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Exploitation of the Chloroplast *trn*L (UAA) Intron Polymorphisms for the Authentication of Plant Oils by Means of a Lab-on-a-Chip Capillary Electrophoresis System

STELIOS SPANIOLAS, CHRISTOS BAZAKOS, MURAD AWAD, AND PANAGIOTIS KALAITZIS*

Department of Horticultural Genetics and Biotechnology, Mediterranean Agronomic Institute of Chania (MAICh), Alsyllio Agrokepiou, P.O. Box 85, Chania 73100, Crete, Greece

Methods to discriminate plant oils facilitate the detection of either deliberate or accidental adulteration. To this direction, the variability in length among plant species of the chloroplast *trn*L intron was exploited for the authentication of edible and cosmetic plant oils, with an extra emphasis on olive oil. The methodology was based on the combinatorial use of a PCR assay with a capillary electrophoresis system such as the lab-on-a-chip technology. Application of the assay on DNA extracted from different oil producing plant species, including olive oil and sesame oil, indicated the ability of the *trn*L intron to be used as an analytical target. Furthermore, this assay could be used for the detection of adulteration of olive oil with various other plant oils, with the exception of avocado and sesame oil.

KEYWORDS: Food forensics; oils authenticity; olive oil; lab-on-a-chip; chloroplastic DNA

INTRODUCTION

The authenticity, labeling, and traceability of raw and processed food are of major concern for the food analysis and quality assurance sectors due to socio-economic and public health considerations. The food commodities which command a premium price in the market are expected to be labeled accurately because they are most vulnerable to fraud due to deliberate adulteration with similar products of lower price and nutritional value (*1*). The need for monitoring misdescription and food fraud as well as determining food authenticity resulted in the development of appropriate analytical approaches and technological platforms (*2*).

Olive oil is one such commodity which is adulterated with other edible oils originated from plant species such as sesame (*Sesamum indicum*), soya (*Glycine max*), sunflower (*Helianthus annus*), maize (*Zea mays*), hazelnut (*Corylus avellana* and *C. americana*), and cotton (*Gossypium hirsutum* and *G. barbadense*) due to the price differential (*3*). Most of the latter are potential food allergens, while soya, maize, and cotton cause additional concerns due to the presence of genetically modified organism (GMO) varieties in the market. Therefore, appropriate directives and regulations have also been developed for food commodities that contain GMOs or ingredients originated from allergenic plant species. In addition, the cosmetics industry uses oils derived from plant species such as almond (*Prunus dulcis*), avocado (*Persea americana* and *P. gratissima*), and walnut (*Juglans regia* and *J. nigra*), which are also potential targets for adulteration. As a consequence, there is a need for a reliable, simple, and sensitive analytical method to detect the plant species from which the oils and/or oil mixtures were originated.

DNA-based analytical techniques are considered appropriate for the detection of the plant species present in a food commodity (*1*). However, the isolation of acceptable quality DNA from the food commodity is the most crucial step for the successful application of such approaches. In this context, the ability to recover DNA of acceptable quality from plant oils is no longer considered to be a difficult task due to the availability of a wide range of commercial DNA extraction kits, published protocols, and comparison studies (*4, 5*). Although several

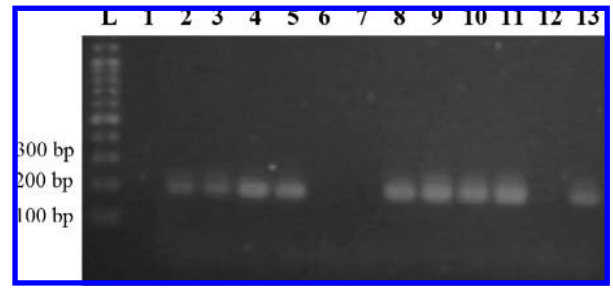

Figure 1. Agarose gel electrophoresis (2% v/v) of PCR amplifications on DNA samples extracted from sunflower oil with three different extraction methods, using primers for the *rbc*L gene: (L) 100 bp DNA ladder; (1) nontemplate control; (2−3) undiluted DNA from DNA*Extractor Fat* kit; (4−5) undiluted DNA from Stool kit; (6−7) undiluted DNA from Wizard kit; (8−9) diluted DNA from DNA*Extractor Fat* kit; (10−11) diluted DNA from Stool kit; (12−13) diluted DNA from Wizard kit.

* To whom correspondence should be addressed. Phone: +30 28210 35000. Fax: +30 28210 35001. E-mail: panagiot@maich.gr.

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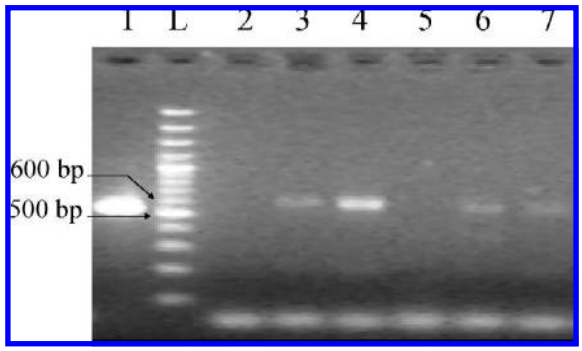

Figure 2. Agarose gel electrophoresis (2% v/v) of PCR amplifications on DNA samples from filtered olive oil using the (c) and (d) primers. The oil was stored for up to 6 months in transparent plastic bottles at supermarket conditions up to six months: (1) positive control based on DNA from olive leaves; (L) 100 bp DNA ladder; oil stored for (2) 2 days; (3) 10 days; (4) 20 days; (5) 40 days; (6) 90 days; (7) 180 days.

reports have been written on the development of molecular markers for the discrimination of olive cultivars (*6–8*) and their application in determining the authenticity of olive oil (*9, 10*), there are no similar reports on the identification of plant species from which various oils are derived. However, several studies on the molecular evolution (*11*) and plant DNA barcoding (*12–14*) have indicated regions from the plastid genome that could potentially be used for the above purpose.

The polymorphic chloroplast *trn*L intron was exploited for the development of a PCR assay combined with capillary electrophoresis in order to discriminate plant species which are used as a source for plant oil production. Particular emphasis was given to olive oil authentication and adulteration with edible plant oils. The Experion lab-on-a-chip capillary electrophoresis system was used to simplify and increase the reliability of the proposed methodology following previous reports on the authenticity of fish species (*15*), raw (*16*) and processed coffee beans (*17*), and more recently on the varietal identification of olive oils using microsatellites (*18*). The resolution of the system and its potential efficiency to detect and discriminate a range of oil-producing plant species were tested, and issues such as DNA extraction protocols on the PCR assay were also discussed.

MATERIALS AND METHODS

Plant Tissue and Oil Samples. Olive, sunflower, avocado, walnut, hazelnut, and almond leaves and sesame, soy, corn, and cotton seeds

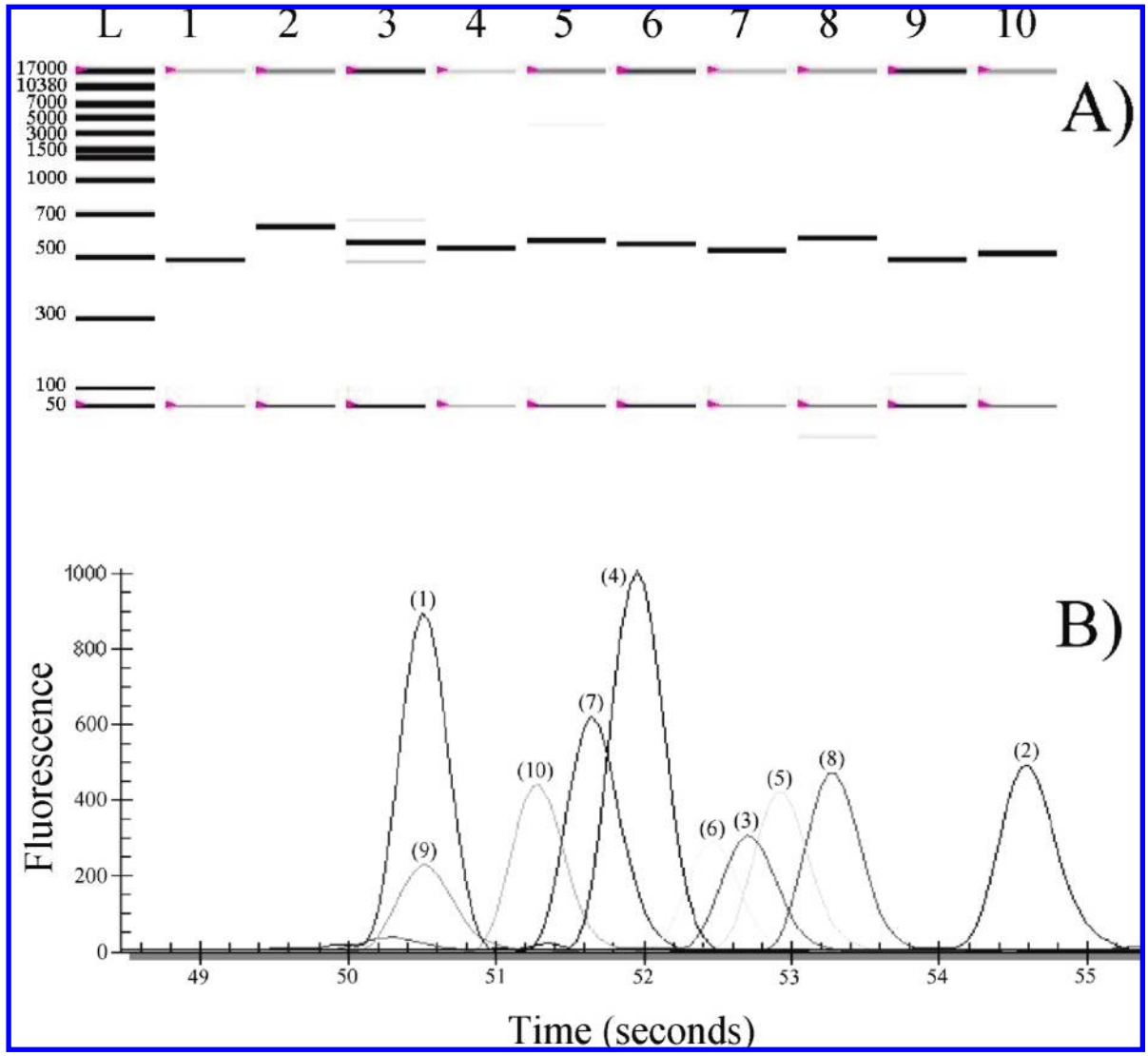

Figure 3. Electrophoretic mobility of PCR amplicons, using (c) and (d) primers from leaf or seed DNA of 10 plant species tested, as detected on lab-on-a-chip capillary electrophoresis. (**A**) Virtual gel and (**B**) superimposed electropherograms: (1) sunflower; (2) cotton; (3) soya; (4) sesame; (5) walnut; (6) almond; (7) avocado; (8) hazelnut; (9) corn; (10) olive.

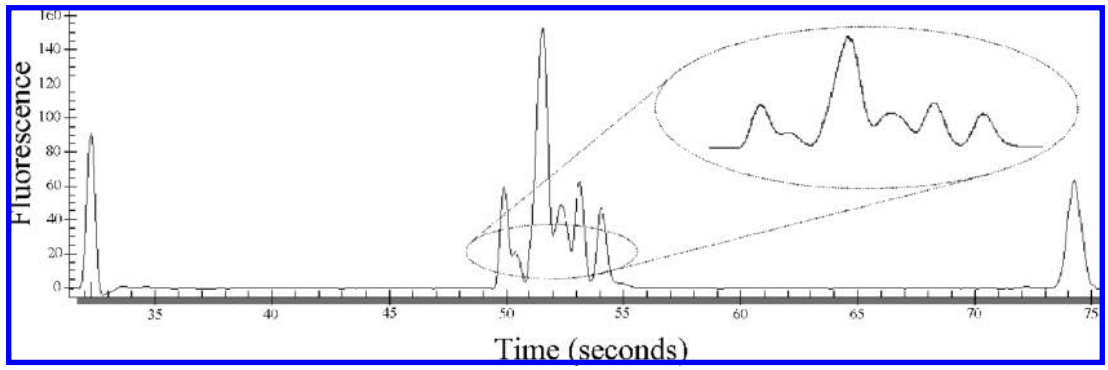

Figure 4. Electropherogram of pooled PCR products using the (c) and (d) primers on leaf or seed DNA of 10 plants species tested, as detected on lab-on-a-chip capillary electrophoresis.

Table 1. Comparison of Amplicons' Sizes as Measured with Experion Capillary Electrophoresis and Sequencing of Cloned PCR Products

	measured size (bp)[a]	STDEV[b]	CV%[c]	actual size (bp)[d]	difference between actual and measured size	% error
walnut	577	8	1.4	600	23	3.8
sesame	539	5	0.9	563	24	4.3
olive	522	6	1.1	548	26	4.7
hazelnut	591	10	1.6	M.V.[e]	M.V.[e]	M.V.[e]
almond	561	8	1.3	590	29	4.9
cotton	633	9	1.3	651	18	2.8
corn	496	5	1.0	533	37	7.0
avocado	532	4	0.7	558	27	4.7
soya	568	2	0.3	585	17	3.0
sunflower	492	1	0.1	511	19	3.7

[a] Average from three replicates as given from Experion's software. [b] Standard deviation. [c] Coefficient of variation. [d] As given by sequencing results generated in this study. [e] Missing value.

found in the herbarium and the seed bank of the Mediterranean Agronomic Institute of Chania (Greece) were used for DNA extraction. Filtered extra virgin olive oil packaged in a tin can was provided by the industrial olive oil cooperative (ABEA) "Anatoli" based in Chania (Greece). This oil sample (one lot) was stored for 6 months in a growth chamber under controlled conditions of 25 °C and 12 h photoperiod as described elsewhere (*19*), in order to simulate retailer standard storage conditions. DNA extractions and PCR amplifications were carried out in triplicates.

DNA Extraction from Plant Tissue and Oil Samples. DNA was extracted from 100 mg of plant leaf or seed tissue based on a previously published CTAB protocol (*20*). Phenol chloroform extraction and ethanol precipitation were carried out as described elsewhere (*21*).

DNA was extracted from plant oil samples using the DNA*Extractor Fat* kit (Genescan Analytics, GmbH, Germany) and the QIA*amp* DNA Stool mini kit (Qiagen, Valencia, CA) according to manufacturers' instructions and the Wizard Magnetic DNA Purification System for Food (Promega, Madison, WI) with minor modifications (*22*). The QIA*amp* kit was applied on a pellet that was obtained after centrifuging 50 mL of oil sample at 14000*g* for 15 min at 4 °C.

PCR Amplification of DNA from Plant Tissue and Oil Matrix and Detection with Capillary Electrophoresis. The PCR reactions were carried out using the following reagents and conditions unless otherwise stated: 1X AmpliTaqGold PCR buffer, 2.5 mM $MgCl_2$, 200 μM for each dNTP, 30 nM for forward and reverse primer, 1.0 unit of AmpliTaqGold *polymerase* (Applied Biosystems, Foster City, CA) and 5 μL of undiluted DNA template per 50 μL of reaction volume. Nuclease free water (Sigma-Aldrich Corporation, St. Louis, MO) was also added up to the appropriate volume. After an initial denaturation step at 95 °C for 10 min, 40 PCR cycles were followed with the denaturation step at 95 °C for 30 s, the annealing step at 60 °C for 30 s and the extension step at 72 °C for 60 s. A final extension step at 72 °C for 10 min was also included, and the PCR samples were stored at −20 °C prior to any further analysis. The PCR primer pairs used for the analytical assay were the (c) CGAAATCGGTAGACGCTACG and (d) GGGGATAGAGGGACTTGAAC oligonucleotides (*13*), whereas those used for the assessment of the quality of the extracted DNA were the 5′-AATCTTCTACTGGTACATGGA-3′ and 5′-GTAAACATGT-TAGTAACAG-3′ (MWG-Biotech, GmbH Germany). The latter primer pair amplified a chloroplast fragment of 174 bp length from the rubisco (*rbc*L) gene. The quality as well as the presence of PCR inhibitors on the extracted DNA was also tested using PCR amplification of a yeast DNA fragment with yeast specific primers (*19*). All PCR reactions were carried out in two replicates for evaluation of DNA extraction methods and in three replicates for the analytical assay in the capillary electrophoresis system.

PCR amplicons were then applied on a 12 K DNA LabChip and detected with the Experion automated capillary electrophoresis system (Biorad, Hercules, CA). All virtual gels and electropherograms were analyzed using the Experion software, *System Operation and Data Analysis Tools* (version 1.0). According to the *Instruction Manual* (Biorad), the data analysis is based on use of the DNA 12K ladder along with two internal markers. The ladder generates 13 peaks in the electropherogram, whereas the first and the last ones are identified by the software as the lower and the upper alignment markers, respectively. The two internal markers are present in the Experion DNA 12K loading buffer and are used for the alignment of every sample lane to the corresponding ladder. The upper marker is also used for quantifying the DNA peaks of the sample.

The PCR products were sequenced after initial extraction from the agarose gel and purification with the StrataPrep DNA Gel Extraction kit (Stratagene, La Jolla, CA). The purified products were then inserted into pCR II-TOPO plasmid vectors (Invitrogen, Carlsbad, CA) with TOPO TA Cloning kit (Invitrogen) and transformed into One Shot Mach 1-T1R chemically competent *E. coli* cells (Invitrogen). Finally, plasmids were isolated using the QIAGEN Plasmid mini kit (Qiagen) and subjected to sequencing reactions on both orientations with M13 primers using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems), and sequencing traces were detected through an ABI 310 capillary electrophoresis DNA sequencer (Applied Biosystems) according to manufacturer's instructions.

RESULTS AND DISCUSSION

Evaluation of DNA Extraction Methods. A prerequisite for the use of DNA-based food authentication techniques is the extraction of DNA of acceptable quality without PCR inhibitors. Therefore, an indicative comparison study among different extraction protocols should precede any attempt to develop a DNA-based analytical technique for a particular food commodity (*23–25*). To this direction, three different DNA extraction systems were tested on sunflower oil and olive oil: the DNA*Extractor* Fat kit, the Wizard Magnetic DNA Purification System for Food, and the QIA*amp* DNA Stool mini kit. Sunflower oil was chosen for two reasons: (i) the strict processing steps required for its extraction and (ii) its frequent use in olive oil adulteration (*3*).

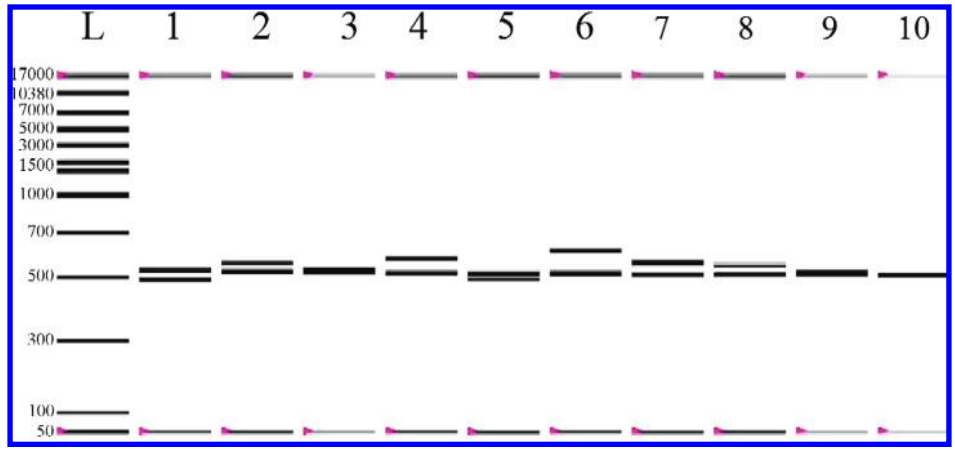

Figure 5. Virtual gel of PCR amplicon pairs, using (c) and (d) primers from leaf or seed DNA of 10 plants species tested, as detected on lab-on-a-chip capillary electrophoresis: (1) olive and sunflower; (2) olive and soya; (3) olive and sesame; (4) olive and hazelnut; (5) olive and corn; (6) olive and cotton; (7) olive and walnut; (8) olive and almond; (9) olive and avocado; (10) olive.

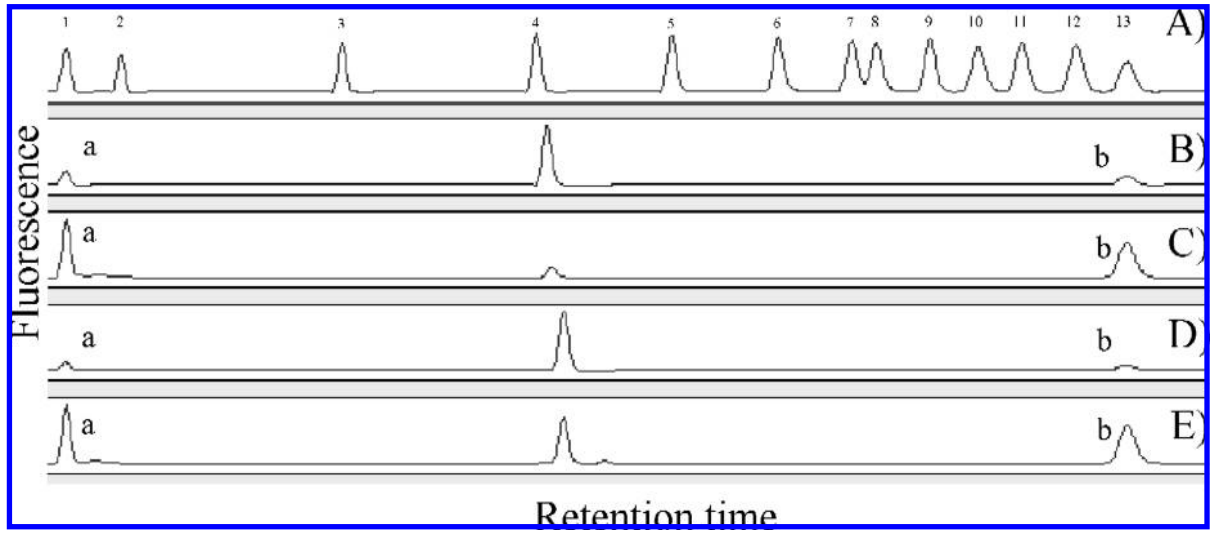

Figure 6. Electropherograms of PCR products, using (c) and (d) primers on DNA samples from olive leaves (**B**), olive oil (**C**), sesame seeds (**D**), and sesame oil (**E**). The lengths of the DNA fragments (**A**) that constitute the ladder used are as follows: (1) 50 bp; (2) 100 bp; (3) 300 bp; (4) 500 bp; (5) 700 bp; (6) 1000 bp; (7) 1500 bp; (8) 2000 bp; (9) 3000 bp; (10) 5000 bp; (11) 7000 bp; (12) 10380 bp; (13) 17000 bp. Internal markers (a and b) are also used for a correct alignment of the electropherograms.

The DNA samples were used as templates to amplify a 174 bp long chloroplastic PCR fragment from the Rubisco (*rbc*L) gene in order to assess the quality of the extracted DNA. Undiluted DNA template extracted with the QIA*amp* Stool kit (lanes 4 and 5) gave a stronger PCR amplification signal compared to the DNA extracted with the DNA*Extractor* kit (lanes 2 and 3), whereas the Wizard kit (lanes 6 and 7) gave no amplification signal (**Figure 1**). DNA diluted with water at a ratio of 1:5 v/v improved the PCR amplification efficiency of the DNA*Extractor* kit (lanes 8 and 9) and the Wizard kit DNA templates (lanes 12 and 13), although only one of the two DNA extraction replicates was successful using the Wizard kit. The positive effect of diluted DNA on PCR amplification has also been reported in food commodities such as olive oil (*22*) and roasted coffee beans (*17*).

PCR amplification of a 250 bp nuclear target using olive oil DNA as template (variety *Chondrolia Chalkidikis* which was extracted using the three different kits) indicated that the QIA*amp* system gave the stronger amplification signal (data not shown). However, diluted olive oil DNA extracted with the QIA*amp* and the DNA*Extractor* kits gave chloroplastic amplicons of similar band intensity. It is very likely that the more the DNA is diluted prior to PCR amplification, the greater the limitation of detection of an adulterant, thus giving QIA*amp* a potential edge over the other DNA extraction methods. Efforts to quantify the sunflower and olive oil DNA were not successful due to the low DNA yield recovered from such matrices (*26*).

Conclusively, the QIA*amp* DNA Stool mini kit proved to be probably the most efficient for isolation of acceptable quality DNA for PCR amplification. However, its efficiency needs to be tested in the other oils of plant origin.

Use of the *trn*L (UAA) Intron as Template for PCR Amplification. The chloroplast *trn*L (UAA) intron (548 bp) was amplified with universal primers using olive oil DNA. This template was extracted using the QIAamp DNA Stool mini kit, and the amplification was successful for oil samples stored from 2 days to up to 6 months (**Figure 2**). However, this result contradicts that of a previous study carried out by the same authors (*19*) in which a 415 bp amplicon could not be amplified from olive oil stored for more than 20 days. This contradiction can be attributed to the nature of the amplification target used in the two studies. In the former case, the amplified target was chloroplastic DNA, whereas in the latter case the target was spiked λDNA. This difference could be explained by the high copy number of chloroplasts found in the plant cell (*27*). The ability to amplify this chloroplastic region seemed to be

consistent regardless of the variation in the source of the DNA template such as olive oil age, variety, and/or lot (data not shown). These results indicate a particular trend which was not observed in terms of reproducibility with the amplification of spiked λDNA.

An additional advantage of the use of chloroplastic DNA as a target for amplification is the presence of universal primers, which can possibly discriminate plant species according to their length (*11*). The same DNA region has been successfully used in the past for the authentication of coffee (*16*) and the detection of potential allergens (*28*). The failure of the amplification signal for lanes 2 (2 days of storage period) and 5 (40 days of storage period) (**Figure 2**) can be explained by the lack of reproducibility of PCR amplifications on DNA from plant oils as was observed in previous experiments (data not shown) and was previously reported (*19*). This lack of reproducibility could be also attributed to several DNA damage mechanisms, which have been extensively reviewed elsewhere (*20*).

Application of the Assay on Oil Producing Plant Species. The discrimination ability of the PCR assay on the chloroplast *trn*L (UAA) intron using universal primers was tested on DNA samples extracted from the leaves or seeds of 10 oil producing plant species, by analysis of the amplicons on a lab-on-a-chip capillary electrophoresis system.

After PCR amplification, only one product (500−650 bp) was produced from each one of the 10 plant species DNA (**Figure 3**). The only exceptions were the soy (lane 3) and corn (lane 9) DNA samples which gave additional, nonspecific amplicons of lower signal intensity. The length of each amplicon could be characterized as species-specific with the exception of sunflower (lane 1) and corn (**Figure 3**). The corresponding peaks of their amplicons overlapped, indicating similar if not identical lengths (**Figure 3**). These results suggest that the chloroplast *trn*L (UAA) intron is polymorphic enough to discriminate 8 out of the 10 oil-producing plant species. Furthermore, pooling and analysis of the 10 amplicons in the capillary electrophoresis system resulted in six peaks that probably corresponded to at least six amplicons according to their respective length (**Figure 4**). Analysis of multiple PCR products generated using universal primers might be problematic due to the incomplete discrimination ability of the lab-on-a-chip system or alternatively due to the small differences in length among the analyzed amplicons.

The lengths of the amplicons determined using the Experion capillary electrophoresis system were compared with those determined after subcloning and sequencing (**Table 1**). The Experion values were always shorter by approximately 24 ± 6 bp, thus producing an error (%) of 4.3 ± 1.3. It is also interesting to note that the reproducibility of the instrument was such that the CV% never exceeded the value of 1.6.

In order to assess the potential effectiveness of this assay on the authenticity and adulteration of olive oil, olive amplicons were mixed with every other plant species amplicon at a 1:1 (v/v) ratio and analyzed in the capillary electrophoresis system (**Figure 5**). The corresponding peaks illustrated adequate discrimination with the exception of sesame (lane 3) and avocado (lane 9) which seemed to exhibit either the same or at least similar electrophoretic mobility with that of olive (lane 10) (**Figure 5**). These results indicate the potential of the assay to detect adulteration in olive oil with oils extracted from seven different plants species unless the adulterants are sesame or avocado oil.

The ability of the capillary electrophoretic system was also tested on DNA samples extracted from olive and sesame oil (**Figure 6**). Amplicon peaks of equal length were obtained from leaf/seed and their corresponding oil DNA samples, thus confirming the effectiveness of the system. This analytical technology provides several advantages over the standard agarose gel electrophoresis, namely higher resolution, ease of use, fast and straightforward analysis, and avoidance of dangerous solvents and chemicals (*15, 16*).

Exploitation of the chloroplast *trn*L intron polymorphism through the use of capillary systems offers a DNA-based analytical assay for the detection and discrimination of oil producing plant species and their corresponding oils. The limitations of this approach are mainly due to the sizing accuracy as given by the manufacturer (±15% approximately) and due to the inefficient resolution. This might justify the lack of discrimination for sunflower and corn although the lengths of their chloroplastic amplicons differ only by 4 bp. When examining the authenticity and adulteration of olive oil, this assay could serve as a preliminary diagnostic test prior to the use of more advanced technological platforms.

The use of a capillary electrophoresis system for the exploitation of chloroplastic polymorphisms, combined with additional analytical steps, could produce an accurate and reliable analytical assay for authenticating plant oils at the level of species. To this direction, future work is needed to determine whether a restriction digest after PCR amplification is able to produce unique peak profiles for each plant species tested, since there are adequate discriminative restriction sites found in *trn*L that could theoretically lead to improved authentication.

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