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## 6. Authentication of vegetable oils

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**Abstract.** Authenticity of vegetable oils continues to be a challenge and the target of many studies. Consumers expectancy on healthier products that conform to the labelled information, and the vast amount of legislation about the correct characterisation and classification of vegetable oils have boosted a number of scientific works on this subject. Analytical techniques to face this challenge are, at least, as manifold as are the ways of adulteration, ranging from classical determination of chemical parameters to highly sophisticated instrumental and molecular biology techniques. Rather than being an exhaustive revision of published works, the aim of the present chapter is to summarise: i) the analytical methods used in the determination of the main oil components such as fatty acids, triacylglycerols, phytosterols, tocopherols and tocotrienols, phenolic compounds, pigments and volatile compounds, emphasising their importance in authenticity evaluation; ii) the alternative techniques based on spectroscopic

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methods such as Nuclear Magnetic Resonance, Fluorescence Spectroscopy, Mass Spectrometry, Atomic Absorption/Emission Spectrometry, Raman Spectroscopy, Infra-Red Spectroscopy; iii) and the recent developments about the use of DNA markers. An overview of the parameters used for the traceability of geographical origin and for the cultivar identification will be also presented.

## **Introduction**

Vegetable oils are of utmost importance for humans, not only from the nutritional point of view, but also for their use as technical components in chemical, pharmaceutical and cosmetic industries. Recently, they are also used as raw material for renewable energy. The importance of vegetable oils to the global economy becomes clear when considering the amount of vegetable oils produced and consumed worldwide. Based on the Food and Agriculture Organization (FAO) data, during the period of 2000 to 2005, the supply of vegetable oils has increased worldwide from 63.8 to 73.2 million tons [1]. This increase has been supported mainly by the so-called “World developing countries”, while in the considered “World developed countries” the values have been practically constant during this period. Besides, the amount and type of vegetable oils consumed also differ between these two groups. Based on the food supply data from FAO [1], soybean is the most consumed oil in both groups. In the developing countries it is followed by palm oil (food supply of ~11.8 million tons in 2005), rape and mustard oil (~5.1 million tons), groundnut oil (~4.0 million tons) and sunflower seed oil (~2.4 million tons), while in the developed countries it is followed by sunflower seed oil (food supply of ~5.2 million tons in 2005), rape and mustard oil (~3.8 million tons), olive oil (~2.2 million tons) and maize germ oil (1.2 million tons) [1]. The dissimilarities between consumption patterns are probably related to the different vegetable species availability in the different regions around the globe, as well as to cultural and economical reasons. In the last years, in the more developed societies there has been a growing awareness regarding the possible deleterious effects due to overconsumption of fats and oils. As well, the population is becoming more informed concerning the possible beneficial health effects associated with the intake of certain foods. For example, olive oil is one of the most important components of the Mediterranean diet, which is thought to be part of a healthy diet and considered as one of the best in the prevention of coronary heart disease. This point certainly explains the difference in the olive oil consumption between developed and developing countries, being the 4th and 12th most consumed oil, respectively. In the same way, consumers are demanding for high quality food products that combine pleasant flavours

with nutritional benefits [2]. Responding to consumer's expectations, there has been an increase in the number of available products considered as high quality or premium products, such as, for example, those produced in restricted geographical locations, and/or applying traditional production methods. As those products generally have higher economical value, they are more prone to be violated by fraudulent malpractices.

Although in most cases, the adulteration of vegetable oils does not pose a threat to the consumer's health, it represents an economical fraud, disloyal competition among producers and violates the consumer's right to make informed choices regarding the products they acquire. In the context of authenticity evaluation of vegetable oils, two main issues should be considered, namely the profitable adulteration by blending higher economic value oils with cheaper ones, and the fraudulent mislabelling regarding the information about the geographical origin, the cultivars and/or the production methodology [3].

Considering the importance of authentication for food processors, regulatory authorities and consumers, in the last years, different analytical methodologies have been proposed. Instrumental techniques based on chromatographic analysis of different families of compounds are among the most used approaches suggested for monitoring the quality and authenticity of vegetable oils. High performance liquid chromatography (HPLC) or gas chromatography (GC) have been applied for obtaining qualitative and quantitative data regarding different compounds such as fatty acids, triacylglycerols, phytosterols, tocopherols and tocotrienols, hydrocarbons, phenolic compounds, pigments and volatile compounds. In general, chemical pre-treatment of the sample prior to the chromatographic analysis is required, making some of these methodologies time consuming and labour intensive. Several other alternative approaches mainly based on spectroscopic techniques, such as near-infrared (NIR), mid-infrared (MIR), Fourier transform infrared (FTIR), front face fluorescence (FFF) and nuclear magnetic resonance (NMR) spectroscopy, are also being increasingly used for evaluating vegetable oils identity [4]. Compared to chromatographic methodologies, these techniques are considered faster, simpler and less expensive. In both chromatographic and spectroscopic techniques, multivariate analysis is usually required for classification treatment of the obtained data. With the technological advances, more powerful equipments are becoming available, generating huge amounts of data, making chemometrics an essential tool for authenticity assessment.

Among other approaches described in the literature for the authentication of vegetable oils are included the electronic nose analysis (sensor technology), the differential scanning calorimetry and the use of molecular markers. Since the chemical composition of oil, seeds and fruits can differ

among seasons and growing areas, there has been an increasing interest towards the application of DNA-based markers once they are independent from environmental conditions [5]. With the increasing commercial use of genetic modified plants for vegetable oil production, DNA analysis also occupies a place of utmost importance as being the technique of choice for genetically modified organism detection.

The aim of this chapter is to present an overview of the methods currently used for authentication of vegetable oils. For their importance, the parameters used to accomplish traceability of geographical origin and cultivar identification will also be presented at the end of this chapter.

## **Analytical tools for authentication of vegetable oils**

In the last decade, the technological advances have greatly contributed to control and fight against adulteration of food products. Conversely, it is also true that the same advances and knowledge are also being used by defrauders, resulting in more sophisticated adulterations and making them more difficult to detect. Olive oil is undoubtedly one of the oils more prone to fraudulent practices as it commands a higher price than other vegetable oils. The peculiar organoleptic characteristics of olive oil associated to its proved beneficial health effects have increased its popularity and demand in the last years. Owing to its chemical similarity (e.g. in the composition of fatty acids (FA), triacylglycerols (TAG), sterols and tocopherols) adulterations of olive oil with hazelnut oil are frequent and difficult to detect, being a good example of widely practiced frauds. Authentication of vegetable oils can be carried out by a variety of methods, from the classical physico-chemical techniques to more recent chromatographic, spectroscopic and molecular-based methodologies, among others. The most relevant methodologies are further detailed in the following sections.

### **Chromatographic methods**

#### **Fatty acids**

Fatty acids comprise a major portion of the saponifiable fraction of the vegetable oils. Most of FA are in the esterified form [esters of glycerol in triacylglycerides (90%), diacylglycerides, and monoacylglycerides (0.5-4%), as esters of other polar lipids such as lecithin (0.5-5%), as sterol esters (up to 1%), as esterified forms with natural aliphatic alcohols in waxes], and only a small part is found as free FA (up to 1%). Generally, FA are determined by GC as fatty acid methyl esters (FAME). Different methods based on alkaline

and/or acid catalysis [2] can be used to convert FA to FAME, depending on the presence of free FA, short-chain FA, or highly polyunsaturated fatty acids (PUFA) [6]. The detection in routine analysis is usually performed by flame ionisation detection (FID) since it is able to respond linearly to non-oxidised carbon.

Fatty acid composition has been widely used to discriminate and detect adulteration in vegetable oils. As referred in the review of Aparicio and Aparicio-Ruíz [2], the palmitic acid can be used as an indicator of adulteration of cottonseed oil by palm olein, since cottonseed oil has a palmitic acid content of 21-26% whereas palm olein contains around 40%. The presence of linoleic acid is a good indicator of adulteration of groundnut and sunflower oils, which might be adulterated with cheaper soybean or rapeseed oils. Sunflower and groundnut oils contain less than 0.1% of this FA vs. 10% in rapeseed and soybean oils [2]. Several other studies demonstrated that the comparison on FA composition is suitable for detecting adulteration of virgin olive oils (VOO) with sunflower oil [7], deodorised olive oil [8] or vegetable refined oils [9].

Other advantage of using the FA profile, beyond the discrimination and detection of adulteration in vegetable oils, is its usefulness as an indicator of geographical origin. Stefanoudaki et al. [10] showed, using canonical discriminant analysis, that the FA composition of olive oil samples depended on the altitude where the olives were grown, as well as other environmental factors such as relative humidity and rainfall. Additionally, FA composition of VOO varies from year-to-year and with the harvest date, which is probably due to differences in the amount of rainfall and temperature during the summer, and is determinant for the fruit ripening and oil biosynthesis [11]. Regardless, the FA composition of olive oil consists primarily of oleic acid (*cis* 18:1n-9), ranging from 55.0-83.0 % of total FA in the sample [12] and where specific studies have found it to range from 74.7 to 81.0 % [10,11]. Morelló et al. [13] noted that after 12 months of VOO storage in sealed bottles in the dark, the proportion of oleic acid tended to increase whereas the trend for polyunsaturated FA (PUFA), i.e. linoleic (18:2n-6) and linolenic (18:3n-3) acids both decreased. This trend was observed in both oils that were made from olives harvested “first” (i.e. from November to January) and “last” (i.e. January and beyond) [13]. Regarding other FA found in olive oil, the palmitic (16:0) and linoleic acids (18:2n-6) account for 7.5-20.0 % and 3.5-21.0 % of the FA content in olive oils, respectively [12].

### **Triacylglycerols**

Edible vegetable oils consist predominantly of TAG that, generally, follow a unique and typical pattern in the glycerol molecule, being

characteristic in the different oilseeds. The advantage of using TAG profile comparing to FA in the discrimination of vegetable oils is related to the stereo-specific distribution of FA in the glycerol molecule, which is genetically controlled and thus, the information of intact TAG is usually higher [2]. Different analytical techniques have been used for studying TAG profile of vegetable oils: thin-layer chromatography (TLC), reversed-phase HPLC (RP-HPLC), RP-HPLC combined with silver chromatography (Ag-RP-HPLC) and high-temperature GC [2,14,15]. Although Ag-RP-HPLC permits the separation of TAG based solely on the degree of unsaturation, it cannot separate TAG that differ only in the length of the chains of their FA. However, in RP-HPLC, the TAG are separated according to increasing carbon number (CN) and decreasing unsaturation (DB), i.e. according to equivalent carbon number ( $ECN = CN - 2DB$ ), providing better separation of individual TAG molecules [16]. The detection in both cases using Ag-RP-HPLC or RP-HPLC can be performed by ultraviolet (UV), refractive index (RI) or evaporative light scattering detectors (ELSD). The latter can be considered as a good alternative since no baseline drifts occur, without limitations on the use of mobile phase solvents compared with the other detectors [17]. It is noteworthy that, out of the entire chromatogram obtained by the HPLC analysis of TAG, the only peaks that are taken into consideration are those of trilinolein (LLL) and  $ECN=42$ . The LLL content was useful to detect the addition of soybean oil in olive oil from levels as low as 4%. Usually the LLL content in VOO is lower than 0.3%, while in peanut, corn, oat, whole rice, barley, soybean, tomato, sunflower, sesame, cotton, grape, tobacco, and hazelnut oils, is present at higher levels [18]. The parameter  $ECN=42$  is a very useful and effective tool in the detection of the presence of the most common vegetable oils. More specifically, the established limit for the  $ECN=42$  TAG in olive oil is satisfactory for the detection of adulteration of an olive oil with sunflower, soybean, cotton, corn, walnut, sesame, safflower and rapeseed oils. The use of this limit allows the detection of even very low levels of adulteration. The established limit for the  $ECN=42$  is, however, not satisfactory for detecting percentages lower or equal to 5% of hazelnut, almond, peanut and mustard oils in mixtures with olive oil [19].

TAG profile coupled with chemometric analysis may also be used for classification of the vegetable oils. Cunha and Oliveira [20] used the profile of TAG combined with principal component analysis (PCA) for the identification and discrimination of eight vegetable oils (sunflower, corn, peanut, soybean, hazelnut, walnut, sesame and olive oil). The same authors, based on the same profiles of three olive oil cultivars, were able to discriminate these monovarietal olive oils using PCA statistical analysis.

Amaral et al. [21] showed by canonical discriminant analysis of monovarietal walnut oil samples that the profile of TAG depended on the cultivar as well as other environmental factors such geographical localization and year of production. Similar behaviour was verified for monovarietal hazelnut oils [22]. The adulteration of vegetable oils (palm, palm kernel oil and canola) with animal fats (lard, beef tallow and chicken) in levels as low as 2% was easily verified using TGA profile and chemometric analysis [23].

The structural characterisation of TAG and, in particular, of the linked FA chains is becoming increasingly important in the detection of adulteration in vegetable oils. HPLC coupled to mass spectrometry (MS) is a powerful tool in this field, enabling the identification of individual FA groups without the need for authentic reference standards. The identification of TAG positional isomers by HPLC coupled with atmospheric pressure chemical ionisation mass spectrometry (APCI-MS) allowed the discrimination of vegetable oils from eight different sources (blackcurrant, blue poppy seed, evening primrose, olive oil, hazelnut, maize and rapeseed) [24]. Also Jakab et al. [25] used HPLC-APCI-MS combined with linear discriminant analysis to distinguish different kinds of vegetable oils (almond, avocado, corn germ, grape seed, linseed, olive, peanut, pumpkin seed, soybean, sunflower, walnut, wheat germ) based on their TAG composition.

The determination of both TAG and FA combined with chemometric analysis has been often applied to identify the origin of VOO [26,27], for identification of vegetable oils [28] and for detection of adulteration [29].

## Phytosterols

Phytosterols comprise a major portion of the unsaponifiable fraction of vegetable oils, occurring both in the free and esterified forms. The proportions of these fractions can strongly vary among different vegetable oils. In some oils, such as soybean, olive and sunflower, free sterols are the most representative form (ranging from 57-82%) while in others, such as canola and corn oils, can represent only a small proportion (33-38% of total sterols) [30]. Based on the chemical structure, they can be grouped into 4-desmethyl, 4-monomethyl and 4,4-dimethyl sterols [30], being the most representative group in vegetable oils. The standard method described on the ISO 12228:1999 [31] for phytosterol analysis involves the saponification of the oil sample followed by extraction of the unsaponifiable matter, clean-up of the extract using TLC, derivatisation to trimethylsilyl ethers and analysis by GC-FID or GC-MS. The resulting qualitative and quantitative phytosterol profiles have been used to authenticate different vegetable oils. For example,  $\Delta^7$ -stigmastenol and campesterol have been used to detect olive oil adulteration with sunflower



and soybean oil, and brassicasterol has been used to detect olive oil adulteration with rapeseed oil [32]. Nevertheless, this methodology only provides information on the total phytosterol composition, not discriminating among the free and esterified forms and, consequently, losing potential useful information. In other studies, the esterified and free sterols are separated by TLC or, more recently, using solid phase extraction cartridges, and analysed separately. Gordon and Miller [33] studied the steryl ester content and composition of ten different vegetable oils, allowing their classification into three major groups: high steryl esters content (corn and rapeseed), medium content (sunflower and high-oleic sunflower) and low content (safflower, soybean, cottonseed, groundnut, olive and palm oils). Although some oils, e.g. olive oil, showed a large variation in steryl ester content, the same authors concluded that the developed method was particularly suitable for detecting admixtures of low levels of corn and rapeseed oils. Based on the evaluation of sterol profiles by direct infusion mass spectrometry, Lerma-García *et al.* [34] were able to perfectly classify samples belonging to eight different botanical origins (hazelnut, sunflower, corn, olive, soybean, avocado, peanut and grapeseed). The evaluation of sterol profiles and total sterol contents, sometimes in combination with compositional data from other compounds, have been used for assessing oil authenticity and, according to some authors, can be a useful tool for the control of olive oil adulterations. Following the regulations established by the European Union (Regulation No 2568/91/EEC and later amendments) [35], olive oil must not exceed the upper limit of 4% of campesterol, 0.5% of cholesterol, 0.5% of  $\Delta^7$ -stigmastenol, should present a lower value of stigmasterol compared to campesterol, should present an apparent  $\beta$ -sitosterol ( $\Delta^{5,23}$ -stigmastadienol +  $\Delta^{5,24}$ -stigmastadienol + clerosterol +  $\beta$ -sitosterol +  $\beta$ -sitostanol +  $\Delta^5$ -avenasterol)  $\geq 93\%$  and should have a total sterol content  $\geq 1\text{g/kg}$  of oil. VOO can be adulterated by blending lower grade olive oils, such as refined olive oils, or by adding other vegetable oils, either crude or refined. Hazelnut oil is one of the VOO adulterants most difficult to detect due to the similarities of FA and TAG profiles in both oils. Considering that sterol profiles present important quantitative differences [36] in hazelnut and olive oils, the evaluation of phytosterol composition can provide helpful information to detect the adulteration of VOO with hazelnut oil. Based on the composition of three 4-desmethylsterols (campesterol,  $\Delta^7$ -stigmastenol and  $\Delta^7$ -avenasterol), Mariani *et al.* [37] suggested the calculation of a ratio ( $R_1 = \%\text{campesterol} \times [(\%\Delta^7\text{-stigmastenol})^2 / (\%\Delta^7\text{-avenasterol})]$ ) to detect this kind of adulteration. Depending on the olive oil variety, the limit of detection of the adulteration was between 5 and 10%, with the exception of mixtures based on African olive oils in which the algorithm was not able to identify hazelnut oil in levels as high as 20%. Due to the low accuracy of this ratio, other

analytical parameters were suggested to be additionally tested to assess the authenticity of olive oils [38]. Latter, another method was proposed by the same authors [38] based on free and esterified sterols ( $R_2 = \text{free } \Delta^7\text{-stigmastenol (mg/kg)} \times [(\% \Delta^7\text{-stigmastenol free}) / (\% \Delta^7\text{-stigmastenol esterified})]$ ). Those authors reported that, independently of the olive cultivars used to produce virgin and refined olive oils and the hazelnut oil samples analysed, when using both ratios the adulteration could be detected at a level of 6-8%, and in some cases even lower. The methodology for olive oils authentication based on both ratios ( $R_1$  and  $R_2$ ) was subsequently tested in an interlaboratorial study to determine its usefulness in detecting the presence of low quantities of any kind of hazelnut oil in olive oils [29]. In this study, two methods were compared, one based on both sterol ratios proposed by Mariani et al. [39] and another based on the difference between theoretical and empirical TAG calculation. It should be noticed that the main interest of this study was not to determine the quantity of adulterant oil but to give a result in terms of presence/absence of adulteration. This study evidenced poor results for the method based on the phytosterol ratios since it showed a maximum efficiency (81.8%) at a cut-off of 10% of hazelnut oil, while the maximum efficiency of the TAG method (90.3%) was obtained at a cut-off of 8% of hazelnut oil. The lower performance of the method based on sterols was associated with the high variability of the relative standard deviations (RSD) obtained for the compounds involved in  $R_1$  and  $R_2$  calculation. As the sterols involved in both ratios are minor components of the sterol fraction, higher RSD can be expected.

Other sterols, besides 4-desmethylsterols, have also shown their usefulness to detect olive oils adulteration. The composition of 4,4-dimethylsterols has been used to detect VOO adulteration with olive pomace oil (OPO) at levels of 5% [40]. Differences between the composition of 4,4-dimethylsterols in hazelnut and olive oils were described by Azadmard-Damirchi et al. [32,41,42], with lupeol and an unknown compound with a lupine skeleton found to be present only in hazelnut oil. The authors suggested that those two compounds can be used as markers for detecting olive oil adulteration with hazelnut oil. Crude hazelnut oil, at levels as low as 3.5%, was detected in adulterated VOO, but in refined hazelnut oil it was more difficult to obtain since significant losses of both compounds can occur. In opposition, the adulteration of olive oils with 2% of fully refined hazelnut oil was still detected by tracing total and esterified lupeol [43].

### **Tocopherols and tocotrienols**

The four tocopherols ( $\alpha$ -T,  $\beta$ -T,  $\delta$ -T, and  $\gamma$ -T) and four tocotrienols ( $\alpha$ -T3,  $\beta$ -T3,  $\delta$ -T3, and  $\gamma$ -T3) are collectively called Vitamin E. All this eight

forms contain a chromanol ring and a hydrophobic side chain, a phytyl in the case of tocopherols and an isoprenyl with three double bonds in tocotrienols. Tocopherols are the major Vitamin E components present in most vegetable oils, while tocotrienols are present especially in palm oil.

The determination of tocopherols and tocotrienols in vegetable oils can include liquid-liquid extraction without saponification or solvent extraction after saponification before a chromatographic analysis by HPLC or GC [44]. GC analysis is normally disregarded due to the nonvolatile nature of these compounds, requiring derivatisation prior to the quantification step. Thus, HPLC using both normal (NP) and reversed phases (RP) is the most common methodology used for the analysis of tocopherols and tocotrienols. When comparing RP to NP columns for separation, the latter show the main advantage over the former by completely separating all isomers [20,44]. HPLC used in the analysis of these compounds include UV, fluorescence, ELSD, electrochemical, and amperometric detection. Fluorescence detection is described as more sensitive and selective than UV. ELSD has been successfully applied in the analysis of different compounds and, consequently, has been increasingly used in many analytical laboratories [20].

The distribution of tocopherols varies widely among different vegetable oils, representing a useful parameter to discriminate them. In VOO,  $\alpha$ -T is nearly 95% of the total tocopherol content. Quantitative determination of tocopherols has been suggested as a method to determine adulterations of olive oil with less expensive soybean, cottonseed or hazelnut oils [37,18]. Peanut oil exhibits a relatively high amount of  $\gamma$ -T, whereas safflower oil contains only trace amounts. Thus, even in an aged or partially refined safflower oil it should be possible to detect peanut oil at a level of 10%. Sesame seed oil contains very low levels of  $\beta$ -T, which can be used as a parameter of adulteration of this type of oils, if found at high levels. Soybean oil can be detected in sunflower and peanut oils due to the high contents of  $\gamma$ -T and  $\delta$ -T, respectively. The high concentration of  $\alpha$ -T in sunflower seed and wheat germ could be used to detect these oils in lower  $\alpha$ -T containing oils, such as in grape seed oil [18]. Tocopherols have also been described to be used in the detection of the sophisticated adulteration of soybean oil with linseed oil [45]

Tocopherol and tocotrienol contents are not constant on the fruits, depending frequently on the cultivar, stage of ripening, edafoclimatic conditions, and olive-growing techniques. Psomiadou *et al.* [46] showed that the content of  $\alpha$ -T in Greek VOO ranged widely among the samples from the same year of harvest and was high for the majority of the samples.

$\alpha$ -Tocopherol is traditionally considered to be the major antioxidant of olive oil and its concentration varies from a few ppm up to 300 ppm, depending the cultivar used [47]. Concentrations of  $\beta$ -,  $\gamma$ - and  $\delta$ -tocopherols have also been reported to range from trace amounts to 25 ppm in 18 samples of olive oils from two regions of Portugal [48].

### **Volatile compounds**

Volatile compounds are low molecular weight substances that are easily vaporised at ambient temperature, belonging to diverse chemical families such as hydrocarbons, aldehydes, alcohols, ketones and esters. Crude vegetable oils contain volatile compounds that may confer characteristic aroma to some oils, which is considered one of the parameters that largely contributes to the acceptance/rejection of a food product. Different techniques have been used to evaluate the volatile compounds of vegetable oils, being mostly based on the headspace analysis, either after chromatographic separation on analytical columns and subsequent identification, or by the direct analysis of the unresolved mixtures of the volatiles in the headspace, such as the case of electronic nose technology [49]. A chemical fingerprint obtained by analysing the whole volatile fraction can be used for vegetable oil authentication. Peña et al. [50] suggested the direct coupling of headspace with MS, in combination with multivariate pattern-recognition and regression techniques for data treatment, to detect the adulteration of olive oil with hazelnut oil. The method allowed the detection of 7 and 15% of crude hazelnut oil in refined and virgin olive oils, respectively. The electronic nose technology coupled to chemometrics has been proposed by Oliveros et al. [51] for the detection of olive oil adulteration with sunflower oil and OPO at levels as low as 5%. Recently, Mildner-Szkudlarz and Jelén [49] compared the effectiveness of three methods applied to the analysis of volatile compounds, namely solid phase micro-extraction (SPME) of volatiles followed by GC-MS analysis, electronic nose based on metal oxide sensors (HS-Enose) and direct coupling of SPME to mass spectrometry (SPME-MS). The three methods allowed the detection of 5% (v/v) of virgin hazelnut oil in extra VOO. The authors pointed out that the SPME-MS and electronic nose methods presented the advantage of being much faster than SPME coupled to GC-MS. Although the whole volatile fraction analysis can be useful for authentication of vegetable oils, it should be noticed that natural variations in the volatile composition can occur among samples from different production years, geographical origins and/or obtained from different varieties. Consequently, chemometric tools as well as large data sets are generally required. For this reason, the analysis of characteristic compounds to serve as

markers can be very useful for authentication purposes. The presence of (*E*)-5-methylhept-2-en-4-one (filbertone) in the volatile fraction is considered to be the main responsible for the hazelnut characteristic aroma. As this compound is absent in olive oil, it could be used as a chemical marker to detect olive oil adulteration with hazelnut oil [52-57]. Considering the low levels of filbertone in adulterated samples, pre-concentration steps are frequently required. The most used methods consist on the simultaneous distillation-solvent extraction (DSE), SPME, ultrasonically assisted solid-phase extraction and supercritical fluid extraction [53]. The sample is subsequently analysed, generally by using on-line coupled RP-HPLC and GC with MS or FID. In the last case, the use of chiral columns is advisable since it allows the enantiomeric separation of the two isomers, *R*(-)- and *S*(+)-filbertone. The analysis of both enantiomers is especially useful when overlapping one of the enantiomers with unknown compounds occurs using the FID detector [54,55]. A method based on the identification of *R*(-)- and *S*(+)-filbertone enantiomers involving the use of DSE and subsequent GC analysis with a chiral stationary phase has been proposed by Caja *et al.* [56]. The method allowed the detection of adulterations of olive oil with hazelnut oil at levels of 5%. In another work, filbertone analysis was used as a chiral marker to detect the same adulteration at identical levels by direct RP-HPLC/GC analysis [57] using a programmable temperature vaporiser (PTV). The method presented the advantage of allowing the direct and rapid analysis of the oil samples without requiring any type of sample pre-treatment. Although filbertone detection seems promising in detecting olive oil adulteration with hazelnut oil, it should be noticed that it is mainly useful when the crude oil is involved in the adulteration, as most volatile compounds are eliminated during the deodorisation step in the refining of vegetable oils. Nevertheless, depending on the conditions used during the deodorisation step, the lost can be only partial and minute amounts of filbertone can still be present in the refined hazelnut oil. Blanch *et al.* [54] demonstrated that filbertone is not completely removed during deodorisation and, consequently, its determination could also be useful for establishing the presence of refined hazelnut oil in olive oil. The authors also pointed out that, although the adulteration of VOO with refined hazelnut oil could be more difficult to detect by filbertone analysis, the use of harsh refining conditions would probably give rise to olive oils with quality and purity characteristics (such as sterol composition and UV absorbance) outside legal tolerances [54]. According to the same authors, the proposed method based on filbertone analysis seems to be advantageous regarding both reliability and selectivity. On-line coupled RP-HPLC/GC by means of a horizontally positioned PTV was also used by Flores *et al.* [52], allowing the detection of adulteration of

olive oil with crude and refined hazelnut oil at levels of 5 and 12%, respectively. However, the reported levels were shown to depend on the concentration of filbertone in the hazelnut oil, since in the case of low concentration of filbertone in the blending hazelnut oil it was difficult to confirm the adulteration. Recently, a method using a headspace autosampler in combination with GC equipped with a PTV and a MS detector was suggested by Pavón et al [53]. The proposed method did not require any sample pre-treatment (filtration, extraction or pre-concentration) as it just consisted on placing the olive oil sample in the analysis vial. This allowed the reduction of the time of analysis as well as the reduction of all possible experimental errors associated to sample pre-treatment. Moreover, the limit of detection of filbertone in olive oil was about 25 times lower than in other previous works.

### **Phenolic compounds and pigments**

Phenolic compounds are secondary metabolites of plants, including a wide range of molecules (mainly grouped as phenolic acids and flavonoids) that have been associated to numerous health benefits [58]. Both phenolic compounds and pigments are minor constituents that can be found in crude edible oils. They are generally associated to the quality and organoleptic characteristics of the oils. The phenolics are associated to bitterness and antioxidant properties, delaying oxidation processes. The chlorophyll and pigments influence the consumer's acceptability of oils since they are associated to colour perception [59]. Gandul-Rojas et al. [60] studied the composition of chlorophyll and carotenoids of monovarietal VOO and showed that those pigments varied according to the olive variety. Nevertheless, independently of pigment content, the ratio between chlorophyll and carotenoid fractions remained constant. Latter, the same group analysed the chlorophyll and carotenoid profiles of 50 mono varietal VOO to develop an index of authenticity [60]. They concluded that, for VOO in general, independently of the variety, the ratio of chlorophyll/carotenoid should be around 1 and that the ratio of minor carotenoids/lutein should be around 0.5. Furthermore, it was referred that the percentage of lutein, violaxanthin and total pigments could be used as a tool to distinguish among mono varietal VOO.

Different phenolic compounds have been reported in VOO, such as simple phenols (tyrosol and hydroxytyrosol), phenolic acids, secoiridoids such as oleuropein, among others [61]. The feasibility of using the profile of phenolic compounds applied to cultivar classification was studied by Gomés-Alonso et al. [61] throughout the analysis of several commercial Cornicabra

VOO during five consecutive crop seasons (1995 to 2000). The variability of the content distribution of three compounds (1-acetoxypinoresinol, 4-(acetoxyethyl)-1,2-dihydroxybenzene, and listroside aglycon) seemed to be related to the factor “cultivar”, thereby, suggesting the feasibility of cultivar classification based on the profile of phenolic compounds, in particular for Arbequina and Picual varieties.

Although the usefulness of the analyses of phenolic compounds and pigments in the case of olive oils has been demonstrated, they have no utility for most other vegetable oils that are submitted to refining processes decreasing/removing many minor compounds, including phenolics and pigments [59].

## **Spectroscopic methods**

Spectroscopic methods represent one of the main tools of modern chemistry for the determination of the authenticity of edible oils, and the detection of adulteration. Most spectroscopic methods for adulteration detection are based on NMR spectroscopy of  $^1\text{H}$  and  $^{13}\text{C}$ , infrared spectroscopy, Raman spectroscopy, fluorescence spectroscopy and mass spectrometry. The primary advantage of these methods is related to the non-destructive character, simplicity (relative easy sample preparation and adaptation for the use by untrained personnel), rapidness and moderate cost instrumentation. One important factor in the successful use of such methods is the requirement for spectral databases, which include most or all of the significant variations of parameters likely to be found in the material under evaluation. The creation of spectra databases can be expensive, although useful models can be obtained for the identification of adulterations in vegetable oils [62]. To build up, test and validate such models, appropriate multivariate statistical tools are required, among which PCA, discriminant analysis (DA) and canonical analysis (CA) occupy a very important position.

### **NMR spectroscopy**

NMR spectroscopy is based upon the measurement of absorption of radiofrequency radiation by atomic nuclei with non-zero spins under a strong magnetic field [63]. The absorption of the atomic nuclei is affected by the surrounding atoms, which cause small local modifications to the external magnetic field. In this way, detailed information about the molecular structure of a food sample can be obtained. Among nuclei with non-zero spin, the isotopes of hydrogen-1 (spin = 1/2) and carbon-13 (spin = 1/2) are the most used in NMR, although other isotopes as nitrogen-15 (spin = 1/2),

oxygen-17 (spin = 5/2), fluorine-19 (spin = 1/2), or phosphorous-31 (spin = 1/2) are also frequently employed [64].

$^1\text{H}$ ,  $^{13}\text{C}$  and  $^{31}\text{P}$  NMR spectroscopy were reported to be useful in the detection of adulterations of olive oils with seed oils (soybean, peanut, maize, hazelnut, sunflower, walnut, coconut, almond, rapeseed, etc.) by measuring the levels of n-3 linolenic acid [65] or the ratio of 1,2-diglycerides to total diglycerides (1,2-diglycerides and 1,3-diglycerides) combined with acidity, iodine value and FA composition [66]. The major FA (oleic and linoleic) of oils of three different botanical origins (olive, hazelnut and sunflower) were analysed by  $^1\text{H}$  NMR spectroscopy, which allowed the detection of their mixtures [67]. García-Gonzalez [68], presented an Artificial Neural Network based on  $^1\text{H}$  and  $^{13}\text{C}$  NMR data, which resulted in a mathematical model capable of detecting the admixture of 0.8% of hazelnut oil to olive oil. Extra virgin, refined and lampante olive oils were discriminated using multivariate statistical analysis applied to  $^{31}\text{P}$  NMR spectra, enabling the detection of admixtures of 5% of refined and lampante grade in VOO [69].

### **Infrared spectroscopy**

Infrared (IR) spectroscopy deals with electromagnetic spectrum in the region of IR. The IR portion of the electromagnetic spectrum is divided into three regions: the near-, mid- and far- IR, named for their proximity to the visible spectrum. The far-infrared spectroscopy (FIR) is based on the absorption of electromagnetic radiation at wavenumber in the range of 200-10  $\text{cm}^{-1}$  (wavelengths 50-1.0  $\mu\text{m}$ ). The MIR spectroscopy is related to the absorption of electromagnetic radiation at wavenumber in the range of 4000-200  $\text{cm}^{-1}$  (wavelengths 50-2.5  $\mu\text{m}$ ). The NIR spectroscopy is based on the absorption of electromagnetic radiation at wavenumber range of 13000-4000  $\text{cm}^{-1}$  (wavelengths 2.5-0.78  $\mu\text{m}$ ).

MIR spectra of vegetable oils comprise the vibrations of polymethylene chains of TAG. Two distinct regions are present in a MIR spectrum. The first region (3100-1700  $\text{cm}^{-1}$ ) is formed by well-resolved peaks from the absorption due to the C-H stretching vibration of *cis* FA ( $-\text{CH}-\text{CH}=\text{}$ ) that appears near 3005  $\text{cm}^{-1}$  in triolein and shifts towards higher frequencies as the degree of unsaturation increases. The corresponding *trans* FA absorb near 3025  $\text{cm}^{-1}$ . The second part of a MIR spectrum (1500-700  $\text{cm}^{-1}$ ) is called the fingerprint region and shows overlapping peaks. The fingerprint region is closely related to the degree and type of unsaturation, and also to the content of *cis* and *trans* isomers. The intensity of the band near 1400  $\text{cm}^{-1}$  depends on the percentage of monounsaturated acyl groups, while that of the band near 1160  $\text{cm}^{-1}$  depends on the content of saturated acyl groups [70].



In the last 15 years the original MIR instruments of the dispersive type have been successfully substituted by FTIR. The main advantages of Fourier-transform instruments over MIR equipments are the increased sensitivity, the allowance of much higher energy throughput and the dramatic improvement of spectral acquisition speed.

FTIR was employed by Guillén and Cabo [71] to detect and quantify adulteration of olive oil with other vegetable oils. They verified that adulterant oils (sunflower, corn, walnut, rapeseed, soybean, safflower, peanut, wheat germ, and sesame oil) exhibited absorption bands around  $913\text{-}914\text{ cm}^{-1}$ , which differed in intensity and position for olive oil showing very low or no intensity. The potential of FTIR was also investigated in the works of Ozen and Mauer [72], for discriminating adulterated hazelnut oil with sunflower oils at levels of 2%. Yang *et al.* [73] discriminated among 10 different edible oils and fats (coconut, soybean, canola, safflower, olive, corn, pea, cod liver, butter and lard). Vlachos *et al.* [74] quantified the adulteration of extra VOO with corn or sesame oils (at levels of 9%) and sunflower or soybean oils (at levels of 6%). More recently, Gurdeniz and Ozen [75] used MIR spectra combined with chemometrics to detect and quantify olive oil adulteration with different vegetable oils such as corn, sunflower, cottonseed and rapeseed. The model identified the adulterants, cottonseed and rapeseed oils, in olive oil at a level of 5% and the detection of corn-sunflower oil mixtures in olive oil at the same level.

NIR spectra generally contain a number of broad and overlapping bands, arising from the overtones (first and second) and combinations of functional groups present in oil samples. The most intense bands in the oil spectra can be found at  $4260$  and  $4370\text{ cm}^{-1}$ , and are characteristic of the combinations of C–H stretching vibrations of  $-\text{CH}_3$  and  $-\text{CH}_2-$  with other vibrations. The two bands at  $5700$  and  $5750\text{ cm}^{-1}$  correspond to the first overtone of the C–H stretching vibration of  $-\text{CH}_3$ ,  $-\text{CH}_2-$  and  $-\text{HC}=\text{CH}-$ . The absorption band near  $6010\text{ cm}^{-1}$  is due to C–H vibration of *cis*-unsaturation. Fatty acids with *cis* double bond exhibit strong absorption bands in the region around  $6010\text{ cm}^{-1}$ , whose intensity augments with increasing unsaturation. In the region between  $7700$  and  $9100\text{ cm}^{-1}$ , the second overtone of the C–H stretching vibration of  $-\text{CH}_3$ ,  $-\text{CH}_2-$  and  $-\text{HC}=\text{CH}-$  can be found [70].

NIR spectroscopy has been successfully applied to detect adulteration of VOO and extra VOO with refined olive oils and other vegetable oils such as corn and sunflower [76,77], or to classify soybean, corn, rice bran, peanut, rapeseed, sesame olive and cottonseed oils [78]. Downey *et al.* [79] applied NIR to discriminate adulterated VOO with sunflower oils at levels of 1%. Still using the same approach, Christy *et al.* [80,81] were able to quantify the

adulteration of VOO with oils from soyabean, sunflower, corn, walnut and hazelnut obtaining low error limits.

### **Raman spectroscopy**

Raman spectroscopy is based on vibrational, rotational and other low-frequency modes in a system. It has been effectively used in the detection of adulterants in vegetable oils without the need of sample pre-treatments. Lopez-Diéz et al. [82] and Heise et al. [83] applied this technique in the discrimination of vegetable oils and in the detection of hazelnut and sunflower oils in VOO, respectively. This technique can provide unambiguous results when combined with adequate statistical analysis. Heise et al. [83], using Fourier Transform (FT) Raman spectroscopy, concluded that sunflower oil could be detected even at a level of 1%. Baeten et al. [84] were able to detect the addition of hazelnut oil to olive oil above 8% by FT-Raman spectroscopy. El-Abassy et al. [85] applied FT-Raman in combination with chemometrics to detected adulteration of VOO with sunflower oil at levels of 1%.

### **Fluorescence spectroscopy**

Fluorescence spectroscopy has proved to be a useful technique for monitoring the authenticity of vegetable oils, with the advantages of high sensitivity for a wide range of potential analytes and, in general, the avoidance of consumable reagents and extensive sample pre-treatment [86]. In the last few years, the instrumental improvements and the availability of specific software for fluorescence spectra analysis have contributed to the development of this technique [87]. Particularly, the possibility of using the excitation emission matrices (EMM) – a set of emission spectra recorded at several excitation wavelengths – has boosted research in the area. The detection of hazelnut crude or refined oil in virgin and refined olive oils was tested using EMM methodology [88]. The adulteration of VOO with refined olive oil and OPO was also studied by interpreting EMM data using several statistical discrimination techniques [87]. Sikorska et al. [86] used both total luminescence and EMM techniques to characterise and differentiate edible oils, including soybean, sunflower, rapeseed, peanut, olive, grapeseed, linseed and corn oils. Both methods were able to provide a high discrimination level of the oil classes with low classification error. The potential of these techniques has been demonstrated, but a strong statistical background is needed to achieve satisfactory results.

## Mass spectrometry

Several advanced MS methods such as Headspace-MS (HS-MS), Isotope Ratio MS (IRMS), Electrospray Ionisation MS (EIS-MS) and Inductively Coupled Plasma MS (ICP-MS) have been applied in authentication of vegetable oils. The HS-MS analysis consists in the introduction of the global volatile fraction present in the HS of a sample, without prior chromatographic separation, into the ionisation chamber of a mass spectrometer. Normally, chemometric methods are necessary to analyse spectral data and convert them into useful information. HS-MS has been used to detect the addition of hazelnut, sunflower [50] and OPO [89] to VOO.

IRMS consists of measuring the isotope ratio ( $^2\text{H}/^1\text{H}$ ,  $^{13}\text{C}/^{12}\text{C}$ ,  $^{15}\text{N}/^{14}\text{N}$  and  $^{18}\text{O}/^{16}\text{O}$ ) of an analyte isotopically representative of the original sample. It has been used to classify different vegetable oils (olive, pumpkin, sunflower, maize, rape, soybean, and sesame oils) based on  $^{13}\text{C}$  values of FA [90]. Kelly *et al.* [91], based on  $^{13}\text{C}$  values of the principal FA (palmitic, stearic, oleic and linoleic acids), were able to discriminate groundnut, palm, rapeseed and sunflowers oils.  $^{13}\text{C}$  values of FA, sterols and tocopherols were used to detect the adulteration of maize oil down to a level of 5% [92].

Soft ionisation MS methods are required to analyse both small molecules and biomacromolecules, as in the case of very informative whole-food fingerprinting techniques used in metabolomics. ESI-MS can provide soft ionisation and has been used to produce informative and discriminating mass spectra of VOO and its common adulterants [93]. Catharino *et al.* [94] used the same approach applied to polar components of several oils extracted with a mixture of methanol/water (1:1). The authors refer the capability of the method, not only to detect adulterants (soybean oil in olive oil), but also the aging of vegetable oils. Using a further ESI-MS technique – the ESI Fourier Transform Ion Cyclotron Resonance MS (ESI FT-ICR MS) – Wu *et al.* [95] have resolved and identified FA, di- and tri-acylglycerols and tocopherols in canola, olive and soybean oils without the need of any sample pre-treatment. The same authors have proven the effectiveness of the technique in detecting the addition of soybean oil to olive oil.

## DNA-based methods

In the last years, there has been a growing interest towards the application of methodologies based on the analysis of DNA regarding food authentication [96]. DNA analysis presents several advantages such as, a high thermal stability of DNA molecules compared to other compounds such as proteins, high specificity and high sensitivity, associated with the ubiquity of

DNA molecules in most cells. These advantages make the use of DNA makers as effective targets independent of geographical, climatic or agronomical factors.

Most DNA-based methods rely on the specific amplification of one or more DNA fragments by means of polymerase chain reaction (PCR). The PCR amplification is based on the hybridisation of specific primers and synthesis, *in vitro*, of millions of copies of the fragments flanked by those primers [96]. Several papers are available reporting the application of different DNA fingerprinting methods to olive oil traceability and cultivar identification, namely: Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Single-Nucleotide Polymorphism (SNP) and microsatellites, also known as Simple Sequence Repeats (SSR) [97-101].

One critical point regarding the successful application of DNA-based methods is, undoubtedly, the effectiveness of DNA extraction. In the specific case of vegetable oils, one problem to overcome is the minute amounts of DNA in the sample to be extracted [102]. Moreover, in refined vegetable oils, DNA can undergo degradation during processing, making DNA analysis even more difficult to accomplish. Different works have reported specific protocols for DNA isolation from olive oil [99-101,103], demonstrating the subsequent successful DNA amplification. Compared to VOO, which is obtained from the olive fruit exclusively by mechanical means without any further treatment, the refined vegetable oils are submitted to several steps along processing (such as degumming, neutralisation, bleaching and deodorisation). These steps, especially those comprising heat treatment and severe pH variations, may affect the quantity and integrity of the remaining DNA in the fully refined oil [102]. Thus, reports concerning the successful DNA extraction and amplification from fully refined vegetable oils are still scarce.

Breton et al. [104] showed that DNA traces are present in olive oil samples, even refined oil, whose quantity depended on the technology of processing and conservation conditions of oil. The authors tested several DNA extraction protocols, including available commercial kits, such as the Wizard Food kit (Promega). The results showed that the highest DNA yields were obtained with the method that used magnetic beads and that was successfully amplified using SSR primers, being considered a suitable protocol for routine use. Costa et al. [105] tested different extraction protocols for the isolation of DNA from commercial refined vegetable oils: the in-house prepared Wizard and CTAB methods, and the methods based on the use of the commercial kits Wizard<sup>®</sup> Magnetic DNA purification (Promega) system for food and Nucleospin<sup>®</sup> food kit (Macherey-Nagel). The latter was the only one that allowed obtaining amplifiable DNA from the

tested refined oils, which was verified by using both end-point PCR and real-time PCR targeting the soybean lectin gene [105]. The selected extraction protocol was further applied to detect genetically modified soybean along all steps of oil refining in an industrial unit [102]. That was achieved by the specific amplification of the soybean lectin gene from all the DNA extracts corresponding to the different refining steps (including crude, degummed/neutralised, washed, bleached and deodorised oil) [102]. DNA amplification from commercial sunflower and maize oils was also reported by Doveri and Lee [106], when applying different protocols for DNA extraction on a range of processed foods.

Beyond the crucial importance of selecting an adequate DNA extraction protocol, it should also be noticed that, when degradation of DNA is expected to occur, as in the case of vegetable oils, the selection of primers towards small amplification products (generally smaller than 200 bp) and high PCR efficiency is also of extreme importance [5,102]. For authentication purposes, quantitative tools can sometimes be of major importance. In this case, conventional end-point PCR is not adequate and quantitative real-time PCR is required. Other advantages of using real-time PCR technique are the increased sensitivity, fastness and, especially when using specific oligonucleotide probes, enhancing the specificity and, consequently, the reliability of the analysis.

Recently, a methodology based on the combinatorial use of a PCR with a lab-on-a-chip capillary electrophoresis system was proposed for the authentication of plant oils [107]. The results indicated that the methodology could potentially detect the adulteration of olive oil with oils extracted from seven different plant species (sunflower, walnut, hazelnut, almond, soybean, corn and cotton), although without discriminating sesame or live avocado oil. The authors suggested that the proposed assay could be useful as a preliminary diagnostic test for authentication of olive oil.

## **Traceability: Geographical origin determination**

The determination of the geographical origin is a critical aspect in the authenticity and quality evaluation of vegetable oils. There are many categorised vegetable oils, sold at high prices, declaring local high quality characteristics especially relevant for VOO. Therefore, legal instruments to protect the consumers and the producers have been introduced in the EU [108]. To be eligible the use of Protected Designation of Origin (PDO) or Protected Geographical Indication (PGI), an agricultural product or foodstuff must comply with strict specifications.

During the last years, a wide number of chemical and sensory parameters such as FA, TAG phenolic acids, waxes, sterols and hydrocarbons have been combined with comprehensive statistical data analysis, though they do not provide a secure classification of geographical origin. Thus, the on-site inspections by the control authorities are currently the only accepted way to safeguard the PDO/PGI label.

One of the most promising approaches in this field seems to be the IRMS based on the pattern of naturally occurring isotopes as influenced by biochemical properties of plants and geoclimatic conditions, making it dependent on the geographical and botanical origin of plants [109]. IRMS has been used to identify the geographical origin of VOO sold with certified origin appellation based on  $^{13}\text{C}$  values of palmitic and oleic acids to detect the blending of other edible oils with similar FA covariations [90]. Woodbury et al. [110] showed that the variability in  $^{13}\text{C}$  values was related to the geographical origin of the oil, year of harvest and the particular variety of oil.  $^{18}\text{O}/^{16}\text{O}$  and  $^{13}\text{C}/^{12}\text{C}$  values of the whole oil fraction, sterols and aliphatic alcohols were used to identify the geographical origin of the olive oil produced in Greece, Morocco, Spain, Italy, Tunisia, and Turkey [111].

NMR allowed the simultaneous detection of several low mass components in complex mixtures of oils using a simple sample preparation. Several authors have shown that  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectroscopy is useful to distinguish the variety and origin of VOO by informing about the acyl composition and the positional distribution of the glycerol moiety combined with TAG and FA composition [112,113]. A quantitative high-resolution  $^{13}\text{C}$  NMR method was also able to discriminate between Italian olive oils by cultivar and geographical origin. Multivariate analysis was carried out on the 35 carbon signals obtained. By using variable reduction techniques, coupled with standard statistical methods (partial least-squares (PLS) and PCA), it was possible to separate samples according to variety and region of origin. Thus, three regions with great representation in the data set, all except one of a test set of 34 samples, were correctly predicted [114]. McKenzi and Koch [115] have developed a rapid method (less than 20 minutes) by  $^{13}\text{C}$  NMR spectroscopy for extra VOO authentication based on the determination of the major FA (oleic, linoleic and saturated acids), demonstrating the high potentiality of this technique for region and cultivar identification.

FTIR spectroscopy has been applied successfully to classify monovarietal olive oils obtained from three different cultivars and to differentiate the mixture from monovarietal oils [116]. For geographical origin confirmation of sixty extra VOO samples from producers of four different countries (Greece, Italy, Spain and Portugal), FTIR spectroscopy in combination with multivariate analysis, was successfully applied [117].

Hennessy *et al.* [118] combined FTIR spectroscopy with germanium attenuated total reflectance and multivariate analysis (PCA and factorial DA) to confirm the origin of authentic Liguria extra VOO.

The potential of NIR spectroscopy was assessed for the quantification of FA and TAG in VOO samples ( $n = 125$ ) and for their classification (PLS1-DA) into five very close regions of France (Aix-en-Provence, Haute-Provence, Nice, Nyons and Vallée des Baux) assigned as PDO [119]. The spectroscopic interpretation of regression vectors showed that each PDO was correlated to one or two specific components of VOO according to their cultivar compositions. The results were quite satisfactory, in spite of the similarity of cultivar compositions between two denominations of origin (Aix-en-Provence and Vallée des Baux) [119]. Casale *et al.* [4] classified 195 extra VOO from Liguria using NIR and class modelling techniques such as potential functions techniques, soft independent modelling of class analogy and multivariate range modelling. Woodcock *et al.* [120], also using NIR spectroscopy (1100-2498 nm), confirmed the geographical origin of Ligurian extra VOO. A total of 913 extra VOO samples (210 Ligurian and 703 non-Ligurian) were collected over three consecutive harvests (2005, 2006 and 2007). A multivariate spectral fingerprint for Ligurian olive oil was developed and deployed to confirm or refute a claim that any given sample was Ligurian. The best models identified correctly the origins of samples in the prediction set up to 92.8 and 81.5% for Ligurian and non-Ligurian olive oil samples, respectively, using a first-derivative data pre-treatment.

A number of aromatic volatiles compounds have been investigated to discriminate varieties and differentiate geographic origins through different prototypes of electronic noses combined with multivariate analysis [121,122]. Although some promising results have been obtained, the analytical approach is in its infancy and much effort should be done to give a reliable origin indication.

## **Traceability: Cultivar identification**

The determination of the cultivar can be a decisive aspect regarding the authenticity and quality of some vegetable oils, such as the case of olive oil. Olive (*Olea europaea* L.) has a considerable number of different cultivars, which present differences concerning chemical composition and sensorial characteristics. Moreover, among different countries and even in different regions of the same country, genetically identical cultivars are sometimes designated by different names [123,124]. The chemical composition and sensorial descriptors outlining each cultivar are also affected by climatic and

agronomic aspects, together with olive ripeness and the olive extraction system [125,126]. As already referred in the last section, the EU has created legislation to establish and protect several PDO olive oils, ensuring both consumer's rights and fair commercial trade. In this context, the determination of the olive cultivar(s) used in olive oil production is of high importance for the final product authentication as, depending on the PDO olive oil, only certain cultivars are allowed to be used. Therefore, several analytical techniques have been suggested to ensure PDO olive oil authentication regarding the cultivar. The analysis of different chemical components of olive oil coupled to chemometric techniques for data exploitation has been reported by several authors as a possible approach. To differentiate monovarietal olive oils obtained from four different Italian cultivars, Brescia et al. [112] compared the feasibility of two methodologies, one based on the FA, TAG and sterols differences determined by chromatography coupled to chemometrics, and another based on  $^{13}\text{C}$  NMR spectroscopy. The authors concluded that the models obtained from applying discriminant analysis to the data sets of chromatographic and  $^{13}\text{C}$  NMR results were statistically similar as the predictability of the constructed models was 92 and 88%, respectively. Based on the major FA and chemometrics, Bucci et al. [127] were able to correctly predict which cultivar was used in a set of oils comprising five Italian cultivars (Carboncella, Frantoio, Leccino, Moraiolo and Pendolino).

The characterisation of four cultivars (Arbequina and Picual from Spain, Coratina from Italy and Koroneiki from Greece, collected in two years in the respective countries) based on the chemical composition comprising 31 non-volatile compounds (FA, sterols, alcohols and methylsterols) and 65 volatile compounds was proposed by Aparicio et al. [128]. From an exclusively chemical point of view, esters and furans (volatile compounds) and alcohols, both aliphatic and triterpenic, could be used to characterise the cultivars, as Koroneiki and Arbequina showed the maximum concentration of esters (responsible for the green/grass perception) and of furans (sweet/ripe fruit perception), respectively. The total concentration of furans was found to distinguish Picual and Koroneiki cultivars from Coratina and Arbequina as the former cultivars are picked when they are completely black, while the latter are still green when harvested [128]. Extra VOO of the cultivars Nocellara, Biancolilla and Cerausola were effectively discriminated using HPLC-MS combined with chemometrics, allowing the detection over 50 compounds in the samples, from which minor tri- and diacylglycerols proved to be very useful for discrimination [129]. Commercial Cornicabra VOO from five successive year crops (1995 to 2000) were analysed by Gomez-Alonso et al. [130] for their profile of phenolic compounds. The authors



found that the content distribution of some phenolics differed among the different cultivars evaluated, suggesting the feasibility of cultivar classification based on the phenolic profile, in particular for Arbequina and Picual cultivars.

Although spectroscopy has been mainly used for tracing the geographical origin of olive oils, in some works it has been used for cultivar determination. FTIR spectrometer equipped with a ZnSe attenuated total reflection system and a deuterated tri-glycine sulfate detector allowed the successful classification of monovarietal extra VOO of the Turkish cultivars Erkence, Ayvalik and Nizip, as well as the detection of different monovarietal oil mixtures (2-20%) [116]. Gurdeniz *et al.* [131] compared the results obtained for FA composition and for MIR spectra analysis of Turkish olive oils regarding to their cultivars, growing location and harvesting years. Although MIR spectroscopy allowed cultivar and seasonal discrimination to some extent, better results were obtained based on the FA analysis.

Recently, several works based on DNA analysis applied to cultivar recognition have been reported. Pasqualone *et al.* [132] used DNA microsatellite analysis for cultivar identification of different olive oils. The DNA from olive oils produced using olives of ten different cultivars was extracted and used as a template targeting seven different microsatellite sequences. The electrophoretic pattern obtained for each oil sample was identical to the corresponding leaves and fruits. Other works reported the utilisation of SSR markers to distinguish VOO from different cultivars [132], and the same technique was able to identify the presence of one cultivar in one PDO olive oil [133]. The use of advanced techniques based on DNA analysis coupled to a microarray platform has been recently reported by Consolandi *et al.* [103]. The application of a microarray-based assay for single nucleotide polymorphic analysis (SNP) distinguished alleles in a ligation detection reaction (LDR) with subsequent fluorescent detection by hybridisation on a universal array (UA). The LDR-UA approach was used to detect 17 SNP in olive genomic sequences previously amplified by PCR from fresh olive leaves. The methodology was adequate to discriminate 49 olive varieties selected among the most widely cultivated for olive oil production in the Mediterranean area [134]. Later on, the same authors reported and improvement of the assay, using a simple and robust protocol to extract amplifiable DNA from olive oil, followed by the simultaneous amplification of SNP-containing sequences by multiplex PCR [103]. Again, the 49 olive oil cultivars were unequivocally discriminated, but with 13 out of the 17 investigated SNP. The authors pointed out several advantages of the developed method, namely the suitability of the extraction protocol for large scale applications and the high-throughput capabilities of the combined use

of a multiplex PCR assay with and UA for olive precise and accurate genotyping assays.

## Conclusion

Many studies have been performed concerning the authenticity of vegetable oils, as this remains an actual and challenging issue. The main reason for vegetable oils adulteration is the increased profit and trading advantages. Consequently, most studies focus on the detection of adulteration/substitution of one oil with another less expensive or deal with false claims regarding geographical origin and cultivar identification. Due to several technological advances in the last years, novel and powerful analytical tools are becoming available. Considering the importance of authentication issues for trade industries, regulatory authorities and consumers, in parallel with the technological advances, different analytical methodologies have been proposed for vegetable oils authentication purposes. Nevertheless, due to the natural variations associated with biological, edaphoclimatic and agronomical factors, authenticity assessment of vegetable oils still remains a very difficult task. Given the complexity of the problem, the measurement of different parameters can be required as well as the use of multivariate statistical tools. Recently, there has been an increasing interest towards the application of DNA-based markers once they are independent from environmental conditions and proved to be effective for cultivar identification of olive oil. Continued growth in this area is therefore expected, especially for monitoring and ensuring legislation concerning specific labelling claims, such as the ones regarding the use of specific cultivars and/or geographical origin.

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