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An Overview of the Authentication of Olive Tree and Oil

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Abstract: Adulteration of virgin olive oil with less expensive oils is a serious problem for the public and quality control evaluators of olive oil. That is why olive oil authenticity has become a major issue for producers, consumers, and policy makers. In order to avoid fraud to consumers, it is crucial to study the traceability of olive oil. This review covers 2 important techniques, analytical, and molecular methods, used to characterize olive oil and detect possible adulteration. Several analytical techniques are discussed for the detection of olive oil adulteration by analyzing minor and major compounds of olive oil. However, the chemical composition of olive oil can dramatically change due to the environmental and processing conditions. For this reason, the DNA-based technologies are gaining greater attention now because they are not influenced by environmental conditions and provide an opportunity for direct comparison of different genetic materials. In this review, we emphasize the great potential of different authenticity methods and discuss their practical implementation in olive oil traceability.

Introduction

Olive oil is recognized worldwide for its nutritional value, health benefits, and is appreciated for its particular aroma and taste (Elloumi and others 2012). Olive oil is considered an economically important crop, especially for producing countries in the Mediterranean area. The olive oil varieties consumed in the Mediterranean basin retain virtually all their natural nutritional properties because they are usually obtained from their respective plant sources through either physical crushing or pressing. Thus, olive oil keeps on attracting the interest of scientists due to the health benefits associated with its consumption. Olive oil, unlike seed oils (soya, colza, and so on.), is not a commodity with one world reference price due to the heterogeneity of the physical product and its quality categorization. Virgin olive oil is considered a price premium product compared to other vegetable oils. It is very prone to adulteration leading to economical losses, disloyal competition among producers, and break in consumer trust.

Recent crises in the food sector, such as with dioxin in poultry (Vellinga and Van Look 2002), bovine spongiform encephalopathy (BSE) (Willesmith 1996), and the controversy about genetically modified organisms (GMO) have highlighted the need for more strict food quality control, which should include determination of the origin of a product and the raw materials used in it. That is why a well-documented traceability system has become a requirement for quality control in the food chain.

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The definition of traceability according to the European Council Regulation EEC 178/2002 is the ability to identify and trace a product or a batch of products at all stages of production and marketing. Traceability is important for commercial reasons and plays a considerable role in the assurance of public health.

Olive oils command a premium price in the market, leading to great temptation to adulterate them with vegetable seed oils (Cercaci and others 2003). Some cultivars of olive oil are recognized as being of higher quality because they derive from well-defined geographical areas, command better prices, and generally are legally protected. Indeed, the aim of protected designations of origin (PDO) is to add value to certain specific high-quality products from a particular origin.

Over the last 10 y, research and technology have experienced great progress in the fight against forgery of extra virgin oils. Nevertheless, the knowledge of fraudsters has also followed this trend, which allowed the introduction of more sophisticated frauds that require the use of novel approaches for detection. Biochemical techniques have been employed for the classification and authenticity of olive oils using a high number of variables such as triacylglycerides (TAGs) composition, phenolic fraction, and unsaponifiable components monitoring by statistical and mathematical analyses in order to facilitate the evaluation of the results. More recently, the suitability of DNA markers in providing unequivocal identification for authentication and traceability of foods has been a subject of great interest. In fact, many researchers have applied biomolecular methodologies for olive/olive oil genetic characterization, identification, and authentication. Molecular markers allow the detection of DNA polymorphism and enable to effectively distinguish different cultivars in an effective way, without any environmental influence (Busconi and others 2003).

The aim of this review is to describe the different methods (biochemical and molecular techniques) used to study the

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cultivar designation of olive oils, and to illustrate the importance of molecular markers to study the composition and the traceability of olive oils.

Compositional analysis and authenticity of olive oil

Olive oil can be traded as a blend from different cultivars or from different provenances. Some olive oils are a blend of high and low grade; yet, they are traded as being of high quality. For these reasons, several analytical techniques are used to detect adulteration of virgin olive oil and to establish its authenticity.

Olive oil chemical components can be divided into major and minor compounds as briefly described later. The macro- and microcomponents of oils are semivolatile, that is why chromatographic instruments can be utilized for their measurement. In fact, numerous chromatographic methods such as high-performance liquid chromatography (HPLC) and gas chromatography (GC) techniques offer the possibility for reliable and rapid separation and also quantitative determination of major and minor compounds (Nollet 2003; Cserháti and others 2005).

In the last few years, spectroscopic approaches associated with multivariate analysis have been used to detect adulteration of virgin olive oil. Among these spectroscopic methods are vibrational techniques such as near-infrared spectroscopy (NIR) (Christy and others 2004), Fourier transform infrared (FT-IR) and Fourier transform Raman (FT-Raman) (Ozen and Mauer 2002), and nuclear magnetic resonance (NMR) spectroscopes (Alonso-Salces and others 2010).

Major components

Fatty acid (FA) analysis. FAs are important to characterize quality and a specific oil. The FA composition of olive oils was strongly influenced by several factors such as cultivar, maturation, stage of fruit, and zone of origin (Stefanoudaki and others 1999; D'Imperio and others 2007).

FAs are usually converted to FA methyl esters (FAMEs) for gas–liquid chromatography (GLC) analysis using capillary columns that are widely accepted (Aparicio and Aparicio-Ruiz 2000). According to the Intl. Olive Oil Council, the characterization of FAs in olive oils was usually performed by GC coupled with flame ionization detector (FID). This technique was used by several authors such as Brescia and others (2003), Cerretani and others (2006), Matos and others (2007), Baccouri and others (2007), D'Imperio and others (2007), and Issaoui and others (2010). The FAs mainly studied in olive oils are included in Table 1. Moreover, in order to obtain satisfactory results to describe the characteristics of oils, classify them, and also to select the best variables some researchers have required the use of chemometric approaches (Matos and others 2007; Lerma-Garcia and others 2008).

TAG analysis. The most important group of compounds in olive oils is represented by triglycerides (TGs), which in chemical terms are trihydric alcohols esterified with FAs.

The triacylglycerols can be examined (Table 1) by reversed-phase HPLC (RP-HPLC), or mass spectrometric detection (HPLC-MS) (Ranalli and others 2002), or NMR (HPLC-RID and ^{13}C NMR) (Brescia and others 2003; Mannina and others 2003). However, HPLC equipped with a refractive index detector (HPLC-RID) combined with chemometric tools was the most widely used method for the analysis of triglycerides in olive oils (Aranda and others 2004; Ollivier and others 2003; Cerretani and others 2006; Issaoui and others 2007). Characterization of the TAGs fraction in oils can be performed by GC combined with electron ionization mass spectrometry (EI-MS) (Aparicio and Aparicio-Ruiz 2000; Lorenzo and others 2002; Pereira and others 2002; Mannina and others 2003; Ollivier and others 2004; Rotondi and others 2004) or flame ionization detector (FID) (Pereira and others 2002; Rotondi and others 2004). Other researchers have actually used HPLC equipped with evaporative light scattering detector (HPLC-ELSD) for the analysis of triglycerides in olive oils (Baccouri and others 2007; Guerfel and others 2012).

The yield of TAG and FAs is greatly dependent on the cultivar and, perhaps, on the geographical origin.

Minor components or unsaponifiable fraction

The unsaponifiable fraction is made up of minor constituents (1% to 2%). It plays an indispensable role in quality and purity analyses and more recently in the studies of authentication and olive oil traceability. The quality and the yield of the unsaponifiable matter can be varied and depends on vegetal species, climatic condition, extraction and refining procedures, and storage conditions (Alonso-Salces and others 2010).

The unsaponifiable fraction is constituted of a heterogeneous group of compounds and the most studied minor components have been hydrocarbons, sterols, tocopherols, pigments, volatile compounds, and phenolic compounds (Garcia-Gonzalez and others 2008).

Hydrocarbons. Hydrocarbons are the least polar compounds of the unsaponifiable fraction of vegetable oils including olive oil (virgin olive oil may contain up to 0.7% hydrocarbons).

Squalene ($C_{30}H_{50}$) is an important constituent of the unsaponifiable matter of virgin olive oils. The separation of squalene has been achieved by thin-layer chromatography (TLC) of the unsaponifiable matter (Angerosa and others 1999; Moreda and others 2001; Nagy and others 2005). For the analysis of squalene products, GC-based methods were reported (Moreda and others 2001).

During the refining process squalene isomerizes (Moreda and others 2001), that is why squalene analysis can be used to distinguish between refined olive oil and crude olive oil.

Volatile compounds are responsible for the taste and especially the aroma of olive oil. Several GC-based approaches have been developed in order to analyze volatile fractions (Table 1), such as GC-FID (Angerosa and others 1999; Cert and others 2000; Cavalli and others 2003; Koprivnjak and others 2005) and GC-MS (Vichi and others 2003; Nagy and others 2005; Torres Vaz-Freire and others 2009). Other groups (Lorenzo and others 2002; Nagy and others 2005) used headspace MS.

Phenol analysis. The phenolic fraction of virgin olive oil consists of a heterogeneous mixture of compounds, each of which varies in chemical properties and in the impact it has on the quality of virgin olive oil (Psomiadou and others 2003). In fact, the antioxidant potential of phenolic compounds in olive oil has also been a subject of considerable interest because it is a major factor in the high stability of olive oils (Caponio and others 2001). Polyphenols are also responsible for the organoleptic characteristics of virgin olive oil (Ryan and Robards 1998).

For all of these reasons, the identification and quantification of the individual components of virgin olive oil are of great interest. Many analytical procedures directed toward determination of the complete phenolic profile have been proposed. Many authors have studied the phenolic fraction using the spectrophotometric determination of total phenols after treatment with sodium-molibdate solution (Gómez-Alonso and others 2002; Rotondi and others 2004).

Table 1–Detection of major and minor components in olive oils by using analytical methods.

Compounds studied	Analytical technique	Areas of origin	References
Fatty acids	GC-FID	Italy, Spain	D'Imperio and others 2007; Brescia and others 2003
		Portugal	Matos and others 2007
		France, Tunisia	Cerretani and others 2006; Baccouri and others 2007; Issaoui and others 2010
		Greece	Stefanoudaki and others 1999
	MS	Spain	Lerma-Garcia and others 2008
	GC-FID and ^{13}C NMR	Italy	Brescia and others 2003
Triglycerides	HPLC-RID	Italy, Spain, France, Tunisia	Cerretani and others 2006, Aranda and others 2004; Issaoui and others 2007 ; Ollivier and others 2003
	HPLC-RID and ^{13}C NMR	Italy	Brescia and others 2003; Mannina and others 2003
	HPLC-MS	Greece	Alves and others 2005; Ranalli and others 2002
	HPLC-ELSD	Tunisia	Baccouri and others 2007; Guerfel and others 2012
Sterols	GC-FID	Italy, Spain, Portugal	Brescia and others 2003; Matos and others 2007; Galeanzo Diaz and others 2005
	HPLC-MS	Italy and Greece	Nagy and others 2005
Phenolic compounds	HPLC-DAD	Spain and Turkey	Ocakoglu and others 2009; Garcia and others 2003
	HPLC-DAD and HPLC-MS	Italy, France, Tunisia	Cerretani and others 2006; Baccouri and others 2007
	MS	Spain	Lerma-Garcia and others 2008
Volatile compounds	GC-FID	Italy, Spain, Greece, Tunisia	Angerosa and others 1999; Tena and others 2007; Tura and others 2008
	GC-FID and GC-MS	Tunisia, Greece, Italy, Spain, Algeria, France	Manai and others 2008
	GC and GC-MS	Portugal	Torres Vaz-Freire and others 2009
Pigments	HPLC-DAD	Spain, Italy	Roca and others 2003; Cerretani and others 2008
	UV-Vis spectrophotometry	Italy and France	Cerretani and others 2006
Hydrocarbons	GC-FID	Tunisia, Spain, Italy, Croatia	Baccouri and others 2007; Osorio Bueno and others 2005; Koprivnjak and others 2005; Cert and others 2000; Cavalli and others 2003
	GC-MS	Italy and Greece	Nagy and others 2005
Tocopherols	HPLC-DAD	Tunisia, Italy	Baccouri and others 2007
	HPLC-LIF	Spain	Garcia and others 2003
	HPLC-FLD	Italy and France	Cerretani and others 2006

GD-FID, gas chromatography-flame ionization detector; ^{13}C NMR, carbon-13 nuclear magnetic resonance spectroscopy; MS, mass spectrometry; HPLC-RID, high-performance liquid chromatography refractive index detection; HPLC-MS, high-performance liquid chromatography-mass spectrometry detector; HPLC-DAD, high-performance liquid chromatography diode array detector; GC-MS, gas chromatography-mass spectrometry detector; HPLC-LIF, high-performance liquid chromatography-laser-induced fluorescence detection; HPLC-FLD, high-performance liquid chromatography-fluorescence detector;

Many researchers have identified the phenolic compounds by using sophisticated analytical techniques such as MS (Lerma-Garcia and others 2008), GC-MS (Angerosa and others 1996; Owen and others 2000; Tasioula-Margari and Okogeri 2001; Okogeri and Tasioula-Margari 2002), and GC-MS/MS as trimethylsilyl derivatives. One problem in gas chromatographic technique is that the use of high temperature could damage the analytes (Saitta and others 2002).

In recent times, HPLC-based methods were used for determination of phenols (Bendini and others 2003; Rotondi and others 2004), and HPLC with tandem mass spectrometry (LC-MS-MS) and negative atmospheric pressure chemical ionization (APCI) was used for the analysis of phenolic acids (tyrosol and oleuropein derivatives) (Bianco and others 2001). In instances where mass spectral data are insufficient to establish a definitive structure, NMR spectrometry is a powerful complementary technique for structure assignment. The limitation of this technique is that it requires large amounts of the compounds.

For all of the reasons explained, HPLC is the most commonly employed technique for analysis of the polar fraction of olive oil. However, GC and HPLC could be complementary. In fact, several recent studies on the isolation and the characterization of virgin olive oil phenolic compounds have been done using HPLC and GC simultaneously (Tasioula-Margari and Okogeri 2001; Romani and others 2001).

Actually, Bonoli and others (2003, 2004) showed that capillary zone electrophoresis (CZE) can be successfully applied in the semiquantitative analysis of phenolic compounds.

The composition of phenolic compounds in virgin olive oil is strongly affected by the oil extraction process (Giovacchino and others 2002) and the agronomic conditions of its production (Uceda and others 1999).

Phytosterol analysis. Phytosterols are natural components of edible vegetable oils. The dominant olive oil phytosterols are β-sitosterol, Δ5-avenasterol, campesterol, and other minor sterols such as stigmasterol (Cunha and others 2006).

The separation and the knowledge of the sterol cases are very useful in the quality control of olive oils as well as in the detection of fraudulent admixtures of cheaper oils from other plants like soybean or sunflower oils. For instance, Kamm and others (2001) claimed that the presence of a large amount of stigmasterol reveals an adulteration with lower priced soybean or cottonseed oils. Subsequently, another group (Verleyen and others 2002) declared that the knowledge of olive oil sterol composition could be employed regarding the refining procedure, which increases esterified and decreases free sterol contents. In fact, lampante olive oils can be distinguished from extra virgin olive oils by the lower percentage of free campesterol and free stigmasterol (Verleyen and others 2002). More recently, Azadmard-Damirchi (2010) studied the adulteration of virgin olive oil with less expensive oils such as hazelnut oil by analyzing the phytosterol classes. They concluded that various classes of phytosterols in these 2 oils are assessed as possible markers to detect adulterated olive oil.

Analysis of sterol oxidation products has recently been done by both HPLC and GC methods (Table 1) after saponification and solid-phase extraction (SPE) (Lercker and Rodriguez-Estrada 2000) or by GC-MS (Brescia and others 2003; Galeano Diaz and others 2005; Matos and others 2007) after the separation of free and esterified sterols by means of SPE columns, transesterification, and silylation (Cunha and others 2006). Nagy and others (2005)

analyzed sterol components by HPLC-MS detector. The sterol profile can be affected by many factors such as agronomy, geography (Sánchez Casas and others 2004), harvesting (Gutiérrez and others 1999), technological means (Gutiérrez and others 2000), and processing (Piironen and others 2000). These factors are so diverse that it is very difficult to completely characterize the sterol profile.

Tocopherols. Tocopherols are heteroacid compounds with high molecular weight that have been designated α, β, γ, and δ-tocopherols. α-Tocopherol comprises about 90% of the total tocopherol in olive oils. Tocopherols contribute to the antioxidant properties of olive oil (Deiana and others 2002) and their profile and composition are often criteria of purity.

Several HPLC- and GC-based methods have been reported for the detection of tocopherols in olive oils (Table 1).

α-Tocopherol and the total tocopherol content can be determined either by HPLC diode array detector (HPLC-DAD) (Baccouri and others 2007), by HPLC-fluorescence detector (HPLC-FLD) (Aparicio and Aparicio-Ruiz 2000; Cerretani and others 2006), or by HPLC-laser-induced fluorescence (HPLC-LIF) detection (Garcia and others 2003). The gas chromatographic method is also being used. Tocopherol analysis can be used to study adulteration of olive oils. In fact, tocopherols vary from one edible vegetable oil to another. Therefore, analyses of such samples of olive oil can detect any kind of other edible oil. It has been found that tocopherol content is affected by olive variety (Issaoui and others 2007; Dabbou and others 2011).

Pigments. The pigments are other constituents of the unsaponifiable matter which impact the characteristic color of an olive oil. They are made up of carotenoids and chlorophyll. They can be quantified by HPLC-DAD (Roca and others 2003; Cerretani and others 2008) or by UV-Vis spectrophotometry (Cerretani and others 2006) (Table 1).

Chlorophyllic derivatives present in olive oil extracts were analyzed using an HPLC method. β-Carotene can be quantified by HPLC (Lercker and Rodriguez-Estrada 2000) or TLC with colorimetric detection (Ranalli and others 2001). The quantity of pigments depends significantly on olive ripeness, the processing system, the cultivar, and storage conditions.

Several techniques based on olive oil composition (such as GC and HPLC) have been applied to detect adulteration. However, some difficulties have been found in distinguishing olive cultivars based on both drupes and oils of different cultivars because their characteristics are strongly influenced by environmental conditions. Recently, DNA-based markers, which are independent from environmental conditions, have been successfully applied to overcome this problem.

DNA-based tools in olive oil authentication

When we blend olive oils of the same category, but from different provenances, most chemical analyses are of limited significance. Due to their high variability according to environmental conditions, neither morphological characteristics of different groups nor the analyses of chemical composition of FA and secondary metabolites can provide reliable results for oil traceability (Ben-Ayed and others 2009).

For this reason, genetic identity seems to be the most appropriate method for identifying the cultivar from which the olive oil under study derives. In fact, DNA in oil is not affected by the environment and is identical to the mother-tree DNA since the oil-containing tissues are formed by diploid somatic cells of the tree. However, extra-alleles can be detected in the oil that do not correspond to the mother-tree allele but to the pollinator alleles contained in the embryo, itself located inside the seed (see, for example, Ben-Ayed and others 2009, 2012).

The use of DNA-based technology in the field of food authenticity is gaining increasing attention (Rasmussen and Morrissey 2008). The use of DNA-based methods for olive oil presents a number of advantages over biochemical-based methods, including increased specificity, sensitivity, and reliable performance with highly processed samples (Montealegre and others 2010).

This technique makes use of molecular markers that mostly use polymerase chain reaction (PCR) and are thus easy to genotype. Even in a complex matrix, such as olive oil, molecular marker techniques such as random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and simple sequence repeat (SSR) are very useful in the study of the traceability of olive oil. In the following sections we will discuss the potential of these classes of markers in oil authenticity.

DNA extraction from olive oil

All the studies published so far have shown that the reliability and reproducibility of molecular marker profiles are determined by the quality of the DNA extracted from the oil (Breton and others 2004; Testolin and Lain 2005; Muzzalupo and others 2007; Ben-Ayed and others 2009). DNA extraction from oil is a key step and was addressed in detail by Agrimonti and others (2011). In fact, the amount of DNA isolated from olive oil is low and highly degraded by the nuclease present in olive oil (Muzzalupo and Perri 2002; Testolin and Lain 2005; Pafundo and others 2007; Ben-Ayed and others 2009). For this reason, the extraction of DNA from olive oil is a difficult task. Additionally, phenolic compounds and residual polysaccharides can inhibit the activity of DNA polymerases and provide irregular PCR amplifications (Testolin and Lain 2005).

Several techniques of DNA preparation and immobilization for subsequent sample analysis have been developed. These methods utilize such supports as silica, hydroxyapatite, magnetic beads, and spin columns. These supports enable the DNA to be amplified and analyzed using various quantities of oil. In particular, magnetic beads in conjunction with additional processing have proved useful. However, the defined procedure needs 2 × 40 mL of virgin olive oil, and the preparation of DNA regularly takes 5 h (Breton and others 2004). Besides, other authors (Testolin and Lain 2005; Ben-Ayed and others 2009) have tried various protocols of DNA extraction from olive oil such as: Wizard kit (Promega, Lyon, France), CTAB protocol extraction, QIAamp DNA stool extraction kit (Qiagen) (Biomaghreb, Ariana, Tunisia). They concluded that the most reproducible results were obtained when the template DNA was recovered from the olive oil using the QIAamp DNA stool extraction kit (Qiagen).

Random amplified polymorphic DNA (RAPDs) (Table 2)

In this technique, a PCR amplification of genomic DNA is performed using a set of arbitrary primers (Williams and others 1990). For each primer a large number of bands is generated. The presence/absence of a band can distinguish between individuals and each individual is expected to have a specific fingerprint of bands. This molecular technique has several advantages. It is simple, cheap, requires small amounts of DNA (Fritsch and Rieseberg 1996), and it can be applied without prior genetic information about the organism. Besides, it is fast, and does not require radioactivity. However, this analysis has several limitations including dominance, sensitivity to the reaction conditions, uncertain locus homology, and lack of good reproducibility.

Table 2–Genetic markers and their use to study olive and olive oil cultivars.

Genetic marker	Objective of the study	Reference
RAPD	Discrimination of olive cultivars	Cresti and others 1996; Khadari and others 2003
	Genetic diversity of inter- or intracultivar	Wiesman and others 1998; Mekuria and others 1999, 2002, Roselli and others 2002, Belaj and others 2002, 2003a, b; Gemas and others 2004
	Genetic relationships between cultivars	Belaj and others 2002, 2003b; Besnard and others 2001a; Khadari and others 2003
	Genetic differentiation in the olive complex	Besnard and others 2001b; Martins-Lopes and others 2007
	Genetic relationships between varieties	Claros and others 2000
	Authentication and traceability of olive oil	Pasqualone and others 2001; Muzzalupo and Perri 2002
AFLP	Genetic diversity within and among a range of Spanish and Italian olive cultivars	Sanz-Cortés and others 2003; Sensi and others 2003
	Identification of olive cultivars	Grati-Kamoun and others 2006
	Genotyping olive species	Busconi and others 2003; Pafundo and others 2005
	Traceability of the origin and authenticity of olive oil	Pafundo and others 2005
SCAR	Cultivar identification	Busconi and others 2006
	Olive oil traceability	De la Torre and others 2004 ; Pafundo and others 2007
ISSR	Discrimination between intracultivar variability of 201 accessions belonging to 11 Portuguese cultivars	Gemas and others 2004
	Cultivar traceability in olive oil	Pasqualone and others 2001
	Phylogenetic analysis	Hess and others 2000; Vargas and Kadereit 2001
SNP	Genetic diversity of olive trees	Reale and others 2006
	To discover 9 new SNPs by using a direct sequencing of the lupeol synthase (OEW) and cycloartenol synthase (OEX) genes in 16 Tunisian olive cultivars	Rekik-Hakim and others 2010
	To identify 5 SNPs in the partial sequence of the gene for alternative oxidase *Oe*AOX2	Santos Macedo and others 2009
	To establish several SNPs in the phytochrome A gene	Muleo and others 2009
SSR	Analysis of intracultivar variability	Cipriani and others 2002; Lopes and others 2004
	Linkage mapping	Wu and others 2004
	Characterization of olive germplasm resources	Belaj and others 2003a; Montemurro and others 2005
	Genetic diversity within cultivated olive germplasm	Owen and others 2005
	Identification of cultivars	Sarri and others 2006
	Genetic diversities of olive cultivar	Tamalli and others 2006, 2007; Rekik and others 2008; Muzzalupo and others 2009
	Paternity analysis	Rallo and others 2000; Mookerjee and others 2005, Diaz and others 2006; Rekik and others 2008
	Traceability issues to define the olive oil origin and to detect the presence of prohibited cultivars	Muzzalupo and others 2007; Testolin and Lain, 2005; Ben-Ayed and others 2009

RAPD, random amplified polymorphic DNA; SCAR, sequence-characterized amplified region; AFLP, amplified fragment length polymorphism; ISSR, inter simple sequence repeats; SNP, single nucleotide polymorphism; SSR, simple sequence repeat.

RAPDs thus combine the advantages of low technical input with almost an unlimited number of markers. They have proven to be very useful in the characterization of genetic diversity of plants for which few genomic data are available (Qian and others 2001; Bandelj and others 2002). RAPD markers were the first ones to be implemented to study diversity of the species *Olea europaea* (Belaj and others 2001), to discriminate olive cultivars (Cresti and others 1996; Khadari and others 2003), to study inter- or intracultivar genetic diversity (Wiesman and others 1998; Mekuria and others 1999, 2002; Roselli and others 2002; Belaj and others 2002, 2003b; Gemas and others 2004), to establish genetic relationships between cultivars (Claros and others 2000; Besnard and others 2001a; Belaj and others 2002, 2003b; Khadari and others 2003), and to study genetic differentiation in the olive complex (Besnard and others 2001b; Martins-Lopes and others 2007).

As early as their use in genetic studies, RAPD markers have been used for the authentication and traceability of olive oil (Pasqualone and others 2001; Muzzalupo and Perri 2002). However, numerous authors (Claros and others 2000; Pasqualone and others 2001; Sanz-Cortés and others 2001) concluded the nonreproducibility of RAPD markers in the authentication of olive oil, which resulted in inconsistent electrophoretic patterns. These unsuccessful attempts were due to the bad quality of DNA extracted from the oil (Pasqualone and others 2001; Muzzalupo and Perri 2002).

Amplified fragment length polymorphism (AFLPs) (Table 2)

AFLP was described by Vos and others (1995) as a more reproducible alternative to RAPD for the genetic identification of crop plants. This technique is based on the selective PCR amplification of restriction fragments from total digests of genomic DNA.

In olive, AFLP markers have been used for genetic diversity studies and cultivar identification. In fact, AFLP technology has been used by Angiolillo and others (1999) to obtain a large number of markers for olive. This has been used in addressing genetic relationships among wild and cultivated varieties, as well as among *O. europaea* L. and other species from the genus (within the *Olea* complex).

This technique has also been used to study the genetic diversity within and among a range of Spanish and Italian olive cultivars (Sanz-Cortés and others 2003; Sensi and others 2003). Owen and others (2005) used AFLP markers to evaluate the structure of genetic diversity among common olive varieties cultivated in the eastern Mediterranean area. Additionally, AFLP analysis, as previously described, has been used in genetic variability studies for about 29 varieties (including oil and table olive cultivars originating from Tunisia and other Mediterranean countries) of the genus *Olea* using 9 AFLP primer combinations (Grati-Kamoun and others 2006).

More recently, different studies (Busconi and others 2003; Pafundo and others 2005) have reported that it is possible to use AFLP markers for genotyping olive species.

As far as oil traceability is concerned, Busconi and others (2003) reported that the AFLP fingerprint of olive oil was only partially superimposable with that of the cultivar from which the oil was made. However, in more recent studies, Pafundo and others (2005) and Montemurro and others (2008) concluded that AFLP profiles

of DNA purified from leaves and the monovarietal oil of the same cultivar were comparable. These latter authors evaluated the possibility of identifying virgin olive oil from 10 different cultivars by the analysis of AFLP markers using 6 AFLP primer combinations.

For the AFLP, as well as for RAPDs, the quality of DNA isolated from olive oil seems again to be problematic (very low quantity, high degradation, and richness in polysaccharides and phenolic compounds). Poor quality of DNA is responsible for inconsistent results and low reliability of AFLP profiles due to the inhibition of the restriction enzymes and the DNA polymerase activity.

Sequence-characterized amplified region (SCAR) (Table 2)

An SCAR is a genomic DNA fragment at a single genetically defined locus that is identified by PCR amplification using a pair of specific oligonucleotide primers. The SCARs are inherited in a codominant fashion in contrast to RAPDs, which are inherited in a dominant manner. SCAR technique that was introduced by Paran and Michelmore (1993) converted RAPD or AFLP markers into SCARs by the development of specific primers. In *O. europaea*, these kinds of markers have been used for DNA fingerprinting of cultivars (Busconi and others 2006) and in cultivar traceability (de la Torre and others 2004; Pafundo and others 2007).

Inter simple sequence repeats (ISSRs) (Table 2)

The ISSR molecular markers are semi-arbitrary. Single forward primers with 16 to 18 nucleotide length comprise repetitive units and anchor 2 to 4 arbitrary nucleotides at the 3' or 5' end. This method did not require information about genomic sequences and therefore, by means of these primers, a high level of polymorphism could be realized (Zietkiewicz and others 1994). In olive, ISSR markers were successfully used to distinguish 10 Italian varieties by studying genomic DNA extracted from olive fruit (Pasqualone and others 2001). These markers were also applied in phylogenetic analysis in olive cultivar identification (Vargas and Kadereit 2001) and within the *O. europaea* species (Hess and others 2000). ISSRs have been used for intracultivar variability of 201 accessions belonging to 11 Portuguese cultivars (Gemas and others 2004).

Simple sequence repeats (SSRs) (Table 2 and 3)

SSRs are a class of DNA markers that consist of short tandem repeat sequences (2 to 6 bp), which have become one of the most successful and the most interesting markers for genotype identification due to their good properties; in addition to their high specificity, they are highly polymorphic, codominant, locus-specific, ubiquitous, widely distributed throughout the genome, and easily amenable to automated PCR-based analysis. At present, they are the most reliable DNA profiling method in forensic investigations (Jobling and Gill 2004). SSRs also are highly informative and reproducible tools because they use longer primer sequences (Vicente and Fulton 2003).

In olive, SSRs have shown high potential for resolving issues of synonymies, homonymies, and misnamings. Many SSRs have been developed for olive and applied with success (Sefc and others 2000; Carriero and others 2002; Cipriani and others 2002; De la Rosa and others 2003; Sabino Gil and others 2006). All these characteristics make them ideal markers for applications in analysis of intracultivar variability issues (Cipriani and others 2002; Lopes and others 2004), linkage mapping (Wu and others 2004), and for characterizing olive germ plasm resources (Belaj and others 2003b; Montemurro and others 2005; Pasqualone and others 2007; Poljuha and others 2008; Doveri and others 2008). Sarri and others (2006) confirmed the power of SSR markers in the identification of 118 cultivars from different Mediterranean areas.

In addition, several other papers used SSR technologies to study the genetic diversities of olive cultivars. In fact, Tamalli and others (2006) investigated the diversity of 25 Tunisian olive cultivars using 10 SSR markers, and Tamalli and others (2007) studied the diversity of the 2 major Tunisian cultivars (*Chemlali* and *Chetoui*) using 11 SSR markers. Another group (Rekik and others 2008) used 10 SSR markers to differentiate 20 Tunisian olive cultivars. A recent report by Muzzalupo and others (2009) characterized 211 Italian olive cultivars by using 11 loci microsatellites in order to study and to establish relationships of geographically related olive tree cultivars. Microsatellites are also very useful markers for paternity analysis (Rallo and others 2000; Mookerjee and others 2005, Diaz and others 2006; Rekik and others 2008). Martins-Lopes and others (2008) examined 11 Portuguese monovarietal olive oil cultivars and 12 commercial olive oils (combination of RAPD and ISSR and SSRs markers) to allow olive variety differentiation in olive oils

Recently microsatellites have become available and reliable molecular markers for traceability issues to define olive oil origin and to detect the presence of prohibited cultivars (Testolin and Lain 2005; Doveri and others 2006; Muzzalupo and others 2007; Ben-Ayed and others 2009, 2012). Most of these publications addressed optimization of the extraction of high-quality DNA from olive oils and to identify the most interesting SSR markers in cultivar discrimination.

Indeed, Pasqualone and others (2004) used 7 microsatellites (3 DCA, 3 GAPU, and 1 UDO) to analyze the oil obtained from 7 different varieties and to study the genetic diversity of 7 Italian varieties. Testolin and Lain (2005) used DNA obtained from 3 monovarietal virgin oils and amplified 6 microsatellite loci of the UDO series with original and nested primers, in order to increase the sensitivity of the procedure. Recently we used 6 SSR loci to compare the profile of oil and the leaves of the 2 major Tunisian cultivars (Chemlali and Chetoui), designated to study the varietals composition of olive oils and the pollinization phenomenon (Ben-Ayed and others 2009). More recently, we used 8 SSR loci to characterize 22 virgin olive oils grown in different geographical region cultivars and to establish a classification key with the minimum number of markers (GAPU59 and GAPU71A) that discriminate all the studied cultivars (Ben-Ayed and others 2012).

Single nucleotide polymorphism (SNP) (Table 2)

In recent years, a novel class of markers, namely SNP, has emerged as an important tool in genomics and is increasingly being used as molecular markers in various laboratories for diverse applications. The development of this type of markers needs a high level of genome sequence information. Therefore, only a few SNP markers have been reported in olive since only very few sequence data were available before the year 2010. SNP markers have been recently developed in olive and utilized to study the genetic diversity of olive trees (Reale and others 2006; Rekik-Hakim and others 2010).

Reale and others (2006) identified 8 SNP in olive by using both a sequence-based and an arbitrary approach. Rekik-Hakim and others (2010) used direct sequencing of the lupeol synthase (OEW) and cycloartenol synthase (OEX) genes in 16 Tunisian olive cultivars to discover 9 new SNPs. In addition, Santos Macedo and others (2009) identified 5 SNP in the partial sequence of the gene for alternative oxidase *Oe*AOX2. Besides, Muleo and others (2009) established several SNP in the phytochrome A

Table 3–Microsatellite markers and their usefulness for studying the genetic diversity of olive cultivars or traceability of olive oil varieties.

Nr. SSRs markers	Objective of the study	References
-	Genetic diversity	Owen and others 2005
-	Genetic diversity of 118 cultivars from different Mediterranean countries	Sarri and others 2006
10	Genetic diversity of 25 Tunisian cultivars	Tamalli and others 2006
11	Diversity of the 2 major Tunisian cultivars (*Chemlali* and *Chetoui*)	Tamalli and others 2007
10	Genetic diversity of 20 Tunisian cultivars	Rekik and others 2008
11	Characterized 211 Italian olive cultivars	Muzzalupo and others 2009
7	Oil analysis of 7 Italian varieties	Pasqualone and others 2004
6	Analysis of monovarietal oil	Testolin and Lain, 2005
6	Compare the profile of olive oil and leaves of the 2 major Tunisian cultivars (*Chemlali* and *Chetoui*)	Ben-Ayed and others 2009
8	Characterization, identification, and discrimination of 17 olive cultivars	Doveri and others 2008
7	Compare the commercial monovarietal olive oil profile and the profile of the reference leaf	Doveri and others 2006
14	Discrimination among 19 olive cultivars	Bandelj and others 2002
12	Assessment of genetic diversity of 27 Istrian olive germplasm and solve the question of synonymousness of 9 Istrian olive cultivars	Poljuha and others 2008
10	Differentiation of the main olive variety used to elaborate an olive oil	Breton and others 2004
4	Examination of 11 Portuguese monovarietal olive oil cultivars and 12 commercial olive oils (combination of RAPD and ISSR and SSRs data in a PCA analysis allowing olive variety differentiation in olive oils)	Martins-Lopes and others 2008
8	Characterization of 22 olive oil varieties originating from Mediterranean countries	Ben-Ayed and others 2012
7	Discrimination of 1 olive variety *Collina di Brindisi* in a PDO olive oil	Pasqualone and others 2007
6	Correspondence between purified oil DNA and leaf DNA of the same cultivar	Muzzalupo and others 2007

gene by means of high-resolution melting (HRM) analysis of DNA.

However, to our knowledge only 1 study has used the SNP markers in traceability of olive oils and has been published (Consolandi and others 2008). Recently, the most interesting way to analyze SNP markers is the simultaneous detection of multiple SNP from a single DNA sample. Consolandi and others (2007) adopted the "ligation detection reaction-universal array" (LDR-UA) to successfully genotype a panel of 49 varieties with respect to 17 SNP, 12 amplicons containing these SNP were successfully amplified from oil-derived DNA, and the resulting profiles were fully consistent with those obtained from the leaf-derived template (Consolandi and others 2008).

To conclude, it is important to mention that some of these methods are being widely used for several purposes, like characterization and identification of olive species or authenticity and traceability of olive oil. Interestingly, the quality of olive oil has also been related to several factors, including processing, ripeness of fruit, and storage of olive oil.

Conclusions

Olive oil is a key component of the Mediterranean diet, which keeps on attracting the interest of scientists due to the health benefits associated with its consumption. Oil quality is of great importance in relation to consumer acceptability, but also from an industrial point of view. In recent years interest in the study of the authenticity of olive oil using molecular markers has been revived. Some studies conducted in the last few years indicate that traceability and authenticity of olive oil using chemical properties of major and minor compounds would not be reliable because of dependence on environmental conditions. These problems could be overcome by analysis of olive oil samples utilizing DNA-based markers. These molecular markers can be used not only to define the varietal origin of a particular olive oil, but also to estimate the admixture of the detection of cheaper vegetable oils for quality control purposes.

Traceability of olive oil is an important issue for consumers who demand and pay for high-quality oils. In this sense, future trends point to the use of more sensitive technologies such as DNA microarray chips and quantitative real-time PCR methods. In fact, these are based on DNA analysis and even are able to distinguish between different cultivars. Furthermore, the use of databases has become increasingly important in this field by providing a compilation of genetic information on a variety of olive oil cultivars, which is of great interest and can be applied for quality control purpose and authenticity confirmation.

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