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Title: Multi-method study of the impact of fermentation on the polyphenol composition and color of Grenache, Cinsault, and Syrah rosé wines

Cécile Leborgne[a,b], Marie-Agnès Ducasse[d], Emmanuelle Meudec[a,c], Stéphanie Carrillo[a], Arnaud Verbaere [a,c], Nicolas Sommerer[a,c], Matthias Bougreau[b], Gilles Masson[b], Aude Vernhet[a], Jean-Roch Mouret[a], and Véronique Cheynier[a,c,*]

[a] SPO, INRAE, Univ Montpellier, Institut Agro, Montpellier, France; emmanuelle.meudec@inrae.fr (E.M.); stephanie.carrillo@inrae.fr (S.C.) ; arnaud.verbaere@inrae.fr (Ar.V.); nicolas.sommerer@inrae.fr (N.S.) ; aude.vernhet@inrae.fr (Au.V.); jean-roch.mouret@inrae.fr (J.-R.M.); veronique.cheynier@inrae.fr (V.C.)

[b] Institut Français de la Vigne et du Vin, Centre du rosé, Vidauban, France; cecile.leborgne@inrae.fr (C.L.); matthias.bougreau@vignevin.com (M.B.); gilles.masson@vignevin.com (G.M.)

[c] INRAE, PROBE research infrastructure, Polyphenol Analytical Facility, Montpellier, France;

[d] Institut Français de la Vigne et du Vin, UMT Oenotypage, Domaine de Pech Rouge, France; marie-agnes.ducasse@vignevin.com (M-A.D.)

Corresponding author: Véronique Cheynier (veronique.cheynier@inrae.fr)

Abstract:

Rosé wines show large color diversity, due to different phenolic pigment compositions. However, the mechanisms responsible for such diversity are poorly understood. The present work aimed at investigating the impact of fermentation on the color and composition of rosé wines made from Grenache, Cinsault, and Syrah grapes. Targeted MS analysis showed large varietal differences in must and wine compositions, with higher concentrations of anthocyanins and flavanols in Syrah. UV-visible spectrophotometry and size exclusion chromatography data indicated that Grenache and Cinsault musts contained oligomeric pigments derived from hydroxycinnamic acids and flavanols which were mostly lost during fermentation due to adsorption on lees. Syrah must color was mainly due to anthocyanins which were partly converted to derived pigments through reactions with yeast metabolites with limited color drop during fermentation. This work highlighted the impact of must composition, reflecting varietal characteristics, on changes occurring during fermentation and consequently wine color.

Keywords: wine; polyphenols; fermentation; rosé wine color; HPSEC; UPLC-QqQ-MS

1. Introduction

Color is one of the key elements for the marketing of rosé wines (Peres et al., 2020). Their broad range of color is due to the presence of pigments belonging to phenolic compounds extracted from grapes or formed during wine-making. The major pigments in *Vitis vinifera* grapes and wines are red anthocyanins, represented mainly by malvidin-3-O-glucoside. The specific rosé wine-making process, involving controlled maceration of skins in the juice before a liquid phase alcoholic fermentation, limits extraction, resulting in different proportions of phenolic compounds in rosé wines compared to red wines. Indeed, studies on red and rosé wines made from Grenache showed that hydroxycinnamic acids are the main phenolic compounds of rosé wines whereas flavanols are predominant in red wines (Wirth et al., 2012). Moreover, a recent study performed on 268 commercial rosé wines showed that, although color intensity depends on extraction of phenolic compounds from grape skins, the different nuances of light and more intense rosé wines are due to different pigment compositions (Leborgne et al., 2022).

Hydroxycinnamic acids and their derivatives are colorless. Nevertheless, enzymatic oxidation of hydroxycinnamoyltartrates, especially caftaric acid, is known to be involved in the browning of white musts and may also occur in rosé wine-making, as pressing takes place before fermentation when polyphenoloxidase is still active. This process involves oxidation of hydroxycinnamoyltartrates to caftaric acid *o*-quinone, followed by addition of glutathione to form Grape Reaction Product (GRP), i.e. 2-S-glutathionyl caftaric acid (Cheynier et al., 1986). After glutathione depletion, *o*-quinones can proceed to brown oxidation products (Sarni-Manchado et al., 1997). Caftaric acid o-quinones can also add to anthocyanins, generating anthocyanin-caftaric adducts (Sarni-Manchado et al., 1997). Free hydroxycinnamic acids or vinylphenols resulting from their decarboxylation react with anthocyanins to form phenylpyranoanthocyanin pigments (Fulcrand et al., 1996; Schwarz et al., 2003).

Flavanols found in grapes and wines include monomers (catechins) and oligomers and polymers, i.e. proanthocyanidins (PA). They are colorless but can be involved in oxidative browning (Es-Safi et al., 1999; Guyot et al., 1996) and react with anthocyanins to form derived pigments (Cheynier et al., 2006; Leborgne et al., 2022).

In particular, flavanol and anthocyanin condensation with acetaldehyde, which can be produced by yeast metabolism and but also by oxidation, leads to purple ethyl linked anthocyanin-flavanol adducts (Somers, 1971). These molecules are unstable and yield flavanylpyranoanthocyanin (Mateus et al., 2002) and other products through random reaction cascades (Vallverdú-Queralt et al., 2017).

During alcoholic fermentation, the reactions of anthocyanins with yeast aldehyde metabolites such as acetaldehyde or pyruvic acid generate orange pigments, pyranoanthocyanins (e.g. vitisin B, i.e. pyranomalvidin 3-glucoside) and carboxy-pyranoanthocyanins (e.g. vitisin A, i.e. carboxypyranomalvidin 3-glucoside) respectively (Bakker & Timberlake, 1997; Benabdeljalil et al., 2000; Fulcrand et al., 1998).

Adsorption of phenolic compounds and in particular of flavanols and anthocyanin pigments on yeasts is also known to impact wine color, depending on the strain (Morata et al., 2003). Preferential interactions of acylated anthocyanins (Morata et al., 2005) along with oligomeric proanthocyanidins (Vernhet et al., 2020) with yeast cells have been demonstrated, accounting for 2 to 8% of the color loss observed during liquid phase fermentation of red must (Morata et al., 2003, 2005; Vernhet et al., 2020).

All above mentioned studies have been performed in red wine or model solutions mimicking red wines while very limited data is available on rosé wines. The few investigations performed on rosé wines showed that their phenolic composition is highly variable, close to that of red wines for the darker rosés but very different for the light ones (Lambert et al., 2015; Leborgne et al., 2022; Wirth et al., 2012). Moreover, large variations in the extent of color loss taking place during fermentation have

87 been reported to be highly variable but the mechanisms responsible for the observed color loss and
88 causes of such variability are unknown.
89 The hypothesis of the present work was that the color and composition of light and darker rosé wines
90 are driven by different mechanisms occurring during alcoholic fermentation, depending on the must
91 composition. To test this hypothesis, the reactions and adsorption on yeast lees of phenolic
92 compounds and their role in color and composition changes during alcoholic fermentation of rosé
93 musts were investigated. To this aim, three different *Vitis vinifera* grape varieties commonly used for
94 the elaboration of rosé wines in French Provence area were selected for their different color potential:
95 Grenache, Syrah, and Cinsault (Cayla et al., 2011).
96
97 ## 2. Material and methods
98 ### 2.1. Chemicals
99 HPLC grade formic acid, methanol and N,N-dimethyformamide were purchased from VWR Prolabo
100 (Fontenay-sous-Bois, France). Acetaldehyde, acetic acid, acetone, hydrochloric acid, lithium chloride,
101 phloroglucinol, L-ascorbic acid, sodium hydrogen sulfite and ammonium formiate were purchased
102 from Sigma Aldrich. Deionized water was obtained from a Milli-Q purification system (Millipore,
103 Molsheim, France).
104 Standards of caffeic acid (>99%), vanillic acid (>99%) and ferulic acid (>99%) were purchased from Fluka
105 (Buchs, Switzerland). Standards of syringic acid (>99%), *p*-coumaric acid (>98%),-quercetin (>95%), (+)-
106 catechin (>99%), (-)-epicatechin (>98%), (-)-epicatechin 3-*O*-gallate (>97.5%), protocatechuic acid
107 (>95%), reduced and oxidized glutathione (>98%) were purchased from Sigma-Aldrich (St. Louis, MO,
108 USA). Standards of malvidin 3-*O*-glucoside chloride (>95%) and malvidin 3,5-diglucoside chloride
109 (>97%) were purchased from Extrasynthese (Geney, France). Standards of trans-caftaric acid (>94%),

quercetin 3-*O*-glucoside (>90%) and procyanidin dimer B2 (>90%) were purchased from Phytolab (Vestenbergsgreuth, Germany).

2.2. Oenological analysis

Free sulfites, total sulfites, sugar and assimilable nitrogen contents were measured with a Fourier-transform infrared (IRTF) spectrometer (Foss, Nanterre, France) following the OIV method (OIV, 2019). A pHmeter (Mettler Toledo®, Viroflay, France) was used to measure pH in samples. A turbidimeter (Mettler Toledo®, Viroflay, France) was used to measure turbidity.

2.3. Must preparation

The three different *Vitis vinifera* grape varieties Cinsault, Grenache and Syrah were harvested and processed by the Centre du Rosé (IFV, Vidauban, France) in September 2018. A hundred kg of grapes were hand harvested in 20 kg grape containing bins in order to avoid grape crushing and uncontrolled maceration. At the arrival to the cellar, grapes were kept at 12 °C and processed the day after for all grapes to be treated at the same temperature. Grapes were destemmed and crushed with addition of pectolytic enzyme (Kzym 4 g/100 kg, 26800 polygalacturonase units/100 kg, Laffort, France) and added with 3 g/100 kg potassium hydrogen sulfite for protection against oxidation and microbial contamination (4 g/100 kg more potassium hydrogen sulfite was added to the Syrah must as harvest contained visual microbial contamination on grapes). Musts were then submitted to controlled maceration at 18 °C during 2 hours for Syrah, 4 hours for Grenache and 12 hours for Cinsault, according to standardized wine-making process developed by the Centre du Rosé. After maceration, grapes were pressed with addition of 1.5 g/100 kg potassium hydrogen sulfite to protect from oxidation during the press cycle. The free run juice and the press juice were recovered separately. Twenty liters of free-run and first press juice were sampled to constitute musts with “low” extraction level (Syrah1, Grenache1, and Cinsault1). Then, 10 L of that juice was pooled with 10 L of end press juice to elaborate musts with “high” extraction level (Syrah2, Grenache2, and Cinsault2). Musts were clarified by cold settling at 12

°C before racking. Analysis of the musts were performed at the Centre du Rosé. Clarified musts and solid particles were stored at -20 °C until use.

After thawing and centrifugation (4000 *g*, 5 °C, 10 min) (Jouan, Nantes, France) of each must, pH was adjusted to 3.5 using hydrochloric acid (50%, *v/v*) or sodium hydroxide (32%, *w/w*). Solid particles collected after racking were added to the musts to reach a turbidity of 135 NTU (Nephelometric Turbidity Unit) and sugar concentration was normalized at 250 g/L by the addition of a fructose and glucose mixture (50:50, v/v). Assimilable nitrogen content was at 163 mg/L for both Grenache musts, 148 mg/L for both Syrah musts and 185 mg/L for both Cinsault musts. Musts were conditioned in 2L buckets before distributing into fermenters (Legallais, Montferrier-sur-Lez, France). For each must, three aliquots of 50 mL were taken for color analysis that was performed on the same day at the experimental unit of Pech Rouge (IFV, Gruissan, France). Twenty 1 mL aliquots were sampled for polyphenol analysis and stored at -80 °C until analysis.

2.4. Fermentation

Grenache and Cinsault fermentations were conducted in quadruplicate in 300 mL fermenters filled with 250 mL of must (controlled by weighing). Syrah fermentations were performed in duplicate in the same containers. All fermenters and vials were protected from oxidation using inert gas Argon. Fermenters were equipped with fermentation airlock systems allowing to maintain anaerobiosis. All fermenters were placed in a thermoregulated room set at 16 °C and stirred at 150 rpm. Sulfites were added to Cinsault and Grenache musts using a potassium hydrogen sulfite solution (222 g/L) to reach 50mg/L of total sulfite content, our target value. Syrah1 and Syrah2 were respectively at 101 mg/L and 89mg/L of total sulfite content before fermentation. All fermenters were maintained at 16 °C for 24h before inoculation.

Active dry yeasts (K1 strain, ICV, France) were rehydrated for 20 min at 37 °C using a glucose solution in water (50 g/L) before inoculation at 0.4 g/L. Fermentation kinetics were manually monitored by

158 weight losses induced by CO_2 release. Fermentations were considered achieved when no weight loss
159 higher than 0.1 g per 24 hours was observed. All wines were fermented to dryness (less than 2 g/L
160 residual sugar) with no interruption in around 20 days. Yeasts were collected by centrifugation (4000
161 g, 10 °C, 15 min), frozen and stored at -20 °C until analysis. For each fermenter, 50 mL of wine were
162 taken for color analysis and 5 samples of 1 mL sampled for polyphenol analysis and stored at -20 °C
163 until analysis.
164 To provide control yeast samples, fermentations in synthetic grape must were carried out under the
165 same conditions in triplicate. The synthetic must contained 425 mg/L of assimilable nitrogen, 200 g/L
166 of sugar (glucose:fructose), salts, vitamins and weak acids as described by Bely et al., 1990.

167 2.5. Desorption from lees

168 The method used for polyphenol desorption from lees was adapted from Vernhet et al., 2020. Yeasts
169 recovered from each 250 mL fermentation were rinsed with 10 mL of distilled water, freeze-dried and
170 weighed to determine the dry mass per mL. Three successive extractions were performed on 80mg of
171 dry yeast. Each extraction was performed by addition of 0.25 mL volume of solvent A (methanol, 1%
172 formic acid) followed by 5 minutes of sonication. Then, 1.75 mL volume of solvent B (acetone/water
173 (60/40), 1% formic acid) was added and the extraction carried out during 30 minutes alternating 5
174 minutes of sonication and 10 minutes of stirring before centrifugation (10000 *g*, 10 °C, 10 min). The
175 three extracts (3 x 2 mL) were pooled and separated in three 2 mL aliquots, evaporated to dryness
176 under vacuum using a Genevac (SP Scientific, Warminster, PA, USA) and stored at -80 °C.

177 2.6. UV-visible spectrophotometry

178 A Shimadzu UV-1900 UV–visible spectrophotometer was used to perform UV-Visible absorbance
179 measurements following the protocols described by Atanasova et al., 2002, using a 1 cm path length
180 cell and adapting dilution to obtain absorbance values between 0.01 and 1.

Direct absorbance measurements were achieved on wines and musts from 230 nm to 800 nm. The three CIELab color space parameters were calculated from the spectrum according to OIV method OIV-MA-AS2-11 (OIV, 2019) along with the hue angle ($H = \text{arctg}\frac{b*}{a*}$). Absorbance values at 520 nm (A_{520}), 420 nm (A_{420}), and 620 nm (A_{620}) were extracted from each spectrum and used to calculate color intensity ($CI = A_{420} + A_{520} + A_{620}$).

Total polyphenol index (TPI) at 280 nm, total red pigments (TRP) at 520 nm, total yellow pigments at 420 nm (TYP) and absorbance at 320 nm (A_{320}) were measured 20 minutes after dilution of wines, musts and lees extracts in 1 M HCl solution (TPI = $A_{280} \times F_D$ (dilution factor); TRP= $A_{520\text{-HCl 1M}} \times F_D$; TYP = $A_{420\text{-HCl 1M}} \times F_D$ and $A_{320} = A_{320\text{-HCl 1M}} \times F_D$). Dry lees extracts, were dissolved in 1 mL of MeOH:H_2O (1:1, v/v), 1% formic acid prior to dilution in 1 M HCl solution and absorbance values of control were subtracted from those of samples.

Absorbance at 520 nm was measured 5 minutes after addition of 60 µL of sodium hydrogen sulfite solution (200 g/L) to 4 mL of wine or must, to determine the sulfite bleaching resistant fraction of pigments (NBP= $A_{520\text{-SO2}}$). A_{520} was also determined on musts and wines 40 minutes after addition of 40 µL of a 12.6 % (v/v) acetaldehyde aqueous solution to 4 mL of samples to trap sulfites and convert bisulfite adducts to flavylium ions. This enabled calculation of the concentrations of bisulfite adducts (BA = $A_{520\text{-acetaldehyde}} - A_{520}$), flavylium forms (AH= $A_{520} - A_{520\text{-SO2}}$) and hydrated forms (HF = TRP - $A_{520\text{-acetaldehyde}}$) of pigments susceptible to sulfite bleaching.

2.7. High Performance Size-Exclusion Chromatography (HPSEC)

Wines (1 mL) and yeast extracts (2 mL, corresponding to 27 mg dry yeast) were evaporated to dryness under vacuum using a Genevac (SP Scientific, Warminster, PA, USA) and the residue dissolved in 500 µL of the HPSEC mobile phase composed of N,N-dimethylformamide (DMF), 1% (v/v) glacial acetic acid, 5% (v/v) water, and 0.15 M lithium chloride. To eliminate sugar, must samples were previously purified by solid phase extraction on 500 mg/3 mL tC18 cartridges (Strata®, 55 µm, 140 Å, Phenomenex, Le

205 Pecq, France), according to the method described by (Oszmianski et al., 1988). After loading the sample
206 (1 mL), elution of sugars was achieved using 3 mL of water containing 1% (v/v) formic acid and
207 monitored by refractometry until no longer detectable (< 0.5% Brix). Phenolic compounds were then
208 desorbed using 3 mL of acetone/water (60/40, v/v) acidified with 1% (v/v) formic acid and taken to
209 dryness under vacuum using a Genevac (SP Scientific, Warminster, PA, USA). The dry residue was
210 dissolved in 500µL of the HPSEC mobile phase. Polyphenol loss was evaluated by comparing
211 absorbance values obtained on the fractions with those of the initial musts.
212 Separation was achieved on an Agilent 1260 HPLC system (Agilent, France) constituted of a vacuum
213 degasser, an autosampler chamber set at 15 °C, an isocratic pump, a column heater set at 60 °C, and a
214 diode array detector and equipped with a guard column containing highly cross-linked polystyrene-
215 divinylbenzene (50 x 7.8 mm, 5 µm) and 3 PLgel columns (Phenomenex, Le Pecq, France) composed of
216 the same material (300 x 7.8 mm, 5 µm and 50, 1000 and 1000000 Å pore size) connected in series
217 (Kennedy & Taylor, 2003). The flow rate was set at 1 mL/min and the polyphenol elution time was
218 calibrated using commercial standards (caffeic acid (>99%), *p*-coumaric acid (>98%), catechin (>99%),
219 malvidin 3-*O*-glucoside chloride (>95%), procyanidin dimer B2 (>97.5%) and A2 (>99%) and tannin
220 fractions with different polymerization degrees previously purified in the laboratory (Vernhet et al.,
221 2014). Data were extracted and analyzed using the Excel software.
222 2.8. Polyphenol targeted analysis
223 Polyphenols in musts and wines were analyzed by ultra high-performance liquid chromatography
224 coupled to triple-quadrupole mass spectrometry (UHPLC-QqQ-MS) in the multiple reaction monitoring
225 (MRM) mode, according to the method described by (Lambert et al., 2015). UHPLC-QqQ-MS analyses
226 directly performed on the musts/wines allowed detection and quantification of 106 phenolic
227 compounds belonging to the following groups: hydroxybenzoic acids, hydroxycinnamic acids,
228 flavonols, flavanol monomers, dimers, and trimers, anthocyanins and their derivatives, namely

pyranoanthocyanins, carboxypyranoanthocyanins, phenylpyranoanthocyanins (catechyl, hydroxyphenyl, and guaiacylpyranoanthocyanins), flavanol-anthocyanin (F–A) and anthocyanin-flavanol (A–F) dimers, and anthocyanin-flavanol dimer adducts (F-ethyl-A), and ethyl linked flavanol dimers (F-ethyl-F). The list of phenolic groups, variables, and MRM transitions used are provided in supporting information S1. Results are expressed as the sums of concentrations of all variables in each group.

Constitutive units of proanthocyanidins were analyzed by UHPLC-DAD after acid-catalyzed depolymerization in the presence of phloroglucinol. The residue obtained after taking to dryness 1 mL of must or wine using a Genevac (SP Scientific, Warminster, PA, USA) was dissolved in 250 µL of a solution containing phloroglucinol (50 g/L) and ascorbic acid (1 g/L) in methanol at 0.2 N HCl. The phloroglucinolysis reaction was carried out for 20 minutes at 50 °C, and stopped by addition of 200 mM ammonium formiate buffer (250 µL), according to the method described by (Kennedy & Jones, 2001). An internal standard, 50 µL of *p*-hydroxybenzoic methyl ester in methanol was added in both wines and musts samples (0.3 g/L) after the reaction was completed. For yeasts, the depolymerisation reaction was performed directly on dry yeasts (27 mg) using 500 µL of phloroglucinolysis reagent and the same volume of ammonium formiate buffer at 200mM. Constitutive units of flavanol derivatives released after acid-catalyzed depolymerization in the presence of phloroglucinol were analyzed by UHPLC using a Vanquish UHPLC equipped with an Ethylene Bridged Hybrid C18 (1.0 x 150 mm, 1.7 µm) and a diode array detector (DAD) used for quantification. Standards of catechin and epicatechin were used for the quantification of flavanols and their phloroglucinol adducts. Total flavan-3-ol units released by phloroglucinolysis were calculated. The average degree of polymerisation (mDP) was also evaluated as the ratio of all flavan-3-ol units after phloroglucinolysis to the sum of terminal units.

2.9. Statistical analysis

253 ANalysis Of VAriance were performed with RStudio version 4.0.3 software (www.rstudio.com (2020-
254 10-10)) using Student-Newman-Keuls post-hoc test in Agricolae package. ANOVA results were
255 considered significant at p-value < 0.05.
256
257 3. Results and discussions
258 3.1. Musts
259 3.1.1.Spectrophotometry analysis

Table 1: UV-visible spectrophotometry analysis performed on musts, wines and lees under various conditions. All values are given in absorbance units except for L*,a*,b* and hue. Absorbance values of lees were reported to the initial dry mass of lees and wine volume. Different superscript letters indicate significant differences between all samples (wines, musts and lees) for a given parameter (ANoVa with SNK test for *p-value* < 0.05).

	Must					
	Cinsault1	Cinsault2	Grenache1	Grenache2	Syrah1	Syrah2
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
L*	69.95 ± 2.90 ^{g}	52.43 ± 1.07 ^{h}	89.75 ± 1.66 ^{c}	85.18 ± 3.45 ^{d}	78.80 ± 0.99 ^{e}	73.15 ± 0.92 ^{fg}
a*	20.50 ± 1.25 ^{e}	39.70 ± 0.23 ^{b}	2.59 ± 0.33 ^{i}	5.84 ± 0.95 ^{g}	31.60 ± 0.98 ^{d}	36.49 ± 0.47 ^{c}
b*	38.97 ± 0.91 ^{a}	34.84 ± 0.37 ^{b}	26.84 ± 0.66 ^{c}	35.49 ± 2.85 ^{b}	1.55 ± 0.13 ^{g}	3.47 ± 0.57 ^{f}
Hue angle (H)	1.09 ± 0.03 ^{e}	0.72 ± 0.01 ^{f}	1.47 ± 0.01 ^{a}	1.41 ± 0.01 ^{b}	0.05 ± 0.00 ^{i}	0.09 ± 0.02 ^{h}
Color Intensity (CI)	1.73 ± 0.16 ^{b}	2.65 ± 0.08 ^{a}	0.69 ± 0.07 ^{de}	1.00 ± 0.20 ^{c}	0.79 ± 0.05 ^{d}	1.06 ± 0.05 ^{c}
Total Polyphenol Index (TPI)	11.58 ± 1.11 ^{de}	16.18 ± 0.28 ^{a}	8.62 ± 0.11 ^{g}	10.03 ± 0.22 ^{f}	13.62 ± 0.15 ^{c}	15.31 ± 0.13 ^{b}
Total Red Pigments (TRP)	1.25 ± 0.19 ^{f}	2.42 ± 0.05 ^{e}	0.42 ± 0.02 ^{h}	0.57 ± 0.07 ^{g}	6.50 ± 0.08 ^{b}	7.47 ± 0.09 ^{a}
Total Yellow Pigments (TYP)	1.41 ± 0.17 ^{cd}	1.83 ± 0.06 ^{b}	0.61 ± 0.02 ^{f}	0.83 ± 0.13 ^{e}	1.77 ± 0.00 ^{b}	2.06 ± 0.02 ^{a}
A_{320}	7.33 ± 0.77 ^{bc}	11.37 ± 0.18 ^{a}	6.28 ± 0.06 ^{d}	6.81 ± 0.11 ^{cd}	7.34 ± 0.06 ^{bc}	7.90 ± 0.10 ^{b}
A_{520}	0.55 ± 0.05 ^{d}	1.07 ± 0.02 ^{a}	0.16 ± 0.02 ^{g}	0.24 ± 0.05 ^{f}	0.48 ± 0.03 ^{e}	0.63 ± 0.01 ^{c}
$A_{520\text{-acetaldehyde}}$	0.49 ± 0.04 ^{f}	0.96 ± 0.01 ^{c}	0.18 ± 0.00 ^{i}	0.28 ± 0.02 ^{g}	1.88 ± 0.01 ^{b}	2.08 ± 0.01 ^{a}
Flavylium cations (AH)	0.29 ± 0.12 ^{e}	0.75 ± 0.01 ^{a}	0.07 ± 0.02 ^{g}	0.09 ± 0.04 ^{g}	0.42 ± 0.03 ^{d}	0.52 ± 0.01 ^{c}
Bisulfite Adducts (BA)	-0.06 ± 0.06 ^{f}	-0.10 ± 0.02 ^{g}	0.02 ± 0.02 ^{e}	0.04 ± 0.05 ^{de}	1.39 ± 0.04 ^{b}	1.46 ± 0.01 ^{a}
Hemiketal Forms (HF)	0.76 ± 0.15 ^{g}	1.46 ± 0.05 ^{e}	0.23 ± 0.02 ^{hi}	0.29 ± 0.06 ^{h}	4.63 ± 0.07 ^{c}	5.38 ± 0.09 ^{a}
Non Bleachable Pigments (NBP)	0.26 ± 0.10 ^{b}	0.32 ± 0.02 ^{a}	0.09 ± 0.00 ^{cde}	0.15 ± 0.02 ^{c}	0.06 ± 0.00 ^{de}	0.10 ± 0.00 ^{cd}

	Wine					
	Cinsault1	Cinsault2	Grenache1	Grenache2	Syrah1	Syrah2
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
L*	93.48 ± 0.33 ^{b}	86.28 ± 0.40 ^{d}	97.93 ± 0.10 ^{a}	97.93 ± 0.10 ^{a}	75.35 ± 0.21 ^{f}	72.30 ± 0.28 ^{fg}
a*	4.19 ± 0.37 ^{h}	15.77 ± 0.54 ^{f}	0.81 ± 0.15 ^{j}	1.14 ± 0.12 ^{j}	39.82 ± 0.04 ^{b}	42.41 ± 0.29 ^{a}
b*	9.69 ± 0.63 ^{d}	7.77 ± 0.21 ^{e}	4.85 ± 0.24 ^{f}	4.39 ± 0.10 ^{f}	4.24 ± 0.29 ^{f}	5.50 ± 0.16 ^{f}
Hue angle (H)	1.16 ± 0.05 ^{d}	0.46 ± 0.02 ^{g}	1.40 ± 0.03 ^{b}	1.32 ± 0.02 ^{c}	0.11 ± 0.01 ^{h}	0.13 ± 0.00 ^{h}
Color Intensity (CI)	0.32 ± 0.02 ^{f}	0.54 ± 0.02 ^{e}	0.11 ± 0.00 ^{g}	0.11 ± 0.00 ^{g}	0.98 ± 0.01 ^{c}	1.13 ± 0.02 ^{c}
Total Polyphenol Index (TPI)	6.63 ± 0.09 ^{h}	10.11 ± 0.09 ^{f}	5.01 ± 0.10 ^{i}	5.11 ± 0.14 ^{i}	11.03 ± 0.10 ^{e}	11.89 ± 0.01 ^{d}
Total Red Pigments (TRP)	0.44 ± 0.04 ^{h}	1.17 ± 0.02 ^{f}	0.15 ± 0.01 ^{i}	0.16 ± 0.01 ^{i}	5.03 ± 0.03 ^{d}	5.65 ± 0.03 ^{c}
Total Yellow Pigments (TYP)	0.25 ± 0.02 ^{h}	0.45 ± 0.02 ^{g}	0.09 ± 0.01 ^{i}	0.09 ± 0.01 ^{i}	1.35 ± 0.00 ^{d}	1.53 ± 0.03 ^{c}
A_{320}	3.98 ± 0.09 ^{d}	7.57 ± 0.05 ^{a}	4.09 ± 0.13 ^{d}	4.00 ± 0.08 ^{d}	6.59 ± 0.11 ^{c}	6.84 ± 0.02 ^{b}
A_{520}	0.10 ± 0.01 ^{h}	0.25 ± 0.01 ^{f}	0.03 ± 0.00 ^{i}	0.03 ± 0.00 ^{i}	0.63 ± 0.00 ^{c}	0.71 ± 0.01 ^{b}
$A_{520\text{-acetaldehyde}}$	0.10 ± 0.01 ^{j}	0.24 ± 0.01 ^{h}	0.03 ± 0.00 ^{k}	0.03 ± 0.00 ^{k}	0.74 ± 0.00 ^{e}	0.79 ± 0.00 ^{d}
Flavylium cations (AH)	0.06 ± 0.00 ^{g}	0.19 ± 0.01 ^{f}	0.02 ± 0.00 ^{g}	0.03 ± 0.00 ^{g}	0.56 ± 0.00 ^{bc}	0.62 ± 0.01 ^{b}
Bisulfite Adducts (BA)	0.00 ± 0.00 ^{ef}	-0.01 ± 0.01 ^{ef}	0.00 ± 0.00 ^{ef}	0.00 ± 0.00 ^{ef}	0.11 ± 0.01 ^{c}	0.08 ± 0.01 ^{cd}
Hemiketal Forms (HF)	0.34 ± 0.03 ^{h}	0.93 ± 0.02 ^{f}	0.12 ± 0.01 ^{i}	0.13 ± 0.01 ^{i}	4.28 ± 0.03 ^{d}	4.86 ± 0.03 ^{b}
Non Bleachable Pigments (NBP)	0.04 ± 0.00 ^{de}	0.06 ± 0.00 ^{de}	0.01 ± 0.00 ^{e}	0.01 ± 0.00 ^{e}	0.07 ± 0.00 ^{de}	0.09 ± 0.00 ^{cde}
	Lees					
	Cinsault1	Cinsault2	Grenache1	Grenache2	Syrah1	Syrah2
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Total Polyphenol Index (TPI)	0.62 ± 0.07 ^{bc}	1.41 ± 0.16 ^{a}	0.48 ± 0.13 ^{c}	0.67 ± 0.05 ^{bc}	0.85 ± 0.04 ^{b}	0.69 ± 0.06 ^{bc}
Total Red Pigments (TRP)	0.13 ± 0.01 ^{b}	0.28 ± 0.06 ^{a}	0.11 ± 0.05 ^{b}	0.15 ± 0.02 ^{b}	0.18 ± 0.00 ^{b}	0.18 ± 0.01 ^{b}
Total Yellow Pigments (TYP)	0.20 ± 0.02 ^{ab}	0.30 ± 0.07 ^{a}	0.19 ± 0.07 ^{ab}	0.24 ± 0.02 ^{a}	0.10 ± 0.00 ^{bc}	0.07 ± 0.01 ^{c}
A_{320}	0.64 ± 0.07 ^{bc}	1.44 ± 0.09 ^{a}	0.59 ± 0.12 ^{bc}	0.74 ± 0.05 ^{b}	0.48 ± 0.01 ^{c}	0.46 ± 0.01 ^{c}

260

Total polyphenol index (TPI) presented in Table1 showed differences between all six musts with significantly higher values for Syrah compared to Grenache musts. Cinsault1 was intermediate while Cinsault2 had higher absorbance values than Syrah musts. For all varieties, musts containing press juice showed higher TPI values than free-run juice, confirming higher polyphenol extraction rates. The total red pigment (TRP) levels were much higher in Syrah musts with values of 6.51 and 7.47, respectively, for Syrah1 and Syrah2, followed by Cinsault2 (2.41) and Cinsault1 (1.25) while values were very low in Grenache musts (0.42 and 0.57, respectively for Grenache1 and Grenache2). The same trend was observed for Total Yellow Pigment (TYP) values, ranging from 0.61 for Grenache1 to 2.06 for Syrah2. All TRP and TYP values were also significantly higher in musts containing press juice, again indicating higher pigment extraction rates. This was also observed on the A_{520} values measured directly on musts but these values were significantly higher in Cinsault than in Syrah. When A_{520} values were measured after addition of acetaldehyde ($A_{520\text{-acetaldehyde}}$), the impact of grape variety was very similar to that observed on TRP values. The concentration of bisulfite adducts was high in Syrah musts, representing about 20% of TRP, while it was very low in Grenache and Cinsault musts. Thus, Syrah musts contained larger concentrations of anthocyanin pigments than Cinsault but appeared less colored at wine pH because of the presence of higher concentrations of sulfites, causing sulfite bleaching. Values measured after addition of sulfite were significantly higher in Cinsault2 than in Cinsault1 and in both Cinsault musts compared to Grenache and Syrah musts. However, non-bleachable pigments represented a higher proportion of red pigments contributing to rosé must color (A_{520}) in Grenache (60%) than in Cinsault (47% and 30%, respectively in Cinsault1 and Cinsault2), and in Syrah (12% and 16%, respectively in Syrah1 and Syrah2). NBP represented 13 to 26% of total red pigments in Grenache and Cinsault musts but only 1% in Syrah musts.

Values of the L* parameter ranged from 50 to 90 and were negatively correlated with A_{520} measured directly on wine (R^2= -0.98) and with color intensity (R^2=-0.93). Must color also showed qualitative

differences. Syrah musts were red with high values of a*, low values of b* and a reddish hue while Grenache musts appeared as light salmon rosé with high values of b* compared to a* and a higher hue value. Cinsault musts showed high values of both a* and b* giving an orange hue.

3.1.2.Polyphenol targeted analysis

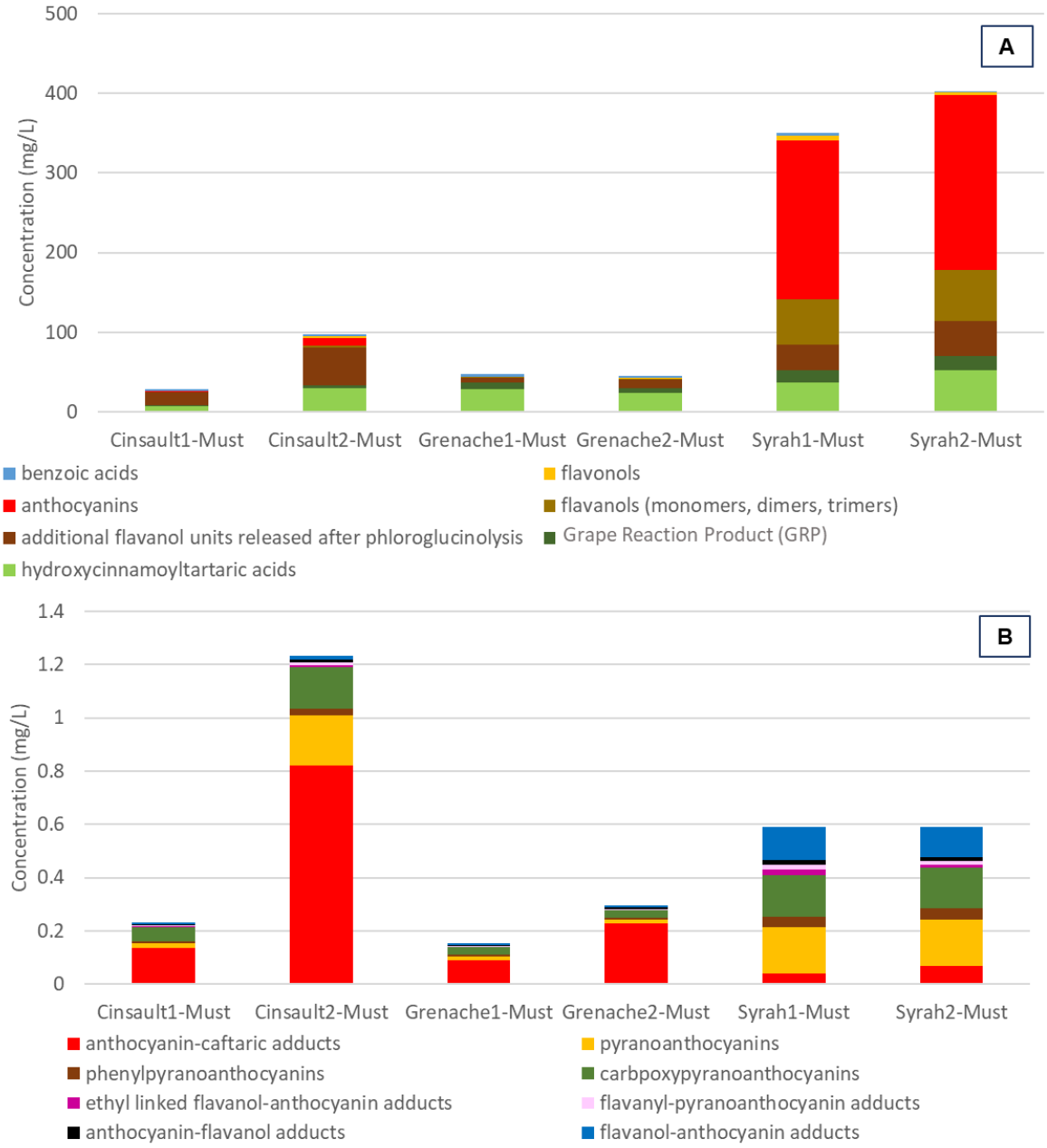


Fig. 1: Concentration of native (A) and derived (B) polyphenolic compounds in the six musts

291 Targeted MS analysis (Fig. 1 and supporting information S2) showed large composition variations
292 between the musts, with much lower amounts of polyphenols in Cinsault and Grenache musts than in
293 Syrah musts.
294 Concentrations were also significantly higher in Cinsault and Syrah musts containing press juice than
295 in free run juice while those of Grenache1 and Grenache2 were not significantly different.
296 Hydroxycinnamic acid derivatives, represented mostly by caftaric acid and GRP, were the most
297 abundant phenolic compounds in Grenache and Cinsault musts, with significantly lower amounts in
298 Cinsault1. These molecules were also found in large amount in Syrah musts which however contained
299 much higher concentrations of anthocyanins and flavanols monomers and dimers. Anthocyanins were
300 the major polyphenolic compounds of Syrah musts while almost absent in Grenache musts. Significant
301 differences were also observed between the two levels of extraction for Cinsault and Syrah musts, with
302 higher levels of all groups of phenolic compounds in musts containing press juice.
303 The musts also contained different concentrations and proportions of anthocyanin derived pigments.
304 These pigments were particularly abundant in Cinsault2, which contained significantly higher amounts
305 of caftaric-anthocyanin adducts, resulting from enzymatic oxidation. These compounds were the
306 predominant derived pigments in Cinsault and Grenache musts while Syrah musts contained
307 significantly higher amounts of phenylpyranoanthocyanins, flavanylpyranoanthocyanins and flavanol-
308 anthocyanin adducts. The concentrations of carboxypyranoanthocyanins and pyranoanthocyanins
309 were also significantly higher in Cinsault2, Syrah1 and Syrah2 than in the three other musts. These
310 molecules resulting from reactions of anthocyanins with pyruvic acid (Fulcrand et al., 1998) and
311 acetaldehyde (Benabdeljalil et al., 2000) have been reported in grape, as well as flavanol-anthocyanin
312 adducts (Pinasseau et al., 2017).
313 Comparison of the total concentrations of phenolic compounds measured by the MRM method with
314 TPI and TYP values suggests that a large proportion of compounds contributing to absorbance at 280

315 nm and 420 nm in Cinsault and Grenache musts are missing in the targeted MRM method. These
316 compounds likely correspond to larger proanthocyanidin oligomers and polymers (DP>3) or derived
317 polyphenols formed in the early stages of must preparation, in particular by enzymatic oxidation.
318 Indeed, enzymatic oxidation of flavanol monomers yields yellow pigments (Guyot et al., 1996).
319 Moreover, although procyanidin oligomers are not substrates for grape PPO, they are readily oxidized
320 by caftaric acid quinone through coupled oxidation mechanisms (Cheynier et al., 1988). The higher
321 concentrations of caftaric-anthocyanin adducts found in Cinsault and Grenache musts also reflect
322 enzymatic oxidation of these musts.
323 Flavanol concentrations determined by phloroglucinolysis (supporting information S2) were much
324 higher than the concentrations of monomers and dimers measured by MRM, indicating the presence
325 of larger flavanol oligomers or derived tannins. In addition, the excess of flavanol units released from
326 these oligomeric compounds compared to monomers and oligomers measured by MRM were
327 significantly different in the three types of musts. Indeed, flavanol concentration after
328 phloroglucinolysis was 1.5 times higher than before in Syrah musts, whereas it was increased by about
329 10-fold in Grenache musts and more than 20-fold in Cinsault musts.
330 Mean degree of polymerisation (mDP) values calculated from the phloroglucinolysis data were low
331 (1.2 - 1.7) for Grenache and Cinsault musts, suggesting that a large proportion of polymer extension
332 units are derived units which are not taken into account in the calculation. These values were
333 significantly higher for Syrah musts (about 4), illustrating the structural differences between oligomers
334 of Syrah musts compared to those of Grenache and Cinsault. However, the flavanol units released by
335 phloroglucinolysis accounted for only part of the high TPI values measured in these musts and
336 especially in Cinsault2, meaning that they also reflect the presence of unknown compounds, covalently
337 linked or not to flavanols.

Flavonols and benzoic acids were found in low quantities for all musts. No significant difference was observed for benzoic acids while flavonol concentrations were significantly higher in Syrah musts and in Cinsault2 musts than in the other three.

3.1.3. High performance size-exclusion chromatography

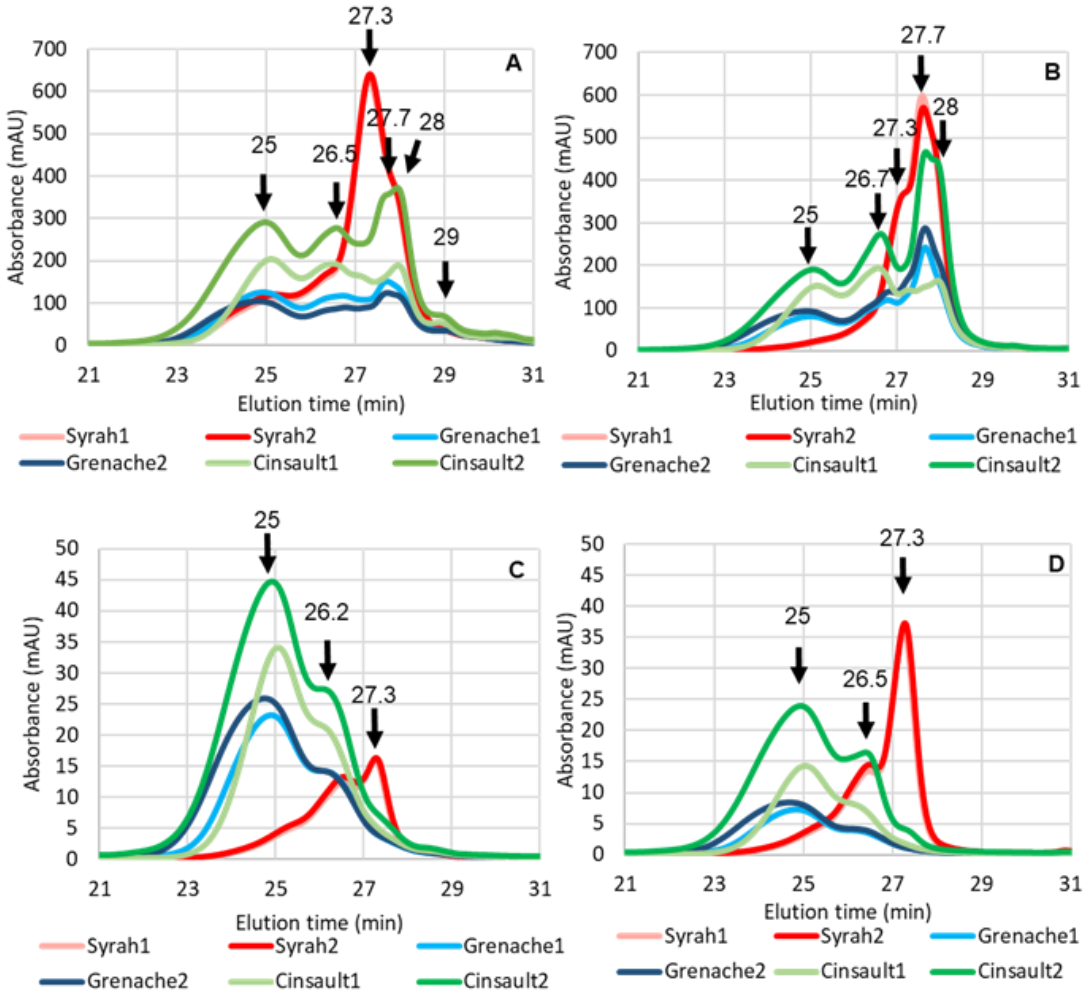


Fig. 2: Analysis of polyphenols from musts by HPSEC. (A) at 280 nm (B) at 320 nm (C) at 420 nm (D) at 520 nm. Values and arrows indicate the elution time of different polyphenolic compounds. The elution time of different polyphenols were determined first (supporting information S3) and were as follows:

347 *p*-coumaric acid, 29.6 ± 0.13 min; caffeic acid, 29 ± 0.13 min; catechin, 28 ± 0.13 min; oenin chloride,
348 27.5 ± 0.13 min; aDP6 apple tannins, 25 ± 0.13 min; aDP 40 apple tannins, 23.7 ± 0.13 min)
349 Polyphenol losses on the SPE column used to eliminate sugars prior to SEC analysis were evaluated by
350 UV-visible spectrophotometry. Losses measured at 280 nm, 320 nm, 420 nm and 520 nm were below
351 10%, except for Cinsault must pigments for which higher losses (up to 30% at 420 nm and 40% at 520
352 nm) were recorded, indicating the specific composition of these musts compared to Syrah and
353 Grenache musts.
354 HPSEC profiles of the musts are displayed in Fig. 2 Elution times of different phenolic
355 compounds/fractions are also provided (supporting information S3). The profiles showed the presence
356 of different populations in size, eluted between 22 and 31 min. Those recorded for both Syrah musts
357 were similar, showing a major peak eluted around 27-28 min and a smaller one eluted around 26 min,
358 and completely different from those of Cinsault and Grenache musts, with three distinct peaks.
359 In Syrah musts, the major peak eluted at 27.3 absorbing at all wavelengths and close to the retention
360 time of A2 dimer and malvidin 3-glucoside can be attributed to red pigments such as anthocyanin
361 monoglucosides found in large quantities in Syrah musts. Similarly, the peak eluted at 26.5 min, visible
362 at A_{520}, A_{280} and A_{420}, corresponds to pigments with slightly larger hydrodynamic diameters, such as
363 acylated anthocyanins. It is worth emphasizing that under the elution conditions used in SEC, i.e. low
364 acidity of the mobile phase, most anthocyanin pigments are not under their flavylium cation form and
365 thus do not absorb in the visible range (Vernhet et al., 2020). Two peaks eluted at 27.7 min and 28 min
366 and detected only at A_{280} and A_{320}, were visible in all musts. Their retention time and their particular
367 ratio of A_{320} to A_{280} superior to 1, along with their concentrations higher in Syrah than in Cinsault2 and
368 lower in the other three must samples, allowed to attribute them to caftaric and coutaric acids.
369 Compounds with the highest hydrodynamic diameter are eluted at 25 min, corresponding to the
370 elution time of higher molecular weight species present in a fraction of apple procyanidins with an

371 average polymerization degree of 6. The profiles recorded at 280 nm show that these polymeric
372 compounds are much more abundant in Cinsault than in the other musts and in Cinsault2 than in
373 Cinsault1, in agreement with the results of spectrophotometry and phloroglucinolysis analysis.
374 Moreover, the profiles recorded at the other wavelengths indicate that they have different structures
375 in the different musts. Indeed, the polymers detected in Syrah absorb mostly at 280 nm and much less
376 in the visible range, suggesting that they consist of flavan-3-ol oligomers and derivatives absorbing
377 only at 280 nm, along with some derived pigments. Among these pigments, flavanol-anthocyanin
378 adducts, like their anthocyanin precursors, are mostly under hydrated colorless form in mildly acidic
379 media such as the SEC elution phase (Salas et al., 2004) while ethyl-linked flavanol-anthocyanin
380 adducts and pyranoanthocyanins are colored (Bakker & Timberlake, 1997; Somers, 1971). In the
381 Cinsault and Grenache musts, compounds eluted between 23 and 27 min showed higher absorbance
382 values than the Syrah musts at 420 and 520 nm indicating that they also contain yellow and red
383 chromophores.
384 The width of the peak eluted at 26.5 min (Fig. 2A) suggests a coelution of several compounds
385 corresponding to larger hydrodynamic diameters than B2 dimer. The high absorbance values at 320
386 nm (A280/A320 below 1) at 26.7 min, suggest the presence of molecules containing hydroxycinnamic
387 acids moieties that are not matching GRP quantification results. The caftaric-anthocyanin adducts
388 detected by LC-MS in higher amounts in the Cinsault and Grenache musts may contribute to the peak
389 eluted at 26.2 min. These compounds show absorbance at 280 and 320 nm but are mostly colorless
390 under the SEC elution conditions (Sarni-Manchado et al., 1997).
391 Taken together, these results indicate very different compositions for the six musts. Syrah musts
392 exhibited higher TPI and TRP values and contained larger concentrations of anthocyanins, flavanols
393 and hydroxycinnamic acids, and of pigments derived from these molecules, namely flavanol-
394 anthocyanin adducts, flavanylpyranoanthocyanins and phenylpyranoanthocyanins, than the other

musts. In contrast, Cinsault musts (especially Cinsault2) and, to a lesser extent, Grenache musts contained large amounts of hydroxycinnamic acids and of phenolic compounds derived from enzymatic oxidation, including caftaric-anthocyanin adducts but also a much larger proportion of unknown products involving hydroxycinnamic acid units (as shown by comparison of the SEC profiles at 280 and 320 nm), flavanol units (released after phloroglucinolysis), along with some yellow and red chromophores. These compounds are not present in the targeted method for polyphenol composition, which explains the discrepancy between TPI values and targeted polyphenol content, especially in Cinsault2. The major derived pigments detected by MRM, namely caftaric-anthocyanin adducts in Grenache and Cinsault versus pyranoanthocyanins, carboxypyranoanthocyanins, and flavanol-anthocyanin derived pigments in Syrah can be considered as markers of the different families of polyphenol derivatives evidenced by HPSEC analysis. These composition differences are related to the extent of extraction, increasing with pressing intensity. The concentrations of anthocyanins and flavanols could not be explained by maceration duration which was shorter for Syrah compared to Grenache and Cinsault. This may be due to varietal differences as high concentrations of anthocyanins as well as high extractability of both anthocyanins (Romero-Cascales et al., 2005) and proanthocyanidins (Bautista-Ortín et al., 2016) have been reported for Syrah grapes compared to other varieties. The observed differences in pigment composition (i.e. pigments derived from hydroxycinnamic acids versus anthocyanins) can also reflect the higher extent of enzymatic oxidation in Grenache and Cinsault compared to Syrah musts which contained higher concentration of sulfur dioxide.

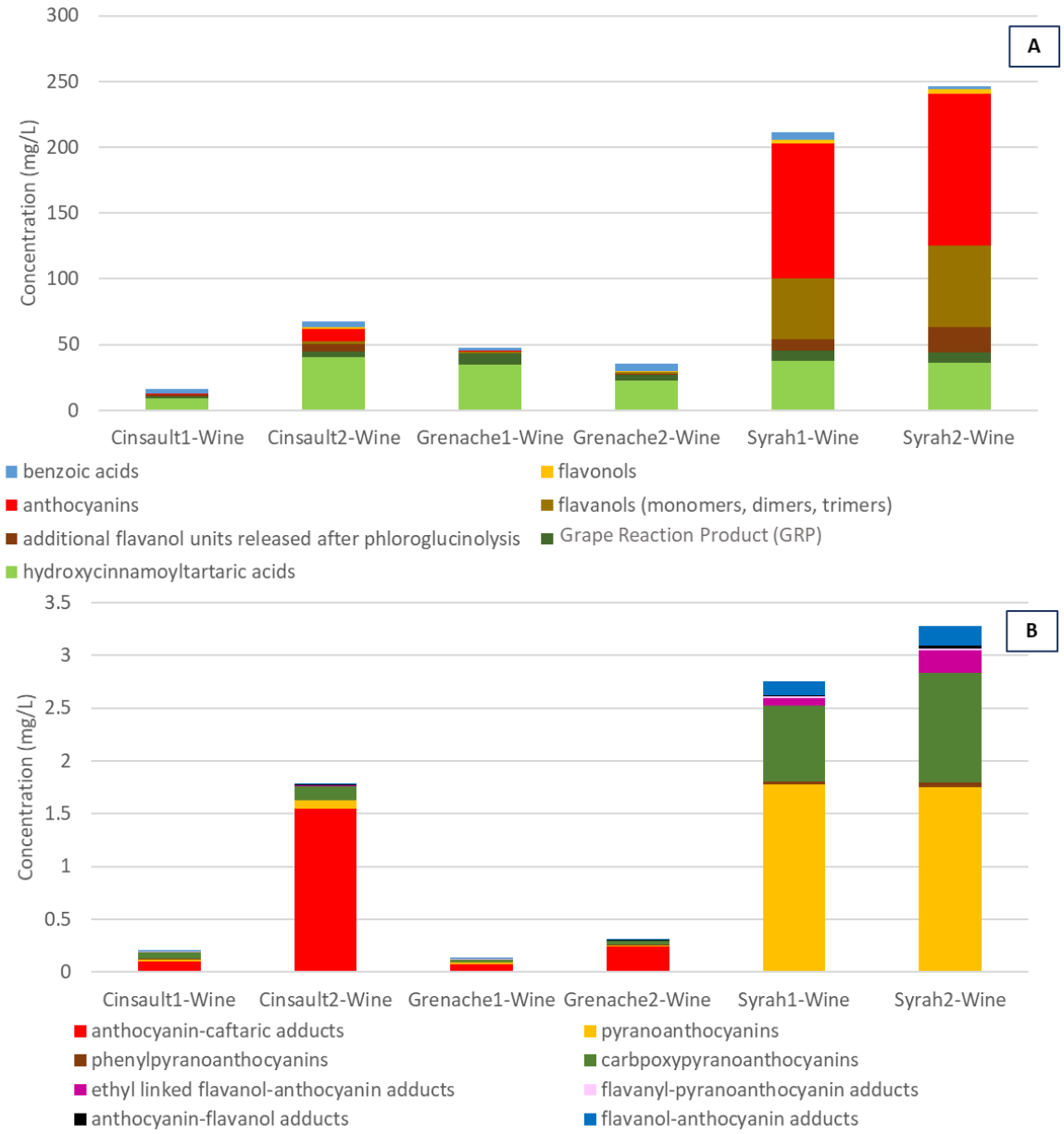
3.2. Wines

3.2.1.Spectrophotometry analysis

After fermentation, Syrah wines showed significantly higher total polyphenol index values than Cinsault wines while those of Grenache wines were lower (Table 1). TPI values of Syrah and Cinsault

419 wines made from free run juice were significantly lower compared to wines made with free run and
420 press juice, while those of Grenache1 and Grenache2 wines were not significantly different. The same
421 pattern was observed for TYP, TRP, CI, and A_{520} values measured directly on wine or after addition of
422 acetaldehyde, with Syrah wines showing significantly higher absorbance values, followed by Cinsault2,
423 then Cinsault1, and both Grenache wines (Table 1). No significant difference was observed on the
424 content of sulfite bleaching resistant pigments. However, they represented a higher proportion of red
425 pigments contributing to wine color (A_{520}) in Cinsault1 (NBP/A520=42%) than in Cinsault2
426 (NBP/A520=25%) and Grenache (NBP/A520=29% and 24%, respectively in Grenache1 and Grenache2),
427 and in Syrah (NBP/A520=11% and 13%, respectively in Syrah1 and Syrah2). Bisulfite adduct
428 concentrations were very low, but significantly higher in Syrah wines, reflecting the higher levels of
429 sulfur dioxide. Most absorbance values were significantly lower in wine than in the corresponding
430 must, indicating losses of phenolic compounds (TPI) and pigments in the course of fermentation. The
431 only exceptions were higher A_{520} values in Syrah wines than in musts, reflecting the release of
432 anthocyanins from bisulfite adducts during fermentation, due to acetaldehyde production by yeast.
433 Color intensity values were also reduced by 80-90% after fermentation of Grenache and Cinsault while
434 they were increased in Syrah, owing to the increase in A_{520} values.
435 TPI values were reduced by 20% and TRP and TYP values by about 25 % during fermentation of Syrah.
436 Losses were much more important for the other wines, representing 40-50 % of the must value for TPI,
437 50 -70 % for TRP and 75-90% for TYP, the lowest losses being observed for Cinsault2. Losses in
438 absorbance values at 320 nm were also lower (10%) for Syrah than for the other four musts (about
439 40%). These differences are probably related to differences in the initial must composition although
440 the presence of higher levels of sulfites may also have contributed.
441 Values of the CIEL*a*b* system parameters for wines also showed significant differences as their
442 corresponding musts. Cinsault wines were significantly darker than Grenache wines and lighter than

443 Syrah wines; Cinsaut2 was darker than Cinsault1. Syrah wines appeared red with high values of a*
444 parameter and a reddish hue angle while Grenache wines and Cinsault1 appeared as light salmon rosé
445 with higher values of b* compared to a* and a light orange yellowish hue (OIV, 2019). Cinsault2 showed
446 an a* value intermediate between those of Syrah and other wines but higher than b* parameter giving
447 a dark orange hue. There is no definition of rosé wine color. However, colorwise, Grenache and
448 Cinsault1 wines were similar to the light group described by Leborgne et al. (2022) whereas Syrah and
449 Cinsault2 were qualified as dark rosé wines.
450 L* values increased while a* and b* values much decreased during fermentation of Grenache and
451 Cinsault. These changes were not observed on Syrah, which contained high levels of both anthocyanin
452 pigments and sulfur dioxide. In such media, the loss of polyphenols was compensated by the
453 conversion of colorless bisulfite adducts to anthocyanin flavylium cations, due to accumulation of
454 acetaldehyde released by yeasts during fermentation, as above mentioned.
455
456 3.2.2.Polyphenol targeted analysis



457

458 Fig. 3: Concentration of native (A) and derived (B) polyphenolic compounds in the six wines

459

460 Targeted MS analysis of the wines (supporting information S2) showed that the differences observed
461 at must stage were maintained in the wines (Fig. 3A). Indeed, Syrah wines contained significantly
462 higher levels of all grape phenolic compounds than Cinsault and Grenache wines. The major
463 compounds in the latter group were hydroxycinnamic acids, showing significantly lower

concentrations in Cinsault1 than in all other wines, while Syrah wines contained significantly higher concentrations of anthocyanins, flavonols, and flavanol monomers and dimers. Cinsault2 was also significantly enriched in anthocyanins and flavanol monomers and dimers compared to Cinsault1 and Grenache wines. The concentrations of hydroxycinnamic acids and flavanol monomers and dimers were similar in wines and musts while anthocyanins and GRP suffered significant losses of about 50% during fermentation in Syrah.

Derived pigments (Fig. 3B) were also detected in wines. Caftaric-anthocyanin adducts were the predominant derived pigments in Grenache and Cinsault wines and particularly abundant in Cinsault2 while they were present only in trace amounts in Syrah wines. Pyranoanthocyanins and carboxypyranoanthocyanins were the major derived pigments in Syrah wines along with lower amounts of flavanol-anthocyanin and ethyl-linked flavanol-anthocyanin adducts. Pyranoanthocyanins and carboxypyranoanthocyanins were significantly accumulated during fermentation in Syrah but not in the other wines, suggesting that their formation is determined by the concentration of anthocyanins rather than that of pyruvic acid and acetaldehyde. Flavanol-anthocyanin and ethyl-linked flavanol-anthocyanin dimer adducts were also present in significantly higher amounts in Syrah2 wine than in Syrah2 must, consistent with the high concentration of flavanols in this must. An accumulation of syringic acid and protocatechuic acid, resulting from degradation of malvidin based anthocyanins and of (epi)catechin and procyanidins, respectively, was observed during fermentation. Syringic acid formation represented less than 10% of the malvidin 3-*O*-glucoside losses during fermentations.

Flavanol units measured by phloroglucinolysis were significantly higher in Syrah wines than in the other wines. They were also generally higher than the total concentrations of monomers, dimers, and trimers, especially in Cinsault wines, indicating that these wines contained large proportions of higher molecular weight proanthocyanidins or flavanol derivatives. The concentration of flavanol units

measured by phloroglucinolysis was much lower in wines than in musts, losses from must to wine representing 80 to 90% in Cinsault and Grenache, 40 % in Syrah1 and 25% in Syrah2.

3.2.3. High performance size-exclusion chromatography

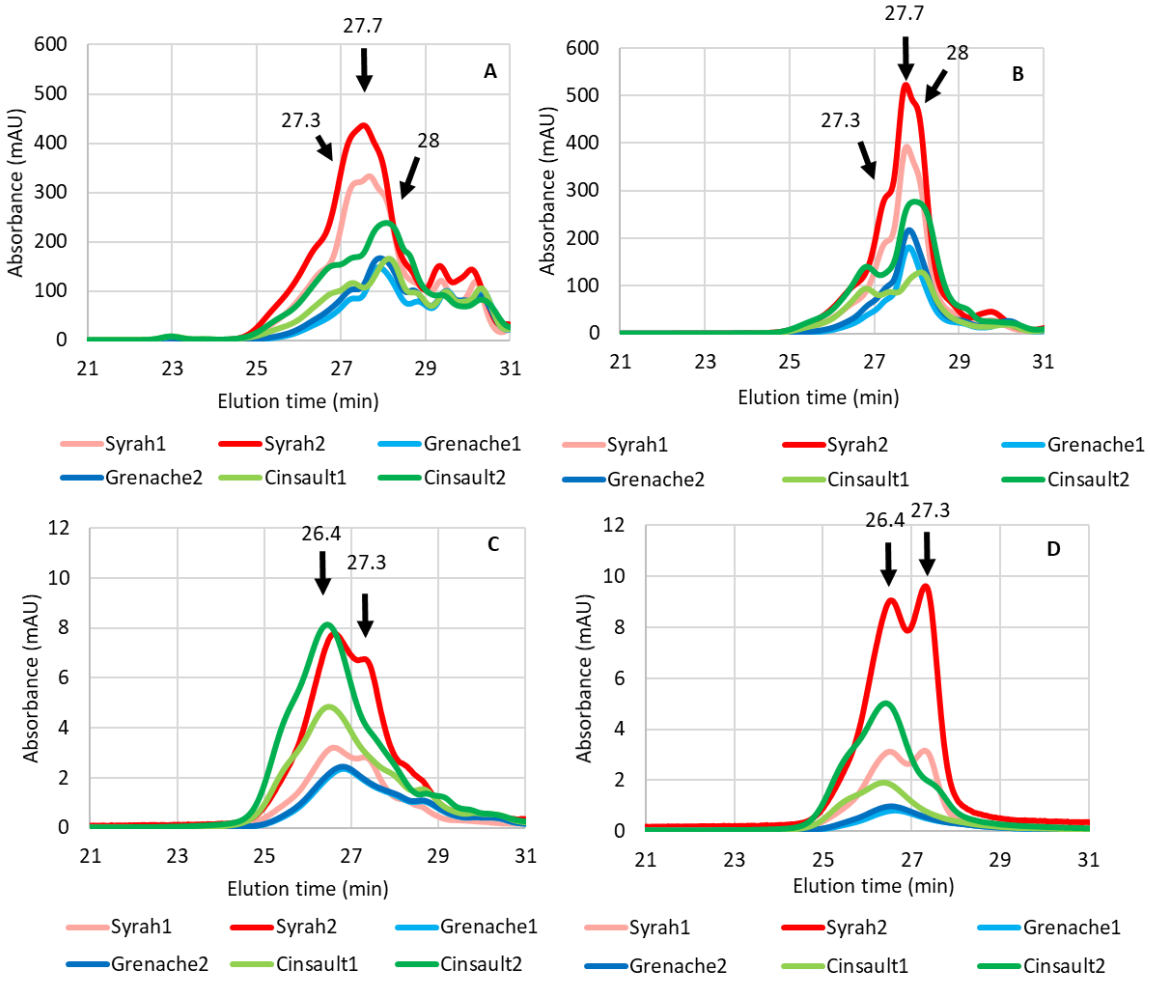


Fig. 4: Analysis of polyphenols from wines by HPSEC. (A) at 280 nm (B) at 320 nm (C) at 420 nm (D) at 520 nm. Values and arrows indicate the elution time of different polyphenolic compounds. The elution time of different polyphenols were determined first (supporting information S3) and were as follows: *p*-coumaric acid, 29.6 ± 0.13 min; caffeic acid, 29 ± 0.13 min; catechin, 28 ± 0.13 min; oenin chloride, 27.5 ± 0.13 min; aDP6 apple tannins, 25 ± 0.13 min; aDP 40 apple tannins, 23.7 ± 0.13 min)

As illustrated in figure 4, the phenolic profiles of Syrah, Grenache and Cinsault wines were more similar than those of the musts. Comparison of the size exclusion chromatographic profiles obtained for musts and wines shows that the population eluted at 25 min, corresponding to compounds with higher hydrodynamic diameter, disappeared after fermentation. The loss of these compounds may be related to the loss of color and TPI measured by spectrophotometric analysis which could not be explained with the targeted mass spectrometry analyses used in this study. All wines contained equivalent amounts of compounds with smaller hydrodynamic diameter than *p*-coumaric acid (eluted at 29 min), detected at 280 nm. These peaks which were not present in musts likely correspond to lower molecular weight yeast metabolites such as tyrosol or peptides (Fig. 4A). Syrah wines presented large quantities of native anthocyanins (27.3 min and 26.4 min) and hydroxycinnamoyl tartaric acids (i.e. caftaric acid and coutaric acid eluted at 27.7 min and 28 min) which were the major compounds in Grenache and Cinsault wines. A small shoulder between 25 min and 26 min is noticeable at 280 nm but also at 420 and 520 nm, corresponding to procyanidin oligomers and derived pigments. The profiles of Cinsault and Grenache wines showed higher proportions of hydroxycinnamoyltartaric acids and of oligomeric compounds eluted between 25 and 27 min that contain structural motifs absorbing at 320 nm such as hydroxycinnamic acid units, especially in Cinsault, and contribute to wine color.

During Syrah must fermentations, absorbance values and anthocyanin contents dropped by 10% and 50% respectively, indicating the conversion of native anthocyanins to new derived pigments. These include pyranoanthocyanins, carboxypyranoanthocyanins and flavanol-anthocyanins adducts that are detected by our MRM method. In contrast, the important drops in absorbance values observed after alcoholic fermentation of Grenache and Cinsault were not related to the concentrations of phenolic compounds measured by targeted analysis, which remained stable during fermentation. Losses of flavanol units measured after phloroglucinolysis were observed in all wines and much higher for Grenache and Cinsault, confirming that absorbance of these musts was due to flavanol derivatives not

520 targeted by the MRM method. In addition, HPSEC analysis revealed that the oligomeric compounds
521 detected in musts were missing in the wine profiles, suggesting their precipitation or adsorption on
522 lees during alcoholic fermentation.

523 3.3. Adsorption on lees

524 3.3.1. Spectrophotometry analysis

525 Absorbance values at 280, 420 and 520 nm corresponding to polyphenols desorbed from lees were
526 similar for all modalities. However, extracts from Cinsault2 lees which showed significantly higher TPI
527 and TRP values than all other modalities while extracts from Syrah lees exhibited lower TYP values.
528 The TPI values obtained after desorption from lees represented less than 10% of those measured in
529 musts in all modalities and accounted for 10 % (in Grenache and Cinsault1) to 30 % (in Syrah1) of the
530 losses measured after fermentation. Absorbance recovered from Syrah lees at 320, 420 and 520 nm
531 also represented less than 10% of the must values but large proportions of the losses from must to
532 wine (43-64%, 12-25%, 10-12% respectively for A_{320}, TYP, and TRP, with higher percentages for Syrah1).
533 Absorbance values at 320 nm measured on the lees extracts from Grenache and Cinsault represented
534 about 10% of the must values and 19 to 38% of the losses after fermentation. TYP and TRP desorbed
535 from lees represented 15% and 10%, respectively, in Cinsault, and 30% and 25%, respectively, in
536 Grenache, of the initial must values. Thus, they explained about 20% of the losses in Cinsault and 40%
537 in Grenache.
538 Except for TRP in Grenache and TYP in Grenache and Cinsault, the absorbance values recovered from
539 lees were much lower than those of wine, as observed for TRP values in earlier studies performed on
540 red wines (Morata et al., 2003; Vernhet et al., 2020). They did not explain the losses measured after
541 fermentation which can be due either to irreversible adsorption or to chemical changes impacting UV-
542 visible spectra. The presence of residual color on the lees after extraction indicates incomplete

543 desorption of phenolic compounds so that these values are likely underestimated, as observed by
544 other authors (Mazauric & Salmon, 2006; Vernhet et al., 2020).

545 3.3.2.Phloroglucinolysis analysis

546 Phloroglucinolysis performed directly on the lees (supporting information S2) allowed to recover all
547 flavanol derivatives lost after fermentation of Grenache and Syrah, meaning that the losses were due
548 to precipitation and /or adsorption on yeast cells. In the case of Cinsault, the recovery was only about
549 40%, indicating incomplete desorption of the derived flavanols present in these wines, likely due to
550 structural differences, as observed on SPE.

551 The proportions of flavanol units recovered from lees represented about 85% of the total amount
552 present after fermentation in Grenache, 76% in Cinsault1, 64% in Cinsault2, 43% in Syrah 1 and 27% in
553 Syrah2. These variations can be attributed to saturation of the lees or to various affinities of different
554 oligomeric flavanol derivatives toward yeast cell wall.

555 3.3.3. High performance size-exclusion chromatography

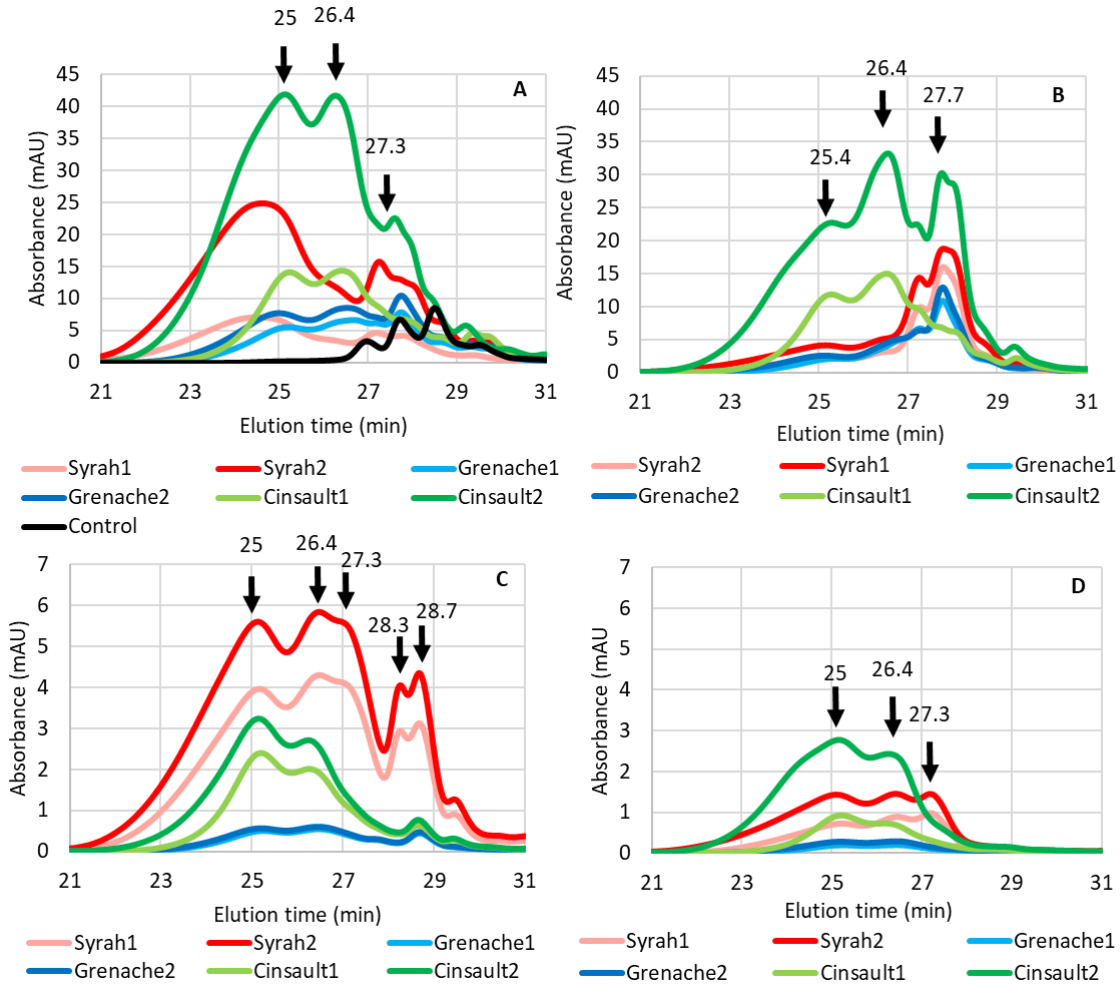


Fig. 5: Analysis of polyphenols from lees by HPSEC (A) at 280 nm (B) at 320 nm (C) at 420 nm (D) at 520 nm. Values and arrows indicate the elution time of different polyphenolic compounds. The elution time of different polyphenols were determined first (supporting information S3) and were as follows: *p*-coumaric acid, 29.6 ± 0.13 min; caffeic acid, 29 ± 0.13 min; catechin, 28 ± 0.13 min; oenin chloride, 27.5 ± 0.13 min; aDP6 apple tannins, 25 ± 0.13 min; aDP 40 apple tannins, 23.7 ± 0.13 min)

As illustrated in figure 5, lees seemed to preferentially adsorb higher molecular weight compounds lost during alcoholic fermentation. Control sample revealed absorbance only at 280 nm and at retention times corresponding to low molecular weight yeast metabolites (Fig. 5A). Much higher concentrations of phenolic compounds were desorbed from Cinsault lees than from those of other modalities. The

oligomeric compounds eluted at 26.4 min and 25 min in Grenache and Cinsault lees showed high absorbance values at 280 nm and 320 nm and also absorbed at 520 nm, suggesting the presence of hydroxycinnamoyltartaric acid and anthocyanins moieties in their structures.

Hydroxycinnamoyltartrates, i.e. caftaric acid and coutaric acid eluted at 27.7 and 28 min, respectively, were desorbed from all lees (Fig. 5A&B) but in higher amount in Cinsault2 and lower amount in Cinsault1. The profiles recorded at 520 nm shows three peaks eluted at 27.3 min, 26.4 min, and 25 min. The first one coelutes with malvidin 3-glucoside found in larger amount in Syrah wines. However, its much higher absorbance at 420 nm suggests that it also contains other pigments. The other two peaks are present in higher concentrations in the extracts from Cinsault2 lees. The populations present in lee extracts from Syrah wines also show much higher ratios of A420 to A520 and of A280 to A320 than those eluted at the same retention times in the extracts from Cinsault lees, again suggesting different compositions. Moreover, additional peaks eluted at 28.3 and 28.7 min in the Syrah profiles at 420 nm also indicate the presence of lower molecular weight yellow pigments.

Desorption from the lees allowed partial recovery of the oligomeric fraction observed by HPSEC on musts profiles. Absorbance values were similar for all modalities even though significantly higher levels of phenolic compounds were desorbed from Cinsault2 lees. Adsorption of phenolic compounds on lees has been previously reported for red fermentation but their impact on color was negligible, corresponding to less than 10% of the final wine color (Morata et al., 2003; Vernhet et al., 2020). Similar results were obtained in the present study for Syrah: the proportion of red and yellow pigments adsorbed on lees representing less than 10% of those of rosé wines and musts. On the opposite, these proportions were much higher for the light rosé wines: TRP and TYP recovered from lees accounting for about 30% of Grenache musts values and representing 72-92% of the wine values for TRP and exceeding them (200-280%) for TYP.

4. Conclusion

In order to understand the drivers of rosé wine color, six rosé musts exhibiting large color differences were fermented and their pigment composition was analyzed before and after fermentation, using a combination of complementary analytical methods. Our results showed that major phenolic compounds in Grenache and Cinsault musts were hydroxycinnamic acid derivatives, including GRP and caftaric-anthocyanin adducts that result from enzymatic oxidation, but also higher molecular weight compounds whereas Syrah musts contained larger amounts of anthocyanins and flavanols.

Must composition impacts changes that take place during fermentation and final wine color. Syrah must anthocyanins were partly converted to derived pigments through reactions with yeast metabolites and flavanols, with limited drop in color and total polyphenol content (about 20 %) during fermentation. Losses were much more important for Cinsault and Grenache wines, representing 50 % of the must total phenol content and 50-90% of pigments. HPSEC analysis indicated that they were mostly due to adsorption of higher molecular weight compounds on lees.

In summary, this study analyzed for the first time the phenolic composition of rosé musts and wines and of lees collected after fermentation. Taking advantage of the complementary information provided by HPLC-MS targeting specific phenolic compounds, UV-visible spectrophotometry, and HPSEC-DAD analysis, which have seldom been combined for wine analysis and never applied to rosé wine fermentation, it demonstrated that the color of light and dark rosé wines and especially color changes taking place during fermentation are driven by different phenolic reactions and interactions, depending on the initial must composition. It also confirmed that the pigments of light and dark rosés wines are structurally different and provided evidence of the presence of higher molecular weight pigments and hydroxycinnamic derivatives in light rosé musts and wines. Further work is needed to determine the nature of these pigments and the role of grape varietal characteristics and pre-fermentative and fermentation conditions on their formation.

Supporting information: S1: List of phenolic compounds, MRM parameters and calibration ranges.pdf; S2: Concentrations of phenolic compounds with ANoVa statistics.xls, S3: Standard elution in HPSEC.ppx

CRediT: Conceptualization: G.M., V.C. and J.-R.M.; methodology: E.M., S.C., Ar.V., C.L. and N.S.; formal analysis: C.L., S.C., E.M., Ar.V. and M.-A.D.; investigation: C.L. and V.C.; resources: G.M.; data curation: Ar.V., E.M., M.-A.D. and C.L.; writing—original draft preparation, C.L.; writing—review and editing: M.-A.D., M.B., Au.V., J.-R.M. and V.C.; visualization: C.L.; supervision: Au.V., N.S., J.-R.M. and V.C.; project administration and funding acquisition, G.M., M.B., Au.V., J.-R.M. and V.C. All authors have read and agreed to the published version of the manuscript."

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Figure Captions

Figure 1: Concentration of native (A) and derived (B) polyphenolic compounds in the six musts

Figure 2: Analysis of polyphenols from musts by HPSEC. (A) at 280 nm (B) at 320 nm (C) at 420 nm (D) at 520 nm. Values and arrows indicate the elution time of different polyphenolic compounds. The elution time of different polyphenols were determined first (supporting information S3) and were as follows: *p*-coumaric acid, 29.6 ± 0.13 min; caffeic acid, 29 ± 0.13 min; catechin, 28 ± 0.13 min; oenin chloride, 27.5 ± 0.13 min; aDP6 apple tannins, 25 ± 0.13 min; aDP 40 apple tannins, 23.7 ± 0.13 min)

Figure 3: Concentration of native (A) and derived (B) polyphenolic compounds in the six wines

Figure 4: Analysis of polyphenols from wines by HPSEC. (A) at 280 nm (B) at 320 nm (C) at 420 nm (D) at 520 nm. Values and arrows indicate the elution time of different polyphenolic compounds. The elution time of different polyphenols were determined first (supporting information S3) and were as follows: *p*-coumaric acid, 29.6 ± 0.13 min; caffeic acid, 29 ± 0.13 min; catechin, 28 ± 0.13 min; oenin chloride, 27.5 ± 0.13 min; aDP6 apple tannins, 25 ± 0.13 min; aDP 40 apple tannins, 23.7 ± 0.13 min)

Figure 5: Analysis of polyphenols from lees by HPSEC (A) at 280 nm (B) at 320 nm (C) at 420 nm (D) at 520 nm. Values and arrows indicate the elution time of different polyphenolic compounds. The elution time of different polyphenols were determined first (supporting information S3) and were as follows: *p*-coumaric acid, 29.6 ± 0.13 min; caffeic acid, 29 ± 0.13 min; catechin, 28 ± 0.13 min; oenin chloride, 27.5 ± 0.13 min; aDP6 apple tannins, 25 ± 0.13 min; aDP 40 apple tannins, 23.7 ± 0.13 min)

Table Caption

Table 1: UV-visible spectrophotometry analysis performed on musts, wines and lees under various conditions. All values are given in absorbance units except for L*,a*,b* and hue. Absorbance values of lees were reported to the initial lees dry mass and wine volume. Different superscript letters indicate significant differences between all samples (wines, musts and lees) for a given parameter (ANoVa with SNK test for *p-value* < 0.05).