Food Research International 120 (2019) 26–32

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Mass spectrometry based proteomics for the molecular fingerprinting of Fiano, Greco and Falanghina cultivars

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ARTICLE INFO

Practical application: Thanks to our approach, it could be possible to outline a proteomic fingerprinting between different cultivars of *Vitis vinifera* peculiar of south Italy (Campania) used for white wine production (Fiano, Greco and Falanghina) by advanced biomolecular mass spectrometry approach. Our data show a new way for the biomolecular signature of vines.

Keywords:
Vine cultivars
Molecular characterization
Typization
High throughput proteomics
Label free proteomics
LC-MS/MS
Orbitrap

ABSTRACT

The official methodologies used for the identification and comparison of vine cultivars are ampelography and ampelometry. These methodologies are essentially based on qualitative assessments or biometric dependent morphological features of the plant.

The heterogeneity of cultivars and consequently the increasing demand for a more detailed product typization, led to the introduction of new methodologies for the varietal characterization. In this scenario, proteomics has already proved to be a very useful discipline for the typization of many kinds of edible products.

In this paper, we present a proteomic study carried out on three cultivars of *Vitis vinifera* peculiar of south Italy (Campania) used for white wine production (Fiano, Greco and Falanghina) by advanced biomolecular mass spectrometry approach.

Our data highlight variations in the proteomic profiles during ripening for each cultivar and between analyzed cultivars, thus suggesting a new way to outline the biomolecular signature of vines.

1. Introduction

Over the centuries, the diversification of vine varieties has required the development of new descriptive criteria for the classification and recognition of different cultivars. The official methodologies used for the identification and varietal comparison are ampelography (Walker & Boursiquot, 1992) (used since 1800) and ampelometry (Martinez, Boursiquot, Grenan, & Boidron, 1997). These methods are based on qualitative assessments and on biometric dependent morphological features of the plant which although specific for different cultivars they are heavily influenced by many variables (Bokulich, Thorngate, Richardson, & Mills, 2014); therefore, their use for varietal characterization requires to be implemented by molecular methods, although their importance remains undisputed.

Actually, in the last decade, new molecular methodologies have been developed to meet this need (Flamini & De Rosso, 2006; Moretti & Berg, 1965) and the new frontier for wine chemistry is nowadays represented by the obtainment of a specific molecular fingerprinting for each cultivar also by the use of the "omics" science. Among all these disciplines, proteomics has already been widely applied to many kinds of edible products offering a powerful tool in the varietal typization (Galano, Imbelloni, Chambery, Malorni, & Amoresano, 2015; Pepe et al., 2010). A huge amount of proteomics data can be collected to be used for multiple purposes e.g. the discovery of frauds and alterations, or to provide an identity card of a product strictly related to a specific geographical location and farming conditions. Each proteome is in fact deeply connected to a complex mixture of external (temperature, farming condition, response to stress, soil etc) and internal (genome, post-translational modifications) factors, which characterize the growth and the development of an organism (Vannini et al., 2016). Therefore, a proteomic approach based on differential analysis (Carpentieri, Marino, & Amoresano, 2007) can greatly explain at a molecular level the response of the biological system to changes (for example) in environmental conditions (Abdi, Holford, & McGlasson, 2002; Lamikanra, 1993; Pigllucci, 1996; Turesson, 1922).

Given the crucial role of proteins in a biological system (Grimplet

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https://doi.org/10.1016/j.foodres.2019.02.020
Received 1 October 2018; Received in revised form 20 December 2018; Accepted 8 February 2019
Available online 10 February 2019

et al., 2009; Palma, Corpas, & Luís, 2011; Sarry et al., 2004), the proteome represent probably the best way to specifically identify and characterize a cultivar (Grimplet et al., 2009).

In general, the synthesis of proteins starts with veraison of grapes with greater relevance for red grapes varieties rather than in white ones. During this stage the main chemical changes, within the berry, are represented by a decrease in acidity (resulting in accumulation of sugars) and for red grapes in particular, by an accumulation of polyphenols (Ribereau-Gayon, 2007). As for wine proteins, they are mainly produced by the vine itself but they can derive from yeast and other microorganisms too (Hsu & Heatherbell, 1987). The lower concentration of proteins in the final product is due to the drastic reduction of their content by events occurring during the fermentation phases e.g. pH decrease, interaction with tannins, degradation due to proteases, ethanol production (Ferreira et al., 2000; Waters, Wallace, & Williams, 1992).

The varietal richness of the heritage of South Italy and in particular of Campania region represents one of the best scenario for the purposes of the research presented in this paper. Moreover, the limited studies related to the identification and characterization of the different varieties located in this territory, opens the way to the application of next-generation techniques for the characterization of local vines (Marangon, Van Sluyter, Haynes, & Waters, 2009).

In this paper, we present a proteomic study carried out on three cultivars (Fiano, Greco and Falanghina) of *Vitis vinifera* peculiar of south Italy (Campania).

Thanks to our approach, a proteomic fingerprinting between different cultivars and for each cultivar during ripening process (which was monitored by the techniques widely used among enologists) could be defined.

Our results, performed on pool of different grape lots, show that the proteomic profile is different among analyzed samples and it is deeply connected to internal and external factors, underlying the potentiality of our approach to define biomolecular signatures for each cultivar.

2. Materials and methods

2.1. Cultivar sampling

Different grape lots of each variety were purchased by local producers; each analyzed vine is typical of South Italy (Campania region):

- Pool1: Falanghina Del Sannio DOP
- Pool2: Fiano DOP Sannio
- Pool 3: Greco Sannio DOP

The sampling took place in the vineyards with 10-day intervals for each grape variety examined. Samples were then pooled, and further analyses were performed on the pools.

Each sample was obtained choosing the same row and pick the grapes from the first cluster-bearing plant. For each sample, about 50 grapes were harvested taking care to remove, from each cluster selected, the grape together with the pedicel in order to obtain the whole grapes that were immediately frozen. Each sampling was repeated in duplicate, an aliquot of the sample was used for monitoring the state of ripening of its variety while the other one was used for protein analysis.

2.2. Chemicals

Urea, thiourea, dithiothreitol (DTT), 3-[(3-Cholamidopropyl) dimethylammonio]- 1-propanesulfonate (CHAPS), tris-(2-hydroxymethyl)-aminomethano clorhidrato (Tris-HCl), iodoacetamide (IAM), ammonium bicarbonate (AMBIC), Trypsin TPCK-treated from bovine pancreases were from Sigma-Aldrich.

Acetonitrile, formic acid, chloroform and methanol were purchased from Baker and Bovine serum albumin (BSA) from Fluka.

Ziptip pipette tips were purchased from Millipore, Comassie Brilliant Blue from Bio-Rad.

2.3. Total acidity

The total acidity is the sum of grape acid components which can be titrated by addition of a known concentration alkaline solution ("Progetto di REGOLAMENTO (CEE) N. 2676/90 DELLA COMMISSIONE del 17 settembre 1990 che determina i metodi d'analisi comunitari da utilizzare nel settore del vino, 2018"); the carbon dioxide is not included in the total acidity.

We performed a potentiometric titration using a calomel-saturated potassium chloride electrode. The instrument was calibrated by standard solutions of known pH and, next, a known volume of a sodium hydroxide solution (NaOH) 0.1 M was slowly added under constant stirring, up to neutral pH. Both calibration and titration were performed at 20 °C. The total acidity, expressed in grams of tartaric acid per liter, is proportional to NaOH solution milliliters added.

2.4. pH

Three measurements were performed for each sample, by directly reading the pH value on the pH-meter. Results are reported as the arithmetical average of the three determinations.

2.5. Determination of the sugar content

A small amount of sample was placed on the lower prism of the refractometer, to cover the glass surface evenly, and three measurements were performed.

In Table 1, the refractive index at 20 °C (expressed in absolute value or as percent by mass of sucrose), is reported ("Progetto di REGOLAMENTO (CEE) N. 2676/90 DELLA COMMISSIONE del 17 settembre 1990 che determina i metodi d'analisi comunitari da utilizzare nel settore del vino, 2018").

2.6. Proteins extraction

Proteins were directly extracted from grape pools. 10 g of berry were frozen using liquid nitrogen and reduced to powder using a mortar. Proteins were then extracted using a buffer containing Urea 8 M, Thiourea 2 M, CHAPS 1%, Tris-HCl 100 mM, overnight at 4 °C under stirring.

2.7. Reduction and carbamidomethylation

Reduction was performed by using a 10:1 (DTT: cysteins) molar ratio. After 2 h incubation at 37 °C, iodoacetamide was added to perform carboxyamidomethylation using an excess of alkylating agent of 5:1 to the moles of thiolic groups. The mixture was then incubated in the dark at room temperature for 30 min. The alkylation reaction was stopped by addition of formic acid to an acidic pH.

2.8. Enzymatic digestion

The excess of salts and reagents were removed by Chloroform/Methanol precipitation before enzymatic digestion. Digestion was carried out in AMBIC 50 mM pH 8.0 buffer using trypsin at a 50:1 protein: trypsin weight ratio. The sample was incubated at 37 °C for 18 h.

2.9. LC-MS/MS analysis

Peptide mixtures were directly analyzed by LTQ Orbitrap XL™ Hybrid Ion Trap-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, Bremen, Germany). C-18 reverse phase capillary column 75 μm*10 cm (Thermo Fisher Scientific), was performed using a flow rate of 300 nl/

Table 1
Official monitoring parameters for three different varieties: Greco, Falanghina and Fiano in 6 different sampling.

	Date	Sugars (Brix)	Total acidity (g/lt)	pH	Berries average weight (g)
Greco					
1° sampling	20/07/2014	3.7	27.28	2,69	0,87
2° sampling	30/07/2014	4.0	27.05	2.70	0.93
3° sampling	09/08/2014	6.0	26.62	2.73	0.95
4° sampling	19/08/2014	9.5	21.37	2.83	1.15
5° sampling	29/08/2014	12.1	18.70	2.93	1.20
6° sampling	08/09/2014	15.8	16.93	3.01	1.29
Falanghina					
1° sampling	20/07/2014	3.8	30.18	2.58	0.97
2° sampling	30/07/2014	4.4	29.97	2.60	1.08
3° sampling	09/08/2014	4.6	28.56	2.59	1.08
4° sampling	19/08/2014	6.5	26.27	2.63	1.19
5° sampling	29/08/2014	10.1	21.12	2.78	1.23
6° sampling	08/09/2014	15.9	17.56	2.91	1.28
Fiano					
1° sampling	20/07/2014	3.8	29.81	2.69	0.83
2° sampling	30/07/2014	6.3	27.56	2.68	0.94
3° sampling	9/08/2014	11.9	20.75	2.81	1.15
4° sampling	19/08/2014	12.7	12.87	3.10	1.30
5° sampling	29/08/2014	14.9	10.21	3.22	1.41
6° sampling	08/09/2014	16.2	8.75	3.31	1.49

min, with a gradient from eluent A (0.2% formic acid in 2% acetonitrile) to eluent B (0.2% formic acid in 95% acetonitrile). The following gradient conditions were used: $t = 0$ min, 5% solvent B; $t = 10$ min, 5% solvent B; $t = 90$ MIN, 50% solvent B; $t = 100$ min, 80% solvent B; $t = 105$ min, 100% solvent B; $t = 115$ min, 100% solvent B; $t = 120$ min; 5% solvent B. Peptides analysis was performed using data-dependent acquisition of one MS scan followed by CID fragmentation of the five most abundant ions. For the MS/MS experiment, we selected the three most abundant precursors and subjected them to sequential CID-MS/MS acquisitions. For the MS scans, the scan range was set to 400–1800 *m/z* at a resolution of 60,000, and the automatic gain control (AGC) target was set to 1 × 106. For the MS/MS scans, the resolution was set to 15,000, the AGC target was set to 1 × 105, the precursor isolation width was 2 Da, and the maximum injection time was set to 500 ms. The CID normalized collision energy was 35%; AGC target was set to 1 × 105.Data were acquired by Xcalibur™ software (Thermo Fisher Scientific). Each analysis was repeated in triplicate.

2.10. MASCOT identification

Acquired MS/MS spectra were transformed into *Mascot generic* format (.mgf) and used for protein identification, with a licensed version of MASCOT software (http://www.matrixscience.com) version 2.4.0. Proteins identifications were performed using a customized database of *Vitis vinifera* (29,838 sequences; 10,757,043 residues).

Additional MASCOT search parameters were: peptide mass tolerance 10 ppm, fragment mass tolerance 0.6 Da, allowed trypsin missed cleavages up to 3, carbamidomethylation of cysteines as fixed modification, oxidation of methionine, pyro-Glu N-term Q, as variable modifications. Only doubly and triply charged ions were considered.

2.11. Label-free proteomics data analysis

Mascot identifications were then processed with Scaffold (Proteome Software, Inc., Portland, OR 97219, Oregon, USA) software.

Scaffold (Proteome Software, Inc., Portland, OR 97219, Oregon, USA) was used to validate protein identifications derived from MS/MS sequencing results and for label free quantification. Scaffold verifies peptide identifications assigned by Mascot using the X!Tandem database searching program (Craig & Beavis, 2003; Searle, Turner, & Nesvizhskii, 2008). Then the software probabilistically validates peptide identifications using PeptideProphet (Keller, Nesvizhskii, Kolker, & Aebersold, 2002) and derives corresponding protein probabilities using ProteinProphet (Nesvizhskii, Keller, Kolker, & Aebersold, 2003; Searle, 2010).

The Scaffold LFQ default method was used for label-free relative quantification. This method uses the sum of all the spectra associated with a specific protein within a sample, which includes also those spectra that are shared with other proteins and is referred to as the Total Spectrum Count.

Multivariate statistical analysis by using the principal component analysis (PCA) and heat maps were performed by XLStat 2016.5 version.

3. Results and discussion

Because of the importance of proteins in many biological processes as well as in organoleptic characteristics of food products e.g. sensorial quality and foamability, proteomic studies are of crucial relevance. Grapevine (*Vitis vinifera*) cultivars have recently been subjected to extensive genomic and proteomic investigation due to the strong economic impact worldwide for both fruit and beverages.

Even if our study was essentially focused on grape proteins and no investigation on proteins retained in the final wine has been performed, our data could represent a preliminary step in wine characterization and possibly suggest new strategies for wine making and new targets for quality control and wine typization.

In this paper, we also present data obtained using the methodologies officially recognized to monitor berries ripening and results are summarized in Table 1. The overall trend of parameters measured for each cultivar under investigation (berries average weight, free sugars content, etc) is in perfect agreement with the different phenological stages considered. As evinced in Table 1, the berries weight increases during ripening as long as the free sugars content while total acidity coherently decrease; if related to proteins, these data are even more interesting.

In order to understand the trend of protein expression along all of sampling period, an aliquot of berries lot was in parallel subjected to an extraction protocol for the subsequent proteomics analysis.

The protein component constitutes a minor part with respect to other biomolecules present in berries. For our study, we used 10 g of berries for each cultivar pool. Grapes were frozen using liquid Nitrogen and pulverized as described in the previous section.

Proteins were then precipitated by chloroform/methanol and subsequently quantified by Bradford assay (using BSA as a standard

Table 2
Quantity of proteins extracted in the 6 samples of Fiano.

Fiano	20/07/14 1°sampling 39 µg	30/07/14 2°sampling 55 µg	09/08/14 3°sampling 72 µg	19/08/14 4° sampling 110 µg	29/08/14 5°sampling 142 µg	08/09/14 6° sampling 198 µg

protein). The amount of extracted proteins resulted to be increased following the progress of the ripening as reported (as an example) for Fiano sample in Table 2.

After quantification, equal amounts of proteins were reduced alkylated and hydrolyzed with trypsin for further proteomic analyses. Peptide mixtures thus obtained were analyzed by high-resolution nLC/MS-MS. Taking advantage of the high resolution of the Orbitrap and thanks to the remarkable speed of the linear ion trap coupled to this analyzer, we could collect a very large data set for proteins identifications, which was later performed by Mascot software, as reported in Materials and Methods section. Identified proteins are reported in in Tables S1 (Fiano), Table S3 (Falanghina) and Table S5 (Greco).

Thanks to our approach, we identified about 400 proteins for each sample; our data sets were analyzed by Scaffold software (as described in Materials and Methods section) to compare results.

Fig. 1 reports Venn diagram showing the comparison among identified proteins in the last samplings for the three cultivars. The diagram clearly shows a considerable overlap of the three datasets, with only a relatively small number of unique proteins for each cultivar. Definite sets of unique proteins were identified, namely 35 for Fiano, 19 for Greco and 4 for Falanghina. Unique proteins identified for each cultivar as well as the information obtained from SwissProt database are reported in Supplementary material (Tables S2, S4 and S6). Differences among the three cultivars might be related to different farming (use of fertilizer or pesticides) or growing (soil composition, severe weather and extreme temperatures) conditions. As for example, in Fiano cultivar three different aquaporins (accession number: Q0MX16, Q5PXH0, Q0MX09) were identified. These proteins are usually related to adaptation to various environmental stress (Verkman, 2013), suggesting that this cultivar have been exposed to more severe weather condition when compared to the other two. These sets of unique proteins could represent a first step to define a pool of proteins to be used as potential biomarkers for each cultivar.

We then performed a label free quantification of extracted proteins by the total spectrum count (see Materials and Methods) for each identified protein. Information thus obtained were used to build heat maps reported in Figs. S1, S2 and S3 (Fiano, Falanghina and Greco respectively).

Since the major amount of proteins is expressed in the latest sampling for each cultivar (see Table 2), in order to make a final comparison among different cultivars, we used proteomic data to build the heat map shown in Fig. 3. A better clusterization (indicating a higher similarity) between Greco and Falanghina cultivars can be deduced from the map with Fiano one showing major differences.

To obtain the overall images of the data interpretation and better understand proteomic data as well as to visualize the aforementioned differences, we performed a principal component analysis (PCA) for each cultivar (Fiano, Falanghina and Greco) and among cultivars in the latest sampling and results are reported in Figs. S4, S5, S6 and Fig. 4 respectively. PCA of all proteins in the three different cultivars is perfectly in agreement with Venn diagram showing a clusterization for the major part of identified proteins with only few proteins distinctive for each cultivar; in particular we could observe a higher similarity for Falanghina and Greco proteomes whilst more significant differences were observed for Fiano (Fig. 4).

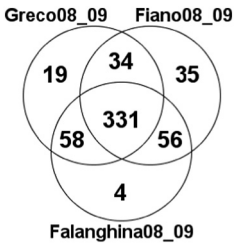

Fig. 1. Venn diagram showing a comparison between protein identifications of last sampling for each cultivar.

Among identified proteins, we could detect those responsible for the plant self-defense, such as thaumatin-like protein (TLP) (accession number: F6HUH1) and Class IV endochitinase (accession number: D7UCJ5).

Chitinase belongs to the pathogen related-3 (PR-3) family; this type of protein is produced by the plant following fungal attacks, and it is responsible for the hydrolysis of the main fibrillar polysaccharide of the mushroom wall (Ferreira et al., 2007). Recent studies have shown that chitinase activity is influenced by other factors, such as phenological status and ripening, and it has also been observed that the amount of chitinase increases proportionally with the one of sugars (Salzman, Tikhonova, Bordelon, Hasegawa, & Bressan, 1998).

TLPs are quite common in plants as they play a fundamental role against environmental stresses, including pathogen/pest invasion, drought, wounding and cold hardiness (Liu, Sturrock, & Ekramoddoullah, 2010).

As our data clearly show, in Fiano cultivar for example, both kind of defense proteins show a growing trend during the ripening phase, in particular, they have the maximum concentration in the sixth sampling (Fig. 2).

Unlike in the Greco cultivar, a decreasing trend is observed in the first stage of ripening followed by a progressive increase until the end of ripening (Fig. 2).

As for Falanghina samples, the presence of these proteins has only been found in the last maturation phase, especially IV endochitinase showing a progressive increase in the maturation phase, while the Thaumatin-like proteins decrease (Fig. 2).

We could also identify at the end of ripening (Fig. 4) in the three cultivars, polygalacturonase inhibitor (accession number: F6HL98): this protein plays a role in the plant defense and its expression is compatible with parasites attacks, most likely because pesticide treatments were not carried out precisely, probably because of heavy rainfall.

Finally, we could observe peroxidases (accession number: D7SJL8, F6HUD1, D7SKR5) for Fiano cultivar (Fig. S4). The role of this protein can be related to its activity as scavengers of reactive oxygen species (ROS) (a grapevine peroxidase that detoxifies H_2O_2 oxidizes flavonoids in response to light). More specifically, peroxidases can be induced and/or enhanced in response to stress as high temperature (Movahed et al., 2016) and this would explain data obtained, in which an increase in the expression of this enzyme can be noted in periods of the year (August) when temperatures are considerably high, especially in South Italy.

As for berries accretion proteins, we identified vacuolar invertase (accession number: F6HAU0); this protein belongs to a family of 32 glycoside hydrolases and is one of the most abundant glycoproteins found in grapes. It is a key enzyme in the sugar metabolism of grape berries, responsible for the hydrolysis of sucrose into glucose and fructose and is involved in the ripening process (Hovasse et al., 2016). Our data show that its content increases during maturation period for each cultivar. Though, at the end of ripening its level decreases, concomitantly with the increase in glucose and fructose content (see

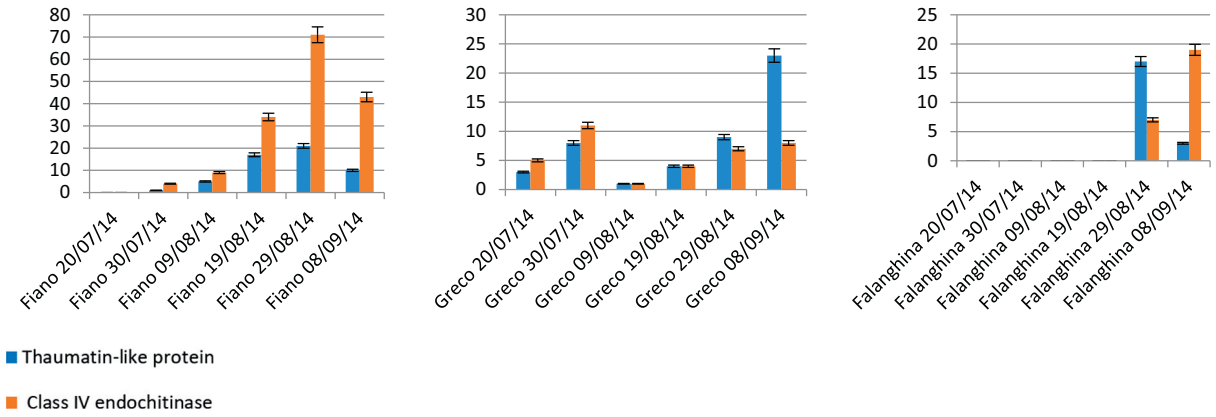

Fig. 2. Class IV endochitinase and thaumatin-like protein trends in analyzed cultivars during ripening.

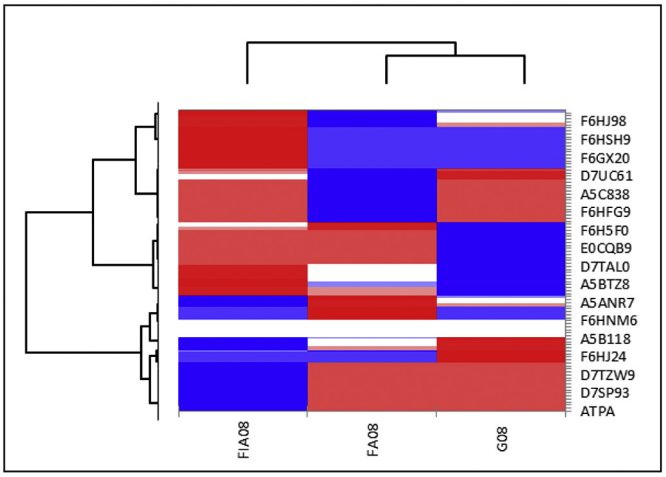

Fig. 3. Heat maps showing a diverse proteins profile between the three cultivars in the same ripening phase.

Table 1).

Many of the structural polysaccharides in the plant-cell wall undergo enzymatic hydrolysis, trans glycosylation reactions, solubilization and depolymerization, which are thought to contribute to wall loosening therefore on grape softening (Deytieux-Belleau, Vallet, Donèche, & Geny, 2008).

Fasciclin-like arabinogalactan protein 2 (accession number: F6HHQ0) and fasciclin-like arabinogalactan protein 10 (accession number: F6I327) are arabinogalactan proteins (AGPs) involved in this process (Moore, Fangel, Willats, & Vivier, 2014). Arabinogalactan proteins (AGP) increased at veraison and this variation is related to changes (in berry metabolism) aimed at the growth of the berry.

Similarly, the disassembly of the cell wall structure occurs through the action of different cell wall-modifying enzymes (hydrolases) including pectinases like pectinmethylesterase (or pectinesterase) (PME) (Accession number: F6HZ64) and polygalacturonase (PG) (Accession number: F6HL98) (Ciardiello et al., 2008; Deytieux-Belleau et al., 2008; Nunan, Sims, Bacic, Robinson, & Fincher, 1998) which were identified thanks to our approach. All the aforementioned accretion related proteins seem to have the same trend in all analyzed cultivars.

As a whole, our data (even if not exhaustive) represent a first step in a deep molecular characterization of selected cultivar, which could be used for the varietal characterization of vines.

4. Conclusion

Proteomic is a very useful methodology for large-scale analysis of protein extracts. In this paper, we focused our attention on three cultivars from the south of Italy, used to make three of the best white wines (in terms of organoleptic properties) in the Italian market (Greco, Fiano and Falanghina); taken together, our results show a different proteomic profile for each cultivar which can be used for many purposes.

Our data show the strict relation between the parameters commonly accepted to monitor grape ripening (the average weight and the free sugars content) and variations in proteomic profile. Thanks to our results (for example) we can conclude that among analyzed cultivars, one (Fiano) was exposed to major stress conditions, probably because of the severe weather that were mainly related to heavy rains.

Metabolomes and proteomes are interesting tools to specifically identify and characterize a biological system and thus a cultivar. Proteomic data obtained in this study represent an interesting key to be used for the finding of specific proteins to be used for species typization by defining a specific set of characteristic proteins in a cultivar. Even interesting, the finding of specific proteins to be used as biomarkers for a single cultivar requires a higher number of samples to be investigated. However, our strategy paves the way to a new approach for the characterization of vines moving the attention from the usual parameters measured to control the quality, to more specific ones (such as proteins) which could return more exhaustive information about the general conditions of a plant.

We could in fact identify characteristic protein patterns for a single cultivar related not only to the physiology of a plant (for example the response of a plant to stress conditions such as weather adversity or parasite attack) but also to farming conditions.

Because of all the efforts addressed at enhancing foods and beverages production (including genetic engineering as long as new pesticides and herbicides) by producers, it is nowadays becoming more and more difficult to identify new targets for quality control In this contest, the bio molecular fingerprinting of edible products represent probably the new frontier for food chemistry for quality assessment and as anti-fraud.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2019.02.020.

Conflict of interest

All the Authors declare no conflict of interest.

Author contributions

Carpentieri Andrea collected data and drafted the manuscript.
Sebastianelli Angelo prepared samples for mass spectrometry analysis.
Melchiorre Chiara performed mass spectrometry analysis and data interpretation by Scaffold and XLStat softwares.

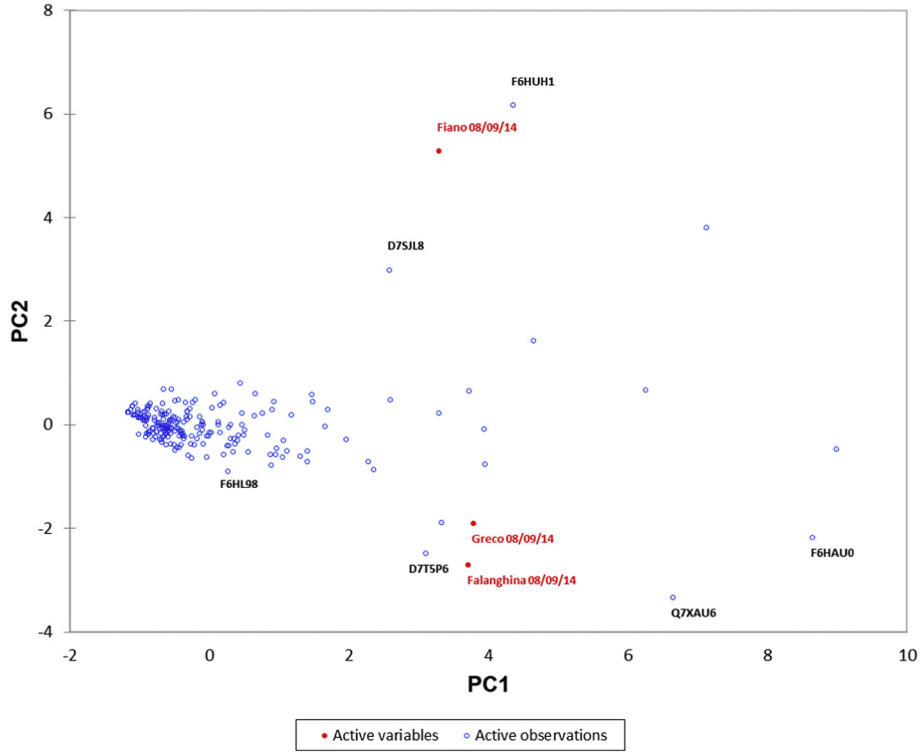

Fig. 4. PCA score biplot of protein percentage data of Fiano, Greco and Falanghina samples (Active observations) in the last ripening (Active variables) analyzed according to the proposed procedure. Cumulative data variance on first two PCs 95%.

Pinto Gabriella performed mass spectrometry.
Staropoli Alessia prepared samples for mass spectrometry analysis.
Trifuoggi Marco interpreted the results.
Amoresano Angela designed the study and interpreted the results.

Acknowledgments

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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