A NEW METHOD OF PROTEIN EXTRACTION FROM RED WINES

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Abstract

Aim: Wine is a very complex medium and is often evaluated according to its main components like alcohol, sugar, tannins, and acid levels. Proteins are rarely considered in this evaluation because their concentrations are only a few mg/L of wine. However, in an enological context, proteins appear to be more and more important, in particular for the stability of wines with protein haze problems. The study of proteins is less obvious in red wines than in white wines because the proteins are strongly tied to tannins, which makes their extraction and analysis even more difficult.

Methods and results: This article describes a technique for the separation of proteins from tannins thanks to a methanol/chloroform emulsion in an acid solution. The protein extract, obtained after 4 hours, was later analyzed by SDS-Page and the protein profile of the wine established. Experiments showed that the protein profiles remained the same during the different stages of the winemaking process, whereas the overall amount of proteins decreased. Characteristic protein profiles of different grape varieties were established, and it was also possible to visualize the presence of exogenous proteins from fining agents like albumin and casein.

Conclusion: This procedure allowed the extraction of proteins from 8 red wine samples within 4 hours. It also made it possible to analyze the extracted proteins by SDS-Page without tannin interference within 2 hours.

Significance and impact of the study: This method shows in a very promising manner how proteins might be extracted from red wines after being separated from their tannins. The extracted proteins are then available for analysis using even more advanced techniques such as ESI-QTof or ELISA.

Key words: protein, SDS-Page, cultivar, fining, red wine

Résumé

Objectif : Le vin est un milieu très complexe souvent évalué en fonction de ses éléments principaux comme l'alcool, le sucre, les tanins et la teneur en acide. Les protéines sont rarement retenues dans cette évaluation car elles ne sont présentes qu'à quelques mg/L de vin. Cependant, elles apparaissent de plus en plus importantes en œnologie, en particulier pour la stabilité des vins face aux troubles en bouteille. En vin rouge leur étude est plus problématique qu'en vin blanc car les protéines sont fortement liées aux tanins, ce qui les rend difficiles à extraire et à analyser.

Méthodes et résultats : Cet article propose une technique permettant la séparation des protéines des tanins grâce à une émulsion méthanol/chloroforme en solution acide. L'extrait protéique obtenu en 4 heures a ensuite été analysé par SDS-Page et le profil protéique des vins établi. Des essais ont démontré que le profil des protéines restait stable au cours de la vinification alors que la quantité globale diminuait. Des profils protéiques caractéristiques ont été établis en fonction des cépages et il a été possible de visualiser dans des vins collés en laboratoire la présence de protéines exogènes comme l'albumine et la caséine.

Conclusion : Cette technique a permis d'extraire les protéines de 8 vins rouges en 4 heures et de les rendre analysables en SDS-Page sans interférence avec des tanins en 2 heures.

Signification et impact de l'étude : Très prometteuse, cette méthode d'extraction met à la disposition de techniques d'analyse aussi pointue que le ESI-QTof ou l'ELISA des extraits de protéines de vin rouge jusqu'alors trop liées aux tanins pour être analysées.

Mots clés : protéine, SDS-Page, cépage, collage, vin rouge

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INTRODUCTION

Proteins are found at relatively low concentrations in grapes (0.05% of the pulp (Giribaldi and Giuffrida, 2010)) and at even lower concentrations in wines (100 mg/L of wine (Okuda *et al.,* 2006)). About 90% of the proteins found in wines are those that originated from grapes (skins and seeds) and resisted alcoholic fermentation (Fukui and Yokotsuka, 2003; Okuda *et al.,* 2006; Yokotsuka *et al.,* 1994). The other wine proteins originate from yeasts (Fukui and Yokotsuka, 2003), some from bacteria and molds (Kwon, 2004). They are mainly mannoproteins (Dambrouck *et al.,* 2003).

For a long time, the role of proteins in wines was underestimated and very little studied because of their low concentration, even though it was known that their nature varies a lot and that up to 160 different proteins were found in a Chardonnay, for instance (Okuda *et al.,* 2006). Today, it is established that proteins contribute importantly to the quality of wines and play a major role in the stabilization of the foam in Champagne (Cilindre *et al.,* 2007; Dambrouck *et al.,* 2005). Certain authors point out that proteins contribute to the varietal differentiation of musts and wines (Dambrouck *et al.,* 2005; Vincent *et al.,* 2006). Proteins may also cause technological problems during the winemaking process(e.g., during filtration, clarification and cold stabilization (Weiss and Bisson, 2001)) and may be responsible for protein haze in bottled wines (Kwon, 2004; Okuda *et al.,* 2006; Waters *et al.,* 1992). Such problems seem to be less related to protein concentration than to the nature of the proteins.

Proteins may be used in wine as fining agents for wine clarification, lowering of phenolic components, or correction of certain defaults. Such proteins (casein, albumin, isinglass) are potentially allergenic and analysis methods to detect traces of these proteins are still in the development stage (Rolland *et al.,* 2008; Weber *et al.,* 2009). On the other hand, some allergic reactions to grape-derived proteins in wines are also reported in the literature (Pastorello *et al.,* 2003).

The presence of protein components in red wines was questioned for many years because of their tannin concentrations. In fact, tannins and proteins form complexes (Poncet-Legrand *et al.*, 2007; Siebert *et al.,* 1996; Siebert and Lynn, 2005) and generally precipitate. However, the composition and the structure of certain proteins may limit these precipitations and lead to tannin/protein complexes in colloidal stable forms (Fukui and Yokotsuka, 2003). Due to these interactions, protein extraction from red wines is particularly difficult (Vincent *et al.,* 2006). Recently, it has been demonstrated that yeast protein (namely Hsp12) can increase the sweetness of red wines (Marchal *et al.,* 2011). The main objective of this article was to describe an optimized protein extraction technique for red wines using a methanol/chloroform emulsion developed by Ducruet (2000). The protein mix obtained can be analyzed by SDS-Page. The interest of studying proteins is illustrated in various contexts: e.g., protein evaluation at different stages of winemaking, analysis of protein profiles for several grape varieties (Nakopoulou *et al.* 2006) and finding traces of fining agents in wines.

MATERIALS AND METHODS

1. The wines

Several red wines from the 2009 vintage (Gamay, Gamaret, Garanoir, Carminoir, Cabernet Sauvignon, Syrah, and Cornalin) with different tannin levels were selected during or at the end of the winemaking process. The wines were made in the experimental winery of the Agroscope Changins-Wädenswil Research Station (ACW) and at the Engineering School of Enology (EIC) in Changins, Switzerland. All wines were mono-cultivar and followed standard winemaking procedure (without fining agents), allowing a comparison of their protein profiles.

2. Extraction procedure

Wine (16 mL) was added to 8 mL of a chloroform/methanol (Merck) mixture (1/1) (Vertommen *et al.* 2010). Then 2 mL of formic acid (Réactolab) was added to acidify the mixture and promote the separation of the tannins. A fixed quantity of Bovine Serum Albumin (BSA, 5.8 µg per extraction, BioRad) was also added to each tube as internal standard. The mixture was emulsified through vigorous shaking for 30 sec. Decanting was done by centrifugation (10 min at 4500 g). Proteins were then retrieved at the interface between the methanol and chloroform phases as an emulsion. The mixture was emulsified one more time to increase the amount of proteins extracted from the wine.

This emulsion was washed twice with 4 mL of a methanol/water mixture (1/1). The washing procedure was carried out by gently shaking the tubes 10 times before centrifugation for 5 min at 4500 g. The emulsification was put in an Eppendorf® tube for a final wash with 1 mL of the methanol/water mixture (1/1). The protein extract was mixed with 500 µL of acetone (Sigma-Aldrich) to remove solvent traces and centrifuged (10 min at 4500 g), then 300 µL of ether (Merck) was added to eliminate the last traces of water

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and acetone. Ether was then evaporated under a stream of air or nitrogen. The extracted proteins were mixed with 40 µL of Tris HCl (0.16 M, pH 6.8) (BioRad) and dissolved using ultrasound for 60 min at 50 °C. After determining the protein concentration of the samples, 10 µL of buffer (4 mL of anhydrous glycerol (Fluka), 4 mL of SDS 10 % (Serva), and 4 mL of Bromophenol blue 0.5 % (Fluka)) was added. Proteins were denatured for 10 min at 95 °C. These protein samples were sometimes acidic, which changed the Bromophenol blue dye in the buffer to yellow. It was therefore necessary to increase the pH with 27 % ammonia vapor, until samples turned blue.

Proteins (1 µg, corresponding to 1-20 µL of protein extracts) were loaded onto the gel in order to ensure a good readability of the gels and a good comparison of profiles between wines. A modified Bradford method at dual wavelength was used for protein quantification (Zor and Selinger 1996). Before analysis, the proteins were diluted in Tris buffer and water only, because of specific interferences between the Coomassie blue dye reagent, used to quantify, and the SDS detergent, used to help the solubilization of the extracted proteins.

Note that whenever the volume of the interface is too small or the concentration of proteins too low (< 1 µg), several extracts of the same wine might be mixed or the extractions might be repeated on a larger volume of wine.

3. Migration

Proteins were analyzed by SDS-Page on a 15 % acrylamide gel (BioRad), which allowed an optimal separation of small proteins. The concentration gel was 4 % acrylamide. Migration through the stacking gel lasted 20 min at 15 mA, followed by 1 h at 25 mA through the separation gel. After, the electrophoresis gels were stained for 10 min with a 25 % Coomassie blue solution (Merck) before being rinsed several times with a 7 % acetic acid solution (Fluka).

4. Gel analysis

The gels were scanned and interpreted with the help of an image analysis system (Phoretix Advanced V 4.01 and Kodak® Analysis Software), which allowed the calculation of the molecular weights by matching them to markers of known size (BioRad) and the densitometric analysis of band intensity. The concentration of each wine protein was then expressed in BSA equivalents by comparing the intensity of the band with that of the internal standard.

5. Fining of the wines

For these experiments, the wines were fined in the laboratory with 10 g/hL of egg albumin (Ovicolle Oenofrance®) and 40 g/hL of potassium caseinate (Casei Plus Laffort®). These fining agents were diluted in water according to the manufacturer's recommendations. 100 mL of wine was fined with the appropriate amount of agent and settled for 36 h at 10 °C. The wines were then centrifuged for 5 min at 500 g to eliminate the remaining suspended particles, and finally, the proteins were extracted. The wines used for these tests were Gamay and Gamaret (EIC).

RESULTS AND DISCUSSION

1. Extraction

The initial extraction procedure (Ducruet 2000) presented several major disadvantages (e.g., extraction time, amount of reagents used, and difficulties to dry the samples), which generated higher costs and environmental concerns. The standardization of the emulsification step was very delicate and a success key for the extraction technique. The emulsification was initially done by mixing 100 mL of methanol/chloroform (1/1) and 200 mL of wine with an Ultraturrax® homogenizer for 3 min, followed by settling for 8 h at ambient temperature. Here, the emulsion was prepared in tubes containing only 16 mL of wine and 8 mL of the chloroform/methanol mixture (1/1). The settling was accelerated by centrifuging the mixture for 10 min at 4500 g. The use of formic acid, tested during the initial steps, has been generalized to decrease the sample pH, which allowed a better recovery of proteins (quantitatively and qualitatively) by decreasing tannin/protein interactions.

The volumes used for the extraction procedure were greatly reduced from 100 to 8 mL in these trials compared to the initial protocol, which saved solvents and reduced the volume of chlorinated waste. After the last washing procedure, acetone was added to the proteins to remove water and methanol. Then, this acetone was removed again by centrifugation and replaced with ether to remove the last traces of acetone. The procedure allowed extracting proteins from 8 wine samples within 4 hours.

The internal BSA standard gave an indication of the percentage of protein recovery during the extraction procedure. Because it was added to wines before extraction, BSA was instantly tied to wine tannins and extracted like other proteins. BSA has been chosen because of its high proline content, mimicking best the interaction of the tannins with the wine proteins.

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However, BSA is non-glycosylated and therefore does not behave exactly like certain wine proteins that are glycosylated (Dambrouck *et al.* 2003; Nakopoulou *et al.* 2006).

The protein quantification procedure, which is least dependent on traces of tannin contents in the dry extracts, was the modified Bradford Coomassie Brilliant Blue Assay (Zor and Selinger 1996). The only drawback is its non-compatibility with the SDS concentration needed for a good solubility of the protein extracts. Results were reported as theoretical concentrations of BSA and expressed in mg of BSA equivalents per liter of wine.

Extraction results were very different between grape varieties (Table 1 and Table 2). Gamay and Humagne were the grape variety with the highest wine protein concentration. They are varieties with very little tannin content. From more tannic varieties like Syrah, Gamaret and Garanoir, only few proteins were extracted. Several tests were initiated to verify if the tannins were in part responsible for these variations. Depending on their concentration and their reactivity, tannins are able to precipitate wine proteins and/or make them less available for extraction (Schmauch 2010).

2. Repeatability and recovery rate

To determine the repeatability of the extraction and the recovery rate, proteins were extracted four times from the same wine sample (respectively with 67 µg/L and 134 µg/L of BSA as internal standard) (Figure 1).

The quantitation of the proteins was performed by calculating the area of each band in the gel using the densitometric profile of the lane as shown in Figure 2.

The results are expressed as µg of BSA equivalents (Table 3). The standard deviation for the 4 extractions was between 3 and 24 %. It was particularly low when the band intensity was high (e.g., band 5), but higher and less consistent with low-intensity bands.

To determine the recovery rate of proteins, BSA was added to the gel, extracted and quantified using the densitometric profile. The BSA signal was easily distinguishable from that of wine proteins (Figure 2).

The mean recovery was 43 % (Table 4), which seems acceptable considering the numerous extraction steps.

Protein-tannin interaction is highly dependent on the proline content, the glycosylation degree and the pI of the proteins. The use of an internal standard allows the calculation of the recovery rate of proteins, but it

Table 1 - Protein concentrations in wines made from different grape varieties expressed in mg of BSA equivalents per liter of wine (mg BSA-eq./L) (n=1).

	Gamay (EIC)	Gamaret (EIC)	Cabernet Sauvignon	Syrah	Cornalin	Carminoir	Garanoir
mg BSA-eq./L	22.3	3.8	0.9	1.2	1.1	0.2	1.3

Table 2 - Protein concentrations in wines made from different grape varieties expressed in mg of BSA equivalents per liter of wine (mg BSA-eq./L) (n=2).

	Pinot noir	Cornalin	Humagne	Syrah	Diolinoir
mg BSA-eq./L	1.7 ± 0.2	3.5 ± 2.1	18 ± 0.7	8.2 ± 1.3	1.9 ± 0.3

Table 3 - Protein concentrations (µg/L) in one red wine sample extracted with different concentrations of BSA (lanes 3 and 5, 67 µg/L and lanes 2 and 4, 134 µg/L).

µg BSA-eq./L	Lane 2	Lane 3	Lane 4	Lane 5	Mean	± SE	± (%)
BSA	134	67	134.3	67.3	-	-	-
Band 2	832	468	455	509	***566***	***133***	***24%***
Band 3	795	484	575	579	***608***	***93***	***15%***
Band 4	355	237	242	277	***278***	***38***	***14%***
Band 5	2 150	2 075	2 000	2 150	***2094***	***57***	***3%***
Band 6	472	272	294	335	***343***	***64***	***19%***
Band 7	231	182	179	182	***194***	***19***	***10%***
Band 8	472	339	453	359	***406***	***57***	***14%***
Band 9	346	282	285	354	***317***	***33***	***10%***
Sum	6 148	4 875	4 892	5 378	***5323***	***440***	***8%***

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seems more accurate to express the results as mg of BSA equivalents per liter of wine extracted.

3. Follow-up of the winemaking process

The total amount of proteins decreased during the different stages of the winemaking process. However, protein profiles remained identical (Figure 3). Gamay and Gamaret (EIC) kept the same protein profile from the pressing of the juices through the bottling of the wines, independent of the tested winemaking methods (e.g., pre-fermentation maceration, carbonic maceration, oak barrel).

Very high protein concentrations in wines after pressing and MLF (malo lactic fermentation) resulted in BSA bands with proportionally low intensity. This low intensity of the bands used as internal standards made the precise quantification of the extracted proteins with the available software impossible (data not shown).

4. Grape variety analysis

The proteins contained in wines differed qualitatively and quantitatively according to the grape variety. Figure 4 shows the protein profiles of the 7 different varieties.

Each variety showed a characteristic profile that was different from the others. The presence of BSA in each well at 65 kDa was a proof of an effective extraction. For the Swiss grape cultivar Carminoir (cross of Pinot noir and Cabernet-Sauvignon), it was almost the only band observed and a sign that the extraction went well but that the cultivar's proteins were difficult to extract or too low in concentration. Gamay showed a clearer profile with 3 bands between 15 and 22 kDa (marked 1) and one band at 11 kDa (marked 2). The profile of the neighboring Gamaret was very close but distinguished by a band at more than 250 kDa (marked 4) and the absence of the band just below 25 kDa found for Gamay (marked 3). Garanoir had also a fairly similar pattern but had only 2 bands between 15 and 22 kDa (marked 5). Garanoir and Gamaret are two grape varieties obtained from a Gamay x Reichensteiner cross, which may explain the proximity of their protein profiles.

The profiles of the cultivar Syrah, Cornalin, and Cabernet-Sauvignon were close but very different from those described above. They did not have those intense bands between 15 and 22 kDa. However, each one showed one band at 13 kDa (marked 6). The distinctiveness of the grape variety Syrah was visible by the presence of a band at 70 kDa (marked 7). Cabernet-Sauvignon and Cornalin had bands at 19 and 32 kDa (marked 8 and 9, respectively) in common. Nevertheless, Cornalin may be distinguished thanks to the presence of a band at more than 250 kDa (marked 10). This last band was found in almost all the wines.

The proteins in Gamaret wines (ACW), grown on different soils but strictly made in the same way, were extracted and analyzed in order to assess the influence of production site on protein profiles (Figure 5).

From a quality point of view, all profiles were very close. The intensity of the bands differed slightly but all the bands were present. Profile C showed only the BSA band (technical reasons did not allow a more significant loading).

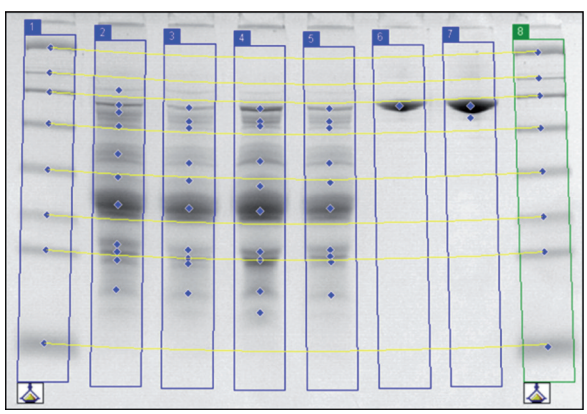


Figure 1 - Protein profile of one red wine sample extracted with 67 µg/L (lanes 3 and 5) and 134 µg/L (lanes 2 and 4) of BSA. Lanes 6 and 7: equivalent to 67 µg/L and 134 µg/L of BSA, respectively, lanes 1 and 8: ladder.

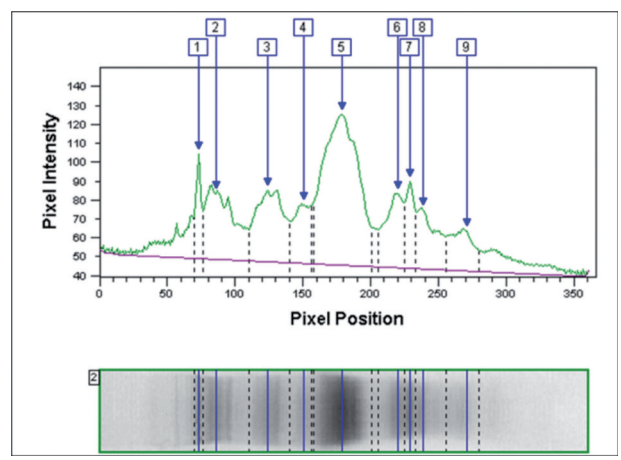


Figure 2 - Densitometric profile of band intensities and measurement of peak area from a wine protein extract (lane 4 of Figure 1). The BSA is the band 1.

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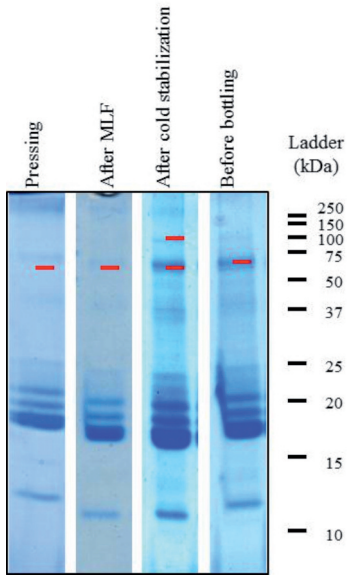


Figure 3 - Protein profile of Gamay wine (EIC research winery) at different winemaking stages. Red bands = BSA; MLF, malo lactic fermentation.

5. Albumin and casein

Albumin (Figure 6) showed 3 distinct bands, formerly identified by Restani *et al.* (2010) as being the ovotransferrin band A3 (60 kDa), the ovalbumin band A1 (40 kDa) and the lysozyme band A2 (12 kDa). Casein (Figure 6) showed 4 bands, namely C1 (36 kDa), C2 (33 kDa), C3 (21 kDa) and C4 (13.5 kDa), with very high intensity, corresponding to the bands obtained by Restani *et al.* (2009) and identified as being the α, β and γ (2 bands) casein. The bands with lower molecular weights were probably due to the partial hydrolysis of casein. The γ casein is already a fragment of the β casein.

An experiment with albumin and casein as fining agents was conducted with Gamay and Gamaret (EIC, no fining agent used previously; Figure 6). After settling for 36 hours at 10°C followed by centrifugation (5 min at 500 g), fining with 10 g/hL of albumin gave two additional bands at 35 kDa (marked A1) and 14 kDa (A2) in the 2 wines. One band at 71 kDa (A3) also appeared for Gamaret but not for Gamay, probably because of the quantity of proteins loaded onto the gel. These 3 bands were the same as those found for albumin only (at 14, 38 and 68 kDa).

In identical conditions, fining with 40 g/hL of casein showed 3 additional bands for Gamaret at 32 kDa (C1), 28 kDa (C2) and 13 kDa (C3). These 3 bands corresponded to the bands obtained with the casein control. C1 and C2 appeared in Gamay but not C3.

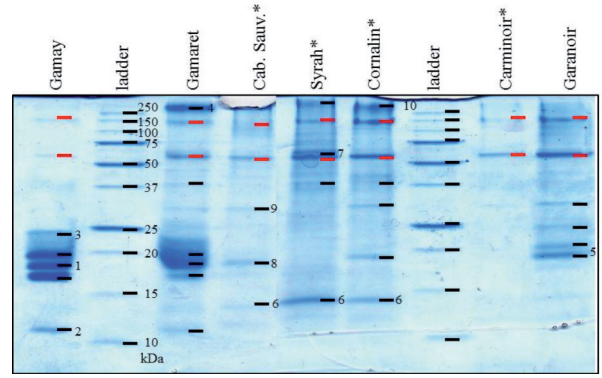


Figure 4 - Protein profiles of 7 mono-cultivar wines. An * indicates < 1 µg extract/well. Red bands = BSA; for bands marked 1 – 10, please refer to the main text for explanation.

The 21 kDa band observed in the casein control sample (C4) was not found in any of the wines.

In addition, it was apparent on the SDS-Page that fining did not decrease the intensity of specific bands of proteins compared to the control.

CONCLUSION

Proteins may be extracted effectively from red wines with a methanol/chloroform emulsion and analyzed on SDS-Page gel. Protein concentrations were very different from one grape variety to another. Tannins may have been in part responsible for these variations because, according to their concentration and reactivity, they precipitate wine proteins or make them less available for extraction.

During winemaking, the total amount of proteins decreased but the protein profile remained the same, from pressing (grape-derived proteins) through bottling, as visualized by SDS-Page.

Each grape variety seemed to have its own protein signature. The protein profile analysis may be used to verify the authenticity of the grape varieties in a wine. This protein analysis could also be an interesting tool to study their direct or indirect organoleptic influences in red wines.

In the present study, extracted proteins were analyzed using SDS-Page, however, the described extraction technique also allows the use of other and more advanced techniques such as ELISA or the ESI-QTof. For instance, the detection of allergens in red wines is problematic because tannins prevent their analysis with conventional immunological tests. The literature provides little information for red wines. Restani *et al.* (2009, 2010) developed an ELISA test that failed to

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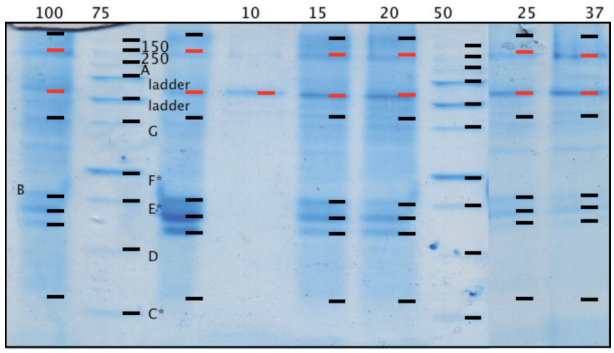


Figure 5 - Protein profiles of Gamaret (ACW) from different soils in Switzerland (A: Ollon, B: Yvorne, C: Blonay, D: Rivaz, E: Echichens, F: St Prex, G: Cote de l'Orbe, H: Bonvillars). An * indicates < 1 µg extract/well. Red bands = BSA.

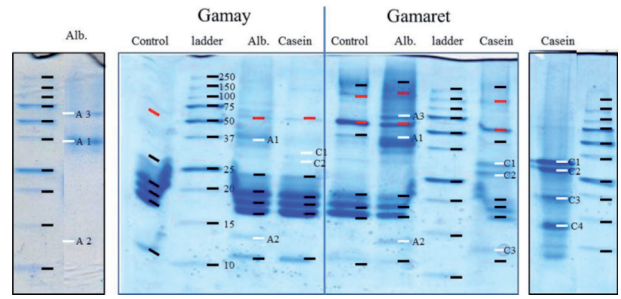


Figure 6 - Identification of protein fining agent residuals in red wines made from Gamay or Gamaret. Fining was done with 10 g/hL albumin or 40 g/hL casein. Red bands = BSA; for white bands, please refer to the main text for explanation. Control lane is protein extract from non treated wine.

Table 4 - Calculation of the recovery rate of BSA.

	Lane 2	Lane 3	Lane 4	Lane 5
Quantity of BSA added (µg)	9.38	4.69	9.38	4.69
Peak area of BSA	14 134	8 168	16 322	7 337
BSA quantified (µg)	4.12	1.90	4.99	1.61
Recovery rate	*44%*	*41%*	*53%*	*34%*

detect added egg whites in pure or diluted (1/2) red wines. However, Weber *et al.* (2009) were able to show corresponding results for white wines.

Proteins extracted from red wines were also usable for proteomic analyses. Indeed, preliminary ESI-QTof tests showed that the protein extracts obtained with this extraction method may be analyzed after a simple tryptic digestion (data not shown). The described analysis method may allow the identification of proteins extracted from red wine and even the finding of endogenous allergens, since it has been shown that some proteins in wines, such as chitinases and lipid transfer protein (LTP), may be responsible for allergic reactions in humans (Pastorello *et al.* 2003).

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