

Fining white wine with plant proteins: effects of fining on proanthocyanidins and aroma components

Tiziana Mariarita Granato · Antonella Nasi ·
Pasquale Ferranti · Stefania Iametti ·
Francesco Bonomi

Received: 24 July 2013 / Revised: 12 September 2013 / Accepted: 23 September 2013 / Published online: 8 October 2013
© Springer-Verlag Berlin Heidelberg 2013

Abstract Plant-derived insoluble proteins (wheat gluten, and isolates from pea, lentil, and soybean) were used as fining agents in model white wine (made from *Catalanescas* grapes) after cold stabilization. Plant proteins were effective in giving a fast and remarkable decrease in turbidity. GC/MS and HPLC/MS approaches indicated that individual proteins had a different impact on the levels of compounds relevant to wine stability. Protein stability of wine was not affected by fining with plant proteins. Lentil proteins and gluten gave the best removal of monomeric and dimeric flavonol. Both caused a decrease in the total content of fermentative aroma compounds, such as ethyl esters, acetate esters, and alcohols. Lentil proteins had the highest impact on the aroma components, giving a marked decrease in aroma components. Gluten may thus be regarded as giving the best balance between fining efficacy and retention of aroma compounds. Also, gluten in the treated wines remained well below the suggested threshold for gluten-free foods. This study provides a methodological frame for thorough characterization of the impact of specific interventions on key wine components.

Keywords Aroma compounds · Fining treatment · Flavan-3-ol monomers and polymers · Mass spectrometry · Plant proteins · White wine

Abbreviations

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
SDS–PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
ELISA	Enzyme-linked immunosorbent assay
FDA	Food and Drug Administration, USA

Introduction

Wine clarity is an essential quality required by consumers, especially for white wines in clear glass bottles. Particles in suspension, either forming a haze or dispersed through the liquid, not only spoil the appearance, but usually also affect the flavor. Wine may be clarified by eliminating these particles, but the obtained effect is not necessarily permanent, due to the many chemical events that naturally occur in wine and are often accompanied by the development of turbidity or the formation of deposits. The objective of wine stabilization procedures is to ensure long-term clarity and to prevent deposits, possibly regardless of storage conditions.

The fining process relies on adding substances that induce flocculation and settling in turbid wines or in wines with colloidal instability. Unlike filtration and spontaneous settling, which can remove only particulates (such as dead yeast and grape fragments), fining can remove soluble

T. M. Granato · S. Iametti · F. Bonomi (✉)
DeFENS, University of Milan, Via Celoria 2, 20133 Milan, Italy
e-mail: francesco.bonomi@unimi.it

A. Nasi · P. Ferranti
Department of Agraria, University of Naples ‘Federico II’,
Portici, Naples, Italy

substances, including polymerized tannins and coloring matter in red wines. Protein-based fining products are often a mixture of denatured or partially denatured proteins that aggregate on contact with tannins and cations or as a consequence of pH changes and entrap the undesired species in the resulting precipitate. Proteins used for fining have been typically of animal origin: egg albumin, serum albumin, casein (milk), isinglass (fish), and gelatin (collagen, from various species). The issues involved with bovine spongiform encephalopathy (BSE) in animals and its possible transmission to humans have led to restrictions in the use of products of animal origin for wine fining. Legislation in several countries, particularly in the European Union, has been updated, banning the use of dried blood powder and of serum albumin. The use of gelatin also has been challenged, regardless of the source, but gelatin is still widely used for its good clarification and stabilization capacities, particularly in red wines. Winemakers are asking for substitute products with properties and application protocols similar to the traditional animal-derived ones, making the use of plant-derived proteins in fining a practically viable possibility.

Plant proteins have shown good potential in this context [1, 2]. Malt proteins were shown to interact with tannins in beer [3], and sorghum prolamins were reported to interact with tannins and to precipitate them [4, 5]. Marchal et al. [6] showed that gluten proteins used at concentrations between 6 and 18 g/hL allowed very good clarification of Burgundy wine, and some gluten preparations (at 12–18 g/hL) gave turbidity values close to those obtained with animal proteins. Maury et al. [7] carried out a study using protein extracts from white lupine in comparison with two gluten-based preparations, and two chemical hydrolysates of gluten. In these studies, each preparation behaved in a specific way, making it necessary to test a large number of products to determine which ones give the best results with a given wine, and to define the most effective doses (typically, around 10–20 g protein/hL).

The present work extends previous studies on the molecular basis of interaction of plant proteins [soy (*Glycine max*), pea (*Pisum sativum*), lentil (*Lens culinaris*), and gluten (*Triticum aestivum*)] with polyphenols from winelike model solutions [8] to real white wine (from *Catalanesca* grapes), evaluating the effect of these fining agents on a number of parameters indicative of wine quality. In this frame, the levels of residual gluten proteins in the treated wine were also tested, given the gluten toxicity to celiac individuals and the ensuing necessity to ensure its complete removal after clarifying. New labeling legislation by Food Standards Australia New Zealand also requires that wine labels identify any detectable potentially allergenic processing aid, additive, or ingredient. Similar regulations are under consideration in Canada, in the European

Union, and in the USA. From the winemakers' 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, or on whether any of these could cause any allergic reactions, making further investigations worthwhile.

Materials and methods

Wine production

Young white wines of 2009 vintage, made from *Catalanesca* grapevine variety (*Vitis vinifera*) from Regione Campania, Italy, were used in this study. Grapes were hand-harvested at full maturation (20.50°Brix), destemmed, crushed, and pressed, and potassium metabisulfite (8 g/hL) and pectic enzyme (2 g/hL, Aspergillus AGAZYM EXT, Garzanti Specialties S.p.a., Milano) were added. The grape skins were macerated in their own juice for 7 days before fermentation at 12 °C. Must was then cooled to 10 °C and allowed to decant for 24 h. Inoculation was carried out at 20 g/hL (MAURIVIN, AWRI FUSION, hybrid *S. cerevisiae* × *S. cariocanus*, Queensland, Australia) after yeast rehydration in warm water for 30 min, as described by the supplier. Fermentation took place in stainless steel tanks at 18 °C. At the end of cold stabilization, the wines were treated with protein fining agents and immediately submitted to spontaneous settling. Analytical data of wine are reported in Table 1.

Wine-fining trials

Chemical composition data and hydrophobicity properties of the protein preparations used in this work have been reported elsewhere [8]. Fining tests were carried out in 100-mL graduated cylinders, which were completely filled and hermetically closed to avoid oxidation events. As the clarifying activity of the materials used here was unknown prior to this study, they were used at the maximum dose commonly used

Table 1 Properties of *Catalanesca* white wine before fining

Sugar content (g/L)	<2
Total acidity (g/L as tartaric acid)	5.2
pH	3.32
Volatile acidity (g/L as acetic acid)	0.54
Alcohol content (% v/v)	12.50
Free SO ₂ (mg/L)	10.88
Total SO ₂ (mg/L)	38.4
Turbidity (NTU)	55

for animal proteins (20 g/hL, gelatin and egg proteins). An untreated sample was used as a negative control. Following thorough mixing, they were left in contact with the wine for 7 days at 20 °C. All experiments were done in duplicate.

Clarification kinetics and conventional enological parameters

Conventional parameters for wine clarity and color were determined according to OIV methods (International Organization of Vine and Wine, 2005) [9]. Turbidity was measured in nephelometric turbidity units (NTU) using a turbidimeter (LP 2000, Hanna Instruments, Laval, Quebec, Canada). Optical density at 420 nm, turbidity, and the volume of lees were measured 1, 4, 10, 20, 32, 48, 60, and 168 h at room temperature (20 ± 2 °C) after the addition of fining agents. The absorbance was determined at 420 nm in a 10-mm cell by an UV–VIS Spectrophotometer (Shimadzu mod. 1601, Shimadzu Italia, Milan, Italy). All measurements were taken in triplicate.

Structural characterization of phenolic compounds after wine fining

A wine aliquot (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 % (v/v) aqueous ethanol containing 0.1 % (v/v) TFA. LC/MS analysis was carried out on a single quadrupole instrument (HP1100-MSD, Agilent Technologies, Santa Clara, CA, USA). A C18 column (Vydac, Hesperia CA, USA; 2.1 × 250 mm) was eluted with HPLC-grade water (solvent A) and acetonitrile (solvent B), both containing 0.1 % (v/v) TFA. OPCs were separated at a constant flow rate of 0.2 mL/min, with a linear (v/v) gradient of solvent B as follows: 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 at 280 nm. LC/ESI-MS analysis of proanthocyanidins was carried out according to the conditions used for wine model solutions [8]. To increase the sensitivity of the ESI-MS measurements, the samples were assayed twice, scanning separately from m/z 100 to 1,000 and from m/z 1,000 to 2,000, at a scan cycle of 4.90 s per scan and 0.1-s interscan 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 at 25 V, according to different experiments. N₂ was used as both drying and spraying gas. Calibration curves for tannins were prepared using (+)-catechin in the 50–250 mg/L concentration range, as reported in [8].

MALDI-TOF spectra were recorded in positive-ion mode, using a Voyager DE-Pro spectrometer (PerSeptive BioSystems, Framingham, MA) equipped with a N₂ laser (337 nm). Alpha-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 reflection mode. External mass calibration was performed with peptide standards (Sigma).

Determination of volatile compounds in wines after fining

Analysis of volatile compounds (varietal molecules such as terpenes, volatile phenols, and nonvarietal compounds such as acids, esters, aldehydes, and lactones) was performed by solid-phase microextraction (SPME) and gas chromatography–mass spectrometry [10]. SPME holder and fibers (PDMS, CAR/PDMS, CAR/DVB/PDMS) were from Supelco (Aldrich, Bornem, Belgium). The fibers were placed in the sample headspace (HS) of 120 mL of wine until equilibrium conditions were reached. 1-Octanol (0.500 mg/L) was added to wine as an internal standard before the extraction. 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 for 1 min. Samples were analyzed with a gas chromatograph (HP 7890, Agilent Technologies, Santa Clara, CA, USA) coupled to a quadrupole mass spectrometer (HP 5975C, Agilent Technologies). The gas chromatograph was equipped with a capillary column (HP-5 MS, 30 m × 0.32 mm ID, Agilent Technologies), and He was used as the carrier gas. For the analysis of aroma compounds, the GC oven temperature was increased from 40 (held for 7 min) to 180 °C at 5 °C/min. The mass spectrometer was operated in electron mode (EI, 70 eV), and the masses were scanned over a m/z range of 45–350 amu. A SIM method was used for terpene compounds (m/z 93, 12, 136). The identification of odorous components was effected through the NIST library and by comparison with spectra and retention times of standards. Quantitative determinations were obtained by means of calibration curves in the concentration range typical for each compound in wine. Seven concentration levels and five replicates per level were used, and the average correlation coefficient was in the 0.99–0.98 range for all the compounds. Samples were analyzed in multiple replicas ($n = 2$ –3).

Wine protein stability assay

The fined wine was filtered through a cellulose acetate membrane (0.45 µm pore size) using a vacuum system.

Heat tests were conducted in triplicate by incubating 20-mL tubes of the sample at 80 °C for 2 h. At the end of the incubation period, the samples were immediately chilled on ice and were then let to equilibrate to room temperature. Turbidity was measured using a turbidimeter (LP 2000, Hanna Instruments, Laval, Quebec, Canada). Samples were considered to be protein unstable when the difference between the heated samples and the corresponding unheated controls was greater than 2 NTU [11].

Extraction and SDS–PAGE of wine proteins

Proteins were precipitated by the addition of 400 mL of absolute ethanol to 100 mL of wine, and the samples were left for 48 h at 4 °C. The samples were then centrifuged (3,000g, 15 min, 4 °C). The protein pellets were suspended in MilliQ water. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out on 12 % (33:1 acrylamide–bis-acrylamide) polyacrylamide gels using a mini protein apparatus (Bio-Rad Laboratories, Berkeley, California). The gels were stained with 0.1 % Coomassie blue R-250 in methanol–acetic acid–water 40:10:50 (v/v) followed by destaining with methanol–acetic acid–water (40:10:50, v/v). High molecular weight markers (Precision Plus Protein™ All Blue standards, Bio-rad) were used for calibration.

Determination of gluten content in white wines by ELISA

The gluten content in wine samples was measured using a commercial ELISA kit (Fast Gliadin R-Biopharm, Darmstadt, Germany). The kit is based on a direct sandwich ELISA, with a reported detection limit of 5 ppm gliadin (10 ppm gluten). Assays were performed according to the standard procedure suggested by the supplier. Prior to analysis, wines were treated with a clarifying solution (5 % w/v isinglass, 2 % PVPP, 60 % ethanol) in order to eliminate any interference of the phenolic-rich matrix with the ELISA procedure [12]. 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 was 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.

Results and discussion

The experimental design used in this study was aimed at testing the action of plant-derived insoluble proteins on white wine in terms of fining efficiency, and at evaluating the ability of various proteins in removing phenolic

compounds responsible for oxidative events, while preserving wine aroma composition. These parameters were evaluated by fining trials on white wine after cold stabilization (initial turbidity $\approx$ 50 NTU).

For these purposes, we identified an Italian wine that could represent a suitable model, made from *Catalanesca* grapes. According to traditional protocols, *Catalanesca* grape skins are macerated in their own juice for 7 days before fermentation, resulting in phenolic levels much higher than most other white wines. Thus, *Catalanesca* wine was chosen as the “worst case,” as its high phenolics content makes it very susceptible to oxidative browning [13], and performing the fining process after cold stabilization could result in improved stability, in decreased astringency, and in an overall softer sensory trait.

The time course of turbidity changes (NTU) in *Catalanesca* wine during fining with different proteins is shown in Fig. 1, along with turbidity data at the end of the treatment. Spontaneous settling of control wine at the end of the kinetics became very slow, suggesting that a fining treatment was appropriate for stabilization. Lentil flour was the most effective fining agent (turbidity decreased to 16 % of initial), followed by pea, soybean, and gluten proteins (18, 26, and 28 % of untreated wine turbidity, respectively). Treatment with lentil proteins gave a very low turbidity after just 36 h, suggesting that lentil proteins could be advantageous when a quick racking is required. Lentil and gluten proteins produced also a lower lees volume than the other matrices (data not shown). In principle, the smaller the lees volume, the lower the loss of wine in the process.

Effect of the various fining agents on browning-relevant phenols

The function of protein fining is not only related to immediate wine stabilization, but also involves the removal by adsorptive precipitation of compounds that may lead to turbidity or to changes in color in later steps of wine life. Browning of white wines is a relevant stability issue related to oxidation of phenolic compounds and depends largely on the nature of the polyphenols. The monomeric catechins and the dimeric procyanidins brown more intensely than other phenolics [14].

We therefore set out to study how the various proteins influenced the proanthocyanidin composition in the *Catalanesca* wine, which is characterized by a high level of phenolics (860 mg/L as gallic acid) and catechins (180 mg/L) that make it particularly sensitive to oxidation. The main effect of the treatment with proteins was a general decrease in the total concentration of all the polyphenolic species (5–15 %) (data not shown). Figure 2 shows the polyphenolic profile of *Catalanesca* wine obtained by means of ESI LC–MS analysis. The MS total ion chromatogram

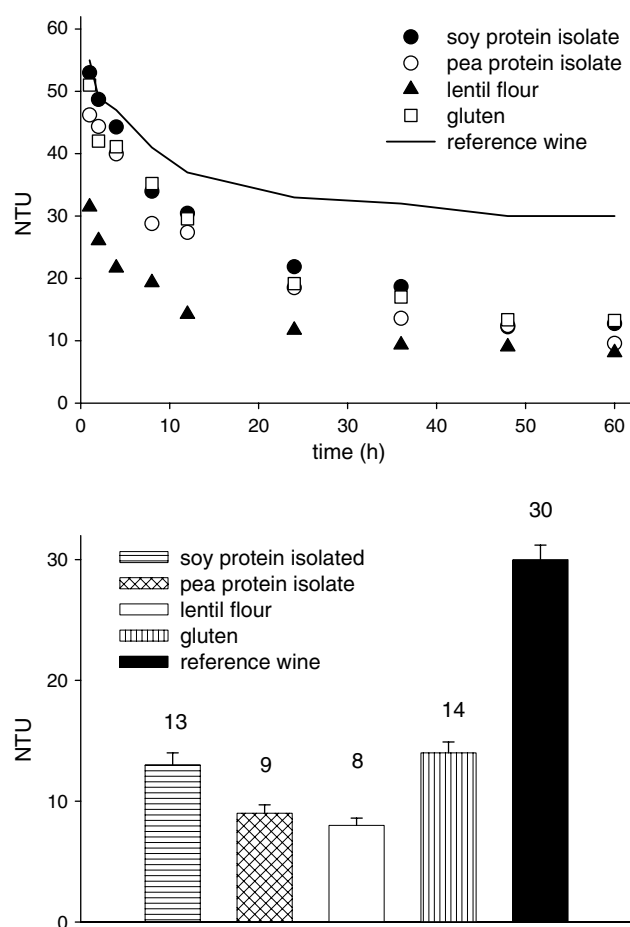


Fig. 1 *Top*: Time course of clarification for *Catalanesca* white wine. All treatments were performed at 20 °C, at a fining agent concentration of 20 g protein/hL. Reference wine was obtained from spontaneous settling at low temperature without any addition of proteins. *Bottom*: Effect of different fining agents on the final turbidity (60 h) of *Catalanesca* white wine

(TIC) of the positive molecular ions of *Catalanesca* wine in the 100–1,000 m/z range indicated the presence of catechin and epicatechin (m/z 291), dimeric (m/z 579) and trimeric (m/z 867) condensed tannins, and their gallic acid ester derivatives (m/z 731.1; m/z 1,019.0).

Figure 3 compares the content of individual low molecular weight proanthocyanidins (the most important flavonoid compounds with respect to white wine oxidation) in the treated wines with their respective level in the unfined one.

The fining agents that removed most effectively monomeric and dimeric flavanols as a whole were lentil proteins and gluten. This observation was in agreement with the results of previous experiments in model wine [8] that also showed that lentil proteins and gluten had the highest ability to interact with tannins. To confirm this finding and investigate the complexity of interaction between individual phenolic species and protein finings, additional

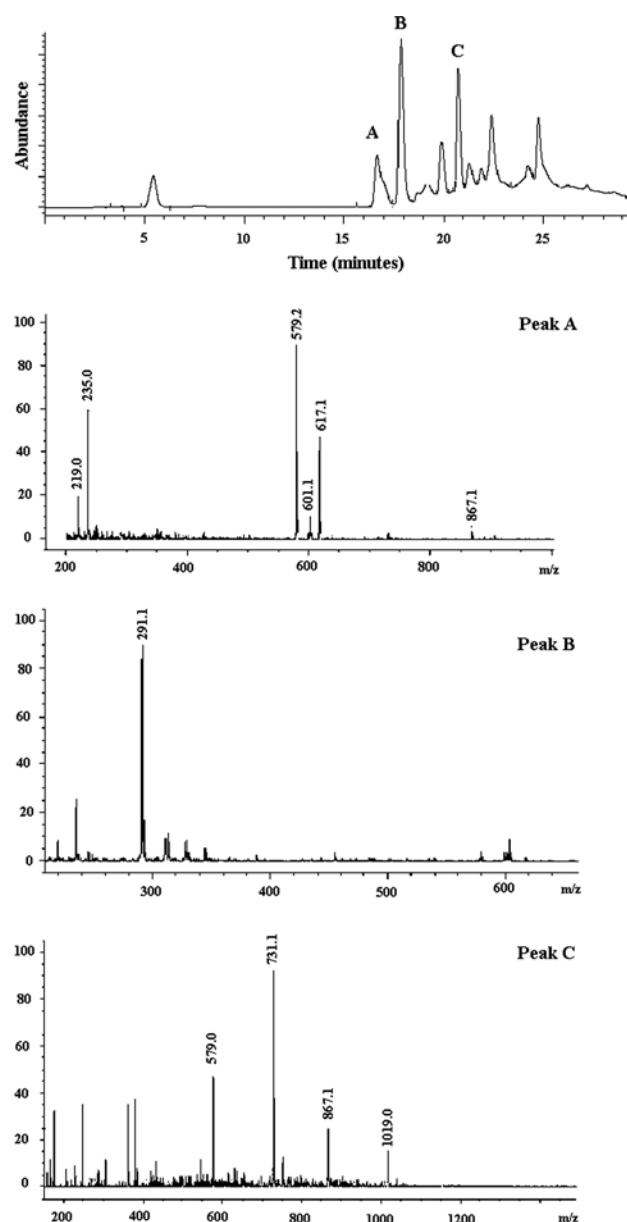


Fig. 2 Total ion current (TIC) chromatogram obtained in positive-ion mode by injection of phenolic compounds extracted by untreated *Catalanesca* wine. ESI mass spectra obtained from the TIC chromatogram for 16.66 (peak A), 17.85 (peak B), 21.55 (peak C), elution time (minutes), respectively, showing the $[M-H]^+$ signals of dimeric catechin (m/z 579), catechin (m/z 291), and dimeric (m/z 579) and trimeric compounds (m/z 867) with their acid gallic ester derivatives (m/z 731 and m/z 1,019)

characterization of oligomeric proanthocyanidins was carried out by MALDI-TOF mass spectrometry. Figure 4 compares the MALDI-TOF spectra of unfined wine and of wine fined with lentil proteins and indicates that lentil proteins removed proanthocyanidins with high molecular weight (up to tetramer) to make them not detectable after treatment. The same behavior was observed when using gluten

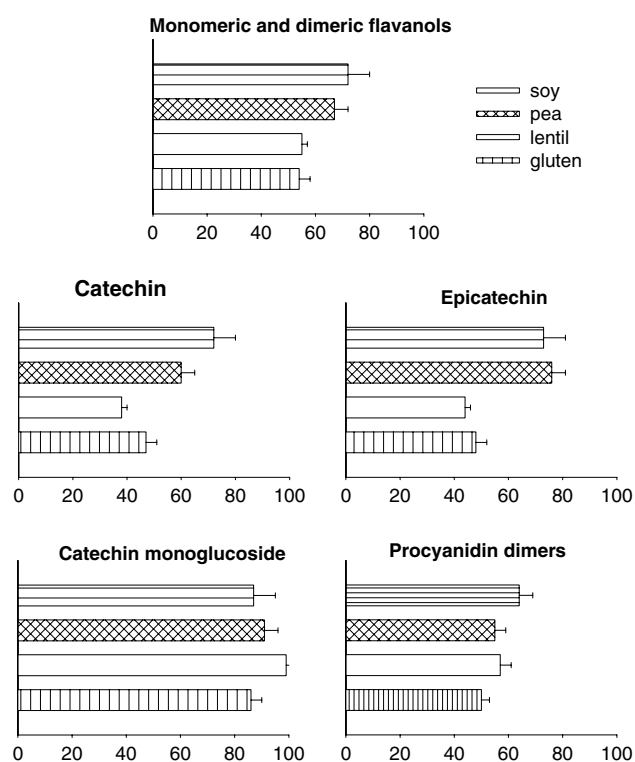


Fig. 3 Percentage loss of individual low molecular weight proanthocyanidins in fined *Catalanesca* wine, as assessed by LC/ESI MS analysis. The content of each species in unfined wine was taken as 100

as the fining agent (not shown). On the contrary, it was observed that a protein from soy and pea did not change the qualitative composition of the oligomeric flavanols, suggesting that these proteins had minor impact on polyphenols (not shown).

These findings mean that the characteristics of the proteins (amino acid composition, structure, size, charge, etc.) play a relevant role in governing these interactions even in a complex medium such as white wine. Proteins with high proline content, such as lentil and gluten proteins (proline content was about 8 and 15 % of total proteins for lentil and gluten, respectively [15, 16]), had high affinity for tannins, also because proline impedes formation of secondary structure, thus leaving vast regions of the protein open and accessible to tannin. The lower proline content of soy and pea proteins (6 and 2 % of total protein content, respectively [17, 18]) seems to relate to their lower efficiency in OPC removal from wine. This is in agreement with previous observations by several authors [14, 19, 20] that also pointed out the efficiency of poorly structured proline-rich proteins in these processes.

Aside from the accessibility issue, a balance between the characteristics of protein molecules and those of the wine plays a major role in determining molecular repulsion or association. For instance, the presence of Na^+ , K^+ ,

Ca^{2+} , Mg^{2+} , and especially Fe^{3+} cations—both in the protein extracts and in wine—seems to affect their wine-finishing ability because these species are involved in flocculation and precipitation of tannins and proteins.

When considering each of the flavan-3-ol molecules in detail, individual fining agents showed a different ability in removing monomeric compounds [(+)-catechin and (–)-epicatechin] with respect to the 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 to interact with the more polymerized proanthocyanidin 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 a colloidal form capable of maximizing formation of hydrogen bonds between some protein functional groups ($-\text{COOH}$, $-\text{NH}_2$, $-\text{OH}$, 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 [19, 20].

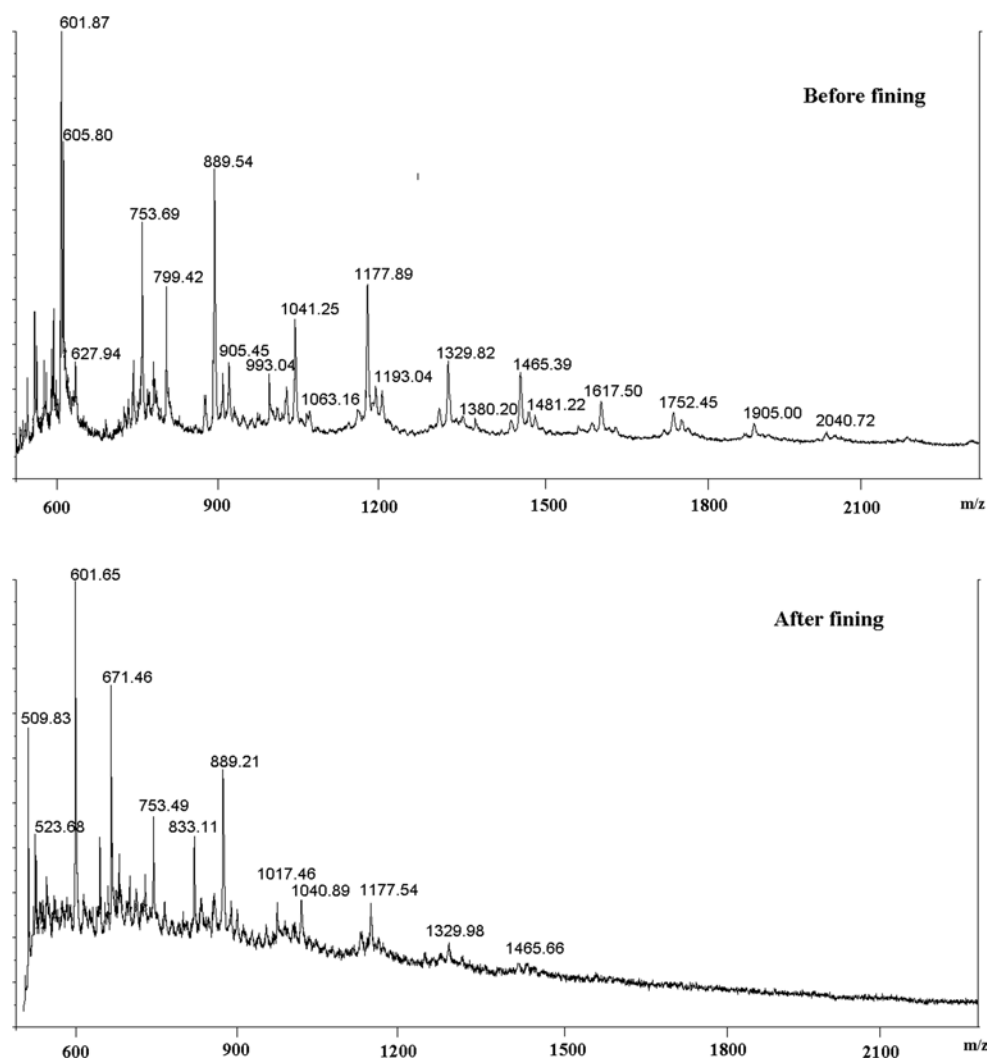
Influence of protein finings on volatile composition

Removal of polyphenols through fining treatment and precipitation has been shown to produce flavor balance modifications [21]. 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, thus compromising the quality and the original potential varietal expression of a wine.

The interaction between fining agents and free or bound aroma compounds depends on several factors, such as the physical–chemical characteristics of the fining agent, the chemical features of the target compound, and the possible interactions between volatiles and other macromolecules previously linked to the fining agent [22–24]. The loss of aroma compounds can be direct (by adsorption of aromas to the protein) [25] or indirect (the aromas are fixed by proteins, and when they are eliminated, they drag with them part of volatile compounds) [22, 23]. Therefore, the fining agent has to be selected to eliminate the risks of oxidation and protein haze, but without excessive effects on the aromatic profile.

The differences generated in the aroma composition of *Catalanesca* white wine when proteins of vegetable origin were used as fining agents were studied here on a laboratory scale. The aroma characterization of wines required qualitative and quantitative determination of different components

Fig. 4 MALDI-TOF MS spectrum of oligomeric proanthocyanidin extracted from *Catalanesca* wine before and after fining with lentil proteins



(varietal and nonvarietal). Solid-phase microextraction (SPME) and GC/MS were used here for the identification of odorous molecules of wines. Figure 5 shows the TIC chromatograms obtained by means of headspace–SPME–GC/MS analysis of treated and untreated *Catalanesca* wine.

No differences in the qualitative profile of volatile compounds of fermentative origin were observed in control vs treated wines, regardless of the fining agent. However, quantitative analysis showed an important decrease in the total concentration of the volatile families responsible for fermentative aroma upon fining, meaning that the aroma of untreated wines could hardly be preserved. The effects of protein fining agents on volatile molecules depended on both the protein type and the chemical nature of the involved compounds. Figure 6 reports the loss of volatiles most affected by fining, as a percentage of what observed in the same wines in the absence of treatment.

Lentil and pea proteins had the highest affinity toward the considered fermentative aroma compounds, such as ethyl and methyl esters of mid-chained fatty acid (ethyl

heptanoate, ethyl octanoate, ethyl decanoate, and methyl octanoate), of acetate esters and of long-chain alcohols, giving a loss between 40 and 60 % of their initial content. Soy and gluten proteins had a less dramatic effect, giving relative 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.

These results clearly show the need to investigate the influence of the nature and strength of the protein–aroma interactions in wine and to relate them to the protein ability at affecting the flavor perception. Indeed, hydrophilic odor-active compounds undergo weak hydrogen binding onto protein surfaces, while more hydrophobic odorant molecules can link to sites at the protein interior with a stronger affinity for hydrophobic substances. Flavor balance modifications are more likely to be perceived upon wine tasting when these interactions are irreversible (or reversible, but very strong).

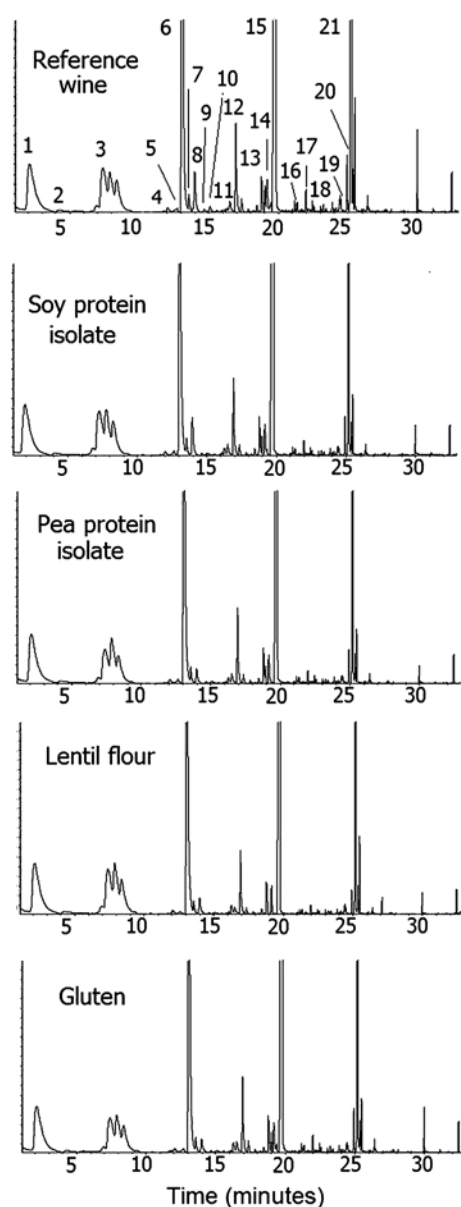


Fig. 5 TIC chromatograms obtained with SCAN method (m/z 45–350) by means of HS–SPME–GC/MS analysis on untreated and fined *Catalanesca* wine. Components are identified by numbers: 1, 3-methyl-1-butanol; 2, ethyl butanoate; 3, 3-methyl-1-butanol acetate; 4, benzaldehyde; 5, hexanoic acid; 6, ethyl hexanoate; 7, limonene; 8, 2-ethyl-1-hexanol; 9, benzene-acetaldehyde; 10, ethyl-2-hexenoate; 11, 1-octanol; 12, phenyl ethyl alcohol; 13, 2-nonanone; 14, octanoic acid; 15, ethyl octanoate; 16, 2-phenyl-ethyl acetate; 17, ethyl nonanoate; 18, methyl decanoate; 19, methyl decanoate; 20, decanoic acid; 21, ethyl decanoate

All these studies were carried out under equilibrium conditions, where protein addition affects the headspace partitioning of volatile and potentially odorous compounds. The presence of residual proteins in wine after fining and/or the presence of soluble complexes formed by volatile compound–protein interactions is indeed expected to shift

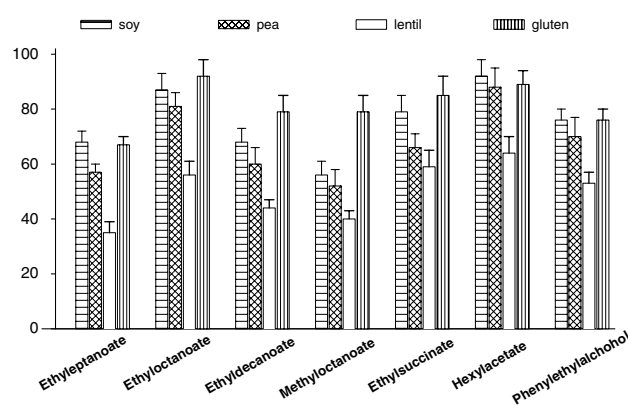


Fig. 6 Percentage loss of fermentative aroma compounds after treatment of *Catalanesca* wine, as assessed by HS–SPME–GC/MS analysis. The content of each species in unfined wine was taken as 100

the partition equilibrium of volatile molecules between the liquid and gas phases of the sample. The consequent changes in the volatile partitioning could cause a decreased presence of aroma compounds in the sample headspace, but not necessarily have a marked effect on aroma perception under the conditions of wine tasting. Previous work indeed demonstrated that the APCI-MS signal of methyl ketones was not influenced by the presence of milk proteins in wine samples as a whole, whereas headspace analysis of the same samples indicated that only a fraction of methyl ketones was present in the gas phase [26].

Protein stability of fined wines

To evaluate the suitability of plant protein finings in affecting haze development after storage, the fined wines were subjected to heat stability test as described in the “Materials and methods” section. The treated wine samples were exposed to a high temperature for a short period of time, followed by cooling, in order to simulate the precipitation of proteins at a proper cool storage temperature over the lifespan of the wine.

All performed tests indicated that fined wines were more stable than unfined one, showing an increase in turbidity after heat treatments lower than 2 NTU. It is important to point out that gelatin and other proteins used as fining agents against overextracted seed or skin tannins may contribute to protein instabilities and are often used in conjunction with other fining agents—such as silica gel—that can precipitate any excess fining proteins that did not bind to wine tannins [11].

Research of residual gluten proteins in wines

Since plant-derived proteins were very promising fining agents, it seemed important to quantify their residual

amount in the fined wine, as plant proteins could cause severe immunological responses or chronic intolerance. Although not a typical allergen, gluten is of particular relevance in this frame, given the high prevalence of gluten-related enteropathies, such as celiac disease, and the additive effects of gluten uptake by sensitive individuals. Given the associated difficulties, there are no studies suggesting thresholds for the biological effects of plant-derived proteins, if not for gluten. Indeed, lower limits for biological reactions are difficult to assess because of the number and variety of involved factors, including individual sensitivity, age, gender, genetic constitution, dietary habits, and other—as yet largely unidentified—environmental factors.

Residual amounts of soluble fining agents were similarly low in the various samples (all at 0.050–0.053 mg/L, regardless of the treatment), likely because all tested proteins are reportedly very scarcely soluble at the pH of wine [10]. SDS–PAGE tracings for fined wines were identical to those obtained for untreated wine (Fig. 7). This identity, together with the results of protein stability tests, confirms the absence of nongrape proteins in fined wines.

In the case of gluten, under the USA-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, so that the final food or drink product contains <20 parts per million (ppm or mg/kg) gluten. Gluten content of treated white and red wines was measured here by means of a commercial sandwich ELISA based on the R5 monoclonal antibody that recognizes the potentially celiac toxic epitope and some closely related sequences that are present in wheat gliadins, rye secalins,

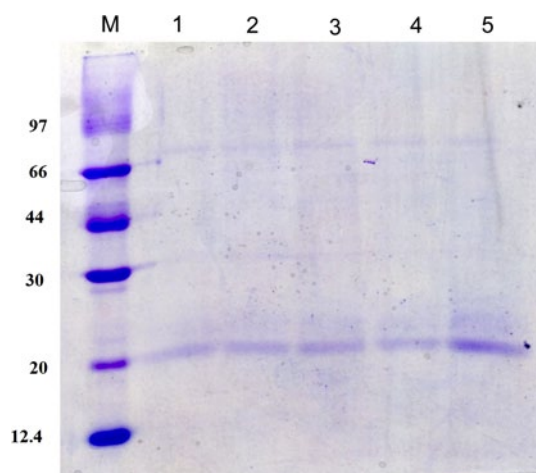


Fig. 7 SDS–PAGE of protein extracts of *Catalanesca* wine before and after fining. (M: molecular weight markers. 1, 2, 3, 4: wine fined with pea, soy, lentil, and gluten proteins, respectively; 5: unfined wine)

Table 2 Gluten content in gluten-fined *Catalanesca* white wine

	Gluten concentration in wine (ppm)	
	Expected	Found
Reference wine	0	<0.02 ^a
Reference wine + 0.05 ppm gliadin	0.10	0.14 ± 0.02
Gluten-fined wine	200 ^b	0.49 ± 0.06

Gliadin content was assumed to represent 50 % (w/w) of gluten. The standard gliadin solution supplied for calibration of the assay was used for spiking the reference wine. Dilution of individual samples prior to ELISA was carried out to ensure that absorbance readings were within the linearity limit of the calibration curve. Both calibration and actual assays were performed in triplicate, and the assay standard deviation is given

^a Below the sensitivity threshold of the assay

^b Calculated from the addition of 0.2 g gluten/L wine in the fining step

and barley hordeins [27–29]. The results in Table 2 indicate the feasibility of such a measurement in this complex matrix and provide evidence that the gluten concentration in the treated wine is by far below even the most restrictive legal threshold for gluten-free drinks. This is in accordance with previous results [30], who also verified that gluten-treated wine presented no risk of triggering a response in sensitive individuals.

Conclusions

All the proteins tested in this study were effective in decreasing turbidity and optical density of white wine. None of the added protein was present in significant amounts in the fined wine, and protein stability of wine was not affected by treatments. Lentil and gluten proteins were most effective in removing monomeric and dimeric flavanols, but lentil proteins caused a drastic decrease in the total content of compounds responsible for fermentative aroma. For the other matrices tested here (pea, soy, and gluten proteins), the loss of aroma compounds caused by treatment was comparable to those reported for other commonly used fining agents (gelatin, bentonite) [23].

The methodological approaches used in this investigation were able to provide information on the impact of fining agent on the phenolic and volatile compounds of the oxidation-prone *Catalanesca* white wine. The complexity of the emerging picture makes it clear that further studies should address the behavior of free aroma compounds and of bound aroma precursors using wines made from different grape varieties and in different steps of the winemaking process in order to verify the conclusions drawn here and their general significance.

Conflict of interest None.

Compliance with Ethics Requirements This article does not contain any studies with human or animal subjects.

References

- Gambuti A, Rinaldi A, Moio L (2012) Use of patatin, a protein extracted from potato, as alternative to animal proteins in fining of red wine. *Eur Food Res Technol* 235:753–765
- Cosme F, Capão I, Filipe-Ribeiro L, Bennett RN, Mendes-Faia A (2012) Evaluating potential alternatives to potassium caseinate for white wine fining: effects on physicochemical and sensory characteristics. *Food Sci Technol* 46:382–387
- Asano K, Shinagawa K, Hashimoto N (1982) Characterization of haze-forming proteins of beer and their roles in chill haze formation. *J Am Soc Bre Chem* 40:147–154
- Hagerman AE, Butler LG (1980) Condensed tannin purification and characterization of tannin-associated proteins. *J Agric Food Chem* 28:947–952
- Hagerman AE, Robbins CT (1987) Implications of soluble tannin-protein complexes for tannin analysis and plant defense mechanisms. *J Chem Ecol* 13:1243–1259
- Marchal R, Marchal-Delahaut L, Lallement A, Jeandet P (2002) Wheat gluten used as a clarifying agent of red wines. *J Agric Food Chem* 50:177–184
- Maury C, Sarni-Manchado P, Lefebvre S, Cheynier V, Moutounet M (2003) Influence of fining with plant proteins on proanthocyanidin composition of red wines. *Am J Enol Vitic* 54:105–110
- Granato TM, Piano F, Nasi A, Ferranti P, Iametti S, Bonomi F (2010) Molecular basis of the interaction between proteins of plant origin and proanthocyanidins in a model wine system. *J Agric Food Chem* 58:11969–11976
- International Organisation of Vine and Wine (2005) Compendium of international methods of wine and must analysis
- Nasi A, Ferranti P, Amato S, Chianese L (2008) Identification of free and bound volatile compounds as typicalness and authenticity markers of non-aromatic grapes and wines through a combined use of mass spectrometric techniques. *Food Chem* 110:762–768
- Pocock KF, Waters EJ (2006) Protein hazes in bottled white wines: how well do stability tests and bentonite fining trials predict haze formation during storage and transport? *Aust J Grape Wine Res* 12:212–220
- Méndez E, Vela C, Immer U, Janssen FW (2005) Report of a collaborative trial to investigate the performance of the R5 enzyme linked immunoassay to determine gliadin in gluten-free food. *Eur J Gastroenterol Hepatol* 17:1053–1063
- Barroso CG, Lopez-Sanchez L, Otero JC, Cela R, Perez-Bustamante JA (1989) Studies on the browning of fino sherry and its relation to polyphenolic compounds. *Z Lebensm Unters Forsch* 189:322–325
- Lee CY, Jaworski AW (1988) Phenolic and browning potential of white grape grown in New York. *Am J Enol Vitic* 39:337–340
- Bhatty RS (1988) Composition and quality of lentil (*lens culinaris medik*): a review. *Can Inst Food Sci Technol J* 21:144–160
- Wieser H (2007) Chemistry of gluten proteins. *Food Microbiol* 24:115–119
- Bian Y, Myers DJ, Dias K, Lihono MA, Wu S, Murphy PA (2003) Functional properties of soy protein fractions produced using a pilot-scale process. *J Am Oil Chem Soc* 80:545–549
- Leterme P, Monmart T, Baudart E (1990) Amino acid composition of pea (*Pisum sativum*) proteins and protein profile of pea flour. *J Sci Food Agric* 53:107–110
- Castillo-Sanchez JX, Garcia-Falconc MS, Garrido J, Martinez-Carballo E, Martins-Dias LR, Mejuto XC (2008) Phenolic compounds and colour stability of vinhao wines: influence of wine-making protocol and fining agents. *Food Chem* 106:18–26
- Cosme F, Ricardo-da-Silva JM, Laureano O (2008) Interaction between protein fining agents and proanthocyanidins in white wine. *Food Chem* 106:536–544
- Dufour C, Bayonove CL (1999) Interaction between wine polyphenols and aroma substances. An insight at molecular level. *J Agric Food Chem* 47:678–684
- Lubbers S, Leger B, Charpentier C, Feuillat M (1993) Effet colloïde-protecteur d'extraits de parois de levures sur la stabilité tartrique d'une solution hydro-alcoolique modèle. *J Int des Sciences de la Vigne et du Vin* 27:13–22
- Lubbers S, Charpentier C, Feuillat M (1996) Study of the binding of aroma compounds by bentonite in must, wine and model systems. *Vitis* 35:59–62
- Moio L, Ugliano M, Gambuti A, Genovese A, Piombino P (2004) Influence of clarification treatment on concentration of selected free varietal aroma. *Am J Enol Vitic* 55:7–12
- Volley A, Lamer C, Dubois P, Feuillat M (1990) Influence of macromolecules and treatments on the behavior of aroma compounds in a model wine. *J Agric Food Chem* 38:248–251
- Le Guen S, Vreeker R (2003) Interactions between flavour compounds and milk protein under static and dynamic conditions. In: Le Quére JL, Etiévant P (eds) *Flavour research at the dawn of the twenty first century*. Lavoisier Tec & Doc, Paris
- Valdés I, Garcia E, Llorente M, Méndez E (2003) Innovative approach to low-level gluten determination using a novel sandwich enzyme-linked immunosorbent assay protocol. *Eur J Gastroen Hepat* 15:465–474
- Osman AA, Uhlig HH, Valdés I, Amin M, Méndez E, Mothes T (2001) A monoclonal antibody that recognizes a potential coeliac-toxic repetitive pentapeptide epitope in gliadins. *Eur J Gastroen Hepat* 13:1189–1193
- Kahlenberg F, Sanchez D, Lachmann I, Tuckova L, Tlaskalova H, Méndez E, Mothes T (2006) Monoclonal antibody R5 for detection of putatively coeliac-toxic gliadin peptides. *Eur Food Res Technol* 222:78–82
- Lefebvre S, Gerland C, Maury C, Gazzola M (2000) Nouvelles colles vegetales: origines, proprietes et performances. *Rev Fr Oenol* 184:28–31