Food Control 80 (2017) 1–10

Contents lists available at ScienceDirect

Food Control

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

Review

Wine varietal authentication based on phenolics, volatiles and DNA markers: State of the art, perspectives and drawbacks

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ARTICLE INFO

Article history:
Received 1 March 2017
Received in revised form
13 April 2017
Accepted 13 April 2017
Available online 17 April 2017

Keywords:
Grape
Volatilome
Chemometrics
Mislabeling
Aglianico
Fingerprinting

ABSTRACT

Due to the worldwide importance of wine and frequent cases of fraud and mislabeling, in recent years there has been growing interest in developing analytical methods to determine the varietal origin of wines with some precision. In the first part of this paper we review varietal authentication based on the analysis of volatiles and phenolics, the compounds most involved in the sensory identity of wines. After illustrating their potential and limitations, we introduce the possibility of exploiting DNA analysis as a further strategy to ascertain the varietal origin of wines. In the second part of the paper we present some results of our efforts to differentiate wines from three Aglianico biotypes, based on the molecules previously discussed. Aglianico is one of the most ancient red grapes cultivated in southern Italy, producing three highly prized PDO wines. Finally, we present and briefly discuss, through a case study, the negative effects of counterfeit wines on consumer demand and producers' economic returns.

Contents

1. Introduction

Wine production is a major economic agricultural activity worldwide. Demand for wine is steadily increasing, with production growth estimated at about 8.6% in the last 10 years (Perini & Camin, 2013). Due to its economic importance, wine is one of the most common beverages subject to fraud and mislabeling. Manipulations can be ascribed to both its intrinsic (e.g. dilution of wines with water, addition of alcohol, coloring or flavoring substances) and its extrinsic properties (e.g. fraudulent misrepresentation of the cultivar and geographic origin) (Holmberg, 2010). The European Union protects agricultural products strictly linked to the origin area and to specific methods of

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http://dx.doi.org/10.1016/j.foodcont.2017.04.020
0956-7135/

production by conferring designations of origin, namely Protected Designation of Origin (PDO), Protected Geographical Indication (PGI), and Traditional Specialties Guaranteed (TSG) (European Commission, 2003). In Europe, most wines of high economic value belong to the PDO category. Each PDO wine is produced according to a set of specific regulations governing such aspects as grape varieties to be used, cultivation and vinification methods, aging, physical/chemical parameters and sensory characteristics. Several PDO wines are produced by a single variety (monovarietal wines). European legislation is now in place to regulate and guarantee the authenticity and geographical traceability of food (Regulation (EU) No. 1151/2012; No. 664/2014; No. 668/2014), with the main instruments being analytical controls and analysis of mandatory documents related to PDO wine production. However, such measures are often not enough to avoid fraud or false labeling declarations, and to ensure honest market competitiveness. Various examples of wine fraud and mislabeling have been recorded over the years, highlighting the need for improvements in authentication of labeling information. Cavicchi and Santini (2011) reported the case of the Brunello wine crisis that occurred in 2008 in Montalcino (Tuscany, Italy), when producers were accused of not adhering to the production standards established by the appellation system. Indeed, grape varieties other than Sangiovese (the only one admitted by the production regulations) were used to make a softer wine, more appreciated by American consumers. Another case occurred in France in 2010, when producers were convicted of replacing Pinot Noirwine with cheaper Merlot and Syrah wines. Approximately 13.5 million liters of the mislabeled Pinot Noir wine were sold to Gallo Winery in the US (Takeoka & Ebeler, 2011).

To prevent fraud, a critical aspect is the ability to assess whether the wine in question conforms with the label information in terms of grape variety and geographical origin. Fraud is often discovered through auditing or whistle-blowers, but might also be detected (or confirmed) chemically. In the last few years, there has been growing interest in developing analytical methods for wine authentication. Stable isotopic ratio analysis of wine can allow its geographical origin to be authenticated thanks to the existence of an official European database (EU-Wine DB). However, instrumentation and running costs, along with the requirement of highly trained analysts, make this investigation burdensome. Alternative rapid and cost-effective methodologies are currently being explored to determine the geographic origin of foodstuffs (Danezis, Tsagkaris, Brusic, & Georgiou, 2016). As regards varietal authentication, controls against counterfeiting are left to the analysis of mandatory documentation in the vineries and photographic records of the vineyards. Unfortunately, the analytical parameters and sensory characteristics laid down in wine production regulations are not designed to identify the grape variety used. Indeed, the chemical parameters for which a minimum value is established are not specifically linked to a grape variety (total alcohol degree, total acidity, non-reducing extract) and very generic sensory terms are used to describe the wine (e.g., fruity, fresh, characteristic, harmonic, dry). In addition, there are no accredited methods to evaluate whether a wine fulfills the legal requirements in terms of sensory characteristics, and evaluation relies on the opinion, training and experience of expert tasters (Etaio et al., 2010).

In this scenario, since food authentication methods are in the phase of exponential growth, effective control for the varietal authenticity of a wine is an excellent opportunity, yet still a challenge. The objective of this paper is not to review recent findings and approaches to prevent fraud in winemaking, but to focus on varietal authentication based on the analysis of those compounds most involved in the sensory identity of wine: volatiles and phenolics. After reporting the current state of the art on the authentication of varietal wines based on the analysis of these compounds, along with potential and limits, we review varietal authentication based on DNA analysis, another promising strategy for varietal authentication. We then provide a case study in wines from Aglianico biotypes and conclude with some economic implications of counterfeit wine and its effect on consumer choices.

2. Metabolomic and bio-molecular approaches for wine varietal authentication: a review

2.1. Varietal authentication based on volatilome

Volatile compounds of different origin (grapes, microorganisms, wood, aging) and belonging to different chemical classes determine the odorous notes perceived at wine tasting. Even though many originate during fermentation and aging, some come from the grapes themselves. In some wines, the peculiar odorous notes are produced by volatile compounds deriving from grapes and characterizing the variety. Examples are the floral notes of Gewürztraminer and Muscat, mainly determined by *cis*-rose oxide and linalool, respectively, the green pepper odor, due to 3-isobutyl-2-methoxypyrazines in Cabernet Sauvignon, and the box-tree odor of Sauvignon Blanc determined by 4-mercapto-4-methylpentan-2-one (Polášková, Herszage, & Ebeler, 2008). For these wines, a control of varietal authenticity by sensory analysis could be attempted thanks to the presence of grapevine-specific and well-recognizable odorous notes. However, sensory control by a selected and qualified panel is a time-consuming approach, as such panels require extensive training and only a limited number of samples per day can be analyzed. In recent decades, the scientific community has tried to develop analytical strategies to perform a varietal control based on volatile compounds. Robust statistical methods (such as multivariate analyses) analyzing a large set of quantitative data of volatiles - an approach known as chemometrics – are employed. As in the case of sensory control, also instrumental authentication can be considered a more achievable task when wines are made from varieties with grape-derived aroma markers. For example, chemometrics was successfully applied to differentiate 90 German aromatic wines (Müller-Thurgau, Riesling, Silvaner, Scheurebe, Weiβburgunder, Gewürztraminer) on the basis of terpenes (García et al., 1998).

In the case of wines from neutral grapes, the use of sensory analysis and volatile compounds to classify wines according to grape variety is more challenging. In these cases, the tendency is to consider the whole volatile fraction. Few studies take into account the olfactory sensory role of volatiles besides their concentrations. For example, the application of principal component analysis (PCA) simultaneously to 101 volatile compounds and sensory quantitative data relative to odor profile allowed Italian wines from Primitivo and Aglianico grapes to be differentiated from Merlot and Cabernet Sauvignon (Genovese, Dimaggio, Lisanti, Piombino, & Moio, 2005, Genovese, Lisanti, Gambuti, Piombino, & Moio, 2007). In another study the multivariate analysis of OUV data (OUV = concentration/odor perception threshold) allowed correct varietal differentiation among wines from four Galician white varieties (Albariño, Loureira, Treixadura and Dona Branca) (Falqué, Fernández, & Dubourdieu, 2001). In most of the studies, only the concentrations of volatile compounds were considered. Using a chemometric method based on major volatile concentrations, 62 South African wines from different grape varieties (red and white, different vintages and winemaking) were differentiated (Tredoux et al., 2008). A study conducted on Spanish aged commercial wines (240 samples) considered only fermented-derived secondary volatile metabolites. Accordingly, monovarietal Cabernet Sauvignon wines proved to have higher concentrations of 2-phenylethanol, ethyl 2-phenylacetate and diethyl succinate, while Cencibel had the

highest concentration of hexanoic and octanoic acids (Garde-Cerdán et al., 2009). Varietal differentiation of several Spanish monovarietal wines (Cabernet Sauvignon, Tempranillo, Monastrell and Bobal) was achieved by applying discriminant analysis to volatile compounds and base chemical parameters simultaneously (Aleixandre, Lizama, Alvarez, & García, 2002). Recently, the suitability of applying different machine learning techniques for varietal classification purposes was investigated. A perfect classification accuracy among four Spanish monovarietal wines (Albariño, Treixadura, Loureira and Dona Branca) was obtained by the Random Forest algorithm using all the volatile compounds determined in the wines (Gómez-Meire, Campos, Falqué, Díaz, & Fdez-Riverola, 2014).

The electronic nose (e-nose) technology represents an additional opportunity. The systems used are an evolution of the traditional e-nose (array of sensors, each detecting a certain substance), handling the chromatographic peaks of the volatile compounds of a wine as the response of a "virtual" sensor, without their identification (Antoce & Namolosanu, 2011). Using this approach, wines from the Cabernet Sauvignon and Merlot varieties, and the two hybrids Dornfelder and Rondo were differentiated (Antoce & Namolosanu, 2011). Interestingly, the method was also able to sense differences of 10% in the blending proportion of Merlot and Rondo. A similar approach used a mass spectrometry-based e-nose (MS e-nose). In this case, mass spectra collected from wine headspace without chromatographic separation are considered as a "volatile fingerprint" of wines. With this technique, Australian Riesling and unwooded Chardonnay were successfully discriminated (Cozzolino, Smyth, Cynkar, Dambergs, & Gishen, 2005). Very recently, the application of MS e-nose to the volatile fraction isolated by solid phase microextraction (SPME), coupled with multivariate analysis, led to a model with a classification and prediction ability of 100% in discriminating among red (Cabernet Sauvignon and Merlot) and white (Chardonnay and Sauvignon Blanc) varietal wines (Ziółkowska, Wąsowicz, & Jeleń, 2016).

The varietal authentication of wine based on the application of chemometrics to volatile composition could be promising. However, some limits emerge for its application on a large scale. First, the compounds discriminating for a certain monovarietal wine differ in the various studies. This makes it difficult to identify "varietal markers". Second, most of the methods (analytical determination + chemometrics) showing a good efficacy were developed on large wine samples of the same vintage. This appears a major constraint to their validation, as vintage may affect classification and prediction power (Martí, Busto, & Guasch, 2004) and should be taken into account. Finally, winemaking techniques employed should be considered, as some oenological practices, whether conventional, such as clarification (Lisanti, Gambuti, Genovese, Piombino, & Moio, 2014, 2017), or novel, such as membrane techniques (Lisanti, Gambuti, Genovese, Piombino, & Moio, 2013), may modify both volatile composition and sensory profiles.

2.2. Varietal authentication based on phenolic compounds

Phenolic compounds are a large group of molecules that can be divided into two major classes based on their carbon skeletons: flavonoids and non-flavonoids. Flavonoids include anthocyanins, flavanols (simple monomeric catechins and polymeric proanthocyanidins) and flavonols. The main non-flavonoid phenolics include cinnamic acids, benzoic acids and stilbenes. These compounds are responsible for important sensory qualities of wines related to their color (principally anthocyanins for red wines and flavanols and cinnamic acids for white wines) and mouth feel characteristics (e.g. flavanols for wine bitterness and astringency). They are also responsible for wine longevity and for the health benefits associated with moderate red wine consumption (Xia, Deng, Guo, & Li, 2010). Among phenolics, anthocyanins are the most successful compounds for red wine varietal authentication. These red pigments, mainly located in grape skins of black varieties, are transferred to wine during maceration of berries and, given their chemical nature and related properties, are easily separated and characterized by HPLC/photodiode array detection (Hong & Wrolstad, 1990). Anthocyanins have sugar residues, which can be acylated with aromatic (such as *p*-coumaric, caffeic, ferulic and sinapic acids) or aliphatic acids (such as acetic, malic, malonic, oxalic and succinic acids) at the glucose moiety. Since the relative proportion of acylated and nonacylated anthocyanins is typical of each cultivar (De Villiers, Vanhoenacker, Majek, & Sandra, 2004; Garcia-Beneytez, Cabello, & Revilla, 2003; Von Baer et al., 2008), wine anthocyanin profiles supply several indications on grape variety, as extensively reviewed by Versari, Laurie, Ricci, Laghi, and Parpinello (2014). The most notable example of a variety, which is easy to discriminate thanks to the analysis of such native pigments is Pinot Noir, which possesses only the five basic anthocyanidin 3-glucosides (Van Leeuw, Kevers, Pincemail, Defraigne, & Dommes, 2014). These pigments have been mainly used to discriminate among young wines. For example, González-Neves, Favre, Piccardo, and Gil (2016) observed that Syrah wines had the highest proportions of malvidin and acetylated glucosides, while Tannat wines were richer in delphinidin, petunidin and non acylated glucosides. Although anthocyanin profiles were also effective at differentiate Tannat wines produced in different years with different winemaking procedures (González-Neves, Favre, Gil, Ferrer, & Charamelo, 2015), their use as markers is limited by the fact that during wine aging they are involved in numerous reactions of degradation and formation of more stable pigments, changing their original distribution and concentration (He et al., 2012).

In addition to anthocyanins, other polyphenols can be used for the authenticity of both red and white wines. Soleas, Dam, Carey, and Goldberg (1997) performed one of the earliest studies for using non anthocyanic phenolic compounds for wine fingerprinting. They evaluated 15 polyphenols belonging to different classes. Effective varietal markers for red wines were flavanols, catechin, epicatechin and flavonols, whereas phenolic acid profiles allowed the discrimination of white wines. In recent years, several publications have documented similar results (Castillo-Munoz, Gomez-Alonso, Garcia-Romero, & Hermosin-Gutierrez, 2007; Ferrandino, Carra, Rolle, Schneider, & Schubert, 2012; Heras-Roger, Díaz-Romero, & Darias-Martín, 2016; Mattivi, Guzzon, Vrhovsek, Stefanini, & Velasco, 2006). Shikimic acid, a key precursor during biosynthesis of phenolic acids and flavonoids, is another non-anthocyanic polyphenol used in testing wines from red varieties such as Carmenère, Shiraz, and Pinot Noir (Tessini, Mardones, Rivas, & von Baer, 2009). Its use proved successful only in combination with anthocyanin profiles, and as such has been proposed as a complementary tool to improve the classification certainty of red wines. Medina, Salagoity, Guyon, and Gaye (2013) successfully used shikimic content and protein/anthocyanin profiles to classify both white and red wines, reporting the possibility of a satisfactory prediction of blends. Several chemometric tools, such as partial least square discriminant analysis (PLS-DA) and PCA, were successfully applied for varietal authentication through these secondary metabolites (Salvatore, Cocchi, Marchetti, Marini, & de Juan, 2013; Sen & Tokatli, 2016).

As in the case of varietal authentication based on the volatilome, some limits exist due to the fact that during wine production and aging almost all wine phenolics are involved in numerous reactions among themselves and with other wine components such as acetaldehyde and pyruvic acid (Remy, Fulcrand, Labarbe, Cheynier, & Moutounet, 2000; Waterhouse, 2002). Storage conditions,

containers and closures used during wine aging may also determine significant changes in phenolic composition (Gambuti, Capuano, Lisanti, Strollo, & Moio, 2010, Gambuti et al., 2017; Sanza & Domínguez, 2006). Since several specific appellations of high quality wines impose a minimum period of wood and bottle aging, the use of phenolic compounds has to be necessarily validated on more aged wines and on a larger number of samples. Another limit is due to the analytical techniques used for phenolic compound analysis. Indeed, HPLC, in combination with an appropriate detector (usually ultraviolet–visible, diode array detector and mass spectrometry), allows the best identification and quantification of a wide range of grape and wine polyphenols. However, not all wine phenolic compounds can be easily separated and the method can prove costly and time-consuming. For this reason, alternative techniques such as color image analysis and near infrared hyperspectral imaging (Nogales-Bueno, Rodríguez-Pulido, Heredia, & Hernández-Hierro, 2015) or the combination of UV–visible spectra and color parameters with orthogonal PLS-DA (Sen & Tokatli, 2016) have been recently proposed.

2.3. Varietal authentication based on DNA technology

DNA-based methods are attractive solutions for accurate and efficient variety identification of wines as alternatives to, or in combination with, chemical profiling. Indeed, DNA presents a high stability even under the high temperature, low pH and chemical treatments used during wine processing. Moreover, it is independent of external conditions, such as soil composition, environmental conditions, vintage and cultural practices. DNA-based methods rely on identification of a suitable DNA sequence (known as molecular marker) characterizing a specific region of the entire DNA molecule that is also unique to a grape variety. Leveraging on the high stability of DNA, the sequence can be recovered from a wine sample and, if it matches the candidate variety, the identification can be made. A large number of grapevine genetic profiles have been published in the last ten years (Zdunić et al., 2015; Carka, Maul, & Sevo, 2015; Schneider et al., 2015), and open-access databases (e.g. European Vitis Database; Swiss microsatellite database; Greek Vitis Microsatellite Database; Grape Microsatellite Collection; Italian Vitis Database) are currently available for both international and native varieties. Grapevine varietal identification is easily guaranteed with the use of simple sequence repeat (SSR) or microsatellite markers, approved and supported by the International Organization of Vine and Wine (OIV, 2007). They are monotonous repetitions of very short (one to five) nucleotide DNA motifs, which occur as interspersed repetitive elements in all eukaryotic genomes and offer some advantages over other molecular markers, including their codominant Mendelian inheritance, reproducibility, simple data interpretation, and hyper-variability (This et al., 2004). In addition, once developed, they are easy to use and inexpensive, and data can be readily compared among laboratories (Tomić, Štajner, Jovanović-Cvetković, Cvetković, & Javornik, 2012). These markers were first applied for varietal authentication in musts by Faria, Magalhaes, Ferreira, Meredith, and Monteiro (2000) and then in wine by Siret, Boursiquot, Merle, Cabanis, and This (2000). In both studies, varietal identification succeeded in musts, but drawbacks were reported for wines due to the low amount of DNA isolated. Subsequent studies were carried out to improve DNA extraction protocols and managed to increase both the yield and quality of the extracted nucleic acids (Garcia-Beneytez, Revilla, & Cabello, 2002; Baleiras-Couto & Eiras-Dias, 2006; Savazzini & Martinelli, 2006; Nakamura, Haraguchi, Mitani, & Ohtsubo, 2007; Pereira, Guedes-Pinto, & Martins-Lopes, 2011; Işçi, Kalkan Yildirim, & Altindisli, 2014). Based on these results, several reports are now available on the feasibility of varietal authentication of finished experimental and/or commercial wines, although reproducibility problems are often reported. For example, Boccacci, Akkak, Marinoni, Gerbi, and Schneider (2012) assessed the usefulness of microsatellites for the genetic traceability of the monovarietal wines Asti Spumante and Moscato d'Asti using a set of seven SSR markers specific to chloroplast sequences (cpSSR). The authors amplified all cpSSR loci both in musts and in commercial wines of Moscato bianco, thereby obtaining a unique genetic profile for this variety with respect to the other nine possible ones used as fraudulent contaminants in producing Asti Spumante wines. Similarly, Bigliazzi, Scali, Paolucci, Cresti, and Vignani (2012) amplified a panel of 16 SSR loci from DNA of seven wines originating from Merlot, Pinot Noir, Zinfandel, Riesling, Sauvignon Blanc, Sangiovese, and Alicante, and the alleles obtained matched those of source grapevines. Marking an improvement in the methodology, other authors (Rodríguez-Plaza et al., 2006; Recupero et al., 2013) used a combination of SSR sequences and the lab-on-chip non-denaturing capillary micro-electrophoresis technique. However, the data highlighted contradictory results both in musts and wines.

As an alternative to SSRs, single nucleotide polymorphism (SNP) markers are also being considered. They concern polymorphisms caused by a single nucleotide mutation at a specific locus in the DNA sequence. SNPs are considered the newest type of molecular markers for traceability purposes since they are abundant in the genome, genetically stable, able to be combined with other markers (Villano, Miraglia, Iorizzo, Aversano, & Carputo, 2016) and allow the use of high to ultra-high throughput detection platforms (Mammadov, Aggarwal, Buyyarapu, & Kumpatla, 2012). These markers have also become extremely popular in grapevine genotyping and breeding. For instance, SNPs have helped to assign first-degree genetic relationships among 950 grapevine accessions representing the grape diversity in the world (Myles et al., 2011) and to verify the true-to-typeness of a collection of 101 genotypes belonging to 21 biotypes of 10 Sicilian cultivars (e.g. Catarratto, Nero d'Avola, Zibibbo, and Grecanico) (Mercati et al., 2016). Nonetheless, little is known about the strengths of SNPs in discriminating grape varieties starting from wine matrix. Results are often variable and technique- or variety-dependent. For example, Pereira, Sediyama, de Moura, da Silva and de Souza (2016) successfully discriminated 13 international and native Portuguese varieties using a set of SNPs associated to three DNA loci starting from leaves, musts and wines. By contrast, Catalano, Moreno-Sanz, Lorenzi, and Grando (2016) were unable to authenticate the Sangiovese cultivar from Brunello di Montalcino wine through SNPs.

Albeit very promising, the molecular approach still presents some issues that need to be solved before its practical application for varietal authentication. A major problem is that nucleic acids from wine undergo some degree of degradation due to microclimatic factors (e.g. fermentation, aging, wine composition, production and storage conditions) which together lead to DNA fragmentation caused by acid catalysis and depurination (An et al., 2014) and hence to reductions in the overall yields and limitations on the size of analytical fragments. Furthermore, the presence of potential polymerase chain reaction (PCR) inhibitors (e.g., polyphenols, polysaccharides or RNA), which derive from the above-cited factors, further reduce the possibility of marker amplification. Comparing the results obtained in the last ten years (Table 1), it seems evident that the best approach to obtain the most reliable results is the use of solution-based protocols (not commercial kits). In addition, researchers are currently developing their analyses to focus on the amplification of short fragments of DNA using PCR, which allows access to minute traces of nucleic material in a very specific manner.

Table 1
Scientific reports published in the last ten years on DNA extraction from wine. Used methods, wine starting volumes, grape varieties, DNA quantification, DNA amplificability and reference are reported.

Method	Starting volume	Varieties	DNA quantification	Amplificability	Reference
CTAB-based					
Siret, Gigaud, Rosec, & This, 2002 modified	400 mL	Merlot, Pinot noir, Zinfandel, Riesling, Sauvignon blanc, Sangiovese, Alicante	3,18 ± 0,93 ng/mL	YES, 16 nSSR	Bigliazzi et al., 2012
Pereira et al., 2011	10 mL	Tinta Roriz, Fernão Pires, six commercial blended wines	302 ± 115 ng/μL	YES, 1 nSSR	Pereira et al., 2011
Lefort and Douglas (1999) modified	40 mL	Welsh Riesling, Fruehroter Veltliner, Saint Laurent, Blaufraenkisch	YES	Not tested	Drábek, Stávek, Jalúvková, Jurček, & Frébort, 2008
Siret et al., 2000 modified	100 mL	Cabernet Sauvignon, Merlot, Sauvignon Blanc	27,9 ± 18,1 ng/μL	Not tested	Işçi et al., 2014
Işçi et al., 2014	45 mL	Cabernet Sauvignon, Merlot, Sauvignon Blanc	36,5 ± 22,6 ng/μL	Not tested	Işçi et al., 2014
Boccacci et al., 2012	30 mL	Moscota bianco	NO	NO, nSSR; YES, cpSSR	Boccacci et al., 2012
Mulcahy et al., 1993	dry residues	Archeological, recentwines	NO	YES,6 nSSR	Milanesi, Bigliazzi, Faleri, Caterina, & Cresti, 2011
Bigliazzi et al., 2012	400 mL	Vernaccia di San Gimignano	2 ng/mL	YES, 13 nSSR	Scali, Elisa, Jacopo, Mauro, & Vignani, 2014
Savazzini and Martinelli (2006)	10 mL	Sangiovese	208,8 ng/μl	unknown	Catalano et al., 2016
Pereira et al., 2011	100 mL	Sangiovese, Brunello, Trento, Lambrusco Grasparossa	490,6 ± 632,3 ng/μl	NO	Catalano et al., 2016
TEPC, Bigliazzi et al., 2012	400 mL	Sangiovese, Brunello, Lambrusco Grasparossa	4,05 ng/μl	NO	Catalano et al., 2016
Pereira et al., 2011	100 mL	Sangiovese, Brunello, Lambrusco Grasparossa	268,3 ± 193,7 ng/μl	NO	Catalano et al., 2016
Pereira et al., 2011 modified	100 mL	Sangiovese, Brunello, Lambrusco Grasparossa	68,9 ± 43,3 ng/μl	NO	Catalano et al., 2016
Pereira et al., 2011 modified	100 mL	Aglianico Taburno, Aglianico Taurasi, Aglianico Vulture	NO	YES,7 nSSR	Our study
Commercial kits					
Dneasy Plant Minikit, Qiagen	400 mL	Touriga Franca, Tinta Barroca, TintoCão, Marselan, Fernão Pires	NO	NO	Baleiras-Couto & Eiras-Dias, 2006
Dneasy Plant Minikit, Qiagen	8 mL	Merlot, Pinot noir, Zinfandel, Riesling, Sauvignon blanc, Sangiovese, Alicante	0,32 ± 0,19 ng/mL	YES,6 nSSR	Bigliazzi et al., 2012
Dneasy Plant Minikit, Qiagen	750 mL	Cabernet Sauvignon, Merlot, Sauvignon Blanc	27 ± 27,7 ng/μL	Not tested	Işçi et al., 2014
kit ROCHE modified	30, 43, 45 mL	Sangiovese, Brunello, Trento, Lambrusco Grasparossa	102,7 ± 63,4 ng/μL	NO	Catalano et al., 2016
MO-BIO soil	30, 43, 45 mL	Cabernet Sauvignon, Merlot, Teroldego	4,2 ± 0,8 ng/μL	NO	Catalano et al., 2016
MO-BIO soil modified	30, 43, 45 mL	Sangiovese, Brunello, Trento, Lambrusco Grasparossa	22,7 ± 9,2 ng/μL	NO	Catalano et al., 2016
PowerPlant Pro DNA isolation Kit (MO-BIO)	30, 43, 45 mL	Sangiovese	2,6 ng/μL	unknown	Catalano et al., 2016
QIAmp DNA Stool for human DNA analysis	30, 43, 45 mL	Sangiovese, Cabernet Sauvignon, Merlot, Teroldego	5,1 ± 4,7 ng/μL	unknown	Catalano et al., 2016
QIAmp DNA Stool for human DNA analysis modified	500 mL	Sangiovese	8,8 ng/μL	unknown	Catalano et al., 2016
QDEK, Bigliazzi et al., 2012	30, 43, 45 mL	Sangiovese, Brunello, Trento, Lambrusco Grasparossa	13,2 ± 7,6 ng/μL	NO, 1 nSSR	Catalano et al., 2016
Other methods					
Thomas, Matsumoto, Cain, & Scott, 1993 modified	400 mL	Touriga Franca, Tinta Barroca, Tinto Cão, Marselan, Fernão Pires	NO	NO	Baleiras-Couto & Eiras-Dias, 2006
Sambrook, Fritsch, & Maniatis, 1989	400 mL	Touriga Franca, Tinta Barroca, TintoCão, Marselan, Fernão Pires	NO	NO	Baleiras-Couto & Eiras-Dias, 2006
Nakamura et al., 2007	30 mL	Chardonnay, Cabernet Sauvignon, Sauvignon Blanc, Riesling, Merlot, Pinot noir, Koshu	YES	YES	Nakamura et al., 2007
Hârţa, Pamfil, Pop, & Vicaş, 2011	750 mL	Tămâioasă Românească, Galbenă de Odobeşti, Fetească Neagră, Busuioacă de Bohotin	231,4 to 0,2 ng/μL	YES, 4 nSSR	Hârţa et al., 2011

3. Metabolomic and bio-molecular approaches to wine varietal authentication: a case study

Based on the potential and limits of the molecules discussed above, it is clear that wine authentication based on grape variety might be affected by the level of metabolomic (aroma and phenolic) and bio-molecular markers and how they are unambiguously related to a variety. In this context, distinguishing wines made from biotypes (clones of the same variety which look different from each other) might be considered the highest level of varietal authentication. In other words, if we can differentiate biotypes we can certainly distinguish between varieties. Therefore, as a case study

we provide results from our works on Aglianico biotype authentication based on metabolomic and bio-molecular approaches. Aglianico is a red grape cultivated in southern Italy, mainly in the regions Campania and Basilicata. It is one of the most ancient grapes, probably introduced by Greeks in pre-Roman times. It is a late-ripening variety, being harvested in October–November. This grape variety is noteworthy for its great intra-variety phenotypic variability, probably as a cumulative effect of selection operated overtime by farmers in its different growing areas (Boselli, 2003; Muccillo et al., 2014). This has led to at least three different biotypes, corresponding to as many distinct production areas and PDO wines, namely Taburno and Taurasi in Campania and Vulture in Basilicata. The strong antioxidant activity confers high positive nutriceutical properties to these grapes and wines (De Nisco et al., 2013). Moreover, the high content of polyphenols (mainly tannins) is responsible for the harsh and astringent character of the wines. Despite these common characteristics, wines obtained from the three biotypes are known by experts and winemakers for having different and distinctive olfactory characteristics. For this reason, we first attempted to differentiate the above biotypes by using volatilome (Lisanti, Genovese, Piombino, Gambuti, & Moio, 2011). For each biotype, wines were obtained following the same winemaking process. PCA was applied to the quantitative data of 57 volatile compounds, belonging to different chemical classes (esters, alcohols, ketones, acids, phenols, lactones, and norisoprenoids) (Fig. 1). Volatiles were extracted by Solid Phase Extraction (SPE), identified and quantified by Gas Chromatography/Mass Spectrometry (GC/MS) as reported in Lisanti et al. (2013). Given the spread of the compounds in the plane defined by the first two components and their relative odors, PC1 identified a "fruity" direction (negative semi axes) and a "spicy-sweet" direction (positive semi axes). The positive PC2 semi-axis was characterized by "floral" compounds, while the negative by "fruity" ones. Of three replicates analyzed for each biotype, the results showed the grouping of two of them, namely V2 and V3 for Aglianico Vulture, T1 and T3 for Aglianico Taurasi, and Tab 2 and Tab 3 for Aglianico Taburno. The composition of the volatile fraction showed an intravarietal variability, while, except for V1 and Tab 1, it showed a good intra-biotype homogeneity. To integrate volatilome studies, the same Aglianico wines were subjected to the analysis of phenolic compounds by means of high performance liquid chromatography (Goldberg, Karumankiri, Diamandis, Soleas, & Ng, 1996; Muccillo et al., 2014). All wine samples were filtered through 0.45 μm, before the injection in the column. The levels of four native anthocyanins (malvidin 3-glucoside, malvidin 3-(6II-acetyl)-glucoside, malvidin 3-(6II-coumaroyl)-glucoside, peonidin 3-(6II-coumaroyl)-glucoside), low molecular weight phenolics (LMWP), the ratio between two flavanols (catechin/epicatechin ratio, cat/epicat) and two esterified anthocyanidins (malvidin 3-(6II-coumaroyl)-glucoside/malvidin 3-(6II-acetyl)-glucoside ratio) were detected and used as dataset for PCA analysis. Projection of samples in the first 1–2 planes gave the respective location of the wines and showed the main characteristics of the three Aglianico biotypes with regard to their phenolic composition (Fig. 2). The first two principal components retained 82.3% of total variance. The three biotypes fall into three main groups: the Vulture biotype was ranked along the first component (64.19%) and showed a higher correlation with high levels of malvidin 3-ace and LMWP. All Taurasi biotypes were well separated from their Vulture counterparts and had high values of the Mv-3-cum/Mv-3-ace ratio.

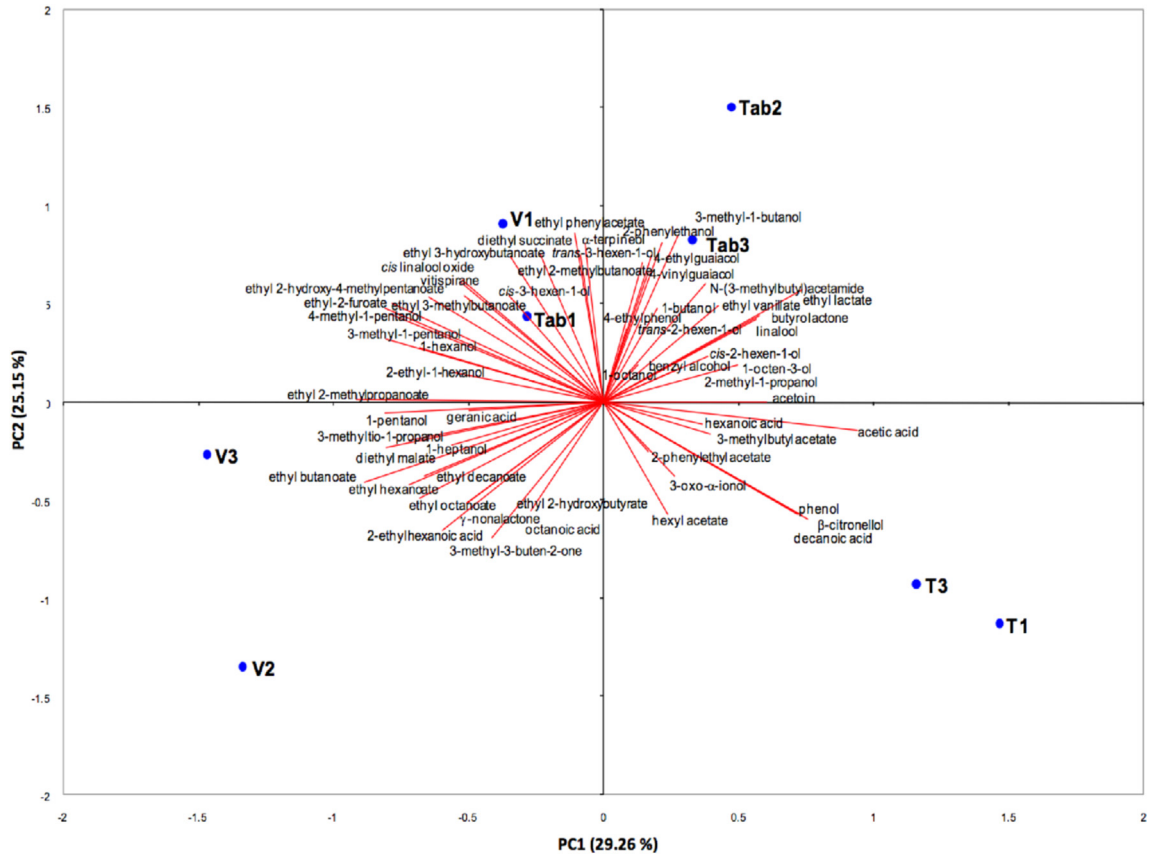

Fig. 1. PCA of volatile compounds of the three Aglianico biotypes (T1, T2, T3 = Aglianico Taurasi; V1, V2, V3 = Aglianico Vulture; Tab1, Tab2, Tab3 = Aglianico Taburno, each from 3 different vineyards 1-2-3). Adapted from Lisanti et al., 2011.

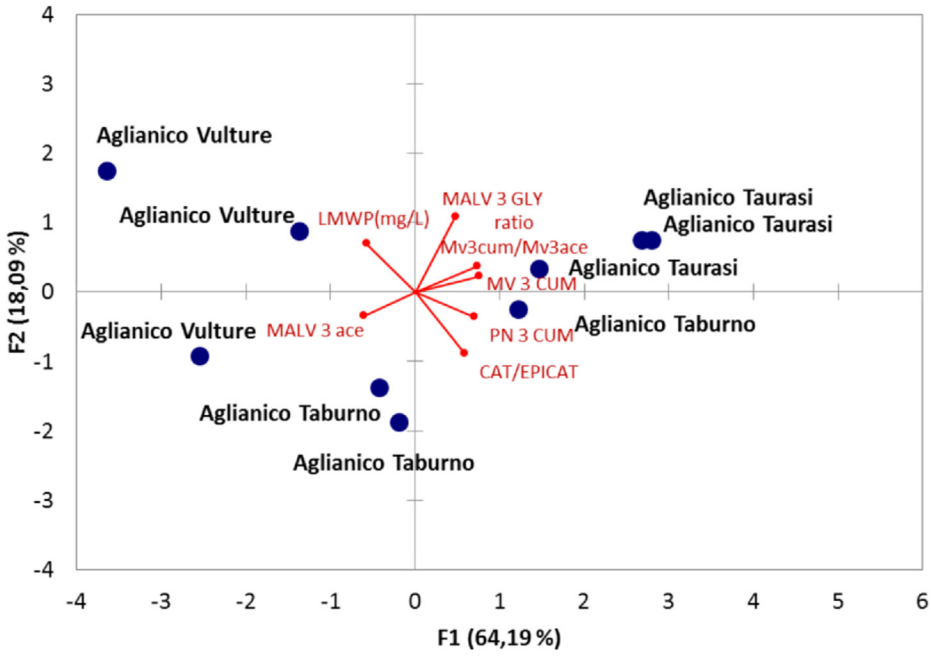

Fig. 2. Principal component analysis applied to data of selected phenolic compounds and phenolic ratios of three Aglianico biotypes: Vulture, Taurasi and Taburno. Mv3gly = malvidin 3-glucoside, Mv3ace = malvidin 3-(6^{II}-acetyl)-glucoside, Mv3cum = malvidin 3-(6^{II}-coumaroyl)-glucoside, Pn3cum: peonidin 3-(6^{II}-coumaroyl)-glucoside, LMWP: low molecular weight phenolics, CAT/EPICAT: catechin/epicatechin ratio; Mv-3-cum/Mv-3-ace: malvidin 3-(6^{II}-coumaroyl)-glucoside/malvidin 3-(6^{II}-acetyl)-glucoside ratio.

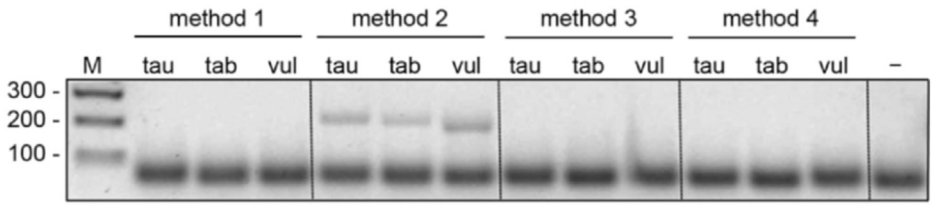

Fig. 3. Agarose gel image showing amplification patterns of VrZAG21 marker in Aglianico Taburno, Aglianico Taurasi and Aglianico Vulture whose DNA was extracted using four different methods (1, 2, 3, 4). The negative control (−) consisted in a PCR reaction without DNA template. M, 100 bp plus ladder (Thermo Scientific).

Taburno biotypes ranked on the negative semi-axis of the second component (18.09%), and though characterized by a higher cat/epicat ratio, did not separate from the other two biotypes.

In our study on Aglianico biotypes we also tried to solve a major limitation in the molecular approach for varietal authentication, namely DNA quality, quantity and purity. Therefore, in the same Aglianico wines we sought to set up a reliable protocol for DNA extraction and analysis. For this purpose, in these three biotypes we first verified the presence of genomic differences through SSR markers, identifying biotype-specific SSR alleles (Villano, Carputo, Frusciante, Santoro, & Aversano, 2014). As the next step, we tested four DNA extraction methods (Fig. 3). The presence of grape DNA in the total DNA extracted was confirmed by the amplification of the biotype-specific SSR alleles previously found. Results indicated that, using Method 2, good amplifications were obtained with seven markers (VrZAG79, Vvlp60, VVMD27, Vvlb01, VrZAG21, VrZAG29 and VrZAG47). This method is based on the protocol developed by Pereira et al. (2011) with some modifications, such as the use of a greater starting volume of wine (100 mL) for DNA extraction and its 1:30 dilution before the SSR marker amplifications. Fig. 3 shows the amplification patterns of VrZAG21 marker in the three Aglianicos, namely Taburno, Taurasi and Vulture. Expected allelic sizes (ranging between 200 and 300 bp) were obtained only in samples extracted with method 2, meaning that good amplification relies on the extraction protocol used. This method will be used in the future to compare the DNA profiles of our Aglianico biotypes with those of other wines and to integrate molecular fingerprinting with chemical analysis.

The possibility shown in this case study of discriminating the three Aglianico biotypes by phenolics and volatiles, as well as the success in extracting grape DNA from wines obtained from the biotypes, is encouraging. The use of metabolic markers to discriminate the three biotypes has to be necessarily validated on a greater dataset of samples and more aged wines - the specific appellation of high-quality Aglianico wines imposes a minimum period of aging in wood and bottles. However, the models obtained for varietal wines could lead the way to authenticating wines from neutral grapes or from blends of different grape varieties.

4. Conclusions: economic implications and future perspectives

The existence of wines with false designations and fake indications on labels generates considerable economic damage in terms of both direct and indirect costs. Counterfeit wines reduce revenues for the producers and distributors of authentic wines, eroding potential market shares. In addition, there are supplementary costs associated with fraud prevention, audits and investigation, time spent with regulators and customs, pressure from legitimate distributors, and increased customer service costs. At the

same time, counterfeiting lowers the brand quality in the eyes of the consumer, and exposes the producer to liability risks, harming the collective and institutional reputation of the product and the individual reputation of the manufacturers (Nickel & Sang, 2007). This damage represents one of the most significant for the wine market, as the reputation of the designation of origin is a core attribute in the choices of wine consumers and their willingness to pay, operating as a quality cue to assist purchasing decisions (Chaney, 2000; Lockshin, 1997; Hall, Lockshin, & Barry O'Mahony, 2001; Schamel, 2006). Collective and institutional reputation thorough wine origin and designation have already been shown to play an important role in both purchase decisions and wine quality formation (Martínez, Mollá-Bauzá, Gomis, & Poveda, 2006). In the presence of information asymmetry or incomplete information, reputation can be effective in reducing decision-making costs for consumers (Schamel, 2006). At the same time, a good reputation allows high-quality producers to sell their wines at a premium price (Schamel, 2006). Nevertheless, firms engaging in moral hazard behavior or free-riding foster activation of the adverse selection phenomenon, i.e. consumer awareness of not being able to distinguish higher quality wines through signals or reputation, and reduce their quality expectations and hence their willingness to pay. Furthermore, adverse selection weakens the correlation between real quality and price, and between good reputation and premium prices, damaging not only consumers but also (and especially) small producers. Finally, the reputation-building processes may be history-dependent especially in the case of past bad behavior, which increases the risk of being stuck in a "bad reputation trap" (Tirole, 1996). Whilst the current analysis does not allow quantification of the economic impact of collective reputation damage due to counterfeit wines, it may be inferred that the adverse monetary consequences are considerable, judging from the significant amount of resources spent by wineries on marketing to establish their brand image, intrinsically connected to collective reputation. Furthermore, the reputation value in awards, independent evaluations, and personal recommendations is critical to establishing a strong market presence.

Clearly, counterfeiting has become a significant economic phenomenon. What is the effect of counterfeit wines on consumer purchasing choices? A survey with a convenience sample of 300 Italian regular wine consumers was performed using an online platform, the results being reported in Suppl. Tables 1 and 2. The results confirm that the region of origin and the presence of a designation of origin (collective brand) are perceived as the main attributes in influencing wine choices, together with price. The findings also revealed that most respondents are aware of the counterfeit problem of Italian wines and the same awareness affects their wine purchasing choices. Almost one-third of the sample (32%) stated that they had recently reduced their average willingness-to-pay for wines with designations of origin and fine wines in general. A probit binary response model was performed with "counterfeit wines are affecting my purchasing choices" as the dependent variable. The regressors were eight attitudinal variables and six socio-demographic variables. This model shows that eight of the 14 regressors yielded a statistically significant contribution to the model. The strongest determinant was the type of wine generally consumed (DOC/DOCG grouped under the PDO according to European legislation), followed by awareness of the counterfeit problem in the wine market and wine consumption frequency. Considering the socio-demographic variables, higher education level is the variable with the strongest impact, followed by age and household average annual income above € 30,000.

In conclusion, a key insight from the literature analyzed in this paper is that the future of wine authentication depends on combining the information from different analytical techniques and interpreting it through statistical predictive models. In this perspective, the establishment of databases containing comprehensive information about different wine profiles is urgently required. Toward this goal, experiments and workflows need to be standardized, and reporting guidelines developed. This is deemed particularly critical for wine production, which is affected by many sources of variability (i.e. vintage, climate, soil, winemaking, and aging). Therefore, the scientific community active in fighting against abuses, unfair trade practices, counterfeiting, and other illegal practices should work to setup and validate officially accepted methods of analysis for the determination of molecular and sensory markers should be developed and validated. As discussed recently by Danezis et al. (2016), this will ensure that studies are performed in accordance with minimum standards that include biological context, chemical analysis, data processing, ontology, and data exchange format. Adherence to these operating procedures will ensure a minimal quality of experiment design and will enable results from different studies to be compared. We believe this is the direction, which will shape the field of wine varietal authentication in years to come.

Acknowledgements

We are grateful to Carmine Lanzillo for his technical support in DNA analysis.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.foodcont.2017.04.020.

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