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Application of a DNA Analysis Method for the Cultivar Identification of Grape Musts and Experimental and Commercial Wines of *Vitis vinifera* L. Using Microsatellite Markers

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A DNA-based method has been applied to the identification of several musts and wines using microsatellite markers. DNA was extracted from the solid phases of sixteen monovarietal and five multivarietal musts (mixtures of two musts down to a 4:1 proportion) and they were genotyped at seven microsatellites through a multiplex PCR reaction and automated fluorescent detection. PCR multiplexing was successful in monovarietal musts, but should be used with caution with at least some markers and in multivarietal musts. The same extraction and detection methods were unsuccessfully applied to the solid and liquid phases of five monovarietal commercial wines, even after using different concentration procedures. Nucleic acids presence was then studied in a recent must, during the fermentation process, and during the subsequent steps of winemaking. Genotyping was possible in the resulting experimental wine until decanting, when the particles in suspension were removed. These results suggest that wine authentication through DNA analysis is not possible in commercial wines, in the tested conditions.

KEYWORDS: Microsatellites; grape must; wine; cultivar identification; DNA analysis; *Vitis vinifera*

INTRODUCTION

In the last several years, premium wines have acquired a great relevance at a worldwide level because of the specific characteristics of the grapes used. These wines are chosen as representatives of important winery zones assigned to Appellations of Origin, where an exhaustive control to guarantee their authenticity is required. The search of precise methods to identify the cultivars employed in winemaking is, in this way, a main objective of Regulatory Councils to ensure the fulfillment of legal dispositions and the final quality of the product.

In the literature, few methods of cultivar identification of grape musts and wines are described, due to the complexity of this purpose. One of the most successful methods is the native electrophoretic analysis of total grape must proteins in polyacrylamide gels (native-PAGE) (*1*−*3*). Other methods are based on the analysis of phenolic profiles (*4*), amino acid profiles (*5*, *6*), trace elements and isotopes (*7*, *8*), or terpens and other aroma compounds (*9*−*11*). Through these methodologies it is possible, in most of the cases, to determine the grapevine variety used to obtain the must, but these methods are time-consuming and do not always give definitive results. Sometimes they provide only information that is complementary to that obtained using other techniques, such as biochemical or morphological analyses performed on vegetative plant tissues.

Molecular markers based on DNA analysis have been successfully applied to the cultivar identification of *Vitis vinifera*, especially those that are PCR-based, such as RAPDs (*12*−*14*), sequence-tagged microsatellite sites (STMSs) (*15*−*17*), and AFLP (*18*−*20*). These are the most reliable and powerful techniques with which to tackle the cultivar identification. So, it seems to be possible to use them to guarantee the authenticity of wines. STMSs are the markers of choice for varietal identification for several reasons: they are codominant, highly reproducible, and easy to score, and they allow the establishment of interchangeable databases among different laboratories. At the moment, different tissues have been used as sources of DNA material. Leaves are the preferred tissues (*21*), but others such as roots (*22*), shoot tips (*16*), in vitro plantlets and calluses (*23*), cambium (*24*), and berries and raisins (*25*) have been used. Recently, two research groups have applied the STMS analysis to the study of grape products, such as grape juice (*26*) and fermenting musts, and experimental wines immediately after the end of the fermentation process (*27*). These are the preliminary works for the cultivar identification in wines by DNA analysis. Both research groups obtained positive results, studying five and six grape varieties, respectively, with a low number of markers (4), and concluded that the main limiting factor for wine authentication is DNA extraction.

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Table 1. Musts Employed in STMS Analysis

cultivar	vintage	color
Airén	1999	white
Albillo	1999	white
Chardonnay	1999	white
Garnacha	2000	red
Macabeo	1999	white
Malvar	2000	white
Moscatel grano menudo	1999	white
Napoleón	2001	rosé
Parellada	2000	white
Pedro Ximénez	1999	white
Red Globe	2001	rosé
Riesling	1999	white
Sauvignon Blanc	1999	white
Tempranillo	2000	red
Torrontés	1999	white
Ugni Blanc	1999	white

In this work, the goodness of STMS analysis for the varietal identification of musts and wines has been evaluated. Seven microsatellite markers have been applied to the analysis of 16 monovarietal and 5 multivarietal musts (mixtures of 2 musts in different proportions), 5 monovarietal wines, and 1 must during the fermentation process and subsequent steps of winemaking (decanting process) until its complete transformation into a wine. Siret et al. (*27*) provided some suggestions to improve the method they have developed, such as to increase the volume and to concentrate the samples, to use other microsatellite loci, and to employ more sensitive methods such as automatic sequencers. In this work, all these suggestions have been followed and applied to commercial wines. It may be considered as an important step toward wine authentication, because, up to now, no reports about authentication of experimental or commercial wines are available.

MATERIALS AND METHODS

Samples. *Musts*. A total of 16 monovarietal musts have been analyzed (**Table 1**). The list includes white, rosé, and red grape cultivars. The influence of sulfur dioxide addition to musts on DNA analysis has been studied by comparing musts from 3 cultivars (Garnacha, Napoleón, and Tempranillo) supplemented with $K_2S_2O_5$ (80 mg/L) with their corresponding controls. Multivarietal musts were prepared in the laboratory by combining different proportions (v/v) of Tempranillo (T) and Garnacha (G) musts (100/0, 80/20, 60/40, 50/50, 40/60, 20/80, and 0/100) in final volumes of 20 mL. Once prepared, all the musts were stored in 1.5-mL aliquots at −20 °C until analysis.

Commercial Wines. Five varietal wines of Malvar, Viura, Cabernet Sauvignon, Garnacha, and Tempranillo cultivars were produced in the experimental cellar of the IMIA (Madrid, Spain). Grapes were collected at maturity from vines grown at the Germplasm Bank (BGV) of the IMIA (vintage 2000), and crushed in a dishes press. Juices were transferred into stainless steel tanks (from 50 to 100 L), and a sulfited solution was added to stabilize the juice. The fermentation process started spontaneously, as no yeasts were added. Temperature and density were monitored daily. Juices from Cabernet Sauvignon, Garnacha, and Tempranillo were treated as red wines (i.e., with maceration of skins and seeds), whereas juices from Malvar and Viura were treated as white wines (i.e., without maceration of skins and seeds). Once fermentation was finished, decantings were performed, and the newly made wines were kept at 10 °C in the same tanks at least 6 months before their transfer into bottles, which occurred after the end of malolactic fermentation in the case of red wines. After the wines were bottled, they were stored at 4 °C until analysis. Conventional chemical analyses (total acidity, volatile acidity, alcohol content, free and total SO_2, and reducing sugars) were carried out in wines according to OIV methods (*28*).

Experimental Wines − Microvinification Experiment. A must of Red Globe (16 L, vintage 2001) was divided into 4 containers (A_1, A_2, B_1, and B_2) with identical volumes. Two of them (A_1 and A_2) were kept as controls at 4 °C with sulfur dioxide addition to prevent fermentation, whereas containers B_1 and B_2 were submitted to a typical red vinification process. Fermentation started spontaneously at day 2, and finished day 6. The fermented musts were separated from skins and seeds on day 9, and decantings were performed on day 15. After this, containers were stored at 4 °C until the end of the experiment. Aliquots of 1.5 mL of each container (both controls and fermenting musts) were collected daily during a 22-day period, and were stored at −20 °C for subsequent DNA analysis. Temperature and density were monitored daily in the four containers during this period. Conventional analyses were carried out in these experimental wines following the OIV methods (*28*).

Sample Preparation. DNA extractions were directly carried out in musts. Wine samples, however, were subjected to different treatments prior to extraction in order to concentrate the nucleic acids present in the solution and to reduce sample volumes.

Solid-Phase Obtention. Wines (750 mL) were centrifuged at 15300*g* for 10 min at 4 °C. Pellets were transferred to a microtube and stored at −20 °C for posterior DNA analysis.

*Precipitation with 2-Propanol.*2-Propanol (0.7 vol) was added to supernatants obtained in the previous centrifugation. After homogenization, the mixture was kept for 1 h at 4 °C, and then centrifuged at 15300*g* for 10 min at 4 °C. The pellet was stored at −20 °C until analysis.

Dialysis. Wines (750 mL) were dialyzed against running water on 6000−8000-Da Cellu Sep T1 membranes (Membranes Filtration Products, Inc., San Antonio, TX) for 48 h.

Concentration under Low Pressure. After dialysis, wines were concentrated under vacuum to approximately 20 mL, and precipitated with 0.7 vol of 2-propanol.

Concentration by Lyophilization. Dialyzed wines were lyophilized and stored at −20 °C until analysis.

DNA Extraction. DNA extraction was carried out in duplicate in all samples using the method described by Faria et al. (*26*). Must samples (1.5 mL) were first centrifuged to obtain the solid phases from which DNA was obtained. For wine samples, the initial volume was always 750 mL. After being treated as described above, DNA extraction procedure was performed in both liquid and solid phases. When indicated, DNA was further purified using the Dneasy Plant Mini Kit (Qiagen, Hilden, Germany) column, according to the manufacturer's instructions.

DNA quality and quantity was determined on a 0.8% agarose gel stained with ethidium bromide in TAE 1× (40 mM Tris−acetate, 1 mM EDTA) pH 8.1 buffer, by visual comparison with known quantities of lambda DNA.

Microsatellite Amplification. Musts and wines were genotyped at the following microsatellite loci: VVS2, VVS5 (*15*), VVMD5, VVMD7 (*16*), ssrVrZAG47, ssrVrZAG62, and ssrVrZAG79 (*17*). Microsatellite VVS29 (primer sequences taken from a CSIRO web page, now not available) was also included in the analysis of DNA extracted from sulfited musts. In monovarietal musts, multiplex PCRs including all the microsatellite primers were carried out. The reaction mix was as follows: Biotools buffer 1× (75 mM Tris−HCl pH 9.0; 50 mM KCl; 20 mM $(NH_4)_2SO_4$), 2 mM $MgCl_2$; 250 μM of each dNTP; 0.075 U/μL of Biotools DNA Polymerase; 2.5 μL of DNA extract and the primers: VVS5 primers 0.28 μM each; VVMD7 primers 0.05 μM each; VVS2 primers 0.12 μM each; VVMD5 primers 0.30 μM each; ZAG 47 primers 0.15 μM each; ZAG 62 primers 0.05 μM each; ZAG 79 primers 0.20 μM each; and primers VVS29 0.10 μM each (when included). A PTC-100 MJ Research thermocycler (Watertown, MA) was programmed for the following: 1 cycle [95 °C, 5 min], 39 cycles [94 °C, 45 s; 50 °C, 1 min; 72 °C, 1 min 30 s], 1 cycle [72 °C, 7 min]. In multivarietal musts and wines (commercial and experimental) also individual PCRs were performed on some of the microsatellites not amplified in multiplex PCR, using the same program and the following mix reaction: Biotools buffer 1×, 2 mM $MgCl_2$; 200 μM of each dNTP; 0.025 U/μL of Biotools DNA Polymerase; 2.5 μL of extracted DNA, and 0.20 μM of each primer. The amplification process was checked by running 7 μL of each sample in a 2% agarose gel in TAE 1× at 200V during 1 h. Bands were detected after staining with ethidium bromide, and gels were photographed under UV light. The separation

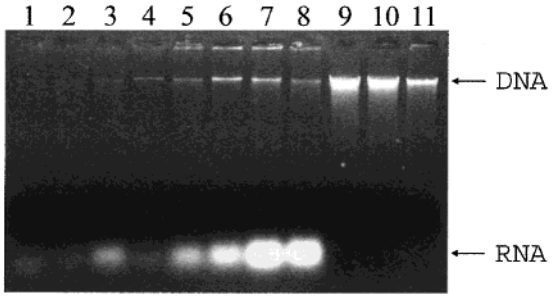

Figure 1. Nucleic acids extracted from grape musts. Lanes 1–8, one-third of the total must extracts; lanes 9–11, standard lambda DNA (lane 9, 150 ng; lane 10, 100 ng; lane 11, 50 ng). The corresponding samples are as follows: lanes 1–4, different extracts of Napoleón must; lanes 5–8, different extracts of sulfited Napoleón must. A faint DNA band is visible in all lanes (more clearly in lanes 4–8). A lower molecular weight smear of degraded RNA is present in all must extracts.

of the amplified fragments was carried out through capillary electrophoresis in an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems). Electrophoresed fragments were sized with the GENESCAN software, using TAMRA 500 as an internal marker. For each cultivar, all loci were analyzed together in the same electrophoresis since they had different size ranges and/or were labeled with different fluorochromes: HEX (VVS2, VVS29, and ZAG79), 6-FAM (VVS5, VVMD5, and ZAG47), and TET (VVMD7 and ZAG62).

RESULTS AND DISCUSSION

Monovarietal Musts. The quantity of extracted DNA, estimated by visual comparison with known quantities of standard DNA, was always lower than 150 ng (**Figure 1**). In some cases DNA could not be estimated by ethidium bromide staining because of the low quantity present. In such cases, the presence of a low-molecular-weight band or smear, consisting essentially of degraded RNA, was useful to evaluate the efficiency of the extraction procedure, as a rough direct relationship between DNA and RNA band intensities could be established in these musts. This was the main reason not to perform RNA digestions, like in others protocols (*27*), after checking that RNA does not interfere with the PCR reaction.

In this work, seven microsatellites have been analyzed, including the six markers selected by European experts of a grapevine characterization and identification project (GENRES-081 research project, www.genres.de/vitis/vitis.htm). A multiplex PCR with the 7 STMS was first carried out. This multiplex PCR had been previously developed using leaf DNA. No differences were found between the multiplex and the corresponding simplex PCRs. An example of the results obtained is shown in **Figure 2A**. This procedure allows genotyping of a given sample in one PCR reaction, with a considerable saving of DNA (the main limiting factor in this work), time, and money. **Table 2** shows the results obtained for monovarietal musts. Each one has been genotyped for 5, 6, or 7 loci. Seven musts out of the total 16 were perfectly characterized by a unique multiplex PCR, but the nine remaining musts needed various PCR reactions. Generally, loci VVS2, VVS5, ZAG62, and ZAG79 were properly amplified in multiplex PCR, whereas for ZAG47 irregular results were obtained. On the contrary, genotyping was not possible with VVMD5 in 9 must samples and with VVMD7 in 5 must samples, not even repeating PCR reactions. These two markers give successful results when they are applied to the analysis of different vegetative plant tissues, such as leaves (*29*), but not when analyzing musts of several cultivars; suggesting that these microsatellites could not be suitable markers for DNA analysis in musts. However, this idea does not agree with results obtained in musts by Faria et al. (*26*), and in fermenting musts by Siret et al. (*27*), where locus VVMD5 amplified efficiently. These differences could be attributed to two factors: on one hand, the PCR protocols used are different. The procedure established by Siret et al. (*27*) was optimized for must samples and differs from that employed in this work, also applied for leaf samples analysis (*29*). On the other hand, there could be varietal factors expressed in musts influencing the efficiency of some of the microsatellite markers in these kind of samples.

Cultivar identification of musts were carried out by comparing the results obtained after STMS analysis with those of the genotypes collected in a database elaborated by Borrego et al. (*29*), where young leaves were used as source material for DNA extraction. As expected, the genotypes obtained from musts did match totally with those obtained from leaves for all the considered varieties. These results guarantee the botanical origin of the 16 studied musts, and validate STMS analysis for the cultivar identification of musts.

To assess the effect of sulfur dioxide addition on DNA analysis, as is a common practice in wineries, 80 mg/L of $K_2S_2O_5$ was added to 3 musts from Tempranillo, Garnacha, and Napoleón varieties. Locus VVS29 was included in this experiment. Multiplex PCRs with the 8 STMS were performed in both controls and sulfited musts. No main differences were detected, neither in quality nor in quantity of the extracted DNAs, and genotypes obtained were exactly equal in all cases, as expected. Thus, it may be concluded that SO_2 addition has no influence on STMS analysis.

Multivarietal Musts. Nucleic acid extractions were successfully carried out in all mixtures of Tempranillo and Garnacha musts, as RNA was always visible in the 0.8% agarose gels. Results obtained after STMS analysis are shown in **Table 3**. The presence of more than one cultivar was revealed in all the samples studied, as 2, 3, or 4 alleles were obtained for each locus (that is to say, two different genotypes). These "two genotypes" were identified, at least, at 3 loci in all samples. These results confirm that it is possible to prove the presence of more than one cultivar in an unknown must through STMS analysis, as described in the literature (*26*). Nevertheless, amplifications were not as easily performed as in monovarietal musts. Multiplex PCR gave successful results only in samples in which Tempranillo was the major component (T/G 80/20, T/G 60/40, and T/G 50/50). In these samples, 6 loci were amplified (VVS2, VVS5, VVMD7, ZAG47, ZAG62, and ZAG79) by multiplex PCR, obtaining two genotypes for each one, except in the case of ZAG47, where the allele from Garnacha was only amplified through simplex PCR (**Figure 2B**). On the contrary, in those mixtures in which Garnacha was in a major proportion (T/G 40/60 and T/G 20/80) multiplex PCR was unsuccessful. In these samples, even with individual PCRs, amplification was obtained for only 3 loci (VVS2, VVS5, and ZAG62). Microsatellite VVMD5 did not amplify in any mixture of musts. Considering both Tempranillo and Garnacha monovarietal musts amplified properly, it seems that PCR reaction is inhibited by a combination between chemical components present in the musts.

To improve these results, the volume of DNA was reduced in the PCR mix reaction, from 2.5 μL of DNA extract to 1 μL, in an attempt to reduce the concentration of possible contaminants. After this modification, the quality of results was improved, as the allelic peaks obtained were cleaner. Nevertheless, those microsatellites not amplified previously, were not amplified. In addition, a DNA purification step was included

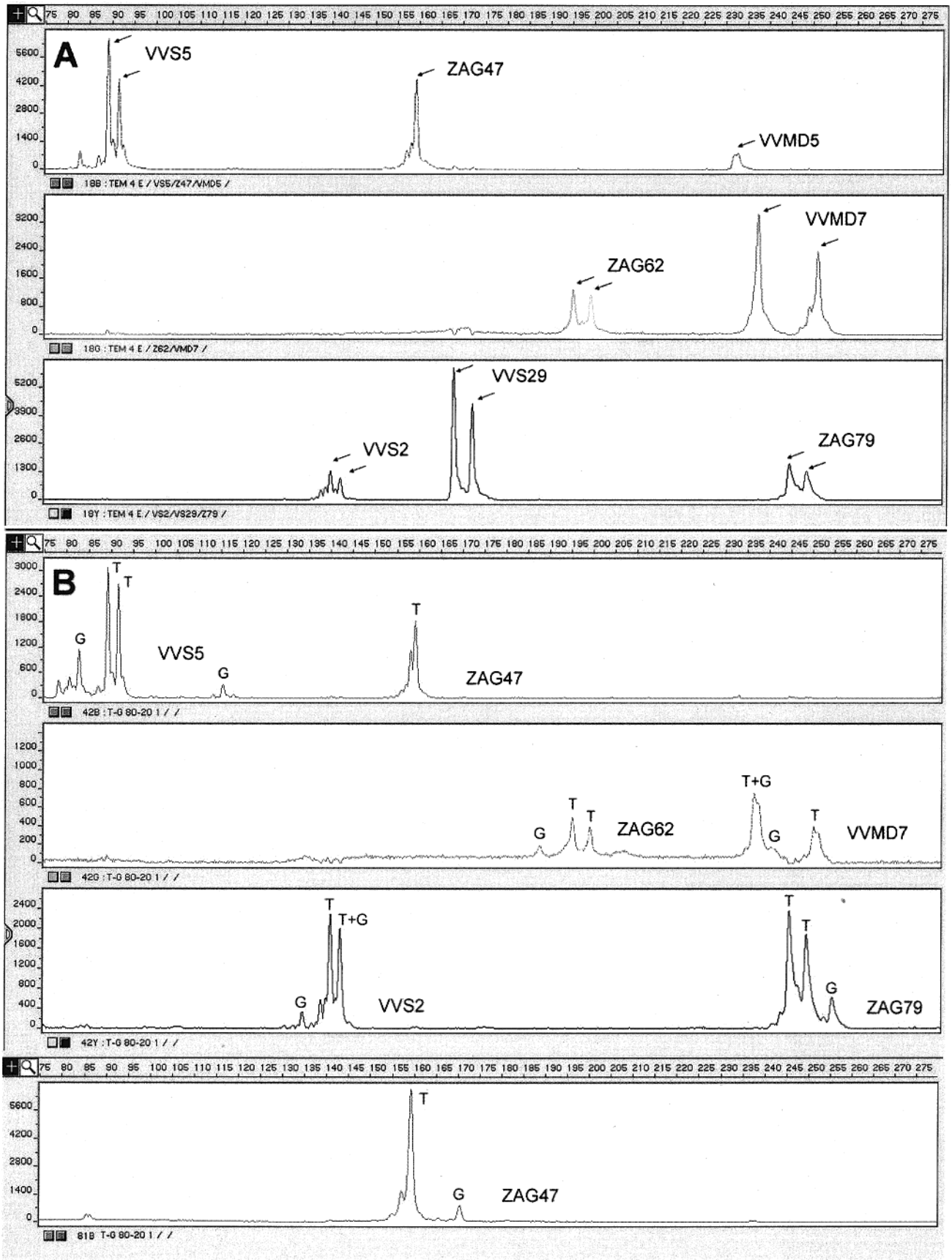

Figure 2. (A) Electropherogram profile provided by GENESCAN software for a must of the cultivar Tempranillo after STMS analysis. Peaks (alleles) corresponding to eight loci can be seen: VVS5, ZAG47, and VVMD5 (first window); ZAG62 and VVMD7 (second window); VVS2, VVS29, and ZAG79 (third window). (B) Electropherogram profile provided by GENESCAN software for a mixture of musts of Tempranillo and Garnacha (80/20). T, alleles from Tempranillo; G, alleles from Garnacha. VVMD5 did not amplify. All the alleles of the remaining six microsatellites were detected for both cultivars in a unique multiplex PCR reaction, except in the case of ZAG47, where the allele of Garnacha was not amplified (upper window). This locus was only amplified in a simplex PCR reaction (lower window).

Table 2. Genotypes Obtained from Musts after STMS Analysis, Expressed as the Size of the Alleles in Base Pairs

	microsatellite							
cultivar	VVS2	VVS5	VVMD5	VVMD7	ZAG47	ZAG62	ZAG79	total
Airén	140:142	149:149	223:231	241:251	157:171	187:199	245:257	7
Albillo	132:154	83:92	ND[a]	237:251	159:171	185:199	249:255	6
Chardonnay	134:140	149:149	231:235	237:241	157:165	187:195	241:243	7
Garnacha	134:142	83:116	ND	237:241	171:171	187:187	255:255	6
Macabeo	130:142	104:149	ND	237:237	165:171	187:187	241:255	6
Malvar	140:142	104:149	233:237	237:241	155:171	185:187	249:255	7
Moscatel	130:148	83:106	ND	231:247	155:171	185:195	249:253	6
Napoleón	130:132	92:116	231:235	247:249	159:171	187:203	245:255	7
Parellada	130:140	106:149	ND	ND	155:157	183:203	245:245	5
Pedro X.	130:142	116:149	225:231	241:247	155:157	183:203	245:245	7
Red Globe	134:150	83:116	ND	ND	159:159	185:187	245:257	5
Riesling	142:150	83:98	ND	ND	159:167	193:203	241:243	5
Sauvignon B.	132:150	83:98	225:231	237:255	153:167	187:193	243:245	7
Tempranillo	140:142	90:92	233:233	237:251	159:159	195:199	245:249	7
Torrontés	140:142	98:148	ND	ND	161:171	185:187	249:255	5
Ugni Blanc	130:140	116:149	ND	ND	155:159	193:199	243:249	5

[a] ND, nondetected.

Table 3. Alleles (Expressed in Base Pairs) Obtained from Multivarietal Musts (T, Tempranillo; G, Garnacha, v/v) after STMS Analysis

	microsatellite						
mixture	VVS2	VVS5	VVMD5	VVMD7	ZAG47	ZAG62	ZAG79
T/G 100/0	140:142	90:92	233:233	237:251	159:159	195:199	245:249
T/G 80/20	134:140:142	83:90:92:116	ND[a]	237:241:251	159:171	187:195:199	245:249:255
T/G 60/40	134:140:142	83:90:92:116	ND	237:241:251	159:171	187:195:199	245:249:255
T/G 50/50	134:140:142	83:90:92:116	ND	237:241:251	159:171	187:195:199	245:249:255
T/G 40/60	134:140:142	83:90:92:116	ND	ND	ND	187:195:199	ND
T/G 20/80	134:140:142	83:90:92:116	ND	ND	ND	187:195:199	ND
T/G 0/100	134:142	83:116	ND	237:241	171:171	187:187	255:255

[a] ND, nondetected.

Table 4. Results of Conventional Analysis Carried out in Commercial and Experimental Wines

	commercial wines					experimental wines	
	Malvar	Viura	Cabernet S.	Garnacha	Tempranillo	container B1	container B2
alcohol content (%)	14.0	14.5	13.9	14.6	13.3	10.4	10.5
ph	3.6	3.1	3.4	3.7	4.0	3.4	3.2
total acidity (g tartaric acid/L)	4.0	4.9	6.3	4.3	4.6	5.5	4.9
volatile acidity (g acetic acid/L)	0.2	0.48	0.7	0.9	1.0	0.8	0.7
reducing sugars (g/L)	1.6	4.3	1.9	2.1	2.3	2.7	2.4
total SO_2	84.6	148.1	57.3	37.4	54.8	44.8	39.8

in the procedure, after standard extraction, using the DNeasy Plant Mini Kit, but no improvements were achieved. Faria et al. (*26*) successfully identified individual varieties in multivarietal musts from Portuguese grape cultivars with microsatellites VVS2, VVMD5, VVMD6, and VVMD7. In contrast, in our case, amplifications of locus VVMD5 did not occur in any must combination, and amplifications of locus VVMD7 did not occur when Garnacha was present in a major proportion. Methods of DNA extraction and analysis were similar in both works. Therefore, as discussed above, these differences could be attributed to varietal factors influencing the efficiency of some of the microsatellite markers.

Commercial Wines. Results of conventional analyses performed in the five wines studied were consistent with commercial ranges (**Table 4**). Different kinds of sample concentration were performed before DNA extraction (as described in Material and Methods), because DNA could be degraded or in a very low concentration after fermentation and the winemaking process (*27*). All procedures carried out gave similar results. Nucleic acids were not visible in agarose gels from either the solid or the liquid phases of wines, even after the concentration procedures. Despite this, the extractions were used in PCRs, because PCR is much more sensitive than ethidium bromide staining and UV−visualization, but GENESCAN analyses corroborated the absence of microsatellite amplification in all samples using multiplex PCR. For this reason some of the extractions were further purified using the DNeasy Plant Mini kit, and simplex PCRs of VVS2 were carried out, but the results were identical: absence of amplification. To corroborate that this result was due to the absence of DNA and not to the presence of contaminants, the simplex PCRs were repeated after adding a foreign DNA of the variety Ondarrabi zuri to the reaction mixes in five wine extracts. In the five samples Ondarrabi zuri alleles were amplified properly (loci VVS2 and ZAG62), which demonstrate the absence of DNA in those extracts. This result and the absence of nucleic acids visible bands in the agarose gels indicate clearly that there is no DNA in any of the commercial wine extracts. Siret et al. (*27*) proposed that additional steps of DNA concentration could be necessary to improve STMS analyses in wines. Results obtained here suggest that concentration techniques employed in this work may not be adequate, probably because they are not selective,

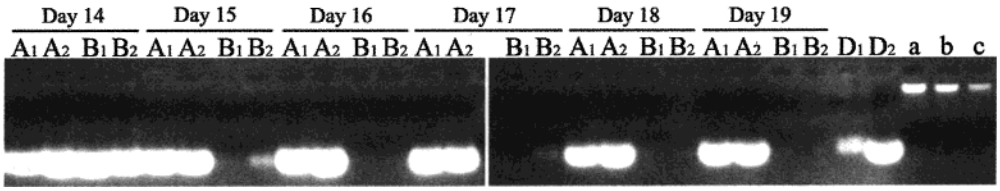

Figure 3. Nucleic acids extracted from both control musts (A_1 and A_2) and fermented musts (B_1 and B_2) from day 14 to day 19 of the microvinification experiment. D_1 and D_2 samples correspond to extracts of the sediments obtained after decanting containers B_1 and B_2, respectively. Lanes a, b and c correspond to 200 ng, 100 ng, and 50 ng of the standard lambda DNA, respectively. The picture was taken in exposing conditions that allowed notice of the disappearance of RNA in fermented musts. DNA bands are not visible here because of their much lower intensity, although they were present in some lanes.

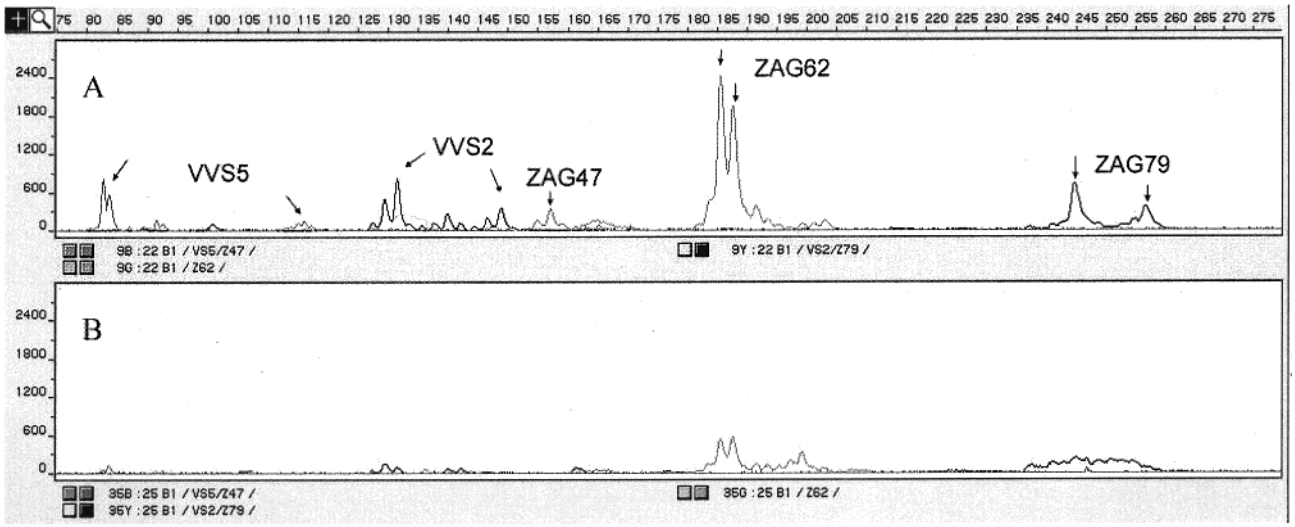

Figure 4. Electropherogram profile provided by GENESCAN software for fermented musts (container B_1) from day 13 (A) and day 16 (B) of the microvinification experiment. Peaks (alleles) corresponding to five microsatellite loci, amplified through simplex PCR, are indicated by arrows.

and other major components of wines, such as polyphenols and polysaccharides, are also concentrated, and act as inhibitors of PCR reaction (*30*, *31*). The above-mentioned "varietal factor" may also have influence in the results obtained, although 5 different varieties have been used. Nevertheless, the most plausible explanation is simply that there is no DNA present in the elaborated wines. Positive results obtained with musts, prior to negative results obtained with wines, suggests that DNA is degraded or lost at some intermediate point during winemaking. A microvinification experiment was proposed to confirm this hypothesis.

Microvinification Experiment. Experimental Wines. Conventional analyses in the experimental wines gave normal values, consistent with commercial ranges (**Table 4**).

DNA extractions were performed all days of the experiment in the four containers (**Figure 3**). As expected, nucleic acids were correctly extracted along the 22-day period in both control musts (containers A_1 and A_2). A faint high-molecular-weight DNA band was generally observed joined to a much more intense low-molecular-weight smear of degraded RNA. In the musts submitted to fermentation (containers B_1 and B_2) nucleic acids were also extracted during the complete fermentation process (days 1 to 6) and after, until the decanting day (day 15). However, after day 18, nucleic acids became undetectable in agarose gels. It should be noted that, especially in this experiment, PCR amplifications are more significant than DNA visualization after ethidium bromide staining, for two reasons: first, PCR is much more sensitive than DNA visualization, and amplification can be achieved from samples showing no DNA in the agarose gel; second, during the fermentation process, the nucleic acids extracted and visualized could come from yeasts as well as from grapes, while the PCR is specific for grape DNA. For these reasons, five microsatellites were analyzed in all samples from days 1, 4, 7, 10, 13, 16, 19, and 22 by individual PCR: VVS2, VVS5, ZAG47, ZAG62, and ZAG79. VVMD5 and VVMD7 gave irregular results in musts and were discarded for these analyses. After PCR, genotypes were correctly established in all analyzed samples of control musts, until the end of the experiment. Only ZAG79 failed to amplify in several samples. For samples corresponding to fermenting musts, amplifications were successful only until day 13, 7 days after the end of alcoholic fermentation (**Figure 4A**). From day 16, no more clear amplifications of the 5 microsatellites analyzed were obtained. These results are consistent with those mentioned above for DNA extraction. After day 16, microsatellites VVS5 and ZAG47 did not amplify, and VVS2, ZAG62, and ZAG79 markers showed various peaks of similar molecular size, making it difficult to identify the true alleles and preventing genotyping (**Figure 4B**).

To evaluate the relationship between the nucleic acids disappearance and the winemaking steps that follow fermentation, DNA extraction was performed in sediments resulting from decanting of containers B_1 and B_2 (**Figure 3**, lanes D_1 and D_2). Agarose gels revealed the presence of nucleic acids (and supposed DNA) in these samples. This suggests that the sediments, basically composed of traces of cellular tissues of grapes, seeds, skins, etc., are also the principal source of DNA in a fermented must. Then, the decanting process removes the main, if not the unique, source of DNA, preventing the use of DNA analysis for wine authentication in commercial wines.

In this work, STMS analysis is revealed as a useful tool for the cultivar identification of both monovarietal and multivarietal

musts. DNA analysis is also possible in wines in the very few days after the end of fermentation. Several steps follow the fermentation process to obtain a final, commercial wine: decanting, clarification, and filtration. During these steps, particles in suspension are removed, eliminating the main source of DNA present. This fact and the DNA degradation that occurs during winemaking process since the berries are crushed, prevent the use of STMS analysis for wine authentication in the tested conditions.

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