Chapter 7

DNA-Based Approaches for Traceability and Authentication of Olive Oil

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Additional information is available at the end of the chapter

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Abstract

Authentication and traceability of extra virgin olive oil is a challenging research task due to the complexity of fraudulent practices. Various chemical and biochemical techniques have been developed for determining the authenticity of olive oil and in recent years non-conventional methods based on DNA analysis have gained attention, due to high specificity, sensitivity and reliability. DNA analyses have very high discriminating power because ultimately the unique identity of a variety or species is to a great extent genetically dependent. Polymorphisms are genetic variations which refer to the variation in populations or species. Molecular markers provide information on genetic variations and are valuable tools to determine olive oil authenticity. Recently several DNA-based methods have been developed to authenticate olive oil, since analysis of the residual oil DNA with the use of molecular markers can lead to the identification of the variety or the plant species from which it was extracted. The aim of this chapter is to provide an overview of the current trends and critical issues on DNA-targeted approaches used for traceability and authenticity of olive oil. This is considered a rapidly expanding field with significant challenges and prospects which shall be discussed thoroughly.

Keywords: olive oil and plant oils authentication, traceability, DNA, molecular markers, identification of varietal origin, SNPs, SSRs, biotechnological approaches

1. Introduction

Olive oil is the main component of the Mediterranean diet and one of the most valuable food products of the agro-food industry, not only due to taste, but also due to high nutritional value

[1]. Authentication and traceability of extra-virgin olive oil is a challenging endeavour that requires persistent efforts and continuous progress due to the complexity and advancement of novel fraudulent practices. Authentication uses methodologies, tools and technological platforms aiming at detection, prevention and exposure of adulteration and mislabelling of food commodities. Traceability is the ability to trace a food commodity from the production until the distribution stage (European Council regulation-EC 178/2002).

The various ways of olive oil adulteration mainly comprise (i) economic adulteration that refers to either the mixing of lower-grade vegetable oils with extra-virgin olive oil or with minimally processed olive oils such as non-refined or cold-pressed olive oils and (ii) mislabelling and misleading origin of protected designation of origin (PDO) and protected geographical indication (PGI) olive oils that are legally protected. One of the aims of PDO and PGI is to provide added market value high-quality olive oils that are derived from well-defined geographical regions. In most of the cases, the higher quality might be attributed to either the cultivar from which the olive oil was extracted or the edaphoclimatic conditions of the area, which is known as the 'terroir' in wine industry.

Therefore, adulteration of extra-virgin olive oil occurs not only by accidental contamination during the stages of oil processing, but is an act of deliberate addition of less expensive olive oils by fraudsters for financial profit.

Plethora of chemical and biochemical techniques have been developed for detecting the adulteration while ensuring authenticity and traceability of extra-virgin olive oil [2–6]. However, neither conventional analytical chemistry methods nor the analysis of biomorphological traits is always able to accurately detect the region of origin and/or the olive oil cultivar due to the diverse climatic conditions in which olive trees are exposed every year and their impact on the chemical composition of olive oil [7, 8].

The authenticity of olive oil, especially the extra-virgin, has been extensively studied by using several analytical approaches such as chromatography, stable isotope analysis, spectroscopy and nuclear magnetic resonance [9, 10]. In an interesting review [11], an extensive analysis of the most relevant compounds used as target analytes for olive oil characterisation and authentication was presented. Among others, the division of all analytical methods for olive oil authentication into 'targeted analysis' and 'profiling or non-targeted analysis' was suggested. The targeted analysis is based on the analysis of chemical compounds that appear only in the adulterant oil species (i.e. seed oils) and not in olive oil samples. Whereas the non-targeted analysis refers to the simultaneous detection of many known or unknown analytes belonging to a pre-defined metabolic pathway; usually, there is no differentiation among them. Although the aim is to rapidly determine the genuineness of olive oils, complicated multivariate statistical procedures are needed.

Recently, non-conventional methods based on DNA analysis have gained attention due to their high specificity, sensitivity and accuracy to detect the varietal origin of olive oil as well as the botanical origin of plant oils [12–15]. Moreover, DNA-based methods overcome deficiencies of conventional methods such as denaturation of proteins due to the heating and processing of food commodities [16]. Therefore, non-conventional methods offer an alternative, comple-

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mentary approach since they rely only on the analysis of the DNA. Plenty of biomolecular methodologies have been developed for the authentication of olive oil using DNA markers. Molecular markers provide information on polymorphisms within DNA regions and are considered valuable tools to determine olive oil authenticity. Polymorphisms are genetic variations that can be detected either in nuclear, ribosomal or mitochondrial genomes as well as in the genomes of other organelles, such as chloroplasts.

Several DNA-based methods have been developed to authenticate olive oil, since analysis of the residual oil DNA with the use of molecular markers can lead to the identification of the variety or the species from which it was extracted regardless of environmental conditions during olive fruit's growth [13]. Plethora of studies took advantage of molecular markers for the identification of the varietal origin of olive oil such as SNPs [14, 17], microsatellites [18–20], SCARs [21] and AFLPs [22, 23]. Recent advances in olive genome and transcriptome sequencing increased the analytical targets and enriched the olive molecular markers database.

However, the reliability and reproducibility of these techniques are strictly dependent on the quality of the DNA extracted from oil samples [12, 19, 24, 25]. Trace amount of DNA is found in olive oil, which is highly degradable. Therefore, a high number of DNA isolation protocols were published while several commercial kits dedicated to olive oil DNA extraction are available [26].

DNA-based reaction chemistry was combined with existing detection methods such as capillary electrophoresis, high-resolution melting (HRM), TaqMan probes, qRT-PCR and single-base extension (SNaPshot) resulting in numerous analytical approaches with particular advantages and disadvantages.

The chapter provides an overview of the current trends and critical issues on DNA-targeted approaches used for traceability and authenticity of olive oil. This is a rapidly expanding field with significant challenges and prospects that shall be discussed thoroughly. Moreover, issues of adulteration with oils of plant origin will be thoroughly discussed while recent advances in authentication and traceability of herbs and medicinal plants will be taken into consideration and compared with oil matrices.

2. Recent advances in DNA extraction from olive and other plant oil matrices

A pre-requisite for the development and improvement of polymerase chain reaction (PCR) based DNA fingerprinting methods is the isolation of adequate quality and quantity DNA. Although the quantity of recoverable DNA from plant oils is hardly detectable by any means, either nano-spectrophotometer or agarose gel electrophoresis, most of the times it is sufficient for molecular markers analysis. However, nuclease activity and the presence of PCR inhibitors such as fats, residual polysaccharides and polyphenols might inhibit PCR amplification [19, 21, 24, 27, 28].

The purity of extracted DNA is the most crucial and significant step in DNA fingerprinting methods ensuring the validity and the reproducibility of plant oil forensic methodologies. Therefore, the need for a reliable and reproducible protocol for DNA isolation from plant oils is mandatory for either research or industrial applications. The ideal plant oil DNA extraction method should be reproducible, simple, relatively cheap and capable to recover stable, free of PCR inhibitors DNA either from filtered and/or heavily processed plant oils or from unfiltered olive oils.

In the last 15 years, many extraction methods and commercial kits were used and further improved valuable tools were provided. Commercial kits were used in several reports with positive results in most cases. Testolin and Lain compared the performance of a variety of commercial kits on DNA isolation from filtered and non-filtered olive oil and concluded that most consistent results were obtained using the QIAamp DNA Stool Mini Kit [24]. The potential of this commercial kit to isolate DNA of acceptable quality from oils of plant origin such as sesame oil was validated by Spaniolas et al. [29]. However, the high cost of this commercial kit motivated researchers to develop or modify CTAB (Cetyltrimethylammonium bromide)-based protocols. Muzzalupo and Perri [28] introduced the use of Proteinase K during malaxation to prevent DNA degradation. Intact DNA was then isolated from non-filtered olive oil sediments using the CTAB method of Doyle and Doyle [30]. The main limitation of this method was the use of Proteinase K during malaxation that is not applicable commercially.

The addition of this enzyme prior to the storage of olive oil improved the amount of recovered DNA [24], although this is not recommended for commercial application. Therefore, Consolandi et al. tested the efficiency of Proteinase K by adding it to the pellet and the oily phase after hexane treatment and centrifugation of oil sample, with positive results [31]. However, the innovation of this method relies on the disruption of neutral micelles by adding the surfactant Tween-20 after hexane extraction. Busconi et al. isolated intact DNA from unfiltered laboratory and commercial olive oils, using a CTAB method on 0.5 g of pellet [32]. They were able to amplify up to 1942 bp fragments. Martins-Lopes et al. used the same method, but their starting material was 6 ml unfiltered olive oil and not 0.5 g oil sediment [33]. The various CTAB-based methodologies indicated that the strategy of using common plant DNA extraction protocols can lead to oil matrix DNA isolates of adequate quality.

Alternatively, Breton et al. extracted DNA from variable amounts of olive oil using several techniques such as magnetic beads, silica extraction, spun column and hydroxyapatite [25]. The most efficient approach which that provided free of PCR inhibitors DNA was the magnetic beads. This technique was further applied in many commercial virgin and crude olive oils leading to the claim by the authors that although it needs further improvement, it could be routinely applied in any olive oil sample. Doveri et al. [34] used the official Swiss method for lecithin extraction [35] from 15 ml unfiltered olive oils, resulting to less than 50% successful amplifications due to the low quantity and quality of extracted DNA.

Giménez et al. compared four DNA isolation methods for commercial mono-varietal olive oils [26]. The study included a CTAB-based [36] and a hexane method [31] as well as two modified CTAB-methods, CTAB-hexane and CTAB-hexane-chloroform. The CTAB-hexane-chloroform methodology comprising a washing step with chloroform/isoamyl alcohol in combination

with the addition of linear acrylamide prior to the precipitation proved to have a beneficial effect on recovering DNA of adequate quality and quantity. It is worth mentioning that the starting olive oil volume was reduced to less than 1 ml. This protocol was successfully used to extract DNA from several plant oils indicating the potential of this method for oil DNA forensic applications [37, 38]. Ramos-Gomes et al. improved this protocol by increasing the concentration of Tween-20 in the lysis buffer and reducing the lysis time to 15 minutes while the precipitation time was limited to 1 h without the addition of linear acrylamide [38]. This improved protocol increased the yield and the PCR amplification efficiency of olive and seed oils DNA [38]. Raieta et al. [39] further modified the CTAB-hexane-chloroform protocol [26] by adding more purification steps with more critical excision of major DNA band from 1% agarose gel aiming to remove most of the PCR inhibitors such as residues of organic components or other contaminants. This protocol was successfully applied on various mono- and multi-varietal commercial filtered olive oils.

Recently, Muzzalupo et al. developed an innovative and simple method that bypasses the DNA extraction/purification steps by using KAPA3G Plant DNA polymerase that enables the direct DNA amplification from virgin filtered and unfiltered olive oils [40]. Definitely, this method seems more efficient and faster than the traditionally DNA isolation protocols. However, the success of this approach remains to be tested in lab and industrial applications.

Considering that in the 1990s only a couple of olive oil DNA extraction protocols were reported, the current availability of numerous protocols ensures the positive prospects of DNA-based olive oil authentication and traceability.

3. Olive oil and varietal origin

The olive cultivar together with the region of production directly affects the quality traits of olive oil. Therefore, mono-varietal extra-virgin olive oils or blends of specific cultivars grown in certain regions are considered as premium olive oils of higher value due to specific quality characteristics. These premium olive oils are protected by the European Commission through certification labels of PDO and PGI in order to ensure authenticity and protection of consumers. Therefore, there is a pressing need for reliable identification of the genetic identity of extra-virgin olive oils.

Several attempts were carried out to relate chemical composition of olive oils with the cultivar of origin such as the mono-saturated fatty acids that are major constituents of olive oils and confer high nutritional value. In this effort, many reports used the fatty acids content to discriminate olive cultivars [41–43] while Mannina et al. performed a study in a well-defined and limited geographical region achieving a relationship between the fatty acid composition and some Sicilian cultivars [42]. These studies revealed that although the varietal effect was important for olive oil discrimination purposes based on fatty acid composition, the geographical and environmental effects were strongly affecting this approach [13]. Recently, Laroussi-Mezghani et al. were able to predict the varietal origin of six Tunisian cultivars by

their fatty acid composition and near-infrared spectra associated with chemometric treatment. Other olive oil constituents such as the triacylglycerols have also been widely studied for their discriminatory efficiency on cultivar origin [41, 44–46]. In addition, several attempts were also made to correlate a long list of minor components such as sterols, pigments, phenolic and volatile compounds, hydrocarbons and tocopherols of olive oils with the cultivar origin of olive oil. Although Matos et al. [47] were able to discriminate three olive cultivars based on sterol composition; sterol was mostly studied in combination with other chemical compounds such as fatty acids and triacylglycerols [48, 49].

The volatile compounds are known to be strongly related to the genotype and the geographic origin of olive fruit, giving the unique flavour and quality of olive oil [13]. The volatile fraction in virgin olive oil consists of more than 100 compounds, while the most important substances for olive variety discrimination are the products of the lipoxygenase pathway (LOX) [50].

Despite the plethora of biochemical methods and analytical tools that were developed for the identification of olive oil cultivar, the main issue is the significant effect of climatic conditions and olive oil processing on chemical composition of virgin olive oil.

DNA-based methods can be used as complementary approaches for olive oil authentication and traceability considering a number of advantages over conventional methods such as reliability, specificity and sensitivity without any influence by the environmental conditions.

Currently, there are plenty of reports on the discrimination of olive cultivars using various molecular markers such as amplified-fragment length polymorphism [51, 52], random amplified polymorphic DNA (RAPD) [53], sequence-characterised amplified regions [54], simple sequence repeats (SSRs) [55, 56], inter-simple sequence repeats (ISSRs) [57] and single-nucleotide polymorphisms [58, 59]. However, only few of them were tested on the identification of the varietal origin of olive oil. This can be attributed to the low quality of olive oil DNA due to severe fragmentation as a result of the degradation process.

A study showed that the DNA quality is affected during storage of filtered olive oil in retail store conditions [27]. The amplification of 107 bp DNA fragments was successful for olive oil samples stored up to 1 year, whereas DNA fragments longer than 415 bp were successfully amplified only after 20 days of storage and no longer [27]. Moreover, Montemurro et al. and Pafundo et al. employed AFLP molecular markers to study the effect of olive oil storage length on the use of DNA as an analyte for molecular traceability [28, 60]. They showed a significant deterioration of DNA quality within a month while the AFLP profiles of leaf and oil DNA were not similar after 9 and 12 months of storage. These reports indicate the absolute requirement for short DNA template molecular markers in order to be used for olive oil DNA forensic applications.

One of the first efforts to fingerprint four Italian olive oils was made by Pafundo et al. using AFLP markers [23]. AFLP markers are based on the detection of restriction fragments by PCR amplification using a genome as template [61]. The origin of polymorphism in AFLP is based on base substitutions within restriction sites or deletions/insertions between two adjacent restriction sites. Therefore, the high-quality DNA is a pre-requisite for such a molecular marker. The identity between AFLP profiles in leaves and oils reached a maximum of 70% due

to differences in the level of DNA degradation between leave and oil samples [23]. Three years later, Montemurro et al. reported enhanced AFLP profiles of 10 Italian cultivars by optimising the DNA extraction protocol and restriction/ligation conditions [22]. They also suggested the setting up of an olive oil reference data bank with AFLP profiles, which could be used as an identity card of mono-varietal olive oils. However, the requirements of high-quality DNA and short-storage time for reliable traceability lead to the advancement of other molecular markers such as SSRs and SNPs in combination with high-performance analytical platforms.

The efficiency of RAPD, ISSR and SSR molecular markers for varietal identification of olive oil was evaluated by Martins-Lopes et al. in 23 Portuguese olive oil samples [33]. This study demonstrated that the ISSR marker system was more informative as compared to the inadequate efficiency of RAPD primers in olive oil samples. SSR analysis was performed to compare the profile of DNA samples isolated from olive oil with that of leaves with satisfactory results.

SSRs are also called microsatellites and consist of 1–10 bp tandem repeats, with a variable number of repetitions [62]. They are highly polymorphic due to the variation in the number of repeats. Microsatellites can be detected by PCR amplification using specific primers annealed to the unique flanking sequences providing high discrimination power. So far they are the most popular molecular markers for olive oil fingerprinting purposes.

Although many studies reported identical olive oil and leaf profiles [18, 24, 34, 63–65], the validity of this approach due to occurrence of repeatability problems is mostly dependent on the SSR sequence and the olive oil DNA quality. Another significant concern on the interpretation of the results is related to differences in amplicon size and allele drop-out in olive oil DNA analysis. These issues might be probably attributed to the low quality and quantity of olive oil DNA, which affect the allele amplification.

In several studies, the appearance of additional than the expected alleles was reported. Muzzalupo et al. and Ben Ayed et al. showed the allele contribution of the pollinator cultivar that is present in seed embryo [12, 19]. This finding contradicts many studies that appeared thereafter. However, additional alleles might appear either by accidental mixing with other cultivars during harvesting or by mixing with traces of olive oils originated from other cultivars during oil-milling processes.

The initial attempts to identify olive cultivars in commercial olive oil samples using SSR markers were reported by Pasqualone et al. and Breton et al. [25, 65]. The SSR profiles of olive oil DNA were identical to those obtained from leaves and drupes, showing the potential of SSR marker to be used for forensic applications. Pasqualone et al. demonstrated the effectiveness of SSR analysis in verifying the identity of a PDO olive oil by the genotyping of a limited number of DNA microsatellites [20]. More recently, Pasqualone et al. showed that a single microsatellite marker was sufficient to discriminate Leucocarpa olive oil from six other mono-varietal olive oils providing an identification key based on the PCR amplification profiles [63]. Additionally, Alba et al. demonstrated the potential of a single, highly polymorphic SSR marker to discriminate seven Italian PDO mono-varietal olive oils [18]. Vietina et al. studied the traceability of 21 mono-varietal olive oils using microsatellites, concluding that in parallel with the improvement of olive oil DNA extraction methods, the implementation of authenti-

cation procedures might require selection of highly polymorphic SSR markers that display the same alleles in different laboratories and the robustness of the method should be assessed in an inter-laboratory ring trial [66].

Recently, Montemurro et al. demonstrated the applicability of SSR markers coupled with high-resolution melting analysis for the identification of olive varieties constituting the 'Terra di Bari' PDO extra-virgin olive oil: Cima di Bitonto, Coratina and Ogliarola among a panel of nine cultivars widespread in Apulia region [67]. This assay provided a flexible, cost-effective, and closed-tube microsatellite genotyping method for authentication analysis in olive oil.

Although genetic traceability using microsatellites is a proven, powerful technique, the identification of an unknown mono-varietal virgin olive oil cultivar is not possible without a reference database. Therefore, Ben Ayed et al. constructed the Olive Genetic Diversity Database (http://www.bioinfo-cbs.org/ogdd/) which is a genetic, morphological and chemical database of about 200 worldwide cultivars [68]. This reference database not only enables the identification of unknown olive cultivars based on their microsatellite allele size(s), but it also provides additional morphological and chemical information for each cultivar.

Single-nucleotide polymorphisms are the most abundant type of mutation. SNPs are the most abundant markers in the genome. They are stably inherited, bi-allelic in most cases, co-dominant, and they require short DNA amplicons for genotyping [69]. Moreover, no other molecular marker has such diverse and numerous methods of analysis as SNPs. Therefore, the recent development of low-cost, high-throughput sequencing technologies which reduced the cost of SNP identification in plant species will expand the use of SNP markers in various industrial applications including olive oil DNA forensics.

Reale et al. and Consolandi et al. bypassed the lack of olive genomic sequences and explored the potential of SNPs to discriminate olive cultivars from Europe and Australia [58, 59]. One year later, Consolandi et al. demonstrated the improvement of their previously developed assay for the genotyping of 49 Mediterranean olive cultivars by ligation detection reaction (LDR)/universal array (UA) [31]. In this analytical assay, a ligation detection reaction distinguishes the alleles that are subsequently detected by hybridisation onto a universal array.

Bazakos et al. developed a simple and efficient assay to identify the varietal origin of olive oils using SNPs [14]. A large number of SNPs were identified, and they used those residing in restriction sites as the basis for the development of a PCR-RFLP assay coupled with capillary electrophoresis. Capillary electrophoresis has a much lower limit of detection for DNA fragments compared to agarose gel electrophoresis and can easily discriminate fragments that differ only by few nucleotides in length [15]. Three SNPs were adequate to discriminate five mono-varietal olive oils. Neither paternal contribution of embryos was detected in olive oil samples nor did additional peaks in leaf samples as was the case of additional alleles observed in leaf samples when certain SSR markers were used [18]. This can be attributed to the single-locus nature of SNPs compared to SSR markers.

Kalogianni et al. took advantage of the discriminatory potential of these three SNPs, and they developed the first multiplex SNP genotyping assay for olive oil cultivar identification that is performed on a suspension of fluorescence-encoded microspheres [70]. The developed

analytical assay could be particularly useful in industrial sector and/or in laboratories involved in official control, that is, laboratories that require methods offering high sample throughput.

Uncu et al. developed an SNP-based identification key to ascertain the cultivar origin of Turkish olive oils [17]. They demonstrated a cleaved amplified polymorphic (CAP) DNA assay for SNPs that reside in restriction sites. Five CAPs were adequate to discriminate 17 olive cultivars. Furthermore, the efficiency and limit of their approach for detecting olive oil admixtures was down to a limit of 20%.

Bazakos et al. enriched the Greek SNP database with Lebanese and Tunisian varieties that resulted in the discrimination of six among 13 mono-varietal olive oils, three Greek and three Tunisian, using PCR-RFLP assay combined with capillary electrophoresis [71]. The highlight of this study was the detection of olive oil admixtures down to a limit of 10%. The authors attributed the better limit of detection to the use of different DNA extraction protocols for the olive oil samples between the two studies that might result in higher quality and quantity of isolated DNA. Although PCR-RFLP is a reliable and simple assay, the main limitation is the requirement of SNPs that reside in restriction sites.

4. Adulteration with oils of plant origin: trends and lessons learned

4.1. Introduction

Incidences of olive oil adulteration refer to the mixing of extra-virgin grade with refined olive oils or/and with cheaper oils of other than olive botanical origin. However, apart from the consumers' deception issue, the adulteration of olive oil with other plant oils could possibly introduce a health risk for consumers allergic to plant species from which the adulterant oils are originated [72–74].

The authenticity of olive oil, especially the extra-virgin, has been extensively studied by using several analytical approaches such as chromatography, stable isotope analysis, spectroscopy and nuclear magnetic resonance [9, 10]. In an interesting review, Aparicio et al. presented an extensive analysis of the most relevant compounds used as target analytes for olive oil characterisation and authentication [11]. Among others, it was suggested the division of all analytical methods for olive oil authentication into 'targeted analysis' and 'profiling or non-targeted analysis'. Targeted analysis is based on the analysis of chemical compounds that appear only in the adulterant oil species (i.e. seed oils) and not in olive oil samples. Whereas the non-targeted analysis refers to the simultaneous detection of many known or unknown analytes belonging to a pre-defined metabolic pathway and usually, there is no differentiation among them. Although the aim is to rapidly determine the genuineness of olive oils, complicated multi-variate statistical procedures are needed.

4.2. DNA target analytes

DNA-based methods target analytes that are characterised in terms of DNA length and/or nucleotide sequence that can be species specific, thus indicating whether an olive oil is

adulterated with oil of other botanical origin. A DNA-based analytical approach usually involves the analysis of species-specific DNA fragments or polymorphisms, the genetic variations between or within species. The characteristic of species-specific DNA fragments is the discrimination of rather distantly related organisms such as the case of food allergens [75, 76] and of genetically modified organisms (GMOs) [75, 76]. The characteristic of polymorphism detection is the discrimination of varieties or closely related species through molecular markers. However, when it comes to the exploitation of polymorphisms of organelle DNA, there is great potential for the discrimination of plant species in food matrices.

Chloroplastic DNA (cpDNA) targets have been widely used in the past for plant phylogenetic studies and nowadays have become valuable tools for the authentication studies of plant origin foods. One of the most extensively studied cpDNA fragment is the *rbcL* gene that encodes the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase and has been sequenced from 500 species [77]. Gielly and Taberlet indicated that *rbcL* coding region usually does not contain enough genetic variation to resolve relationships among closely related genera [78]. Therefore, the analysis of non-coding regions of cpDNA was suggested, such as the *trnL* (UAA) intron and the intergenic spacer between *trnL* (UAA) 3′-exon and *trnF* (GAA) gene, since these regions evolve more rapidly than coding regions do, and thus, are expected to be more useful for discrimination purposes.

For food authentication purposes, the cpDNA target of choice is amplified through PCR assay by using universal primers, thus resulting in an amplified cpDNA fragment ready to be analysed for its length. Due to the presence of insertions/deletions, a species-specific PCR product length exists for each plant species. For food mixtures of more than one species, the number of generated PCR products will correspond to the number of species that constitute the mixture. Consequently, the analysis of the length of the PCR product(s) by using standard gel electrophoresis would lead to the identification of the mixture comprising species. For validation purposes or/and in case that higher resolution is needed due to likelihood of almost similar length amplicons, more sophisticated analytical instruments can be used.

The *trnL* (UAA) intron has been used in the past as an analyte molecule in order to trace specific food crops through PCR amplification including sources of potential allergens, such as canola, corn, potato, soybean, rice, peanut and wheat [79] as well as rye, barley, oat, rice, wheat and maize through an oligonucleotide hybridisation array [80]. The same region was employed to develop a simple PCR-based approach to detect olive oil adulteration with other plant oils such as sunflower, sesame, hazelnut, walnut, cotton, soya, almond, avocado and corn using a lab-on-a-chip capillary electrophoresis system [29]. Preliminary results exhibited efficient discrimination potential except for sesame and avocado [29]. This approach was successfully used in previous authentication studies for the analysis of coffee [81] and while it was further improved by using a DNA capillary electrophoresis platform [82]. As a proof of concept, sesame oil could be easily discriminated from olive oil in a reliable way that was validated through a single-base primer extension assay [15]. The lack of discrimination between olive and avocado oils could be overcome by employing polymorphic SNPs within the cpDNA using a single-base primer extension approach [15].

In addition to insertions/deletions, the presence of single-nucleotide polymorphisms within the PCR target can lead to the validation of the plant species as a constituent of the food matrix. A large number of SNP-based analytical methodologies are available to scientists involved in genotyping approaches.

There are numerous reports on new methodologies, enzymatic tools, platforms and optimised protocols for SNP detection methods and analyses. All these SNP genotyping methods have been classified by Syvanen in several groups/assays, based on the main biochemical principle, thus facilitating their study and in-depth comprehension [83]. Recently, a very promising analytical approach appeared such as the high-resolution melting analysis that has been used for olive oil authentication and was first described by Reed and Wittwer [84].

HRM analysis was performed with PCR primers for the *rbcL* gene in order to detect the presence of maize and sunflower oils in artificial mixture of olive oils and particularly in olive-maize oil mix and olive-sunflower oil mix in ratios (v/v) 50/50, 70/30, 80/20 and 90/10. The HRM results showed that both maize oil and sunflower oil could be detected down to 10% limit of detection [85]. The same approach was applied by Ganopoulos et al. to identify the botanical origin of main vegetable oils and their quantitative detection in mixed oils [37]. The adulteration of olive (*Olea europaea*) oil with canola (*Brassica napus*) oil was selected as a case study. The results showed that the universal *rbcL* region is efficient enough to discriminate plant oil species and to detect the existence of 1% of canola oil admixed into olive oil. In another study, the PCR-CE-SSCP method was applied for the first time to detect cheap oils blended into olive oil, by using the chloroplastic *rbcL* gene as DNA target for PCR amplification. Olive along with six other commonly used oil plants was successfully discriminated [86].

In general, SNPs are advantageous molecular markers because of their high density in genomes that permit high-capacity discriminatory power. SNPs should be considered as the marker of choice to trace and authenticate highly degraded DNA extracted from complex food matrices such as oils of plant origin, since they can be genotyped within low size range amplicons [87]. This is very important since the possibility to amplify PCR products of around 80 bp is much higher than that of 200 bp considering that the template DNA was extracted from highly processed vegetable oils [26, 29]. In this way, the chance to detect a potential adulterant species is much higher.

4.3. Limitations

Nowadays, there are enough analytical platforms, instrumentation and discriminative DNA targets/markers for the analysis of olive oil authenticity and the detection of adulteration with plant oils of other botanical origin. However, there are still limitations related to the DNA extraction step. The availability and extractability of residual DNA is an issue that has to be thoroughly studied particularly from a physical chemistry point of view. Further progress on this issue will strongly impact the olive oil authenticity and traceability industry and the pertinent commercial applications.

5. Conclusions

The research field of DNA-based authentication and detection of adulteration of olive oil has been transformed into a very active area of olive oil-related research with substantial progress. This was accomplished through the established reliability of SSR markers and the continuously increasing use of SNPs markers for olive oil DNA forensic purposes. Moreover, the plethora of available technological platforms to support high-throughput SNP genotyping has significantly contributed towards this direction. This would not have been feasible without the unequivocal advancement of new methodologies for the extraction of high-quality DNA from olive oil and oils of plant origin matrices. The conditions are now mature for a significant boost towards industrial applications of DNA-based approaches for olive oil authentication and traceability.

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References

[1] Wahrburg U, Kratz M, Cullen P. Mediterranean diet, olive oil and health. Eur. J. Lipid Sci. Technol. 2002;104:698–705.

[2] Aroca-Santos R, Cancilla J, Matute G, Torrecilla J. Identifying and quantifying adulterants in extra virgin olive oil of the picual varietal by absorption spectroscopy and nonlinear modeling. J. Agric. Food Chem. 2015;63:5646–5652.

[3] Bevilacqua M., Bucci R., Magri AD, Magri AL, Marini F. Tracing the origin of extra virgin olive oils by infrared spectroscopy and chemometrics: A case study. Anal. Chim. Acta. 2012;717:39–51.

[4] Giacalone R, Giuliano S, Gulotta E, Monfreda M, Presti G. Origin assessment of EV olive oils by esterified sterols analysis. Food Chem. 2015;188:279–285.

[5] Korifi R, Le Dr´eau E, Molinet J, Artaud J, Dupuy N. Composition and authentication of virgin olive oil from French PDO regions by chemometric treatment of Raman spectra. J. Raman Spectrosc. 2010;42:1540–1547.

[6] Portarena S, Gavrichkova O, Lauteri M, Brugnoli E. Authentication and traceability of Italian extra-virgin olive oils by means of stable isotopes techniques. Food Chem. 2014;164:12–16.

[7] Alessandri S, Cimato A, Modi G, Mattei A, Crescenzi A, Caselli S, Tracchi S. Univariate models to classify Tuscan virgin olive oils by zone. La Rivista Italiana Delle Sostanze Grasse. 1997;74:155–164.

[8] Barone E, Di Marco L, Motisi A, Caruso T. The Sicilian germplasm and its characterization by using statistical method. Acta Hortic. 1994;356:66–69.

[9] Aparicio R, Aparicio-Ruiz R. Authentication of vegetable oils by chromatographic techniques. J. Chromatogr. Anal. Chim. Acta. 2000;881:93–104.

[10] Osorio MT, Haughey SA, Elliott CT, Koidis A. Evaluation of methodologies to determine vegetable oil species present in oil mixtures: Proposition of an approach to meet the EU legislation demands for correct vegetable oils labelling. Food Res. Int. 2014;60:66–75.

[11] Aparicio R, Morales M, Aparicio-Ruiz R, Tena N, Garcıa-Gonzalez D. Authenticity of olive oil: Mapping and comparing official methods and promising alternatives. Food Res. Int. 2013;54:2025–2038.

[12] Muzzalupo I, Pellegrino M, Perri E. Detection of DNA in virgin olive oils extracted from destoned fruits. Eur. Food Res. Technol. 2007;224:469–475.

[13] Montealegre C, Alegre MLM, Garcia-Ruiz C. Traceability markers to the botanical origin in olive oils. J. Agric. Food Chem. 2010;58(1):28–38.

[14] Bazakos C, Dulger AO, Uncu AT, Spaniolas S, Spano T, Kalaitzis P. A SNP-based PCR–RFLP capillary electrophoresis analysis for the identification of the varietal origin of olive oils. Food Chem. 2012;134:2411–2418.

[15] Spaniolas S, Bazakos C, Spano T, Zoghby C, Kalaitzis P. The potential of plastid trnL (UAA) intron polymorphisms for the identification of the botanical origin of plant oils. Food Chem. 2010;122:850–856.

[16] Lockley AK, Bardsley RG. DNA-based methods for food authentication. Trends Food Sci. Technol. 2000;11:67–77.

[17] Uncu AT, Frary A, Doganlar S. Cultivar origin and admixture detection in Turkish olive oils by SNP-based CAPS assays. J. Agric. Food Chem. 2015;63:2284–2295.

[18] Alba V, Sabetta W, Blanco A, Pasqualone A, Montemurro C. Microsatellite markers to identify specific alleles in DNA extracted from monovarietal virgin olive oils. Eur. Food Res. Technol. 2009;229:375–382.

[19] Ben Ayed R, Grati-Kamoun N, Moreau F, Rebai A. Comparative study of microsatellite profiles of DNA from oil and leaves of two Tunisian olive cultivars. Eur. Food Res. Technol. 2009;229:757–762.

[20] Pasqualone A, Montemurro C, Summo C, Sabetta W, Caponio F, Blanco A. Effectiveness of microsatellite DNA markers in checking the identity of protected designation of origin extra virgin olive oil. J. Agric. Food Chem. 2007;55:3857–3862.

[21] Pafundo S, Agrimonti C, Maestri E, Marmiroli N. Applicability of SCAR markers to food genomics: Olive oil traceability. J. Agric. Food Chem. 2007;55:6052–6059.

[22] Montemurro C, Pasqualone A, Simeone R, Sabetta W, Blanco A. AFLP molecular markers to identify virgin olive oils from single Italian cultivars. Eur. Food Res. Technol. 2008;226:1439–1444.

[23] Pafundo S, Agrimonti C, Marmiroli N. Traceability of plant contribution in olive oil by amplified fragment length polymorphisms. J. Agric. Food Chem. 2005;53:6995–7002.

[24] Testolin R, Lain O. DNA extraction from olive oil and PCR amplification of microsatellite markers. J. Food Sci. 2005;70:c108−c112.

[25] Breton C, Claux D, Metton I, Skorski G, Berville A. Comparative study of methods for DNA preparation from olive oil samples to identify cultivar SSR alleles in commercial oil samples: Possible forensic applications. J. Agric. Food ChemE. 2004;52:531–537.

[26] Giménez MJ, Pistón F, Martín A, Atienza SG. Application of real-time PCR on the development of molecular markers and to evaluate critical aspects for olive oil authentication. Food Chem. 2010;118:482–487.

[27] Spaniolas S, Bazakos C, Ntourou T, Bihmidine S, Georgousakis A, Kalaitzis P. Use of lambda DNA as a marker to assess DNA stability in olive oil during storage. Eur. Food Res. Technol. 2008;227:175–179.

[28] Muzzalupo I, Perri M. Recovery and characterization of DNA from virgin olive oil. Eur. Food Res. Technol. 2002;214:528–531.

[29] Spaniolas S, Bazakos C, Awad M, Kalaitzis P. Exploitation of the chloroplast trnL (UAA) intron polymorphisms for the authentication of plant oils by means of a lab-on-a-chip capillary electrophoresis system. J. Agric. Food Chem. 2008;56:6886–6891.

[30] Doyle JJ, Doyle JL. A rapid isolation procedure for small quantities of fresh leaf tissues. Phytochem. Bull. 1987;19:11–15.

[31] Consolandi C, Palmieri L, Severgnini M, Maestri E, Marmiroli N, Agrimonti C, Baldoni L, Donini P, De Bellis G, Castiglioni B. A procedure for olive oil traceability and authenticity: DNA extraction, multiplex PCR and LDR-universal array analysis. Eur. Food Res. Technol. 2008;227:1429–1438.

[32] Busconi M, Foroni C, Corradi M, Bongiorni C, Cattapan F, Fogher C. DNA extraction from olive oil and its use in the identification of the production cultivar. Food Chem. 2003;83:127–134.

[33] Martins-Lopes P, Gomes S, Santos E, Guedes-Pinto H. DNA markers for Portuguese olive oil fingerprinting. J. Agric. Food Chem. 2008;56:11786–11791.

[34] Doveri S, Donal M, Lee D. Non concordance between genetics profiles of olive oil and fruit: A cautionary note to the use of DNA markers for provenance testing. J. Food Chem. 2006;54:9221–9226.

[35] Waiblinger HU, Pietsch K, Wurz A. DNA-Extraktion aus Sojalecithin und Kosmetika. Lebensmittelchemie. 1999;53:11.

[36] Doyle JJ. DNA protocols for plants. In: Hewitt G, Johnson AWB, Young JPW, eds. Molecular techniques in taxonomy. NATO ASI series H, Cell Biol: Springer-Verlag, Berlin, Germany. 1991;57:283–293.

[37] Ganopoulos I, Bazakos C, Madesis P, Kalaitzis P, Tsaftaris A. Barcode DNA high-resolution melting (Bar-HRM) analysis as a novel close-tubed and accurate tool for olive oil forensic use. J. Sci. Food Agric. 2013;93:2281–2286.

[38] Ramos-Gómez S, Busto MD, Perez-Mateos M, Ortega N. Development of a method to recovery and amplification DNA by real-time PCR from commercial vegetable oils. Food Chem. 2014;158:374–383.

[39] Raieta K, Muccillo L, Colantuoni V. A novel reliable method of DNA extraction from olive oil suitable for molecular traceability. Food Chem. 2015;172:596–602.

[40] Muzzalupo I, Pisani F, Greco F, Chiappetta A. Direct DNA amplification from virgin olive oil for traceability and authenticity. Eur. Food Res. Technol. 2015;241:151–155.

[41] Stefanoudaki E, Kotsifaki F, Koutsaftakis A. Sensory and chemical profiles of three European olive varieties (Olea europaea L): An approach for the characterisation and authentication of the extracted oils. Sci. Food Agric. 2000;80:381−389.

[42] Mannina L, Dugo G, Salvo F, Cicero L, Ansanelli G, Calcagni C, Segre A. Study of the cultivar-composition relationship in Sicilian olive oils by GC, NMR, and statistical methods. J. Agric. Food Chem. 2003;51:120–127.

[43] D'Imperio M, Dugo G, Alfa M, Mannina L, Segre AL. Statistical analysis on Sicilian olive oils. Food Chem. 2007;102:956–965.

[44] Aranda F, Gomez-Alonso S, Rivera del Alamo RM, Salvador MD, Fregapane G. Triglyceride, total and 2-position fatty acid composition of Cornicabra virgin olive oil: comparison with other Spanish cultivars. Food Chem. 2004;86:485–492.

[45] Cerretani L, Bendini A, Del Caro A, Piga A, Vacca V, Caboni MF, Gallina TT. Preliminary characterisation of virgin olive oils obtained from different cultivars in Sardinia. Eur. Food Res. Technol. 2006;222:354–361.

[46] Issaoui M, Dabbou S, Echbili A, Rjiba I, Gazzah N, Trigui A, Hammami M. Biochemical characterisation of some Tunisia virgin olive oils obtained from different cultivars growing in Sfax National Collection. J. Food Agric. Environ. 2007;5:17–21.

[47] Matos LC, Cunha SC, Amaral JS, Pereira JA, Andrade PB, Seabra RM, Oliveira B. Chemometric characterization of three varietal olive oils (cvs. Cobranc-osa, Madural and Verdeal Transmontana) extracted from olives with different maturation indices. Food Chem. 2007;102:406–414.

[48] Bucci R, Magri A.D, Magri AL, Marini D, Marini F. Chemical authentication of extra virgin olive oil varieties by supervised chemometric procedures. J. Agric. Food Chem. 2002;50:413–418.

[49] Diaz GT, Meras DI, Casas SJ, Franco MF. Characterization of virgin olive oils according to its triglycerides and sterols composition by chemometric methods. Food Control. 2005;16:339–347.

[50] Angerosa F, Basti C, Vito R. Virgin olive oil volatile compounds from lipoxygenase pathway and characterization of some Italian cultivars. J. Agric. Food Chem. 1999;47:836–839.

[51] Ercisli S, Barut E, Ipek A. Molecular characterization of olive cultivars using amplified fragment length polymorphism markers. Genet. Mol. Res. 2009;8:414–419.

[52] Owen CA, Bita EC, Banilas G, Hajjar SE, Sellianakis V, Aksoy U. AFLP reveals structural details of genetic diversity within cultivated olive germplasm from the Eastern Mediterranean. Theoret. Appl. Genet. 2005;110:1169–1176.

[53] Nikoloudakis N, Banilas G, Gazis F, Hatzopoulos P, Metzidakis J. Discrimination and genetic diversity among cultivated olives of Greece using RAPD markers. J. Am. Soci. Hortic. Sci. 2003;128:741–746.

[54] Bautista R, Crespillo R, Canovas FM, Claros MG. Identification of olive-tree cultivars with SCAR markers. Euphytica. 2003;129:33–41.

[55] Diaz A, De la Rosa R, Martin A, Rallo P. Development, characterization and inheritance of new microsatellites in olive (Olea europaea L.) and evaluation of their usefulness in cultivar identification and genetic relationship studies. Tree Genet. Genomes. 2006;2:165–175.

[56] Montemurro C, Simeone R, Pasqualone A, Ferrara E, Blanco A. Genetic relationships and cultivar identification among 112 olive accessions using AFLP and SSR markers. J. Hortic. Sci. Biotechnol. 2005;80:105–110.

[57] Terzopoulos PJ, Kolano B, Bebeli PJ, Kaltsikes PJ, Metzidakis I. Identification of Olea europaea L. cultivars using inter-simple sequence repeat markers. Sci. Hortic. 2005;105:45–51.

[58] Reale S, Doveri S, Diaz A, Angiolillo A, Lucentini L, Pilla F., et al. SNP based markers for discriminating olive (Oleaeuropaea L.) cultivars. Genome. 2006;49:1193–1205.

[59] Consolandi C, Palmieri L, Doveri S, Maestri E, Marmiroli N, Reale S., et al. Olive variety identification by ligation detection reaction in a universal array format. J. Biotechnol. 2007;129:565–574.

[60] Pafundo S, Busconi M, Agrimonti C, Fogher C, Marmiroli N. Storage time effects on olive oil DNA assessed by amplified fragments length polymorphisms. Food Chem. 2010;123:787–793.

[61] Vos P, Hogers R, Bleeker M, Reijans M, Van de Lee T, Hornes M, Frijtens A, Pot J, Peleman J, Kuiper M, Zabeau M. AFLP: A new technique for DNA fingerprinting. Nucleic Acids Res. 1995;23:4407–4414.

[62] Tautz D. Hypervariability of simple sequences as general source for polymorphic DNA markers. Nucleic Acids Res. 1989;17:6463–6471.

[63] Pasqualone A, Di Rienzo V, Blanco A, Summo C, Caponio F, Montemurro C. Characterization of virgin olive oil from Leucocarpa cultivar by chemical and DNA analysis. Food Res. Int. 2012;47:188−193.

[64] Corrado G, Imperato A, La Mura M, Perri E, Rao R. Genetic diversity among olive varieties of southern Italy and the traceability of olive oil using SSR markers. J. Hortic. Sci. Biotechnol. 2011;86:461–466.

[65] Pasqualone A, Montemurro C, Caponio F, Blanco A. Identification of virgin olive oil from different cultivars by analysis of DNA microsatellites. J. Agric. Food Chem. 2004;52:1068–1071.

[66] Vietina M, Agrimonti C, Marmiroli M, Bonas U, Marmiroli N. Applicability of SSR markers to the traceability of monovarietal olive oils. J. Sci. Food Agric. 2011;91:1381–1391.

[67] Montemurro C, Miazzi MM, Pasqualone A, Fanelli V, Sabetta W, Di Rienzo V. Traceability of PDO olive oil "Terra di Bari" using high resolution melting. J. Chem. 2015;DOI: http://dx.doi.org/10.1155/2015/496986.

[68] Ben Ayed R, Ben Hassen H, Ennouri K, Ben Marzoug R, Rebai A. OGDD (Olive Genetic Diversity Database): A microsatellite markers' genotypes database of worldwide olive trees for cultivar identification and virgin olive oil traceability. Database. 2016;DOI: 10.1093/database/bav090.

[69] Brookes AJ. The essence of SNPs. Gene. 1999;234:177–186.

[70] Kalogianni DP, Bazakos C, Boutsika LM, Ben Targem M, Christopoulos TK, Kalaitzis P, Ioannou PC. Olive oil DNA fingerprinting by multiplex SNP genotyping on fluorescent microspheres. J. Agric. Food Chem. 2015;63:3121–3128.

[71] Bazakos C, Khanfir E, Aoun M, Spano T, El Zein Z, Chalak L, El Riachy M, Abou-Sleymane G, Ben Ali S, Kammoun N, Kalaitzis P. The potential of SNP-based PCR-RFLP

capillary electrophoresis analysis to authenticate and detect admixtures of Mediterranean olive oils. Electrophoresis. 2016;DOI: 10.1002/elps.201500537.

[72] Ramazzotti M, Mulinacci N, Pazzagli L, Moriondo M, Manao G, Vincieri FF, Degl'Innocenti D. Analytic investigations on protein content in refined seed oils: Implications in food allergy. Food Chem. 2008;46:3383–3388.

[73] Arlorio M, Coisson JD, Bordiga M, Travaglia F, Garino C, Zuidmeer L. Olive oil adulterated with hazelnut oils: Simulation to identify possible risks to allergic consumers. Food Addit. Contam.: Part A. 2010;27:11–18.

[74] De Ceglie C, Calvano CD, Zambonin CG. Determination of hidden hazelnut oil proteins in extra virgin olive oil by cold acetone precipitation followed by in-solution tryptic digestion and MALDI-TOF-MS analysis. J. Agric. Food Chem. 2014;62:9401–9409.

[75] Brezna B, Hudecova L, Kuchta T. A novel real-time polymerase chain reaction (PCR) method for the detection of walnuts in food. Eur. Food Res. Technol. 2006;223:373–377.

[76] Koppel R, Velsen-Zimmerli F, Bucher T. Two quantitative hexaplex real-time PCR systems for the detection and quantification of DNA from twelve allergens in food. Eur. Food Res. Technol. 2012;235:843–852.

[77] Savolainen V, Chase MW. A decade of progress in plant molecular phylogenetics. Trends Genet. 2003;19:717–724.

[78] Gielly L, Taberlet P. The use of chloroplast DNA to resolve plant phylogenies: Noncoding versus rbcL sequences. Mol. Biol. Evol. 1994;11:769–777.

[79] James D, Schmidt AM. Use of an intron region of a chloroplast tRNA gene (trnL) as a target for PCR identification of specific food crops including sources of potential allergens. Food Res. Int. 2004;37:395–402.

[80] Rønning SB, Rudi K, Berdal KG, Holst-Jensen A. Differentiation of important and closely related cereal plant species (Poaceae) in food by hybridization to an oligonucleotide array. J. Agric. Food Chem. 2005;53:8874–8880.

[81] Spaniolas S, May ST, Bennett MJ, Tucker GA. Authentication of coffee by means of PCR-RFLP analysis and lab-on-a-chip capillaryelectrophoresis. J. Agric. Food Chem. 2006;54:7466–7470.

[82] Spaniolas S, Tsachaki M, Bennett MJ, Tucker GA. Toward the authentication of wines of Nemea denomination of origin through cleaved amplified polymorphic sequence (CAPS)-based assay. J. Agric. Food Chem. 2008;56:7667–7671.

[83] Syvanen AC. Accessing genetic variation: Genotyping single nucleotide polymorphisms. Nat. Rev. Genet. 2001;2:930–942.

[84] Reed GH, Wittwer CT. Sensitivity and specificity of single-nucleotide polymorphism scanning by high-resolution melting analysis. Clinical Chem. 2004;50:1748–1754.

[85] Vietina M, Agrimonti C, Marmiroli N. Detection of plant oil DNA using high resolution melting (HRM) post PCR analysis: A tool for disclosure of olive oil adulteration. Food Chem. 2013;141:3820–3826.

[86] Wu Y, Zhang H, Han J, Wang B, Wang W, Ju X, Chen Y. PCR-CE-SSCP applied to detect cheap oil blended in olive oil. Eur. Food Res. Technol. 2011;233:313–324.

[87] Agrimonti C, Vietina M, Pafundo S, Marmiroli N. The use of food genomics to ensure the traceability of olive oil. Trends Food Sci. Technol. 2011;22:237–244.

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