Characterization of suspended solids in thermo-treated red musts

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CHARACTERIZATION OF SUSPENDED SOLIDS IN THERMO-TREATED RED MUSTS

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Abstract

Aims : Thermo-treatment of grapes, followed by pressing and fermentation in liquid phase, is a growing practice in red winemaking to obtain light and fruity wines. Must clarification before fermentation, a key step to get the expected wine profile, is hardly controlled and strongly varies between different musts. To better understand this variability and its potential impact on quality, suspended solids in several red musts were characterized and the performances of different clarification techniques compared.

Methods and results : Results show a large variability in turbidity and total wet suspended solids between different raw and clarified musts, and a lack of correlation between these values. Clarification is always higher for vacuum filtration than for disk-stack or decanter centrifugation, with strong differences between musts for a given process. Despite a large size distribution, most of suspended particles are micronic and sub-micronic. TEM observations and analyses indicate that they are mostly membrane and organelle fragments along with (macro)molecular aggregates formed during juice extraction. Their overall composition differs from that found in white musts.

Conclusion : Particle heterogeneity and size distribution account for the difficulties encountered in red must clarification. Results also raise the question of the relationship between must turbidity and content in compounds likely to affect wine quality.

Significance and impact of the study : This study constitutes a first characterization of suspended solids in thermo-treated red musts. It provides elements to (i) reason their clarification and (ii) identify the technological and qualitative impact of must suspended solids.

Key words : thermo-treated red musts, clarification, suspended solids, composition, size distribution

Résumé

Objectifs : Le thermo-traitement de la vendange, suivi d'une clarification et d'une fermentation en phase liquide, est une pratique qui se développe pour élaborer des vins rouges fuités et légers. La clarification des moûts avant fermentation, étape clé pour obtenir le profil souhaité, est difficile à contrôler et variable selon les moûts. Pour appréhender cette variabilité et son impact qualitatif potentiel, les solides en suspension ont été caractérisés pour différents moûts et les performances de différentes techniques de clarification comparées.

Méthodes et résultats : Les résultats montrent une grande variabilité de la turbidité et des solides en suspension totaux entre différents moûts bruts et clarifiés, et une absence de corrélation entre ces valeurs. La clarification est toujours plus importante par filtration sous vide que par centrifugation ou décantation centrifuge, avec des différences importantes entre moûts pour un procédé donné. En dépit d'une large distribution en taille, la plupart des particules en suspension sont microniques et sub-microniques. Des observations par microscopie électronique et des analyses montrent qu'elles sont essentiellement constituées de fragments de membranes ou d'organites cellulaires et d'agrégats formés lors de l'extraction des jus. Leur composition globale diffère de celle trouvée dans les moûts blancs.

Conclusion : L'hétérogénéité et la répartition en taille des particules expliquent les difficultés rencontrées dans la clarification des moûts de thermo-traitement. Les résultats soulèvent la question de la relation entre turbidité et teneur en composés susceptibles d'affecter la qualité du vin.

Signification et impact de l'étude : Cette étude constitue une première caractérisation des solides en suspension dans les moûts de thermo-traitement. Ceci fournit des éléments pour (i) raisonner leur clarification et (ii) identifier l'impact technologique et qualitatif de ces solides en suspension.

Mots clés : moûts de thermo-traitement, clarification, solides en suspension, composition, distribution en taille

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INTRODUCTION

The extraction of polyphenols before alcoholic fermentation is a growing practice in oenology for the production of light and fruity red wines. The objective is to avoid the maceration step and to achieve fermentation in liquid phase, at low temperatures. Two main processes, thermovinification and flash-release, can be used. The thermovinification process consists of heating berries to temperatures around 70-75 °C for 30-40 min by immersion in a hot grape juice. Grapes are then pressed and the red must is cooled and clarified before alcoholic fermentation. In the flash-release process, grapes are quickly heated at high temperatures (85-95 °C) with vapour at atmospheric pressure and then placed under a high vacuum. This causes an instant vaporization of water in the cells that induces cracks in cell walls and cooling of treated grapes. These are then pressed and the must clarified before fermentation, in the same way as for thermovinification. Whatever the technique, a key step to obtain the expected sensory and aromatic profile of wines is the clarification of musts. Clarification is evaluated by turbidity measurements.

In white winemaking, the degree of must clarification has been shown to influence (i) yeast fermentation kinetics and cell viability (Alexandre *et al.*, 1994; Casalta *et al.*, 2013) and (ii) the aromatic characteristics of finished wines (Karagiannis and Lanaridis, 2002; Singleton *et al.*, 1975). The impact of suspended solids on fermentation and yeast viability is mainly attributed to their lipid content, especially phytosterols and unsaturated long chain fatty acids (Cabanis and Flanzy, 1998; Luparia *et al.*, 2004). Although aroma formation during winemaking is dependent on several other factors (Styger *et al.*, 2011), lipid availability in musts has also been shown to affect the formation of yeast volatiles, *i.e.*, acetate and ethyl esters, higher alcohols and medium chain fatty acids (Ferreira *et al.*, 1995; Rosi and Bertuccioli, 1992; Varela *et al.*, 2012). In practice, low turbidity levels (between 50 and 150 NTU) often favours the formation of fruity aromas, whereas wines elaborated from high-turbidity musts present heavy, herbaceous aromas with sometimes off-odours. High turbidity may also favour browning. On the other hand, excessive clarification may negatively affect fermentation progress. In addition to their effect on the lipid status of yeast, particles serve as nucleation sites for CO_2, which favours CO_2 release from the fermentation medium and decreases its concentration and toxicity (Kuhbeck *et al.*, 2007).

Similar trends in terms of wine aromas are observed for red wines elaborated by thermovinification and flash-release. However, these musts exhibit high turbidity, in the order of 3000 NTU or higher, and are usually difficult to clarify. Clarification is usually favoured by the use of pectolytic enzymes, added before pressing. It is most often achieved by filtration on rotary vacuum filters. Although it provides satisfactory clarification levels with the high throughputs required by process lines (100 to 120 hL/h for a 15-ton/h line), it has some disadvantages. Among the most important are the cost of filtration earths and the high amount of solid wastes related to their use. Despite several trials and recent technical developments, there is at present no efficient alternative to vacuum filtration. The development of new techniques or the optimization of present ones is hampered by a poor knowledge of the amount, nature and physico-chemical characteristics of the particles responsible for the turbidity. Such information is a prerequisite to optimize the clarification techniques as well as to get a better understanding of the impact of thermo-treated must turbidity on wine characteristics.

Characterization studies were conducted during three years to better understand the technical difficulties inherent to the clarification of red musts. The variability of musts from different varieties and technological pathways was determined in terms of turbidity, Total Wet Suspended Solids (TWSS) and particle size distribution. The impact on turbidity and TWSS was compared for different clarification processes: vacuum filter, disk-stack centrifuge and decanter centrifuge. The nature and composition of the particles responsible for the turbidity were determined through transmission electron microscopy (TEM) and biochemical analyses of two Cabernet-Sauvignon red musts obtained from flash-release and thermovinification.

MATERIALS AND METHODS

1. Musts

The musts used in the present study were recovered from different local wineries (Narbonne area, France). They were processed from various grape varieties by conventional thermovinification (TH) or flash-release (FR). Juices were extracted using pneumatic or continuous presses. Must turbidity was determined using a 2100AN IS turbidimeter (Hach, Germany). TWSS were determined by centrifugation of 45 g of homogenised must at 11500 g for 10 min The supernatant was removed and the pellet weighed. Results were expressed in % w/w.

2. Particle size distribution in thermo-treated musts

Particle size distribution was determined by light diffraction using a Malvern Mastersizer 2000

(Malvern Instruments, Worcestershire, UK) equipped with a He/Ne laser. To obtain an adequate obscuration level, musts were diluted in 100 mM NaCl (adjusted to pH 3.5 with HCl). As there was no information concerning the refractive index of suspended particles and considering their heterogeneous composition, results were analysed using the Fraunhofer theory. The size distribution was obtained in % volume, which represents the volume fraction (in %) occupied by particles with a given hydrodynamic diameter D_H. The volume-weighted average hydrodynamic diameter (in µm) is given by:

$$D_{H,V} = \frac{\Sigma n_i D_{Hi}^4}{\Sigma n_i D_{Hi}^3}$$

From this value, the number-weighted average hydrodynamic diameter, which represents the arithmetic diameter of particles in the sample, can be calculated as:

$$D_{H,N} = \frac{\Sigma n_i D_{Hi}}{\Sigma n_i}$$

Particle size analyses were completed by dynamic light scattering experiments performed on diluted musts (in 100 mM NaCl, pH 3.5) after removal of the visible debris by static decantation. Particle average hydrodynamic diameters (D_H) and polydispersity indexes of the suspensions were measured using a Zetasizer 3000 HS (Malvern Instruments) equipped with a He/Ne laser and APD detection.

3. Clarification of musts

The different technologies compared for the clarification of musts were centrifugation, using either a disk-stack centrifuge or a decanter centrifuge, and filtration (rotary vacuum filtration). Two disk-stack centrifuges were used: an Alfa-Laval CLARA 80 (2009-2010, flow-rate 6 hL/h, 8400 rpm) and an Alfa-Laval CLARA 15 (2011, flow-rate 2 hL/h, 9500 rpm). The decanter centrifuge (Alfa-Laval Foodec 209) was used with the following operating parameters: 10 hL/h, 4000 rpm, differential speed 5. Rotary vacuum filtration was achieved using Randalite W28 as filtration earth (2.5-3.5 Da).

4. Characterization of suspended solids by microscopy and biochemical analyses

Musts and fractionation of suspended solids

Suspended solids were recovered from two Cabernet-Sauvignon grape juices, obtained from two different wineries of Languedoc-Roussillon in 2011. The first one, called FR must, was elaborated by flash-release. The harvest was heated to 90-95 °C in 5 min before being submitted to high vacuum (> 80-100 mbar). A pneumatic press was then use for juice extraction. The second one, called TH must, was obtained by thermovinification (65 °C, 1-hour maceration). The juice was extracted with a continuous press. No enzymatic treatments (pectolytic enzymes) were performed. Conventional oenological parameters (reducing sugars, total acidity, pH and Total Polyphenol Index) were determined according to the Vine and Wine International Organisation methods. Anthocyanins were estimated by absorbency measurement at 520 nm after dilution in a pH 3.5 aqueous solution. The dilution factor was chosen to avoid co-pigmentation effects.

Total suspended solids in the initial musts (1.4 L) were recovered by centrifugation (30 min, 17000 g, 20 °C). The supernatant was removed and the pellets were weighed, pooled and distributed into 250-mL centrifuge tubes. They were then weighed again to determine losses and washed three times with 20 mM NaCl pH 3.5 (adjusted with HCl) by successive dispersion/centrifugation (12000 g, 15 min) steps. Pellets were weighed before and after freeze-drying to determine the total suspended solids and their humidity. Fine particles were separated from the initial musts by static settling of 10 L of must at 2 °C for 18 h. The upper phase (4.5 L), containing mostly fine particles, was recovered (decanted must). An aliquot (1.0 L) was centrifuged (17000 g, 30 min), the supernatant was discarded and the pellets were weighed, pooled, and washed three times with 20 mM NaCl pH 3.5 as described previously. They were then freeze-dried to get the so-called fine particles.

Transmission Electron Microscopy (TEM)

Freeze-dried fine particles recovered from the two grape juices were rehydrated (48 h) and the aqueous solution removed by centrifugation. Particles were then fixed with 6 % glutaraldehyde in 50 mM cacodylate buffer (pH 7.2) containing 1 % caffeine for 6 h at ambient temperature. The glutaraldehyde solution was removed by centrifugation and particles washed twice with water before being post-fixed in 1 % osmium tetraoxide for 1 h. After washing twice with water, fixed particles were dehydrated in successive ethanol baths (30 to 100 %) and embedded in Epon. Thin sections (60-nm thickness) were contrasted with 1 % Oolong tea (Brillouet *et al.*, 2013) and observed with an Hitachi 7100 electron microscope. TEM observations were performed at the Centre de Ressources en Imagerie Cellulaire (CRIC/IURC, Montpellier, France).

Lipids

Total lipids were extracted from fine particles according to the protocol proposed by Folch (Folch *et al.*, 1957), using 500 mg of dried sample. Methanol (6.67 mL), chloroform (13.3 mL) and KCl 0.8 % w/w (4.2 mL) were progressively added to the

samples and incubated under agitation for 1 h. The solid phase (particles), the aqueous phase and the organic phase, which contains extracted lipids, were then separated by centrifugation. The aqueous phase was removed and replaced by an equivalent volume of methanol/$CHCl_3$/KCl 0.8 % (48/3/47 v/v/v). The solid phase was resuspended and the mixture separated again by centrifugation. This step was repeated three times in order to wash the solid phase. The organic phase was then recovered and removed by evaporation under vacuum. The dried extract was weighed to determine the total lipid concentration, and dissolved at 10 g/L in $CHCl_3$/methanol (2/1 v/v) for further analyses. The extracts (10 µL) were used to analyse polar and apolar lipids by Thin Layer Chromatography (TLC), performed with silica gel as stationary phase (Fuchs *et al.*, 2011). Standards were purchased from Sigma Aldrich (glycerides, phospholipids, sterols) and Larodan (glycolipids).

Nitrogen

Nitrogen content (about 50 mg) was determined by the Kjeldahl method, using a Büchi digestion unit K-435 system and a Büchi distillation unit K-314 system.

Polyphenols

Polyphenols were extracted from 10 mg fine particles (dry weight) using successively 2 mL methanol (2 min) and 6 mL acetone/TFA/H_20 solvent (60/0.05/40, 1 hour under shaking) (Fournand *et al.*, 2006). Samples were then centrifuged (15 min, 10000 rpm) and supernatants (extracts) recovered. Aliquots of the supernatants (4 x 400 µL) were dried under vacuum at 35 °C and either redissolved in pure methanol (200 µL) for direct HPLC analysis (free anthocyanins, phenolic acids, flavonols and flavan-3-ol monomers) or used for the analysis of proanthocyanidins (PAs) by acid-catalysed cleavage in the presence of phloroglucinol (Kennedy and Jones 2001). Anthocyanin quantification was carried out from peak areas at 520 nm using malvidin-3-*O*-glucoside as an internal standard. For phloroglucinolysis, the dried extracts were redissolved in 100 µL MeOH containing 0.2 N HCl, 50 g/L phloroglucinol, and 10 g/L ascorbic acid and then heated for 20 min at 50 °C. The reaction was stopped by the addition of 100 µL of 200 mM sodium acetate (Kennedy and Jones 2001). HPLC was used to analyse terminal subunits, released as such, and extension subunits, released as phloroglucinol adducts. The average Degree of Polymerization (aDP) of PAs and all qualitative data were calculated on a molar basis. HPLC analyses were performed using two Waters-Millennium systems equipped with reversed-phase Atlantis dC18 columns (Waters, Milford) following temperature, elution and detection conditions described previously (Fournand *et al.*, 2006). PAs were also quantified by the Bate-Smith method (Porter *et al.*, 1985) using 5 mg of dried particles. PAs, released in the form of anthocyanidins, were quantified by absorbency measurements at 550 nm. Absorbency was measured before (A_0) and after heating (A) to subtract the colour related to the extraction of free anthocyanins. PAs purified from white grape skins (60 µg) were used as a standard for quantification.

Carbohydrates

The neutral glycosyl-residue composition of polysaccharides in the fine particles was determined after hydrolysis with 2 M trifluoroacetic acid (120 °C, 75 min), by converting the monosaccharides into their alditol acetate derivatives (Harris *et al.*, 1984). Hydrolysis was performed directly on a given mass (between 10 and 15 mg) of the dried particles. Inositol and allose (100 mg) were added after hydrolysis as internal standards. Residual solids were removed by centrifugation before reduction and acetylation. The separation of alditol acetates was achieved by gas chromatography (GC) on a silica DB-225 column (30 m x 0.32 mm, J&W Scientific, USA), using a Shimadzu GC 2010-plus gas chromatograph under the following elution conditions : injector temperature 250 °C, oven temperature 210 °C, carrier gas H_2 (65 kPa). Neutral monosaccharides in particles were also analysed by GC for their alditol acetate derivatives after Saeman hydrolysis (Hoebler *et al.*, 1989, Saeman *et al.*, 1954). Saeman hydrolysis was used to evidence the presence of polysaccharides resistant to TFA hydrolysis, especially cellulose. Briefly, particles (10-15 mg) were submitted to 3-hour pre-hydrolysis in 72 % w/w sulfuric acid (150 µL) at 25 °C, followed by 2-hour hydrolysis in 2 N sulfuric acid at 100 °C. The medium was then neutralized with a saturated barium hydroxide solution (Merck) and residual sulfate ions were removed as barium sulfate precipitate. The precipitate and residual solids originating from particles were removed by centrifugation (10 min, 8000 g). Supernatants were brought to dryness by vacuum evaporation and residues dissolved in 1 mL milli-Q water for further reduction and acetylation of the released neutral sugars. Inositol (added before 2 N sulfuric acid hydrolysis) and allose (added before reduction and acetylation) were also used as internal standards.

Ash

Ash content was determined by burning a given mass (between 150 and 250 mg) of dried particles in an electric muffle furnace set at 550 °C. Results,

expressed in % dry weight, are a mean of two separate experiments.

RESULTS

1. Turbidity and particle size distribution in red musts – Impact of different clarification processes

The turbidity of raw red musts obtained by thermovinification and flash-release varied from 1000 to 4500 NTU. Strong variations were observed depending on the raw materials (grape variety and maturity) and technological pathways (TH versus FR and continuous versus pneumatic pressing). Performances in terms of clarification were strongly dependent on the process and, for a given process, on the considered must (Figure 1A). From final turbidity and TWSS values, the different processes could be ranked by decreasing order of efficacy as: RVF > DSC > DC. The initial turbidity did not allow predicting the final level of clarification: a must with a high initial turbidity (for example 2010_FR_Cab Sauv, 4478 NTU) was found to be easier to clarify than a must with a medium initial turbidity (for example 2008_FR_Merlot, 2000 NTU).

The turbidity of raw and clarified red musts is plotted Figure 1B as a function of their TWSS (w/w). If the two values were related, there was no direct and simple relationship between them: musts with very different turbidity presented yet close TWSS values. This indicated different nature and/or size distribution of suspended solids in these musts. Indeed, turbidity does not only depend on the volume fraction of suspended particles but also on their size distribution, refractive index and shape (Benitez *et al.*, 2007, Davies-Colley and Smith 2001).

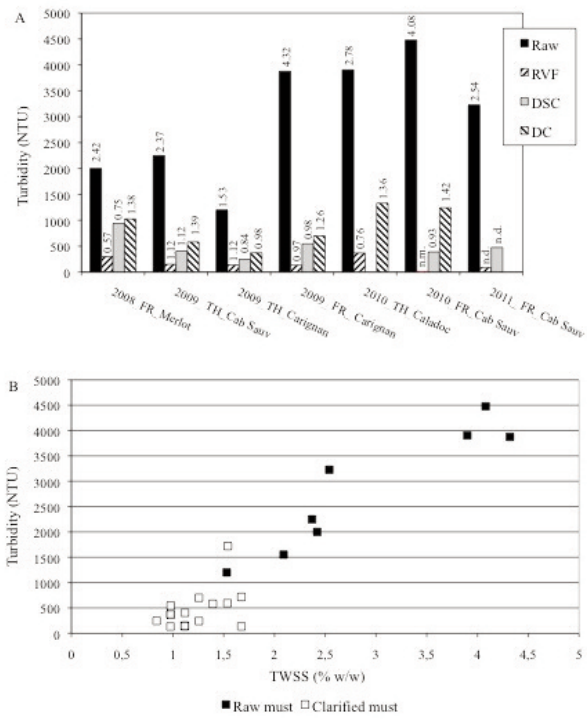

Figure 1 - (A) Impact of different clarification processes on the turbidity and total wet suspended solids (TWSS) of several red musts; (B) turbidity of different red musts as a function of their TWSS.
RVF: rotary vacuum filter;
DSC: disk-stack centrifuge; DC: decanter centrifuge.
n.d.: not determined; n.m.: not measurable (too low).

Musts were diluted for particle size analyses. Visual observations performed on several diluted raw musts showed that visible large cell debris (few hundred µm and higher) represented only a negligible fraction of the whole suspended solids. Most of these suspended solids formed an opalescent dispersion that did not settle during the time of the experiment. Light diffraction experiments indicated a very broad size distribution in all raw musts, with hydrodynamic diameters of the particles ranging from one to several thousand µm. An example of such size distribution is presented in Figure 2. The volume-weighted average hydrodynamic diameters ($D_{H,V}$) of particles in the different raw musts varied between 84 and 531 µm. As expected, these values were decreased in clarified musts ($D_{H,V}$ between 3 and 130 µm). However, the distribution remained very large (Figure 2).

The problem with very polydisperse samples such as those studied here is that $D_{H,V}$ is strongly influenced by the presence of even small numbers of large particles and may not reflect the size of the majority of the particles in the sample. In addition, the expression of D_H in % volume hides the presence of small particles. Although only indicative, the number-weighted average hydrodynamic diameters values suggested that most of the particles in musts were small ($D_{H,N}$ around 1 µm). As granulometry is no more well adapted when dealing with such small particles, this micronic/sub-micronic size of the majority of the particles was confirmed for some musts (raw and clarified) by DLS (Table 1).

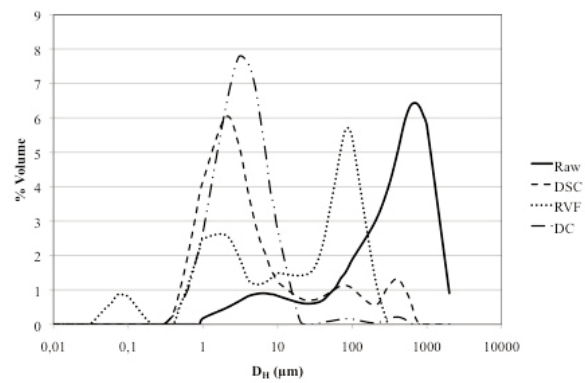

Figure 2 - Particle size distribution in the 2008_FR_Merlot raw and clarified (DSC, RVF, and DC) musts.

These results are in accordance with those obtained by Davin and Sahraoui (1993) in a white must. These authors used sieving (removal of large particles > 63 µm) and impedance measurements with a Coulter counter to determine the size distribution of suspended solids in a white must. Large particles were not quantified but did not represent an important proportion of the suspended solids. Most of the particles (92 %) in the sieved white must were found to have hydrodynamic diameters below 2 µm.

2. TEM observation of suspended solids and biochemical analyses

Two Cabernet-Sauvignon musts, obtained by flash-release and thermovinification, were used for the fractionation and characterization of suspended solids (Table 2). Analyses evidenced a much higher turbidity and suspended solid concentration in the TH must, along with a much higher soluble polyphenol concentration. The characterization of suspended solids was performed on particles recovered from decanted musts (fine particles).

TEM observations

Examination by TEM of must fine particles from either thermovinification or flash-release did not reveal clear differences between the two musts (Figures 3A,B). In accordance with light scattering results, most of these particles were micronic and sub-micronic and exhibited a large polydispersity in terms of size (between few tens nm and few µm), shape and nature. Membranes from various origins and numerous amorphous particles with more or less spherical structures, which can be altered organelles or aggregates formed during extraction, were the main particles in number. Other different elements were easily identified: chloroplasts (Figure 3C) with swollen thylakoid lumen forming tannins (Brillouet 2013), tannin accretions (3D) (Brillouet *et al.*, 2013), a dividing yeast cell (3E) and fragments of grape berry cell walls (3F).

Table 2 - Characteristics of the two musts obtained by thermovinification (TH) and flash-release (FR).

	TH must	FR must
Reducing sugars (g/L)	224	226
Total acidity (g H_2S0_4/L)	4.71	3.93
pH	3.6	3.5
Total Polyphenol Index	80.5	30.3
A_{520}	7.6	3.1
Initial must		
Turbidity (NTU)	> 10000	3227
Total suspended solids (dry, g/L)	6.5	3.5
Decanted must		
Turbidity (NTU)	4134	1406
Total suspended solids (dry, g/L)	1.2	0.9

Suspended solid composition

The average composition of fine suspended solids in the TH and FR musts is summarized in Table 3. Kjeldahl analyses evidenced high amounts of nitrogen in these fines. As the latter were extensively washed before analysis, nitrogen is most likely related to proteins. Protein analysis was attempted using different methods, including extraction ones, but was hampered by the large polyphenol contents. Other organic compounds were polyphenols,

Table 1 - Turbidity and average particle size in some raw and clarified red musts, as evaluated by granulometry and Dynamic Light Scattering.

Must	Turbidity (NTU)	$D_{H,V}$ (µm)	$D_{H,N}$ (µm)	D_H* (DLS) (µm)
2008_FR_ Syrah				
Raw	3100	84	0.9	0.8 (1)
DSC	2900	32	0.8	0.9 (1)
2008 _FR_Merlot				
Raw	2000	531	1.4	1.4 (1)
RVF	299	45	0.1	n.d.
DSC	940	46	0.9	0.7 (1)
DC	1026	94	0.9	n.d.
2010_TH_Caladoc				
Raw	3904	156	n.d.	0.50 (1)
RVF	369	n.m.		0.40 (0.3-0.4)
DC	1331	35		0.46 (0.5)
2010_FR_Cab Sauv				
Raw	4478	358	1.4	n.m.
DSC	390	69	0.5	0.30 (0.5)
DC	1242	41	0.9	n.m.

The values in parenthesis represent the polydispersity index of the suspension.
n.m : not measurable (too small particles); n.d. : not determined

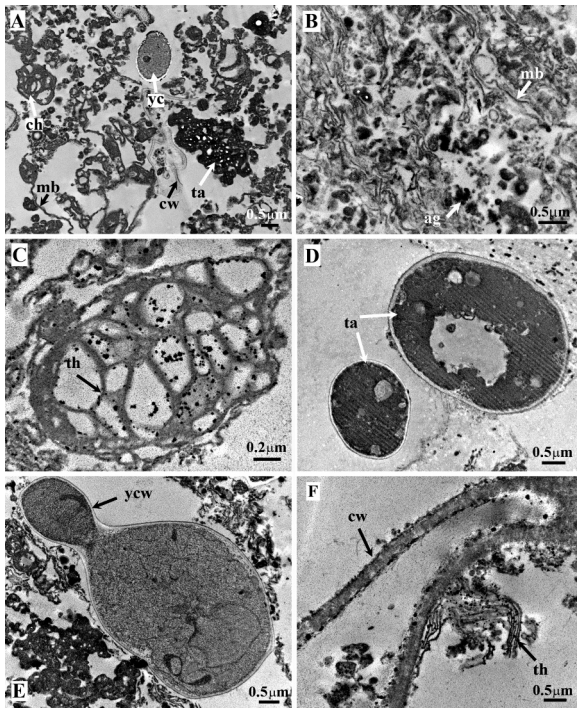

Figure 3 - Aspects of the particles found in thermo-treated red musts (flash-release or thermovinification) under transmission electron microscopy. (A, B) General views of particles showing a high concentration of membranes along with cell debris and numerous sub-micronic and micronic more or less spherical elements ; (C) an inflated chloroplast with swollen thylakoids ; (D) two tannin accretions ; (E) a dividing yeast cell ; (F) grape cell walls and thylakoids. (cw) grape cell wall ; (ch) chloroplast ; (mb) membrane ; (ta) tannin accretion ; (th) thylakoids ; (ycw) yeast cell wall ; (ag) aggregates.

polysaccharides and lipids. Ash represented about 3 % of the dry weight in both cases. All these compounds may be associated with the cell organelles and debris (membranes, chloroplasts, tannosomes, cell walls, etc) evidenced by TEM. They can also be found in the form of aggregates, formed during juice extraction and also evidenced by TEM, or be adsorbed on suspended solids. Aggregation phenomena are expected between proteins due to their extraction in the acidic must and heat treatment, as well as between solubilised PAs and proteins (McManus *et al.*, 1985; Oh *et al.*, 1980). The propensity of PAs to adsorb on grape cell wall materials is also well established (Bindon *et al.*, 2010, Hanlin *et al.*, 2010). In addition, the membrane fragments evidenced by TEM were osmiophilic, indicating that PAs also adsorb on them.

Polyphenols in suspended solids were anthocyanins (free anthocyanins and their acylated and *p*-coumaroylated derivatives) and PAs. No phenolic acids, flavan-3-ol monomers and flavonols were detected. PAs were quantified either by HPLC after their extraction using methanol and acetone/water (60/40 v/v) or by the Bate-Smith method, directly performed on dried particles. They accounted for 10-12 % of the dry mass. However, it must be kept in mind that PA interactions with other biopolymers, as well as their involvement in chemical reactions, lead to a decrease in the yield of acid-catalysed cleavage reaction (Aron and Kennedy 2007, Bindon *et al.*, 2010, Poncet-Legrand *et al.*, 2010). In addition to physico-chemical interactions, chemical reactions are expected to be favoured due to the heat-treatment of grapes. Thus, PA contents in suspended solids are likely underestimated here. The aDP of PAs was found to be in the order of 40. This is high by comparison to the aDP of tannins found in wines elaborated from usual maceration or thermo-treatment techniques (mostly within the range 5-10) (Morel-Salmi *et al.*, 2006) but consistent with the aDP of Cabernet-Sauvignon skin tannins (Hanlin *et al.*, 2011). The presence of epicatechin-gallate units in higher amounts indicates a higher seed tannin extraction in the TH than in the FR must. Anthocyanins were much less abundant in the particles of the FR must than in those of the TH must. Considering their solubility, it can be hypothesised that anthocyanins are adsorbed on suspended solids and that the differences observed between the two musts are related to their different content in soluble polyphenols, including pigments (Table 2). Anthocyanins in grape juices may also be found in vesicle-pectin complexes with size between 10 and 200 nm (Jacob and Paliyath, 2008).

Neutral sugars were characteristic of water-soluble polysaccharides of grape berry cell wall, *i.e.*, glucose, rhamnose, arabinose, galactose and xylose (Table 3). These soluble polysaccharides only represent a small fraction of the whole non-cellulosic grape polysaccharides and are mainly type II Arabinogalactan-proteins (AGPs), which are not structural components of the cell walls, along with galacturonans, arabinans, type II arabinogalactans and to a lower extent xyloglucans (Vicens *et al.*, 2009; Vidal *et al.*, 2000; Vidal *et al.*, 2001). The lack of glucose resistant to trifluoroacetic acid hydrolysis and the low amount of xylose found in fines indicated that insoluble cell wall fragments, rich in cellulose and xyloglucans, were poorly represented (Nunan *et al.*, 1998; Vidal *et al.*, 2001). Neutral sugar analyses performed on large particles concentrated by successive settling/decantation steps (results not shown) evidenced the presence of such cell wall fragments. However, even after concentration, these were only poorly represented : glucose and xylose released by Saeman hydrolysis represented 7.2 and

Table 3 - Composition of fine suspended solids in the thermo-treated red musts, expressed in % dry weight. PAs : proanthocyanidins.

	Fine suspended solids Thermovinification	Fine suspended solids Flash-release
N (%)	5.4 ± 0.1	5.7 ± 0.14
Anthocyanins[†] (%)	1.2	0.3
PAs phloroglucinolysis[†] (%)	12.6	9.0
aDP	40.0	42.3
% Epigallocatechin	20.4	26.1
% Epicatechin-Gallate	7.0	4.1
PAs Bate-Smith (%)	13.9 ± 0.1	10.7 ± 0.9
Neutral sugars (%)	6.5 ± 0.3	8.5 ± 0.9
Rhamnose	0.2	0.2
Fucose	0.1	0.0
Arabinose	1.6	2.3
Xylose	0.1	0.1
Mannose	1.0	1.6
Galactose	1.7	2.0
Glucose from Saeman [§]	2.0	1.8
from TFA hydrolysis	2.0	1.8
from cellulose or β-glucans	0.0	0.0
Lipids (%)	13.4	12.7
Ash (%)	3.1 ± 0.1	3.1 ± 0.1
Total (%)	*72.8*	*71.1*

[†] Extractable pigments and tannins

[§] Glucose was quantified after TFA and Saeman hydrolysis. The difference between the two values is mainly attributable to cellulosic glucose but some may also arise from yeast b-glucans, also partly resistant to TFA hydrolysis in the applied conditions. Glucose content attributable to anthocyanins has been subtracted.

10.4 % of the dry weight of large particles in the FR and TH must, respectively. Galacturonic acid, and more generally uronic acids, could not be analysed here. Uronic acids represent, however, 40 to 45 % of the total sugars from insoluble skin and pulp cell walls, and 30 to 40 % of the soluble grape polysaccharides (Vicens *et al.*, 2009, Vidal *et al.*, 2001). As for tannins, polysaccharide contents in suspended solids are then likely underestimated. Particles also contained significant amounts of mannose, attributed to yeast cells evidenced by TEM. Thus, some of the glucose in fines also likely arises from yeast cell walls.

Water-soluble polysaccharides were probably involved in aggregates or adsorbed on aggregates or other cell debris. The heat-treatment of grapes has been shown to strongly enhance the extraction of pectic polysaccharides, especially homogalacturonans (Vidal *et al.*, 2000). Besides proteins and tannins, aggregation may then also involve proteins and soluble pectic polysaccharides (Beaulieu *et al.*, 2005 ; Beveridge, 2002; Jones *et al.*, 2010), tannins and soluble pectic polysaccharides (Le Bourvellec *et al.*, 2012), or proteins, tannins and pectic polysaccharides (Soares *et al.*, 2012). Recent experiments by isothermal titration microcalorimetry have evidenced that in solution, procyanidins show significant affinity for pectins (Le Bourvellec *et al.*, 2012). The latter is, however, much lower than that measured between different proteins and PAs.

Lipids represented between 9 and 14 % of the particle dry mass. Their presence in must suspended solids is in accordance with earlier studies performed in the context of white wine clarification : it has been evidenced that must clarification induces important losses in fatty acids and sterols (Bertrand and Miele, 1984; Cocito and Delfini, 1997) and lipids were found to represent 8 % dry mass of fine suspended solids in a white must (Alexandre *et al.*, 1994). Lipid classes in particles were identified by TLC. TLC

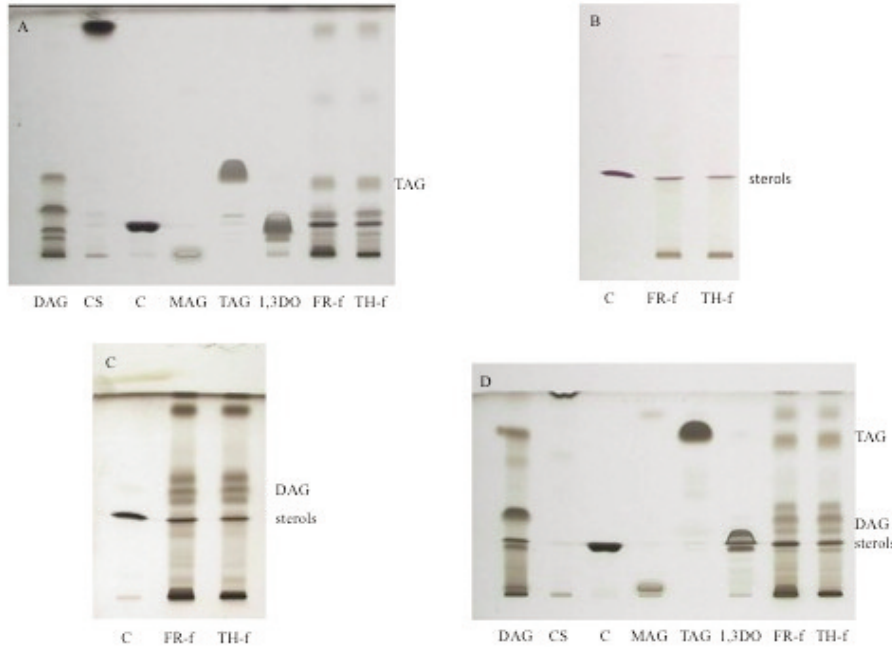

Figure 4 - Analysis of apolar lipids in fine suspended solids by TLC. (A) 90/10/1 hexane/diether/acetic acid solvent, dying with cooper sulfate ; (B, C) 60/40/1 hexane/diether/acetic acid solvent, dying with ferric chloride (B, sterols and sterol esters) and ferric chloride + copper sulfate (C, DAG) ; (D) 70/30/1 hexane/diether/acetic acid solvent, dying with cooper sulfate.

Standards were DAG : racemic mixture of 1,2 and 1,3 diolein ; CS : cholesteryl stearate ; C : cholesterol ; MAG : oleyl-rac-glyceride ; TAG : glyceryl trioleate ; 1,3DO : 1,3 diolein. FR-f: fines, flash-release ; TH-f: fines, thermovinification.

using first a 90/10/1 hexane/diether/acetic acid solvent showed the presence of triacylglycerides (Figure 4A). Sterols and diacylglycerides were evidenced with less polar solvents (60/40/1 and 70/30/1 hexane/diether/acetic acid, Figures 4C,D) and specific dying of sterols with ferric chloride (Figure 4B). Sterol esters were not detected. Polar lipids were glycolipids and phospholipids (Figures 5A,C). Glycolipids were mainly steryl glycosides and esterified steryl glycosides, as shown by specific dying (Figure 5B).

Glycolipids, phospholipids and sterols are especially abundant in the membranes of plant cells. Their presence is consistent with the numerous membrane fragments observed by TEM. Di and triacylglycerides accounted for a major part of the apolar lipids. Their biosynthesis occurs in the endoplasmic reticulum (ER), and they accumulate in droplets located on the outer leaflet of this reticulum. In must particles, they may either be in the form of droplets associated with RE fragments or be adsorbed on/co-aggregated with other organic debris.

DISCUSSION

Up to now, the question of the impact of must clarification and its residual turbidity on fermentation kinetics and wine aromas has mostly been studied in relation to the elaboration of white wines. In recent years, this question has also been of interest for the elaboration of red wines. This is due to the development of new practices, and especially the pre-fermentative treatment of grapes by thermovinification or flash-release that allows polyphenol extraction from skins, followed by pressing and fermentation in liquid phase. In either case, only little is known about the exact nature, size distribution and composition of must particles. Such information is needed to understand the technological and qualitative impact of the residual turbidity, and to control its level.

When dealing with red musts, clarification needs to be realized with high throughputs. It is achieved through filtration (RVF), centrifugation (DSC or DC) or flotation rather than through settling at low temperature, which is not adapted in this case. Our results illustrate the variability of the performances of a given clarification technique (RVF, DSC and DC in the present study) depending on the considered must. They also illustrate how difficult it is to obtain a targeted turbidity range : starting from different musts, the final turbidity and TWSS values obtained with a given technique can be quite different. This observation is consistent with the characteristics of the suspended particles. Light scattering experiments

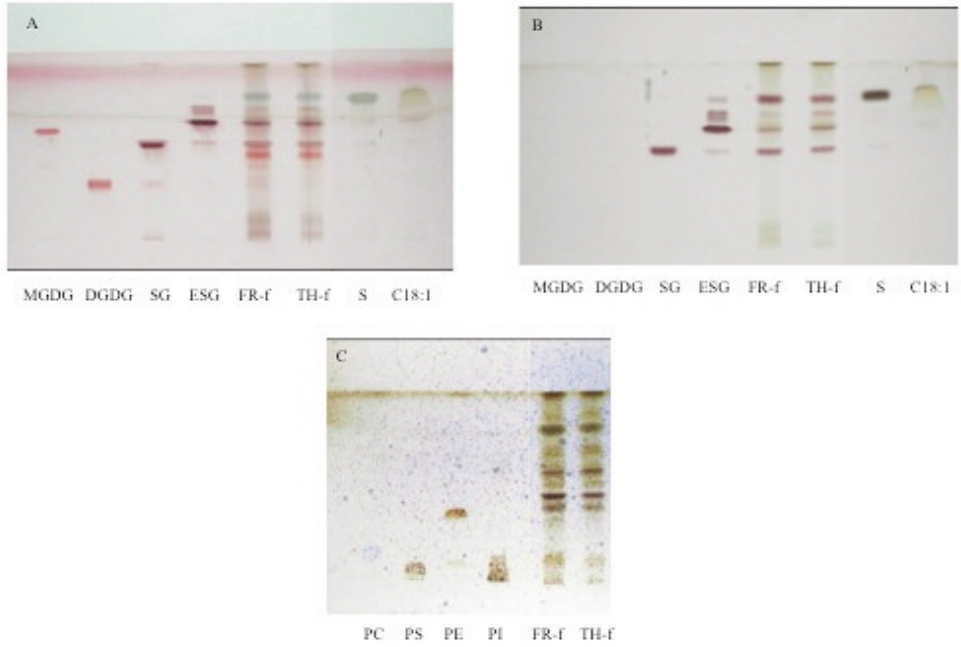

Figure 5 - Analyses of polar lipids in fine suspended solids by TLC using chloroform/methanol/water (95/20/2.5) as migration solvent. (A) dying with thymol (glycolipids); (B) dying with ferric chloride (esters); (C) dying with molybden blue (phospholipids) followed by cooper sulfate.

Standards were: MGDG: monogalactosyl diglyceride; DGDG: digalactosyl diglyceride; SG: steryl glucoside; ESG: esterified steryl glucoside; S: stigmasterol; C18:1: oleic acid; PC: phosphatidylcholine; PS: phosphatidyl serine; PE: phosphatidyl ethanolamine; PI: phosphatidyl inositol. FR-f: fines, flash-release; TH-f: fines, thermovinification.

showed that particles in red musts present a very broad size distribution, with hydrodynamic diameters up to several hundred µm, but that most of them are colloidal particles in the micron-size range. TEM observations also highlighted the diversity of these fine particles in terms of nature and shape. This diversity suggests different physico-chemical properties (density for example) and thus behaviours depending on the clarification technique. RVF, which is the most common, acts through surface-deposition and, to a lower extent, in-depth trapping of suspended solids. The thickness and the characteristics of the deposited layer, which acts itself as a filter, strongly impact the filtration performances. Thus, high initial turbidity is usually associated with high particle retention when using RVF (Figure 1A). Our results show that DSC and DC do not allow reaching the clarification levels obtained by RVF. Size-distribution analyses well account for this observation, as neither DC nor DSC commonly used in wineries are adapted to the removal of sub-micronic particles. The variability of the clarification is likely related to the diversity of suspended particles not only in terms of size, but also in terms concentration and density, which are other important parameters in centrifugation. The heterogeneity of particles can also be an issue for the efficiency of the different fining agents that could be used to favour their aggregation and get better clarification by centrifugation. This is also the case when dealing with flotation (not studied here). This efficiency is strongly dependent on interactions between fining agents and suspended particles, which determine the final size distribution and density of flocculates, and then on the physico-chemical properties of suspended particles. Mechanisms involved remain poorly understood and would deserve further investigations.

Present results also underline the difficulty to relate turbidity and TWSS, or turbidity and amount of compounds likely to impact fermentation and/or wine aroma. Among these compounds, special attention has been paid to lipids (Alexandre *et al.*, 1994; Casalta *et al.*, 2013; Karagiannis and Lanaridis, 2002, Varela *et al.*, 1999). Lipids (di and triglycerides, glycolipids, phospholipids and sterols) represented about 10 % of the dry weight of fine particles in the two studied red musts. This value is of the same order of magnitude than that found by Alexandre *et al.*, (1994) in suspended solids obtained from a white must. In both cases, analyses were performed on particles recovered after the removal of the largest ones by settling. However, the exact impact of the clarification level and process used on the lipid content in suspended solids and their availability for

yeasts still needs clarification. Besides their effect on the final TWSS in musts, different clarification techniques may affect differently the nature and composition of suspended solids, and thus lipid availability (Bertrand and Miele, 1984; Cocito and Delfini 1997). Lipids may be found in the membrane fragments evidenced by TEM, but are also likely present in other forms, especially when dealing with di and triglycerides.

Furthermore, although the total lipid contents found for white and red must fine particles were close, analyses showed quite different compositions in terms of carbohydrates, polyphenols and nitrogen. The overall composition found for suspended solids in white must was (in % dry weight) : 72 % total sugars, 8 % lipids, 5.5 % minerals and 2.6 % nitrogen (Alexandre *et al.*, 1994). By comparison to that found here in red must solids, the main differences were a much higher amount of carbohydrates and a much smaller amount of proteins (factor 2). Polyphenols were not analysed. On the basis of their composition, it was concluded that solid particles in white musts consisted mostly of cell wall fragments. In red musts, composition and TEM observations led to a very different conclusion. Neutral sugar analyses indicated that cell wall fragments only accounted for a minor proportion of fine particles and that most polysaccharides were water-soluble cell wall polysaccharides. Besides cell debris, TEM observations showed the presence of numerous amorphous colloidal size-range aggregates. Differences in suspended solid composition between white and red musts could then be related to the presence of higher amounts of aggregates in red musts as compared to white ones. Indeed, aggregation can be largely enhanced in red grape juices due to the simultaneous presence of proteins and PAs, and to the impact of heat on protein aggregation and on the solubilisation of polyphenols and water-soluble pectic polysaccharides. To confirm this hypothesis, TEM observations should be performed on white must particles and the composition compared with the same methods.

CONCLUSION

This study provides a first characterization and analysis of suspended solids in thermo-treated red musts. Results underline the diversity in terms of size and nature of suspended particles in raw red musts, the variability of the degree of clarification that can be achieved for different musts with a given clarification technique, and the differences between the techniques studied. They underline how difficult it can be to establish the turbidity range to reach, as this value does not necessarily reflect a given content and composition in suspended solids, and thus a potential impact on quality. If lipids are really decisive, the exact impact of the clarification level and process used on their status and content in suspended solids still needs clarification. Future works will be required to evaluate their respective impact on fermentation and/or wine sensory characteristics and to define effective technologies to control their content in red musts.

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