Technical Brief

An Enhanced Method for *Vitis vinifera* L. DNA Extraction from Wines

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Abstract: Wine quality and value largely depend on grape variety, which is of primary importance in wine identification. The aim of the present work was to enhance a wine DNA extraction protocol and, subsequently, grapevine variety identification. This enhanced method is an outcome from several previously developed extraction methods and effectively allows obtaining large amounts of high-quality DNA exhibiting an optimal 260/280 ratio. Grapevine variety DNA extracted from wine was amplifiable with a specific SSR primer. This procedure was applicable for monovarietal and older commercial red and white wines. The potential of this enhanced method relies on its use for traceability as part of protecting both consumer and producer interests.

Key words: wine, DNA extraction, grapevine identification, SSR marker, traceability

Wine quality and market value depend on grapevine variety, together with other factors such as terroir and winemaker. Wine is usually made from one or more varieties of the European species *Vitis vinifera* L. Depending on the wine production region, especially if it is a Denomination of Origin (DO) wine, only a limited number of varieties are allowed. The inclusion of other varieties is only permitted under legally defined percentages. The fact that different grape varieties may be used in wine production is in itself an attraction for fraudulent practices. Scientific techniques and legislative guidelines have been developed for grape, must, and wine traceability to guarantee product origin and detect fraud and mislabeling.

Methods used for grapevine varietal identification or grape geographical origin determination comprise several features, such as must protein profiles (Moreno-Arribas et al. 1999, Pueyo et al. 1993, Gonzalez-Lara et al. 1989), anthocyanins (Garcia-Beneytez et al. 2003, Revilla et al. 2001), amino acids (Vasconcelos and Chaves das Neves 1989), aromatic compounds (Muñoz-Organero and Ortiz 1987), and chemical elements (Coetzee and Vanhaecke 2005, Almeida and Vasconcelos 2003, Monaci et al. 2003). These methods are time-consuming and influenced by various parameters such as soil composition, weather conditions, vinification methodologies, and wine aging.

Grapevine DNA can be extracted from any part of the plant, although the preferred material is young leaves (Lodhi et al. 1994). Several studies have reported the ability to extract and genotype DNA from different grapevine products, including grape juice (Faria et al. 2000), grape must (Faria et al. 2008, Baleiras-Couto and Eiras-Dias 2006, Rodríguez-Plaza et al. 2006), experimental wines collected immediately after the end of fermentation (Baleiras-Couto and Eiras-Dias 2006, Garcia-Beneytez et al. 2002, Siret et al. 2002), and aged wine samples (Drábek et al. 2008, Nakamura et al. 2007, Savazzini and Martinelli 2006). Nevertheless, efficient DNA extraction and amplification from must and wine samples remains difficult. Previous studies hypothesized that these difficulties could be due to various processes involved in winemaking, such as decanting, clarification, and filtration, which completely remove grapevine DNA (Garcia-Beneytez et al. 2002).

Developing DNA extraction protocols using wine as a sample for grapevine varietal identification and/or differentiation is a worthwhile pursuit. The detection of *V. vinifera* variety by polymerase chain reaction (PCR) using wine DNA is hampered by the insufficient quantity and quality of template DNA obtained after extraction, given the degradation that plant DNA suffers during the fermentation process (Savazzini and Martinelli 2006, Faria et al. 2000). There are other constraints, including the interference of polysaccharides and polypeptides in beverages and the coexistence of pigment substances such as polyphenols, all of which interfere with or even inhibit DNA polymerase during PCR (Garcia-Beneytez et al. 2002, Siret et al. 2000). Nevertheless, several studies have attempted to overcome these difficulties (Drábek et al. 2008, Nakamura et al. 2007, Savazzini and Martinelli 2006). The strategy selected for this study included gathering reported

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protocols and choosing the most suitable approach to define a DNA extraction protocol from wine that would be suitable for PCR amplification.

Materials and Methods

DNA extraction. Leaves from *V. vinifera* varieties Tinta Roriz and Fernão Pires were obtained from the vineyards of the University of Trás-os-Montes and Alto Douro, Vila Real, Portugal, and used as a standard. An established CTAB method (Doyle and Doyle 1987) was used for leaf DNA extraction.

Two monovarietal wines produced from Tinta Roriz (red) and Fernão Pires (white) varieties were collected at the end of the fermentation process. Three commercial blended red wines (2006 Torre de Ferro, 2005 Vinha do Côro Reserva, and 2004 Estremadouro Reserva) and three commercial blended white wines (2007 Porta da Ravessa, 2006 Caves Santa Marta, and 2005 Monte Novo) were purchased in a local market. The labels of these wines identified that Fernão Pires and Tinta Roriz varieties, among others (Tinta Barroca, Tinta Amarela, Touriga Franca, Roupeiro, Arinto, Malvasia Fina, Gouveio e Cerceal), were used in their production, although no information was given about the percentage of each variety.

The wine samples were precipitated in a plastic centrifugation tubes (35 mL), using 10 mL of sample and 0.7 vol of 2-propanol (Merck, Darmstadt, Germany) and maintained at -20°C for 2 weeks, after which crude DNA was collected as a precipitate by 30 min of centrifugation at 4,000 *g* at room temperature (Hettich Universal Zentrifugen D-7200, Tuttlingen, Germany). The pellet was dissolved in 750 µL preheated extraction buffer [20 mM ethylenediaminetetraacetic acid (EDTA), 10 mM tris-(hydroxymethyl) aminomethane–hydrochloride (Tris-HCl) pH 8.0, 1.4 M sodium chloride (NaCl) and 2% (w/v) hexadecyltrimethylammonium bromide (CTAB; Sigma-Aldrich, St. Louis, MO), included just prior to use, 1% (v/v) 2-mercaptoethanol (Sigma-Aldrich), 2% (w/v) polyvinylpyrrolidone (PVP; Sigma-Aldrich), and Proteinase K (20 mg/mL; Sigma-Aldrich)] by briefly vortexing. The samples were incubated at 65°C for 60 min. An equal volume of chloroform:isoamyl alcohol (24:1) (v/v) was added to the sample, followed by centrifugation at 13,000 *g* for 15 min at 4°C (Biofuge Fresco Heraeus, Kendro Laboratory Products, Hanau, Germany). The upper layer was transferred to another tube and the samples were treated with RNase (10 mg/mL; MBI Fermentas, Burlington, Canada) at 37°C for 30 min. DNA was precipitated with 0.6 vol of cold 2-propanol and incubated at -20°C overnight. After precipitation, DNA was collected by centrifugation at 10,000 *g* for 15 min at 4°C. DNA was dissolved in 300 µL TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). An equal volume of neutral phenol (Sigma-Aldrich) was added and homogenized. The upper layer was transferred to another centrifuge tube after centrifugation at 13,000 *g* for 15 min at 4°C. DNA was precipitated with 0.6 vol of cold 2-propanol and incubated at -20°C overnight. After precipitation, DNA was collected by centrifugation at 10,000 *g* for 15 min at 4°C. The supernatant was discarded and the DNA pellet was washed with buffer (76% ethanol, 10 mM ammonium acetate) for 5 min. The DNA pellet was dried at room temperature, eluted in 50 µL TE, and maintained at -20°C until use.

One commercial DNA extraction method, DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), five academic methods (Drábek et al. 2008, Nakamura et al. 2007, Baleiras-Couto and Eiras-Dias 2006, Rodríguez-Plaza et al. 2006, Savazzini and Martinelli 2006), and the protocol described in this study were used to test each of the commercial wine samples. These methods were compared in terms of wine sample starting volume, average DNA concentration, and total DNA yield.

DNA quantification and quality. Nucleic acid concentration and extract quality was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). All measurements were repeated three times, presenting the average value.

Grape microsatellite analysis. Analyses of DNA extracted from grape leaves, monovarietal wine, and commercial wine were performed using VrZAG79 microsatellite loci (Sefc et al. 1999). Forward primer was labeled at the 5′-end with a specific fluorochrome (5–carboxy-fluorescein; 5-FAM) compatible with the Beckman analysis system (Beckman Coulter, Fullerton, CA), synthesized by Sigma-Genosys (Woodlands, TX).

PCR conditions. PCR reactions were performed in a 20 µL vol containing 10 × PCR buffer containing NH_4SO_2, 2.5 mM $MgCl_2$, 10 µM each of dATP, dCTP, dGTP, and dTTP, 0.5 U Taq polymerase (MBI Fermentas), 0.3 µM of each VrZAG79 primer, and 40 ng DNA extracted from leaves and monovarietal and commercial wines. PCR was performed in a T Gradient 96 cycler (Whatman-Biometra, Göttingen, Germany). PCR conditions for DNA from leaves were as follows: initial denaturation at 95°C/5 min, followed by 35 cycles with a temperature profile of 95°C/20 s, 61°C/30 s, and 72°C/30 s, and a final extension step at 72°C/5 min. For DNA analysis from monovarietal and commercial wines, 45 cycles were applied.

Allelic size determination. Separation of the amplified products was carried out through capillary electrophoresis on a Beckman Coulter automated sequencer with the help of internal size standards (CEQ DNA Size Standard Kit-400; Beckman Coulter) using the software package CEQ 8000 fragment analysis system (Beckman Coulter).

Results and Discussion

DNA extraction. A consistent wine DNA extraction method is the basis for any marker-based assessment of wine varietal composition. To overcome the difficulties found throughout the extraction process, the development or improvement of a DNA extraction protocol from wine is essential. As this is an improved protocol, it is necessary to justify some options. Initially, wine samples (commercial) were precipitated over several time scales: 24 and 48 hours and one and two weeks. The present protocol was applied to all precipitation times and and results showed that for commercial white wines there was a pronounced decrease in yield (31%), while for commercial red wines the decrease was only 7% when comparing 25 hours and two weeks (Table 1). If in red wine samples no significant

differences were found in terms of total DNA yields, regarding the time-scale used for precipitation, in what concerns PCR amplification there were clear differences found among white and red wine samples; all white wine samples were amplifiable by PCR, whereas red wine samples were only amplifiable when precipitated for two weeks. Thus, precipitations for two weeks were preferred to all other time-scales.

The constitution and the concentration of the extraction buffer components varied widely among the protocols reviewed. Only one cationic detergent (CTAB) was chosen, for its capacity to dissolve the membranes and to increase DNA precipitation with high NaCl concentrations; CTAB also reduces sample polysaccharide contamination. DNase performance was inhibited by the addition of Tris-HCl and EDTA. 2-Mercaptoethanol was chosen to promote protein denaturation and to eliminate polyphenols. PVP was used because of its antioxidant effect and ability to eliminate polysaccharides. The concentrations used in the present protocol were adapted to sample characteristics. The protocol involves the addition of chloroform:isoamyl alcohol and includes phenol and proteinase K to facilitate the separation of chromatin proteins.

The present protocol was compared to six other DNA extraction methods (Table 2). The Qiagen DNeasy Plant Kit was not adequate for the wine matrix, and although the results are shown, they are not entered in the following presentation of results. The protocol described in this study presented the highest yield and DNA concentrations, even though it began with the smallest volume sample. The present protocol needs only a 10 mL wine sample for DNA extraction, a great advantage when compared with other methods that require a sample volume ranging from 30 to 400 mL (Drábek et al. 2008, Nakamura et al. 2007, Baleiras-Couto and Eiras-Dias 2006, Savazzini and Martinelli 2006). Additional information regarding 260/280 and 260/230 ratios are supplied. Once again the present protocol presents the optimal values for DNA quality (Table 2), which may explain why the samples are amplifiable, as none of the other samples had low values for 260/280 and 260/230 ratios were amplifiable by PCR.

In terms of total extraction time, this method describes entirely the steps required. In all the protocols available in the literature, some specifications are not given (e.g., incubation, precipitation, centrifuge, and washing times), and for that reason the extraction time is variable and cannot be accurately determined. Therefore, comparison between methods is not possible.

Quantification and purity of DNA templates. The measurements performed with the NanoDrop spectrophotometer revealed high genomic DNA concentrations and purity of all samples extracted from grapevine leaf and monovarietal and commercial blended wines (Table 3). This study therefore demonstrates that DNA from *V. vinifera* remains available in wine samples even after fermentation and other winemaking processes, given that wine samples were either collected at the

Table 1 Wine precipitation in 2-propanol over 2 weeks, 1 week, 48 hours, and 24 hours in white and red commercial wines. The results refer to genomic DNA concentration and yield, considering average values.

Sample	DNA (ng/μL)	Yield
2 weeks		
White wine	229	11,450
Red wine	481	24,050
1 week		
White wine	200	10,000
Red wine	483	24,150
48 hours		
White wine	189	9,450
Red wine	480	24,100
24 hours		
White wine	157	7,850
Red wine	447	22,350

Table 3 Nucleic acid quantification and extract purity evaluation from grapevine leaf and monovarietal and commercial wine samples.

Sample	DNA (ng/μL)	260/280 ratio	260/230 ratio
Leaf			
Tinta Roriz	716	1.93	2.10
Fernão Pires	981	1.94	2.12
Monovarietal wine			
Tinta Roriz	465	1.71	1.94
Fernão Pires	343	1.79	1.98
Red commercial wine			
Torre de Ferro, 2006	452	1.81	1.79
Vinha do Côro Reserva, 2005	330	1.71	1.81
Estremadouro Reserva, 2004	260	1.73	1.77
White commercial wine			
Porta da Ravessa, 2007	206	1.74	1.66
Caves Santa Marta, 2006	197	1.72	1.97
Monte Novo, 2005	164	1.73	1.82

Table 2 Comparison of seven different DNA extraction methods, evaluating initial quantity of wine, DNA concentration average, 260/280 and 260/230 ratios, and total DNA yield.

Method	Starting vol (mL)	[DNA]g (ng/μL)	260/280 ratio	260/230 ratio	Yield (ng)
Baleiras-Couto and Eiras-Dias 2006	400	25.7	1.34	0.36	2,570
Rodríguez-Plaza et al. 2006	15	13.2	1.23	0.29	264
Drábek et al. 2008	40	11.5	1.19	0.18	575
Nakamura et al. 2007	30	148.4	1.37	0.32	4,452
Savazzini and Martinelli 2006	45	98.3	1.43	0.38	4,915
Qiagen DNeasy Mini Plant Kit	2	1.6	0.98	0.34	80
Present protocol	10	268.2	1.74	1.79	13,410

end of fermentation process or two to six years after bottling, contrary to previous reports (Drábek et al. 2008, Nakamura et al. 2007, Baleiras-Couto and Eiras-Dias 2006, Savazzini and Martinelli 2006, Garcia-Beneytez et al. 2002, Siret et al. 2002).

Phenol is frequently used in DNA extraction methods and increases contamination risk. Nevertheless, this reagent has a maximum absorbance of 270 to 275 nm, which is close to that of DNA, and phenol contamination at times mimics both higher yields and purity because of an upward shift in the A_{260} value, giving misleading results. Other contaminants such as polysaccharides, proteins, solvents, and salts are also present and absorbed at 280, 270, and 230 nm. Considering the toxicity and contamination properties of phenol, it was eliminated from the DNA extraction protocol in a first approach, but the results revealed both poor DNA yield and quality. Thus, phenol extraction was shown to be crucial for good DNA extraction from wine samples.

UV spectra performed with the NanoDrop spectrophotometer on two samples of genomic DNA extracted from wine, one presenting phenol contamination and other using the method reported here without contamination, demonstrated that the enhanced protocol overcame the phenol contamination problem (Figure 1). The UV spectrum also showed that other contaminations usually present when DNA is extracted from these matrices were removed efficiently from the DNA template, supported by the DNA purity from monovarietal and commercial wine samples, which presented values between 1.71 and 1.81 for red wines and between 1.72 and 1.79 for white wines (Table 3).

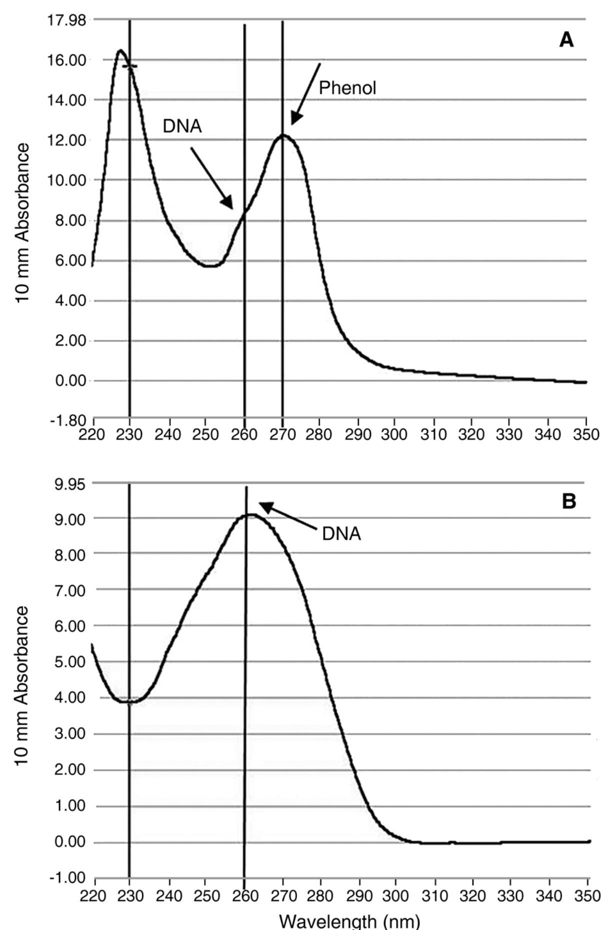

Figure 1 UV spectrum performed by NanoDrop ND-1000 spectrophotometer showing (**A**) phenol contamination and (**B**) the absence of phenol and/or other contaminations in DNA wine samples.

Amplification of genomic DNA from wine. PCR reaction can be inhibited by several factors. In wine samples, the presence of phenolic compounds and polysaccharides are the main cause of PCR impasse (Garcia-Beneytez et al. 2002, Siret et al. 2000, 2002). Other difficulties related to PCR failure are the small DNA quantities and its degradation status (Drábek et al. 2008, Savazzini and Martinelli 2006). Several strategies have been adopted to overcome these problems, such as improving DNA extraction methods and the use of chloroplast microsatellite markers in wine (Baleiras-Couto and Eiras-Dias 2006) and real-time PCR (Drábek et al. 2008, Savazzini and Martinelli 2006). In this protocol, both concentration and purity were high. The combination of 2-propanol precipitation, enzyme treatment, phenol and chloroform extraction, and several washes was positive and favorable for PCR reaction, eliminating all possible contaminants found in both white and red wine samples.

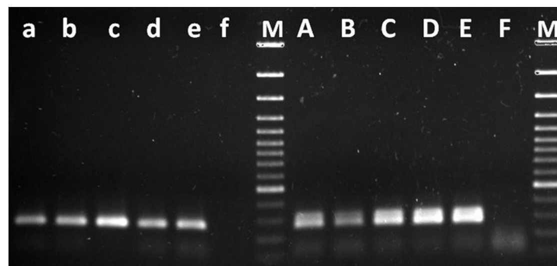

Figure 2 Amplification profile obtained with VrZAG79 nuclear primer. Lanes a, b, c: commercial white wines; d: monovarietal white wine; e: Fernão Pires variety (leaf), f: negative control. Lanes A, B, C: commercial red wines; D: monovarietal red wine; E: Tinta Roriz (leaf); F: negative control; M: molecular marker GeneRuler 100 bp DNA Ladder (MBI Fermentas).

In order to confirm the presence of *V. vinifera* DNA, a suitable primer combination for the PCR reaction was selected. The first condition for primer selection was that it would not amplify the DNA of microorganisms responsible for alcoholic and malolactic fermentation, such as yeasts and lactic acid and/or acetic acid bacteria, and be specific for grapevine varietal identification. Although six SSR nuclear primers have been accepted as universal markers for grapevine genotyping (GENRES 081 research project; www.genres.de/vitis/vitis.htm), only one primer was chosen (VrZAG79), given that the main purpose of this study was to prove that the improved DNA extraction method was efficient and the extracted DNA was suitable for PCR amplification of specific *V. vinifera* DNA segments. All samples amplified successfully (Figure 2). The microsatellite VrZAG79 allele sizes were obtained on an automatic sequencer and are expressed in base pairs (Table 4). The microsatellite allelic sizes found in the monovarietal wine

Table 4 Microsatellite genotype using VrZAG79 nuclear locus, expressed as allele size and size range, in base pairs obtained with DNA from grape leaves, monovarietal wines, and commercial wines.

Sample	Microsatellite VrZAG79 Allele size (bp)	Size range (bp)
Leaf		
Tinta Roriz	245 : 249	—
Fernão Pires	245 : 245	—
Monovarietal wine		
Tinta Roriz	245 : 249	—
Fernão Pires	245 : 245	—
Red commercial wine		
Torre de Ferro, 2006	—	243–251
Vinha do Côro Reserva, 2005	—	243–251
Estremadouro Reserva, 2004	—	243–249
White commercial wine		
Porta da Ravessa, 2007	—	243–251
Caves Santa Marta, 2006	—	243–251
Monte Novo, 2005	—	243–247

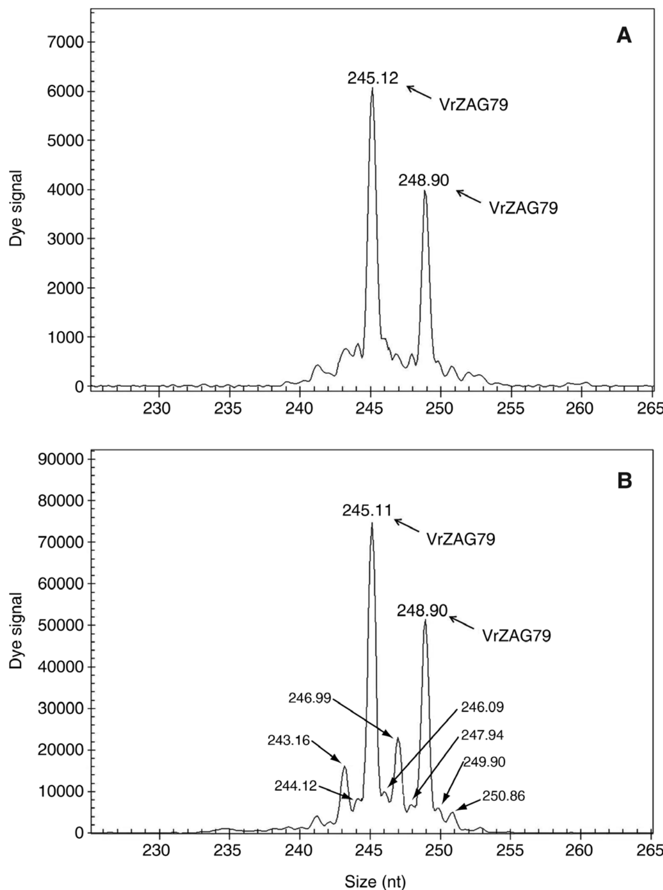

Figure 3 Plots of the dye signal traces provided by CEQ 8000 Fragment Analysis Software for microsatellite amplification of DNA at VrZAG79 loci for monovarietal (**A**) and commercial (**B**) red wine using Tinta Roriz variety.

samples (red and white) correspond to those detected in the leaf samples. For commercial blended wines, a size range was found instead of a specific allelic size, which is acceptable for commercial wines, given that several grapevine varieties were used in their production. Furthermore, VrZAG79 profiles provided by the automatic sequencer for monovarietal and commercial blended red (Figure 3) and white (Figure 4) wine samples are presented. Apart from the expected alleles (Tinta Roriz in red wines and Fernão Pires in white wines), the commercial blended wines presented other peaks, possibly belonging to other varieties present in these blends. It was not possible to associate the intensity of the peaks (alleles) with the relative proportion of each variety in the wine, since they are unknown. Several authors have reported PCR amplification from experimental wine samples collected immediately after fermentation (Garcia-Beneytez et al. 2002, Siret et al. 2002), from stabilized wines 8 months after fermentation and ready for bottling (Baleiras-Couto and Eiras-Dias 2006), and from 24-month-old monovarietal wines (Savazzini and Martinelli 2006). Nevertheless, difficulties were found in all of the reports when amplifying genomic DNA due to low DNA quantity and integrity. To our knowledge, this is the first report on PCR amplification using DNA extracted from 6-year-old bottled wine.

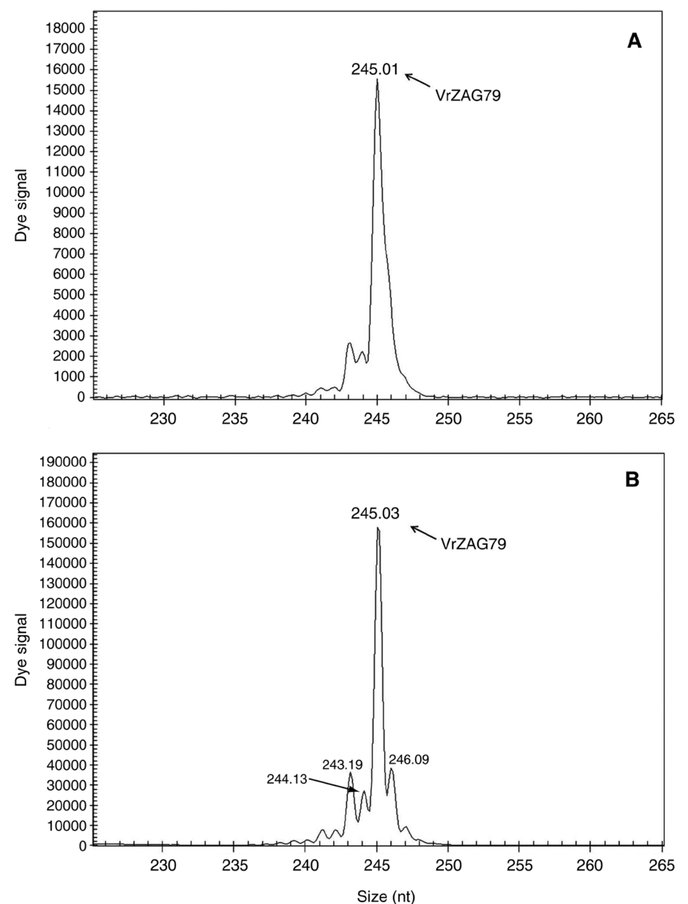

Figure 4 Plots of the dye signal traces provided by CEQ 8000 Fragment Analysis Software for microsatellite amplification of DNA at VrZAG79 loci for monovarietal (**A**) and commercial (**B**) white wine using Fernão Pires variety.

Conclusions

This study reports on an efficient *V. vinifera* DNA extraction method from wine samples suitable for amplification.

The main steps for a successful DNA extraction protocol from wine were 2-propanol precipitation, enzyme treatment, phenol and chloroform extraction, and several washes. Although these steps were performed equally on red and white wine samples, they should be strictly followed for red wine samples. With white wine samples the precipitation time can be reduced significantly without affecting PCR efficiency.

In regard to previously published data in the literature, the method described here presents several advantages: type of sample—allows for efficient extraction and consequent amplification of genomic DNA extracted from wines after several years of bottling; initial volume of sample—allows for grapevine DNA extraction from a wine sample volume of only 10 mL, which is advantageous compared to the volumes presented in the current literature; DNA quantity and purity—quantification of genomic DNA extracted from wine samples revealed a high concentration and purity level, thereby showing that *V. vinifera* DNA remains after the entire vinification process; PCR reaction inhibition—removes all possible contaminants, providing an unambiguous amplification profile; and DNA amplification—all DNA samples were amplifiable with a specific marker, thereby demonstrating that the DNA corresponded to a grapevine variety and excluding the hypothesis of belonging to a microorganism.

Wine quality, value, and price depend on several factors, but one of the main characteristics is grapevine variety. Thus, it is important that consumers trust wine labeling. An efficient traceability system is imperative. In the wine market the correspondence between the final product and grapevine varieties may be established through molecular marker technology, which requires a reliable and reproducible DNA extraction method. This protocol can provide the basis for a successful traceability methodology to guarantee product origin and to detect fraud and mislabeling. DNA extraction is the most relevant step for further analysis, such as *V. vinifera* detection in wine.

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