

Highlighting protein fining residues in a model red wine

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ABSTRACT

Many studies have dealt with fining treatments, focusing on their impact on phenolic precipitation or sensory properties of the treated wines. Previous articles suggested the presence of soluble complexes with tannins and fining proteins, and probably wine polysaccharides too. However, no study has quantified these possible protein residues in wine. The analyses performed on a model red wine to highlight the residual fining proteins were the measurement of viscosity, the quantification of amino acid/proteins of the samples and the radioactivity of the supernatants obtained after fining with a radioactive protein. This work has clearly shown, both qualitatively and quantitatively, the presence of fining residues in a model red wine that had been fined with a gelatin and two hydrolyzed plant proteins. These results have significant implications, yet to be confirmed with commercial fining agents and 'real' wines for allergen residues in treated wines.

1. Introduction

Fining treatments are widely used to clarify wines since consumers expect limpid wines, the clarity having to be stable with time (Ribéreau-Gayon, Glories, Maujean, & Dubourdieu, 2012). Among fining agents, proteins such as gelatin or casein are commonly added to precipitate tannins and prevent haze formation. They are classified as adjuvants in enology and have to be eliminated with the lees after treatment (Moreno-Arribas & Polo, 2009). Many research works have focused on the impact of fining on color (González-Neves, Favre, & Gil, 2014), turbidity (Iturmendi, Durán, & Marín-Arroyo, 2010), phenolic content (Sarni-Manchado, Deleris, Avallone, Cheynier, & Moutounet, 1999; Yokotsuka & Singleton, 1995) and sensory properties (Scollary, Pásti, Kállay, Blackman, & Clark, 2012) of the treated wines. For example, protein fining treatments induce the reduction of astringency in some cases (Maury, Sarni-Manchado, Lefebvre, Cheynier, & Moutounet, 2001) by precipitation of tannins, or proanthocyanidins, and particularly highly polymerized and galloyled tannins. If fining proteins are added to wine in order to precipitate tannins, it is crucial to check that the products added have the expected behavior, in short that they are totally precipitated out of the wine after treatment. This question is relevant since tannin/protein interactions first involve formation of soluble complexes that, depending on the protein to tannin ratio and medium composition, can remain in solution (Maury, Sarni-Manchado, Poinsaut, Cheynier, & Moutounet, 2016). Formation of soluble and colloidal complexes between tannins and proteins has indeed been

demonstrated (Canon et al., 2013; Poncet-Legrand et al., 2006). Introducing a SDS treatment increased proanthocyanidin recovery after fining with gelatins (Maury et al., 2001) or plant proteins (Maury, Sarni-Manchado, Lefebvre, Cheynier, & Moutounet, 2003), in particular in the supernatant, thus suggesting that part of the fining proteins remain in the wine after treatment as tannin-protein complexes. Earlier studies on allergenic proteins did not detect any residue from casein, ovalbumin, or peanut in 153 Australian wines using enzyme-linked immunosorbent assays (ELISA) tests (Rolland, Apostolou, De Leon, Stockley, & O'Hehir, 2008). Peanut was tested since peanut related-proteins could induce severe clinical reactions; non-grape derived tannins are also used in enology with the potential cross-reactivity with tree nuts and peanut allergens. In the same way, no residue of egg white proteins was detected by immunoblotting and ELISA test in the 85 wines from different countries (Uberti et al., 2014). These observations however do not imply that different kinds of proteins, such as gelatins and plant proteins, would have a similar behavior. Moreover, if a tannin-protein complex was present, it might be not detected by these tests that are specific for proteins.

Most studies focused on the identification of a method allowing the detection of allergenic proteins (Peñas, di Lorenzo, Uberti, & Restani, 2015) like caseins (Monaci, Losito, Palmisano, & Visconti, 2010) or/and egg white proteins (De Angelis, Pilolli, & Monaci, 2017; Koestel, Simonin, Belcher, & Rösti, 2016; Pilolli, De Angelis, Godula, Visconti, & Monaci, 2014) in wines or the possible allergenic reaction of such proteins (Pilolli, Chaudhari, Palmisano, & Monaci, 2017). Uberti et al.,

Abbreviations: AA, amino acid; FT, fining treatment; mWi, model wine; P, pellet; RA, radioactivity; S, supernatant; TCA, trichloroacetic acid

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2014; Vassilopoulou et al., 2011; Vasilescu, Nunes, Hayat, Latif, & Marty, 2016), and none of these address the quantification of gelatin or plant protein residues in a wine or a model wine. Quantification of residual proteins in wine after fining is therefore of importance for several reasons: it could have an impact on sensory properties of wines, which could be less astringent because of these tannin-protein complexes; the classification as adjuvant could be reconsidered and last, the presence of these proteins could have a negative health impact like the presence of allergenic plant proteins in recent wines.

In our earlier work (Maury et al., 2016), we showed that tannin precipitation by gelatin was reduced in the presence of wine polysaccharides, presumably owing to the formation of soluble ternary complexes. Therefore we decided to investigate the extent of protein precipitation in a model wine after fining with different methods using different properties of these proteins: viscosity measurement, amino acid determination and protein labelling quantification. The main objective is the quantification of the potential residues of the fining agents in the model wine.

2. Materials and methods

2.1. Materials

2.1.1. Model wine (mWi)

A model wine was prepared from a Syrah wine from Nîmes (Gard, France) as previously described (Maury et al., 2016). Briefly, a polyphenolic powder (746 g of powder from 200 L of wine) was prepared from this wine at the INRA Unité Expérimentale d'Enologie de Pech Rouge (Gruissan, Aude, France). The model wine was prepared by dissolving 3.73 g/L of the polyphenolic powder in an aqueous solution containing potassium hydrogen tartrate 0.015 M, pH 3.7 and 12.3% ethanol. The analysis of the model wine before fining is considered as a control. The Syrah extract contained mainly phenolic compounds since its polysaccharide level was only 80 mg/L and the proteins only 2 mg/L (Maury et al., 2016).

2.1.2. Fining treatments

2.1.2.1. Plant proteins. Commercial plant proteins HG1 and HG2 were provided by Martin Vialatte (Epernay, France). HG1 and HG2 were a powder preparation from enzymatically and chemically hydrolyzed wheat gluten, respectively. These two preparations were mainly composed of 10–16 kDa proteins but contained some of larger size, in particular 50 kDa proteins (Maury et al., 2003). HG1 and HG2 have a similar protein profile. The purity of proteins was assessed on the basis of amino acid determination: total hydrolysis (24 h at 120 °C with HCl 6 N) of the samples, followed by HPLC analysis with a post-column derivatization of the amino acids using ninhydrin (Maury et al., 2001). Thus, HG1 and HG2 contained respectively 88% and 86% of proteins (Maury et al., 2003). Plant protein preparations were suspended in water to obtain a final concentration of 10 g of protein per liter. The sampling of HG suspension was achieved under agitation.

2.1.2.2. Gelatin. A gelatin fraction (G16) was obtained from a liquid commercial one (Gélisol) by sequential ammonium sulfate precipitations (Maury et al., 2001). Proteins of this preparation had a molecular mass centered on 16 kDa (mainly from 10 to 25 kDa). Amino acid analysis performed as described above for plant proteins showed that G16 contained 72% of proteins with a concentration of 35 g of proteins per liter (Maury et al., 2016).

2.2. Methods

The experimental design flow chart is presented in Fig. 1.

2.2.1. Fining experiments

Fining experiments were performed by adding proteins (66.7 µL of

gelatin or 230 µL of plant proteins) resulting in negligible dilution (0.29% for G16 and 1% for plant proteins) to 23 mL of sample (model wine) to obtain a final protein concentration of 0.1 g/L, in the 0.05–0.2 g/L range classically used for gelatin in fining red wines (Karamanidou, Kallithraka, & Hatzidimitriou, 2011; Maury et al., 2001, 2003). The mixture was maintained at 25 °C for 48 h. There was no attempt to exclude oxygen during fining as its exclusion is not normal winery practice. The samples were then centrifuged at 4 °C at 1900g for 10 min (SORVALL® Ultraspeed Centrifuge RC5 B, Dupont de Nemours, Les Ullis, France; rotor SA-600) giving a pellet and a supernatant (treated wine). All experiments were done in triplicate, and one analysis was performed per sample. When fining with radioactive proteins, a mixture of radioactive and non-radioactive proteins were used for the fining experiment since the method chosen allowed the labelling of 250 pmol of proteins only.

2.2.2. Viscosity measurement

The measurement of viscosity was made with the Schott Geräte AVS 400 viscometer and with the capillary Schott Geräte 29138, type 53610/I. The kinematic viscosity was assessed based on the time of flow between two points as: $\eta = K.T$, where η is the kinematic viscosity in mm²/s, K is a constant and T is the time of flow in s.

After 15 min of temperature equilibrium (25 °C), 5 measurements were realized on each sample filtered at 5 µm (Millipore).

2.2.3. Amino acid determination (from Benson, Manuel, Gordon, & Patterson, 1967; Maury et al., 2001)

A preliminary experiment dealt with the separation and analyses of free amino acids and amino acids from proteins and peptides of the samples following precipitation with ethanol (3 mL of wine with 15 mL of ethanol 95% at –20 °C during 48 h) and these conditions were used in subsequent experiments. However, gelatins did not precipitate under these conditions. Therefore, only global analyses of all amino acids were performed after hydrolysis, without the precipitation step, except for the model wine. A previous study showed that tannin-protein complexes created as a consequence of protein fining were sufficiently strong and to withstand the hydrolysis conditions (Maury et al., 2016). A 10%SDS treatment allowed the recovery of proteins and tannins (Maury et al., 2016). Thus, supernatants and pellets from fining treatments were previously treated by 10% SDS at 90 °C for 5 min in order to dissociate proteins from tannins. The samples were then dried under vacuum and submitted to hydrolysis. Samples were hydrolyzed with 2 mL of HCl 6 N at 120 °C during 24 h. Samples were: 6 mL of model wine or supernatants (treated by SDS), the content of 2 pellets pooled (in order to get enough raw material for the analyses) after fining treatment, or 1 mg of fining proteins. When the samples had cooled, they were dried and then solubilized in 0.5 mL of lithium citrate 0.20 M at pH 2.2. Amino acid analyses were performed using a Biochrom 20 analyzer (Pharmacia LKB Biotechnology AB, Uppsala, Switzerland) controlled with the EZChrom software. The amino acid separation was realized with a cation-exchange column, lithium Ultropac 8 (particules 9 µm ± 0.5) with a lithium citrate gradient from 0.2 M to 1.65 M, pH from 2.80 to 3.55 and temperatures from 35 °C to 78 °C. The flow rate was 0.42 mL/min. Ninhydrin (Sigma) was introduced for post-column derivatization at a flow-rate of 0.33 mL/min. The detection and the quantification of the amino acids derivatized by ninhydrin were made at 570 nm, except for proline and hydroxyproline which were detected at 440 nm, on the basis of their retention times in comparison with standards and calibration graphs.

2.2.4. Protein labelling

PKace kit (Novagen) was used for labelling the fining proteins G16 and two hydrolyzed wheat gluten HG1 and HG2. The sub-units of the protein kinase AMPc-dependent (protein kinase A, PKA) targeted the specific amino acid sequence Arg-Arg-Ala-Ser-Val present in the fining agents; the serine units were then labelled with [γ -³²P]. Each fining

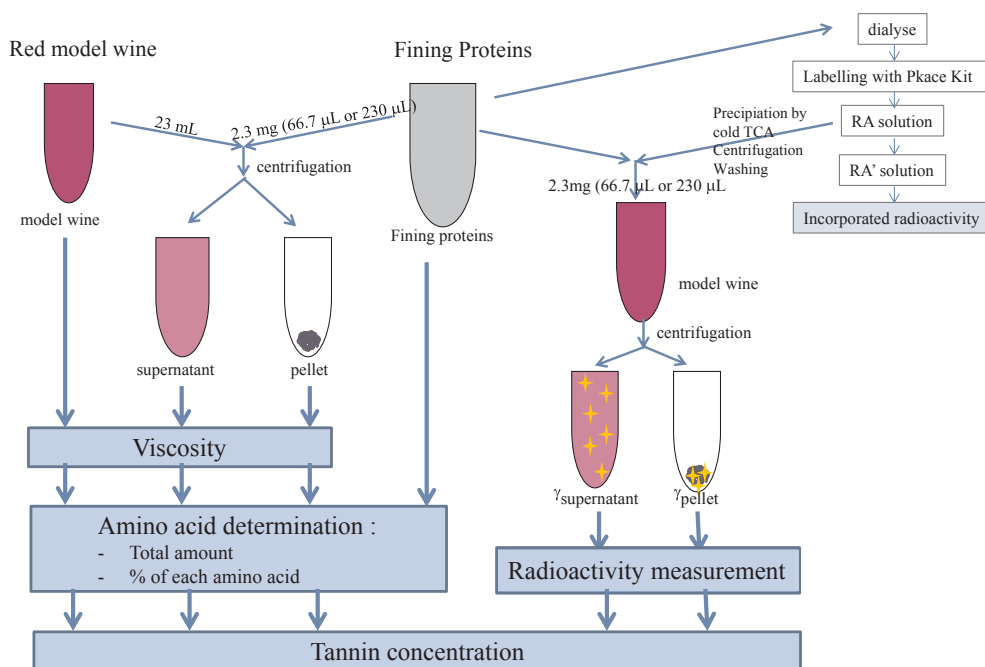


Fig. 1. Experimental design flow chart. RA: Total scintillation of the solution composed by proteins, buffer, protein kinase, [γ - ^{32}P]-ATP; RA': scintillation of the proteins labelled after removing the free [γ - ^{32}P]-ATP.

agent was labelled in triplicate. The labelling protocol consisted in mixing deionized water, PKA buffer, protein kinase, [γ - ^{32}P]-ATP, controlled protein, with or without fining agent. These solutions were named RA. An aliquot of RA (2 µL) were diluted with deionized water (98 µL) in 900 µL of TCA 25%. The total radioactivity RA' was then counted after the addition of the scintillation mixture (FluorSafe, Scintan BHD) with a scintillation counter LS 6500 (Beckman).

2.2.5. Recovery of labelled proteins

The radioactive proteins present in the remaining RA solution were separated from the free [γ - ^{32}P] by 25% cold TCA precipitation in ice followed by a centrifugation step. A previous verification step showed that 25% cold TCA precipitated 70% of G16 and 84 and 82% of respectively HG1 and HG2. The radioactive proteins recovered were then washed with ether/ethanol (3:1, v/v) twice then incorporated in the protein part which was used for fining the model wine. After a contact of 48 h (see Section 2.2.1), pellets and supernatants were separated and their radioactivity was determined.

2.2.6. Statistical analysis

Analysis of variance was conducted with Statgraphics Plus® 5.0 software.

3. Results and discussion

3.1. Tannin concentration of model wines after fining with gelatin and plant proteins

The objective of the fining treatment is clarifying the wine and thus precipitating the colloids present in the wines, which could lead to haze and precipitates. Fig. 2 presents the evolution of tannin concentration due to fining treatments. As already shown (Maury et al., 2016), fining treatments significantly decreased the tannin concentration. No significant difference in tannin concentration of supernatants was observed between the fining agents used in this study.

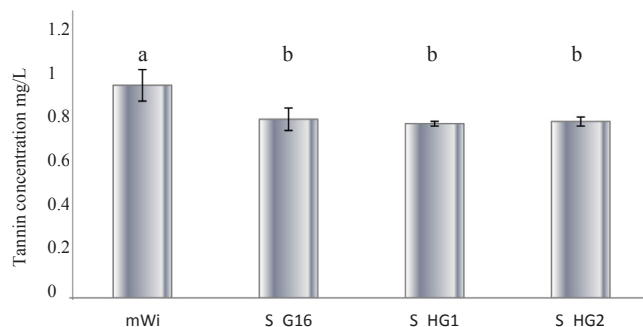


Fig. 2. Tannin concentration (mg/L) of the model wine (mWi) and the supernatants after fining with G16, HG1 and HG2. Values $\pm$ standard deviation (3 repetitions per treatment). Different letters denote significant differences between the samples ($p < 0.05$).

3.2. Viscosity of treated wines

The viscosity of the model wine was analyzed before and after fining. Fig. 3 shows that the supernatants obtained after fining with G16 and the two HG had significantly higher viscosity than the model wine, and the supernatant from the gelatin fining had a higher viscosity than those from the hydrolyzed gluten treatments. The higher viscosity of the solutions after fining can only be explained by the presence of soluble tannin-protein complexes in the supernatants since the viscosity of the fining proteins at 0.1 g/L in the hydroalcoholic solution used in this study reached only 1.33 mm²/s. The fining agents are indeed the only additives of the model wine in this experiment and previous results showed that at the concentration used, the fining agents themselves do not participate in the viscosity of the model wine (Maury et al., 2016). Moreover, as shown in Fig. 2, the tannin concentration decreased after fining thus the increase of viscosity cannot be explained by the evolution of the phenolic content.

3.3. Amino acid determination

Table 1 shows the amino acid concentration of the fining proteins,

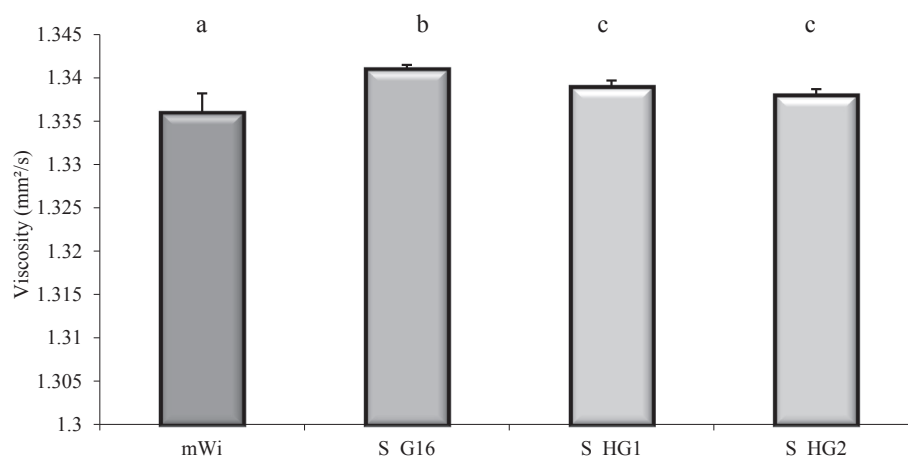


Fig. 3. Viscosity of the model wine before fining (mWi) and of the supernatants after fining with G16 (S_G16), HG1 (S_HG1), and HG2, (S_HG2). Values $\pm$ standard deviation (3 repetitions per treatment). Different letters denote significant differences between the samples at the 95% confidence level.

Table 1

Amino acid concentration in mg/L of fining treatment (FT), model wine (mWi), and pellets (P) and supernatants (S) obtained after fining with G16, HG1 and HG2. Different superscript letters in a row indicate significant differences ($p < 0.05$), a,b: comparison between the mWi and the supernatants, α,β : comparison between the FT and the corresponding pellet; * means not significantly different from the mWi at 95% confidence but significant at 90%.

Amino acid (mg/L)	Fining treatments		
	G16	HG1	HG2
mWi	86.5 $\pm$ 3.1 ^a		
FT	69.1 $\pm$ 7.8 ^a	81.3 $\pm$ 6.8 ^a	86.3 $\pm$ 1.3 ^a
total1	161.5	167.8	172.8
pellet	61.3 $\pm$ 10.9 ^a	58.1 $\pm$ 4.10 ^b	59.8 $\pm$ 1.7 ^b
supernatant	107.9 $\pm$ 13.3 ^{ab*}	107.5 $\pm$ 1.1 ^b	123.1 $\pm$ 24.8 ^{ab*}
total2	169.2	165.6	183.0
%Recovery	105%	99%	106%
%(S-mWi)/mWi	25	24	42
%(S-mWi)/FT	29	26	24
%P/FT	82	72	69

and of the model wine before and after fining treatment. The mWi had 86.5 mg/L of total amino acids, corresponding to 11% of the amount found in the initial wine and only 8 mg/L of free amino acids (data not shown). The composition in amino acids of the model wine and the initial wine showed globally that the amino acid profile of the hydrolyzed model wine was closer to composition of wine proteins than to the free amino acids (Supp.1). More than 80% of the amino acids of the

mWi analyzed were recovered in the ethanol extract after hydrolysis. This suggests that these components were probably peptides as they were not precipitated by ethanol. These proteins/peptides may have been co-eluted with phenolics during the preparation of the model wine, the main part of the proteins being washed and removed from it. It is important to note that the precipitation with ethanol did not work well with the mWi (nor with the gelatins) and thus the different analyses presented in this work are based on the total hydrolysis of samples. The first two lines of Table 1 show that FT and model wine contribute similar amounts of amino acids in the samples. The next two lines of the table present the amino acid content of the pellets and supernatants after fining. Comparison of the amino acid concentration of (model wine + fining proteins) and (pellets + supernatants) showed that the proteins were quantitatively recovered from all samples. The fact that the supernatants were richer after fining than the untreated model wine indicates that part of the proteins/peptides added in the fining treatment remain in the fined wine. The enrichment reached 25, 24 and 42% respectively for G16, HG1 and HG2. The presence of fining residues in the supernatants was also reinforced by the fact that the amino acid concentration in the pellets represented only 82, 72, and 69% of that added by fining with G16, HG1 and HG2, respectively. The extra amino acid quantities in the supernatants after fining were calculated ($\text{Supernatant} - \text{mWi}$)_{mg/L} and compared to the amount of fining treatment added. Therefore, excess of amino acids (in mg/L) corresponded to 29, 26 and 24% respectively for G16, HG1 and HG2.

The amino acid determination allowed a differentiation of the protein content of the model wine before and after fining with G16, HG1 and HG2 (Table 2). The differences observed when fining with

Table 2

Differences of amino acid concentrations between the mWi after fining with G16, HG1 and HG2 and the mWi. mWi: model wine, S: supernatant, %/mWi = $(S - \text{mWi}) \times 100/\text{mWi}$, FT: fining treatment, %/FT = $(S - \text{mWi}) \times 100/\text{FT}$. Differences were calculated on the mean of 3 analyses. Main amino acids are in bold. Statistics: * and ** means that values are significantly different from those of the model wine respectively at the level of 90 and 95% of confidence.

Amino acids	Concentration in mmol/L				S_G16 – mWi			S_HG1 – mWi			S_HG2 – mWi		
	mWi	S_G16	S_HG1	S_HG2	Δ mmol/L	%/mWi	%/FT	Δ mmol/L	%/mWi	%/FT	Δ mmol/L	%/mWi	%/FT
α -Ala	53.57	83.00**	63.00*	62.33*	29	55	28	9	18	3	9	16	3
Arg	20.33	29.33**	26.00**	27.33**	9	44	25	6	28	3	7	34	4
Asx	61.17	84.00	85.33	82.00	23	37	63	24	40	9	21	34	8
Glx	93.87	115.00	153.00*	263.67**	21	23	38	59	63	2	170	181	6
Gly	123.23	195.00**	148.33**	155.00**	72	58	35	25	20	6	32	26	7
Hydroxypro	22.67	47.67**	26.33	26.00	25	110	33						
Lys	34.93	41.00	40.67	42.67	6	17	23	6	16	5	8	22	7
Pro	57.77	83.33**	66.00**	70.00**	26	44	35	8	14	1	12	21	1
Ser	54.00	69.00	63.33	65.33	15	28	51						
Thre	48.17	58.33	53.33	56.67	10	21	68						

G16 corresponded to an enrichment of the amino acids of the model wine of 110, 58 and 55% respectively for Hydroxypro, Gly and α -Ala due to the treatment. If that enrichment is calculated on the basis of the content of the fining proteins, that means that, in the treated wine, 34% of Hydroxypro, 35% of Gly and 28% of α -Ala of the fining proteins are recovered in the supernatants. The percentage calculated in Table 2, %/mWi, showed that the model wine was enriched in amino acid after any fining treatment (positive values). Similarly, an enrichment of 63 and 181% of the Glx can be observed in the model wine, for HG1 and HG2, respectively, which corresponds to the presence of 2 and 6% Glx of HG1 and HG2 respectively, in the treated model wines. The model wines were enriched with the three main amino acids (Glx, Asx and Gly) of the fining treatment (Supp.2), basically at a level corresponding to 6–7% of the amount of the fining agent added (HG1 and HG2). The pellets produced by the fining treatments showed similar amino acid proportions to the fining fraction as expected (Supp.2).

This analysis showed that a part of the fining proteins were recovered in the supernatants obtained after fining the model wine. This part corresponded in average to 5–40% of the fining proteins added in the model wine. More protein residues were found in the supernatants after fining with the gelatin than with the plant proteins.

3.4. Presence of radioactive proteins in wine after fining

The previous methods clearly showed an enrichment of proteins in the model wine after fining with an increase of the percentage of amino acids specific of the fining proteins. In order to use a method based specifically on proteins from the fining treatments, another method was applied, consisting in labelling a part of the fining proteins and counting the radioactivity in the treated wine after fining.

3.4.1. Incorporation of ATP_γ in the fining treatments

The enzyme used for the labelling was efficient (controls) and the fining proteins were labelled (Supp.3). The incorporated radioactivity was rather low (2–4%) but sufficient to use the labelled proteins as markers in the wine treatment. Since the labelling was specific on serine residues, the total incorporated radioactivity could not be higher than the content of serine units. The incorporation of the radioactivity of G16 corresponded at 4%, which was also the percentage of serine residues in the protein amino acid composition indicating that the labelling step was efficient. The amino acid analysis showed that HG1 and HG2 contained 7% of serine. In these cases, the RA incorporation was only of 2%, suggesting that only part of the serine was labelled (Supp.3). Even if the labelling is focusing on serine residues, a bigger zone, constituted by the amino acids sequence “Arg-Arg-Ala-Ser-Val” (PKace kit (Novagen)) is involved in the recognition of the substrate by the enzyme, as commonly found in protein kinases action (Kennelly & Krebs, 1991). That induced probably a lower labelling yield compared to the potential given by the single serine residue number.

3.4.2. Radioactivity in the supernatants after fining

The presence of ATP in the fining treatment did not modify the behavior of phenolic compounds, and therefore did not influence the fining step itself (Supp.4). Radioactivity was found both in pellets and in supernatants after fining the model wine with the labelled fining proteins (Table 3). The presence of this radioactivity in supernatants

was further clear evidence for the presence of ATP and thus of fining protein residues in the treated wine. The incorporated RA corresponded to the radioactivity of the labelled fining agents. This radioactivity was shared in two, a part being recovered in the pellets and another part in the supernatants. The radioactivity found in the supernatants corresponded between 26 and 58% of the labelled fining proteins.

In these experiments with the radiolabelled fining proteins, the question about the homogeneity of the labelling of the different types of protein present in the fining agents arose. If the labelling were homogeneous, the results would correspond to the actual content of the fining proteins remaining in the model wine after fining. If not, depending on which proteins/peptides were actually labelled and their efficiency to precipitate tannins, the minor or major ones, the results would respectively under or over-estimate the amount of protein remaining in wine after fining. Furthermore, the methodology would have been improved if a higher level of ATP incorporation had been achieved as this would provide a more representative assessment of the fining treatment.

Two different methods have been used to highlight and quantify the presence of fining residues in a red model wine: amino acid content and quantification of radiolabelled fining proteins in the model wine after the fining treatment. Both methods showed the presence of fining residues. The wide range of values obtained (24–58% of the initial amount used for fining) suggests that each method may be quantifying different types of proteins (Supp.5). The assessment of the global concentration of amino acids is probably the most reliable method for measuring residual protein after fining since there is no reason for this method to induce a bias. It is important to note that, after total hydrolysis, the amino acid concentration of (wine + fining) corresponded to that of (supernatant + pellet), showing good recovery. Moreover, the comparison of the percentage of each amino acid in the sample before and after fining indicates enrichment in some amino acids specific of the fining proteins. It seems that all proteins present in the fining agents do not react in the same way, some precipitated as expected while others remained in solution after fining, probably as a consequence of their involvement in forming soluble complex with tannins (Carvalho et al., 2006; Gazzola, Van Sluyter, Curioni, Waters, & Marangon, 2012; Maury et al., 2016; Scollary et al., 2012). These interactions may depend on the composition, structure and molecular weight of both fining proteins and other wine macromolecules (Canon et al., 2013; Poncet-Legrand, Gautier, Cheynier, & Imberty, 2007). It would be interesting to go further in the analyses and understand the structure and characteristics of such fining proteins and of the supramolecular assemblies in which they are involved.

In contrast, quantification of labelled proteins may be biased as the labelling specifically involves some amino acids and the proteins which were labelled may not correspond to average/most representative fining proteins. These results are in agreement with the fact that the use of SDS treatment increased the recovery of tannins in the fined wines (Maury et al., 2003). This treatment induced a recovery of +14% and +3% of the tannins respectively of supernatants and pellets after fining with G16, and +4% and +3% of supernatants and pellets after fining with HG2. Moreover, it is noticeable that the amount of protein recovered from the supernatant is similar to or higher than the part in the pellets. This information supports the hypothesis of complexes between tannins and fining proteins, non-soluble for a part but soluble (in the

Table 3

Radioactivity (RA) after protein fining treatments (FT). S: supernatant, P: pellet. Experiments were realized in triplicate.

Fining treatment	RA (.10 ⁴ cpm)		Incorporated RA in the FT (.10 ⁴ cpm)	%S/incorporated RA	%P/incorporated RA
	S	P			
G16	31.43 ± 24.18	53.57 ± 3.85	122.77 ± 44.91	26	44
HG1	21.55 ± 4.12	19.23 ± 2.65	40.67 ± 6.44	53	47
HG2	22.52 ± 0.48	7.16 ± 2.00	38.89 ± 9.11	58	18

supernatant) for the other part.

4. Conclusion

Although the two methods used gave different quantitative results, they were all in agreement with the fact that fining protein residues could be detected in the model wine after fining with gelatin and hydrolyzed wheat gluts. The reason why each method gave different quantitative results is because they were based on different ways to count proteins: i/the addition of the concentration of all amino acid of the sample, ii/the evolution of the percentage of each amino acid in the sample before and after fining, and iii/the quantification of labelled proteins specifically, knowing that the proteins which were labelled may not correspond to an average/most representative fining proteins. The detection of these proteins in the fined wine corresponded to 24 to 58% of the initial fining proteins added to the model wine. These results were obtained using a model wine and similar experiments need to be performed on actual wines as the more complex matrix of wines may impact the fining process and thus vary the observation found with the model system. For example, the presence of polysaccharides could play a major role in influencing the aggregation of the tannin/fining protein thereby affecting the extent of residual fining protein. Since polysaccharides limit tannin precipitation (Maury et al., 2016), protein residues could be detected with higher levels in wine.

From a consumer healthcare perspective, there would be little risk from residual protein after fining except possibly for the wheat gluten. Other allergenic proteins are used in oenology as clarifying agents like eggs and isinglass. From 2012, the European legislation (2007/68/EC) requests the information of the presence of milk, wheat, eggs or lysozyme in food and wine when the concentration is higher than 0.25 mg/L. OIV recommends the mention of the presence of any allergens found in wine.

Based on our results in a model wine, it is reasonable to suggest the residual protein remaining in the wine is in a complex form with other wine components, especially tannins. This needs to be in context of the results of Rolland et al. (2008) and Uberti et al. (2014) who did not detect allergenic proteins in wine after fining using ELISA or immunoblotting techniques, respectively. However, any residual protein that is bound or in an aggregate with other wine compounds may not be recognized by antibodies and furthermore may be not available to react in human bodies as allergens if the complexes are not separated in the digestive tract. Lifrani et al. (2009) hypothesized that the complex between allergenic proteins and tannins could prevent an anaphylactic reaction. Vassilopoulou et al. (2011) found that patients diagnosed with fish allergy were able to drink moderate amounts of wine fined with isinglass without developing a positive skin prick test. Endly, Peñas et al. (2017) conclude their review with the fact that some allergenic proteins did not react in immunoassays but were found analytically in wine. If that hypothesis is confirmed, that would mean that drinking wine while eating allergenic proteins from food may limit the reactivity of these persons.

From a regulatory point of view, if the presence of a protein fining residues is confirmed in wine, the fining preparations could not be considered as adjuvants but rather as additives, thus requiring a change in their enological classification. Before going to this regulatory change, other enological processes need to be checked for their impact on removing these proteins such as centrifugation, filtration and the use of bentonite. Lifrani et al. (2009) showed, in the case of other protein fining treatments, such as ovalbumin and isinglass, that ELISA tests were positive during the winemaking process, but negative in the commercial wines that had undergone filtration. On the contrary, although bentonite treatment and successive filtration did contribute significantly to the decrease of casein residues in wines, there were casein residues remaining in white wines (Weber, Steinhart, & Paschke, 2009). OIV recommends indeed a filtration after using plant proteins (RESOLUTION OIV – OENO – SECSAN 520 – 2014). Given the success

of this model wine study in pointing towards the formation of tannin/protein aggregates, the next phase is to apply the concepts developed here to real wines.

Declaration of interests

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.2018.11.141>.

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