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An enhanced analytical procedure to discover table grape DNA adulteration in industrial musts

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ABSTRACT

The need of accurate and reliable methods for DNA isolation and plant species identification in foodstuffs is of great importance, especially in the protection of high added value products. Fresh foods, which are not subjected to any modifications, are suitable for many kind of analysis; for processed products, such as musts, wines, olive oils, and pasta, the situation may be more complicated due to DNA fragmentation and, in the worst case, by its degradation. This work aimed to establish an exhaustive and reproducible analytical procedure for table grape DNA tracing in industrial musts. Three different DNA extraction methods were initially compared and DNA was tested in PCR for its suitability for the amplification reaction of microsatellite markers or simple sequence repeats (SSRs). An optimized DNA extraction method for microsatellite amplification was developed and adapted for industrial musts. Two SSR-based molecular methods, High Resolution Melting and capillary electrophoresis, were tested and the markers VrZAG62 and VrZAG79 were found to be the most informative. High Resolution Melting analysis, here applied for the first time on musts, proved to be the method of choice for a preliminary screening using four cultivars chosen as references and different DNA mixtures prepared in laboratory. Capillary electrophoresis, providing allele size, allowed a fine genotyping of musts in comparison with reference cultivars. The LOD_6 of a single grape cultivar in mixture with other varieties was also determined at 2.5 ng. Merging the information of the two molecular analyses applied to real samples, we demonstrated that is possible to discover case of musts adulterated with table grapes, and we propose our procedure in controlling musts quality and origin certification.

1. Introduction

Italy is the second European wine producer after France, with yearly production accounting for 5.82 billion kg of wine in 2012 (http://www.istat.it/it/files/2011/06/Italia_in_cifre_20132.pdf), although in recent years, the Italian wine sector underwent a gradual reduction in market share. This loss is partially due to an increased proportion of high quality wines, as certified by the European Commission (EC) marks PDO (Protected Designation of Origin) and PGI (Protected Geographical Indication) (http://www.istat.it/it/files/2011/06/Italia_in_cifre_20132.pdf). In this context, Apulia region is one of the most important producing areas for both wine (Sangiovese, Trebbiano and Montepulciano) and table grapes (Italia, Regina dei Vigneti, and Michele Palieri). Being one of the main productive activities, the wine sector is subjected to an extensive legislative discipline, according both to the EC Regulations no. 822/87 (European Commission, 1987) and 823/87 (European Commission, 1996), and to the Italian Decree no. 260 of 2000, which forbids the use of table grapes for wine-making. Table grapes are sometimes illicitly used in wine making, especially when a surplus of production occurs (Del Nobile et al., 2007).

The detection of adulteration in wine making by the addition of table grapes could be a crucial step when one has to certify the authenticity of the product, especially in upholding the "Made in

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Italy" as warranty of a quality product (Galimberti et al., 2015). Until few years ago, the characterization of musts and wines was mostly based on the analysis of chemical and biochemical compounds (Gonzàlez-Lara, Correa, Polo, Martın-Alvarez, & Ramos, 1989; Soufleros, Bouloumpasi, Tsarchopoulos, & Biliaderis, 2003), which are not in themselves enough to provide definitive and comprehensive results (Garcıa-Beneytez, Moreno-Arribas, Borrego, Polo, & Ibanez, 2002). Nowadays, DNA molecular markers represent a much more reliable tool for varietal characterization, not being influenced by environment conditions (İşçi, Yildirim, & Altindişli, 2009). Among them, microsatellites (SSRs) have proved to be the markers of choice due to: i) their ability to detect small-sized fragments of DNA; ii) their codominant nature; iii) their specie-specificity; iv) their high degree of polymorphism; and, v) their high reproducibility (Tautz, 1989). Such markers have been successfully employed for varietal traceability and adulteration detection in many processed foods that may have undergone DNA degradation, including: bread, semolina and pasta (Pasqualone, Montemurro, Grinn-Gofron, Sonnante, & Blanco, 2007 and 2010; Sonnante et al., 2009), tomato products (Sardaro, Marmiroli, Maestri, & Marmiroli, 2013; Turci et al., 2010), sweet cherry preserves (Ganopoulos, Argiriou, & Tsaftaris, 2011a), olive oil (Muzzalupo, Pellegrino, & Perri, 2007; Pasqualone et al., 2012; Scarano, Montemurro, Corrado, Blanco, & Rao, 2012), and fermented table olives (Pasqualone et al., 2013).

Up to now, research assessing the varietal origin of musts and wines using molecular markers have mainly used experimental samples produced in laboratory (Baleiras-Couto & Eiras-Dias, 2006; Faria, Magalhaes, Ferreira, Meredith, & Monteiro, 2000, 2008; Siret, Boursiquot, Merle, Cabanis, & This, 2000, 2002). Only a few authors have worked on commercial musts and wine samples collected at different winemaking steps; unfortunately, they were not able to successfully amplify markers either from musts halfway through fermentation and or from finished wines (Boccacci, Akkak, Marinoni, Gerbi, & Schneider, 2012; Garcia-Beneytez et al., 2002).

For effective marker testing in must, which is rich in DNA polymerase inhibitors such as polyphenols, polysaccharides, and proteins, a reliable DNA extraction protocol is needed. Furthermore, it is important to set up a robust analytical method for resolution of SSR polymorphisms (Madesis, Ganopoulos, Sakaridis, Argiriou, & Tsaftaris, 2014). The standard method for allele discrimination is capillary electrophoresis (CE) due to the ability of automatic sequencers to resolve size differences as low as one base pair. Nowadays, an alternative approach is provided by high resolution melting (HRM) analysis, a widespread technology of mutation scanning and genotyping that has been used in several scientific fields since its introduction in 2003 (Gundry et al., 2003). First adopted in clinical chemistry and human pathology, HRM has been adopted in plant sciences for several uses, such as varietal identification (Mackay, Wright, & Bonfiglioli, 2008), polymorphism detection and microsatellite genotyping (Distefano, Caruso, La Malfa, Gentile, & Wu, 2012; Mader, Lukas, & Novak, 2008), food adulteration (Ganopoulos, Argiriou, & Tsaftaris, 2011b; Mader, Ruzicka, Schmiderer, & Novak, 2011; Vietina, Agrimonti, & Marmiroli, 2013), and pathogen identification (Sanzani, Montemurro, Di Rienzo, Solfrizzo, & Ippolito, 2013), but not previously in analysis of must. Now, HRM is an attractive technique that allows the quick and high-throughput verification of specific DNA amplicons that are characteristic of a particular genotype. The analysis is sensitive, stable, and reliable, allowing closed-tube and homogeneous genotyping without fluorescently labelled probes. Moreover, unlike CE, no additional post-PCR handling is necessary, making the method fast with results generated in less than two hours (Ganopoulos et al., 2011b).

In our study we used HRM on industrial musts as a pre-screening to discover adulterations and CE for an accurate genotyping. The aims of this work were: 1) the development of a reliable DNA extraction method applicable to industrial musts; 2) the identification of table grape cultivars added in must samples using two molecular methods; and, 3) the analysis of different mixtures of table and wine grape cultivars in order to define the lowest threshold of a single grape cultivar detectable by DNA analysis.

2. Materials and methods

2.1. Sample collection

Ten industrial must samples were collected from different local wineries in Apulia, Italy. A total of 6 L for each sample was collected and stored in three bottles of 2 L each. All the musts were sampled after 10 min of stirring, and immediately stored at +4 °C. An official documentation, accompanying the ten samples, stated them to be mono-cultivar Trebbiano musts. In addition, it was possible to recovery the indirect information about the grape varieties cultivated present in the vineyard farms that have conferred the materials to the wineries, by examining the register of goods loaded and unloaded.

Leaves of the certified grapevine (*Vitis vinifera*, L.) cultivars Trebbiano, Regina dei Vigneti, Michele Palieri, and Italia, kindly provided by the certified grapevine collection field of CRSFA (Centro di Ricerca, Sperimentazione e Formazione in Agricoltura Basile Caramia) located in Locorotondo, (Italy), were used as reference. The cultivar Trebbiano is used for white wine production, whereas the other three are table grape cultivars.

2.2. DNA extraction

Three different DNA extraction methods were tested on ten different musts: (i) the protocol described by Li, Yang, Chen, Zhang, and Tang (2007) for sunflower leaves, as modified by Sabetta, Alba, Blanco, and Montemurro (2011) (method 1); (ii) the protocol proposed by Pereira et al. (Pereira, Guedes-Pinto, & Martins-Lopes, 2011) for wines, with some modifications (method 2); (iii) the commercial kit for genomic DNA extraction Nucleospin® Food (Macherey-Nagel, Düren, Germany) (method 3). These methods were applied to the pellet recovered (400 mg) after centrifuging 150 mL of must at 7000 × *g* for 30 min. Method 1 was used both for DNA extraction from must and young lyophilized leaves. Method 2 was slightly modified: since after incubation of the musts at −20 °C for 2 weeks, DNA was collected as a precipitate by 30 min of centrifugation at 7000 × *g* instead of 4000 × *g*. The pellet was dissolved in 1.5 mL preheated extraction buffer instead of 750 μL and proportional volumes were used in the following steps. Method 3 was applied to four aliquots of about 100 mg each, although the manufacturer's instructions suggested 200 mg, which were pooled at the end of the whole procedure. In fact, because of the very sticky characteristics of the pellet, starting quantity of 200 mg overloaded the mini-columns and hampered their full functionality.

After extraction, DNA of the reference cultivars was mixed to obtain various blends according to Table 1.

Genomic DNA concentration and quality were analysed both by using a Nano-Drop™ 1000 Spectrophotometer (Thermo Scientific, Waltham, MA) measuring the DNA absorbance at 260 nm and the sample purity ratios at 260/280 nm and 260/230 nm, and 0.8% gel electrophoresis along with 50, 100, 200 ng of λ-DNA.

2.3. DNA purification

DNA extracted from musts according to the three procedures,