Chapter 16
Wine Quality

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16.1 Introduction

One of the most ancient legends about the origins of wine tells of a Persian princess who tried to poison herself so as to put an end to her love pangs. So she drank some juice from the bottom of a jar containing rotten grapes, and, instead of being killed by them, she forgot all her pain, and soon thereafter she regained her lover's heart. The King himself tried the prodigious drink, and began to spread its fame throughout his kingdom. Many other legends regarding the prodigious powers of wine can be found in literature, and even in the main world religions, of which the episode of Noah's drunkenness in the Old Testament is an example.

The domestication of the grapevine can be traced back to the third millennium BC: since then, the cultivation of *Vitis* ssp. has expanded to all the continents, with the exception of Antarctica. According to the International Organization of Vine and Wine (O.I.V. Report 2008), in 2007 the surface area given over to grape cultivation accounted for almost 7.8 million hectares, Europe being the main producer, transformer, and consumer of grapes for winemaking. Among the products derived from grapes, wine is the most significant. Wine production touched 266 million hL in 2007 (O.I.V. Report 2008), showing a sharp decrease (more than 20%) since the beginning of the 1980s. This decrease has been accompanied by increasing attention on the part of consumers towards wine quality. Fraud concerning adulterated wines (Frank in 2007 reported that up to 5% of the wine sold in secondary markets could be counterfeit) leads to important economic losses in the wine trade, mostly due to the producers rightly or wrongly losing credibility, as well as causing severe safety alarms.

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The importance of the wine trade and the growing attention paid by modern consumers towards food quality has raised interest in recent years among the scientific community in defining quality markers for wine. The quality of a food is not unambiguous: it is made up of safety issues, organoleptic features, technological aspects, and nutritional requirements, among other characteristics. All these features have been investigated in wines, and almost all of them have been studied with proteomic means, mostly in the last two decades.

The scientific reviews on wine protein analysis published to date can be grouped into two main categories: those devoted to methodological issues (Moreno-Arribas et al. 2002; Flamini and De Rosso 2006; Curioni et al. 2008; Le Bourse et al. 2010) and those focused on the application of proteomics to specific wine issues (Ferreira et al. 2002; Giribaldi and Giuffrida 2010; Pedreschi et al. 2010; D'Alessandro and Zolla 2012). Moreno-Arribas and colleagues mainly described the methods used for the extraction, quantification, and separation of wine proteins (Moreno-Arribas et al. 2002). Interestingly, mass spectrometry (MS) in their report was briefly described as a tool for identifying proteins, and only a few years later the review published by Flamini and De Rosso (2006) focused on its use for the separation, quantification, and identification of grape and wine proteins, thus paving the way for the leading role acquired by MS in the analysis of foods and beverages in recent years. In 2002, Ferreira and collaborators revised the state of the art on wine proteins by providing an overview of their origin and function, and focused on the turbidity issues caused by them, including possible solutions to remove them. More recently, Curioni et al. (2008) and Le Bourse et al. (2010) provided updated reviews of the latest trends in the analytical techniques used for the study of grape juice and wine proteins. In 2010, Giribaldi and Giuffrida updated the proteomic studies published since 2005, covering aspects of grape physiology and grape berry ripening, as well as protein function in wines (Giribaldi and Giuffrida 2010). In the same year, wine proteomics was included in a review devoted to the application of proteomics to various important food industry sectors (Pedreschi et al. 2010). The most recent survey on the issue is the review by D'Alessandro and Zolla (2012) on proteomics applications in the field of wine safety and traceability.

The present chapter briefly and exhaustively describes the papers that recount proteomic means to study wine proteins in recent decades. However, the authors strongly recommend consulting these reviews in order to achieve a more complete understanding of wine protein science.

16.2 Methodological Aspects in Wine Proteomics

In recent decades, the application of new techniques to the characterization of wine proteins has brought the number of wine proteins identified by proteomics to over 100 (D'Amato et al. 2011). Traditional techniques, such as electrophoresis and chromatography, originally allowed for the detection of the most abundant proteins (accounting for only about 30% of the total protein species in wine, according to D'Amato and collaborators).

One major step towards achieving these goals was made in 2007, when two independent studies, one supported by a French–Italian consortium (Jaillon et al. 2007) and the other by an Italian–American initiative (Velasco et al. 2007), published the genome sequence of one non-cultivated highly homozygous and of one cultivated highly heterozygous Pinot Noir clone, respectively. The annotation of the grapevine genome is still in progress, and although it may sometimes complicate the interpretation of research findings, the availability of genome sequences has significantly boosted grape and wine proteomics.

16.2.1 Quantification of Protein Content in Wines

Typically, protein concentrations have been reported in a range from 15 to 230 mg/L (Monteiro et al. 2001; Ferreira et al. 2002; Waters et al. 2005). Proteins are thus considered as minor components of both white and red wines, with little nutritional relevance. Several techniques are currently available for total protein quantification in foods and beverages, but wine is typically rich in compounds that may interfere with normal quantification techniques, such as phenolics, ethanol, and organic acids (Marchal et al. 1997; Moreno-Arribas et al. 2002; Le Bourse et al. 2010).

The standard protein quantification method in the food industry is based on the evaluation of total proteins by conversion of nitrogen measured by Kjeldahl assay (multiplying nitrogen content by 6.25) (AOAC method 960.52), but this method is known to cause frequent overestimations of wine protein content (Vidigal et al. 2012). The reliable quantification of wine proteins may have an impact on the stability of the wine itself, as they are major causes of white wine clouding (Waters et al. 2005). The most common methods used for protein quantification in wines to date are based on spectrophotometric detections, such as the Bradford (Bradford 1976), Lowry (Lowry et al. 1951), Biuret (Gornall et al. 1949), or Smith (Smith et al. 1985) tests, which have often been used due to their ease and speed (Vidigal et al. 2012). Nevertheless, the presence of interfering compounds and the absence of standard wine proteins may lead to erroneous quantification with these methods (Moreno-Arribas et al. 2002; Le Bourse et al. 2010, 2011; Vidigal et al. 2012).

In recent years, Vincenzi et al. (2005) and Smith et al. (2011) have provided comparisons between the more widely used protein quantification methods and developed improved procedures for protein recovery and quantification in white and red wines. Vincenzi et al. (2005) concluded that potassium dodecyl sulphate (KDS) precipitation coupled with Smith's assay gave the most accurate results, consistent with those obtained by densitometric quantification of SDS-PAGE protein bands. Smith et al. (2011) concluded that, in red wines, protein precipitation with cold trichloroacetic acid/acetone and quantification based on Bradford's assay absorbance using a yeast mannoprotein invertase standard gave results similar to those obtained by micro-Kjeldahl analysis.

Some authors have suggested using HPLC-based techniques to achieve reliable quantification of wine proteins (Peng et al. 1997; Pocock and Waters 2006).

Marangon et al. (2009) developed a quantification method for protein fractions separated by hydrophobic interaction chromatography based on comparison with the HPLC peak area of two standard proteins (cytochrome c and bovine serum albumin, BSA), but this method may suffer from limitations due to the use of non-wine standard proteins (Le Bourse et al. 2010). To circumvent this major drawback, Le Bourse et al. (2011) published a method for purifying grape juice class IV chitinase and thaumatin-like (TL) proteins by liquid chromatography. The pure protein fractions were then used to build reliable calibration curves for ultra-HPLC and for ELISA quantification of these proteins in different grape juices and wine samples, thus providing a valuable tool for future oenological studies.

The densitometric quantification of wine protein bands from SDS-PAGE gels (after staining with Coomassie Brilliant Blue) was frequently used in the past (Marchal et al. 2000; Hsu and Heatherbell 1987a, b). Recently, Sauvage et al. (2010) and Dufrechou et al. (2010, 2012) reported a quantification method based on absorption/desorption of proteins with excess bentonite, a clay with protein absorption capacity commonly used in wine fining, followed by SDS-PAGE, image analysis, and quantification of wine protein bands using a standard BSA band. This method, although it is said to provide an estimate of total wine proteins, fails to take into account low and high molecular weight proteins, which are not visualized in the SDS-PAGE gel. Moreover, it relies on the staining intensity of a non-wine protein, BSA, similarly to most of the previously cited techniques, loaded in a single concentration on the gel, and it thus fails to build a standard curve, with a regression and a correlation coefficient. In addition, similarly to all the methods based on Coomassie Brilliant Blue protein staining, including the Bradford method, the composition of some proteins may not give a linear response (Fountoulakis et al. 1992).

The most recent advance in the field of protein quantification in white wines is the modified Lowry assay coupled with solid-phase extraction recently developed by Vidigal et al. (2012). The method is based on the retention of proteins in the solid support, nitrilotriacetic acid Superflow beads (Qiagen), charged by Cu^{2+}, and on the absorbance at 500 nm after addition of Folin–Ciocalteu's reagent. Results from Vidigal's work represent a significant advance with respect to current methods in terms of sample volume required, poor pre-processing before analysis, and automation (Vidigal et al. 2012). The limitations due to the use of non-wine standard protein should be evaluated carefully, and further investigations into its reliability for wine glycoprotein quantification are needed.

16.2.2 Methods Used in Wine Protein Characterization

As brilliantly summarized in several reviews (Moreno-Arribas et al. 2002; Flamini and De Rosso 2006; Curioni et al. 2008; Le Bourse et al. 2010), the main techniques used for grape and wine proteome analysis can be grouped into chromatography, electrophoresis, and MS-based methods.

Electrophoresis and 2-D-electrophoresis have been widely used in the past and are still in use to characterize the protein fraction of wines, often in association with

chromatographic techniques. Early works mainly used electrophoretic techniques, in the form of both native gel electrophoresis (Bayly and Berg 1967; Pueyo et al. 1993; Moreno-Arribas et al. 1999) and denaturing gel electrophoresis (Yokotsuka et al. 1991; Waters et al. 1992, 1993; Pueyo et al. 1993; Dorrestein et al. 1995; Santoro 1995; Marchal et al. 1996). Wine proteins have often been separated according to their isoelectric point as a preparatory or analytical step (Murphey et al. 1989; Yokotsuka et al. 1991; Pueyo et al. 1993; Dawes et al. 1994; Marchal et al. 1996; Luguera et al. 1998). The coupling of isoelectrofocusing and denaturing electrophoresis led to detailed screening of wine proteins ever since its early application to wine proteomics (Hsu and Heatherbell 1987a, b; Hsu et al. 1987). Protein immunoblotting has been widely used as a tool for investigating the origin and the structural similarity of wine proteins from different varieties (Hsu and Heatherbell 1987a, b; Hsu et al. 1987; Marchal et al. 1998; Monteiro et al. 1999; Ferreira et al. 2000; Monteiro et al. 2001, 2003a, b; Dambrouck et al. 2003; Manteau et al. 2003; Monteiro et al. 2007). In more recent years, the coupling of electrophoresis with protein identification by mass spectrometry has allowed researchers to unravel the complexity of the wine proteome in different conditions, and thus improved wine science (Okuda et al. 2006; Cilindre et al. 2008; Wigand et al. 2009; Sauvage et al. 2010; Vincenzi et al. 2011). Moreover, the introduction of enrichment technologies, such as the use of combinatorial peptide ligand libraries (CPLL), has increased the detection of low-abundance constitutive or contaminating proteins in wines (Cereda et al. 2010; D'Amato et al. 2010, 2011).

Capillary electrophoresis has been applied in the past to wine protein analysis (Moine Ledoux et al. 1992; Luguera et al. 1997, 1998; Dizy and Bisson 1999), and one recent example of its potential is represented by varietal differentiation recently obtained by high-performance capillary electrophoresis of wine proteins and shikimic acid quantification by Chabreyrie et al. (2008).

Chromatographic approaches used in wine proteomics for protein purification, separation, and characterization include FPLC (Waters et al. 1992, 1993; Dawes et al. 1994; Dorrestein et al. 1995; Waters et al. 1995; Luguera et al. 1998; Monteiro et al. 1999, 2001, 2003a, b, 2007; Esteruelas et al. 2009), HPLC (Tyson et al. 1981; Santoro 1995; Yokotsuka and Singleton 1997; Girbau et al. 2004), size exclusion chromatography (Pellerin et al. 1993; Gonçalves et al. 2002), affinity chromatography (Pellerin et al. 1993; Waters et al. 1993; Marchal et al. 1996; Gonçalves et al. 2002; Vanrell et al. 2007), and more recently hydrophobic interaction chromatography (Falconer et al. 2010; Marangon et al. 2009, 2011a, b), hydrophilic interaction chromatography, titanium dioxide enrichment, and hydrazide chemistry enrichment (Palmisano et al. 2010).

Mass spectrometry and N-terminal sequencing have greatly increased our understanding, allowing the identification of several wine proteins. In recent years, MS has become a useful tool for wine traceability, allowing for varietal fingerprinting and contaminant detection in both red and white wines (Szilágyi et al. 1996; Weiss et al. 1998; Kwon 2004; Catharino et al. 2006; Carpentieri et al. 2007; Chambery et al. 2009; Monaci et al. 2010, 2011; Simonato et al. 2011; Tolin et al. 2012; Nunes-Miranda et al. 2012).

16.3 The Origin of Wine Proteins

One of the main aspects investigated by wine proteomics has been the elucidation of the origin of wine proteins. Early investigations declared wine proteins to be derived exclusively from the grape berry (Luguera et al. 1998; Ruiz-Larrea et al. 1998; Ferreira et al. 2000). One reason could be the fact that, according to almost all the reports, grape-derived proteins, and especially the class named pathogenesis-related (PR) proteins (Linthorst 1991), represent the vast majority of the protein components found in all studied wines (Waters et al. 1996; Monteiro et al. 2001; Okuda et al. 2006; Wigand et al. 2009; Dufrechou et al. 2010; Sauvage et al. 2010; Vincenzi et al. 2011; Dufrechou et al. 2012). According to other hypotheses, the poor visualization of yeast proteins from wines may be due to the revelation method used, as their sugar moiety makes them poorly detectable by Coomassie and silver-based stains (Waters et al. 1993; Dambrouck et al. 2003; Wigand et al. 2009).

Currently, most authors agree on the mixed origin of wine proteins, as yeast-deriving proteins (mostly cell wall components) have been demonstrated to occur in wines by several methodological approaches, such as chromatography (Marchal et al. 1996; Yokotsuka and Singleton 1997; Monteiro et al. 2001; Gonçalves et al. 2002; Palmisano et al. 2010), electrophoresis (Waters et al. 1993; Dupin et al. 2000; Kwon 2004; Cilindre et al. 2008; Wigand et al. 2009; D'Amato et al. 2011; Marangon et al. 2011a, b), immunostaining (Monteiro et al. 2001; Dambrouck et al. 2003), and mass spectrometry (Simonato et al. 2011; Tolin et al. 2012). The functions of parietal yeast mannoproteins include adsorption of ochratoxin A, combination with phenolic compounds, increased growth of malolactic bacteria, inhibition of tartrate salt crystallization, interaction with the yeast-derived superficial film (*flor*) formed in the manufacture of sherry-type products, reinforcement of aromatic components, and wine enrichment during aging on fine lees (Caridi 2006; Blasco et al. 2011). Their major roles, being haze-protective factors in white wines and foam aids in sparkling wines, are detailed in the following chapter.

Other fermentative agents in wines include several types of bacteria. The presence of bacterial proteins in wines used to be excluded by immunostaining (Dambrouck et al. 2003) until recently. Simonato et al. (2011) and Tolin et al. (2012), by means of LC-MS/MS profiling, were the first, to the author's knowledge, to detect one 60 kDa chaperonin from *Oenococcus oeni,* a lactic acid bacterium involved in malolactic fermentation.

Fungal pathogens are responsible for considerable economic losses for wine-makers, and the costs of field prevention against their occurrence are a major expense for agriculture. The presence of proteins deriving from fungal infection of grape clusters on the vine has been demonstrated by Western blot and electrophoresis in recent years (Kwon 2004; Cilindre et al. 2007, 2008). One recent investigation of a commercial Valpolicella red wine revealed the presence of proteins from several fungal pathogens (D'Amato et al. 2011).

16.4 The Role of Proteins in Wines

Although they are minor constituents in wines, proteins are widely recognized to have a significant impact on wine quality. They are thought to contribute to wine taste and body (Jones et al. 2008), and to the foaming properties of sparkling wines (Vanrell et al. 2007; Blasco et al. 2011; Coelho et al. 2011). They are known to be detrimental for wine quality on some occasions, causing turbidity in white wines (Waters et al. 1992, 2005). Moreover, some of the wine proteins have been found to be allergenic for some susceptible individuals (Pastorello et al. 2003; Vassilopoulou et al. 2007; Giribaldi and Giuffrida 2010; Gonzalez-Quintela et al. 2011).

Profiling by nano-HPLC/tandem MS of a Sauvignon Blanc wine led to the identification of 20 major proteins, including several yeast proteins (Kwon 2004). The identified grape-derived proteins were vacuolar invertase, TL proteins, class IV endochitinase, and β-glucanase (Kwon 2004). The 2DE pattern of one Chardonnay wine confirmed the occurrence of several vacuolar invertase protein spots, as well as the presence of PR proteins such as osmotins and thaumatins, and detected for the first time one low molecular weight lipid transfer protein (Okuda et al. 2006). The presence of these proteins has since been confirmed in red and white wines by other published reports (Sauvage et al. 2010; Dufrechou et al. 2012; Lambri et al. 2012), with minor differences, such as the detection of grape ripening-related proteins and of PR4 proteins (chitin-binding proteins) in some cases (Cilindre et al. 2008; Esteruelas et al. 2009; Marangon et al. 2009; Wigand et al. 2009).

The application of more sensitive techniques such as direct MS analysis, or the use of enrichment strategies such as CPLL (Righetti et al. 2011) and glycopeptide enrichment (Palmisano et al. 2010), has helped to identify several other low-abundance proteins present in red (Simonato et al. 2011; Tolin et al. 2012) and white wines (D'Amato et al. 2011). Although there is detailed knowledge of the type of proteins that wine may contain, there is still little understanding of the role they may play in wine, especially with regard to low-abundance proteins.

16.4.1 Heat-Unstable Proteins and Haze Formation

Wine proteins may cause a common white wine defect called "casse protéique." During bottle storage, occasional extremes of temperature may lead to protein aggregation and flocculation, which causes turbidity (Waters et al. 2005). A haze or deposit in bottled wine can reduce or invalidate its commercial value, and winemakers usually perform fining treatments, typically with bentonite, to avoid this turbidity. The occurrence of this defect led to early studies on wine proteins (Koch and Sajak 1959; Moretti and Berg 1965; Bayly and Berg 1967; Hsu and Heatherbell 1987b; Waters et al. 1992).

Although total protein quantity may have an impact on the probability of haze development in white wines (Mesquita et al. 2001), not all wine protein fractions

seem to share the tendency to flocculate, as some are more heat-labile than others (Moretti and Berg 1965; Bayly and Berg 1967; Hsu and Heatherbell 1987b; Hsu et al. 1987; Moine Ledoux et al. 1992; Waters et al. 1992). One recent application of CPLL to the soluble fraction and sediment of one white wine revealed a very limited overlap between the two types of proteins (D'Amato et al. 2011), thus contributing to the hypothesis of differential haze-forming tendencies for different wine proteins.

The heat-unstable protein fraction is mainly made up of grape PR proteins (Waters et al. 1996; Esteruelas et al. 2009; Sauvage et al. 2010; Marangon et al. 2011a, b; Vincenzi et al. 2011; Dufrechou et al. 2012). These proteins are able to persist through the winemaking process (Vincenzi et al. 2011), mainly due to their resistance to proteolysis and to their stability at acid pH (Linthorst 1991). The major contributors to natural wine haze to date have been identified as β-glucanases, class IV chitinases, and TL proteins (Waters et al. 1996; Esteruelas et al. 2009; Falconer et al. 2010; Marangon et al. 2011b; Sauvage et al. 2010; Dufrechou et al. 2012). These proteins were characterized recently for their haze-forming tendency and absorbance by bentonite (Sauvage et al. 2010). A progressive sensitivity to heat-induced precipitation, and a concomitant increased susceptibility to bentonite absorption, was found for β-glucanases, class IV chitinases, and a fraction of TL proteins, with invertases and the other fraction of TLs being less affected. These results confirmed previous findings on the thermal stability of purified chitinase, invertase, and TL protein, which reported that chitinase is the major player in heat-induced wine haze formation, probably due to its low melting temperature (Falconer et al. 2010).

The aggregation kinetics in white wines at different temperatures were determined by dynamic light-scattering experiments (Dufrechou et al. 2010). At low temperature (40 °C), aggregation took place during the heating phase, whereas at higher temperatures (60 °C and 70 °C) protein aggregation mainly developed during the cooling phase. Results confirmed the differential heat sensitivity of diverse TL protein fractions and the haze-forming tendency at low temperatures of β-glucanases and class IV chitinases (Sauvage et al. 2010), but a temperature lower than that reported in model wine solutions (Falconer et al. 2010) was found to be necessary for invertase aggregation and precipitation. Recently, Marangon and co-workers (2011b) found that the natural haze of white wine consisted mainly of class IV chitinase, with the contribution of β-glucanase and, for the first time, of one yeast cell wall glucantransferase. Using a model wine solution, they found that haze in the presence of purified chitinase and TL protein was formed only when sulphate was present. Similar results were found in a protein-free wine added with isolated chitinase, TL proteins, and sulphate. As reported for invertase by Dufrechou et al. (2010), the wine proteins tested by Marangon and co-workers (2011b) had lower unfolding temperatures in real wine than in model wine solutions.

Because different wines with different haze potential usually contain very similar protein fractions (Ferreira et al. 2000; Monteiro et al. 2001; Wigand et al. 2009), one or more unknown non-proteinaceous wine component(s) (termed X factors) are thought to be needed to cause visible haze formation (Mesquita et al. 2001; Waters

et al. 2005; Batista et al. 2009). Candidate factors that may play a modulating role in wine haze formation include the sulfate anion (Pocock et al. 2007; Marangon et al. 2011a, b), pH value (Batista et al. 2009; Dufrechou et al. 2012), ionic strength (Dufrechou et al. 2010, 2012; Marangon et al. 2011a), phenolic compounds (Waters et al. 1995; Marangon et al. 2010; Esteruelas et al. 2011), and organic acids (Batista et al. 2010). To date, the identity of the X factor remains unclear.

Marangon et al. (2011a) studied the impact of ionic strength and sulfate upon thermal aggregation of purified grape chitinases and TL proteins in a model wine solution. They reported that, although TL proteins are not very susceptible to ionic strength changes, chitinase isoforms behave differently, one being precipitated above 21 mM, the other above 100 mM. Sulphate, even at low concentration, increased the instability of both chitinase isoforms, and it had no effect on TLs. Very recently, Dufrechou et al. (2012) published a report on the effects of ionic strength, pH, and temperature on wine protein instability, using both model and real wines. By screening aggregation kinetics, they proposed a model for heat-induced haze formation which includes a balance between pH-induced unfolding, leading to conformational changes responsible for colloidal aggregation of wine proteins at low pH, and heat-induced unfolding, leading to denaturation and aggregation at higher temperatures.

The validity of the experiments on wine protein instability involving the analysis of wine model solutions containing organic acids has been recently questioned: Batista et al. (2010) found a dramatic reduction in the haze potential of wine proteins when measured in the presence of organic acids normally encountered in wines. This reduction was also observed in real wines when added with organic acids. They suggested this phenomenon may be linked to the removal of considerable amounts of phenolics, which are apparently involved in protein haze formation (Waters et al. 1995; Marangon et al. 2010; Esteruelas et al. 2011).

Glycosylated proteins are known to play an important role in wine turbidity, as they may interact with tannins, polyphenols, and other proteins (Siebert et al. 1996). Moine-Ledoux et al. (1992) showed that wines aged on yeast lees were less prone to haze formation and were stabilized by the addition of less bentonite than wines aged without lees. Subsequently, they were able to demonstrate that this protection from haze was due to a 32-kDa fragment of glycosylated yeast invertase (Moine-Ledoux and Dubourdieu 1999). Dupin et al. (2000) proposed a competitive mechanism between yeast mannoprotein and wine proteins for unknown wine components, otherwise required for the formation of large insoluble aggregates of denatured protein (the X factor?). Other glycoproteins showing haze-protective activity include whole yeast invertase (Moine-Ledoux and Dubourdieu 1999), Arabinogalactan proteins (Waters et al. 1994b; Pellerin et al. 1993), and high molecular weight yeast mannoproteins (Waters et al. 1993; Waters et al. 1994a). To date, several studies have demonstrated that modified yeast strains overproducing mannoproteins significantly contributed to improved white wine stability (Brown et al. 2007; Gonzalez-Ramos et al. 2008). The recent screening of wine glycoproteins published by Palmisano and co-workers (2010) may increase the possibilities for the characterization of yeast and grape glycoproteins, which may have a technical

application in the reduction of white wine haziness. To this intent, one predictive assay for wine haze tendency based on the separate recovery and quantification of wine proteins and glycoproteins was recently developed (Fusi et al. 2010). The authors showed that protein content and glycoprotein concentrations are different in wine, whereas their electrophoretic patterns are almost superimposable. They also demonstrated a straightforward connection between their assay and prediction of haze as measured by traditional assays.

16.4.2 Foam Formation and Stability

Wine proteins have been shown to play an important role in the sparkling wine industry because they are known to promote foam formation and stability. A positive correlation between protein concentration and foam formation in sparkling wines has been reported since the earliest studies (Brissonet and Maujean 1993; Malvy et al. 1994; Andres-Lacueva et al. 1996; Marchal et al. 1996; Luguera et al. 1997, 1998). The occurrence of protein degradation in sparkling wines has been shown to reduce their foamability. Dambrouck et al. (2005) found that a significant decrease in both the total protein and the grape invertase contents of Champagne-base wines was correlated with the loss of wine foaming properties.

Several studies have investigated the detrimental effect on foam stability of infection by fungal pathogens, such as *Botrytis cinerea.* The reduction in foamability registered in the presence of botrytized grapes was due to fungal proteases able to significantly hydrolyze wine proteins (Girbau et al. 2004; Marchal et al. 2006; Cilindre et al. 2007, 2008).

Vanrell and colleagues demonstrated that the use of bentonite fining treatments on sparkling wines caused a significant reduction in foam formation and stability. This effect was due to the registered depletion of all the protein fractions by bentonite, except for the high molecular mass fraction, which probably contains glycoproteins and polysaccharides (Vanrell et al. 2007).

As previously described, during alcoholic fermentation and aging on lees, glycosylated proteins (mannoproteins) are released by the yeasts. These proteins were recently shown to have potential foam-active properties in wine and also in beers (Blasco et al. 2011). Very recently, one experiment on molecular reconstituted model sparkling wines demonstrated that foam height and foam stability increased exponentially with the concentration of high molecular weight mannoproteins (Coelho et al. 2011).

Due to the contribution to foam formation and stability of some wine proteins, especially the high molecular weight glycosylated proteins, the investigation of fining methods other than bentonite, with a more selective removal capacity, is still one of the major needs of the oenological industry.

16.5 Wine Proteins as a Tool for Traceability

16.5.1 Varietal/Geographical Differentiation

The transformation of the wine market into a global market has pushed producers and legislators towards the approval of protection policies for several high-quality wines. To this end, labeling policies have been created throughout the world. The two main types of labeling policy are derived from the American and the French model. In the first model, wines are labeled according to the most abundant grape variety used (minimum 75%). In the French policy, the system of Protected Designations of Origin, geographical criteria are as important as varietal ones. Regardless of the system used, wines bearing protected labels are considered of higher quality and are generally more expensive than non-labeled wines. It is thus not surprising to see efforts being made by scientists to develop new techniques to prove wine authenticity. Most of these techniques rely on DNA typing (Siret et al. 2000; García-Beneytez et al. 2002), biochemical characterization of both volatile and non-volatile compounds (Rebolo et al. 2000; Moret et al. 1994), and analysis of stable isotopes (Day et al. 1995; Di Paola-Naranjo et al. 2011).

In more recent years, the study of compounds that can be used in grape traceability has expanded to nitrogenous compounds, including proteins. The first steps towards varietal differentiation of wines based on protein profiling were taken by Pueyo et al. (1993), who found differences in the native electrophoresis patterns of musts obtained from different grape varieties, and by Moreno-Arribas et al. (1999), who analyzed 41 musts made from a mixture of grapes from large vineyards and were able to group them according to the grape variety using the same approach.

In 2002, Rodriguez-Delgado and collaborators used capillary gel electrophoresis of wine proteins in order to differentiate between different wines from the Canary Islands, and found that, although similar, the relative amounts of specific protein fractions allowed differentiation among them, due to the different grape varieties used, the soil in which the vines grew, and the climatic conditions. One recent application of high-performance capillary electrophoresis to varietal differentiation of still white wines, based on protein profiling coupled with shikimic acid quantification, has been proposed (Chabreyrie et al. 2008). Comparison of the SDS-PAGE patterns of commercial red, rosé, and white wines from different varieties revealed great similarities among the analyzed wines, although some differences could be found (Wigand et al. 2009). The protein band identified as lipid transfer protein, for example, was not detected in most of the commercial red wines, although it was fully detected in the Dornfelder red wine, less in the rosé wine, and not in white wines, probably due to the shorter contact times between wine and skins (Wigand et al. 2009).

MS analysis of wine proteins has been proposed as a tool for wine authentication since 1996, when Szilágyi and colleagues published their results on the application of MALDI for distinguishing wines and musts. MALDI and SELDI (surface-enhanced laser desorption/ionization) were then used for the fingerprinting of proteins in different wines (Weiss et al. 1998), and ESI-MS was used on directly infused musts

and wines, and proved to be able to reveal the addition of unfermented must or sugar (Catharino et al. 2006). MALDI-TOF-MS profiling of peptides obtained by tryptic digestion has been recently proposed as a tool for differentiating high-quality white wines from the Campania region (Chambery et al. 2009).

To promote the use of MS profiling of wine proteins as a tool for differentiation, more methodological and technical evidence is needed. To this end, one recent study has been published by Nunes-Miranda et al. (2012), taking into account the type of matrix, the number of bottles of white wine, the number of technical replicates, and the number of spots, as well as the classification algorithm used. In their report, the best conditions for the reliable profiling of unprocessed wine proteins were found to be the use of α-Cyano-4-hydroxycinnamic acid matrix, mixed 0.75:1 with analyzed wine, with three spots from five different bottles of each wine as minimum requirements, with the Bayes-Net algorithm performing the best in these conditions (Nunes-Miranda et al. 2012). Although expensive both in terms of costs and time, more studies in this direction are urgently needed to move from the field of research to real applications of MS for proteins and peptides in the field of varietal/geographical differentiation of wines.

16.5.2 Detection of Contaminating Proteins

One major issue for wine traceability in the last decade has been the detection of residual proteins deriving from fining treatments. Some winemakers usually add protein-based fining agents (milk casein, egg ovalbumin, fish gelatin, gluten) in order to reduce or eliminate potential sediments of grape and yeast proteins during long-term bottle storage (D'Alessandro and Zolla 2012). These fining proteins may cause severe problems for wine commercialization, as most of them are potentially allergenic, and are now subjected to mandatory labeling. The techniques used to date for detection of contaminating proteins of animal and/or plant origin in wines mainly relied on the antibody/antigen reaction, such as ELISA (Rolland et al. 2008; Weber et al. 2009; Lacorn et al. 2011) and Western blotting (Weber et al. 2009). The detection limit of these methods is often considered too high (100 μg/L for Weber et al. 2009, 2010). One recent clinical work by Vassilopoulou et al. (2011) reported that, although no allergen was detected by traditional methods in the fined wines, positive skin prick test reactions and basophil activation to the treated wines were observed in the majority of patients with allergy to milk, egg, or fish, correlating with the concentration of the fining agents used.

Mass spectrometry has been applied in recent years to the detection of these fining proteins to wines. Capillary LC combined with ESI-Q-TOF-MS was used by Monaci et al. (2010) for the detection of caseins in white wines, with a declared limit of detection (50 mg/L) which is still much higher than approved ELISA methods. Very recently, one commercial ELISA kit has been validated for detection of caseins in white wine with a declared detection limit as low as 1 ppm (Restani et al. 2011).

Mass spectrometry has also been used for the detection of gluten-derived proteins in red wines by LC-MS/MS analysis (Simonato et al. 2011), and the method

proved to be significantly more sensitive (LOD: 1 mg/L) than the usual ELISA methods (LOD: 50 mg/L). The same research approach has recently been used for egg protein detection in red wines, and again in this case proved to be more effective than immunochemical methods, achieving an LOD of 5 mg/L of egg white (Tolin et al. 2012).

The application by Cereda et al. in 2010 of the CPLL to white wines allowed the detection of amounts of added caseins as low as 1 μg/L. The same research group performed screening of commercial Italian red wines using the same approach, and was able to detect the use of milk proteins for red wine fining instead of the expected occurrence of egg ovalbumin (D'Amato et al. 2010). Major criticisms of the cited method for the detection of allergenic fining proteins in wines are the poor quantitative results, mainly due to the limited dynamic range of electrophoresis and staining techniques. Nevertheless, their significant improvement in the detection limit of contaminating proteins in wines have boosted the chances of preventing frauds that can seriously damage consumer health.

16.6 Concluding Remarks

Wine proteomics has recently achieved new relevance, and the number of surveys devoted to oenological aspects influenced by the wine proteins has exponentially increased since the accomplishment of grape genome sequencing. Nevertheless, more efforts towards absolute protein quantification and standardization of the methods are currently needed in the field of wine proteomics, particularly for its use in quality assessment and in traceability.

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