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Review

Heard it through the grapevine: Proteomic perspective on grape and wine

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ABSTRACT

Grapevine (*Vitis* ssp.) is currently considered as the most important fruit plant throughout the world, both due to its economic importance and to its role as a non climacteric model species. The relevance of the studies devoted to the dissection of grapevine biology and biochemistry underlines the great amount of attention that this plant has attracted over the last decade. The milestones among these studies are represented by the accomplishment of the genome sequencing programmes in 2007 [1,2]. Since then, the investigation of grape OMICS has been implemented, and the number of reports published on grape and wine protein investigations using proteomic techniques have significantly improved knowledge in the field.

Contents

1. Introduction

Grapevine (*Vitis* ssp.) is the most cultivated fruit plant throughout the world and represents one of the most important crops from an economic point-of-view [3]. Its landscape value is mentioned in the worldwide famous song "Heard it through the grapevine", released by Marvin Gaye in 1968. Grapevine is becoming more important for scientists as a model plant [4,5]. This is partly due to the high value of the fruit and, most of all, to the importance of producing juices, liquors, and wines. Grapevine is also considered a source of health-promoting secondary metabolites [6,7], the most important being the antioxidant resveratrol [8]. The evaluation process of the commercial maturity

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of grapes includes observation of changes in skin colour, and the measurement of titratable acidity, soluble solid content and concentration of volatile aroma compounds. Among the parameters considered for the assessment of harvest time, the protein content and quality are not evaluated by winemakers. In spite of their low concentrations, about 0.05% of the pulp fresh weight [9,10], and less than 100 mg/L in wines, the study of grape and wine proteins is becoming more and more important.

Proteins are responsible for the majority of biological transformations that affect the plant and fruit development: they are involved in the general metabolism, such as cell rescue and defence, production of important metabolites, and transduction of signals. A deeper knowledge of the changes in protein biosynthesis following different conditions could represent a new chance to control plant response. From this point of view, many efforts have been devoted to unravelling the secrets of protein synthesis in grape over the last few decades.

As some grape berry proteins are known to resist fermentation and to cause turbidity in wines, their study has been widely exploited by several authors. The detailed knowledge of the protein content and characteristics of grape berries and juices is important for winemakers, since protein precipitation is a major cause of haze formation in wines, and especially in white wines [11–13]. The denaturation and subsequent aggregation of proteins can lead to amorphous sediment or flocculate, causing turbidity. A haze or deposit in bottled wine indicates that the product is unstable, has a low commercial value and is therefore unacceptable for sale, and winemakers usually perform some kind of fining, such as bentonite absorption, to avoid this defect. Moreover, some of the proteins detected in both wine and in grape berries, such as chitinase and lipid transfer protein (LTP), are known to be allergenic [14–16], and this may be a severe threat to its commercialization. In addition, some of the fining agents traditionally used in winemaking are also allergenic (e.g.: albumin, casein). Nevertheless, proteins could also be considered as important constituents in wines, for example in the sparkling wine industry, because they promote foam formation and stability [17–19].

Two state of art reviews on grape and wine protein research were published in 2002. One of them focused mainly on the methods that are used to separate and analyse wine proteins [20], while the other one by Ferreira and co-workers reported on the identification and the potential physiological role of the proteins identified up to date in grapes and wine [21]. The most recent update is represented by the article published in 2006 by Flamini and De Rosso on the use of mass spectrometry for grape protein characterization [22].

Since the publication of these reviews, researchers have increased their efforts, and the accomplishment of *Vitis* genome sequencing programmes in 2007 helped speed up the possibilities of protein analysis in this model plant [1,2]. Since then, a consistent number of papers concerning *Vitis* proteins have been published, especially from a proteomic perspective. In Table 1 we list the research papers about grapes and wine proteomics published after the year 2005 and quoted in the present review.

2. Grapevine physiology under the proteomic lens

Grapevine is an interesting plant from a physiological point of view: it is tolerant to different abiotic and biotic stresses, and it is one of the oldest cultivated plants, and thus very adaptive, as demonstrated by its widespread cultivation environment. Due to its high value, and to the high costs of pesticide treatments for this species, many efforts have been devoted to the investigation of its natural resistance.

One of the first studies on grape adaptability to abiotic stress was published in 2005 by Castro and colleagues [23], who studied the time course response to herbicide treatment on in vitro cultivated shoots, roots and leaves using proteomic techniques (Table 1). The results indicated that Rubisco, one of the main proteins of the plant leaves, is subjected to fragmentation after treatment, and antioxidant proteins are induced, such as those belonging to photorespiration. The carbon flux is altered, and plant defences are stimulated, as revealed by the increase in the pathogenesis related protein 10 (PR10) isoforms. Rubisco stability in grapevine leaves has also been studied in ex vitro transplanted grapevine plantlets [24]. The authors compared different acclimatization conditions, and found that a high irradiance and higher CO_2 levels than atmospheric conditions are needed to reduce Rubisco degradation, as detected by 2D gel immunoblotting, thus inducing a faster acquisition of autotrophy in ex vitro plantlets. These findings have important implications in the optimization of transplanting protocols, thus improving their efficiency and the subsequent outcome of practices such as the meristem cultures used in micropropagation and sanitation treatments.

A PR10 has been found to be modified during a proteomic comparison of stressed and unstressed stems, roots and leaves of the salt-tolerant grapevine cultivar Razegui [25]. This PR10 spot was increased to a great extent in the stems and leaves after the addition of NaCl, and is thought to be involved in the salt-stress resistance displayed by the cultivar.

In 2007, water and salt stresses were investigated by Vincent and coworkers, who published a proteomic comparison of two *Vitis vinifera* cultivars, which displayed different tolerance to abiotic stress [26]. They found that the plants displayed different protein content after stress, that the stress response depended on the developmental stage and on the duration of the stress itself, and that several proteins were cultivar specific in their response. Fifteen percent of the spots were modified by abiotic stresses: the main changes involved the reduction of photosynthesis-related proteins and of protein synthesis and fate, the latter mostly being expressed by Cabernet Sauvignon, which also suffered from a reduction in shoot elongation. Most of the spots subjected to mass spectrometry were identified as hypothetical proteins, or as proteins with an unknown function, and this made it very difficult for the authors to characterize the plant responses. In this study, a PR10 was found to increase after stress in Cabernet Sauvignon shoots, which is considered the most susceptible cultivar, while it was stable in Chardonnay, the most resistant one, in contrast to what previously reported concerning the role of this protein in stress tolerance [23,25].

Table 1 – Synthetic summary of the published papers on grape and wine proteomics since 2005.

Year	Authors	Cultivar	Tissue	Conditions	Protein separation	Identification
2005	Carvahlo et al. [24]	Touriga Nacional	Leaves from *ex vitro* transplanted plantlets	Variable amounts of irradiance and CO_2	2D immunoblots	Polyclonal antibodies
2005	Castro et al. [23]	Chardonnay	Shoots, roots and leaves cultivated *in vitro*	Herbicide treatment	2D gels	LC-MS/MS
2006	Okuda et al. [49]	Chardonnay	Wine	Proteome profiling	2D gels	N-term sequencing
2006	Vincent et al. [35]	Cabernet Sauvignon	Whole clusters	Different extraction protocols	2D gels	MALDI-TOF/TOF
2007	Cilindre et al. [17]	Chardonnay	Wine	Champagne proteins after *Botrytis cinerea* infection	2D gels and immunoblots	Polyclonal antibodies
2007	Deytieux et al. [37]	Cabernet Sauvignon	Skins	Three stages of ripening	2D gels	LC-MS/MS
2007	Giribaldi et al. [42]	Nebbiolo	Deseeded berries	Seven stages of ripening	2D gels	MALDI-TOF
2007	Negri et al. [40]	Cabernet Sauvignon	Cell wall fraction and cytosolic fraction of seeds and skins	Different extraction protocols	2D gels	LC-MS/MS
2007	Sauvage et al. [31]	*Not specified*	Leaves	Transformation on alcohol dehydrogenase	2D gels	MALDI-TOF and LC-MS/MS
2007	Vanrell et al. [19]	Macabeu, Xarel.lo, Parellada, Chardonnay and Pinot Noir	Wine	Bentonite treatment	FPLC	–
2007	Vincent et al. [26]	Cabernet Sauvignon and Chardonnay	Shoots	Water and salt stresses	2D gels	MALDI-TOF-TOF
2008	Cilindre et al. [18]	Chardonnay	Wine	Champagne proteins after *Botrytis cinerea* infection	2D gels and immunoblots	LC-MS/MS
2008	Jeloulli et al. [25]	Razegui	Stems, roots and leaves	Salt treatment	2D gels	N-term sequencing
2008	Marsoni et al. [32]	Thompson seedless	Calluses	Embryogenesis and non embryogenesis	2D gels	LC-MS/MS
2008	Negri et al. [39]	Barbera	Skins	Five stages of ripening	2D gels	LC-MS/MS
2008	Pesavento et al. [34]	Raboso Piave, Prosecco and Malvasia Nera	Seeds	Varietal differentiation	MALDI	–
2008	Rolland et al. [60]	Various Australian wines	Wine	Ovalbumin, casein and peanut-related protein detection	ELISA	Antibody
2008	Zhang et al. [41]	Cabernet Sauvignon	Grape berry plasma membrane	Three stages of ripening	2D gels	MALDI-TOF
2009	Basha et al. [28]	*Vitis* ssp.	Sap	Pierce's disease	2D gels	LC-MS/MS
2009	Batista et al. [58]	Arinto	Wine	Haze formation and pH effect	2D gels	–
2009	Esteruelas et al. [54]	Sauvignon blanc	Wine	Haze formation	Chromatography; SDS PAGE; Native PAGE; IEF	MALDI-TOF/TOF
2009	Ferri et al. [29]	Barbera	Cell suspensions	Chitosan treatment	2D gels	MALDI-TOF and MALDI-TOF-TOF
2009	Grimplet et al. [27]	Cabernet Sauvignon	Skin, flesh and seeds	Water stress	2D gels	MALDI TOF/TOF
2009	Martinez-Esteso et al. [30]	Gamay	Cell suspensions	Methylated cyclodextrins and methyl jasmonate treatments	2D gels	MALDI-TOF and LC-MS/MS
2009	Van Sluyter et al. [52]	Semillon and Sauvignon blanc	Juice	Proteome profiling	SDS PAGE; chromatography; X-ray crystallography	LC-MS/MS
2009	Wang et al. [44]	Sangiovese and Trebbiano	Skin and flesh	β-1,3-glucanase profiling	2D immunoblots	MALDI-TOF
2009	Weber et al. [60]	Various white wines	Wine	Casein detection	ELISA, SDS–PAGE, Western Blot immunostaining	antibody
2009	Wigand et al. [16]	Portugieser, Dornfelder, Pinot Noir, Riesling, Portugieser rosé, Cabernet Sauvignon, Shiraz, Chianti, Bordeaux	Wine	Proteome profiling	SDS PAGE	LC-MS/MS
2009	Zhang et al. [33]	Cabernet Sauvignon	Calluses	Necrosis or not following transformation with *Agrobacterium*	2D gels	MALDI-TOF
2010	Falconer et al. [53]	Semillon and Sauvignon blanc	Juice	Thermal treatment	Scanning calorimetry	LC-MS/MS
2010	Marangon et al. [4]	Semillon	Juice and wine	Proteome profiling	SDS PAGE; chromatography	LC-MS/MS
2010	Parrotta et al. [5]	Sangiovese	Flowers and buds	Bud development	2D immunoblots	anti-α and anti-β tubulin antibodies
2010	Sauvage et al. [38]	Chardonnay	Wine	Thermal treatment and bentonite treatment	2D gels and SDS PAGE	MALDI-TOF and LC-MS/MS

The results of one investigation on water stress effects on grapes were published in 2009 by Grimplet and co-workers [27], who profiled proteins, as well as main secondary metabolites, in both irrigated and stressed grape clusters. This study represented the first proteomic mapping of grape berry constituting tissues (Table 1). By dissecting the pericarp (skin and flesh) and the seeds, the authors were able to describe the major differences in terms of expressed proteins: the skin tissue mainly contained PR proteins and chaperones, and proteins involved in light and dark photosynthetic reactions, phenylpropanoid and amino acid biosynthesis; the flesh tissue contained several proteins involved in oxidative and abiotic stress responses, and ripening-related proteins; finally, the seeds were richer in globulins and Late Embryogenesis Abundant proteins. As expected, water stress had little effect on the seed proteins, while it induced modifications on several spots in the skin and pulp, but without any evident correlation between the response proteins in the two tissues.

The first in vivo study of proteins induced by biotic infection, that is, by Pierce's disease, was published in 2010 by Basha and co-workers [28]. The sap proteins of different grapevine species displaying different tolerance levels to the xylematic pathogen *Xylella fastidiosa* revealed a wide variation in expression. A set of proteins, including β-1,3-glucanase (already known to be involved in pathogen response), a class III secretory peroxidase (involved in H_2O_2 regulation and oxidation of toxic compounds), and a subunit of oxygen-evolving enhancer protein 1, were differentially expressed in the tolerant species (*Vitis* ssp.), but were absent in the susceptible ones (belonging to *Vitis vinifera* ssp.), which also displayed a higher content of soluble sugars and free amino acids, thus being more attractive for *Xylella fastidiosa*. The study of Basha et al. [28] paves the way to the investigation of the mechanism of action of biotic infections in grapevine, which is a rather poorly understood topic from a proteomic point of view, despite the impressive costs of disease and pest control for this plant.

Recently, the elicitation effect of chitosan on the proteome of Barbera cell suspensions was studied by Ferri and colleagues [29]. Together with a stronger trans-resveratrol endogenous accumulation, the chitosan treatment induced several proteins belonging to the stilbene biosynthetic pathway, such as stilbene synthase isoforms and chalcone–flavanone isomerase, while a general decrease was observed in proteins belonging to the general metabolism and to the energy metabolism. It is worth noting that the PR10 isoforms were induced by the chitosan treatment, thus confirming their importance in response to different stresses. The elicitor effect on grapevine cell suspensions has also been studied by Ref. [30]. The authors used methylated cyclodextrins and methyl jasmonate on cells from a fungal susceptible cultivar (Gamay). They found that methyl jasmonate strongly decreases protein synthesis to a great extent, probably as a consequence of the reduced cell growth in *V. vinifera* cell suspensions. Methylated cyclodextrins alone or in combination with methyl jasmonate increased the expression of several spots with basic pI, including secretory peroxidases, chitinase-III, β-1,3-glucanase, SGNH plant lipase-like, NtPR27-like, xyloglucan endotransglycosylase and subtilisin-like protease, thus inducing a similar response to the one that is derived from pathogen invasion, as reported by Ref. [28].

In 2007, Sauvage and coworkers [31] performed a proteomic analysis of the effects of grapevine transformation for alcohol dehydrogenase activity. They found 14 spots among the sense and antisense transformants and control leaves that were altered, but, curiously, none of these spots was identified as alcohol dehydrogenase, although an increased enzyme activity had been detected. The main changes were concentrated in chloroplastidic proteins, such as Rubisco, thus suggesting that alcohol dehydrogenase transformation could lead to alterations in photosynthetic reactions and in carbon metabolism.

Somatic embryogenesis in *V. vinifera* has been studied by Marsoni et al. [32]. Embryogenetic and non embryogenetic grape calluses were found to differentially express 35 spots, that were mostly upregulated by somatic embryogenesis. The upregulated proteins included ascorbate peroxidase and glutathione-S-transferase (involved in the oxidative stress response), some chaperones, some proteins linked to cell division and structuring (such as actin), and proteins involved in the general and energy metabolisms, while PR10 was up regulated in non embryogenetic cells, thus indicating the stressed status of these cells. Similar purposes were pursued by Zhang et al. [33], which investigated the different attitude of embryogenetic and non embryogenetic grape calluses to develop necrosis after *Agrobacterium tumefaciens* mediated transformation. They focused their attention on the stress response, and reported the predominant and/or exclusive activation of specific stress response pathways in the two types of cells. They confirmed the upregulation of the PR10 spot in the non embryogenetic calluses, and stressed the accent on the different ROS detoxification mechanisms which are activated in the two types of cells, underlying the shift among the different isoforms of ascorbate peroxidase and of catalase as a determining factor in the necrosis following *Agrobacterium* mediated transformation.

Grape seed proteins were analyzed in 2008 through MALDI mass spectrometry (Table 1), in order to provide a new and reliable varietal differentiation method [34]. The authors tested different seed protein extraction and purification protocols, as well as different matrices for MALDI. The most suitable protocol included extraction in water–0.1% trifluoroacetic acid, defatting with hexane and dialysis, and MALDI with 2,5-dihydroxybenzoic acid as the matrix. This novel approach was used to compare three varieties, and some differences that are useful for varietal differentiation were easily detected. In order to assess the reliability of these differences, the authors profiled one of these varieties in different environments and over different years, and found that the characterizing peptide of the analyzed cultivar was detectable in all conditions, thus providing a useful marker for the identification of the cultivar itself through mass spectrometry of seed extracts. This work pointed out the need for fast and reliable methods for the certification of origin, which is becoming a main task in food and beverage industry, especially when the Protected Designation of Origin (PDO) is involved.

An anti-tubulin 2D immunoblotting method (Table 1) has been recently set up to profile the changes in microtubules during grape bud development [5]. The authors identified eight α-tubulin and seven β-tubulin isoforms, with an

expression that differs according to the different tissues and ages: more acidic α and β-tubulin isoforms were detected in the buds, while more basic α and β-tubulin isoforms increased in the tendrils and flowers. Moreover, they also found that the occurrence of a specific post translational modification (namely, tyrosination) was characteristic of the developmental stage, in large and bursting buds, thus confirming previous reports on other plants and confirming the use of *Vitis vinifera* as a model plant to study the accumulation of specific tubulin isoforms in microtubule arrays.

3. The dynamic evolution of proteins during grape berry ripening

One of the main tasks in grape berry research has always been the investigation of berry development and ripening, since producers are interested in improving cultivating practices and in increasing yield and quality of the final product. Since 2007, several authors have contributed to the debate by publishing proteomic surveys on protein profile changes in grape berries, regarding both the whole fruit and the subproteomes. A first important step was made in 2006, when Vincent and coworkers [35] published a comparison of the different protein extraction protocols (Table 1) on whole clusters of Cabernet Sauvignon. The authors excised and identified 81 spots from the different gels in order to characterize the specificity of each extraction procedure. The protocol based on phenol extraction followed by methanol–ammonium acetate precipitation [36] which they first performed on grapes is still the most commonly used in grape berry proteomics, due to its high protein extraction efficiency and to its ability to eliminate interfering compounds such as anthocyanins.

The first proteomic analysis of grape ripening was conducted by Deytieux and coworkers [37], who compared the proteome of grape skins over three ripening stages from the beginning of véraison (i.e. the stage of growth when the colour changes) until full maturity. Using the protocol described by Ref. [36], they were able to characterize proteins isolated from the skin tissue of Cabernet Sauvignon. Proteins involved in photosynthesis, carbohydrate metabolisms and stress response were identified as being over-expressed at the beginning of véraison, while the end of the colour-change was accompanied by increases in anthocyanin biosynthesis related proteins. At harvest, the dominant proteins were involved in defence, and the quantity of PR proteins, chitinase and β glucanase isoforms was of particular interest, since they are also known to be detectable in wine [16–18,38].

Negri et al. [39] also focused attention on protein evolution during skin ripening. Although their study was similar to Ref. [37], they focused on a different cultivar, Barbera, and analyzed two more stages of development. The analysis highlighted a clear differentiation over the first weeks after véraison and the other three stages: most of the changes occurred in the first phase, and were mainly involved in response to stress, general metabolism and amino acid metabolism. In 2007, the same authors published a survey on different protocols for protein extraction (Table 1) applied to the enrichment of the cell wall fraction in Cabernet Sauvignon skin and seed samples [40]. They also compared the proteins extracted in cell wall enriched fraction with those obtained from the cytosolic fraction using the same protocol. Their work paved the way for the subsequent proteomic analysis performed by Zhang et al. [41] on plasma membrane proteins of grape berries during ripening. As expected, according to the analyzed tissue, most of the identified proteins belong to transport and signal transduction. Moreover, only a small portion of the identified proteins were found to be differentially expressed in the three ripening stages, and, in spite of the accomplishment of genome sequencing for *V. vinifera* in 2007 [1,2], only 62 of the 200 spots subjected to mass spectrometry were identified [41].

A different approach was performed by Giribaldi and colleagues [42], who used a Rabilloud-derived protocol [43] to profile the protein changes in whole Nebbiolo berries from one month after flowering up to the ripe stage. They found 118 protein spots, which were mostly involved in general metabolism, energy metabolism and protein synthesis and fate, to be differentially expressed during development. Their results suggest a general decrease in glycolysis during ripening, and an increase in PR proteins in the 20–35 kDa range (proteins in the same range have been reported in wines). They also reported a decrease in oxidative stress related proteins and extensive cytoskeleton rearrangements during ripening. In Fig. 1 we report 2DE gels from Ref. [42] representing the proteome of whole Nebbiolo berries before véraison (panel A), at véraison (panel B), and at full maturity (panel C), including the protein spots identified by MALDI MS.

One of the noticeable aspects of these studies is that, despite the different extraction procedures, the different analyzed cultivars and the different sampling stages, most of the identified spots represent the same proteins, such as thaumatins, chitinases, ATPases, glyceraldheide phosphate dehydrogenase, enolase and alcohol dehydrogenase. These are the most abundant proteins in the berry, and they are prone to developmental regulation.

The expression of one of these proteins, β-1,3-glucanase, has recently profiled in different grape berry tissues through proteomic analysis [44] (Table 1). After providing evidence of the advantages of acidified PVPP (polyvinylpolypyrrolidone) cleanups of berry extracts, the authors profiled the β-1,3-glucanase expression through 2D immunoblots in different tissues, and found that it consisted of two isoforms, with different pI, which were more abundant in the ripe berry skins than in the flesh, and in the red varieties than in the white ones. These findings are very important because, according to previous reports [45–47], β glucanase activity appeared to be low or undetectable in ripe berries, while several proteomic studies have reported the existence of two isoforms induced during berry ripening [10,37,39,42], and an increase in β glucanase activity in ripening Cabernet Sauvignon skins [37].

4. Wine proteins: to be or not to be?

One of the key points in the investigation of grapevine proteins is that they could survive vinification and cause serious damage to the final product. Many works in the past were devoted to the analysis of the so-called haze proteins, and more recently proteomic approaches have been used to have a better understanding of their characteristics.

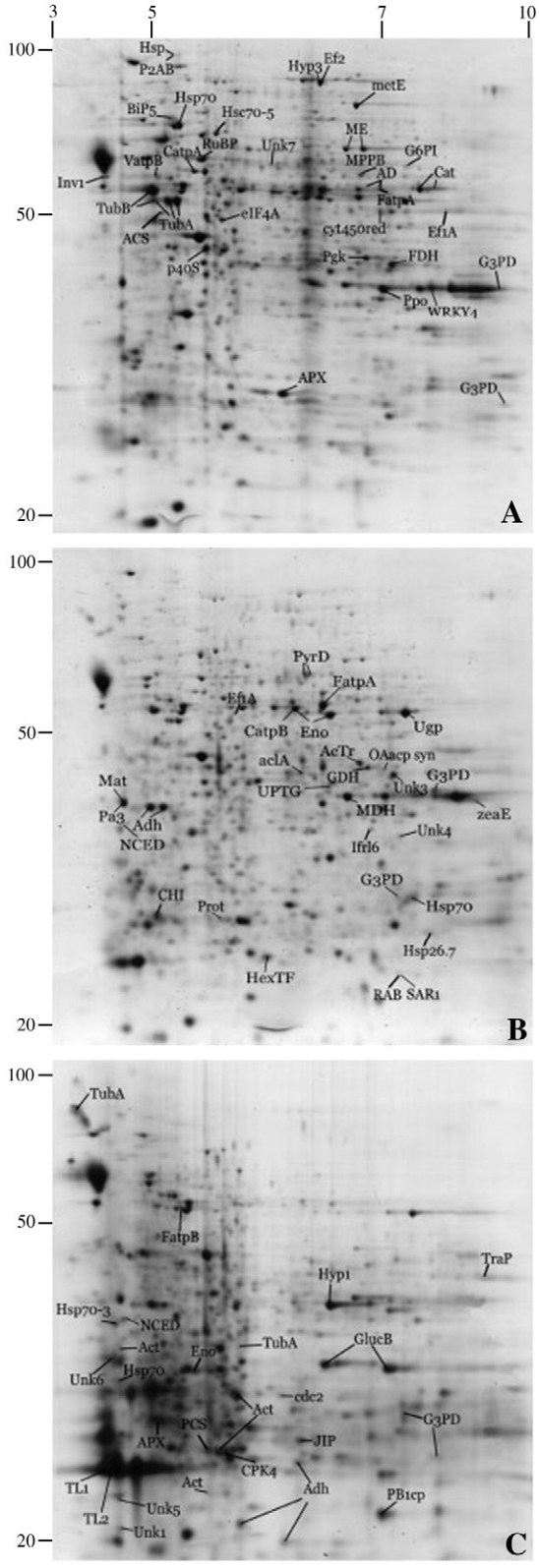

Fig. 1 – Evolution of whole Nebbiolo berry proteome from green stage (A) to véraison (B) and maturity (C). Reprinted from Giribaldi et al. [42] by permission.

Haze forming proteins have been known for some time to be mostly plant PR proteins, although the occurrence of yeast proteins has also been demonstrated [22]. PR proteins are stable at acid pH and highly proteolytically resistant [48]. Proteins are low concentration components in wines, and they are thus very difficult to isolate, since the wine is rich in polyphenols and other compounds that interfere with the protein extraction. For this reason, most proteomic studies have been performed on white wines, which have no anthocyanins, and are easier to manage.

A 2D electrophoresis approach (Table 1) was used in 2006 to characterize the Chardonnay wine proteins [49]. The authors used ammonium sulfate to precipitate the proteins, and then fractionated them through chromatography. The first fraction mainly contained invertase and proteoglycans, while the second, more abundant, one contained glycoproteins. Several spots were identified as vacuolar invertase 1, which is one of the main spots in grape berry flesh [10]. Due to the different molecular masses and pI of the spots, the authors suggested that the enzyme is cleaved during vinification, but grape berry proteome studies report the existence of at least one cleavage product already in the whole fruit [4,42]. Osmotins and thaumatins were also found. One important novelty was represented by the identification of an LTP spot, which is a major allergen in grape and wine [14,15,50]. Because of its molecular mass, lower than the predicted mass (13 kDa), the LTP spot was considered as a cleavage product by the authors [49]. This migration behaviour has already been reported [14] and is probably due to the LTP characteristics, rather than its degradation.

Two more proteomic surveys on Chardonnay proteins were published by Cilindre and coworkers [17,18]. These authors profiled Champagne wine proteins and their changes after *Botrytis cinerea* infection, which is a fungal pathogen known to have a deleterious effect on the foam properties of champenois based wine. As those properties also rely, to some extent, on wine proteins [13,51], the proteolytic effect of *Botrytis* was also investigated. The number of protein spots was decreased to a great extent by the infection, although new spots also appeared. Among the proteins to be identified, the authors found vacuolar invertase, several PR proteins and some yeast proteins, but also two pectinolytic enzymes secreted by *Botrytis cinerea*, involved in plant cell wall disruption [17,18].

A study on the effects of bentonite on protein quality and foam characteristics in sparkling wines was published in 2007 [19]. Using FPLC (Table 1), the authors fractionated base wines and sparkling wines treated or untreated with bentonite, and found a decrease in low molecular mass proteins for some non-fined sparkling wines in comparison to the base ones. The bentonite treatment was shown to cause a great reduction in all protein fractions, except the high molecular mass fraction, which probably contains glycoproteins and polysaccharides. Recently, Sauvage and coworkers studied Chardonnay proteome, in order to study the efficacy of bentonite fining and the termostability of wine proteins [38]. The identified proteins were chitinases, invertases, glucanases and thaumatins, and they showed different sensitivity to heat-induced precipitation and bentonite fining, with a minor susceptibility for invertases and a fraction of thaumatins [38]. These results agree with those obtained by the research team headed by Elizabeth Waters, one of the first researchers involved in the study of wine proteins

[13]: the 2010 papers by Marangon et al. [4] and Van Sluyter et al. [52] described the use of strong cation exchange and hydrophobic interaction chromatography (Table 1) to isolate juice and wine proteins from Semillon and Sauvignon blanc cultivars. Their approach led to the identification of chitinases, thaumatins, invertase, LTP and PR-4 type proteins [4], and to the crystallization (Table 1) of chitinase and thaumatin fractions, in order to characterize them by X-ray analyses [52]. They also reported a reduction in chitinase content during the passage from juice to wine, while the thaumatin content seemed unaffected [4]. The same research team investigated the thermal stability of thaumatin-like protein, chitinase, and invertase using scanning calorimetry (Table 1), and found chitinase to be a major player in heat-induced haze in unfined wines, because of its low melting temperature [53]; although obtained through completely different approaches, these results are surprisingly similar to those obtained by Ref. [38]. Consistent results were reported in 2009 by other authors [16], who investigated the diversity of proteins from red, rosé and white wines through SDS PAGE (Table 1). They identified a number of yeast derived proteins, that were mainly located in the cell walls. They also identified classical wine proteins (invertase, LTP, etc.), and compared their levels in several wines: they found that, in spite of the great similarities, differences were detectable among coloured and non coloured wines. The LTP protein band, for example, was not detected in most of the red wines purchased in supermarkets, despite the variety, while it was clearly detected in the Dornfelder variety, and less in Portugieser rosé wine. They found LTP to be absent in white wine, in contrast of what had previously been reported by Refs. [4] and [49], and suggested this could be due to the lower time of contact of wine and skins. In our opinion, the absence of LTP could be due to the smaller amount of white wine that was analyzed (1 mg instead of the 7.5 mg of rosé wine). The consistently simpler protein pattern of commercial wines compared to Dornfelder wine purchased from the winemaker could be linked to the results previously obtained [4,38,53], concerning the thermostability and resistance of invertase and of a fraction of thaumatin-like proteins, which are the most detectable bands in commercial wines, to bentonite fining. As LTP and a class IV chitinase have been demonstrated to be grape and wine allergens [14], bentonite fining and some sort of thermal treatment would probably reduce the risk of allergy for consumers.

Haze active proteins in Sauvignon white wine have been studied by Esteruelas and coworkers [54]: apart from the already known thaumatins and β-1,3-glucanase, they were the first to identify a GRIP 22 precursor (grape ripening induced protein) in wine haze proteins. This protein shares a 73% homology with kiwellin, a protein isolated from *Actinidia deliciosa*, and with kissper, a peptide derived from kiwellin, both of which known for their allergenic power and their resistance to proteolysis [55–57].

The protein haze formation mechanism and its dependence upon pH have been investigated by Batista et al. [58], who used 2D electrophoresis (Table 1) to visualize protein pattern changes after heat stability tests on Arinto white wine. Their results indicate a change in protein flocculate particle size, according to increasing pH, even without a significant change in the total haze formation. Moreover, they highlighted the influence of the isoelectric properties of single wine proteins on heat induced flocculate formation. At lower pH, the prevalent mechanism of haze formation seems to require the presence of one or more low molecular mass wine components to induce protein denaturation and precipitation, although the authors were not able to characterize the involved mechanism.

An important aspect of the cited works on the wine proteins is that, despite the detailed knowledge of which proteins are more prone to haze formation and why, the investigation of fining methods other than bentonite has been poorly exploited by proteomic studies. Nevertheless, there is the need for new methods, able to avoid the haze formation without an excessive removal of those proteins which can contribute to the foam and wine stability.

Finally, two recent articles indicated that there is a possibility that allergenic proteins used for wine fining could be detected in commercial wines [59,60]. The first screening on commercial Australian wines for the detection of ovalbumin, casein, and peanut-related proteins using ELISA methods (Table 1) gave interesting results: only two red wines, which were fined using whole eggs instead of ovalbumin alone, gave positive reactions [59]. In a 2009 study, the authors demonstrated that caseins can be detected in white wines by ELISA method, although below the 0.9 mg/L value considered as dangerous following a literature search on allergenic levels of bovine caseins, and considering a daily maximum consumption of 1 L volume. Moreover, they found that further steps of fining such as bentonite absorption and membrane filtration are able to decrease the quantity of detected caseins, thus reducing the risk of allergies [60].

5. Conclusions

The large number of publications on grape and wine protein profiling published over the last few years has highlighted many aspects. First of all, from a scientific point of view, in addition to its economic role, grapevine is considered more and more as a model plant, thanks to the efforts in grape genome sequencing [1,2]. Physiological issues, apart from grape berry ripening, are becoming more important. Efforts are now required to characterize red wine proteins, with a special effort towards consumer safety, and to product quality.

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