Università degli Studi di Milano

Facoltà di Agraria

Graduate School in Molecular Science and Plant, Food and Environmental Biotechnology

PhD Program in Food Science, Technology and Biotechnology

XXIII cycle

Interaction between proteins of plant origin and wine components: molecular-based choice of protein fining agents for organoleptic improvement

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R07421

2009/2010

“I've dreamt in my life dreams that have stayed with me ever after, and changed my ideas; they've gone through and through me, like wine through water, and altered the colour of my mind”

- Emily Bronte -

ACKNOWLEDGEMENTS – RINGRAZIAMENTI

La stesura di questa tesi di dottorato mi ha portato necessariamente ad analizzare in maniera approfondita quanto fatto durante il tempo avuto a disposizione e, forse maggiormente, a riflettere su quanto avrei potuto fare di più, o semplicemente in modo diverso. Quando però mi guardo indietro e considero le difficoltà, anche personali, che hanno caratterizzato questi tre anni, non sono i risultati o le scelte compiute a emergere in modo più vivo dai miei ricordi, ma le persone che proprio nelle difficoltà mi hanno saputo aiutare, talvolta accompagnandomi per mano e altre volte lasciandomi il tempo necessario per riflettere e maturare. Al termine di questo percorso sento il desiderio di ringraziare tutti coloro che mi hanno sostenuto con la loro competenza ed il loro appoggio morale, riponendo in me una enorme fiducia e incoraggiandomi nell'intraprendere un nuovo progetto di ricerca, oggetto del presente lavoro. Desidero innanzitutto ringraziare il mio Tutor, il Prof. Francesco Bonomi, e la Prof.ssa Stefania Iametti per avermi offerto la possibilità di intraprendere questo percorso, per la loro disponibilità, per i preziosi consigli e il supporto fornitomi in questi anni. Ringrazio il Prof. Luciano Piergiovanni per essere stato una preziosa e paziente guida per tutte le attività da svolgere in questo percorso formativo.

Un ringraziamento particolare va al Prof. Pasquale Ferranti, già mio relatore di tesi di laurea, per avermi dato la possibilità di svolgere parte della ricerca nei laboratori del DSA dell'Università Federico II di Napoli, per avermi accompagnato nelle fasi significative del presente lavoro con estrema pazienza e disponibilità, ma allo stesso tempo per avermi dato la possibilità portare avanti l'attività di ricerca in piena autonomia. Insieme a lui ringrazio tutto il gruppo di lavoro del DSA, le mie amiche-colleghe Antonella, Libera, Chiara, Carmen ed in particolare la mia ex-tesista Sara, per aver partecipato con estremo entusiasmo ad alcune attività di questa ricerca ed aver allietato anche i momenti più bui (comprese le prove di chiarifica durante le feste natalizie e le analisi all'LC-MS fino a notte fonda). Un doveroso ringraziamento va al mio compagno di ricerca Federico per il valido supporto fornito all'attività di ricerca, per i continui suggerimenti, gli spunti e le interessanti chiacchierate. Ringrazio tutto il team di lavoro del DISMA, in particolar modo Mauro e Matteo per la loro cordialità e disponibilità. Ringrazio ancora tutti i miei compagni di Dottorato, in particolare a Daniela, per aver condiviso le tante esperienze e al contempo le ansie che questo percorso ha previsto.

Non posso di certo tralasciare tutte le persone speciali che mi sono state accanto, contribuendo a rendere unici questi anni e condividendo con me successi e fallimenti, in particolare tra tutte la mia "spalla" Dolores. Infine, anche se non per ultimi, desidero ringraziare di cuore la mia famiglia, mia nonna e mio marito per non avermi mai fatto mancare il loro affetto e il loro sostegno in questi tre anni e per avermi sopportato con amorevole pazienza nelle fasi finali della preparazione di questo lavoro, che dedico loro con profondo amore.

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CHAPTER 0. PREFACE

After alcoholic fermentation wine is a colloidal solution and suspension. Clarification treatments are generally performed to remove suspended material like yeast residues, macromolecular compounds with colloidal behaviour, and to reduce the risks of formation of tartar crystals and colored solids in the bottle. Fining is also employed to improve stability and control browning and over-oxidation of white wines during storage and aging. Darkening of white wines is due primarily to chemical reactions involving phenolic compounds, in particular catechins, proanthocyanidins and hydroxycinnamic acids. These processes affect colour and colloidal properties of wine, leading to a decrease of the wine's visual and sensory qualities. Spagna G et al. (2000), therefore recommended the removal of polyphenols with the objective of marketing clear and stable products and of reducing the potential for browning.

Nowadays a wide range of protein fining agents are commonly used including gelatine, casein, egg albumin, and, more recently, some proteins of vegetable origin, able to replace the animal ones. In the last decade, numerous cases of bovine spongiform encephalopathy really caused a situation of crisis, and winemakers have been encouraged to stop using bovine gelatin. In Europe, the fear of transmitting this disease to man led to the interdiction of the use of bovine plasma and blood cells (regulation EC no. 2087/97, Council of October 20, 1997). Some winemakers also hesitate to use egg albumin because of their animal origin.
In response to winemakers' interest in replacing fining agents of animal origin with plant-based products, the Martin Vialatte research company (BP 1031, 51319 Epernay, France) started studying the properties of plant proteins and assessing the possibilities of using them as fining agents for wine (Lefebvre et al., 2000). Initial results have been found to be promising with several powdered products. In 2003, Maury C et al. carried out a study using a protein extracted from white lupine, two wheat gluten-based preparations, and two chemical hydrolysates of gluten. Experiments were carried out using two unfiltered wines and a model solution prepared with phenolic compounds extracted from Syrah wine. Fining efficiency of all the fining agents tested was related to their capability to precipitate relatively condensed tannins. Molecular weight was a major factor in the effectiveness of these proteins. Gelatin generally fined the wine more efficiently, although some plant proteins precipitated galloylated tannins under the same conditions.

Although the influence of protein-based finings on the wine composition and the organoleptic perception have been reported in several scientific and practical studies, fining treatments are yet very empirical. All these studies have focussed attention on the wine phenolic composition but not on structural characteristics of protein fining agents responsible for their ability to interact with polyphenolic compounds (Sarni-Manchado P et al.1999). The various protein fining agents could behave differently,

depending on their composition, their origin and wine characteristics. It has been shown that tannins combine with proteins by intermolecular binding dominated by stacking of polyphenolic rings onto protein hydrophobic surfaces and strengthened by multiple cooperative binding of polyphenolic rings. The formation of protein- tannin aggregates produced the expected flocculation. Nowadays, there is a lack of information on the molecular bases of the interactions, and, above all, on the structural characteristics of oligomeric and polymeric proanthocyanidins (mean degree of polymerisation, galloylation, cis/trans ratio and the percentage of prodelphinidins) remaining in wine after fining as a function of the type of fining protein added. A better knowledge of all the molecules involved in fining could lead to an enhanced control and thus to an optimisation of this treatment.

0.1 References

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CHAPTER 1. STATE OF THE ART

1.1. Clarification and stabilization treatments

Clarity is an essential quality required by consumers, especially for white wines in clear glass bottles. Particles in suspension, either in forming a haze or dispersed through the liquid, not only spoil the presentation but usually also affect the flavor. Turbidity is undeniably a major negative factor in assessing a wine. Turbidity in a liquid results from an optical phenomenon known as the Tyndall effect, caused by the presence of particles in suspension that deflect light from its normal path. The measurement of clarity is, therefore, related to estimations of turbidity, depending on the number and size of particles in suspension. Wine may be clarified by eliminating these particles, but the obtained effect is not necessarily permanent, due to the many naturally occurring in wine that are often accompanied by the formation of turbidity or deposits. The objective of stabilization is to ensure long-term clarity and prevent deposits, whatever the temperature, oxidation or lighting conditions where the wine is stored. Efficient treatments are available for stabilizing wines, when necessary, before bottling. Spontaneous clarification, i.e. through settling, is due to the sedimentation, by gravity, of the particles in suspension and their adsorption on container walls. Natural settling is relatively fast in red and dry white wines, but occurs much less readily in sweet white wines and certain red wines made from grapes affected by rot.

Fining consists of adding a substance that induces flocculation and settling in turbid wines or wines with colloidal instability (colouring matter in red wines). This substance captures the particles responsible for turbidity or instability in the wine, thus clarifying and stabilizing it. Fining products are often a mixture of denatured proteins that precipitate on contact with tannins, cations or acidity. They may also be of mineral origin and flocculate on contact with cations in wine. From an organoleptic standpoint, fining leads to either positive or negative changes. According to the type and quantity of fining agent used, it may make a wine softer and more elegant or, on the contrary, thinner and less attractive.

1.2. Protein fining agents

Traditionally, products used for fining are proteins of animal origin: egg albumin, blood albumin, casein (milk), isinglass (fish) and gelatins (collagen). Several inorganic products (bentonite and siliceous earth) are also used in clarification and stabilization. Every product used in protein fining has a specific action, according to its origin, and therefore its composition. The issues involved with bovine spongiform encephalopathy (BSE) in animals and its possible transmission to humans have led to a restriction in the use of products of animal origin for fining wine. Legislation in several countries, particularly the European Union, has been updated, banning the use of dried blood powder and blood albumin. Egg and milk albumin are now the only

animal albumins permitted. The use of gelatin has also been challenged, even though it is mainly a pork by-product. It is, however, still widely used for its good clarification and stabilization capacities, particularly in red wines. Winemakers would like to have substitute products with similar qualities,
so there are incentives for developing alternative fining agents and plant-based proteins currently represent a possibility to explore.
Previous data suggest that plant proteins may be good candidates; malt proteins are able to interact with tannins in beer (Asano K et al., 1982) and sorghum proteins (prolamins) have been reported to interact with tannins and precipitate them (Hagerman EA et al., 1980; Hagerman AE & Robbins CT, 1987). The Martin Vialatte research company (BP 1031, 51319 Epernay, France) started studying the properties of plant proteins and assessing the possibilities of using them as fining agents for wine (Lefebvre et al., 2000). Initial results have been found to be promising with several powdered products. Marchal and coworkers (2002) showed that gluten proteins used at concentrations between 6 and 18 g/hL allowed very good clarification of the Burgundy treated wine. After wine fining, some glutens used at 12 and 18vg/hL gave turbidities situated between the minimal and maximal values obtained with animal proteins. A study made in 2001 with more than 10 red wines, from different French areas and different varieties, showed that efficiencies were always better with enzymatically hydrolyzed glutens than with deamidated glutens. In 2003, Maury et al. carried out a study using a protein extracted from white lupine, two wheat gluten-based preparations, and two chemical hydrolysates of gluten.
Nowadays, literature data suggest that it is possible to use plant proteins as fining agents in wine, but each preparation behaves in a specific way. It will, therefore, be necessary to test a large number of products to determine which ones give the best results with different types of wine and define the most effective doses, likely to be around 10–20 g/hl.

1.3. Legume and cereal proteins

These seed proteins are termed storage proteins.
Proteins in legume seeds represent from about 20% (dry weight) in pea and beans up to 38–40% in soybean and lupin (Duranti M, 2006). Therefore legume seeds are among the richest food sources of proteins and amino acids for human and animal nutrition. Traditionally, the classification of storage proteins is based on their solubility properties: albumins are soluble in water, globulins are soluble in salt water solutions and prolamins are soluble in ethanol/water solutions. This old classification scheme still has an operative validity, especially in relation to the techno/functional properties of these proteins.
The most abundant class of storage proteins in grain legumes are the globulins. They are generally classified as 7S and 11S globulins according to their sedimentation coefficients (S). The 7S and 11S globulins of pea are named vicilin and legumin,

respectively, so that the corresponding proteins of other seeds are often indicated as vicilin- and legumin-like globulins (Kriz AL, 1999; Kriz and Schwartz, 1986; Kriz and Wallace, 1991; Wallace and Kriz, 1991).

The 7S proteins are oligomeric proteins (usually trimers) of Mr ~150,000 to 190,000 that lack cysteine residues and hence cannot form disulfide bonds.

The 11S legumins are the major storage proteins not only in most legumes but also in many other dicots (for example, brassicas, composits, and cucurbits) and some cereals (oats and rice). The mature proteins consist of six subunit pairs that interact noncovalently. Each of these subunit pairs consists in turn of an acidic subunit of Mr ~40,000 and a basic subunit of Mr ~20,000, linked by a single disulfide bond.

Under dissociating conditions, both the 7S and 11S globulins liberate their constituent subunits. These polypeptide chains are naturally heterogeneous, being the selective pressure on them scarce. Heterogeneity is evident at both size and charge levels, and arises from a combination of different factors, including the multigene origin of each storage globulin and the post-translational modifications of relatively few expression products (Wallace NH and Kriz AL, 1991). The mutual contribution of these factors vary significantly intra- and inter-generically.

All legume storage proteins are relatively low in sulphur-containing amino acids, methionine, cysteine and tryptophan, but the amounts of another essential amino acid, lysine, are much greater than in cereal grains (Lending CR et al., 1989). Therefore, with respect to lysine and sulphur amino acid contents, legume and cereal proteins are nutritionally complementary. The degree of mutual supplementation may also depend, however, on the contents of the second limiting amino acids, i.e., threonine in cereals and tryptophan in legumes.

The prolamins are most prominent in cereal seeds, in which they usually account for approximately half of the total grain nitrogen. Exceptions to this general rule are cuts and rice, in which the major storage proteins are 11s globulin-like and prolamins are present at low levels (-5 to 10Vo of the total grain protein). The prolamins of cereal (barley, wheat, and rye) are highly polymorphic mixtures of components whose Mr values range from -30,000 to 90,000. These prolamins are classified into three groups (Shewry PR and Tatham AS, 1990): -the S-rich, S-poor, and high molecular weight (HMW) prolamins-based on their amino acid sequences.

The S-rich prolamins are the quantitatively major prolamin group species, accounting for 80 to 90% of the total prolamin fractions. They include polymeric (that is, with interchain disulfide bonds) and monomeric (with intrachain disulfide bonds) components and consist of at least two families in each species: the β and γ-hordeins of barley; two types of γ-secalin of rye; and the α-gliadins, γ -gliadins, and low molecular weight (LMW) glutenin subunits of wheat. Their amino acid sequences consist of two separate domains: an N-terminal domain composed of repeated sequences, and a nonrepetitive C-terminal domain. The repetitive domain consists of tandem or interspersed repeats based on one or two short peptide motifs rich in proline and glutamine. For example, the repetitive domain of the γ-gliadin is based on a Pro-Gln-

Gln-Pro-Phe-Pro-Gln heptapeptide. This domain forms a secondary structure containing β-reverse turns and poly-L-proline II helix (Shewry PR and Tatham AS, 1990). In contrast, the nonrepetitive domain appears to have a globular structure rich in a-helix. This domain also contains most or all of the cysteine residues. Eight cysteines are present in the monomeric γ-gliadin, which form four intrachain disulfide bonds (Shewry PR and Tatham AS, 1997). Six of these cysteine residues are also present in the monomeric α-gliadins (based on sequence context); additional "unpaired" cysteine residues present in the polymeric LMW glutenin subunits may be responsible for polymer formation.
The S-poor prolamins include C hordein of barley, the ω-secalins of rye, and the ω-gliadins of wheat. This proteins consist almost entirely of repeats of the octapeptide motif Pro-Gln-Gln-Pro-Phe-Pro-Gln-Gln that are flanked at the N-terminal side by short unique sequences of 12 residues and at the C-terminal side by short unique sequences of either six or four (ω-secalin) residues. The S-poor prolamins generally lack cysteine residues and therefore cannot form oligomers or polymers.
The HMW prolamins are typified by the HMW subunits of wheat glutenin. Extensive repeated sequences are present, flanked by nonrepetitive N- and C-terminal domains. The repeated sequences are based on the motifs Gly-Tyr-Tyr-Pro-Thr-Ser-Pro or LewGln-Gln, Pro-Gly-Gln-Gly-Gln-Gln, and, in some subunits only, Gly-Gln-Gln. Differences in the number of repeated peptides are largely responsible for
variation in HMW subunit size. Although the repeated sequences present in the HMW subunits are not related to those in the S-poor prolamins, they appear to adopt a similar spiral secondary structure.

Due to their three-dimensional structures, these storage proteins could be involved in many technological processes. Protein fining represents only one of them. Over the years, food chemists have been trying to elucidate the mechanism of protein functionality. Hydrophobic, steric, and electrical parameters are the most important variables that affect the structure of proteins. Among these factors, hydrophobicity is known to be significantly related to the functional properties of proteins (Nakai S, 1983). The tendency of nonpolar solutes to adhere to one another in an aqueous environment is called hydrophobicity. Shen (1981) explained the formation of insoluble precipitate of soy protein as a combination of two reactions, i.e., reversible conversion of soluble monomers to aggregates followed by the irreversible conversion further into the insoluble precipitate. The balance between charge frequency and hydrophobicity of protein molecules can create forces for molecular repulsion and association, respectively.
Although many papers have been published to emphasize the importance of hydrophobicity and hydrophobic interactions in protein functionality, the quantitative administration of this parameter is not easy. The quantitation of protein hydrophobicity can be an essential step for accurate prediction of protein functionality.

One approach to quantify protein hydrophobicity is through fluorescent probe methods. The quantum yields of fluorescence and wavelength of maximum fluorescence emission of these compounds depend on the polarity of their environment (Li-Chan E C Y, 1999). Several fluorescent probes such as 1-anilinonaphthalene-8-sulfonic acid (ANS) and cis-parinaric acid (CPA) have been widely used to measure protein hydrophobicity. These probes have low quantum yield of fluorescence in aqueous solution. Upon binding of the probes to accessible hydrophobic regions of proteins, an increase in fluorescence is observed, which is used as a measure of protein surface hydrophobicity. However, due to the possible contribution of both electrostatic and hydrophobic interactions to the binding of these anionic probes, the interpretation based on these probes has not been easy.

1.4. Theory of protein fining: mechanism of flocculation of macromulecolar colloids

In view of the complex behavior of proteins in wine, many theories have been advanced to provide a chemical interpretation of the fining mechanism. The first theoretical approach to fining wine presented fining as a series of charges and discharges of colloidal particles. Protein particles were positively charged at the pH of wine while the particles responsible for turbidity were negatively charged. The result of fining depended on the reciprocal discharge of the particles present. Flocculation and clarification were more efficient if there was a full discharge. Research by Ribéreau-Gayon, starting in 1934 (summarized by Ribèreau-Gayon et al., 1977), showed that fining mechanisms were much complex.

The process can be divided into two stages: (a) flocculation, produced by interactions between tannins and proteins, (b) clarification, by eliminating matter in suspension from the wine. In the first stage, flocculation was held to result from the reaction between proteins in the fining agent (e.g. gelatin) and tannins in wine. This converted proteins, positively charged hydrophilic colloids, into negatively charged hydrophobic colloids (figure 1.1).

Complexes were formed between proteins and tannins, depending on many factors (pH, temperature, tannin and protein concentrations, etc.). These complexes were stable in a clear solution but precipitated in the presence of metal cations that caused discharges. Tannin-protein reactions produced flocculation, by associating particles and forming flakes that grew, clumped together and precipitated. The phenomenon depended on two parameters: electrical neutralization and dehydration.

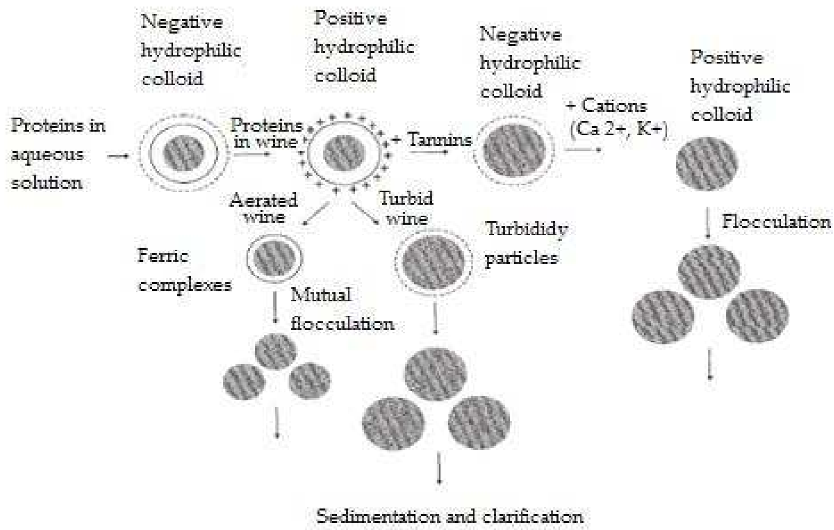


Figure 1.1. Diagram of the flocculation mechanism of proteins in wine during fining (Ribèreau-Gayon et P al., 1977)

Proteins that have not yet reacted with tannins may combine with particles in suspension or in colloidal solution, most of which are negatively charged. This mutual flocculation occurs during clarification in the absence of tannins.

The formation of aggregates of tannin particles, or tannins and proteins, may be inhibited by the presence of polysaccharides (macromolecular colloids). This observation has been confirmed by several authors (figure 1.2).

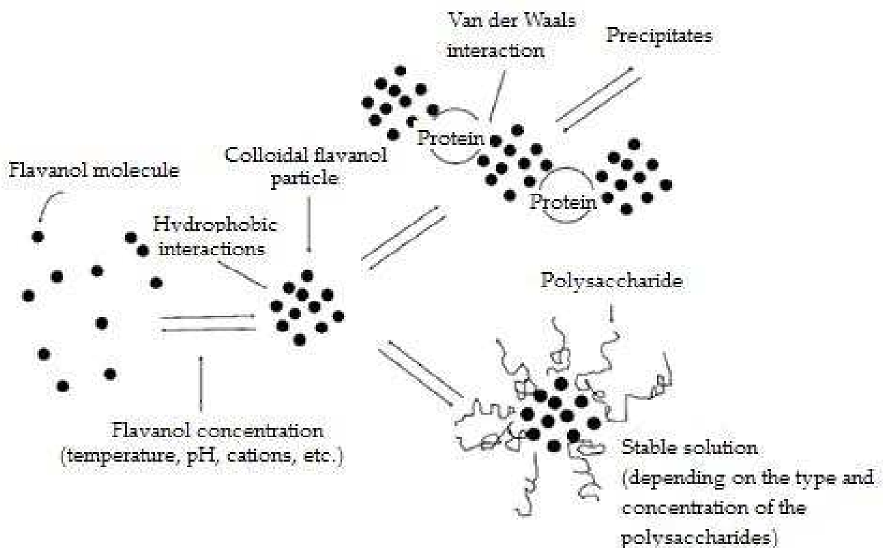


Figure 1.2. Model of the colloidal properties of flavanols (tannins) (Saucier, 1997)

1.5. Molecular bases of protein fining: flavonoid - protein interactions

Many researchers have shown that tannins combine with proteins by hydrogen bonds and hydrophobic interactions, depending on the characteristics of the tannins, those of the proteins and conditions in the medium. Intrinsically, the phenolic nucleus is a structural unit that is favourable to molecular (non-covalent) interactions with proteins. These interactions can be divided into two classes:
– Van der Waals interactions: the non-polar polarizable aromatic ring can develop strong dispersion interactions with amino acid residues displaying similar properties. Hydrophobic effects occur between tannins and the non-polar regions of the proteins (Ozawa T et al., 1987). Many authors (Oh et al., 1980) even consider that this is the predominant interaction mode, due to the hydrophobic nature of the tannins. These reactions seem to be the origin of the complexation reinforced by hydrogen bonds, for example, between the carbonyl group of the secondary amine function of the proline and the phenol OHs (Haslam, 1996). These surface phenomena depend not only on the number of phenol groups on the periphery of the molecule (Haslam E and Lilley T H, 1988) but also on the relative proportions of each of the two families.
– Electrostatic interactions: in the case of phenols, hydrogen bonding is probably the most important interaction falling in this category. Indeed, the OH group of the phenolic nucleus can act as a hydrogen bond donor (via its acidic proton) and a hydrogen bond acceptor toward polar amino acid residues.
The main molecular interactions involved in phenol–protein interactions are represented in Figure 1.3.

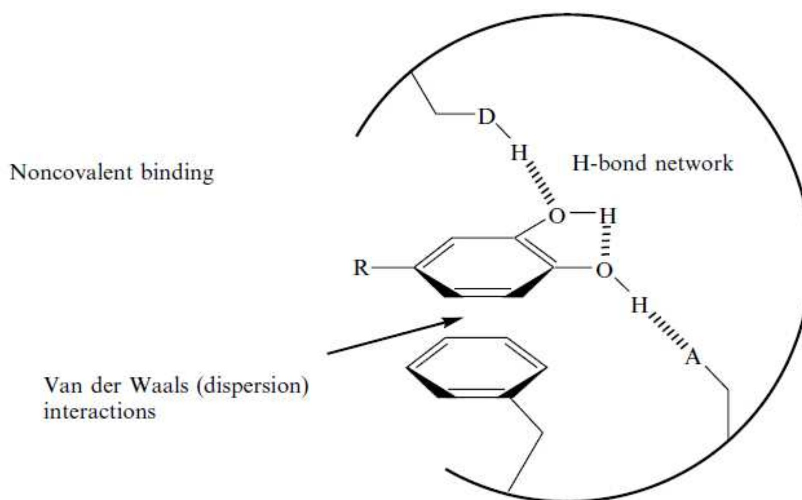


Figure 1.3. A schematic representation of molecular interactions at work in flavonoid–protein binding

Finally, in addition to these noncovalent and reversible interactions, flavonoid–protein redox reactions and oxidative covalent coupling may result from one- or two-electron oxidation of the flavonoid brought about by such mechanisms as: autoxidation (oxidation by dioxygen catalyzed by metal ion traces), scavenging of reactive oxygen species (ROS) (antioxidant activity) eventually produced by the protein itself, and enzymatic oxidation. Being both electrophilic and oxidizing, flavonoid oxidation products (aryloxyl radicals, quinones, and quinonoid compounds) may react with nucleophilic or oxidizable amino acid residues, thereby irreversibly modifying the protein by covalent coupling or oxidation (Figure 1.4).

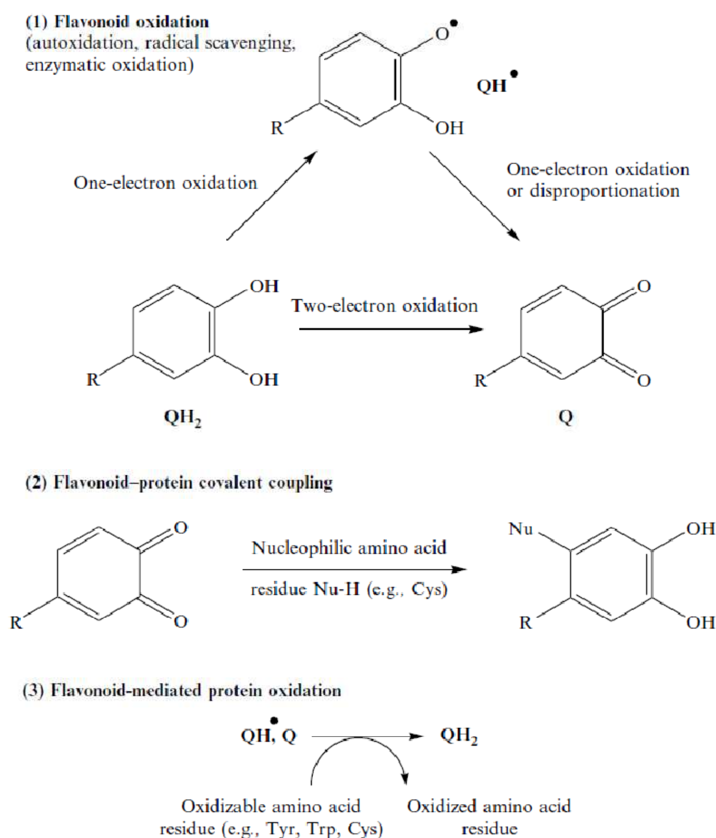


Figure 1.4. Flavonoid oxidation and protein modifications.

The model of interactions between tannins and proteins, described by Haslam in 1980, are illustrated Figure 1.5. In the case of small quantities of proteins, the polyphenols spread over the surface in a single layer, thus decreasing their hydrophilic character. The proteins clump together and, eventually, precipitate. When the protein concentration increases, phenolic compounds spread over their surface act as 'ligands'

or cross-linking agents between the various molecules. The superficial hydrophobic layer then recombines and causes the proteins to precipitate. Therefore, the relative concentrations of tannins and proteins affect the formation and precipitation of tannin-protein complexes, although the non-stoichiometry of the tannin-protein reaction observed by many authors (Ribèreau-Gayon P et al., 1977).

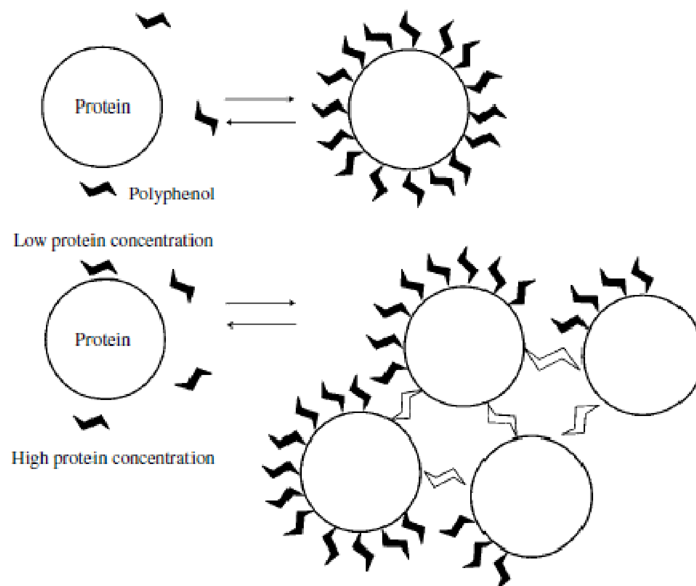


Figure 1.5. Model of protein precipitation by polyphenols (Haslam E, 1980)

Tannin-protein complexation is reversible, provided that covalent bonds are not involved and that both condensation and aggregation are limited. If this is not the case, quinoid intermediaries are formed. These are highly reactive with proteins and the combinations formed are insoluble and irreversible.

1.6. Specificity of flavonoid–protein interactions

Given the intrinsic propensity of the phenolic nucleus for developing molecular interactions, it is no surprise that examples of flavonoid–protein complexation are numerous and concern a wide variety of proteins. However, the question of their specificity deserves examination. In the case of conformationally open proteins (random coils) with multiple binding sites for polyphenols such as proline-rich salivary proteins, binding constants are quite low for small polyphenols (gallates, catechin) but increase sharply when the number of polyphenolic nuclei increases (flavanol-3-O-gallates, oligomeric procyanidins, polygalloylglucose), thus allowing multiple molecular contacts along the protein chain with a preference for the hydrophobic proline residues. (Charlton A J et al., 2002; Baxter N J et al., 1997)

Such trends reflect the approximately additive character of hydrogen bonding and Van der Waals interactions and suggest rather unspecific binding along an extended protein chain or at the surface of globular proteins (Haslam E, 1996; Spencer C M et al., 1988). By contrast, structure–affinity relationships with various globular proteins having well-defined binding cavities (enzymes, receptors) clearly point to specific interactions with properly substituted flavonoids (generally, flavone, isoflavone, or flavonol aglycones) reaching dissociation constants in the range 1 nM to 1 mM.

1.7. Influence of the Medium on Tannin-Protein Interactions

A number of factors, including pH, reaction time, temperature, solvents and ionic strength, have an influence on the formation of tannin-protein complexes.
Furthermore, the type and molecular weight of the proteins seem to play a major role in the formation of insoluble complexes. Hagerman and Butler (1980) showed that proteins with a high proline content had a great affinity for condensed tannins. This property has an impact on the organoleptic qualities of tannins in red wine and plays an important role in fining wine (Lagune L, 1994), thus explaining the significance of the fining agent's protein composition.
When a standard quantity of proteins is added, the quantity of tannins taken up generally increases with the tannin concentration of the wine, with certain exceptions. For example, Lagune L (1994) showed that 5 g/hl of gelatine eliminated 120 mg/l of tannin from a red wine that initially contained 1.72 g/l. Only 40 mg/l was eliminated from another Bordeaux red wine with a much higher tannin content (3.54 g/l).
In general, the larger the quantity of proteins added, the more tannins are eliminated. However, the reaction depends on the type of proteins, and no direct correlation has been observed between the quantity of protein added and the quantity of tannin eliminated. Turbidity (Siebert K J et al., 1996), as well as the type and quantity of tannin-protein precipitates, depend on the relative concentrations of the various components (Calderon P et al., 1968).
At pHs ranging from 2 to 4, tannin-protein flocculation is faster and the particles precipitate better at lower acidity (Ribéreau-Gayon P et al., 1977). When the same dose of fining agent is added, the quantity of tannins eliminated increases according to the wine's pH. In red wine, this amount almost doubles between pH 3.4 and 3.9.
The presence of Na^+, K^+, Ca^{2+}, Mg^{2+} and especially Fe^{3+} cations is involved in flocculation and the precipitation of tannins and proteins (Ribéreau-Gayon P, 1934). Dissolved oxygen promotes flocculation, as it facilitates the formation of trivalent iron. Thus, the aeration resulting from racking improves the effectiveness of fining.
Different types of polysaccharides have highly variable effects. These polymers may have a 'protective' action that prevents flocculation and precipitation, and, therefore, clarification. This is true of glucane and gum arabic, which may even make fining impossible. Polysaccharides may also have an 'activating' effect. The presence of

pectins, arabinogalactans and polygalacturonic acids increases the intensity of turbidity and is favorable to fining, while neutral polysaccharides have no effect.
Calderon P et al. (1968) reported a decrease in the affinity of tannins for gelatin in media with a high alcohol content, and stated that the complexes they formed were soluble. Personally, we did not find any significant differences at alcoholic strengths between 11 and 13% (by volume).
A low temperature (15◦C) enhances precipitation and clarification, due to the decrease in Brownian movement that facilitates flocculation of the colloids. It is generally recommended to carry out fining in winter.

1.8. Effect of fining on the organoleptic properties of wine: phenolic and aroma compound

As described above, fining a red or white wine with proteins means clarify and stabilize it by eliminating unstable colloidal coloring matter. The influence of treatments on the wine composition and the organoleptic perception depends on protein origin, composition and preparation condition and also on the nature and structure of the possible interacting compounds. Phenolic and aroma molecules represent the most important compounds that could be affected by treatment, causing changes in wine quality. In order to understand the phenomena involved in the process, it is important elucidate the chemistry and reactivity of this compounds.

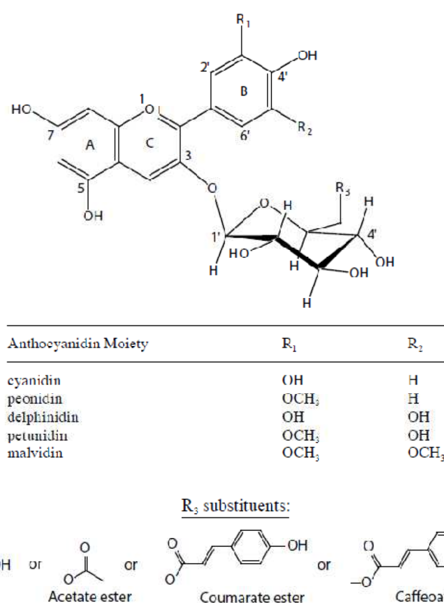
1.9. Phenolic compounds and their role in flavour and quality of wine

Flavonoids are important components of grapes and essential to wine quality. They are responsible for the color and astringency of red wines as well as for the yellow hue of oxidized white wines, and are also involved in the development of haze and precipitates, and other technological problems (e.g., clogging of filtration membranes, adsorption on tank surface). Grape flavonoid composition has been extensively studied: it consists primarily of anthocyanins (in red varieties) and flavanols, along with smaller amounts of flavanols and dihydroflavonols. Wine composition depends not only on the type of grape used as raw material, which is influenced by varietal and agricultural factors, but also on the wine-making process, which determines extraction of flavonoids into the liquid phase and their subsequent reactions. The reactions of anthocyanins and proanthocyanidins play a major role in organoleptic changes taking place during wine aging:

- conversion of grape anthocyanins to other pigments responsible for color changes, from the purple nuance of young wines toward the red-brown tint of aged wine
- the decrease of astringency due to reactions of proanthocyanidins, based on their characteristic C–C bond-breaking and bond-making processes and on oxidation mechanisms

1.9.1. Anthocyanins

Anthocyanins constitute a large family of polyphenols in plants and are responsible for many of the fruit and floral colours observed in nature. Their structure contains the benzopyrilium ion as base molecule, responsible for the colour of grapes and wines, and a β-ring substituted by different groups. On the basis of the number and position of methoxyl and hydroxyl groups on the β-ring, five anthocyanins, delphinidin (Dp), cyanidin (Cy), petunidin (Pt), peonidin (Pn) and malvidin (Mv), are formed. Anthocyanin pigments from grape, especially *V. Vinifera*, have been extensively studied. Anthocyanins were reported to be 3-glucosides, 3-acetylglucosides, 3-coumaroylglucosides, 3-caffeoylglucosides, 3,5-diglucosides, 3-acetyl-5-diglucosides, 3- coumaroyl-5-diglucosides, and 3-caffeoyl-5-diglucosides of cyanidin, delphinidin, peonidin, petunidin, and malvidin (Baldi A et al., 1995; Revilla I et al., 1999).The structures and molecular weights of these six major aglycones are listed in Figure 1.6.



Anthocyanidin Moiety	R_1	R_2
cyanidin	OH	H
peonidin	OCH_3	H
delphinidin	OH	OH
petunidin	OCH_3	OH
malvidin	OCH_3	OCH_3

Figure 1.6. Anthocyanins found in *Vitis Vinifera*.

The color of these pigments depends on conditions in the medium (pH, SO_2), as well as the molecular structure and the environment. On the one hand, substitution of the lateral cycle leads to a bathochrome shift of the maximum absorption wavelength (towards violet). On the other hand, glucose fixation and acylation shift the colour in the opposite direction, i.e. towards orange. These molecules are mainly located in the skin cells, with a concentration gradient from the inside towards the outside of the grape (Ros Barcelo A et al., 1994). All grape varieties have the same basic

anthocyanidin structures, but there are a few small variations in composition. Indeed, among the five anthocyanins, malvidin is the dominant molecule in all grape varieties, varying from 90% (Grenache) to just under 50% (Sangiovese). Malvidin monoglucoside (malvine) may be considered to form the basis of the colour of red grapes and, by extension, red wine. On the other hand, the quantity of acylated monoglucosides is highly variable according to the grape variety. Concentrations vary a great deal according to the age of the wines and the grape varieties.
The majority of these pigments combine and condense with tannins in wine to form another, more stable, class of color molecules, responsible for color in wine. Another relatively small fraction of the anthocyanins, however, disappears, either broken down by external factors (temperature, light, oxygen, etc.) or precipitated in colloidal coloring matter. The elimination of these pigments is particularly detrimental to the quality of the wine, as it leads to loss of color.

1.9.2. Tannins

Flavan-3-ols are encountered in grape as monomers, oligomers, and polymers. Within the grape berry, they are localized mostly in seeds and skins although trace amounts of monomers and dimers have been detected in pulp, especially in the teinturier variety Alicante Bouchet. Major monomers are (þ)-catechin, (_)-epicatechin. Grape seed proanthocyanidins are based on catechin, epicatechin, and epicatechin 3- gallate units and thus partly galloylated procyanidins (Prieur C et al., 1994; Ricardo da Silva J M et al., 1991; Czochanska Z et al., 1979). A number of B-type procyanidins dimers and trimers, including some galloylated derivatives, have been identified in grape in addition to the C4–C8 linked dimers (B1–B4) (Weinges K and Piretti M V, 1971).

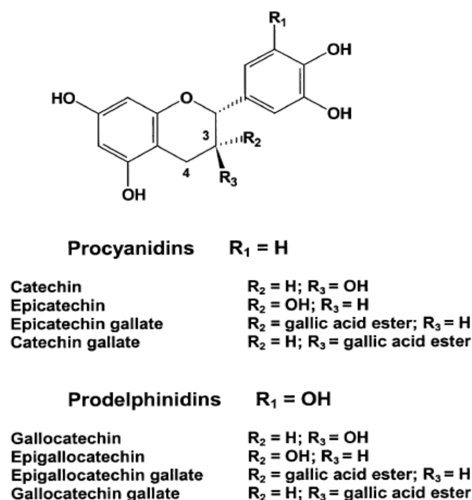


Figure 1.7. Structures of the flavan-3-ol units

Additional dimers based on both prodelphinidin and procyanidin units, presumably arising from grape skins, were also found in wine (figure 1.7). Gallocatechin–gallocatechin, gallocatechin–catechin, and catechin–gallocatechin were tentatively identified in wine on the basis of their mass spectra and relative retention times in HPLC whereas epigallocatechin–catechin, epicatechin–gallocatechin, and epicatechin–epigallocatechin were characterized by mass fragmentation and thiolysis (Remy S, 1999).

The lower molecular weight compounds make up only a relatively small proportion of grape proanthocyanidins, which consist mostly of higher oligomers and polymers, as in most other plant species (Prieur C et al., 1994; Souquet J M et al., 1996; Czochanska Z et al., 1980; Cheynier V et al., 1997). Heterogeneity of proanthocyanidins increases with their chain length, due to the diversity of constitutive units, linkage positions, and possible sequences. This results in poorer resolution in all separation methods and renders isolation and formal identification of individual compounds almost impossible beyond the tetramer.

Application of thiolysis to grape proanthocyanidin polymers showed that those extracted from seeds are partly galloylated procyanidins (Prieur C et al., 1994) whereas those of skins (Souquet J M et al., 1996) and stems (Souquet, J M et al., 2000) consist of both procyanidins and prodelphinidins, confirming earlier results obtained by 13C NMR (Czochanska Z et al., 1979). The major constitutive units of grape skin proanthocyanidins are epicatechin and epigallocatechin. Their 3-gallates are also encountered as extension units whereas catechin and gallocatechin are relatively more abundant in the terminal positions. Much higher average degrees of polymerization were calculated in skins (about 30) than in seeds and stems (around 10). The proportions of galloylated units are also quite different in skins (5%), stems (15%), and seeds (30%).

1.9.3. Reactivity of flavonoid compounds in wine

Changes in flavonoid composition involve both enzymatic and chemical processes. The former is restricted to the early stages of wine-making whereas the latter rapidly becomes prevalent as the enzymes become inactivated, and continues throughout aging. Whether they are biochemical or chemical, these processes rely primarily upon the reactivity of phenolic compounds, which is based on the reactivity of the phenol hydroxyl group itself but can be modulated by the presence of substituents. Additional reactions involve substituents or substitution bonds (e.g., enzymatic or acid-catalyzed hydrolysis of the glycosidic or ester linkages).

The reactivity of polyphenolic compounds is due, on the one hand, to the acidity of their phenolic hydroxyl groups and, on the other hand, to the resonance between the free electron pair on the phenolic oxygen and the benzene ring, which increases electron delocalization and confers the position of substitution adjacent to the hydroxyl group a partial negative charge and thus a nucleophilic character. The A-ring shared

by all grape flavonoids possesses two nucleophilic sites, in C8 and C6 positions, due to activation by the hydroxyl groups of its phloroglucinol (1,3,5-trihydroxy)-type structure. Anthocyanins are usually represented as the red flavylium cations (Figure 1.8, left). However, this form is predominant only in very acidic solvents (pH < 2) such as those used for HPLC analysis. In mildly acidic media, the flavylium cations undergo proton transfer and hydration reactions, respectively, generating the quinonoidal base and the hemiketal (syn carbinol) form (Figure 1.8, right) that can tautomerize to the chalcone (Brouillard R and Delaporte B, 1977; Brouillard R and Dubois, J E, 1977).

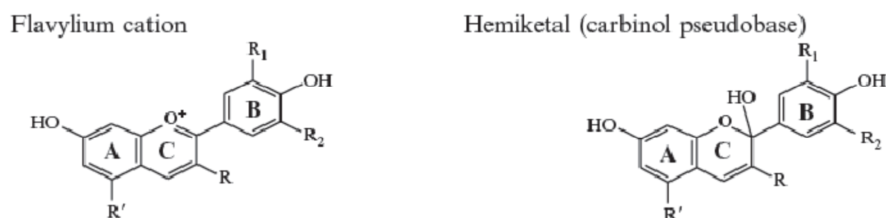


Figure 1.8. Structures of grape anthocyanins

Thus, at wine pH, malvidin 3-glucoside occurs mostly as the colorless hemiketal (75%), the red flavylium cation, yellow chalcone, and blue quinonoidal base being only minor species. The phloroglucinol A-ring of the anthocyanin hemiketal is nucleophilic whereas the C-ring of the flavylium form, bearing a cationic charge in C2 or C4, reacts as an electrophile. Classical examples of nucleophilic addition reactions onto the flavylium cation are those of water and bisulfite that have long been known to result in anthocyanin bleaching. NMR studies demonstrated that addition of water occurs mostly in C2 position, the 4-carbinol being only a minor product (Cheminat A and Brouillard R, 1986) whereas addition of sulfur dioxide yields the two C4-sulfonate adducts (Berke B et al., 1998). Another reaction of the flavylium cation has recently been demonstrated.(Cheynier V et al., 1997; Lu Y and Foo Y, 2001; Fulcrand H et al., 1996; Fulcrand H et al., 1998). It involves the addition of compounds possessing a polarizable double bond on the electron-deficient site C–4 and the oxygen of the 5-hydroxyl group of the anthocyanin. The new pigments thus formed, showing a second pyran ring, have been referred to as vitisins, but the term pyranoanthocyanins proposed by Lu and Foo (Lu Y and Foo Y, 2001) is preferred (figure 1.9). Flavanols also react both as nucleophiles, through their A-ring, and as elecrophiles, through the carbocations formed after acid-catalyzed cleavage of the interflavanoid linkages. The latter reaction, restricted to oligomers and polymers, was shown to occur spontaneously at wine pH values (Haslam E, 1980; Vidal S et al., 2002).

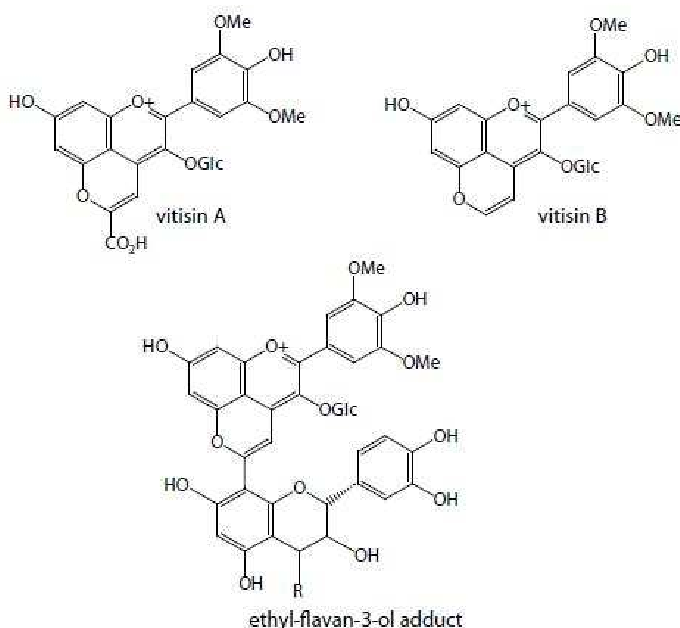


Figure 1.9. Examples of malvidin-3-O-glucoside-based pyranoanthocyanins that have been identified in red wine.

Colour and taste changes taking place during wine aging have long been ascribed to conversion of grape anthocyanins to polymeric pigments through addition reactions with flavanols. Three mechanisms have been postulated.

The first one involves nucleophilic addition of flavanols (in C8 or C6) onto the C4 position of the anthocyanin flavylium ion yielding 4-flavanyl-anthocyanins, which are also referred to as anthocyanin–flavanol (A–F) or anthocyanin–tannin (A–T) adducts (Figure 1.10).

The second mechanism is based on nucleophilic addition onto the carbonium ion formed in acid solutions from flavan 3,4-diols or by cleavage of procyanidins (Thompson R S et al., 1972; Geissman T and Dittamr H, 1965). Yellow products formed after acid treatment of this adduct were postulated to be xanthylium salts, resulting from oxidation of an intermediate xanthene (Jurd L and Somers T C, 1970). According to these authors, a similar mechanism may explain the formation of so-called phlobaphene pigments from proanthocyanidins. Acid-catalyzed degradation of proanthocyanidins was also shown to take place at wine pH values (Haslam E, 1980; Vidal S et al., 2002). In the presence of large amounts of flavanol monomers, proanthocyanidin losses were much reduced and oligomeric species gradually replaced higher molecular weight polymers as the monomers added to the intermediate carbocation released by acid-catalyzed cleavage. Finally, addition of

anthocyanins, either in the flavene form or in the hemiketal form, onto the carbonium ion, leading to F–A adducts was described (Ribéreau-Gayon P, 1982). The resulting flavene (F–A) or hemiketal (F–AOH) adducts both generate the corresponding flavylium, through oxidation or dehydration reactions, respectively.

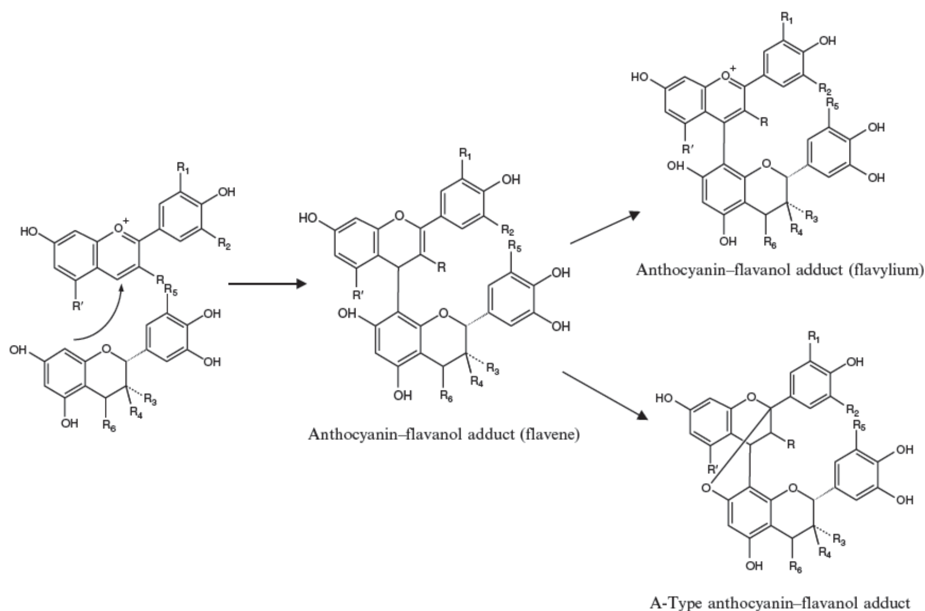


Figure 1.10. Postulated mechanism for formation of anthocyanin–flavanol adducts (flavanyl–anthocyanins).

Reactions of anthocyanins and flavanols take place much faster in the presence of acetaldehyde (Timberlake C F and Bridle P, 1976; Baranowski J D and Nagel C W, 1983; Bakker J et al., 1993) that is present in wine as a result of yeast metabolism and can also be produced through ethanol oxidation, especially in the presence of phenolic compounds. The third mechanism proposed involves nucleophilic addition of the flavanol onto protonated acetaldehyde, followed by protonation and dehydration of the resulting adduct and nucleophilic addition of a second flavonoid onto the carbocation thus formed (Timberlake C F and Bridle P, 1976). The resulting products are anthocyanin–flavanols adducts in which the flavonoid units are linked in C6 or C8 position through a methylmethine bond, often incorrectly called ethyl-link in the literature.

Earlier investigations relied upon model solution studies starting with grape components or related molecules but none of these structures or reactions had been formally demonstrated in wine until recently. The development of more sensitive and selective analytical techniques, such as HPLC coupled to diode array detector and MS, has enabled the characterization of various wine components and to postulate the reaction mechanisms generating them. These mechanisms can then be investigated in model solution studies and characteristics of the resulting products compared with

those of wine constituents. Conversely, products obtained in wine-like solutions serve to develop specific analytical tools as well as chromatographic and spectral data that are used for determination of new products in wine.

1.9.4. Impact of flavonoid reactions on wine colour

The color of an anthocyanin solution is determined by the proportions of the different anthocyanin forms, namely red flavylium cation, violet quinonoidal bases, colorless water or sulfite adducts, and, finally, yellow chalcones. At wine pH, the C2-water adduct (hemiketal or carbinol and its open-chain cis-retrochalcone isomer) is actually the predominant form of malvidin 3-glucoside and other grape anthocyanin monoglucosides (Brouillard R and Delaporte B, 1977). These species do not contribute red color. Thus, the intense red wine color and its preservation over years require some pigment stabilizing mechanisms to take place. Such stabilization is achieved, on the one hand, through complexation of the anthocyanin chromophores with other species and, on the other hand, through conversion of labile anthocyanins to more stable derived pigments. The former mechanism may be the first step leading to the latter (Brouillard R and Dangles O, 1994).

Molecules involved in association with anthocyanins can be an identical anthocyanin molecule, an aromatic acyl substituent in the anthocyanin itself, or another molecule, the processes referred to as self-association, intramolecular copigmentation, and intermolecular copigmentation, respectively. Their mechanisms have been thoroughly investigated and are described in detail in excellent reviews (Goto T and Kondo T, 1991; Brouillard R and Dangles O, 1993). The major driving force is hydrophobic vertical stacking to form π–π complexes from which water is excluded. Both the flavylium cations and quinonoidal bases but not the hemiketal form are planar hydrophobic structures that can stack to protect themselves from the water environment. The enhanced color intensity resulting from self-association or copigmentation is due to a shift of the hydration balance toward the pigment forms involved in these stable complexes. It can thus be expected to be particularly important in the wine pH range where hydrated forms normally predominate. The bathochromic effect often associated with copigmentation is attributed to the larger amount of quinonoidal base formed by deprotonation of the flavylium.

The conversion of anthocyanins to the various pigments mentioned above increases the range of available colors. Moreover, substitutions of the C-ring as encountered in some of the derivatives impede nucleophilic addition of sulphite or water thus increasing color stability (Brouillard R et al., 1982; Mazza G and Brouillard R, 1987).

Pyranoanthocyanins are orange pigments but further substitution with vinylbenzyl derivatives yield blue colors (Roehri-Stoeckel C et al., 2001) These pigments are remarkably resistant to sulfite bleaching and hydration compared to anthocyanins. Pyranoanthocyanins are also more stable over time than anthocyanins themselves, so

that their contribution to wine color is expected to increase during aging (Sarni-Manchado P et al., 1996).
The UV–visible spectra of anthocyanin oligomers and anthocyanin–flavanol adducts resulting from condensation with aldehydes are bathochromically shifted compared to those of their precursors (10 nm for linear substituents, 20 nm for branched substituents).
The methylmethine-catechin derivative is much more resistant to discoloration through hydration and sulfite bleaching than genuine grape anthocyanins (Pissara, J et al., 2003). Since the C-ring of the anthocyanin moiety in the dimer is not substituted, its greater protection against nucleophilic attack of water (and sulfites) may be due to stabilization through sandwich-type stacking as demonstrated for similar products obtained from a synthetic anthocyanin (Escribano-Bailon M et al., 1996).

The influence of controlled oxygenation on color characteristics of red wine was studied and correlated with changes in flavonoid composition (Atanasova V et al., 2002). Pigments formed during aging were less red and more yellow and showed higher resistance to sulphite bleaching than their anthocyanin precursors whereas those resulting from oxygenation were more purple. Higher levels of pyranoanthocyanins and methylmethine-linked pigments were associated with aging and oxidation, respectively, suggesting that both types of derivatives play a part in the observed color changes.
Browning of white wines was shown to be correlated to their flavanol content (Cheynier V et al., 1989). Flavanol auto-oxidation and glyoxylic acid-mediated condensation resulting from oxidation of tartaric acid may contribute to the browning process. The latter mechanism yields much more intense xanthylium yellow pigments and may also be involved in pinking of white wine, since some of the products resulting from glyxoxylic acid-mediated reactions are purple pigments (Es-Safi N E et al., 2000).

1.9.5. Impact of flavonoid reactions on wine taste properties

The major organoleptic character associated with flavonoids is astringency although the lower molecular weight flavanols have also been reported to contribute bitterness (Noble A, 1990; Gacon K et al., 1996).
The physiological grounds of astringency that is described as drying, roughing, or puckering of the mouth mucosa are still obscure. However, it is generally accepted that it is not a taste perceived through recognition by taste receptors, but a tactile sensation (Breslin P A et al., 1993; Green B G, 1993)
Astringency of tannins results from their interactions with salivary proteins and glycoproteins, in particular proline rich proteins, causing a loss in the lubricating power of the saliva, or with the glycoproteins of the mouth epithelium. Briefly, the affinity of polyphenols for proteins depends primarily on the number of phenolic

moieties, which are the major interactions sites in the molecule, the presence of several phenolic rings in a tannin molecule enabling it to build bridges between the proteins or with other polyphenols. All flavonoids can precipitate proteins if present in sufficient amounts but precipitation increases with the degree of polymerization and the number of galloyl units in the polyphenol structure(McManus J P et al., 1985; Baxter N J et al., 1997; Okuda T et al., 1985). Nevertheless, precipitation does not necessarily reflect astringency that might also be related to conformational changes in the protein structure induced by formation of soluble complexes with tannins. Spectroscopic methods such as NMR, MS, and light scattering have been used to study auto-association of flavonoids and their complexation with peptides in solution (Sarni-Manchado P and Cheynier V, 2002; Riou V et al., 2002). Mechanisms involving hydrophobic interactions and hydrogen bonding were thus proposed. In addition, colloidal particles derived from flavanol aggregation might play an important role in tannin associations with macromolecules.

Within a series of flavanol monomers and dimers, self-association and formation of soluble complexes with peptides, detected by MS, increased with the chain length and with the presence of galloyl substituents. Aggregation of lower molecular weight flavanols increased with their molecular weight but particle size decreased with larger polymers (Riou V et al., 2002).

Proanthocyanidin astringency has been reported to increase with chain length, up to the decamer level, and to decrease beyond this value, as the polymers become insoluble. However, higher molecular weight proanthocyanidins (mDP > 20) were shown to be present in a red wine and selectively precipitated by proteins used as fining agents, meaning that they were soluble and presumably astringent (Sarni-Manchado P et al., 1999; Maury C et al., 2001).

Assessment of taste is achieved by sensory analysis, from very simple experiments such as triangular tests aiming at determining detection thresholds to complex descriptive analysis approaches. A method referred to as time–intensity that consists in recording continuously the intensity of a given sensation over time under standardized conditions has been applied to study flavonoid bitterness and astringency properties (Fischer U and Noble A C, 1994; Guinard J X et al., 1986).

Recent studies performed using this method have shown that flavanol bitterness decreases from monomer to trimer. Epicatechin was perceived more bitter than catechin and the C4– C6-linked catechin dimer more bitter than other procyanidin dimers with C4–C6 linkages. This may be due to the higher lipophilic character of these molecules facilitating their diffusion to the gustatory receptor.

The decrease of astringency occurring during wine aging is usually ascribed to the conversion of proanthocyanidins to less astringent and eventually insoluble derivatives through polymerization reactions.

Anthocyanins contributed neither bitterness nor astringency. Whether incorporation of anthocyanin moieties in tannin-derived structures affects their interactions with proteins and taste properties remains to be investigated. Taste perception of flavanols

is also greatly affected by other constituents of the medium. In particular, lowering of pH leads to a significant increase in astringency whereas increasing the level of ethanol enhances bitterness (Noble A, 1998; Noble A C, 2002). The gustatory perception of tannins may also be altered by the presence of polysaccharides and proteins. Polysaccharides isolated from wine inhibited aggregation of flavanols. Similarly, analyses of the wines before and after protein fining suggested that the reduction of astringency induced by fining was due to the presence of soluble tannin–protein complexes, along with removal of highly polymerized and highly galloylated tannins (Maury C et al., 2003).

1.10. Aroma compounds

Formation of wine aroma during winemaking is an extremely complex process, in which a large array of chemical and enzymatic reactions contributes to the final volatile composition of wine, in conjunction with the odour-active compounds directly deriving from the grape. In the case of the so-called non-aromatic grape varieties, free forms of aroma compounds occur in the grapes at concentration lower than their odour thresholds, and their contribution to the aroma characteristics of non-aromatic wines is therefore negligible.

The typical aroma characters of these wines result mainly from the transformation of odourless precursors into odour-active compounds during winemaking. Glycosidically bound volatile compounds have been identified in many grape varieties as a group of aroma precursors possibly responsible for some of the varietal attributes of wines (Ugliano M and Moio L, 2008). Several powerful groups of odorants, such as monoterpenes, norisoprenoids, benzenoids, and lactones can be present in grapes as glycosides [1–3]. They can be released during winemaking through the action of endogenous or exogenous glycosidase enzymes or due to the mild acid conditions of grape juice and wine (Berger R G, 1995; Chrisholm M G, 1994). While in some cases the formation of a single powerful odorant through these hydrolytic processes determines the appearance of specific sensory varietal attributes (i.e. 1,1,6-trimethyl-1,2-dihydronaphthalene for aged Riesling), in most cases the balance between the various odorants or groups of odorants deriving from glycoside hydrolysis determines specific aroma nuances that contribute to the varietal character of wines.

Flavour is considered as one of the most important attributes determining the acceptance of wine by the consumer. It has been shown (Armada L and Falqué E, 2007) that wine clarification and stabilization processes exert a negative influence upon sensory properties when the amount of eliminated macromolecules reaches 30%. When the macromolecule content of the wine is reduced by filtration, losses of aroma intensity and flavour persistence are observed. Protein agents have little flavour of their own, but they are known to bind and trap aroma compounds. Depending on the nature and the strength of the binding, the release of aroma compounds in the gas phase will be more or less decreased and this will have a significant impact on the

overall aroma perception, due to changes in the aromatic balance. In the following paragraphs, the flavour binding by proteins as well as the effect of differences in type of protein on this binding will be discussed. Next, the effect of the medium on protein–flavour interactions will be described and finally attention will be given to the impact of these interactions on flavour perception.

1.10.1. Flavour biding by proteins

The protein interacts with many flavour compounds, such as aldehydes and ketones, ionones, and esters. Results reported by different authors for the same flavour compounds are often difficult to compare, because different protein batches may have been used and differences in experimental conditions may also induce changes in binding capacities of the protein. However, binding constants determined by static headspace analysis and equilibrium dialysis are always comparable. Data obtained by liquid- chromatography, dynamic coupled liquid chromatography or affinity chromatography take into account reversible binding and seem to be more receptive to investigate ligand exchanges in ingested food products (Guichard E and Langourieux S, 2000).
In most cases the interactions between protein and flavour compounds are reversible, involving hydrophobic and hydrogen bonding (Lubbers S et al., 1998). However, aliphatic aldehydes are also connected by covalent irreversible bonds to proteins. In the case of well-structured proteins such as β-lactoglobulin, the role of specific interaction involving polar groups seems to be significant. It has been demonstrated that within one chemical class, affinity for β-lactoglobulin increases with hydrophobic chain length or overall hydrophobicity, except for terpenes.
However, it was not possible to find a simple explanation of the strength of binding for aroma compounds from different chemical classes. Indeed, three-dimensional Quantitative Structure–Activity Relationships (3- D QSAR) molecular modelling studies were done, based on modelling the aroma–protein interaction from the point of view of protein, but using information only derived from the aroma compound. They allowed to show the existence of two groups of ligands, confirming the presence of at least two binding sites on the b-lactoglobulin and put forward the role of hydrogen bonding (Tromelin A and Guichard E, 2004). This original approach appears thus to be appropriate to identify binding sub-sites. The advantage is that no information is needed on the structure of the protein, which means that this approach can be applied to any protein, if there exists a sufficient amount of data on aroma–protein binding constants.
Among the other fining proteins, α-lactalbumin, caseins, bovine serum albumin and soy proteins are studied to a lower extent for their binding properties towards flavour compounds. α-Lactalbumin was found to bind ketones and aldehydes but with a poor flavour binding capacity compared to other whey proteins. Casein is the major milk protein. All caseins exhibit a similar amphiphilic character, the polypeptide chain

folded in such a way that the nonpolar part is buried in the interior of micelle-like structure. The association of bovine casein with small hydrophobic molecules involves an inclusion mechanism within the hydrophobic interior of this micelle-like structure (Farrell et al., 2002).
Soy protein and bovine serum albumin (BSA) exhibited the same binding properties. In contrast to the binding properties of β-lactoglobulin, they are rather independent of pH and temperature. The interactions are characterized by weak and unspecific binding forces, but a practically unlimited binding capacity.
BSA binds carbonyl compounds with a high affinity; the binding induces conformational changes of the protein (Damodaran S and Kinsella JE, 1980). The chemical reduction of disulfide bridges reduces the affinity for carbonyl compounds. Fatty acids bind to BSA, probably by hydrophobic interactions, the carbonyl group playing only a minor role in this interaction. Proteins of leguminous plants comprise two major components, vicilin and legumin. The role of the soybean protein structure in binding and release of aroma was investigated in the case of hexyl acetate and revealed that the native legumin molecules possess the higher binding affinity for this compound compared to vicilin molecules.

1.10.2. Effect of medium on protein–flavour interactions

The addition of proteins to flavour compounds results in effects that also depend on the medium and the matrix. Protein concentration, pH, chemical classes and chain length of aroma compounds have a non-negligible effect on the protein–flavour interactions. The effect of pH on the retention of aroma compounds by β-lactoglobulin depends on the aroma compound (Jouenne E and Crouzet J, 2000). For limonene and myrcene, a “salting out effect” was noticed for acid pH, whereas methylketones and esters were bound by the protein, with a higher retention at pH 9, due to a modification of the structure of the protein.
The temperature seems to have an important effect on binding properties, but only when the structure of protein is modified. This point is particularly true for heat denaturation. The heat treatment induces the unfolding of the protein, which modifies the nature of the binding, resulting in a decrease in the association constant and an increase in the number of binding sites (Damodaran S and Kinsella JE, 1981).
The addition of salts modifies the ionic force of the medium, and may modify the interactions between proteins and flavour compounds. For example, the retention of 2-octanone by β-lactoglobulin increases with increasing sodium chloride concentration. The presence of chemical agents such as urea can also influence the interactions. Binding of 2-nonanone to soy proteins decreases when urea concentration decreases. Urea denaturates the protein, thus inducing an increase of the number of binding sites, but with a lower global affinity for 2-nonanone (Damodaran S and Kinsella JE, 1981).

1.10.3. Impact of interactions on flavour perception

The flavour intensity of benzaldehyde, limonene and citral as experienced by panelists were determined in the presence of casein and whey protein by quantitative descriptive analysis deviation from reference (Hansen AP and Heinis JJ, 1992). In the presence of whey protein, the benzaldehyde flavour intensity declined, on the other hand, casein had no effect on benzaldehyde flavour intensity. For limonene, the flavour intensity decreased when the protein concentration (whey protein or casein) increased. For citral, panellists detected no effect on flavour intensity in presence of whey protein or casein. The decrease of benzaldehyde and limonene flavour intensity in the presence of whey protein or casein may be due to non-polar interaction for casein and interaction with non-polar binding sites, cysteine–aldehyde condensation, or Schiff base formation with whey protein. Addition of β-lactoglobulin to water induced a significant decrease in odor intensities of methyl ketones and eugenol, whereas no significant effect was found for vanillin, the compound with the lowest affinity for the protein. Also, it appeared that only an increase in aroma retention of more than 20% was noticeable as a significant decrease in odor perception (Guichard E, 2000).

All these studies were done using in vitro physicochemical experiments under equilibrium conditions. More recently, the development of a new technique, (Atmospheric Pressure Chemical Ionisation-Mass Spectrometry (APCI-MS), allowed the study of the protein– flavour interactions in vivo, by measuring the amount of flavour released in the nasal cavity (Le Guen S and Vreeker R, 2003). The in vivo APCI-MS signal of methyl ketones was not influenced by the presence of milk protein, whereas the in vitro headspace analysis showed a great retention of methylketones. The hypothesis of the authors for the in vivo aroma release process is that not only all free compounds in the film will be released into the exhaled air but also all of those reversibly bound to the whey protein. In the case of aldehydes, which are irreversibly bound to protein, a decrease in their concentration in the nasal cavity was observed.

These results clearly show the influence of the nature and strength of the protein–flavour interactions on flavour perception.

1.11. Allergenic potential of wines fined with various proteinogenic fining agents

Hidden allergens are a common problem in food safety that has been known for many years (Miller J B, 1978). The main issue is that the average consumer does not expect these allergens in the food and, thus, they present a potential high risk for allergic individuals. The main reasons for hidden allergens in foods are contaminations from previously or simultaneously produced products ("cross-contact" or "carry-over"), the use of allergenic materials as processing aids, or simply the lack of or misleading labelling declaration of food products (Miller J B, 1978; Deibel K et al., 1997).

For that reason, the European Parliament adopted Directive 2003/89/EC amending 2000/13/EC. In addition to specific foods, Directive 2003/89/EC also requests the declaration of specific substances that were used in the production and could present a risk for allergic individuals. Not only ingredients or contaminations but also processing aids are affected. Annex IIIa specifies a list of substances that are known to trigger allergic reactions for which no labelling exemptions are allowed. This list also includes products derived from egg, milk, or fish. Because no scientific data exist, a temporary exemption of labelling has been granted by the European Community to provide research data when it is investigated whether these processing aids can cause adverse reactions in allergic individuals (EFSA, 2005).

Directive 2003/89/EC affects wine manufacturers not only within the European Community but also in several other countries, for example, Australia, New Zealand, or the United States, where similar regulations have been introduced or are already taken into consideration. Because egg, milk, and fish products, such as isinglass and, more recently, plant proteins are used as fining agents to clarify wines or as stabilizers, they need to be listed on the wine label.

So far, no cases of allergic reactions after wine consumption have been reported due to the content of fining agent residues. This could be due to the absence of allergenic amounts of fining agents in wines or because allergic reactions usually are not associated with the consumption of wine as the average consumer does not expect allergens such as egg, milk, or fish proteins in wines (EFSA, 2005). Indeed, cases of allergy against wines have been reported particularly in the Mediterranean area, but it has also been proven that those adverse reactions were triggered by wine proteins or intolerances to compounds such as sulfites or histamine (Schad S G et al., 2005; Pastorello E A et al., 2003; Borghesan F et al., 2004; Kalogeromitros D C et al., 2006). Therefore, it could be assumed that fining agents are almost completely removed during the manufacturing process, for example, by filtration and adsorption to processing aids, such as bentonite, or by precipitation with tannins in wine. This has been confirmed for isinglass in beer clarification (Leiper K A et al., 2002), but, until now, there has been no evidencefor wine.

Furthermore, some countries use fining agents after filtration or adsorption steps. Thus, the analysis of fining agent residues in fined wines is important to evaluate the possible risk of fined wines for allergic individuals and to evaluate the need of labeling according to Directive 2003/89/EC. First efforts have been published by Rolland et al. on Australian wines with double-blind, placebo-controlled food challenges (DBPCFC) and basophile activation analysis (Rolland J M et al., 2006). There was no anaphylaxis or symptom or sign of an adverse reaction that could be attributable to the consumption of wine made using the food allergens fish or egg. However, this study considered a panel of 5 egg-allergic, 1 milk-allergic, and 10 fish-allergic patients, indicating problems of the statistical reliability especially for egg- and milk-allergic persons. Furthermore, regional differences in wine treatment may affect the transferability of those results and make the investigation of European wines

necessary. The determination of hidden allergens is an ambitious intention. In addition to the high specificity of those methods, sufficient sensitivity is essential to detect trace amounts in foods that could trigger adverse reactions. The enzyme-linked immunosorbent assay (ELISA) is a well-known and the most promising tool for this type of analysis (Besler M et al., 2002). Although ELISAs are commercially available to detect major egg and milk allergens, no suitable ELISA kit was found to determine lysozyme or fish proteins, especially gelatin or collagen. The detection limits of hidden allergens are described in some studies. On the basis of the comprehensive DBPCFC of Morisset et al. (Morisset M et al., 2003) with 125 egg-allergic and 59 milk-allergic humans, detection limits should be 2 ppm for egg proteins and 12 ppm for milk proteins to evaluate the risk for sensitive individuals based on the lowest observed adverse-effect level (LOAEL).

However, other publications describe the sensitivity to be ≤ 10 ppm (Holzhauser T et al., 2003). Although fish is a widely known and well-reported cause of food allergy (Hamada Y et al., 2001), oral challenge studies especially for fish gelatin or collagens which is the main protein of isinglass, are rare, and threshold doses have not been established yet. Minimal doses for codfish have been identified by Hansen and Bindslev- Jensen at 6 mg, indicating that the minimal eliciting dose for fish appears to be in the milligram range for the most sensitive patients (Hansen T K and Bindslev-Jensen C, 1992) and, therefore, making a sensitivity of 10 ppm inevitable. But, generally, no adverse reactions especially to gelatin have been reported (Taylor S L et al., 2004). Indeed, antibodies against fish gelatin were detected in fish-allergic patients (André F et al., 2003; Sakaguchi M and Inouye S, 2000; Hamada Y et al., 2003), but an oral DBPCFC with 30 codfish-allergic patients showed no allergic reactions to doses up to 3.6 g of fish gelatin (Hansen T K et al., 2004).

A study still in progress is examining the treatment of white wine with pea and lupine extracts. The results indicate that, from a health standpoint, there are no objections to using plant proteins for fining wine. However, because of the rare information about allergic reactions to plant proteins it is necessary to determine the fining agent residues in wine after treatment in order to evaluate the possible risk for allergic patients.

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CHAPTER 2. AIMS OF THE STUDY

Proteins have been used as wine fining agents for a long time. They not only allow to clarify colloidal suspensions, but the precipitation of complexes between tannins and proteins in the process known as fining softens the gustatory appraisal and can reduce the astringency of otherwise rough wines. Fining also improves wine stability with respect either to the colour of red wine or to browning and over-oxidation in white wine (Cosme F et al., 2008; Spagna G et al., 2000; Tschiersch C et al., 2010), as well as to bitterness and roughness in both red and white wines during ageing.

A broad range of animal proteins have been used as fining agents, but the bovine spongiform encephalopathy pandemic in the recent past led to prohibiting the use of bovine plasma and blood cells (regulation EC no 2087/97). Winemakers have been encouraged to stop using bovine gelatine as well, whereas there are reservations on using egg albumin because of its animal origin (Marchal R et al., 2000). In this scenario, use of plant-derived proteins as wine fining agents has become of much interest.

The influence of treatments with proteins, both of animal or vegetable origin, is related to proteins-polyphenolic compounds associations, in which hydrogen bonds and hydrophobic interactions are responsible for the expected flocculation and clarifying (Versari A et al., 1999; Sarni-Machado P et al., 1999; Yokotsuka K and Singleton V L, 1995). Improved knowledge of the functional properties of proteins used as fining agents and of the structure of polyphenolic compounds interacting with various classes of clarifying agents is expected to take the whole protein-based fining process beyond the empiricism that has characterized it so far.

The three-dimensional protein structure is dependent on a broad range of factors, which must be taken into account in a synergistic way to explain the functional properties of proteins relevant to the food business (Li-Chan E, 1991). Among these factors, surface hydrophobicity is known to be significantly related to the functional properties of food and non-food proteins (Nakai S and Li-Chan E, 1988). Fluorescent probes are often used to measure the number and relative affinity of hydrophobic groups on the protein surface that are able to bind the probe. One of the most valuable and widely used non-covalent hydrophobicity probes is 1,8-anilinonaphtalenesulfonate (ANS). ANS has been used in studies concerning process-induced modification of isolated food proteins (Nakai S and Li-Chan E, 1988; Bonomi F and Iametti S, 1991; Iametti S. and Bonomi F, 1993; Cairoli S et al., 1994) and of complex food systems undergoing processes of various nature (Iametti S et al., 1998; Bonomi F et al., 1988; Pagliarini A et al., 1990; Iametti S et al., 1991).

In this PhD thesis project was studied the molecular basis of non-covalent interactions between proteins of plant origin and polyphenolic compounds, known for

their role in organoleptic as well as stability properties of wines. Surface hydrophobicity of proteins of plant origin was investigated in wine-like model systems by studying changes in the binding properties of ANS, used as extrinsic fluorescent probe. Hydrophobic interactions between phenolic compounds and proteins were evaluated by the study of competition of phenolic compounds with probe for the same binding sites. Polymer chain length and the chemical nature of interacting phenols was also addressed by carrying out a qualitative and quantitative characterization of phenolic compounds retained by fining agents by means of mass spectrometry techniques (LC-ESI MS, MALDI-TOF MS). Finally, the presence of residues of protein fining agents was investigated by ELISA assay.

2.1. References

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CHAPTER 3. RESULTS AND DISCUSSION

3.1. TOPIC 1: Study of the interaction between proteins of plant origins and proanthocyanidins in a model wine system

The first part of this PhD research project was aimed at studying the molecular basis of non-covalent interactions between proteins of plant origin and polyphenolic compounds, known for their role in defining organoleptic and stability properties of wines. Surface hydrophobicity of proteins of plant origin was investigated in wine-like model systems by studying changes in the binding properties of 1,8-anilinonaphtalensulphonate (ANS), used as extrinsic fluorescent probe. Hydrophobic interactions between phenolic compounds and proteins were evaluated by competition studies of phenolic compounds with the probe for the same binding sites. Polymer chain length and the chemical nature of interacting phenols was also addressed by carrying out a qualitative and quantitative characterization of phenolic compounds retained by fining agents by means of mass spectrometry techniques (LC-ESI MS, MALDI-TOF MS).

3.2. MATERIALS AND METHODS

3.2.1. Chemicals, reagents and oenological products

Water was purified with a Milli-Q system (Millipore, Bedford, MA). The wine-like model solution used was ethanol/water (10:90 v/v), buffered to pH 3.5 by adding tartaric acid. Oligomeric Proanthocyanidin Complexes (OPCs) from *Vitis vinifera* seeds were supplied by International Nutrition Company (INC, Loosdrecht, The Netherlands). The fining agents for experimental activities included commercial protein extracts from soybean and pea, lentil flour, and gluten proteins (Table 1).

3.2.2 Probe binding studies

Protein surface hydrophobicity was assessed by using 1,8-anilinonaphtalensulphonate (ANS) as a fluorescent probe. Spectrofluorimetric measurements were performed in a Perkin-Elmer Luminescence LS 50 Spectrometer using 2.5 band-widths for both excitation and emission. Spectrofluorimetric titration of protein samples with the hydrophobic fluorescent marker ANS was performed at 25°C with magnetic stirring. Binding of ANS was monitored at λ_{ex} 390 nm and λ_{em} 460 nm; multiple addition of the fluorescent probe were done up to saturation with the probe (constant fluorescence response). Titration results were analyzed by standard binding algorithms that allowed to estimate the overall binding capacity of the proteins for the probe (given as

fluorescence at saturating ANS, F_{max}) and the apparent dissociation constant of the supposedly equimolar protein-ANS complex (K_d^{app}). The overall binding capacity (F_{max}) was then corrected for the total protein content of each sample. A protein surface hydrophobicity index was calculated as [F_{max}(corrected for the protein content) x $(K_d^{app})^{-1}$] (Bonomi et al., 2004).

The ability of insoluble proteins to bind ANS was measured by adding an excess of the fluorescent probe (> 2 × K_d^{app}) to a suspension of proteins in a wine-like solution. The suspension was then centrifuged (3000*g*, 10 min, 20°C). An aliquot of the supernatant was mixed with a detergent solution (Triton X-100 2% w/w), that incorporated free ANS and ANS bound to soluble proteins (19). The amount of ANS in the micellar phase was quantitated spectrofluorimetrically by adding ANS as an internal standard.

3.2.3 Competition studies

Hydrophobic interactions between polyphenol compounds and proteins of plant origin were evaluated by competition studies. Excess ANS (> 2xK_d^{app}) was added to protein suspensions. The decrease in ANS fluorescence due to probe displacement or by quenching was measured as a function of added polyphenolics (catechin or oligomeric proanthocyanidins). Concentration of oligomeric proanthocyanidins was expressed as catechin equivalents. Titration with polyphenolics was continued until no further changes in fluorescence were observed.

In a different approach, the disappearance of ANS binding sites in the insoluble fraction of the various plant protein preparation after interaction with polyphenols was studied. Polyphenols were added to protein suspensions at concentrations corresponding to those where no more fluorescence changes were observed in the ANS-displacement experiments presented above. Excess ANS (> 2xK_d^{app} for each individual protein system) was then added, and the amount of ANS remaining in the soluble fraction was quantitated after centrifugation by the detergent/internal standard procedure depicted in the section above.

3.2.4 High-Performance Liquid Chromatography/Electrospray Ionization-Mass Spectrometry (HPLC/ESI-MS)

Proteins (200 mg/L) were added to OPC solutions (1 mg/mL) in 20 mL of wine-like buffer. Each sample was mixed for 30 minutes, and centrifuged (3000*g*, 15 min, 20°C). Both the supernatant and the pellet were analyzed by LC ESI-MS. Pellets were taken up either in wine-like buffer or in a 2:1 mixture of acetonitrile and 0.1% TFA in water, and centrifuged before the analysis.

LC ESI-MS was carried on a single quadrupole instrument (HP1100-MSD, Agilent Technologies, Santa Clara, CA, USA) and by using C18 columns (Vydac, Hesperia, CA, USA; 2.1 × 250 mm). The eluents were 0.1% (v/v) TFA in HPLC-grade water (solvent A) and 0.1% (v/v) TFA in acetonitrile (solvent B). OPCs were separated at a constant flow-rate of 0.2 ml/min, with a linear gradient of solvent B in the following proportions (v/v): 4 min, 0% B; 4–14 min, 0–18% B; 14–22 min, 18–28% B, 22–24 min, 28% B; 24–26 min, 28–60% B; 26–27 min; 60–80% B; and 27–30 min, 80–100% B. The total run time was 30 min with UV detection at 280 nm. Calibration curves were prepared using flavan-3-ol monomers ((+)-catechin, (-)-epicatechin and (-)-epigallocatechin-3-O-gallate), in the 50-250 mg/L concentration range. Five different concentrations were used for each analyte, the experiments being performed in triplicate.

3.2.5 MALDI-TOF MS

MALDI-TOF spectra were recorded in positive-ion mode, using a Voyager DE-Pro spectrometer (PerSeptive BioSystems, Framingham, MA) equipped with a N_2 laser (337 nm). Alpha-ciano-4-hydroxycinnamic acid (Fluka, Buchs, Switzerland) was used as the matrix and prepared by dissolving 5 mg in 1 ml of aqueous 50% acetonitrile (v/v)/0.1% TFA (v/v). The instrument operated with an accelerating voltage of 20 kV. Mass spectrum acquisition was performed in both positive linear and reflectron mode. External mass calibration was performed with peptide standards (Sigma).

3. 3 RESULTS AND DISCUSSION TOPIC 1

3.3.1 Protein surface hydrophobicity

Surface hydrophobicity plays an important role in protein functionality. Several studies have reported the use of ANS to characterize the surface hydrophobicity of soluble and insoluble proteins, such as those in cereal-based products (Genot C et al., 1992a; Genot C et al., 1992b; Sironi et al., 2001). Binding parameters for ANS may be inferred directly from titration experiments regardless of the presence of heterogeneous phases, and offer a rather accurate description the surface properties of proteins (Bonomi et al., 2004).

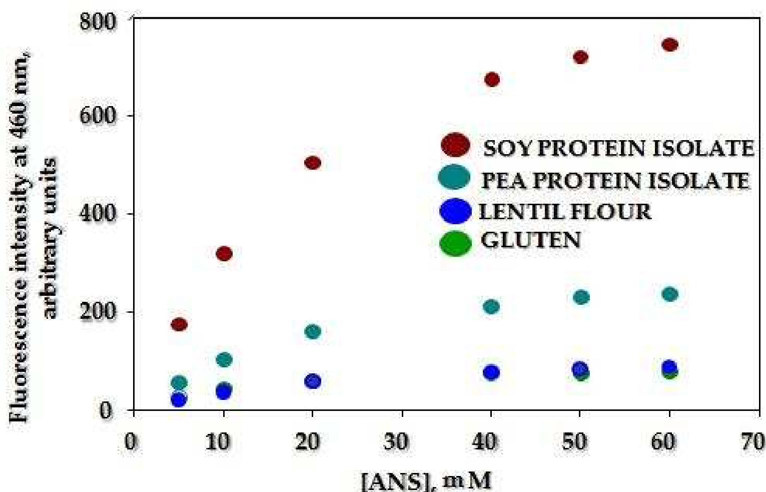

Figure 3.1. Fluorometric titration with 1-anilino-8-naphtalene sulphonate (ANS) of soybean, pea, lentil flour, and gluten proteins (each at 1 mg protein/ml in a wine-like model solution (10% ethanol (v/v), tartrate buffer, pH 3.50)).

Spectrofluorimetric titrations with increasing ANS of protein suspensions in wine-like buffer are presented in Figure 3.1, and confirm the general applicability of this procedure also to particulate and multiphase systems. Figure 3.1 also shows that the various preparations of plant proteins had evident differences in their overall binding capacity towards the probe. The number of surface sites available for binding of the probe is expressed by F_{max}, the fluorescence at saturating probe concentration corrected for the protein content of individual preparations. As listed in Table 1, soybean proteins were characterized by the highest number of binding sites per unit mass protein. followed by pea proteins, by gluten, and by proteins in lentil flour.

From the titration curves in Figure 3.1 it was possible also to calculate also the apparent dissociation constants of the protein-ANS complexes (K_d^{app}), that were similar in the various samples (Table 3.1).

Table 3.1. Surface hydrophobicity properties of proteins in the various fining agents

fining agent	total protein content, mg/g	soluble protein, % of total	F_{max}, fluorescence at saturating probe concn × (mg of protein)$^{-1}$	K_d^{app}, μM	PSH
soy protein isolate	918 ± 12	0.56	1033 ± 16	30.09 ± 1.2	186.73 ± 3.7
pea protein isolate	900 ± 22	0.94	676 ± 32	26.53 ± 0.2	65.39 ± 3.2
lentil flour	315 ± 18	0.24	278 ± 12	24.11 ± 0.7	20.26 ± 1.1
gluten	975 ± 31	<0.10	432 ± 16	33.23 ± 2.2	17.80 ± 2.1

[a] Data and standard deviations are from a minimum of three determinations.

The ANS binding properties of individual protein preparations may conveniently be expressed – for comparative purposes - by combining the number of sites available for binding of the probe and their average affinity in a single surface hydrophobicity index (PSH = [F_{max}/prot]/K_d^{app} (Bonomi et al., 1998; Pagliarini et al. 1990). As summarized in Table 1, PSH increased in the order: gluten<lentil flour<<pea protein isolate<soybean protein isolate.

ANS partition studies were carried out to discriminate between binding of the probe to insoluble and insoluble proteins that are simultaneously present in all the preparations used here but gluten. Binding of hydrophobic compounds to the insoluble protein fraction is obviously of paramount relevance to the wine fining process. These studies were also meant to set up conditions suitable for carrying out the competition experiments reported in a following section. A slight excess (~ 2 × K_d^{app}) of ANS was added to individual protein suspensions, and the amount of ANS remaining in solution after centrifugation was assessed by a detergent-stripping method ((Bonomi et al., 2004). As shown in Figure 3.2, the insoluble protein fraction in all preparations had a remarkable capability of retaining the probe.

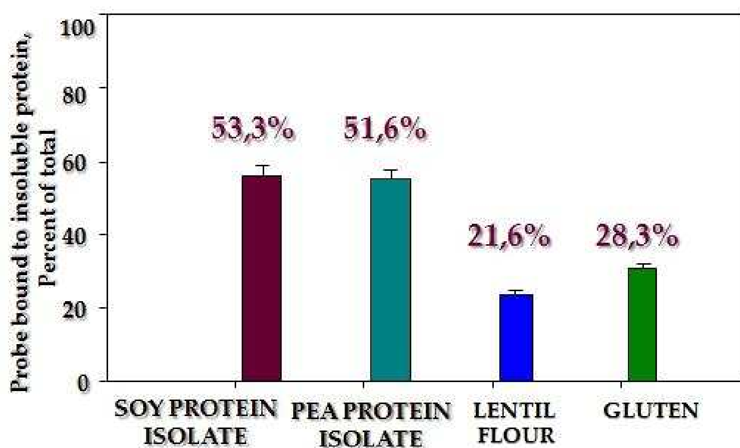

Figure 3.2. Percent fraction of ANS associated with the insoluble fraction of various proteins (1 mg/ml in 10% ethanol (v/v), tartrate buffer, pH 3.50) after interaction with 60 μM ANS.

Insoluble proteins in soybean and pea preparations (accounting for 99.44 and 99.06 % of total proteins, respectively, in the wine-like buffer used in these studies, see Table 3.1) captured almost 50% of the fluorescent probe initially present, whereas the almost completely insoluble gluten and insoluble proteins in lentil flour (99.76 % of the total proteins) managed to capture about 30% and 20%, respectively, despite the modest overall affinity of these proteins for the probe as assessed by the titration studies shown in Figure 3.1.

3.3.2 Competition studies

The specificity of the interactions between polyphenols and proteins of plant origin was evaluated by competition studies, in which polyphenols were tested for their ability to displace protein-bound ANS. As shown in Figure 3.3, the addition of increasing amounts of either catechin or oligomeric proanthocyanidins resulted in a decrease of ANS fluorescence in all cases. At the concentrations used here, both catechin and oligomeric proanthocyanidins had little effect on the very low fluorescence of free ANS. Thus, the fluorescence decrease in Figure 3.3 stems from detachment of the hydrophobic probe from the protein and seems to confirm that the interactions between proteins and phenolic compounds are governed by hydrophobic forces. Indeed, for both catechin and oligomeric proanthocyanidins, the ability to compete with the probe was higher for those proteins having the lower surface hydrophobicity.

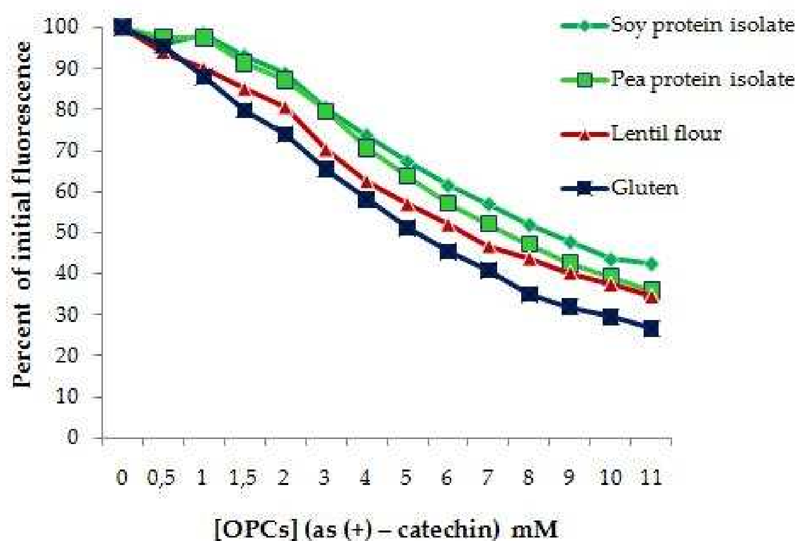

Figure 3.3. Decrease of ANS fluorescence upon addition of increasing amounts of oligomeric proanthocyanidins to wine-like model solutions (10% ethanol (v/v), tartrate buffer, pH 3.50) containing 60 μM ANS and 1 mg/ml of proteins of various origin. Concentration of oligomeric proanthocyanidins is given as catechin equivalents.

The same competition approach was used to assess the amount of ANS remaining bound to the insoluble fraction after incubation of each protein system in the presence of fixed concentrations of ANS (0.1 mM) and oligomeric proanthocyanidins (10 mM as catechin equivalents). As shown in Figure 3.4, the 100-fold excess of proanthocyanidins was unable to prevent binding of ANS to the insoluble proteins in any of the systems. The fluorescence decrease observed in Figure 3.3 may be explained as due to ANS displacement from the soluble fraction of these proteins, but this hypothesis can not justify what observed in the case of the totally insoluble gluten. Thus, a more fitting molecular-based explanation of the experiments in Figures 3.3 and 3.4 more likely implies that polyphenols bind to proteins "on top" of the bound ANS, quenching its fluorescence and simulating its displacement. Therefore, a more direct approach is required to assess the extent and specificity of the binding of polyphenols to plant-derived proteins, and to verify whether the fining process may be finely tuned by an appropriate choice of the involved proteins.

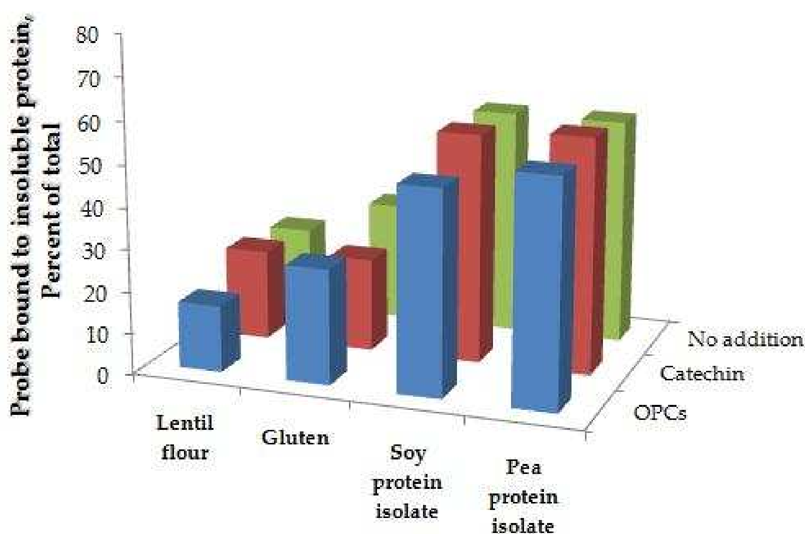

Figure 3.4. Percent fraction of ANS associated with the insoluble fraction of various proteins (1 mg/ml in 10% ethanol (v/v), tartrate buffer, pH 3.50) after interaction with 60 μM ANS in the absence (no addition) or in the presence of 10 mM catechin and 10 mM proantocyanidins (OPC, concentration given as catechin equivalents).

3.3.3 Structural characterization of phenolic compounds before and after interaction with proteins used as fining agents

In order to investigate the molecular basis of tannin-protein associations, oligomeric proanthocyanidins were incubated with each of the various proteins in the same wine-like model solution used above. The identification of newly formed compounds and

the changes in composition and concentration of OPCs were monitored by HPLC in combination with electrospray mass spectrometry (ESI-MS). To increase the sensitivity of the ESI-MS measurements, the samples were assayed twice, scanning from m/z 100 to 1000, and from m/z 1000 to 2000, respectively. Proanthocyanidin solution, without addition of fining agents, were used as control solution.

The MS total ion chromatogram (TIC) of the positive molecular ions of OPCs standard solution (0.1 mM) in the range m/z 100–1000 indicated the presence of P1 (m/z 291), P2 (m/z 579), P3 (m/z 867), and of some proanthocyanidin gallates (PnGn), including P1G1 (m/z 443), P2G1 (m/z 731), P2G2 (m/z 883). Higher polymerized OPCs, in the range m/z 1000 –2000, are predominantly distributed at HPLC retention times ranging 20–28 min. The mass spectra obtained from the TICs of the extract showed the molecular-ion peaks of P4–P6 as well as those of the gallate derivatives P3G1 (m/z 1019), P3G2 (m/z 1172), P4G1 (m/z 1308), P4G2 (m/z 1460), P5G1 (m/z 1595), P5G2 (m/z 1748), P6G1 (m/z 1884).

More complete data about higher polymerized tannins were obtained by MALDI-TOF MS analysis in positive-ion linear and reflectron modes. MALDI-TOF is able to measure masses in complex mixtures of low and high molecular weight compounds. In model white wine were detected an oligomeric series of catechin/epicatechin units and of their gallic acid ester derivatives (sodium adduct ions $M+Na^+$), up to the decamer (see Table 3.2). Additional masses corresponding to a series of poly-galloyl poly-flavans were also detected.

Table 3.2. Oligomeric proanthocyanidin composition of wine-like model solution (10% ethanol (v/v), tartrate buffer, pH 3.50, 10 mM proanthocyanidins) obtained after fining with soybean, pea, lentil flour, and gluten proteins (200 mg/l) by MALDI-TOF MS. OPCs: oligomeric proanthocyanidins; n.d.: not detected.

	Number of galloyl units (g)	*Calculated M + Na+*	*OPCs (M + Na+)*	*MODEL WINE*			
				SOY PROTEIN ISOLATE	*PEA PROTEIN ISOLATE*	*LENTIL FLOUR*	*GLUTEN*
Dimer	0	601.3	602.5	602.1	602.4	601.8	602.2
	1	753.3	754.3	753.8	754.1	753.4	754.0
	2	905.3	907.7	905.6	905.9	905.2	905.7
Trimer	0	889.8	890.9	889.7	889.9	889.5	889.9
	1	1041.9	1042.4	1041.4	1041.6	1040.8	1041.4
	2	1194.0	1194.2	1193.0	1194.5	1194.0	1193.3
	3	1346.1	1347.4	1344.8	1345.6	n.d.	1346.3
Tetramer	0	1178.0	1178.0	1177.2	1177.7	1176.7	1178.2
	1	1330.1	1331.8	1329.0	1329.3	1329.9	1329.6
	2	1482.2	1483.0	1481.2	1483.1	1480.9	1483.0
	3	1634.4	1634.6	n.d.	1633.4	1636.3	n.d.
	4	1786.5	1787.9	1785.7	1785.7	1784.5	n.d.
Pentamer	0	1466.3	1466.1	1465.9	1466.4	1465.0	1466.0
	1	1618.4	1618.8	n.d.	1617.4	1615.3	1617.8
	2	1770.5	1770.8	n.d.	1771.0	1769.0	1769.1
	3	1922.6	1922.9	1919.0	1920.9	n.d.	1920.8
	4	2074.7	2074.6	n.d.	2075.8	2077.9	2074.7

Hexamer	0	1754.5	1754.9	1752.7	1752.1	1751.1	1754.5
	1	1906.7	1907.6	1905.0	1905.9	1903.0	1905.9
	2	2058.8	2059.9	2057.4	2057.4	2056.5	2055.8
	3	2210.9	2211.3	n.d.	2210.0	n.d.	2209.9
	4	2363.0	2363.6	n.d.	n.d.	n.d.	n.d.
	5	2515.1	n.d.	n.d.	n.d.	n.d.	n.d.
	6	2667.2	n.d.	n.d.	n.d.		n.d.
Heptamer	0	2042.8	2042.3	2042.4	2041.0	2043.3	2042.8
	1	2194.9	2194.7	n.d.	2192.4	n.d.	2194.9
	2	2347.0	2347.9	2346.5	2345.0	n.d.	2344.0
	3	2499.1	2499.4	n.d.	2495.0	n.d.	2494.4
	4	2651.2	2650.6	n.d.	n.d.	n.d.	n.d.
	5	2803.3	2802.5	n.d.	2805.9	n.d.	2800.2
	6	2955.4	2956.2	n.d.	2956.2	n.d.	2959.1
	7	3107.5	3106.3	3106.7	3104.8	3103.3	n.d.
Octamer	0	2331.1	2330.5	n.d.	2332.2	2327.8	n.d.
	1	2483.2	2481.7	n.d.	2482.1		n.d.
	2	2635.3	2634.0	2634.4	2632.5	n.d.	2634.2
	3	2787.4	2786.4	n.d.	2876.5	2786.4	n.d.
	4	2939.5	2936.8	n.d.	2939.5	2941.7	n.d.
	5	3091.0	3093.6	n.d.	3088.8	n.d.	3089.9
	6	3243.0	3241.0	n.d.	3244.4	n.d.	n.d.
	7	3395.0	n.d.	3394.2	3397.2	n.d.	n.d.
Nonamer	0	2619.3	2619.1	2619.0	n.d.	n.d.	n.d.
	1	2771.4	2771.1	n.d.	n.d.	n.d.	n.d.
	2	2923.5	2922.6	n.d.	n.d.	n.d.	2924.8
	3	3075.6	3072.6	n.d.	n.d.	n.d.	n.d.
	4	3227.7	3228.3	n.d.	3229.6	n.d.	3226.7
	5	3379.8	3377.3	n.d.	n.d.	3378.6	n.d.
Decamer	0	2907.6	2905.0	n.d.	n.d.	n.d.	2909.3
	1	3059.7	3056.0	n.d.	3053.8	3059.3	n.d.
	2	3211.8	3209.5	n.d.	n.d.	n.d.	n.d.
	3	3363.9	3360.7	n.d.	n.d.	n.d.	n.d.
	4	3516.0	n.d.	n.d.	n.d.	n.d.	n.d.
	5	3668.1	n.d.	n.d.	n.d.	n.d.	n.d.

Fining model systems were set up by adding each of the fining agents (20 g/100 l, corresponding to a 200 mg/l protein concentration) to OPC wine-like solution, that were stirred for 30 minutes and centrifuged. The resulting supernatants and the pellets, taken up in wine-like model solution to dissociate weakly bound tannins, were analyzed by LC-ESI MS. TICs of all treated wine-like systems resembled those obtained for OPC standard solution, suggesting an identity of small oligomeric flavan-3ols (Figure 3.5). This finding was supported by MALDI TOF mass spectrometry results, as summarized in Table 3.2.

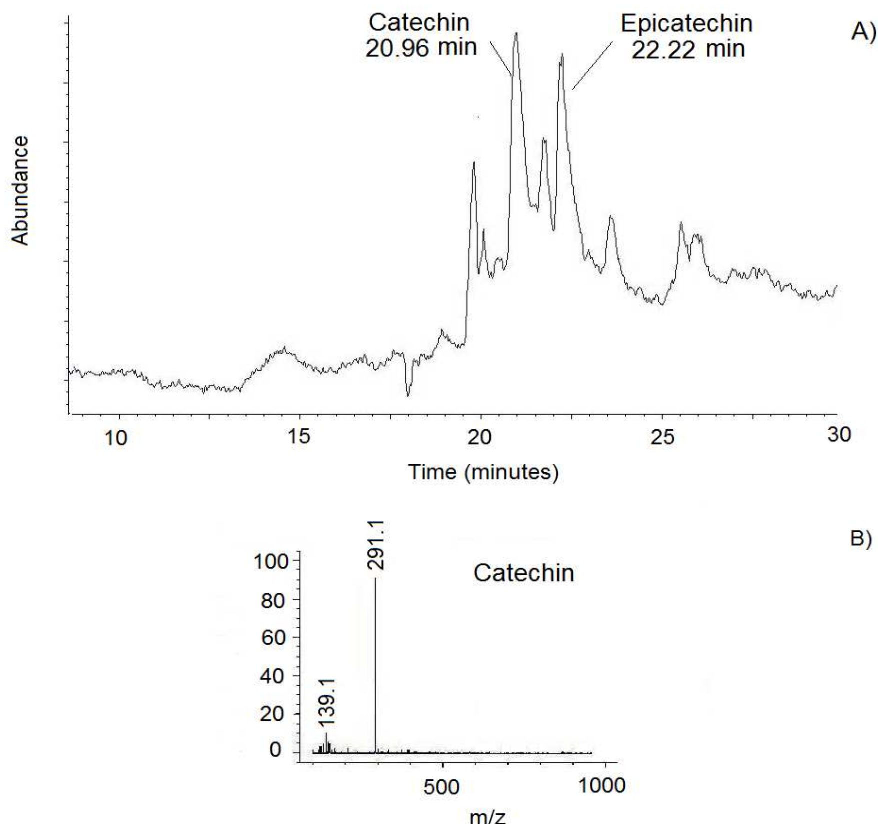

Figure 3.5. A) TIC (total ion current) chromatogram obtained in positive ion mode by injection of wine-like model solution after fining with pea protein isolate (1 mg/ml in 10% ethanol (v/v), 10 mM proantocyanidins, tartrate buffer, pH 3.50). B) ESI mass spectrum obtained from the total ion current chromatogram for 20.92 minute elution time, showing the $[M\text{-}H]^+$ peaks of catechin (m/z 291).

The results of MALDI-TOF analysis of fined wine-like systems also suggest that all tested protein fining agents selectively removed polymeric proanthocyanidins, lowering their mean degree of polymerization in fined model wine with respect to the unfined one. These results are in accordance with previous reports, which assume that the largest proanthocyanidin molecules are precipitated first in fining experiments (Ricardo-Da-Silva JM et al., 1991). This effect could be due to the higher number of phenolic rings present in the more polymerised proanthocyanidins, that increases their hydrophobicity and allows for more effective removal (Baxter NJ et al., 1997).

Characterization and estimates of the relative amounts of polyphenols precipitated from wine-like model system were made by LC-ESI MS analysis of pellets after fining treatment. All the pellets showed the presence of newly formed products. For instance, we observed the presence of vinyl-catechin and vinyl-epicatechin (m/z 316), eluting

later than their unmodified compounds (figure 3.6), and originating by catechin/epicatechin auto-polymerization induced by acetaldehyde.

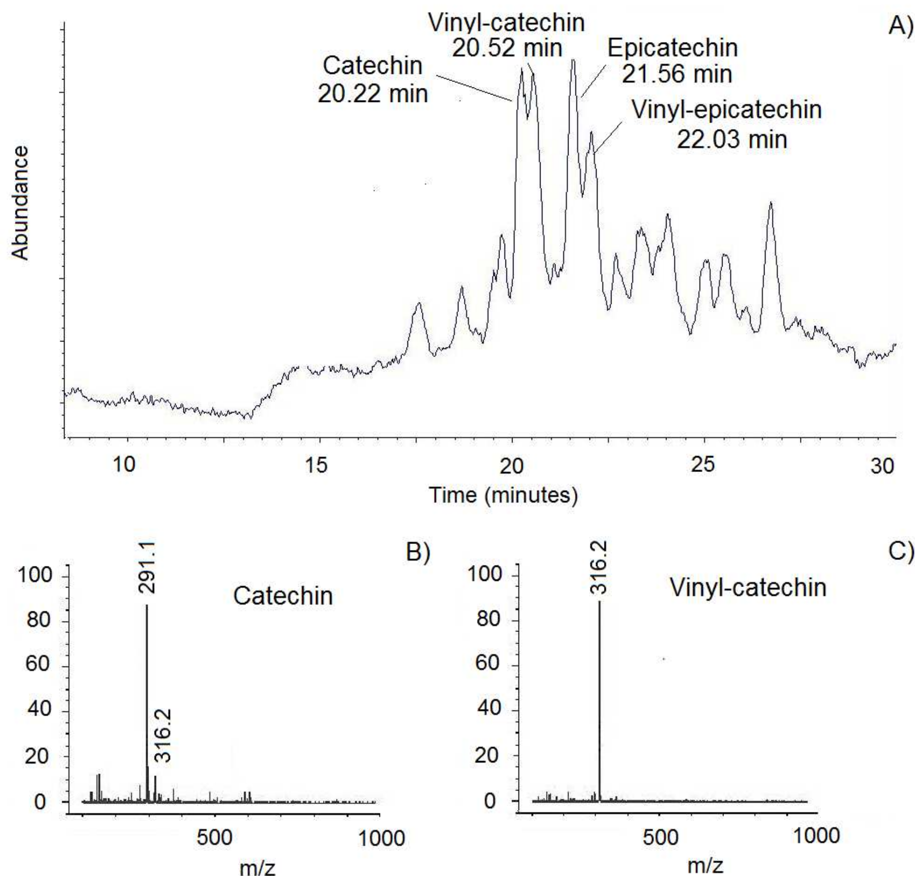

Figure 3.6. A) TIC (total ion current) chromatogram obtained in positive ion mode by injection of insoluble fraction of pea protein isolate (1 mg/ml in 10% ethanol (v/v), 10 mM proantocyanidins, tartrate buffer, pH 3.50). B-C) ESI mass spectra obtained from the total ion current chromatogram for 20.22 and 20.52 minute elution time respectively, showing the $[M-H]^+$ peaks of catechin (m/z 291) and vinyl-catechin (m/z 316).

The acetaldehyde present in fining model systems derives from the oxidation of ethanol, catalyzed by transition metals such as iron and copper (Ross ARS et al., 2000,) (that reportedly are found associated with plant-derived proteins) or through coupled oxidation of phenols. The reaction starts with the nucleophilic addition of the protonated form of acetaldehyde to the flavanol. The new formed ethanol adduct, losing a water molecule, is attacked by a second nucleophilic flavanol unit to yield an ethyl-linked flavanol dimer. The ethyl linkages generated by acetaldehyde in the polycondensated tannins are not stable and cleave into vinylflavanol monomers and

oligomers (Fulcrand H et al., 2006). Compared to direct condensation between flavanols, the rapid polymerization mediated by acetaldehyde gives rise to instability and precipitation (Es-Safi NE et al., 2002; Es-Safi NE et al., 2003). This could explain the presence of vinylflavanol products in the pellets precipitated by finings and not in the supernatant of treated samples. In addition to these compounds, various dimeric and oligomeric ethyl-bridged molecules were also detected by MALDI-TOF analysis of pellets. including adducts of trimers and their gallic acid derivatives (m/z 923, 1075.9).

We also carried out a detailed quantitative LC-MS analysis of the flavonoid compounds most important with respect to white wine oxidation (monomeric and dimeric proanthocyanidins), in order to evaluate which molecules were most easily removed by the various proteins. The browning capacity of white wines depends largely on the nature of polyphenols. Due to their catechol (o-diphenol) structure, most of them are rather readily oxidised in winemaking processes, with the monomeric catechins and the dimeric procyanidins browning more intensely than other phenolics (Lee CY & Jaworski AW, 1988), and there is strong evidence of epicatechin being the most relevant browning agent among redox-active polyphenols (Sioumis N et al., 2006).

In the OPCs control solution (5 mg/ml) the calculated total concentration of monomeric and dimeric molecules (catechin, epicatechin, monomers gallate, dimers, dimers gallate, dimers digallate) was 2.9 mg/ml. A general decrease in the concentration of all these species – considered as a whole - was observed after treatment with proteins. Lentil flour was the most effective removal agent, giving a 16.4% decrease in OPCs, followed by gluten, soy, and pea proteins, that gave decreases of 12.6%, 9.26% and 8.44%, respectively. These differences in clarifying efficiency is likely related to the molecular composition, the biochemical characteristics, and the conformation of proteins relevant to the complex interactions that ultimately lead to flocculation of their complexes with polyphenols and to clarification of the model wine-like solutions used here.

At first sight the interactions brought forward by the clarification ability of proteins from the various sources seem to be in disagreement with the surface hydrophobicity data and with the competition experiments. Based on these latter data, we expected that proteins characterized by the highest surface hydrophobicity should have given the highest removal of OPC from the wine-like medium, whereas the direct measurements reported above indicate that the actual ranking was reversed, at least when OPC are considered as a whole.

However, if the loss and recovery is examined in terms of individual molecules, there is evidence that this discrepancy is more apparent than substantial, and that molecular specificity plays a role in governing the interaction between hydrophobic sites on the protein surface and the molecules considered here. Figures 3.7 presents quantitative data as for the removal of individual species after treatment with the various proteins, obtained through HPLC analysis. For instance, the efficiency of the various proteins in removing (+) catechin, and (-) epicatechin, which differ only on the spatial position of one OH group with respect to the ring, was remarkably different. In particular, (+) catechin was more specifically removed than (-) epicatechin by all of the protein fining agents tested and especially by the lentil flour and the gluten proteins. Moreover, the levels of galloylated proanthocyanidins precipitation appeared to be higher than the other phenols for all protein fining agents (from 28% loss with gluten proteins to 36% loss with soy proteins), indicating that the more galloylated proanthocyanidins were removed in a preferential way.

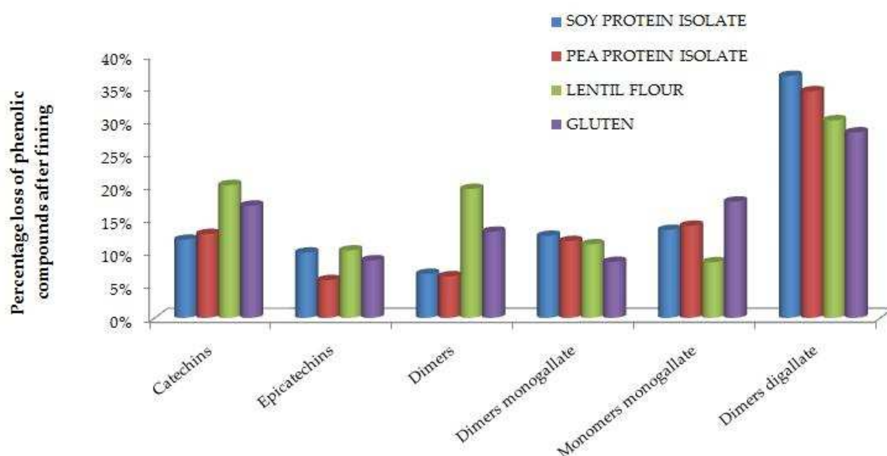

Figure 3.7. Percentage loss of individual flavan-3-ols species in fined wine-like samples obtained by means of LC-ESI MS analysis.

Pellets obtained from the fining processes carried out with the various proteins also were treated with water and with more apolar solvents to assess the nature and intensity of the forces involved in the interaction. Pellets were dissolved in acetonitrile/ water 0.1%TFA (2:1) to dissociate soluble and insoluble tannin-protein complexes. The percentage recovery of phenolic compounds from pellets (ratio between the concentration of each molecules in the pellet and their loss in fined wine-like sample) are compared for each of the tested proteins in Figure 3.8.

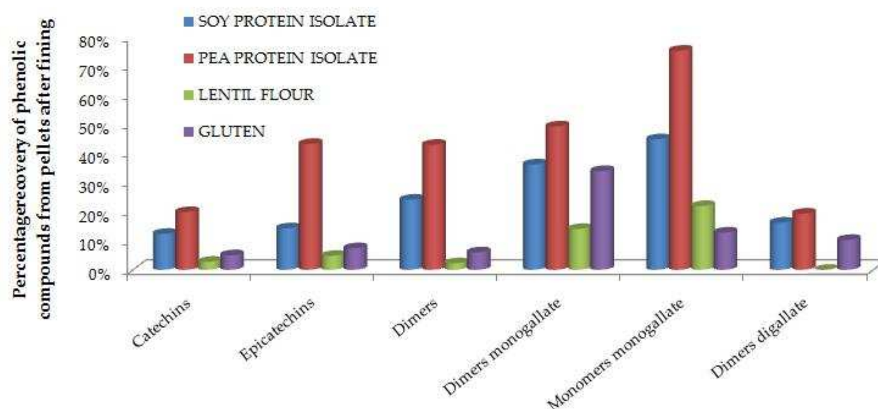

Figure 3.8. Percentage recovery of phenolic compounds from pellets obtained after fining processes carried out with the various proteins (ratio between the concentration of each molecules in the pellet and their loss in fined wine-like sample).

Pea and soy proteins were the fining systems in which proanthocyanidins were more easily recovered from pellets. This is in accordance with the evidence gathered from competition studies, and confirm that "weak" forces, such as hydrophobic ones, were most relevant to the interactions responsible for precipitation by these fining agents. The interaction between polyphenols and gluten proteins and above all lentil flour systems (characterized by higher loss in treated wine-like samples, lower surface hydrophobicity and low recovery from the pellets) could be instead governed by other types of attractive forces such as hydrogen or covalent bonds (cross linkages between proteins) which may impair the release of polymerized tannins.

3.4. CONCLUSION

The work of structural characterization allowed to asses molecular properties of protein of plant origin in terms of surface hydrophobicity. The resulting binding parameters provided a comparative estimate of the number and of the affinity of binding sites on the surface of the various proteins, and the indications provided by these studies were confirmed to a large extent by competition/displacement experiments. However, a straightforward interpretation of the displacement experiment was made difficult by simultaneous binding of the fluorescent probe and of polyphenols. This indicates the existence of multiple binding sites on the protein surface, with a possible different specificity.

This observation implies that proteins of different origin may selectively bind peculiar fractions in a complex mixture of polyphenols, as indeed was found to be the case when LC-MS analysis was used to assess the pattern of bound and residual polyphenols in mixtures simulating the actual fining process. This observation is of possible practical interest, in what it paves the way to a selective use of protein agents for "fine tuning" the properties of the finished product with respect to important organoleptic properties and to their stability.

However, it was also noticed that treatment of simulated wine with the commercial protein preparations used here favours some chemical reactions among some of the polyphenols and other wine components. The significance of these reactions in a real wine (where they may be affected by other wine components) remains to be evaluated.

The possible requirement for plant-protein based fining agents designed and prepared "ad hoc" for this particular purpose also remains to be evaluated. Should this be necessary, the methodologies presented here could be fruitfully exploited to assess whether these material will be suitable for this particular use, for instance by testing their surface hydrophobicity properties, prior to resorting to exceedingly laborious, time consuming, and expensive experimentation in actual wine-making applications.

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3.6. TOPIC 2: Study of the effects of plant protein fining agents on the quality parameters of white and red wines

In the second part of this PhD thesis project, the study of mechanism of interaction of plant proteins and polyphenols was extended to real wine in order to evaluate the effect of finings on all parameters responsible for wine quality and its sensorial appraisal. The last point concerns the research of residual gluten proteins in the treated wine. Gluten indeed causes sometimes severe intolerance (celiac disease) and it is then very important to know if it is completely eliminated after clarifying.

3.7. MATERIALS AND METHODS

3.7.1 Experimental wines

Young white and red wine of vintage 2009 was used in this study made from various grapevine varieties (all *Vitis vinifera*) from Regione Campania, Italia. Fiano grapes were hand-harvested at full maturation (20.50°Brix) in vineyards located in the town of Lapio (AV), a DOCG area in the Campania Region. Grapes were destemmed, crushed and potassium metabisulfite (8 g/hl) and pectic enzyme (2 g/hl) were added (Aspergillus AGAZYM EXT, Garzanti specialties S.p.a., Milano). Must was immediately cooled to 10 °C and was submitted to static decantation for 24 h. Inoculation was carried out at 20 g/hl (MAURIVIN, AWRI FUSION, hybrid *S.cerevisiae* x *S. cariocanus*, Queensland, Australia) after yeast rehydratation in warm water for 30 minutes, as described by the manufacturer. Fermentation took place in stainless steel tanks at 18 °C: at the end of alcoholic fermentation, the wines were treated with protein fining agents and immediately submitted to spontaneous settling.

For the production of *Catalanesca* white wines the winemaking procedure used was similar to that employed for *Fiano* wine with the exception that the fining treatments were carried out after cold stabilization. The usual wine analyses have been reported in table 3.3.

Table 3.3. Analytical characteristic of Fiano and Catalanesca white wine before fining

	Catalanesca wine	Fiano wine
Sugar content (g/l)	< 2	5.6
Total acidity (g/l of tartaric acid)	5.2	8
Ph	3.32	3.2
Volatile acidity (g/l of acetic acid)	0.54	0.3
Alcohol content (% v/v)	12.50	11.8
Free SO_2 (mg/l)	10.88	13.6
Total SO_2 (mg/l)	38.4	30.2
Turbidity (NTU)	55	545.35

The young red wine used in this study (Aglianico del Taburno) was produced from *Aglianico* grapes harvested in vineyards located in the town of Torrecuso (BN), a DOC area in the Campania Region. They had the following chemical characteristics: alcohol content 15.10% (v/v), titratable acidity 6.71 g/L expressed as tartaric acid, volatile acidity 0.29 g/L expressed as acetic acid, pH 3.31, free sulphur dioxide 18 mg/L and total sulphur dioxide 30 mg/L. The fining treatments of red wines were performed including also two preparations obtained from enzymatically hydrolyzed pea proteins.

3.7.2 Wine fining trials

Fining tests were carried out by adding protein fining agents in graduated cylinders (volume, 100 mL). As enological products clarifying activity was totally unknown, doses used for the tests were 20 g/hl: these value are the maximum dose commonly used for animal proteins (gelatin and egg proteins). An untreated sample was used as a control. The fining agents were freshly prepared just before addition into the wine, were thoroughly mixed and allowed to remain in contact with the wine for 7 days at 20°C. All experiments were done in duplicate. A gelatin soluble in cold water (PULVICLAR S, Enartis, Italy) that is commonly used in clarification of red wines was included for comparative purposes.

3.7.3 Clarifycation kinetics and analysis of conventional oenological parameters

Classic parameters of wines were determined according to the international methods of the OIV (International Organization of Vine and Wine, 2005). Turbidity was measured in Nephelometric Turbidity Units (NTU) using a turbidimeter (LP 2000 HANNA INSTRUMENTS). Optical density at 420 nm (for white wines), color intensity index (A420 + A520 + A620) and tonality (A420/A520) for red wines, turbidity and the volume of lees generated were measured 1, 4, 10, 20, 32, 48, 60 and 168 h after the addition of fining agents. Kinetics were studied at room temperature (20°C ± 2°C). The absorbance was determined at 420, 520, 620 nm in a 10 mm cell by an UV-VIS Spectrofotometer (mod. 1601, Shimadzu). All analyses were carried out in triplicate.

3.7.4 Analytical method used for structural characterization of phenolic compounds of wine after fining

The wine (5 ml) was loaded on a Sep-Pak C18 cartridge (Waters, Milford, MA, USA) previously conditioned by sequential washing with 5 ml of methanol and 5 ml of water. The cartridge was washed three times with 10 ml of water and then eluted with 70% ethanol 0,1% TFA.

The LC/MS analysis was carried out by means of a LC/MS instrumentation (HP1100-MSD, Agilent Technologies, Santa Clara, CA, USA) with single quadrupole by using

C18 column (Vydac, Hesperia CA, USA; 2.1 * 250 mm). The eluent was HPLC-grade water containing 0.1% (v/v) TFA (solvent A) and acetonitrile–0.1% (v/v) TFA (solvent B). OPCs were separated at a constant flow-rate of 0.2 ml/min, with a linear gradient of solvent B in the following proportions (v/v): 4 min, 0% B; 4–14 min, 0–18% B; 14–22 min, 18–28% B, 22–24 min, 28% B; 24–26 min, 28–60% B; 26–27 min; 60–80% B; and 27–30 min, 80–100% B. The total run time was 30 min and UV detection was carried out at 280 nm for tannins and 520 nm for anthocyanins and their copigmentation products.

For LC/ESI-MS analysis, the proanthocyanidins were characterized according to the conditions used for wine model solution; calibration curves for tannins were prepared using (+)-catechin in the 50-250 mg/L concentration range. For anthocyanin compounds, the ESI mass spectra were scanned from m/z 1000 to 200 at a scan cycle of 4.90 s per scan and 0.1 s inter-scan delay. The source temperature was 180 °C. Spectra were acquired in the positive ion mode; the capillary voltage was 3.6 kV and the cone voltage was maintained either at 40 or 25 V, according to different experiments. N_2 was used as both drying and spraying gas. Anthocyanins were quantified using malvidin-3-glucoside as external standard. Five different concentrations were used for each analyte, the experiments being performed in triplicate.

3.7.5 Analytical method used for the aromatic characterization of wines after fining

In order to obtain more complete data as possible for the composition of the treated wines, the quali-qualitative analysis of odorous compounds (varietal molecules such as terpenes, volatile phenols and non varietal compounds such as acids, esters, aldehydes, lactones, etc) was performed by the analytical approach of solid phase micro-extraction (SPME) and gas chromatography-mass spectrometry (Nasi A et al., 2008). SPME holder and fibers (PDMS, CAR/PDMS, CAR/DVB/PDMS) were purchased by Supelco (Aldrich, Bornem, Belgio).

SPME was carried out with the following conditions: the fibers were immersed in the headspace (HS) of the samples using 120 ml of wine until equilibrium conditions. 1-Octanol (0.500 mg/l) was added to wine before the extraction as an internal standard.

Thermal desorption of the analytes from the fiber inside the GC injection port was carried out in the split mode (1/10) at a desorption temperature of 250 °C during 1 minute. For gas chromatography-mass spectrometric analysis, all samples were analysed with an HP 6890 coupled to a 5973N quadrupole HP mass spectrometer. The gas chromatograph was equipped with an HP-5 ms capillary column (30m x 0.32 mm ID) and the carrier gas used was helium.
For the analysis of aroma compounds, the GC oven temperature was increased from 40°C (held for 7 minutes) to 180 °C at 5 °C/min . The mass spectrometer was operated in electron mode (EI, 70 eV) and the masses were scanned over an m/z range of 45-350 amu. In other cases a SIM method was used (for terpene compounds m/z 93, 12, 136).

The identification of odorous components was effected by NIST library and/or by comparison with spectra and retention times of standards.

3.7.6 Determination of gluten content in white and red wines by ELISA

The gluten content in wine samples was measured by using a commercial ELISA kit (Fast Gliadin R-Biopharm, Darmstadt, Germany). The kit is based on a direct sandwich ELISA and the distributor gives a detection limit of 5 ppm ol gliadin. Given the general consensus on 50% of gluten being gliadin, this represents a limits of 10 ppm of gluten. The solid support of the reaction is a microtiter plate coated with the R5 monoclonal antigliadin antibody. The ELISA assay was performed according to the standard procedure suggested by the supplier. For the preparation of the sample, red wines were previously treated with a clarifying solution (5% w/v isinglass, 2% PVPP, 60% ethanol) in order to eliminate any interference of the phenolics-rich matrix with the ELISA procedure. At the end of the assay procedure, the optical density in each well was measured at 450 nm in a microplate reader (BioRad Laboratories, Hercules, CA). Absorbance figure were corrected for average blank readings, and BioRad software was used to calculate absorbance values and to correlate them with the gliadin content in the samples.

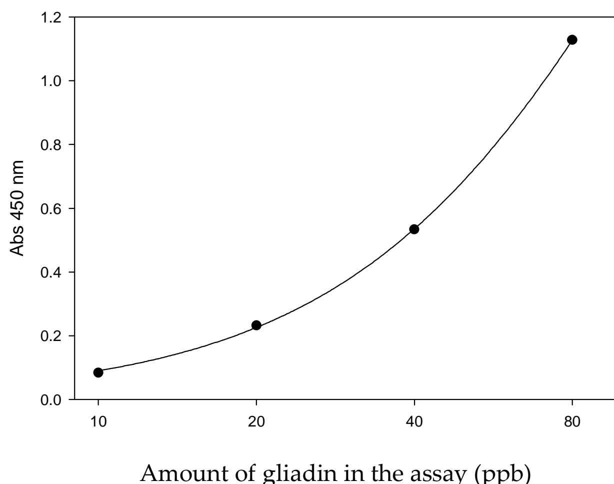

Figure 3.9. ELISA response curves for a standard solution of gliadin (ppb)

2. 8 RESULTS AND DISCUSSION TOPIC 2

3.8.1 Effects of fining agents on the technological parameters of white wines

The efficiency of proteins as fining agents was evaluated by fining experiments on white wines, made from *Fiano* and *Catalanesca* varieties, at two different steps of winemaking process, at the end of alcoholic fermentation (initial turbidity 545 NTU) and after cold stabilization (initial turbidity 55 NTU) respectively. Seven days after protein addition, measurements of principal clarification parameters were taken.

The reason of this experimental design was to compare the action of protein finings on must and wine in terms of fining ability and evaluate what was the step of winemaking process more suitable to treat the wines preserving their quality. Previous study (Wehrung 1996) suggested that fining agents, in white vinification, should be successfully used to clarify the must during fermentation because the process was less drastic in juice than in wine. Furthermore, they were able to act as insoluble solids that promote yeast growth and allow the fermentation process to be completed faster (Groath & Ough, 1978; Sims et al., 1995).

Results of *Catalanesca* wine turbidity reduction and final turbidity, in NTU units, are shown in figure 3.10 and 3.11.

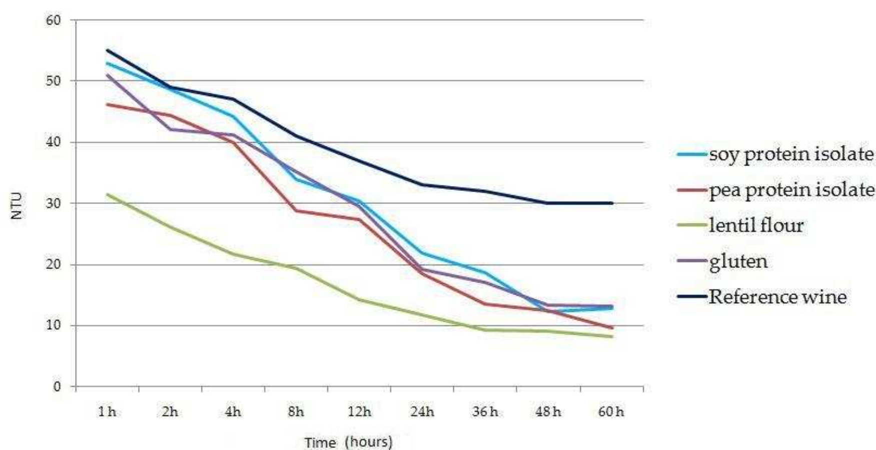

Figure 3.10. Time course of clarification for *Catalanesca* white wine. All treatments were performed at 20g protein/hl. Reference wine was obtained from spontaneous settling at low temperature without any addition of proteins.

For both white wines tested, all proteins fining agents significantly decreased the initial turbidity and optical density. Lentil flour was the most effective fining agent for treatment of *Catalanesca* wine (wine turbidity decrease to 15% of untreated wine), followed by pea, soy and gluten proteins (17%, 23% and 24% of untreated wine

turbidity respectively). For control wine, we observed that at the end of the kinetic, when the wine reached 35 NTU, the spontaneous settling became very slow, showing that fining treatment was really necessary.

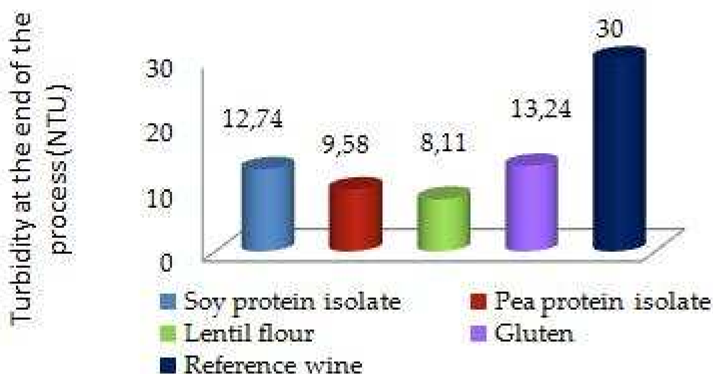

Figure 3.11. Effect of different fining agents on the final turbidity (60 h) of Catalanesca white wine.

Treatment with lentil proteins gave a very low turbidity after 36 h only. This means that they present a certain advantage for fining in cases in which a quick racking (a few days) is required. Lentil and gluten proteins produced also less lees volume than the other matrices (data not shown): this parameter has to be considered when choosing the fining agent because it is related to the loss of wine.

As observed when working with model wine having a controlled composition, the differences in clarifying efficiency and kinetic of protein matrices were related to their molecular composition, biochemical characteristics (protein surface hydrophobicity and pHi values) and the conformation of proteins responsible for the interactions with polyphenols and consequent flocculation and clarification. Moreover, also the wine composition was very important. The fining efficiencies of proteins could be affected by the amount of phenolic compounds present in white wine (including catechins, proanthocyanidins, hydroxycinnamic acids and their derivatives), the nature of tannins (low or high degree of polymerization) and also by their susceptibility to maderization and browning.

In our cases, despite differences in chemical-physical characteristics of *Catalanesca* and *Fiano* wines (in particular their initial turbidity), we found that the protein fining at doses of 20 g/hl gave relatively similar results at the end of the treatments. Also for *Fiano* wine the clarifying efficiencies of lentil flour and pea proteins were situated between those measured for soy and gluten proteins (figure 3.12). Differences were observed for the clarifying rate (seven days of treatment for *Fiano* wine against 2.5 days for *Catalanesca* one) probably because of higher initial turbidity of *Fiano* wine.

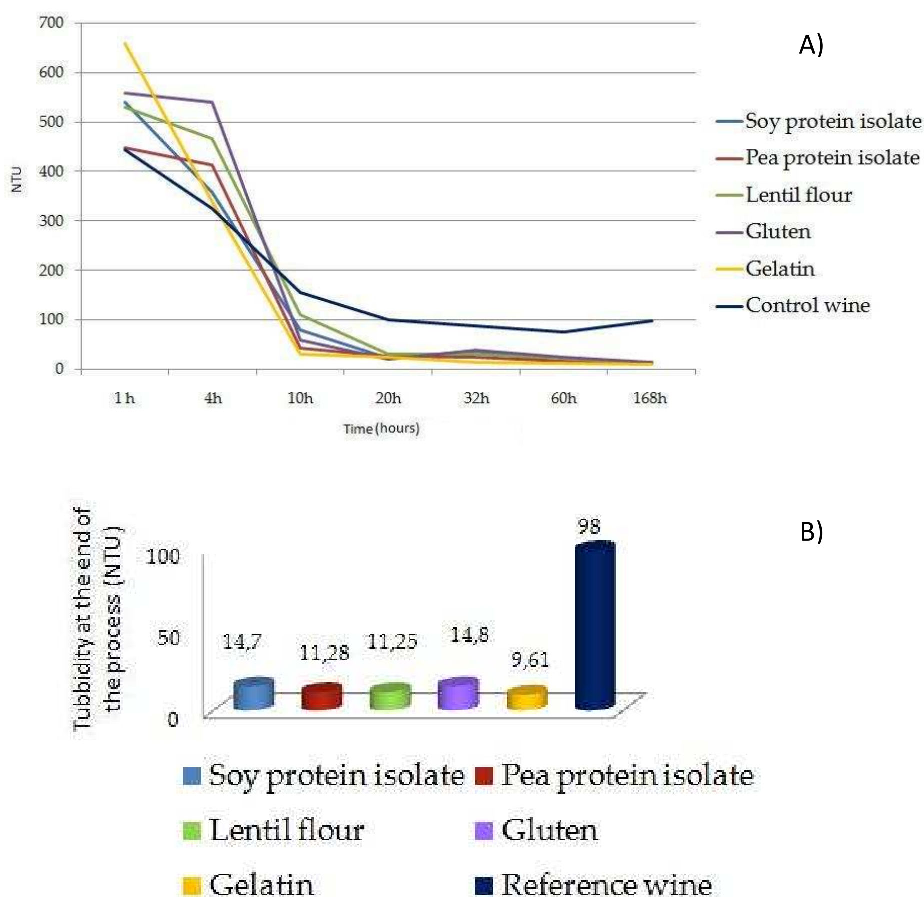

Figure 3.12. A) Time course of clarification for *Fiano* white wine. All treatment were carried out at 20 g protein/hl. Reference wine was obtained from spontaneous settling at low temperature without any addition of proteins. B) Effect of various fining agents on the final (168 h) turbidity of *Fiano* white wine.

The function of protein fining is mainly to clarify the wine but also to remove by adsorptive precipitation those compounds that lead to turbidity or to changes in color. The browning of white wine represents an important stability problem, related to oxidation of phenolic compounds. The browning capacity of white wines depends largely on the nature of polyphenols: the monomeric catechins and the dimeric procyanidins brown more intensely than other phenolics (Lee et al., 1988).

We also monitored the most important flavonoid compounds with respect to white wine oxidation (monomeric and dimeric proanthocyanidins) by a detailed quantitative LC-MS analysis, in order to evaluate which molecules were most prefencially removed by the various proteins. Figure 3.13 showed the percentage of monomeric and dimeric proanthocyanidins of the treated *Catalanesca* wine with respect to the unfined wine. This variety was chosen for its high level of phenolic compounds (860 mg/l as gallic acid) and catechins (180 mg/l) that make it particularly sensitive to oxidation.

A general decrease in the concentration of all these species – considered as a whole - was observed after treatment with proteins. The fining agents that removed monomeric and dimeric flavanols most effectively as a whole were lentil and gluten proteins. This observation was in agreement with the results of experiments in model wine which showed the higher ability to interact with tannins for these proteins. As we assessed by studying the interaction in model solution, the characteristics of the proteins (amino acid composition, structure, size, charge, etc.), as well as those of the tannins, obviously play a major role in these reactions. Indeed, proteins with a high proline content have a great affinity for tannins (Hagerman and Butler, 1980; Charlton et al., 2002; Charlton et al., 1996; Poncet-Legrand C et al., 2006). The importance of proline is probably due to its incapacity to form helixes, leaving the protein open and accessible to tannins (Hagerman and Butler, 1981). On the other hand, small, compact proteins have a low affinity for tannins (Hagerman and Butler, 1980). The presence of Na^+, K^+, Ca^{2+}, Mg^{2+} and especially Fe3+ cations in the protein extracts could affect their ability to fine the wine, because they are involved in flocculation and precipitation of tannins and proteins. Dissolved oxygen promotes flocculation, as it facilitates the formation of trivalent iron. Thus, the aeration resulting from racking improves the effectiveness of fining.

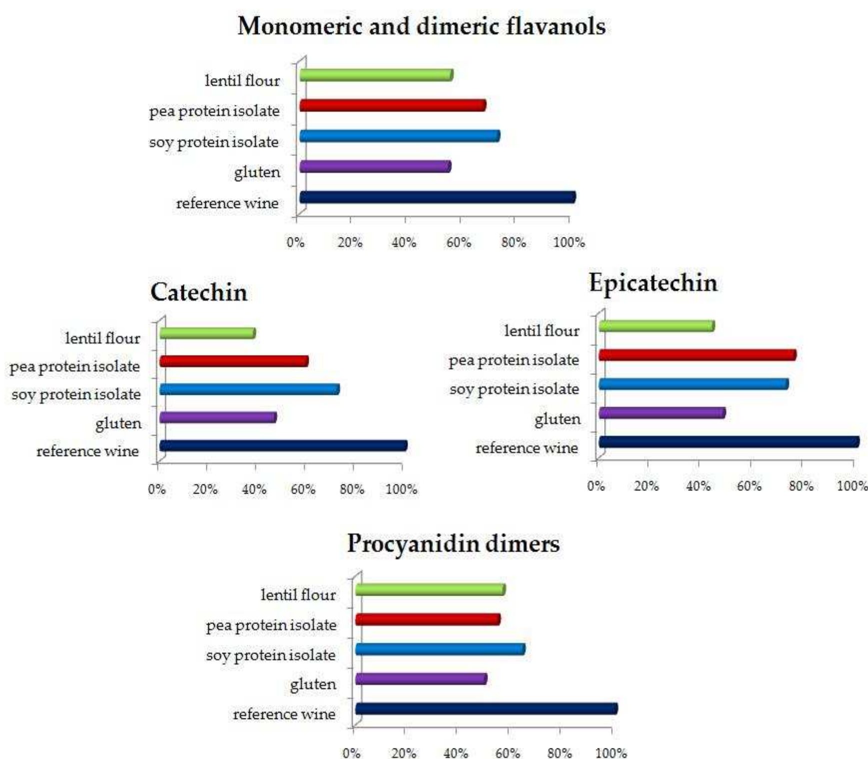

Figure 3.13. Percentage loss of individual low molecular weight proanthocyanidins in fined *Catalanesca* wine, as assessed by LC-ESI MS analysis.

Considering the removal of flavan-3-ols in terms of individual molecules, there was evidence that the fining agents had different efficiencies in removing the isomers (+)catechin and (-)epicatechin respect to the individual dimeric procyanidins (B1, B2, B3 and B4). This could be due to different attractive forces being involved in the interaction and to consequent formation of complexes between these compounds and proteins. Hydrophobic interactions could be probably the most relevant forces responsible for the protein ability of stitching the more polymerized proanthocyanidins molecules and to precipitate them. As for the more hydrophilic monomeric compounds, the mechanism of interaction could be different: the protein fining agents, in the acidity and composition conditions typical of wine, precipitate in the colloidal form of floccules giving complexes capable of adsorbing them by setting up hydrogen bonds between its functional groups (-COOH, -NH2, -OH, etc.) and the hydroxyl groups of the polyphenols. Moreover, the presence of aromatic groups both in the proteins and in the polyphenols may favor the formation of π- π type bonds as well.

3.8.2 Influence of proteins finings on volatile composition of white wines

As we could see above, fining agents were able to increase the efficiency of settling white must and wine, and to make the precipitation of suspended solids easier. Removal of polyphenols through fining treatment and precipitation have be showed to produce flavor balance modifications (Dufour C & Bayonove CL, 1999). From an organoleptic standpoint, fining could lead to either positive or negative changes. According to the type and quantity of fining agent used, it may make a wine softer and more elegant or, on the contrary, thinner and less attractive. This fact could mean compromise the quality and original potential varietal expression of a wine.

The interaction between fining agents or free and bound aromatic compounds depends on several factors, such as such as physical-chemical characteristics of the agent, chemical nature of the compound, and possible interactions between volatiles and other macromolecules previously linked to the fining agent (Lubbers S et al., 1993; Lubbers S et al., 1996; Moio et al., 2004). The loss of aromas can be direct (by adsorption of aromas to the protein) (Volley et al.1990) or indirect (the aromas are fixed by proteins and, when they are eliminated, they drag with them part of volatile compounds) (Lubbers S et al., 1993; Lubbers S et al., 1996).

Therefore, the fining agent has to be selected to eliminate the risks of oxidation and protein haze, but without affecting the aromatic profile excessively. Here we studied the differences generated in the aromatic composition of *Catalanesca* and *Fiano* white wines when proteins of vegetable origin were used as fining agents on a laboratory scale. The aromatic characterization of wines required qualitative and quantitative determination of different components (varietal and non varietal); for the identification of odorous molecules of wines the analytical approach of solid phase micro-extraction

(SPME) and GC MS were used. In figure 3.14 the TIC chromatograms obtained by means of headspace-SPME-GC/MS analysis of reference and treated *Catalanesca* wine are shown. The odorous molecules detected were hexylacetate, ethyl hexanoate, ethyl heptanoate, phenyl ethyl alcohol, ethyl and methyl octanoate, ethyl decanoate, ethyl succinate, and terpenes such as limonene, 3-carene and 4-carene.

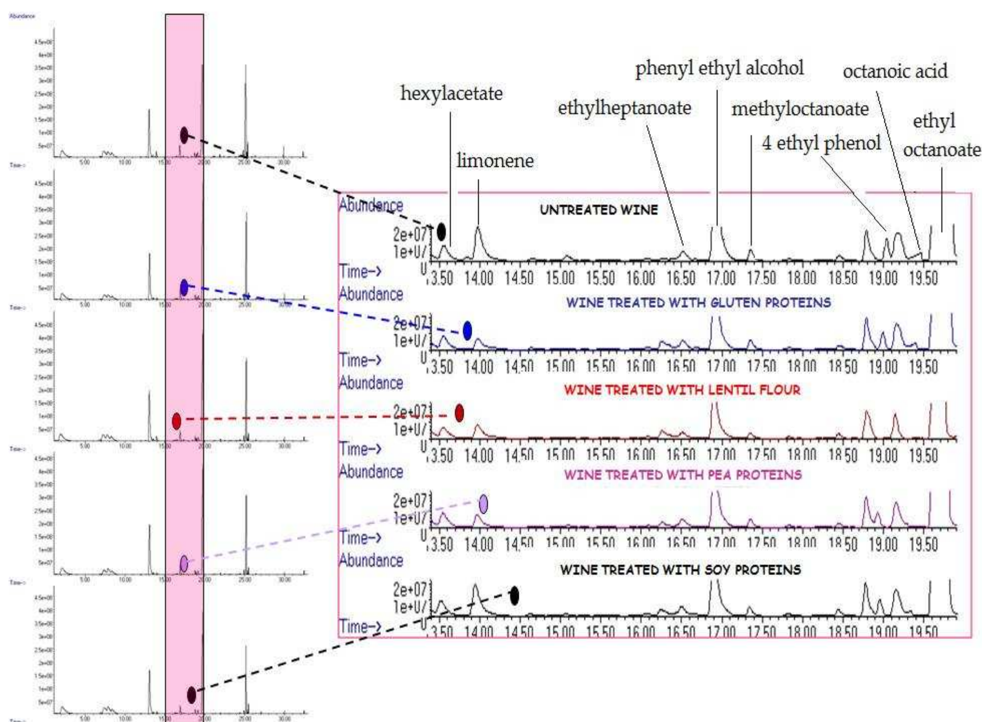

Figure 3.14. TIC chromatograms obtained with the SCAN method (m/z 45–350) by means of static headspace-GC/MS analysis on untreated and fined *Catalanesca* wine.

No differences in qualitative aroma profile were observed for control and experimental wines. In the wine treated with proteins the aromatic substances typical of the variety as well as those deriving from fermentative process were observed, meaning that the aroma of wine is preserved.

Although the use of protein fining agents did not produce appreciable changes in the overall profile, quantitative analysis showed an important decrease in the total concentration of the aromatic families responsible of fermentative and varietal aroma. The effects of protein fining agents on aroma substances varied with the chemical nature of volatile compounds and of proteins. Figure 3.15-3.16 showed the normalized peak areas of higher fermentation alcohols and esters of *Catalanesca* and *Fiano* treated wines, expressed in percentage respect to the unfined ones. Ethyl acetates of fatty acids

are produced by yeast during alcoholic fermentation and have very pleasant odors of wax and honey which contribute to the aromatic finesse of white wines. They are present at total concentrations of a few mg/l.

We observed that lentil flour had the highest affinity for fermentative aroma compounds, giving a loss which ranged between 40 and 60% of the initial content, followed by pea, soy and gluten proteins (losses between 10% and 40%). This behavior paralleled turbidity decrease, indicating that the proteins characterized by the highest ability to clarify the wine were also leading to highest loss of fermentative aroma compounds through intermolecular interactions.

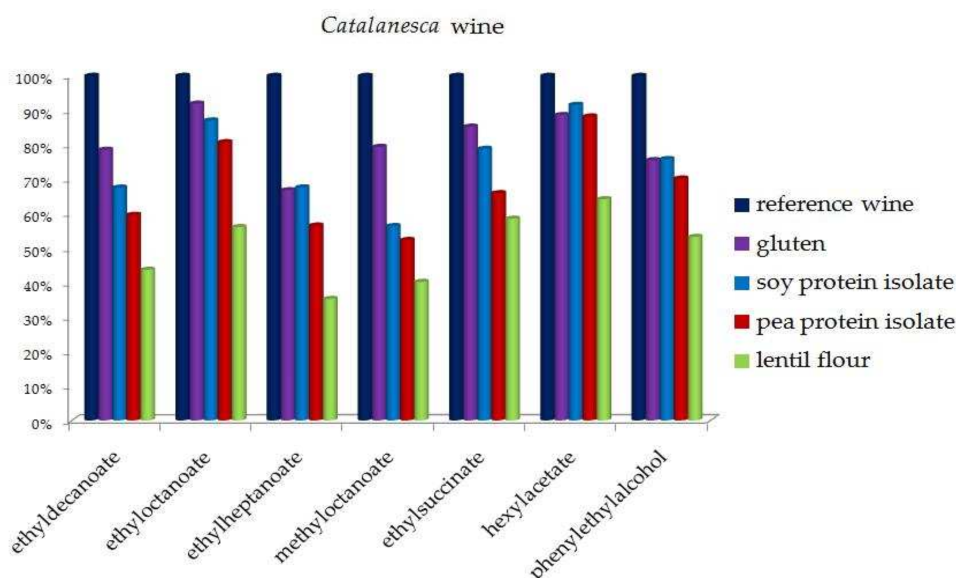

Figure 3.15. Percentage loss of fermentative aroma compounds after treatment of *Catalanesca* wine, as assessed by static headspace-GC/MS analysis

Moreover, we have to consider also the effect of protein addition on the release of aroma compounds in the headspace of wine. The presence of residual proteins in wine after fining and/or the presence of soluble complexes formed by volatile compound-protein interactions shift the partition equilibrium of the esters between the liquid phase of the sample and the gas phase. This change in the aromatic balance could cause the decrease of aroma compounds extracted in the headspace of sample.

Figure 15 shows the effect of plant proteins on fermentative aroma compounds of *Fiano* wine in comparison to those of gelatin, the most common fining agent of animal origin used in wine-making. In this case, we observe that for each fining agents the loss of volatile compounds was less drastic respect to *Catalanesca* wine, varying between 20% and 30% of that of untreated wine, and that there were not important differences in the effects caused by the use of various clarifying agents on fermentative aroma compounds.

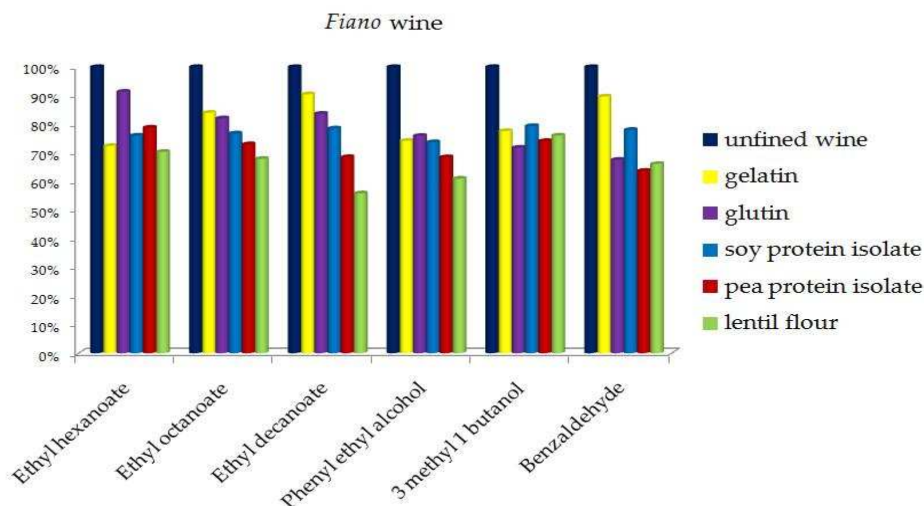

Figure 3.16. Percentage loss of fermentative aroma compounds after treatments of *Fiano* wine, as assessed by static headspace-GC/MS analysis

This finding confirmed that the effect of fining treatment on aroma was related not only to the type of fining agents but also to the type of wine, being related to its chemical- physical characteristic, its composition, and also to the moment of winemaking process in which the treatment was done. In our case, we observed that finings of vegetable origin may be more successfully used for clarify the wine at the end of alcoholic fermentation than after cold stabilization, because of the less drastic effect on fermentative aroma compounds.

Anyway, it is important to notice that the decrease of fermentative aroma compounds after fining was in the same range of gelatin for most of assayed proteins.

However, the complexity of wine flavour is made up by the proper combination of fermentative and varietal aroma compounds. These compounds have to be considered in order to evaluate the effect of fining treatment on volatile profile of wine. The quali-quantitative composition of terpenes, which are odorous molecules with low perception threshold, is strictly related to the varietal origin (Camara J S et al., 2004; Carro N et al., 1996; Mateo J J and Jimenez M, 2000; Flamini R, 2005; Petka, J et al. ,2006; Rapp A, 1998; Lopez R et al., 2002; Rosillo L et al., 1999; Oliveira J M et al., 2004). These compounds are responsible for the characteristic aroma in the called "aromatic grapes" and wines, although they are also present (at low concentrations) in simply-flavored varieties, such as *Catalanesca* and *Fiano* ones. The low quantities of varietal molecules could not provide immediate evidence of the presence of these compounds among many other odorous molecules at high concentrations in the headspace of the

wine; in fact sometimes the background spectra and the presence of coeluting chromatographic peaks calls for a very accurate analysis of spectrometric data.

In particular, we detected and quantified two monoterpenes in the form of simple hydrocarbon (limonene) and alcohol (linalool) in *Fiano* wine. Figure 3.17 shows the amount of these compounds in control and treated wine. As we can see, all protein fining agents decreased the concentration of the assayed terpenes after wine treatment, confirming their capability to interact with them. Each protein shows a different affinity for these two compounds, and also, different behavior related to fermentative aroma compounds. This finding could be explained by the different chemical nature of this compounds, responsible for their specific properties such as polarity and volatility that form the molecular basis of their interaction with protein finings.

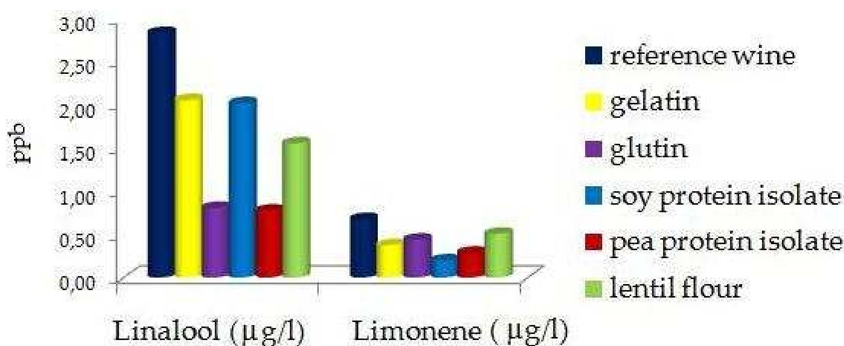

Figure 3.17. Concentration of linalool and limonene after fining

When the effects of finings on varietal and fermentative aroma compounds were evaluated, we had to consider that the olfactory perception thresholds of these substances may vary quite considerably. Certain compounds, present in trace amounts, in the order of ng/l, may play a major role in aroma, whereas more plentiful compounds may make only a minor contribution. Monoterpenes (hydrocarbons and alcohols) are among the most odoriferous compounds, especially linalool, which has a floral aroma reminiscent of rose essence. The olfactory perception thresholds of these compounds are rather low, typically at a few micrograms per liter, and the olfactory impact of terpene compounds is synergistic.

In table 3.4 are reported the amounts of fermentative and varietal aroma compounds in control and experimental *Fiano* wines and the relative odor threshold. In our case, we have to consider that the low level of fermentative aroma compounds observed was probably due to the fact that the tested wines were treated with finings ones the alcoholic fermentation had already occurred. Consequently their fermentative aroma compounds composition and concentration were still unstable.

Table 3.4. Effects of fining on the main aroma compounds in *Fiano* wine. (GL: gluten; PI: pea protein isolate; GE: gelatin; LE: lentil flour; SI: soy protein isolate).

	Odour threshould (µg/l)	Odour	UW	GE	GL (µg/l)	SI	PI	LE
Ethyl hexanoate	14 (Ferreira et al, 2000)	Apple	323 ± 12	232 ± 8	292 ± 9.3	243 ± 7.5	252 ± 5.5	225 ± 9
Ethyl octanoate	580 (Maarse et al, 1991)	Pineappl e	185 ± 7.3	157 ± 4.5	153 ± 3.7	143 ± 4.9	136 ± 4.3	127 ± 3.3
Ethyl decanoate	200 (Ferreira et al, 2000)	Flower	72 ± 23	70 ± 9	65 ± 20	61 ± 8	53 ± 11	43 ± 18
Phenyl ethyl alcohol	10000 (Guth, 1997)	Rose	2234 ±225	1661 ±234	1699 ±298	1650 ±198	1533 ±165	1365 ±113
3-Methyl-1-butanol	30000 (Guth, 1997)	Grass	705 ± 8.5	635 ± 42	587 ± 13	552 ± 78	481 ± 56	392 ± 44
Benzaldehyde	350-3500	Almond	16.7 ±1.2	14 ±0.95	11 ± 0.52	12 ± 0.26	10 ± 0.88	11 ± 0.74
Linalool	15 (Guth, 1997)	Orange flowers	3 ±1	2 ± 0.1	<1	2 ± 0.1	<1	2 ± 0.2
Limonene	10	Citrus fruit	< 1	< 1	< 1	< 1	< 1	< 1

3.8.3 Effects of fining agents on the technological parameters of young red wine

Fining ensures the stabilization and clarification of white wines through the addition of substances capable of inducing flocculation and consequently sedimentation of partially soluble components from the wine. In red wines, fining is important also because it eliminates those tannin molecules that react most readily with proteins, and are the most aggressive from an organoleptic point of view.

Astringency is one of the most important organoleptic qualities of numerous beverages, including red wines. Astringency is generally thought to originate from interactions between tannins and salivary proline-rich proteins. This oral sensation was described by ASTM as a "complex of sensations due to shrinking, drawing or puckering of epithelium as result of exposure to substances such as alums or tannins" (Gawel, R, 1998). These sensations are usually attributed to friction-based mechanisms induced by salivary protein precipitation (Bennick A, 2002). Precipitates themselves may cause a loss of lubrication of the oral epithelium (Bennick, A, 1982; Butler L G and Mole S, 1988). Excessive astringency is considered a negative characteristic for red wines. Fining treatments, removing molecules that contribute to the impression of body and volume on the palate, reduce astringency and bitterness giving softer and thinner wines.

Here we evaluated the effect of non animal proteins, once employed for white wines clarification, on *Aglianico* red wines right after winemaking, and after twelve or

twenty-four months of ageing. In fining trials were included also two preparations obtained from enzymatically hydrolyzed pea proteins in order to evaluate if the hydrolytic step, that modifies some biochemical characteristics of proteins such as molecular weight and hydrophobicity, could increase their fining efficiency while minimizing possible negative effects on wine quality.

Figure 3.18 A) and B) show the time course of clarification and the final turbidity values for control and treated wines. Lentil flour, soy proteins and one pea protein hydrolyzate showed a very good fining behaviour in the young red wine tested. The hydrolyzed pea proteins had a slower fining rate than other matrices, probably because of the time needed for the flocculate formation, but at the end of the treatment their fining efficiency was comparable to - if not better than- the native proteins and gelatin. At contrast, gluten gave a very low turbidity in the first 24 hours, but we observed an increase of turbidity at the end of the process (after seven days of treatment). This indicated a faster clarification kinetics compared to the other fining agents.

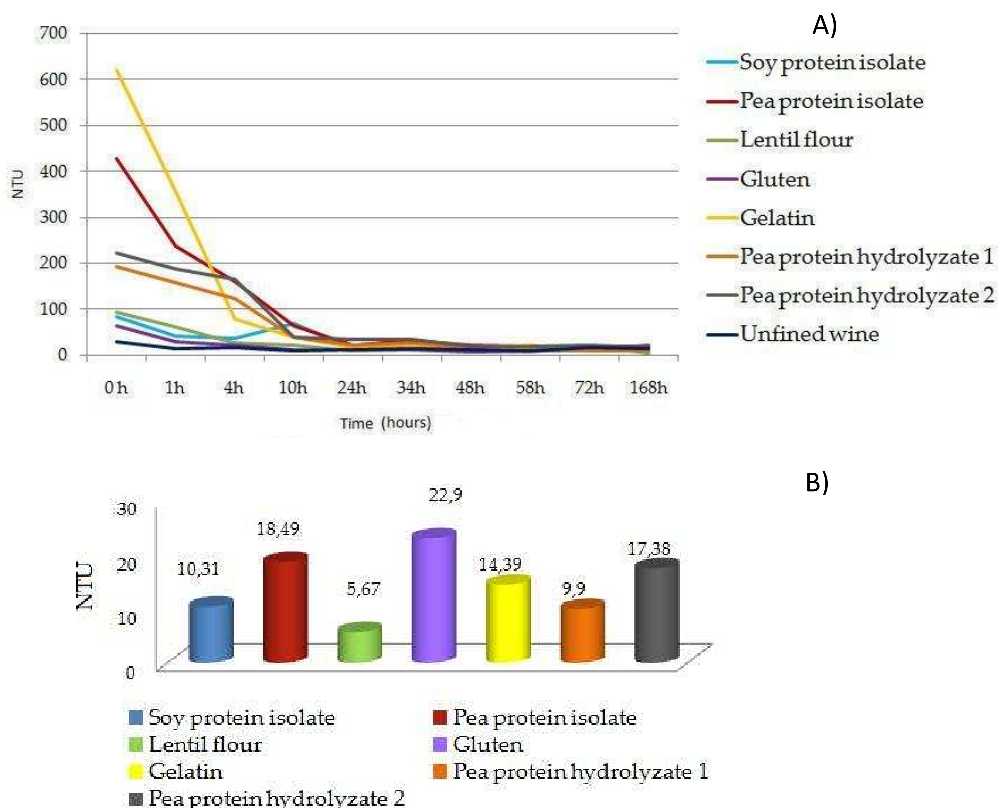

Figure 3.18. A) Time course of *Aglianico* red wine clarification. All treatment were done at a 20g protein/hl. Reference wine was obtained from spontaneous settling at low temperature without any addition of proteins. B) Effect of different fining agents on the final turbidity (168 h) of *Fiano* white wine.

The effect of fining treatments on the flavan-3-ols components in red wine was evaluated by a quantitative LC-ESI MS analysis and is summarized in figure 3.19. Lentil proteins confirmed also in red wine the high affinity for proanthocyanidins observed both in wine-like model solutions and in white wines, causing an important decrease in tannins (from 40% for monomeric compounds to 70% for proanthocyanidins trimers). Soy and gluten proteins were the fining systems which gave the lowest decrease in phenolic compounds but also the worst final turbidity.

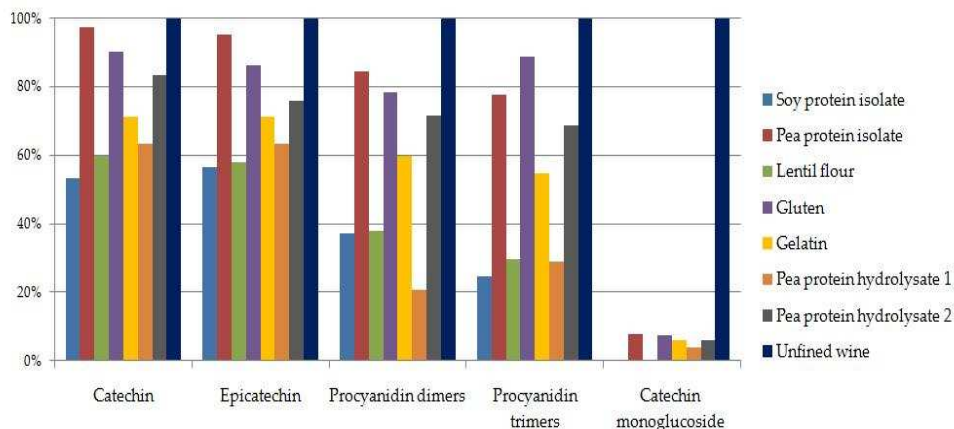

Figure 3.19. Percentage loss of individual low molecular weight proanthocyanidins in fined *Aglianico* red wine, obtained by means of LC-ESI MS analysis.

The different capability of various proteins to interact with proanthocyanidins paralleled their fining efficiencies, and it was probably related to their different amino acidic composition and conformation. The large native proteins could impede accessibility to phenolic binding sites or allowed binding to phenolic compounds, but not precipitation (for example because stabilization of adducts by hydrogen bonds may be impaired). Enzymatic hydrolysis opens the protein structure, thus improving the binding site accessibility and favoring cross linking interactions leading to precipitation. This could justify the better behavior of hydrolyzed pea proteins with respect to the corresponding native proteins.

To achieve a more complete dataset about the effects of the fining on phenolic compounds, we focused above anthocyanin profiling by using liquid chromatography combined with electrospray ionization mass spectrometry (LC-ESI MS). The anthocyanins occurring in the tested red wines are listed in table 3.5. The eight HPLC profiles were qualitative similar, and forty-four anthocyanin derivatives were identified. The simultaneous occurrence of two or more components in a single HPLC peak was a common event, as clearly shown in table 3.5. As an example, by examining the TIC profile of control wine in figure 3.20, the prominent peak at retention time 21.2 min was found to be composed of malvidin- and peonidin-3-O-glucoside (spectrum in fig. 3.20b). In addition, the molecular ions of the coumaric acid and caffeic acid esters of the 3-O-glucosides were also detected.

Table 3.5. Mean values of concentration (mg/l) and standard deviations (n = 2) for anthocyanic phenolics belonging to different chemical families (monomeric anthocyanins, anthocyanin-flavan-3-ol adducts mediated by acetaldehyde, pyranoanthocyanins and hydroxyphenyl-pyranoanthocyanins) as identified by HPLC-MS in *Aglianico* wine either unfined (UW) or fined with various plant proteins (GL: gluten; PI: pea protein isolate; PH1: Pea protein hydrolysate1; PH2: Pea protein hydrolysate2; GE: gelatin; LE: lentil flour; SI: soy protein isolate; n.d.: not detected).

	RT	*m/z*	*UW*	*GL*	*PI*	*PH2*	*GE*	*LE*	*PH1*	*SI*
de-3-O-glu	19.8	465	5,47 ±0.09	5,74 ±0.05	6,61 ±0.03	4,92 ±0.08	4,70 ±0.01	2,29 ±0.03	2,37 ±0.02	2,12 ±0.08
cy-3-O-glu	20.5	449	0,40 ±0.04	0,44 ±0.01	0,37 ±0.01	0,34 ±0.02	0,34 ±0.04	0,20 ±0.03	0,23 ±0.02	0,19 ±0.00
pe-3-O-glu	21.90	463	4,69 ±0.22	4,82 ±0.19	5,41 ±0.03	3,69 ±0.13	4,04 ±0.09	3,01 ±0.00	2,81 ±0.05	0,31 ±0.15
mv-3-O-glu	21.90	493	77,08 ±1.09	72,68 ±0.15	87,18 ±0.55	67,63 ±0.25	60,87 ±0.26	41,91 ±0.03	36,68 ±0.16	41,03 ±0.35
pt-3-O-glu	20.90	479	11,06 ±0.45	11,39 ±0.22	12,85 ±0.05	9,18 ±0.00	10,15 ±0.68	5,48 ±0.28	5,44 ±0.00	5,59 ±0.48
pt-3-O-(6-O p-cumaril)-glu	26,90	625	0,91 ±0.08	0,73 ±0.04	0,62 ±0.00	0,53 ±0.05	0,59 ±0.04	0,98 ±0.01	0,53 ±0.02	0,51 ±0.03
de-3-O-(6-O p-cumaril)-glu	25,70	611	0,43 ±0.11	0,56 ±0.08	0,43 ±0.01	0,23 ±0.12	0,28 ±0.04	0,63 ±0.01	0,23 ±0.00	0,17 ±0.03
cy-3-O-(6-O p-cumaril)-glu	26,70	595	0,35 ±0.08	0,27 ±0.00	0,12 ±0.01	0,10 ±0.00	0,12 ±0.00	0,31 ±0.01	0,10 ±0.01	nd
pe-3-O-(6-O p-cumaril)-glu	28,11	609	1,69 ±0.38	1,49 ±0.05	1,16 ±0.10	1,02 ±0.06	1,13 ±0.08	1,68 ±0.40	1,02 ±0.16	1,13 ±0.25
mv-3-O-(6-O p-cumaril)-glu	28,16	639	11,03 ±1.25	10,99 ±0.80	7,87 ±0.95	6,50 ±0.45	6,57 ±0.22	12,34 ±0.70	6,50 ±0.31	6,91 ±0.11
pt-3-O-(6-O p-caffeoil)-glu	28,16	641	1,06 ±0.10	0,98 ±0.21	0,81 ±0.00	0,73 ±0.09	0,59 ±0.13	1,14 ±0.35	0,73 ±0.40	0,67 ±0.00
pe-3-O-(6-O p-caffeoil)-glu	27,90	625	0,17 ±0.09	0,14 ±0.00	0,11 ±0.13	0,08 ±0.05	0,07 ±0.00	0,17 ±0.00	0,08 ±0.07	0,11 ±0.05
mv-3-O-(6-O p-caffeoil)-glu	26,40	655	0,18 ±0.03	0,24 ±0.01	0,17 ±0.03	0,13 ±0.02	0,16 ±0.00	0,22 ±0.01	0,13 ±0.01	0,15 ±0.02
cy-3-O-(6-O p-caffeoil)-glu	28,00	611	0,10 ±0.01	n.d.	0,10 ±0.00	0,16 ±0.03	0,14 ±0.01	0,14 ±0.01	0,16 ±0.02	0,12 ±0.01
mv-4-vinylphenol	20,44	447	0,54 ±0.22	0,24 ±0.13	0,18 ±0.06	0,13 ±0.04	0,19 ±0.00	0,41 ±0.01	0,13 ±0.00	0,16 ±0.09
mv-3-O-glu-4-vinylphenol	27,28	609	0,32 ±0.05	0,21 ±0.01	0,07 ±0.00	0,10 ±0.01	0,14 ±0.00	0,27 ±0.02	0,10 ±0.01	0,10 ±0.00
pe-3-O-glu-4-vinylguaiacol	29,01	609	0,32 ±0.00	0,26 ±0.01	0,19 ±0.03	0,20 ±0.00	0,21 ±0.01	0,31 ±0.00	0,20 ±0.00	0,22 ±0.08

mv-3-O-glu-4-vinylguaiacol	28,16	639	11,10 ±0.98	10,90 ±1.09	7,87 ±0.10	6,42 ±0.55	6,63 ±0.23	12,15 ±0.46	6,42 ±0.55	4,47 ±0.94
mv-3-O-glu pyruvic acid (visitin A)	25,89	561	0,24 ±0.09	0,24 ±0.05	0,22 ±0.00	0,14 ±0.00	0,24 ±0.10	0,10 ±0.05	0,14 ±0.06	0,15 ±0.01
pe-3-O-glu pyruvic acid	25,16	531	2,61 ±0.17	2,02 ±0.24	2,05 ±0.21	1,40 ±0.14	1,41 ±0.41	2,25 ±0.12	1,40 ±0.13	1,52 ±0.52
de-3-O-glu pyruvic acid	19,90	533	1,86 ±0.35	1,72 ±0.12	1,44 ±0.22	1,22 ±0.01	1,41 ±0.14	2,06 ±0.44	1,22 ±0.15	1,29 ±0.10
mv-3-O-coumaroil- glu pyruvic acid	25,50	707	1,11 ±0.41	0,95 ±0.11	0,83 ±0.09	0,22 ±0.00	0,19 ±0.00	1,08 ±0.12	0,22 ±0.09	0,16 ±0.01
(epi)cat-mv-3-O-glu	19,80	781	2,44 ±0.52	2,66 ±0.12	2,28 ±0.21	0,65 ±0.15	0,83 ±0.29	2,75 ±0.10	0,65 ±0.01	0,75 ±0.09
(epi)cat-pe-3-O-glu	19,80	751	0,25 ±0.08	0,23 ±0.00	0,20 ±0.00	0,06 ±0.01	0,05 ±0.01	0,26 ±0.03	0,06 ±0.01	0,04 ±0.01
(epi)cat-mv-3-O-couglu	25,03	927	0,27 ±0.05	0,28 ±0.00	0,27 ±0.01	0,05 ±0.02	0,05 ±0.00	0,31 ±0.01	0,05 ±0.00	0,06 ±0.01
di(epi)cat-mv-3-O-glu	20,82	1069	0,15 ±0.02	0,14 ±0.00	0,12 ±0.05	0,02 ±0.00	0,01 ±0.01	0,14 ±0.01	0,02 ±0.00	0,01 ±0.01
mv-3-O-glu-8-ethyl-(epi)cat	23,97	809	4,92 ±0.52	4,73 ±0.35	4,14 ±0.25	2,03 ±0.12	1,99 ±0.45	5,93 ±0.21	2,03 ±0.44	2,01 ±0.11
mv-3-O-glu-8-ethyl-(epi)cat	24,58	809	8,27 ±0.78	6,56 ±0.35	6,14 ±0.32	3,19 ±0.12	1,17 ±0.09	8,90 ±0.41	3,19 ±0.35	3,57 ±0.23
mv-3-O-glu-8-ethyl-(epi)cat	25,04	809	2,82 ±0.21	3,06 ±0.15	1,48 ±0.08	1,02 ±0.35	3,02 ±0.17	3,26 ±0.19	1,02 ±0.12	1,02 ±0.04
mv-3-O-glu-o-ethyl(epigallo)gallocat	23,24	821	0,55 ±0.01	0,46 ±0.12	0,56 ±0.22	0,13 ±0.05	0,10 ±0.01	0,60 ±0.05	0,13 ±0.01	0,10 ±0.01
mv-3-O-glu-o-ethyl(epigallo)gallocat	25,00	821	0,37 ±0.05	0,29 ±0.01	0,32 ±0.00	0,06 ±0.00	0,05 ±0.01	0,31 ±0.01	0,06 ±0.00	0,03 ±0.01
pe-3-O-coumaroil-glu-8-ethyl-(epi)cat	27,45	925	0,25 ±0.06	0,17 ±0.04	0,16 ±0.03	0,08 ±0.03	0,07 ±0.01	0,24 ±0.01	0,08 ±0.00	0,04 ±0.00
mv-3-O-coumaroil-glu-8-ethyl-(epi)cat	27,45	955	2,63 ±0.25	2,21 ±0.16	1,80 ±0.32	0,87 ±0.10	0,97 ±0.26	2,26 ±0.23	0,87 ±0.15	0,88 ±0.30
mv-3-O-glu-4-vinyl(epi)cat	23,24	805	1,45 ±0.35	1,17 ±0.12	1,20 ±0.15	0,71 ±0.05	0,68 ±0.13	1,36 ±0.15	0,71 ±0.09	0,71 ±0.25
mv-3-O-glu-4-vinyl(epi)cat	25,00	805	0,87 ±0.20	0,80 ±0.15	0,78 ±0.09	0,44 ±0.12	0,42 ±0.19	0,88 ±0.18	0,44 ±0.02	0,45 ±0.05
mv-3-O-glu-4-vinyl-di(epi)cat	21,14	1093	0,01 ±0.00	n.d.	0,01 ±0.00	nd	nd	0,02 ±0.00	nd	0,01 ±0.00
mv-3-O-glu-4-vinyl-di(epi)cat	24,31	1093	0,09 ±0.01	0,08 ±0.01	0,09 ±0.00	0,02 ±0.00	0,01 ±0.01	0,09 ±0.00	0,02 ±0.00	0,02 ±0.01

mv-3-O-couglu-4-vinyl-(epi)cat	28,60	951	0,77 ±0.10	0,61 ±0.01	0,40 ±0.02	0,30 ±0.02	0,34 ±0.09	0,76 ±0.00	0,30 ±0.01	0,31 ±0.01
mv-3-O-couglu-4-vinyl-(epi)cat	29,30	951	0,19 ±0.09	0,18 ±0.01	0,18 ±0.01	0,10 ±0.09	0,16 ±0.02	0,26 ±0.01	0,10 ±0.00	0,11 ±0.01
mv-3-O-acglu-4-vinyl-(epi)cat	25,00	847	0,11 ±0.01	0,13 ±0.01	0,11 ±0.01	0,08 ±0.00	0,03 ±0.00	0,11 ±0.01	0,08 ±0.00	0,05 ±0.00
mv-3-O-acglu-4-vinyl-(epi)cat	26,29	847	0,41 ±0.14	0,33 ±0.01	0,30 ±0.20	0,12 ±0.15	0,18 ±0.01	0,37 ±0.09	0,12 ±0.11	0,12 ±0.01
mv-3-O-(6-O-ac)-glu	25,43	535	9,25 ±0.90	8,51 ±0.50	6,83 ±0.20	5,31 ±0.15	6,15 ±0.29	9,43 ±0.45	5,31 ±0.12	6,11 ±0.10
mv-3-O-glu acetaldehyde	23,15	517	1,91 ±0.21	1,54 ±0.12	1,36 ±0.10	1,08 ±0.12	1,83 ±0.09	1,93 ±0.04	1,08 ±0.10	1,97 ±0.20
TOTAL ANTHOCYANIN CONTENT			***171,41***	***161,80***	***166,01***	***121,67***	***118,51***	***129,95***	***83,43***	***85,66***
RESIDUAL, % OF INITIAL				***94,39***	***96,85***	***70,98***	***69,14***	***75,81***	***48,67***	***49,97***

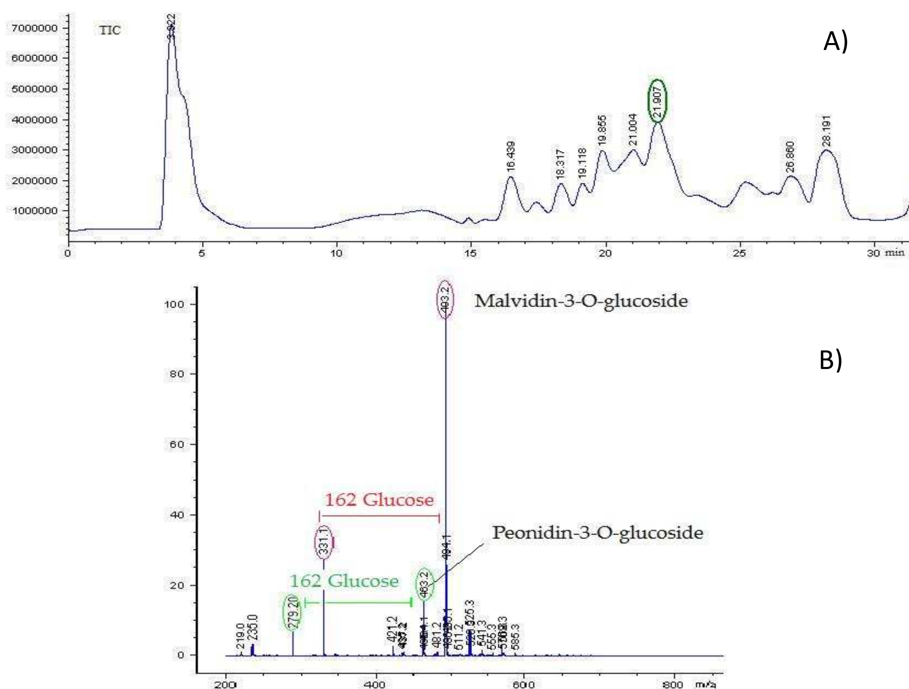

Figure 3.20. A) TIC (total ion current) chromatogram obtained in positive ion mode by injection of unfined Aglianico wine; B) ESI mass spectrum obtained from the total ion current chromatogram for 21.2 minute elution time, indicating the co-presence of malvidin-3-O-glucoside (m/z 493) and peonidin-3-O-glucoside (m/z 463).

The other anthocyanin compounds identified included adducts with pyruvic acid and acetaldehyde, and catechin derivatives formed by copigmentation. Copigmentation involves complexation phenomena, generally at low bond energy (hydrogen bonds and hydrophobic interactions), either between the various forms of anthocyanins or between anthocyanins and other, mostly colorless, phenolic compounds. A mechanism for cycloaddition on anthocyanins consists of a cycloaddition between a flavylium ion and compounds with a polarized double bond. In particular, the detected vinyl-phenol derivatives result from the decarboxylation of p-coumaric acid by yeast decarboxylases and by the consequent reaction with malvidin, either as a monoglucosides or in the form of an acylated monoglucosides (p-coumarylglucoside). The double bond is added between the carbon 4 of the anthocyanin and the oxygen on carbon 5, forming a new oxygen heterocyclic species. The resulting compound is colorless and recovers unsaturated structure and color upon oxidation.

Another group of pigments identified in *Aglianico* reference wine corresponded to the addition of pyruvic acid onto anthocyanins (Bakker J and Timberlake C F, 1997). Compared with other pigments in wine, these copigmented products were present in very small quantities (table 3.5). However, they have been reported to be relatively stable and their concentration changes very slowly during ageing.

By comparing the estimate amount of anthocyanin compounds of control wine with experimental ones, we found that all protein fining agents, except the first pea protein hydrolyzate and soy proteins, caused a general decrease of the total anthocyanins content lower than that caused by gelatin (table 3.5). However, taking into account the decrease of pigments in terms of individual molecules, we observed that the relative amount of some compounds increased after treatment. This was the case for some anthocyanin monoglucosides (delphinidin, peonidin and malvidin-3O-glucoside) upon treatment with gluten and pea proteins, and for some copigmented anthocyanins following treatment with lentil proteins. This could be justified by the capability of these fining agents to interact with some pigments and to prevent them from become bound to those solids that were removed by the racking step after the spontaneous settling of control wine.

Anthocyanins are the main agents responsible for the color of red grapes and of the wines produced from them. Colour is one of the most important parameters of the quality of wine because it is the first sensory property perceived by both the consumer and the winemaker. Many factors responsible for the color of red wine could be affected by fining treatments (such as the anthocyanin content), but also the presence of other components that cause both a color shift towards violet (bathochromic effect) and an increase in color intensity (hyperchromic effect). In particular, metal cations (mainly Al^{3+}, Fe^{3+}, Cu^{2+}, Mg^{2+}, that reportedly are found associated with plant-derived proteins), are able to form complexes with anthocyanins that have two phenol moieties in the ortho-position on the B nucleus (delphinidin, petunidin and cyanidin). These are held responsible for bathochromic effects of varying intensity. For this reason, changes

in composition of red wines colour could occur after fining treatments and we have to consider them. The color analysis was carried out by means of optical density measurements at 420 and 520 nm, with an additional measurement at 620 nm to include the blue component of young red wines.

Figure 3.21 A) and B) shows changes in the colour intensity index and in tonality during wine treatments. Although fining agents decreased the color intensity at the end of the fining, the tonality remained stable, as expected in view of the previous reports of the same effects.

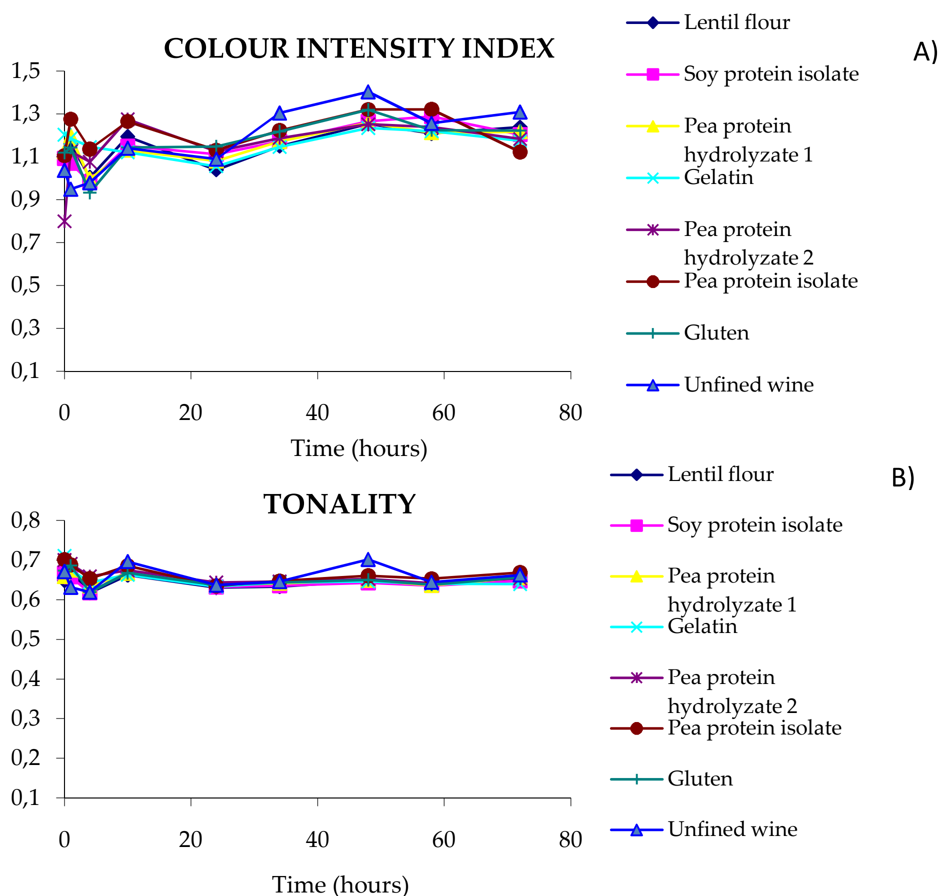

Figure 3.21. Time course of changes in colour intensity index (A) and tonality (B) of A*glianico* red wine during the fining treatment.

The variation in absorbance (420, 520 and 620 nm) responsible for the yellow, red and blue colours respectively, is represented in figure 3.22. These data provide information about the influence of protein substances on colour compounds. In general, treatments slightly lowered the intensity of the three colours. The effect on the yellow and red colours was more pronounced than on the blue one. The blue colour in young red wines is due to the association between pigments and copigments, and also involves

other substances, mainly derivatives of the flavonol and the flavone subgroups (Boulton R, 2001).

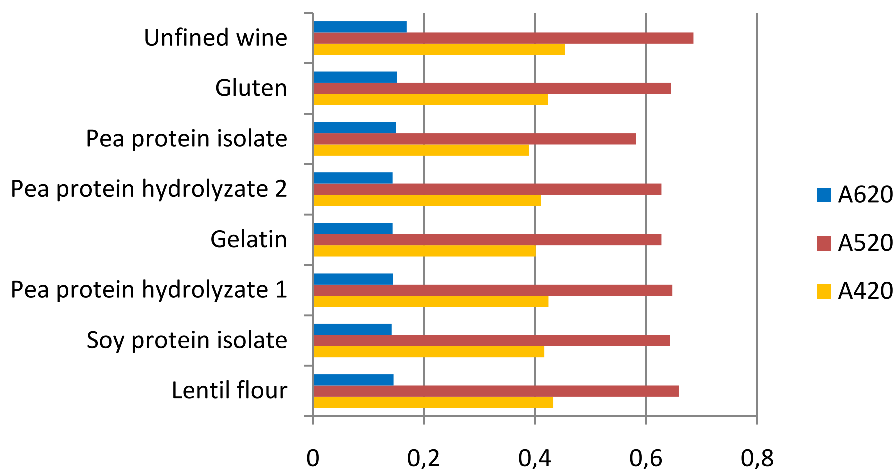

Figure 3.22. Absorbance at 420, 520, 620 nm as a function of the fining agent

3.8.4 Effects of fining agents on volatile composition of young red wine

The analysis of volatile compounds represented a preliminary step in order to obtain more data about the effect of fining treatments with plant proteins on sensorial quality of young red wine. Varietal and fermentative aroma molecules detected in *Aglianico* wine are indicated in fig. 3.23, where the TIC chromatogram obtained by means of static headspace-GC/MS analysis (SCAN method) is reported.

The terpene composition of *Aglianico* (table 3.6) wine appeared more complex in comparison with other wines made with non-aromatic autochthonous grapes: some terpenes such as α-pinene, β-pinene, limonene, 3-carene, and geraniol were now detectable. The presence of β-damcenone, norisoprenoid compound related to floral and fruit odorous nuances, with odor threshold 0.05 ppb (Lopez et al., 2002) was also observed.

In the TIC chromatogram (figure 3.23) peaks with the largest areas correspond to the fermentation compounds such as ethyl ester, acetate esters, fatty acids, and alcohols. They all probably play a significant role in the remarkable fruity notes of this wine.

Table 3.6. Fermentative and varietal aroma compounds in Aglianico red wine.

tr	AGLIANICO WINE	ODOROUS DESCRIPTOR
2.7	3-methyl, 1-butanol	Malt
4.86	Ethyl butanoate	Fruit
5.3	2-3 butanediol	
7.37	Ethyl pentanoate	
8.04	1-hexanol	
8.39	Isoamyl acetate	Banana
10.46	Methyl hexanoate	/
10.70	α-pinene	Pine oil
11.68	Benzaldehyde	/
12.34	1-heptanol	/
12.47	b-pinene	Pine oil
13.44	Ethyl hexanoate	Apple
13.95	Esyl acetate	Fruit
14.00	3-carene	Orange
14.4	Limonene	Citrus fruit
14.76	Benzyl alcohol	/
14.86	Benzeacetaldehyde	/
15.00	Ethyl-2-hexenoate	/
15.97	1-octanol	/
16.89	Ethyl eptanoate	Fruit
17.3	Phenyl ethyl alcohol	Rose
17.72	Methyl octanoate	/
17.99	Limonene oxide cis	/
18.15	Limonene oxide trans	/
19.45	Butanoic acid, diethyl ester	/
19.7	7-ethyl octenoate	/
19.85	Methyl salicylate	/
20.05	Ehtyl octanoate	Pineapple
21.52	Isopentyl hexanoate	/
21.65	Geraniol	Rose
21.68	2-phenyl ethyl acetate	Fruit
22.83	Ethyl nonanate	/
23.59	Mehtyl decanoate	/
24.7	b-damascenone	/
25.5	Ehtyl decanoate	Fruit, grape
26.42	α-ionone	Flower
26.78	3-methyl, butyl octanoate	/
28.48	BHT	/

As we can see from TIC chromatograms, qualitative composition of wines fined with different proteins was the same found for untreated wine (figure 3.23).

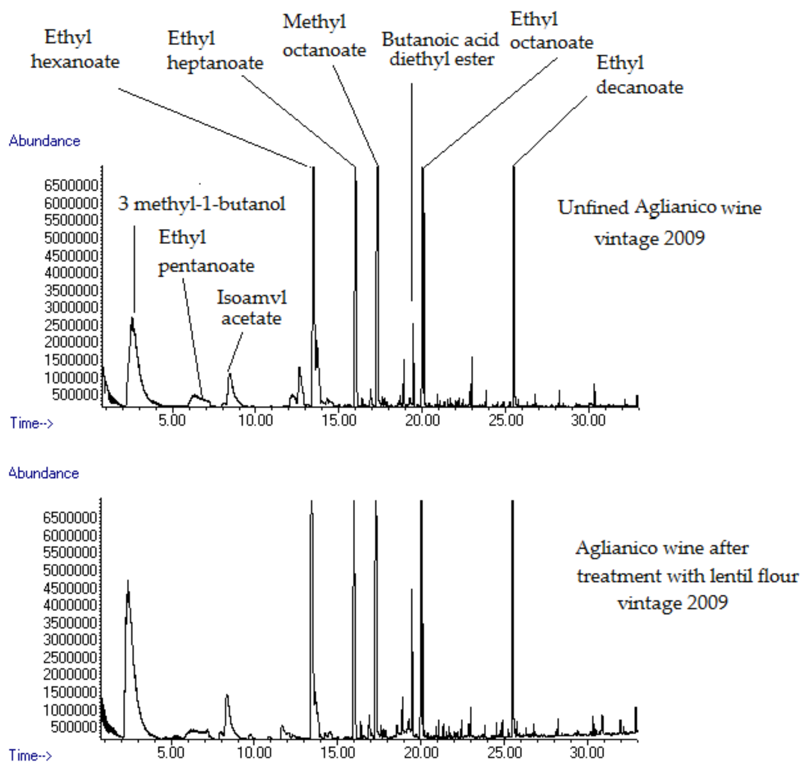

Figure 3.23. TIC chromatograms obtained with SCAN method (m/z 45–350) by means of static headspace-GC/MS analysis on *Aglianico* wine either unfined (A) or fined with lentil proteins (B).

After treatments with non animal proteins the losses of fermentative aroma compounds of this wine, in particular of esters, were found relatively limited with respect to what observed for white wines (figure 3.24). This result was not surprising and could be explained by the presence in red wines of one or more components (such as tannin, anthocyanins and their copigmented compounds) able to interact with hydrophobic compounds (such as esters) and to prevent them from being fixed on solids which were removed by racking.

Each fining agent showed different affinity for fermentative and aromatic compounds. On the basis of the protein chemical composition and biochemical characteristics (and of aroma volatility) it is not possible at the moment to understand why one protein affected aroma less than other. Red wines are a very complex biochemical medium containing much components which could have a “protective action” against the loss of volatiles. To explain the differences observed among proteins, it will be necessary to work with a model wine having a controlled composition.

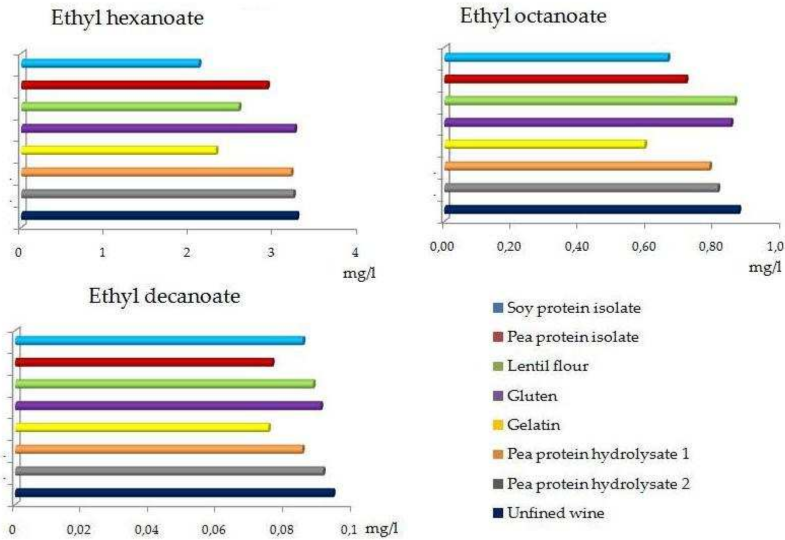

Figure 3.24. Percentage loss of esters in Aglianico wine after various treatments, as obtained by means of static headspace-GC/MS analysis

It is important to point out that the decrease of aroma compounds in this wine was less drastic for plant proteins than for gelatin (figure 3.24-3.25). Lentil flour, which affected aroma compounds of white wines more than other matrices, had a behaviour comparable to the other tested protein when used on red wine. This finding confirm the influence of the wine matrix on the fining efficiency.

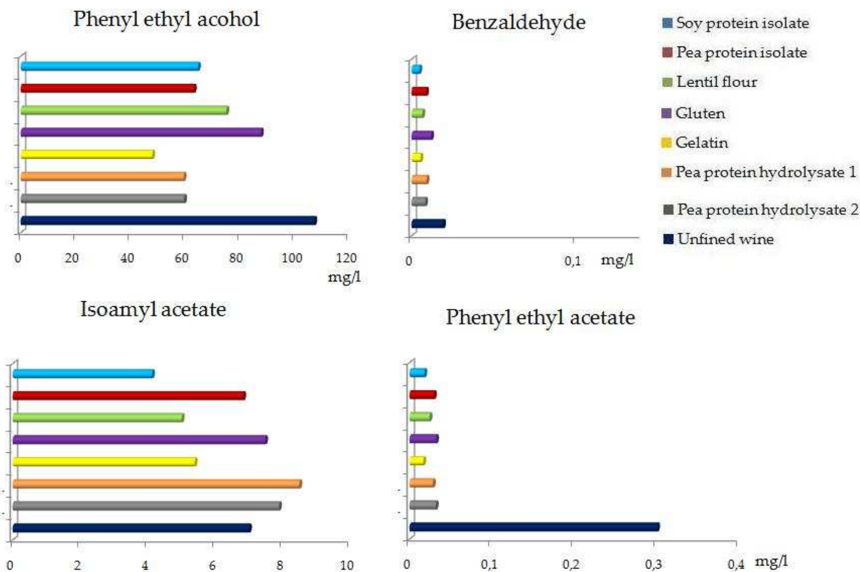

Figure 3.25. Percentage loss of various aroma compounds in *Aglianico* wine after treatments, obtained by means of static headspace-GC/MS analysis

3.8.5 Effects of fining agents on aged red wines

In the final part of this PhD project, the plant proteins tested on white and young red wines were used to fine red wine after twelve and twenty-four months of storage, thus allowing to evaluate the effect of tratments on aged wine. All protein were able to reset to zero the turbidity of wine after treatment, obtaining the same fining efficiency of the traditionally used animal protein (data not shown).

The approach used to understand the positive or negative effects of finings on wine quality was focused on the implication of the treatment on reactions involving phenolic compounds, particularly anthocyanins. The presence of acetaldehyde-mediated anthocyanin-tannin condensation products and of pyranoanthocyanins compounds is important for improvement and stabilization of wine colour, in what these species are more resistant to pH variations, to SO_2 bleaching, and to further anthocyanin oxidation (Escribano-Bailon T et al., 2001). In table 3.7 and table 3.8 are reported the effect of finings on anthocyanic phenolic compounds of *Aglianico* red wine treated after twelve and twenty-four months of ageing respectively. The observed decrease in total anthocyanin content of aged wines respect to the young one is due to combination of reactions with various other compounds in the wine, especially tannins, as well as to breakdown reactions.

Table 3.7. Mean values of concentration (mg/l) and standard deviations (n = 2) of anthocyanic phenolic compounds identified by HPLC-MS in 2008 vintage *Aglianico* wine either unfined (UW) or fined with various plant proteins (GL: gluten; PI: pea protein isolate; PH1: Pea protein hydrolysate1; PH2: Pea protein hydrolysate2; GE: gelatin; LE: lentil flour; SI: soy protein isolate; n.d.: not detected).

	UW	*GL*	*PI*	*PH2*	*GE*	*LE*	*PH1*	*SI*
cy-3-O-glu	0,24±0.12	0,44 ± 0.11	0,63± 0.01	0,81± 0.09	0,14± 0.02	0,23± 0.02	0,25± 0.06	0,67± 0.01
pe-3-O-glu	2,36±0.36	1,93± 0.19	3,41±0.25	2,76± 0.14	1,58± 0.20	1,49± 0.21	2,07± 0.26	2,34± 0.30
mv-3-O-glu	10,65±0.40	7,22± 0.22	9,71± 0.39	7,15± 0.25	8,46± 0.19	7,81± 0.22	8,99± 0.32	8,61± 0.20
pt-3-O-glu	0,82±0.12	0,83± 0.21	1,02± 0.26	1,04± 0.09	0,69± 0.09	0,62± 0.01	0,72± 0.05	0,78± 0.15
pe-3-O-(6-O p-cumaril)-glu	0,70±0.10	0,49± 0.08	0,52± 0.10	0,39± 0.02	0,49± 0.12	0,44± 0.16	0,54± 0.09	0,46± 0.04
mv-3-O-(6-O p-cumaril)-glu	2,40±0.26	1,64± 0.12	1,63± 0.20	1,20± 0.18	1,65± 0.04	1,58± 0.07	1,87± 0.12	1,43± 0.19
pe-3-O-(6-O p-caffeoil)-glu	1,79± 0.16	1,16± 0.02	1,36± 0.21	0,81± 0.01	1,41± 0.05	1,17± 0.07	1,35± 0.12	0,92± 0.15
pe-3-O-glu-4-vinylguaiacol	0,97± 0.10	0,73± 0.12	0,72± 0.07	0,49± 0.06	0,68± 0.19	0,68± 0.16	0,68± 0.17	0,55± 0.09
mv-3-O-glu-4-vinylguaiacol	0,14± 0.12	n.d.	n.d.	n.d.	0,12± 0.12	n.d.	n.d.	n.d.
pe-3-O-glu pyruvate	0,26± 0.03	0,19± 0.01	0,22± 0.01	0,14± 0.00	0,20± 0.02	0,16± 0.02	0,21± 0.03	0,16± 0.00
de-3-O-glu	0,34± 0.09	0,23± 0.03	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

pyruvate								
mv-3-O-couglu pyruvate	0,47± 0.07	0,27± 0.03	0,45± 0.04	0,21± 0.01	0,28± 0.02	0,22± 0.02	0,50± 0.00	0,26± 0.01
mv-3-O-(6-O-ac)-glu	2,75± 0.46	1,81± 0.13	2,18± 0.32	1,56± 0.19	2,01± 0.14	1,86± 0.17	2,32± 0.22	1,62± 0.14
mv-3-O-glu acetaldehyde	0,70± 0.08	0,55± 0.12	0,50± 0.04	0,38± 0.03	0,47± 0.04	0,55± 0.02	0,48± 0.01	0,42± 0.09
TOTAL ANTHOCYANIN CONTENT	***26,45***	***18,52***	***23,43***	***18,04***	***19,26***	***17,80***	***21,30***	***19,54***
RESIDUAL, % OF INITIAL		***70,0***	***88,6***	***68,2***	***72,8***	***67,3***	***80,5***	***73,9***

The *Aglianico* red wine of 2008 vintage showed a less complex composition in all anthocyanin compounds than the same young red wine, and also than the wine of previous vintage (2007). This results are expected because anthocyanin molecules are not very stable, so their concentration in wine drops sharply during the first few months of aging. Many changes occur in the composition of the wine during aging, accompanied by the development of color, aroma and flavor. The way in which a wine ages depends on both the conditions and the wine's specific characteristic. Its phenol composition, characterized by the total quantity of phenols, by the ratio of the various pigments (tannins/anthocyanins), and by the type of tannins (seed tannins consisting of procyanidins polymerized to varying degrees, whereas skin tannins have more complex structures). Among the conditions that affect the process are oxidation–reduction phenomena that take place in the wine itself, temperature, and time. Differences in even one of these factors could explain the different final composition in anthocyanin compounds of the three wines considered here.

Table 3.8. Mean values of concentration (mg/l) and standard deviations (n = 2) for anthocyanic phenolic compounds identified by HPLC-MS in 2007 vintage *Aglianico* wine either unfined (UW) or fined with various plant proteins (GL: gluten; PI: pea protein isolate; PH1: Pea protein hydrolysate1; PH2: Pea protein hydrolysate2; GE: gelatin; LE: lentil flour; SI: soy protein isolate; n.d.: not detected).

	UW	*GL*	*PI*	*PH2*	*GE*	*LE*	*PH1*	*SI*
de-3-O-glu	0,27 ± 0.01	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
cy-3-O-glu	0,30 ± 0.01	n.d.	n.d.	0,17 ± 0.04	n.d.	n.d.	0,15 ±0.00	0,16 ± 0.01
pe-3-O-glu	0,65 ± 0.03	n.d.	n.d.	0,44 ± 0.03	0,29 ± 0.01	0,34 ± 0.01	0,50 ± 0.05	0,70 ± 0.009
mv-3-O-glu	9,64 ± 0.51	2,30 ± 0.41	2,53 ± 0.20	2,29 ±0.15	2,15 ± 0.20	2,61 ± 0.29	2,29 ± 0.19	2,46 ± 0.21
pt-3-O-glu	0,84± 0.009	n.d.	0,18 ± 0.03	0,20 ± 0.04	n.d.	0,19 ± 0.01	0,30 ± 0.02	0,20± 0.03

de-3-O-(6-O p-cumaril)-glu	0,16± 0.02	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
cy-3-O-(6-O p-cumaril)-glu	0,17± 0.01	n.d.	0,17± 0.0	0,12± 0.0	n.d.	n.d.	0,14±0.01	n.d.
pe-3-O-(6-O p-cumaril)-glu	2,08 ± 0.23	n.d.	n.d.	1,11 ±0.11	0,21 ± 0.0	n.d.	n.d.	n.d.
mv-3-O-(6-O p-cumaril)-glu	0,52± 0.07	0,16 ± 0.01	0,23 ± 0.00	n.d.	0,17 ± 0.0	0,16± 0.01	n.d.	0,17± 0.01
pe-3-O-(6-O p-caffeoil)-glu	2,27± 0.27	1,12 ± 0.19	n.d.	0,92 ± 0.09	n.d.	1,05 ± 0.11	0,96 ± 0.07	0,70 ± 0.11
mv-3-O-(6-O p-caffeoil)-glu	0,14± 0.01	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
pe-3-O-glu-4-vinylguaiacol	2,08± 0.24	1,32 ± 0.14	0,15 ± 0.01	1,11 ± 0.12	n.d.	1,08± 0.24	1,29 ± 0.14	0,78 ± 0.15
mv-3-O-glu-4-vinylguaiacol	0,20± 0.03	0,13 ± 0.0	0,20 ± 0.02	n.d.	1,67 ± 0.19	n.d.	n.d.	0,11 ± 0.01
mv-3-O-glu piruvic acid	0,20± 0.01	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
pe-3-O-glu piruvic acid	1,79± 0.20	0,25 ± 0.01	0,32 ± 0.03	0,23 ± 0.01	0,21 ± 0.02	2,29 ± 0.23	0,24 ± 0.01	0,17 ± 0.01
de-3-O-glu piruvic acid	1,49± 0.19	0,24	0,43	0,27	0,23	0,30	0,25	0,25
mv-3-O-couglu piruvic acid	0,11± 0.00	0,17 ± 0.01	n.d.	0,14 ± 0.02	0,2 ± 0.01	0,28 ± 0.02	n.d.	0,19 ± 0.01
(epi)cat-mv-3-O-glu	0,70± 0.03	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
di(epi)cat-mv-3-O-glu	0,01± 0.00	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
mv-3-O-glu-8-ethyl-(epi)cat	0,18± 0.00	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
mv-3-O-glu-8-ethyl-(epi)cat	0,36± 0.01	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
mv-3-O-glu-8-ethyl-(epi)cat	0,06± 0.02	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
mv-3-O-glu-o-ethyl(epigallo)gallocat	0,16± 0.01	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
mv-3-O-coumaroil-glu-8-ethyl-(epi)cat	0,14± 0.01	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
mv-3-O-glu-4-vinyl(epi)cat	0,45± 0.07	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
mv-3-O-glu-4-vinyl(epi)cat	0,18± 0.01	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
mv-3-O-couglu-4-vinyl-(epi)cat	0,19± 0.01	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
mv-3-O-(6-O-ac)-glu	1,17± 0.14	0,48 ± 0.04	0,54 ± 0.09	0,45 ± 0.04	0,43 ± 0.01	0,42 ± 0.02	0,05 ± 0.02	0,44 ± 0.05
TOTAL ANTHOCYANIN CONTENT	***16,16***	***2,20***	***1,77***	***2,06***	***2,54***	***2,20***	***1,99***	***2,20***
RESIDUAL, % OF INITIAL		***13,6***	***10,9***	***12,7***	***15,7***	***13,6***	***12,3***	***13,6***

After fining, both aged wines showed a decrease of total anthocyanin content (table 6 and table 7) without any change in colour characteristic of wine (data not shown). The

percentage loss of these compounds was much less drastic in the wine aged for twelve months than in the one for twenty-four months.

The observed effects could be only partially related to the interaction of protein finings with phenolic compounds. They could be also caused by the aeration and consequent oxidation phenomena that unavoidably occurred when the fining and following racking are carried out on a laboratory scale. The molecular oxygen introduced with this operation may react with redox-sensitive species (Fe^{2+} and Cu^{+}) present in the medium but also in protein isolates, forming Fenton-related unstable peroxides that, in turn, may oxidize other redox-active substances that are not directly oxidized by molecular oxygen, which is a slowly reacting oxidant (Wildenradt and Singleton, 1974). The reactions involved in oxidative transformations of phenols mainly involve ethanal (formation of an ethyl cross-bond between anthocyanin and tannin molecules or a cycloaddition to the anthocyanins, producing tannin-pyranoanthocyanins). It has been demonstrated that it is possible to produce ethanal by oxidizing ethanol in the presence of phenols and Fe^{3+} or Cu^{2+} ions (Wildenradt and Singleton, 1974; Ribereau-Gayon et al., 1982). Ribereau-Gayon and coworkers observed also that the proportion of anthocyanin combined with tannins in colored forms increases regularly with aeration. All these phenomena could explain because the decrease in total anthocyanin content of wine after treatment did not affect the colour characteristic of treated wines.

3.8.6. Research of residual gluten proteins in wines

Known allergenic food proteins are traditionally used in wine-making as fining agents (milk, evaporated milk, casein, potassium caseinate, isinglass, egg white). Between them, also proteins of vegetable origins, and in particular gluten and pea proteins, could cause severe responses or chronic intolerance (celiac disease), making it very important to know if they are completely eliminated after clarifying.

New labeling legislation by Food Standards Australia New Zealand requires that wine labels identify any detectable potentially allergenic processing aids, additives, or other ingredients. Similar regulations are under consideration by Canada, by the European Union, and by the US. From the wine-makers’ perspective, only minimal residual protein should remain after fining to avoid visible precipitates. There is no published literature on whether any proteins derived from fining agents are present in the finished wine, and whether these could provoke an allergic reaction, making further investigations essential.

We measured the total level of proteins before and after treatment by Bradford assay (table 3.9) and characterized residual proteins of wine by SDS PAGE (data not shown). No detectable amounts of soluble fining agents was measured in white wines, likely because all tested proteins are almost totally insoluble at pH of the wine.

Table 3.9. Total protein content of Fiano white wines after treatment with plant protein.

	mg/ml
Reference wine	0,053
Pea proteins fined wine	0,052
Soy proteins fined wine	0,050
Lentil folur fined wine	0,053
Gluten proteins fined wine	0,052
Gelatin fined wine	0,051

There are no studies suggesting thresholds for plant proteins allergens, if not for gluten. Indeed, the exact lower limit for avoiding an allergenic reaction is difficult to assess because of the number and variety of involved factors, such as individual sensitivity, age, gender, genetic constitution, dietary habits, and other - as yet largely unidentified - environmental factors.

Under the FDA's proposed rule for gluten-free labeling, a food or drink may be labeled gluten-free if the final product contains ingredients derived from wheat, barley, rye, and triticale that have been processed to remove gluten (eg, wheat starch) and the final food or drink product contains < 20 parts per million (ppm or mg/kg) gluten. In view of this fact, it was important to verify and ensure that no residues were left in wine fined with gluten and, that there was no risk of triggering a response in sensitive individuals.

In this study, we assessed gluten content of treated white and red wines by means of commercial assay sandwich R5 ELISA. This method is based on the R5 monoclonal antibody that recognizes the potentially celiac toxic epitope (ie, the antibody-binding site) QQPFP (Gln-Gln-Pro-Phe-Pro), and some closely related sequences that are present in wheat gliadins, rye secalins, and barley hordeins (10, 18, 19). According to the manufacturers specification, this assay is able to detect gluten quantitatively, with a detection limit of 2 ppm gliadin and a quantification limit of 5 ppm gliadin (corresponding to 10 ppm gluten) (21). In the case of our matrix (native gluten), prolamins were not heat processed and could be directly extracted with 60% ethanol.

The results obtained for white wine clearly indicated that if there were residual gluten in treated wine, their concentration was lower than the limit of quantification by this assay (table 3.10). For the Codex Alimentarius this wine - even if clarified with wheat gluten - may be considered as a gluten-free drink.

Table 3.10. Gluten content in Fiano white wines after treatment.

Samples	Abs Average	Concentration on standard curves (ng/g)	Dilution Factor	Concentration in wine (ppm)
Fiano reference wine + 0.05 ppm gliadin	0.864	55.8	100	0.14
Fiano wine treated with gluten	0.609	39.4	500	0.49

The same procedure was used to quantify gluten proteins in red wines. In this case, the high content of phenolic compounds made necessary to treat preliminary the wine with a clarifying solution able to reduce the interference of this compounds with the ELISA protocol. Red wines, fined with 20 g/hl of gluten, did not contain any detectable wheat gluten residues. Results obtained was in accordance with Lefebvre et al. (2003), who tested also the fined wine for immunoreactivity and verified that they presented no risk of triggering a response in sensitive individuals.

3.9. CONCLUSION

Four commercial preparations of plant protein (soy, pea, lentil and gluten proteins) were studied as alternatives to gelatin for the clarification of white and red wines. The results of fining trials showed that all proteins were able to decrease turbidity and optical density of both white and red wines in percentages comparable to the effect of gelatin and produced also less lees volume than did the conventional fining agent. For *Catalanesca* white wine, clarified after cold stabilization, lentil and gluten proteins were the fining agents that removed monomeric and dimeric flavanols most effectively but also caused an important decrease of the total content of compounds responsible for the varietal and prefermentative aroma. For *Fiano* white wine, treated at the end of alcoholic fermentation, the loss of volatile compounds for each fining agents was less drastic respect to *Catalanesca* wine. Further studies should be conducted on the behaviour of free aroma compounds and bound aroma precursors using wines from different grape varieties and in different steps of winemaking process in order to verify and generalize these first conclusions.
Enzymatically hydrolyzed pea proteins were included in red wine fining trials. Results of fined red wine showed that each protein preparations presented a distinct interaction and precipitation capacity respect to the different anthocyanin compounds (monomeric anthocyanins, anthocyanin-flavan-3-ol adducts mediated by acetaldehyde, pyranoanthocyanins and hydroxyphenyl-pyranoanthocyanins). Also, color intensity and molecules related to wine color can be selectively decreased by specific fining proteins. These results suggest that the enologist's choice of protein fining agent for clarification and for decrease of particular phenolic compounds is important and should be very carefully considered.

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Appendix I. Abstract (in English)

Gelatine, casein, egg albumin, and, more recently, proteins from plant sources are commonly used in winemaking as fining agents to remove particles responsible for turbidity, to improve stability, and to control browning, over-oxidation, and bitterness during ageing (Spagna et al., 2000; Cosme et al., 2008). The formation of covalent and non-covalent interactions (hydrogen bonds and hydrophobic interactions) between the protein matrix and wine polyphenolics is the basis of the flocculation and of the consequent clarification which results in an overall improvement of wine quality parameters (Versari et al., 1999; Sarni-Machado et al., 1999).

In accordance with the PhD thesis project, we studied the molecular basis of the interactions between selected plant proteins (soybean, pea, lentil and gluten proteins) and polyphenolic and aroma compounds by using spectrometric and mass spectrometry methodologies (LC-ESI MS, MALDI TOF MS). Protein surface hydrophobicity was investigated in a wine-like model system by spectrofluorimetric determination of changes in the binding properties of 1,8-anilinonaphthalenesulfonate (ANS), used as extrinsic fluorescent probe. Hydrophobic interactions between phenolic compounds and protein finings were evaluated by the study of competition of phenolic compounds with the ANS probe for the same binding sites. Structural characterization of phenolic compounds (polymer chain length and chemical structure and composition of individual chains), as well as their interactions with the plant proteins, essential for the definition of protein binding affinity, was performed by means of mass spectrometry techniques. Differences among interactions between polyphenols with the various protein matrices have been related with the quality parameters of the resulting wines.

Appendix II. Abstract (in Italian)

La trasparenza, la limpidezza e la stabilità nel tempo di un vino sono alcune delle caratteristiche che il consumatore esige al momento del consumo. Numerosi sono i fattori che influiscono sulla qualità di un vino e che possono comprometterne l'aroma, il colore e il sapore. Tra questi l'ossidazione dei composti fenolici può causare l'imbrunimento del vino bianco oppure l'eccessiva presenza di tali composti può determinare la comparsa della sensazione di astringenza, dovuta all'interazione delle sostanze polifenoliche con le proteine salivari, nei vini rossi.

Questi fenomeni si possono prevenire controllando parametri quali la temperatura, il pH e l'assorbimento di ossigeno da parte del prodotto, ma anche utilizzando sostanze chiarificanti e stabilizzanti di natura proteica (gelatina, albumina, caseina) o di natura minerale (silice e bentonite). La chiarifica mediante collaggio proteico rimuove dal vino le sostanze di natura colloidale responsabili della torbidità o di intorbidamenti, e migliora la filtrabilità e le caratteristiche organolettiche, controllando l'imbrunimento e la polimerizzazione ossidativa di composti polifenolici e riducendo la sensazione di astringenza.

Le proteine animali sono state per anni le più utilizzate; negli ultimi anni la comunità scientifica ha spostato l'attenzione sulla ricerca di proteine di chiarifica di origine vegetale. Ciò è avvenuto principalmente sotto la spinta dell'opinione pubblica, allarmata sui possibili pericoli derivanti dal consumo alimentare di proteine animali, in seguito al fenomeno dell'encefalopatia spongiforme bovina (BSE ovvero Bovine Spongiform Encephalopathy). In particolare, è stato vietato l'utilizzo di albumina e colla di pesce in campo viti-vinicolo in seguito all'emanazione del Regolamento CE 2087/97, Consiglio di Ottobre 20, 1997.

Per tale motivo, negli questi ultimi anni si è verificata una graduale e costante tendenza all' abbandono dell'impiego di prodotti di origine animale in enologia. In tale scenario si colloca il presente progetto di tesi di Dottorato, che si è posto l'obiettivo mettere a punto una metodica analitica per lo studio delle interazioni non covalenti tra molecole idrofobiche responsabili di note organolettiche nei vini, quali polifenoli e molecole odorose, e chiarificanti proteici di origine vegetale, da leguminose (soia, pisello e lenticchia) e da cereali (frumento), per consentirne un utilizzo più selettivo e razionale. L'indagine è partita da soluzioni idroalcoliche modello e è stata estesa a vini trattati con i chiarificanti selezionati a concentrazioni ottimali. I composti coinvolti nell'interazione ed i complessi generati sono stati caratterizzati combinando tecniche separative e di spettrometria di massa (LC-ESI MS, MALDI TOF MS). Poiché il collaggio proteico può provocare una riduzione più o meno marcata delle componenti cromatiche ed aromatiche di un vino, nella fase successiva dello studio è stata valutata l'incidenza del trattamento di chiarifica su tali componenti di vini bianchi e rossi.

Appendix III. List of abbreviations

ANS	1-anilinonaphthalene-8-sulfonic acid
CPA	cis-parinaric acid
ROS	reactive oxygen species
PSH	protein surface hydrophobicity
Fmax	maximum fluorescence intensity
Kdapp	apparent dissociation constant of the protein-ANS complex
LC-MS	liquid chromatography-mass spectrometry
ESI	electrospray ionization
MALDI-TOF	matrix-assisted laser desorption ionization time-of-flight
TFA	trifluoroacetic acid
TIC	total ion current
OPCs	oligomeric proanthocyanidins complexes
PnGn	proanthocyanidin gallates
SPME	solid phase micro-extraction
HS	headspace

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Table 3.8. Mean values of concentration (mg/l) and standard deviations (n = 2) for anthocyanic phenolic compounds identified by HPLC-MS in 2007 vintage *Aglianico* wine either unfined (UW) or fined with various plant proteins (GL: gluten; PI: pea protein isolate; PH1: Pea protein hydrolysate1; PH2: Pea protein hydrolysate2; GE: gelatin; LE: lentil flour; SI: soy protein isolate).

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Appendix VI. Scientific production

WINE

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Sulphite-free organic additives to be used in wine making process

Key words: white and red wines, sulphites, additives, vinification process

SUMMARY

Sulphites are extensively used as additives in many foodstuffs including wines, beer, cider, fruit juices, dried fruits, biscuits and vegetables. Currently all the wine produced in the world involves the use of sulphur dioxide and/or sodium, potassium salts of hydrogen-sulphite in various stage of wine-making process for their technological efficacy (antioxidant power, antimicrobial agents, enzyme inhibitors, control of enzymatic and non-enzymatic browning reactions, pro-fermentative and colourstabilising effect). Sulphite additives were also implicated as the major cause of wine induced severe asthma attacks and anaphylactic reactions and presented some cytossic, mutagenic and antinutritional effects: for these reasons winemakers and researches are trying to obtain an added sulphites free wines by using modern wine making techniques (hyperoxigenation, flotation and tangential microfiltration). Nowadays neither validated methodology has been developed.
The final object of this research work was to develop a natural additive able to mimic the SO_2 effects and to preserve the typical organoleptic characteristics of white and red wines. Qualitative and quantitative composition of the new formulation (organic acids, flavonoids, catechins, and oligomeric proanthocyanidins) was defined on the basis of microbiological, chemical, biochemical, sensory and toxicological data by using an innovative analytical approach based on the "Experimental Design", a methodology allowing to minimize the number of experiments and maximise the resources. The effectiveness of the designed mixture was tested "on field" in real vinification process of several variety of white and red grapes. Polyphenols, tannins, procyanidins and other components and their influence on wine stability was assayed by conventional tests as well as advanced instrumental techniques (HPLC and HPLC-MS). The odorous compounds were analyzed by the analytical approach of solid phase micro-extraction (SPME) and headspace GC MS. All field tests showed a success in the use of this formulation to preserve wine avoiding oxidation and microbial spoilage. Also sensory analysis did not show defects and/or alterations in experimental wines.

1. INTRODUCTION

The winemaking process includes multiple stages at which microbial spoilage can occur, altering the quality and hygienic status of the wine and rendering it unacceptable (1). The faults caused include bitterness and off-flavours (mousiness, ester taint, phenolic vinegary, buttery, geranium tone), and cosmetic problems such as turbidity, viscosity, sediment and film formation.
These spoilage organisms can also affect the wholesomeness of wine by producing biogenic amines and precursors of ethyl carbamate. The judicious use of chemical preservatives, mainly sulphur dioxide during the winemaking process decreases the risk of microbial spoilage. Currently, the forms employed include sulfur dioxide gas, and the sodium and potassium salts of sulfide, bisulfite or metabisulfite. In aqueous solution sulfur dioxide and sulfite salts form sulfurous acid, and ions of bisulfite and sulfite (2).

$$SO_2 + H_2O = H_2SO_3$$

$$2H_2SO_3 = H^+ + HSO_3^- + 2H^+ + SO_3^{2-}$$

The relative proportion of each form depends on the pH of the solution, and at pH 4.5 or lower the HSO^{3-} ion and undissociated sulfurous acid predominate. It has been shown that sulfur dioxide is most effective as an antimicrobial agent in acid media, and this effect is believed to result from undissociated sulfurous acid, which is the dominant from below pH 3.0.
The enhanced antimicrobial effect of sulfur dioxide at low pH values may result because undissociated sulfurous acid can more easily penetrate the cell wall. Sulfurous acid inhibits yeasts, molds, and bacteria, but not always to the

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same degree. This is particularly true at high pH values, where it has been suggested that the HSO^{3-} ion is effective against bacteria but not against yeasts (2). Postulated mechanisms by which sulfurous acid inhibits microorganisms include the reaction of bisulfite with acetaldehyde in the cell, the reduction of essential disulfide linkages in enzymes, and the formation of bisulfite addition compounds that interfere with respiratory reactions involving nicotinamide dinucleotide.

Of the known inhibitors of non-enzymatic browning in wine, sulfur dioxide is probably the most effective. The chemical mechanism by which sulfur dioxide inhibits nonenzymatic browning is not fully understood, but it probably involves bisulfite interactions with active carbonyl groups.

Bisulfite combines reversibly with reducing sugars and aldehydic intermediates, and more strongly with a-dicarbonyls and a,b-unsaturated aldheydes. These bisulfite addition products appear to retard the browning process, which when coupled with the bleaching action of sulfur dioxide on pigments, results in effective inhibition of non-enzymatic browning.

Sulfur dioxide also inhibits certain enzyme-catalyzed reactions, notably enzymic browning. The production of brown pigments by enzyme-catalyzed oxidation of phenolic compounds can lead to a serious quality problem in winemaking.

Sulfur dioxide also functions as an antioxidant in wine and beer, avoiding the developments of oxidized flavours during storage. Also, sulfur dioxide in combination with buffering agents is applied to prevent browning and to induce oxidative bleaching of nathocyanin pigments: the resulting properties are desired in products, such as those used to make white wines and maraschino cherries (3).

Sulfur dioxide and sulfites are metabolized to sulphate and are excreted in the urine without any obvious pathological results. However, the safety-related aspects of sulfur dioxide and its derivatives are undergoing extensive reviews because of reports of severe reactions in some asthmatics upon consumption of wine, and also because of potential mutagenicity.

For these reasons, there is mounting consumer bias against chemical preservatives and the subsequent request of use of natural preservatives in complying with the consumers' demand for "clean and green" products (1-5-7).

All food additives must have a demonstrated useful purpose and undergo a rigorous scientific safety evaluation before they can be approved for use: the aim of this work was the study the molecular and functional characteristics of natural additives and the definition of the strategies for employing these compounds in the technological process of winemaking. The natural vegetable products for wine processing should be able to mimic the effect of the SO_2 in white wine making process, thus guaranteeing the antioxidant and antibacterial action. The use of such products could decrease the amount of added sulphites during vinification and to obtain, as final result, a concentration of volatile sulphites lower than 10 ppm in white wines.

Both critical chemical and biochemical parameters of new formulation and experimental wines were assayed by conventional tests as well as analytical methods based on high resolution chromatographic techniques in combination with structural analysis by mass spectrometry (GCMS, HPLC and HPLC-MS).

2. EXPERIMENTAL ACTIVITIES AND RESULTS

The additives used for experimental activities included two main class of natural compounds:

- the first class included aqueous extracts from plants (ADD 1) composed by mixtures of vegetable extracts, organic acids, tannins, pectines, etc. used as stabilizer for the winemaking process with high antioxidant power. These products can replace the traditional chemical treatments, thus enabling the improvement of organoleptic characteristics and the preservation of wine;
- the second class was made of OPCs, Oligomeric Proanthocyanidin Complexes (ADD 2,3,4), purified from *Vitis vinifera* seeds, which are primarily known for their antioxidant activity. These compounds have also been reported to have antibacterial actions so they may be a useful additive in the treatment of wine.

The formulations were preliminarily tested on water/ethanol solutions (90/10 v/v) mimicking the wine composition and two reference wines (a white wine and a red wine for comparison). The tests included solubility, stability (precipitation reaction), chemical physical parameters (total acidity, pH, turbidity, colour, polyphenolic composition and structural characterization, aromatic compounds), compatibility with SO_2.

The preliminary tests on ADD 2-3 were performed using different concentrations ranging from 100 to 400 mg/L. This was because of the brown colour of these compounds that could alter the colour of white wines.

The three additives were completely soluble both in ethanol solution (pH 3.5) and in wine, but only ADD 1 was stable at precipitation reactions during time. The pH, total acidity, total phenolic content, colour and turbidity of reference white and red wine were not modified by the addition of the ADD 1 both in the absence and in the presence of a reduced amount of SO_2.

ADD 2-3 showed a high content of total polyphenolics (316.27 and 556.55 mg/L) and optical density. ADD 2 test-

ed at lower concentration, modified the turbidity of wine both in combination of SO_2 and without SO_2. By increasing the concentration of this additive, white wine became darker after storage; the influence on turbidity and colour became more evident when the additive was used in combination with SO_2.

ADD 3 had the same effects on the colour of wine, but showed a positive effect by reducing the turbidity of wine. The above mentioned effects could be observed when the additive was used in combination with SO_2, although it was less evident.

Taking into account the effect of OPCs additives on the optical density (colour) of the reference wine, new sample of proanthocyanidins with a much lighter colour than the already proanthocyanidins studied (ADD 4), was preliminary tested by the same analytical approach of the other three formulations. The optical density of this additive in model solution was lower than the other OPC samples. The pH, total acidity, total phenolic content, colour were not modified by their addition in the absence and in the presence of a reduced amount of SO_2; it showed also a positive effect by reducing the turbidity when it was used in combination with SO_2.

In order to obtain more complete data, the polyphenolic and the odorous compounds of the polymeric tannin extracts were determined.

The extraction of polyphenols was carried out using a Sep-pak C18 cartridge and characterized by a number of techniques including RP-HPLC, HPLC-MS and matrix assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS).

MALDI-TOF MS of additive 4 (**Fig. 1**) shows peaks with m/z values corresponding to an oligomeric series of catechins/ epicatechin/gallo(epi)catechin units and their gallic acid ester derivatives, up to the dodecamer.

Anthocyanins, in traces, were also detected by LC-ESI MS analysis (tr: 21.90, 25.19 and 27.68 min). They were identified as the 3-O-monoglucosides of malvidin and peonidin on the basis of their UV-Vis and mass spectra (493 and 463 m/z, shown in **Fig. 2**) and by comparison with reference compounds. Additionally, 3-O-acetylglucosides and 3-O-p-coumaroylglucosides of malvidin were detected (535 and 639 m/z).

The odorous compounds were analyzed by the analytical approach of solid phase microextraction (SPME) and GC MS.

The additives showed the presence of terpenes (such as limonene, whose aromatic descriptor is citrus fruit, 3- and 4-carene) and their derivatives, esters (such as ethyl octanoate and decanoate) and alcohols.

The aromatic profile of reference wine

Fig. 1 - MALDI MS spectrum of additive 4.

Fig. 2 - ESI mass spectra of the peaks eluted at (a) 21.90, (b) 25.19 and (c) 27.68 min of th additive 4. The mass spectra corresponded to (a) malvidin and peonidin-3-O-glucoside, (b) malvidin-3-O-(6-Oacetyl)-glucoside and (c)Malvidin-3-O-(6-O-p-coumaryl) glucoside.

WINE

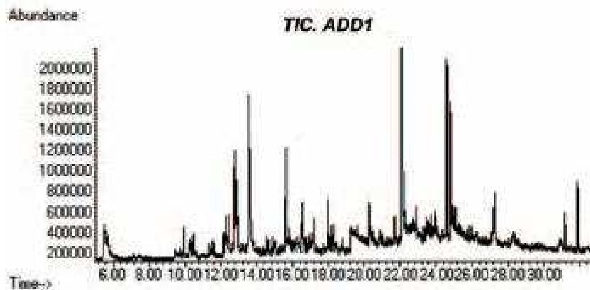

Fig. 3 - Total Ion Current Chromatogram from SPME analysis of additive 1.

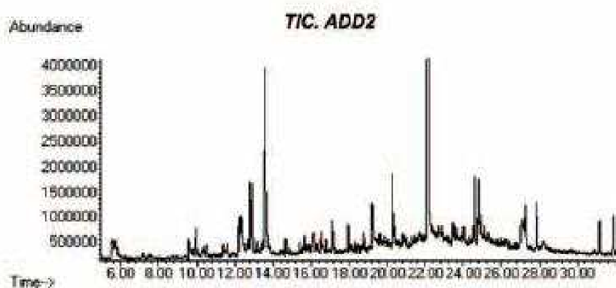

Fig. 4 - Total Ion Current Chromatogram from SPME analysis of additive 2.

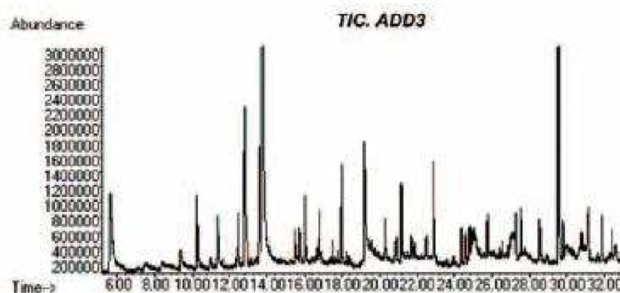

Fig. 5 - Total Ion Current Chromatogram from SPME analysis of additive 3.

was compared with the same wine added with one of two proanthocyanidins formulations (**Fig. 6**): there are evident similarities among them and so it is clear that the aromatic profile was not altered by the additives.
However, interaction effects were observed between wine and aromatic compounds from additives: some odorous compounds present in the additives, such as alpha-pinene and limonene, were not observed in wine after addition of the additive, while they are observed in model solution at the same concentration. This was due to the presence in wine of compounds which interfere with the volatility of the compounds, probably by adsorption.
In order to test the capability of additive to be used in winemaking process, they were employed in experimental microvinification of several white and red grapes:

- Falanghina grape variety grown in Campania. Today Falanghina is used to make fresh and lively white wines with aromas and flavors of green apples and citrus fruits;
- Tempranillo grapes, vinificated by Bodegas Roda (Spain);
- White grapes vinificated by Argiolas for the production of S'ELEGAS wine (Sardinia, Italy).

The experimental vinification was carried out using the three additives alone and the same with a reduced amount of SO_2, equivalent to 20% of the amount normally used.
Chemical-physical characterization of experimental wines at different stage of vinification process confirmed the possibility of using new selected formulations in winemaking. Data obtained for each step of each fermentation were analyzed in three ways:
1. Correlation in each chemical property for each formulation to find the differences between formulations in each variable. From this analysis we could say that the chemical data obtained through the different stages of the vinification process does not de-

pend on the formulation. So the experimental data set is trusty and a the chemical analysis are accurate;
2. Correlation in time for each formulation and similarity with traditional sulphites addition to find the most similar additive;
3. Correlation between different formulations to find the experimental difference between formulations. The relationship between different formulations is found in all four different stages of the vinification process. The results are presented in different matrices in **Fig. 7**.
It could be seen that the second additive (second variable of the matrix) behavior is almost similar to SO_2 (the first variable of the same matrix). It could be seen that this formulation is the most related to the SO_2 while the must characteristics are measured. Besides this, the correlation after the vinification process evolve is higher than 0.8, meaning that the chemical characteristics of wine with all formulations are similar to the wine with SO_2.
The results of the experimental work are summarized in the following sentences:
- OPCs are not compatible with SO_2, on the basis of different chemical-physical parameters (volatile acidity, polyphenols, optical density and sensory analysis).
- OPCs darken white wines.
This fact can be explained by the grapes maderization or by too high concentration of additive. Lower concentration may be suitable for the vinification of white wines. Further tests will be performed.
Colour darkening during storage of experimental white wines resulted essentially from chemical reactions involving phenolic compounds, in particular the oxidation of flavan-3-ol derivatives. For this reason, these additives, that are oligomeric proanthocyanidin complexes, darkens the white wines and may shorten their com-

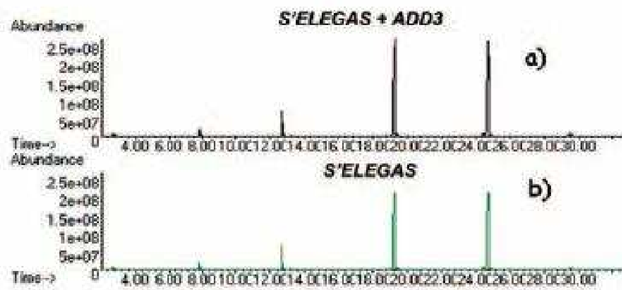

Fig. 6 - Total Ion Current Chromatogram from SPME analysis of S'ELEGAS alone and S'ELEGAS added with ADD 3.

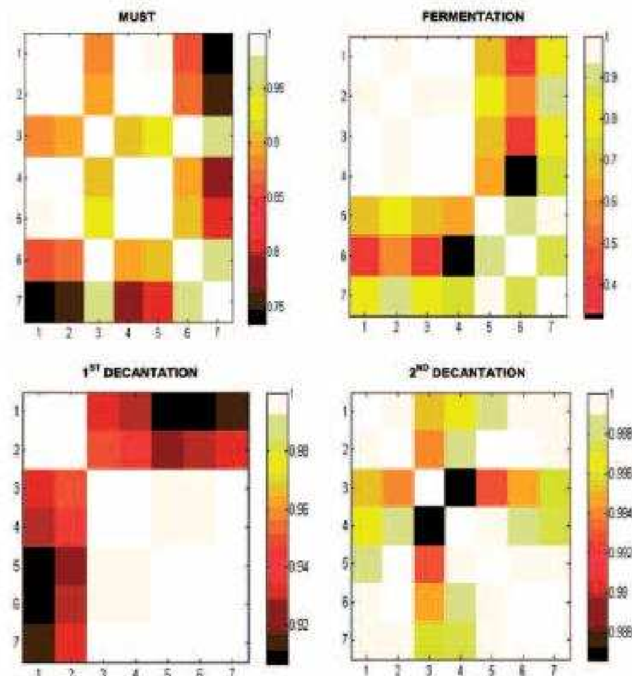

Fig. 7 - Correlation between different formulations.

WINE

Table 1 - Total Ion Current Chromatogram from SPME analysis of additive 2.

Compounds	Aromatic descriptor
-pinene	pine oil
Limonene	citrus fruit
,-mircene	balsamic
ethyl octanoate	ananas
3-carene	citrus fruit
BHT (antioxidamt compound)	-

Table 2 - Aromatic compounds of additive 2.

Compounds	Aromatic descriptor
-pinene	pine oil
2-ethyl, 1-hexanal	-
2-ethyl, 1-hexanol	rose
Limonene	citrus fruit
,-mircene	balsamic
ethyl hexanoate	apple peel
ethyl octanoate	pineapple
ethyl decanoate	grape, fruit
3-carene	citrus fruit
BHT (antioxidamt compound)	-

Table 3 - Aromatic compounds of additive 3.

Compounds	Aromatic descriptor
Limonene	citrus fruit
2-ethyl, 1-hexanol	rose
ethyl octanoate	pinapple
Isobornyl acetate	pine oil
ethyl decanoate	grape, fruit
BHT (antioxidamt compound)	-
3-carene	citrus fruit

Table 4 - Chemical-physical parameters of Tempranillo experimental wines.

Tempranillo	Stored wine		
	SO_2	ADD 4	ADD 2
Total Acidity (g/L)	4.80	5.32	6.45
pH	3.94	3.88	4.11
Free SO_2	38.40	35.20	22.40
Total SO_2	115.20	102.40	86.40
A 420	0.42	0.48	0.46
A 520	0.59	0.61	0.63
A 620	0.12	0.14	0.14
Colour Dendity	11.36	12.31	12.32
Tint	0.71	0.79	0.74
Total Anthocyanins (mg/L)	484.46	389.80	371.90

mercial life. During storage and aging of wine, polyphenolic compounds are gradually modified. Reactions among flavan-3-ol, proanthocyanidins and other compounds, such as glyoxylic acid, piruvic acid and acethaldehyde, and also between flavanols themselves are responsible for the appearance of new pigments, and hence for the disappearance of oligomeric proanthocyanidins from solution.

- However the above mentioned processes do not affect the colour and colloidal properties of red wine. Red wines vinificated with OPCs are good and there are no significant differences of chemical parameters and organoleptic properties between the reference wine and the experimental ones (**Table 4**). The study of the ageing is still in progress.

Sensory analysis do not show defects and/or alterations: additive 2, used in absence of SO_2, provides the best sensory characteristics.

All wine treated with additives 2-4 showed a residual value of free and bound SO_2, even when sulphites were not added, probably due to the interference of phenolic compounds with the procedure for SO_2 determination. Alternative analytical methods are under evaluation for further tests.

- ADD 1 shows synergetic effects with sulphure dioxide.

The vinification of S'ELEGAS wine with ADD 1, performed with a reduced amount of SO_2 (about 20% of the amount normally used), provides the better results for white wines. Figure shows experimental wines after 3 months of storage: no alteration in chemical parameters (pH, volatile acidity, free SO_2, total SO_2, optical density, etc.) and also in sensory analysis were observed during the time.

Antioxidant power assays showed that OPCs additives have optimal behaviour at the beginning of vinification, while the ADD 1 formulation preserve and stabilize the wine during storage. These findings suggest the

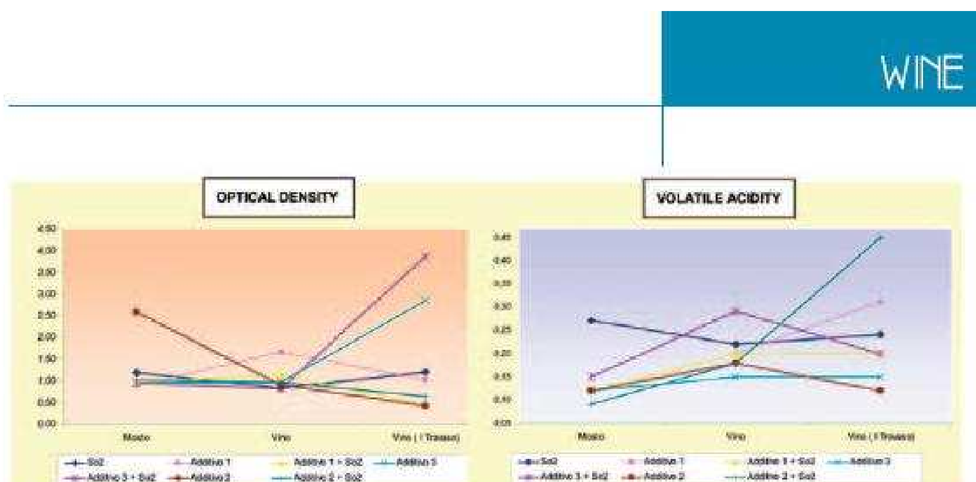

Fig. 8 - Optical density and volatile acidity of experimental wines.

Table 5 - Chemical-physical parameters of Tempranillo experimental wines.

S'ELEGAS	STORED WINE 23/02/2007		
	SO_2	ADD 1	ADD 4
Total Acidity (g/L)	6.83	7.58	8.63
pH	2.92	2.92	2.78
Free SO_2	15.36	6.40	8.32
Total SO_2	56.32	21.76	30.72
Optical density	0.0592	0.0680	1.6510
Catechins	33.54	35.37	142.10
Total Poliphenolics	103.71	45.00	619.00

possibility of using the two classes of additives in a differential way, i.e. OPCs are best suited in the early steps of vinification (grape pressing, alcoholic fermentation) whereas ADD 1 can be used in the last phases as stabilizer (wine ageing, bottling, refining).

CONCLUSION

A natural additive able to mimic the SO_2 effects on wine making process is pursued. Both, aqueous extracts from plants and OPCs additives were tested. Colour, aromatic and antioxidant

Fig. 10 - S'ELEGAS experimental wines.

Fig. 9 - Tempranillo experimental wines.

WINE

effects were analyzed in different microvinifications of several white and red grapes. The chemical-physical characterization of experimental wines at different stages of vinification process confirmed the possibility of using the new selected formulation in wine making. All field tests showed a success in the use of these formulation to preserve wine avoiding oxidation and microbial spoilage. Also sensory analysis did not show defects and/or alterations in experimental wines, even more one additive shows a synergetic effect with sulphur dioxide suggesting the possibility of using two classes of additives in different wine making process stages.

ACKNOWLEDGEMENTS

This research has been performed within SULPHREE, a CRAFT project (Contract no. COOPCT- 2004-512721) funded under the VI European Framework Programme.

Proceedings of the 3rd CIGR Section VI International Symposium on "Food and agricultural products: processing and innovations" - Naples, Italy, 24-26 September 2007

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PAPER

DETERMINATION OF VARIETAL VOLATILES AS QUALITY AND SHELF-LIFE MARKERS/ORIGIN AND TYPICALNESS TRACERS IN SOUTHERN ITALIAN WINES

DETERMINAZIONE DI COMPOSTI VARIETALI VOLATILI QUALI TRACCIATORI DI QUALITÀ E TIPICITÀ IN VINI DELL'ITALIA MERIDIONALE

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ABSTRACT

The composition of wine varietal volatiles (terpenes, norisoprenoids, etc.), expressions of distinctive vine genetic characteristics, was determined in some southern Italian wines. The aim was to tentatively identify specific quality and shelf-life molecular tracers, among compounds related to the grapevine biodiversity, and to observe their modifications under different storage conditions. During the shelf-life of bottled Fiano wine samples, high temperatures produced an increase of terpene interconversion and degradation. p-Cymene and some terpenyl oxides (linalool oxide and limonene oxide) were also produced which appeared as shelf-life molecular markers. In Aglianico wine samples, some terpene compounds (α-pinene, β-myrcene, 3-carene, 4-carene, limonene and linalool) acted as possible process markers during the ageing step. In autochthonous Ischia varieties, terpene compounds also acted as suitable indicators of geographical origin. The data obtained showed that some volatile compounds could be potential analytical tools for checking, saving and improving the quality of typical wines.

RIASSUNTO

È stata determinata la composizione di composti volatili varietali (terpeni, norisoprenoidi, ecc.), espressione delle caratteristiche genetiche distintive dei vitigni, in alcuni vini dell'Italia Meridionale, al fine di determinare potenziali tracciatori molecolari di qualità e di shelf-life tra i composti correlabili alla biodiversità dei vitigni autoctoni, e di osservarne possibili modificazioni sotto differenti condizioni di conservazione. Durante la shelf-life in bottiglia dei campioni di vino Fiano, le temperature più alte hanno prodotto un incremento dell'interconversione e degradazione dei terpeni, ed inoltre la produzione di p-cimene e di alcuni ossidi terpenici (ossido di linalolo e di limonene), i quali possono essere considerati possibili tracciatori molecolari di shelf-life. Nei campioni di vino Aglianico alcuni composti terpenici (α-pinene, β-mircene, 3-carene, 4-carene, limonene, linalolo) possono agire come marker di processo durante la fase di 'invecchiamento'. In varietà autoctone ischitane i terpeni costituiscono possibili tracciatori di origine geografica. I dati ottenuti indicano che nuovi potenziali strumenti analitici possono essere considerati per salvaguardare e migliorare la qualità delle produzioni enologiche tipiche.

- Key words: aging, terpenes, shelf-life, varietal volatile compounds, wine quality -

INTRODUCTION

High quality wines acquire a meaningful importance on the market through the diversification of their specific identifiable qualitative characteristics. During the last 30 years, particularly in the traditional winemaking countries (Italy, France, Spain, etc.) consumer preferences have become more oriented towards products with a strong cultural and territorial identity. In particular, consumer preferences have been oriented towards authochthonous wines with quality trademarks such as the European Appellation of Origin designations (e.g., Italian Controlled and Guaranteed Denomination of Origin, i.e. DOCG, or Controlled Denomination of Origin, i.e. DOC, etc.). The typicalness of these wines is related to the grape variety and specific characteristics of the *terroir* (i.e. soil, location, climate, and specific vineyard-environment interaction).

Several studies of viticultural zoning have been carried out by using a multidisciplinary approach to define the best qualitative potential of the oenological production. As high quality products have always been the main aim, technological innovation in the oenological sector has been coupled with tradition and typicalness (CHRISTAKI and TZIA, 2002).

Using analytical tools to identify quality, authenticity and typicalness, molecular markers provide new information and when combined with conventional analytical parameters give a better description of the typicalness and quality of food products.

For this reason several studies have been carried out to determine the trace elements in order to correlate the composition of these elements and the origin, *terroir* and authenticity of the wine (THIEL *et al.*, 2004). Several studies have identified and quantified the molecular typicalness markers in wines such as anthocyanins, norisoprenoids, pyrazines and terpenes (CÁMARA *et al.*, 2004; CARRO *et al.*, 1996; ROSILLO *et al.*, 1999; OLIVEIRA *et al.*, 2004; SÁNCHEZ-PALOMO *et al.*, 2005; MAMEDE *et al.*, 2006; MATEO *et al.*, 2000; FLAMINI, 2005; MAZZUCA *et al.*, 2005; NASI *et al.*, 2008, 2006; VILANOVA and SIEIRO, 2006).

The quali-quantitative composition of terpenes, which are odorous molecules with low perception threshold, is strictly related to the varietal origin (CÁMARA *et al.*, 2004; CARRO *et al.*, 1996; MATEO *et al.*, 2000; FLAMINI, 2005; PEŤKA *et al.*, 2006; RAPP, 1998; LÓPEZ *et al.*, 2002; ROSILLO *et al.*, 1999; OLIVEIRA *et al.*, 2004). Specific genes encode for enzymes that catalyse the monoterpene biosynthesis that produce these compounds that show a large structural diversity (LUCKER *et al.*, 2001; HARBORNE, 1991).

Conservation modalities during the ageing step and storage conditions during the shelf-life of wines could affect the quality and original potential varietal expression of a wine. Some researchers have investigated the modifications that occur in the volatile components in wines during shelf-life (PÉREZ-COELLO *et al.*, 2003; LAMBROPOULOS and ROUSSIS, 2007). Very few studies have reported the possible modification and interconversion of terpenes due to the effects of temperature and pH in aqueous solutions (MAICAS and MATEO, 2005). No study has been reported that is specifically aimed at identifying potential shelf-life and quality markers from among varietal volatiles and related degradation products.

In view of the potential contribution of varietal volatile compounds to wine quality and aroma, the varietal volatile components of some southern Italian autochthonous red and white wines (Fiano white wine and Aglianico red wine) were investigated under different conditions of storage during the shelf-life in bottle and the ageing step. The aim was to identify potential quality markers and other modifications which could potentially affect their specific characteristics in order to save and reinforce the quality characteristics of these wines that are well known on the international market.

The composition of volatile varietal markers of autochthonous wines (Don Lunardo and Arilla white wines) from different areas on the Isle of Ischia (specifically chosen because of its small, defined geographical area) was also determined in order to identify potential tracers of geographical origin.

MATERIALS AND METHODS

After fermentation following a traditional winemaking protocol in a winery in Taurasi (Avellino, Italy), the Aglianico wine was separated into 3 different tanks for ageing (9 months): a) a 5000 L inox tank; b) a 3000 L oak barrel; and c) a 228 L oak barrel.

The Fiano wine was produced in a winery in Atripalda (Avellino, Italy). After bottling the sample was divided into 5 parts (each part= 11 bottles of 750 mL), each part was stored at 4 different temperatures (5, 14, 20 and 35 °C) and at two different light exposures: light and dark.

The Don Lunardo and Arilla grapes and wines were produced in different areas on the Isle of Ischia. The same winemaking conditions were used to produce the wines.

Liquid/liquid extraction of volatile molecules was carried out with 2.5 mL of dichloromethane and 50 mL of wine using a vortex for 1 h.

The SPME (Solid Phase Micro-Extraction) analysis was effected with the holder and fibres (50/30 µm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS)) purchased from Supelco (Aldrich, Bornem, Belgium). It was carried out on 100 mL of sample (wine and must) with 30 g of NaCl added and also on 3 g of skins homogenised in 10 mL of water with 4 g of NaCl. Thermal desorption of the analytes from the fibre inside the GC injection port was carried out in the split mode (1/10) at a desorption temperature of 250°C for 1 min.

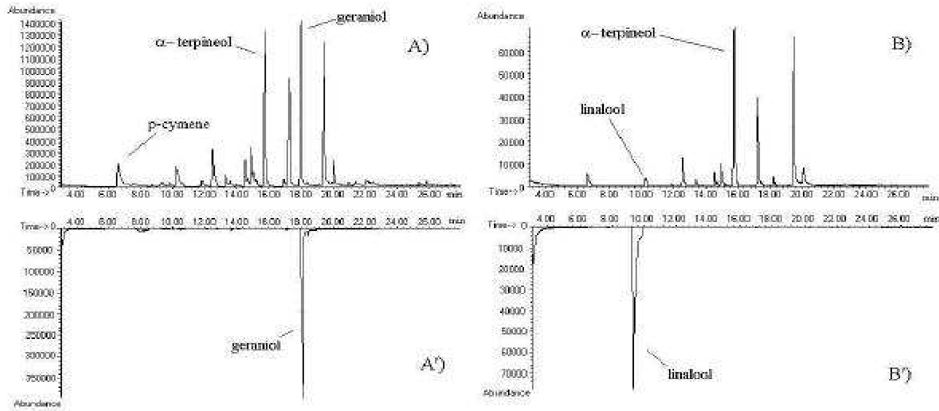

Fig. 1 - TIC chromatograms obtained through GC/MS analysis on an extract in dichloromethane from hydroalcoholic solution containing geraniol and linalool after (A, B) and before (A', B') acid hydrolysis (pH 3) at 35°C.

All samples were analysed with an HP 6890 coupled to a 5973N quadrupole HP mass spectrometer (Agilent Technologies, Palo Alto, CA, USA). The gas chromatograph was equipped with an HP-5ms capillary column (30 m x 0.25 mm ID; 0.25 μm Film Thickness) and the carrier gas used was helium.

For the analysis of the volatile molecules, the GC oven temperature was programmed from 40°C (held for 7 min) to 180°C at 5°C/min (6°C/min for the analysis in wine model hydroalcoholic solutions (pH=3)). The masses were scanned on m/z range of 45-350 amu. In other cases, a SIM method was used (for terpene compounds m/z 59, 69, 93, 121, 136). The NIST library and comparison with spectra and retention times of standards (Sigma-Aldrich; Acros Organics) were used to identify the odorous compounds.

Quantitative determinations of terpenes were obtained by means of calibration curves, in the concentration ranges typical of wines for each compound. In the range of linearity verified seven concentration levels and five replicates per level were used. Multiple replicates (n=3-6) of the samples were analysed.

RESULTS AND DISCUSSION

Varietal volatiles as shelf-life tracers in Fiano wines

The composition of the varietal volatile component was investigated during the shelf-life (seven months) of the white DOCG Fiano wine.

Some modifications of the terpene compounds were observed during the storage of Fiano wine bottles. These changes were also detected *in vitro*, i. e. in hydroalcoholic wine model solutions (pH=3), containing terpene molecules and stored at 35°C, for 5, 35 and 45 days; Fig. 1). The relative concentration of the α-terpineol increased and the relative concentration of the other ter-

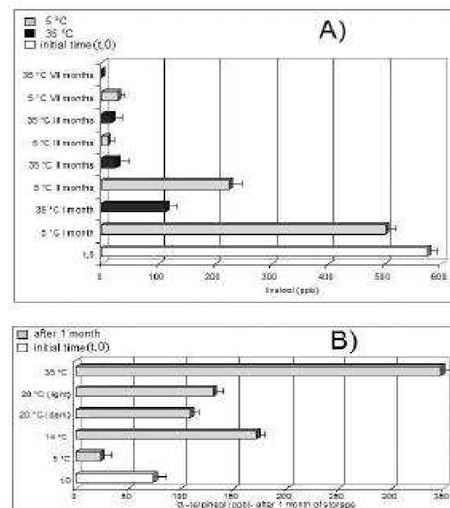

Fig. 2 - Concentrations in ppb of linalool (A) at 5°C and 35°C during different storage times, and α-terpineol (B) after the first month of storage at different storage temperatures.

Table 1 - Qualitative terpene component in Fiano wine samples stored under different conditions for different times. * Potential shelf-life markers (present only for higher storage temperatures and for longer storage times); L Light exposure, D. Dark exposure, tr. in traces.

Storage Temperature (°C)	Storage time (months)	limonene	linalool	α-terpineol	geraniol	α-ionone	p-cymene	limonene epoxide	linalool oxide
5	1	+	+	+	+	+	-	-	-
5	2	+	+	+	+	+	-	-	-
5	3	+	+	+	+	+	-	-	-
14	1	+	+	+	+	+	-	-	-
14	2	+	+	+	+	+	-	-	-
14	3	+	+	+	+	+	-	-	-
20	1 (L)	+	+	+	+	-	-	-	-
20	2 (L)	+	+	+	+	+	+ (tr)*	-	-
20	3 (L)	+	-	+	-	+	+ (tr)*	-	-
20	1 (D)	+	+	+	+	-	-	-	-
20	2 (D)	+	+	+	+	+	+ (tr)*	-	-
20	3 (D)	+	+	+	+	+	+ (tr)*	-	-
35	1	+	+	+	+	+	+*	-	-
35	2	+	+	+	+	+	+*	-	-
35	3	+	+	+	+	+	+*	+*	+*
Initial conditions		+	+	+	+	+	-	-	-

penes decreased in the wine samples during the storage time. These modifications, which derived presumably from an interconversion and degradation of the terpenes, were particularly complex at higher temperature, longer storage times and under light conditions (Fig. 2, Table 1).

p-Cymene was only detected at higher temperatures and longer storage times (Fig. 3); p-cymene has already been identified as a degradation product in some other matrices such as tea-tree oil formulations used in therapeutic products (SHABIR, 2005).

Linalool and limonene oxides were also present only at higher temperature and after longer storage times. These compounds (p-cymene, linalool and limonene oxides) could serve as potential shelf-life molecular markers which are strictly related to terpene degradation depending on the time, temperature and light exposure (Fig. 2, 3, Table 1).

After 7 months of storage, the terpene molecules were only present above their odour threshold in the samples stored at 5°C and 14°C (25 μg/L, 10 μg/L, 250 μg/L, 130 μg/L, for linalool, limonene, α-terpineol and geraniol respectively; LÓPEZ *et al.*, 2002). In the samples stored at 20°C, in the light and in the dark, and at 35°C, these compounds were present but at concentrations below the reported odour threshold, Fig. 2.

These data appeared to correspond to the sensory evaluation carried out through a panel test. The results indicated that the varietal aroma composition of the Fiano wine samples was maintained better at 4°C during the shelf-life (data not shown).

Varietal volatiles as process markers during the ageing step in Aglianico wines

Different winemaking conditions can affect the varietal volatile component of a wine. This study investigated possible modifications in an autoch-

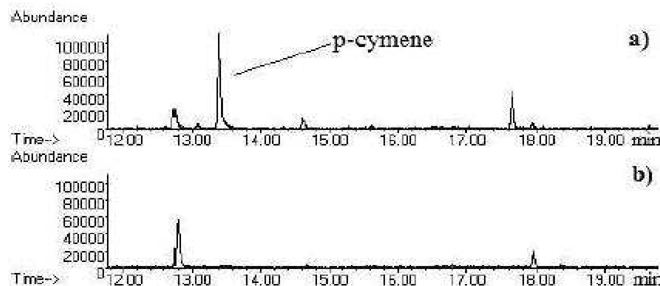

Fig. 3 - TIC chromatogram (ion trace at m/z 119) obtained through HS-SPME-GC/MS analysis on a wine sample stored at 35°C (a) and at 5°C (b) after 3 months of storage.

thonous red wine (Aglianico wine) obtained under different ageing conditions [inox tanks (5000 L) and oak barrels (3000 L and 228 L)].

The quantitative composition of varietal molecules in the Aglianico wine aged (9 months) in inox (5000 L), in a 3000 L oak barrel and in a 228 L oak barrel, is reported in Fig. 4. The wine aged in inox had the highest quali-quantitative level of varietal volatile components in comparison with the wine aged in oak barrels; some of the volatile compounds in the oak barrel wine were present but below the reported odour threshold (e. g. β-myrcene; PLOTTO *et al.*, 2004). The lower concentration of varietal compounds in the oak barrel wines is in agreement with the results obtained in previous studies carried out *in vitro* on model wine, i. e. hydroalcoholic solutions containing terpene molecules in contact with wood of oak barrel (RAMIREZ *et al.*, 2001; 2004). In these previous *in vitro* studies the wood surface/solution volume ratio appeared to be crucial.

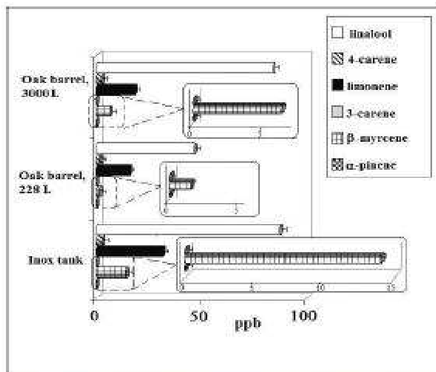

Fig. 4 - Concentrations in ppb of some terpenes detected in Aglianico wine samples aged for 9 months in different modalities: in oak barrels (3000 L and 228 L) and in inox tanks (5000 L).

The quantitative differences observed between the oak barrel wines (Fig. 4) can be attributed to the internal surface/volume ratio which is smaller in the 3000 L oak barrel than in the 228 L oak barrel. The differences could be due to possible gas exchange or the adsorption process of the volatile molecules effected by the wood surface.

The sensory evaluation of the Aglianico wine samples indicated that the varietal aroma was better preserved in the inox tanks compared to the oak barrels (data not shown). The greatest quantities of varietal components were found in the wine aged in the inox tanks.

Varietal volatiles as typicalness and origin tracers in Don Lunardo and Arilla grapes and wines

The terpene composition of Don Lunardo grapes (b) and wines (a) from Cuotto (Isle of Ischia), and Arilla grapes (b) and wines (a) from Cuotto and Spadara (two geographical areas on the Isle of Ischia) is reported (Fig. 5).

As expected, the most notable differences in the terpene composition were related to the varietal origin. In fact, Don Lunardo grapes and wines had a more complex qualitative and quantitative terpene composition in comparison to the Arilla grapes and wines.

The quantitative differences observed for the Arilla grapes and wines from Cuotto and Spadara were related to their different geographical origins, and were presumably due to different salinity stress and water deficit conditions and to different ripening rates. The terpene compounds in Arilla grapes and wines that came from Spadara tended to have higher concentrations compared to those from Cuotto. This result was related to earlier ripening in Spadara in comparison to Cuotto (data not shown). In Arilla wines the dominant terpene was linalool which was not

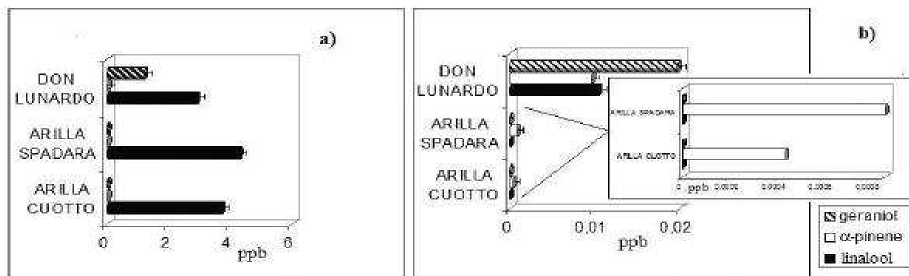

Fig. 5 - Concentrations in ppb of some terpenes detected in some autochtonous grapes and wines from Ischia (Italy): Don Lunardo grapes (b) and wines (a) from Cuotto area, Arilla grapes (b) and wines (a) from Cuotto and Spadara areas.

detected in the grapes but was produced in the wine during the winemaking process through acid and enzymatic hydrolysis from its glycoside precursors. The dominant terpene in Arilla grapes was α-pinene but it was lost during winemaking because it is not present in the glycoside form.

The sensory evaluation of the samples analysed indicated that the aroma expression was different for the Arilla wines produced from the two different geographical areas of the Isle of Ischia even though the same winemaking conditions were used to make them (data not shown).

CONCLUSIONS

Suitable quality and authenticity molecular markers of typical foods and drinks can furnish useful analytical tools which can provide quality control and commercial protection. The metabolomic approach carried out in this study on some varietal volatile compounds (terpenes and norisoprenoids) of autochthonous wines from the Italian region of Campania indicated that the varietal metabolites identified which express the distinctive vine genetic characteristics and grapevine biodiversity, could act as quality molecular tracers for typical oenological products.

Further work and statistical treatments are being conducted in our laboratories in order to verify systematically the effective suitability of these compounds to serve as tracers of quality and typicalness. If suitable, these tracers could be new tools for checking and improving the quality of autochthonous oenological products.

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Revised paper received March 31, 2009 Accepted September 9, 2009

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 11969–11976 11969
DOI:10.1021/jf102803r

Molecular Basis of the Interaction between Proteins of Plant Origin and Proanthocyanidins in a Model Wine System

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Plant proteins are being used as a replacement for animal proteins in wine fining. The surface hydrophobicity of plant proteins in four commercial preparations differing for their origin and processing was assessed by using a fluorescent hydrophobic probe in wine-like media. Displacement of the probe by addition of wine phenolics was measured as a way to compare and predict to some extent the efficiency of these proteins in wine fining. It was found that the binding of polyphenols was much more specific than that of the hydrophobic probe. Further analysis of the polyphenol pattern in protein-treated wine-like solutions pointed out two relevant facts: (1) proteins may interfere with the chemistry of the interactions between polyphenols and other wine components; (2) individual protein preparation having different surface hydrophobicities also have different specificities in binding different polymeric forms of the polyphenols and in their substitution products. These findings are related to the possible carry-over of transition metals and may be worth exploring for custom tailoring the fining process. Whether the practical application of the latter finding will call for production and/or screening of plant-derived proteins with features appropriate to this task remains to be investigated. However, the approaches presented in this study may be used for large-scale screening of protein suitability for fining application under laboratory conditions, providing guidelines for their use in actual winemaking applications.

KEYWORDS: Wine fining; plant proteins; anthocyanidins; protein hydrophobicity; mass spectrometry

INTRODUCTION

Proteins have been used as wine fining agents for a long time. They not only allow clarification of colloidal suspensions but the precipitation of complexes between tannins and proteins in the process known as fining, which softens the gustatory appraisal and can reduce the astringency of otherwise rough wines. Fining also improves wine stability, limiting browning and overoxidation in white wine (*1, 2*) and stabilizing the color of red wine (*3*), as well as acting on bitterness and roughness in both red and white wines during aging.

A broad range of animal proteins have been used as fining agents, but the recent bovine spongiform encephalopathy pandemic led to the prohibition of the use of bovine plasma and blood cells (EC regulation 2087/97). Winemakers have been encouraged to stop using bovine gelatin as well, and there are also reservations on using egg albumin because of its animal origin (*4*). In this scenario, the use of plant-derived proteins as wine fining agents has become of much interest.

The influence of treatments with proteins, of both animal and vegetable origins, is related to protein–polyphenolic compound associations, in which hydrogen bonds and hydrophobic interactions are responsible for the expected flocculation and clarifying action (*5–7*). Improved knowledge of the functional properties of proteins used as fining agents and of the structure of polyphenolic compounds interacting with various classes of clarifying agents is expected to take the whole protein-based fining process beyond the empiricism that has characterized it so far.

The three-dimensional protein structure is dependent on a broad range of factors, which must be taken into account in a synergistic way to explain the functional properties of proteins relevant to the food business (*8*). Among these factors, surface hydrophobicity is known to be significantly related to the functional properties of food and nonfood proteins (*9*). Fluorescent probes are often used to measure the number and relative affinity of hydrophobic groups on the protein surface that are able to bind the probe. One of the most valuable and widely used noncovalent hydrophobicity probes is 1-anilino-8-naphthalenesulfonate (ANS). ANS has been used in studies concerning process-induced modification of isolated food proteins (*10–13*) and of complex food systems undergoing processes of various natures (*14–18*).

Here we studied the molecular basis of noncovalent interactions between proteins of plant origin and polyphenolic compounds, known for their role in organoleptic as well as stability properties of wines. Surface hydrophobicity of proteins of plant origin was investigated in wine-like model systems by studying changes in the binding properties of ANS, used as extrinsic fluorescent probe. Hydrophobic interactions between phenolic compounds and proteins were evaluated by the study of competition of phenolic compounds with probe for the same binding sites. Polymer chain length and the

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Published on Web 10/28/2010 pubs.acs.org/JAFC

Table 1. Surface Hydrophobicity Properties of Proteins in the Various Fining Agents[a]

fining agent	total protein content, mg/g	soluble protein, % of total	F_{max}, fluorescence at saturating probe concn × (mg of protein)$^{-1}$	K_d^{app}, μM	PSH
soy protein isolate	918 ± 12	0.56	1033 ± 16	30.09 ± 1.2	186.73 ± 3.7
pea protein isolate	900 ± 22	0.94	676 ± 32	26.53 ± 0.2	65.39 ± 3.2
lentil flour	315 ± 18	0.24	278 ± 12	24.11 ± 0.7	20.26 ± 1.1
gluten	975 ± 31	<0.10	432 ± 16	33.23 ± 2.2	17.80 ± 2.1

[a] Data and standard deviations are from a minimum of three determinations.

chemical nature of interacting phenols was also addressed by carrying out a qualitative and quantitative characterization of phenolic compounds retained by fining agents by means of mass spectrometry techniques (LC-ESI-MS, MALDI-TOF MS).

MATERIALS AND METHODS

Chemicals and Reagents. Water was purified with a Milli-Q system (Millipore, Bedford, MA). The wine-like model solution used was ethanol/water (10:90 v/v) containing 20 mM tartaric acid and buffered to pH 3.5 with NaOH. Unless otherwise specified, all chemicals were from Sigma Chemical Co. (St. Louis, MO). Oligomeric proanthocyanidin complexes (OPCs) from *Vitis vinifera* seeds were supplied by International Nutrition Co. (INC, Loosdrecht, The Netherlands). The fining agents for experimental activities included commercial protein extracts from soybean and pea, lentil flour, and gluten proteins (all from Prodotti Gianni, Milan, Italy). Chemical data for the various protein preparations are given in Table 1.

Probe Binding Studies. Protein surface hydrophobicity was assessed by using ANS as the fluorescent probe. Spectrofluorometric measurements were performed in a Perkin-Elmer Luminescence LS 50 spectrometer using 2.5 nm bandwidths for both excitation and emission. Spectrofluorometric titration of protein samples with negligible volumes of aqueous solutions of the hydrophobic fluorescent marker ANS was performed at 25 °C with magnetic stirring, as done before on protein suspensions (*10−12*, *16*). Binding of ANS was monitored at λ_{ex} 390 nm and λ_{em} 460 nm. Multiple additions of the fluorescent probe were done up to saturation with the probe (no increase in fluorescence intensity upon further addition of the probe). The increase in fluorescence intensity (F) upon progressive addition of ANS was analyzed by binding algorithms based on the relationship

$$F = F_{max}[\mathrm{ANS}]/(K_d^{app} + [\mathrm{ANS}])$$

that allowed estimation of the overall binding capacity of the proteins for the probe (given as fluorescence intensity at saturating ANS, F_{max}) and the apparent dissociation constant of the proteins−ANS complex (K_d^{app}). The overall binding capacity (F_{max}) was then corrected for the total protein content of each sample. A protein surface hydrophobicity index (PSH) was calculated as [F_{max} (corrected for the protein content) × $(K_d^{app})^{-1}$] (*15*).

The ability of insoluble proteins to bind ANS was measured by adding an excess of the fluorescent probe (> $2K_d^{app}$) to a suspension of proteins in a wine-like solution. The suspension was then centrifuged (3000*g*, 10 min, 20 °C). An aliquot of the supernatant was mixed with a detergent solution (aqueous Triton X-100, 2% w/v), which incorporated free ANS and ANS bound to soluble proteins (*19*). The ANS content in the micellar phase was then quantified spectrofluorometrically by adding appropriate amounts of ANS as an internal standard.

Competition studies. Hydrophobic interactions between polyphenol compounds and proteins of plant origin were evaluated by competition studies. Excess ANS (>$2K_d^{app}$) was added to protein suspensions. The decrease in ANS fluorescence due to probe displacement or by quenching was measured as a function of added polyphenolics [catechin or oligomeric proanthocyanidins, (OPCs)]. Concentration of OPCs was expressed as catechin equivalents. Titration with polyphenolics was continued until no further changes in fluorescence were observed.

In a different approach, the disappearance of ANS binding sites in the insoluble fraction of the various plant protein preparation after interaction with polyphenols was studied. Polyphenols were added to protein suspensions at concentrations corresponding to those at which no more fluorescence changes were observed in the ANS displacement experiments presented above. Excess ANS (> $2K_d^{app}$ for each individual protein system) was then added, and the amount of ANS remaining in the soluble fraction was measured after centrifugation (3000*g*, 10 min, 20 °C) by the detergent inclusion/internal standard procedure described in the section above.

High-Performance Liquid Chromatography−Electrospray Ionization−Mass Spectrometry (HPLC-ESI-MS). Proteins (200 mg/L) were added to OPC solutions (1 mg/mL) in 20 mL of wine-like buffer. Each sample was mixed for 30 min and centrifuged (3000*g*, 15 min, 20 °C). Both the supernatant and the pellet were analyzed by LC-ESI-MS. Pellets were taken up either in wine-like buffer or in a 2:1 mixture of acetonitrile and 0.1% TFA in water and centrifuged as above before analysis of the supernatant.

LC-ESI-MS was carried on a single-quadrupole instrument (HP1100-MSD, Agilent Technologies, Santa Clara, CA) and by using C18 columns (Vydac, Hesperia, CA; 2.1 × 250 mm). The eluents were 0.1% (v/v) TFA in HPLC-grade water (solvent A) and 0.1% (v/v) TFA in acetonitrile (solvent B). OPCs were separated at a constant flow rate of 0.2 mL/min, with a linear gradient of solvent B in the following proportions (v/v): 4 min, 0% B; 4−14 min, 0−18% B; 14−22 min, 18−28% B; 22−24 min, 28% B; 24−26 min, 28−60% B; 26−27 min, 60−80% B; and 27−30 min, 80−100% B. The total run time was 30 min with UV detection at 280 nm. Calibration curves were prepared using flavan-3-ol monomers [(+)-catechin, (−)-epicatechin, and (−)-epigallocatechin-3-*O*-gallate] in the 50−250 mg/L concentration range. Five different concentrations were used for each analyte, and experiments were performed in triplicate.

MALDI-TOF MS. MALDI-TOF spectra were recorded in positive-ion mode, using a Voyager DE-Pro spectrometer (PerSeptive BioSystems, Framingham, MA) equipped with a N_2 laser (337 nm). α-Cyano-4-hydroxycinnamic acid (Fluka, Buchs, Switzerland) was used as the matrix and prepared by dissolving 5 mg in 1 mL of aqueous 50% acetonitrile (v/v)/0.1% TFA (v/v). The instrument operated with an accelerating voltage of 20 kV. Mass spectrum acquisition was performed in both positive linear and reflectron mode. External mass calibration was performed with peptide standards (Sigma Chemical Co., St. Louis, MO).

RESULTS AND DISCUSSION

Protein Surface Hydrophobicity. Surface hydrophobicity plays an important role in protein functionality. Several studies have reported the use of ANS to characterize the surface hydrophobicity of soluble and insoluble proteins, such as those in cereal-based products (*20−22*). Binding parameters for ANS may be inferred directly from titration experiments regardless of the presence of heterogeneous phases (*10−12*, *16*) and describe rather accurately the surface properties of proteins (*18*).

Spectrofluorometric titrations with ANS of protein suspensions in wine-like buffer are presented in Figure 1 and confirm the general applicability of this procedure also to particulate and multiphasic systems. Figure 1 also shows that the various preparations of plant proteins had evident differences in their overall binding capacity toward the probe. The number of surface sites available for binding of the probe is expressed by F_{max}, the fluorescence at saturating probe concentration corrected for the protein content of individual preparations. As listed in Table 1, soybean proteins were characterized by the highest number of binding sites per unit mass protein, followed by pea proteins, gluten, and proteins in lentil flour.

From the titration curves in Figure 1 it was possible also to calculate the apparent dissociation constants of the protein−ANS complexes (K_d^{app}), which were similar in the various samples (Table 1). The ANS binding properties of individual protein

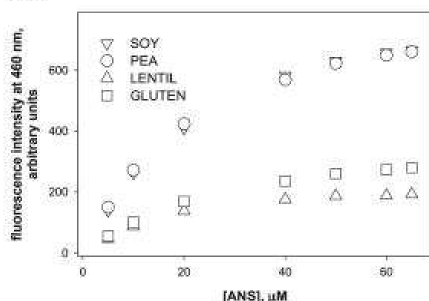

Figure 1. Fluorometric titration with ANS of soybean, pea, lentil flour, and gluten proteins (each at 1 mg of protein/mL in a wine-like model solution). Data are the average of at least triplicate measurements. Symbol size is larger than the standard deviation.

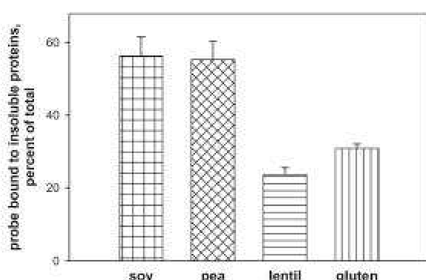

Figure 2. Percent fraction of ANS associated with the insoluble fraction of various proteins (1 mg/mL in a wine-like model solution) after interaction with 60 μM ANS.

preparations may conveniently be expressed, for comparative purposes, by combining the number of sites available for binding of the probe and their average affinity in a single surface hydrophobicity index (PSH = $[F_{max}/\text{prot}]/K_d^{app}$ (*14, 15*). As summarized in Table 1, PSH increased in the order gluten < lentil flour ≪ pea protein isolate < soybean protein isolate.

ANS partition studies were carried out to discriminate between binding of the probe to soluble proteins and binding of the probe to insoluble proteins that are simultaneously present in all of the preparations used here but gluten. Binding of hydrophobes to the insoluble protein fraction is obviously of paramount relevance to the wine fining process. These studies were also meant to set up conditions suitable for carrying out the competition experiments reported in a following section.

A slight excess (~ $2K_d^{app}$) of ANS was added to individual protein suspensions, and the amount of ANS remaining in solution after centrifugation was assessed by a detergent-stripping method (*19*). As shown in Figure 2, the insoluble protein fraction in all preparations had a remarkable capability of retaining the probe.

Insoluble proteins in soybean and pea preparations (accounting for 99.44 and 99.06% of total proteins, respectively, in the wine-like buffer used in these studies, see Table 1) captured almost 50% of the fluorescent probe initially present, whereas the almost completely insoluble gluten and insoluble proteins in lentil flour (99.76% of the total proteins) managed to capture about 30 and 20%, respectively, despite the modest overall affinity of these proteins for the probe as assessed by the titration studies shown in Figure 1.

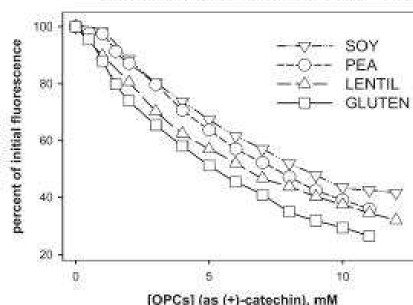

Figure 3. Decrease of ANS fluorescence upon addition of increasing amounts of oligomeric proanthocyanidins (OPCs) to wine-like model solutions containing 60 μM ANS and 1 mg/mL of proteins of various origin. Concentration of OPCs is given as cathechin equivalents. Data are the average of duplicate measurements. Symbol size is larger than the standard deviation.

Competition Studies. The specificity of the interactions between polyphenols and proteins of plant origin was evaluated by competition studies, in which polyphenols were tested for their ability to displace protein-bound ANS. As shown in Figure 3, the addition of increasing amounts of OPCs resulted in a decrease of ANS fluorescence in all cases. The results of similar experiments carried out with catechin (not shown) were overlapping those obtained with OPCs. At the concentrations used here, both catechin and OPCs had little effect on the very low fluorescence of free ANS. Thus, the fluorescence decrease in Figure 3 stems from detachment of the hydrophobic probe from the protein and seems to confirm that the interactions between proteins and phenolic compounds are governed by hydrophobic forces. Indeed, for both catechin and oligomeric proanthocyanidins, the ability to compete with the probe was highest for those proteins having the lowest ability to bind ANS (see Figures 1 and 2) and, thus, the lowest surface hydrophobicity.

The same competition approach was used to assess the amount of ANS remaining bound to the insoluble fraction after incubation of each protein system in the presence of fixed concentrations of ANS (0.1 mM) and oligomeric proanthocyanidins (10 mM as cathechin equivalents). As shown in Figure 4, the 100-fold excess of OPCs was unable to prevent binding of ANS to the insoluble proteins in any of the systems. The fluorescence decrease observed in Figure 3 may be explained as being due to ANS displacement from the soluble fraction of these proteins, but this hypothesis cannot explain what was observed in the case of the totally insoluble gluten. Thus, a more molecular based explanation of the experiments in Figures 3 and 4 more likely implies that polyphenols bind to proteins "on top" of the bound ANS, quenching its fluorescence and simulating its displacement. Therefore, a more direct approach is required to assess the extent and specificity of the binding of polyphenols to plant-derived proteins and to verify whether the fining process may be finely tuned by an appropriate choice of the involved proteins.

Structural Characterization of Phenolic Compounds before and after Interaction with Proteins Used as Fining Agents. To investigate the molecular basis of tannin–protein associations, OPCs were incubated with each of the various proteins in the same wine-like

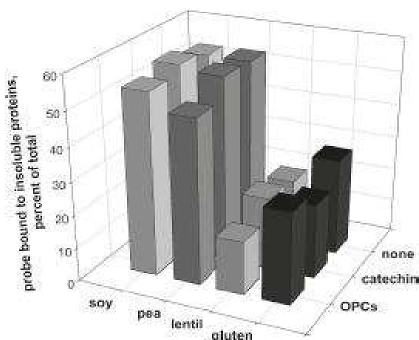

Figure 4. Percent fraction of ANS associated with the insoluble fraction of various proteins (1 mg/mL in a wine-like model solution) after interaction with 60 μM ANS in the absence (no addition) or in the presence of 10 mM catechin and 10 mM OPCs. Concentration of OPCs is given as cathechin equivalents. Data are the average of at least duplicate measurements. In all cases, standard deviation (not given for the sake of clarity) was within 5% of the measured value.

model solution used above. The identification of newly formed compounds and the changes in composition and concentration of OPCs were monitored by HPLC in combination with electrospray mass spectrometry (ESI-MS). To increase the sensitivity of the ESI-MS measurements, the samples were assayed twice, scanning from m/z 100 to 1000 and from m/z 1000 to 2000, respectively. Proanthocyanidin solutions, without addition of fining agents, were used as controls.

The MS total ion chromatogram (TIC) of the positive molecular ions of OPC standard solution (0.1 mM) in the range m/z 100−1000 (not shown) indicated the presence of P1 (m/z 291), P2 (m/z 579), P3 (m/z 867), and some proanthocyanidin gallates (PnGn), including P1G1 (m/z 443), P2G1 (m/z 731), and P2G2 (m/z 883). Polymerized OPCs, in the range m/z 1000−2000, are predominantly distributed at HPLC retention times of ca. 20−28 min. The mass spectra obtained from the TICs of the extract showed the molecular ion peaks of P4−P6 as well as those of the gallate derivatives P3G1 (m/z 1019), P3G2 (m/z 1172), P4G1 (m/z 1308), P4G2 (m/z 1460), P5G1 (m/z 1595), P5G2 (m/z 1748), and P6G1 (m/z 1884).

More complete data about polymerized tannins were obtained by MALDI-TOF MS analysis in positive-ion linear and reflectron modes. MALDI-TOF is able to measure masses in complex mixtures of low and high molecular weight compounds. In model white wine were detected an oligomeric series of catechin/epicatechin units and their gallic acid ester derivatives (sodium adduct ions $M + Na^+$), up to the decamer (see Table 2). Additionally, masses corresponding to a series of polygalloyl polyflavans were also detected.

Fining model systems were set up by adding each of the fining agents (at the commonly used concentration of 20 g/100 L, corresponding to a protein concentration of 200 mg/L) to OPC solutions in our wine-like buffer, which were stirred for 30 min and centrifuged. The resulting supernatants and the pellets, taken up in a wine-like solution to dissociate weakly bound tannins, were analyzed by LC-ESI-MS. Representative TIC and MS tracings from one of these experiments are shown in Figure 5. The TICs of all treated wine-like systems resembled those obtained for standard solutions of OPCs, suggesting an identity of small oligomeric flavan-3ols. This finding was also supported by MALDI-TOF MS results, as summarized in Table 2.

The results of MALDI-TOF analysis of fined wine-like systems also suggest that all tested protein fining agents selectively removed polymeric proanthocyanidins, lowering their apparent average degree of polymerization in fined model wine with respect to the original untreated solution of OPCs. These results are in accordance with previous studies, which assume that the largest proanthocyanidin molecules are precipitated first in fining experiments (23). This effect could be due to the higher number of phenolic rings present in the more polymerized proanthocyanidins, which increases their hydrophobicity and allows for more effective removal (24).

Characterization and estimates of the relative amounts of polyphenols precipitated from wine-like model system were made by LC-ESI-MS analysis of pellets after fining treatment. All of the pellets showed the presence of newly formed products. For instance, we observed the presence of vinylcatechin and vinylepicatechin (m/z 316), eluting later than their unmodified forms (Figure 6), and originating by catechin/epicatechin autopolymerization induced by acetaldehyde.

The acetaldehyde present in fining model systems derives from the oxidation of ethanol, either catalyzed by transition metals such as iron and copper (that reportedly are found associated with plant-derived proteins) or through coupled oxidation of phenols (25). The reaction starts with the nucleophilic addition of the protonated form of acetaldehyde to the flavanol. The newly formed ethanol adduct, losing a water molecule, is attacked by a second nucleophilic flavanol unit to yield an ethyl-linked flavanol dimer. The ethyl linkages generated by acetaldehyde in the polycondensated tannins are not stable and cleave into vinylflavanol monomers and oligomers (26). Compared to direct condensation between flavanols, the rapid polymerization mediated by acetaldehyde gives rise to instability and precipitation (27, 28). This could explain the presence of vinylflavanol products in the pellets precipitated by finings and not in the supernatant of treated samples. In addition to these compounds, various dimeric and oligomeric ethyl-bridged molecules were also detected by MALDI-TOF analysis of pellets. These newly formed species included adducts of trimers and their gallic acid derivatives (m/z 923; m/z 1075.9).

We also carried out a detailed quantitative LC-MS analysis of the flavonoid compounds most important with respect to white wine oxidation (monomeric and dimeric proanthocyanidins) to evaluate which molecules were most easily removed by the various proteins. The browning capacity of white wines depends largely on the nature of polyphenols. Due to their catechol (*o*-diphenol) structure, most of them are rather readily oxidized in winemaking processes, The monomeric catechins and the dimeric procyanidins contribute to the browning more intensely than other phenolics (29), and there is strong evidence of epicatechin being the most relevant browning agent among redox-active polyphenols (30).

In the OPCs control solution (5 mg/mL) the calculated total concentration of monomeric and dimeric molecules (catechin, epicatechin, monomers gallate, dimers, dimers gallate, dimers digallate) was 2.9 mg/mL. A general decrease in the concentration of all these species, considered as a whole, was observed after treatment with proteins. Lentil flour was the most effective removal agent, giving a 16.4% decrease in OPCs, followed by gluten, soy, and pea proteins, which gave decreases of 12.6, 9.26, and 8.44%, respectively. These differences in clarifying efficiency are likely related to the molecular composition, the biochemical characteristics, and the conformation of proteins relevant to the complex interactions that ultimately lead to flocculation of their

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Table 2. Oligomeric Proanthocyanidin Detected by MALDI-TOF MS before and after Fining Wine-like Model Solutions (10 mM Proanthocyanidins) with Soybean, Pea, Lentil Flour, and Gluten Proteins (200 mg/L)[a]

		molecular mass, Da					
				fined wine-like solutions			
	no. of galloyl units	calcd (M + Na$^+$)	original OPCs (M + Na$^+$)	soybean	pea	lentil flour	gluten
dimer	0	601.3	602.5	602.1	602.4	601.8	602.2
	1	753.3	754.3	753.8	754.1	753.4	754.0
	2	905.3	907.7	905.6	905.9	905.2	905.7
trimer	0	889.8	890.9	889.7	889.9	889.5	889.9
	1	1041.9	1042.4	1041.4	1041.6	1040.8	1041.4
	2	1194.0	1194.2	1193.0	1194.5	1194.0	1193.3
	3	1346.1	1347.4	1344.8	1345.6	nd	1346.3
tetramer	0	1178.0	1178.0	1177.2	1177.7	1176.7	1178.2
	1	1330.1	1331.8	1329.0	1329.3	1329.9	1329.6
	2	1482.2	1483.0	1481.2	1483.1	1480.9	1483.0
	3	1634.4	1634.6	nd	1633.4	1636.3	nd
	4	1786.5	1787.9	1785.7	1785.7	1784.5	nd
pentamer	0	1466.3	1466.1	1465.9	1466.4	1465.0	1466.0
	1	1618.4	1618.8	nd	1617.4	1615.3	1617.8
	2	1770.5	1770.8	nd	1771.0	1769.0	1769.1
	3	1922.6	1922.9	1919.0	1920.9	nd	1920.8
	4	2074.7	2074.6	nd	2075.8	2077.9	2074.7
hexamer	0	1754.5	1754.9	1752.7	1752.1	1751.1	1754.5
	1	1906.7	1907.6	1905.0	1905.9	1903.0	1905.9
	2	2058.8	2059.9	2057.4	2057.4	2056.5	2055.8
	3	2210.9	2211.3	nd	2210.0	nd	2209.9
	4	2363.0	2363.6	nd	nd	nd	nd
	5	2515.1	nd	nd	nd	nd	nd
	6	2667.2	nd	nd	nd		nd
heptamer	0	2042.8	2042.3	2042.4	2041.0	2043.3	2042.8
	1	2194.9	2194.7	nd	2192.4	nd	2194.9
	2	2347.0	2347.9	2346.5	2345.0	nd	2344.0
	3	2499.1	2499.4	nd	2495.0	nd	2494.4
	4	2651.2	2650.6	nd	nd	nd	nd
	5	2803.3	2802.5	nd	2805.9	nd	2800.2
	6	2955.4	2956.2	nd	2956.2	nd	2959.1
	7	3107.5	3106.3	3106.7	3104.8	3103.3	nd
octamer	0	2331.1	2330.5	nd	2332.2	2327.8	nd
	1	2483.2	2481.7	nd	2482.1	nd	nd
	2	2635.3	2634.0	2634.4	2632.5	nd	2634.2
	3	2787.4	2786.4	nd	2876.5	2786.4	nd
	4	2939.5	2936.8	nd	2939.5	2941.7	nd
	5	3091.0	3093.6	nd	3088.8	nd	3089.9
	6	3243.0	3241.0	nd	3244.4	nd	nd
	7	3395.0	nd	3394.2	3397.2	nd	nd
nonamer	0	2619.3	2619.1	2619.0	nd	nd	nd
	1	2771.4	2771.1	nd	nd	nd	nd
	2	2923.5	2922.6	nd	nd	nd	2924.8
	3	3075.6	3072.6	nd	nd	nd	nd
	4	3227.7	3228.3	nd	3229.6	nd	3226.7
	5	3379.8	3377.3	nd	nd	3378.6	nd
decamer	0	2907.6	2905.0	nd	nd	nd	2909.3
	1	3059.7	3056.0	nd	3053.8	3059.3	nd
	2	3211.8	3209.5	nd	nd	nd	nd
	3	3363.9	3360.7	nd	nd	nd	nd
	4	3516.0	nd	nd	nd	nd	nd
	5	3668.1	nd	nd	nd	nd	nd

[a] OPCs, oligomeric proanthocyanidins; nd, not detected.

complexes with polyphenols and to clarification of the model wine-like solutions used here.

At first sight the interactions brought forward by the clarifying ability of proteins from the various sources seem to be in disagreement with the surface hydrophobicity data and the competition experiments reported in other sections of this work. On the basis of these latter data, we expected that proteins characterized by the highest surface hydrophobicity should have given the highest

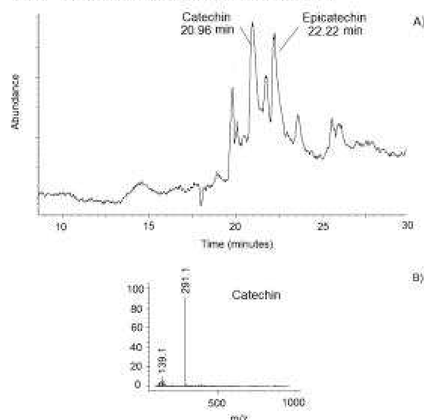

Figure 5. (A) Ttotal ion current (TIC) chromatogram obtained in positive ion mode by injection of wine-like model solution after fining with pea protein isolate (1 mg/mL protein, 10 mM proantocyanidins). (B) ESI mass spectrum obtained from the TIC chromatogram for 20.92 min elution time, showing the $[M - H]^+$ peaks of catechin (*m/z* 291).

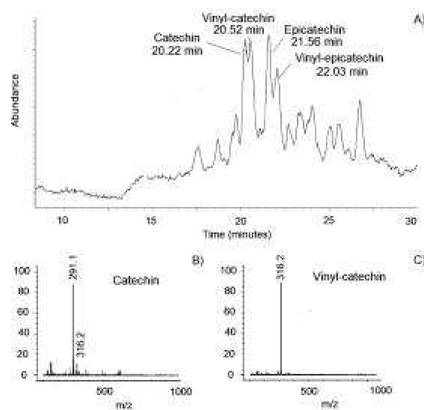

Figure 6. (A) Total ion current (TIC) chromatogram obtained in positive ion mode by injection of insoluble fraction of pea protein isolate (1 mg/mL protein, 10 mM proantocyanidins). (B, C) ESI mass spectra obtained from the TIC chromatogram for 20.22 and 20.52 min elution time, respectively, showing the $[M - H]^+$ peaks of catechin (*m/z* 291) and vinylcatechin (*m/z* 316).

removal of OPC from the wine-like medium, whereas the direct measurements reported above indicate that the actual rank was reversed, at least when OPCs are considered as a whole.

However, if the loss and recovery are analyzed in terms of individual molecules, there is evidence that this discrepancy is more apparent than substantial and that molecular specificity plays a role in governing the interaction between hydrophobic sites on the protein surface and the molecules considered here.

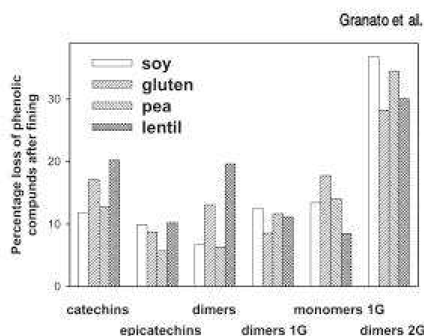

Figure 7. Percentage loss of individual flavan-3-ols species in fined wine-like samples, as assessed by means of LC-ESI-MS analysis.

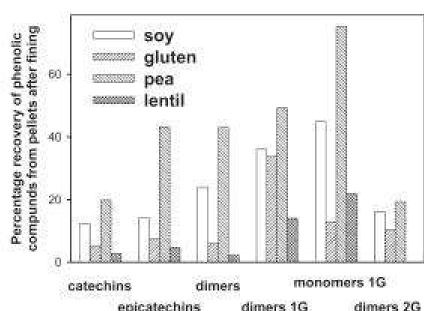

Figure 8. Percentage recovery of phenolic compounds from pellets obtained after fining processes carried out with the various proteins (ratio between the concentration of each molecules in the pellet and their loss in fined wine-like sample).

Figure 7 presents quantitative data as for the removal of individual species after treatment with the various proteins, obtained through HPLC analysis. For instance, the efficiency of the various proteins in removing (+)-catechin, and (−)-epicatechin, which differ only on the spatial position of one OH group with respect to the ring, was remarkably different. In particular, (+)-catechin was more specifically removed than (−)-epicatechin by all of the protein fining agents tested, especially by the lentil flour and the gluten proteins. Moreover, the levels of galloylated proanthocyanidin precipitation appeared to be higher than those of the other phenols for all protein fining agents (from 28% loss with gluten proteins to 36% loss with soy proteins), indicating that the more galloylated proanthocyanidins were removed in a preferential way.

Pellets obtained from the fining processes carried out with the various proteins also were treated with water and with more apolar solvents to assess the nature and intensity of the forces involved in the interaction. Pellets were dissolved in acetonitrile/water 0.1% TFA (2:1) to dissociate soluble and insoluble tannin−protein complexes. The percentage recoveries of phenolic compounds from pellets (ratio between the concentration of each molecules in the pellet and their loss in fined wine-like sample) are compared for each of the tested proteins in Figure 8.

Pea and soy proteins were the fining systems in which proanthocyanidins were more easily recovered from pellets. This is in accordance with the evidence gathered from competition studies and confirms that "weak" forces, such as hydrophobic ones, were most relevant to the interactions responsible for precipitation by these fining agents. The interaction between polyphenols and gluten proteins and, above all, lentil flour systems (characterized by higher loss in treated wine-like samples, lower surface hydrophobicity, and low recovery from the pellets) could be instead governed by other types of attractive forces such as hydrogen or covalent bonds (cross-linkages between proteins), which may impair the release of polymerized tannins.

In conclusion, the structural characterization of proteins of plant origin in terms of surface hydrophobicity provided a comparative estimate of the number and of the affinity of binding sites on the surface of the various proteins. The indications provided by these studies were confirmed to a large extent by competition/displacement experiments. However, a straightforward interpretation of the displacement experiments was made difficult by simultaneous binding of the fluorescent probe and of polyphenols. This indicates the existence of multiple binding sites on the protein surface, with a possible different specificity for different molecules, as observed for many other food and nonfood proteins (*31*, *32*). This implies that proteins of different origin may selectively bind peculiar fractions in a complex mixture of polyphenols, as confirmed by LC-MS analysis of the pattern of bound and residual polyphenols in mixtures simulating the actual fining process. This finding is of possible practical interest, in that it paves the way to a selective use of protein agents for "fine tuning" the properties of the finished product with respect to important organoleptic properties and their stability.

However, it was also noted that treatment of simulated wine with the commercial protein preparations used here favors some chemical reactions among some of the polyphenols and other wine components. The significance of these reactions in a real wine (in which they may be affected by other wine components) remains to be evaluated. The possible requirement for plant-protein-based fining agents chosen and/or processed ad hoc for this particular purpose also remains to be evaluated. Should this be appropriate or necessary, the methodologies presented here could be fruitfully exploited to assess whether these materials will be suitable for this particular use, for instance, by testing their surface hydrophobicity properties, prior to resorting to exceedingly laborious, time-consuming, and expensive experimentation in actual winemaking applications.

ABBREVIATIONS USED

ANS, 1-(anilino)naphthalene-8-sulfonate; PSH, protein surface hydrophobicity; F_{max}, maximum fluorescence intensity; K_d^{app}, apparent dissociation constant of the protein−ANS complex; LC-MS, liquid chromatography−mass spectrometry; ESI, electrospray ionization; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; TFA, trifluoroacetic acid; TIC, total ion current; OPCs, oligomeric proanthocyanidins complexes; (PnGn), proanthocyanidin gallates.

ACKNOWLEDGMENT

The assistance of Dennis Van der Vlies at INC in preparation of the proanthocyanidins used in this study is gratefully acknowledged.

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Received for review July 7, 2010. Revised manuscript received October 4, 2010. Accepted October 11, 2010. Work supported in part by PRIN2008 "Evoluzione di composti solforati varietali nella trasformazione enologica".

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ADDITIVI DI ORIGINE NATURALE SOSTITUTIVI DELL'ANIDRIDE SOLFOROSA NELLA VINIFICAZIONE

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I solfiti sono ampiamente utilizzati come additivi in diverse matrici alimentari quali vino, birra, succhi di frutta, frutta secca, biscotti e prodotti vegetali. Attualmente l'anidride solforosa costituisce uno strumento indispensabile nella pratica di cantina grazie alle sue peculiari e complesse proprietà tecnologiche (potere antiossidante, agente antimicrobico, attività antiossidasica e defecante, azione pro-fermentativa e di stabilizzazione del colore). Ciò nonostante, essa rappresenta anche uno degli additivi più discussi per gli inconvenienti che causa quando impiegata in dosi troppo elevate: sintomi di intossicazione (nausea, vomito, irritazione gastrica e cefalea), reazioni allergiche caratterizzate da manifestazioni di asma, difficoltà respiratoria, tosse ed inoltre effetti antinutrizionali e citotossici. Per molti anni produttori di vino e ricercatori hanno cercato una valida alternativa all'impiego dei solfiti in vinificazione, ma attualmente non è stata individuata ancora nessuna valida alternativa.

Questo studio si è proposto di

TESTARE NUOVE FORMULAZIONI DI COMPOSTI NATURALI DA IMPIEGARE NEL PROCESSO DI VINIFICAZIONE IN SOSTITUZIONE E/O A COMPLEMENTO DEL BIOSSIDO DI ZOLFO, IN GRADO DI MIMARNE QUANTO PIÙ POSSIBILE L'EFFICACIA TECNOLOGICA

ADDITIVI TESTATI

ADDITIVO 1

Estratto vegetale dalla pianta di Acerola (ciliegia delle Barbados), di consistenza liquida, incolore. Composizione: acidi organici, carotenoidi, flavonoidi, composti e derivati solforati, tannini e pectine.

ADDITIVO 2
ADDITIVO 3
ADDITIVO 4

Proantocianidine oligomeriche (OPCs), polimeri dei flavan-3-oli, purificati da *Vitis vinifera*. Consistenza polverosa, colore variabile dal rossiccio al bruno.

DISEGNO SPERIMENTALE

CARATTERIZZAZIONE DEGLI ADDITIVI

Tutti gli additivi sono solubili in soluzione idroalcolica modello, ma solo l'ADD 1 mostra stabilità nel tempo alla reazione di precipitazione; gli OPCs determinano la formazione di un corpo di fondo pochi giorni dopo essere stati solubilizzati. Gli additivi 2, 3 e 4 incrementano la densità ottica e la torbidità della soluzione, probabilmente a causa della loro natura (elevato contenuto in polifenoli totali).

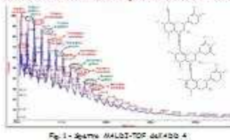

Fig. 1 - Spettro MALDI-TOF dell'ADD 4

La caratterizzazione molecolare degli OPCs è stata condotta mediante analisi LC-ESI/MS e MALDI-TOF MS: dallo spettro di massa MALDI-TOF dell'ADD 4 è possibile evidenziare polimeri della catechina fino all'eptamero e della catechina gallato fino all'esamero.

TABELLA 1 - COMPOSTI AROMATICI E CORRISPONDENTI DESCRITTORI ODOROSI DEL VINO DI PROVA E DELLO STESSO + ADD1

VINO	VINO + ADD1	DESCRITTORE ODOROSO
3-metil-1-butanolo	3-metil-1-butanolo	Malto
2,3-butandiolo	2,3-butandiolo	Frutta
Etilbutanoato	Etilbutanoato	Frutta
1-esanolo	1-esanolo	Erba
Isoamilacetato	Isoamilacetato	Banana
Stirene	Stirene	Balsamico
Etilesanoato	Etilesanoato	Buccia di mela
Esilacetato	Esilacetato	Frutta
Limonene	Limonene	Agrumi
3-carene	3-carene	Buccia d'arancia
β-mircene	β-mircene	Balsamico
Linalolo	Linalolo	Limone
Feniletilalcol	Feniletilalcol	Rosa
Succinato di etile	Succinato di etile	Frutta, vino
Etilottanoato	Etilottanoato	Ananas
Isopentilesanoato	Isopentilesanoato	/
9-decenoato di etile	9-decenoato di etile	/
Etildecanoato	Etildecanoato	Uva, frutta
Etildodecanoato	Etildodecanoato	/
/	α-pinene (tracce)	Olio di pino

VINO DI PROVA + SO_2

VINO DI PROVA + ADD1

Fig. 2 - Cromatogramma TIC ottenuto mediante analisi HS-SPME-GC/MS del vino di prova e dello stesso + ADD 1

L'analisi HS-SPME-GC/MS di un campione di vino di prova, prima e dopo l'aggiunta degli additivi nelle concentrazioni di utilizzo, mostra che la composizione qualitativa in molecole odorose resta la stessa. I profili cromatografici sono sovrapponibili.

I saggi di tossicità *in vitro* mostrano che l'ADD 1 non altera la normale crescita cellulare, mentre l'ADD2 può determinarne l'arresto se la dose impiegata è troppo elevata.

MICROVINIFICAZIONI SPERIMENTALI

Fig. 4 - Vino bianco sperimentale varietà Nuragus di Cagliari

L'efficacia tecnologica degli additivi in esame è stata testata nella microvinificazione sperimentale di una varietà di uva a bacca bianca tipica della Regione Campania (*Falanghina*), della Sardegna (*Nuragus di Cagliari*) e di una varietà a bacca rossa spagnola (*Tempranillo*) nel triennio 2005-2007.

La miscela di estratti vegetali (ADD1) ha fornito i risultati migliori per la vinificazione in bianco, mostrando anche un effetto sinergico qualora utilizzata in presenza di una ridotta quantità di anidride solforosa, pari al 20% di quella normalmente aggiunta.

La caratterizzazione chimico-fisica e sensoriale dei mosti e dei vini sperimentali ha evidenziato che gli OPCs (ADD 2-3-4) sono inadeguati per la vinificazione in bianco, a causa del contributo negativo dato al colore del vino alle concentrazioni di utilizzo.

Gli OPCs (in particolare ADD2 e ADD4) sono stati utilizzati con successo nella vinificazione in rosso dell'uva *Tempranillo*

Fig. 6 - TEA test dei vini sperimentali a fine fermentazione, al primo travaso e al secondo travaso

L'analisi del potere antiossidante dei vini rossi ha mostrato che l'ADD2-3-4 hanno maggiore potere antiossidante all'inizio della vinificazione al contrario dell'ADD1 che sembra svolgere meglio la funzione antiossidante nella fase di conservazione del vino.

CONCLUSIONI

Tutti i vini sperimentali non hanno evidenziato difetti e/o differenze sensoriali rispetto al controllo: risultati ottimali per la vinificazione in bianco sono stati ottenuti con l'impiego dell'ADD1 ed una ridotta quantità di anidride solforosa, pari al 20% di quella normalmente impiegata.

Le due classi di additivi hanno mostrato un diverso comportamento in funzione della fase del processo di vinificazione in cui sono state impiegate: ciò suggerisce la possibilità di utilizzarle in modo differenziato nella vinificazione in rosso.

EFFETTO DEL TRATTAMENTO CON PROTEINE VEGETALI SULLE COMPONENTI AROMATICHE E TANNICHE DEL VINO: SELEZIONE SU BASE MOLECOLARE DI COADIUVANTI PROTEICI PER IL MIGLIORAMENTO DELLA QUALITÀ ORGANOLETTICA DEI VINI

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La chiarificazione mediante collaggio proteico rimuove dal vino le sostanze di natura colloidale responsabili della torbidità o di intorbidamenti, e migliora la filtrabilità e le caratteristiche organolettiche, controllando l'imbrunimento e la polimerizzazione ossidativa di composti polifenolici e riducendo la sensazione di astringenza. Le proteine animali sono state per anni le più utilizzate, ma le restrizioni normative rispetto ai coadiuvanti enologici di origine animale, rendono interessante l'impiego di proteine vegetali, da cereali o leguminose. Il presente lavoro si pone l'obiettivo di mettere a punto una metodica analitica per lo studio delle interazioni non covalenti tra molecole idrofobiche responsabili di note organolettiche nei vini, quali polifenoli e molecole odorose, e chiarificanti proteici vegetali, per consentirne un utilizzo più selettivo e razionale. L'indagine è partita da soluzioni idroalcoliche modello e si è estesa a vini trattati con i chiarificanti selezionati a concentrazioni ottimali. I composti coinvolti nell'interazione ed i complessi generati sono stati caratterizzati combinando tecniche separative e di spettrometria di massa (LC-ESI MS, MALDI TOF MS). Poiché il collaggio proteico può provocare una riduzione delle componenti aromatiche di un vino, nella fase successiva dello studio si valuterà l'incidenza aromatica della chiarifica mediante microestrazione in fase solida e analisi in GC-MS.

CARATTERIZZAZIONE DI METABOLITI GENERATI DURANTE LA MATURAZIONE DEL SALAME TIPO NAPOLI: CONFRONTO TRA PRODOTTO INDUSTRIALE ED ARTIGIANALE

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La proteolisi e la lipolisi che avvengono durante la maturazione del salame tipo Napoli sono di fondamentale importanza nella definizione delle caratteristiche organolettiche e strutturali tipiche di questo prodotto: la frazione lipidica subisce cambiamenti idrolitici ed ossidativi, coinvolgenti la liberazione di acidi grassi liberi (FFA) che determinano la formazione diretta di composti volatili o la formazione di precursori di molecole odorose. Allo stesso tempo l'azione proteolitica da parte di enzimi endogeni, calpaine e catepsine, e di enzimi della specie *Lactobacillus* determina la formazione di peptidi e amminoacidi che fungono da precursori di composti aromatici. Lo scopo del seguente lavoro è stato quello di caratterizzare i metaboliti generati durante la maturazione del salame tipo Napoli al fine di identificare marcatori molecolari in grado di evidenziare eventuali differenze connesse alla tecnologia di produzione (artigianale o industriale). L'analisi proteomica è stata effettuata mediante l'utilizzo combinato di tecniche cromatografiche e di spettrometria di massa (MALDI-TOF, ESI-MS-MS); l'analisi metabolomica della componente lipidica ed aromatica è stata condotta mediante tecniche GC-MS. Tale approccio metodologico ha permesso di rilevare differenze nel profilo aromatico e proteico tra il prodotto industriale e quello artigianale correlabili alla presenza di colture starter nel prodotto industriale.

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Italian Proteomics Association
Congresso Nazionale 2010
Firenze, Chiostro del Maglio – via Venezia
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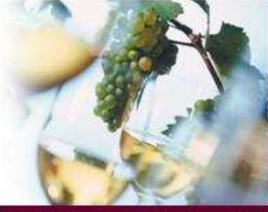

INTERACTION BETWEEN PROTEINS OF PLANT ORIGIN AND WINE COMPONENTS: MOLECULAR-BASED CHOICE OF PROTEIN FINING AGENTS FOR ORGANOLEPTIC IMPROVEMENT

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Proteins have been used in wine as fining agents for a long time to improve stability of wines; they modify and stabilize the colour of red wine and control browning and over-oxidation in white wine (Spagna et al., 1996; Spagna et al., 2000; Cosme et al., 2008) and bitterness and roughness both in red and white wines during ageing. Nowadays a wide range of animal protein as fining agents are used; more recently cases of bovine spongiform encephalopathy caused a situation of crisis leading to the interdiction of the use of bovine plasma and blood cells (regulation EC no 2087/97) and winemakers have been encouraged to stop using bovine gelatine; some winemakers also hesitate to use egg albumin because of their animal origin (Marchal et al., 2002). In this scenario some proteins of vegetable origin as clarifying agents have become more interesting. The influence of treatments with proteins is related to proteins-polyphenolic compounds interactions, which are responsible for the expected flocculation and clarifying (Versari et al., 1999; Sarni-Machado et al., 1999; Yokotsuka et al., 1995). A better knowledge of the functional properties of proteins used as fining agents and of the structure of polyphenolic compounds kept by clarifying products can lead to less empirical finings.

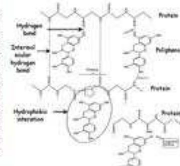
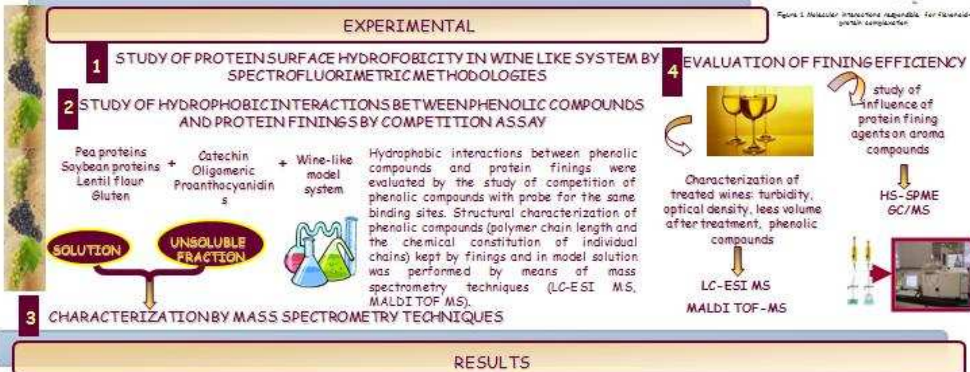


Figure 1 Molecular interactions responsible for flavonoid-protein complexation



RESULTS

SPECTROFLUORIMETRIC ASSAYS

Protein surface hydrophobicity was investigated in wine-like model system by spectrofluorimetric determination of changes in the binding properties of the anilinonaphthalenesulfonate (ANS), used as extrinsic fluorescent probe.

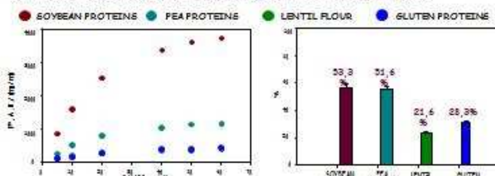


Figure 2. Fluorimetric titration with 1-anilino-8-naphtalene sulphonate (ANS) of soybean, pea, lentil and gluten proteins

Figure 3. Percent fraction of ANS associated with the insoluble fraction of various after interaction with 60 µM ANS.

Soybean proteins were showed the highest number of binding sites per unit mass protein, followed by pea proteins, gluten and by proteins in lentil flour (Fig. 2).

Insoluble proteins in soybean and pea preparations (99.44 % and 99.06 % of total proteins, respectively) captured almost 50% of the fluorescent probe initially present, whereas the almost completely insoluble gluten and insoluble proteins in lentil flour (99.76 % of the total proteins) managed to capture about 30% and 20%, respectively, despite the modest overall affinity of these proteins for the probe (Fig. 3).

The addition of increasing amounts of phenolic compounds to the protein-ANS complex quenched the probe fluorescence, indicating a competition for the same binding sites. Proteins characterized by higher surface hydrophobicity (soy and pea preparations) showed a higher competition for binding sites in wine-like medium (Fig. 3). The capability of insoluble fractions to bind hydrophobic probe was instead not modified by the addition of polyphenolic compounds employed.

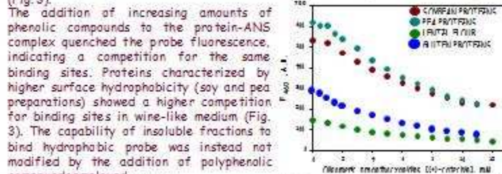
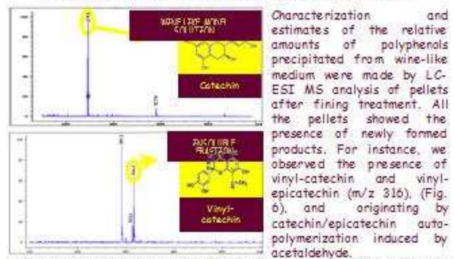


Figure 4. Decrease of ANS fluorescence upon addition of increasing amounts of oligomeric proanthocyanidins to wine-like model solutions containing 60 µM ANS and 1 mg/ml of proteins.

MASS SPECTROMETRY CHARACTERIZATION

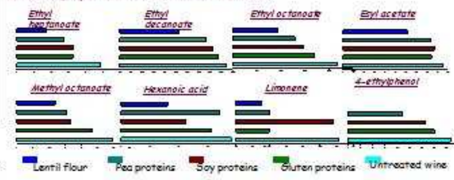


Characterization and estimates of the relative amounts of polyphenols precipitated from wine-like medium were made by LC-ESI MS analysis of pellets after fining treatment. All the pellets showed the presence of newly formed products. For instance, we observed the presence of vinyl-catechin and vinyl-epicatechin (m/z 316), (Fig. 6), and originating by catechin/epicatechin auto-polymerization induced by acetaldehyde.

Figure 5. LC-ESI MS spectrum obtained in positive ion mode by injection of a) wine-like model solution (10% v/v ethanol, tartrate buffer, pH 3.5, oligomeric proanthocyanidins) and b) insoluble fraction of gluten proteins

FINING EXPERIMENTS

The study of mechanism of interaction of finings and polyphenols was extended to real wine with fining experiments on Falanghina white wines. All fining agents tested allowed very fine clarification of wine, but the use of lentil proteins caused a decrease of global content of te responsible compound for the varietal and prefermentative aroma (Fig. 6). The other fining agents seems more suitable when the wines are prepared from aromatic grapes such as Moscato or Malvasia.



DETERMINATION OF SECONDARY METABOLITES AS QUALITY AND TYPICALNESS TRACERS IN AUTOCHTHONOUS VITIS VINIFERA GRAPES AND WINES FROM ISCHIA ISLE

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ABSTRACT

In this study a "metabolomic" approach carried out on secondary metabolites of autochthonous vinegrapes from a terroir with very specific and distinctive characteristics, such as the Ischia isle, permitted us to obtain the characterization on molecular basis of some typical oenological productions. The analysis started from white and red grapes (Arilla grapes from Cuotto and Spadara; Forastera, Don Lunardo, Livella from Panza and Spadara; Guarnaccia from Spadara and Fango), and extended to wines, was effected by means of advanced mass spectrometry techniques (GC/MS, MALDI/TOF-MS; LC/ESI-MS). The characterization of secondary metabolites (volatile compounds and anthocyanins) allowed identification of molecular tracers which can be important for the fingerprinting and protection of qualitative characteristics of these typical productions. Possible relationships between the quantitative composition of these metabolites and some environmental characteristics such as hydric stress conditions were also investigated.

KEYWORDS: wine and grape quality and biodiversity; secondary metabolites; "terroir".

INTRODUCTION

High quality wines acquire a meaningful importance on the market through the diversification of their specific identifiable qualitative characteristics. During the last 30 years, consumer preferences have been oriented towards authochthonous wines with quality trademarks such as the European Appellation of Origin designations (e.g., Italian Controlled and Guaranteed Denomination of Origin, i.e. DOCG) or Controlled Denomination of Origin, i.e. DOC, etc.). The typicalness of these wines is related to the grape variety and specific characteristics of the *terroir* (i.e. soil, location, climate, and specific vineyard-environment interaction). Several studies of viticultural zoning have been carried out by using a multidisciplinary approach to define the best qualitative potential of the oenological productions (Nasi *et al.*, 2010). In grapes and wines the composition of odorous molecules and antocyanin compounds is often used for varietal differentiation being the rationale of these studies that these compounds are constituted by several molecules whose concentration can vary depending on the grape variety (Mamede, Pastore, 2006; Nasi *et al.*, 2008; Oliveira *et al.*, 2004).

Analytical tools can provide new information on molecular basis and when combined with conventional analytical parameters can give a better description of typicalness and quality of food products. Molecular approaches by means of a combined use of mass spectrometric techniques can be important in order to open new possibilities in the differentiation and

defense of typical products. In this study carried out through a combined use of techniques based on mass spectrometry (GC/MS, MALDI-TOF-MS), the characterization of secondary metabolites (volatile compounds and antocyanins) in autochthonous grapes and wines from isle of Ischia, allowed identification of molecular tracers which can be important for the fingerprinting and protection of qualitative characteristics of these typical productions. Possible relationships between the quantitative composition of these metabolites and some environmental characteristics such as hydric stress conditions were also investigated.

MATERIALS AND METHODS

Determination of volatile compounds

The SPME (Solid Phase Micro-Extraction) analysis was effected with the holder and fibres (50/30 µm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS)) purchased from Supelco (Aldrich, Bornem, Belgium) on 130 mL of sample (wine and must). Thermal desorption of the analytes from the fibre inside the GC injection port was carried out in the split mode (1/10) at a desorption temperature of 250 °C for 1 min. All samples were analyzed with an HP 7890A coupled to a 5975C quadrupole HP mass spectrometer (Agilent Technologies, Palo Alto, CA, USA). The gas chromatograph was equipped with an HP-5ms capillary column (30 m x 0.25 mm ID; 0.25 µm Film Thickness) and the carrier gas used was helium. For the analysis of the volatile molecules, the GC oven temperature was programmed from 40 °C (held for 7 min) to 180 °C at 5 °C/min. The masses were scanned on m/z range of 45-350 amu. In other cases, a SIM method was used (for terpene compounds m/z 59, 69, 93, 121, 136, 154). The NIST library and comparison with spectra and retention times of standards (Sigma-Aldrich; Acros Organics) were used to identify the odorous compounds. Quantitative determinations were obtained by means of calibration curves, in the concentration ranges typical of wines for each compound. In the range of linearity verified seven concentration levels and five replicates per level were used. Multiple replicates (n=3-6) of the samples were analyzed.

Determination of antocyanin compounds

4 g of skins were homogenised in 10 mL of acidified methanol (0,1%, v/v HCl in methanol) and the extraction was effected overnight. The extracted samples were filtered with sodium sulphate anhydrous and analyzed through MALDI/ToF/MS.

MALDI-TOF spectra were recorded using a Voyager DE-Pro spectrometer (PerSeptive BioSystems, Framingham,MA) equipped with a N2 laser (337 nm); α-ciano-4-hydroxy-cinnamic acid (Fluka, Buchs SG, Switzerland) was used as matrix prepared by dissolving 5 mg in 1 ml of aqueous 50%, v/v, acetonitrile/0.1%, v/v.

RESULTS

Varietal volatile compounds detected in grape and wine samples are listed in Tab. 1, 2.

The most notable differences in the terpene and norisoprenoid composition were related to the varietal origin.

Among the white grapes and wines considered Don Lunardo grapes and wines had a more complex qualitative and quantitative terpene composition in comparison to the Arilla and Forastera grapes and wines. Identified dominant terpene and norisoprenoid compounds for these varieties were: for Don Lunardo variety, limonene, geraniol, linalool, isobornylacetate, α-pinene, β-myrcene, β-damascenone; for Arilla variety, limonene, isobornylacetate and menthol; for Forastera variety α-pinene, limonene, isobornylacetate, menthol and β-damascenone. Don Lunando grapes are also characterized by the presence of benzaldehyde with potential almond odour. In Arilla wines the dominant terpene was linalool which was not

detected in the grapes but was produced in the wine during the winemaking process through acid and enzymatic hydrolysis from its glycoside precursors.

Table 1. Dominant varietal compounds in white varieties. +. Present; (tr). Present only in traces; -. Not detectable

		GRAPE				WINE			
VARIETAL COMPOUND	RT (min)	Arilla Cuotto	Arilla Spadara	Don Lunardo	Forastera	Arilla Cuotto	Arilla Spadara	Don Lunardo	Forastera
α-pinene	9.1	(tr)	(tr)	+	+	(tr)	(tr)	+	+
benzaldehyde	10.9	—	—	+	—	—	—	+	—
β-myrcene	11.6	—	—	+	—	—	—	+	—
limonene	12.8	+	+	+	+	+	+	+	+
linalool	15.4	—	—	+	(tr)	+	+	+	+
menthol	17.6	+	+	(tr)	(tr)	+	+	(tr)	—
geraniol	20.3	—	—	+	—	—	—	+	—
β-damascenone	20.6	—	—	+	+	—	—	+	+
isobornylacetate	20.8	+	+	+	+	+	+	+	+

The quantitative differences observed for the Arilla grapes and wines from Cuotto and Spadara were related to their different geographical origins, and were presumably due to different salinity stress and water deficit conditions (as confirmed by the determination of the quantities in grapes and wines of proline, which is considered a suitable molecular stress indicator; data not shown) and to different ripening rates. In fact the terpene compounds in Arilla grapes and wines that came from Spadara tended to have higher concentrations compared to those from Cuotto (Fig. 1).

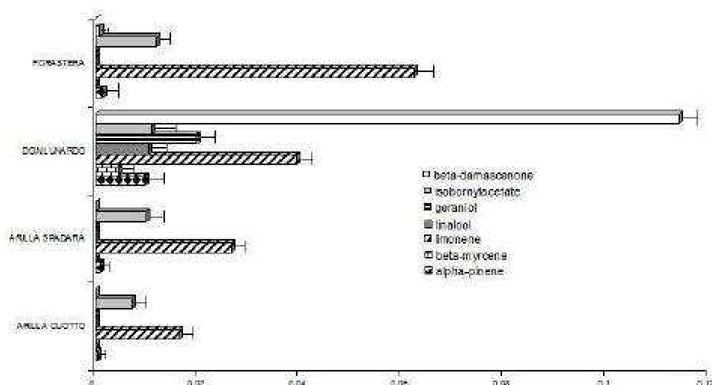

Figure 1. Quantitative data (ppb) for terpene and norisoprenoid compounds in white grapes.

For red varieties, identified dominant terpene and norisoprenoid compounds were: for Livella variety limonene, α-pinene, β-myrcene, isobornylacetate, α-damascenone e di β-

damascenone; for Guarnaccia variety limonene, isobornylacetate, α-damascenone, β-damascenone, α-ionone.

Table 2. Dominant varietal compounds in red varieties. +. Present; (tr). Present only in traces; -. Not detectable.

		GRAPE				WINE		
VARIETAL COMPOUND	RT (min)	Guarnaccia Fango	Guarnaccia Spadara	Livella Panza	Livella Spadara	Livella Panza	Livella Spadara	Guarnaccia Fango
α-pinene	9.1	—	—	+	+	+	+	—
benzaldehyde	10.9	+	+	+	+	+	+	+
β-myrcene	11.5	—	—	+	+	+	+	—
limonene	12.8	+	+	+	+	+	+	+
linalool	15.5	(tr)	(tr)	—	—	—	—	(tr)
α-terpineol	18.1	—	—	(tr)	(tr)	(tr)	(tr)	—
β-damascenone	20.6	+	+	+	+	+	+	+
isobornylacetate	20.8	+	+	+	+	+	+	+
α-damascenone	23.4	+	+	+	+	+	+	+
α-ionone	24.5	+	+	—	—	—	—	+

Anthocyanins extracted from red grapes and wines are listed in Tab. 3 and 4.
M/z 507 and M/z 533 signals, corresponding to delphinidin-3-O-(6-O acetyl)-glucoside and delphinidin-3-glucoside piruvic compound respectively, are present in Livella grapes and wines but absent in Guarnaccia grapes and wines: these anthocyanin compounds can act as varietal molecular markers.Livella and Guarnaccia appeared to be different for composition of diglucosides also: petunidin-3,5-O-diglucoside can act as varietal marker for Livella variety (Tab. 3 and 4).

Table 3. Anthocyanin compounds identified in the metanolic extract of red grape skins.

ANTOCYANIN COMPOUNDS	M+	LIVELLA SPADARA	LIVELLA PANZA	GUARNACCIA SPADARA	GUARNACCIA FANGO
malvidin	331	+	+	+	+
cyanidin-3-O-glucoside	449	+	(tr)	+	+
peonidin-3-glucoside	463	(-)	(-)	+	+
petunidin-3-O-glucoside	479	(tr)	+	+	+
malvidin-3-O-glucoside	493	+	+	+	+
peonidin-3-O-(6-O acetyl) glucoside	505	+	+	+	+
delphinidin-3-O-(6-O acetyl)-glucoside	507	+	+	(-)	(-)
peonidin-3-glucoside piruvic compound	531	+	+	+	+
delphinidin-3-glucoside piruvic compound	533	+	+	(-)	(-)
malvidin-3-acetylglucoside	535	+	+	+	+
cyanidin-3,5-O-diglucoside or delphinidin-3-O-(6-O p-coumaroyl)-glucoside or cyanidin-3-O-(6-O p-caffeoyl)-glucoside	611	+	+	+	+
malvidin-3glucoside-4-vinylguaiacyl	639	+	+	+	+
petunidin-3,5-O-diglucoside	641	(-)	(-)	+	+
peonidin-3-(p-coumaroyl)-glucoside piruvic compound	677	+	+	+	+
+ present; (-) not detectable; (tr) only in traces					

Table 4. Anthocyanin compounds identified in the metanolic extract of red wines.

ANTOCYANIN COMPOUNDS in WINES	M+	LIVELLA SPADARA	LIVELLA PANZA	GUARNACCIA FANGO
malvidin	331	+	+	+
malvidin-4-vinylphenyl	447	+	+	+
cyanidin-3-O-glucoside	449	+	+	(-)
peonidin-3-O-glucoside	463	+	+	+
petunidin-3-O-glucoside	479	+	+	+
cyanidin-3-O-glucoside	491	+	+	+
malvidin-3-O-glucoside	493	+	+	+
peonidin-3-O-(6-O acetyl) glucoside	505	+	+	+
delphinidin-3-O-(6-O acetyl)-glucoside	507	+	+	(-)
malvidin-3-glucoside-4-vinyl	517	+	+	+
peonidin-3-glucoside piruvic compound	531	+	+	+
delphinidin-3-glucoside piruvic compound	533	+	+	(-)
malvidin-3-acetylglucoside	535	+	+	+
malvidin-3-glucoside piruvic compound	561	+	+	+
malvidin-3-glucoside-4-vinylphenyl or peonidin-3-glucoside-4-vinylguaiacyl	609	+	+	+
cyanidin-3,5-O-diglucoside or delphinidin-3-O-(6-O p-coumaroyl)-glucoside or cyanidin-3-O-(6-O p-caffeoyl)-glucoside	611	+	+	+
petunidin-3-(p-coumaroyl)-glucoside	625	+	+	+
delphinidin-3,5-O-diglucoside or delphinidin-3-O-(6-O p-caffeoyl)-glucoside	627	+	+	+
malvidin-3-glucoside-4- vinylguaiacyl	639	+	+	+
petunidin-3,5-O-diglucoside	641	+	(tr)	(-)
malvidin-4 vinyl-catechin or epicatechin	643	+	+	+
malvidin-3-(p-coumaroyl)glucoside-4-vinylphenyl	755	+	+	(-)
malvidin-3-glucoside4-vinylcatechin or epicatechin	805	+	+	+
malvidin-3-glucoside-4-ethyl-catechin or epicatechin or malvidin-3-glucoside-8-ethyl-catechin or epicatechin	809	+	+	(-)
malvidin-3-(acetyl)glucoside-4-vinylcatechin or epicatechin	847	(-)	(-)	+
unknown	889	+	+	+
malvidin-4-vinyl-procyanidin dimer	931	+	+	+
malvidin-3-(p-coumaroyl)-glucoside-4-vinylcatechin or epicatechin	951	(-)	(-)	+
malvidin-3-glucoside-4-vinyl- procyanidin dimer	1093	+	+	+

+ present; (-) not detectable; (tr) only in traces.

CONCLUSIONS

Suitable quality and authenticity molecular markers of typical food products can furnish useful analytical tools which can provide quality control and commercial protection. The metabolomic approach carried out in this study on autochthonous grapes and wines from Isle of Ischia indicated that the varietal metabolites identified, which express the distinctive vine genetic characteristics and grapevine biodiversity, could act as quality and origin tracers for these typical oenological products.

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IL PAESAGGIO DELLE ALBERATE AVERSANE ED IL VINO ASPRINIO

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RIASSUNTO

Nel corso del 2009, in alcuni vigneti allevati ad alberata in provincia di Caserta (Italia), è stata avviata una ricerca per valutare la variabilità genetica della popolazione del vitigno 'Asprinio', la condizione sanitaria delle piante e le caratteristiche del vino sia rispetto alla forma di allevamento (alberata tradizionale e controspalliera) che all'altezza della fascia produttiva. I primi risultati indicano la totale omogeneità genetica della popolazione del vitigno 'Asprinio', non essendo stati ritrovati campioni vegetali riferibili a biotipi diversi. I saggi immunoenzimatici ELISA hanno rilevato la presenza di GLRaV 1, GLRaV 3 e GVA in tutti i campioni, mentre l'analisi delle molecole aromatiche delle uve e dei vini, condotta mediante analisi SPME-GC/MS, ha messo in evidenza che le uve 'Asprinio', prodotte sulla fascia più bassa delle alberate, presentano una maggiore potenzialità aromatica, rispetto a quelle della fascia più alta o delle controspalliere. I vini prodotti con diversi protocolli mostrano parametri enologici (grado alcolico, livelli di pH a acidità totale) simili tra di loro ed a quelli riportati da autori della metà del XX secolo.

PAROLE CHIAVE

Asprinio – alberata – DNA – profilo aromatico

ABSTRACT

During 2009, in some vineyards grown on trees (*alberata)* in the province of Caserta (Italy), a study is carried out to assess the genetic variability of the 'Asprinio' grapevine population, the health condition of the plants and the features of the wine in relation to the breeding system (traditional *alberata vs* horizontal training system) and to the heigth of fertile shoots. The first results point out the genetic identity of the 'Aprinio' grapevine population, because no different bio-types were found. The immunoenzymatic essays ELISA revealed that all the accessions were infected by GLRaV 1, GLRaV 3 and GVA; whereas the determination of the aromatic molecules from grapes and wines, performed by SPME- GC/MS analysis, indicated that the 'Asprinio' grapes, grown on lower area of the *alberata*, show greater aromatic potential than those from highest level of the same or those from vertical training system. The wines, produced by different procedures, show oenologycal parameters (alcohol degree, pH and total acidity level) similar to each other and to those reported by some authors of the mid-twentieth century

KEYWORD

Asprinio – *alberata* – DNA – aromatic profile

INTRODUZIONE

Le alberate aversane, antica e storica forma di allevamento della vite in Campania, maritata a tutori vivi come pioppo e olmo e utilizzate per coltivare il vitigno 'Asprinio', costituiscono un elemento fortemente caratterizzante la pianura alluvionale compresa tra le province di Napoli e Caserta, non solo per l'imponenza delle strutture ma anche per le particolari pratiche agronomiche ad esse associate. Infatti, la potatura, effettuata in maniera radicale ogni 3-5 anni, veniva realizzata staccando tutta la parete produttiva, poggiandola al suolo dove veniva potata e calpestata e poi riportata sulla struttura portante. La raccolta viene ancora oggi effettuata da pochissimi vendemmiatori superstiti con l'uso di scale strettissime, costruite in base alle dimensioni della loro gamba e alte come l'alberata.

Ritenute da molti di origine etrusca, nel corso dei secoli hanno subito radicali adattamenti a condizioni ambientali ed economiche diverse. Infatti, l'altezza (9 – 15 metri) e l'interfila (anche 20 metri) delle alberate trovano la loro ragion d'essere, nel primo caso, nell'allontanamento dei grappoli dal suolo per la presenza di notevole umidità e, nel secondo caso, nella coltivazione sottochioma di specie ortive e di fruttiferi. La produzione d'uva era quindi considerata accessoria e complementare alla formazione del reddito complessivo. Tuttavia, questo tipo di coltivazione secolare del vitigno 'Asprinio' ha dato origine alla produzione di un vino, conosciuto fin dal 1500, caratterizzato da una notevole acidità, come si deduce dallo stesso nome, e da un tenue aroma agrumato, che ha suscitato giudizi contrastanti nella letteratura e nella critica enologica del secolo scorso.

MATERIALI E METODI

L'indagine è stata condotta in diversi comuni della provincia di Caserta, tutti compresi nell'area alluvionale dell'agro aversano, caratterizzato da suolo sabbioso profondo, permeabile e sciolto. Nel corso della ricerca, sono stati selezionati numerosi ceppi di alberate di età non inferiore ai 60 anni coltivate su piede franco e di una controspalliera di impianto recente. I relativi campioni vegetali sono stati sottoposti ad analisi genetica con 8 marcatori molecolari microsatelliti (VVS2, MD5, MD7, MD25, MD27, MD31, Zag62, Zag79) secondo la metodologia riportata in Costantini *et al.*, 2005.

Campioni vegetali degli stessi ceppi ma replicati in base all'altezza della fascia produttiva dell'alberata sono stati sottoposti ad analisi virologica con saggi immunoenzimatici ELISA nei confronti di 8 virus della vite (GFLV; GFkV; GLRaV 1, 2, 3, 7; GVA; GVB), con l'obiettivo di verificare la condizione sanitaria e di procedere al risanamento di uno o più ceppi selezionati tra quelli meno affetti. A tale scopo alcune talee di un ceppo, affetto solo da GVA, sono state inviate all'Istituto di Virologia vegetale del CNR – U.O.S di Grugliasco (Torino) per la produzione di materiale risanato.

La determinazione del profilo aromatico, realizzata sia sulle uve - prelevate a differente altezza (1,20 e 6 metri circa) delle alberate di siti diversi e di una controspalliera di confronto - che sui vini, è stata effettuata mediante analisi HS-SPME-GC/MS (Nasi *et al.*, 2008; Nasi *et al.*, 2010).

Per la valutazione delle caratteristiche enologiche delle uve, circa 4 quintali di uve di alberate e spalliera dei vari siti, sono stati vinificati utilizzando diversi protocolli, tipologie di lievito e dosaggi dell'enzima di chiarifica. In particolare, rispetto ai protocolli relativi al post-fermentazione sono state realizzate le seguenti sperimentazioni: a) induzione di fermentazione malolattica; b) affinamento su feccia "totale" con tempi di sosta variabili; c) affinamento su feccia "fine" con tempi di sosta variabili.

RISULTATI E DISCUSSIONE

Riguardo la variabilità genetica della popolazione del vitigno Asprinio, nessuna differenza allelica è emersa dall'analisi con marcatori microsatelliti (SSR). Nella Tab. 1 vengono riportati i dati genetici relativi ad alcuni dei ceppi selezionati, realizzati presso la Fondazione E. Mach di S. Michele all'Adige (Trento).

Tabella 1 – Risultati dell'analisi con marcatori 8 microsatelliti (SSR) di alcuni campioni

Comuni (Caserta)	VVS2	VVMD5	VVMD7	VVMD25	VVMD27	VVMD31	Zag62	Zag79
Cesa	130 155	227 231	248 250	240 242	183 187	212 216	201 204	244 251
Gricignano	130 155	227 231	248 250	240 242	183 187	212 216	201 204	244 251
Casal di Principe	130 155	227 231	248 250	240 242	183 187	212 216	201 204	244 251
Villa Literno	130 155	227 231	248 250	240 242	183 187	212 216	201 204	244 251
S.Arpino	130 155	227 231	248 250	240 242	183 187	212 216	201 204	244 251

Rispetto allo stato sanitario dei ceppi selezionati, verificato dall'Istituto per la Patologia vegetale di Roma, nella Tab. 2 si nota che solo in 3 casi è stata rilevata la presenza del solo GVA (complesso del legno riccio); negli altri campioni, sono stati rilevati GLRaV 1 e GLRaV 3 (complesso dell'accartocciamento fogliare). Per la procedura di risanamento sanitario sono state prelevate alcune talee, durante la potatura invernale, dal ceppo "S.Arpino".

Tabella 2 – Risultati dei saggi immunoenzimatici ELISA nei confronti di 8 virus della vite (GFLV; GFkV; GLRaV 1, 2, 3, 7; GVA; GVB)

Comuni	Virus identificati
Cesa 1	GLRaV 1, 3 GVA
Gricignano 1	GLRaV 1, 3 GVA
Cesa 2	GLRaV 1 GVA
Cesa 3	GLRaV 3 GVA
Gricignano 2	GVA
Cesa 3	GLRaV 1, 3 GVA
Casal di Principe 1	GLRaV 1, 3 GVA
Casal di Principe 2	GVA
Casal di Principe 3	GLRaV 1, 3 GVA
Villa Literno 1	GLRaV 1, 3 GVA
Villa Literno 2	GLRaV 1, 3 GVA
Villa Literno 3	GLRaV 1, 3 GVA
Gricignano 3	GLRaV 1, 3 GVA
S.Arpino	GVA

Il profilo delle molecole volatili e potenzialmente odorose delle uve alla raccolta e dei vini, in relazione ai siti, altezza della fascia produttiva e forma di allevamento, è indicato nella Tab. 3. Tra le molecole varietali delle uve, i terpeni alfa-pinene e beta-pinene (note odorose di olio di pino), limonene (note odorose di agrumi), linalolo (note odorose di agrumi) e due norisoprenoidi (note odorose floreali, tabacco) sono risultate comuni a tutti i campioni. Nei vini il linalolo, presente solo in tracce nelle uve, risulta il terpenolo dominante, in quanto ulteriormente prodotto durante la vinificazione per azione idrolitica e glicosidasica (Martino *et al*., 2000; Mateo, Di Stefano, 1997). Risultano inoltre rilevabili due terpeni (3-carene e 4-carene) ed un altro norisoprenoide (alfa-ionone), non riscontrati nelle uve. Dal confronto dei profili aromatici dei vini si rilevano differenze più di tipo quantitativo che qualitativo.

Tabella 3 – Composizione qualitativa delle molecole aromatiche delle uve e dei vini

tr (min)	Molecola			UVE					VINI			
		1 b	1 a	2b	2a	3b	3a	3S	1	2	3	3S
2.2	3-metil-1-butanolo								+	+	+	+
3.7	acido butanoico								+	+	+	+
4.1	etilbutanoato								+	+		+
4.6	esanale	+	+	+	+	+	+	+				
6.9	2-esenale	+	+	+	+	+	+	+				
7.4	1-esanolo						+		+	+	+	+
7.9	isoamilacetato								+	+	+	+
9.9	2,4-esadienale			+	+		+	+				
10.4	alfa-pinene	+	(tr)	+	(tr)	(tr)	+	+	+	+	+	+
11.4	benzaldeide			+		+			+	+	+	+
11.5	acetofenone			+								
11.6	1-eptanolo								+	+	+	+
11.8	etill 2-metillbutenoato	+										
12.1	beta-pinene	+	(tr)	(tr)	(tr)	(tr)	(tr)	(tr)	+	+	+	+
12.2	1-otten-3-olo			+			+	+				
12.4	acido esanoico						+		+	+	+	+
12.6	6-metil-5-epten-2-one						+					
12.7	3-ottanone											+
12.8	2-pentilfurano	+		+			+	+				
13.0	etilesanoato			(tr)	(tr)	+	+	+	+	+	+	+
13.1	ottanale	+	+									
13.6	esilacetato	+	+			+	+	+	+	+	+	+
13.7	2-esen-1-ol acetato	+	+				+	+				
13.9	limonene	+	+	+	+	+	+	+	+	+	+	+
14.1	2-etil-1-esanolo	(tr)	(tr)	+	+	+	+					+
14.5	benzeneacetaldeide						+					
14.6	etil 2-esenoato								+	+	+	
15.0	3-carene								+	+	+	+
15.5	1-ottanolo		+			+	+					
15.8	2,5-furandicarbossialdeide						+					
16.1	4-carene								+	+	+	+
16.5	etileptanoato								+	+		+
16.6	linalolo	(tr)	(tr)	(tr)	(tr)	(tr)	(tr)	(tr)	+	+	+	+
16.7	nonanale	+	+	+	+	+	+					
16.9	feniletilalcol					+	+	+	+	+	+	+
17.4	metilottanoato								+	+		+
17.7	4-etilfenolo	+	+									
18.6	acido benzencarbossilico					+						
18.8	3-etilfenolo										+	
18.9	acido ottanoico					+			+	+	+	+
19.1	etilsuccinato								+	+	+	+
19.5	metil 2-idrossibenzoato			+	+	+	+	+				
19.6	etilottanoato			(tr)	(tr)	+	+	+	+	+	+	+
19.8	decanale	+	+	+	+	+	+					
21.1	2-metilfenolo			+	+	+						
21.2	isopentilesanoato								+	+	+	+
21.3	2-feniletilacetato					+	+	+	+	+	+	+
22.0	alfa-ionone								+	+	+	+
22.4	etilnonanoato								+	+	+	+
23.2	metildecanoato								+	+	+	+
24.2	3,7-dimetil-6-otten-1-ol acetato			+		+						
24.3	acido decanoico								+	+	+	+
24.4	4-metossi-3-metilfenolo				+							
24.9	etil 9-decenoato								+	+	+	+
25.1	etildecanoato			+		+			+	+	+	+
25.9	beta-damascenone	+	+	+	+	+	+	+	+	+	+	+
26.9	beta-ionone	+	+	+	+	+	+	+	+	+	+	+
28.1	BHT	+	+	+	+	+	+	+	+	+	+	+

(siti: 1 - Casal di Principe, 2 – Gricignano, 3 - Cesa; altezza: a - alta; b - bassa; s: spalliera: tr- tracce)

Dalla Tab. 4, che mostra sinteticamente le differenze quantitative nella composizione in molecole aromatiche delle uve di alberate in relazione ai siti ed altezza, si nota che i siti Casal di Principe e Gricignano presentano un andamento molto simile, al contrario il sito Cesa ha un maggior

Tabella 3 – Composizione qualitativa delle molecole aromatiche delle uve e dei vini

tr (min)	Molecola	UVE							VINI			
		1 b	1 a	2b	2a	3b	3a	3S	1	2	3	3S
2.2	3-metil-1-butanolo								+	+	+	+
3.7	acido butanoico								+	+	+	+
4.1	etilbutanoato								+	+		+
4.6	esanale	+	+	+	+	+	+	+				
6.9	2-esenale	+	+	+	+	+	+	+				
7.4	1-esanolo						+		+	+	+	+
7.9	isoamilacetato								+	+	+	+
9.9	2,4-esadienale			+	+		+	+				
10.4	alfa-pinene	+	(tr)	+	(tr)	(tr)	+	+	+	+	+	+
11.4	benzaldeide			+		+			+	+	+	+
11.5	acetofenone			+								
11.6	1-eptanolo								+	+	+	+
11.8	etill 2-metillbutenoato	+										
12.1	beta-pinene	+	(tr)	(tr)	(tr)	(tr)	(tr)	(tr)	+	+	+	+
12.2	1-otten-3-olo			+			+	+				
12.4	acido esanoico						+		+	+	+	+
12.6	6-metil-5-epten-2-one						+					
12.7	3-ottanone											+
12.8	2-pentilfurano	+		+			+	+				
13.0	etilesanoato			(tr)	(tr)	+	+	+	+	+	+	+
13.1	ottanale	+	+									
13.6	esilacetato	+	+			+	+	+	+	+	+	+
13.7	2-esen-1-ol acetato	+	+				+	+				
13.9	limonene	+	+	+	+	+	+	+	+	+	+	+
14.1	2-etil-1-esanolo	(tr)	(tr)	+	+	+	+					+
14.5	benzeneacetaldeide						+					
14.6	etil 2-esenoato								+	+	+	
15.0	3-carene								+	+	+	+
15.5	1-ottanolo		+			+	+					
15.8	2,5-furandicarbossialdeide						+					
16.1	4-carene								+	+	+	+
16.5	etileptanoato								+	+		+
16.6	linalolo	(tr)	(tr)	(tr)	(tr)	(tr)	(tr)	(tr)	+	+	+	+
16.7	nonanale	+	+	+	+	+	+					
16.9	feniletilalcol					+	+	+	+	+	+	+
17.4	metilottanoato								+	+		+
17.7	4-etilfenolo	+	+									
18.6	acido benzencarbossilico					+						
18.8	3-etilfenolo										+	
18.9	acido ottanoico					+			+	+	+	+
19.1	etilsuccinato								+	+	+	+
19.5	metil 2-idrossibenzoato			+	+	+	+	+				
19.6	etilottanoato			(tr)	(tr)	+	+	+	+	+	+	+
19.8	decanale	+	+	+	+	+	+					
21.1	2-metilfenolo			+	+	+						
21.2	isopentilesanoato								+	+	+	+
21.3	2-feniletilacetato					+	+	+	+	+	+	+
22.0	alfa-ionone								+	+	+	+
22.4	etilnonanoato								+	+	+	+
23.2	metildecanoato								+	+	+	+
24.2	3,7-dimetil-6-otten-1-ol acetato			+		+						
24.3	acido decanoico								+	+	+	+
24.4	4-metossi-3-metilfenolo				+							
24.9	etil 9-decenoato								+	+	+	+
25.1	etildecanoato			+		+			+	+	+	+
25.9	beta-damascenone	+	+	+	+	+	+	+	+	+	+	+
26.9	beta-ionone	+	+	+	+	+	+	+	+	+	+	+
28.1	BHT	+	+	+	+	+	+	+	+	+	+	+

(siti: 1 - Casal di Principe, 2 – Gricignano, 3 - Cesa; altezza: a - alta; b - bassa; s: spalliera: tr- tracce)

Dalla Tab. 4, che mostra sinteticamente le differenze quantitative nella composizione in molecole aromatiche delle uve di alberate in relazione ai siti ed altezza, si nota che i siti Casal di Principe e Gricignano presentano un andamento molto simile, al contrario il sito Cesa ha un maggior

contenuto di alcuni terpeni nella fascia alta della produzione e di norisoprenoidi nella fascia bassa.

Tabella 4 – Contenuto di molecole aromatiche delle uve in relazione all'altezza dell'alberata

Casal di Principe	basso	alto
Alfa-pinene	↑	↓
Beta-pinene	↑	↓
Limonene	↑	↓
Gricignano	basso	alto
Alfa-pinene	↑	↓
Beta- pinene	↑	↓
Limonene	↑	↓
Cesa	basso	alto
Alfa-pinene	↓	↑
Beta-pinene	↓	↑
Limonene	↑	↓
Norisoprenoidi	↑	↓

I campioni di vino ottenuti dalle microvinificazioni presentano parametri di alcool, pH ed acidità molto simili tra loro ed in linea con quelli desumibili dalla letteratura scientifica degli anni '60 (Violante, Bordignon, 1959) che riferisce di livelli contenuti di grado alcolico (tra 8,5° e 10,5°), di valori bassi del pH (circa 3), di elevato tenore di acidità totale (7 – 9 g/l) e di acido malico (4.5 – 5 g/l). Indipendentemente dalla fermentazione malolattica, è stata rilevata in tutti i campioni la presenza di anidride carbonica disciolta.

CONCLUSIONI

I risultati preliminari della ricerca condotta hanno messo in evidenza una notevole omogeneità genetica della popolazione del vitigno 'Asprinio' nell'areale tipico di coltivazione, riferibile a due condizioni agronomiche principali: la consuetudine di propagare le piante per talea o propaggine, tanto che tutte le alberate ancora presenti sono a piede franco, e la rarefazione dei vigneti che non ha permesso una sufficiente diversificazione genetica.

L'estesa e diffusa presenza di complessi virali nelle piante è da attribuire a varie cause, prima tra tutte l'avanzata età dei ceppi delle alberate, in qualche caso superiore ai 60 anni. In secondo luogo, proprio il tipo di propagazione utilizzato può essere ritenuto responsabile dell'accumulo e della diffusione dei virus. Infine, negli ultimi anni si è assistito all'abbandono della coltivazione di ortaggi e legumi sottochioma o di alcune pratiche colturali come il sovescio e la letamazione che, migliorando la generale condizione nutritiva delle piante e del suolo, avevano in qualche misura contenuto e circoscritto lo sviluppo delle virosi. Alla luce dei risultati immunoenzimatici, è stato avviato, dall'azienda, un progetto di risanamento del materiale prelevato da uno dei ceppi.

Il profilo aromatico delle uve e soprattutto dei vini, caratterizzato da note aromatiche varietali agrumate, mandorlate e speziate, mostra un andamento molto simile nei diversi siti considerati ma differente rispetto sia all'altezza della fascia produttiva che alla forma di allevamento. In effetti, le condizioni microclimatiche che si determinano a diverse altezze dell'alberata, con ristagno di umidità e minore penetrazione della luce nella fascia più bassa, hanno influenzato il profilo aromatico delle uve e dei vini. Sotto l'aspetto sensoriale, come risultato da alcuni panel di degustazione, i vini si presentano leggeri, di grande freschezza e molto "sorbevoli"; di colore bianco/paglia con riflessi verdolini, molto limpidi, dal profumo tenue leggermente agrumato, dal sapore di erba di campo e mela limoncella, dotati di buona mineralità, estremamente secchi e leggermente "mossi".

RINGRAZIAMENTI

Gli autori desiderano ringraziare i viticoltori dell'area aversana, il sig. Umberto Guarino (Assessorato Agricoltura Regione Campania), il dott. Francesco Faggioli (Istituto per la Patologia vegetale di Roma), la dott.ssa Maria Stella Grando (Fondazione E. Mach di S. Michele all'Adige) per la preziosa collaborazione.

Si ringrazia inoltre il dott. Riccardo Cotarella per i preziosi suggerimenti circa i protocolli di vinificazione.

La Tenuta Adolfo Spada, promotrice della ricerca, dedica questo lavoro a Luigi Veronelli e Mario Soldati che tanto decantarono le virtù di questo "piccolo, grande vino".

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