acid may not be.

5. Quantification of skin volatile compounds

Hydrolysed D- GRE model solution was unavailable (for technical reasons). The results are reported in supplementary material. The PCA of logged intensity highlighted an outlier (model solution / hydrolysed / GRE / D+ / replicate 2) that was subsequently removed from the dataset for the rest of the statistical analysis (results not shown). After removing this sample, the intensity (logged) averaged across replicates of the different samples was represented on a heatmap (Figure S3). The resulting sample clustering was consistent with existing classifications: the first level of classification comprised berry and model solution samples that were completely separate, and the second level showed a clear distinction between the varieties GRE or CAR. The third level showed a differentiation between hydrolysed and non-hydrolysed samples.

The differentiation between the berries and model solutions can primarily be attributed to the presence of alcohols, esters and acids in the model solutions, which were obtained from fresh skin berries, and the fact that the berries were characterised as being rich in aldehydes. Alcohols and esters are well-known compounds identified in hydro-alcoholic solutions like our model solution (Genovese *et al.*, 2013b). While aldehydes are found in both skin and pulp, their concentrations and their number are higher in the pulp (Wu *et al.*, 2016).

The 4-way ANOVA showed the differences between GRE and CAR in terms of volatile organic compounds (Table 3) to be significant. The CAR grape variety is widely used in blended wines. Consequently, it is unsurprising that GRE was found to be the most aromatic in our study, despite being considered a neutral grape variety. Significant volatile organic compounds associated with the GRE variety have primarily been linked to citrus notes (such as nonanal, citronellol, limonene and hex-3-en-1-ol), floral and fruity aromas (including 2-nonanol, ethyl acetate and 2-phenylethanal), and green/woody notes (such as heptanol) (Carpena *et al.*, 2021; Cullere *et al.*, 2011). This delicate fruity-citric-floral aroma profile results from the combination of these compounds present at low levels (Ferreira and Lopez, 2019). The presence of 2- or 3-methylbutanol observed in the CAR variety originates from the amino acids isoleucine and leucine respectively through the yeast Ehrlich pathway (Gutierrez-Gamboa & Moreno-Simunovic, 2019). In high concentrations, these alcohols can adversely affect wine quality.

The significance of hydrolysis was primarily demonstrated for vitispirane and linalool (Table 5; $p < 0.001$). Vitispirane is a C13-norisoprenoid resulting from the oxidative enzymatic degradation of carotenoids, while linalool is a monoterpene. Present in grapes as glycosidic precursors, their hydrolysis, whether enzymatic or chemical, results in the formation of fragrant compounds that contribute to wine aroma (Perestrelo *et al.*, 2011). The commonly identified compounds β -ionone, geraniol, TDN and eugenol were not detected in this study, because CAR and GRE are neutral varieties and are low in glycosidic precursors.

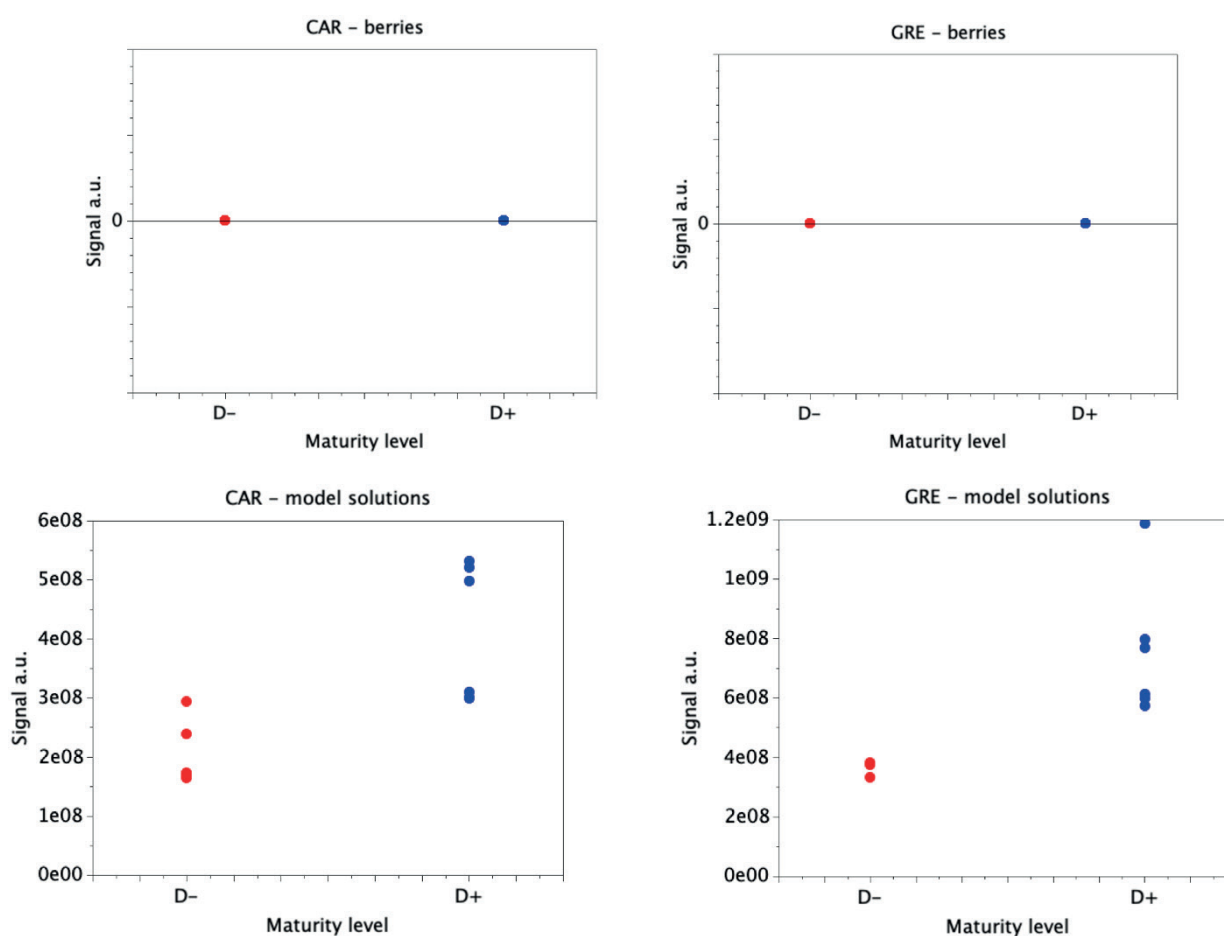
Finally, the significance of the maturity effect (D+/D-) is summarised in Table 3. Octanol, octanoic acid and hex-2-enal were more intense in the D- berries. C6 compounds like hex-2-enal, are lipoxygenation compounds, characteristic of immature berries (Carlomagno *et al.*, 2016). Grape-based alcohols and fatty acids, such as octanol and octanoic acid, are also more prevalent in immature berries (Barbara *et al.*, 2020; Arias *et al.*, 2019). 2,4,5-trimethyl-1,3-dioxolane was not detected in the berries, but it was observed in the model solutions (Figure 3). This acetal is a product of fermentative processes, formed by the condensation reaction between glycerol and acetaldehyde in acidic conditions (Coetzee, 2014), and it contributes to the 'old port-like' aroma perceived in some wines. However, our model solutions did not undergo a fermentation step and did not contain glycerol, suggesting another origin for this compound. Maturation led GRE D+ and CAR D+ to higher concentrations of dioxolane.

6. Skin and seed phenolic composition

Tannins and anthocyanins were the predominant phenolic compounds in all the grape berries, which were accompanied by lower amounts of hydroxycinnamic acids and flavonols, as is generally observed for red varieties (Table S3). However, large differences between the varieties were observed: GRE skins contained significantly higher levels of hydroxycinnamic acids and lower concentrations of anthocyanins and flavonols

TABLE 3. Significant volatile organic compounds (VOCs) for three effects: variety, degree and hydrolysis.

Variety effect				
VOCs	pvalue	CAR	GRE	higher in
2/3-methylbutanol	$7.22 \cdot 10^{30}$	$1.50 \cdot 10^9$	0	CAR
nonanal	$4.55 \cdot 10^9$	$2.11 \cdot 10^7$	$3.69 \cdot 10^7$	GRE
citronellol	$3.16 \cdot 10^7$	$4.38 \cdot 10^7$	$9.26 \cdot 10^7$	GRE
heptanol	$5.04 \cdot 10^7$	$2.20 \cdot 10^7$	$2.76 \cdot 10^7$	GRE
2-nonanol	$8.00 \cdot 10^7$	$5.54 \cdot 10^6$	$23.8 \cdot 10^6$	GRE
limonene	$1.72 \cdot 10^6$	0	$2.37 \cdot 10^7$	GRE
ethyl acetate	$3.48 \cdot 10^6$	$1.02 \cdot 10^9$	$1.16 \cdot 10^9$	GRE
2-phenylethanal	$1.84 \cdot 10^5$	$1.66 \cdot 10^6$	$11.8 \cdot 10^6$	GRE
hex-3-en-1-ol	$2.66 \cdot 10^5$	$1.43 \cdot 10^7$	$2.38 \cdot 10^7$	GRE
Degree effect				
VOCs	pvalue	D-	D+	higher in
octanol	$2.87 \cdot 10^2$	$3.99 \cdot 10^7$	$2.90 \cdot 10^7$	D-
hex-2-enal (unknown isomer)	$3.44 \cdot 10^2$	$3.44 \cdot 10^8$	$3.29 \cdot 10^8$	D-
octanoic acid	$4.36 \cdot 10^2$	$3.17 \cdot 10^9$	$2.84 \cdot 10^9$	D-
2,4,5-trimethyl-1,3 dioxolane	$4.79 \cdot 10^2$	$1.09 \cdot 10^8$	$2.52 \cdot 10^8$	D+
Hydrolyse effect				
VOCs	pvalue	H	NH	higher in
vitispirane	$3.65 \cdot 10^{-13}$	$4.78 \cdot 10^7$	$2.00 \cdot 10^7$	H
linalool	$4.64 \cdot 10^6$	$3.41 \cdot 10^7$	$1.58 \cdot 10^7$	H

**FIGURE 3.** Ion intensities of dioxolane for D-/D+ effect per variety and matrix (berry vs. model solution).

than CAR skins. A significant increase in the concentrations of non-acylated anthocyanins was also observed with the ripening of both varieties, while flavonols accumulated only in GRE. Regarding anthocyanins, there was a higher proportion of coumaroylated anthocyanins in CAR than in GRE, but their concentration significantly decreased with the ripening of CAR. The concentration of coumaroylated anthocyanins is known to depend on the variety, but it is also affected by vine growing conditions (Downey *et al.*, 2003): in particular, the accumulation of these compounds has been found to increase with heat (Spayd *et al.*, 2002) and with water stress after veraison (Olle *et al.*, 2011). Losses of anthocyanins during overripening have also been reported (Fournand *et al.*, 2006). Here, the concentration of seed flavanols was higher in GRE than in CAR and decreased with ripening, but the differences were only significant between GRE D- and CAR D+. Flavanols were abundant both in the skins and seeds, with much higher concentrations in the seeds. Skin flavanols contained higher proportions of epigallocatechin units (10–12 % Egc) and showed higher degrees of polymerisation (mDP 20) than seed flavanols (0.1 % Egc, mDP 6), which were more galloylated (22–25 % Ec-G), as reported earlier. There was no significant impact of variety or ripening stage on flavanol composition, except for the proportion of galloylated units (2 % Ec-G in CAR, 5.5 % in GRE D+, 6.3 % in GRE D-), and epigallocatechin units (higher in skins and negligible or missing in seeds). Phenolic compounds were also analysed by size exclusion chromatography (SEC), as shown in Figure 4. The concentrations of skin tannins (DP > 3) measured by SEC were similar to those determined by phloroglucinolysis, except for CAR D-, for which they were higher; this suggests that part of the higher molecular weight species determined by SEC were derived tannins that are not taken into account by phloroglucinolysis, such as tannin anthocyanin adducts, which were also present in significantly

higher amounts in this sample. The concentration of seed tannins measured by SEC also exceeded that measured by phloroglucinolysis, especially in CAR, suggesting a higher extent of seed oxidation in these samples, since browning of the seed coat has been attributed to oxidation markers (Rousserie *et al.*, 2019).

7. Classical wine analysis

The classical wine analysis results are reported in supplementary material (Table S4). As expected, the D+ batches showed higher alcohol content than the D- batches. The highest value was obtained for GRE D+, with 14.2 % ethanol. Also as expected, malolactic fermentation did not take place, since malic acid was still present and lactic acid was missing. This is not usual in traditional red winemaking. However, in the context of our experiment, any side effects of malolactic fermentation were thereby avoided, giving a better picture of the extraction process.

8. Wine polyphenol quantification

Total pigments, total polyphenol index and colour intensity, as well as the concentrations of anthocyanins and flavonols, were significantly higher in CAR wines than in GRE wines, and they increased with ripening. The concentrations of hydroxycinnamic acids and flavanols were significantly lower in CAR wines and increased with ripening in GRE wines only. The percentage of epicatechin-gallate units was higher in wine than in skins, indicating some contribution of tannins from the seeds or perhaps the pulp. The differences observed in the wines generally reflect those observed in the grapes. However, the comparison of wine composition with grape berry potential indicates large differences in recovery rates between the four experimental batches (GRE D-, D+ and CAR D-, D+) for different groups of compounds. Significantly higher proportions of grape anthocyanins were recovered in GRE wines and these

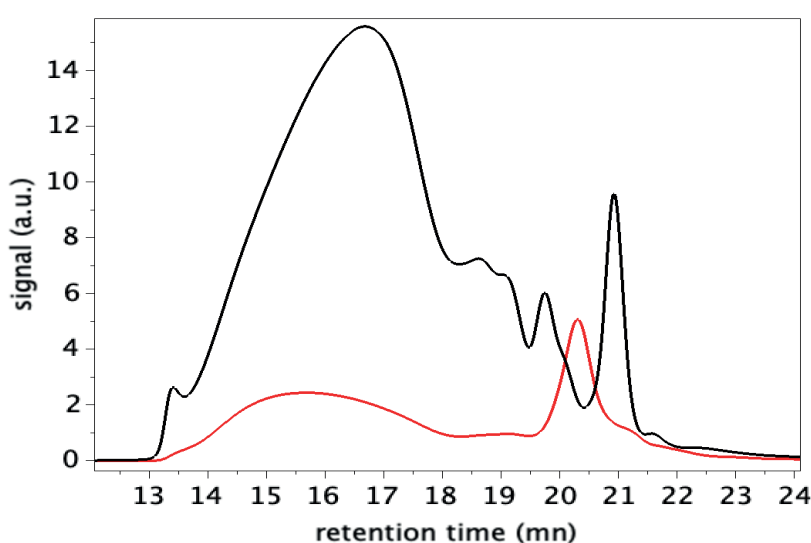


FIGURE 4. Size exclusion chromatogram (SEC) of GRE skins (red) and seeds (black). Skin anthocyanins correspond to the red peak after 20 min. For seeds, tannins (flavanols) of mDP ≥ 3 were eluted before 19 mn, non-galloylated dimers and galloylated monomers of flavanol just before 20 mn, and catechin and epicatechin (flavanol monomers) around 21 mn.

proportions also increased with ripening. The recovery of coumaroylated anthocyanins was much lower than that of non-acylated anthocyanins. The lower extractibility of coumaroylated anthocyanins has been reported in the literature (Downey *et al.*, 2003; Abi-Habib *et al.*, 2021) and has been attributed to the higher affinity of these compounds for grape cell walls (Goncalves *et al.*, 2012; Abi-Habib *et al.*, 2021). In the present study, only a small proportion of grape tannins was recovered in the wines, being higher in GRE, especially when considering only skin tannins. The mDP values of the wines were significantly higher in GRE than in CAR, but much lower than in the skins and seeds. This suggests that larger molecular weight tannins were not extracted or were selectively adsorbed onto plant cell walls (Fournand *et al.*, 2006; Bindon *et al.*, 2010; Abi-Habib *et al.*, 2021; Abi-Habib *et al.*, 2022) and/or lees (Vernhet *et al.*, 2020) as shown earlier. However, reactions undergone by flavanol monomers and proanthocyanidins during winemaking can lead to a misestimation of mDP values, as some of the derived units are not taken into account by phloroglucinolysis. The concentrations of polymers measured by SEC were higher than those of total flavanol units determined by phloroglucinolysis, confirming that polymers comprise additional units that were not identified by the latter method. The significantly higher excess of polymers in CAR wines indicates a higher conversion of flavanols and anthocyanins to derived tannins and pigments, which may have contributed to the lower recovery rate of anthocyanins and tannins calculated for these wines.

9. Wine polysaccharide quantification

The proportions of the main neutral sugars derived from polysaccharides are reported in supplementary material (Table S4). No difference was observed between D- and D+. Slight differences were observed between GRE and CAR: CAR contained more rhamnose, arabinose, xylose and galactose, while GRE contained more glucose, mannose, and galacturonic acid. However, this global composition is not accurate enough to identify the original polysaccharides.

DISCUSSION

The large number of analyses which was undertaken was not balanced with the number of observations; with only two grape varieties x two levels of maturity, it is the main weakness of this work. Therefore, the results should be regarded as observations rather than as evidences. However, while they may not have provided answers to our questions, they brought to light several new relevant questions.

Data related to the composition of the waxes on the skin cuticule are relatively sparse in the literature, since the cuticule is not expected to contribute to the final wine composition; however, it has been found to contribute to the growth of *Saccharomyces cerevisiae* (Flanzy, 1998). Waxes are also involved in the mechanical properties of the berries (Chang & Keller, 2021), with possible consequences regarding the extraction of polyphenols, polysaccharides and aromas, since the cuticule is the first protective layer of the berry.

In the present study, few differences in wax composition were observed between the two varieties GRE and CAR, and between the D-/D+ maturation levels, maybe because they are both from the *Vitis vinifera* species. However, as resistance to downy and powdery mildew in the new and disease-resistant varieties is supported by genes from a non-*Vitis* genus (*e.g.*, *Muscadinia rotundifolia*), modifications in the phenotype are to be expected. It would be of great interest to characterise the skin cuticule in order to evaluate its contribution to resistance.

Dioxolane is usually formed during fermentation, thus its absence in the berries was expected. It was therefore surprising to find it in the model solutions following extraction from the berries. The experiment lasted a few hours while ethanol was being added, thus it was not likely that fermentation occurred. Moreover, significantly higher values of dioxolane were observed in the ripest berries in both GRE and CAR. One possible explanation is that dioxolane was already present in the berries; rather than being the result of the sample preparation process before analysis, its extraction may be due to the presence of ethanol and may have been facilitated by the degradation of the cell walls during maturation.

¹³C solid-state NMR on alcohol insoluble residues (AIRs) from cell walls from skins or pulps revealed the presence of large quantities of tartaric acid, while malic acid was missing. Tartaric and malic acids are the two main acids found in must. Tartaric acid is also present in caftaric (caffeoyl-tartaric), p-coutaric (para-coumaroyl-tartaric) and fertaric (feruloyl-tartaric) acids, caftaric being the most important non-flavonoids of the must, at a concentration of around 100 mg/L (Cheynier *et al.*, 1989). All three are located in the vacuole, hence the extraction process leading to AIRs should have removed them, as was observed for other acids, such as malic acid. Thus, the presence of a strong tartaric signal in the NMR suggests that caftaric, p-coutaric and/or fertaric acids were bound to the AIRs. Effectively, p-coutaric acid are found mainly in skins, due to stronger bonds, while caftaric and fertaric acid are mainly found in musts (Garrido & Borges, 2013). It would be necessary to explain such an apparently high signal from tartaric acid, if it were due only to these three acids. If another form of tartaric acid had been present in the AIRs, then it would likely have played a role in the interactions and thus in the extraction process.

Sugars are located in the pulp. NMR water mobility by ¹H MRI showed lower sugar concentrations in the vicinity of the skin. This region corresponds to the hypoderm, which is between the epiderm (skin) and the pulp. During maturation, hypoderm cells evolve into pulp cells, and cell walls become thinner and decrease in lower polysaccharide content. They also loose their tannins located in the vacuoles (Fougere-Rifot *et al.*, 1996). Hypoderm thickness could help to characterise the different stages involved in the maturation. Measurements can be performed using images, such as those in Figure 1.

The present work drew our attention to two p-coumaroylated families of compounds: p-coumaroylated anthocyanins (*e.g.*,

with malvidin-3-glucoside) and p-coutaric acid (with tartaric acid). All these compounds are more difficult to extract from the skins to which they are bound than their counterparts, non acylated or caffeoyl anthocyanins (Abi-Habib *et al.*, 2021; Abi-Habib *et al.*, 2023) and caftaric or fertaric acids (Garrido & Borges, 2013). This indicates that the p-coumaroyl part of these molecules plays a specific role in the adsorption mechanism, which warrants further study.

Seeds were not expected to contain epigallocatechin; however, small quantities were detected due to the sensitivity of HRMS analysis.

CONCLUSION

The phenolic composition of berries has been investigated in depth. Polysaccharidic composition is usually analysed following depolymerisation, giving the main families (pectins, RGII, etc). ¹³C solid state NMR can also give a general picture of cell wall composition, contributing to its characterisation.

However, in addition to cell wall composition, an in-depth knowledge of the functioning of cell walls is necessary for an in-depth understanding of phenolic extraction. What happens at a molecular level (*e.g.*, polyphenol binding onto pectins) depends on the degradation state of cell walls, which evolves during maturation; for example, cell wall pectins tend to form a gel, thus impacting water mobility. The degradation state could be assessed by imagery using NMR water mobility, provided the accuracy of the method is improved. Other imagery approaches would also need further evaluation. Investigations at tissue scale could be complementary to chemical composition for a better understanding and prediction of phenolic extraction and stabilisation.

ACKNOWLEDGEMENTS

This work was funded by the INRAE Transform division (PF4CEPIA-AIC project) and by the French Research Agency as part of the Interfaces project (ANR-10-LABX-001-01).

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