Eur Food Res Technol (2006) 223: 625–631
DOI 10.1007/s00217-005-0244-2

ORIGINAL PAPER

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Combining microsatellite markers and capillary gel electrophoresis with laser-induced fluorescence to identify the grape (*Vitis vinifera*) variety of musts

Received: 4 October 2005 / Revised: 20 December 2005 / Accepted: 22 December 2005 / Published online: 27 January 2006

Abstract In this work, a new method that combines the use of microsatellite markers (VVMD5 and ZAG79) together with capillary gel electrophoresis with laser-induced fluorescence (CGE–LIF) is developed and applied to the identification of Albariño and Moscatel Grano Menudo musts. The GCE-LIF method uses commercially available products including polymers, DNA-intercalating dyes and bare fused silica capillaries to provide reproducible and sensitive separations of DNA fragments for grapevine characterization. The CGE–LIF procedure offers highly resolved separations of DNA fragments from 48 to 1031 bp in ca. 30 min with efficiencies up to 1.8×10^6 plates/m allowing the separation of fragments that differ in 4 bp. The use of different DNA standards (i.e., 100 bp ladder, $\Phi\times174$ and pBR322) and their effect on size assignment of the amplified DNA is also investigated. It is demonstrated that the microsatellite markers (VVMD5 and ZAG79) provide DNA amplification patterns specific for Albariño and Moscatel Grano Menudo grapes that can be adequately differentiated by using CGE–LIF. Moreover, the DNA sizes determined by this CGE–LIF method are corroborated using a more standard procedure (i.e., an automatic genetic analyzer with a commercial kit) demonstrating the usefulness of this new methodology.

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Keywords Grapevine characterization · LIF · Capillary gel electrophoresis · Polymerase chain reaction · DNA · Microsatellites

Abreviations
CGE: capillary gel electrophoresis · CTAB: *N*-cetyl-*N*,*N*,*N*-trimethyl-ammoniumbromide · EDTA: ethylenediamine tetraacetic acid · HEC: hydroxyethylcellulose · LIF: laser-induced fluorescence · PCR: polymerase chain reaction · PVA: poly(vinyl alcohol) · TRIS: tris[hydroxymethyl]aminomethane

Introduction

The genome of eukaryotic organisms contains a variety of repetitive sequences that constitute the basis for the development of molecular methods exploiting DNA polymorphism for the identification and detection of subtaxonomic groups between animal, plant and biological species (i.e. strains, serotypes, varieties, etc.). That is the case for microsatellites, also known as simple sequence repeats (SSRs) or short tandem repeats (STRs). These are tandem repeats of short sequences (usually 1–4 bp), abundant, highly polymorphic and evenly distributed over the genome. Microsatellites can be used in several ways for the study of eukaryotic DNA polymorphism, including hybridization, polymerase chain reaction (PCR) fingerprinting and *locus*-specific PCR [1]. Microsatellites have also become especially useful as sequence-tagged microsatellite sites (STMS) for the identification and genetic characterization of agronomic species. STMS are developed by identifying microsatellite flanking sequences either from databases or by screening microsatellite sequences from genomic libraries. These flanking sequences are used for the design of *locus*-specific primers. To be useful, STMS must reveal some degree of polymorphism and the primer pair must show enough specificity for their cognate *locus*.

Identification of grapevine varieties have rapidly progressed since the development of the above-mentioned DNA-based methods [2]. However, accuracy of the results provided by these procedures may depend among other factors on the resolution and efficiency of the separation technique used to analyze the DNA fragments obtained after PCR amplification. Additionally, considerable genetic information can be lost if the sensitivity of the separation technique is insufficient. In this sense, although microsatellite markers have been applied to the varietal identification of grape must as well to detect DNA during must fermentation [2–10], very few works have analyzed the possibilities of the combined use of molecular techniques and non-denaturing capillary gel electrophoresis (CGE) for the genetic analysis of grapevine varieties [11]. Thus, practically, in all these applications electrophoresis in a classical slab-gel format [3, 4, 6–8] or electrophoresis under denaturing conditions in an automatic genetic analyzer [5, 12] were used to analyze the DNA fragments from grapes or grapevine material. However, the use of slab-gel electrophoresis is known to provide much lower resolution and sensitivity than capillary electrophoresis what can even give rise to misleading conclusions [13]. Although the use of automatic genetic analyzers (also based on capillary electrophoresis of denatured DNA fragments) can overcome these limitations [14], these systems also show some drawbacks as, for instance, the effect of laboratory temperature on genotyping error [15] or the important differences observed in assigned allele sizes when using different instruments, separation matrices and size standards [16, 17]. In this sense, the development of alternative procedures, as the one proposed in this work, can be a useful strategy to corroborate the analysis of DNA fragments. Moreover, CGE–LIF analysis of DNA fragments under non-denaturing conditions could provide electrophoretic profiles that can be used as fingerprints of a given variety [11].

The routinely authentication analysis in the food industry, using DNA markers, demands simple, fast and inexpensive methods. To this end, in this work we describe the development of a method that combines the use of microsatellite markers together with non-denaturing capillary gel electrophoresis with laser-induced fluorescence (CGE–LIF) for the differentiation of Albariño and Moscatel Grano Menudo musts. To develop this method, two informative markers, VVMD5 and VrZAG79, were selected among the six reference markers recently proposed in the GENRES081 European Union research project because the combination of these two markers allowed the differentiation of multiple Spanish grape varieties [18]. The results are further corroborated by using an automatic genetic analyzer demonstrating the usefulness of our approach.

Materials and methods

Chemical and samples

All chemicals were of analytical reagent grade and used as-received. Tris[hydroxymethyl]aminomethane (TRIS), Tris-HCl, and ethylenediaminetetraacetic acid (EDTA) from Sigma (St. Louis, MO), 2-hydroxyethyl cellulose (HEC) (M_w 90,000) poly(vinyl alcohol) (PVA) (M_w 50,000) from Aldrich (Milwaukee, WI), chloroform from Scharlau (Barcelona, Spain), isoamilic alcohol, *ortho*-phosphoric acid and *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide (CTAB) from Merck (Darmstadt, Germany), NaCl, 2-propanol and ethanol from Panreac (Barcelona, Spain) were used. YOPRO-1 from Molecular Probes (Leiden, Holland) were added as intercalating dyes to CE running buffers. Buffers were stored at 4 °C and warmed at room temperature before use. Milli-Q water (Millipore, Bedford, MA) was used in all experiments.

The following DNA ladders were used as standards to determine the size of the amplified fragments: DNA 100 bp (0.5 mg/ml), pBR322 (0.5 mg/ml) digested with *Bsu*RI, Φ×174 (0.5 mg/ml) digested with *Hin*fI, from Biotools (Madrid, Spain), and 8–587 bp (pBR322) digested with *Bsu*RI, from Roche (Indianapolis, IN); all ladders were diluted to a final concentration of ca. 25 ng/μl. AmpliTaq DNA polymerase, including reaction buffer and $MgCl_2$, dATP, dCTP, dGTP, dTTP were from Perkin-Elmer (Madrid, Spain), RNase was from Roche (Indianapolis, IN), oligonucleotides were synthesized by Bonsai Technologies Group (Alcobendas, Spain).

Albariño and Moscatel Grano Menudo grapes have guaranteed identity and were from the experimental vineyard of the Mision Biológica de Galicia (CSIC), in the north of Spain. Albariño variety is autochthonous of the north of Spain while Moscatel Grano Menudo variety is very ancient and expanded to the whole Europe. These varieties have been selected for this study by its economic interest since they produce a wine of great quality. The grapes were pressed and sulfur dioxide (SO_2) was added to a final concentration of 40 mg/l to stabilize the juice. Musts were stored at –20 °C until DNA extractions.

Leaves from the same cultivars were collected from plants cultivated in the Mision Biológica de Galicia (CSIC) ampelographic collection and stored at –80 °C until use.

DNA extraction

Grape genomic DNA was extracted from musts following the method of García-Beneytez et al. [5]. Briefly, 15 ml of monovarietal must were centrifuged (5600 *g* for 15 min at 4 °C), the pellet was washed once with 2 ml of clear must containing 2% β-mercaptoethanol (5600 *g* for 2 min) and once with TE buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA) in the same conditions. The pellet was then suspended in 600 μl of extraction buffer (2% CTAB, 1.4 M NaCl, 10 mM Tris-HCl, pH 8, 20 mM EDTA, and 2% β-mercaptoethanol) supplemented with 6% PVPP (polyvinylpolypyrrolidone) after 5 min of vigorous agitation, the samples were incubated at 65 °C with agitation. After 1 h of incubation the mixture was extracted twice with chloroform/isoamyl alcohol (24:1), and nucleic acids were precipitated from the final aqueous phase with 0.7 volumes of ice-cold 2-

Table 1 Repeatability and accuracy of size assignment of the DNA fragments obtained for the two grape varieties investigated in this work (Albariño and Moscatel Grano Menudo) depending on the microsatellite marker (VVMD5 or ZAG79) and the ladder used as standard (100 bp, Φ×174 or pBR322)

				Ladder used					
				100 bp		Φ×174		pBR322	
Grape	Microsatellite	Theoretical size	Peak name	Experimental $size_{av}$	$\%RSD_{(n=3)}$	Experimental $size_{av}$	$\%RSD_{(n=3)}$	Experimental $size_{av}$	$\%RSD_{(n=3)}$
Albariño	VVMD5	218	A_1^V	221.5	1.38	218.5	0.50	217.9	0.15
		228	A_1^V	230.8	2.22	228.8	0.46	228.7	0.19
Moscatel	VVMD5	224	M_1^V			220.6	0.15	220.4	0.12
		232	M_2^V			230.6	0.13	230.4	0.17
Albariño	ZAG79	245	A_1^Z			242.9	0.29	244.0	0.10
		249	A_2^Z			247.7	0.07	249.4	0.08
Moscatel	ZAG79	249	M_1^Z			248.0	0.15	248.0	0.12
		253	M_2^Z			251.6	0.15	251.7	0.27

propanol. The resulting pellet was dissolved in 300 µl of TE buffer and precipitated again with 0.5 volume of 5 M NaCl and 2 volumes of ice-cold ethanol. After drying the pellet was recovered in 20 µl of TE with RNase (25 µg/ml).

PCR amplification

Musts were genotyped at the VVMD5 [19] (primers VVMD5F: 5′-CTAGAGCTACGCCAATCCAA-3′, VVMD5R: 5′-TATACCAAACATATTCCTAAA(AGC)-3′) and ZAG79 [20] (primers ZAG79F: 5′-AGATTGTGGAGGAGGGAACAAACC-3′, ZAG79R: 5′-TGCCCCCATTTTCAAACTCCCTTCC-3′) microsatellite loci. Each PCR reaction contained 1× AmpliTaq reaction buffer, 0.16 mM $MgCl_2$, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.2 mM dTTP, 0.2 µM each primer, 1 µl genomic DNA (1–10 ng), and 1.25 U of AmpliTaq DNA polymerase. The following thermal parameters were used for DNA amplification of ZAG79 loci: first denaturation, 5 min at 95 °C, 40 cycles of 45 s at 95 °C, 30 s 50 °C and 1 min 72 °C; terminal elongation, 7 min at 72 °C. For VVMD5 the amplification conditions were first denaturation, 5 min at 95 °C; 40 cycles of 45 s at 95 °C, 30 s 55 °C and 1 min 72 °C; terminal elongation, 7 min at 72 °C. Amplifications were performed in a Mastercycler® thermocycler.

CGE–LIF

The analyses were carried out in a P/ACE-MDQ equipped with an Argon laser working at 488 nm (excitation wavelength) and 520 nm (emission wavelength), from Beckman Instruments (Fullerton, CA). Bare fused silica capillaries with 75 µm i.d. were purchased from Composite Metal Services (Worcester, England). Injections of the PCR products were directly made at the cathodic end using N_2 pressure of 1 psi for 24 s. Coinjections of PCR products and DNA ladders were consecutively carried out at the cathodic end using N_2 pressure of 1 psi for 24 s from each vial. The P/ACE-MDQ was controlled by a PC running the 32 Karat Software also from Beckman.

Before the first use, any uncoated capillary was preconditioned by rising with 0.1 M HCl for 30 min. Between injections, capillaries were rinsed using 0.1 M HCl for 4 min, 1% PVA for 2 min, and separation buffer for 4 min. At the end of the day, the capillary was rinsed with Milli-Q water for 5 min and stored overnight with water inside.

Automatic genetic analyzer

Amplified PCR products corresponding to microsatellite loci VVMD5 and ZAG79 were also resolved using an ABI-Prism 3700 DNA sequencer and data were analyzed using GENESCAN v 3.7 (Appleva Hispania, Madrid, Spain).

Results and discussion

PCR–CGE method development

The first step of method development consisted in the optimization of the annealing temperature during thermal cycling for each STMS markers, using independently Albariño and Moscatel Grano Menudo genomic DNA as templates. The annealing temperatures shown in Materials and methods section for VVMD5 (55 °C) and ZAG79 (50 °C) is the result of this optimization procedure. The analysis of some samples from the annealing temperature optimization experiment of Albariño and ZAG79 suggested a correlation between the relative amounts of alleles A_1^Z and A_2^Z and the annealing temperature. This could be explained considering that one of the ZAG79 *loci* in this specific variety showed imperfect matching with the primers. However, after repeating the analysis with 10 samples amplified using annealing temperatures ranging from 50 to 65 °C (eight annealing temperatures tested) this correlation could not be confirmed.

The usefulness of the combined use of VVMD5 and ZAG79 microsatellite markers and CGE–LIF to adequately characterize Albariño and Moscatel Grano Menudo cultivars directly from musts can be deduced from Fig. 1. Thus, Fig. 1 shows that the experimental procedure described under Materials and methods section, in which VVMD5 and ZAG79 are used as microsatellite markers, makes possible to extract DNA from musts, to amplify it using the proposed PCR protocol and to obtain adequate DNA profiles by CGE–LIF for the two grape varieties. Moreover, from the electropherograms of Fig. 1 it can be concluded that the microsatellite markers VVMD5 and ZAG79 seem to provide different CGE–LIF profiles for Albariño and Moscatel Grano Menudo grapes. Hence, comparing in Fig. 1 the profiles obtained using VVMD5 as microsatellite marker (A and B) with those obtained using ZAG79 (C and D), it can be deduced that the use of VVMD5 provides much cleaner amplifications than ZAG79. Also, as expected, the DNA fragments obtained after amplification using the selected microsatellite markers are very similar in size [10]. Thus, VVMD5 y ZAG79 should provide amplicons for Albariño and Moscatel Grano Menudo with sizes ranging from 218 to 253 bp [10] what is corroborated by the very similar analysis times observed for the eight DNA fragments shown in Fig. 1. Moreover, the CGE–LIF method brings about adequate separation of the amplified DNA fragments in less

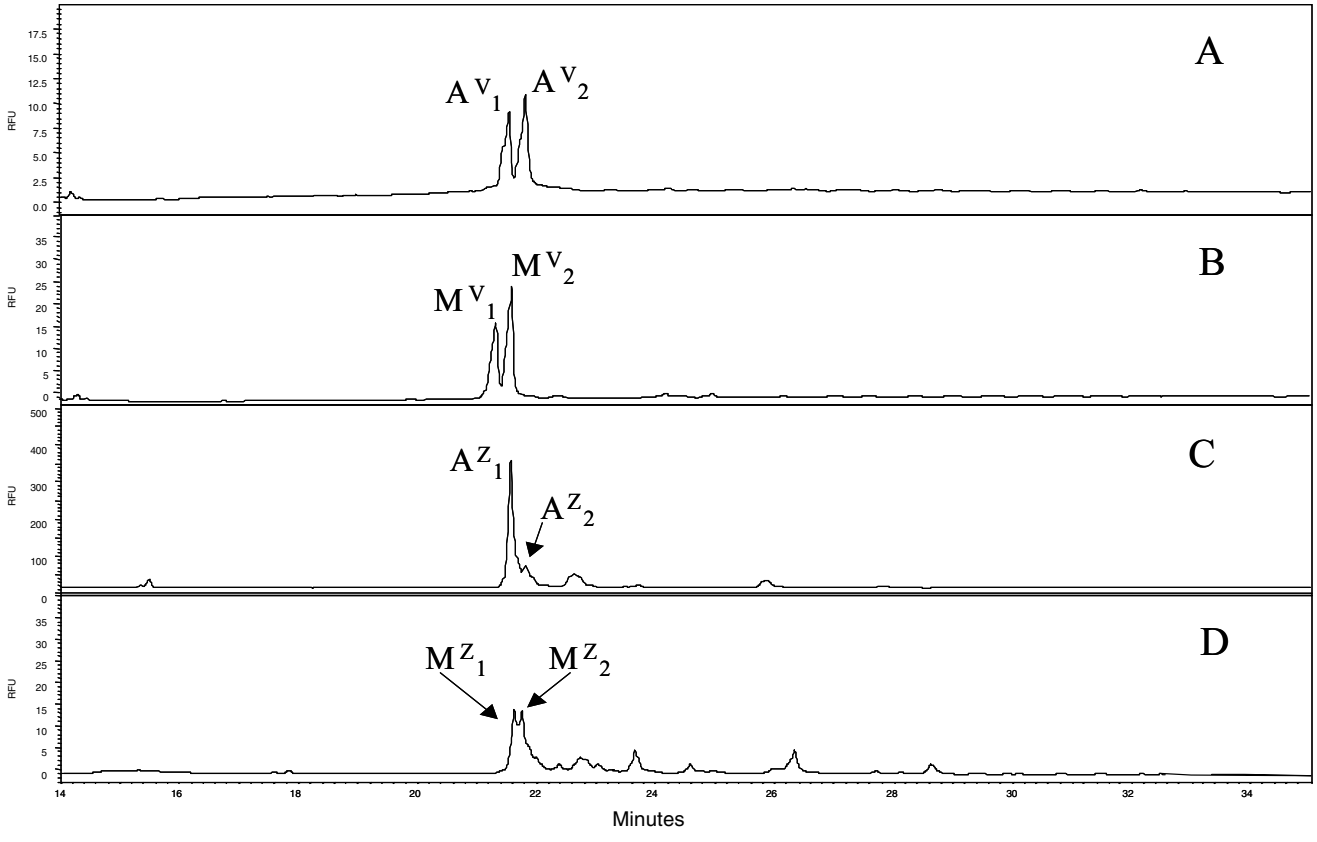

Fig. 1 CGE–LIF electropherograms obtained for the PCR amplifications done using the DNA from Albariño and Moscatel musts together with VVMD5 or ZAG79 microsatellite markers. **A** Albariño and VVMD5 (218, 228); **B** Moscatel and VVMD5 (224, 232); **C** Albariño and ZAG79 (245, 249); **D** Moscatel and ZAG79 (249, 253). CGE–LIF conditions: uncoated fused silica capillary with 60 cm of total length, 50 cm of effective length, and 75 μm i.d.; separation voltage of –13 kV; running buffer: 20 mM Tris, 10 mM ortophosphoric acid, 2 mM EDTA and 4.5% HEC at pH 7.3 plus 500 nM YOPRO 1 as DNA intercalating. Injection for 24 s using N_2 pressure (1 psi) of the PCR amplification. For peaks assignment, see text

than 30 min with efficiencies up to 1.8×10^6 plates/m. In this sense, an additional prove of the high resolving power of the CGE–LIF method is the baseline separation of amplicons A_1^V and A_2^V (see Fig. 1A) that correspond to DNA fragments whose difference in size is expected to be only 10 bp. Similarly, DNA fragments M_1^V and M_2^V could be almost baseline separated as demonstrated in Fig. 1B (in this case, the size difference is only 8 bp). Moreover, Fig. 1C and D show that some separation of M_1^Z from M_2^Z and A_1^Z from A_2^Z is also obtained even when in this case the expected difference between these fragments is expected to be as low as 4 bp. It is interesting to remark in this point that the use of 2% agarose slab-gel electrophoresis did not provide, in any case, observable separation of these alleles (data not shown), what confirms the usefulness of CGE–LIF over the most classical slab gel electrophoresis procedures.

Under these experimental conditions, the typical CGE–LIF method used to determine the size of these DNA fragments (i.e., obtention of a calibration curve, plotting for each DNA fragment the logarithm of its number of base pairs vs. the inverse of its analysis time, after injecting a DNA external standard mixture) was tested. However, the reproducibility of the size assignment using this procedure was very poor what precluded the correct variety determination. Moreover, CGE–LIF migration time was observed to depend on the ionic strength of the sample injected what precluded the direct comparison between migration times from standard DNAs and PCR amplifications. To overcome this limitation, co-injection of the PCR amplifications plus three different DNA ladders used as internal standards were tested by CGE–LIF, comparing such ladders in terms of size assignment repeatability and accuracy.

Effect of the DNA ladder used as internal standard on the size assignment by PCR–CGE–LIF

Three different DNA ladders were tested as internal standards (namely, 100 bp, Φ×174 and pBR322 ladders) by co-injecting each one together with the PCR product obtained after amplifying DNA from must of Albariño variety with the primer pair VVMD5. The three ladders were compared in terms of repeatability and accuracy, using as reference the expected size for the amplified DNA fragments [10]. Typical electropherograms are shown in Fig. 2, while the size repeatability and accuracy results are given in the second row of Table 1 for the three different DNA ladders used. As can be deduced from Table 1, the use of a 100 bp ladder provides the worst results in terms of size repeatability with RSD values (n=3) up to 2.22%. Higher repeatability was obtained by using the Φ×174 ladder, with $RSD_{n=3}$ values lower than 0.50%, while the best results were obtained by using pBR322 ladder, which provided $RSD_{n=3}$ values lower than 0.19% (see Table 1). This different behavior can be explained through the electropherograms of Fig. 2. Thus, as can be seen in Fig. 2A, the DNA fragments from the PCR sample migrates too far from the peaks of the 100 bp ladder used as size marker what must logically reduce the repeatability and accuracy of the peak assignment. This is corroborated by comparing the expected size for A_1^V and A_2^V (218 and 228 bp, respectively) with the values experimentally obtained (221.5 and 230.8 bp) by using the 100 bp ladder. As can be seen, the size assignment clearly improved by using the Φ×174 ladder that provided experimental sizes of 218.5 and 228.8 bp (see Table 1), or by using the pBR322 ladder that provided experimental sizes of 217.9 and 228.7 bp (see Table 1). Therefore, considering the similar expected size for the amplified DNA fragments from Albariño and Moscatel Grano Menudo, the latter two size markers (Φ×174 and pBR322) were used for the rest of assignments. The results obtained for the amplification of the DNA from the two grape varieties using the two microsatellite markers are also given in Table 1. As can be seen, in general, slightly better repeatability was obtained by using the pBR322 ladder as size marker, while similar size assignments for the DNA fragments were obtained by using Φ×174 ladder or pBR322 ladder as internal standard.

To corroborate the size assignment provided by the CGE–LIF method to the amplified DNA fragments, these values were compared with those provided by an automatic genetic analyzer using DNA from leaves of the two considered varieties. Table 2 shows the comparison among these results. In general, a good agreement was observed among the results provided by the two experimental procedures (CGE–LIF vs. automatic genetic analyzer) and the allele sizes theoretically expected for Albariño and Moscatel Grano Menudo using VVMD5 and ZAG79 [10]. As can be seen in Table 2, the higher differences were observed comparing the CGE–LIF values and the automatic analyzer values for M_1^V and M_2^V. Namely, the theoretical size values for M_1^V and M_2^V were 224 and 232 bp, respectively, and although they were confirmed by the automatic genetic analyzer (224.8 and 233.4 bp, respectively), the CGE–LIF method provided slightly lower values (220.4 and 230.4 bp, respectively). At the moment, a clear explanation for this (small) difference is not available, although it is noteworthy that smaller values were systematically found using CGE–LIF for M_1^V and M_2^V (e.g., see in Table 2 that independent of the ladder used similar lower sizes were obtained for M_1^V and M_2^V). Interestingly, the agreement observed for the other allele sizes (i.e., A_1^V, A_2^V, M_1^Z, M_2^Z, A_1^Z and A_2^Z) was good as can be deduced from the values given in Table 2. For instance, the theoretical values for A_1^V and A_2^V are 218 and 228 bp, respectively, obtaining 217.9 and 228.7 by CGE–LIF and 218.8 and 228.9 bp using the automatic genetic analyzer (see Table 2), corroborating the usefulness of this analytical approach. An additional consideration is that the separation time required by the genetic analyzer was almost 50 min for each sample, while the CGE–LIF separation only required ca. 30 min. However, some genetic analyzers can run simultaneously several samples in parallel, although their cost is higher than a standard CGE–LIF instrument [21]. On the other hand, both systems provide similar sensitivity.

In conclusion, in this work, we have demonstrated that by selecting adequate separation conditions, CGE–LIF can

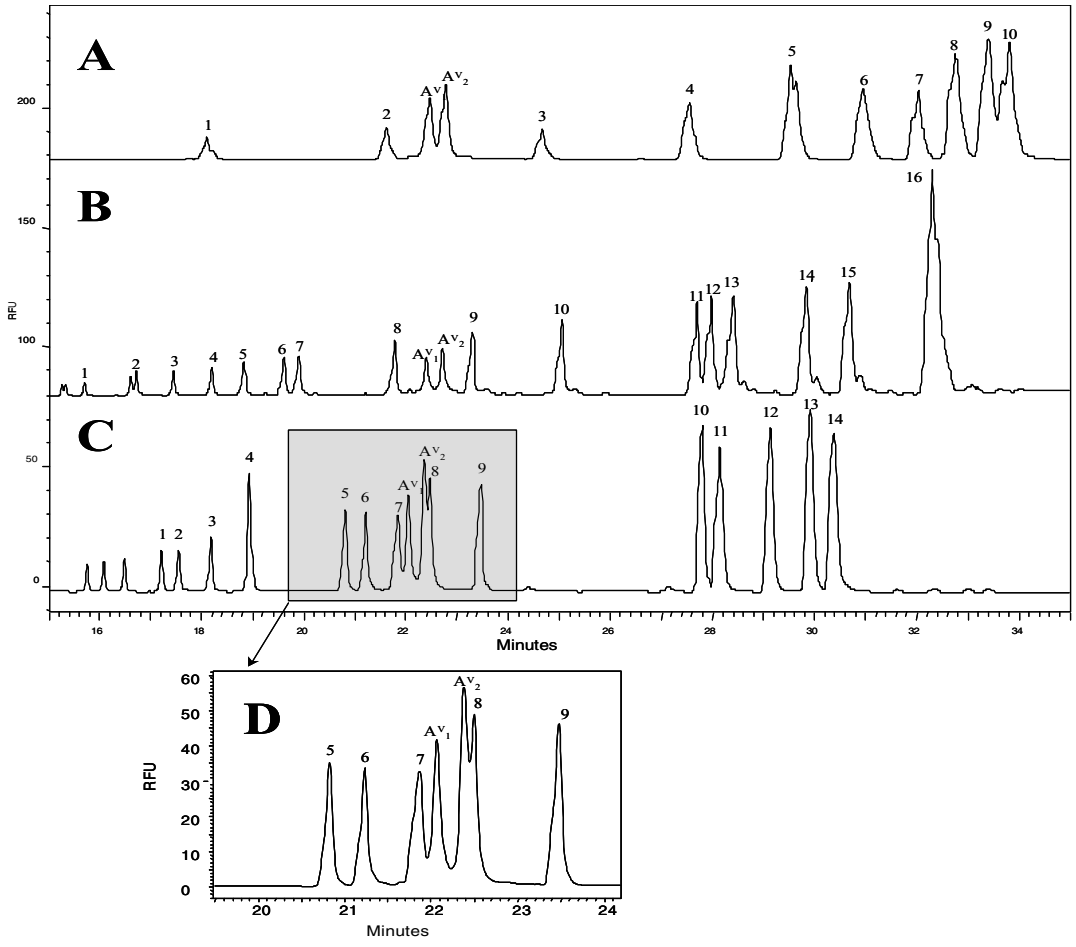

Fig. 2 CGE–LIF electropherograms obtained for the PCR amplification using DNA of Albariño variety and the primer pair VVMD5 co-injected with three different ladders. Co-injection for 24 s using N_2 pressure (1 psi) of the PCR amplification ((A_1^V) Albariño 218 and (A_2^V) Albariño 228) plus: **A** 100 bp ladder ((1) 100, (2) 200, (3) 300, (4) 400, (5) 500, (6) 600, (7) 700, (8) 800, (9) 900, (10) 1031); **B** Φ×174 ladder ((1) 48, (2) 66, (3) 82, (4) 100, (5) 116, (6) 140, (7) 151, (8) 200, (9) 249, (10) 311, (11) 413, (12) 417, (13) 427, (15) 500, (16) 553 and (17) 713, 726 bp); **C** pBR322 ladder ((1) 80, (2) 89, (3) 104, (4) 123, 124, (5) 184, (6) 192, (7) 213, (8) 234, (9) 267, (10) 434, (11) 458, (12) 504, (13) 540, and (14) 587 bp); **D** the enlargement of electropherogram **C**. CGE–LIF conditions are indicated in Fig. 1

Table 2 Comparison of the size assignment (bp) provided to the different amplified DNA fragments by the CGE–LIF method and an automatic genetic analyzer

Grape	Microsatellite	Theoretical size	Peak name	Experimental size (CGE–LIF)	Experimental size (automatic genetic analyzer)
Albariño	VVMD5	218	A_1^V	217.9	218.8
		228	A_1^V	228.7	228.9
Moscatel	VVMD5	224	M_1^V	220.4	224.8
		232	M_2^V	230.4	233.4
Albariño	ZAG79	245	A_1^Z	244.0	245.6
		249	A_2^Z	249.4	249.5
Moscatel	ZAG79	249	M_1^Z	248.0	249.5
		253	M_1^Z	251.7	253.6

be a suitable analytical technique to differentiate Albariño and Moscatel Grano Menudo musts based on the use of microsatellite markers, providing reproducible and efficient separations and, as a result, adequate size assignments. Because of its simplicity, rapidity and cost-effectiveness, this CGE–LIF procedure can be proposed as an alternative and straightforward method that can be implemented in any analytical laboratory that owns a CE instrument.

Acknowledgement This study was supported by a CSIC-Intramural Project 200470F037.

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