Food Chemistry 199 (2016) 229–237

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Quantitative analysis of Bordeaux red wine precipitates by solid-state NMR: Role of tartrates and polyphenols

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ARTICLE INFO

Article history:
Received 12 May 2015
Received in revised form 2 December 2015
Accepted 2 December 2015
Available online 7 December 2015

Keywords:
Wine coloring matter
Precipitate
Solid- and solution-state NMR
Wine ageing
Polyphenols
Glycerol
Polysaccharides

ABSTRACT

Stability of wines is of great importance in oenology matters. Quantitative estimation of dark red precipitates formed in Merlot and Cabernet Sauvignon wine from Bordeaux region for vintages 2012 and 2013 was performed during the oak barrel ageing process. Precipitates were obtained by placing wine at −4 °C or 4 °C for 2–6 days and monitored by periodic sampling during a one-year period. Spectroscopic identification of the main families of components present in the precipitate powder was performed with ^{13}C solid-state CPMAS NMR and 1D and 2D solution NMR of partially water re-solubilized precipitates. The study revealed that the amount of precipitate obtained is dependent on vintage, temperature and grape variety. Major components identified include potassium bitartrate, polyphenols, polysaccharides, organic acids and free amino acids. No evidence was found for the presence of proteins. The influence of main compounds found in the precipitates is discussed in relation to wine stability.

1. Introduction

Maintaining stability and clarity in wine is an important issue in oenology and methods are being developed to produce stable and clear wine that cope with exportation and reduced ageing periods. Loss of clarity in red wine is observed as a precipitation of red coloring material that is undesirable in terms of visual perception and loss in taste and flavor (Ribéreau-Gayon, Glories, Maujean, & Dubourdieu, 2006). There are many physico-chemical mechanisms that could lead to instability, flocculation and finally precipitation. In fact one may differentiate two main types of precipitates, tartrate precipitates and coloring matter precipitates. In very young red wines (at the start of barrel ageing process), it is well known that high concentration of potassium bitartrate and other tartrate salts lead to instability due to their low solubility in alcoholic solutions (Ribéreau-Gayon et al., 2006). This leads to tartrate salts precipitation along with some coloring matter in red wines. Traditionally, a cold stabilization at −4 °C is performed to eliminate bitartrate precipitation (Lasanta & Gomez, 2012). More recently, methods have been developed to either remove bitartrate, by use of electro dialysis and ion exchange resins, or inhibit its precipitation with substances like meta-tartaric acid, carboxymethyl cellulose (CMC) and yeast mannoprotein extracts (Lasanta & Gomez, 2012; Moine-Ledoux & Dubourdieu, 1999). The other precipitate is called colored matter and may happen after a few months of barrel ageing. It may be sometimes gelatinous and strongly red colored. If the red wine is aged and bottled, the precipitate may occur later in the form of a thin leaf, lining the inner side of the bottles (Waters et al., 1994). A method commonly used in wineries to reduce wine turbidity and stabilize the coloring matter is fining. Fining is performed by addition of a protein (albumin, casein or gelatin) that promotes flocculation or precipitation before the bottling process resulting in clarification of the wine (Glories, 1979). However, in some cases, precipitate formation can still be observed in the later stages of the ageing process. Two assays have been proposed in the literature to predict coloring matter instability: a heat assay performed at 84 °C for 16 h and then cooling down at 23 °C for 4 h (Peng, Waters, Pocock, & Williams, 1996a) and a cold assay at 4 °C for 48 h (Lagune-Ammirati & Glories, 2001). In both cases, measuring the turbidity after the assay is correlated with coloring matter instability; the more turbidity observed, the less stable the wine.

The chemical composition of wine may trigger flocculation and precipitate formation. Wine composition depends on numerous parameters that include grape berry, vintage, 'terroir', fermentation processes and ageing processes and makes it a complex mixture, containing a large number of molecules ranging from organic acids,

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http://dx.doi.org/10.1016/j.foodchem.2015.12.013
0308-8146/

phenols to macromolecules like polysaccharides and proteins (Ribéreau-Gayon et al., 2006). Predominant components in red wine are presented in Fig. 1. Some compounds are known to be poorly soluble such as procyanidins (condensed tannins) originating from grape seeds; resulting in generation of colloidal-sized particles (Pianet et al., 2008). It has also been reported that grape polysaccharides, depending on their structures, may lead to complexes with procyanidins (Ducasse et al., 2010; Poncet-Legrand, Doco, Williams, & Vernhet, 2007).

To assess the composition of this complex mixture and therefore the origin of the precipitate is a challenging task. Analytical techniques such as high performance liquid chromatography (HPLC), mass spectrometry (MS), infrared (IR), and nuclear magnetic resonance (NMR) spectroscopies provide alternative methods for chemical analysis on food and beverages without performing time-consuming extraction processes (De la Cruz et al., 2012; Karoui, Downey, & Blecker, 2010; Robinette, Bruschweiler, Schroeder, & Edison, 2012). In identifying the molecular components of an intact solid mixture, NMR spectroscopy has advantages over other techniques. NMR spectroscopy is a well-established tool for determining molecular structures in a non-destructive manner and allowing the study of samples both in solution and in the soft or solid-state. Solution-state NMR spectroscopy has been extensively used to analyze the soluble compounds in grape berry, juice and wine (Lee et al., 2011; Pereira et al., 2006; Son et al., 2009). Precipitates from red wine have not been extensively studied using NMR spectroscopy. One notable exception is a ^{13}C solid-state MAS NMR study on a red wine bottle precipitate that reported on the presence of tannins, anthocyanins, polysaccharides and amino acids (Waters et al., 1994).

The aim of the present work was to identify and quantitate the different families of components present in the precipitates from the Bordeaux red wine sampled during the ageing process in oak barrels. Precipitate formation was experimentally controlled by chilling wine at either −4 °C (imitating the cold stabilization already mentioned) or 4 °C (mimicking the cold assay, *vide supra*) for 2–6 days with solid-state NMR. Solution-state NMR will complement the analysis on partially solubilized precipitates. Rather than making a statistical analysis on a large number of wines, we selected only a few and followed them as a function of time in barrels; the focus of this study was hence to investigate whether NMR spectroscopy applied on entire/genuine precipitates could be used to identify as far as possible their composition.

2. Materials and methods

2.1. Sources

All the deuterated solvents (DMSO-d6, D_2O) were purchased from Cambridge Isotope Laboratories (Saint Aubin, France). Potassium bitartrate was provided by Laffort, (Bordeaux, France). All chemicals were of analytical grade from Sigma–Aldrich (Paris, France) and were used without further purification.

2.2. Precipitate isolation

Red wines from Merlot and Cabernet Sauvignon (Château Reynon 2012 and 2013, Premières Côtes de Bordeaux appellation, Bordeaux, France) were fermented with the same *Saccharomyces cerevisiae* yeast and the same *Oenococcus oeni* bacteria in tank.

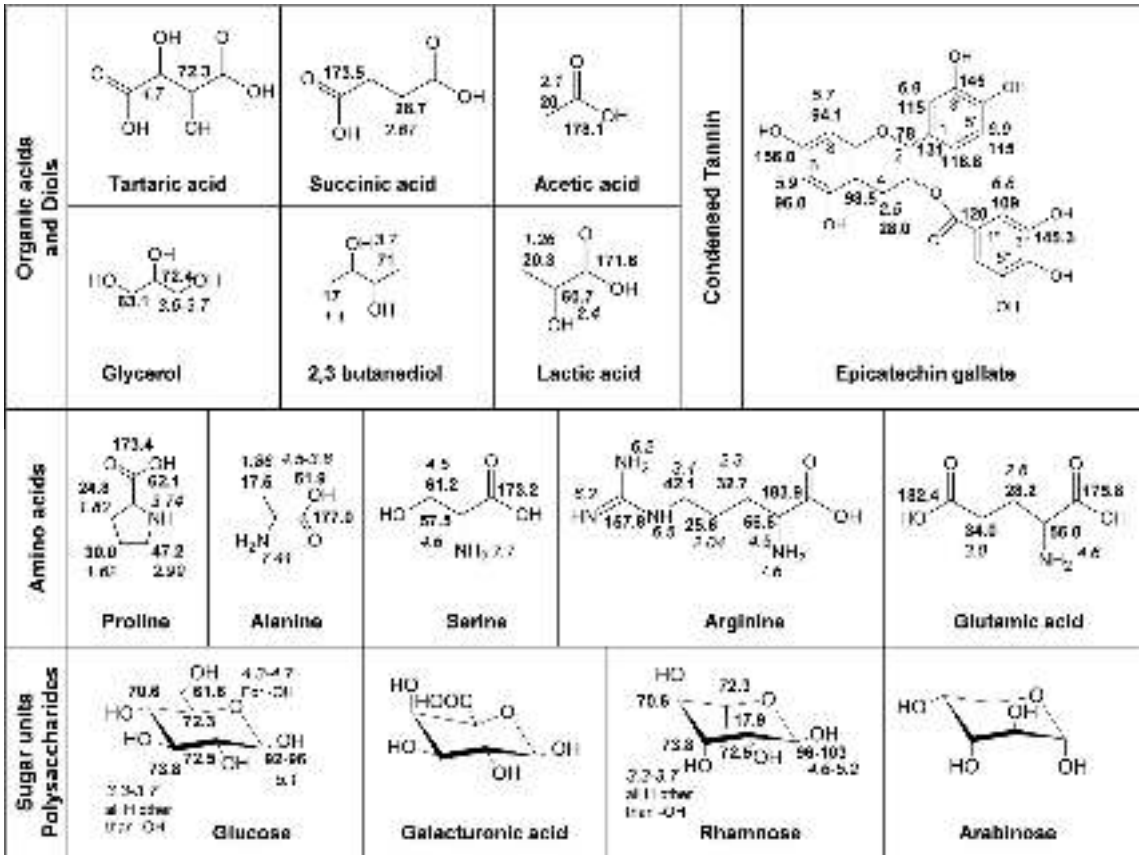

Fig. 1. Components in red wine. Table showing some of the predominant components or families of components present in red wine with their respective ^{13}C and ^{1}H NMR chemical shifts (Newman et al., 1987). ^{13}C chemical shifts are given in bold and ^{1}H chemical shifts in italics. Epicatechin gallate is a typical example of polyphenol molecules since it covers the chemical shifts values for catechin, epicatechin and malvidin-3-O-glucoside units that belong to the family of polyphenol molecules.

After malolactic fermentation, wine was racked and transferred into oak barrel for ageing. Precipitates from Bordeaux red wine (Chateau Reynon, 2013 and 2012) were prepared as follows and as outlined in Scheme S1 (Supporting Information). A volume of 4–10 L from Merlot and Cabernet Sauvignon varieties was collected at times, t = 1 and 3 months for the 2012 Merlot and Cabernet Sauvignon vintage and t = 1 and 4 months for the 2013 Merlot and Cabernet Sauvignon vintage, where t = 0 month is the beginning of barrel ageing. A blended wine (65% Merlot + 20% Petit Verdot + 15% Cabernet Sauvignon, v/v/v) was collected only for year 2012 at times t = 10 and 13 months. All the wine samples were taken from the upper layers of wine in the oak barrels and placed into a flask. All wines were pre-filtered by glass fiber filters (Millipore, AP2504700, Ireland) and filtered through a 0.65 μm nitrocellulose membrane (Millipore, DAWP04700, Ireland) to remove microorganisms and solid particles. An aliquot from each sample (500 mL) was removed for wine analysis (*vide infra*). Three precipitates were obtained from the remaining volume at two temperatures according to the following (see Scheme S1): (i) Pre-freezing (Pf) precipitate: 2–5 L of wine in a flask were placed at −4 °C for six days. On the seventh day, in most cases a formation of red colored flaky precipitate, sticking to the bottom of the flask could be observed. The flask was centrifuged for 30 min at 4 °C, 5000 rpm in a JLA 8.100 rotor (Beckman Coulter, USA) yielding a dark red colored solid pellet. (ii) Short Cold Storage (SCS) precipitate: 2 L of red wine were placed at 4 °C and processed as in (i). (iii) Long Cold Storage (LCS) precipitate: supernatant (2 L) from the Short Cold Storage (SCS) precipitate was kept for further 6 days at 4 °C. On the seventh day it was processed as above. All three precipitates were dispersed in a minimum amount of distilled water and lyophilized overnight yielding a dry red-dark powder.

2.3. Sample preparation for NMR

About 3–50 mg of the lyophilized powder were used for solid-state NMR measurements. The fine powder was placed into 4 mm Zirconia rotors (Cortecnet, Paris) of various volumes ranging from 12.5 to 70 μL and closed with a Kel-F cap. For solution NMR measurements, 3.7–5 mg of the lyophilized Pf and LCS precipitates from Merlot and Cabernet Sauvignon 2013 were placed in (90:10) v/v H_2O/D_2O. 5 mg of the SCS precipitate from Merlot 2012 and 10 mg of the lyophilized SCS precipitate from Cabernet Sauvignon 2012 were placed in 500 μL of (i) (90:10) v/v H_2O/D_2O or (ii) DMSO-d6. The red wine precipitates were only partially soluble in each of the solvents. As a consequence, the solution was centrifuged and the supernatant was taken for measurements using a 5 mm NMR glass tube. The remaining precipitates were lyophilized, weighed and analyzed by solid-state NMR. In case of DMSO, the remaining precipitate was washed with water before lyophilizing.

2.4. NMR measurements

^{13}C Cross-polarization magic angle sample spinning (CPMAS, rotation frequency of 10 kHz) NMR measurements on the precipitates were performed on a 800 or 500 MHz Bruker Avance III NMR spectrometer (Wissembourg, France) using a dual 4 mm CPMAS probe. Additional one-dimensional and two-dimensional solution ^{1}H, ^{13}C and ^{15}N NMR measurements were performed on the same system using a 5 mm TCI cryo-probe (1H–13C/15N/2H). Specific details of these experiments are available in Supplementary materials.

2.5. Wine analysis

Alcohol content (% v/v), pH, total acidity (expressed in tartaric acid g/L), volatile acidity (expressed in H_2SO_4 g/L), and total tannins (g/L) by Bate-Smith reaction (Bate-Smith, 1973) were determined at SARCO laboratory (COFRAC). The Total Polyphenolic Content was determined by spectrophotometric method (UV-180, Shimadzu, Canby, Oregon, USA,) and expressed as a total phenolic index (TPI = $A_{280} * 100$, where A_{280} is the absorbance at 280 nm). The coloring matter instability was determined by a 'cold test' in which difference in turbidity of wine is measured before and after the wine is placed for 48 h at 4 °C (Lagune-Ammirati & Glories, 2001). The difference in turbidity of the coloring matter instability values, ΔNTU values were used to classify red wines. Wines with turbidity increment below or equal to 5 ΔNTU are considered stable, 5–10 ΔNTU are slightly unstable, 10–20 ΔNTU are unstable, 20–50 ΔNTU are very unstable and above 50 ΔNTU are highly unstable. Turbidity of the wine was measured with a Hach 2100P nephelometer (Loveland, Colorado, USA) calibrated using formazine standards solution and expressed in Nephelometric Turbidity Units (NTU). The tartaric stabilization was measured by the Degree of Tartaric Instability (DTI,%) and the Critical Tartaric Stability Index by solubilizing 50 mg of bitartrate in 100 mL of wine (50 g/hL, CTSI50, μS) (Saint-Pierre, Battle, Escudier, & Moutounet, 1998). DTI expresses the potentially tartaric instability in percentage. Stable red wines have DTI values below 5% and those slightly unstable from 5% to 11%. CTSI50 expresses the real tartaric stability; values below 5 μS indicate a stable red wine from the tartaric stability viewpoint.

3. Results

3.1. Mass and aspect of precipitates

Precipitates obtained after centrifugations were weighed. The amount and aspect of precipitates obtained for each grape variety for vintages 2012 and 2013 are presented in Table S2. Except for the unusually high amount of Pf precipitate for Merlot 2012 at t = 1 month, the amount of precipitates for vintage 2012 varies between 25–70 mg/L and for vintage 2013 between 0–75 mg/L. In general, vintage 2012 wines gave more precipitates than the vintages 2013 wines. It is also interesting remarking that 2012 blended wines give SCS precipitates even after one year of ageing. One may also notice that the grape variety Merlot gives in general less precipitate than Cabernet Sauvignon; for vintage 2013, t = 1 month there is indeed no precipitate for the SCS. All the precipitates were red in color, with precipitates from vintage 2012 wines in general being clearer than those from vintage 2013 wines.

3.2. ^{13}C CPMAS NMR of wine Pf, SCS and LCS precipitates

To identify families of components in all three types of precipitates, ^{13}C CPMAS NMR spectra were recorded. Spectra of the Pre-freezing (Pf) precipitates obtained from Merlot 2012, at t = 1 and 3 months are shown in Fig. 2A and B respectively. The two pairs of sharp signals at 75.3, 73.8 ppm and 181.2, 176.2 ppm are readily assigned to potassium bitartrate (spectrum of pure compound in Fig. S5). These sharp signals dominate the Pf precipitate at the start of the ageing process and are still visible at t = 3 months with a significant reduction in intensity making it possible to detect other peaks present in the precipitate. Partial resonance assignment was accomplished by comparison with established ^{13}C chemical shift values in literature on standard compounds (see Fig. 1). The region from 140 to 160 ppm contains peaks from procyanidins and anthocyanins (Newman, Porter, Foo, Johns, & Willing, 1987). The region from 10 to 50 ppm shows contributions from methyl groups in organic acids, free amino acids and amino acid side chains. The peak at 32.7 ppm can be assigned to succinic acid, that at 21.8 ppm to acetic and lactic acids and the peak at

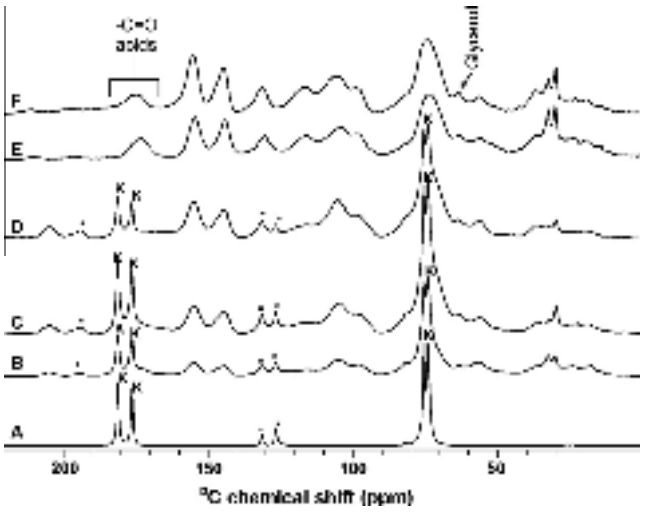

Fig. 2. ^{13}C CPMAS NMR spectra of Pf precipitates from 2012 and 2013 vintages, T = 298 K. (A) Merlot 2012, t = 1 month. (B) Merlot 2012, t = 3 months. (C) Merlot 2013, t = 1 month. (D) Cabernet Sauvignon 2013, t = 1 month. (E) Merlot 2013, t = 4 months. (F) Cabernet Sauvignon 2013, t = 4 months. Lorentzian filtering of 100 Hz was applied to all spectra. Intensity of spectrum (A) is scaled by ×0.12 respective to other spectra. Residual spinning side bands are marked with (∗) and peaks from potassium bitartrate with (K).

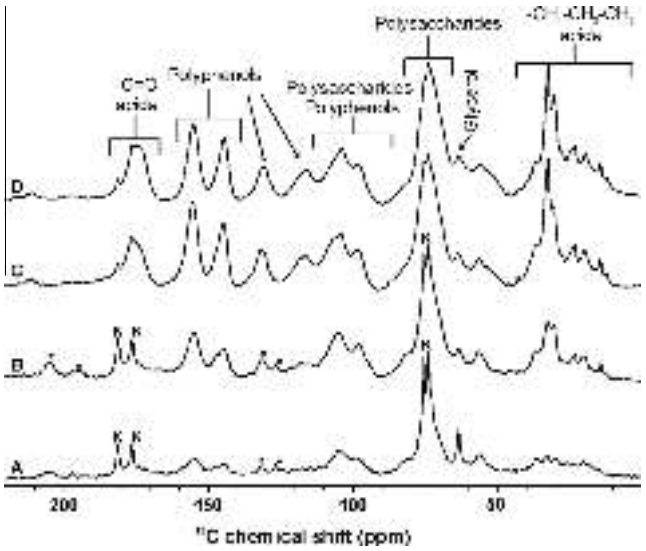

Fig. 3. ^{13}C CPMAS NMR spectra of SCS precipitates from 2012 and 2013 vintages T = 298 K. (A) Merlot 2012, t = 1 month. (B) Cabernet Sauvignon 2013, t = 1 month. (C) Merlot 2013, t = 4 months. (D) Cabernet Sauvignon 2013, t = 4 month. Lorentzian filtering function of 100 Hz was applied to all spectra. Intensity of spectra (A), (C) and (D) has been scaled by ×2.55, 0.7 and 0.9 respectively to (B). Residual spinning side bands are marked with (∗) and peaks from potassium bitartrate with (K).

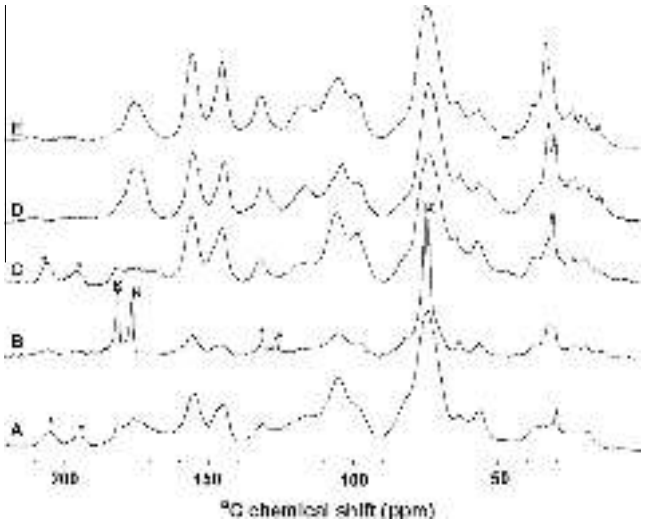

Fig. 4. ^{13}C CPMAS NMR spectra of LCS precipitates from 2012 and 2013 vintages T = 298 K. (A) Merlot 2012, t = 1 month. (B) Merlot 2013, t = 1 month. (C) Cabernet Sauvignon 2013, t = 1 month. (D) Merlot 2013, t = 4 months. (E) Cabernet Sauvignon 2013, t = 4 months. Lorentzian filtering function of 100 Hz was applied to all spectra. Intensity of spectra (A), (B) and (D) has been scaled by 0.1 and (C) by 0.25 to (E). Residual spinning side bands are marked with (∗) and peaks from potassium bitartrate with (K).

30.7 ppm is a contribution from the C^{β} of amino acids (Pretsch, Buehlmann, & Affolter, 2000).

Spectra of the Pre-freezing (Pf) precipitates obtained at t = 1 month for vintage 2013 with Merlot and Cabernet Sauvignon varieties are reported in Fig. 2C and D, respectively. Those obtained at t = 4 month are seen in Fig. 2E and F. Although the sharp signals from bitartrate are still present in spectra at t = 1 month, they have completely disappeared in spectra at t = 4 months. Further assignments can be performed, especially for the 170–180 ppm region of carbonyl groups and the glycerol resonances above 60 ppm. ^{13}C CPMAS spectra obtained for Short Cold Storage (SCS) precipitates from Merlot and Cabernet Sauvignon for vintages of 2012 and 2013 are presented in Fig. 3.

Spectra resemble those obtained for the Pf precipitate though the amount of potassium bitartrate is much less important at time t = 1 month (Fig. 3A and B). At time t = 4 months its resonances almost completely disappeared allowing assignment of all the families of compounds as already detected for the Pf precipitate (Fig. 3 top). Additional assignment may be done (Fig. 3); the peak centered at 145.0 ppm can be assigned to the quaternary carbon C3′ (carbon numbering as shown in Fig. 1), 155.3 ppm to C5, C7 and C9, 131 ppm to C1′, 147 ppm to C4′ and 116.7, 118.7 ppm to C2′, C5′, C6′ of catechin and a malvidin-3-O-glucoside unit. The region from 65 to 90 ppm is characteristic for the contribution from the sugar moieties in the polysaccharides. However, the broad peak at 76 ppm makes it difficult to identify specific sugar units. Spectra for Long Cold Storage (LCS) precipitates are reported in Fig. 4.

Similar to Pf precipitate NMR analysis, potassium bitartrate resonances from the LCS precipitates are detected at t = 1 month and are absent at t = 4 months. ^{13}C CPMAS spectra were also obtained from the SCS precipitates of a 2012 blended Bordeaux wine, at t = 10 months and t = 13 months, and are presented in the Supplementary materials, Fig. S6. The wine is a blend (v/v) of 65% Merlot, 15% Cabernet Sauvignon and 20% Petit Verdot. ^{13}C CPMAS spectra from SCS precipitates of Merlot and Cabernet Sauvignon 2012 are presented for comparison. In general, the spectra from the SCS precipitates from the blend have the same profile as the SCS precipitates of Merlot and Cabernet Sauvignon 2012 for t = 1 and t = 3 months respectively. Chemical shift values of assigned components from all the spectra together with a comparison with the literature values on standard compounds are presented in Table 1 and Table S10 (Supplementary materials). In these tables resonance signal assignments obtained from solution-state NMR spectroscopy (*vide infra*) are also listed.

3.3. Identification of components in the soluble fraction of precipitates with solution NMR

Although classes of components present in precipitates could be identified with ^{13}C CPMAS NMR, the low spectral resolution made it difficult to identify and assign specific molecules. In order to

Table 1
Chemical shift assignments of the major components in wine coloring matter.

Class of components		Carbon numbering[a]	Literature chemical shift[b]		Measured chemical shift [c]	
			^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)
Organic acids (Pretsch et al., 2000)	Succinic acid	2&3	28.7	2.67	**32.7**, *29.72*	*2.40–2.52*
	Lactic acid	2	60.7	4.04	*67.65*	*4.06*
		3	20.3	1.26	**21.8,** *19.70*	*1.27*
	Acetic acid	2	20.0	2.1	*21.8*	
	Carbonyl groups		172–178		*174.2, 179.7*	
Diols (Pretsch et al., 2000)	Glycerol	1 & 3	63.1	3.6–3.7	**62.3**, *62.55*	*3.46–3.55*
		2	72.4		*72.16*	*3.68*
Polysaccharides Sugar units (Beier, Mundy, & Strobel, 1980)	Glucose/Mannose	1	92–96	4.9–5.3	*92.17*	*5.21*
		6	62	3.7	*68.10*	*3.73*
		2–5	70–79	3.3–3.7	**76.0**, *69.30*	*3.82*
Polyphenols (Watanabe, 1998)	Catechin/cyanidin	3′, 5, 7, 4′	145–155		**145.0, 155.3, 147.0**	
Procyanidins/Anthocyanins		1′	131		**131.0**	
		2′, 5′, 6′	115–119	6.9	**116.7, 118.7**, *115.20–116.70*	*6.78–6.84*
Amino acids (Pretsch, Buehlmann, & Affolter, 2000)	Proline	3, 4, 5, 2	24.8, 30.0, 47.2, 62.4	1.62, 1.82, 2.99, 3.74,	**30.7**, *23.73, 28.92, 46.15, 61.27*	*1.92, 2.25, 3.23, 3.32, 4.03*
	Valine	3′, 4, 3, 2	18.5 18.5, 30.3, 59.8	1.25, 2.6, 4.32	**30.7,** *16.50, 15.60, 29.20, 61.80*	*0.77, 0.84, 1.87, 4.07*
	Leucine	4′, 5, 4, 3, 2	21.8, 22.9, 25.1, 40.7, 54.4	1.1, 1.1, 2.0, 2.0, 4.3	*23.85, 38.91*	*1.66, 2.94, 4.72, 7.49*
	Alanine	3, 2	17.5, 51.9	1.86, 3.8–4.5	*19.09, 59.20*	*1.34, 4.32*
	Carbonyl groups		173–182		*174.2, 179.7*	
Salts	Potassium bitartrate				**75.3, 73.8, 181.2, 176.2**	

[a] According to IUPAC rules and with reference to Fig. 1.
[b] Chemical shift values of the components from the literature.
[c] Assignments determined from the ^{13}C CPMAS spectra are presented in bold and those from solution NMR in *italics*.

increase the spectral resolution precipitates were dispersed in (90:10) v/v H_2O/D_2O or DMSO-d6 and the soluble fraction collected and examined with solution-state NMR. Unfortunately, precipitates were not completely soluble in water or DMSO, the residue was dried and weighed, reported in Table S4. We observed that Pf precipitates are less soluble in water than LCS precipitates and that coloring matter from Merlot variety is more soluble than that of Cabernet Sauvignon. Of course DMSO can solubilize more precipitate but not entirely, there remains residues that are neither soluble in water nor in DMSO. The soluble part of precipitates was analyzed by solution-state NMR (*vide infra*) whereas CPMAS NMR was conducted on the remains and shows the dominance of polyphenols (Fig. S10).

^{1}H NMR spectra from the water soluble fraction of Pf and LCS precipitates from Merlot 2013 and Cabernet Sauvignon 2013 at t = 1 month are shown in Fig. 5A, B and C, D respectively. The major components identified and assigned in the soluble fraction of the precipitates include glycerol, succinic acid and lactic acid. Contribution from succinic acid can be seen at 2.5 ppm, lactic acid at 1.27 and 4.06 ppm and the –CHOH and CH_2OH groups of glycerol between 3.4 and 3.8 ppm. In addition, the peaks from the polyphenolic components are detected in the region 6–8 ppm. The region from 0.5 to 3 ppm contains the aliphatic contribution from organic and amino acids. Glycerol, succinic acid and lactic acid are predominant components in red wine (Fig. 1) and have been identified previously in red wine with ^{1}H NMR metabolomics studies (Son et al., 2009). Spectral normalization with respect to the mass that is soluble in the NMR tube affords quantitative comparison of compounds identified in the coloring matter. It appears clearly that the LCS precipitate (soluble fraction) from Merlot 2013 contains the largest amount of glycerol and succinic acid, the Pf precipitate from Merlot 2013 contains the most polyphenols and LCS precipitate from Cabernet Sauvignon 2013 contains the most lactic acid. ^{1}H NMR spectra from the soluble fraction of the SCS precipitates from Merlot 2012 at t = 1 month and Cabernet Sauvignon 2012 at t = 3 months in water are shown in Figs. S7A and S7B respectively. The peak due to alanine at 1.34 ppm is clearly visible in SCS precipitates from Merlot 2012, but not present in Cabernet Sauvignon 2012. In order to make a conclusive assignment of the peaks, 2D NMR spectroscopy was performed on the soluble fractions of the precipitates.

Expansions of selected areas of the 2D ^{1}H–^{1}H TOCSY spectrum of the soluble portions of the precipitates is presented with the assignments in Fig. S8. All the detailed chemical shift assignments are presented in Table 1 (soluble fraction in (90:10) v/v H_2O/D_2O) and Table S10 (soluble fraction in DMSO-d6). A comparison of reported values from the literature is also presented in tables. Glycerol and lactic acid could be clearly identified and assigned by cross peaks present in the TOCSY and HSQC spectra (Supplementary Figs. S11 and S13). ^{1}H and ^{13}C chemical shifts are assigned at 3.46, 3.55, 3.68 ppm and 62.55, 72.16 ppm for glycerol, respectively, while chemical shifts for lactic acid are observed at 1.27, 4.06 ppm and 19.7, 67.6 ppm, respectively. Amino acids could also be identified; Proline dominates the ^{1}H–^{1}H TOCSY spectrum of the soluble fraction of the SCS precipitate from Merlot 2012 in water with peaks at 1.92, 2.25, 3.23, 3.32 and 4.03 ppm for ^{1}H and corresponding ^{13}C peaks at 23.73, 28.92, 46.15, 61.27 ppm. Other amino acids that could be identified include valine, alanine and leucine. Peaks are observed from C6–C3–C6 flavonoid rings of catechin/malvidin-3-O-glucoside units of polyphenols in the aromatic region of the TOCSY and HSQC maps. Correlations were observed for 2′, 5′ and 6′ carbons of catechin/malvidin-3-O-glucoside units (numbering corresponds to the structure in Fig. 1) at 115.9 and 115.2 ppm for ^{13}C and the corresponding ^{1}H peaks at 6.78 and 7.07 ppm. A six carbons sugar molecule, possibly glucose or mannose and another five carbons sugar molecule, which could be arabinose, were identified. The anomeric proton for glucose/mannose and arabinose are detected at 5.21 and 4.76 ppm respectively. The TOCSY spectrum exhibits connections between these anomeric protons and the other protons of the

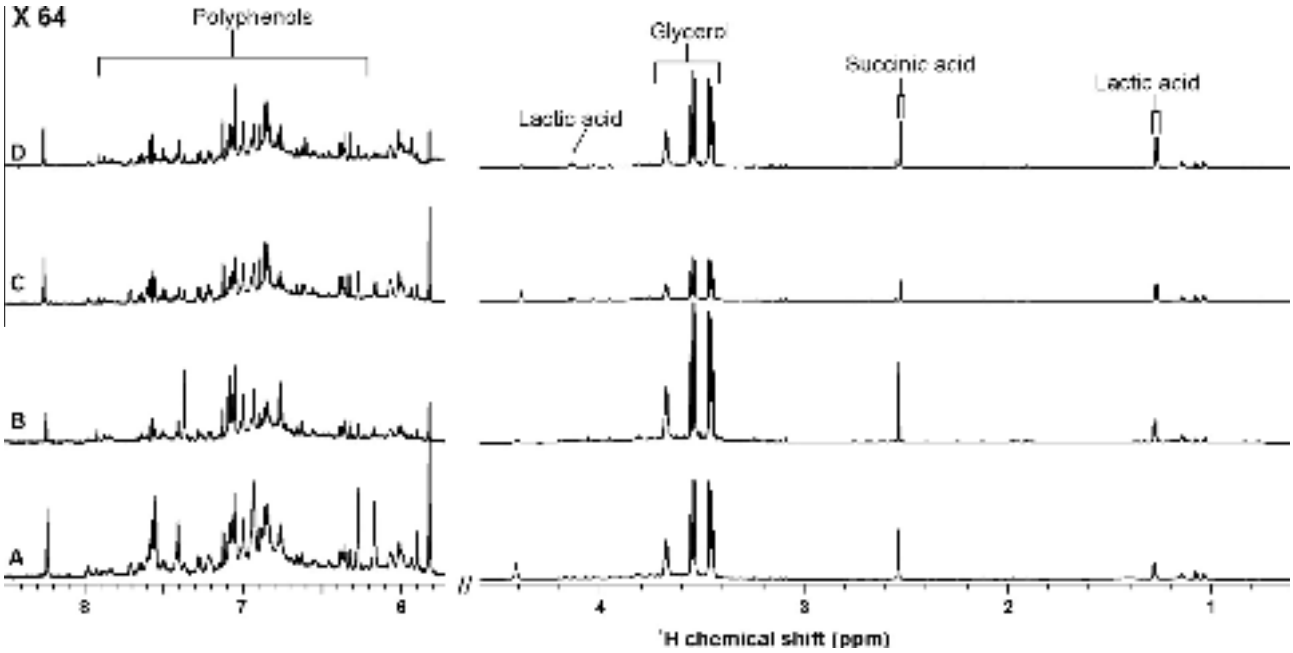

Fig. 5. ^{1}H NMR spectrum of the water-soluble part of Pf and LCS precipitates for Merlot 2013 and Cabernet Sauvignon 2013 at t = 1 month, T = 298 K, excitation sculpting pulse sequence to suppress the water signal (see Supplementary materials). (A) Pf precipitate from Merlot, (B) LCS precipitate from Merlot, (C) Pf precipitate from Cabernet Sauvignon and (D) LCS precipitate from Cabernet Sauvignon. Intensities of all the spectra have been normalized to the soluble fraction of Pf precipitate from Merlot water (Table S4).

sugar. The corresponding ^{13}C chemical shift values for the anomeric carbons of glucose or mannose and arabinose were obtained at 92.17 and 107.6 ppm respectively from the HSQC spectrum. Another sugar molecule, possibly rhamnose, is identified in the soluble fraction of the SCS precipitate of Cabernet Sauvignon in DMSO by the connections of the methyl group at 1.1 ppm to the –CH_2OH groups at 3.4, 3.5 and 3.8 ppm in the TOCSY spectrum. Identification of amino acids in the precipitate was also conducted by natural abundance ^{15}N NMR measurements using 2D ^{1}H–^{15}N HSQC experiments. Threonine or arginine could be identified. An expansion of the spectrum showing these signals is given in Fig. S9.

3.4. Wine analysis

Standard analysis was performed on all the wines for the two vintages, before the precipitate preparation, as described in the methods section. The wines were analyzed for their alcohol content and pH. Degree of Tartaric Instability (DTI,%), Critical Tartaric Stability Index (CSTI), Total Phenolic Index (TPI), coloring matter instability and total tannins were also determined for some of the samples. Results are presented in Table S1 in Supplementary materials. In general, Merlot wine contained higher alcoholic content than Cabernet Sauvignon. Cabernet Sauvignon wines have in general a greater tannin content than Merlot (5 vs. 3.5 g/L). Marked differences are found between vintages 2012 and 2013. Vintage 2012 is less acidic with average values around 3.6, compared with 2013 with an average pH of 3.4. Alcohol content is also different especially with values above 14% for merlot 2012 whereas vintage 2013 ranges around 13.4%. The 2012 vintage was considered as highly unstable from the coloring matter instability viewpoint with values of the turbidity increment, ΔNTU, around 70 or 80, whereas the vintage 2013 was slightly unstable but after winter, it became stable. From the tartaric stability viewpoint indicators, the situation appears different. In October 2013, the blended wine 2012 is already stable, even though the Degree of Tartaric Instability is slightly above 5%, the Critical Tartaric Stability Index is below 5 μS. Merlot wines from 2013 are slightly tartaric unstable in both tests in 2014. However Cabernet Sauvignon wine from 2013 is at the stability limit in January 2014 and completely stable in April 2014.

4. Discussion

Precipitation in wines is a central problem in wine stability and has been evidenced in our study. Appearance of precipitates occurs both at −4 °C and 4 °C, two key temperatures that are used in the winemaking processes. NMR spectra of solid precipitates derived from chilled Bordeaux red wine provide important information regarding the nature of precipitates and their composition. The main findings can be summarized as follows:

(a) A method to quantitatively estimate precipitate formation in red wines at −4 °C and 4 °C can be proposed.
(b) Solid-state ^{13}C NMR can be considered a robust analytical method for semi-quantitative determination of wine precipitate components.
(c) The amount of precipitate is highly dependent on temperature, vintage and grape variety relative to the ageing time of wine.
(d) Precipitates are dominated by potassium bitartrate, tannins, and polysaccharides.

All these points are discussed and parameters that are important in wine stability are proposed in relation with the wine industry.

4.1. A method to produce colored matter precipitates for analytical purposes

Investigating the amount of colored matter that may precipitate from bottled wines is important in wine production, as precipitates are an undesired occurrence for marketed wines. We have shown that precipitation of tens of mg/L of coloring matter occurs both

at −4 °C and 4 °C, two temperatures that are used in the winemaking process. Under conditions used for the current study, precipitation is obtained after keeping the wine several days at low temperature. It is noteworthy that the coldest temperature favors the largest amount of precipitate formation. This "pre-freezing", Pf, precipitate is rich in tartrate salts and this procedure is well known in the wine making process as it is a necessary step to remove salts from wine during the so-called cold stabilization. Interestingly, the NMR signature of potassium bitartrate disappears from precipitates after 3–4 months. This is clearly indicative of its sedimentation over the winter period at the bottom of the barrel. This is well known and is indeed used as a simple predictive test for tartrate precipitates: placing wine at 0 °C for 4–6 days and checking for tartrate crystals (Ribéreau-Gayon et al., 2006). However it is more surprising to detect bitartrate at 4 °C as the bitartrate solubility in a hydro-alcoholic solution increases with temperature (Descotes, Rochard, & Valade, 1992), as an example it varies from 1.21 g/L at −4 °C to 1.75 g/L at 5 °C in 10% ethanol solution. Interestingly, a test to follow the disappearance of potassium bitartrate, at the top of the barrel, could be proposed for preparations both at −4 °C or +4 °C by running ^{13}C CPMAS NMR on the precipitates.

After the disappearance of bitartrate from precipitates after 3–4 months, the peaks due to the amorphous coloring matter precipitates can be identified and assigned. It is worth mentioning that precipitates obtained after keeping the wine at 4 °C for 2 days give practically the same amount of information than that obtained by keeping the wine for 6 additional days at 4 °C. Of course the more one keeps the wine at low temperature, the more precipitate will occur, but the qualitative information does not vary much. Our results are in agreement with the 'cold test' proposed by Lagune-Ammirati & Glories, 2001 as a predictive test for coloring matter instability. A quantitative NMR "fingerprint" of coloring matter precipitate could be obtained after letting the wine rest for 2 days at 4 °C. In our study, it would be interesting to compare this NMR "fingerprint" for coloring matter precipitates after a few years of ageing for vintages of 2012 and 2013. However, this is not possible in the present experiment time scale. Of interest, this short cold storage precipitate may not be present as for Merlot 2013 or may be present in small quantities (10 mg/L for Cabernet Sauvignon 2013). As discussed below, this is associated with wine stability; the less precipitate the more stable the wine.

4.2. Solid-state ^{13}C NMR as a new method to analyze genuine colored matter in wines

^{13}C CPMAS NMR spectra recorded from the various precipitates of wine coloring matter, obtained either at −4 °C or 4 °C, give a complete assessment of their carbon composition. Spectra are obtained with only a few mg of isolated residue, in a powder form, which is not altered by isolation or the experiment. It is possible to readily identify families of components and estimate their relative amounts. For example it is noteworthy that after a few months of ageing in oak barrels the NMR signature of potassium bitartrate has completely disappeared, thus allowing determination of the presence or absence of this compound in colored matter. ^{13}C CPMAS NMR can also differentiate between tartrate crystals (Hill, Zens, & Jacobus, 1979), making it possible to identify potassium bitartrate from other tartrate crystals. More importantly, families of major compounds such as polyphenols, polysaccharides, glycerol and acids can also be identified at a glance or by comparison with chemical shifts compiled in Table 1. ^{13}C CPMAS NMR has been applied previously to study bottle precipitates from Australian red wine (Waters et al., 1994). Polyphenol peaks in the region from 110 to 160 ppm for bottle precipitates are very similar to the spectra obtained in our study for the coloring matter precipitates. The relative quantities of the components differ in both the cases. No organic acids and glycerol were identified in the bottle precipitates in contrast to our study, whereas we did not identify any proteins (*vide infra*). These differences could be due to a variety of reasons ranging from the age of the precipitate to the region of wine production. The solid-state NMR technique can be complemented by solution-state NMR on partially solubilized precipitates. The additional resolution brought by the technique allows further identification of compounds. It is quantitative but is limited by the amount of precipitate that can be solubilized.

It is interesting comparing the NMR method, to other analytical techniques used for estimation of food components such as GC–MS and HPLC-MS. Although the latter techniques are more sensitive than NMR spectroscopy and require less material, they require either a solvent (HPLC-MS) or derivatisation (GCMS) (Di Stefano et al., 2012) and are therefore difficult to apply directly on coloring matter precipitates without solubilization. UV–VIS spectroscopy that is commonly used in oenology for expressing quantities of polyphenols (Peng et al., 1996a) might also be used for precipitates. It is unfortunately not specific to molecules but to certain chemical groups and must be conducted on solubilized components. Solid-state NMR thus appears to be a rather simple and robust method to work on entire wine precipitates without further sample manipulation. The ^{13}C CPMAS NMR technique is per say not entirely quantitative. It would require the use of so-called "build up" experiments (Hartmann & Hahn, 1962) to determine the precise "mixing time" at maximum build-up curve to allow more accurate integration of peaks and therefore full quantification of proportions of families of compounds in precipitates. Such experiments are beyond the scope of the present work.

The amount of precipitate depends on temperature, vintage, grape variety, and not much on ageing

As discussed above, the cooler the temperature, the more precipitate is obtained; from Table S2 one may derive the following relationship for masses of precipitates, $M_{Pf} > M_{SCS} + M_{LCS}$. This is linked to the presence of large amounts of bitartrate in the first moments of the ageing process (Figs. 2–4).

Vintage 2012 was determined to be a less stable wine than vintage 2013, in terms of both tartrate and coloring matter stability according to oenological analysis (tartrate stability and turbidity measurements, Table S3). This was quantitated in our study for both the tartrate and coloring matter precipitates by the presence of greater amounts of all precipitates; for instance the mass of SCS + LCS Merlot precipitates (57 mg/L) for 2012 is twelve times higher than the corresponding masses (5 mg/L) for 2013. Cabernet Sauvignon has a larger amount of precipitate than Merlot as can be seen for vintage of 2013. This may be linked to the higher total amount of tannins, *i.e.*, ∼5.0 g/L vs. ∼3.5 g/L, respectively (Table S3), as polyphenols are indeed known to have limited solubility in water solutions (Pianet et al., 2008).

As already commented, there seems to be little effect of ageing on the amount of precipitate, at least on a one-year scale. Caution must however be taken because our time scale is only limited to one year for vintage 2012 and to 4 months for 2013, so wines are still considered as "young" by oenologists and may continue evolving.

4.3. Precipitates are dominated by potassium bitartrate, tannins, and polysaccharides

As discussed above, the presence of bitartrate appears clearly in the first month of ageing in barrels. A previous study (Peng, Waters, Pocock, & Williams, 1996b) on red wine precipitates looked at the influence of cold stabilization at two different temperatures of −4 °C and 2 °C. The study stated that the composition of the precipitates formed at −4 °C is very similar to the bottle

precipitates observed after few years of ageing. This is in apparent contradiction with our study where we found bitartrate in the very first moments of ageing only. However, in this study, the precipitate was dialyzed against water before lyophilizing and since bitartrate is soluble in water, up to 6.17 g/L, its presence in coloring matter precipitation could not be detected.

Tannins are present in large amounts in wines (3–5 g/L) and are of first importance for taste and feelings such as astringency and bitterness while drinking. It is clear from the NMR data that tannins are also strongly present at all stages of precipitate formation. This is not surprising since these polyphenolic molecules have limited solubility in a water alcohol medium and may aggregate as micelles when there are above their critical micelle concentrations that are in the mM range (Pianet et al., 2008). However the tannins content in precipitates is limited to few tens of mg per liter at best, Merlot leading to less precipitate than Cabernet sauvignon, as mentioned above. The predominant peaks observed from polyphenols in the ^{13}C CPMAS NMR spectra are from catechin, epicatechin and malvidin-3-O-glucoside units. It is however difficult to differentiate individual tannins species from oligomeric tannins and no difference at this stage can be made in between polyphenols coming from Merlot and those from Cabernet Sauvignon. It is interesting relating the variability in precipitates obtained from different grape varieties to their water solubility. In general, ~70% of precipitates from Merlot are soluble in water as compared to 20–40% of Cabernet Sauvignon. DMSO helps increasing solubility by ca. 20% but precipitates cannot be entirely solubilized, especially those coming from Cabernet Sauvignon. The difference in solubility suggests a difference in composition of the two precipitates. Merlot contains less tannins than Cabernet Sauvignon and one may conjecture that more polyphenols with low CMC, *i.e.*, that are prone to aggregate, may be found in precipitates coming from Cabernet Sauvignon. This solubility difference could also be due to the mean polymerization degree (mDP) of wines since Cabernet Sauvignon tannins are more polymerized than Merlot ones (Chira, Jourdes, & Teissedre, 2012).

Solid-state NMR clearly identified polysaccharides that may come from grape cell walls and or from yeast membranes. The grape polysaccharides originate from breakdown of grape skin and cell walls. The major soluble grape polysaccharides constitute the arabinogalactan proteins. We have identified arabinose units by 2D NMR spectroscopy that could be indicators of the presence of arabinogalactan proteins in the precipitate. On the other hand, mannose was tentatively identified in precipitates and as the main polysaccharides released by yeast are mannoproteins, this would suggest the presence of mannoproteins in the precipitate. However, no signature of peptidic NH resonances, indicative of protein structure, was found in the 2D HSQC ^{15}N–^{1}H experiment. Rough calculations by assuming that 10% of the water soluble fraction is composed of polysaccharides indicates that for the LCS precipitate, Merlot 2013 (Table S3), the protein content would be ca. 0.3 mg/L. By considering arabinogalactan-like proteins as the only source of polysaccharides present in the precipitate we would obtain a ca. 3 μM concentration in the NMR tube (taking an average molecular weight of 190 kDa). Because the polysaccharidic part makes most of the protein molecular weight, the peptidic part (3–4% (Pellerin et al., 1996)) would be 20 times less, making it almost impossible to detect in our experimental conditions. A previous study on red wine precipitate (Waters et al., 1994) proposed a leading role for proteins in precipitate formation due to their interactions with procyanidins. Unfortunately, we do not find any evidence for the presence of large amounts of proteins, if any, in the precipitate. Amino acids that are predominant in the precipitate are therefore free from peptidic linkage and include proline, alanine, leucine and arginine. In all the amino acids present in the precipitate, proline seems to be the most abundant. This is in fact not surprising because this amino acid is not converted during fermentation.

To our surprise is also the finding of glycerol in the precipitates. Its resonances are unambiguously assigned by 2D NMR and one may wonder how such a water-soluble molecule may appear in appreciable amounts in precipitates. Organic acids such as succinic and lactic acids that are produced during fermentation are also present in large quantities in the precipitates. It is however surprising to find them as they are hydrophilic molecules and very soluble in alcohol–water solutions. As this stage one may only conjecture that all the above soluble compounds they are trapped in colloidal complexes with tannins and may be polysaccharides. Acetic acid was also detected although the measured volatile acidity is low and ranges from 0.2 to 0.4, that is to say, below the threshold of 0.5 g/L of H_2SO_4 above which wine are considered to show high volatile acidity.

4.4. Implications for wine stability

Stability of wines as a function of time is an important aspect of the wine process. It is not only important from a market point of view but also from the desire of controlling wine evolution during the ageing process. In this study we investigated two extreme cases, the vintages 2012 that is considered as unstable and that of 2013 that was ranked as stable. It is worth mentioning that winemaking and ageing were classical of traditional management for Bordeaux red wine. Alcoholic and malolactic fermentation were carried out in tanks and then the wine was transferred into used oak barrels. There were not lees ageing as such because wines were racked from tanks to barrels. However it is difficult not to transfer trace amounts of microorganisms, so fine lees ageing might occur. As this wine making process is quite classical, our study may therefore be used to provide some general observations. Under cold treatment (−4 °C or 4 °C) vintage 2012 produced much more precipitate than that of 2013. Even though polyphenols have been proposed to help tartaric stabilization (Ribéreau-Gayon et al., 2006), we clearly identified two main components in wine precipitates: tartrates and polyphenols. Potassium bitartrate crystals have been clearly assigned in this work by solid-state NMR spectroscopy. Wine instability due to the presence of tartrates and the influence of wine components on tartrate crystals has been well studied in oenology (Boulangé-Petermann, Vernhet, Dupré, & Moutounet, 1999; Gerbaud, Gabas, Blouin, Pellerin, & Moutounet, 1997; McKinnon, Scollary, Solomon, & Williams, 1995) and it is not surprising to see that vintage 2012 that contains more tartrate than 2013 is more unstable form this viewpoint. Changes in total acidity and alcohol content can also cause tartrate precipitation and differences in acidity and alcohol content for vintage 2012 as compared to vintage 2013 could be the reason for more bitartrate presence in vintage 2012. The disappearance of bitartrate in our samples after 3–4 months ageing is due to the natural precipitation of potassium tartrate during the first months (January to March) of barrel ageing in cellars of chateaux. No observation was however made of the influence of wine components on bitartrate crystals since a change in the crystals can be detected with ^{13}C CPMAS NMR spectroscopy (*vide supra*). It has been reported that metals and inorganic salts could create wine instability (Ribéreau-Gayon et al., 2006). NMR spectroscopy of the metals could in principle be used to detect their presence, this is however beyond the scope of the present work. Polyphenols have been also found in large quantities at all sampling stages along one year. As discussed earlier, grape variety and type of polyphenols could play an important role in forming coloring matter precipitate after cold treatment. The usual oenological practice for stabilization of coloring matter is to wait until

the end of ageing process in order to obtain a total natural stabilization of the coloring matter through chemical processes evolving in wine. A more recent study (Arapitsas, Speri, Angeli, Perenzoni, & Mattivi, 2014) observed that chemical composition of wine evolved at a slower rate at 4 °C as compared to higher temperatures over the duration of two years. In our study, we observe precipitate formation over shorter time periods and at colder temperatures. This may indicate that physico-chemical parameters of pH, acidity and alcohol content (related to vintage) could drive rapid precipitate formation as opposed to chemical processes that may occur over a much more longer time scale.

5. Conclusion

Solid-state NMR has been shown to be a technique of choice to semi quantify components in complex mixtures of natural products, such as wine precipitates that cannot be solubilized completely. Crystals of potassium bitartrate are readily identified in the early stages of wine precipitation whereas amorphous mixtures of polyphenols, polysaccharides, amino acids and glycerol are involved in further precipitate formation. Analyzing precipitates as a function of wine ageing, vintage and grape variety allows identification of general behaviors and highlights the role of potassium bitartrate, polyphenols in precipitation phenomena. Procedures for separation of components and their solubilization in solvent mixtures are under investigation in our laboratories. This opens a new avenue for further characterization of precipitates, and in particular their exact composition that may help wine makers in producing wines of greater stability.

Acknowledgments

Financial support from the TGIR-RMN-THC Fr3050 (French High-Field NMR network) is acknowledged. The Aquitaine Region is also thanked for financial support for NMR equipment. Dr. Vanessa Zhendre and Estelle Morvan from IECB are thanked for help with NMR and Philippe Louazil from Laffort for wine analysis. Finally, the authors would especially like to thank Château Reynon and Denis Dubourdieu (Béguey, Gironde, France) for generously providing wine samples.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.12.013.

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