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

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

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21

22

23 **Abstract:**

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

36

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

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39

40 1. Introduction

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

53 Hydroxycinnamic acids and their derivatives are colorless. Nevertheless, enzymatic oxidation of  
54 hydroxycinnamoyltartrates, especially caftaric acid, is known to be involved in the browning of white  
55 musts and may also occur in rosé wine-making, as pressing takes place before fermentation when  
56 polyphenoloxidase is still active. This process involves oxidation of hydroxycinnamoyltartrates to  
57 caftaric acid *o*-quinone, followed by addition of glutathione to form Grape Reaction Product (GRP), i.e.  
58 2-S-glutathionyl caftaric acid (Cheynier et al., 1986). After glutathione depletion, *o*-quinones can  
59 proceed to brown oxidation products (Sarni-Manchado et al., 1997). Caftaric acid *o*-quinones can also  
60 add to anthocyanins, generating anthocyanin-caftaric adducts (Sarni-Manchado et al., 1997). Free  
61 hydroxycinnamic acids or vinylphenols resulting from their decarboxylation react with anthocyanins to  
62 form phenylpyranoanthocyanin pigments (Fulcrand et al., 1996; Schwarz et al., 2003).

63 Flavanols found in grapes and wines include monomers (catechins) and oligomers and polymers, i.e.  
64 proanthocyanidins (PA). They are colorless but can be involved in oxidative browning (Es-Safi et al.,  
65 1999; Guyot et al., 1996) and react with anthocyanins to form derived pigments (Cheynier et al., 2006;  
66 Leborgne et al., 2022).

67 In particular, flavanol and anthocyanin condensation with acetaldehyde, which can be produced by  
68 yeast metabolism and but also by oxidation, leads to purple ethyl linked anthocyanin-flavanol adducts  
69 (Somers, 1971). These molecules are unstable and yield flavanlypyranoanthocyanin (Mateus et al.,  
70 2002) and other products through random reaction cascades (Vallverdú-Queralt et al., 2017).

71 During alcoholic fermentation, the reactions of anthocyanins with yeast aldehyde metabolites such as  
72 acetaldehyde or pyruvic acid generate orange pigments, pyranoanthocyanins (e.g. vitisin B, i.e.  
73 pyranomalvidin 3-glucoside) and carboxy-pyranoanthocyanins (e.g. vitisin A, i.e.  
74 carboxypyranomalvidin 3-glucoside) respectively (Bakker & Timberlake, 1997; Benabdeljalil et al.,  
75 2000; Fulcrand et al., 1998).

76 Adsorption of phenolic compounds and in particular of flavanols and anthocyanin pigments on yeasts  
77 is also known to impact wine color, depending on the strain (Morata et al., 2003). Preferential  
78 interactions of acylated anthocyanins (Morata et al., 2005) along with oligomeric proanthocyanidins  
79 (Vernhet et al., 2020) with yeast cells have been demonstrated, accounting for 2 to 8% of the color loss  
80 observed during liquid phase fermentation of red must (Morata et al., 2003, 2005; Vernhet et al.,  
81 2020).

82 All above mentioned studies have been performed in red wine or model solutions mimicking red wines  
83 while very limited data is available on rosé wines. The few investigations performed on rosé wines  
84 showed that their phenolic composition is highly variable, close to that of red wines for the darker  
85 rosés but very different for the light ones (Lambert et al., 2015; Leborgne et al., 2022; Wirth et al.,  
86 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-dimethylformamide 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 formate 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%),

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

## 112 2.2. Oenological analysis

113 Free sulfites, total sulfites, sugar and assimilable nitrogen contents were measured with a Fourier-  
114 transform infrared (IRTF) spectrometer (Foss, Nanterre, France) following the OIV method (OIV, 2019).

115 A pHmeter (Mettler Toledo<sup>®</sup>, Viroflay, France) was used to measure pH in samples. A turbidimeter  
116 (Mettler Toledo<sup>®</sup>, Viroflay, France) was used to measure turbidity.

## 117 2.3. Must preparation

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

134 °C before racking. Analysis of the musts were performed at the Centre du Rosé. Clarified musts and  
135 solid particles were stored at -20 °C until use.

136 After thawing and centrifugation (4000 *g*, 5 °C, 10 min) (Jouan, Nantes, France) of each must, pH was  
137 adjusted to 3.5 using hydrochloric acid (50%, *v/v*) or sodium hydroxide (32%, *w/w*). Solid particles  
138 collected after racking were added to the musts to reach a turbidity of 135 NTU (Nephelometric  
139 Turbidity Unit) and sugar concentration was normalized at 250 g/L by the addition of a fructose and  
140 glucose mixture (50:50, *v/v*). Assimilable nitrogen content was at 163 mg/L for both Grenache musts,  
141 148 mg/L for both Syrah musts and 185 mg/L for both Cinsault musts. Musts were conditioned in 2L  
142 buckets before distributing into fermenters (Legallais, Montferrier-sur-Lez, France). For each must,  
143 three aliquots of 50 mL were taken for color analysis that was performed on the same day at the  
144 experimental unit of Pech Rouge (IFV, Gruissan, France). Twenty 1 mL aliquots were sampled for  
145 polyphenol analysis and stored at -80 °C until analysis.

#### 146 2.4. Fermentation

147 Grenache and Cinsault fermentations were conducted in quadruplicate in 300 mL fermenters filled  
148 with 250 mL of must (controlled by weighing). Syrah fermentations were performed in duplicate in the  
149 same containers. All fermenters and vials were protected from oxidation using inert gas Argon.  
150 Fermenters were equipped with fermentation airlock systems allowing to maintain anaerobiosis. All  
151 fermenters were placed in a thermoregulated room set at 16 °C and stirred at 150 rpm. Sulfites were  
152 added to Cinsault and Grenache musts using a potassium hydrogen sulfite solution (222 g/L) to reach  
153 50mg/L of total sulfite content, our target value. Syrah1 and Syrah2 were respectively at 101 mg/L and  
154 89mg/L of total sulfite content before fermentation. All fermenters were maintained at 16 °C for 24h  
155 before inoculation.

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



158 weight losses induced by CO<sub>2</sub> 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.

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

186 Total polyphenol index (TPI) at 280 nm, total red pigments (TRP) at 520 nm, total yellow pigments at  
187 420 nm (TYP) and absorbance at 320 nm ( $A_{320}$ ) were measured 20 minutes after dilution of wines,  
188 musts and lees extracts in 1 M HCl solution ( $TPI = A_{280} \times F_D$  (dilution factor);  $TRP = A_{520-HCl\ 1M} \times F_D$ ;  $TYP =$   
189  $A_{420-HCl\ 1M} \times F_D$  and  $A_{320} = A_{320-HCl\ 1M} \times F_D$ ). Dry lees extracts, were dissolved in 1 mL of MeOH:H<sub>2</sub>O (1:1,  
190 v/v), 1% formic acid prior to dilution in 1 M HCl solution and absorbance values of control were  
191 subtracted from those of samples.

192 Absorbance at 520 nm was measured 5 minutes after addition of 60  $\mu$ L of sodium hydrogen sulfite  
193 solution (200 g/L) to 4 mL of wine or must, to determine the sulfite bleaching resistant fraction of  
194 pigments ( $NBP = A_{520-SO_2}$ ).  $A_{520}$  was also determined on musts and wines 40 minutes after addition of 40  
195  $\mu$ L of a 12.6 % (v/v) acetaldehyde aqueous solution to 4 mL of samples to trap sulfites and convert  
196 bisulfite adducts to flavylum ions. This enabled calculation of the concentrations of bisulfite adducts  
197 ( $BA = A_{520-acetaldehyde} - A_{520}$ ), flavylum forms ( $AH = A_{520} - A_{520-SO_2}$ ) and hydrated forms ( $HF = TRP - A_{520-}$   
198  $acetaldehyde$ ) of pigments susceptible to sulfite bleaching.

## 199 2.7. High Performance Size-Exclusion Chromatography (HPSEC)

200 Wines (1 mL) and yeast extracts (2 mL, corresponding to 27 mg dry yeast) were evaporated to dryness  
201 under vacuum using a Genevac (SP Scientific, Warminster, PA, USA) and the residue dissolved in 500  
202  $\mu$ L of the HPSEC mobile phase composed of N,N-dimethylformamide (DMF), 1% (v/v) glacial acetic acid,  
203 5% (v/v) water, and 0.15 M lithium chloride. To eliminate sugar, must samples were previously purified  
204 by solid phase extraction on 500 mg/3 mL tC18 cartridges (Strata<sup>®</sup>, 55  $\mu$ m, 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

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

235 Constitutive units of proanthocyanidins were analyzed by UHPLC-DAD after acid-catalyzed  
236 depolymerization in the presence of phloroglucinol. The residue obtained after taking to dryness 1 mL  
237 of must or wine using a Genevac (SP Scientific, Warminster, PA, USA) was dissolved in 250  $\mu$ L of a  
238 solution containing phloroglucinol (50 g/L) and ascorbic acid (1 g/L) in methanol at 0.2 N HCl. The  
239 phloroglucinolysis reaction was carried out for 20 minutes at 50 °C, and stopped by addition of 200  
240 mM ammonium formate buffer (250  $\mu$ L), according to the method described by (Kennedy & Jones,  
241 2001). An internal standard, 50  $\mu$ L of *p*-hydroxybenzoic methyl ester in methanol was added in both  
242 wines and musts samples (0.3 g/L) after the reaction was completed. For yeasts, the depolymerisation  
243 reaction was performed directly on dry yeasts (27 mg) using 500  $\mu$ L of phloroglucinolysis reagent and  
244 the same volume of ammonium formate buffer at 200mM. Constitutive units of flavanol derivatives  
245 released after acid-catalyzed depolymerization in the presence of phloroglucinol were analyzed by  
246 UHPLC using a Vanquish UHPLC equipped with an Ethylene Bridged Hybrid C18 (1.0 x 150 mm, 1.7  $\mu$ m)  
247 and a diode array detector (DAD) used for quantification. Standards of catechin and epicatechin were  
248 used for the quantification of flavanols and their phloroglucinol adducts. Total flavan-3-ol units  
249 released by phloroglucinolysis were calculated. The average degree of polymerisation (mDP) was also  
250 evaluated as the ratio of all flavan-3-ol units after phloroglucinolysis to the sum of terminal units.

251

252 2.9. Statistical analysis

253 ANalysis Of VAriance were performed with RStudio version 4.0.3 software ([www.rstudio.com](http://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 <sub>320</sub>	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 <sub>520</sub>	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 <sub>520</sub> -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
Flavylum 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 <sup>b</sup>	86.28	± 0.40 <sup>d</sup>	97.93	± 0.10 <sup>a</sup>	97.93	± 0.10 <sup>a</sup>	75.35	± 0.21 <sup>f</sup>	72.30	± 0.28 <sup>fg</sup>
a*	4.19	± 0.37 <sup>h</sup>	15.77	± 0.54 <sup>f</sup>	0.81	± 0.15 <sup>j</sup>	1.14	± 0.12 <sup>j</sup>	39.82	± 0.04 <sup>b</sup>	42.41	± 0.29 <sup>a</sup>
b*	9.69	± 0.63 <sup>d</sup>	7.77	± 0.21 <sup>e</sup>	4.85	± 0.24 <sup>f</sup>	4.39	± 0.10 <sup>f</sup>	4.24	± 0.29 <sup>f</sup>	5.50	± 0.16 <sup>f</sup>
Hue angle (H)	1.16	± 0.05 <sup>d</sup>	0.46	± 0.02 <sup>g</sup>	1.40	± 0.03 <sup>b</sup>	1.32	± 0.02 <sup>c</sup>	0.11	± 0.01 <sup>h</sup>	0.13	± 0.00 <sup>h</sup>
Color Intensity (CI)	0.32	± 0.02 <sup>f</sup>	0.54	± 0.02 <sup>e</sup>	0.11	± 0.00 <sup>g</sup>	0.11	± 0.00 <sup>g</sup>	0.98	± 0.01 <sup>c</sup>	1.13	± 0.02 <sup>c</sup>
Total Polyphenol Index (TPI)	6.63	± 0.09 <sup>h</sup>	10.11	± 0.09 <sup>f</sup>	5.01	± 0.10 <sup>i</sup>	5.11	± 0.14 <sup>i</sup>	11.03	± 0.10 <sup>e</sup>	11.89	± 0.01 <sup>d</sup>
Total Red Pigments (TRP)	0.44	± 0.04 <sup>h</sup>	1.17	± 0.02 <sup>f</sup>	0.15	± 0.01 <sup>i</sup>	0.16	± 0.01 <sup>i</sup>	5.03	± 0.03 <sup>d</sup>	5.65	± 0.03 <sup>c</sup>
Total Yellow Pigments (TYP)	0.25	± 0.02 <sup>h</sup>	0.45	± 0.02 <sup>g</sup>	0.09	± 0.01 <sup>i</sup>	0.09	± 0.01 <sup>i</sup>	1.35	± 0.00 <sup>d</sup>	1.53	± 0.03 <sup>c</sup>
A <sub>320</sub>	3.98	± 0.09 <sup>d</sup>	7.57	± 0.05 <sup>a</sup>	4.09	± 0.13 <sup>d</sup>	4.00	± 0.08 <sup>d</sup>	6.59	± 0.11 <sup>c</sup>	6.84	± 0.02 <sup>b</sup>
A <sub>520</sub>	0.10	± 0.01 <sup>h</sup>	0.25	± 0.01 <sup>f</sup>	0.03	± 0.00 <sup>i</sup>	0.03	± 0.00 <sup>i</sup>	0.63	± 0.00 <sup>c</sup>	0.71	± 0.01 <sup>b</sup>
A <sub>520</sub> -acetaldehyde	0.10	± 0.01 <sup>j</sup>	0.24	± 0.01 <sup>h</sup>	0.03	± 0.00 <sup>k</sup>	0.03	± 0.00 <sup>k</sup>	0.74	± 0.00 <sup>e</sup>	0.79	± 0.00 <sup>d</sup>
Flavylium cations (AH)	0.06	± 0.00 <sup>g</sup>	0.19	± 0.01 <sup>f</sup>	0.02	± 0.00 <sup>g</sup>	0.03	± 0.00 <sup>g</sup>	0.56	± 0.00 <sup>bc</sup>	0.62	± 0.01 <sup>b</sup>
Bisulfite Adducts (BA)	0.00	± 0.00 <sup>ef</sup>	-0.01	± 0.01 <sup>ef</sup>	0.00	± 0.00 <sup>ef</sup>	0.00	± 0.00 <sup>ef</sup>	0.11	± 0.01 <sup>c</sup>	0.08	± 0.01 <sup>cd</sup>
Hemiketal Forms (HF)	0.34	± 0.03 <sup>h</sup>	0.93	± 0.02 <sup>f</sup>	0.12	± 0.01 <sup>i</sup>	0.13	± 0.01 <sup>i</sup>	4.28	± 0.03 <sup>d</sup>	4.86	± 0.03 <sup>b</sup>
Non Bleachable Pigments (NBP)	0.04	± 0.00 <sup>de</sup>	0.06	± 0.00 <sup>de</sup>	0.01	± 0.00 <sup>e</sup>	0.01	± 0.00 <sup>e</sup>	0.07	± 0.00 <sup>de</sup>	0.09	± 0.00 <sup>cde</sup>
	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 <sup>bc</sup>	1.41	± 0.16 <sup>a</sup>	0.48	± 0.13 <sup>c</sup>	0.67	± 0.05 <sup>bc</sup>	0.85	± 0.04 <sup>b</sup>	0.69	± 0.06 <sup>bc</sup>
Total Red Pigments (TRP)	0.13	± 0.01 <sup>b</sup>	0.28	± 0.06 <sup>a</sup>	0.11	± 0.05 <sup>b</sup>	0.15	± 0.02 <sup>b</sup>	0.18	± 0.00 <sup>b</sup>	0.18	± 0.01 <sup>b</sup>
Total Yellow Pigments (TYP)	0.20	± 0.02 <sup>ab</sup>	0.30	± 0.07 <sup>a</sup>	0.19	± 0.07 <sup>ab</sup>	0.24	± 0.02 <sup>a</sup>	0.10	± 0.00 <sup>bc</sup>	0.07	± 0.01 <sup>c</sup>
A <sub>320</sub>	0.64	± 0.07 <sup>bc</sup>	1.44	± 0.09 <sup>a</sup>	0.59	± 0.12 <sup>bc</sup>	0.74	± 0.05 <sup>b</sup>	0.48	± 0.01 <sup>c</sup>	0.46	± 0.01 <sup>c</sup>

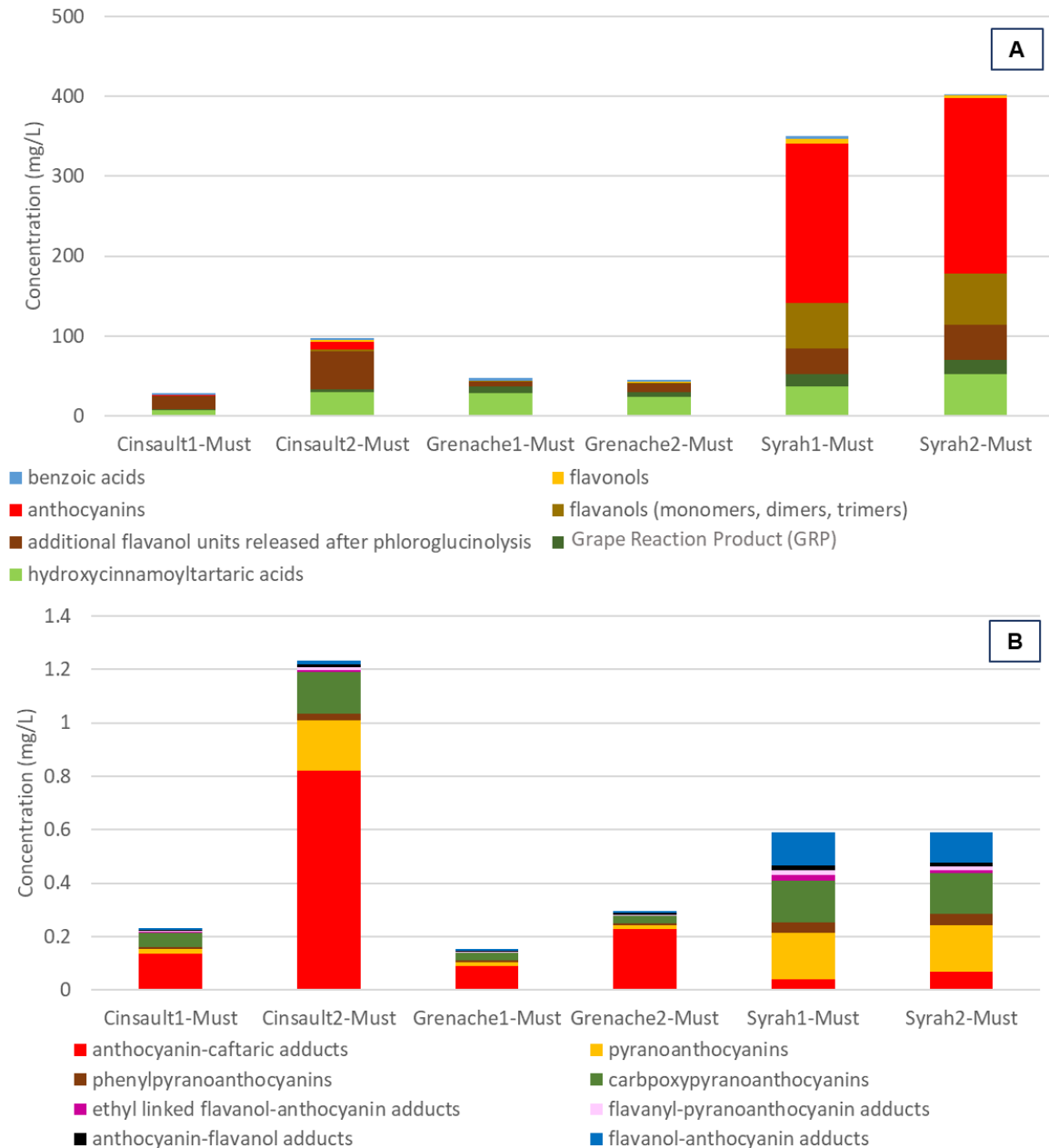
261 Total polyphenol index (TPI) presented in Table1 showed differences between all six musts with  
262 significantly higher values for Syrah compared to Grenache musts. Cinsault1 was intermediate while  
263 Cinsault2 had higher absorbance values than Syrah musts. For all varieties, musts containing press juice  
264 showed higher TPI values than free-run juice, confirming higher polyphenol extraction rates. The total  
265 red pigment (TRP) levels were much higher in Syrah musts with values of 6.51 and 7.47, respectively,  
266 for Syrah1 and Syrah2, followed by Cinsault2 (2.41) and Cinsault1 (1.25) while values were very low in  
267 Grenache musts (0.42 and 0.57, respectively for Grenache1 and Grenache2). The same trend was  
268 observed for Total Yellow Pigment (TYP) values, ranging from 0.61 for Grenache1 to 2.06 for Syrah2.  
269 All TRP and TYP values were also significantly higher in musts containing press juice, again indicating  
270 higher pigment extraction rates. This was also observed on the  $A_{520}$  values measured directly on musts  
271 but these values were significantly higher in Cinsault than in Syrah. When  $A_{520}$  values were measured  
272 after addition of acetaldehyde ( $A_{520\text{-acetaldehyde}}$ ), the impact of grape variety was very similar to that  
273 observed on TRP values. The concentration of bisulfite adducts was high in Syrah musts, representing  
274 about 20% of TRP, while it was very low in Grenache and Cinsault musts. Thus, Syrah musts contained  
275 larger concentrations of anthocyanin pigments than Cinsault but appeared less colored at wine pH  
276 because of the presence of higher concentrations of sulfites, causing sulfite bleaching. Values  
277 measured after addition of sulfite were significantly higher in Cinsault2 than in Cinsault1 and in both  
278 Cinsault musts compared to Grenache and Syrah musts. However, non-bleachable pigments  
279 represented a higher proportion of red pigments contributing to rosé must color ( $A_{520}$ ) in Grenache  
280 (60%) than in Cinsault (47% and 30%, respectively in Cinsault1 and Cinsault2), and in Syrah (12% and  
281 16%, respectively in Syrah1 and Syrah2). NBP represented 13 to 26% of total red pigments in Grenache  
282 and Cinsault musts but only 1% in Syrah musts.

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



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

288 3.1.2. Polyphenol targeted analysis



289

290 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, flavanlypyranoanthocyanins and flavanol-  
308 anthocyanin adducts. The concentrations of carboxypyrananthocyanins and pyrananthocyanins  
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 (Cheyner 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.

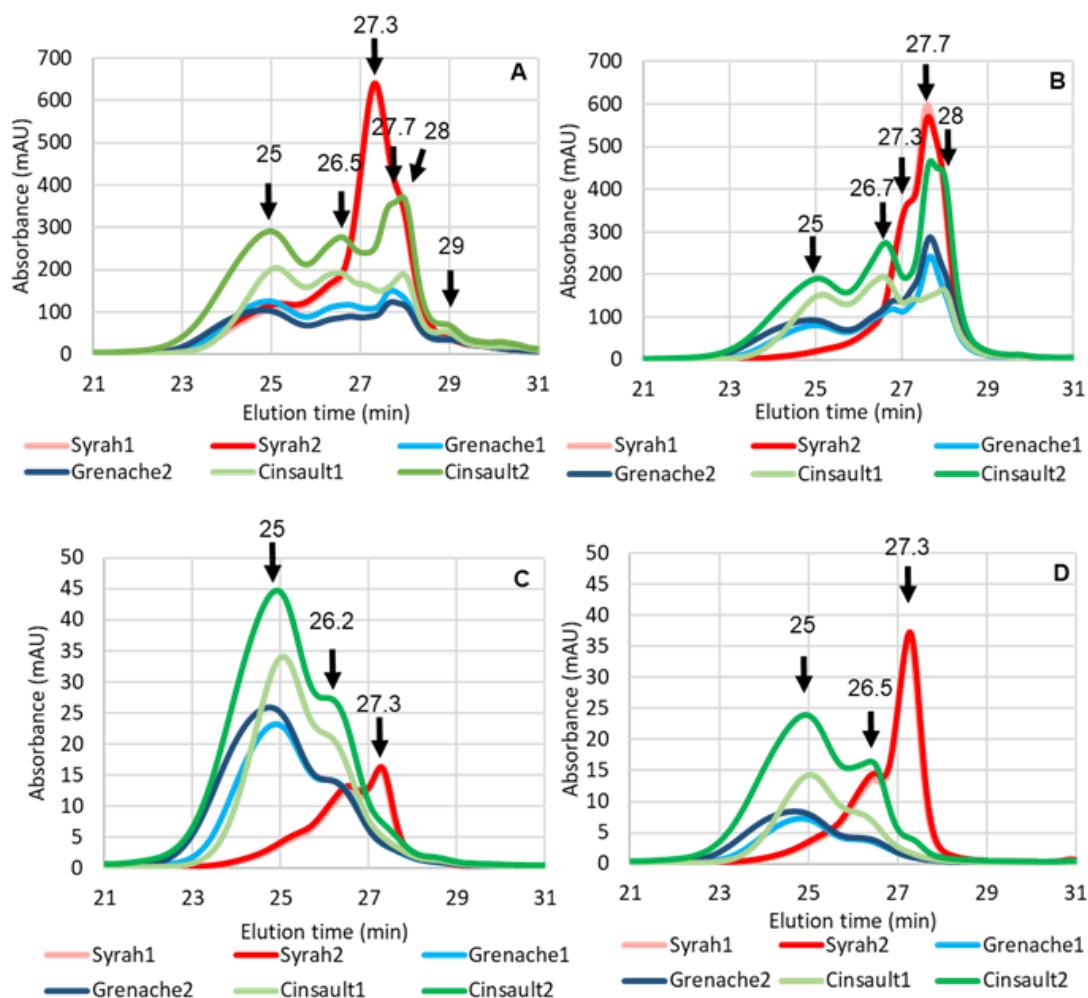
323 Flavanol concentrations determined by phloroglucinolysis (supporting information S2) were much  
324 higher than the concentrations of monomers and dimers measured by MRM, indicating the presence  
325 of larger flavanol oligomers or derived tannins. In addition, the excess of flavanol units released 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.

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

341

342 3.1.3. High performance size-exclusion chromatography



343

344 Fig. 2: Analysis of polyphenols from musts by HPSEC. (A) at 280 nm (B) at 320 nm (C) at 420 nm (D) at  
 345 520 nm. Values and arrows indicate the elution time of different polyphenolic compounds. The elution  
 346 time of different polyphenols were determined first (supporting information S3) and were as follows:

347 *p*-coumaric acid,  $29.6 \pm 0.13$  min; caffeic acid,  $29 \pm 0.13$  min; catechin,  $28 \pm 0.13$  min; oenin chloride,  
348  $27.5 \pm 0.13$  min; aDP6 apple tannins,  $25 \pm 0.13$  min; aDP 40 apple tannins,  $23.7 \pm 0.13$  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 coumaric 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 ( $A_{280}/A_{320}$  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, flavanilpyranoanthocyanins and phenylpyranoanthocyanins, than the other

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

## 415 3.2. Wines

### 416 3.2.1. Spectrophotometry analysis

417 After fermentation, Syrah wines showed significantly higher total polyphenol index values than  
418 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/ $A_{520}$ =42%) than in Cinsault2  
426 (NBP/ $A_{520}$ =25%) and Grenache (NBP/ $A_{520}$ =29% and 24%, respectively in Grenache1 and Grenache2),  
427 and in Syrah (NBP/ $A_{520}$ =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 flavylum cations, due to accumulation of  
454 acetaldehyde released by yeasts during fermentation, as above mentioned.

455

456 3.2.2. Polyphenol targeted analysis

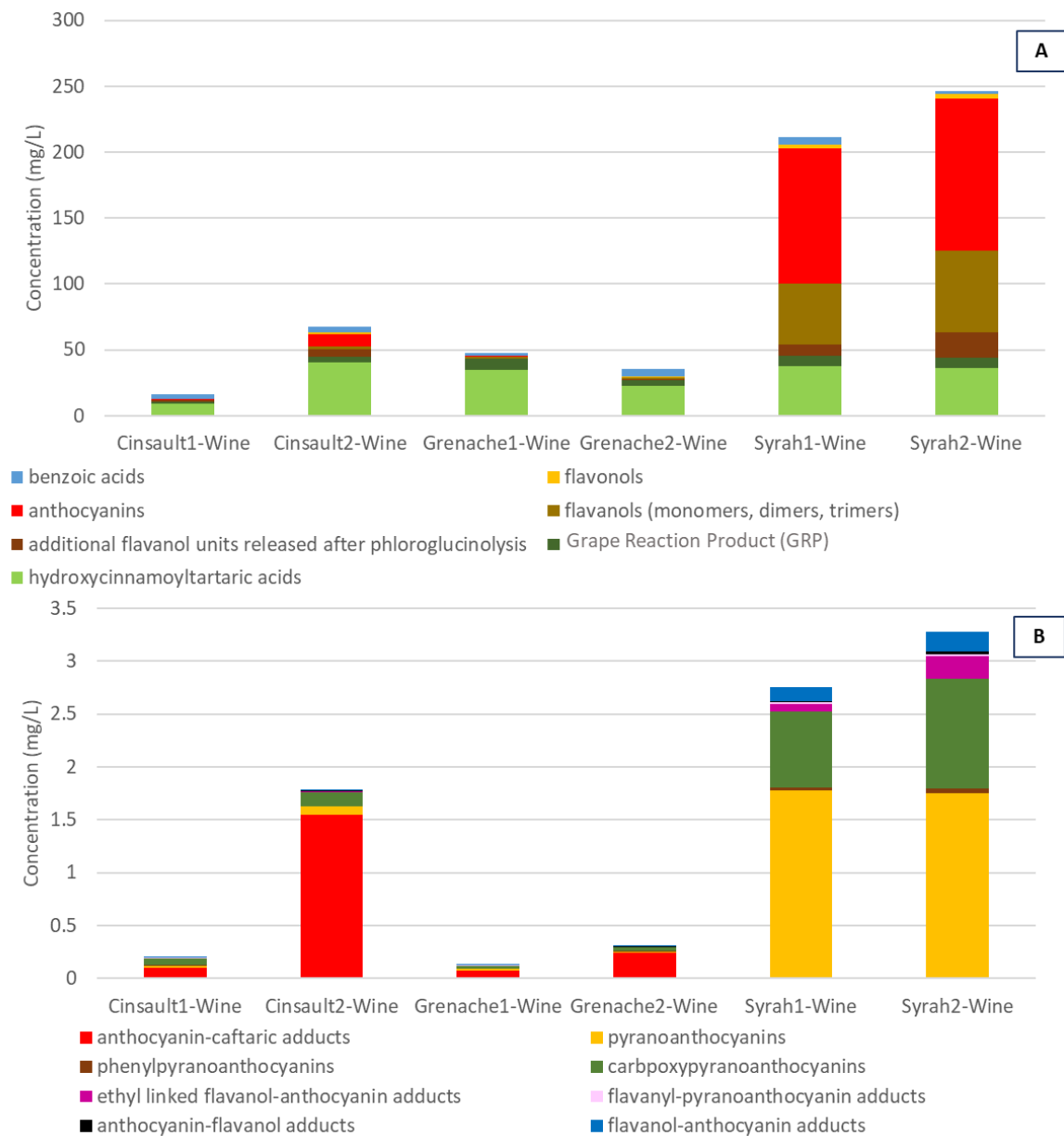


Fig. 3: Concentration of native (A) and derived (B) polyphenolic compounds in the six wines

457

458

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

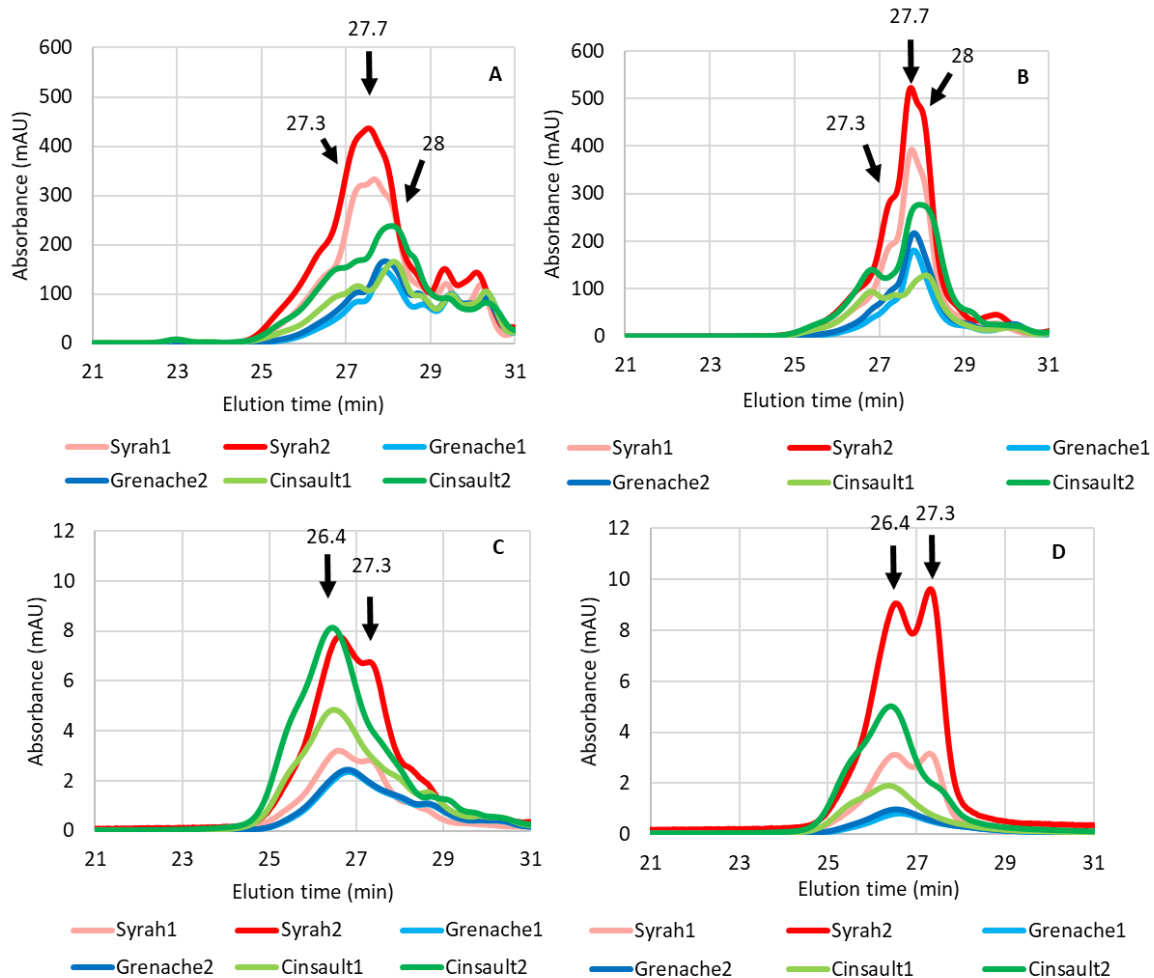
464 concentrations in Cinsault1 than in all other wines, while Syrah wines contained significantly higher  
465 concentrations of anthocyanins, flavonols, and flavanol monomers and dimers. Cinsault2 was also  
466 significantly enriched in anthocyanins and flavanol monomers and dimers compared to Cinsault1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

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

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

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

489 3.2.3. High performance size-exclusion chromatography



490  
 491 Fig. 4: Analysis of polyphenols from wines by HPSEC. (A) at 280 nm (B) at 320 nm (C) at 420 nm (D) at  
 492 520 nm. Values and arrows indicate the elution time of different polyphenolic compounds. The elution  
 493 time of different polyphenols were determined first (supporting information S3) and were as follows:  
 494 *p*-coumaric acid, 29.6 ± 0.13 min; caffeic acid, 29 ± 0.13 min; catechin, 28 ± 0.13 min; oenin chloride,  
 495 27.5 ± 0.13 min; aDP6 apple tannins, 25 ± 0.13 min; aDP 40 apple tannins, 23.7 ± 0.13 min)

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

512 During Syrah must fermentations, absorbance values and anthocyanin contents dropped by 10% and  
513 50% respectively, indicating the conversion of native anthocyanins to new derived pigments. These  
514 include pyranoanthocyanins, carboxypyrananthocyanins and flavanol-anthocyanins adducts that are  
515 detected by our MRM method. In contrast, the important drops in absorbance values observed after  
516 alcoholic fermentation of Grenache and Cinsault were not related to the concentrations of phenolic  
517 compounds measured by targeted analysis, which remained stable during fermentation. Losses of  
518 flavanol units measured after phloroglucinolysis were observed in all wines and much higher for  
519 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

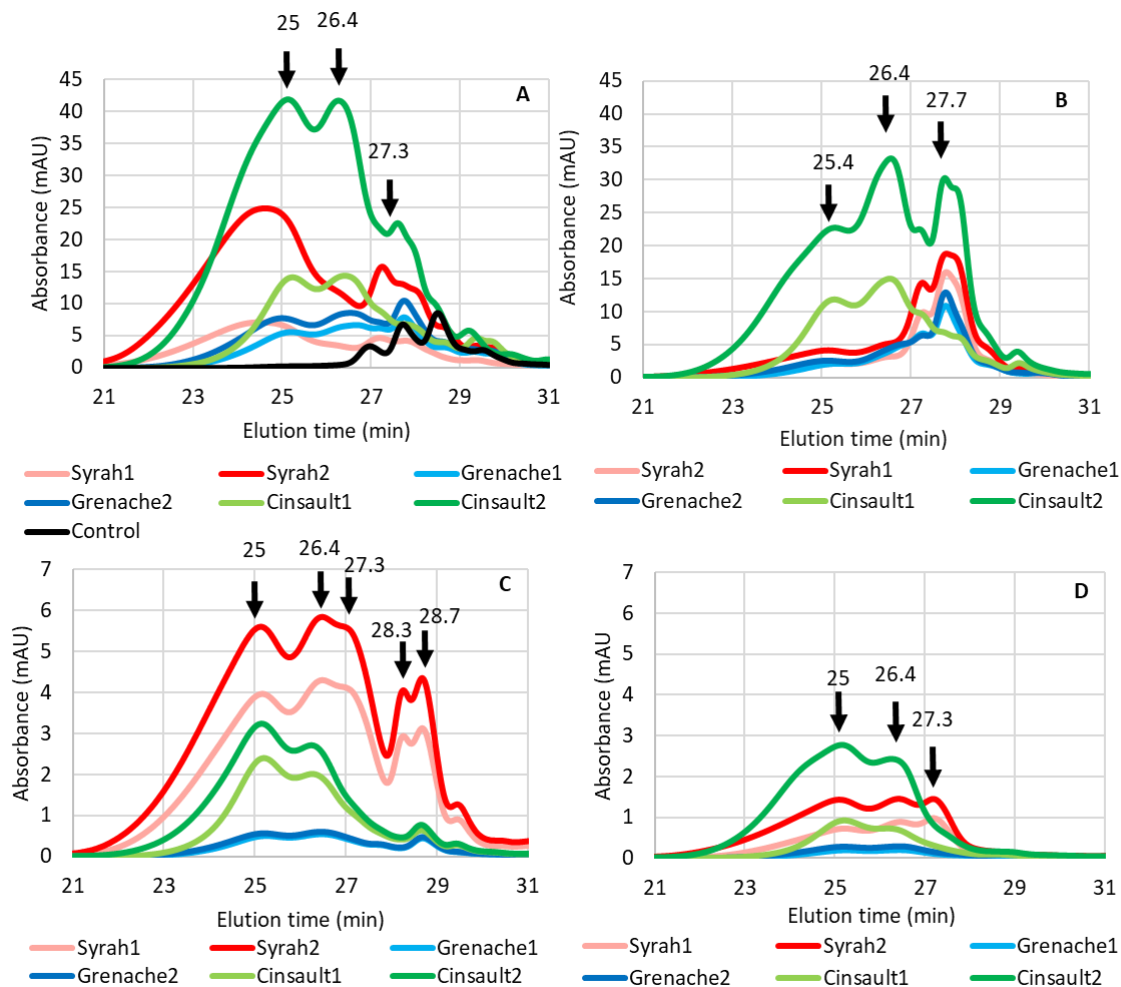
543 desorption of phenolic compounds so that these values are likely underestimated, as observed by  
544 other authors (Mazauric & Salmon, 2006; Vernhet et al., 2020).

### 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



556

557 Fig. 5: Analysis of polyphenols from lees by HPSEC (A) at 280 nm (B) at 320 nm (C) at 420 nm (D) at 520  
 558 nm. Values and arrows indicate the elution time of different polyphenolic compounds. The elution  
 559 time of different polyphenols were determined first (supporting information S3) and were as follows:  
 560 *p*-coumaric acid,  $29.6 \pm 0.13$  min; caffeic acid,  $29 \pm 0.13$  min; catechin,  $28 \pm 0.13$  min; oenin chloride,  
 561  $27.5 \pm 0.13$  min; aDP6 apple tannins,  $25 \pm 0.13$  min; aDP 40 apple tannins,  $23.7 \pm 0.13$  min)

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



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

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

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

589 4. Conclusion

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

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

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

613

614 **Supporting information:** S1: List of phenolic compounds, MRM parameters and calibration ranges.pdf;  
615 S2: Concentrations of phenolic compounds with ANOVA statistics.xls, S3: Standard elution in HPSEC.ppx  
616

617 **CRedit:** Conceptualization: G.M., V.C. and J.-R.M.; methodology: E.M., S.C., Ar.V., C.L. and N.S.; formal  
618 analysis: C.L., S.C., E.M., Ar.V. and M.-A.D.; investigation: C.L. and V.C.; resources: G.M.; data curation:  
619 Ar.V., E.M., M.-A.D. and C.L.; writing—original draft preparation, C.L.; writing—review and editing: M.-  
620 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  
621 administration and funding acquisition, G.M., M.B., Au.V., J.-R.M. and V.C. All authors have read and  
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623

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628

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631

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735

736



737 **Figure Captions**

738

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

740 **Figure 2:** Analysis of polyphenols from musts by HPSEC. (A) at 280 nm (B) at 320 nm (C) at 420 nm (D)

741 at 520 nm. Values and arrows indicate the elution time of different polyphenolic compounds. The

742 elution time of different polyphenols were determined first (supporting information S3) and were as

743 follows: *p*-coumaric acid,  $29.6 \pm 0.13$  min; caffeic acid,  $29 \pm 0.13$  min; catechin,  $28 \pm 0.13$  min; oenin

744 chloride,  $27.5 \pm 0.13$  min; aDP6 apple tannins,  $25 \pm 0.13$  min; aDP 40 apple tannins,  $23.7 \pm 0.13$  min)

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

746 **Figure 4:** Analysis of polyphenols from wines by HPSEC. (A) at 280 nm (B) at 320 nm (C) at 420 nm (D)

747 at 520 nm. Values and arrows indicate the elution time of different polyphenolic compounds. The

748 elution time of different polyphenols were determined first (supporting information S3) and were as

749 follows: *p*-coumaric acid,  $29.6 \pm 0.13$  min; caffeic acid,  $29 \pm 0.13$  min; catechin,  $28 \pm 0.13$  min; oenin

750 chloride,  $27.5 \pm 0.13$  min; aDP6 apple tannins,  $25 \pm 0.13$  min; aDP 40 apple tannins,  $23.7 \pm 0.13$  min)

751 **Figure 5:** Analysis of polyphenols from lees by HPSEC (A) at 280 nm (B) at 320 nm (C) at 420 nm (D) at

752 520 nm. Values and arrows indicate the elution time of different polyphenolic compounds. The elution

753 time of different polyphenols were determined first (supporting information S3) and were as follows:

754 *p*-coumaric acid,  $29.6 \pm 0.13$  min; caffeic acid,  $29 \pm 0.13$  min; catechin,  $28 \pm 0.13$  min; oenin chloride,

755  $27.5 \pm 0.13$  min; aDP6 apple tannins,  $25 \pm 0.13$  min; aDP 40 apple tannins,  $23.7 \pm 0.13$  min)

756

757

758 **Table Caption**

759

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

765