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# Isolation of DNA from olive oil and oil sediments: Application in oil fingerprinting

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**Abstract**

A lot of plant species are well characterized by using molecular markers. One of them is the olive-tree, although its main product, the olive oil, was refractory to characterization with such technologies. We report here an extraction method that isolates DNA from olive oil that serves as template for PCR reactions. This DNA has been used to identify and characterize olive oils based on specific molecular markers. The method has been developed with internal controls to ensure that contaminant DNA from other sources will not affect the results. The combination of SCAR markers and the DNA extracted from olive oil and sediments allows the fingerprint of different olive oils from different manufacturers. The DNA pattern obtained was (i) the same when DNA was extracted from olive oil or from oil sediments, and (ii) consistent with the one obtained using olive-tree genomic DNA. Finally, we observed that the specific fingerprint of each olive oil is a consequence of the olive-tree cultivars used during the olive milling. Then, our methodology represents the starting point to control the industrial production of olive oils to avoid fraud by mixing oils from cultivars not declared or by mixing other oils with olive oil. It also opens a new way to authenticate the olive oil in order to support Labels of Origin as well as Preserved Origin Denominations.

***Key words:*** Molecular markers, fingerprinting, SCAR, olive oil, *Olea europaea.*

## Introduction

Olive tree (*Olea europaea* L.) is one of the most important oleaginous crops in the Mediterranean area and the oil obtained from its drupes is an economically important product. Olive oil is almost unique among oils in that it can be consumed in crude form without refining, possessing increased stability as well as nutritional and healthy features with respect to other vegetable oils. Additionally, it has a fine aroma and a pleasant taste. All these properties make olive oil commands to rise a higher price than most other edible plant oils. This in turn means that there is a great temptation to adulterate it with cheaper oil. To avoid adulteration and promote the authentication of this oil, the attribution of labels of origin are in expansion. Labels of Origin as well as Preserved Origin Denominations are the main way to control the quality during olive-milling and commercial transactions. The obtainment of a label of origin implies to identify both the oils and the drupe stocks with particular regard to the utilization of typical cultivars from different areas. A lot of research has been performed to assure the authenticity of olive oil analyzing its contents of sterols, phenols, fatty acids, alcohols... [1-5]. However, some difficulties have been encountered in distinguishing olives and olive-oils from different cultivars because their characteristics are strongly influenced by environmental conditions. By the other side, accurate and rapid identification of cultivars is especially important to obtain a reliable label of origin. Differentiation among olive-tree cultivars is traditionally supported by numerous phenotypic traits in maturity for trunk, leaf, flower and fruit shape [6, 7], and more recently, with allozymes [8-10]. The weakness of both criteria to identify oils and cultivars has been put in evidence by the demonstration that chemical and morphological changes in olive-trees, as well as other plants, are subjected to environmental conditions and induced by domestication [11]. Therefore, there is an urgent need for the development of methods that identify oils and cultivars easily, rapidly and at an early stage of development. The use of analytic parameters independent from environmental fluctuations, such as the DNA-based molecular markers, is the most recommended.

Olive-tree cultivar identification has been recently improved by using DNA as molecular marker in a PCR based technique. PCRs are generally quick and straight forward to perform, and the fact that PCR requires only small amounts of DNA makes it very useful. Since there is little sequence information of the olive genome available [12, 13], most of the studies have been performed using "arbitrary" primers [14-21], although the best differentiation has been obtained using specific molecular markers [22]. Some unsuccessful attempts have been carried out regarding the DNA fingerprinting of olive oils since the isolated DNA was highly degraded and its amplification mainly produced inconsistent electrophoretic patterns [23, 24]. In order to understand what happened in these works, it is necessary to know that olive oil is obtained by grinding olives in a mill as quick as possible to keep down oxidation and acidity [25]. This results into a paste that is malaxed (slow mixed) to allow the oil-water emulsion to coalesce: small microscopic oil droplets join together into large drops that can be easily separated in the next step. The malaxed paste is pressed or centrifuged at a "cold" temperature (around 30 to 37°C) to separate the olive oil from a fibrous olive cake (pomace) and a wastewater. This wastewater, so called vegetable water, is a brown watery liquid

that still contains valuable trace elements, oxidants and other organic compounds like most of the cell DNA and proteins. We then hypothesize that when olive drupes are grinded, the cell compartmentalization is destroyed and everything is mixed in the aqueous solution (the wastewater) where most DNA and proteins went. Hence, the resultant olive oil is the water-insoluble fraction mainly devoid of DNA and proteins, but enriched in organic compounds that would interfere with DNA polymerase reactions. In previous works, we have developed a number of specific molecular markers based on the SCAR technology [26] that served to identify and characterize olive-tree cultivars in Spain [17, 22]. In this work we report the extraction of high quality DNA from olive oil and the utilization of the described SCAR markers in the identification of olive oils.

## Materials and Methods

***Samples and olive-tree cultivars:*** Oil-manufacturers currently introduce the pressed olive oil in a decanter or a clarifier to remove particles from oil before final filtering. This clarification produces a waste residual that will be called "oil sediments" in this study. Oil sediments consisted in cells and small tissue pieces from olive drupes that were not separated with pomace. The resulting oil without these solids will be called "filtered oil". It is important to note that some brands produce "unfiltered olive oil" that has been incompletely clarified and will be processed like oil sediments and filtered oils, these bottles being the most desirable for genotyping. Olive-tree leaves, oil sediments and filtered oils (the same that can be purchased in supermarkets) were provided by oil-manufacturers and stored at room temperature and dark (as in the manufacturer's stores) until use. Samples called "Axarquía" were obtained from Aceites Montosa (Valle Niza, Spain) and the olive oil is mainly produced by Verdial de la Axarquía, Nevadillo Blanco and Picudo de la Axarquía cultivars. Samples called "Ronda" were obtained from Suragro (Ronda, Spain) and the olive oil is mainly produced by Morisco, Verdial de Ronda, Picudo de El Burgo, Lechín de Sevilla and Picudo de Ronda cultivars. Finally, samples called "Guaro" were obtained from "S.C.A. El Molino de Guaro" (Guaro, Spain) and the olive oil is mainly produced by Manzanillo and Hojiblanca cultivars [17, 27].

***DNA extraction:*** The DNA from leaves was used as a source of genomic DNA for positive controls in PCR experiments. It was prepared following previously described methods [17].

DNA from oil sediments was obtained as follows: 25 to 500 g of olive oil were centrifuged at 30,000xg for 15 min at 20°C to obtain 1 g of sediments. The supernantant (olive oil) was discarded and the pellet was homogenized with mortar and pestle in the presence of liquid nitrogen. The fine powder was vigorously mixed with TEA (100 mM Tris, 10 mM EDTA, 0.1% ascorbic acid, pH 8.0) and chloroform in the proportion 1:1:1 (w/v/v). After centrifugation at 9,000xg for 30 min at 15°C, the aqueous phase was precipitated with 2 volumes of ethanol in presence of 0.3 M sodium acetate and 4 µg/mL of glycogen at -20°C for 2 h, and subsequently centrifuged at 35,000xg for 30 min at 4°C. The pellet was resuspended in 1 mL of sterilized distilled water and incubated with 10 µg/mL RNase for 20 min at 37°C. After that, the sample was treated with 6% insoluble PVP [MW 360000] for 1 h at 75-80°C. PVP was removed by centrifugation and the treatment was repeated with 4% insoluble PVP. The aqueous phase was precipitated with 1.6 M NaCl and 13% PEG for 10 min and centrifuged at 20,000xg for 30 min at 4°C. The pellet was washed with 70% ethanol and recovered in 50 µL of sterilized distilled water. The sample was electrophoresed in 1% low melting point agarose gel for 5 minutes at 7 V/cm and the gel was sliced to purify the DNA using Qiaquick Gel Extraction Kit (Qiagen) and following the manufacturer's instructions. The purified DNA was finally resuspended in 50 µL of 10 mM Tris pH 8.5. One extraction can take around 28 h. DNA from 100 g of olive oil was extracted by magnetic stirring for 1 h at room temperature with 20 mL of TEA. The phases were separated by centrifugation at 35,000 xg for 30 min at 20°C. The aqueous phase was utilized to extract 100 g of new olive oil. This process was repeated to finally process 500 g of olive oil. The resultant aqueous phase was extracted with an equal volume of chloroform and centrifuged at 9,000xg for 15 min at 20°C. The upper phase was collected and precipitated overnight with 0.3 M sodium acetate, ethanol, and 4 µg/mL glycogen and, then, subjected to the same treatment as DNA from olive oil sediments as previously described. The only important difference is that DNA extracted from olive oils was purified only once with 4% PVP. DNA concentration was estimated by ethidium bromide staining using the stained drop method [28].

***DNA amplification and detection:*** RAPD analyses were carried out using 200 µL extra-thin tubes in a volume of 20 µL containing 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1 mM each of dATP, dCTP, dGTP and dTTP, 1 µM primer and 1 U of EcoTaq (EcoGen, Spain), and 1 µL of DNA (from 2 to 0.05 ng). The primers used in this study were OPF6 and OPAH2 (Operon Technologies, USA) that amplify at least one band in all cultivars [17]. Amplification reactions were performed in a Biometra thermal cycler programmed for 1 cycle of 1 min at 91°C followed by 42 cycles of 1 min at 91°C, 1 min at 36°C and 1.5 min at 72°C, for denaturing, annealing and primer extension phases respectively. The last cycle was followed by 7 minutes at 72°C. The SCAR assay was carried out in standard PCR reaction that consisted in 0.5 min denaturation at 94°C followed by 50 cycles of 40 s at 94°C, 50 s at the annealing temperature (58°C), 1 min extension at 72 °C, and a final extension of 10 min at 72°C. In the RAPD and SCAR reactions, the Mg concentration revealed critical since concentrations of 2 mM or higher $MgCl_2$ provided mainly non-specific amplifications. All reactions were performed in duplicate, one with DNA and another without it as negative control. In cases where amplification was observed without DNA, the result of its companion was discarded. Amplification products were resolved electrophoretically in a 1.5% agarose gel in 1X TAE buffer and stained with 0.5 µg/mL of ethidium bromide. Electrophoresis was carried out for 1 h at 7 V/cm and the image was taken with the Imagestore 5000 (Ultra Violet Products).

## Results

***Both olive oil and sediment contain DNA:*** We set up a method that can extract DNA from olive oil and oil sediments with enough purity to be amplified by using specific molecular markers. Due to the small amount of DNA isolated from oil,

ethanol precipitation was helped with glycogen as a carrier. The DNA extracted was significantly contaminated with RNA since the sample treatment with 10 µg/mL RNase removes an intense band in the front of the gel (Figure 1). The small pieces of RNA as well as other small molecules were removed with a precipitation in the presence of PEG. However, the resuspended DNA was a coloured solution that produced unrepeatable results and imposes the utilization of small volumes of DNA to perform PCRs. This suggested that polymerase inhibiting substances were still present since further purification steps were needed to use this DNA as a template.

An additional purification on agarose gel with Qiaquick kit was introduced to remove contaminant molecules and to produce a high quality DNA for specific and reproducible amplifications. In these conditions, we have been able to produce from 0.2 to 2 ng of DNA per gram of olive oil sediments. The yields suggested that we have to extract at least 1 g of sediments to produce enough DNA for a few PCR reactions. The DNA sizes shown in Fig 1 ranged from 15 to 0.2 kbp, limiting the use of molecular markers because amplifications longer of 1000 bp are difficult to produce. For example, the PF84 marker has never produced the 0.8 kbp band, and RAPDs fail to amplify bands over 1 kbp (results not shown).

As filtered olive oils do not contain any particle, DNA must be solubilized in an aqueous solution by means of oil emulsion. Due to fractionation coefficient of DNA, the amount of DNA dissolved in oil is expected to be low. Hence, we decided to emulsion a volume of oil with a 1/5 volume of an aqueous solution that will be re-used to extract further quantities of olive oil. Pure water as well as solutions with different pH and ionic strength were assayed as aqueous solutions, but the best yields and purities were obtained with the TEA buffer (results not shown). 0.1 to 0.5 pg of DNA were isolated from 1 g of olive oil, indicating that we have to extract at least 500 g of olive oil to obtain enough DNA for several PCR assays. The same results were obtained with olive oils from the current year as well as olive oil from the previous years, suggesting that provided that olive oil is not oxidized or get rancid [5], DNA is not modified during storage in the dark.

***DNA from olive oil and sediments produce specific and concordant amplifications:*** In addition to agarose gel electrophoresis, the presence of DNA was initially put in evidence by RAPD amplification with the decamer OPF6 (result not shown). However, the non-specific amplification performed by PCR, the low amount of DNA obtained from filtrated olive oil, and the extensive manipulation of samples can account for the amplification provided that a contaminant DNA were present. The molecular marker GF10, which is olive-tree specific and amplifies a single, common DNA fragment only in olive tree cultivars [22], was assayed. It produced the same amplification using genomic DNA obtained from leaves, olive oil sediments and filtered olive oil (Figure 2). The same agreement was obtained with other SCAR markers like GX4B (result not shown). It is clear that amplification of DNA from sediments produced the most similar results to genomic DNA, while DNA from olive oil produced a little difference, always amplifying the characteristic GF10 band of 358 bp. The presence of other kind of amplifications can be explained by the low concentration of DNA obtained from olive oil. We can

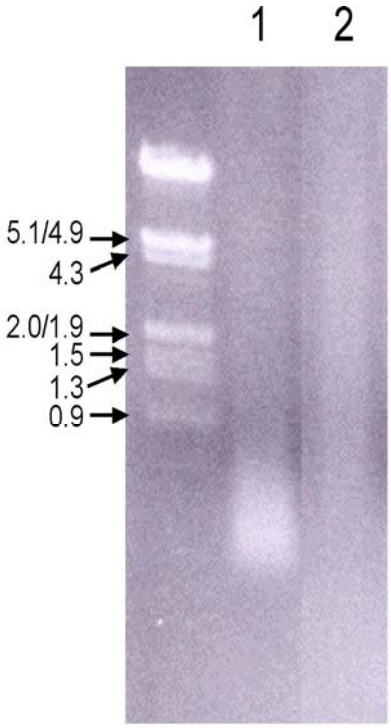


***Figure 1.*** Nucleic acid isolation from olive oil sediments. Lane 1 contains the total nucleic acids purified from 10 g of oil sediments without RNase treatment. Lane 2 shows the same sample treated with RNase and precipitated with PEG. The molecular weight marker is lambda DNA digested with *Hin*dIII and *Eco*RI.

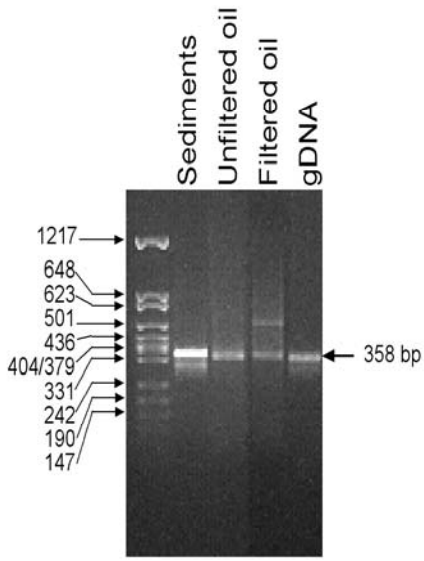


***Figure 2.*** Typical ethidium bromide-stained agarose gel of amplification products using Ronda samples with the SCAR marker GF10. 1 ng of DNA from sediments, 10 pg of DNA from olive oil, and 2 ng of genomic DNA were used. gDNA refers to genomic DNA obtained from leaves from cultivar Hojiblanca. The same amplified band of 358 bp was obtained in all DNAs. Unfiltered olive oil was centrifuged at 30,000xg for 30 min to isolate the sediments and treat them as described in materials and methods; the supernatant was also extracted like filtered olive oil; the resulting samples were pooled to isolate DNA. The molecular weight marker is plasmid pFL61 DNA digested with *Hpa*II [17].

suggest that molecular markers producing small DNA fragments can be used to fingerprint olive oils and to compare this fingerprint with the results obtained in cultivars used to produce such oil. Whenever possible, fingerprinting with DNA obtained from sediments should be preferred.

SDS-PAGE analysis of these samples and further silver staining showed that DNA is accompanied of a few proteins (R. Sampalo, F de la Torre and M. G. Claros, unpublished results), usually less than described for seed oils [29, 30]. Further work will be carried out to identify such proteins since they can be used to strengthen the authentication of olive oils.

***Olive oil can be fingerprinted from itself as well as from its sediments:*** Figure 2 proved that SCAR markers could be used to amplify specific DNA fragments from DNA prepared from sediments and from olive oil. Next is to set up a method founded on the application of SCARs to distinguish oils prepared from different olive-tree cultivars. The SCAR markers previously

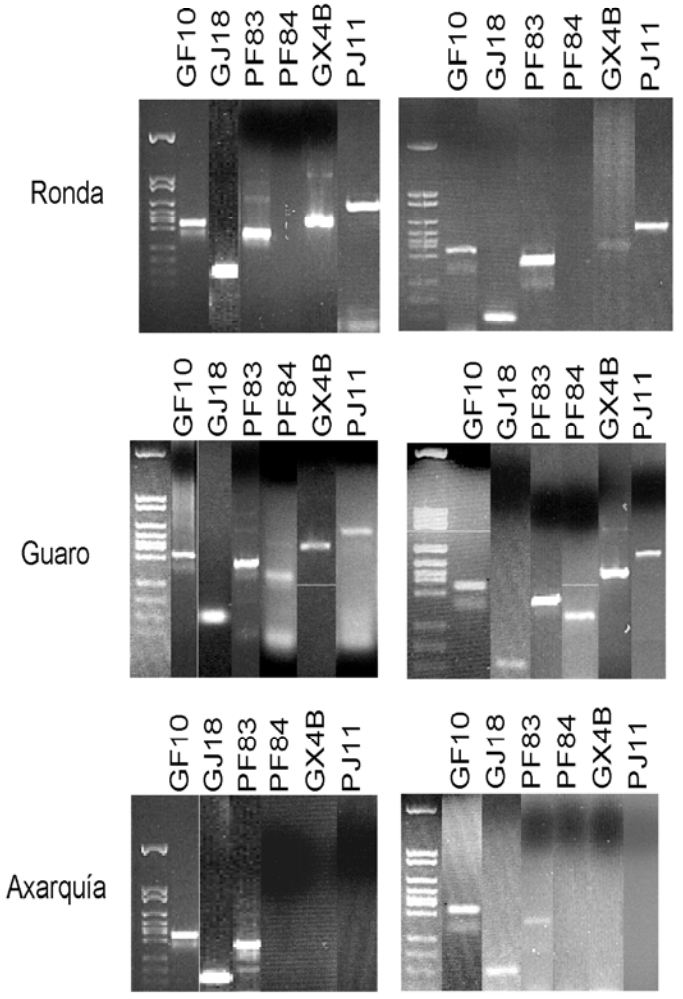


***Figure 3.*** DNA fingerprinting of the Ronda, Guaro and Axarquía olive oils and their sediments using the 6 SCAR markers GF10, GJ18, PF83, GX4B and PJ11. All amplifications were repeated at least three times in order to guarantee the negative amplifications. Every amplification was accompanied of a negative control that do not contain DNA (lanes not shown); only amplifications that provided a blank negative control were used to elaborate the pattern. The molecular weight marker is plasmid pFL61 DNA digested with *Hpa*II (see Figure 2).

olive oils prepared by the manufacturers using different olive-tree cultivars. Leaf DNA was used as a positive control in all experiments. The six SCAR markers assayed provide positive and repetitive results on the three olive oils, giving a complex pattern that enabled one to differentiate the olive oils patterns by visual inspection (Figure 3). The 358 bp band of GF10 is common in all cases since it was described as a positive control for olive tree DNA [22]. The cases of GJ18 (which provides a 150 bp fragment) and PF83 (giving a 310 bp DNA band) are similar: they show a constant pattern since they are present in all cultivars considered in our study [22]. The main differences can be seen in the cases of markers PF84, GX4B and PJ11. PF84 does not amplify anything although a slight 284 bp fragment was shown in Guaro samples. GX4B provides a 379 bp DNA fragment in Ronda and Guaro samples while it does not amplify anything in Axarquía samples. The same behaviour was observed with PJ11, which amplifies a band of 447 bp only in Ronda and Guaro samples. In fact, PF84, PJ11 and GX4B can be used as a specific marker to distinguish the oils.

## Discussion

The degree of unsaturation in oils and fats is an important indicator of the likely development of rancidity in foods. It is also used to predict physico-chemical oil properties. In the same way, the estimation of the proportions of the different types of acyl groups in edible oils is of great interest both from a nutritional and technological point of view. Currently, only studies like the gas chromatography, the Iodine Value or $^{1}H$ NMR allow determination of chemical and organoleptic properties [5], while DNA should serve to fingerprint olive oils. Although DNA is water-soluble, a small fraction of this macromolecule is retained in oil during the olive grinding, since some laboratories have been able to produce RAPD results from olive oil [23, 24]. Other studies have also been able to isolate intact proteins from seed oils [29-32]. The aim of our study was to find out whether commercial olive oils could be fingerprinted and if these fingerprints can be correlated to the pattern obtained from sediments or residuals and the olive tree cultivars utilized for its obtention. The first part of our work set up the DNA extraction methods from olive oils and oil sediments. Next is the fingerprinting of the extracted DNA.

***A reliable method to extract DNA from olive oil:*** The olive oil was stored at room temperature instead of 4°C to follow the same storage conditions than the manufacturers. This guarantees that if we are able to obtain positive results with our samples, the same result ought to be obtained from the manufacturer's stored oils. The protocol described in this study permits to extract DNA from filtered olive oil as well as from the sediments found in bottles or storing containers at the manufacturer's place. It is thought that the manufacturing process, especially the degree of refinement, may influence the biomolecule content of olive oil samples. However, the purified DNA is suitable for PCR characterization (using both RAPD and SCAR) of olive oils in a repetitive way. This method has also been tested successfully with refined olive oil as well as seed oils (that have to be refined before commercialization). One can perform RAPD as well as SCAR amplifications on these DNAs (J.M. Fernández y M.G. Claros, unpublished

results).

It could be reasonable to think that RAPD amplification could be explained by contaminant DNA due to the low DNA concentration and the extensive treatment. The specific amplification of GF10 marker as well as other SCAR markers derived from olive tree DNA guarantees that amplification band analyzed in this work were obtained from genomic olive oil DNA and not from contaminating DNA. There is also an internal limitation that could serve as a control: if amplification of high molecular weight bands (more than 1 kbp) is detected, one should suspect that the template was a contaminant DNA, since no amplification band use to be obtained over 1 kbp when olive oil DNA was extracted.

Hence, the described protocol enables the DNA extraction from olive oil in enough amount and purity to permit a fingerprinting analysis. Moreover, we have tested this protocol in other oily solutions. The main difficulty is the low concentration of DNA found in oils, since we can see that the better cases provide 0.5 pg of DNA per millilitre of olive oil. On the other side, the presence of lipids, that can bind to DNA and mask its structure, is a source of inhibitors of the DNA polymerase reactions. This was suggested by the fact that DNA that was not purified with Qiaquick produced arbitrary results.

***A reliable olive oil fingerprint:*** In contrast to previously described [23, 24], amplification of DNA templates from sediments or from oils produced concordant results among themselves as well as with the genomic DNA (Figures 2 and 3). This could be due to the level of purity provided by the method described in this work, since we have demonstrated that extracted DNA without affinity column purification and RNase treatment (Figure 1) is hard to amplify. In all cases, DNA extracted from sediments provide more consistent results with those obtained from genomic DNA: sediments are composed of cells and tissue fragments that have not pelleted during centrifugation or the decanter process while olive oil only contains DNA that was not dissolved in the wastewater during the olive oil obtention. This could explain the low amount of DNA obtained from olive oil. Moreover, this low amount of DNA could also explain why in a few cases the SCAR amplification provides slightly different pattern with respect to genomic DNA or DNA from sediments.

Molecular markers based on PCR amplification are widely used and their number is continuously increased. This is due to their capability to detect extremely low amounts of DNA and the easiness of use. Currently, these markers are tested against genomic DNA for plant breeding programmes, genetic relations, or identification of varieties or species for legal rights or scientific knowledge. We have been able to use these markers with oil to fingerprint and differentiate olive oils produced by different manufacturers (Figure 3). The differences found in the SCAR patterns shown in Figure 3 can be used as a characteristic fingerprint of each oil with the certainty that these amplifications are only obtained with olive DNA and not with DNA from any other contaminant origin (as could be the case if RAPD are utilized). The agreement among the main amplification bands obtained using genomic DNA, sediment DNA or olive oil DNA strengthens the reliability of our method.

The main risk of this method is to identify a negative result as an absence of amplification where olive oils are used as DNA source. In this case, the extremely low amount of DNA obtained from filtered olive oil could not be enough to amplify a SCAR marker. This false negative can only be discarded when a similar result is repetitive and agrees with the one obtained with DNA extracted from sediments (Figures 2 and 3). The task can be facilitated increasing the number of SCAR markers used to characterize the olive oils: new markers for seed oil ought to be developed.

The presence of proteins in olive oil extracts could open the study of any allergenic activity in olive oil, since allergy to olive-tree affects more than 30% of the population in the Mediterranean area [33]. We have confirmed the presence of proteins in filtered olive oils. Development of antibodies against these proteins could open a new approach or a kind of verification to authenticate the olive oils and detect any kind of mixing with seeds oils, where antibodies against them have been described [29, 30, 32, 34].

In addition to the scientific supply of these results, an important industrial utility has been put in evidence: the method described in this paper can be adapted to control the olive-tree cultivars that were used in the elaboration of olive oils to avoid fraud by mixing olive oil with seed oils. We report success where previous works always refer an inconsistent pattern [23, 24]. Thereafter, we have to focus on the development of additional specific markers that should increase the reliability of the method used to identify olive tree cultivars and olive oils, certify their origin and use it as quality control for Labels of Origin or Preserved Origin Denominations. Multiplex PCR could be used to simplify the final diagnostic.

## Conclusions

The study presented here reports a suitable method to obtain DNA from olive oil as well as its sediments. The main fact is that, for the first time, this DNA can be used for fingerprinting: it can be efficient in enabling easy discrimination of the olive oils with a reasonable number of markers. The protocol could permit a reliable authentication (it provides consistent and nearly identical results using DNA from olive leaves, oil sediments and olive oil) of olive tree cultivars used for olive oils protected by a label of origin although more molecular markers have to be developed to increase the accuracy and extend the amount of olive oils analyzed successfully. Since we have described how to fingerprint oil from their sediments, this protocol can be used to follow the utilization of the same containers for different oils at the manufacturer place.

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