Chapter 13

# Olive Oil Traceability

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## 1. Introduction

The market for imported, premium priced foods has increased dramatically over recent years, as consumers become ever more aware of products originating from around the world. There are many food products that are of superior quality (taste, texture, fragrance etc.) because of the locale in which they are cultivated. Environmental conditions, such as local climate and soil characteristics, combine to yield crops that exhibit specific traits. Clearly, higher quality products demands higher market prices, therefore unscrupulous traders may attempt to increase profits by deliberately mislabelling foods, or by increasing the volume of a good quality batch through adulteration with sub-standard produce.

In recent years, there has been increasing legislation to protect the rights of both the consumer and honest producers. On 2009 the European Union (EU) Member States agreed to require origin labelling for virgin and extra virgin olive oils (EC Regulation 182/2009) to defend consumers need about true characteristics and origin. To enforce these laws, a measure of the authenticity of samples must be made, most often in the form of proving the presence/absence of adulterants, or verifying geographical or cultivar origin by comparison with known and reliable samples.

The term traceability is often used in modern language, however its usage is applied for different meanings. In fact, in certain cases it is used to describe the origins of the raw materials, whilst in others to describe from which geographical region a foodstuff originates. It is also used more generically to identify a particular producer or from where it has been purchased. Many consider it as a guarantee of food safety and therefore obligatory for all food products; others however as a guarantee of quality and therefore a voluntary value.

Also the applied legislation is highly complex, with lawmakers giving different functions to traceability depending on the application and environment of the law. With the event of the food crisis in the 90's it was evident that new guidelines were urgently required to simplify and streamline the processes which could also be applied to food contamination. For this reason, the new laws of the European Union were considered as an umbrella and termed

"General Laws for food safety" and was not applied only to food safety but also: to introduce the concept of traceability where food companies (producers, manufacturers and importers) need to guarantee to be able to demonstrate the traceability of every food, animal feed and ingredient, showing the chain from producer to consumer; create the " European Food Safety Authority" (EFSA) to unify the various commities and to make public the scientific process and risks; to strenghthen the ealry warning system adopted by European governments and the European Commission to enable rapid interventions in cases of food safety in the human and animal food chains.

In this chapter, we will only consider the "traceability" as a process that allows us to track "from downstream to upstream" the informations distributed along the olive-oil production chain, from producer to consumer (from farm to fork), and to include a evaluation of analytical methods useful in ascertaining what is claimed on the label, in according to the Regulation (CE) 178/2002.

## 2. Traceability in regulation (EC) 178/2002

Since 1 January 2005, a rule has required that EU countries implement labelling and identification procedures for products sold by farmers, producers and first importers to the EU to enable and facilitate their traceability when they are put on the market. The main purpose is to be able to initiate a withdrawal and/or recall procedure for products in the event of a food crisis. The quality of traceability will enable targeted and precise withdrawals. It will also limit the extent of recalls and ensure the removal of holds on products that are not involved.

The law (EC Regulation No. 178/2002) defines traceability as: "The ability to reconstruct and follow a food, feed, a food-producing animal or substance intended to be, or to join a food or feed, through all stages of production, processing and distribution" (Article 3, paragraph 15).

Many use the terms tracking and traceability synonymously. In reality, these two terms identify two inverse processes:

- tracking identifies the location of a product from upstream to downstream in the chain and at every stage of the journey;
- traceability or tracing is the inverse process, which allow us to gather the information previously issued. It is evident that the two processes are strongly related and based on the same system.

Traceability does not refer to the production of a generic good. It makes each unit of production physically identifiable, managing production processes that are determined by "lots", and manage traceability means identifying each group of products and following the path.

It is necessary to record information relating to inputs (products and companies), processing (product lots, which lots and what end products) and outflows (products/companies /clients). The key is to define the composition of a set of products that have undergone the same process of transformation.

Moreover, the amount of information that identifies a batch may vary and of course the complexity of the whole system increases with what information the company chooses to include in the identification of a lot.

Internal traceability, then, helps to express the internal procedures of each company to trace the origin of materials used.

The label is the instrument through which information is transferred to consumers. It's easy to understand why this essential requirement is effective.

Finally, the information given in the production lot should be able to trace all the links along the chain, back to the first producer and/or supplier of the product or substance to whom it belongs. Traceability chain is an inter-company process, resulting from internal processes of every operator in the industry. These systems should be linked by efficient information systems. It is not governed by a single person in the chain, the relations between the operators allow the tracing of the chain.

## 3. Analytical methods for the traceability of extra virgin olive oil

The increase in the demand for high-quality *olive oil*s has led to the appearance in the market of *olive oil*s elaborated with specific characteristics. They include oils of certain regions possessing well-known characteristics, that is, *olive oil*s with a denomination of origin, or with specific olive variety composition, that is, coupage or monovarietal *olive oil*s. Monovarietal *olive oil*s have certain specific characteristics related to the olive variety from which they are elaborated (Montealegre *et al*., 2010). However, coupage *olive oil*s are obtained from several olive varieties to achieve a special flavor or aroma.

The appearance of denominations and protected indications of origin has promoted the existence of oils labeled according to these criteria. Regulation 2081/92, to promote and protect food products, created the systems known as:

- Protected Designation of Origin (PDO);
- Protected Geographical Indication (PGI);
- ''Traditional Speciality Guaranteed" (TSG).

An *olive oil* with a PDO denomination requires meeting precise definition of several parameters such as cultivar, geographical origin, agronomic practice, production technology, and organoleptic qualities (Giménez *et al*., 2010), and all of these parameters have to be investigated to study its traceability and to certify its quality. The introduction of certifications of origin and quality for virgin *olive oil* as PDO makes necessary the implementation of traceability procedures.

Any research dealing with *olive oil* traceability is focused on investigating the botanical or geographical origin. In both cases, the selection of the markers (compounds with discriminating power) to be studied is complicated because the composition of extra virgin *olive oil*s is the result of complex interactions among olive variety, environmental conditions, fruit ripening, and oil extraction technology (Araghipour *et al*., 2006). The verification of the

cultivars employed to produce an *olive oil* sample may contribute to address the oil origin. This fact may have commercial interest in the case of monovarietal *olive oil*s or *olive oil*s with PDO because these high-quality *olive oil*s may be adulterated by other oils of lower quality, using anonymous or less costly cultivars (Sanz-Cortes *et al*., 2003).

Unfortunately, morphological traits have been difficult to evaluate, are affected by subjective interpretations, and are severely influenced by the environment and plant developmental stage (Japon-Lujan *et al*., 2006). Nowadays, several efforts have been focused on the investigation of one or several compounds present in *olive oil*s usable to differentiate olive varieties (Sanz-Cortes *et al*., 2003).

Compositional markers (substances that take part of the composition of the olive oils) include major and minor components. Major components such as sterols, phenolic compounds, volatile compounds, pigments, hydrocarbons, tocopherols, fatty acids and triglycerides may provide basic information on olive cultivars. Minor components can provide more useful information and have been more widely used to differentiate the botanical origin of *olive oil*s (Howarth and Vlahov, 1996; Lanteri *et al*., 2002).

In recent years, there has been increasing legislation to ensure consumer confidence and to protect the rights of both the consumer and honest producers.

It is of great importance the development of analitycal methods to verify the correspondence between what is stated on the label and what is contemplated in the documents (EC Regulation 182/2009 and Reg. 834/2007) in relation to the production of olives and extra virgin olive from organic farming. Moreover, to enforce these laws, a measure of the authenticity of samples must be made, most often in the form of proving the presence/absence of adulterants, or verifying geographical or cultivar origin by comparison with known and reliable samples. The latter method often includes the use of multivariate statistical techniques such as principal components analysis, linear discriminant analysis, canonical variance analysis and partial least squares regression to investigate sample data (Giménez *et al*., 2010).

## 3.1. Chromatographic techniques for the traceability of olive oil

In the last decade, gas chromatography (GC), high performance liquid chromatography (HPLC), supercritical fluid chromatography (SFC), and capillary electrophoresis (CE) have been widely used in the authentication analysis of olive oil. The fat, sterols, protein, carbohydrate or other natural compound profiles (Aparicio-Ruiz, 2000; Benincasa *et al*., 2003; Vichi et al., 2005; Lopez Ortiz et al., 2006; Temime et al., 2006; Canabate-Diaz et al., 2007; Cavaliere et al., 2007; Haddada et al., 2007; Vichi et al., 2007; Lazzez et al., 2008) have been used to provide species and geographical differentiation. In several cases, enantiomeric composition has been utilised (Rossmann et al., 2000). These methods of discrimination rely on samples of different species and/or different geographical origin having different chemical compositions. This is not always the case, samples from the same location have been found to contain different components and conversely samples from different regions may display identical chemical composition.

Detectors usually used, in combination with chromatographic techniques (GC and HPLC), may be more or less selective and sensitive, but lack information about the identity of compounds. Therefore, the coupling of chromatographic techniques and mass spectrometry (MS) overcomes this drawback (Cavaliere *et al.,* 2007; Lazzez *et al.,* 2008).

MS is a sensitive and selective detector, sometimes allowing preparation steps to be avoided. GC–MS is a robust technique, used routinely in many laboratories for food analysis; for example for the determination of aroma compounds and pesticide analysis. More recently, LC coupled to quadrupoles, magnetic sectors or time-of-flight (TOF) detectors, has also had a great expansion into the field of food analysis.

The recently introduced spray methods (ESI) have fostered qualitative and quantitative analysis of medium to high polar analytes by mass spectrometry. The designing of ion source houses for ESI has also fostered the rediscovery of atmospheric pressure ionization (API) methods such APCI where the chemical ionization (CI) is achieved at atmospheric pressure. Both techniques produce soft ionization, but additional fragmentation can be achieved by performing in-source collision induced dissociation (CID) in tandem or trap instruments. MS/MS in space (tandem, sectors, quadrupoles, TOFs, etc.) and in time (traps) provide additional and unique information on the structure of analytes. ESI is useful for polar and ionic solutes ranging in molecular weight from 100 to $150\times10^3$ dalton. APCI is applicable to non-polar and medium polarity molecules with a molecular weight from 100 to 2000 dalton. Although the choice of the right interface, as well as the detection polarity are based mostly on the compounds polarity and thermal stability, and the HPLC operating conditions, many classes of compounds can give good response with both ionization techniques. In certain circumstances both positive and negative ionization modes are needed, while in most of the cases the choice of only one operation mode is enough. The number of applications of HPLC–API-MS to food analysis has rapidly increased in recent years. ESI is much more widespread than APCI, but for both techniques the trend is towards an increase in the number of applications (Sindona et al., 1999; 2000; Di Donna et al., 2001).

### 3.2. Stable isotope techniques for the traceability of olive oil

Stable isotope techniques enable differentiation of chemically identical substances through alterations in their isotopic fingerprint, and have been used in authenticity studies for many food products. The isotopic composition of light elements (lighter than calcium) in plant material can vary depending on location but, the dominant factor is the influence of latitude on the fractionation of the elements in groundwater.

Fractionation occurs during physical processes such as evaporation. Lighter isotopes evaporate very slightly faster than their heavier counterparts, therefore in warmer regions where the amount of evaporation is higher, the isotopes are fractionated to a greater degree. The discrimination between isotopes in such physical processes is only significant for light elements, with a high relative mass difference between the isotopes. Thus hydrogen ratios, measured by site-specific natural isotope fractionation nuclear magnetic resonance (SNIF-NMR), and carbon, nitrogen, oxygen and sulphur isotope ratios measured by isotope ratio mass spectrometry (IRMS) have been applied to the authentication of foods.

The elemental composition of vegetation reflects (to a certain extent) that of the soil in which it has grown (Kelly *et al*., 2002) which in turn will depend on the topography, geology and soil characteristics. Therefore, no two countries will have identical soil maps, and the concentration of elements in food product, can then be used to assign their geographical origin. As with other methods of authentication, a database of samples of known origin must be available, against which unknown samples can be compared. The most useful elements for the assignment of origin are those that are not homeostatically controlled. Elements such as K, Ca and Zn are actively absorbed by organisms and will therefore be present in samples at similar levels, regardless of the environmental conditions experienced.

Elements that have no role in normal physiological processes, such as the rare earth elements (REEs) and the heavy metals, are passively absorbed and the concentration of these elements in an organism will strongly reflect the environmental levels to which the organism has been exposed.

### *3.2.1. Inductively coupled plasma mass spectrometry technique*

Several studies have used multi-element concentration profiles in the determination of food authenticity, either alone or in combination with chromatographic or stable isotope ratio data. Elemental concentrations were determined by atomic absorption spectroscopy (AAS), inductively coupled plasma - atomic emission spectroscopy (ICP-AES) or inductively coupled plasma – mass spectrometry (ICP-MS) (Dugo *et al.,* 2004; Benincasa *et al.,* 2007; Cindric *et al*., 2007).

Several trace elements have variable natural isotopic abundance due to the decay of radioactive isotopes. These include Li, B, Cu, Sr, Nd, Hf, Pb and U. The composition and age of the local rocks dictates the abundance of the radioactive precursors and their daughter species. Elements are taken up into plants in the same isotopic proportions as they occur in the soil and in precipitation.

Therefore isotope ratios in plant-derived food products depend on the geology of the region in which the source crop was grown, and are different in produce of different geographical origin. There have, however, been relatively few reports of the use of heavier stable isotope ratio measurements for the authentication of foodstuffs. For many years thermal ionization mass spectrometry (TIMS) was the only technique capable of performing isotope ratio measurements with sufficient precision to allow geographical assignment of food products based on trace element isotopic composition. Samples for TIMS analysis must be loaded in the form of the pure element, meaning that extensive sample preparation is required if this technique is to be applied.

ICP-MS is now a well established technique for isotopic trace element determinations. ICP-MS allows rapid analysis of a large range of sample types, requires minimal sample preparation and due to the ionising power of the ICP, can be applied to a wider range of elements than TIMS. The precision of isotope ratio analyses by ICP-MS has only recently matched that achievable by TIMS, through the application of double focusing mass analysers coupled to multi-collector detection arrays. The increasing availability of multi-

collector ICP-MS is likely to lead to wider application of heavier stable isotope ratio measurements to the authentication of food products.

### *3.2.2. $^{13}C$ compound-specific isotope analysis*

One of the most powerful techniques to be used in food authenticity studies is stable isotope ratio mass spectrometry (SIRMS). SIRMS has found application in the authentication of a wide range of foodstuffs, especially for the detection of added cane sugar in fruit juices (Bricout and Koziet, 1987), wines (Dunbar and Schmidt, 1984), spirits (Parker *et al*., 1998), honey (White *et al*., 1998) and to detect the adulteration of flavour compounds with synthetic analogues (Culp and Noakes, 1990). The majority of the reported research has focused on bulk or global stable carbon isotope ratios ($^{13}C/^{12}C$).

Approximately 98.89% of all carbon in nature is $^{12}C$ and 1.1% is $^{13}C$. The amount of $^{13}C$ present in a sample is expressed as a ratio to the amount of $^{12}C$ present, relative to an international standard using the δ notation. This notation has the units per mil (‰). The $\delta^{13}C$ value of plants varies because of isotopic fractionation during physical, chemical and biological processes.

Photosynthetic fixation of $CO_2$ by plants takes place by three different routes, depending on the nature of the plant. Most terrestrial plants photosynthesise using the Calvin or C3 pathway in which $CO_2$ is fixed via the carbon cycle to from a three-carbon compound. Some plants, mainly tropical grasses, such as maize and sugar cane use the Hatch-Slack or C4 pathway in which the initial "Hatch-Slack" step is via a dicarboxylic acid, a four carbon compound. A third photosynthetic class of plants uses the CAM (Crassulacean acid metabolism) pathway.

Typical CAM plants are succulents and are of minor importance in the oleochemical industry. In the C3 pathway carbon dioxide is fixed via the carboxylation of ribulose-1,5-diphosphate to form phosphoglyceric acid (a 3-carbon molecule). This enzyme catalysed reaction discriminates against $^{13}C$ and so proceeds with a relatively large isotope effect (O'Leary, 1981). This means that less $^{13}CO_2$ is incorporated into C3 plants than into C4 plants. C3 plants have bulk $\delta^{13}C$ values in the range -24 to -30‰ whereas C4 plants have bulk $\delta^{13}C$ values from -9 to –14‰.

Olive oil shows a $\delta^{13}C$ values of 28.7‰.

The most widely used technique to assess the authenticity of edible oils is to measure the fatty acid composition. However it is not always possible to detect adulteration by this technique because of the natural variation in fatty acid profiles and because blends of oils with fatty acid compositions similar to the authentic oil may be prepared relatively easily. A more sophisticated approach is to determine the fatty acid composition at the 2 position of the triacylglycerol, since this is known to differ from the overall fatty acid composition. Woodbury *et al*., (1998) used this technique to obtain the compound-specific $\delta^{13}C$ values of the fatty acids specifically at the 2-position of the triacylglycerol.

Royer *et al.,* (1999) examined 188 olive oils produced mainly in Greece during 1993 to 1996. The concentration and $\delta^{13}C$ value of individual fatty acids present in the olive oils were determined by gas chromatography and GC-C-IRMS respectively. The results were examined in terms of geographical, temporal, and botanical factors. French and Italian olive oils were securely classified at the 99.9% confidence interval using the $\delta^{13}C$ values of the principal fatty acids palmitic (C16:0), oleic (C18:1) and linoleic (C18:2). Regional classifications for the Greek olive oils were also achieved on the basis of differences in the $^{13}C$ abundance of oleic acid compared to linoleic acid and palmitic acid.

Glycerol is a primary metabolite in plants. It is nominally present in its ester form as glycerolipids in fats and oils (Kiritsakis and Christie, 1999). Glycerol is bio-synthesised relatively early in the lipidic metabolic pathway compared to fatty acids (Weber *et al*., 1997; Harwood and Sánchez, 1999).

Consequently, it may be expected that the isotopic distribution in glycerol is a better indicator of the botanical and environmental influences on any given plant. A number of compound specific IRMS studies on glycerol have been performed, some of which include data derived from vegetable oils.

### *3.2.3. $^{18}O$ and $^{2}H$ pyrolysis CF-IRMS*

The abundance of the stable isotopes oxygen-18 ($^{18}O$) and deuterium ($^{2}H$) are particularly interesting isotopic probes for both botanical and geographical identification of a variety of different food products. The primary source of all organic hydrogen and oxygen is the hydrosphere. The meteoric water that has passed through the meteorological cycle of evaporation, condensation and precipitation finally constitutes the groundwater and exhibits a systematic geographical isotope variation. Decreasing temperatures cause a progressive heavy-isotope depletion of the precipitation when the water vapour from oceans in equatorial regions moves to higher latitudes and altitudes. Evaporation of water from the oceans is a fractionating process that decreases the concentration of the heavy isotopomers of water ($^{1}H^{2}H^{16}O$, $^{1}H^{1}H^{18}O$) in the clouds compared to the sea. As the clouds move inland and gain altitude further evaporation, condensation and precipitation events occur decreasing the concentration of deuterium and oxygen-18.

Consequently, the ground water reflects this isotopic gradient from the coast to inland areas. For land plants, a further pre-assimilation affects the isotopic composition of the water substrate. The hydrogen and oxygen present in plant material originates from the water taken up by the roots. The water is transported through the plants xylem system. The isotopic composition of the xylem water is the same as that of water taken in by the roots, and the water is taken into the leaves without a change in isotopic composition. Evapo-transpiration of water through the leaf enriches the remaining water in the heavier isotopomers. Therefore, it is expected that growing regions with relatively low humidity, where the rate of evaporation from the leaf is higher, result in plant materials with relatively enriched $\delta^{2}H$ and $\delta^{18}O$ values.

Over the past 5 years there has been a marked increase in the use and application of $^{2}H$ and $^{18}O$ stable isotopes in many areas of food research. This has been facilitated by recent developments in on-line gas preparation devices that proceed by high temperature pyrolysis of organic products and the availability of commercial IRMS analysers capable of measuring $^{2}H/^{1}H$ ratios in the presence of a helium carrier gas. These innovations have, to a large extent, overcome the difficulties associated with offline gas preparation for DI-MS and greatly increased the applicability of this measurement. It is now possible to routinely measure $^{2}H$ and $^{18}O$ abundances in organic samples by Pyrolysis-Continuous Flow-Isotope Ratio Mass Spectrometry (Py-CF-IRMS).

Therefore, measurements of stable isotope ratios of the light elements (H, C ,N ,O, S and bioelements) and of the heavy element stronzium, in natural cycles, have provided geographical fingerprints (Roßmann *et al.*, 2000).

Preliminary investigations into the application of $^{18}O$-pyrolysis continuous-flow IRMS to obtain information about the geographical origin of olive oil samples has been conducted by Angerosa *et al*. (1999). They measured the $\delta^{13}C$ and $\delta^{18}O$ values of whole olive oil, sterols and aliphatic alcohol fractions from fruits of *Olea europaea* L. produced in Greece, Italy, Morocco, Spain, Tunisia, and Turkey. The results permitted provincial classification of the oils. However, there were some misclassifications observed for oil samples coming from neighbouring countries with similar climates.

A secure geographical classification of an olive oil, in order to ensure that the consumer is not defrauded and that the honest trader is not disadvantaged by having their PDO oils misrepresented by inferior products, can be achieved by performing heavy isotope ratios (e.g. $^{88}Sr/^{86}Sr$) and multi-element analysis.

### *3.2.4. Nuclear magnetic resonance spectroscopy*

During the last ten years, nuclear magnetic resonance spectroscopy (NMR) (Del Coco *et al*., 2012; Mannina & Segre, 2002), has played an ever-increasing role in the study olive oil characterization and autentication. In particular, it has been shown that high-resolution NMR together with statistical analysis constitutes a powerful tool for the geographical characterization of olive oils on Mediterranean, national, regional and PDO scales. On this regard, innovative techniques like NMR spectroscopy seem to be able to distinguish olive oils on the basis of their geographical origin, whereas the conventional analyses suitable for the determination of quality and genuineness seem not to be so appropriate for this type of discrimination (Frankel, 2010; Guillen & Ruiz, 2001).

Important information on the fatty acid distribution on the glycerol moiety can be obtained by $^{13}C$ NMR (Rezzi *et al*., 2005; Petrakis *et al*., 2008; Alonso-Salces *et al.*, 2010b; Mannina *et al*., 2010; Alonso-Salces *et al.*, 2011b). Two groups of resonances are observed in the carbonyl region of the $^{13}C$ NMR spectrum of an olive oil: one group is due to fatty chains in position sn-1,3 of the glycerol moiety, the other one is due to fatty acids in position sn-2.

It must be noted that although gas chromatographic methods give the full composition of fatty chains, no information is given about the fatty chains distribution on glycerol. Thus in this case, gas chromatography and $^{13}C$ NMR methodologies must be considered complementary and not alternative. $^{13}C$ NMR spectroscopy together with discriminant analysis has been proposed by Mavromoustakos et *al.*, to detect the presence of soybean oil, cottonseed oil, corn oil and sunflower seed oil in virgin olive oils. Only double bonds signals have been used for the analysis. Fatty acids composition and fatty chain positional distribution on glycerol moiety determined by $^{13}C$ NMR spectroscopy allow, in combination with the multivariate statistical procedure, the classification of olive oils according to their variety (Marini *et al*., 2004, 2006).

$^{31}P$ NMR spectroscopy has been employed for the detection and quantification of the minor compounds mono- and diacylglycerols, polyphenols, sterols, phospholipids and for the determination of free acidity and moisture (Petrakis *et al*., 2008). This analytical technique requires the derivatization of the labile hydrogens of the hydroxyl and carboxyl groups of the olive oil constituents using the phosphorous reagent 2-chloro-4,4,5,5- tetramethyl dioxaphospholane and the use of $^{31}P$ chemical shifts of the phosphitylated compounds to identify the labile centers. The main phospholipids found in olive oil were phosphatidic acid, lyso-phosphatidic acid and phosphatidylinosotol, although very small amounts of other phospholipids were detected as well. In this way, the content of free fatty acids has been determined showing a good correlation with official titration method. Besides, using the same derivatization method, sn-1,2, sn-1,3-diacylglycerides and monoacylglycerides have been quantified and have showed a characteristic trend during olive oil storage.

The composition of diacylglycerols (sn-1,2- 1,3-diacylglycerols and total) determined by $^{31}P$ NMR derivatization method has been used to characterize olive oils from different Greek areas (Petrakis *et al*., 2008). Some preliminary correlations between the diacylglycerols content and the geographical regions have been observed.

The geographical characterization of monovarietal virgin olive oils from three regions of Southern Greece, namely Peloponnesus, Crete and Zakynthos, has been performed applying both $^{1}H$ and $^{31}P$ NMR (Petrakis *et al*., 2008). The correct geographical prediction at the level of three regions, based on discriminant analysis, has been rather high (87%). Phenolic compounds and free acidity determined by $^{31}P$ NMR spectroscopy have enabled to classify Koroneiki, Athinolia and Kolovi monovarietal Greek extra virgin olive oils using ANOVA and discriminant analysis.

$^{1}H$ NMR spectrum of an olive oil does not provide this information directly; however, it is possible to have an indirect measurement of acidity using the measurable amount of diglycerides and monoglycerides.

Another study carried out by using $^{1}H$ NMR involved the characterization of the phenolic fraction of olive oil from three different areas of the Apulia region (Sacco *et al.,* 2000). Statistical evaluation has showed discrimination between Coast, Hinterland and North samples.

Extra virgin olive oils sampled in three harvesting years and coming from different Italian regions (Tuscany, Lazio, Lake of Garda) have been analyzed by $^{1}H$ methodology. The statistical elaboration applied on the intensity of b-sitosterol, aldehydes and some other volatile compounds has allowed the classification of the olive oils according to the geographical regions.

$^{1}H$-NMR fingerprinting of olive oil is a valuable analytical tool for the traceability of virgin olive oils from different points of view, i.e. food authentication and food quality. As described before, $^{1}H$ and $^{13}C$ NMR techniques provide different information: $^{1}H$ NMR spectrum allows the measurement of minor components of olive oils such as β-sitosterol, hexanal, 2-(E)-hexenal, formaldehyde, squalene, cycloartenol and linolenic acid; the $^{13}C$ NMR spectrum detects major components such as glycerol tri-esters of olive oils, defining also acyl composition and positional distribution on the glycerol moiety. The main sensitivity concern about $^{1}H$ NMR analysis is the dynamic range, which makes the detection of signals below the threshold imposed by the ADC (analogue to digital converter) impossible. Therefore, in a standard extra virgin oil spectrum, trace component detection is limited to signals more intense than 10–5 times the intensity of the fatty acid $CH_2$ signals. Weak signals above the dynamic range threshold are in any case affected by severe baseline distortion. Selective pulses coupled to gradient spin-echo refocusing such as DPFGSE (69), in NMR modern instruments, allow these limitations to be circumvented, paving the way for the easy detection of minor components, especially those presenting resonances away from other interfering resonances.

The power of these new sequences could be demonstrated by the detection of aldehydes, carotenoids or other specific minor components whose resonances fall in a relatively free region. It was performed a specific DPFGSE analysis on the aldehydic components of some extra virgin olive oils, mostly responsible of the sensorial properties of extra virgin olive oil. Comparison with standard $^{1}H$NMR spectra show tremendous improvement of quantitative and qualitative information with drastic instrumental time reduction.

Fingerprinting techniques such as NMR, NIR (Galtier *et a*l., 2007; Mignani *et al*., 2011), MIR (Reid *et al*., 2006), fluorescence (Kunz *et al*., 2011), FT-IR, FT-MIR and FT-Raman (Baeten *et al*., 2005; Lopez-Diez *et al*., 2003; Yang *et al*., 2005) spectroscopies, have been used for the determination of food authenticity (Reid *et al*., 2006).

These types of techniques are particularly attractive since they are non selective, require little or no sample pre-treatment; use small amounts of organic solvents or reagents; and the analysis takes only a few minutes per sample.

### 3.3. Molecular markers for the traceability of olive oil

The food crisis situation seen in last years and the controversy about genetically modified organisms (GMO), with a sharp increase in basic food prices, highlights the extreme susceptibility of the current agricultural and food model and the need for more strict food quality control, which should include determination of the origin of the product and the raw

materials used in it. That's why a well documented traceability system has become a requirement for quality control in the food chain.

Almost 84% from the total olive oil production derives from the European Union, especially from Spain, Italy and Greece. The olive oil is a main constituent of the Mediterranean diet. However there has recently been an increase in olive oil consumption internationally, due to greater availability and the current consideration of high nutritive and health benefits, including a qualified health claim from Food and Drug Administration (FDA, USA).

Molecular markers allow the detection of DNA polymorphisms and enable to effectively distinguish different varieties in an effective way, without any environmental influence.

Olive oil extraction is the process of extracting the oil present in the olive drupes for food use. Olive oil extraction is the process of separating the oil from the other fruit contents. It is possible to attain this separation by physical means alone, *i.e.* oil and water do not mix, so they are relatively easy to separate. The modern method of olive oil extraction uses an industrial decanter to separate all the phases by centrifugation. In this method the olives are crushed to a fine paste. This can be done by a hammer crusher, disc crusher, depicting machine or knife crusher. This paste is then malaxed for about 30 minutes in order to allow the small olive droplets to agglomerate.

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 fatty acid and secondary metabolites can provide reliable results for oil traceability (Ben Ayed *et al.*, 2009; Papadia *et al.*, 2011). For this reason, genetic identity seems to be the most appropriate method for identifying the variety from which the olive oil under study derives.

Molecular markers allow the detection of DNA polymorphisms and enable to effectively distinguish different varieties in an effective way, without any environmental influence. 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 (Muzzalupo *et al.*, 2007). However, depending on the molecular markers used correctly, 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 (Muzzalupo and Perri, 2002; Ben Ayed *et al.*, 2010).

The use of DNA based technology in the field of food authenticity is gaining increasing attention. 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 RAPDs (random amplified polymorphic DNA, Pasqualone *et al.*, 2001; Muzzalupo and Perri, 2002).), AFLPs (amplified fragment length polymorphism, Pafundo *et al.*, 2005; Montemurro *et al.*, 2007) and SSRs (simple sequence repeat, Muzzalupo *et al.*, 2007; Breton *et al.*, 2004; Bracci *et al.*, 2011; Ben Ayed *et al.*, 2009) are very useful in the study of the traceability of olive oil. Single nucleotide polymorphism

(SNPs) markers have been recently developed in olive and utilized to study the genetic diversity of olive trees (Reale *et al.*, 2006). SNP detection can be delivered in a number of ways, but the simultaneous detection of multiple SNPs from a single DNA sample is of particular interest (Consolandi *et al*., 2008). PDO oils are typically not monovarietal, so a method for quantifying the components of the mixture is essential if conformity with certification depends on a prescribed proportion of varietal types. So far, application of Real-Time as a tool for olive oil authentication has been explored by Giménez *et al*., (2010). The authors evidenced that Real-Time PCR is useful to quantify DNA extracted from oil, and thus to assess the yields of different methods of extraction. But the size of amplicon, is critical for the success of analysis. A possibility of utilising qRT-PCR to quantify varieties in PDO oils rests on the use of taqMan probes designed on SNPs specific of varieties entering in the oil composition (Marmiroli *et al*., 2009). Several sequences from noncoding spacer region between *psbA-trnH* and partial coding region of *matK* of plastid genome provided a good discrimination of pure *olive oil* and its admixture by other vegetable oils such as canola and sunflower. The plastid based molecular DNA technology has a great potential to be used for rapid detection of adulteration easily up to 5% in olive oil (Kumar *et al*., 2011).

The knowledge of genome nucleotide sequences also could be useful to identify new sequence polymorphisms, which will be very useful in the development of many new variety-specific molecular markers and in the implementation of more efficient protocols for tracking and protect olive oil origin.

A recent publication reported by Papadia *et al.*, 2011 a systematic effort to obtain genetic characterization by SSR amplification, soil analyses, and 1H-NMR spectra, is carried out in order to make a direct connection between the olive tree variety (genetic information) and the NMR spectra (chemical information) of the extra virgin olive oil produced. The results reported show that a multidisciplinary approach, through the application of multivariate statistical analysis, could be used to set up a method for variety and/or geographic origin certification, based on the construction of a suitable database. Further research will be directed to the growth of an organic genetic/NMR/soil database, in order to improve the prediction ability of the LDA, and furthermore to develop a way to correlate $^{1}$H-NMR spectra of commercial extra virgin olive oils with their geographical and genetic origin.

### 3.4. Electrochemical sensor analysis for the traceability of olive oil

Electronic noses (e-noses) and electronic tongues (e-tongues) for liquid analysis, based on the organizational principles of biological sensory systems, developed rapidly during the last decade. E-noses and e-tongues crudely mimic the human smell and taste sensors (gas and liquid sensors) and their communication with the human brain. The human olfactory system is by far the more complex and contains thousands of receptors that bind odor molecules and can detect some odors at parts per trillion levels (Breer, 1997) and include between 10 and 100 million receptors (Deisingh *et al*., 2004). Apparently some of the receptors in the olfactory mucus can bind more than one odor molecule and in some cases one odor molecule can bind more than one receptor. This results in a mind-boggling amount

of combinations that send unique signal patterns to the human brain. The brain then interprets these signals and makes a judgment and/or classification to identify the substance consumed, based in part, on previous experiences or neural network pattern recognition. The electronic nose often consists of non-selective sensors that interact with volatile molecules that result in a physical or chemical change that sends a signal to a computer which makes a classification based on a calibration and training process leading to pattern recognition. The non-selectivity of the sensors results in many possibilities for unique signal combinations, patterns or fingerprints. The human tongue contains sensors, in the form of 10,000 taste buds of 50–100 taste cells each (Deisingh *et al*., 2004) for sweet, sour, bitter, salty and umami and is much less complicated than the human olfactory system. The e-tongue then uses a range of sensors that respond to salts, acids, sugars, bitter compounds, etc. and sends signals to a computer for interpretation. The interpretation of the complex data sets from e-nose and e-tongue signals is accomplished by use of multivariate statistics. For non-linear responses, artificial neural networks (NAA) can be used for modeling the data. Biosensors are also being developed, but are not yet commercialized. In contrast to chemical sensing materials, that are broad spectrum to generate characteristic response patterns, there are biological systems. The problem with chemical sensors is that these systems are extensive, require large sample sizes for analysis, have low sensitivity and poor specificity compared with the human nose. The bioelectronic nose utilizes olfactory receptors as sensing mechanisms and are cell or protein-based to mimic a mammalian olfactory system (Lee and Park, 2010). Another type of sensing system is based on colorimetric sensor array built in disposable chips (Suslick *et al.,* 2010). These arrays are based on the chemical interactions between the analyte and a chemical dye. They are being developed for volatile and non volatile molecules (Zhang *et al*., 2006; Zhang and Suslick, 2007; Musto *et al.,* 2009) for applications by the food industry.

The advantage of the human sensory system is that the brain can receive signals from both olfactory and tongue receptors and integrate both sets of data to form classifications and/or judgments. The e-nose and e-tongue are not integrated since each has its own software package, but the data from both instruments could be imported into another program and integrated. The disadvantage of the human sensory system is that no two brains are alike (of course from another point of view, this is a good thing), and the same brain may react differently from one day to the next, depending on an individual's health, mood or environment, making the data subjective. On the contrary, e-nose and e-tongue instruments can be calibrated to be reliably consistent and can give objective data for important functions like quality and safety control. These instruments can also test samples that are unfit for human consumption. A disadvantage for the e-nose and e-tongue systems (as with humans) is that they are also affected by the environment including temperature for both e-nose and e-tongue and humidity for e-nose, which can cause sensor drift, although calibration systems and built-in algorithms help compensate for this. There are more or at least not less different types of sensing materials for e-tongue (liquid sensors) compared to e-nose systems, and liquid sensors often possess higher selectivity and significantly lower detection limits compared to the gas sensors (e-nose).

There are several reviews on the subject of e-nose and e-tongue technology, including reviews on e-noses (Di Giacinto et al., 2010; Wilson and Baietto, 2009), biomimetic/biotechnology e-nose and/or e-tongue sensing systems (Rudnitskaya and Legin, 2008; Ghasemi-Varnamkhasti *et al*., 2010)], applications for e-noses and e-tongues (Scampicchio et al., 2008), neural networks for e-noses (Lu *et al*., 2000), pattern recognition techniques (Berrueta *et al*., 2007); meat quality assessment by e-nose (Ghasemi-Varnamkhasti *et al*., 2009) and computational methods for analysis of e-nose data (Jurs *et al*., 2000). This review will concentrate on the recent literature on applications of e-noses and e-tongues in the food industry.

## 4. Conclusions

Some varieties 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), Protected Geographical Indication (PGI) and Traditional Specialty Guaranteed (TSG) is to add value to certain specific high quality products from a particular origin.

The development of accurate analytical fingerprinting methods for the authentication of olive oils and for the certification of the geographical origin is an actual issue and an important challenge. In fact, to protect the rights of both the consumer and honest producers and, to enforce the laws, it is important to develop analytical methods to measure the authenticity of the samples, to verify the geographical or cultivar origin and to provide the presence/absence of adulterants.

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