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A comprehensive study on the effect of bentonite fining on wine charged model molecules

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ABSTRACT

In bottled wines, haze and turbidity are phenomena to be avoided. Since bentonite fining is a common process to clarify wines removing heat unstable proteins, a theoretical study on the adsorption of three Charged Model Molecules (CMMs, egg albumin, polyphenols and riboflavin) was carried out to deep comprehend this chemical phenomenon. Four bentonites were adopted and finely characterized together with the potential release of Na^+ and Ca^{2+} cations, revealing suitable for RT albumin removal within 120 min. Better results in terms of adsorbed quantity were achieved by adopting 12%v/v EtOH/H_2O solvent and by swelling bentonites for 24 h before use. With the most performing sample (Na/Ca_0.27), a comprehensive study on simultaneous adsorption of the three CMMs was performed, resulting in polyphenols adsorption increase due to their interactions with albumin. Notwithstanding the majority of albumin and riboflavin was successfully removed, ca. 40–50% of tested polyphenols was preserved.

1. Introduction

Wine limpidity is a quality of compulsory importance for consumers, especially for white wines in clear glass bottles. The turbidity in wines involves colloidal phenomena (Lambri et al., 2010; Ribéreau-Gayon et al., 2006): it is connected to the presence of particles in suspension that either stop or diffuse part of the incident light rays, thus leading to an opaque solution. These suspended particles forming haze and turbidity do severely spoil the wine appearance (Hsu et al., 1987; Pocock & Waters, 2006; Van Sluyter et al., 2015). Hence, in the wine-making industry fining processes, *i.e.* the removal of particular type of compounds from wine solutions, are of primary importance allowing the stabilization of bottled wines (Batista et al., 2010; Sun et al., 2007). Moreover, wine not only must be clear at the time of bottling, but also it should retain its limpidity during aging and storage for an indefinite period, whatever the temperature conditions (Pocock & Waters, 2006). Specifically, proteins characterized by a medium MW (~45000 Da) and low pI (4.1 – 5.8) together with glycoproteins are the major and most important fractions contributing to protein instability in wines (Fig. S1a) (Hsu et al., 1987; Pocock & Waters, 2006; Sun et al., 2007). Hence, clarification processes are highly recommended and, in particular, limpidity is usually achieved by gradual settling, followed by racking to eliminate the solids (Mierczynska-Vasilev & Smith, 2015; Ribéreau-Gayon et al., 2006). Other, more rapid, processes (*i.e.* filtration and centrifugation) may also be used (Ribéreau-Gayon et al., 2006), but these treatments do not guarantee the final protein stability. There are several materials for protein stabilization, among which bentonites (Fig. S1b) (Lambri et al., 2010; Pocock & Waters, 2006; Salazar et al., 2017; Sun et al., 2007), tannic acid (Morris & Main, 1995) and a quite novel continuous process (not yet allowed as winery procedure), contemplating the use of a packed column of zirconium oxide as adsorbent material (Lucchetta et al., 2013; Pashova et al., 2004; Salazar & Achaerandio, 2006), are widely exploited. Among all these compounds, bentonite is commonly accepted owing to its low price (Lambri et al., 2010; Pocock & Waters, 2006). Specifically, this adsorbent material is mainly composed by montmorillonite polymorph, consisting of two tetrahedral sheets (Si-O) separated by an octahedral one (Al-O-OH). Its net negative surface charge is mainly due to the intrinsic acidic behavior of silica (SiO_2) that at pH above ca. 3 is negatively charged (Lambri et al., 2010; Sun et al., 2007). Hence, positively charged molecules can be adsorbed onto the surface of bentonite owing to the strong electrostatic interaction between the negative and positive charges (Cappelletti et al., 2006; Pargoletti et al., 2019). Therefore, nowadays, the fining

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treatment with bentonites is effective for both processes, *i.e.* wines stabilization and clarification (Mierczynska-Vasilev & Smith, 2015; Ribéreau-Gayon et al., 2006). Indeed, the treatment with these materials has been reported to be successful to prevent protein haze in white wines and it is also very effective for clarifying and stabilizing colloidal coloring matter, especially in red wines (Fig. S1c) (Morris & Main, 1995; Ribéreau-Gayon et al., 2006). The use of bentonite shortly after alcoholic fermentation offers long known advantages, but also presents certain more recently discovered inconveniences, related to the removal of substances linked to the final odour, flavor and taste of wines (Mattivi et al., 2000; Ribéreau-Gayon et al., 2006). Hence, the lower the amount used, the better the final organoleptic properties (Vincenzi et al., 2015).

Herein, in order to finely evaluate the efficacy of four different bentonites as clarifying agents, three charged model molecules (CMMs, namely egg albumin, polyphenols and riboflavin) were used, trying to mimic a wine solution (International Oenological Codex, 2015). Indeed, at the white wine pH of ca. 3.2, all these CMMs can electrostatically interact with the negatively charged bentonites. In particular, notwithstanding egg albumin is not normally present in wines, it is routinely considered for the bentonites quality control and consequently for their standardization, as stated by the International Oenological Codex (OIV) (International Oenological Codex, 2015). Besides, notwithstanding wine heat-unstable proteins include chitinase, β-glucanase, thaumatin-like proteins, lipid transfer proteins and invertase ones (Hayasaka et al., 2001; Van Sluyter et al., 2015), their composition strictly depends on the adopted varieties of grape *Vitis vinifera* (Marangon et al., 2014), opening up countless varieties of possible combinations and, thus, making the adsorption study difficult to be reproducible on a large scale. Therefore, in order to standardize our study, egg albumin was chosen as the model protein (International Oenological Codex, 2015; Sun et al., 2007), polyphenols are connected to the organoleptic properties of wines, while riboflavin was considered due to its connection with the possible occurrence of the undesired light-struck taste in white wines. Remarkably, polyphenols (as tannins) are believed not only to have a significant impact on the esters volatility, resulting fundamental for the preservation of the wine flavor (Mitropoulou et al., 2011), but also they may mitigate the light-struck taste of bottled white wine, thanks to their interaction with riboflavin, the main photosensitizer in this unwanted process (Fracassetti et al., 2019). In this context, we suggest herein a novel approach trying to either delineate the optimal experimental conditions to be adopted to increase the final adsorption performances or investigate the adsorption kinetic models for the three CMMs. Moreover, once studied the single molecules adsorption, the kinetics was carried out in a wine-like system, allowing us for the first time to evaluate the possible synergistic effects/competitive adsorption among the model molecules.

2. Experimental

All the chemicals were of reagent-grade purity and were used without further purification. Four different activated bentonites (purchased from Enolife S.r.l.; typically used for wine clarification processes resulting in haze decrease as reported in Table S1) were adopted, having different sodium-to-calcium atomic ratios, namely 0.12, 0.20, 0.27, 0.49 (by energy dispersive x-ray spectroscopy elemental analysis; all the obtained standard deviations, computed on ten different replicates, were lower than ± 0.02). These adsorbent materials were labeled as Na/Ca_x, where x stands for the atomic ratio, accordingly. All the bentonites are extracted from European soils and they fulfill the requirements of the International Oenological Codex-2015 Issue (International Oenological Codex, 2015). The adopted polyphenols come from a Chardonnay extract and they comprise grape flavanols and their dimers. Then, taking into account the extremely wide range of water hardness values, MilliQ water was used for the kinetic tests in order to have a rigorous method for the bentonites standardization.

2.1. Na^+ and Ca^{2+} release from bentonite adsorbent

The release of sodium and calcium ions into wine solutions was determined by both Atomic Absorption Spectroscopy (AAS) and Inductively Coupled Plasma – Atomic Emission Spectroscopy (ICP-AES) analyses. Three different Italian wines (namely *Negroamaro* Rosé, *Verdeca* White and *Primitivo* Red) were adopted and treated by using 0.40 g L^{-1} of bentonite having sodium-to-calcium atomic ratio equal to 0.27 by EDX, as representative sample (*i.e.* Na/Ca_0.27). As reported in the literature, the pH plays a pivotal role in the bentonite-protein interactions (Dordoni et al., 2015). Hence, tests were performed at (25 ± 1) °C, pH 3.2 typical of white wine (pH 3.0–3.5), and for a maximum of 120 min. The data were expressed as a difference in absolute value between the cations concentration in untreated wine and the one at the end of the release procedures. The quantification, in absorption technique (AAS), of both Ca^{2+} and Na^+ ions was made by means of Perkin Elmer Atomic Absorption Spectrometer with UV WINLAB software. Concerning the ICP-AES analyses (by adopting the ICPE-9800 Series, by Shimadzu), a standard method OIV-MA-AS322-13 was used (Compendium of international analysis of methods - OIV Analysis of mineral elements in wines using ICP-AES (inductively coupled plasma / atomic emission spectrometry), 2013). In both cases, five different replicates were performed and the relative average values together with the standard deviations were calculated.

2.2. Bentonites characterization

X-Ray Powder Diffraction (XRPD) analyses were performed on a Philips PW 3710 Bragg-Brentano goniometer equipped with a scintillation counter and 1° divergence slit, 0.2 mm receiving slit, and 0.04° soller slit systems. A graphite-monochromated Cu Kα radiation (Cu $K_{\alpha1}$ λ = 1.54056 Å, $K_{\alpha2}$ λ = 1.54433 Å) was employed at 40 kV × 40 mA nominal X-rays power. Diffraction patterns were collected between 5° and 80° with a step size of 0.1° and a total counting time of about 1 h. A microcrystalline Si-powdered sample was used as a reference to correct for instrumental line broadening effects.

Scanning Electron Microscopy (SEM) was carried out using a SEM Hitachi TM-1000 Microscope equipped with Hitachi ED3000 spectrophotometer for the elemental analysis by Energy Dispersive X-ray Spectroscopy (EDX).

The Brunauer Emmett Teller (BET) surface area was determined by a multipoint BET method using the adsorption data in the relative pressure (p/p_0) range of 0.05–0.20 (Coulter SA3100 apparatus). Desorption isotherms were used to determine the total pore volume using the Barrett-Joyner-Halenda (BJH) method.

ATR-FTIR spectra were recorded using a Nicolet 380 spectrophotometer (Thermo Electron Corporation) between 4000 and 400 cm^{-1}.

The particle size distributions and zeta potential values (ζ) were measured through Dynamic Light Scattering (DLS) using a Malvern Zeta Sizer NanoZS series particle size analyzer and by means of Zetasizer software to process the dimensional distribution graphs.

2.3. Charged model molecules (CMMs) adsorption

As already stated, three different Charged Model Molecules (CMMs) were used to mimic the main compounds present in wine solutions, namely egg albumin, polyphenols and riboflavin. In our

laboratory procedure, the appropriate amount of bentonite (in the range between 0.10 and 0.90 g L^{-1}) was put into a 0.6 L of MilliQ water (Achaerandio, Pacova, & Lopez, 2001; Hwang et al., 2013; Lambri et al., 2010; M.R. Sarmento et al., 1999; Sauvage et al., 2010; Sun et al., 2007). Then, after the CMM addition (1000 ppm in the case of egg albumin (International Oenological Codex, 2015), 30 ppm for polyphenols and 3 ppm for riboflavin), the system was kept under stirring at 600 rpm and at pH 3.2 (adjusted by adding concentrated nitric acid), in a thermostatic bath (25 ± 1 °C). Concerning the adopted CMMs concentrations, the amount of riboflavin used is much higher than the one commonly allowed of ca. 40 ppb (Fracassetti et al., 2019). However, for the feasibility of our analytical detection method, we were obliged to operate at higher concentrations. Subsequently, the samples withdrawn at different times (0, 10, 20, 30, 40, 60, 120, 180, 270, 360, 420 min) were centrifuged at 9500 rpm for 15 min and the UV/Vis absorption spectrum (200–600 nm) of the surnatant was measured using a Shimadzu UV-2600 spectrophotometer. In particular, egg albumin and polyphenols absorption maxima were set at 280 nm, whereas the one at 450 nm was chosen for riboflavin. Hence, the amount of CMMs adsorbed onto the bentonite surface, in terms of q_t (adsorption capacity) was determined by the following equation:

$$q_t = \frac{V_{reactor}\ (c_0 - c_e)}{m_{bentonite}} \quad (1)$$

where c_0 and c_e are the starting and the equilibrium concentration of CMMs in mg L^{-1}; $V_{reactor}$ is the volume of the total solution (0.6 L); $m_{bentonite}$ is the bentonite amount in grams. Notably, the equilibrium concentration was computed after having acquired the calibration plots at the same reaction conditions (see Fig. S2).

Once optimized the kinetics time and bentonite concentration, either the effect of medium (*i.e.* pure water or 12%v/v EtOH in water) or the bentonites swelling were investigated. A specific protocol was adopted (International Oenological Codex, 2015), that consists in dispersing 2 g of bentonite (previously dried at 80 °C for 12 h) into 90 mL of MilliQ water. Then, the sample was covered and left to deposit for 24 h after which was possible to observe and measure the swelling index.

2.4. Charged model molecules adsorption isotherms

In order to deeply investigate the adsorption of CMMs onto bentonite material, the same kinetic tests were carried out, as described previously. In this case, the optimal experimental conditions were adopted, such as 0.40 g L^{-1} of bentonite concentration used in the swelled form, 12%v/v EtOH/H_2O at pH 3.2, for a total time of 120 min. Parallelly, the amount of CMMs was varied depending on the studied molecule: from 200 to 1400 ppm for egg albumin, from 24 to 60 for polyphenols and in the range 1.0–2.4 ppm for riboflavin. The amount of protein adsorbed onto the bentonite surface was determined as reported previously. Hence, the adsorption surface excess (Γ) was quantified as follows:

$$\Gamma = \frac{n_{ads\ protein}\ 100}{w_{bentonite} S_{BET}} \quad (2)$$

where $n_{ads\ protein}$ is the number of molecules moles adsorbed by bentonite, $w_{bentonite}$ is the mass of adsorbent used, S_{BET} is the bentonite specific surface area.

For either egg albumin or polyphenols, Frumkin-Fowler-Guggenheim (FFG) model was adopted, whereas in the case of riboflavin the Freundlich method better fitted the experimental outcomes. Thus, in the former, the following equation was utilized:

$$\ln\left(\frac{\theta}{c_e\ (1-\theta)}\right) = a\theta + \ln K_{FFG} \quad (3)$$

where θ is the coverage degree defined as the ratio between Γ and Γ_{max}, c_e is the adsorbate equilibrium concentration (expressed in mol L^{-1}), a is the lateral interaction parameter and K_{FFG} is the Frumkin-Fowler-Guggenheim constant (Koopal & Avena, 2001). Conversely, the Freundlich model follows the equation:

$$q_e = K_F c_e^{\ bF} \quad (4)$$

in which K_F and *bF* are the Freundlich constants. This expression is characterized by the heterogeneity factor (*bF*) and so the Freundlich isotherm may be used to describe heterogeneous systems (Lambri et al., 2010). Theoretically, using this expression, an infinite amount of adsorption can occur. To determine the constants K_F and *bF*, the linear form of the equation was used (see Equation (5)):

$$\ln(q_e) = \ln K_F + bF \ln(c_e) \quad (5)$$

Finally, the variation of the Gibbs energy (ΔG) was computed by using the Eq. (6):

$$\Delta G = -RT\ \ln K \quad (6)$$

where *K* is the constant according to the adopted models (K_{FFG} or K_F).

2.5. Model wine adsorption kinetics

Once investigated and optimized the single adsorption kinetics of the three CMMs onto bentonite adsorbents, a simplified wine-like system was prepared and studied. This model wine was composed by egg albumin (1000 ppm), polyphenols (30 ppm) and riboflavin (3 ppm) in 12%v/v EtOH/H_2O at pH 3.2. The kinetics lasted 120 min, under constant stirring of 600 rpm, at (25 ± 1) °C, using 0.40 g L^{-1} of bentonite concentration. Since all the three CMMs absorb nearly in the same wavelength range, HPLC analyses were carried out to discern among the three model molecules. An Agilent 1200 Series HPLC System with a Poroshell 120 reversed phase C_{18} column (4.6x100 mm; 2.7 μm) was used in a gradient elution system of TFA-H_2O 0.1% (eluent A) and TFA-CH_3CN 0.1% (eluent B), according to the literature (Muñoz et al., 2008). The gradient elution was: 0 min 100% A, 10 min 91% A – 9% B, 25 min 82% A – 18% B, 30 min 79.4% A – 20.6% B, 5 min for reconditioning the system; the flow rate was 1.0 mL min^{-1}; and the injection volume was 20 μL. The stock solution of standards was diluted in a mixture of 12%v/v EtOH/MilliQ water to obtain the working standard solutions. Analytes were sampled at 0, 40, 80, 120 min and each concentration was calculated from chromatogram peaks areas on the basis of the calibration curves.

Moreover, in order to correctly identify the polyphenols and riboflavin, coupled HPLC-MS analysis (reversed phase C_{18} column 4.6x100 mm; 2.7 μm) was performed, equipped with an ESI detector.

3. Results and discussion

3.1. Na^+ and Ca^{2+} release by bentonites into wines

Prior to assess the effective adsorbent behaviour and the eventual promising selective adsorption features of the adopted bentonites, the possible release of sodium and calcium cations into wine has to be verified. Indeed, a high amount of these two species results in an increase of the ionic strength, thus provoking the possible precipitation of the potassium bitartrate and calcium tartrate that are almost insoluble in the wine medium, especially at low temperatures

(Ribéreau-Gayon et al., 2006). Hence, AAS and ICP-AES analyses were carried out either before or after the cations release with all the four selected bentonites, using three types of Italian wine, *i.e. Negroamaro* Rosé, *Verdeca* White and *Primitivo* Red. Table 1 shows the amount of Na^+ and Ca^{2+} released into solution after 120 min of kinetics. Notably, with both the two analytical techniques, similar values were obtained. Furthermore, we observed a higher exchange of sodium ions with respect to calcium ones for all the three wines. From a technical point of view, it is recommended to limit the concentrations of sodium and calcium below 50 and 100 mg L^{-1}, respectively (Spayd et al., 2010). Therefore, these bentonites can be considered very promising for the wine fining process. Hence, in this respect, the four materials were further finely investigated as adsorbents for three charged model molecules, *i.e.* egg albumin, polyphenols and riboflavin.

3.2. Bentonites physico-chemical properties

Prior to any physico-chemical characterization of the four adsorbent materials, EDX analysis was carried out to define the Na/Ca atomic ratio in order to discern among the studied bentonites. Indeed, each clay shows different features depending on its origin and type. In accordance with some recent literature (Catarino et al., 2008; Lambri et al., 2010), a sodium enriched bentonite (*i.e.* high Na^+/Ca^{2+} ratio) is more efficient for protein removal than analogous compounds having a higher concentration of other interlayer cations, such as calcium ones. Hence, by means of elemental analysis (see Table 2 1st column), we were able to distinguish among the four adsorbents: notably, two of them (Na/Ca_0.49 and 0.27) can be defined as sodium-enriched, whereas the others can be called as calcic ones. Therefore, on the basis of the already reported literature (Catarino et al., 2008; Lambri et al., 2010), we can preliminary infer the affinity of Na/Ca_0.49 and 0.27 towards high molecular weight proteins should be greater with respect to the other two clays.

Table 1
Quantification of the release of sodium and calcium ions in three different Italian wines (Negroamaro, Verdeca and Primitivo) by Na/Ca_0.27 (0.40 g L^{-1}) bentonite after 120 min, by means of Atomic Absorption Spectroscopy (AAS) and Inductively Coupled Plasma (ICP-AES) technique, together with the relative standard deviations computed on five different replicates.

Wine	AAS		ICP-AES	
	ΔNa^+ (mg L^{-1})	ΔCa^{2+} (mg L^{-1})	ΔNa^+ (mg L^{-1})	ΔCa^{2+} (mg L^{-1})
Negroamaro Rosé	30 ± 2	16 ± 2	26 ± 3	10 ± 4
Verdeca White	15 ± 1	13 ± 1	25 ± 2	9 ± 3
Primitivo Red	41 ± 3	11 ± 1	22 ± 5	9 ± 2

Concerning their structural properties, either x-ray diffraction analysis or infrared spectroscopy were performed. As shown in Fig. 1a, all the four bentonites are composed by the same three polymorphs, which are typical of clay-based materials (Sun et al., 2007), namely montmorillonite (JCPDS 13-0135), quartz (JCPDS 46-1045) and calcite (JCPDS 05-0586). Parallelly, FTIR (Fig. 1b) measurements highlight the main stretching mode ascribable to Si-O-Si bonds at about 1000 cm^{-1}, as corroborated by literature data (Sun et al., 2007).

On the morphological point of view, SEM micrographs display two different surface textures. In particular, Na/Ca_0.49 and 0.20 (Fig. 1c,e) have micrometric spherical aggregates with diameters of ca. 500 μm; conversely, Na/Ca_0.27 and 0.12 (Fig. 1d,f) show a dustier morphology (few micrometers in size).

Furthermore, since the surface area is an important parameter that characterizes an adsorbent material, this feature was deeply investigated. Indeed, the adsorption process is strictly related to this value, *i.e.* the higher the surface area, the greater the adsorption of the target molecules. For all the studied bentonites, the specific surface area (S_{BET}) and the total pores volume ($V_{tot.\ pores}$) lie in the range 50–60 $m^2\ g^{-1}$ (Table 2, 2nd column) and 0.100–0.200 $cm^3\ g^{-1}$ (Table 2, 3rd column) respectively, *i.e.* values typical of montmorillonite soils (Vincenzi et al., 2015). Besides, by investigating the pores size distribution, for all the bentonite samples the majority of the pores has very small diameters, centred at around 4 nm (Fig. 1g).

Nevertheless, in order to determine the real size of agglomerates and the clays charge in 12%v/v EtOH/MilliQ water medium, both Dynamic Light Scattering (DLS) and ζ-potential analyses were performed. Both the grain size and the ζ-potential (evaluated at pH of white wines of 3.2; Table 2, 4th–5th columns and Fig. S2) are characterized by a single mode distribution for all the analysed samples, showing a quite narrow distribution and, thus, a small uncertainty degree (see the error values reported in Table 2). Interestingly, at the adopted pH, the calcic bentonites have agglomerates of around 200–350 nm, which are definitely smaller than those of sodium-enriched ones (ca. 600–700 nm). This trend is maintained even by changing the operative pH, resulting in bigger agglomerates with the increasing of this parameter, as clearly visible in Figure S2a. Besides, as concern the particles charge, ζ-potential measurements reveal a net negative charge of ca. −35 mV in the pH range of 2–8 for all the studied bentonites, showing the highest charge at pH typical of white wine systems (ca 3.2, as reported in Fig. S2b) (Lambri et al., 2010).

3.3. Optimization of adsorption kinetics with bentonite towards egg albumin

In clarifying processes, heat unstable protein, especially those having medium molecular weight and pI between 4.1 and 5.8, should be removed from wine since they can cause several undesired

Table 2
Na/Ca atomic ratios by energy dispersive x-ray spectroscopy (EDX) relative to the four investigated bentonites (standard deviations on ten replicates has been reported) alongside with their active surface area (S_{BET}), total pores volume ($V_{tot.\ pores}$), aggregates average diameter by dynamic light scattering ($<D^{DLS}>$) and ζ-potential values at pH 3.2.

		Na/Ca	S_{BET} ($m^2\ g^{-1}$)	$V_{tot.\ pores}$ ($cm^3\ g^{-1}$)	$<D^{DLS}>$ (nm)	ζ-potential (mV)
Bentonites	Calcic	0.12 ± 0.02	52	0.114	340 ± 50	−32 ± 8
		0.20 ± 0.01	60	0.209	170 ± 30	−37 ± 7
	Sodical	0.27 ± 0.04	57	0.113	700 ± 100	−34 ± 8
		0.49 ± 0.02	57	0.106	600 ± 100	−37 ± 5

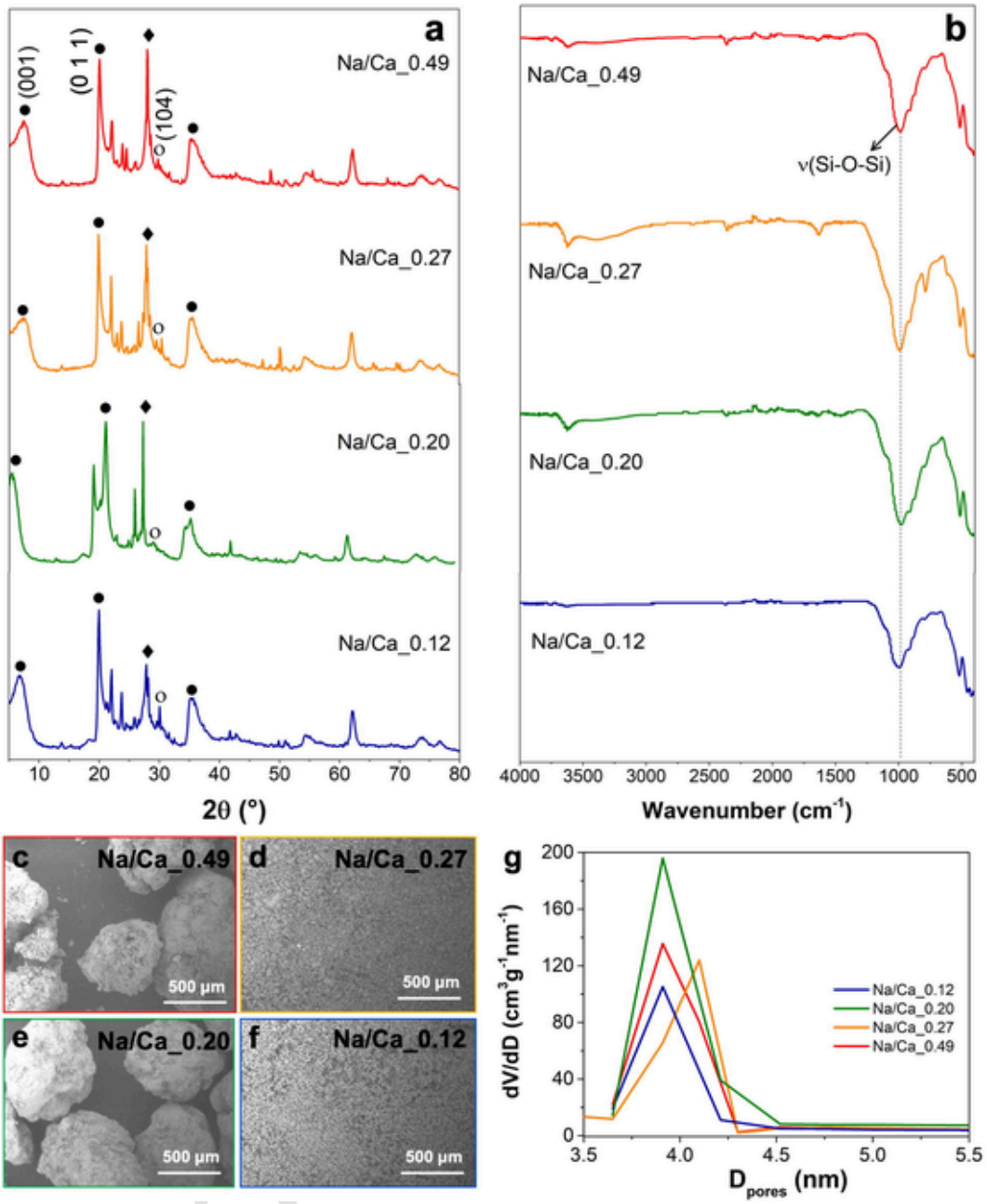


Fig. 1. (a) X-ray diffraction lines and (b) corresponding FTIR spectra of the studied bentonites. The main diffraction peaks (the 100% hkl plane is reported) of montmorillonite (•), quartz (♦) and calcite (o) have been highlighted, together with the main infrared band. (c–f) Relative SEM images and (g) pores volume distribution obtained by BJH method.

phenomena, such as haze and sediments (Hsu et al., 1987; Pocock & Waters, 2006; Sun et al., 2007). As previously stated, adsorbent materials as bentonites bare a net negative charge at the wine pH of 3.2. Hence, in order to succeed in removing the majority of these unstable compounds, the adsorbates should be positively or partial positively charged to guarantee an electrostatic interaction between them and the clarifying agents. Thus, in our study, three different molecules were investigated: egg albumin to simulate high molecular weight unstable proteins (in accordance with the International Oenological Codex (International Oenological Codex, 2015; Sun et al., 2007)), polyphenols, that are connected to the organoleptic properties of wines which therefore should not be entirely eliminated, and riboflavin. Before investigating a wine-like system comprehending all these three compounds, the single adsorption of egg albumin, polyphenols and riboflavin was deeply studied.

Hence, starting from high molecular weight proteins, egg albumin is reported to have an isoelectric point (pI) of 4.7 at a constant ionic strength (Kubiak-Ossowska et al., 2016; Ribéreau-Gayon et al., 2006). The isoelectric point is the pH at which the net charge of a protein is zero and it is the most critical parameter to consider regarding the adsorption phenomena. At the pI of protein, its structure is more hydrophobic, more compact and less stable due to the absence of interparticle repulsive forces. Hence, proteins can easily aggregate and precipitate at their pI. The difference in the isoelectric point values of biomolecules is obviously caused by the different environment modifying the ionic strength, the pH and the ion types. Specifically, at acidic pH (around 3.5), egg albumin is stated to pos-

sess a net positive charge of about 100 (Baler et al., 2014). Therefore, even at our operative conditions (pH 3.2 of white wines), it can be considered positively charged, resulting in its more favorable adsorption onto negative bentonites. Starting from these considerations, by using this CMM, a first screening of the bentonite adsorbent performances was conducted. Fig. 2 shows the results obtained by varying the adsorption time (Fig. 2a), the bentonites concentration (Fig. 2b), the medium (Fig. 2c) and the adsorbent pre-treatment (*i.e.* the eventual materials swelling, Fig. 2d). Regarding the kinetics duration, the adsorption capacity parameter (q_t, expressed by Equation (1) in which the albumin concentration is computed through the calibration plot in Fig. S3a,b) was followed. The relative comparison among the four bentonites is reported in Fig. 2a. Specifically, we can observe that Na/Ca_0.27 and Na/Ca_0.20 samples are the most performing adsorbents towards egg albumin with respect to Na/Ca_0.49 and Na/Ca_0.12. Moreover, the plateau region was reached already after 120 min, therefore this value was chosen to end the further kinetics tests. Analogous measurements were carried out to establish the optimal bentonite concentration at which the highest protein adsorption values can be achieved. Fig. 2b displays the obtained results. Notably, Na/Ca_0.49 does not seem to reach an adsorption plateau value, whereas the other three bentonites from 0.40 g L^{-1} show a constant adsorbed albumin concentration. Once more, Na/Ca_0.20 and 0.27 are the most performing samples. After determining the kinetics duration and bentonite concentration, the reaction medium and the clays swelling were considered. Concerning the former, in order to mimic the wine environment, a mixture of 12%v/v EtOH/MilliQ water was adopted. Moreover, the addition of ethanol is reported to facilitate the achievement of the plateau value, due to the increase of the gap between the interlayer channel of bentonites, also resulting in an enhancement of the protein adsorption (Sun et al., 2007), as demonstrated in Fig. 2c (second histogram). Particularly, in the case of Na/Ca_0.27 bentonite (as a representative sample), about 950 mg g^{-1} of adsorption capacity was reached within 120 min instead of 300 min, passing from pure MilliQ water to 12%v/v EtOH/H_2O solvent. Thus, by using this solvent mixture, an improvement of the bentonite adsorption capacity of about 20% can be obtained. Conversely, as regards the bentonites pre-treatments, the swelling procedure has been introduced since it is commonly used in the wine-making industry (Ribéreau-Gayon et al., 2006). The use of a swelled bentonite (Sun et al., 2007) (see Fig. 2d), in this case Na/Ca_0.27 (as a representative sample), instead of a dried one, increases of about 30–40% the adsorption of the protein (Fig. 2c, third histogram, and Fig. S4). Thus, for the successive studies only swelled bentonites were used.

3.4. Adsorption isotherm models for different CMMs

In order to assess the thermodynamic parameters, the relative adsorption isotherms were elaborated by varying the CMMs starting concentrations. As regards the egg albumin protein, the experimental curves were well fitted by the Frumkin-Fowler-Guggenheim (FFG) model (see Fig. 3a and S5a). Egg albumin has a big molecular structure, thus leading to the possibility of interactions between the adsorbates, after the adsorption takes place. According to literature (Sun et al., 2007), this behaviour could be described by the FFG model, considering either the total charge of the adsorbed molecules or the attractive/repulsive interactions among them. Albumin shows a positive and high *a* value related to the repulsive nature of the lateral interaction parameters: for this reason, the maximum adsorption value, reached after 20–30 min, is about 950 mg g^{-1} instead of the calculated theoretical one of 1200 mg g^{-1}. This can be explained by

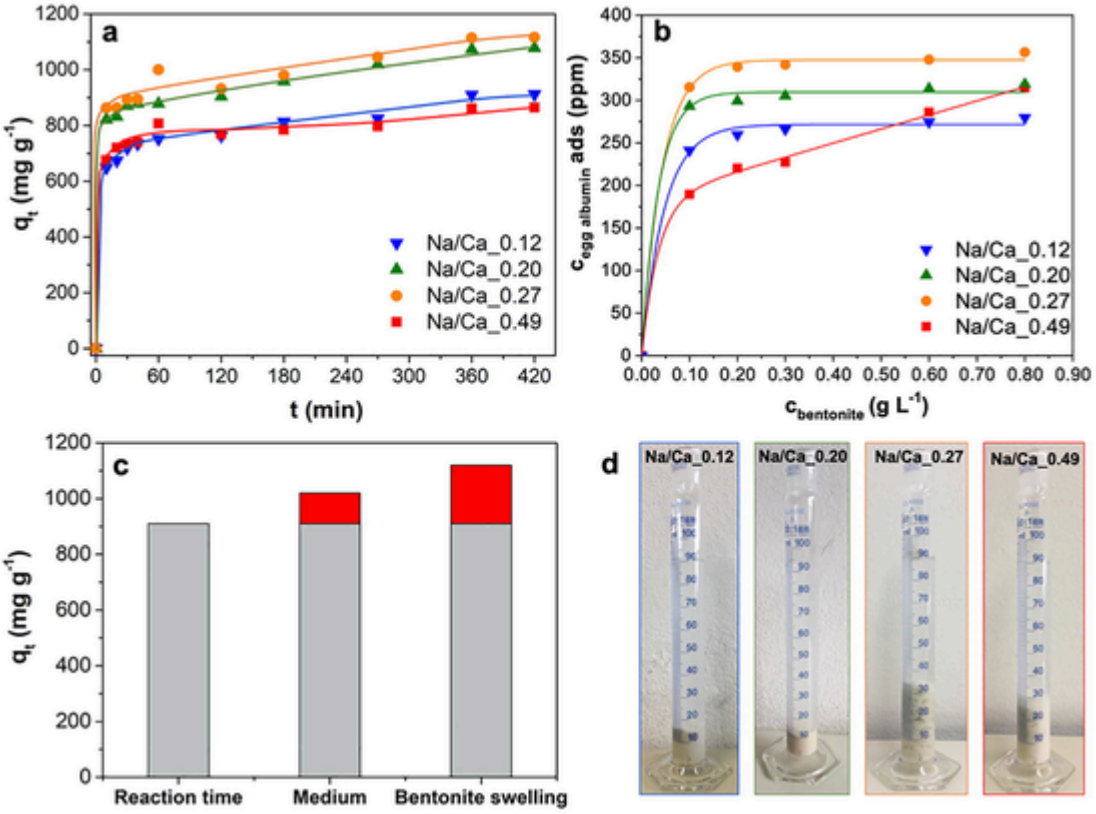


Fig. 2. Investigation of the parameters influencing the adsorption kinetics: (a) reaction time ($c_{bentonite}$ = 0.40 g L^{-1}), (b) adsorbent concentration, (c) effect of medium (12%v/v EtOH in water) and swelling index for Na/Ca_0.27. (d) Pictures of all the four bentonites during the swelling procedure. Egg albumin was used as the model molecule (at 1000 ppm), pH = 3.2, T = (25 ± 1) °C and constant stirring at 600 rpm.

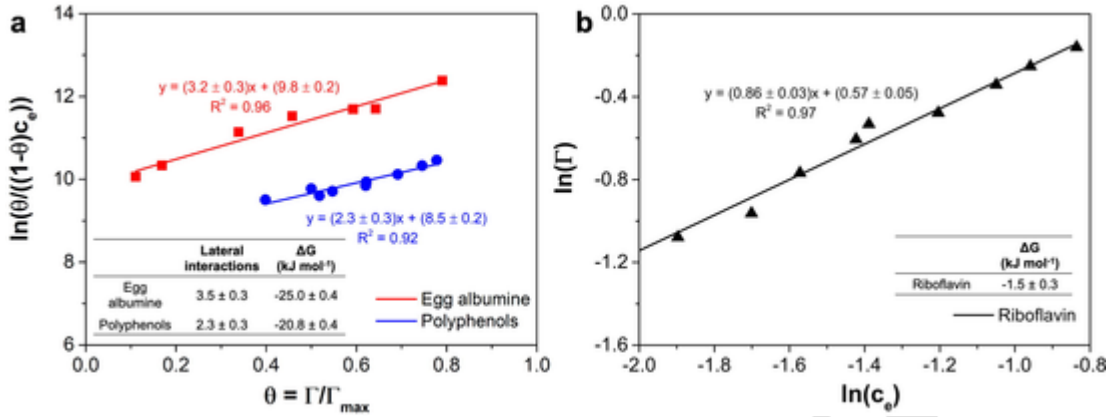


Fig. 3. Adsorption isotherms linearization obtained with Na/Ca_0.27 bentonite towards (a) egg albumin (1000 ppm) and polyphenols (30 ppm) by FFG model, and (b) riboflavin (3 ppm) by Freundlich equation. Solvent 12%v/v EtOH/Milli Q water pH = 3.2, T = (25 ± 1) °C and constant stirring at 600 rpm.

considering the electrostatic repulsions between egg albumin molecules adsorbed onto bentonite surface and free ones in the bulky solution. Hence, the available surface area of bentonites cannot be covered completely. Moreover, all the tested bentonites show similar $\Delta G_{adsorption}$ values (around –25 kJ mol^{-1}) and a strong affinity toward the other molecules (Table in inset in Fig. 3a). Hence, these results are promising since they reveal that the model molecule, used to recreate the undesired heat unstable high molecular weight proteins, is well removed by the adopted bentonite (namely Na/Ca_0.27 as representative adsorbent being one of the most performing bentonites).

Similarly to the previous study, adsorption isotherms were deeply investigated also in the case of polyphenols and riboflavin. Regarding the former, the linearization of the adsorption isotherms, following the Frumkin–Fowler–Guggenheim (FFG) model, was obtained (Figs. 3a and S5b) (Sun et al., 2007). Like egg albumin, polyphenols have quite big molecular structures, due to aggregation phenomena, thus interactions among the adsorbates after the adsorption could occur. This behaviour is scarcely described in the literature, in which a more common Langmuir-type behaviour is usually reported (Marsal et al., 2009; Miguel R. Sarmento et al., 2000; Sun et al., 2007). As reported in Table in inset of Fig. 3a, tested polyphenols show positive *a* value, hence the lateral interaction parameters are of repulsive nature. Conversely, regarding the free energy of adsorption, polyphenols seem to have less affinity towards the bentonite material (Na/Ca_0.27 as representative case), when compared to egg albumin. Instead, for what concern riboflavin, this molecule consists of an isoalloxazine ring to which at N-10 position a ribityl carbohydrate chain is linked to four hydroxyl groups (positions 2′,3′,4′and 5). Being a neutral molecule, depending on the environment pH value, the vitamin B2 may behave as a polyprotic acid or base. In an alkaline environment, for pH exceeding 10, it has acidic properties resulting from the possibility of dissociating protons from the neutral form. On the contrary, in an acidic environment, it becomes a basic compound and may accept protons with the nitrogen atoms of the isoalloxazine ring (Brzezińska et al., 2008). Since the vitamin concentration is quite low (3 ppm), the system can be considered as a dilute solution. Moreover, due to its relatively small dimensions, with respect to either polyphenols or egg albumin structures, the lateral interactions between adsorbate-adsorbate can be considered negligible. Therefore, the best fitting procedure is achieved by exploiting the Freundlich isotherm (Figs. S5c and 3b), as already stated in the literature (Noerpel et al., 2012). The further linearization method allows the evaluation of the free energy adsorption equal to –(1.5 ± 0.3) kJ mol^{-1}. Furthermore, notwithstanding the $\Delta G_{adsorption}$ obtained by different models cannot be compared, it is evident that the $\Delta G_{adsorption}$ of riboflavin is one order of magnitude smaller with respect to egg albumin or polyphenols ones, thus revealing less prone to adsorb onto bentonite materials. Summarizing, by looking at the previous results, egg albumin seems to be preferentially adsorbed to the bentonite powders.

Moreover, HPLC-MS measurements were parallelly performed in order to exactly identify the investigated polyphenols. In the case of egg albumin, this molecule was not successfully detected under our adopted experimental conditions, probably due to its high dimensions with respect to the column pores. Fig. 4 shows the obtained results. Specifically, procyanidin B1, catechin, procyanidin B2, epicatechin, epicatechin-epicatechin-3-O-gallate and epicatechin-3-O-gallate were detected: all of them are flavanols or their relative dimers (Ribéreau-Gayon et al., 2006). Procyanidin B1 (*m/z* 576.93) and B2 (*m/z* 576.92) are stereoisomers between them as catechin (*m/z* 289.04) and epicatechin (*m/z* 289.04). Epicatechin-O-gallate (*m/z* 440.90) has a galloyl group instead of a hydroxyl one as the epicatechin-epicatechin-O-gallate (*m/z* 728.83) even if, the latter, is characterized by two units of epicatechin (Fig. 4b). Hence, Fig. 4a displays the single adsorption of the polyphenols extract showing the relative q_t parameter (black curve), that results in a total of 19% of polyphenols adsorbed in 120 min. Therefore, we can infer that these compounds are slightly absorbed onto bentonites with respect to egg albumin. Furthermore, by focusing on the HPLC results (histograms in Fig. 4a), four samplings at 0, 40, 80 and 120 min were analysed at 280 nm, allowing to identify around the 90% of the polyphenols constituting the original Chardonnay extract. This percentage was divided into the six different polyphenols detected by their different retention times and identified by the MS *m/z* ratios. As can be noted by the histograms in Fig. 4a, some polyphenols are easier to be adsorbed with respect to others, particularly, catechin and epicatechin ones. Moreover, since catechin can be responsible for the spoilage of white wine (Ribéreau-Gayon et al., 2006), its adsorption onto bentonite is beneficial; likely, the other polyphenols present are less adsorbed (Mitropoulou et al., 2011).

Besides for the single adsorption of riboflavin, Fig. 4c shows a final q_t value of about 83% after 120 min, highlighting that riboflavin is well absorbed onto bentonites. This is very promising since vitamin B2 is believed to be the main photosensitizer responsible for the so-called light-struck taste, an undesired process typical of clear glass bottled white wines (Fracassetti et al., 2019). Also, herein, four sampling at 0, 40, 80 and 120 min were analysed by means of HPLC, which confirmed the adsorption value obtained by UV/Vis spectroscopy (Fig. 4c,d).

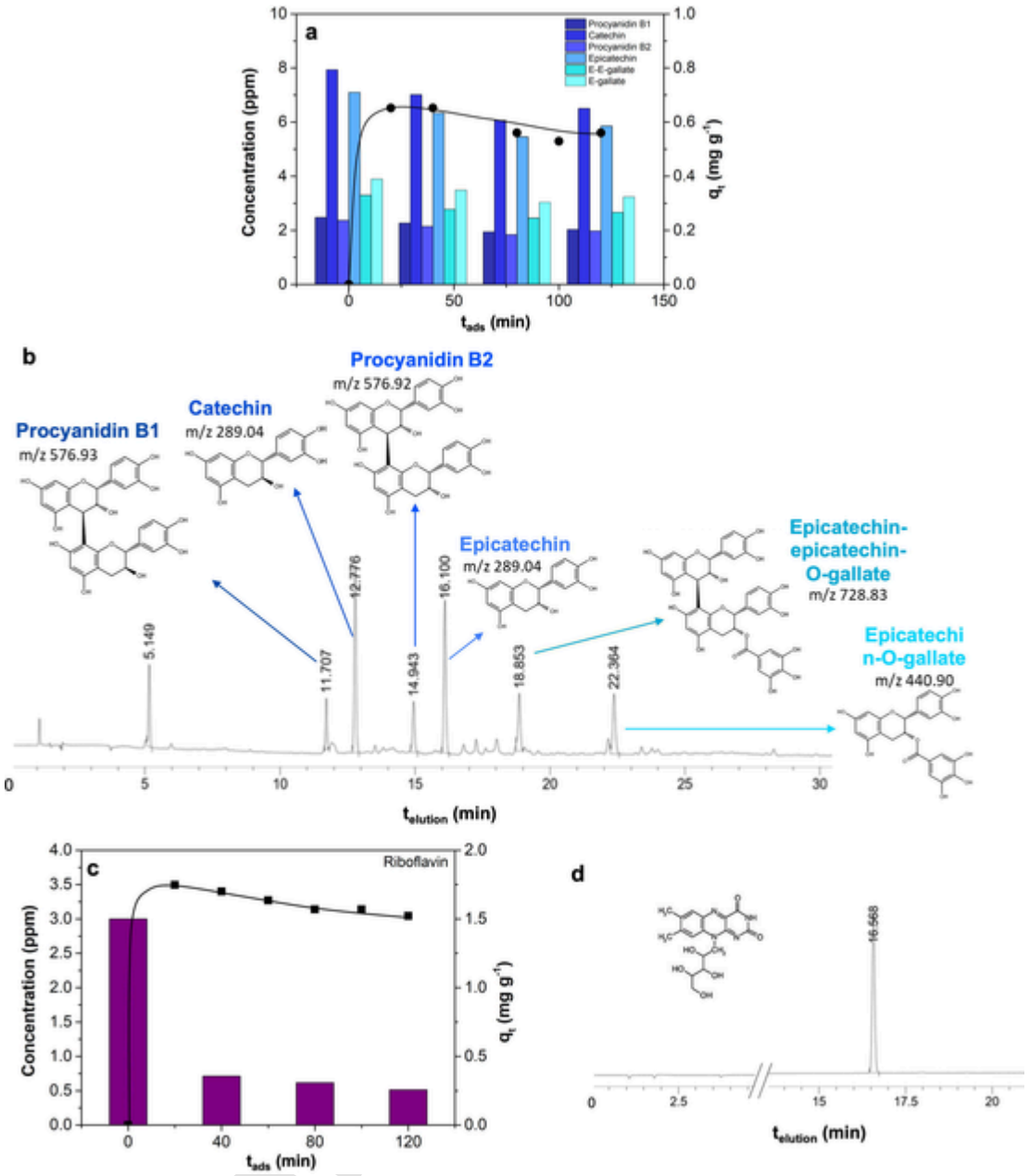
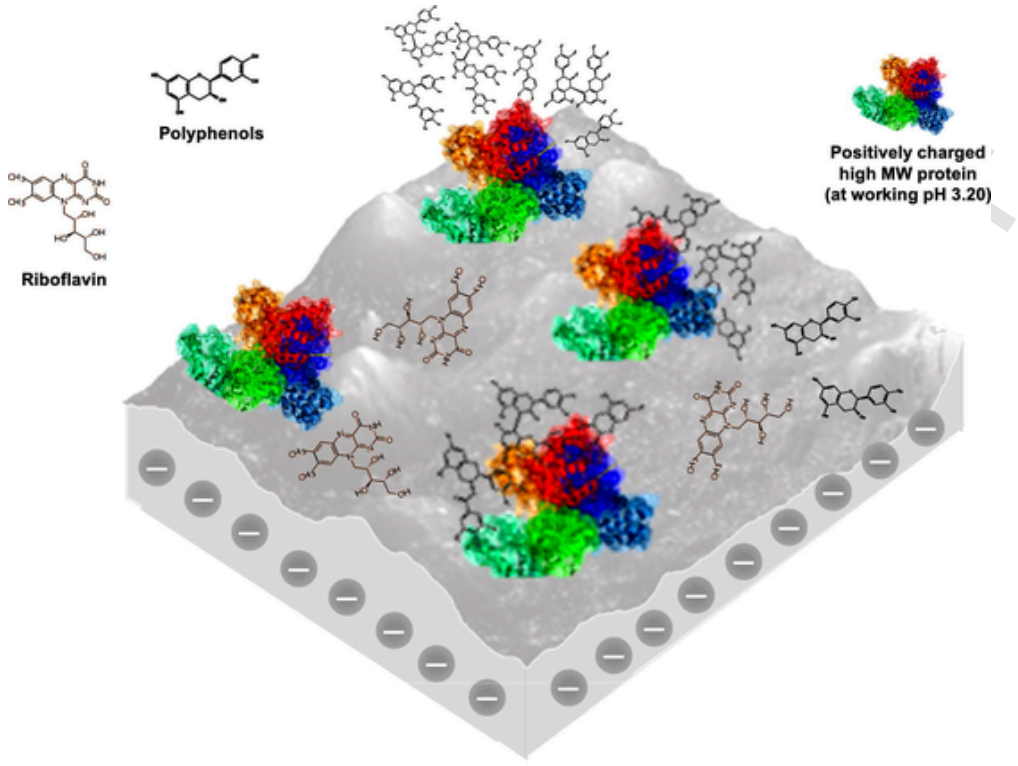


Fig. 4. Adsorption kinetics by HPLC-MS (histograms and MS results) and UV/Vis spectroscopy (q_t parameter) of (a,b) polyphenols and (c,d) riboflavin onto swelled Na/Ca_0.27 bentonite (0.40 g L^{-1}) in 12%v/v EtOH/Milli Q water, at pH 3.2, T = (25 ± 1) °C and constant stirring at 600 rpm.

3.5. Adsorption kinetics in a model wine

Once studied the single CMMs adsorption, a more complex system simulating the wine matrix was investigated. Indeed, proteins and polyphenols can combine together and their coupling is influenced by different factors, such as the type of protein (globular proteins have a stronger affinity with low molecular weight polyphenols) (Brzezińska et al., 2008; Noerpel et al., 2012), the size and flexibility of polyphenols (Brzezińska et al., 2008), the tannin/protein ratio and the medium factors (pH, temperature, ionic strength) (Bandyopadhyay et al., 2012). In particular, tannins bind and precipitate proteins thanks to their ability to behave as a multidentate ligand. As previously stated, egg albumin was too big to be detected by the adopted chromatographic analysis; instead, concerning the other CMMs (*i.e.* polyphenols and riboflavin), they were well separated and identified (see Fig. S6). Notably, either riboflavin or epicatechin were eluted concomitantly and, thus, followed at 280 nm and 450 nm, respectively. Generally, the different compounds can be considered competitors (Bianchi et al., 2004) for their adsorption onto bentonites surface (Fig. 5), therefore, they are adsorbed in a lower quantity with respect to the single adsorption kinetics (70% instead of 89% and 75% instead of 83% for egg albumin and riboflavin, respectively; Table in inset of Fig. 5). Interestingly, in the model wine system, polyphenols interact with egg albumin by hydrogen bonds leading to aggregates well adsorbed onto bentonites (Ribéreau-Gayon et al., 2006; Sun et al., 2007), as clearly represented in Fig. 5. As a result, polyphenols are adsorbed in a more massive way with respect to their single adsorption mechanism (60% instead of 19%; Table in inset of Fig. 5).



	Single adsorption %	Competitive adsorption %
Egg albumin	89	70
Polyphenols	19	60
Riboflavin	83	75

Fig. 5. Schematic representation of egg albumin, polyphenols and riboflavin adsorption in a wine-like system at pH 3.2 mainly through electrostatic interactions. At the bottom, table shows a comparison of the percentages between the case of single molecules adsorption and the competitive one, as in the wine-like system.

4. Conclusions

In the present research work, four bentonites, having different Na/Ca atomic ratios, were finely investigated as adsorbent materials towards three representative charged wine model molecules, *i.e.* egg albumin, polyphenols and riboflavin, to deeply understand their behavior when adopted in real systems. Because of the possible sodium and calcium ions release into wines resulting in possible detrimental effects, these adsorbent materials were primarily studied to assess their ions release into three different wines (*Negroamaro* Rosé, *Verdeca* White and *Primitivo* Red). Since all the Na^+ and Ca^{2+} released concentrations were lower than the threshold value of 30 and 20 mg L^{-1}, respectively, all the bentonites can be considered good candidates for the adsorption kinetics.

Hence, after having deeply characterized them on diverse physico-chemical points of view, the optimization of the adsorption kinetics parameters performed on egg albumin evidenced that Na/Ca_0.20 and 0.27 are the most performing ones, especially in terms of high surface excess within 120 min. This may be due to the slightly higher surface area for the former, and smoothly greater pores dimensions by BJH method, for the latter. Notably, an enhancement in the adsorption performances was achieved by both adding 12%v/v of ethanol into the aqueous medium, mimicking the wine system, and the bentonites swelling. Both the treatments favor the exfoliation of the bentonites sheets thus increasing the probability of contact between the adsorbent and the CMMs. Going deeply into the theory of the adsorption mechanisms, single kinetics revealed that both egg albumin and polyphenols data are well represented by the Frumkin-Fowler-Guggenheim model, whereas riboflavin isotherm is typical of the Freundlich one. Moreover, through these adsorption models the variation of the Gibbs free energy and the lateral interactions parameter were computed. For all the three CMMs, a negative value of $\Delta G_{ad\text{-}sorption}$ was obtained resulting in a thermodynamically favoured process, even if for the riboflavin this parameter was one order of magnitude smaller. Also the *a* factor was positive in each case, denoting that the forces among the charged molecules are of repulsive nature.

Finally, aimed at deeply investigating the physico-chemical mechanisms in wine fining, adsorption tests in a wine-like system composed by the simultaneous presence of the three CMMs were carried out and followed by both UV/Vis spectroscopy and HPLC analysis. Also, here, Na/Ca_0.27 revealed to be the most performing adsorbent leading to a high adsorption of all the three molecules. However, by comparing these results with the ones obtained in single adsorption kinetics, we inferred that the increase of polyphenols adsorption in the wine-like system was due to their interactions with

egg albumin molecules, possibly giving rise to albumin/polyphenols complexes. Therefore, this interaction may have affected the final adsorption. Nevertheless, a percentage of about 40–50% of the tested polyphenols was preserved into the model wine, whereas the majority of the egg albumin, mimicking the medium/high MW heat unstable proteins, and riboflavin was removed from the system, as required.

CRediT authorship contribution statement

E. Pargoletti: Data curation, Validation, Writing - original draft. **L. Sanarica:** Conceptualization, Writing - review & editing. **M. Ceruti:** Data curation, Methodology. **F. Elli:** Data curation, Methodology, Validation. **C. Pisarra:** Methodology. **G. Cappelletti:** Conceptualization, Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.127840.

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