Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

Scienze e Biotecnologie degli Alimenti

Ciclo XXV

Settore Concorsuale di afferenza: 07/F1

Settore Scientifico disciplinare: AGR/15

TITOLO TESI

Analytical methods for evaluating the quality and the genuineness of olive oils

Presentata da: Enrico Valli

Coordinatore Dottorato

Relatore

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Analytical methods for evaluating the quality and the genuineness of olive oils

Ph.D. Thesis by Dr. Enrico Valli
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Ph.D. on Food Science & Biotechnology
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1. SUMMARY AND OBJECTIVES

This Ph.D. thesis was designed to follow a research project related to a 3-year-agreement (2009-2012) stipulated between the University of Bologna (Department of Agricultural and Food Sciences) and the largest consortium of Italian olive oil's producers (UNAPROL). Such an investigation focused on the assessment of the quality and genuineness of different commercial categories of olive oils which can be found in the Italian market. In order to achieve this aim, I collected different sets of samples of oils obtained by olives (*paragraph 3.0.1*), and I adopted a full analytical plan, carrying out and developing many analytical methods (*paragraph 3.0.2* and *chapter 1*). The results I got are fully discussed in this Ph.D. thesis, that has a special focus on the quality and purity markers of olive oils.

On one side, extra virgin olive oil (EVOO) is a foodstuff often subjected to many frauds (*chapter 2*), because of its high nutritional (*paragraph 2.8*), sensorial (*paragraph 2.7* and *chapter 4*) and economical values (*paragraphs 2.1, 2.2, 2.3* and *2.4*). On the other side, within the market it is possible to find EVOOs that share the same labelled denomination, but have a different real quality level, from the so-called "low-cost" products to the high-quality ones. In general, consumers seem not to be apprised of such a wide range of quality, and they usually do not seem to practice a "well-informed" consumption of EVOOs (*chapter 4*), maybe because they cannot appreciate the real quality of products.

In order to fill these gaps, the investigations carried out within this Ph.D. project were realized with different aims, that are all linked together:

- evaluating and studying the chemical parameters recently introduced by the European Union law (EU Reg. 61/2011), such as the fatty acid alkyl esters (FAAEs), in order to evaluate their importance as quality/genuineness marker for EVOOs (*paragraph 3.1.13* and *chapter 2*, see paper "Detection of low-quality…");
- finding innovative, faster, cheaper and more environmentally friendly analytical methods (such as Ft-IR, TDR, NIR) (*chapter 2* and *paragraph 3.1.13*) in order to replace, confirm or simply add values to the official ones and/or to improve them (*paragraph 3.1.10*);
- studying the so-called "unofficial" parameters, like diacylglycerols, phenolic and volatile compounds and water content, because, even if they are still not included in the law, they are nevertheless very important for the assessment of the quality and the purity of oils.

These markers have to be considered together with some traditional parameters, in order to verify their effectiveness (*paragraphs 3.1.3*, *3.1.8*, *3.1.13*, *3.1.14*, *3.1.15* and *chapter 2*);

- promoting the adoption of the objective sensory analysis for EVOOs (Panel test, EC Reg. 640/2008) as a crucial quality tool (*paragraph 2.7*), by focusing on studies involving both the sensorial and the chemical approaches (*paragraph 3.1.14*, *chapters 2* and *3*);

- evaluating the main factors that affect consumers' sensorial perception of EVOOs and their impact and correlation with the objective sensory attributes evaluated by the Panel Test (*chapter 4*);

- proposing and studying new technological systems able to improve the effect of a particular process on the quality of the EVOOs (*chapter 3*).

Thanks to the collaboration with other research groups, I carried out other "parallel" applicative studies, that focused on the shelf-life of olive oils (*chapter 5*) and the effects of thermal stresses on the quality of the product (*chapter 6*).

Moreover, in *annex I* I reported some references to other scientific papers that were realized during my Ph.D., even if they are not related to olive oils, but to other foodstuffs; these papers are interesting too, since they focus on the relation among objective sensory analysis, volatile compounds and consumers' preferences.

In order to avoid redundancy within the text, in this Ph.D. thesis I inserted many references connected to already published manuscripts, submitted papers and unpublished experimental results too.

2. INTRODUCTION

2.1 Global production of olive oils in the world

Considering the world-ranking production of all the vegetable oils in 2008/2009, olive oil was the ninth among them (Gunstone, 2011). Actually, the olive oil production was less than a tenth respect to palm oil and nowadays it represents less than the 3% of the total amount of produced vegetable oils, according to the data provided by the United States Department of Agriculture (Foreign Agricultural Service) (USDA, 2012). Checking the statistical data provided by the International Olive Council (I.O.C.) (IOC statistical results about production of olive oil in 2011*)* and updated to November 2011, the estimated production of olive oils for the 2010/2011 harvest all over the world is about 3 millions tons, of which about 2 millions tons produced in the European Union (E.U.). Spain is the production leader, with a 64% of the whole E.U. production (about 1.4 million tons). Italy has a production near 440 thousand tons (20% of the E.U.). After them, Greece produces 15% of the E.U. production (300 thousand tons). Considering the extra-E.U. countries:

-Turkey, Syria and Morocco have had good increases in the last years;

-Tunisia and Algeria have showed significant decreases in the last years.

Among the new areas of production, it is interesting to observe an increase also in the Australian production of olive oils (in 2010/2011 the estimated production is about 18 thousands tons).

2.2 Exportation of olive oils from Italy

During 2010, Italy exported 252010 tons of virgin olive oil (VOO), with an increase of 17.5% respect to 2009. It is interesting to underline that the exportations from E.U. countries has been decreased during the last four years: in 2006 the exportations from E.U. were 44.6% of the total exportation of VOO, while in 2010 they decreased to 43.1% (ISTAT report, 2011). In Italy, the exportations of olive oils to extra-UE countries were about 160000 tons in 2010-2011, secondary only to Spain (197600 tons) (IOC statistical results about exportations of olive oil in 2011). Actually, the most important market for Italian VOO is USA, with more than 33% of the total exported VOO (and it increased of 9.9% respect to 2009). In the ranking of VOO importers from Italy, the USA are followed by Germany, France, UK and Japan (Federolio, 2011) . Even if it is important to underline that

5 of the 10 most important countries for exportations are extra-E.U. countries, Italy is the main exporter for the most traditionally vocated-VOO countries out of the Mediterranean basin (Germany, North Europe); considering the "emergent-VOO-consumers" countries (Brazil, Russia, China), Spain is the main exporter, with a larger amount of oil exported than Italy (Federolio, 2011).

2.3 Global consumption of oils obtained by olives

Considering results provided by the I.O.C. (IOC statistical results about consumption of olive oil in 2011*)* and updated to November 2011, it is possible to distinguish among two kinds of consumption behaviors: actually, from one side there are the so-called traditionally "devoted" countries located in the Mediterranean basin and on the other side "new emerging" countries, which are expressing a positive interest in this foodstuff, thanks to the migration flows, to an increase knowledge and interest on healthy food-styles, in particular regarding the awareness about the beneficial properties of the Mediterranean diet, of which olive oils is a key-constituent. E.U. is the most important area of consumption of olive oil allover the world, with a 79% of the overall consumption, that was near 3 millions tons in 2010/2011 (IOC statistical results about consumption of olive oil in 2011). In particular, the 22% of the global amount of olive oils is consumed in Italy, followed by Spain (18%) and Greece (7%). American are also good "new" consumers of olive oils, which an amount that is growing year by year (around 9% in 2010/2011). Regarding the "new emerging" areas of consumption, Australia, Japan and Canada are the countries with the highest consumptions in 2011 (around 40 thousand tons). In the last years, also in Russia the consumption of olive oil (22 thousand tons in 2010/2011) has been increased (IOC statistical results about consumption of olive oil in 2011).

2.4 Consumption of different categories of oils obtained by olives sold in 2009 in large retail trade in Italy

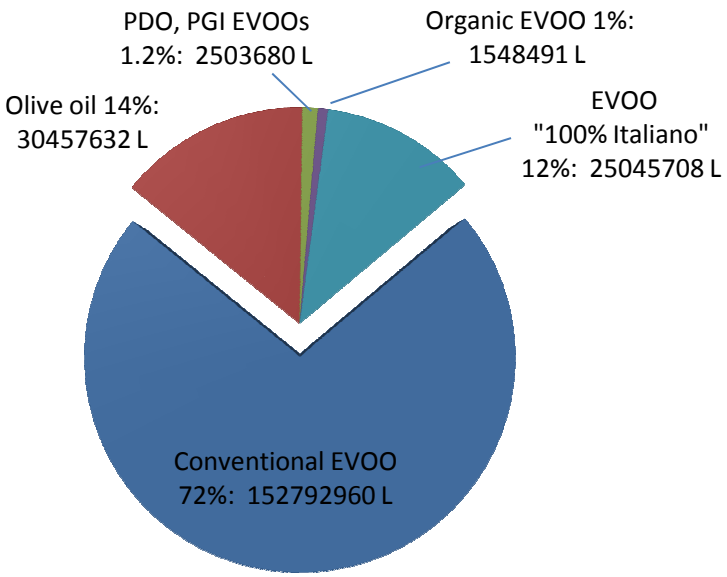

Figure 1 I. Percentage and total volume (L, litre) of oils obtained by olives sold in the retail trade in Italy.

In 2009, more than 212 millions liters of oils obtained by olives were sold in Italy in the large retail trade, for a total value of about 880.4 millions of euros (Falasconi, 2012). The most sold commercial class was the "conventional" EVOO, without specific designation or added-values, with a total amount of consumption near 152 millions liter (72% of the total turnover, see **Figure 1 I**). Olive oils and EVOOs labeled as "100% italiano" (obtained from olives all collected in Italy) reached lower percentages, with a total amount of 14% and 12%, respectively (Falasconi, 2012). The oils characterized by a designation of origin (Protected Geographical Indication (PGI) or Protected Designation of Origin (PDO), see *paragraph 2.5*) and the ones produced by organic farming system represented a very small percentage of the total consumption, both near 1% of the global amount (see **Figure 1 I**).

2.5 Designation of quality: Protected Geographical Indication (PGI) and Protected Designation of Origin (PDO) extra virgin olive oils

In order to certify and guarantee the geographical origin of typical food with excellent characteristics, the E.U. identifies specific food quality denominations, based on their geographical origin: the Protected Geographical Indication (PGI), the Protected Designation of Origin (PDO) and the Traditional Speciality Guaranteed (TSG) (EC Reg. 510/2006). Since the PDO and PGI certifications for EVOOs usually define the area of origin of each product, the varieties of used olives as well as some specific guidelines for the production, the EVOOs included in the same PDO share some common characteristics, including both sensory properties and specific chemical composition. Considering EVOOs, on 31st December 2011, Italy has 239 PDO, PGI and TSS foodstuffs recognized by E.U. (EC Reg. 510/2006), of which 233 result "active": only fruits, vegetables and cereals (94) and cheeses (43) have more protected denominations than EVOOs (ISTAT report, 2011). Actually, Italy is the country with the highest number of protected designations of quality for EVOOs (42, of which 41 PDO + 1 PGI), followed by Greece and Spain. According to ISTAT data related to 2011 (ISTAT report, 2011), an increase in the number of producers of PDO and PGI EVOOs has been observed respect to 2010 (+1.9%), in the olive trees-area where the olives for producing PDO and PGI EVOOs are collected (+2.5%) and in the mills producing them (+2.3%). According to ISMEA elaboration (Adua, 2010), such a production has been strongly increased through the years 2004-2008, from 5000 to 8500 tons (+69,2%). Moreover, the production of PDO and PGI EVOOs is highly oriented (26% of the total production) to foreign markets, with a total turnover of 26.3 millions of euro (Adua, 2010).

2.6 ***References (Summary and Objectives Introduction)***

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2.7 The sensory analysis of virgin olive oil

Sensory analysis is an essential tool for evaluating the quality of oils obtained by olives. The importance, the method and the implications of such an evaluation are detailed in the chapter-review reported in the next pages.

Moreover, relations between sensory analysis and consumer perception is also discussed in *chapter 4*.

My contribution in the realization of this chapter-review focused on finding recent publications and especially on the draft of chapters “2. Flavours and off-flavours of virgin olive oil: The molecules responsible for sensory perceptions” and “3. Sensory methodology for evaluating the quality of VOO: Basic concepts”. I would like to underline that all the chapter was written together with the other co-authors.

Bendini, A., Valli, E., Barbieri, S. & Gallina Toschi, T. (2012). The sensory analysis of virgin olive oil. In: Boskou, D. (Ed.), Olive Oil - Constituents, Quality, Health Properties and Bioconversions. InTech, pp. 109-130.

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Sensory Analysis of Virgin Olive Oil

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1. Introduction

Virgin olive oil (VOO) is the supernatant of the fresh juice obtained from olives by crushing, pressure and centrifugation, without additional refining. Its flavour is characteristic and is markedly different from those of other edible fats and oils. The combined effect of odour (directly via the nose or indirectly through a retronasal path, via the mouth), taste and chemical responses (as pungency) gives rise to the sensation generally perceived as "flavour".

Sensory analysis is an essential technique to characterize food and investigate consumer preferences. International cooperative studies, supported by the International Olive Oil Council (IOOC) have provided a sensory codified methodology for VOOs, known as the "COI Panel test". Such an approach is based on the judgments of a panel of assessors, conducted by a panel leader, who has sufficient knowledge and skills to prepare sessions of sensory analysis, motivate judgement, process data, interpret results and draft the report. The panel generally consists of a group of 8 to 12 persons, selected and trained to identify and measure the intensity of the different positive and negative sensations perceived. Sensory assessment is carried out according to codified rules, in a specific tasting room, using controlled conditions to minimize external influences, using a proper tasting glass and adopting both a specific vocabulary and a profile sheet that includes positive and negative sensory attributes (Dec-23/98-V/2010). Collection of the results and statistical elaboration must be standardized (EEC Reg. 2568/91, EC Reg. 640/08). The colour of VOO, which is not significantly related to its quality, may produce expectations and interferences in the flavour perception phase. In order to eliminate any prejudices that may affect the smelling and tasting phases, panelists use a dark-coloured (blue or amber-coloured) tasting glass.

Many chemical parameters and sensory analyses (EEC Reg. 2568/91 and EC Reg. 640/08), with the latter carried out by both olfactory and gustatory assessments, can classify oils in different quality categories (extra virgin, virgin, lampant). Extra virgin olive oil (EVOO) extracted from fresh and healthy olive fruits (Olea europaea L.), properly processed and adequately stored, is characterized by an unique and measurable combination of aroma and taste. Moreover, the category of EVOO should not show any defects (e.g. fusty, musty, winey, metallic, rancid) that can originate from incorrect production or storage procedures.

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Positive or negative sensory descriptors of VOO have been related to volatile and phenol profiles, which are responsible for aroma and taste, respectively.

The characteristic taste of VOO, and in particular some positive attributes such as bitterness and pungency that are related to important health benefits, is not completely understood or appreciated by consumers. In this respect, it is interesting to consider the degree of acceptability of VOO in several countries based on literature data. In this way, it is possible to lay the foundations for correct instruction of the sensory characteristics of EVOO. The main chemical, biochemical and technological processes responsible for the positive and negative (defects) descriptors of VOO are summarized in this chapter. An overview on the sensory methodologies proposed, applied and modified during the last 20 years is also presented.

2. Flavours and off-flavours of virgin olive oil: The molecules responsible for sensory perceptions

VOOs are defined by the European Community as those "…oils obtained from the fruit of the olive tree solely by mechanical or other physical means under conditions that do not lead to alteration in the oil…" (EEC Reg. 2568/91). This production method renders VOO different from other vegetable oils that undergo refining, which leads to loss of most of the minor components such as volatile molecules and "polar" phenolic compounds.

Many authors (Angerosa et al., 2004; Kalua et al., 2007) have clarified that several variables affect the sensory characteristics and chemical composition of an EVOO. These include environmental factors, cultivation and agronomic techniques, genetic factors (cultivar), ripening degree of drupes, harvesting, transport and storage systems of olives, processing techniques, storage and packaging conditions of the oil.

The sensory attributes of EVOO mainly depend on the content of minor components, such as phenolic and volatile compounds. The independent odours and tastes of different volatile and phenolic compounds that contribute to various and typical EVOO flavours have been extensively studied; the sensory and chemical parameters of EVOO have been correlated in a large number of investigations (Bendini et al., 2007; Cerretani et al., 2008).

Each single component can contribute to different sensory perceptions. It is well established that specific phenolic compounds are responsible for bitterness and pungency (Andrewes et al., 2003; Gutiérrez-Rosales et al., 2003; Mateos et al., 2004). Few individuals, except for trained tasters of EVOO, know that the bitterness and pungency perceived are considered positive attributes. These two sensory characteristics, more intense in oils produced from olives at the start of crop year, are strictly related to the quali-quantitative phenolic profile of EVOO.

Even in small quantities, phenols are fundamental for protecting triacylglycerols from oxidation. Several authors (Gallina Toschi et al., 2005, Carrasco-Pancorbo et al., 2005; Bendini et al., 2006; Bendini et al., 2007) have reported their importance as antioxidants as well as nutracetical components. The major phenolic compounds identified and quantified in olive oil belong to five different classes: phenolic acids (especially derivatives of benzoic and cinnamic acids), flavones (luteolin and apigenin), lignans ((+)-pinoresinol and (+)-

acetoxypinoresinol), phenyl-ethyl alcohols (hydroxytyrosol, tyrosol) and secoiridoids (aglycon derivatives of oleuropein and ligstroside). The latter are characteristic of EVOOs.

Several investigations (Gutiérrez-Rosales et al., 2003; Mateos et al., 2004) have demonstrated that some phenols, and in particular secoiridoid derivatives of hydroxytyrosol, are the main contributors to the bitterness of olive oil; other phenolic molecules such as decarboxy-methyl-ligstroside aglycone, which seems to be a key source of the burning sensation, can stimulate the free endings of the trigeminal nerve located in the palate and gustative buds giving rise to the chemesthetic perceptions of pungency and astringency (Andrewes et al., 2003). Using a trained olive oil sensory panel, some investigators (Sinesio et al., 2005) have studied the temporal perception of bitterness and pungency with a time-intensity (TI) evaluation technique. It has been shown that the bitterness curves had a faster rate of increase and decline than the pungency curves. It was also demonstrated that differences in kinetic perception are linked to the slower signal transmission of thermal nociceptors compared to other neurons.

On the other hand, approximately 180 compounds belonging to several chemical classes (aldehydes, alcohols, esters, ketones, hydrocarbons, acids) have been separated from the volatile fractions of EVOOs of different quality. Typical flavours and off-flavour compounds that affect the volatile fraction of an oil obtained from olives originate by different mechanisms: positive odours are due to molecules that are produced enzymatically by the so-called lipoxygenase (LOX) pathway. Specifically both C_6 aldehydes, alcohols and their corresponding esters and minor amounts of C_5 carbonyl compounds, alcohols and pentene dimers are responsible for pleasant notes. In contrast, the main defects or off-flavours are due to sugar fermentation (*winey*), amino acid (leucine, isoleucine, and valine) conversion (*fusty*), enzymatic activities of moulds (*musty*) or anaerobic microorganisms (*muddy*), and to auto-oxidative processes (*rancid*).

Volatile molecules can be perceived in very small amounts (micrograms per kilogram or ppb) and these compounds do not have the same contribution to the global aroma of EVOO; in fact, their influence must be evaluated not only on the basis of concentration, but also on their sensory threshold values (Angerosa et al., 2004; Kalua et al., 2007). In addition, antagonism and/or synergism among different molecules can occur, affecting the global flavour of EVOO. Chemical factors of molecules (volatility, hydrophobic character, size, shape, conformational structure), type and position of functional groups appear to affect the odour and taste intensity more than their concentration due to their importance in establishing bonds with receptor proteins (Angerosa et al., 2004).

In general, it is correct to surmise that from healthy olives, picked at the right degree of ripening and properly processed, it is always possible to obtain an EVOO, independent of the olive variety. However, from unhealthy olives or from those harvested off the ground it is inevitable to produce an olive oil characterized by unpleasant flavours and sensory defects. Thus, both natural (olive variety, environmental conditions, degree of ripening and health status of olives) and extrinsic (technological processing by olive farmer/mill worker) factors may profoundly influence olfactory and gustative notes.

Several agronomic and climatic parameters can affect the volatile and phenolic composition of VOOs. The genetic characteristics of the olive cultivar are some of the most important

aspects that determine the level of enzymes in fruit (Angerosa et al., 1999) that are involved in synthesis of volatile molecules (LOX pathway) and phenol compounds (biosynthetic pathways via PPO and β-glucosidase) present in VOOs.

Even if enzymatic activity depends on the stage of ripeness (Morales et al., 1996; Aparicio & Morales 1998) agronomic (fertilization, irrigation) and climatic (temperature and rainfall) conditions also play an important role.

2.1 Key points in obtaining a high quality VOO

- Processing of healthy olives:

When the common olive fly (*Bactrocera oleae*) attacks olives (from the beginning of summer to the start of harvesting), damage occurs as a result of larval growth: oils from damaged fruits show changes in both volatile and phenolic compounds that influence negatively the sensory properties and oxidative stability of the product, especially during oil storage (polar phenols have a fundamental role as antioxidants during storage). The bad taste due to these changes caused by the olive fly is well known as a *grubby defect* (Angerosa et al., 1992; Gómez-Caravaca et al. 2008).

In order to obtain a high quality olive oil, it is necessary to process olives that are not overripe. The use of fruits that have partially degraded tissues cause an increase in enzymatic and microrganism activities and oxidative reactions; therefore the produced oil probably will be characterized by an higher free acidity and perceivable sensory defects. When olives are accumulated in piles for many days, the high temperature and humidity inside the mass promotes proliferation of bacteria, yeasts and moulds, producing undesirable fermentation and degradation that give rise to specific volatile molecules responsible for unpleasant odours (i.e. winey, fusty and mouldy).

Winey, the typical pungent sensory note perceptible in oils produced by olives stored in piles or in jute sacks for several days, arises from alcoholic fermentation: *Lactobacillus* and *Acetobacter* have been detected in olives inducing fermentative processes. The main microorganism found in olives depends on the length of storage: at the beginning the enterobacteriaceae genera *Aerobacter* and *Escherichia* prevail, while *Pseudomonas*, *Clostridium* and *Serratia* are predominant after longer periods of time. The activity of these microorganisms results in the presence of low concentrations of biosynthetic volatiles and large amounts of compounds such as the branched alcohols due to degradation of amino acids that lead to the typical undesirable sensory note known as fusty (Angerosa, 2002; Morales et al 2005). The most abundant deuteromycetes found in olives stored at high humidity are several species of the genus *Aspergillus* together with ascomycetes *Penicillium*; these organisms oxidize free fatty acids producing mainly methyl ketones, in contrast to yeasts of the genera *Candida*, *Saccharomyces* and *Pichia* which are able to reduce carbonylic compounds. Enzymes from these microorganisms interfere with the LOX pathway to produce volatile C_8 molecules characterized by very low odour thresholds, and reduce some C_6 compounds. This volatile profile is responsible for the musty defect of EVOO.

- Selection of the most suitable milling conditions

The phenolic content is greatly influenced by this technological step. In general, the use of the more violent crushing systems (i. e. with hammers instead of blades) causes an increase

in extraction of phenolic compounds due to more intense tissue breaking; therefore, a more vigorous milling system should be used to process olive varieties that are naturally low in phenolic compounds, and permit enrichment of bitter and pungency intensities. The use of more violent milling systems also produces a significant increase in olive paste temperature and a corresponding decrease of the activity of enzymes that play a key role in the production of volatile compounds responsible for fruity and other green notes (Salas & Sanchez, 1999; Servili et al., 2002).

Concerning the malaxation phase, which consists in a slow kneading of the olive paste, the time-temperature pair should be carefully controlled to obtain a high quality EVOO. The lipoxygenase pathway is triggered by milling of olives and is active during malaxation. The volatile compounds produced are incorporated into the oil phase to confer its characteristic aroma. Specifically, a temperature above 28°C for more than 45 min should be avoided; in fact, these conditions can lead to the deactivation of enzymes that produce both positive volatile compounds and oxidize the phenolic compounds causing changes in oil flavour (Salas & Sanchez 1999; Kalua et al., 2007). The reduced concentration of oxygen in paste, obtained by replacing air with nitrogen in the headspace of malaxer during processing, can inhibit these enzymes and minimize the oxidative degradation of phenolic compounds during processing (Servili et al., 1999; Servili et al., 2003). Malaxation under erroneous conditions is responsible for the unpleasant flavor known as a "*heated defect*" due to the formation of specific volatile compounds (Angerosa et al., 2004).

- The application of different oil separation systems

One of the main disadvantages of discontinuous mill systems is the possible fermentation and/or degradation phenomena of residues of pulp and vegetation waters on filtering diaphragms; these reactions give rise to a defect termed "*pressing mats*", but also promote winey and fusty defects (Angerosa et al., 2004). It is well known that among continuous systems, discontinuous mill systems with a three-phase decanter need lukewarm water to dilute olive paste in contrast to a two-phase decanter, which has two exits producing oil and pomace and separates the oil phase from the olive paste This latter system has advantages in terms of water reduction and major transfer of phenols from the olive paste to the oil, with a consequent increase in oxidative stability, bitterness and pungency.

The amount of water added determines the dilution of the aqueous phase and lowers the concentration of phenolic substances that are more soluble in vegetable waste water. Consequently, a large amount of antioxidants is lost with the wastewater during processing. In addition to phenolic compounds, some volatile compounds accumulate more in oil from a dual-phase decanter than in oils extracted with three-phase decanters. Therefore, the use of a two-phase decanter promotes greater accumulation of volatile and phenolic compounds that are not lost in the additional water as in a three phase decanter. The higher concentrations of these compounds are related to the high intensities of bitter, pungent, green fruity, freshly cut lawn, almond and tomato perceptions (Angerosa et al., 2000; Angerosa et al., 2004; Kalua et al., 2007).

- Storage of oil under suitable conditions

In unfiltered oil, the low amounts of sugars or proteins that remain for extended times in oil can be fermented or degraded by specific anaerobic microrganisms of the *Clostridium* genus,

producing volatile compounds responsible for an unpleasant muddy odour by butyric fermentation. The filtration of newly-produced oil can avoid this phenomenon. It is known (Fregapane et al., 2006; Mendez & Falque, 2007; Lozano-Sanchez et al., 2010) that EVOO has a low amount of water, and for this reason it can be considered as a water-in-oil emulsion (Koidis et al., 2008)

The orientation of phenolic compounds in the oil-water interface and the active surface of water droplets can protect against the oxidation of oil. According to some researchers (Tsimidou et al., 2004; Gómez-Caravaca et al., 2007), the stability of unfiltered samples is significantly higher than that of the corresponding filtered oils. This coincides with a higher total phenolic content in unfiltered oils due to a greater amount of emulsified water. On the other hand, higher water levels are expected to favour enzymatic catalysis, including lipase, lipoxygenase and polyphenol oxidase activities. Thus, a more rapid oxidation of unfiltered oil is expected. Some authors (Montedoro et al., 1993) observed that hydrolytic processes occurr in parallel with oxidation during long term storage.

Lipid oxidation is an inevitable process that begins immediately after oil extraction and leads to a deterioration that becomes increasingly problematic during oil storage. The presence of a rancid defect, typical off-flavour for the fatty matrices, can be avoided or substantially slowed. The most advanced oxidation stages are characterized by the complete disappearance of compounds arising from the LOX cascade and by very high concentrations of saturated and unsaturated aldehydes together with unsaturated hydrocarbons, furans and ketones that contribute mainly to the rancid defect because of their low odour thresholds (Guth & Grosch, 1990; Morales et al., 1997; Bendini et al., 2009). To avoid the rancid perception, it is fundamental to control factors that promote lipid oxidation. These include a decrease in the availability of oxygen, the protection of the oil from light and storage at a temperature of 12-14°C. Before bottling, it is advisable to maintain the oil in stainless steel tanks under an inert gas such as nitrogen equipped with devices that periodically eliminate sediments from the bottom of the tank.

3. Sensory methodology for evaluating the quality of VOO: Basic concepts

A sensory codified methodology for virgin olive oils, known as the "COI Panel test", represents the most valuable approach to evaluate the sensory characteristics of VOO. The use of statistical procedures to analyze data from assessors' evaluation provides results that can be trusted as well as methods usually adopted in scientific fields. The purpose of this international method is to standardize procedures for assessing the organoleptic characteristics of VOO, and to establish the methodology for its classification. This methodology, incorporated into regulations of the European Union since 1991, uses, as an analysis tool, a group of 8-12 persons selected in a controlled manner, who are suitably trained to identify and measure the intensity of positive and negative sensations (EEC Reg. 2568/91).

A collection of methods and standards has been adopted by the International Olive Oil Council (IOOC or COI) for sensory analysis of olive oils. These documents (IOOC/T.20/Doc. 4/rev.1 and IOOC/T.20/Doc.15/rev.2) describe the general and specific terms that tasters use. Part of the vocabulary is common to sensory analysis of all foods (general vocabulary), while a specific vocabulary has been developed *ad hoc* and established by sensory

experts of IOOC. In addition, the official method (IOOC/T.20/Doc.5/rev.1 and IOOC/T.20/Doc.14/rev.2) includes precise recording of the correct tasting temperature, as well as the dimensions and colour of the tasting glass and characteristics of the test room.

The panel leader is the person responsible for selecting, training and monitoring tasters to ascertain their level of aptitude according to (IOOC/T.20/Doc.14/rev.2). The number of candidates is generally greater than that needed in order to select people that have a grater sensitivity and discrimination capability. Screening criteria of candidates are founded on sensory capacity, but also on some personal characteristics of candidates. Given this, the panel leader will personally interview a large number of candidates to become familiar with their personality and understand habits, hobbies, and interest in the food field. He uses this information to screen candidates and rejects those who show little interest, are not readily available or who are incapable of expressing themselves clearly.

The determination of the detection threshold of the group of candidates for characteristic attributes is necessary because the "threshold concentration" is a point of reference common to a "normal group" and may be used to form homogeneous panels on the basis of olfactory-gustatory sensitivity.

A selection of tasters is made by the intensity rating method, as described by Gutiérrez Rosales (Gutiérrez Rosales et al., 1984). A series of 12 samples is prepared by diluting a VOO characterized by a very high intensity of a given attribute in an odourless and tasteless medium (refined oil or paraffin). The panel leader sends out the candidate, removes one of the 12 tasting glasses from the series, and places the remaining together; the candidate is called back in the room and is asked to correctly replace the testing glass withdrawn from the series by comparing the intensity of this last with that of the others. The test is carried out for fusty, rancid, winey and bitter attributes to verify the discriminating capacity of the candidate on the entire scale of intensities.

The stage training of assessors is necessary to familiarize tasters with the specific sensory methodology, to heighten individual skill in recognizing, identifying and quantifying the sensory attributes and to improve sensitivity and retention with regards to the various attributes considered, so that the end result is precise and consistent. In addition, they learn to use a profile sheet.

The maintenance of the panel is made through continuous training over all duration of life of the same panel, the check of the sensory acuity of tasters, and exercises that allow the measurement of the panel performance.

Every year, all panels must assess a number of reference samples in order to verify the reliability of the results obtained and to harmonize the perception criteria; they must also update the Member State on their activity and on composition changes of their group.

3.1 Evolution of sensory methodology: From old to new

A method for the organoleptic evaluation of olive oils was introduced in the Regulation (EEC) No 2568/91, Annex XII, that is inspired by the COl/T.20/Doc. no.15, published in 1987. In the profile sheet of EEC Reg. 2568/91, a number of positive attributes and defects were evaluated, giving each a score from 0 to 5 (Figure 1).

Drawing on experience, the International Olive Oil Council has devised a new method of organoleptic assessment of VOOs (Decision Dec-21/95-V/07) that is simpler and more reliable than that in EEC Reg. 2568/91. In particular, the EC Reg. 796/2002 introduced a reduction of the attributes of the old profile sheet, asking tasters to consider only the defects of the oil (fusty, mustiness/humidity, winey/vinegary, muddy sediment, metallic, rancid and others) and only the three most important positive attributes (fruity, pungent and bitter). The most important innovation of EC Reg. 796/2002 is the use of continuous scales, from 0 to 10 cm, for evaluating the intensity of perception of the different attributes (positive and negative), as reported in Figure 2. In this way, tasters are free to evaluate the intensity of each attribute by ticking the linear-scale, without having a prefixed choice (as with the discrete scale of EEC Reg. 2568/91, see Figure 1).

Sensory analysis and its application to olive and virgin olive oil

VIRGIN OLIVE OIL

PROFILE SHEET

GRADING TABLE

OLFACTORY-GUSTATORY-TACTILE NOTES

	0	1	2	3	4	5
Olive fruity (ripe and green)						
Apple						
Other ripe fruit						
Green (leaves, grass)						
Bitter						
Pungent						
Sweet						
Other allowable attribute(s) (Specify.....)						
Sour/Winey/Vinegary/Acid						
Rough						
Metallic						
Mustiness/humidity						
Muddy sediment						
Fusty ("Atrojado")						
Rancid						
Other unallowable attribute(s) (Specify.....)						

DEFECTS	CHARACTERISTICS	OVERALL MARK: POINTS
	Olive Fruity	
None	Olive fruity and fruitness of other fresh fruit	9 8 7
Barely perceptible	Weak fruitness of any tipe	6
Slight perceptible	Rather imperfect fruitness, anomalous odours and tastes	5
Considerable, on the border of acceptability	Clearly imperfect, unpleasant odours and tastes	4
Great and/or serious, clearly perceptible	Totally inadmissible odours and tastes for consumption	3 2 1

1 Barely perceptible
2 Slight perceptible
3 Average
4 Great
5 Extreme

REMARKS.....

.....

NAME OF ASSESSOR.....
LEGEND OF SAMPLE.....

DATE.....

Fig. 1. Profile sheet for EVOO used for designation of origin (EEC Reg. 2568/91, annex XII).

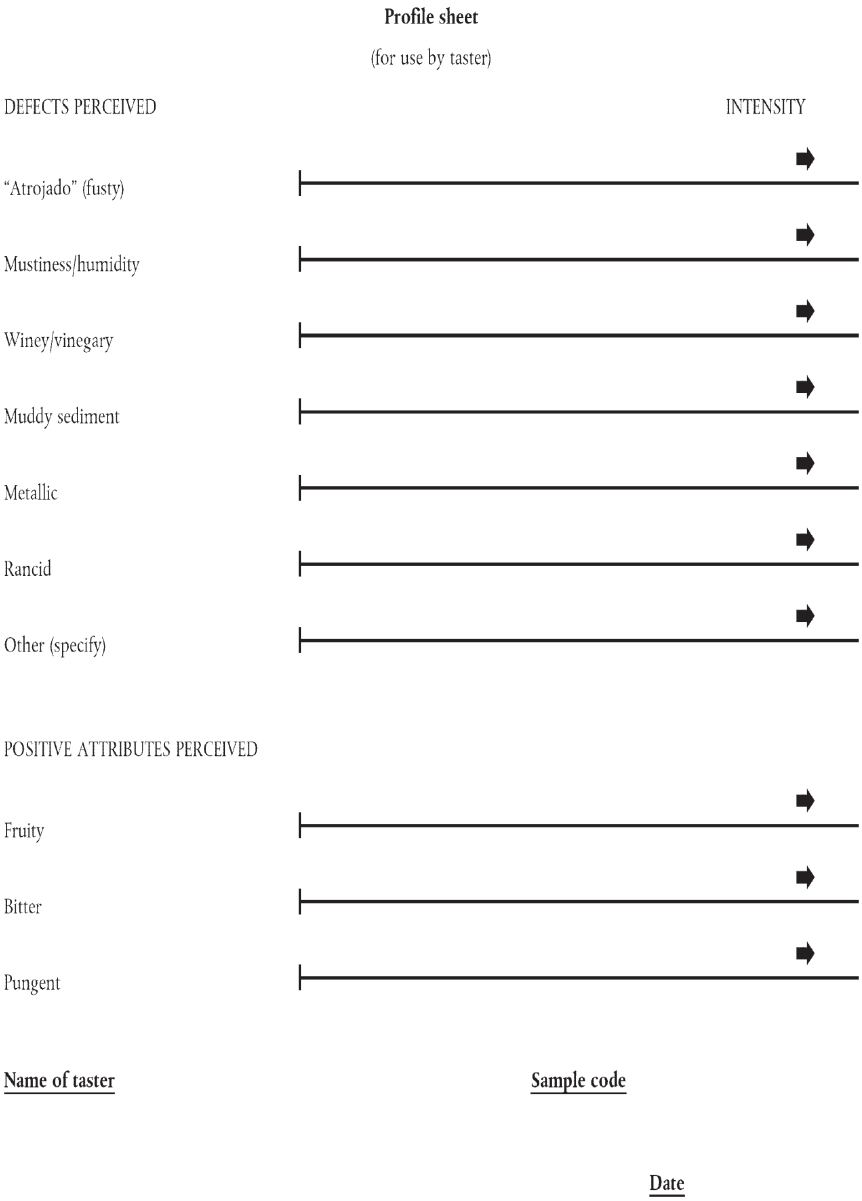

Fig. 2. Profile sheet for VOO assessment currently adopted by the EU (EC Reg. 796/02).

Each attribute is calculated, and the median value of each is used to classify the oil according to the median of the defect perceived with greatest intensity and the median for "fruity". It is important to remember that the value of the robust variation coefficient for this negative attribute must be no greater than 20%.

The classification of olive oils, according to sensory attributes, has also undergone evolution. According to EC Reg. 796/2002, oils are classified as:

a. extra virgin olive oil: the median of the defects is 0, and the median for "fruity" is above 0;
b. virgin olive oil: the median of the defects is above 0, but not above 2.5 and the median for "fruity" is above 0;
c. ordinary virgin olive oil: the median of the defects is above 2.5, but not above 6.0, or the median of the defects is not above 2.5 and the median for "fruity" is 0;
d. lampante virgin olive oil: the median of the defects is above 6.0.

Since November 2003, categories c) and d) have been replaced by (c) "lampante olive oil": the median of defects is above 2.5, or the median of the defects is not above 2.5 and the median for "fruity" is 0.

EC Reg. 640/08 introduced a new upper limit of defect for discriminating between virgin and defective oils: in particular, the evaluation of the median defect ('2.5') was replaced by '3.5'. An important innovation of Reg. 640/08 was also the grouping in only one negative attribute of two different defects: fusty and muddy sediment.

A revised method for the organoleptic assessment of VOO was adopted by the IOOC in November 2007 (Decision No DEC-21/95-V/2007, 16 November 2007) and adopted by the European Community with EC Reg. 640/2008. This revision updated the descriptions of the positive and negative attributes of VOO and the method. It also amended the maximum limit for the perception of defects in VOO. The IOOC's revised method for the organoleptic assessment of VOO also specifies the conditions for the optional use, on labels, of certain terms and expressions relating to the organoleptic characteristics of VOO (optional terminology for labelling purposes).

The most recent change is Decision No Dec-23/98-V/2010 of the IOOC, which defined a new method for assessing the organoleptic properties of VOO and to establish its classification on the basis of those characteristics (IOOC/T.20/Doc. No 15/Rev. 3).

3.2 The method for assigning commercial class: The official profile-sheet and expression of results

The organoleptic assessment of VOO is officially regulated in Europe by a Commission Regulation (EC Reg. 640/2008). This regulation describes the procedures for assessing the organoleptic characteristics of VOOs, the method for classification according to sensory characteristics, the specific vocabulary for sensory analysis of VOOs, including positive and negative attributes, and the optional terminology for labelling purposes. The selection, training and monitoring of skilled VOO tasters, the skills and responsibilities of the panel leader, the specific characteristics of the glass for oil tasting and the test room were also considered, according to previous regulations and IOOC documents (IOOC, 2007 and 2010).

The official profile sheet intended for use by tasters, shown in Figure 3 (EC Reg. 640/08), is quite simple and is formed by an upper section for evaluation of the intensity of defects, and

Profile sheet for virgin olive oil

INTENSITY OF PERCEPTION OF DEFECTS

Fusty/muddy sediment | ——————————→

Musty-humid-earthy | ——————————→

Winey-vinegary — acid-sour | ——————————→

Metallic | ——————————→

Rancid | ——————————→

Other (specify) | ——————————→

INTENSITY OF PERCEPTION OF POSITIVE ATTRIBUTES

Fruity | ——————————→

greenly ☐ ripely ☐

Bitter | ——————————→

Pungent | ——————————→

Name of taster:

Sample code:

Date:

Comments:

Fig. 3. Profile sheet for VOO assessment currently adopted by the EU (EEC Reg. 640/08).

a lower part for the evaluation of the three most important positive sensory attributes (fruity, bitter, pungent). Tasters have to smell the sample, taste the oil (overall retronasal olfactory, gustatory and tactile sensations) and evaluate the intensity with which they perceive each of the negative and positive attributes on the 10-cm scale. If a taster identifies greenly or ripely as fruity attributes, the correct options must be indicated in the profile sheet. Green fruitiness is a characteristic of the oil which is reminiscent of green olives, dependent on the variety of the olive and coming from green, sound, fresh olives. Ripe fruitness is reminiscent of ripe fruit. If any negative attributes not listed in the upper section of the profile are perceived, the taster records them under the "others" heading, using the descriptors among those in the specific vocabulary for the sensory analysis of olive oils (IOOC/T.20/Doc. 4/rev.1).

The panel leader collects the profile sheets and elaborates the results by a statistical approach. In particular, the medians of the greatest perceived defect and fruity attribute are calculated. According to these two parameters, the oil can be graded in different quality categories. Such values are expressed to one decimal place, and the value of the robust coefficient of variation which defines them shall be no greater than 20%. As already mentioned, the classification of the oil is carried out by comparing the medians of the defects and the fruity attribute with the reference ranges established by EC Reg 640/08 for the different categories:

1. Extra virgin olive oil: the median of the defects is 0 and the median of the fruity attribute is above 0;
2. Virgin olive oil: the median of the defects is above 0, but not more than 3.5, and the median of the fruity attribute is above 0;
3. Lampante olive oil: the median of the defects is above 3.5, or the median of the defects is not more than 3.5 and the median of the fruity attribute is 0.

The panel leader can also state that the oil is characterized by greenly or ripely fruity attributes if at least 50% of the panel agrees.

Actually the most important result for sensory analysis of VOO is to identify the presence of defects instead of evaluating the positive attributes, in agreement with the aim of such an analysis, which is essentially to classify the product in different commercial classes.

3.2.1 Optional terminology for labelling purposes

Upon request, the panel head may certify that an oil complies with the definitions and ranges that correspond to the following adjectives, according to the intensity and perception of attributes:

a. for each of the positive attributes mentioned (*fruity* — whether *green* or *ripe* — *pungent* or *bitter*):
   i. the term “intense” may be used when the median of the attribute is greater than 6;
   ii. the term “medium” may be used when the median of the attribute is between 3 and 6;
   iii. the term “light” may be used when the median of the attribute is less than 3;
   iv. the attributes in question may be used without the adjectives given in points (i), (ii) and (iii) when the median of the attribute is 3 or more;

b. the term "well balanced" may be used when the oil does not display a lack of balance, which is defined as the smell, taste and feel that the oil has when the median of the *bitter* and/or *pungent* attributes is two points higher than the median of its *fruitiness*;
c. the term "mild oil" may be used when the medians of the *bitter* and *pungent* attributes are 2 or less.

3.3 Method for organoleptic assessment of EVOO to assign designation of origin: Sensory profile and data processing

In 2005, the IOOC issued a document on methods to be used for the organoleptic assessment of EVOO for granting designation of origin (D.O.) status (IOOC/T.20/Doc. no 22). This document declared that the D.O. authority shall select the characteristic descriptors of the designation of origin (10 at the most) from those defined and reported in Table 1, and shall incorporate them into the profile sheet of the method.

Direct or retronasal aromatic olfactory sensations	
Almond	Olfactory sensation reminiscent of fresh almonds
Apple	Olfactory sensation reminiscent of the odour of fresh apples
Artichoke	Olfactory sensation of artichokes
Camomile	Olfactory sensation reminiscent of that of camomile flowers
Citrus fruit	Olfactory sensation reminiscent of that of citrus fruit (lemon,orange, bergamot, mandarin and grapefruit)
Eucalyptus	Olfactory sensation typical of Eucalyptus leaves
Exotic fruit	Olfactory sensation reminiscent of the characteristic odours of exotic fruit (pineapple, banana, passion fruit, mango,
Fig leaf	Olfactory sensation typical of fig leaves
Flowers	Complex olfactory sensation generally reminiscent of the odour of flours, also known as floral
Grass	Olfactory sensation typical of freshly mown grass
Green pepper	Olfactory sensation of green peppercorns
Green	Complex olfactory sensation reminiscent of the typical odour of fruit before it ripens
Greenly fruity	Olfactory sensation typical of oils obtained from olives that have been harvested before or during colour change
Herbs	Olfactory sensation reminiscent of that of herbs
Olive leaf	Olfactory sensation reminiscent of the odour of fresh olive leaves
Pear	Olfactory sensation typical of fresh pears
Pine kernel	Olfactory sensation reminiscent of the odour of fresh pine kernels
Ripely fruity	Olfactory sensation typical of oils obtained from olives that have been harvested when fully ripe
Soft fruit	Olfactory sensation typical of soft fruit: blackberries,raspberries, bilberries, blackcurrants and redcurrants
Sweet pepper	Olfactory sensation reminiscent of fresh sweet red or green peppers
Tomato	Olfactory sensation typical of tomato leaves
Vanilla	Olfactory sensation of natural dried vanilla powder or pods,different from the sensation of vanillin
Walnut	Olfactory sensation typical of shelled walnuts
Gustatory sensations	
Bitter	Characteristic taste of oil obtained from green olives or olives turning colour; it defines the primary taste associated with aqueous solutions of substances like quinine and caffeine
"Sweet"	Complex gustatory-kinaesthetic sensation characteristic of oil obtained from olives that have reached full maturity
Qualitative retronasal sensation	
Retronasal persistence	Length of time that retronasal sensations persist after the sip of olive oil is no longer in the mouth
Tactile or kinaesthetic sensations	
Fluidity	Kinaesthetic characteristics of the rheological properties of the oil, the set of which are capable of stimulating the mechanical receptors located in the mouth during the test
Pungent	Biting tactile sensation characteristic of oils produced at the start of the crop year, primarily from olives that are still unripe

Table 1. List of descriptors for D.O. of EVOO.

The characteristic descriptors are identified according to the round-table method: the panel supervisor leads a discussion based on a series of samples of known origin that display the most important specific characteristics of the VOO undergoing preparatory analysis. When the descriptor recognition stage is completed, the panel supervisor opens discussions with panel members to establish a list of all descriptors that are considered to be most important and characteristic of the designation that is undergoing preparatory analysis.

Validation should take into account the possible natural variations that may occur in the oil from one crop year to the next. When the profile sheet is completed, tasters shall assess the intensity of perception of the descriptors cited in the profile sheet on the 10-cm scale used for commercial grading of oils. The D.O. authority shall fix the maximum and minimum limits of the median for each descriptor included in the profile sheet and shall establish the limits for the robust coefficient of variation of each descriptor. It shall then enter these values in the *IOOC spreadsheet folder-profile* (software) accompanying this method to define the intervals of the characteristic sensory profile of the designation of origin.

Most of the specifications for the designation of origin of oils before 2005 or those that have not undergone revisions after this date, do not refer to the method IOOC just explained, but to the use of a previous procedure (EEC Reg. 2568/1991) for sensory evaluation of the oils. In Figure 1, the profile sheet according to the old regulation for the commercial grading is shown (EEC Reg. 2568/1991). This method provides a partial description of flavour: tasters are requested to define the fruity type, green or ripe, and recognize the presence of attributes such as grass, leaf, apple and other fruits. For each attribute, a discreet score from 0 to 5 is assigned (0: absence of perception; 1: intensity slightly perceptible; 2: intensity light; 3: average intensity; 4: great intensity; 5: extreme intensity), and there are many positive attributes to evaluate in addition to defects. Tasters rate the overall grading by using a 9-point scale: 9 for oils with exceptional sensory characteristics, and 1 for products with the worst qualities. The mean score identifies the category. An oil could be classified as EVOO if it obtains a final score (expressed as an average of the panel's judgement) of 6.5.

In the case of specifications for the designation of origin of some D.O oils, which have not yet been reviewed according to the new IOOC regulation (IOOC, 2005), it is firstly necessary to verify that the sample has the characteristics provided in the extra virgin category using current methods (EC Reg. 640/08), and to subsequently analyze it according to the old profile sheet (EEC Reg. 2568/1991) to verify the presence of characteristic descriptors. The final score for the D.O must be at least 7, but can be even higher.

4. Consumer acceptability of the sensory characteristics of VOO: An overview of literature data

As previously stated, a virgin oil that is not subjected to any subsequent tecnological refining has a sensory profile standardized by a rich/robust/harmonized regolatory environment (Conte & Koprivnjak, 1997) strongly linked to the quality of the starting olives. Any damage to drupes, which can lead to hydrolysis or fermentation, produces molecules that remain in the product and irreversibly affect its quality. There is no way of correcting

chemical and/or sensory defects in a virgin product. On the other hand, technological refining results in the loss of the superior quality of "extra virgin/virgin" oil, and the transition to a lower category with weaker sensory attributes. The difference in the overall quality between a virgin and a refined oil, the latter adjusted in both quality and the flavour, is not always correctly perceived by the consumer.

Generally, consumers appreciate what is familiar, what is strongly linked to the territory (tradition/origin) or to which they have a precise expectation (brand, other values) (Caporale et al., 2006, Costell et al., 2010). Furthermore, as demonstrated in a recent large study, people do not understand dietary fat, either the importance of the quality or the quantity needed for health and this generally results in consumers adhering to fat choices they are comfortable with (Diekman & Malcolm,2009). In the case of EVOO, for a correct perception of the overall quality the fruity (green or ripe) and bitter and pungent attributes should be perceived by consumers as "healthy" indicators of quality and genuine taste, linked to the raw oil and its richness in pungent and bitter minor components (phenols) (Carluccio et al, 2003). To achieve this purpose, consumers should be made capable, by research dissemination, to appreciate bitterness (primary taste of oil obtained from green olives or olives turning colour) and pungency (biting tactile sensations characteristic of oils produced at the start of the crop year, primarily from olives that are still unripe) (COI/T.20/Doc. no 22) as healthy substances related attributes.

By law, the virgin oil "ideal" sensory profile is quite simple and easy, the fruity attribute is universally recognized as the primary sensory characteristic, and the bitter and pungent aspects are reported as positive attributes (*CODEX STAN 33-1981*). However, due to the superficial knowledge in terms of fat quality, technology (virgin and refined) and sensory characteristics, consumers do not appear to practice an informed/univocal consumption of EVOO. In this regard, research on consumer behaviour has intensified in recent years, and some of the more salient findings are provided below.

A study in Turkey (Pehlivan & Yilmaz, 2010) comparing olive oils originating from different production systems (continuous, organic, stone pressed, refined) declared that, for a sample of 100 consumers, hedonic values of the refined samples were close to the values of the virgin samples. Similar findings were previously reported by Caporale et al (2006), by which consumers are able to differentiate EVOO on their characteristic sensory attributes, but buying intentions (blind test) of the refined samples were as high as the values for the virgin samples. Again, the sensory attributes of EVOO, even if perceived, did not seem to be drivers to purchase it.

In Italy, Caporale et al. (2006) demonstrated that information about origin creates a favourable hedonic expectation, with regards to specific sensory attributes, such as pungency and bitterness. This means that, if familiar with bitter/pungent oils, consumers can have high and positive expectations of bitter and pungency attributes as distinguishing characteristics of typical olive oils (i.e. *Coratina* cv.). To confirm this physiological opportunity to perceive pungent as a positive attribute can be cited an interesting paper on the unusual pungency of EVOO (Peyrot des Gachons et al., 2011), sensed almost exclusively in the throat, suggesting that it is, therefore, perhaps no coincidence if phenols with potent anti-inflammatory properties (oleocanthale, ibuprofen) also elicit such a localized/specific

pungency. In this paper the authors ask what is the functional significance of the pungency to the human upper airways; they suggest that the posterior oral location of toxin and irritant detectors can protect against their intake either by inhalation or ingestion. But if the role of these ion channels, in general, is to protect tissue from harmful compounds, then it is a mystery how one (TRPA1-channel), mediating throat irritation of extra-virgin olive oils, came to be valued as a positive sensory attribute by those who consume them. The authors hypothesize that this pungency, distinguisheing particularly good olive oils in the European Union standards, similarly to other common food irritants (e.g., capsaicin, menthol, and so forth), also important positive components in many cuisines, turns, from a usually negative taste-kinesthetic sensation into positive, because the molecules that elicit it have a body healthy action. This theory requires considerably more investigations to be demonstrated, but is true that many compounds eliciting pungency are also linked to decreased risks of cancer, degenerative and cardiovascular diseases (Boyd et al., 2006; Peng & Li, 2010).

In the case of EVOO, but this is a very general question, the authors suggest that people can transform an inherently unpleasant sensation into a positive one, commonly experienced around the world when consuming pungent EVOO, because it has beneficial health effects (Peyrot des Gachons et al., 2009). If this theory is correct, it means that this kind of pungency colud be easily taught as a positive sensation quality-related, to the unfamiliar consumers.

Infact, it has been reported (Delgado & Guinard, 2011) in the USA, an emergent market, that in a study on 22 samples evaluated in blocks of 5, for the majority of 100 consumers bitterness and pungency were negative drivers of liking.

Descriptive analysis (Delgado & Guinard, 2011) has been proposed as a more effective method to provide a more detailed classification of EVOO; the final method consisted of 22 sensory attributes, some of which were original but infrequent (butter/green tea). But, in the case of EVOO, the challenge for the future does not appear descriptive analysis, which has had the most interesting developments for the characterization/valorization of monovarietal, PDO and PGI (Inarejos-García et al., 2010; Cecchi et al. 2011) with many targeted/robust attributes. Rather it concerns the fact that consumers are actually able to appreciate/perceive its fundamentals of sensory profile (fruity, bitter, pungent) as related to its quality.

Finally, the worldwide problem of two different qualities of EVOO, a high one (expensive) and a "legal" one (less flavour/cheaper), was also highlighted in a means-end chain study (Santosa & Guinard, 2011), explaining that the attributes associated with EVOO generally have high (more flavour, more expensive, smaller size) or, unfortunately, low (cheaper/on sale, big quantity/bulk size, less flavour) levels of product involvement.

5. Conclusion

Sensory analysis of EVOO has been used for classification for more than 20 years. Since 1987, the "COI Panel test" has undergone many revisions, became law in 1991 in Europe and actualy COl/T.20/Doc. no. 15. is the method of analysis accepted by the Codex Alimentarius. Over the years, the profile sheet has undergone simplifications that have

restricted selected specific positive (fruity, bitter, pungent) attributes and defects (fusty/muddy sediment, winey-vinegary-acid-sour, metallic, rancid, others).

On the other hand, in 2005 the IOOC issued document COI/T.20/Doc. no 22 that provides specifics about the methods to be used for sensory assessment of EVOO when granting designation of origin (D.O.) status. The method contains a list of 23 direct or retronasal aromatic olfactory sensations, 2 (bitter, sweet) gustatory sensations, 2 tactile or kinesthetic sensations (fluidity/pungent) and a qualitative retronasal persistence. Even taking into account the recent development of sensory analysis, there is no other food that has such a rich/robust/harmonized regulatory environment regulated by the EU, International Olive Oil Council and, as any food, Codex Alimentarius (FAO-OMS).

At present, origin, tradition and habits, more than sensory profile, are purchase drivers for EVOO and the real challenge for the future is improving consumer education in appreciating the foundamental attributes: fruity, together with taste and tactile sensations of phenols, functional and healthy substances naturally present in EVOO, respectively, bitterness and pungency.

Therefore, nowadays, the key to provide the consumer a truly effective EVOO organoleptic knowledge is the worldwide dissemination of the three basic quality-related and "healthy" sensory attributes.

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2.8 Virgin olive oil in preventive medicine: from legend to epigenetics

This paragraph has been summarized from the publication:

Caramia, G., Gori, A., Valli, E. & Cerretani, L. (2012). Virgin olive oil in preventive medicine: from legend to epigenetics. European Journal of Lipid Science and Technology, 114, 375-388.

This review was realized under Prof. Giuseppe Caramia's supervision, who is an expert pediatrician, President of the International Society "Olive Oil and Health". My contribution focused especially on finding recent publications and translating some parts of the review.

Since the above cited scientific review is very long and complex, I've decided to report in this Ph.D. thesis only the summary, the practical applications and a crucial table (**Table 1 I**), in which benefic effects of a diet rich in EVOO on the human health are highlighted, adding the references that support each one of them.

Summary

Among vegetable oils, extra virgin olive oil (EVOO) has nutritional and sensory characteristics that make it unique and a basic component of the Mediterranean diet. EVOO has always been used over the centuries for its preventive and therapeutic properties, as well as precious and valuable dietary lipidic condiment. Benefic effects of a diet rich in EVOO on the human health, especially in prevention and/or reduction of hypercholesterolaemia, serum lipoprotein levels and atherosclerosis, hypertension, cardiovascular diseases and thrombotic risk, oxidation and oxidative stress, obesity and type 2 diabetes, inflammatory processes and cancer are discussed in these review. Recent studies suggest also its role in regulating the sense of satiety. The chemical compounds of EVOO that may contribute to its overall therapeutic characteristics, the epigenetic and physiological mechanisms involved are focused, taking into account the most important studies in the literature of the last years.

Practical Applications

After many studies on various aspects of nutrition, it is now clear that many human diseases are influenced by lifestyle, in which the diet has an important aspect. The use of

extra virgin olive oil is especially important from early childhood and throughout adult life to contribute to hinder the aging process. The importance of preventive and sometimes curative action, carried out by its various components in several pathological conditions has emerged from clinical, experimental and epidemiological studies which, in many cases, are accompanied by indisputable scientific evidences. Taking into account the most important studies in the literature of the last years, the chemical compounds of extra virgin olive oil and the physiological mechanisms involved behind their curative/health effects are focused.

Table 1 I. Benefic effects of a diet rich in EVOO on the human health.

Benefic effect	Component of EVOO involved	Ref.
Hypercholesterolaemia, serum lipoprotein levels and atherosclerosis		
Reduction of risk factors, such as hypercholesterolaemia, atherosclerosis and hypertension, and mortality for cardiovascular diseases		21-23
Reduction of mortality due to cardiovascular disease by 9%, cancer by 6%, total mortality by 9% and the incidence of Parkinson's and Alzheimer's disease by 13%		20
Reduction of LDL cholesterol		25, 26
Reduction of triglyceridemia and increase in HDL		25, 26
Reduction of LDL oxidation process	Phenolic compounds	61, 62
Arterial hypertension		
Decrease in diastolic and systolic pressure observed in both hypertensive subjects and normotensive subjects		29-33
Prevention of damages to vascular endothelium	Phenolic compounds, hydroxyl-oleic acid derived from oleic acid	37, 38
Thrombotic profile		
Inhibition in the formation of blood clots by decreasing monocyte adhesion and increasing fibrinolysis	Oleic acid	20
Inhibition of platelet aggregation and alteration of the platelet/vascular wall, reduction of fibrinogen, factor VII and the principal suppressant of hemostasis, thereby increasing fibrinolysis	Phenolic compounds	44-49
Reduction of TXB2 and LTB4 in both hyperlipaemic subjects and patients with type 2 diabetes		44, 50
Chemo-protective action and improvement of the endothelium function	Hydroxytyrosol and tyrosol	44, 50
Preventive action against thrombotic and microthrombotic events in patients with type 2 diabetes and hyperlipaemic subjects	Hydroxytyrosol	44, 50

continue…

Benefic effect	Component of EVOO involved	Ref.
Reduction of risks for patients with cardiac pathologies		25
Oxidation and oxidative stress		
Maintainance of cellular integrity and reduction of ageing	High level of oleic acid and lack of excess of linoleic acid	56
Antiinflammatory and vasodilatative action	α-linolenic acid (ALA)	60
Delay of atherosclerosis	Phenolic compounds	16, 44, 59, 60
Prevention of oxidation of cells-membrane lipids and plasma lipoproteins, reducing the risk of atherosclerosis	Tocopherols	43, 60
Reduced production of free radicals and prevention of damages to the cellular membrane, mitochondria, and DNA, with beneficial effects on aging and cancer risk		66, 67
Inflammation		
Antiinflammatory action by non-selectively inhibition of the COX-1 and COX-2 enzymes	Oleocanthal	38, 69
Protection against various pathological conditions (10 types of tumors including colon, stomach, breast, prostate, lung, and Alzheimer's disease)	Oleocanthal	72, 73, 78
Obesity and diabetes		
Reduction of risk by inhibition of the activation of NF-kB at the cellular level	Phenols, carotenoids, and tocopherols	60, 92
Protective action on mitochondria, reduced production of free radicals and protection against DNA oxidation		60, 92
Reduction of insulin requirements, with an improvement of both the lipid profile and the glycemic index	Oleic acid	60, 92
Improvement of sensitivity to insulin, without increasing its secretion		25
Benefits for obese individuals by inhibition of the inflammatory response	ALA	25

continue…

Benefic effect	Component of EVOO involved	Ref.
Neoplasm		
Reduction of the incidence of cancer		16, 93
Antineoplastic activity	oleuropein	96
Inhibition of the oncogenic HER2 gene in the presence of high levels of the enzyme FASN (fatty acid sithase)	oleic acid	98-100
Increase the inhibitory effect of herceptin on breast cancer cells	oleic acid	98-100
Other benefic effect		
Increase in the sense of satiety	OEA	104-113

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3. EXPERIMENTAL SECTION

Chapter 0. Samples and analytical plan

3.0.1 Samples

Different sets of samples of oils obtained by olives were collected within the experimental work carried out in this Ph.D. thesis, in particular:

set 1) 28 samples of EVOOs (F1-F28), all directly collected from Italian mills in 2010;

set 2) 34 samples of EVOOs (C1-C34), all sold at a medium-low price (2-5 €/kg) in the large scale retail trade (supermarkets and discounts) in 2010;

set 3) 6 lampante and "repaso" olive oils (LR1-LR6), obtained by a further extraction of the oils from the paste of olives (usually carried out by adding warm water), and 6 refined olive oils (R1-R6) in 2010;

set 4) 35 samples of EVOOs (S1-S35) with different prices, all from the retail trade, of which: 12 certified from organic farming, 15 with a labeled declaration of Italian origin of the olives ("100% italiano"), 5 Protected Denomination of Origin (PDO) and 1 Protected Geographical Indication (PGI) EVOOs (see *chapter 2,* paper "Sensory and chemical quality…", see Table 1; in this paper, the code of each sample is followed by the class of price, as reported in the caption of Table 1: as example, sample S1 corresponds to sample 1L) in 2011;

set 5) 140 samples of "premium quality" EVOOs coming from 5 countries and 4 different harvest years (2007-2010), all participating at the I.O.O.A. (International Olive Oil Award, Zurich) (see *chapter 4*).

3.0.2 Brief summary of the analytical plan

3.0.2.1 Chemical and sensorial analyses

For the **sets of samples 1-4**, the determination of FAAEs was performed, using analytical procedures that followed the historical evolution of the method, first reported in the literature (Pérez-Camino et al., 2008; Bendini et al., 2009a; Bendini et al., 2009b; Cerretani et al., 2011) and then adopted by the E.U. (EU Reg. 61/2011) (see *paragraph 3.1.13* and *chapter 2,* see paper "Detection of low-quality…").

Some samples of the **sets 1, 2 and 4** were submitted to a more complete plan of analysis, involving different basic quality indexes (free acidity (FA), peroxide value (PV),

spectrophotometric indexes K_{232} and K_{270}, sensory analysis carried out with Panel test method) and other specific analytical determinations (diacylglycerols (DAG), sterols and methylsterols, fatty acids, phenolic and volatile compounds, bitterness index K_{225}, acquisition of the FT-IR spectra). Full and detailed descriptions of the procedures related to all the above mentioned analytical methods and the respective main results are described in *chapter 1* (see "Materials and methods" & "Results and discussion" for each constituent) and *chapter 2* (see paper "Sensory and chemical quality…").

Moreover, the samples included in the **set 5** were sensorially evaluated both by the Swiss Olive Oil Panel and in different consumer test sessions during an important annual food exhibition in Zurich, in order to evaluate the consumers perception and preferences (*chapter 4*).

3.0.2.2 Statistical analysis

A statistical elaboration of all the analytical and sensorial results (ANOVA, PCA, Preference mapping) was performed, by using the software XLSTAT 7.5.2 (Addinsoft, USA).

Chapter 1. Olive oil composition

The composition of olive oil is primarily triacylglicerols and secondarily free fatty acids and some 0.5-1.5% nonglyceridic constituents (Boskou, 2007). The methods for detecting all the most important compounds and the limits related to their presence are fully provided by the International Olive Council (IOC/T.15/NC No. 3/Rev. 6, 2011), the Commission of European Community (EEC Reg. 2568/1991 and successive amendments) and the Codex Alimentarius (CAC/RS 33-1970). Moreover, many reviews have been already written on this topic from the past, implementing the new developments up to nowadays (Gracian Tous, 1968; Fedeli, 1977; Boskou, 1996; Angerosa et al., 2006; Boskou, 2007; Frankel, 2010). For this reason, the aim of this chapter is not to discuss the olive oil chemistry or the different analytical approach theirselves, but to highlight and to summarize some cutting-edge aspects that make this product unique and different from the other vegetable oils.

3.1.1 Fatty acids

The fatty acid composition is peculiar of each botanical species and for olive oils it depends on different parameters, such as the zone of production, the variety of the olives, the latitude, the climatic conditions and the maturity of the fruits. As example, North African products are characterized by lower percentage of oleic acid and higher percentages of linoleic and palmitic acids than oils from the Mediterranean basin (Angerosa et al., 2006). Limits for the typical olive oils are fixed by E.U. Regulation for the main fatty acids (EU Reg 61/2011), and also by the I.O.C. (IOC/T.15/NC No. 3/Rev. 6, 2011) and by the Codex Alimentarius (CAC/RS 33-1970) for the main commercial classes of olive oils. About the composition in fatty acids, the EU Reg 61/2011 reports some limits that are valid for all the edible categories of oils obtained by olives: palmitic acid (C16:0) between 7.5% and 20%, palmitoleic (C16:1) between 0.3% and 3.5%, heptadecanoic (C17:0) ≤ 0.3%, stearic (C18:0) between 0.5% and 5%, oleic (C18:1) between 55% and 83%, linoleic (C18:2) between 3.5% and 21%, linolenic (C18:3) ≤ 1%, arachidic (C20:0) ≤ 0.6%, eicosenoic (C20:1) ≤ 0,4%, behenic (C22:0) and lignoceric (C24:0) ≤ 0.2%. The content in fatty acid have been evaluated for samples of both the set 1) 28 samples of EVOOs (F1-F28), all directly collected from Italian mills and of the set 4), collected at the supermarket (S1-S35). The results are discussed here below and were also reported in Valli et al., 2012.

3.1.1.1 Materials and methods

3.1.1.1.1 Fatty acid composition by gas chromatographic determination

The fatty acid composition of the samples was determined as the corresponding methyl esters (FAMEs) by gas chromatographic (GC) (Clarus 500 GC Perkin Elmer Inc., Shelton, CT, USA) analysis. The FAMEs were prepared by alkaline treatment carried out by mixing 0.05 g of oil dissolved in 2 mL of *n*-hexane with 1 mL of 2 N potassium hydroxide in methanol, according to Christie, 1998. One microliter of the 1:10 (v/v) *n*-hexane diluted upper phase was injected into a split 1:10 GC port set at 250 °C. A fused silica RTX-2330 capillary column (30 m length, 0.25 mm i.d., 0.25 µm film thickness), purchased from Restek (Bellefonte, PA, USA) was utilized. A flow rate of 0.8 mL/min of helium as carrier gas was used. The FID was at 250 °C. The initial oven temperature was kept at 120 °C for 1 min and raised to 240 °C at a rate of 2.5 °C/min and maintained for 4 min. Three replicates were carried out for each sample. Results were expressed as % of each FAME on the total amount of FAMEs.

3.1.1.1.2 Statistical analysis

The software XLSTAT 7.5.2 version (Addinsoft, USA) was used to elaborate data by Analysis of Variance (ANOVA, Fisher LSD, $p < 0.05$).

3.1.1.2 Results and discussion

All the examined oils (F1-F28 and S1-S35, see *paragraph 3.0.1)* showed a typical composition for edible olive oils and were within the legal limits established for them by E.U. (EU Reg 61/2011), with a few exceptions. Actually, some fatty acids were present in percentages a bit higher than the legal limits. In particular, some samples exceeded the legal limits for arachidic acid (C20:0) and for the eicosenoic one (C20:1). Nine samples out of 63 (see **Tables 2 E** and **3 E**: F12, F19, F20, F21, F22; S15, S16, S34, S35) had a value of arachidic acid (C20:0) higher than the limit of 0.6%, while four samples (see **Table 2 E**: F17, F20, F21, F22) were above the limit (0.4%) for the eicosenoic acid (C20:1): only three samples (F20, F21, F22) showed both the values outside the limits. The six samples (F12, F17, F19, F20, F21, F22) of the set collected directly at Italian mills that showed values (of either arachidic or eicosenoic acids) higher than the legal limits came all from Foggia and Barletta (Apulia, South of Italy). The four samples of the set S1-S35 (S15, S16, S34, S35)

with arachidic acid higher than the limit were all Italian EVOOs sold at medium price (*chapter 2*, see paper "Sensory and chemical quality…", Table 1): two were produced with organic farming system and two with the conventional one. It is also interesting to observe that for 54 out of 63 sample, the oleic acid was higher than 70%, while for all the other EVOOs the linoleic acid was lower than 10% (except for samples S15 and S26). This is an interesting aspect, since a ratio between oleic and linoleic acid more or equal than 7 was suggested to assume a good oxidative stability.

3.1.2 Triacylglicerols

Triacylglicerols profile of olive oil strictly depends on the botanical origin of the vegetable oil, so the detection of these compounds is useful to check authenticity of olive oils, in terms of mixture with other oils or re-esterified oils (Casadei, 1987). Their evaluation with a chemometric approach can be also a reliable tool to reveal frauds (Tsimidou et al., 1987). In olive oils the most found triacylglicerols are OOO, POO, OOL, POL and SOO, but smaller amount of OLO, POP, PLO, POS, OLnL, LOL, OLn=, PLL, PLnO and LLL are also detectable (O = oleic acid; P = palmitic acid; S = stearic acid; L = linoleic acid; Ln = linolenic acid) (Boskou, 2007).

Table 2 E. Fatty acids composition of the EVOO samples, directly collected at Italian mills (F1-F28). Data are expressed as percentage on the total fatty acid content. Different letters in the same column indicate significant differences (Fisher LSD, $p < 0.05$). Values in bold are out of the legal limit established by EU Reg. 61/2011.

	C16:0	C16:1	C17:0	C18:0	C18:1	C18:2	C18:3	C20:0	C20:1	C22:0	C24:0
F1	13.2 d,e	1.12 f	0.04 b-g	1.72 l	73.7 k,l	5.78 f,g	0.65 h,i	0.31 m-o	0.30 g	0.09 h-l	0.05 a-c
F2	12.6 g	0.97 j,k	0.05 b-d	1.78 j,k	74.5 h,i	5.70 g	0.65 i	0.32 i-l	0.31 f,g	0.10 g-j	0.02 f
F3	12.6 g	0.98 i,j	0.06 b,c	1.73 l	73.9 j,k	6.18 f,g	0.68 f,g	0.32 j-m	0.32 f	0.10 d-f	0.05 a,b
F4	12.2 h,i	0.87 n	0.03 e-h	1.48 m	74.2 i,j	6.52 e-g	0.80 a	0.32 j-m	0.40 c	0.11 d,e	0.06 a,b
F5	12.2 h	0.88 n	0.03 e-h	1.94 g	75.0 e,f	5.81 f-g	0.62 k	0.33 h,i	0.29 h	0.10 e-g	0.01 f
F6	11.8 j,k	0.97 j,k	0.05 b-e	1.87 h	75.3 e	6.04 f-g	0.57 l	0.29 o-q	0.27 l-o	0.08 m-p	0.03 e-f
F7	12.2 h	1.09 g	0.04 c-g	1.88 h	74.3 h,i	6.23 f-g	0.65 i	0.30 k-m	0.26 o-q	0.08 n-p	0.03 d-f
F8	12.8 f,g	1.06 h	0.04 c-g	1.89 h	73.4 l,m	6.50 e-g	0.71 d	0.31 n-p	0.27 k-n	0.09 k-o	0.05 b-d
F9	11.7 k	1.06 g,h	0.04 c-g	1.83 i	74.6 g,h	6.47 e-g	0.75 b	0.30 n-p	0.27 n-p	0.08 o,p	0.04 b-e
F10	13.0 e,f	0.96 k	0.05 b-e	2.06 d,e	72.6 n	7.24 b-f	0.70 d,e	0.33 h	0.27 n-p	0.09 i-l	0.05 a,b
F11	13.5 b,c	1.22 c	0.04 d-g	1.85 h,i	70.8 p	7.96 a-e	0.73 c	0.33 h-j	0.28 k-n	0.09 j-m	0.05 a,b
F12	13.7 b	1.00 i	0.04 b-g	2.00 f	72.0 o	7.05 c-g	0.34 o	***0.74*** b	0.29 h-j	0.09 h-k	0.05 a,b
F13	12.2 h	0.89 m,n	0.03 f-h	1.86 h,i	74.9 f,g	5.96 f,g	0.70 e	0.31 l-n	0.28 k,l	0.09 k-n	0.07 a
F14	12.1 h-j	0.91 l,m	0.05 b-e	1.75 k,l	74.9 f,g	6.18 f,g	0.69 f	0.31 m-o	0.28 i-k	0.09 k-o	0.05 a,b
F15	13.1 d-f	1.05 h	0.05 b-e	1.88 h	72.5 n	7.00 d-g	0.75 b	0.32 h-k	0.28 k-l	0.09 j-m	0.06 a,b
F16	12.1 h,i	1.19 d	0.01 h	1.85 h,i	73.3 m	7.19 c-g	0.68 g	0.29 p-q	0.26 q	0.08 l-p	0.03 c-f
F17	10.7 m	0.37 q	0.04 b-g	2.07 d	77.1 b	6.42 f,g	0.70 d,e	0.40 f	***0.42*** b	0.12 a,b	0.05 a,b
F18	10.1 n	0.40 p	0.04 b-g	2.18 c	77.5 a	6.65 e-g	0.66 h	0.39 f	0.37 d	0.11 c,d	0.05 a,b
F19	13.8 b	1.25 b	0.03 g,h	2.05 d-f	69.0 r	5.97 f,g	0.34 o	***0.63*** d	0.29 h-i	0.10 e-g	0.05 a,b
F20	10.6 m	0.35 q,r	0.04 b-g	1.96 g	76.6 c	6.59 e-g	0.38 n	***0.70*** c	***0.42*** b	0.11 c,d	0.05 a,b
F21	10.3 n	0.33 r	0.05 b-e	2.01 f	77.1 b	6.55 e-g	0.40 m	***0.76*** a	***0.46*** a	0.12 a	0.06 a,b
F22	10.8 m	0.37 q	0.04 b-f	2.02 e,f	75.8 d	7.13 c-g	0.39 n	***0.74*** b	***0.41*** b	0.12 b,c	0.06 a,b
F23	11.2 l	0.62 o	0.12 a	3.13 a	72.7 n	8.52 a-c	0.69 f,g	0.46 e	0.34 e	0.12 a,b	0.06 a,b
F24	13.3 c,d	1.16 e	0.06 b	2.31 b	69.9 q	8.80 a	0.63 j	0.35 g	0.28 j,k	0.10 f-h	0.05 a,b
F25	13.6 b	1.26 b	0.04 c-g	1.96 g	69.8 q	8.70 a,b	0.62 j,k	0.32 i-l	0.29 h	0.10 f-i	0.05 a-c
F26	14.7 a	1.40 a	0.04 d-g	1.95 g	70.1 q	7.03 c-g	0.63 j	0.32 i-l	0.26 p,q	0.09 h-l	0.04 b-e
F27	13.7 b	1.24 b,c	0.04 c-g	1.85 h,i	70.1 q	8.25 a-d	0.75 b	0.36 g	0.30 g	0.12 a,b	0.06 a
F28	11.9 i-k	0.93 l	0.04 b-g	1.82 i,j	75.3 e	5.95 f,g	0.68 f-g	0.29 p-q	0.27 m-p	0.08 p	0.05 b-d

Table 3 E. Fatty acids composition of the EVOO samples, collected at the supermarket (S1-S35). The description of these oils are reported in *chapter 2*, see paper "Sensory and chemical quality…", Table 1. Data are expressed as percentage on the total fatty acid content and as mean of three replications. Different letters in the same column indicate significant differences (Fisher LSD, $p < 0.05$). Values in bold are out of the legal limit established by EU Reg. 61/2011.

	C16:0	C16:1	C17:0	C18:0	C18:1	C18:2	C18:3	C20:0	C20:1	C22:0	C24:0
S1	10.6 j-n	0.51 q-r	0.05 i-l	2.44 f-i	76.0 b-d	6.50 k,l	0.64 h-m	0.44 d-i	0.39 a	0.15 a	0.04 b-d
S2	10.5 k-n	0.64 m-o	0.06 i-k	2.82 b,c	74.4 e-i	7.21 h-j	0.63 j-m	0.45 d-h	0.33 b-h	0.14 a-c	0.05 b-d
S3	10.9 h-m	0.61 n-p	0.05 i-l	2.29 h-l	75.0 c-f	7.51 f-i	0.63 i-m	0.40 j-m	0.36 a-d	0.12 c-f	< 0.01 d
S4	11.9 e-h	0.78 h-l	0.10 d,e	2.75 c,d	71.7 m-p	8.80 e	0.65 f-l	0.43 e-k	0.32 b-j	0.12 b-e	0.04 b-d
S5	11.2 f-l	0.62 m-o	0.06 h-j	2.46 f-h	74.7 e-g	7.24 g-i	0.67 d-i	0.42 f-l	0.36 a-e	0.11 c-f	0.04 b-d
S6	10.1 m,n	0.44 r	0.05 i-k	2.79 b,c	74.5 e-h	8.83 d,e	0.62 k-m	0.44 d-j	0.37 a-c	0.10 d-g	< 0.01 d
S7	13.2 b,c	1.32 b,c	0.14 a	2.75 c,d	69.0 r	9.03 c-e	0.57 o	0.42 f-l	0.28 g-l	0.13 a-d	< 0.01 d
S8	13.9 b	1.45 a	0.12 b,c	2.59 d-f	67.4 s	9.13 c-e	0.65 g-m	0.45 d-g	0.30 d-k	0.14 a-c	0.08 b
S9	13.3 b,c	1.03 d	0.04 k-l	2.32 h-k	72.3 l-n	6.61 k-l	0.74 b	0.43 f-l	0.31 c-k	0.13 a-d	0.06 b,c
S10	12.5 c-e	0.86 e-h	0.05 j-l	2.12 l-o	71.7 m-p	8.78 e	0.65 f-m	0.36 m,n	0.33 b-i	0.10 e-g	< 0.01 d
S11	11.7 e-i	0.69 l-n	0.04 k-l	2.30 h-l	73.8 g-j	7.59 f-i	0.69 c-h	0.38 l-m	0.34 a-f	0.10 d-g	< 0.01 d
S12	11.3 f-l	0.88 e-g	0.10 c,d	2.91 b,c	74.8 d-g	5.95 m,n	0.64 i-m	0.40 j-m	0.27 i-l	0.12 b-e	< 0.01 d
S13	13.6 b	1.35 a-c	0.14 a,b	3.23 a	67.4 s	9.36 c,d	0.67 d-j	0.47 d	0.27 h-l	0.15 a,b	< 0.01 d
S14	13.0 b,d	1.29 c	0.16 a	2.89 b,c	69.7 q-r	7.87 f	0.57 n-o	0.47 d,e	0.27 i-l	0.14 a-c	0.03 b-d
S15	15.5 a	1.41 a,b	0.14 a	2.22 j-n	62.5 u	12.5 a	0.42 p-r	***0.84*** a	0.31 d-k	0.11 c-f	< 0.01 d
S16	11.5 e-k	0.69 l-n	0.04 k-l	2.00 o	74.2 f-j	7.76 f-h	0.38 s	***0.69*** b	0.37 a-c	0.11 c-f	< 0.01 d
S17	11.2 f-l	0.90 e,f	0.08 e-h	2.96 b	72.2 l-o	8.97 c-e	0.66 d-k	0.43 e-k	0.27 i-l	0.12 c-f	< 0.01 d
S18	11.0 g-m	0.88 e-g	0.09 d-f	2.97 b	72.2 l-o	8.97 c-e	0.66 e-k	0.46 d-f	0.35 a-e	0.11 c-f	0.18 a
S19	10.3 l-n	0.46 r	0.04 k-l	2.18 k-o	77.1 a,b	6.67 j-l	0.70 b-d	0.38 k-m	0.23 l	0.12 c-f	0.01 c,d
S20	9.76 n	0.31 s	0.05 j-l	2.21 j-n	77.8 a	6.60 k,l	0.70 c-e	0.41 g-l	0.40 a	0.07 h	< 0.01 d
S21	11.6 e-j	0.86 e-h	0.08 e-g	2.82 b,c	73.2 j-l	7.51 f-i	0.69 c-g	0.44 d-i	0.30 d-k	0.12 b-e	< 0.01 d
S22	11.8 e-h	0.87 e-h	0.05 i-k	2.73 c-e	74.6 e-h	5.94 m,n	0.69 c-f	0.41 g-l	0.35 a-e	0.12 c-f	< 0.01 d
S23	12.0 d-f	0.73 j-l	0.05 i-l	2.04 n-o	74.8 d-g	6.42 l,m	0.66 e-k	0.40 j-m	0.33 b-i	0.10 e-g	0.03 b-d
S24	10.5 k-n	0.74 i-l	0.06 g-j	3.24 a	76.1 b,c	5.73 n	0.62 k-m	0.40 j-m	0.25 k-l	0.09 e-g	0.05 b-d
S25	12.0 d-f	0.91 e,f	0.07 g-i	2.54 e-g	71.1 o-p	9.47 c	0.66 e-k	0.43 e-j	0.31 d-k	0.11 c-f	< 0.01 d
S26	14.9 a	1.28 c	0.10 d,e	2.26 i-m	65.8 t	10.6 b	0.82 a	0.41 g-l	0.30 d-k	0.09 e-g	< 0.01 d
S27	11.8 e-h	0.80 g-k	0.09 d-f	2.80 b,c	71.3 n-p	8.72 e	0.43 p,q	0.59 c	0.30 d-k	0.13 a-d	< 0.01 d
S28	11.9 e-h	0.82 f-j	0.05 i-k	2.36 g-k	73.2 i-l	7.64 f-h	0.62 l-n	0.40 i-m	0.31 d-k	0.09 f-h	< 0.01 d
S29	13.8 b	0.71 k-m	0.05 i-l	2.08 m-o	72.6 k-m	6.19 l-n	0.39 r,s	0.57 c	0.30 e-k	0.12 c-f	< 0.01 d
S30	12.1 d-f	0.94 d,e	0.08 f-h	2.53 f,g	71.7 m-p	8.69 e	0.61 m,n	0.40 h-l	0.29 f-k	0.10 e-g	< 0.01 d
S31	12.0 d-g	0.83 f-i	0.03 l	2.23 j-n	74.2 f-j	7.05 i-k	0.57 n,o	0.33 n	0.26 k-l	0.08 g,h	< 0.01 d
S32	12.4 c-e	0.95 d,e	0.06 h-j	2.97 b	70.9 p-q	8.67 e	0.73 b,c	0.44 d-j	0.26 j-l	0.11 c-f	0.03 b-d
S33	12.0 d-g	0.52 p-r	0.05 j-l	2.22 j-n	73.5 h-k	7.77 f,g	0.47 p	0.58 c	0.37 a,b	0.13 a-d	< 0.01 d
S34	10.1 m,n	0.51 q-r	0.06 h-j	2.38 g-j	76.7 a,b	6.44 l,m	0.41 q-s	***0.65*** b	0.34 a-g	0.11 c-f	< 0.01 d
S35	10.8 i-m	0.56 o-q	0.05 i-k	2.26 i-m	75.4 c-e	7.33 f-i	0.38 r,s	***0.68*** b	0.37 a,b	0.08 g,h	< 0.01 d

3.1.3 Partial glycerides

The presence in small amount (from 1 to 2.8%) of monoacylglycerols (MAG) and diacylglycerols (DAG) in virgin olive oils is due either to hydrolysis and incomplete biosynthesis of triacylglycerols. In order to evaluate the degree of freshness of olive oils, it is important to consider the ratio between 1,2-DAG and 1,3-DAG (Frega et al., 1993a), even if it is not an official method (*paragraph 3.2.2*). In general, the 1,3-DAG are formed only as a consequence of lipolytic process and they increase during the storage of the oils, mainly after an isomerization reaction that involves 1,2-DAG (Serani et al., 2001). Differently from the free acidity content, that can be decreased with fraudulent practices, the presence of DAG cannot be illegally modified. In particular, Pérez-Camino et al. (2001) reported that the ratio between 1,2-DAG and 1,3-DAG is a useful marker for assessing the genuineness of EVOOs characterized by low acidities during the early stages of storage.

Monoacylglycerols are present in much smaller quantities than DAG (less than 0.25%) (Boskou, 2007). 1- monoglycerides are considerably higher than the respective 2-isomers and their ratio depends on oil acidity (Paganuzzi, 1999). The content in DAG have been evaluated for samples of both the set 1) 28 samples of EVOOs (F1-F28), all directly collected from Italian mills and the samples of the set 4), all collected at the supermarket (S1-S35). The results are discussed here below and were also reported in Valli et al., 2012.

3.1.3.1 Materials and methods

3.1.3.1.1 Gas chromatographic (GC) determination of total amount of DAG *and major 1,2-DAG and 1,3-DAG.*

DAG were determined according to a modified version of the method suggested by Serani et al. (2001). 500 µL of a solution of dilaurin, used as internal standard (20 mg of dilaurin in 10 mL of chloroform), was added to 100 mg of oil. The mixture was vortexed for about 30 s and taken to dryness under a nitrogen stream. The residue was silylated according to Sweeley et al. (1963) with 0.2 mL of silylation reactive, obtained from pyridine, hexamethyldisilazane and chlorotrimethylsilane (3:1:9, v/v) and kept for 5 min at room temperature. Then, this mixture was taken to dryness under a nitrogen stream and the residue was dissolved in 200 µL of n-hexane centrifuged at 2000 rpm for 1 min. A volume (1 µL) of the solution was injected into a GC equipped with a flame ionization detector (FID). The GC was a Carlo Erba MFC500 coupled with a Rtx-65TG (Rested, Bellefonte, PA)

fused silica capillary column (30 m length x 0.25 mm i.d. x 0.10 µm film thickness) coated with 35% dimethyl-65% diphenyl polysiloxane. Oven temperature was programmed from 250 to 320 °C at a rate of 2 °C min^{-1} then raised to 365 °C at a rate of 5 °C min^{-1}. The final temperature was kept for 21 min. The injector and detector temperatures were both set at 360 °C. Helium was used as carrier gas at a pressure of 130 kPa. The split ratio was 1:70. Identification of DAG was carried out by comparing the peak retention times and the GC traces with those of the DAG standards and chromatograms reported in literature. Three replicates were carried out for each sample. Results were quantified respect dilaurin as internal standard and expressed as g of dilaurin in 100 g of oil.

3.1.3.1.2 Statistical analysis

The software XLSTAT 7.5.2 version (Addinsoft, USA) was used to elaborate data by Analysis of Variance (ANOVA, Fisher LSD, $p < 0.05$)

3.1.3.2 Results and discussion

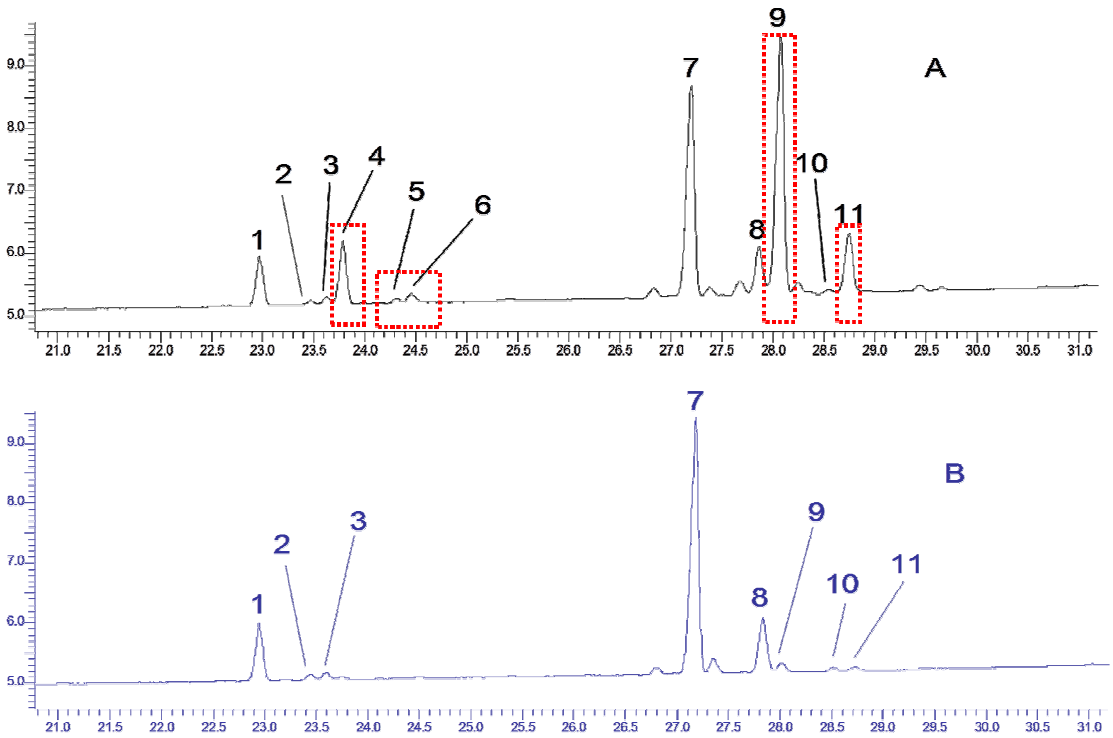

Figure 2 E. Overlay of two gaschromatographic traces related to the fraction of DAG of an EVOO that was stored for one year before the analysis (A) and an EVOO just obtained before the analysis (B). **1**, 1,2-PO; **2**, 1,2-PoO; **3**, 1,2-PL; **4**, 1,3-PO; **5**, 1,3-PoO; **6**, 1,3-PL; **7**, 1,2-OO; **8**, 1,2-OL; **9**, 1,3-OO; **10**, 1,2-LL; **11**, 1,3-OL + 1,3-LL. P = palmitic acido; Po = palmitoleic acid; O = oleic acido; L = linoleic acid.

The **Figure 2 E** depicts an overlay of two chromatograms related to the fraction of DAG respectively of an EVOO that was stored for one year before carryong out the analysis (A) and an EVOO just obtained before the analysis (B). It was possible to identify the main compounds (11 in total, of which some 1,2-isomers and some 1,3-isomers), that were quantified in samples F1-F28 and S1-S35 (see **Table 4 E**).

Table 4 E. Total amount of DAG (DAG TOT), expressed as g of dilaurin per 100 g of oil and ratio between 1,2- and 1,3-DAG (1,2-/1,3-DAG), adimensional, for the EVOOs collected at the supermarkets (S1-S35, see *paragraph 3.0.1*) and directly collected at Italian mills (F1-F28, see *paragraph 3.0.1*). Data are shown as mean of three replications. Different letters in the same column indicate significant differences (Fisher LSD, $p < 0.05$).

	DAG TOT (%)	1,2-/1,3-DAG		DAG TOT (%)	1,2-/1,3- DAG
S1	1.59 g-k	1.09 j-l	**F1**	1.28 g-i	5.18 j
S2	1.69 e-j	1.02 j-n	**F2**	1.39 f-h	5.18 j
S3	1.66 f-j	1.23 i,j	**F3**	1.64 b-d	3.84 k
S4	1.92 c-e	0.70 o-q	**F4**	1.09 k-m	13.55 b
S5	1.53 h-l	2.04 g	**F5**	1.26 h-j	12.90 b
S6	1.60 g-k	11.23 i,j	**F6**	1.12 j-m	13.47 b
S7	1.44 j-m	0.72 o-q	**F7**	1.31 g-i	4.90 j
S8	1.40 k-m	1.22 i,j	**F8**	1.38 f-h	9.71 c
S9	0.96 n	2.57 f	**F9**	1.56 c-e	9.25 c-e
S10	1.40 k-m	1.74 h	**F10**	1.19 i-m	9.22 c-e
S11	1.34 l,m	0.88 m-o	**F11**	1.49 d-f	6.28 i
S12	1.87 d-f	0.56 q,r	**F12**	1.39 f-h	5.13 j
S13	1.69 e-i	0.57 p-r	**F13**	1.32 g-i	9.15 c-f
S14	1.83 d-g	0.50 r	**F14**	1.38 f-h	9.33 c,d
S15	1.95 c,d	0.49 r	**F15**	1.55 c-e	7.72 g,h
S16	2.21 a,b	0.59 p-r	**F16**	1.92 a	7.76 g,h
S17	1.79 d-g	0.73 o-q	**F17**	1.19 i-l	8.21 f-h
S18	1.81 d-g	0.73 o-q	**F18**	1.20 i-l	9.84 c
S19	1.64 f-k	0.75 o,p	**F19**	1.67 b,c	2.41 l
S20	1.51 i-m	0.94 l-o	**F20**	1.08 l,m	9.40 c,d
S21	1.80 d-g	0.63 p-r	**F21**	1.24 h-k	10.10 c
S22	1.83 d-g	1.15 j,k	**F22**	0.90 n	8.60 d-g
S23	1.71 d-i	3.46 e	**F23**	1.04 m,n	1.10 m
S24	1.77 d-h	0.88 n,o	**F24**	1.42 e-g	3.34 k,l
S25	2.42 a	1.39 i	**F25**	1.78 a	6.53 i
S26	2.15 b,c	2.50 f	**F26**	1.28 g-i	7.54 h
S27	1.70 d-i	0.64 p-r	**F27**	1.20 i-l	8.36 e-h
S28	1.61 g-k	2.10 g	**F28**	1.08 l,m	17.04 a
S29	1.40 k-m	5.63 b			
S30	1.28 l,m	5.34 c			
S31	1.51 i-l	1.07 j-m			
S32	1.66 f-j	2.15 g			
S33	1.21 m,n	6.56 a			
S34	1.66 f-j	4.57 d			
S35	1.73 d-i	2.16 g			

The ratios between 1,2-DAG and 1,3-DAG were also considered, as marker of quality and freshness of the samples (Serani et al., 2001; Frega et al., 1993a). For all the oils sampled from Italian mills (F1-F28), except for one sample, this ratio was always more than one, suggesting a good quality and freshness of the samples (**Table 4 E**). For 15 out of 35 samples collected at the supermarket (S1-S35), the ratio was less than 1, indicating both an advanced degree of preservation and a low freshness of such products (**Table 4 E** and see *chapter 2*, paper "Sensory and chemical quality…").

3.1.4 Free fatty acids

The limits for the free fatty acid content, espressed as % of oleic acid, are different for each category of oils obtained by olives, according to the European Community (EEC Reg. 2568/1991 and successive amendments), and also considering the I.O.C. (IOC/T.15/NC No. 3/Rev.6, 2011) and the Codex Alimentarius (CAC/RS 33-1970) standards. The amount of free fatty acids strictly depends on the quality of the raw material (olives) in terms of their freshness and healthy state, and another relevant factor is the storage time before processing (Angerosa et al., 2006). The attack of olives by molds, by bacteria and by a damaging fly (*Bactrocera oleae*), a prolonged preservation of the fruits before processing them or the adoption of not good manufacturing practices during the production of the oil can cause notable increase in free acidity (Angerosa et al., 2006). Free acidity values, evaluated for the set of samples collected at supermarket (S1-S35), are discussed here below and were also reported in *chapter 2* (see paper "Sensory and chemical quality…").

3.1.4.1 Materials and methods

3.1.4.1.1 Titrimetric determination of free acidity

Free acidity was measured according to the official titrimetric method described in EEC Reg. 2568/91. Three replicates were carried out for each sample.

3.1.4.1.2 Statistical analysis

The software XLSTAT 7.5.2 version (Addinsoft, USA) was used to elaborate data by Analysis of Variance (ANOVA, Fisher LSD, $p < 0.05$).

3.1.4.2 Results and discussion

All the examined EVOOs were below the legal limit (0.8 g oleic acid/ 100 g oil) established for EVOOs by the European Community (EEC Reg. 2568/1991 and successive amendments).

3.1.5 Minor constituents

It is possible to split the so-called "minor constituents" into two classes, according to their derivation or not from fatty acids (Boskou, 2007).

Phospholypids, waxes, fatty acid alkyl esters and steryl esters are fatty acid derivatives, while hydrocarbons, free sterols, tocopherols, pigments and polar phenols are not (Boskou, 2000).

3.1.6 Phospholipids

The main phospholipids identified in olive oils are phosphatidylcoline, phosphatidylethanolamine, phosphatitylinositon and phossphatidylserine (Alter & Gutfinger, 1982). They have a detectable antioxidant activity (Pokorny et al., 2001) and they are naturally present at high level in cloudy olive oils, while they are generally at lower concentrations in filtered oils and refined ones (Koidis & Boskou, 2006).

3.1.7 Waxes, fatty alcohols and diterpene alcohols

The most important alcohols present in olive oils are fatty and diterpene alcohols.

Fatty alcohols are mainly linear saturated alcohols with more than 20 carbon atoms, such as docasanol, tetracosanol, haxacosanol (Reiter & Lorbeer, 2001).

Waxes (esters of fatty acids with fatty alcohols) usually found in olive oils are C36, C38, C40, C42, C44, and C46 esters. As they accumulate in the skin of olives, higher amounts of them can be detected in olive pomace oils rather than in virgin olive oils (Bianchi et al., 1994). The diterpene alcohols include phytol and geranylgeraniol, that are two acyclic diterpenoids present in the aliphatic alcohol fraction of olive oil, both in free and esterified form (Boskou, 2007).

3.1.8 Polar phenols

These compounds are well discussed and their importance is pointed out in *chapter 4*, since they are directly involved in the sensorial attributes of bitter and pungent of virgin olive oils. Several analytical methods exist for the quali-quantitative determination of phenolic compounds, both by chromatographic and spectroscopic methods carried out on the phenolic fraction extracted from olive oils (Bendini et al., 2007). Moreover, some researchers (Gutierrez et al., 1992; Beltran et al., 2007) found that bitterness of EVOOs can be estimated by the spectrophotometric measurement of the specific absorbance at 225 nm (K_{225}), carried out on the phenolic extract, too. Neverthless, it is important to underline that the use of such a spectrophotometric index for evaluating the bitterness show some limits, since other phenolic compounds may influence its determination (such as the aldehydic form of oleuropein aglycone, that can absorb at 225 nm, but is not characterized by bitterness) (Inarejos-Garcia et al., 2009). Total amount of phenolic compounds, of *ortho*-diphenols and bitterness index (expressed as K_{225}) were evaluated for the set of samples collected at supermarket (S1-S35). It is also important to highlight that EU Reg. 432/2012 introduced the possibility to report the health claim "*Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress*" in the label, if the olive oil contains at least 5 mg of hydroxytyrosol and its derivatives (e.g. oleuropein complex and tyrosol) per 20 g of product. The analyitical procedures for quantifying them are reported in the EU Reg. 432/2012.

3.1.8.1 Materials and methods

3.1.8.1.1 Extraction of polar phenolic compounds

A liquid-liquid extraction was used to extract the phenolic compounds from EVOOs. According to Carrasco-Pancorbo et al. (2004), 60 g of oil were dissolved in 60 mL of *n*-hexane, and the solution was extracted successively with three 20 mL portions of methanol/water (60:40, v/v) solution. The combined extracts of the hydrophilic layer were brought to dryness in a rotary evaporator under reduced pressure and a temperature of 35 °C. Finally, the residue was redissolved in 5 mL of methanol/water (50:50, v/v), filtered through a 0,45 μm filter (VWR, West Chester, PA). For carrying out the spectrophotometric determinations, the extract was further diluted 1:5 (v/v).

3.1.8.1.2 Determination of total phenols and ortho-diphenols by spectrophotometric methods

The total phenolic content was determined by the Folin-Ciocalteau spectrophotometric method (Carrasco Pancorbo et al., 2004) at 750 nm; the content of total *o*-diphenols was evaluated according to Mateos et al., 2001, at 370 nm and against a reference prepared with ther same procedure, but without adding the EVOO extract. Both the assays were carried out using spectrophotometer UV-VIS 1800 CE 230V (Shimadzu Co., Kyoto, Japan). The total phenol and *o*-diphenol concentrations were calculated by using the respective calibration curves, using gallic acid as standard (from 0.025 to 1 mg/ml, r^2=0.9966 (TP) and r^2=0.9939 (*o*-DPH)). Results were expressed as mg gallic acid kg^{-1} oil and the analysis was repeated three times for each extract.

3.1.8.1.3 Determination of bitterness index (K_{225})

Evaluation of bitterness index (as K_{225}) was carried out spectrophotometrically at 225 nm according to Gutièrrez et al., 1992, with some modifications: the phenolic extract, obtained as described previously (see *paragraph 3.1.8.1.1*), was diluted 1:250 (v/v) with methanol/water (1:1, v/v) solution; then, the absorbance was measured at 225 nm against a reference constitued by the solvent in a 1-cm quartz cuvette. The test was carried out using spectrophotometer UV-VIS 1800 CE 230V (Shimadzu Co., Kyoto, Japan). K_{225} can be calculated on the basis of the read absorbance (1 g of oil in 100 ml of solvent):

$$K_{225} = \frac{1}{g} \times A_{225} \times \frac{V * d}{100}$$

g = grams of oil

A_{225} = assorbance at 225 nm

V = ml used to redissolve the extract

d = dilution

3.1.8.1.4 Statistical analysis

The software XLSTAT 7.5.2 version (Addinsoft, USA) was used to elaborate data by Analysis of Variance (ANOVA, Fisher LSD, $p < 0.05$).

3.1.8.2 Results and discussion

The results concerning the total amount of phenolic compounds, of *o*-diphenols and bitterness indexes (expressed as K_{225}) were fully discussed in *chapter 2* (see paper "Sensory and chemical quality...") for the set of samples collected at the supermarket (S1-S35).

3.1.9 Hydrocarbons

Squalene (2,6,10,15,19,23-Hexamethyl-2,6,10,14,18,22-tetracosahexaene) is present in small amount in olive oils (approximately 0.5% m/m) and it shows very important biological properties, as antioxidant, anticarcinogenic and anti-inflammatory compound (see *paragraph 2.8*). Beta-carotene is another important hydrocarbon of olive oil, which can act also as antioxidant, both as single oxygen quenchers and as free radical trapping agents (Jorgensen & Skibsted, 1993). Other main carotenoids present in olive oil are lutein and other minor xantophylls. Refining process causes the appearance of hydrocarbons not naturally occurring in virgin olive oils, such as alkadienes (mainly *n*-hexacosadiene), stigmasta-3,5-diene, isomerization products of squalene, isoprenoidal olefins from hydroxy derivatives of squalene and steroidal hydrocarbons deriving from 24-methylene cycloartanol (Lanzòn et al., 1994).

3.1.10 Free sterols

The sterol profile of a vegetable oil is a peculiar fingerprint of each botanical species. For this reason, the determination of sterol composition is widely applied as an effective and reliable tool to detect the adulteration of olive oils with other vegetable oils (Angerosa et al., 2006). The content of some sterols, such as campesterol, stigmasterol, and β-sitosterol, decrease during the refining process since they undergo a dehydration: the obtained oils, characterized by a low sterol content (so called "desterolized") can be used to adulterate virgin olive oils for economical purpose (Angerosa et al., 2006). Four different classes of sterols usually occur in olive oil: common sterols (4-alpha-desmethylsterols), 4-alphamethylsterols, tripterpene alcohols (4,4,-dimethylsterols) and triterpene dialcohols. The properties of each class are fully described in Boskou (2007). The above-cited molecules were investigated for samples directly collected at Italian mills (F1-F28). The results are discussed here below and were also reported in Valli et al., 2012.

3.1.10.1 Materials and methods

3.1.10.1.1 Determination of sterols and metylsterols by gaschromatographic analysis

For the determination of sterols and methylsterol, the official method adopted by European Community in EEC Reg. 2568/1991 has been adopted. This method consists in a saponification of the oil added with α-cholestanol as internal standard, a liquid/liquid extraction of the unsaponifiable fraction, a recovery of the sterols fraction by thin layer chromatography (TLC), their "transformation" into trimethyl-silyl esthers derivatives and a quali-quantitative determination by capillary-column gas chromatography. Moreover, after some preliminary tests, it was tested to directly inject the unsaponifiable fraction- previously added with a proper reagent in order to have the formation of trimethyl-silyl esthers derivatives - in the gas chromatographic system, without the need to purify it by TLC. This approach permits to save time and money for buying solvent, lab materials, etc., ensuring good gas chromatographic separation and resolution of the analytes (see **Figures 3 E**, **4 E** and **5 E**). A similar approach was proposed in the past for the screening of different samples of olive oils by Frega et al. (1992, 1993b). For the analysis, a ZB-5MS (30m x 0.25mm i.d., 0.25µm f.t.) column (Phenomenex, Torrence, USA) was used. The gas chromatographic conditions (Agilent 6890N Network GC System, coupled with Agilent 5973 Network MSD; Agilent Technologies, Palo Alto, CA, USA) were: injected volume = 1 µL; flow of the carrier gas = 1 mL min^{-1}; split ratio = 1/15; temperature of the injector = 330 °C. The temperature of the oven was set at 250 °C and then increased at 3 °C min^{-1} up to 325 °C (keep for 10 min); mass scan = 15-800 amu; temperature of the mass source = 230 °C; temperature of the mass quad = 150 °C. The sterols were identified according to their mass spectra (library: NIST '05), a previous experimental work in the literature (Cercaci et al., 2007) and some examples of chromatograms reported in EU Reg. 61/2011. Moreover, overlays among the chromatograms related to the fractions of sterols and methylsterol (collected together from the same sample of oil) and the same separated fractions (collected separately) were useful for the identification of such compounds (see **Figures 4 E** and **5 E**). Three replicates were carried out for each sample. Compounds were quantified by using an internal standard (α-cholestanol) and the results were expressed as total amount of sterols (mg of internal standard kg^{-1} oil) and as percentage of each compound expressed on the total amount of sterols (EU Reg. 61/2011).

3.1.10.1.2 Statistical analysis

The software XLSTAT 7.5.2 version (Addinsoft, USA) was used to elaborate data by Analysis of Variance (ANOVA, Fisher LSD, $p < 0.05$).

3.1.10.2 Results and discussion

The EU Reg. 61/2011 shows the composition in sterols common for all the edible oils obtained by olives: brassicasterol ≤ 0,1%, campesterol ≤ 4,0%, stigmasterol < campesterol, apparent β-sitosterol (sum of Δ5,23-stigmastadienol + clerosterol + β-sitosterol + sitostanol + Δ5-avenasterol + Δ5,24-stigmastadienol) ≥ 93%; total amount of sterols ≥ 1000 mg kg^{-1}.

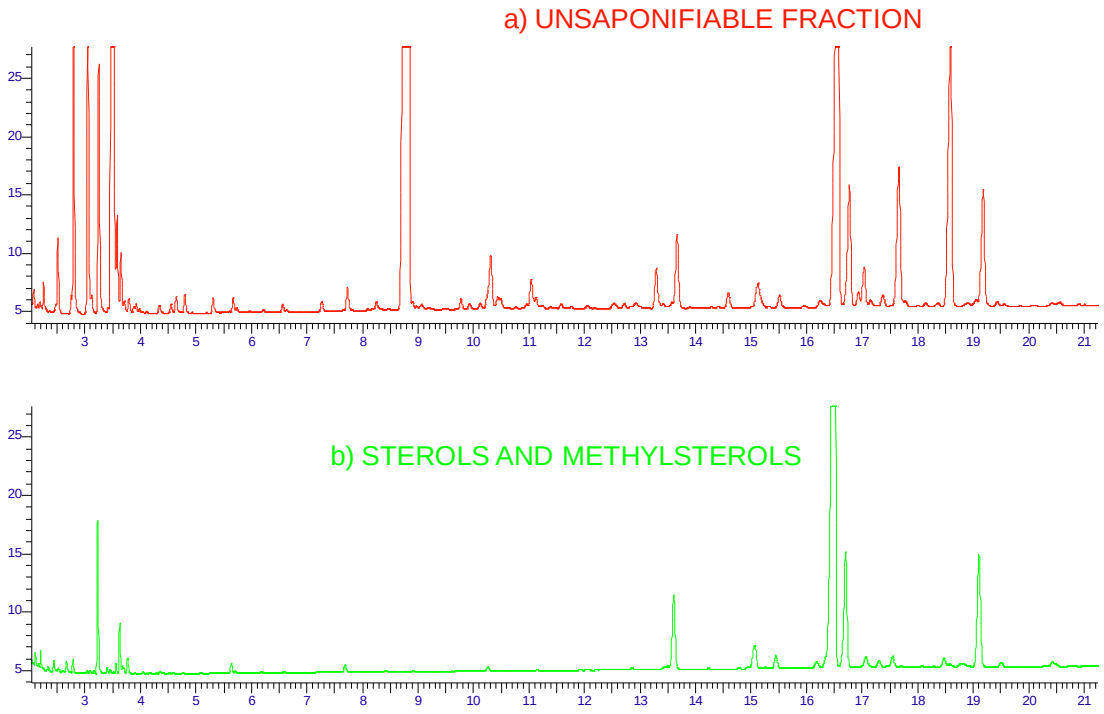

Figure 3 E. Overlay between two chromatograms: a) obtained by direct injection in the gas chromatographic system of the unsaponifiable fraction-previously transformed into trimethyl-silyl esthers derivatives, as reported in *paragraph 3.1.10.1.1*, without the need to purify it by TLC; b) obtained by injection of the fraction of sterols and methylsterols purified by TLC, as reported in *paragraph 3.1.10.1.1*.

The **Figures 4 E** and **5 E** depict two overlays between the chromatograms related to sterols and methylsterols (by recovering together both the bands of the TLC) and respectively the band of sterols (**Figures 4 E**) and methylsterols (**Figures 5 E**). The peaks 9, 11, 13, 14, 15 and 16 (**Figure 5 E**) could be related to methylsterols, since they were not present (or present just in traces) in the band of sterol (**Figure 4 E**).

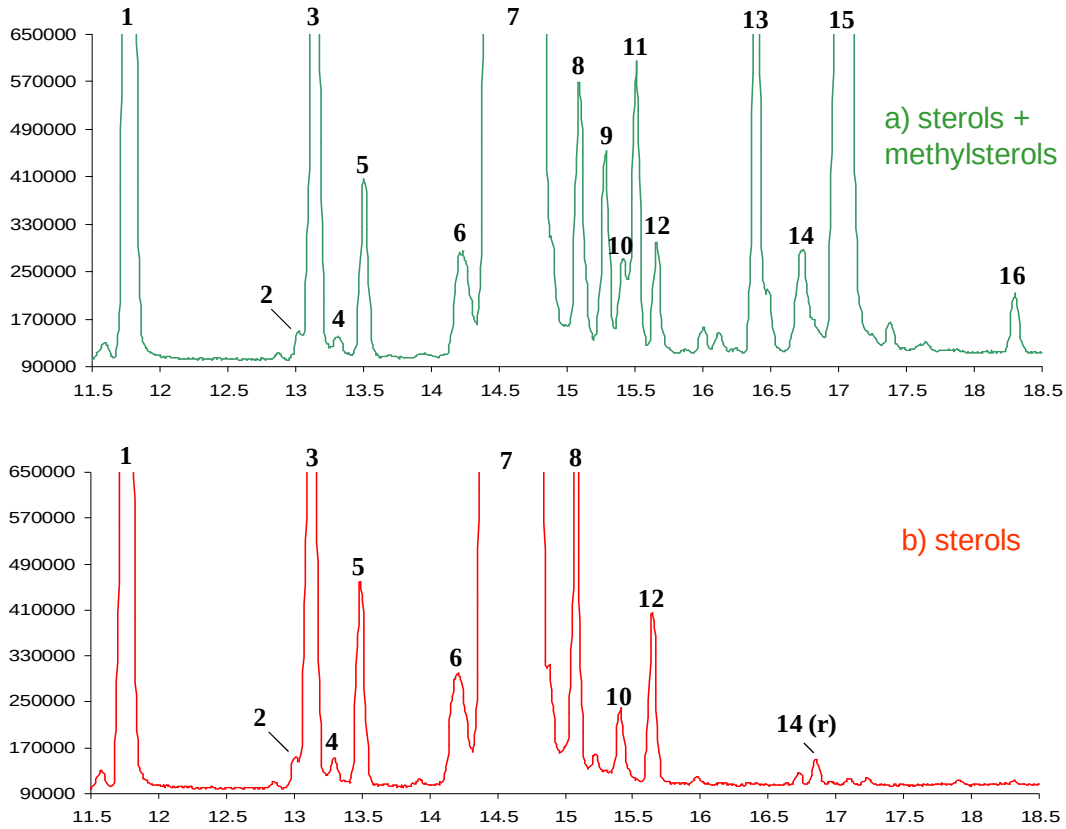

Figure 4 E. Overlay between two chromatograms related to a) both the fraction of sterols and methylsterol (collected together) and b) the sterols fraction (collected separately from the same sample of oil, F25). Identification of compounds, as reported in *paragraph 3.1.10.1.1*: 1, 19- hydroxycholesterol; 2, 24-methylene cholesterol; 3, campesterol; 4, campestanol; 5, stigmasterol; 6, clerosterol; 7, β –sitosterol + Δ5-avenasterol; 8, Δ5, 24-stigmastadienol; 9, methylsterol A; 10, Δ7- stigmastenol; 11, methylsterol B; 12, Δ7-avenasterol; 13, methylsterol C; 14, methylsterol D; 15, methylsterol E; 16, methlsterol F. "(r)" indicates the compounds that could be identified as methylsterols within the fraction of sterols.

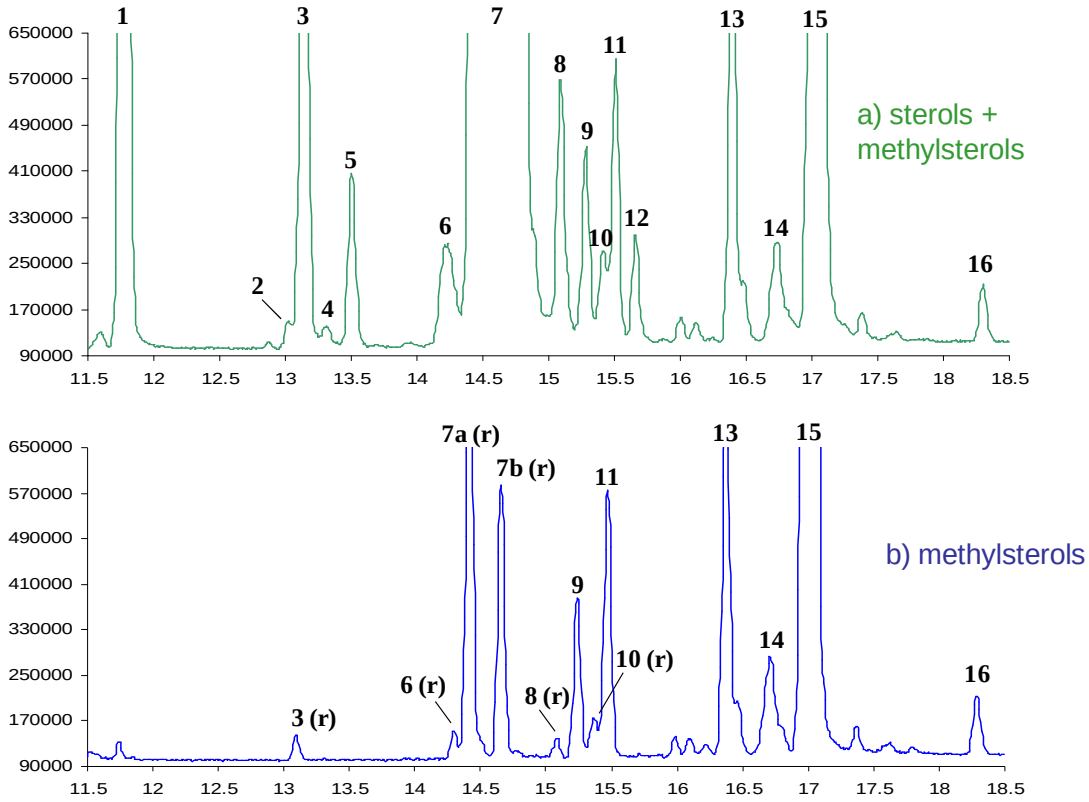

Figure 5 E. Overlay between two chromatograms related to a) both the fraction of sterols and methylsterol (collected together) and b) the methylsterols fraction (collected separately from the same sample of oil, F25). Identification of compounds, as reported in reported in *paragraph 3.1.10.1.1*,: 1, 19-hydroxycholesterol; 2, 24- methylene cholesterol; 3, campesterol; 4, campestanol; 5, stigmasterol; 6, clerosterol; 7, β –sitosterol + Δ5-avenasterol; 8, Δ5, 24-stigmastadienol; 9, methylsterol A; 10, Δ7-stigmastenol; 11, methylsterol B; 12, Δ7-avenasterol; 13, methylsterol C; 14, methylsterol D; 15, methylsterol E; 16, methlsterol F. "(r)" indicates the compounds that could be identified as methylsterols within the fraction of sterols.

Table 5 E. Mean values (calculated on three replicates) related to the amount of each sterol, expressed as % on the total amount of sterols (reported in the last column and expressed as mk kg^{-1} of internal standard) in the samples collected at Italian mills (F1-F28). Different letters in the same column indicate significant differences (Fisher LSD, $p < 0.05$). Values in bold and italic are out of the limits established by EU Reg 61/2011. LOQ: limit of quantification.

	% brassicasterol	*% campesterol*	*% stigmasterol*	*% apparent β-sitosterol*	*total sterols [mg kg^{-1}]*
F1	< LOQ	3.4 j-m	1.2 b,c	94.1 g,h	1362.5 b
F2	< LOQ	3.2 l-o	1.4 a	94.7 b-d	1366.8 b
F3	< LOQ	4.0 f,g	1.4 a	***92.7*** l-n	1117.8 e,f
F4	***0.9*** b	3.3 k-n	0.6 h-k	94.3 f,g	1369.9 b
F5	< LOQ	3.1 n,o	0.7 f-j	95.1 b,c	***991.3*** h,i
F6	0.1	3.4 k-m	0.7 f-j	94.9 b,c	1074.5 f-h
F7	< LOQ	3.1 n,o	0.9 e,f	94.8 b-d	***603.0*** n
F8	0.1	3.0 n,o	0.9 d,e	94.8 c-e	***757.8*** l,m
F9	< LOQ	***4.4*** c,d	0.8 e-g	***92.7*** m,n	1203.4 d,e
F10	< LOQ	***4.6*** a	0.7 e-i	***92.9*** m,n	1070.0 f-h
F11	***1.4*** a	2.7 p	1.3 a,b	***91.6*** p	2309.0 a
F12	< LOQ	***4.1*** e,f	0.9 e,f	93.1 k-m	1087.1 f,g
F13	< LOQ	***4.6*** c,d	0.7 f-j	***92.0*** o	1178.6 e
F14	< LOQ	***4.4*** b,c	0.9 e,f	93.0 k-n	***934.7*** i,j
F15	< LOQ	***4.2*** d,e	0.8 e-g	***92.6*** n	1274.9 c,d
F16	0.1 d,e	3.8 g,h	0.9 d-f	93.2 j-l	***929.4*** i,j
F17	< LOQ	3.3 k-n	0.6 h-k	95.4 a	***503.9*** o
F18	< LOQ	3.3 k-m	0.5 k	95.3 a	***726.9*** m
F19	***0.2*** d	3.6 i,j	0.6 h-k	94.2 h,i	1014.2 g-i
F20	0.1 d,e	3.5 i-k	0.6 h-k	94.5 d-f	***959.7*** i
F21	< LOQ	***4.4*** c,d	0.7 g-k	***91.9*** p	***945.7*** i,j
F22	***0.7*** c	3.4 j-l	0.6 j,k	93.4 j,k	***863.6*** j,k
F23	***0.9*** b	0.6 h,i	0.6 i-k	93.2 j-l	1149.8 e,f
F24	< LOQ	3.8 g,h	0.7 g-k	93.6 i,j	1174.5 e
F25	< LOQ	3.2 m-o	0.8 e-h	95.1 b	1200.2 d,e
F26	< LOQ	4.0 e-g	1.1 c,d	***91.9*** o,p	1350.5 b,c
F27	< LOQ	***4.1*** e-g	0.5 k	94.4 e-g	1337.6 b,c
F28	< LOQ	***4.7*** a,b	0.4 j,k	93.6 i,j	***815.5*** k,l

The examined EVOOs, all collected at Italian mills (F1-F28), showed a tyical composition in sterols, according to the limits proposed by EU Reg. 61/2011, with some exceptions, reported in bold and italic in **Table 5 E**.

3.1.11 Tocopherols

Tocopherols are important fat-soluble vitamins, contributing to the stability of the olive oils and having a benefic biological role as quenchers of free radicals in vivo (Boskou, 2007). Alpha tocopherol is the main homologue of vitamin E forms present in olive oil (95 % of the total); it can act also as singlet oxygen quencher. Beta- and gamma-tocopherols are also typical compounds of olive oils. The presence of tocopherols in olive oils has been

increased in the last years, thanks to an improvement in good manufacturing practices (Boskou, 2007), showing an interesting rather wide range of tocopherols concentrations in virgin olive oils (55-350 mg kg^{-1}) (Cert et al., 1999).

3.1.12 Pigments

The olive oil color is mainly due to clorophylls a and b, pheophytins a and b and carotenoids. The clorophylls have a double and contradictory role in terms of oxidation: in presence of light, they act as strong oxidation promoters, while in dark probably they are weak antioxidants (Boskou, 2000).

3.1.13 Fatty acid alkyl esters

A description of the importance of fatty acid alkyl esters (FAAEs) as quality parameters for EVOOs, due to their mechanism of formation is fully discussed in *chapter 2* (see paper "Detection of low-quality…"). For the sets of samples 1-4 (see *paragraph 3.0.1*), the determination of FAAEs (FAMEs, fatty acid methyl esters and FAEEs, fatty acid ethyl esters) was performed, using analytical procedures that followed the historical evolution of the method, first reported in the literatures (Pérez-Camino et al., 2008; Bendini et al., 2009a; Bendini et al., 2009b; Cerretani et al., 2011), proposed by the International Olive Council (I.O.C.) in IOC/T.20/Doc. No. 28, 2010 and then adopted by the E.U. (EU Reg. 61/2011). The results are discussed here below and were also reported in Valli et al., 2012.

3.1.13.1 Materials and methods

3.1.13.1.1 Determination of FAAEs by gas chromatographic analysis

All the adopted analytical approaches followed the historical evolution of the method and are based on traditional solid-liquid chromatography for isolating the fraction containing the alkyl esters and the waxes and a subsequent gas chromatographic analysis; these methods are full detailed in *chapter 2* (see paper "Detection of low-quality..."). Results were expressed as mg methyl heptadecanoate (MetEsC17:0) per kg of oil, as requested by EU Reg. 61/2011. Moreover, the analytical separation of the FAAEs was improved by using a Fast-GC method, in order to decrease the time of analysis respect to the traditional gas chromatography, giving preliminary satisfactory results: such a method has to be still validated.

3.1.13.1.2 Statistical analysis

The software XLSTAT 7.5.2 version (Addinsoft, USA) was used to create the Box-plot chart (see **Figure 7 E**), by elaborating the mean values of the total amount of FAAEs for each analyzed sample, splitting them on the basis of the sets in which they belong (see *paragraph 3.0.1*).

3.1.12.2 Results and discussion

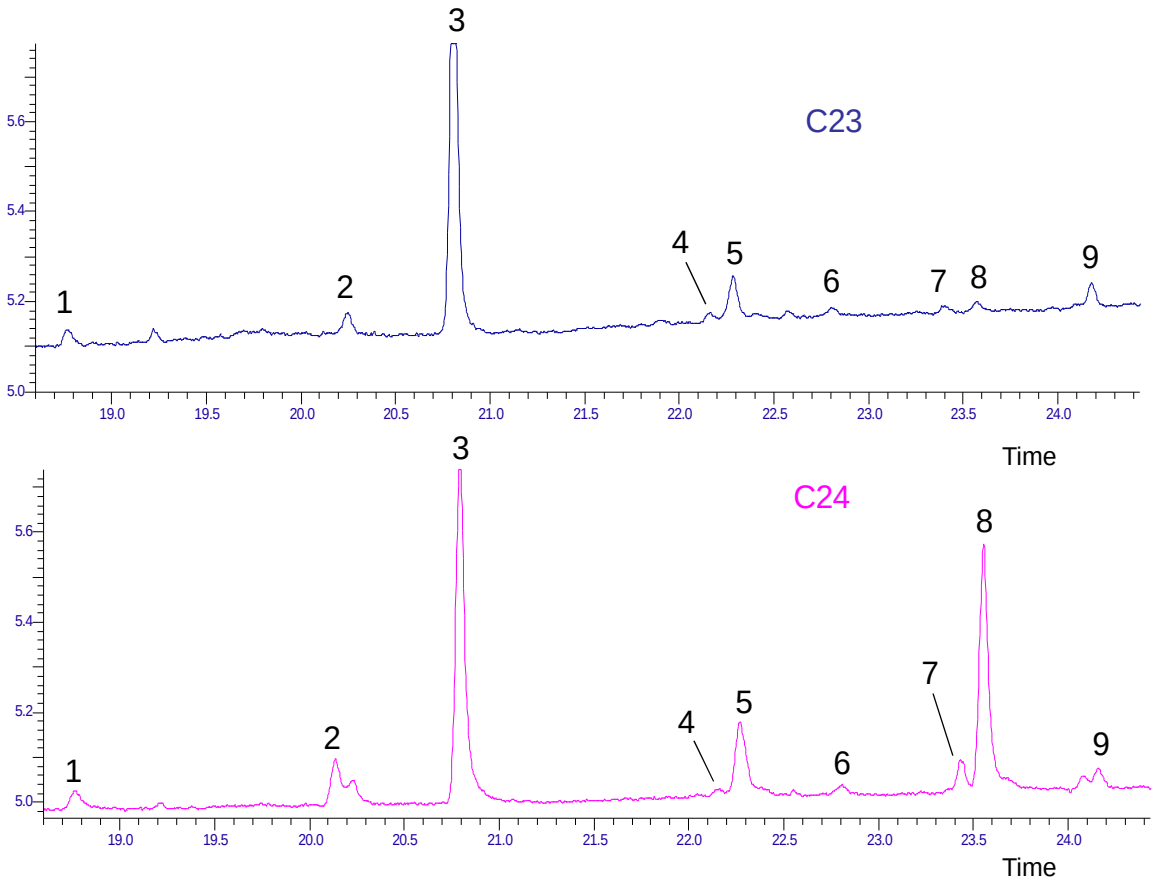

Figure 6 E. GC chromatograms of fatty acid alkyl esters (FAAEs) for samples C23 and C24 (see also Table 6 E). 1, MetEsC16:0; 2, EtEsC16:0; 3, MetEsC17:0 (I.S.); 4, MetEsC18:2; 5, MetEsC18:1; 6, MetEsC18:0; 7, EtEsC18:2; 8, EtEsC18:1; 9, EtEsC18:0.

As reported in **Table 6 E** and in **Figure 7 E**, the samples included in the set C1-C34, purchased at low price at the supermarket, showed a quite wide range in terms of total amount of FAAEs. Many samples (12 out of 34) sold as EVOOs at medium-low price (C1-C34) showed total amounts of FAAEs higher than the legal limit established by the E.U. for EVOOs (EU Reg. 61/2011) (see **Table 6 E**). This results can be due to a very low-quality degree of the raw materials (damaged or bad-preserved olives), and/or to an illegally mixture between EVOOs and low-quality olive oils, such as "mild" deodorized

(Pérez -Camino et al., 2008). It is important to underline that the C1-C34 samples were collected before that the EU Reg. 61/2011 came into effect. In **Figure 6 E** an overlay between two chromatograms related to the FAAEs extracted from the samples C23 and C24 is shown. It is interesting to notice that the sample C24 was richer in these compounds than C23, especially for ethyl oleate (EtEsC18:1), as reported in **Table 6 E**. This latter one could be a useful marker for evaluating lower quality olive oils: sample C24 was also characterized by a sensory defect of winey-vinegary (for this reason, it couldn't be classified as "extra virgin", according to EU Reg. 61/2011) and by olfactory notes of cardboard and eucalyptus, recognized by the majority of the panellists (see **Table 6 E**). Furthermore, the FAAEs were almost not present in the oils sampled directly from Italian mills (F1-F28), suggesting a high quality of the processed olives (**Figure 7 E**). The effectiveness of such a quality parameter is confirmed by the results evidenced for the samples of "repaso" olive oils (LR1-LR6) (see *paragraph 3.0.1*), that were characterized by very high amount of FAAEs (Cerretani et al., 2011 and *paragraph 3.2.8*). The FAAEs are removed from oil by refining process, as seen for samples R1-R6 (**Figure 7 E**). For the samples collected in the second phase of the project (S1-S35), the amounts of FAAEs were lower than the EVOOs previously sampled (**Figure 7 E**). In particular, the EVOOs having a denomination of origin (P.D.O. and P.G.I.) showed a very low content in FAAEs (from 13.42 mg kg^{-1} to 46.57 mg kg^{-1}). Only two samples (S1 and S12) of the S1-S35 group were above the legal limit proposed by the E.U. (EU Reg. 61/2011) for EVOOs: they were both labeled as "not filtered EVOOs" and sold at low price (*chapter 2,* see paper "Sensory and chemical quality…"). Actually, two other samples of the set 4 (S2 and S24) were close to the legal limit of FAAEs and they were also sold at low price. Since the official analytical method for the evaluation of FAAEs (EU Reg. 61/2011) is time/solvent-consuming and expensive, we decided to acquire the FT-IR spectra of the samples, in order to build a statistical PLS model able to discriminate the EVOOs according to their content in FAAEs (see *paragraph 3.2.3* and *chapter 2,* see paper "Detection of low-quality…").

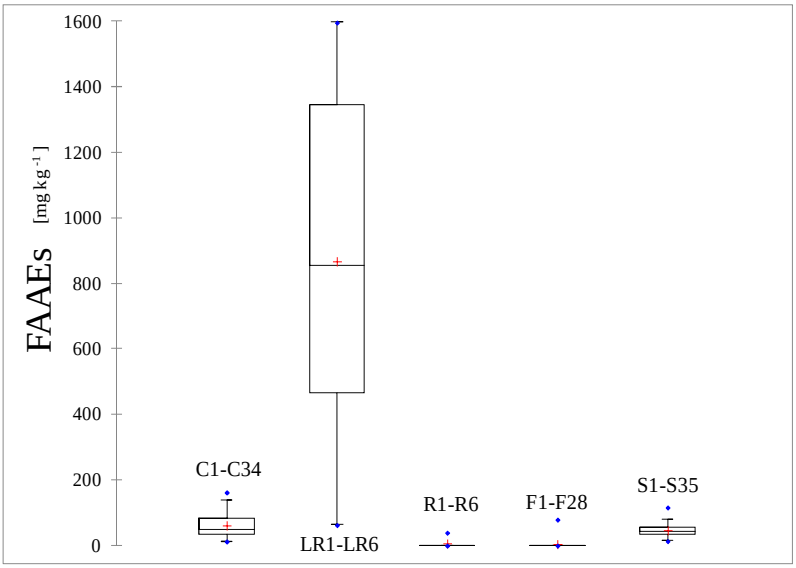

Figure 7 E. Box-plot diagram showing the total amount of FAAEs in all the samples (see *paragraph 3.0.1* for the legend of the sample codes).

3.1.14 Volatile compounds

A full description about the volatile compounds usually found in olive oils, their formation and their role in determining the sensory attributes is reported in *paragraph 2.7*. The results discussed in this Ph.D. thesis are related to the set of 34 samples of EVOOs (C1-C34), all sold at medium-low price (2-5 €/kg) in the large scale retail trade. Moreover, the results of the sensory analysis and their content in fatty acid alkyl esters were also pointed out for the same samples (see *paragraph 3.1.14.2*), in order to highlight some interesting aspects.

3.1.14.1 Materials and methods

3.1.14.1.1 Determination of volatile compounds by SPME-GC/MSD

A 1.5 g amount of each sample was weighed into a 10 mL vial. The oil sample was spiked with 0.15 g of 4-methyl-2-pentanone (internal standard) to a concentration of 5 µg g^{-1}. The vial was fitted with a silicone septum and immersed in a water bath at 40 °C (± 2 °C); the sample was maintained under magnetic stirring. After 2 min of sample conditioning, a divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) SPME fiber (50/30 µm, 2 cm long from Supelco Ltd., Bellefonte, PA, USA) was exposed to the sample headspace for 30 min and immediately desorbed for 3 min at 250° C in the gas chromatograph

injector port. Volatile compounds were identified and quantified by gas chromatography coupled with quadrupolar mass-selective spectrometry, using an Agilent 6890NNetwork gas chromatograph and an Agilent 5973 Network detector (Agilent Technologies, Santa Clara, CA, USA). Analytes were separated on a ZB-WAX column 30 m, 0.25 mm i.d., 1.00 µm film thickness (Phenomenex, Torrence, CA, USA). Column temperature was held at 40 °C for 10 min and increased to 200 °C (held for 2 min) at 3 °C min^{-1}; then the temperature increased at 10 °C min^{-1} up to 250 °C (held for 2 min). The ion source and the transfer line temperatures were set at 230 °C and 250 °C, respectively. Electron impact mass spectra were recorded at 70 eV ionization energy in the 30-250 amu mass range, 2 scans s^{-1}. The identification of the volatile compounds was first carried out by mass spectrometry and later checked with standards, previously injected in the same conditions (Baccouri et al., 2008). Moreover, a confirmation of the identification was obtained by a comparison of their mass spectral data with the information from the National Institute of Standards and Technology (NIST) library (2005 version) and MS literature data. Volatile compounds were also identified using the relative retention times of the standards with respect to the internal standard (4-methyl-2-pentanone) and expressed as mg of internal standard per kg of oil.

3.1.14.1.2 Sensory analysis

Sensory analysis was performed according to the EC Reg. 640/2008, by a fully trained group of 8 expert tasters of the Department of Agricultural and Food Sciences of the University of Bologna, by using an extended profile sheet. A set of positive (fruity and other pleasant attributes such as leaf, grass, artichoke, tomato, almond, apple, others) and negative (winey-vinegary, fusty-muddy, mouldy, rancid and others) sensory attributes were evaluated on a continuous scale from 0 to 10 cm related to the intensity of perception of the flavor stimuli. The median and the robust standard deviation (EC Reg. 640/2008) were calculated for each attribute after tasting the oils. If the values of the robust standard deviations were higher than 20%, the sensory analysis was repeated.

3.1.14.1.3 Determination of FAAEs by gas chromatographic analysis

The extraction of the alkyl esters by traditional liquid chromatography (LC) and their gas chromatographic determination followed the method reported in IOC/T.20/Doc. No. 28,

2010 "Determination Of The Content Of Waxes, Fatty Acid Methyl Esters And Fatty Acid Ethyl Esters By Capillary Gas Chromatography" and recently adopted as official law by the E.U. (EU Reg. 61/2011 and corrigendum). This procedure is fully detailed in *chapter 2* (see paper "Detection of low-quality…"). Results were expressed as mg methyl heptadecanoate (MetEsC17:0) per kg of oil, as requested by EU Reg. 61/2011. Also the ratio between the amount of fatty acid ethyl esters (FAEEs) and fatty acid methyl esters (FAMEs) was calculated (see **Table 6 E**), since it is important for the classification of EVOO if the total amount of FAAEs ranges between 75 and 150 mg kg^{-1} (EU Reg. 61/2011). Three replicates were calculated for each sample.

3.1.14.2 Results and discussion

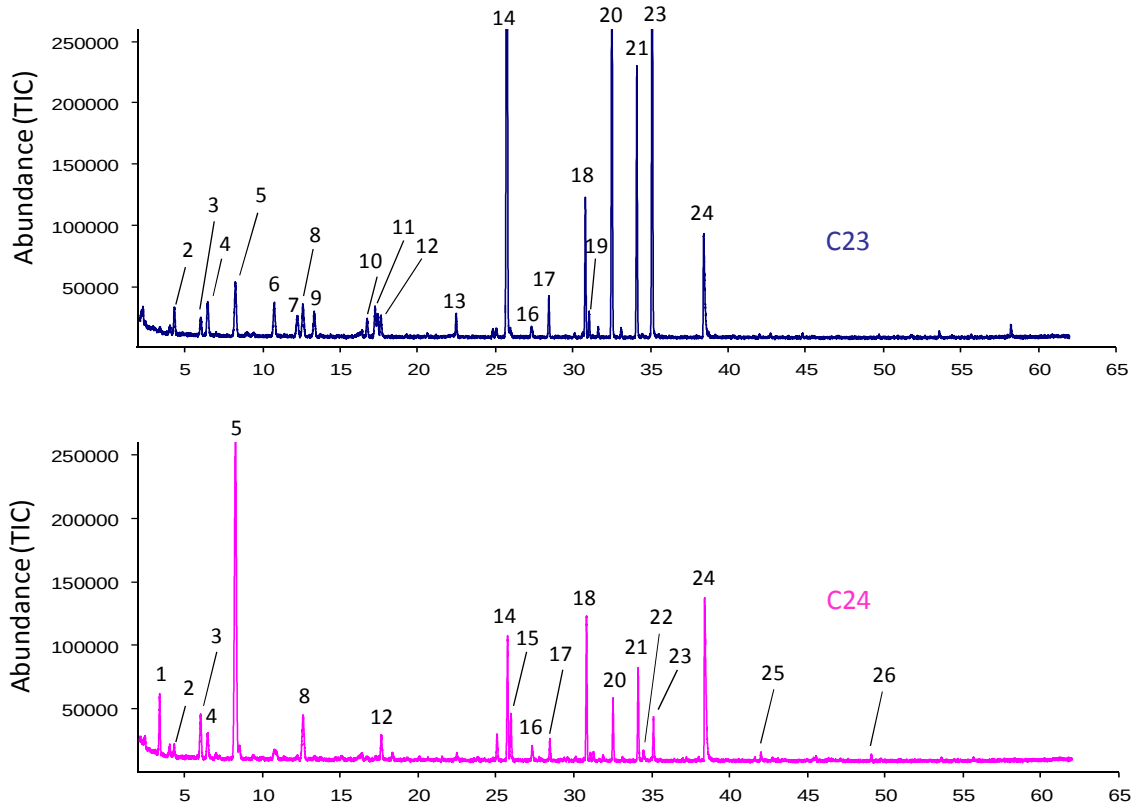

Figure 8 E. GC chromatograms of volatile compounds for samples C23 and C24 (see Table 6 E). 1, octane; 2, methyl acetate; 3, ethyl acetate; 4, methanol; 5, ethanol; 6, 3-pentanone; 7, penten dimer; 8, 4-methyl-2-pentanone (I.S.); 9, penten dimer; 10, (*Z*)-1,9-dodecadiene; 11, 4,8-dimethyl-1,7-nonadiene; 12, hexanal; 13, 1-penten-3-ol; 14, (*E*)-2-hexenal; 15, 2-tridecene; 16, α-pinene; 17, hexyl acetate; 18, (*Z*)-3- hexenyl acetate; 19, 2-penten-1-ol; 20, 1-hexanol; 21, (*Z*)-3-hexenol; 22, nonanal; 23, (*E*)-2-esenolo; 24, acetic acid; 25, propanoic acid; 26, α -farnesene.

In **Figure 8 E**, the chromatograms related to the volatile fraction of the samples C23 and C24 are displayed. It stands to reason that these two samples show rather big differences

in terms of volatile profiles. The same two samples were also compared according to their FAAEs content (see **Figure 6 E**, *paragraph 3.1.13.2*) and they were characterized by very different amount of these latter compounds. The sample C24, that was classified by the Panel as "virgin" for the presence of the sensory defect of winey-vinegar (median value less than 3.5, EU Reg. 61/2011) and also characterized by a higher amount of FAAEs than the legal limit established by EU Reg. 61/2011 (**Table 6 E**), was actually richer than sample C23 (genuine EVOO) in compounds 3, 5, 24 (respectively ethyl acetate, ethanol, actic acid) (see **Figure 8 E**). These latter compounds are actually well-recognized as responsible for the sensory defect of winey-vinegary, if present at concentration above their odour thresholds (Morales et al., 2005). Moreover, the sample C24 showed lower amount than sample C23 of volatile compounds originated by the C6-LOX enzymatic pathway, that are the main responsible of the pleasent fruity olfactory notes (peaks 6, 7, 9, 10, 11, 13, 14, 17, 18, 19, 20, 21, 23 in **Figure 8 E**). The results related to the FAAEs, the volatile compounds and the sensory analysis of the 22 samples named as C1-C22, all sold at a medium-low price (2-5 €/kg) in the large scale retail trade (supermarkets and discounts), were previously reported and discussed in Bendini et al., 2009a. No data have been published yet concerning the results of the 12 samples coded as C23-C34. In **Table 6 E**, all the above-cited results for all the samples C1-C34 are shown. Eleven samples out of 34 (C7, C8, C10, C14, C15, C18 , C20, C23, C25, C28, C31) were judged as "extra virgin olive oils" according to the sensory analysis (EC Reg. 640/2008 and EU Reg. 61/2011) and the majority of the panellists didn't find any other negative olfactory notes or the eucalyptus one. For all of them, the values of the total amount of FAAEs (∑ FAMEs + FAEEs) and the ratio between fatty acid ethyl esters and fatty acid methyl ester (RFF) were within the legal limits established by EU Reg. 61/2011 for EVOOs (see **Table 6 E**). Moreover, the ratio between ethanol and (*E*)-2-hexenal was less than 1 for all the above-cited genuine samples (see **Table 6 E**).

Table 6 E. Results related to the determination of fatty acid alkyl esters, volatile compounds and sensory analysis for the samples C1-C34. EtEsC18:1 = amount of ethyl oleate (mg I.S. kg^{-1} oil); ∑ FAMEs + FAEEs = total amount of FAAEs, as sum of fatty acid methyl and ethyl esters (mg I.S. kg^{-1} oil); RFF = ratio between the amount of fatty acid ethyl esters and fatty acid methyl ester. G = genuine extra virgin olive oil; S.S. = oil strongly suspected to be obtained by adding cheaper and lower quality products, illegally obtained by "mild deodorization".

Samples	*Fatty acid alkyl esters*								*Volatile compounds*		*Sensory analysis*			
	EtEsC18:1		**∑ FAMEs + FAEEs**		**RFF**		**Judjement about FAAEs (Pérez-Camino et al., 2008)**	**Classification (EU Reg. 61/2011)**	**Ethanol / (*E*)-2-hexenal**		**Classification**	**Sensory defects**	**Other negative olfactory notes**	**Other peculiar descriptors**
	mean	sd	mean	sd	mean	sd			mean	sd				
C1	7.26	0.13	26.70	3.92	1.19	0.05	G.	E.V.O.O.	4.07	0.38	E.V.O.O.			eucalyptus
C2	90.39	4.61	162.40	19.68	3.50	0.53	S.S.	NOT E.V.O.O.	2.99	0.17	V.O.O.	winey-vinegary	cardboard	eucalyptus
C3	16.87	0.87	42.39	12.00	1.38	0.14	G.	E.V.O.O.	11.79	1.38	V.O.O.	winey-vinegary		
C4	55.16	5.97	133.43	19.81	1.41	0.04	S.S.	E.V.O.O.	3.29	0.41	E.V.O.O.		cardboard	eucalyptus
C5	8.27	2.65	20.04	4.71	0.76	0.09	G.	E.V.O.O.	4.14	0.24	E.V.O.O.			eucalyptus
C6	36.13	5.33	59.70	15.57	0.79	0.11	G.	E.V.O.O.	3.17	0.43	E.V.O.O.			eucalyptus
C7	6.08	1.27	38.01	4.84	1.50	0.19	G.	E.V.O.O.	0.40	0.07	E.V.O.O.			
C8	4.28	0.77	15.07	2.46	0.56	0.02	G.	E.V.O.O.	0.07	0.00	E.V.O.O.			
C9	56.05	1.12	106.65	9.96	2.22	0.12	S.S.	NOT E.V.O.O.	2.25	0.11	E.V.O.O.			
C10	15.01	1.77	42.88	1.74	2.04	0.17	G.	E.V.O.O.	0.97	0.13	E.V.O.O.			
C11	33.11	0.75	78.14	6.55	4.05	0.63	S.S.	NOT E.V.O.O.	6.08	0.16	V.O.O.	winey-vinegary	cardboard	eucalyptus
C12	51.95	1.74	84.18	7.91	3.26	0.32	S.S.	NOT E.V.O.O.	3.36	0.31	E.V.O.O.		cardboard	eucalyptus
C13	45.20	1.46	79.44	1.53	3.95	0.43	S.S.	NOT E.V.O.O.	4.81	0.00	E.V.O.O.		cardboard	eucalyptus
C14	5.97	1.07	12.48	0.90	0.59	0.05	G.	E.V.O.O.	0.62	0.08	E.V.O.O.			
C15	4.38	0.38	14.22	0.29	0.70	0.15	G.	E.V.O.O.	0.60	0.05	E.V.O.O.			
C16	57.95	4.06	95.26	6.72	5.56	0.29	S.S.	NOT E.V.O.O.	1.45	0.03	E.V.O.O.			
C17	6.16	1.17	18.07	1.90	1.04	0.23	G.	E.V.O.O.	1.27	0.22	E.V.O.O.			eucalyptus
C18	7.46	0.31	19.78	1.34	1.01	0.07	G.	E.V.O.O.	0.29	0.03	E.V.O.O.			
C19	17.58	1.88	35.75	3.65	1.77	0.19	G.	E.V.O.O.	2.91	0.11	V.O.O.	winey-vinegary		
C20	15.81	2.60	31.06	3.64	2.32	0.34	G.	E.V.O.O.	0.39	0.04	E.V.O.O.			
C21	38.76	1.69	73.06	4.78	2.67	0.10	S.S.	E.V.O.O.	1.64	0.26	V.O.O.	rancid	cardboard	eucalyptus
C22	46.15	8.09	76.04	11.77	7.81	0.52	S.S.	NOT E.V.O.O.	1.03	0.09	E.V.O.O.		cardboard	eucalyptus
C23	1.76	0.14	36.40	9.76	0.43	0.09	G.	E.V.O.O.	0.16	0.00	E.V.O.O.			
C24	73.06	4.22	138.27	10.00	3.07	0.13	S.S.	NOT E.V.O.O.	4.41	0.56	V.O.O.	winey-vinegary	cardboard	eucalyptus
C25	10.91	0.71	52.70	2.62	2.02	0.27	G.	E.V.O.O.	0.65	0.10	E.V.O.O.			
C26	32.96	3.56	105.00	17.03	1.96	0.53	S.S.	NOT E.V.O.O.	0.24	0.02	E.V.O.O.	fusty-muddy	cardboard	
C27	26.02	3.80	76.03	10.50	2.00	0.58	S.S.	NOT E.V.O.O.	1.59	0.20	E.V.O.O.			eucalyptus
C28	3.43	0.12	35.45	1.19	1.40	0.16	G.	E.V.O.O.	0.16	0.02	E.V.O.O.			
C29	42.76	1.79	99.89	0.46	1.54	0.22	S.S.	NOT E.V.O.O.	19.21	2.11	V.O.O.		cardboard	
C30	15.58	0.73	45.48	5.47	1.06	0.08	G.	E.V.O.O.	3.30	0.51	E.V.O.O.		cardboard	
C31	16.04	0.60	69.78	9.53	1.41	0.20	G.	E.V.O.O.	0.35	0.02	E.V.O.O.			
C32	16.71	2.30	43.79	5.80	1.76	0.17	G.	E.V.O.O.	0.55	0.00	V.O.O.		cardboard	
C33	11.63	0.80	33.91	2.20	1.51	0.17	G.	E.V.O.O.	0.54	0.08	E.V.O.O.			eucalyptus
C34	34.24	0.12	88.59	2.40	1.57	0.26	S.S.	NOT E.V.O.O.	5.50	0.70	E.V.O.O.		cardboard	eucalyptus

Considering the results reported in **Table 6 E**, it is possible to get some findings that support the effectiveness of the ratio between ethanol and (*E*)-2-hexenal as an interesting innovative and "unofficial" quality maker for EVOOs. In particular, values of ethanol / (*E*)-2-hexenal above 1 were found for:

1) products sold and labelled as EVOOs, but characterized by values of FAAEs outside the actual legal limit established for EVOO (EU Reg. 61/2011) (see **Table 6 E**, samples C2, C9, C11, C12, C13, C16, C22, C24, C26, C27, C29, C34). The high amount of such compounds can be due to the use of low-quality olives for obtaining oils. If the fruits are damaged during the harvest and/or not-well/long preserved before the extraction of the oil, undesirable fermentative and hydrolytic processes may occure, which lead to the formation of low chain alcohols (methanol, ethanol) and a subsequent decrease of C6-LOX volatile compounds, such as (*E*)-2-hexenal (Morales et al., 2005; *paragraph 2.7*). Moreover, the resultant low-quality olive oils could be subjected to an illegal process of deodorization, which is able to decrease volatile compounds but doesn't affect the amount of FAAEs (Pérez-Camino et al., 2008). It is neverthless important to point out that the sampling of this set of sample was realized before that the EU Reg. 61/2011 came into effect;

2) products sold and labelled as EVOOs, but classified as "virgin" according to the sensory analysis (see **Table 6 E**, C2, C3, C11, C19, C24, C29, C32), for the presence of sensory defects (median of the most perceived different from zero and less or equal than 3.5, EU Reg. 61/2011), in particular winey and fusty, both often due to the poor quality of the raw materials (Morales et al., 2005; *paragraph 2.7*);

3) products sold and labelled as EVOO and actually characterized by the median of the most perceived defect equal to zero (as requested by EU Reg. 61/2011 for EVOO), but also characterized by other unpleasant/negative olfactory notes, in particular one that reminds the cardboard for the majority of the panellists (C4, C12, C22, C30, C34);

4) products sold and labelled as EVOO characterized by a peculiar olfactory note of eucalyptus and easily recognizable by the majority of the trained panellists (C1, C2, C4, C5, C6, C11, C12, C13, C17, C21, C22, C24, C27, C33, C34). These latter samples were all characterized by a ratio ethanol/(*E*)-2-hexenal above 1, except for sample C33.

Water

In olive oils, a very small amount of water (range between 500 and 1500 mg kg^{-1} oil) is present in micro-dispersion: such a polar fraction can have a very important and active role. A full description of the role of water and a proposal of a fast and cheap method based on the exploitation of dielectric properties of the oils elaborated with a chemiometric approach is described in the paper reported below.

I would really thank the engeeners' group of the Department of Agricultural and Food Science for their crucial contribution in the realization of such a scientific paper.

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Assessment of the water content in extra virgin olive oils by Time Domain Reflectometry (TDR) and Partial Least Squares (PLS) regression methods

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Partial least squares regression

ABSTRACT

In the present research we discuss a novel way to set up a predictive method for determining the water content of oil based on the Partial Least Squares (PLS) regression analysis of reflectometric signals. Ten different extra virgin olive oils with a water content ranging from 714 to 2008 mg of water/kg of oil were submitted to reflectometric measurement by means of a hand made probe connected to a digital sample oscilloscope with TDR functions interfaced with a PC. Limits of the classical approach based on the TDR electromagnetic theory in the prediction of these small water content differences were also discussed.

The results show that the suggested novel approach is able to predict the water content of very small quantities of oil (1.8 g) in a 3 ml translucent disposable PE cuvette by means of PLS regressions characterized by R^2 value up to 0.984 and a root mean square error of prediction of about 55 mg of water/kg of oil. The temporal region showing the best information content corresponded with the rise of the reflection of the probe end, but information highly correlated with the water content can be extracted from other temporal regions of the entire TDR signal.

1. Introduction

According to the International Olive Oil Council (IOOC, 2009) and the Codex Alimentarius (CODEX STAN, 1981), the level of moisture and volatile matter in extra virgin olive oils (EVOO) should be not higher than 0.2% kg/kg. The amount of water in commercial olive oils (from about 0.03% kg/kg to about 0.2% kg/kg), depends on factors related to the production process technologies such as the extraction and filtration procedures (Cerretani et al., 2010; Lozano-Sánchez et al., 2010). Water in oil is evenly distributed in the form of a stabilized micro-dispersion (Petrakis, 2006). Although it is not clear, the water presence (correlated to the oil bitterness and pungency) has a role in product stability and quality during storage (Lercker et al., 1994; Fregapane et al., 2006; Ambrosone et al., 2007).

Well known standardized methods for the measurements of the moisture content of olive oils are Karl Fischer titration (AOAC, 1998) and mass loss assessment by heating (both moisture and volatile content) (ISO 662, 1998). Since the cited methods are considerably time-consuming, more rapid spectroscopic solutions were studied for both off-line and on-line applications.

To date, due to the rapidity and simplicity of the measurement, Near Infrared (NIR) spectroscopy in combination with powerful chemiometric statistical tools was undoubtedly the most employed technique (Armenta et al., 2010). This solution was especially used for the assessment of the water content in olive fruit (Jimenez et al., 2000), olive pomace (Muik et al., 2004) or in olive oils during production processes (Bendini et al., 2007). Despite showing an appreciable accuracy, the tested predictive models were built from spectra acquired from samples characterized by high levels of water content (greatly higher that 0.2% kg of water/kg of oil).

Others spectroscopic methods focused on the use of NMR (Hatzakis and Dais, 2008) and FT-IR spectroscopy (Cerretani et al., 2010). By using the NMR method, values of the correlation coefficient up to 0.98 for the prediction of EVOO sample moisture content (moisture content ranged from about 0.2% kg/kg to about 0.8% kg/kg) were observed. A coefficient of determination R^2 of 0.89 was obtained through FT-IR spectroscopy for prediction of the water content in virgin olive oils and olive oils (water content ranged from about 290 to 1402 mg water/kg oil).

Since oil and water are characterized by really different levels of dielectric behavior, with dielectric constant ε' values from about 3–3.2 for edible oils and about 77 for water (measured at 1 MHz and 25 °C) (Lizhi et al., 2008), the investigation of the dielectric properties of EVOO can prove useful for the assessment of its water content even if it represents a modest percentage. As shown by the large amount of literature, the analysis of food dielectric properties were mainly used for non-destructive measurement of their moisture content (Nelson, 1991; Sumnu et al., 2005; Tang, 2005; Sosa-Morales et al., 2010). Product dielectric behavior can be studied by

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means of several different methods such as parallel plate capacitors, open ended coaxial probes, transmission lines and resonant cavities (Içier and Baysal, 2004). By using a parallel plate capacitor, Lizhi et al. (2008) investigated the effect of moisture content and fatty acid composition, together with frequency and temperature, on the dielectric properties of different edible oils. For corn oil, the authors showed a second order polynomial relationship between moisture content (ranging from 0.02% kg/kg to 0.31% kg/kg) and the dielectric constant ε', at 1 MHz and 25 °C.

Dielectric behavior can also be observed by analysing in the time domain the reflected signal from a generic load after the application of a fast rise time step from picoseconds to fractions of microseconds; in this way the reflected signal covers a wide range of frequencies (Cole, 1977). The technique, named Time Domain Reflectometry (TDR), was successfully used in the last 20 years for the assessment of soil water content and salinity (Topp et al., 1982; Dalton and Van Genuchten, 1986; Noborio, 2001) and for dielectric permittivity and conductivity measurements of different kinds of liquids such as alcohols (Fellner-Feldegg, 1968), carbohydrate solutions (Van Loon et al., 1995), microemulsions (Nozaki and Bose, 1990) and various materials (Pettinelli and Cereti, 2002). The dielectric parameters can be calculated from the visual or software automated interpretation of the reflected signal as a second order function of the time dependent reflection coefficient ρ. Fourier transforms of the reflected pulse were also used to determine the complex permittivity of beverages and solids foods (Miura et al., 2003; Kent et al., 2004).

Partial Least Squares (PLS) regression technique is a multivariate statistical tool that combines the features of the principal component analysis with the multiple linear regression method (Abdi, 2003). This technique allows us to build predictive models using a large number of data characterized by strong collinearity. The TDR electric signal, characterized by collinear data, can be processed by PLS regression in order to simplify its analysis. The PLS technique was already successfully used for the prediction of egg quality indices from dielectric signals (Ragni et al., 2008, 2010).

An accurate probe design is often required by the TDR technique to obtain a clear and easily readable shape of the reflectometric signal. A good probe construction is a crucial, preliminary condition for the subsequent, correct analysis of the transmission time along the electrical line represented by the probe rods. The manipulation and interpretation of the reflectometric signals is equally important to obtain satisfactory results. The identification of the incoming or the end of a reflexion, for example by means of tracing the tangent to the signal, can drastically compromise the goodness of the calculation of the dielectric parameters. Moreover, some effects, such as the relaxation of the dielectric under test at certain frequencies, can "round" the signal, a phenomenon that also compromises the detection of the end of the reflection (Robinson et al., 2003).

In the present paper, after a discussion according with a classical TDR approach, we suggest a novel method for measuring the water content in extra virgin olive oils based on the analysis of the signals via a PLS algorithm which makes the manipulation and the reading and interpretation of the electrical signal no longer necessary to extract suitable information. This study tests the goodness of the technique by making it able to assess very small differences in water content or in a range less than 1500 mg of water/kg oil.

2. Materials and methods

2.1. Chemical composition of the oil samples

The TDR measurements were conducted on ten different EVOO monocultivar samples from the same Italian region (Tuscany). The water content (WC) of the used oils was estimated in triplicate in a drying oven according to ISO 662, Method B (1998) by weighing 10 g of each sample; the results are shown as mg of water/kg oil. Although this method does not make it possible to distinguish between water and volatile compounds content, it should be accepted since the latter do not involve a substantial error because virgin olive oils only contain levels lower than about 50 ppm (Angerosa et al., 2004).

To chemically characterize the used oils, their fatty acid (FA) composition was also determined. This determination was carried out in triplicate as fatty acid methyl esters (FAMEs) after alkaline esterification and subsequent gas chromatographic analysis according to Bendini et al. (2006). Peak identification was accomplished by comparing the peak retention times with those of the GLC 463 FAME standard mixture (Nu-Chek, Elysian, MN), injected under the same gas chromatographic condition. FA were classified according to their unsaturation degree as saturated (SFA), monounsaturated (MUFA), and polyunsaturated fatty acids (PUFA).

2.2. TDR measurements

The layout of the TDR instrumental chain is shown in Fig. 1. The measurements of the reflectometric signal were carried out by means of a 2-terminal probe made of silver-plated copper wires covered by glass pipes (the contact metal/glass is not continuous along the entire wire length). The insulation with glass was considered necessary to prevent the effects of electrostatic discharge in this very highly sensitive device. The probe, inserted in a 3 ml translucent disposable PE cuvette (Sigma, Milan, Italy) filled with 1.8 g of the EVOO sample, was connected by a coaxial cable (50 Ω; 18 GHz) to a sampling head (Tektronix, SD-24) with TDR function fitted in an oscilloscope (Tektronix, 11801B). The oscilloscope was interfaced to a PC and the capture and data storage was conducted by using a Labview 8.2 program (National Instruments, NI, USA). The oscilloscope has a maximum resolution of 0.01 ps and 5120 stored data. In our case, the acquisition time step was 0.2 ps with a total acquisition time of 790 ps. This makes it possible to capture the reflections due to the interface air–oil, the end of the probe and some multiple reflections. The instrumental chain was switched on 3 h before the test to allow its thermal and electrical stabilization.

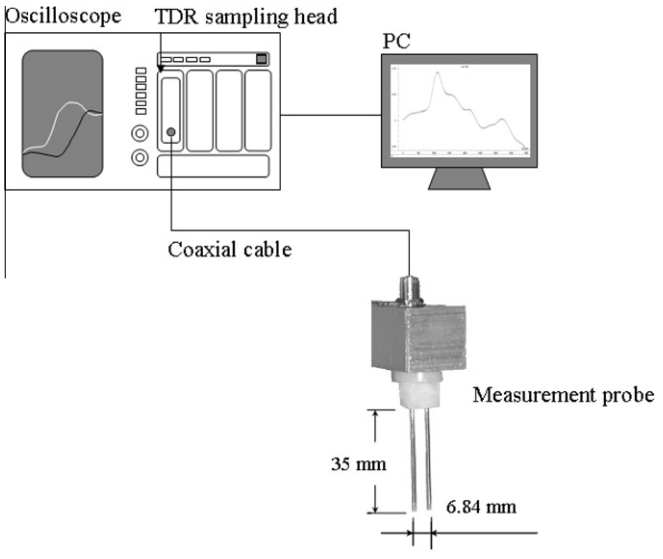

Fig. 1. Layout of the TDR instrumental chain.

For each EVOO sample, five replications were considered. Measurements were carried out both with the probe immerged in oil and in air.

2.3. TDR signal analysis

TDR signals were analyzed by following two different procedures. In the first approach, the classical theory of the time domain response was used to assess the water content (WC) of the EVOO samples. An alternative analysis based on PLS algorithm was considered in a second time in order to overcome possible limits of the classical procedure in the prediction of the WC in EVOO samples characterized by very small quantities and difference in the dielectric behavior.

2.3.1. Classical TDR approach

In this section, the TDR signals were analyzed by following a classical TDR method. The dielectric constants of the system characterized by the EVOO samples in contact with the glass pipes covering the 2-terminal metallic wires were calculated by analysing the voltage reflection coefficient (ρ) waveforms. These waveforms were obtained from the TDR signals according to the following equation:

$$\rho = \frac{V_r}{V_i} \quad (1)$$

where V_i (V) is the incident voltage (measured at the air–oil interface) and V_r (V) is the reflected voltage. The air–oil interface was identified for each EVOO sample in the point where the signal with air diverges from the signal with oil. To do that, the point was arbitrarily chosen where the ratio between the amplitude (V) measured in air and the amplitude (V) measured in oil was higher than 0.005.

By analysing the reflection coefficient waveforms, the TDR electromagnetic theory was used to calculate the dielectric constant κ of the dielectric system characterized by oil and materials surrounding the conductive probe terminal:

$$\kappa = \left(\frac{ct}{2L}\right)^2 \quad (2)$$

where "c" is the velocity of the electromagnetic wave in free space ($\sim 3 \times 10^8$ m s^{-1}), "t" is the travel time of the wave (from the interface air–oil to the end of the probe) and L is the probe length immersed in the oil.

The travel time of the reflection (t) was obtained according to the method described by Robinson et al. (2003). The method suggested that these values can be estimated from the rise of the reflection and from the use of tangent lines to the waveform as showed in Fig. 2.

In order to assess the length (L) of the probe portion inserted in the oil, micro-images were acquired by means of a portable 200× digital microscope. According to the example of the micro image showed in Fig. 3, two different lengths (L_1 and L_2) of the this probe portion was obtained by considering two different procedures. L_1 was calculated by averaging eight measures (from a_1 to a_8) conducted in the intersection points between the concave oil meniscus and the probe; L_2 was the average of two measures (b_1 and b_2) conducted respectively in the highest and the lowest bottom points of the meniscus. Each measure was individually extrapolated from the micro images by considering reference distances.

Two linear regression models between κ and WC was finally calculated and discussed respectively for L_1 and L_2.

2.3.2. Partial Least Square (PLS) regression analysis

The TDR signal (amplitude, V) (reflections from the interface air–oil to the probe end) obtained with each oil was previously subtracted from the signal obtained with only air. This process allows to minimize the unavoidable thermal and electric signal fluctuations in electronic high frequency (GHz) instruments and connection cables. It has no meaning in the classical TDR analysis but has to be assimilated to a correction method, such as the background measurement in NIR (Near-Infrared) spectral analysis (Osborne et al., 1993). The resulting signal, composed of 3950 acquisition points, represented the independent variables of the dataset characterized by a total of 50 signals (five replications for 10 different EVOO samples).

A first analysis was conducted to explore whether this time-domain signal, for the different oils, is linearly (or non-linearly) correlated with their water content. The aim of this analysis was to highlight where the possible correlation is higher and more robust (e.g. permanence of high R^2 values for a long time period). The method should make it possible to identify which temporal region contains more information related to the water content of oil (e.g. region of the reflection due to the probe end).

PLS predictive analysis of the water content (dependent variable) was then carried out for each of these temporal regions and

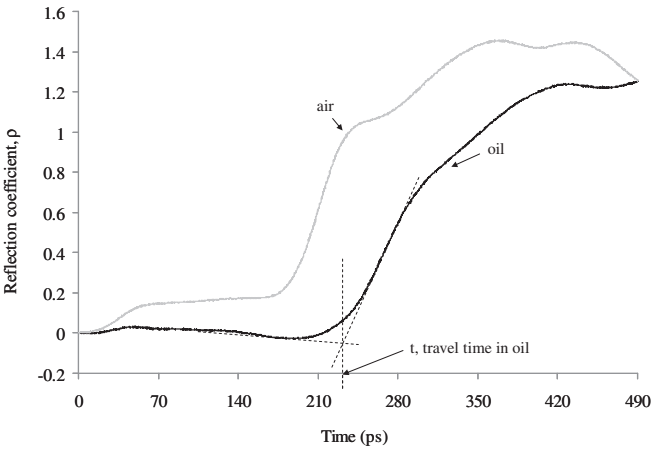

Fig. 2. Estimation of the travel time of the reflection (t) from the rise of the reflection and from the use of tangent lines to the waveform (in the graph only the first 490 ps of the signal are shown).

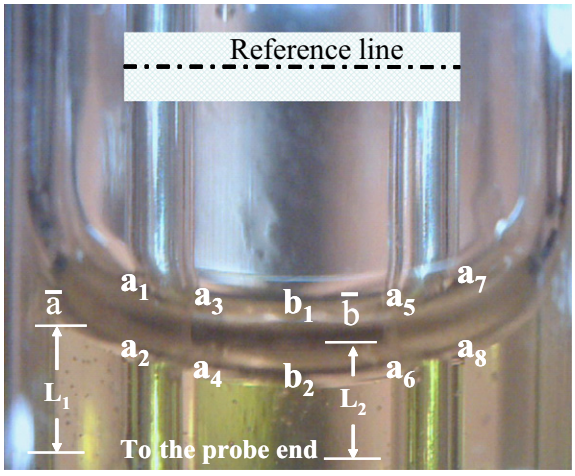

Fig. 3. Micro-image of the oil in contact with the probe used for the measurement of the length of the immersed probe. Note. From a^1 to a^8: distances from the probe end to the intersection points between the concave oil meniscus and the probe; the length L_1 (see Section 2.3.1) was calculated by averaging these eight distances. From b_1 to b_2: distances from the probe end to the highest (b_1) and the lowest (b_2) bottom points of the meniscus; the length L_2 (see Section 2.3.1) was calculated by averaging these two distances.

Table 1
Water content (WA) of the EVOO samples.

EVOO samples	WC (mg of water/kg oil)
A	1354 (33)
B	766 (61)
C	1605 (15)
D	2008 (11)
E	1805 (32)
F	714 (6)
G	1019 (29)
H	714 (16)
I	1278 (49)
L	1463 (3)

Values in brackets are standard deviations.

for the entire signal. The predictive power of the obtained models were tested by analysing the calibration results and by performing the "full cross validation" and the "test set validation". For the "full cross validation", the same data set were used to calibrate and validate the system: a sample of the entire dataset is excluded from time to time by the construction of the model and used to validate it. For the "test set validation", the dataset was randomly divided into two sub-samples, one to calibrate the system (70% of the entire dataset) and the other (30%) to validate it.

3. Results and discussion

3.1. Chemical composition of the oil samples

Water content (WC) and the fatty acid (FA) composition of the EVOO samples used for TDR measurements are respectively summarized in Tables 1 and 2.

The water content of the samples ranged from 714 to 2008 mg of water/kg oil with a minimum and a maximum difference between samples of 52 and 259 mg of water/kg oil, respectively.

In general, oil samples showed similar fatty acid compositions, in particular for those most typically present in extra virgin olive oils, such as oleic acid, palmitic acid and linoleic acid. Relatively high MUFA and low PUFA contents were also found in all the samples. FA percentages were within the range indicated by the Commission Regulation for EVOO category (1513/2001/EEC).

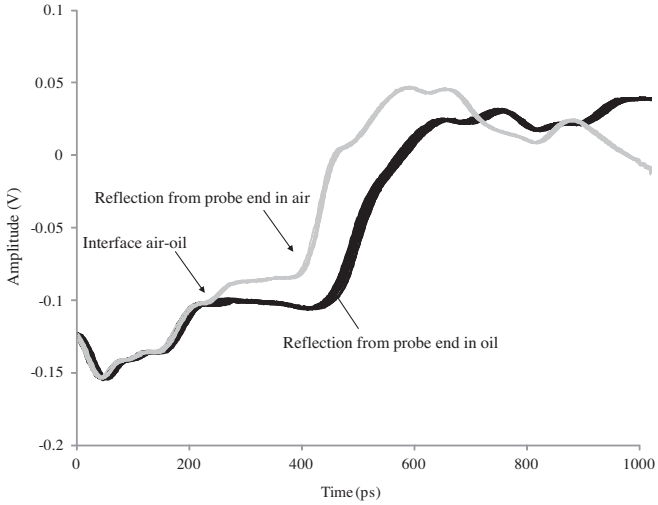

Fig. 4. TDR signals for measurements with air (gray) and with EVOO samples (black).

3.2. TDR signal analysis

TDR signals, both for measurements without and with oil, are plotted, for all the used oils, in Fig. 4. The shape of the signals for the different oils is roughly similar at visual detection with a maximum temporal shift in the order of 20 ps, so that the plotting of the signal for all oils generates a single thick line. The acquisition without oil reveals a moderate undesired shift between subsequent measurements due to unavoidable electrical response of the entire system. At the explored frequency (around 1 GHz), the observed instability of electronic apparata due to electric fluctuations and small air temperature and humidity changes has to be intended as normal (Cataldo et al., 2009). It requires that the measurement with oil is temporally conducted as close as possible to the measurement with air and the subtraction procedure above discussed to minimize the error.

An example of the Fast Fourier Transform (FFT) analysis of the TDR signal from the EVOO sample E (see Table 1) is shown in Fig. 5 (Labview 8.2 program, National Instruments, NI, USA). The frequency analysis was characterized by a peak at about 977 MHz.

Table 2
Fatty acid (FA) composition of the EVOO samples.

EVOO samples										
FA	A	B	C	D	E	F	G	H	I	L
C16:0	12.49 (0.20)	13.65 (0.09)	13.60 (0.79)	13.01 (0.15)	13.38 (0.12)	11.00 (0.13)	14.35 (0.18)	11.23 (0.13)	13 (0.03)	12.2 (0.08)
C16:1a	0.11 (0.02)	0.10 (0.01)	0.11 (0.03)	0.21 (0.01)	0.10 (0.02)	0.08 (0.01)	0.06 (0.00)	0.11 (0.00)	0.09 (0.01)	0.07 (0.00)
C16:1b	0.77 (0.03)	0.89 (0.02)	1.17 (0.33)	0.94 (0.01)	1.12 (0.02)	0.65 (0.02)	1.52 (0.02)	0.76 (0.02)	0.95 (0.01)	0.73 (0.01)
C17:0	0.05 (0.01)	0.05 (0.01)	0.05 (0.01)	0.13 (0.02)	0.06 (0.01)	0.04 (0.01)	0.04 (0.01)	0.10 (0.00)	n.d.	n.d.
C17:1	0.08 (0.01)	0.09 (0.01)	0.10 (0.01)	0.30 (0.03)	0.09 (0.01)	0.06 (0.01)	0.08 (0.00)	0.23 (0.01)	n.d.	n.d.
C18:0	2.27 (0.02)	2.23 (0.02)	2.08 (0.15)	1.86 (0.01)	2.01 (0.01)	2.32 (0.01)	1.90 (0.03)	2.11 (0.01)	2.01 (0.04)	1.99 (0.02)
C18:1 n−9	73.40 (0.23)	72.18 (0.15)	72.22 (2.97)	68.53 (0.15)	73.72 (0.13)	75.46 (0.30)	69.04 (0.22)	74.98 (0.24)	73.5 (0.29)	75.3 (0.08)
C18:1 n−7	2.50 (0.05)	2.55 (0.05)	2.83 (0.57)	3.04 (0.06)	2.86 (0.05)	2.25 (0.10)	3.41 (0.02)	2.54 (0.05)	2.8 (0.12)	2.44 (0.09)
C18:2	6.64 (0.02)	6.72 (0.02)	6.46 (1.49)	10.38 (0.05)	5.28 (0.03)	6.65 (0.01)	8.22 (0.02)	6.29 (0.01)	6.22 (0.00)	5.9 (0.03)
C20:0	0.43 (0.01)	0.39 (0.02)	0.39 (0.01)	0.36 (0.01)	0.36 (0.01)	0.40 (0.03)	0.37 (0.01)	0.44 (0.01)	0.6 (0.08)	0.59 (0.01)
C18:3 n-3	0.74 (0.01)	0.72 (0.00)	0.61 (0.01)	0.79 (0.07)	0.61 (0.03)	0.61 (0.02)	0.60 (0.01)	0.67 (0.00)	0.35 (0.07)	0.33 (0.00)
C20:1	0.35 (0.01)	0.31 (0.01)	0.28 (0.01)	0.32 (0.00)	0.29 (0.00)	0.33 (0.01)	0.30 (0.02)	0.42 (0.00)	0.28 (0.00)	0.32 (0.01)
C22:0	0.16 (0.00)	0.14 (0.00)	0.12 (0.01)	0.12 (0.01)	0.10 (0.00)	0.16 (0.03)	0.11 (0.00)	0.11 (0.07)	0.16 (0.02)	0.13 (0.01)
Monounsaturated	77.21	76.11	76.70	73.36	78.20	78.82	74.42	79.03	77.63	78.85
Polyunsaturated	7.38	7.43	7.07	11.16	5.89	7.27	8.81	6.96	6.57	6.23
Saturated	15.41	16.45	16.23	15.48	15.91	13.91	16.77	14.00	15.80	14.91

Values are in percentage (%) of the total fatty acid composition. Values in brackets are standard deviations. n.d. = not determined.

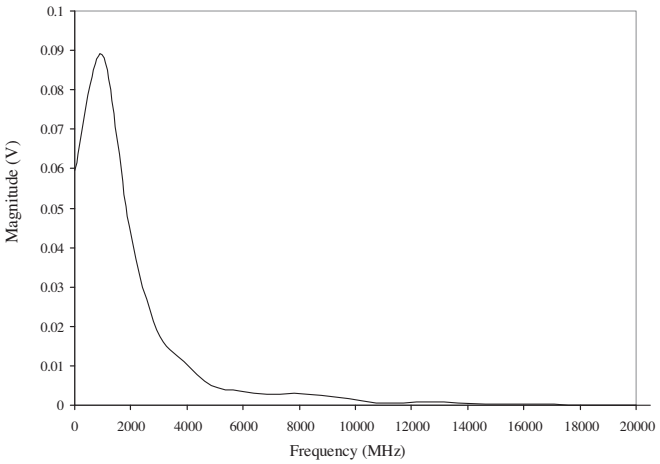

Fig. 5. Fast Fourier Transform analysis of the TDR signal (EVOO sample E).

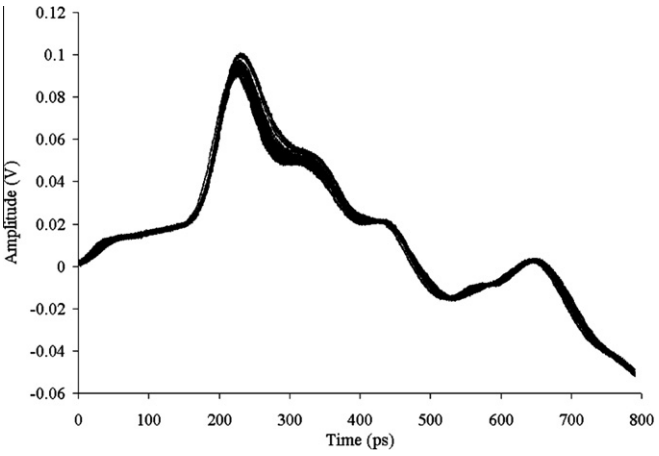

Fig. 8. Spectra obtained by subtracting the signal with oil to the signal with air.

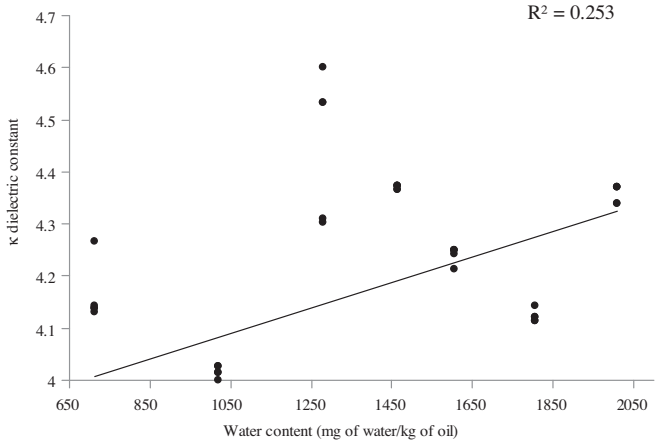

Fig. 6. Dielectric constant (oil and probe covering) calculated by means of L_1 values (see Section 2.3.1) vs water content of EVOO samples.

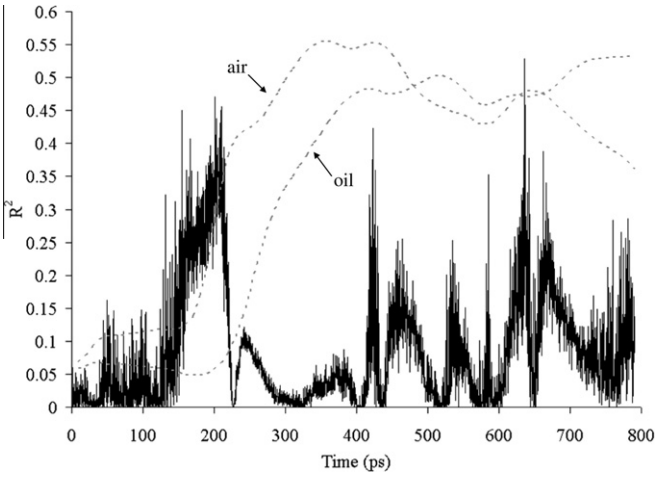

Fig. 9. Trend of the coefficient of determination R^2 for linear correlation with the water content calculated for each acquisition point. The electric signals (in dotted gray lines) are shown overlapped at the correspondence of the R^2 values to assess the dependence of the correlation with the different temporal reflectometric regions.

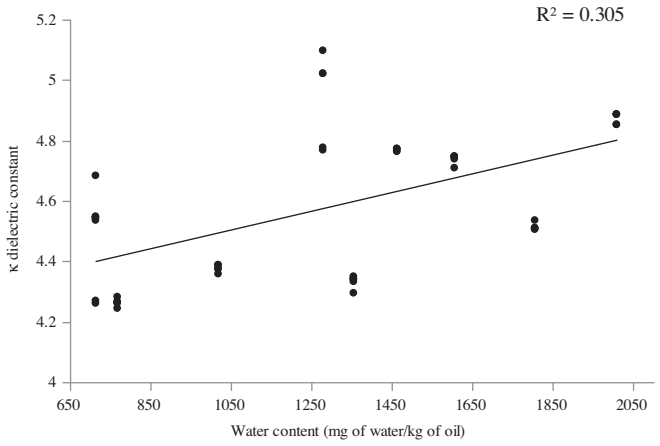

Fig. 7. Dielectric constant (oil and probe covering) calculated by means of L_2 values (see Section 2.3.1) vs water content of EVOO samples.

3.2.1. Classical TDR approach

The results of the linear regressions conducted between the dielectric constant κ of the system (oil and probe covering), and the water content (WC) of the EVOO samples are shown in Figs. 6 and 7. As emerged from the figures, κ appeared positively correlated with the WC of the oil sample. The low level of the accuracy of the model, expressed in terms of coefficient of regression R^2 (0.305 for L_1 and 0.253 for L_2) can be explained by considering the errors occurring in the estimations necessary in this method. We remark again that the insulation of the metallic wires of the probe is recommended to prevent electrical shock in the used high sensitive device to electrostatic discharge. On the other hand, here, we are not interested in the calculation of the dielectric constant of oil but to only estimate its water content.

From these figures it should be noted that the calculated dielectric constant is not of the oil but of the whole probe/oil system where the glass seems to has an important influence.

Determinations of length L, point where the signal with air diverges from that with oil, travel time by geometric method are crucial and different choices of the reference parameter can involve divergent results.

3.2.2. Partial Least Square (PLS) regression analysis

Fig. 8 shows, for the different oils, the data obtained by subtracting the signal with oil from the signal with air and used for the subsequent analysis.

Table 3
PLS model performances for the prediction of the water content (WC) from TDR spectra.

Range	Calibration			Full cross validation			Test set validation		
(ps)	R^2	PCs	RMSE	R^2	PCs	RMSE	R^2	PCs	RMSE
0–790	0.994	6	34.6	0.981	6	62.1	0.958	7	97.6
0–129	0.994	5	32.9	0.947	5	103.4	0.920	6	128.1
129–228	0.992	5	38.5	0.985	5	53.3	0.984	5	55.1
228–790	0.992	6	38.9	0.965	6	84.3	0.916	5	101.2

PCs = number of principal components. RMSE = root mean square error (mg of water/kg oil).

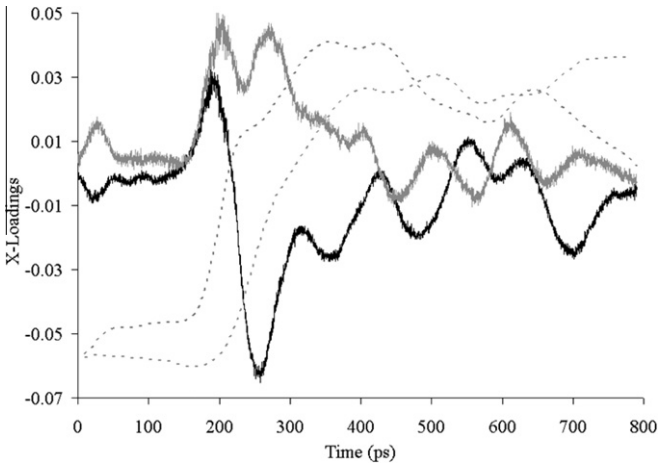

Fig. 10. X-loadings from multivariate analysis for the entire TDR acquisition spectrum (0–790 ps). PC1 (black) (x-exp = 65%), PC2 (gray) (x-exp = 29%). The electric signals (in dotted gray lines) are shown overlapped at the correspondence of the R^2 values to assess the dependence of the correlation with the different temporal reflectometric regions.

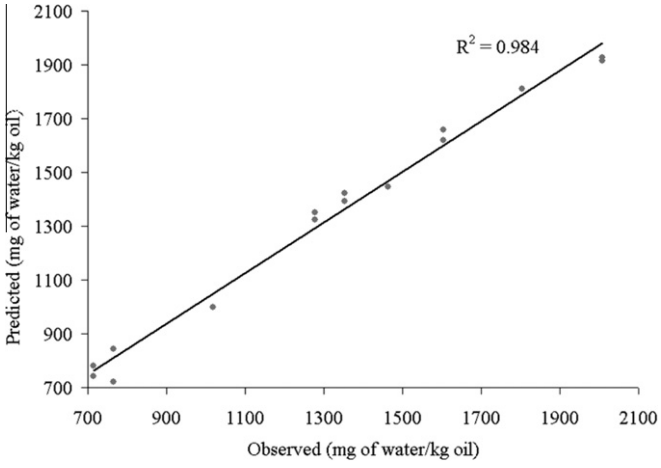

Fig. 11. Predicted values versus the observed value of the water content for test set validation of the PLS model (range = 129–228 ps) (see Table 3).

Fig. 9 shows the trend of the coefficient of determination for linear correlation of the data contained in the signals with the correspondent water content (the trend of the TDR signals without and with oil is overlapped on the graph to make it possible to associate the different temporal zones with their information content). It should be noted that, to be considered as containing information, a signal trait has to show R^2 values that remain high for the entire trait. In fact, a single high value of R^2, appearing as an insulated peak, can easily be casual. Thus, from Fig. 9 one can note that the most reliable information (R^2 from about 0.2 to 0.4) appears in the region of the rise of the reflection from the end of the probe while R^2 up to more than 0.5 in the zone of multiple reflections must be treated as suspicious. High values have to be considered suspicious if they appear as isolated peaks because it means that the correlation can be casual in the signal time history. More time the R^2 values remains high more probable it is due to a robust information in the signal. A quadratic instead of a linear correlation does not improve the fit.

Three temporal zones of the TDR signal were finally roughly identified and used for PLS analysis: from the reflection due to the interface air–oil to the incoming of the rise of the reflection due to the probe end (0–129 ps); from the previous point to the end of the rise (from 129 ps to 228 ps); and from the previous point to the end of the signal (from 228 ps to 790 ps).

The results of the PLS analysis conducted on these signal temporal regions are reported in Table 3.

A plot of the X-Loadings describing the weighting of the individual x-variables with regard to their contribution to the variance for the first two principal components is shown in Fig. 10.

From Table 3, Figs. 9 and 10 it appears clear that, as expected, the most useful information (R^2 = 0.984 in test set validation) for the prediction of the water content is extracted from the intermediate region, within the rise of the reflection from the end of the TDR probe. It is in this temporal trait, in fact, that the different dielectrics (oil and water) surrounding the line represented by the probe rods involve different propagation (reflection) velocities or, in other words, temporal shifts of the rise line. Fig. 11 shows the graph of the observed and predicted water content values. The maximum error in water content prediction is around 93 mg of water/kg oil while the root mean square error is around 55 mg of water/kg oil. The other temporal regions of the TDR signal also contain information that allows a prediction not much worse than the one mentioned above.

4. Conclusions

TDR signals analyzed by means of PLS algorithm appear useful to predict the water content in very small quantities of extra virgin olive oils also for moderate range of variation. The R^2 of the prediction was up to 0.992 and 0.984 in calibration and test set validation, respectively, if the signal corresponding to the rise time of the main reflection is considered. Also by using all the reflectometric signals, or, in other words, without any analysis addressed to select traits of the signal, the prediction remained high (R^2 = 0.958 in test set validation) with a root mean square error within 100 mg of water/kg oil. The PLS analysis of the TDR signal appeared to overcome the limits showed by using the classical approach for the assessment of WC in the EVOO samples where the identification of the reflection starting point and the interpolation curves are necessary. The considerable improvement can be attributed to the power of the multivariate method in the analysis of the entire signals (amplitude) without any subsequent manipulation.

Moreover, the suggested technique allows faster data management that could be advantageously implemented in off or on-line devices.

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Chapter 2. Quality and purity of EVOOS, towards a very recent parameter: the fatty acid alkyl esters

3.2.1 Definitions and classifications

Basically, oils obtained by olives can be distinguished in three main categories: virgin olive oils, olive oils coming from refining treatments (at least in part) and olive pomace oils, obtained by refining of the oil extracted from the olive pomace with a suitable solvent. The legal definitions of each commercial class of oils obtained by olives are reported by European Community (EEC Reg. 2568/1991) and by I.O.C. (IOC/T.15/NC No. 3/Rev. 6, 2011). According to IOC/T.15/NC No. 3/Rev. 6, 2011, virgin olive oils are "the oils obtained from the fruits of the olive tree solely by mechanical or other physical means under conditions, particularly thermal conditions, that do not lead to alterations in the oil, and which have not undergone any treatment other than washing, decantation, centrifugation and filtration". The E.U. regulations do not permit the trade of refined olive oil or refined pomace olive oils, but allow to market their blends with virgin olive oils. Actually, The E.U. (EEC Reg. 2568/1991) fixes only three categories of edible oils: extra virgin, virgin and olive oils, whereas the I.O.C. and the Codex Alimentarius also include the "ordinary" grade. The ordinary virgin olive oils are virgin olive oils which have a free acidity, expressed as oleic acid, not higher than 3.3 grams per 100 grams and other characteristics and analytical parameters in accordance with those fixed for such a category by the standards (IOC/T.15/NC No. 3/Rev. 6, 2011; CAC/RS 33-1970). Moreover, the Codex Alimentarius standard (CAC/RS 33-1970) does not consider oils not fit for human consumption. At now, the European Commission (EU Reg. 61/2011), the International Olive Council (IOC/T.15/NC No. 3/Rev. 6, 2011) and the Codex Alimentarius (CAC/RS 33-1970) provide similar indications and legal limits for almost all the analytical parameters related to quality and genuineness of olive oils. However, some differences between EC regulations and I.O.C. trade standards exist, since the I.O.C. must take into account the specific chemical and physical characteristics of oils obtained by olives produced by all the I.O.C. members all over the world (Angerosa et al., 2006). Some differences in composition may exist respect to olive oils produced in E.U. countries, mainly because of different cultivars and pedo-climatic conditions (Angerosa et al., 2006).

3.2.2 Quality and purity indexes: legal and unofficial chemical parameters

Among foodstuffs, probably EVOO is the one which needs the highest number of analyses for verifying its quality and genuineness. Most of the analytical "conventional" methods are based on the determination of peculiar chemical and sensorial parameters, which values must be within the ranges established by different organisms for assuring the belonging to a specific commercial class, as reported in the *paragraph 3.2.1*. In particular, the European Commission Regulation (EU Reg. 61/2011) is applied for olive oils sold in E.U. countries, while the Codex Alimentarius Standard (CAC/RS 33-1970) and the I.O.C. Trade Standards (IOC/T.15/NC No. 3/Rev. 6, 2011) are recognized and adopted in all the countries belonging to each of them (including E.U. countries). For this reason, all the analytical parameters defined in E.U. regulations or in I.O.C. and Codex Alimentarius standards can be defined as "legal" or "official" ones. Such adopted methods often require both time-consuming and expensive analytical procedures. On the contrary, the EVOO sector demands fast, reliable and affordable systems to monitor - often "on-line"- the product quality and authenticity. Actually, also alternative methods not provided by standardizing bodies, but proposed and carried out by researchers and olive oils experts exist. These latter can be either used to support the results obtained by official analyses or to get a faster and more complete assessment of olive oil quality and purity (Angerosa et al, 2006): we can define them as "unofficial" parameters.

The aim of this chapter is definitively not to consider all the quality and genuineness determinations, since many complete reviews have been already published about this topic (Firestone & Reina, 1996; Kiritsakis et al., 2002; Angerosa et al., 2006; Boskou, 2007; Arvanitoyannis & Vlachos, 2007; Frankel, 2010), but to focus on the most recent and important analytical developments, considering both "legal" and "unofficial" analytical parameters.

3.2.3 Quality evaluation

The quality of olive oils can be assessed on the basis of different physico-chemical and sensorial parameters, strictly due to both the hydrolytic and oxidative processes that take place in the fruits before the extraction, during the technological procedures for producing the oils and also along their preservation. On the basis of such quality parameters, the international organizations (E.U., I.O.C. and Codex Alimentarius) classify the oils obtained

by olives in different merceological classes (Angerosa et al., 2006), as described in *paragraph 3.2.1*. The determinations of free acidity, peroxide value, spectrophotometric absorbances in the UV region, halogenated solvents and the organoleptic assessment are the quality parameters common to all the above mentioned international organizations. In addition, the Codex Alimentarius and the I.O.C. standards include other quality determinations, such as the insoluble impurities and the detection of some metals. Recently, the I.O.C. adopted the "Determination of biophenols in olive oils by HPLC" as harmonized method for evaluating the quality of virgin olive oil (IOC/T.20/Doc. No. 29, 2009). Moreover, lately, both the E.U. regulation (EU Reg. 61/2011) and the I.O.C. standards (IOC/T.15/NC No. 3/Rev. 6, 2011) have included the determinations of fatty acid alkyl esters and waxes as quality index for EVOO (*paragraph 3.1.13*). Other analytical methods, in addition to the official ones, are useful tools to complete the assessment of olive oil quality (*paragraph 3.2.2*): they are related to the quality of the olives before the extraction and to the state of oxidation, the hydrolysis and the shelf-life of the oils (Angerosa et al., 2006). For istance, the determination of volatile compounds, partial glycerides, pigments, contaminants (Polycyclic Aromatic Hydrocarbons, PAHs), pesticides and the accelerated oxidation tests can be also considered as "unofficial" quality determinations.

3.2.4 Purity evaluation

Each category of oils obtained by olives (*paragraph 3.2.1*) has a different quality level, which correspond also to a different value and, subsequently, price; in general, EVOO is characterized by one of the highest economical value among all vegetable oils. Thereby, a temptation for fraudulent action has always been present, such as mixing virgin olive oil with cheaper or inferior quality products (Boskou, 2007). Olive oil, milk, honey, and saffron were the most common targets for adulteration reported in scholarly journals (Moore at al., 2012). The traditional methodologies to detect frauds in olive oils include measurements of some chemical and physical parameters - such as the iodine value and the refractive index determination - the adoption of specific color reactions, which can be useful in revealing adulterations with different seed oils. Nowadays, these methods have been completely replaced by modern chromatographic and spectrometric determinations that provide more information and may lead to more conclusive results (Angerosa et al.,

2006). In order to evaluate olive oil genuineness, basically it is important to consider the differences in olive oil composition respect to the other vegetable oils, but also among different categories of olive oils (such as between virgin and pomace olive oils). Moreover, the changes usually occurring for some specific components of the product due to technological process, such as refining, have to be considered. Chemical compounds that are affected by botanical origin are fatty acids, triacylglycerols and sterols whereas aliphatic alcohols, waxes, and the triterpene dialcohols are influenced by the kind of extraction (mechanical or by solvent). Refining process modifies the natural olive oil composition: some new compounds are produced and others are modified, such as sterols, sterolic hydrocarbons, and *trans* isomers of unsaturated fatty acids (Angerosa et al., 2006). Considering all these genuineness parameters, the limits adopted by the three different international bodies involved in the olive oil sector (E.U., I.O.C. and Codex Alimentarius) are nearly the same within each olive oil category (*paragraph 3.2.1*). Other methodologies to check and to guarantee olive oil genuineness, although not included in official methods, can usefully support attempts to reveal adulteration. These methods are based on the analysis of both triacylglycerols and non-triacylglyceridic components (alcoholic fraction, hydrocarbons, etc.) (Angerosa et al., 2006). Other analytical techniques, such as MIR and NIR spectroscopy, nuclear magnetic resonance (^{13}C-NMR, ^{1}H-NMR, ^{31}P spectra), carbon stable isotope ratio, Ft-IR, Ft-Raman spectroscopy, electronic nose, etc., usually carried out with a chemometric elaboration of data, can be also useful tools to detect adulterations (Angerosa et al., 2006; Arvanitoyannis & Vlachos, 2007). As example, calibration models able to rapidly assess the oil quality and genuineness can be created, allowing the implementations of spectroscopic techniques as in-line and/or on-line screening methods (*chapter 2*, see paper "Detection of low-quality..."). In order to discriminate authentic EVOO samples from adulterated oils, spectral data have to be elaborated by class-modeling and classification techniques. An explorative data analysis can be first carried out in order to identify outliers and to discriminate authentic from adulterated samples. Also the response of the material to applied electromagnetic fields can be exploited to set up instruments and methods for that purpose. Recently, some investigations on dielectric properties have been conducted on food oils aimed to detect differences in composition, presence of water, and adulteration detection (Lizhi et al., 2008; Lizhi et al., 2010). New measurement systems for evaluating the authenticity based

on the interactions between the electromagnetic fields and the EVOOs, followed by the data elaboration with multivariate statistic techniques / neural networks approaches can be investigated and set up using validate analytical techniques as data reference: in particular, impedenzometric, conductivimetric and Time Domain Reflectometry measurements with electrodes probe immersed in the product are very interesting (*paragraph 3.1.15*). Moreover, it is also important to underline that although a large number of analytical methods have been developed in the past decades to determine adulteration of EVOO, the literature in this field is still controversial and confusing (Frankel, 2010).

3.2.5 Recent legislative modifications in the olive oil sector in European Union

In E.U., the two most important recent legislative modifications that has effect of law related to the olive oil sector are the EU Reg. 61/2011 and the EU Reg. 29/2012. The first one is the last amendment of the EEC Reg. 2568/1991 and establishes the different categories of olive oils, supporting it also with new legal parameter (such as the determination of fatty acid alkyl esters, see *chapter 2*, paper "Detection of low-quality" and *paragraph 3.1.13*) for characterizing and classifying them. In January 2012, an important implementing regulation on marketing and labeling standards of olive oil (EU Reg. 29/2012) came also into effect. This regulation sets out special requirements applicable to olive oil, complementing of those set out in the previous E.U. Regulation (EU Reg. 1169/2011) on providing food information to consumers, thanks to the adoption of specific standards for the retail-stage marketing.

3.2.6 Actual incidence of the frauds on the Italian olive oil market

ICQRF (Italian acronym, that means "Ispettorato Centrale della tutela della Qualità e della Repressione Frodi dei prodotti agroalimentari") is the official Italian organism of the Ministry of Agricultural, Food and Forestry Policies with the specific role of monitoring, safeguarding and repressing illegal procedures related to the quality and genuinity of all the foodstuffs. The data provided by ICQRF show the incidence of the frauds related to EVOOs within the Italian market (ICQRF, 2012). It is interesting to underline that only a 6% out of all the monitoring controls carried out inside the olive oil sector within the period 2007-2011, were actually proved as irregularities by law. As reported in **Figure 9 E**, most of

them were related to alterated/aged oils (45.2%), followed by illegal mixture between olive oils and seed oils (25.6%) and illegal mixture between virgin olive oils and refined ones (19.6%). In order of frequency, these latter are followed by illegal mixture with olive oils and pomace oils (5.7%) and by the presence of pesticides in organic oils (3.9%).

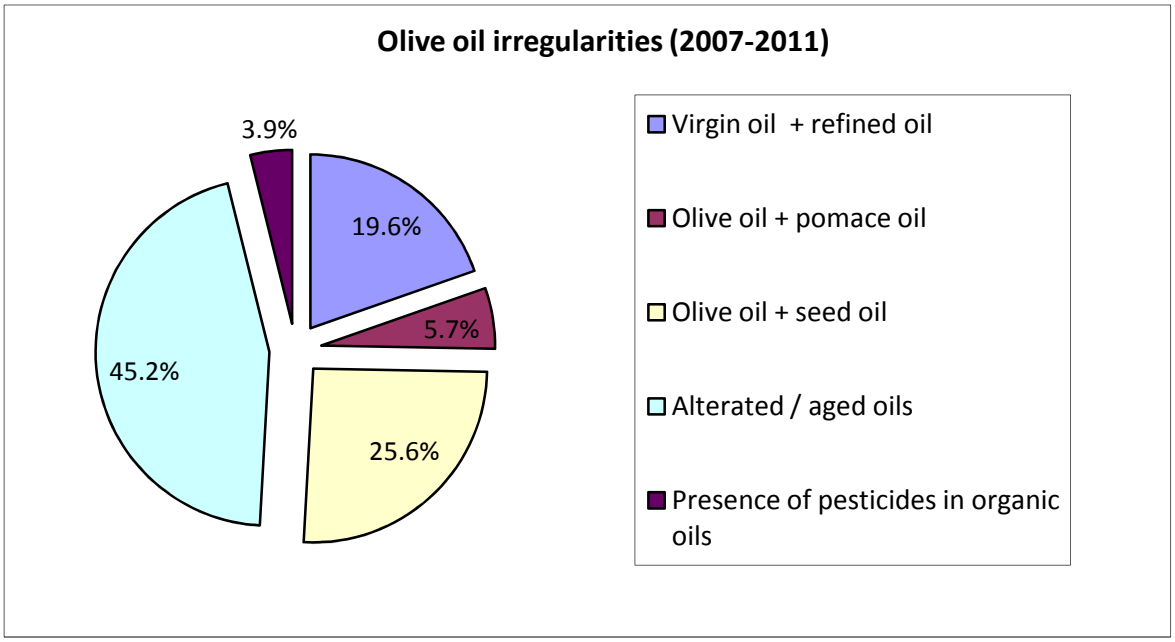

Figure 9 E. *Distribution of the incidence of the irregularities (% on the total) found by ICQRF in Italy between 2007 and 2011. Confidential data provided by ICQRF (ICQRF, 2012).*

3.2.7 References (Chapters 1 and 2)

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Detection of low-quality extra virgin olive oils by fatty acid alkyl esters evaluation: a preliminary and fast mid-infrared spectroscopy discrimination by a chemometric approach

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Summary A set of eighty-one extra virgin olive oils (EVOOs) was analysed according to the new quality parameters relative to the total amount of methyl and ethyl esters of fatty acids [Σ (FAMEs + FAEEs)] and the ratio between ethyl and methyl esters [ratio of FAEEs/FAMEs (RFF)]. Acquisition of the mid-infrared spectra was also performed by Fourier Transform Infrared Spectroscopy (FT-IR). Chemical and spectroscopy data were chemometrically elaborated, and FT-IR coupled by Partial Least Square (PLS) methodology was developed. Results were statistically similar to official procedure in terms of analytical performance for Σ (FAMEs + FAEEs) and RFF in EVOOs: a good agreement between predicted and actual values on calibration data sets was found (0.98 and 0.83, respectively) and the limit of quantification was low enough (29.3 mg kg^{-1}) considering the actual limits for Σ (FAMEs + FAEEs). This new approach, time-saving and environmentally friendly, can be considered as a useful tool for screening procedures.

Keywords Extra virgin olive oil, fatty acid alkyl esters, FT-IR spectroscopy, low-quality oils., mildly deodorized olive oils.

Introduction

Extra virgin olive oil (EVOO) is characterised by one of the highest economic value in comparison with other vegetable oils, thanks to its well-known nutritional and sensory qualities (Velasco & Dobarganes, 2002). Unfortunately, EVOO is also easy to falsify: because of its prestige, it has always been illegally mixed with cheaper and low-quality oils (Harwood & Aparicio, 2000), especially to obtain EVOO sold in supermarkets and discount stores at low cost (Bendini *et al.*, 2009a). The so-called lampante low-quality olive oils cannot be used as raw foodstuff for direct human consumption, as they have an acidity level that is too high, and their volatile profile is characterised by 'soft' off-flavours, derived from low-quality olives or from inappropriate procedures during oil extraction or storage. Deodorization applying a 'mild' technology, developed under vacuum and at low temperature, is able to remove unacceptable defects (mainly winey-vinegary, fusty-muddy sediment and musty), avoiding the formation of chemical traces in the oils exploited as forensic proof of fraud (Cerretani *et al.*, 2008). In the last decades, several analytical methods have been proposed to detect such low-quality EVOOs and their admixtures, such as the determination of diacylglycerols and pheophytin (Serani & Piacenti, 2001a; Serani *et al.*, 2001b), the amount of water present in the micro-emulsion of the oil and study of the volatile profile, especially taking into account of the ratio between ethanol and *E*-2-hexenal (Cerretani *et al.*, 2008; Bendini *et al.*, 2009a). In addition to the proposed analytical methods, one of the most reliable techniques seems to be determination of fatty acid methyl and ethyl esters (fatty acid alkyl esters, FAAEs) as methyl esters of fatty acid

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(FAMEs) and the ethyl esters of fatty acids (FAEEs) which are present in the waxy fraction of olive oils (Mariani & Fedeli, 1986; Mariani *et al*., 1991, 1992; Mariani & Bellan, 2008). In good quality EVOOs, FAMEs and FAEEs are present in very small amounts, while they are present in higher amounts in virgin, lampant olive oils (Mariani & Bellan, 2011) and in second-olive processing oil (the so-called repaso) (Cerretani *et al*., 2011). Actually, these compounds are formed as a consequence of degradation and fermentation processes of low-quality olives, which can be overripe, damaged or simply poorly preserved before they are processed (Biedermann *et al*., 2008). These alterations lead to a production of short chain alcohols by the degradation of the pectins by endogenous pectin-methyl-esterases (methanol) and the aerobic metabolism of microorganisms (ethanol) (Biedermann *et al*., 2008). At the same time, lipolysis of triacylglycerols with liberation of free fatty acids may occur. In these conditions, the formation of FAMEs and FAEEs by esterification can take place: this reaction is catalysed by the temperature reached during the 'mild' deodorization step (Perez-Camino *et al*., 2008), while it does not seem to occur during the storage of high-quality EVOOs (Mariani & Bellan, 2011). In reality, these molecules have two different but complementary meanings: first they are related to the olive (and consequently the olive oil) quality (Biedermann *et al*., 2008), and FAAEs can be considered as 'virtual' markers of possible 'mild-deodorization', as they resist and are not removed by this illegal treatment. In fact, the contemporary presence of a high level of FAAEs, without a clearly perceivable sensory defect, can be reasonably explained by the application of 'mild-deodorization'. Different analytical methods have been performed for the determination of FAMEs and FAEEs in VOO in recent years: the older ones are done by solid phase extraction (SPE) (Perez-Camino *et al*., 2008), while some modifications have been added (Bendini *et al*., 2009b and Cerretani *et al*., 2011). As of April 2011, the determination of alkyl esters, first proposed by the IOC (COI/T.20/DOC. NO. 28, 2009), became an official method adopted by the European Community law (EC Reg. 61/2011 and corrigendum). This method is based on solid–liquid chromatography (LC) by traditional glass column for isolating the fraction containing alkyl esters and waxes, with the aim to assign the evaluated sample to the commercial category of EVOO. Indeed, for EVOO, the concentration of the sum of FAMEs and FAEEs [Σ (FAMEs + FAEEs)] cannot exceed 75 mg kg^{-1} (EC Reg. 61/2011 and corrigendum). If Σ (FAMEs + FAEEs) is between 75 and 150 mg kg^{-1}, the oil can be considered as EVOO only if the ratio of FAEEs/FAMEs (RFF) is ≤ 1.5 (EC Reg. 61/2011 and corrigendum). It is interesting to underline that the European law permits a higher amount of alkyl esters for EVOO (between 75 and 150 mg kg^{-1}) only if RFF is lower or equal than 1.5, as the FAMEs are typically formed with the technological transformation of overripe olive fruits (Biedermann *et al*., 2008).

The determination of food authenticity and the detection of adulteration are current problems of increasing importance in the food industry. EVOO adulteration was extensively studied because it is a high added value product and adulteration employs more sophisticated methods nowadays (Arvanitoyannis & Vlachos, 2007). Traditionally, the chemical treatments of the samples required for determining authenticity of EVOOs are complex, expensive, time-consuming and tedious. On the contrary, FT-IR is a highly useful molecular spectroscopy technique because it is rapid, non-destructive, simple to perform and does not require sample pre-treatment. The employment of several multivariate methods [like principal component analysis, canonical analysis, linear discriminant analysis, cluster analysis, partial least squares (PLS), and surface response methodology] has become a prerequisite for several applications related primarily to food quality control in terms of authentication/adulteration, thanks to a substantial simplification of the classification/grouping task (Tzouros & Arvanitoyannis, 2001). Among the possible analytical approaches, the Fourier transform mid-infrared (FTIR) spectroscopy combined with multivariate chemometric procedures has been used by several authors for predicting the level of authentication/adulteration in EVOO samples based on chemical composition. This approach has been apply to correctly discriminate among genuine and adulterated olive oils containing soybean, corn, olive pomace oils or between pure EVOOs and the same oils adulterated with sunflower oil or with refined oils or with walnut or hazelnut oil (Arvanitoyannis & Vlachos, 2007; Özdemir & Öztürk, 2007; Gurdeniz & Ozen, 2009; Lerma-García *et al*., 2010; Maggio *et al*., 2010). Several chemometric approaches were used to treat variables of olive oil samples to classify extra virgin and ordinary olive oil samples and partial least-squares regression (PLS) resulted in higher prediction success rates (Tzouros & Arvanitoyannis, 2001). Nevertheless, to the best of our knowledge, there are no investigations regarding the application of this technique for a quick check of quality limits for EVOOs introduced by the new regulation. For this aim, the total amount of methyl and ethyl esters [Σ (FAMEs + FAEEs)], and the ratio between ethyl esters and methyl esters (RFF) of a set of eighty-one EVOO samples, sold in Italian supermarkets, were evaluated and compared with the limits proposed by the EC Reg. 61/2011 and corrigendum. Next, correlation between these chemical parameters and spectroscopy data of the oils was performed to detect low-quality EVOOs with a statistical model.

Materials and methods

Samples

A set of eighty-one EVOO samples was purchased from local supermarkets, where they were sold (and labelled) as EVOOs at low cost. The samples were bought in two different years, and the method used to evaluate the alkyl esters followed the historical evolution of the method, first reported in the literatures and then adopted by the European Community law (EC Reg. 61/2011 and corrigendum).

Apparatus

Gas chromatography analyses of alkyl esters were performed using a Carlo Erba MFC 500 (Carlo Erba, Milan, Italy) instrument equipped with a flame ionization detector (FID).

The FTIR spectra were acquired on a Tensor 27™ FTIR spectrometer system (Bruker Optics, Milan, Italy), using a Rocksolid™ interferometer and a DigiTect™ detector system. The coupled attenuated total reflectance (ATR) accessory (Specac Inc., Woodstock, GA, USA) was equipped with a ZnSe 11 reflection crystal.

Materials, reagents and standards

The SPE cartridges (6 mL) STRATA Si-1 Silica (55 μm, 70 Å) packed with silica gel phase (1000 mg) were obtained from Phenomenex (Torrence, CA, USA). The silica gel stationary phase (60–200 mesh) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The standard used for FAAEs quantification [heptadecanoic acid methyl ester (C17:0ME)] was acquired from Sigma-Aldrich, as were methyl pentadecanoate, ethyl pentadecanoate, methyl palmitate, ethyl palmitate, methyl oleate, ethyl oleate, methyl linoleate and ethyl linoleate, which were used to identify the alkyl esters. All solvents used were analytical grade (Merck & Co. Inc., Darmstadt, Germany).

Determination of FAMEs and FAEEs by gas chromatographic analyses

Methyl esters of fatty acids and FAEEs were extracted from oil samples by three different methods (Bendini *et al*., 2009b; Cerretani *et al*., 2011; EC Reg. 61/2011) and quantified by gas chromatographic analyses following the analytical procedure reported in EC Reg. 61/2011. For standardising and harmonisation of the results, FAAEs were referred to the same analytical standard (C17:0 ME) for all samples analysed; moreover, all the response factors related to the GC-FID were set to 1.000.

Extraction of the alkyl esters by traditional liquid chromatography (samples 1–46)

This extraction follows the method reported in COI/T.20/DOC. NO. 28 (2009) 'Determination Of The Content Of Waxes, Fatty Acid Methyl Esters And Fatty Acid Ethyl Esters By Capillary Gas Chromatography' and recently adopted as official law by the European community (EC Reg. 61/2011 and corrigendum). A 0.5 ± 0.0001 g of the sample was mixed with 0.250 mL of standard solution of the internal standard (methyl heptadecanoate, C17:0 ME, 0.02% m/v). Next, 15 g of silica gel were suspended in *n*-hexane and settled spontaneously into a glass column for LC (internal diameter 15 mm, length 30–40 cm). The settling was complete with the aid of an electric shaker to make the chromatographic bed more homogeneous. Then, 30 mL of *n*-hexane were percolated to remove any impurities. The samples were transferred to the chromatography column with the aid of two 2-mL portions of *n*-hexane. The solvent was allowed to flow to 1 mm above the upper level of the absorbent. The alkyl esters were then collected eluting 220 mL of a freshly prepared mixture of *n*-hexane/ethyl ether (99:1, v/v) at a flow of about fifteen drops every 10 s. The resultant fraction was evaporated in a rotary evaporator until the solvent was almost removed, drying the last 2 mL under a weak flow of nitrogen. The fraction containing the methyl and ethyl esters was diluted with 2 mL of *n*-heptane, and 1 μL of this solution was injected.

Extraction of the alkyl esters by SPE (SPE 1) (samples 47–75)

For these twenty-nine samples, the extraction method followed the conditions described by Bendini *et al*. (2009b). A 0.2 ± 0.0001 g of oil sample was mixed with 250 μL of standard solutions of C15:0 EE and C17:0ME (both 50 μg g^{-1}), respectively, for the quantification of ethyl esters and methyl esters, and *n*-hexane was added to obtain a volume of 2 mL. This oil solution was split in two fractions of 1 mL and eluted separately. Silica SPE cartridges (1000 mg) were placed in an automatic vacuum elution apparatus and conditioned by passing 12 mL of *n*-hexane. Next, 1 mL of the oil solution was charged, and the solvent was pulled through at 0.5 mL min^{-1}, leaving the samples and the standards on the cartridge. The elution was made with 7 mL of the solvent mixture *n*-hexane:toluene (85:15, v/v), and this fraction was rejected. Next, the alkyl esters were collected by elution with 10 mL of the same mixture at a flow rate of 1 mL min^{-1}. The eluate was evaporated in a rotary evaporator at room temperature under vacuum until dry. The residue was dissolved in 200 μL of heptane, and a 1 μL of this solution was injected.

Extraction of the alkyl esters by SPE, with a different method (SPE2) (samples 76–81)
For these six samples, the extraction method followed the conditions described by Cerretani *et al.* (2011). A 1 ± 0.0001 g of oil sample was mixed with 500 μL of standard solutions of C17:0 ME, (200 μg g^{-1}) and 500 μL of standard solutions of lauryl arachidate (400 μg g^{-1}). Next, *n*-hexane was added to reach the volume of 5 mL. Silica SPE cartridges (1000 mg) were placed in an automatic vacuum elution apparatus and conditioned by passing 8 mL of toluene. Then, 0.5 mL of the oil solution was charged, and the solvent was pulled through at 0.5 mL min^{-1}, leaving the samples and the standards on the cartridge. The elution was made with 4 mL of the solvent mixture *n*-hexane/toluene (85:15, v/v), and this fraction was rejected. The alkyl esters were then collected by elution with 13 mL of the same mixture at a flow rate of 1 mL min^{-1}. The eluate was evaporated in a rotary evaporator at room temperature under vacuum until dry. The residue was dissolved with 200 μL of *n*-heptane, and 1 μL of this solution was injected.

GC condition of the analyses, followed for both extractions, by SPE and by traditional liquid chromatography
Compared to the official method, a slight modification in the programmed temperature of the oven was introduced to not exceed the maximum temperature of the capillary column. The official method employed a cold injector for direct on-column injection, while we set the temperature of the injector at 325 °C, with a split ratio fixed at 1:30. The capillary column was a ZB–5MS (Phenomenex) (30 m length × 0.25 mm i.d. × 0.25-μm-film thickness). Helium, at a flow rate of 1.2 mL min^{-1}, was the carrier gas. The oven temperature was programmed from 80 °C (kept for 1 min) to 140 °C at a rate of 15 °C min^{-1}, then raised to 325 °C at a rate of 4.5 °C min^{-1} and kept for 20 min. The FID detector was set at 325 °C. The amount of alkyl esters was expressed as mg of C17:0 ME kg^{-1} of oil. The average, for each sample, was calculated from three replicates.

Acquisition of FTIR spectra

Analyses were carried out in triplicate at room temperature. Spectra were acquired (thirty-two scans/sample or background) in the range of 4000–700 cm^{-1} at a resolution of 4 cm^{-1}, using OPUS r. 6.0 (Bruker Optics) software. The absorbance spectrum was collected against a background obtained with a dry and empty ATR cell. Each sample was uniformly spread throughout the crystal surface. Before acquiring each spectrum, the ATR crystal was cleaned with a cellulose tissue soaked in *n*-hexane and then rinsed with acetone.

Data analysis

Data were exported in an ASCII compatible OPUS 6.0 software format (using a OPUS MACRO). PLS models were computed on respective training set samples for each parameter. The calculations were executed by MVC1 routines (Olivieri *et al.*, 2004) written for Matlab (Mathworks Inc., Natick, MA, USA). The moving window of variable size strategy (Ferraro *et al.*, 2001) was also implemented using MVC1. For each parameter, samples were treated independently. The selection of samples for calibration and validation groups was made using the Kennard & Stone algorithm, in both cases.

Results

PLS models for determination of FAMEs and FAEEs

As previously stated, the determination of the Σ (FAMEs + FAEEs) was carried out by previous extraction using three different pre-treatments, namely traditional LC (samples 1–46) and extraction by SPE with two different methods (SPE1, samples 47–75 and SPE2, samples 76–81). Table 1 shows the three groups of samples and their concentrations for Σ (FAMEs + FAEEs) and RFF. To develop and validate a robust PLS, only samples analysed by the official extraction method (EC Reg 61/2011) (LC) were taken into account. The obtained PLS parameters were applied on other groups of samples (SPE1 and SPE2). The content of Σ (FAMEs + FAEEs) for the first group EVOOs, determined by capillary GC, was in the range 13–116 (mg kg^{-1}): seven of the forty-six samples were close to the limits fixed for EVOOs. Figure 1a shows the full IR spectral range 4000–650 cm^{-1} for EVOO

Table 1 Sample groups and related contents for Σ (FAMEs + FAEEs) and RFF

	LC samples	SPE1 samples	SPE2 samples
Number of samples	46	29	6
Mean of Σ (FAMEs + FAEEs) content (mg kg^{-1})	47.1	54.0	437.5
Σ (FAMEs + FAEEs) range (mg kg^{-1})	13–116	9–159	32–749
Σ (FAMEs + FAEEs) SD (mg kg^{-1})	24	41	286
Mean of RFF	1.1	1.4	3.0
RFF range	0.4–1.8	0.3–4.1	1.9–4.1
RFF SD	0.40	1.0	1.1

FAEEs, ethyl esters of fatty acids; FAMEs, methyl esters of fatty acid; LC, liquid chromatography; RFF, ratio of FAEEs/FAMEs; SPE, solid phase extraction.

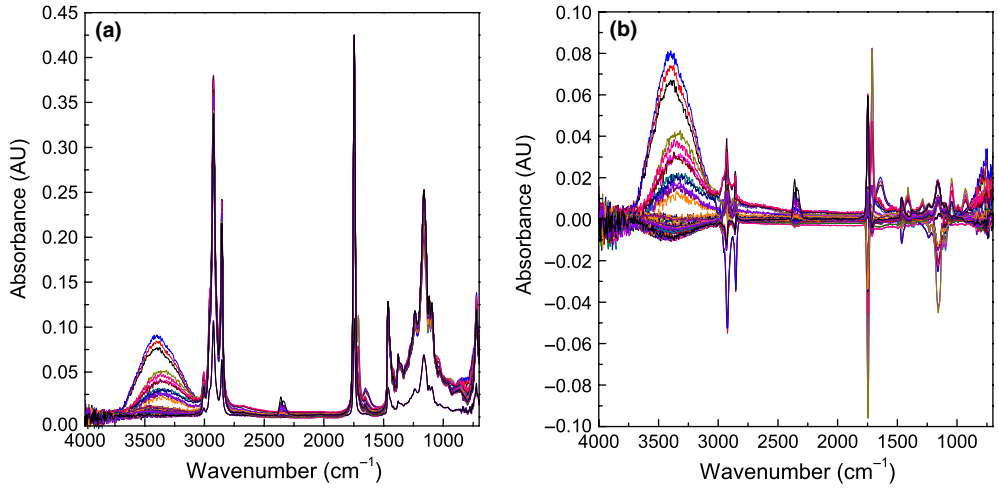

Figure 1 Full spectral range of Fourier transform mid-infrared (FTIR) for extra virgin olive oil (EVOOs) samples (a). Multiplicative Scatter Correction (MSC) and MC pre-treated FTIR spectra of EVOO samples (b).

samples. Different PLS calibration models were initially built by employing full spectra and reduced spectral ranges, obtained by a moving window of variable size strategy. However, none of these models provided an acceptable calibration, and predictions were unsatisfactory. Next, data were Mean-Centred (MC) and Multiplicative Scatter Correction (MSC) was employed to improve the performance of the method during calibration (pre-treated spectra are shown in Fig. 1b). Additionally, the spectral range was shortened to 2839.0–912.3 cm^{-1} to leave out regions where signal-to-noise ratio was very poor. By applying the Haaland and Thomas statistical criterion ($\alpha = 0.75$), the appropriate number of model dimensions was 6 (Haaland & Thomas, 1988). The results regarding calibration models between reduced pre-treated spectra and Σ (FAMEs + FAEEs) contents are reported in Table 2.

Table 2 Method parameters, statistical summary and figures of merits for Σ (FAMEs + FAEEs) PLS models

	LC	SPE1
Method parameters		
PLS factors	6	
Pre-treatment	MC – MSC	
Spectral range (cm^{-1})	2839.0–912.3	
Statistical summary: calibration		
Root Mean Square Deviation (RMSD, mg kg^{-1})	2.60	9.91
Percentage Relative Error In Calibration (REC, %)	5.98	17.35
R^2	0.98	0.95
Statistical summary: validation		
Mean recovery (%)	123	94
Relative Standard Deviation (RSD, %)	27	26
Figures of merit		
Sensitivity	0.00010	0.000026
Analytical sensitivity	0.36	0.19
Minimum detectable difference of concentration		
Σ (FAMEs + FAEEs), mg kg^{-1}	2.78	5.26
LOD Σ (FAMEs + FAEEs), mg kg^{-1}	8.8	7.0
LOQ Σ (FAMEs + FAEEs), mg kg^{-1}	29.3	20.9
Mean spectral residue (AU)	0.00029	0.000080

FAEEs, ethyl esters of fatty acids; FAMEs, methyl esters of fatty acid; LC, liquid chromatography; LOQ, Limit of Quantification; MSC, Multiplicative Scatter Correction; PLS, Partial Least Square; SPE, solid phase extraction.

PLS models for RFF determination

Following the same line of the previous developed models to estimate Σ (FAMEs + FAEEs), a PLS model, using LC samples, was built for detecting RFF. As in the PLS for Σ (FAMEs + FAEEs), MSC and MC spectral data pre-treatments were employed (Fig. 1b) to improve the performance of the model. Table 3 lists the calibration and prediction parameters used. Subsequently, the parameters of the calibration model obtained with LC samples were applied to SPE1 and SPE2 samples. As observed, the PLS model for RFF yields very good correlation coefficients and low Root Mean Square Deviation (RMSD) values; predictions were also very satisfactory, in terms of Relative Error in Calibration (REC) and recovery rate values (Table 3). The validation of the model was also carried out using the independent LC-validation mentioned above.

Table 3 Method parameters, statistical summary and figures of merits for RFF-PLS models

	LC	SPE1
Method parameters		
PLS factors	9	
Pre-treatment	MC – MSC	
Spectral Range (cm^{-1})	2839.0–912.3	
Statistical summary: Calibration		
Root Mean Square Deviation (RMSD, %)	0.15	0.17
Percentage Relative Error in Calibration (REC, %)	14	12
R^2	0.83	0.97
Statistical summary: validation		
Mean recovery (%)	103	107
Relative Standard Deviation (RSD, %)	17	36
Figures of merit		
Sensitivity	0.01	0.00
Analytical sensitivity	20.00	8.80
Minimum detectable difference of concentration	0.05	0.11

LC, liquid chromatography; MSC, Multiplicative Scatter Correction; PLS, Partial Least Square; RFF, ratio of FAEEs/FAMEs; SPE, solid phase extraction.

Discussion

PLS models for determination of FAMEs and FAEEs

The suitability of the proposed method for the objective was evaluated by analysing the model figures of merit and the results of validation samples. The values obtained for both RMSD and REC% were acceptable (Table 2). The Limit of Quantification (LOQ) was low enough considering the limits set by the EU for EVOO. Calibration R^2, which describes the goodness-of-fit of the predicted concentrations to their actual values was 0.98. The validation set exhibited almost quantitative recoveries that contain 100% in its confidence range (123 ± 27%). A Relative Standard Deviation (RSD) value of 27% shows the natural dispersion of the LC extraction method. The results (Fig. 2a) show good agreement between predicted and actual values on calibration and validation data sets. The slopes and intercepts of the curves depicted in this plot were close to unity and zero, respectively, indicating low bias and absence of systematic regression errors.

To demonstrate the inter-changeability and the transferability of the method (possibility of transference to another laboratory), a calibration model using a set of SPE1 samples and previously obtained PLS parameters (pre-treatments, PLS factors and spectral range obtained for PLS-LC model) was built, with satisfactory predictions. Almost quantitative recoveries (mean recovery = 94.38%, Table 2) were found, and high sample dispersion could be attributed to extraction procedure (Table 2 and Fig. 1b). The slope and intercept of the curves were also within required limits

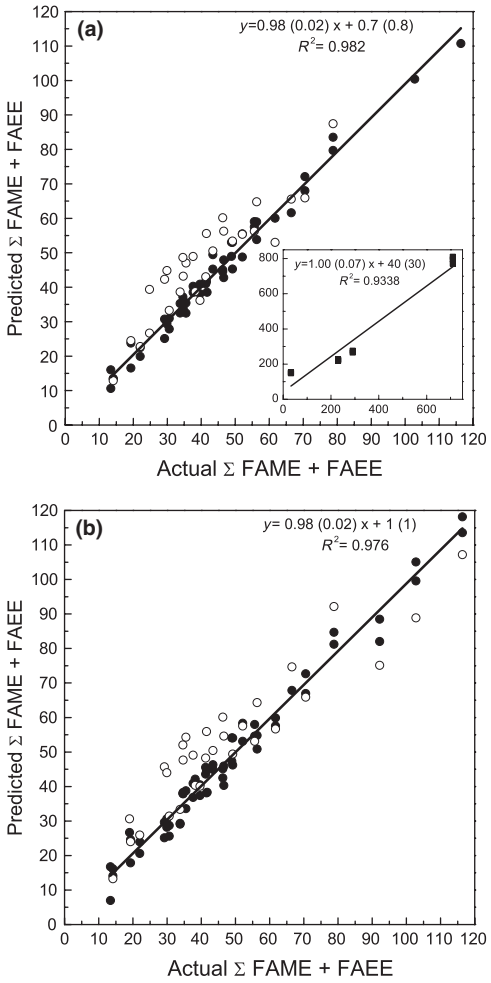

Figure 2 Actual vs. predicted Σ (FAMEs + FAEEs) for liquid chromatography (LC) calibration (●) and validation samples (○) (a). Prediction for SPE2 samples (a, subplot). Actual vs predicted Σ (FAMEs + FAEEs) content for SPE1 calibration (●) and validation samples (○) (b). Equations curves: Predicted = slope (SD_{slope})* Actual + intercept ($SD_{intercept}$). FAMEs, methyl esters of fatty acid; SPE, solid phase extraction.

(1 and 0). On the other hand, very few samples were analysed using the SPE2 method, making the development of a PLS model impossible. Nevertheless, SPE2 samples were analysed using PLS model developed with LC samples obtaining only an estimative prediction (Fig. 2a). As expected, almost all samples in the SPE2 group were out of the calibration range, making both figures of merit and statistical analyses meaningless.

PLS models for RFF determination

Precision and accuracy of RFF-PLS models were accessed by the evaluation of prediction errors of

(17%) and mean recovery (103%, mean value of sample recovery rates), and the latter were close to quantitative. Low bias and absence of systematic errors were demonstrated by the slopes and intercepts of the actual vs. predicted regression lines (Fig. 3a), which had values of unity and zero in their 90% joint confidence interval, respectively.

The same PLS parameters (pre-treatments, PLS factors and spectral range) were used to construct PLS models with SPE1 samples, and satisfactory results were found (Fig. 3b), which demonstrate the interchangeability of the method. Recoveries were similar to that obtained for LC samples (107 ± 37%, Table 2), but dispersion was higher. Consistent with the above, RFF values for SPE2 samples were out of the calibration range, being inappropriate to carry out predictions about this group. In addition, this group was too small to make a division in the validation and calibration groups: for this reason, SPE2 samples were not analysed.

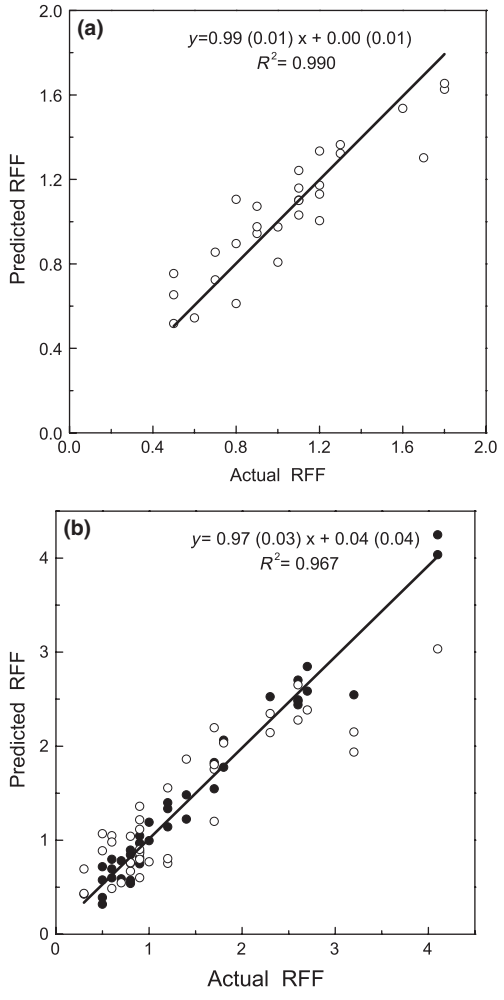

Figure 3 Actual vs predicted ratio of FAEEs/FAMEs (RFF) for liquid chromatography (LC) calibration (●) and validation samples (○) (a). Actual vs Predicted RFF content for solid phase extraction (SPE)1 calibration (●) and validation samples (○) (b). Equations curves: Predicted = slope (SD_{slope})* Actual + intercept ($SD_{intercept}$).

Conclusions

Fourier transform mid-infrared-PLS methodology was developed and demonstrated to be useful for analytical predictions of the Σ (FAMEs + FAEEs) content and RFF in EVOOs. The FTIR-PLS models provided results that were statistically similar to official procedures (LC), in terms of analytical performance, and are thus a useful tool for screening procedures. Moreover, the procedure permits high sample throughput, with significant time-saving, and is more environmentally friendly because no pre-treatment of samples was required. The results obtained here need to be confirmed through the acquisition of a larger set of olive oils, in terms of sample number and Σ (FAMEs + FAEEs) content, to increase the robustness of the model.

Acknowledgments

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Summary

Herein we analyze the quality level of 35 samples labelled as extra virgin olive oil (EVOO) characterized by different quality standards and sold in the Italian market at different prices. The basic quality parameters requested by EU Reg. 61/2011 to belong to the EVOO category (sensory analysis, free acidity, peroxide number, extinction coefficients, alkyl esters of fatty acids) were evaluated. Moreover, additional determinations related to the freshness level of the oils, assessed by the profile in 1,3- and 1,2-diacylglycerols (DAG) and evaluation of minor polar antioxidant compounds, were performed. The results support that a full chemical and sensory analytical approach, involving legal EU and other "unofficial" parameters, such as the ratio between 1,2- and 1,3-DAG, could be useful tools for discriminating high-quality products, sold at higher prices, from low-quality cheaper ones.

1. Introduction

According to EEC Reg. 2568/1991 and related amendments, there are four different commercial categories of oils obtained from olives that are suitable for edible purposes: extra virgin, virgin, blended olive oils composed of refined and virgin olive oils and olive-pomace oils. Among the former, extra virgin olive oil (EVOO) has nutritional and sensory characteristics that make it unique and a basic component of the Mediterranean diet (Caramia et al., 2012). It is sufficient to fulfill the basic minimal chemical and sensorial requirements for EVOO (EU Reg. 61/2011) to be defined, and labelled, as EVOO. In addition, the Codex Alimentarius (CAC/RS 33-1970) and the International Olive Council standards (COI/T.15/NC No. 3/Rev. 6, 2011) include other quality determinations, such as insoluble impurities and detection of some metals. Recently, the IOOC adopted the "Determination of biophenols in olive oils by HPLC" as a harmonized method for evaluating the quality of virgin olive oil (COI/T.20/Doc. No. 29, 2009). Both the EC (EU Reg. 61/2011) and IOOC standards (COI/T.15/NC No 3/Rev. 6, 2011) have also included the determination of fatty acid alkyl esters and waxes as a quality index for EVOO.

It is well-known that a broad variety of products exist within the classification of EVOO (Bongartz and Oberg, 2011), which also have broad range of prices, from 2€/kg (special offers in Italian supermarkets and discounts) up to 30€/kg (usually Protected Designation of Origin (P.D.O.), Protected Geographical Indication (P.G.I.), monovariety, organic EVOOs). This variety is due to diversity of geographical origin and cultivar of olives, period and type of harvest as well differences in the production process (Angerosa et al., 2006). Although P.D.O., P.G.I. and monovariety EVOOs can be considered as "niche" products, they are generally characterized by a higher aromatic complexity and uniqueness of specific sensory descriptors compared to commercial EVOOs sold at low-medium price. On the other hand, lower-priced EVOOs mainly show few unique flavors and rather standard or below-standard harmony, having almost no aromatic complexity (Bongartz and Oberg, 2011). It is also well-known that some olive oils fraudulently sold as EVOOs are mixed with other cheaper olive oils with lower quality and poorer characteristics, such as the so called "mildly deodorized" olive oils (Pérez -Camino et al., 2008). For these reasons, it is important to have an analytical scheme that can characterize the actual quality of an EVOO. Some analytical methods (sensory analysis performed with Panel test, free acidity, peroxide number, extinction coefficients, fatty acids, alkyl esters of fatty acids) are required by the European law (EU Reg 61/2011), while others parameters are "unofficial", but nevertheless important to obtain a complete assessment of the quality of the EVOOs. For example, to evaluate the freshness of an oil, the profile in 1,3- and 1,2-diacylglycerols (DAG) (Serani et al., 2001) can be taken into consideration. The total amount and the peculiar composition in phenolic compounds (Carrasco-Pancorbo et al., 2005) is also an important parameter for evaluating the sensorial and nutritional quality of the product (Bendini et al., 2007). Should be underlined that, recently, the EU Reg. 432/2012 introduced the possibility to report the health claim "Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress" in the label, if the olive oil contains at least 5 mg of hydroxytyrosol and its derivatives (e.g. oleuropein complex and tyrosol), per 20 g of product.

Herein, we evaluated the quality of oils labelled as EVOO and sold in the Italian market at different prices. Firstly, the basic quality parameters requested by EU Reg. 61/2011 to belong to the EVOO category were evaluated. Moreover, both officially recognized sensory and chemical parameters related to the quality of oils, together with the

determination of diacylglycerols profile and the content in phenolic compounds (total and *o*-diphenols) were assessed.

2. Materials and methods

2.1 Samples

Thirty-five oils labelled as EVOO were collected from the Italian retail market in January 2010. As reported in Table 1, samples were characterized by different harvest years, agricultural systems and quality standards. Furthermore, the samples were divided in three different categories according to the purchase prices: low price <5 €/kg, medium price 5-8 €/kg and high price >8 €/kg (respectively with the letters "L", "M" and "H", see the sample codes in Table 1). Before carrying out the chemical and sensory analyses, all EVOOs were stored in closed bottles under dark conditions, protected from light and kept at controlled temperature (± 12 °C). All samples were analyzed before their respective expiry dates.

2.2 Basic quality indices

Free acidity (FA), peroxide value (PV), and ultraviolet spectrophotometric indices (K_{232}, K_{270}) were evaluated according to the official methods described in EEC Reg. 2568/1991. All solvents and reagents were of analytical grade. The absorbance for calculating K_{232} and K_{270} (EEC Reg. 2568/1991) were carried out using spectrophotometer UV-VIS 1800 CE 230V (Shimadzu Co., Kyoto, Japan). Analyses were carried out in triplicate for each sample.

2.3 Determination of alkyl esters of fatty acids (FAAEs) by gas chromatographic analyses

2.3.1 Solvents, reagents standards and apparatus

Ethyl ether, *n*-hexane, and *n*-heptane, all of analytical grade, were purchased from Merck (Darmstadt, Germany). The silica gel (60-200 mesh) and the internal standard (methyl heptadecanoate) were obtained from Sigma-Aldrich (St. Louise, MO, USA). Gas chromatography analyses of alkyl esters were performed using a Carlo Erba MFC 500 (Carlo Erba, Milan, Italy) instrument equipped with a flame ionization detector (FID).

Table 1. Sample codes including an identification number followed by the class of price (L: low, <5 €/kg; M: medium, 5-8 €/kg; H: high, >8 €/kg), origin (Italy or other European countries, EU) and quality standards in terms of farming system (organic or conventional), denomination of origin (P.D.O. or P.G.I.), monovariety olives, for the 35 samples of EVOO.

CODE (NUMBER AND CLASS OF PRICE)	ORIGIN	QUALITY STANDARDS
1L	ITALY	
2L	ITALY	
3L	ITALY	
4M	ITALY	ORGANIC
5M	ITALY	
6M	ITALY	ORGANIC
7M	ITALY	ORGANIC
8M	ITALY	ORGANIC
9M	ITALY	ORGANIC
10M	ITALY	
11M	ITALY	
12L	ITALY	
13M	ITALY	ORGANIC
14M	ITALY	ORGANIC
15M	ITALY	
16M	ITALY	
17L	EU	
18L	EU	
19L	ITALY	
20L	ITALY	
21L	EU	
22L	EU	
23M	ITALY	PGI “TOSCANO”
24L	EU	
25M	ITALY	ORGANIC
26H	ITALY	PDO “MONTI IBLEI”
27M	ITALY	ORGANIC
28H	ITALY	PDO “DAUNO”, ORGANIC
29H	ITALY	PDO “COLLINE DI ROMAGNA”
30H	ITALY	PDO “COLLI MARTANI”
31M	ITALY	PDO “TERRE DI BARI”, ORGANIC
32H	EU	ORGANIC
33M	ITALY	MONOVARIETY “CORATINA”, ORGANIC
34M	ITALY	ORGANIC
35M	ITALY	

2.3.2 Procedure

The extraction of the fraction containing the alkyl esters of fatty acids (FAAEs) as well as their gas chromatographic determination were followed the method reported in COI/T.20/Doc. No. 28, 2010 “Determination Of The Content Of Waxes, Fatty Acid Methyl Esters And Fatty Acid Ethyl Esters By Capillary Gas Chromatography” and recently adopted as official law by the European community (EU Reg. 61/2011 and corrigendum). Analyses

were performed in triplicate for each sample. The amount of each compound was expressed as mg of methyl heptadecanoate (internal standard)/kg of oil.

2.4 Evaluation of the fraction of phenolic compounds

2.4.1 Extraction of polar phenolic extracts

2.4.1.1 Solvents, reagents and standards

Methanol and *n*-hexane, all of analytical grade, were purchased from Merck (Darmstadt, Germany)

2.4.1.2 Procedure

The liquid-liquid extraction (LLE) procedure was carried out according to the method of Valli et al. (2010). Briefly, 60 g of oil was dissolved in 60 mL of *n*-hexane, and the solution was extracted successively with four 20 mL portions of methanol/water (60:40, v/v). The combined extracts of the hydrophilic layer were brought to dryness in a rotary evaporator under reduced pressure at 40° C. This extraction procedure was performed in triplicate, splitting each sample into two fractions of equal amount prior to the evaporation step (thus obtaining six dry extracts for each sample). The dry extracts were first dissolved in 5 ml of methanol/water (50:50, v/v), and then diluted again 1:5 (v/v) and kept at -18 °C before spectrophotometric determinations.

2.4.2 Spectrophotometric assays: total phenolic compounds (TPCs), o*-diphenols and index of bitterness (BI K_{225})*

2.4.2.1 Solvents, reagents standards and apparatus

Gallic acid (purity = 99%) was obtained from Fluka (Buchs, Switzerland). Methanol (analytical grade) and Folin & Ciocalteu's phenol reagent were purchased from Merck (Darmstadt, Germany). Sodium carbonate and sodium molybdate dehydrate (for both, purity ≥ 99.5%) were purchased from Sigma-Aldrich. All the three tests were carried out using a UV-VIS 1800 CE 230V spectrophotometer (Shimadzu Co., Kyoto, Japan).

2.4.2.2 Procedure

The total amount of phenolic compounds (TPCs), *o*-diphenols, and extinction at 225 nm were determined using a UV-Vis 1800 Shimadzu spectrophometer (Kyoto, Japan). TPCs and *o*-diphenols were evaluated according to Singleton et al., 1965 and Mateos et al., 2001, respectively, using EVOO phenolic extracts. TPCs and *o*-diphenols were detected at 750 and 370 nm, respectively, and both were quantified using calibration curves made

with solutions of gallic acid at different concentrations (r^2=0.997 and r^2=0.998, respectively). Data were expressed as mg gallic acid/kg oil (*n* =3). Chemical evaluation of bitterness of polar phenolic extracts was carried out with spectrophotometric evaluation at 225 nm of the phenolic extract (Gutiérrez-Rosales et al., 1992). Three replications were performed for each sample.

2.5 Determination of the fatty acid composition by gas chromatographic analysis

2.5.1 Solvents, reagents, standards and apparatus

Methanol, *n*-hexane, (both of analytical grade) and potassium hydroxide (purity = 90 %) were purchased from Merck (Darmstadt, Germany). The gas chromatographic determination was performed by using a GC equipped with a flame ionization detector (Clarus 500, Perkin Elmer Inc., Shelton, CT, USA).

2.5.2 Procedure

Fatty acid methyl esters (FAME) from the oil samples were obtained by alkaline treatment by mixing 0.05 g of oil dissolved in 2 mL of *n*-hexane with 1 ml of 2 N KOH in methanol (Christie, 1998). Gas chromatographic analyses were carried out according to Rotondi et al. (2004). Results were expressed as % of FAME on the total amount of FAMEs. Moreover, the ratio between oleic acid and linoleic acid (OA/LA) was calculated for each sample. The mean values were calculated considering three replications for each sample.

2.6 Gas chromatographic determination of total diacylglycerols (DAG) and major 1,2-DAG and 1,3-DAG

2.6.1 Solvents, reagents, and standards

Pyridine (purity = 99.8%), hexamethyldisilazane (purity = 99.9%), dilaurin mixed isomers (purity ≥ 99.9%), and chlorotrimethylsilane (purity ≥ 97%) were purchased from Sigma Aldrich (St. Louis, MO, USA). *n*-hexane was purchased from Merck (Darmstadt, Germany). The GC was a Carlo ErbaMFC500 (Carlo Erba, Milan, Italy) equipped with a FID detector.

2.6.2 Procedure

DAG were determined according to a modified version (Bendini et al., 2009) of the method suggested by Serani et al. (2001). The GC column was a Rtx-65TG (Restek, Bellefonte, PA) fused silica capillary column (30 m length, 0.25 mm i.d., 0.10 μm film

thickness) coated with 35% dimethyl-65% diphenyl polysiloxane. Results were quantified with respect to dilaurin as an internal standard and expressed as g of dilaurin in 100 g of oil (*n*=3).

2.7 Sensory analysis

Sensory analysis was carried out in accordance with EEC Reg. 2568/1991 and EC Reg. 640/2008. The samples were tasted under blinded conditions and in two replicates by a panel consisting of 9 fully trained tasters. The profile sheet was the official one established by the EC Reg. 640/2008. Tasters were also requested to evaluate the presence of "other positive attributes", choosing from those established by the IOC for P.D.O. EVOOs (COI/T.20/Doc. No. 22, 2005), and also to indicate the overall-liking for each sample, as required by the old profile sheet reported in EEC Reg. 2568/1991, all on 10-cm continuous scales, from low to high intensity. As requested by EC Reg. 640/2008, the median values of each sensory attribute were considered and the value of the robust variation coefficient that defines them must be no greater than 20 %.

2.8 Statistical analysis

The statistical software XLSTAT 7.5.2 version (Addinsoft, USA) was used to perform one-way analysis of variance (ANOVA) at a 95% confidence level ($p<0.05$). The sensory attributes evaluated by the Panel (see Table 2a and 2b), the chemical results (see Table 3), and the information about the class of price (see Table 1) for all samples were statistically elaborated by performing a Principal Component Analysis (PCA). Before carrying out the PCA, data were also standardized using the biased standard deviation and normalized (rescaled from 0 to 1).

3 Results and Discussion

3.1 Sensory analysis

Sensory analysis, as required by EEC Reg. 2568/1991 and successive amendments, is a fundamental tool to establish the grading of oils obtained from olives, in terms of commercial classes (EC Reg. 640/2008). In this investigation, 34 of 35 samples were classified as EVOO, while one (sample 20L) was a lampante olive oil, since the median of the defect of rancid was above 3.5. According to the median values of the sensory positive

attributes fruitiness, bitterness, and pungency (EC. Reg. 640/2008) (see Table 2a and 2b), it was possible to split the samples in 3 groups that correspond to the following adjectives, on the basis of the ranges of values adopted in the optional terminology used for labeling purposes (EC. Reg. 640/2008):

- “intense” when the median of the attribute is greater than 6;
- “medium” when the median of the attribute is between 3 and 6;
- “light” when the median of the attribute is less than 3.

More than half of samples (1L, 4M, 7M, 8M, 10M, 11M, 12L, 13M, 14M, 15M, 16M, 17L, 18L, 20L, 21L, 22L, 27M, 32H) were characterized by a light intensity of all the three considered positive attributes; a second group, consisting of 15 samples (2L, 3L, 5M, 6M, 9M, 19L, 23M, 24L, 28H, 29H, 30H, 31M, 33M, 34M, 35M) showed medium intensity values of bitter and pungent and among these, almost all also had a medium intensity of fruity (2L, 5M, 6M, 9M, 23M, 28H, 29H, 30H, 31M, 33M, 34M). Moreover, sample 26H was characterized by a medium intensity of fruity but light values of bitter, and sample 25M by low values of pungent and fruity and medium of bitter. Sample 33M showed the highest intensity of bitterness and pungency. It is also interesting to highlight that the trend of fruity for all oils was similar to that observed for overall liking (Figure 1), suggesting that the expert panellists preferred and appreciated oils characterized by high intensities of fruitiness, as confirmed by the high correlations between these two attributes ($r = 0.89$).

According to COI/T.20/Doc. No. 29, 2009, fruity is defined as a “set of olfactory sensations characteristic of the oil that depends on the variety and comes from ”healthy, fresh olives, either ripe or unripe”, and thus is a positive sensation directly linked to fresh and high quality products. Considering the results of the overall liking (Tables 2a and 2b), only 5 samples (23M, 26H, 29H, 30H, 33M) showed mean values higher than 50%; among these, 3 were P.D.O. (26H, 29H, 30H), one was a P.G.I. (23M), and the other one a monovariety (of the cultivar Coratina, typical of the south of Italy, sample 33M). This consideration is related to the fact that the trained panelists preferred and appreciated the EVOOs characterized by well-known and familiar positive attributes.

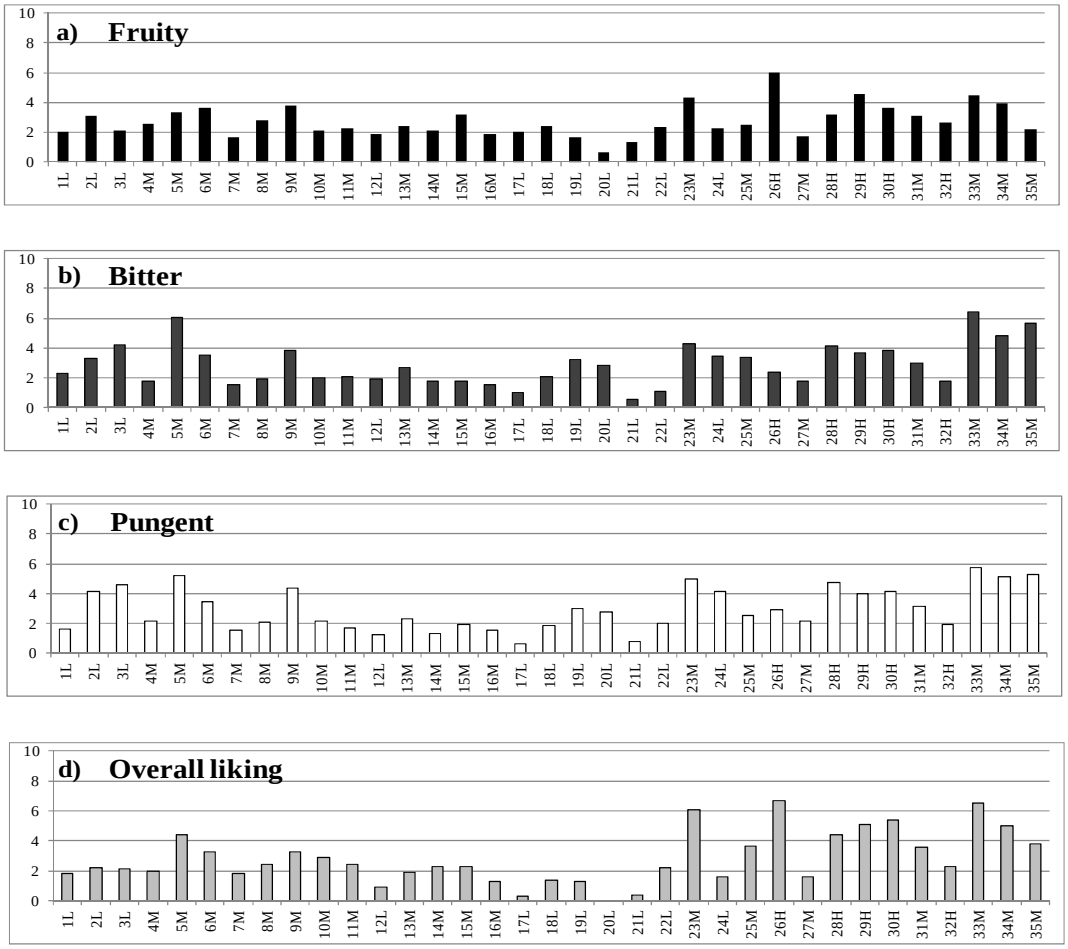

Figure 1. Histograms showing the median values of the intensities of positive attributes: A, fruity; B, bitter; C, pungent; D, overall liking for all the 35 EVOOs.

In the spider web planar graph in Figure 2, the sensory profiles of the two samples (26H and 33M) that scored the highest values of overall liking are shown. Sample 33M showed a medium intensity of fruitiness (4.6) and was also characterized by pleasant green notes (in particular artichoke and aromatic herbs) and by strong bitterness and pungency (respectively of 6.1 and 5.8), whereas the intensity of sweet was low (1.3).

Table 2a. Positive attributes and their intensity values (median values) estimated by QDA® test (from sample 1L to 17L).

Samples	1L	2L	3L	4M	5M	6M	7M	8M	9M	10M	11M	12L	13M	14M	15M	16M	17L
Fruity	2.1	3.1	2.2	2.6	3.3	3.7	1.7	2.8	3.8	2.1	2.3	1.9	2.4	2.2	3.2	1.9	2.0
Green notes (grass/leaf)	0.2	0.7	0.4	1.2	1.6	1.1	0	1.3	1.2	0.4	1.3	0	0	0	1.8	0.6	0
Other positive attributes	0	2.1	1.1	1.6	2.0	2.0	0	1.7	1.1	0.7	0.7	0	1.0	0	2.1	0.5	1.0
Bitter	2.3	3.3	4.3	1.8	6.1	3.5	1.6	2.0	3.8	2.0	2.1	1.9	2.7	1.8	1.8	1.6	1.0
Pungent	1.6	4.1	4.6	2.2	5.2	3.5	1.6	2.1	4.4	2.2	1.7	1.2	2.3	1.3	2	1.6	0.7
Sweet	3.8	3.9	2.9	4.5	1.4	3.1	3.7	3.8	2.1	4.5	3.9	4.2	4.2	4.6	4.0	4.3	5.1
Overall liking	1.8	2.2	2.1	2.0	4.4	3.3	1.8	2.4	3.3	2.9	2.4	0.9	1.9	2.3	2.3	1.3	0.3

Table 2b. Positive attributes and their intensity values (median values) estimated by QDA® test (from sample 18L to 35M).

Samples	18L	19L	20L	21L	22L	23M	24L	25M	26H	27M	28H	29H	30H	31M	32H	33M	34M	35M
Fruity	2.4	1.7	0.7	1.4	2.4	4.4	2.3	2.5	6	1.7	3.2	4.6	3.7	3.1	2.7	4.5	4	2.2
Green notes (grass/leaf)	0	0	0	0	1.7	3.2	0	0.9	5.2	0.2	2.7	2.9	1.8	1.2	0.7	4.1	2.3	1.1
Other positive attribute	2.4	0	0	0	1.2	2.9	2	1.5	4.7	0	2.4	2.9	3.1	2	1.8	3.5	2.5	1.2
Bitter	2.1	3.3	2.9	0.6	1.1	4.3	3.5	3.4	2.4	1.8	4.2	3.7	3.8	3	1.8	6.4	4.8	5.7
Pungent	1.9	3	2.8	0.8	2	5	4.2	2.5	3	2.2	4.8	4	4.1	3.2	1.9	5.8	5.1	5.3
Sweet	4.8	2.8	4.4	5.9	4.2	2.4	3.5	3.9	4.4	4.2	2.6	3.3	2.9	3.5	3.4	1.1	2	1.4
Overall liking	1.4	1.3	0	0.4	2.2	6.1	1.6	3.7	6.7	1.6	4.4	5.1	5.4	3.6	2.3	6.6	5	3.8

Sample 26H was characterized by medium-high intensities of fruitiness (5.6), green notes (5.1), and other positive notes (tomato, almond), whereas the attribute of sweet was predominant and higher compared to both bitterness and pungency. This was confirmed by the higher amount of *o*-diphenols and total phenolic compounds for sample 33M compared to sample 26H (Table 3).

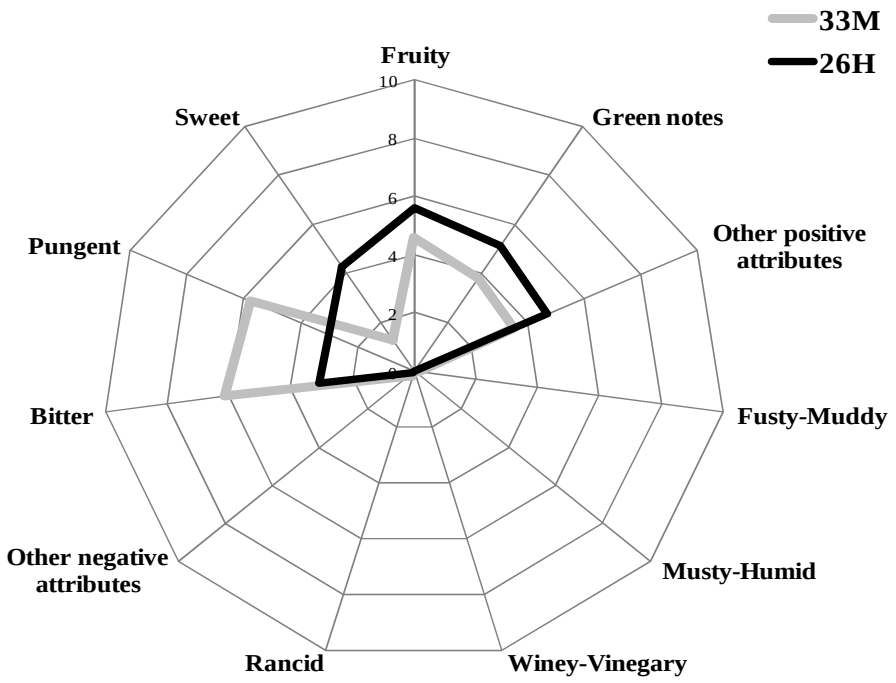

Figure 2. Spider web graph of sensory attributes (expressed as median values on the 10 cm scales) for samples 33M (gray line) and 26H (black line).

3.2 Chemical analyses

Considering the chemical results reported in Table 3, the FA values were below the legal limit established for EVOO (EU Reg. 61/2011) for all samples, while for 9 samples (7M, 8M, 9M, 11M, 12L, 15M, 16M, 32H, 35M) the PV were above the legal limit fixed for EVOOs (EU Reg. 61/2011), suggesting a poor oxidative status. For some samples (2L, 3L, 7M, 12L, 15M, 16M, 17L, 19L, 20L, 21L, 30H), the spectrophotometric extinction coefficients (K_{232} and/or K_{270}) were not within the legal limit established for EVOOs (Table 3, EEC Reg. 2568/1991). In particular, two samples (7M and 15M) with values of K_{232} higher than the limit established for EVOOs (2.50) had a PV higher than the limit for EVOO (20 meq O_2/kg oil), confirming a poor oxidative status, suggesting that they were not fresh, but aged oils. This is also demonstrated by their values of the ratio between 1,2- and 1,3-DAG, which were both very low (Table 3). To evaluate the degree of freshness of oils, it is important to consider this ratio (Serani et al., 2001; Frega et al., 1993), even if it is not an official method reported by EU Reg. 61/2011. In general, 1,3-DAG are not linked to positive characteristic of EVOOs, since they are formed only as a consequence of lipolytic processes and increase during storage, mainly after an isomerization reaction that involves 1,2-DAG (Serani et al., 2001). For 15 of the 35 samples, the ratio was less than 1 suggesting both an advanced degree of preservation and low freshness (Table 3). EVOOs characterized by a denomination of origin (P.D.O. and P.G.I.) (23M, 26H, 28H, 29H, 30H, 31M) showed a very low content in FAAEs (from 13.4 mg/kg to 46.6 mg/kg), suggesting a good quality of olives (Pérez -Camino et al., 2008). For only two samples (1L and 12L), the total amount of FAAEs was outside the legal limit established for EVOOs by the E.U. (EU Reg. 61/2011): interestingly, both were sold at low price (Table 1). Two other samples (2L and 24L), also sold at low price (see Table 1), showed a total amount of FAAEs between 75 and 150 mg I.S. kg $^{-1}$, but the ratio between ethyl ester and methyl ester was less than 1.5, and thus could be classified as EVOOs (EU Reg. 61/2011). Only 10 of 35 samples (1L, 3L, 5M, 6M, 9M, 19L, 20L, 24L, 33M, 34M) showed a total amount of phenolic compounds (TP) between 200 and 500 mg of gallic acid/kg oil, and can be considered as medium rich in phenolic compounds within the analyzed set. All the remaining samples were characterized by low amounts of phenolic compounds (less than 200 mg/kg) (Montedoro et al., 1992). A similar trend was confirmed considering the content of *o*-diphenols (*o*-DPH) and BI K_{225} values (Table 3).

Table 3. FA, free acidity in % of oleic acid; PV, peroxide values in meq. O_2/kg oil; K_{232}, K_{270}, extinction coefficients; BI K_{225}, bitter index as specific coefficient at 225 nm; TP, total phenols, in mg gallic acid/kg oil; o-DPH, orto-diphenols, in mg gallic acid/kg oil; FAAE, total amount of FAAEs, in mg methyl heptadecanoate/kg oil; OA/LA, ratio between oleic acid and linoleic acid; 1,2-/1,3 DAG: ratio between 1,2- and 1,3-DAG. Values in bold are not within the legal limits for an EVOO (EU Reg. 61/2011). Means in the same column shown with different letters are significantly different ($p < 0.05$).

	FA	PV	K_{232}	BI K_{225}	TP	*o*-DPH	FAAE	OA/LA	1,2-/1,3-DAG
1L	0.3 j,k	11 r	1.75 k-o	0.33 d-f	236 e	68 e-g	**92 a-c**	12 c-e	1.09 j-l
2L	0.4 h,i	15 l,m	2.08 d-k	0.22 l-p	176 h,i	59 i-l	103 a,b	10 f,g	1.02 j-n
3L	0.5 c,d	12 r	2.01 e-l	0.31 d-h	226 e,f	68 d-g	49 e-i	10 g	1.23 i,j
4M	0.3 j,k	15 m,n	1.74 k-o	0.28 e-i	198 g	57 j-l	43 f-k	8 i-k	0.70 o-q
5M	0.5 f,g	17 h-j	2.01 e-l	0.34 d	254 d	80 c	36 h-m	11 f,g	2.04 g
6M	0.3 l-n	17 h,i	2.34 b-f	0.35 d	231 e,f	90 b	67 d-f	9 h,i	11.23 i,j
7M	0.5 e-g	**24** c	**2.65 b**	0.21 m-p	127 k,l	50 m-o	71 c-e	8 j,k	0.72 o-q
8M	0.4 g,h	**25** b	2.49 b,c	0.21 m-p	119 k-m	46 o-q	35 h-m	8 j,k	1.22 i,j
9M	0.2 n,o	**28** a	2.29 b-g	0.43 b,c	328 b,c	47 n-p	38 g-m	11 d-f	2.57 f
10M	0.3 l,m	20 f	2.26 b-h	0.35 d	186 g,h	35 r,s	56 d-h	8 i,j	1.74 h
11M	0.4 h,i	**21** e,f	2.18 c-i	0.25 i-n	128 k	40 q,r	40 g-k	10 g,h	0.88 m-o
12L	0.5 d-f	**24** c	2.16 c-j	0.18 p	112 k-n	25 u,v	**79 b-d**	13 a,b	0.56 q,r
13M	0.5 c,d	15 k-m	2.10 d-k	0.24 i-o	177 h,i	69 d-f	34 h-m	8 k	0.57 p-r
14M	0.5 b,c	16 i-k	2.14 c-j	0.19 o,p	96 n,o	29 t,u	43 f-k	9 g,h	0.50 r
15M	0.6 a,b	**27** a	**3.11 a**	0.20 n-p	148 j	40 q,r	42 g-k	5 m	0.49 r
16M	0.6 a	**21** d,e	2.46 b-d	0.21 m-p	106 m-o	20 v	56 d-h	10 g,h	0.59 p-r
17L	0.4 g,h	16 j-l	2.46 b-d	0.25 i-m	190 g,h	53 l-n	46 e-j	8 j,k	0.73 o-q
18L	0.4 g,h	17 h	2.40 b-d	0.23 j-o	160 j	43 p,q	62 d-g	8 j,k	0.73 o-q
19L	0.4 i,j	17 h-j	1.73 k-o	0.41 c	227 e,f	64 f-i	49 e-i	12 c-e	0.75 o,p
20L	0.3 k,l	7 s	1.77 j-n	0.46 a-c	335 b	71 d,e	29 i-m	12 c-e	0.94 l-o
21L	0.5 e,f	12 r	2.32 b-g	0.24 i-o	107 m-o	24 u,v	35 h-m	10 g,h	0.63 p-r
22L	0.3 k,l	14 n,o	2.32 b-g	0.26 h-l	111 l-n	32 s,t	52 e-i	13 a,b	1.15 j,k
23M	0.2 o	13 q	1.62 m-o	0.32 d-g	187 g,h	74 c,d	48 e-i	12 c-e	3.46 e
24L	0.4 i,j	14 n,o	1.38 o	0.33 d,e	218 f	69 d-f	116 a	14 a	0.88 n,o
25M	0.5 c-e	17 h-j	1.99 e-m	0.28 g,k	198 g	65 e-h	30 i-m	8 j,k	1.39 i
26H	0.3 j,k	13 q	1.81 i-n	0.20 n-p	95 o	47 n-p	31 i-m	7 l	2.50 f
27M	0.4 i,j	19 g	1.97 e-m	0.24 i-o	153 j	64 f-i	38 g-l	9 i,j	0.64 p-r
28H	0.3 m,n	16 h-j	1.95 g-m	0.28 f-j	162 i,j	40 q,r	19 k-m	10 g,h	2.10 g
29H	0.3 k,l	11 r	1.46 n,o	0.32 d-g	160 j	61 h-j	13 m	12 b-d	5.63 b
30H	0.2 o	13 p,q	1.69 l-o	0.2 n-p	98 n,o	54 k-m	14 l,m	9 i,j	5.34 c
31M	0.3 l-n	18 g	2.35 b-e	0.21 l-p	191 g,h	35 r,s	47 e-j	11 e,f	1.07 j-m
32H	0.3 j,k	**22** d	1.66 l-o	0.23 k-o	105 m-o	59 i-k	42 g-k	8 i,j	2.15 g
33M	0.3 m,n	13 o-q	1.89 h-m	0.48 a	428 a	114 a	19 k-l	10 g,h	6.56 a
34M	0.3 j,k	17 h-i	1.96 f-m	0.47 a,b	312 c	62 g-j	41 g-k	12 b,c	4.57 d
35M	0.3 k,l	**22** d	2.01 e-l	0.32 d,g	187 g,h	33 s,t	22 j-m	11 f,g	2.16 g

Sample 33M (monovariety from olives of the South Italian cultivar Coratina) was the richest in TP and *o*-DPH, and also had the highest BI K_{225} value. The fatty acid composition of all samples was typical for EVOOs (EU Reg. 61/2011) (data not shown). Most samples showed an oleic acid/linoleic acid ratio higher than 7 (all, except for 15M and 26H, see Table 3), which is a good index of oxidation resistance (Chiavaro et al., 2011). This ratio has been described as being responsible for the stability of virgin olive oils (Aparicio et al., 1999).

3.3 Principal component analysis (PCA)

Considering Pearson correlations among the sensory, chemical, and economic (price) variables, some results are worthy of note (Figure 3): the total amount in FAAEs was negatively correlated with the class of price (r = - 0.60), while the class of price was positively correlated with fruity (r = 0.60) and other positive attributes (r = 0.53). Moreover the class of price positively correlated with overall liking (r = 0.67), suggesting that expert panellists appreciated the more expensive EVOOs over the cheaper ones. Moreover, the ratio 1,2-/1,3-DAG was negatively correlated with the total amount of FAAEs (r = - 0.47), suggesting the aged products were likely obtained with poor quality olives. This ratio was also positively correlated with all the positive sensory attributes, which are high-perceived in fresh olive oils (fruity, r = 0.69; green notes, r = 0.71; other positive attributes, r = 0.66; bitter, r = 0.64; pungent, r = 0.67), and with overall-liking (r = 0.81); a significant correlation was also found between overall-liking and class of price (r = 0.67): this latter also positively correlated with fruity (r = 0.60) and other positive attributes (r = 0.53), suggesting that the belonging to a specific class of price is a crucial parameter affecting the quality of the product. Bitterness also showed high positive correlation with pungency (r = 0.94), since they are related to the presence of similar compounds, especially phenolic compounds that elicited these positive attributes of EVOO (Bendini et al., 2007). Previous investigations (Gutierrez-Rosales et al., 1992; Beltran et al., 2007) found that bitterness of EVOOs can be estimated by the spectrophotometric measurement of absorbance at 225 nm (BI K_{225}), carried out on the phenolic fraction extracted by the EVOO sample. Bitterness and BI K_{225} were positively correlated (r = 0.60), even if it is important to underline that the use of the spectrophotometric index has some limitations, since other phenolic compounds may influence their determination (such as

the aldehydic form of oleuropein aglycone, which can absorb at 225 nm but is not characterized by bitterness (Inarejos-Garcia et al., 2009).

Figure 3 shows a projection on a two-dimensional surface of multidimensionally expressed sensory/chemical attributes, described by orthogonal factors used as dimensions: principal components PC1 and PC2. These factors explain more than 69% of variance among samples having a first component F1 of more than 50%, and F2 explains 19.3% of variance. Moreover, the samples are displayed in the orthogonal surface: considering their location, when close to each other this means that those products are similar (taking into account a multivariate combination of all evaluated attributes), while if far away they differ strongly. An approximate position of the product near certain attribute/chemical parameter vector(s) allows for the conclusion that the product is characterized by this attribute/chemical parameter, since it is particularly expressed.

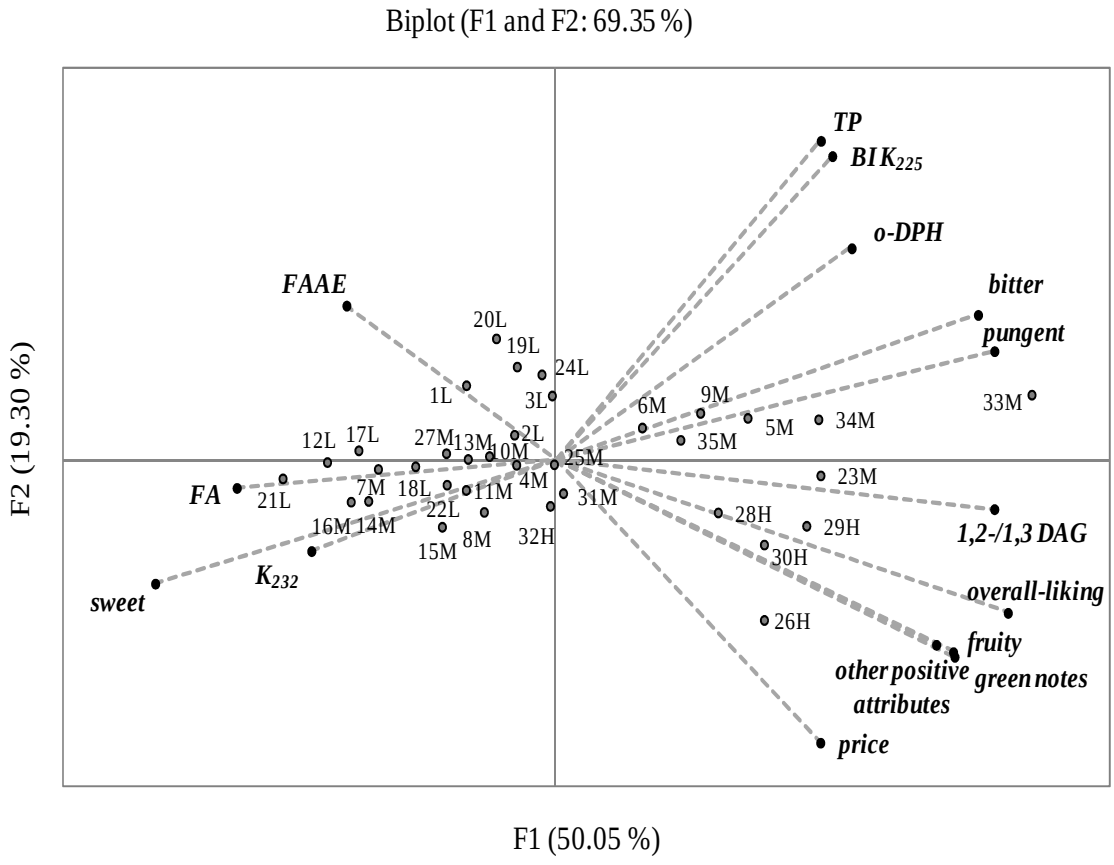

Figure 3. PCA built with chemical (mean values) and sensory (median values) parameters evaluated for the analyzed 35 samples. Acronyms of the variables are reported in the legend of Table 1.

In the top right quadrant of the PCA (Figure 3), all variables related to the phenolic compounds are present, such as total phenols (TP), *o*-diphenols (o-DPH) and K_{225} (BI K_{225}), in addition to the sensory attributes due to phenolic compounds (bitterness and pungency). Considering the location of samples in the factor plane, it should be emphasized that they were arranged on the basis of different levels of quality (Figure 3). All the EVOOs characterized by a P.D.O. (26H, 28H, 29H, 30H, 31M, see Table 1), and also the only P.G.I. (23M), were grouped in the same cluster, located in the bottom right quadrant; they were characterized by freshness, in terms of a high ratio between 1,2- and 1,3-DAG and by a high intensity of fruitiness, green notes and other positive sensory attributes, with good appreciation expressed by panellists (overall liking). The sample P.D.O. "Terre di Bari" (31M) was located between the bottom-left and the bottom right quadrants, since it was not characterized by positive sensory attributes and a ratio 1,2-/1,3-DAG of 1. In the top-left quadrant, there were only samples sold at low-price (except for samples 27M and 10M, which both lie on the axis between top-left and bottom-left quadrants) with a high amount of FAAEs. Four (18L, 21L, 22L and 32H) of the six EVOOs obtained by olives harvested in the European Union (see Table 1) were in the bottom-left quadrant, in the direction of high intensity of sweet, high values of free acidity and K_{232} (primary oxidation products). The two other samples obtained by olives harvested in the European Union (17L and 24L) were in this area of the plane: sample 17L was by the axis between the top-left and the bottom-left quadrants, while sample 24L was in the top-left quadrant, since it was characterized by a high content in FAAEs (116 mg/kg).

4 Conclusions

In this investigation, an overview on 35 differently priced EVOOs sold in the Italian market has been carried out. The range of quality of EVOOs was very wide, in terms of sensory attributes, class of prices and chemical parameters. This investigation supports the fact that a full chemical and sensory analytical approach, involving legal (EU Reg. 61/2012) and other actually "unofficial" parameters, could be useful tool to discriminate high-quality products from low-quality ones. The different class of price was proved to be an important factor linked to quality. Moreover, the freshness of the product was another important quality parameter that was investigated. Except for P.D.O. and P.G.I. EVOOs, from the label it is usually not possible to determine the date of harvesting of olives/production of

the oil, but only the date of bottling, which could be months after pressing. To ensure freshness, which is crucial for EVOOs, it seems important to have official (recognized by EU Regulations) analytical parameters (particularly the ratio between 1,2- and 1,3- DAG) that can define it.

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3.2.8 Other publications in Italian

Here below I report just the references of two papers written in Italian that I realized during the 3-years Ph.D. course, about quality and genuineness of virgin olive oils (the first one) and the so-called “repaso”olive oils, obtained by a further extraction of the oils from the paste of olives, usually carried out by adding warm water(the second one).

Part of the results of these two publications are discussed and cited in chapter 1. For both the papers, my specific contribution was to review the literature, to carry out the analytical plan, to interpret the results and to write the manuscripts, in collaboration with the other co-authors.

Valli, E., Bendini, A., Gallina Toschi, T. & Lercker, G. (2012). Qualità e purezza di oli extravergini di oliva: applicazione di idonei parametri analitici. In: Bungaro, M. (Ed.), Filiera olivicola. Monitoraggio di un campione di imprese e studi di settore, G&G, Unaprol, Rome, Italy, pp. 113-138.

Cerretani, L., Bendini, A., Valli, E., Morchio, G., Chiavaro, E. & Lercker, G. (2011). Caratterizzazione chimica di oli di oliva raffinati e di prodotti di seconda lavorazione (repaso) offerti sui mercati nazionale e internazionale. Rivista Italiana delle Sostanze Grasse, 88, 82-88.

Chapter 3. Innovative technological tool for producing olive oil

Bendini, A., Valli, E., Rocculi, P., Romani, S., Cerretani, L. & Gallina Toschi, T. A new patented system to filter cloudy extra virgin olive oil. Current Nutrition & Food Science, in press.

My contribution to the above cited work focused on carrying out the analytical plan and interpreting the results, also by adopting a statistical approach. I also gave my contribution in writing the paper, under the supervision of Dr. Alessandra Bendini and Prof. Tullia Gallina Toschi.

A New Patented System to Filter Cloudy Extra Virgin Olive Oil

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Abstract: The aim of this investigation was to evaluate the chemical and sensory quality of three extra virgin olive oils (EV1-3) subjected to a new patented system to clarify cloudy oils through the insertion of an inert flow gas (argon) in olive oil mass. For this purpose, several quality parameters were determined on the three clarified (EVC1-3) and untreated (EVNC1-3) samples. In particular, the system patented by the University of Bologna and Sapio (a private company that supplies gas for industrial and research sectors) was applied to a 50 L batch of each oil after its production by a low-scale mill. The EV samples were bottled and stored at room temperature and kept in darkness before analysis. Basic quality indices including free acidity, peroxide value, specific absorption in the conjugated triene region and sensory analysis, as well as the composition of the major (fatty acids) and minor (tocopherols, polar phenols, volatiles, water) compounds were determined after three months of storage. The oxidative stability under stress conditions was also assessed. The main results concern the higher overall quality of the EV samples clarified by the patented system compared to untreated ones. The quantity of water significantly decreased in all clarified samples. Above all, the non-clarified oils showed a tendency to quickly develop off-flavors over time and to decrease their oxidative stability.

Keywords: Clarified edible oil, extra virgin olive oil, filtration systems, inert gas, minor compounds, quality parameters.

INTRODUCTION

Newly produced extra virgin olive oil (EV), obtained solely by mechanical and physical processes, is a turbid and opalescent juice having suspended solid impurities as traces of water in micro-emulsion and dispersed protein type material and mucilages, which can promote enzymatic activity and compromise chemical and organoleptic quality.

The filtration process step of a cloudy EV is generally carried out before storage in order to remove suspended solids and humidity and to make the oil more clear and brilliant for the bottling phase. The most widely used traditional filtration systems in the olive oil industry are filter aids based on inorganic (diatomite) or organic fibrous materials such as cellulose or cotton introduced among the filter plates of the filter press equipment. In recent years, a cross-flow filtration system has been also proposed as an interesting and alternative method that involves oil flow parallel to the membrane instead of in a perpendicular flow in conventional method, using membranes with different characteristics in terms of pore sizes [1-3]. During filtering, quantitative and qualitative changes take place, especially on minor components [4]. In particular, a decrease in polar phenols, molecules of great importance for oil stability and human health [5], has been demonstrated [4, 6-9]. Some traditional filtration systems can also involve problems derived from the disposal of filtering materials. In order to solve these problems and at the same time guarantee the quality level of EV, innovative filtration systems have been recently proposed. In this regard, Italian researchers have developed two new filtration processes: the first, developed by Filterflo (Binasco, Milan, Italy) and previously applied to the enology sector [10], consists in the use of a filter bag system (usually polypropylene) in which the EV is directly transferred from storage tanks to filtration equipment and across the filter bag, allowing removal of suspended solids and a satisfactory level of oil limpidity [11]. The second system, patented by the University of Bologna and Sapio [12] is based on the flow of an inert gas (nitrogen or argon) that is introduced directly in the center of the olive-oil mass. Gas insertion generates a circular movement of the oil mass that facilitates separation of the suspended solids with a significant clarification effect and water decrease. In a recent publication [11], very similar and satisfactory results in terms of chemical composition and sensory characteristics in EV treated by polypropylene filter bag and argon flow gas were reported. Additionally, it is important to underline that the clarification by an inert flow gas avoids the use of organic materials from coming into contact with the olive oil. In order to confirm these first and positive results, in the present work the effect of the patented system, in particular using argon as an inert flow gas, on the chemical and sensory quality of three bottled EV samples compared to non-clarified ones was studied.

MATERIAL AND METHODS

Samples

The analyzed samples were monocultivar EV, all obtained from Correggiolo olives. The olives were harvested in

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the Emilia-Romagna region (Italy) in autumn 2011 at three different ripening levels (25 October, 9 November and 28 November) and processed separately by continuous low-scale plants (Oliomio 150, Tavernelle Val di Pesa, Florence, Italy) equipped with a hammer crusher, vertical malaxator and a two-phase decanter, in order to produce three different samples (EV1-3). 100 kg of olives were processed for each batch and aliquots of the obtained oils were clarified using a filtration system, based on argon gas flow (EVC1-3), while the other aliquots were not clarified (EVNC1-3). The filtration equipment, based on the flow of an inert gas, consisted of a filter tank and inert gas tank developed and patented by University of Bologna and Sapio [12]. Cloudy virgin olive oil was placed in the 50 L filter tank at room temperature and an insertion device for inert gas was connected to the bottom to the tank. The flow rate of argon gas, injected directly into the center of the olive oil mass, was 15 L min^{-1}. All EV samples were stored in several dark glass bottles (0.5 L) at room temperature and kept in darkness before analysis. Analytical determinations started at least 3 months after the production of the samples (from the beginning of February) and the analytical plan was completed within 3 months. All analytical determinations were performed on oil withdrawn from the core of the bottle.

Chemicals

All chemicals were of analytical reagent grade and purchased from Fluka, Sigma-Aldrich (Steinheim, Germany), such as the standards alpha-tocopherol, gallic acid and 4-methyl-2-pentanone.

Basic Quality Parameters

Free acidity, peroxide value and ultraviolet spectrophotometric index (K_{270}) were evaluated according to the official methods described in the EEC Reg. 2568/91 of the Commission of the European Union [13]. All analyses were determined in triplicate for each sample.

Sensory Analysis

Sensory analysis was performed according to the EC Reg. 640/2008 [14] by a fully trained group of 8 expert tasters. A set of positive (bitter, pungent, fruity and other pleasant attributes such as leaf, grass, artichoke, tomato, almond, apple, others) and negative (winey-vinegary, fusty-muddy, mouldy, rancid, others) sensory attributes were evaluated using a continuous scale from 0 to 10 cm related to the perception of flavor stimuli, according to the judgment of assessors. The median and the robust standard deviation (EC Reg. 640/08) were calculated for each attribute. If the value of the robust standard deviation was higher than 20%, the sensory analysis was repeated. For statistical analyses the mean values were considered (n=3).

Fatty Acid Composition

The fatty acid composition of oil samples was determined as the corresponding methyl esters (FAMEs) by gas chromatography (GC) (Clarus 500 GC Perkin Elmer Inc., Shelton, CT, USA) analysis. FAMEs were prepared by alkaline treatment carried out by mixing 0.05 g of oil dissolved in 2 mL of *n*-hexane with 1 mL of 2 N potassium hydroxide in methanol, according to Christie [15]. Chromatographic conditions were previously described by Bendini *et al*. [16]. Results were expressed as % of FAME on the total amount of FAMEs (n = 3). The FAMEs were grouped in three different categories, according to the specific number of double bonds: SFA, Saturated Fatty Acids; MUFA, Monounsaturated Fatty Acids; PUFA, PolyUnsaturated Fatty Acids. Moreover, the OA/LA (ratio between oleic acid and linoleic acid) was calculated for each sample.

OSI (Oxidative Stability Index)

The oxidative stability under forced conditions was determined using an eight-channel oxidative stability instrument (OSI) (Omnion, Decatur, IL). The OSI time was determined according to the analytical protocol described by Jebe *et al*. [17]. Briefly, samples (5 g) were loaded onto each channel and heated at 110 °C under atmospheric pressure. At this stage, the air flow was injected into the center of the sample mass to bubble through the oil at 150 mL min^{-1}, generating an increase in conductivity due to formation of short-chain volatile acids. This increase, measured in distilled water channels directly connected to the sample, determined an induction period (OSI time), expressed in hours and hundreds of hours (n=3).

Determination of Tocopherols

For the quali-quantitative determination of tocopherols, 0.3 g of sample was dissolved in 10 mL of isopropanol and passed through a 0.45 μm filter before HPLC analysis. The chromatographic separation of these compounds was performed on a 150 mm × 4.6 mm i.d., 120 Å, Cosmosil π NAP column (CPS Analitica, Milan, Italy). The injection volume for HPLC was 20 μL. The mobile phases used were methanol:water (90:10 v:v) having water acidified with 0.2% phosphoric acid in isocratic gradient as eluent A and acetonitrile as eluent B to wash the column. The flow rate was 1 mL min^{-1}, and analyses were made at room temperature. The total run time was 35 min. Separated tocopherols were quantified with a DAD detector at 292 nm. A calibration curve was calculated by using six points of alpha-tocopherol at different concentrations, estimated from the amounts of the analytes in samples, and was linear over the range studied (r^2 = 0.999). Results were given in mg of alpha-tocopherol per kg of oil (n=3).

Liquid-liquid Extraction of Phenolic Compounds

Extraction of phenolic compounds from EVOOs was performed following the protocol proposed by Pirisi *et al*. [18] and modified according Rotondi *et al*. [19]. Three replicates were carried out for each sample. The extracts for the spectrophotometric assays were stored at -18 °C before use.

Spectrophotometric Assays: Total Phenols (TP) and o-diphenols (o-DPH)

The TP and *o*-DPH of the extracts were determined using a UV-Vis 1800 Shimadzu spectrophotometer (Kyoto, Japan) and evaluated according to Singleton *et al*. [20] and Mateos

et al. [21], respectively. TP and *o*-DPH were detected at 750 and 370 nm, respectively, and quantified using gallic acid calibration curves (r^2 = 0.993 and r^2 = 0.998, respectively). Data were expressed as mg of gallic acid per kg of oil.

Chemical Index of Bitterness (K_{225})

Chemical evaluation of bitterness of polar phenolic extracts (n=3) was carried out spectrophotometrically at 225 nm using a UV-Vis 1800 Shimadzu spectrophometer (Kyoto, Japan) [22, 23].

Determination of Water Amount (WA)

The water amount was analyzed with a TitroMatic 1S instrument (Crison Instruments, S.A.; Alella, Barcelona, Spain) according to the method described by Gomez-Caravaca *et al*. [4]. Clarified and unclarified EV were dissolved in a solution of chloroform/Hydranal-solvent 2:1 (v/v), and the titrating reagent (Hydranal-Titran 2) was added until the equivalence point was reached. Each sample was introduced three times, and the quantity of the sample was measured with the back-weighting technique. The quantity of water was expressed as mg of water kg^{-1} of oil (n = 3).

Determination of Volatile Compounds

Volatile compounds were identified and quantified by SPME-gas chromatography coupled with quadrupolar mass-selective spectrometry, according to Baccouri *et al*. [24]. The results were expressed as mg of internal standard (4-methyl-2-pentanone) per kg of oil (n=3).

Statistical Analysis

The software XLSTAT 7.5.2 version (Addinsoft, USA) was used to elaborate chemical and sensory data by Analysis of Variance (ANOVA) and Principal Components Analysis (PCA) Before PCA analysis, the data were standardized, normalized and centred.

RESULTS

As detailed in the Materials and methods section, all samples were obtained from a low-scale mill working in continuous system but without a final cleaning of virgin olive oil by a vertical centrifugation as applied in several industrial plants. Therefore, aliquots of newly produced cloudy oils were subjected to clarification by the patented system based on argon flow in a suitable steel tank and then bottled, whereas other aliquots were bottled without any treatment. Consequently, for all samples, the lipid matrix during the storage remained in contact with the dispersed particulate that tended to settle to the bottom over time (see Fig. **1**). It must be emphasized that the experimentation entailed use of non-optimal conditions of storage for the overall quality of an extra virgin olive oil. However, this allowed investigation of the possible effects of clarification by argon flow on the analytical parameters checked. In Fig. (**1**), it can be observed that the patented system led to good separation of the initially dispersed particulate as a thick sediment on the bottom.

Fig. (1). Bottles of clarified (EV3C) and non-clarified (EV3NC) extra virgin olive oils, obtained from Correggiolo olives harvested at the third ripening level.

As shown in Table **1**, according to basic chemical parameters, all samples belonged to the extra virgin olive oil category, as the mean values were within the legal limits for free acidity (FA), peroxide value (PV) and specific extinction at 270 nm (K_{270}). The clarified oils (EV1-3C) did not show any critical differences compared to the nonclarified ones (EV1-3NC) both in terms of hydrolysis and oxidative degradation as suggested by the results of free acidity percentages and peroxide values (primary oxidation products) as well as absorptions in triene conjugated spectrum region (secondary oxidation products).

As already reported in a previous work [11], the clarification process by argon flow had no substantial effect on percentages of the main fatty acids as oleic, palmitic, linoleic, stearic and linolenic (single data not shown) that cover the usual range in olive oils. In fact, the compositions in fatty acids were characterized by high contents in monounsaturated (MUFA) and low in polyunsaturated (PUFA) fatty acids as also seen by the oleic (OA) and linoleic (LA) acids ratios. Small differences among the three pairs of clarified and non-clarified samples were also found as a slight reduction of this ratio was obtained due to the increase of degree of ripening of Correggiolo olives (from EV1 to EV3).

The most important lipophilic phenols quantified by HPLC-DAD in EV samples were alpha and gamma tocopherols. Experimental data showed slightly higher values of both tocopherols in clarified samples (data not shown). As a consequence, the results concerning the TOT TOC also revealed a tendency towards loss of these molecules in the non-clarified oils.

The clarification system did not affect the total polar phenolic content, the sum of all hydrophilic molecules having a phenol group (TP) or all compounds with catecholic

Table 1. Mean values of analytical parameters in oil samples after three months of storage. EV1-3C, monovarietal extra virgin olive oils obtained from Correggiolo olives harvested at three different degrees of ripening were clarified by the patented system with argon gas and bottled; EV1-3NC, monovarietal extra virgin olive oils obtained from Correggiolo olives were harvested at three different degrees of ripening and bottled without previous clarification. FA, Free Acidity expressed as g of oleic acid 100 g^{-1} of oil (≤ 0.8 % is the legal limit for extra virgin olive oil); PV, Peroxide Value expressed as meq of oxygen kg^{-1} of oil (≤ 20 meq O_2 kg^{-1} is the legal limit for extra virgin olive oil); K_{270}, specific extinction at 270 nm (≤ 0.22 is the legal limit for extra virgin olive oil); SFA, Saturated Fatty Acids; MUFA, Monounsaturated Fatty Acids; PUFA, PolyUnsaturated Fatty Acids; OA/LA, ratio between Oleic Acid and Linoleic Acid; TOT TOC, Total amount of Tocopherols expressed as mg of α-tocopherol kg^{-1} of oil; TP, Total Polyphenols by spectrophotometric assay expressed as mg of gallic acid kg^{-1} of oil; *o*-DPH, total amounts of ortho-diphenols by spectrophotometric assay expressed as mg of gallic acid kg^{-1} of oil; K_{225} evaluation of bitterness as specific absorption at 225 nm by spectrophotometric assay; WA, water amount valued by Karl-Fisher automatic titration expressed as mg kg^{-1}; ALD C6, aldehydes with six carbon atoms produced by the lipoxygenase pathway expressed as mg of 4-methyl-2-pentanone kg^{-1} of oil; TOT VOL, total amount of volatile compounds expressed as mg of 4-methyl-2-pentanone kg^{-1} of oil; OSI, time of oxidative stability calculated by Oxidative Stability Instrument and expressed in hours and hundredth of hour. Different letters in the same row indicate significant differences (Fisher LSD, $p < 0.05$).

	EV1C	EV1NC	EV2C	EV2NC	EV3C	EV3NC
Basic quality parameters						
FA	0.3 b	0.3 b	0.4 a	0.5 a	0.5 a	0.5 a
PV	10 e	8 f	14 b	15 a	11 d	12 c
K_{270}	0.11 b, c	0.10 c	0.11 b	0.11 b	0.15 a	0.16 a
Fatty acid categories						
SFA	17.9 a	17.3 a,b	17.0 b, c	17.1 b	16.3 c	17.3 a, b
MUFA	74.1 a	74.1 a	72.6 b	72.9 b	72.5 b	71.7 c
PUFA	7.8 e	8.1 d	9.7 c	9.7 c	10.3 a	10.2 b
OA/LA	9.2 b	9.3 a	7.3 c	7.3 c	6.9 d	6.9 d
Minor components and oxidative stability						
TOT TOC	317 a	273 b	187 d	185 d	271 b	263 c
TP	218 a	236 a	232 a	221 a	204 a	207 a
o-DPH	495.9 a, b	512.9 a	498.1 a, b	484.7 b	474 b, c	455.6 c
K_{225}	0.30 a	0.26 b	0.28 a, b	0.28 a, b	0.31 a	0.26 b
WA	622 c	875 b	238 e	990 a	433 d	901a, b
ALD C_6	7.5 b, c	0.9 d	7.8 a, b	0.1 d	11.3 a	3.9 c, d
TOT VOL	20.5 a	10.7 b	14.6 b	13.3 b	20.0 a	20.4 a
OSI	25.1 a	23.4 b	18.8 c	14.4 d	14.0 e	13.5 f

structure (*o*-DPH). Bitterness is an important sensory attribute of virgin olive oil, usually assessed by tasting and related to phenolic compounds. As suggested by some authors [22, 23], this parameter can be also estimated by the spectrophotometric measurement of the specific absorbance at 225 nm (K_{225}) carried out on the phenolic fraction extracted by the EV sample.

There was a good agreement between the bitterness evaluated by both sensory and chemical tests as shown by the data in Tables **1** and **2** and in Figs. (**2 a-c**).

As expected, and in agreement with previous results [11], for all three pairs of samples the highest water content (WA, Table **1**) was recorded in non-clarified virgin olive oil. This effect seems to be linked to oxidative stability (OSI), and in fact, for all three couples of oils, better stability in terms of oxidative degradation was shown by the clarified oil compared to the untreated one.

Considering the aldehydes with six carbon atoms (ALD C_6) formed by the lipoxygenase enzyme pathway (LOX), which are known to be particularly important for the perception of pleasure green notes in extra virgin olive oils [24], it must be underlined that there was a significantly higher presence of this volatile fraction in oil samples subjected to clarification with argon gas, bottled and stored for few months.

From the data shown in Table **2** and sensory profiles in Figs. (**2 a-c**), there was a clear tendency for the non-clarified

Table 2. Mean values relative to the sensory attributes perceived in samples and evaluated on a 10 cm scales (using the sheet of the Reg. EU 640 2008). EV1-3C, monovarietal extra virgin olive oils obtained from Correggiolo olives harvested at three different degrees of ripening and clarified by the patented system with argon gas before bottling; EV1-3NC, monovarietal extra virgin olive oils obtained from Correggiolo olives harvested at three different degrees of ripening and bottled without clarification. Different letters in the same row indicate significant differences (Fisher LSD, $p < 0.05$).

	EV1C	EV1NC	EV2C	EV2NC	EV3C	EV3NC
Positive attributes						
Fruity	3.5 a	1.9 c	3.1 a, b	2.4 b, c	2.7 a, b	2.3 b, c
Almond	2.9 a	1.4 b-d	2.4 a, b	1.2 b-d	2.3 a-c	0.6 d
Bitter	4.4 a	1.6 c	3.2 b	3.0 b	3.8 a, b	3.2 b
Pungent	4.4 a	1.5 c	4.1 a, b	3.1 b	4.3 a	3.6 a, b
Negative attributes						
Fusty-Muddy	0.2 b	1.4 a, b	0.6 a, b	1.2 a, b	1.5 a, b	1.8 a
Winey-Vinegary	0.0 b	1.2 a	0.0 b	0.5 b	0.6 a, b	0.0 b

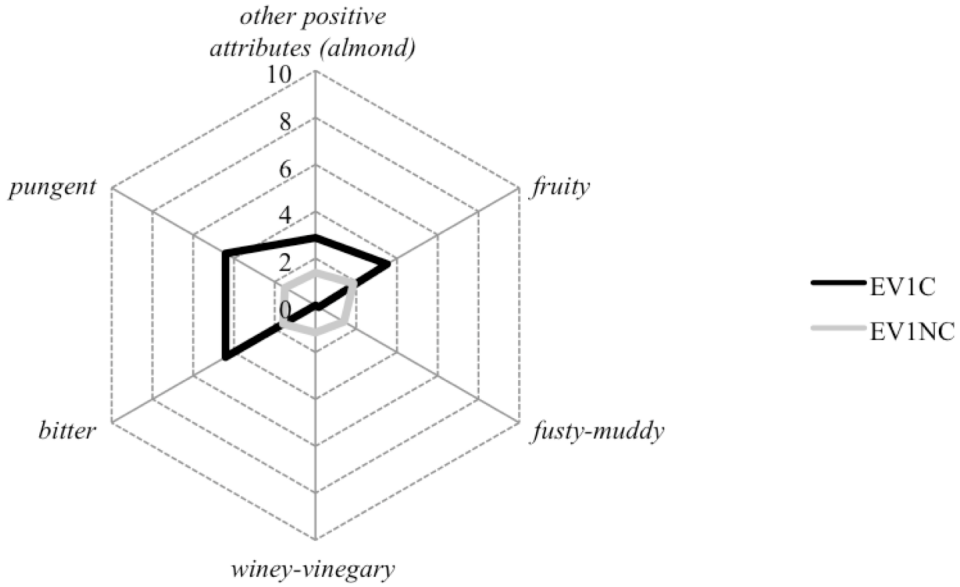

Fig. (2 a). Spider web graph of the sensory attributes (expressed as mean values calculated on the 10 cm scales) of the EV1C (clarified) and EV1NC (non-clarified) pair (see materials and methods section for a description of samples).

oil samples to quickly develop off-flavors over time, in particular fusty-muddy and winey, due to degradation of proteins and sugars present in micro-dispersion into the oil.

In contrast, the corresponding clarified samples were less disqualified for these sensory defects and richer in fruity and almond intensities, probably as a consequence of the sudden deposit of micro-dispersed residuals.

Figure **3** shows the biplot graph obtained from the principal component analysis built using several chemical (Table **1**) and sensory oil characteristics (Table **2**). In general, a PCA picture shows comparison of multidimensionally expressed sensory/chemical product attributes, projected on a two-dimensional surface, described by orthogonal factors used as dimensions: principal components PC1 and PC2. Percentages indicate what % of evaluated product variability is related to each PC. The first two components explained 83.98% of the total variance (55.44% for PC1 and 28.54% for PC2). The sensory attributes and chemical parameters are shown as vectors, the mutual direction of attribute/parameter vectors indicates positive correlation if they are close to each other and go to the same direction, while negative correlation is seen if they are close but in an opposite direction, or unrelated when they are perpendicular. Concerning the location of products on the PC1/PC2 surface, if they are close to each other it means that those products are similar (taking in account a combination of all evaluated attributes), while if they are far away from each other they differ strongly. Approximate position of the product near certain attribute/chemical parameter vector(s) allows for the conclusion that the product has this attribute/chemical parameter particularly expressed. For the construction of Fig. (**3**), two positive (almond and pungent) and two negative (winey-vinegary and fusty-muddy) sensory attributes as well as two chemical pa-

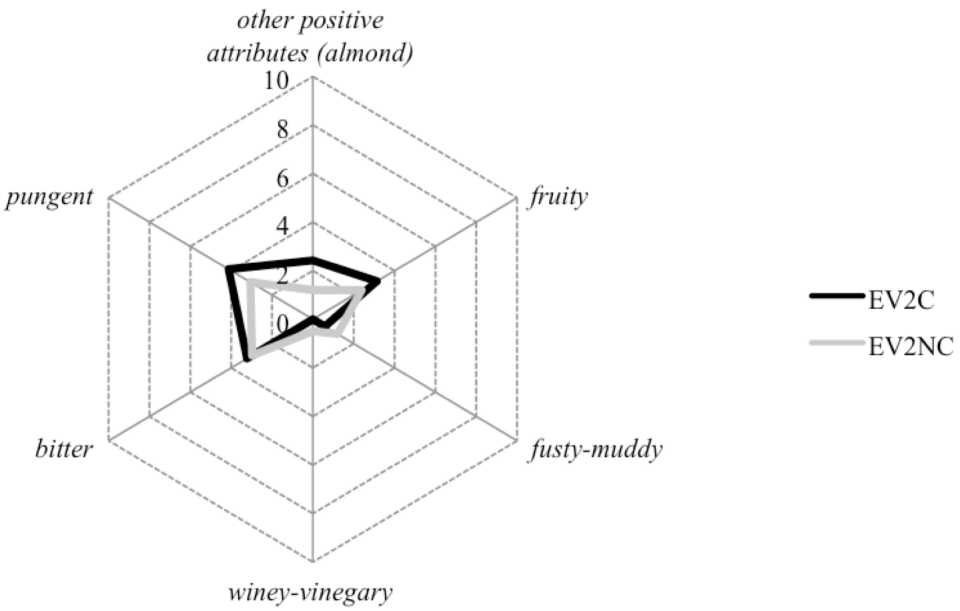

Fig. (2 b). Spider web graph of the sensory attributes (expressed as mean values calculated on the 10 cm scales) of the EV2C (clarified) and EV2NC (non-clarified) pair (see materials and methods section for a description of samples).

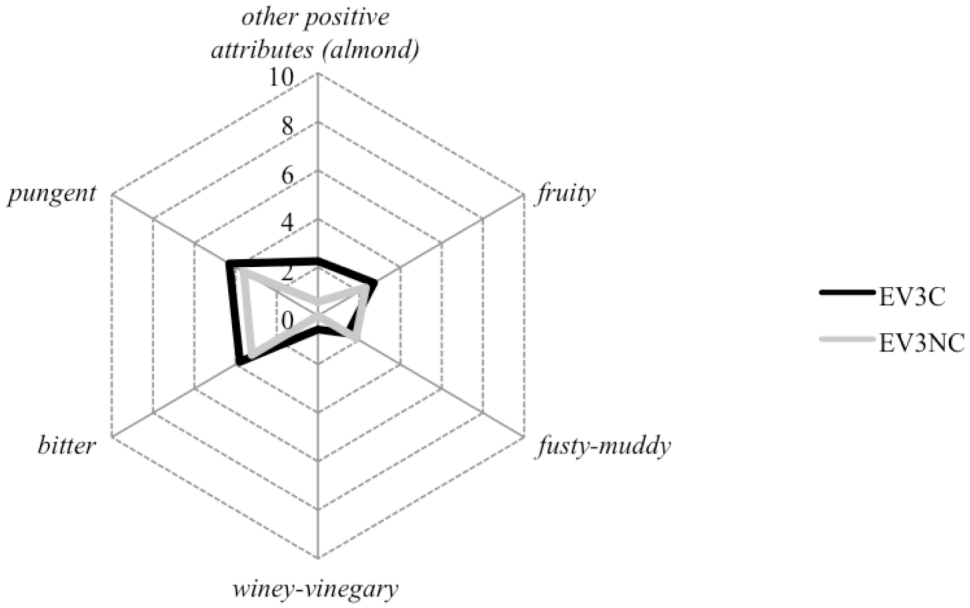

Fig. (2 c). Spider web graph of the sensory attributes (expressed as mean values calculated on the 10 cm scales) of the EV3C (clarified) and EV3NC (non-clarified) pair (see materials and methods section for a description of samples).

rameters (OSI and WA) were chosen. Only parameters with enough high F values (test LSD Fischer, $p<0.05$) and which were not redundant were chosen. In particular, the two pairs EV2C/EV3C and EV2NC/EV3NC located, respectively, in the 2nd and 3rd quadrants were characterized, above all, by the low or high intensities of off-flavors. In fact, the EV2NC/EV3NC pair was disqualified for negative attribute known as fusty-muddy perceived by assessors as defective for the presence of the unpleasant note known as fusty-muddy, whereas the EV2C/EV3C pair did not show the same negative descriptor. Concerning the EV1C/EV1NC couple, EV1C (in the first quadrant) was the best sample in terms of sensory characteristics with a high intensity of almond note (a secondary positive note characteristic of Correggiolo extra virgin olive oils), whereas the EV1NC (in the fourth quadrant) was disqualified by off-flavors recognizable as fusty-muddy and mostly winey-vinegary.

Considering Pearson's correlations, the almond descriptor was positively related to the other pleasant ones as fruity ($r = 0.829$, $p<0.05$). Similarly, the two positive sensations pungent and bitter were highly related to each other ($r = 0.945$, $p<0.05$). Total phenols and *ortho*-diphenols, well known to be mainly responsible for the bitter and pungent sensations in EV [5], also showed a high correlation ($r = 0.879$, $p<0.05$).

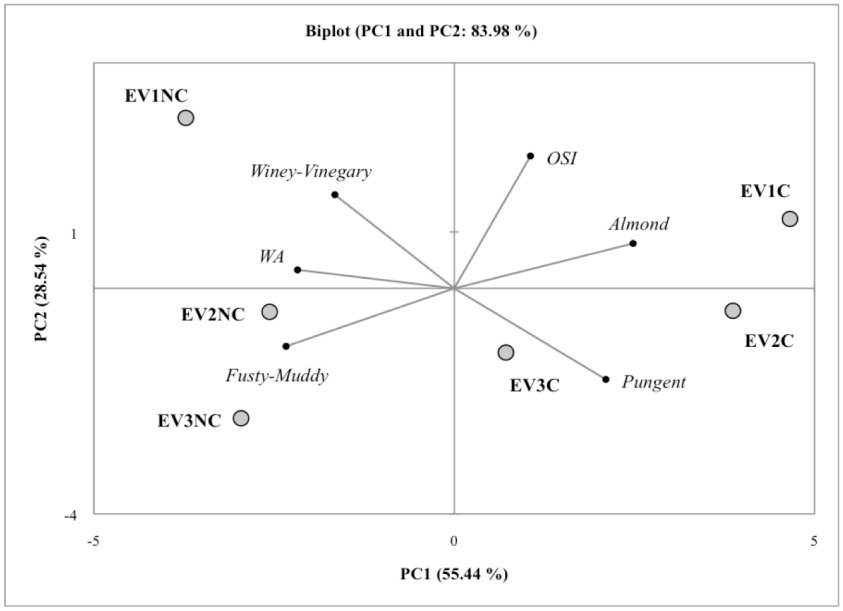

Fig. (3). Biplot graph obtained from the principal component analysis built using the selected chemical and sensory variables.

DISCUSSION

In general, non significant differences were found in clarified oils compared to the untreated ones in terms of hydrolysis and oxidative degradations, and all samples were within the legal limits for the extra virgin olive oil category. The clarification process with argon flow had no substantial effect on the fatty acid composition or total amount of polar phenolic compounds. Probably, during the first months of oil storage, these latter were protected by the presence of tocopherols that, in fact, showed a slightly lower concentration in non-clarified samples. A trend of a greater resistance to forced lipid oxidation for clarified samples compared to untreated oils was observed. This could indicate a greater stability of oils subjected to the argon flow technique prior to storage, perhaps as a consequence of a partial removal of oxygen in the oil mass. It is known that EV oxidative stability is influenced by different physical (temperature, exposure to light, presence or absence of oxygen) and chemical (composition in fatty acids and minor compounds) parameters. The highest values of the OA/LA ratio, in particular observed for oil samples produced by olives harvested at an early degree of ripening (EV1) together with the high content of minor antioxidant compounds, above all tocopherols (TOT TOC), can explain their higher time of oxidative stability (OSI). A high positive Pearson's correlation ($r = 0.947$, $p<0.05$) was found between the OA/LA ratio and OSI values. In the non-clarified oils, a tendency towards loss of TOT TOC was evident. This could be explained assuming that consumption of these antioxidant compounds was able to inhibit the development of the first step of the lipid oxidation [16]. In this regard, a significant negative correlation ($r = -0.833$, $p< 0.05$) was seen between TOT TOC and PV. In agreement with previous results [11], the quantity of water significantly decreased in all clarified samples, and thus the patented system using argon gas can be considered effective in reducing the water content.

Nonetheless, there was a clear tendency for non-clarified oils to quickly develop off-flavors over time, in particular fusty-muddy and winey, and this may be due to degradation of proteins and sugars present in the micro-dispersion. On the other hand, the application of the patented system permitted a rapid deposition of this material on the bottom of the bottles as shown for the EV3C/EV3NC couple (Fig. **1**). Indeed, there was a significant decrease in water content in all three clarified samples compared to the non-clarified oils, which resulted in protection of the positive sensory attributes fruity and almond notes, which are linked to the presence of specific aldehydes with six carbon atoms. In fact, a negative and significant Pearson correlation ($r = -0.844$, $p<0.05$) was found between ALD C_6 and WA.

It can be assumed that the clarified virgin olive oils were less disqualified for sensory defects than the untreated samples as also suggested by the biplot graph obtained from principal component analysis. At the same time, it is important to emphasize that the results of the volatile content and sensory analysis confirmed that clarification of cloudy EV using a flow of inert gas does not reduce the intensity of the main organoleptic attributes that contribute to consumer acceptance.

CONCLUSION

The proposed system permits clarification of extra virgin olive oil, avoiding any contact of the oil with organic or inorganic filtering material. The treatment appears to be very suitable and sustainable for the extra virgin olive oil category, which is declared by law as "…oils obtained from the fruit of the olive tree solely by mechanical or other physical means under conditions that do not lead to alteration in the oil…" [13]. As demonstrated in this short report and the previous investigation by Lozano-Sanchez *et al.* [11], treatment with nitrogen or argon allows persistence of positive attributes in unfiltered oils that are generally more perishable than filtered ones due to the presence of higher quantities of moisture and suspended materials. Moreover, this treatment maintains freshness longer, with less development of off-flavors. This effect could be related to three events: the first is the

quicker separation of moisture and the precipitate from the oil, due to gas bubbling. This phenomenon causes a reduction of the contact area and thus a partial separation from the oil (deposition) of the possible sites of fermentation. The second event is the lowering of the oxygen dissolved in the oil, which is partially washed out/substituted with nitrogen or argon, while the third, and probably the most important, is the depletion of the dispersed water. In fact, it is true that the clarified oil, as previously observed [11], was significantly less rich in water (by around one third and one half, respectively, when nitrogen and argon are used) compared to untreated oils. Furthermore, even if the visible material of the clarified and non-clarified oils has not yet been compared, which will be a focus of future studies, it is reasonable to suppose that the oil separated on the bottom by clarification is also significantly less rich in water, compared to that softly in suspension as in the non-clarified oil (easily visible in Fig. **1**). These three phenomena may help to explain the reason why the clarified oil is less inclined to develop defects over time.

CURRENT & FUTURE DEVELOPMENTS

For an industrial mill, in terms of cost the choice of nitrogen or argon cannot be ignored: nitrogen gas costs about 1 Euro per cubic meter, while argon gas is around three times more expensive. The prices of the same gases in the liquid state, which are less bulky for transportation, but purchasable only for companies with a high work capacity, are also lower even though argon is still about three times more costly (around 0.4 Euro and 1.2 Euro per cubic meter, respectively, for nitrogen and argon).

In the future, some improvements of the patented system need to be made: firstly, a better geometry of the bottom of the oil tank permitting easy discharge of the precipitate and its removal from the clarified oil will be studied, and a vertical window needs to be inserted for better control of turbidity. Secondly, taking into account the possible different sizes of tanks in which to apply the clarification system, correct proportions between the cloudy oil mass and the inert flow rate need to be defined. Finally, possible modifications to the system could help to define the optimal relation between uniformity of gas bubbling, changed by adjusting the rotation speed supply, as well as to reduce its consumption in order to obtain a more compact cake at the bottom of the tank.

CONFLICT OF INTEREST

The authors declare no competing financial interest or conflict of interest.

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LIST OF ABBREVIATIONS

EV	=	extra virgin olive oil
EVC	=	clarified extra virgin olive oil by the patented system
EVNC	=	not clarified extra virgin olive oil
FA	=	Free Acidity
PV	=	Peroxide Value
K_{270}	=	specific extinction at 270 nm
SFA	=	Saturated Fatty Acids
MUFA	=	Monounsaturated Fatty Acids
PUFA	=	Poly-Unsaturated Fatty Acids
OA/LA	=	Oleic Acid and Linoleic Acid ratio
TOT TOC	=	Total Tocopherols
TP	=	Total Polyphenols
o-DPH	=	Total ortho-diphenols
K_{225}	=	specific absorption at 225 nm
WA	=	water amount
ALD C_6	=	aldehydes with six carbon atoms
TOT VOL	=	Total Volatiles
OSI	=	Oxidative Stability Index

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Chapter 4. Objective sensory analysis of EVOOs: relations with chemical composition and consumer perception.

Valli, E., Bendini, A., Popp, M. & Bongartz, A. Sensory evaluation and consumer perception of 140 “premium quality” extra virgin olive oils, submitted to Journal of Sensory Studies.

Abstract

This investigation focused on 140 “premium-quality” extra virgin olive oil (EVOO), participating in four subsequent years at the International Olive Oil Award (Zurich) and characterized by different producing countries, harvest years, agricultural systems and quality standards. The sensory analysis was performed by the Swiss Olive Oil Panel (Regulation (EC) 640/2008, including additional aromatic attributes, sweet, harmony and persistency). The samples were evaluated in consumer tests sessions in Zurich. In the Preference map, the most appreciated samples by consumers were situated in the direction of ripe fruity and sweet axes and opposite to bitter and other attributes (pungent, green fruity, freshly cut grass, green tomato, harmony, persistency) axis, highlighting a different sensorial attitude towards EVOO for consumers and panellists. A grouping, but not discriminatory, effect was evidenced for samples of the same cultivars and produced in the same areas, both characterized by bitterness/pungency and also by sweetness/ripe fruity notes.

Practical applications:

The sensorial profile of EVOOs can be evaluated according to the Regulation (EC) 640/2008, extended with a list of additional positive aromatic attributes as well as the descriptor sweet and the harmony and persistency of the oils. Such an objective profile could be a useful tool to group products according to the cultivar and the geographical origin. Since most of the consumers appear unfamiliar with positive sensorial attributes, like bitterness and pungency, it is very important to teach them about their importance concerning the link with health benefits related to these descriptors. In other words, the crucial research topic in this field is to learn more about the interdependency between relevant parameters determining consumer acceptance and objective sensory characteristics and health benefits of EVOOs. Results should be prepared for

dissemination in the direction of the consumers, in order to make them able to appreciate high quality EVOOs.

Abbreviations: EVOOs, extra virgin olive oils; PDO, Protected Denomination of Origin; SOP, Swiss Olive Oil Panel.

1. INTRODUCTION

1.1 Sensory analysis of virgin olive oil

In combination with chemical parameters, the sensory analysis performed according to the official method reported in Regulation (EC) 640/2008 is an important tool to classify the oils obtained by olives in different quality and commercial categories (extra virgin, virgin, lampante). This official methodology has been established thanks to international cooperative studies, supported by the International Olive Council (IOC), that has provided a sensory codified methodology for extra virgin olive oils (EVOOs), known as the "IOC Panel test" (IOC Decision No Dec-23/98-V/2010). Such an official sensory evaluation takes into account three positive attributes (fruity, perceived both by nose and palate and that could be green or ripe, bitter and pungent) and 5 main defects, extended with a list of additional ones. Actually it can be enhanced by the use of a more detailed profile sheet, which considers additional positive attributes compared to the official profile sheet (Regulation (EC) 640/2008), in particular through the evaluation of the presence and the intensity of aromatic components as well as harmony and persistency. Such a complete and extended objective profiling has already been cross-validated by the German and the Swiss Olive Oil Panel (SOP) (Bongartz and Oberg 2011). The method allows a sensory differentiation of EVOOs in high quality (or "premium quality") compared to standard level products, permitting the identification of excellent quality within the range of EVOOs (Bongartz and Oberg 2011).

1.2 Chemical compounds and sensory attributes: is it possible to discriminate samples?

As it is well known, aromatic notes of EVOOs are strictly linked to many volatile compounds, which are perceptible if their concentration in the aromatic fraction exceeds the odour threshold (Kalua *et al.* 2007). Several factors can influence the composition of the volatile fraction of EVOO, such as genetic, agronomic, processing and storage variables

(Kalua *et al.* 2007; Angerosa *et al.* 2004). Different studies show that it is possible to discriminate oils according to the growing region and the cultivar of the olives, on the basis of the qualitative and quantitative composition of the volatile profile (Luna *et al.* 2006; Pizarro *et al.* 2011). Some previous works about discrimination of EVOOs from different cultivar, have been carried out by the use of sensory analysis in addition with chemical approaches (Guerrero et al. 2001; Rotondi et al. 2011). The combination of sensory and chemical parameters was also useful to discriminate different French PDOs, adopting a chemometric approach (Ollivier et al. 2006). Another interesting study was carried out by Tura et al. 2008, in which volatile compounds were correlated to sensory notes in virgin olive oils from 18 local cultivars in northern Italy assessed for 4 years in the same orchard, in order to characterize them. Concerning the attributes perceived during the tasting phase of EVOOs, the bitter taste (primary taste of oil obtained from green olives or olives turning colour) has been related to the phenolic compounds, especially oleuropein and ligstroside derivatives (Gutierrez-Rosales et al. 2003; Mateos et al. 2004; Bendini et al. 2007) whereas the presence of the dialdehydic form of decarboxymethyl elenolic acid linked to tyrosol (oleocanthal) has been linked to the pungency of the EVOOs (biting tactile sensations characteristic of oils produced at the start of the crop year, primarily from olives that are still unripe) (Andrewes et al. 2003). Literature shows that it is also possible to discriminate oils produced from olive fruits of different cultivar and geographical origin, according to the phenolic composition (Tura et al. 2007; Lerma Garcia et al. 2009). Although several authors pointed out that phenolic compounds of EVOOs can play an important role on human health, due to their antioxidant, anti-carcinogenic and anti-inflammatory properties (Bendini et al. 2007), it is well known that the rejection of bitterness and pungency is a natural reaction for consumers, since poisonous or toxic substances tend to be bitter and pungent: this was confirmed recently by similar results found for American consumers (Delgado and Guinard 2011). This means that by research dissemination, great efforts have to be done in order to make consumers capable to appreciate bitterness and pungency as health- related substances: for example, Peyrot de Gaschos et al. 2011 reported that high-quality EVOOs can be referred to as “one cough” or “two cough” oils (the latter being more highly prized), because of the peculiar pungency. Being successful in such a dissemination will not be an easy purpose, since some authors reported that consumers consider health benefits and flavour (including its use to

enhance the taste of recipes) as main motivators for their food consumption- and in this case for their olive oil consumption-, but nevertheless still second in importance behind packaging, price and size (Martínez et al. 2002; Santosa and Guinard 2011), both in emerging and traditionally located markets. Some researchers suggested that people can transform an inherently unpleasant sensation into a positive one because it has beneficial health effects (Peyrot des Gachons et al. 2011). Moreover, Caporale et al. 2006 reported that giving information to consumers about the origin of the product can lead to a positive expectation regarding specific attributes such as bitter and pungent. For example, if consumers of mono-cultivar Coratina EVOOs usually consider bitter and pungent as characterizing attributes of "familiar" oils, then the information (that the oil is actually made with Coratina olives) creates positive expectations about bitterness and pungency.

1.3 Aims of the investigation

Since the phenolic and the volatile profiles of olive oils depend above all on the geographical origin and on the cultivar of olives (Bendini et al. 2007; Kalua et al. 2007), one aim of this investigation was to verify if also the sensory attributes themselves - which are strictly related to volatile and phenolic compounds - may permit to discriminate EVOOs obtained by olives of different cultivar and/or grown in different regions. A second aim of this work was to evaluate if mono-cultivar EVOOs can be considered of "higher quality" or can be discriminated from the blends made out of more than one cultivar, considering the sensory attributes, the overall liking and the harmony value. The same effect of discrimination was tested also for PDO EVOOs versus EVOOs without PDO certification on the one hand and organic EVOOs versus conventional ones on the other hand. Moreover, another aim of this investigation was to find if some correlations exist among the sensory attributes typical for EVOOs; in particular, it was interesting to investigate if the harmony descriptor evaluated by the SOP is linked to other sensory attributes (aromatic and gustatory). Another purpose was to evaluate whether the consumers' overall liking can be correlated to the harmony or to other sensory attributes evaluated by the trained panel, in order to understand which type of EVOO is preferred by consumers. In other words, the last goal was to investigate the overall liking of consumers that are unfamiliar with bitterness and pungency, for EVOOs that actually were all judged as "medium-high" for the intensities of these descriptors by the trained panel.

2. MATERIALS AND METHODS

2.1 Samples

This investigation focused on 140 EVOO samples, all participating in the IOOA (International Olive Oil Award – Zurich). The samples came from 5 different producing countries: Italy (74 oils, of which 52 came from Sicily), Spain (43 oils, of which 24 came from Andalusia), Greece (15), Portugal (5) and Turkey (3). The samples were collected from 4 different harvests/crop years (2007-2010), so 35 samples were analyzed per year (**Table 1**). All oils belonged to the category of premium/very high quality EVOOs, without defects evaluated by the SOP (see *paragraphs 2.2* and *3.1*). Among the samples, 55 oils were characterized by a PDO, 87 were mono-cultivar EVOOs, which means that they were obtained by only one cultivar of olives (22 different cultivars), 37 were produced by organic farming systems (**Table 1**). Before the sensory analysis, all EVOOs were stored in bottles under dark conditions, protecting them from light and kept under controlled temperature.

Table 1. Samples of EVOOs. The table describes, for each oil: the year of production, the region/province, the cultivar of the olives, the presence of a designation of origin (Protected Designation of Origin, PDO), the farming system (conventional/organic).

Sample	Crop year	Country	Region/Province	Cultivar	PDO	Farming system
1	2007	Italy	Campania	40% Ravece, 30% Leccine, 20% Frantoiane, 10% Ogliarola		org
2	2007	Italy	Campania	100% Ravece		org
3	2007	Italy	Sicily	70% Nocellara del Belice, 15% Cerasuola, 15% Biancolilla		conv
4	2007	Italy	Apulia	100% Coratina	PDO Dauno	org
5	2007	Greece	Peloponnese	100% Manaki	PDO Kranidi	org
6	2007	Italy	Sicily	100% Tonda Iblea		org
7	2007	Italy	Sicily	100% Tonda Iblea	PDO Monti Iblei	conv
8	2007	Italy	Sicily	100% Nocellara del Belice		conv
9	2007	Italy	Sicily	100% Nocellara del Belice	PDO Valle del Belice	conv
10	2007	Italy	Sicily	100% Tonda Iblea	PDO Valle del Belice	conv
11	2007	Italy	Liguria	100% Taggiasca	PDO Riviera Ligure	conv
12	2007	Italy	Tuscany	70% Frantoio, 20% Leccino, 10% Pendolino		conv
13	2007	Spain	Murcia	100% Arbequina		org
14	2007	Italy	Sicily	100% Nocellara Etnea		conv
15	2007	Italy	Sicily	100% Cerasuola		conv
16	2007	Italy	Marche	100% Ascolana		conv
17	2007	Spain	Andalusia	100% Hojiblanca	PDO Priego de Cordoba	org
18	2007	Spain	Andalusia	60% Picudo, 40% Hojiblanca	PDO Priego de Cordoba	conv
19	2007	Greece	Kreta	100% Koroneiki		conv
20	2007	Spain	Andalusia	90% Hojiblanca, 10% Picudo	PDO Priego de Cordoba	conv
21	2007	Greece	Kreta	100% Koroneiki	PDO Sitia	conv
22	2007	Spain	Andalusia	70% Picudo, 30% Hojiblanca	PDO Priego de Cordoba	conv
23	2007	Italy	Tuscany	Frantoio, moraiolo, leccino, raggiale		conv
24	2007	Greece	Kreta	100% Koroneiki	PDO Sitia	conv
25	2007	Spain	Catalonia	80% Arbequina, 10% Hojiblanca, 10% Koroneiki		conv
26	2007	Spain	Andalusia	100% Arbequina	PDO Mallorca	conv
27	2007	Spain	Andalusia	80% Hojiblanca, 20% Picudo	PDO Priego de Cordoba	conv
28	2007	Italy	Sicily	80% Cerasuola e Nocellara del Belice, 20% Biancolilla	PDO Valli Trapanesi	org
29	2007	Spain	Andalusia	80% Picudo, 20% Hojiblanca	PDO Priego de Cordoba	conv
30	2007	Italy	Sicily	100% Nocellara del Belice		conv
31	2007	Spain	Aragon	50% Arbosana, 40% Frantoio, 10% Arbequina		org
32	2007	Greece	Kreta	100% Koroneiki		conv
33	2007	Spain	Catalonia	100% Arbequina	PDO Siurana	conv
34	2007	Italy	Sicily	50% Biancolilla, 40% Cerasuola, 10% Nocellara del Belice		org
35	2007	Turkey	Golf region	100% Edremit		org
36	2008	Italy	Sicily	100% Biancolilla		conv
37	2008	Greece	Kreta	100% Koroneiki		conv
38	2008	Turkey	Izmir	100% Memecik		conv
39	2008	Italy	Sicily	100% Tonda Iblea		org
40	2008	Italy	Sicily	100% Tonda Iblea	PDO Monti Iblei	conv
41	2008	Italy	Marche	100% Ascolana		conv
42	2008	Italy	Sicily	100% Tonda Iblea		conv

continue…

Sample	Crop year	Country	Region/Province	Cultivar	PDO	Farming system
43	2008	Spain	Catalonia	100% Arbequina		conv
44	2008	Italy	Sicily	100% Tonda iblea		conv
45	2008	Italy	Sicily	100% Tonda Iblea	PDO Monti Iblei	org
46	2008	Italy	Sicily	50% Biancolilla 40% Cesaruola 10% Nocellara del Belice		conv
47	2008	Italy	Sicily	100% Tonda Iblea	PDO Monti Iblei	conv
48	2008	Italy	Sicily	100% Cerasuola		conv
49	2008	Greece	Peloponnese	100% Koroneiki		conv
50	2008	Spain	Aragon	40% Arbosana, 30% Arbequina, 30% Frantoio		conv
51	2008	Spain	Andalusia	100% Hojiblanca	PDO Priego de Cordoba	conv
52	2008	Spain	Andalusia	50% Picudo, 50% Hojiblanca	PDO Priego de Cordoba	conv
53	2008	Spain	Andalusia	100% Hojiblanca	PDO Priego de Cordoba	conv
54	2008	Italy	Abruzzo	70% Dritta, 20% Frantoio, 10% Leccino	PDO Apruntino	org
55	2008	Italy	Calabria	100% Dolce di Rossano	PDO Bruzio (Joniche	conv
56	2008	Italy	Tuscany	70% Frantoio, 20% Moraiolo, 10% Leccino		conv
57	2008	Italy	Sicily	33.3% Nocellara del Belice, 33.3% Biancolilla, 33.3% Cerasuola	PDO Valli Trapanesi	org
58	2008	Italy	Sicily	100% Tonda Iblea		conv
59	2008	Italy	Sicily	90% Cerasuola, 10% Biancolilla & Nocellara del Belice	PDO Valli Trapanesi	conv
60	2008	Portugal	Ribatejo	15% Galega, 10% Arbequina, 75% Cobrançosa		conv
61	2008	Italy	Sicily	100% Nocellara del Belice		conv
62	2008	Spain	Andalusia	100% Vidueña		conv
63	2008	Greece	Kreta	100% Koroneiki	PDO Sitia	conv
64	2008	Greece	Peloponnese	100% Koroneiki	PDO Kalamata	conv
65	2008	Spain	Catalonia	100% Siurana	PDO Siurana	conv
66	2008	Greece	Peloponnese	70% Athinochia, 20% Koroneiiki, 10% Mourtoeilia		org
67	2008	Spain	Castile-La Mancha	Arbequina, Hojiblanca, Picudo, Ocal		conv
68	2008	Spain	Andalusia	100% Hojiblanca		conv
69	2008	Italy	Apulia	100% Peranzana		conv
70	2008	Greece	Kreta	100% Koroneiki		Org
71	2009	Italy	Sicily	50% Cerasuola, 45% Biancollila, 5% Nocellara del Belice		conv
72	2009	Italy	Sicily	50% Frantoio, 44% Moraiolo, 5% Leccino, 1% Pendolino		org
73	2009	Italy	Marche	100% Ascolana		conv
74	2009	Spain	Andalusia	100% Hojiblanca	PDO Priego de Cordoba	org
75	2009	Spain	Andalusia	80% Picudo, 20% Hojiblanca	PDO Priego de Cordoba	org
76	2009	Italy	Sicily	60% Biancolilla, 40% Cerasuola		conv
77	2009	Italy	Sicily	100% Tonda Iblea	PDO Monti Iblei	org
78	2009	Spain	Castilla y Leon	40% Manzanila, 40% Picual, 20% Madurol		org
79	2009	Greek	Peloponnese	100% Koroneiki	PDO Kalamata	conv
80	2009	Portugal	Ribatejo	85% Cobrançosa, 10% Arbequina, 5% Galega		conv
81	2009	Spain	Navarra	100% Arbequina		org
82	2009	Italy	Sicily	80% Cerasuola, 10% Nocellara, 10% Biancolilla	PDO Valli Trapanesi	conv
83	2009	Italy	Sicily	90% Cerasuola, 5% Nocellara, 5% Biancolilla	PDO Valli Trapanesi	conv
84	2009	Italy	Sicily	100% Biancolilla		conv
85	2009	Italy	Sicily	100% Cerasuola		conv
86	2009	Italy	Sicily	100% Nocellara del Belice		conv

continue…

Sample	Crop year	Country	Region/Province	Cultivar	PDO	Farming system
87	2009	Italy	Sicily	100% Cerasuola	PDO Valli Trapanesi	conv
88	2009	Italy	Sicily	100% Nocellara del Belice		conv
89	2009	Spain	Extremadura	100% Cornezuelo		conv
90	2009	Spain	Andalusia	100% Hojiblanca	PDO Priego de Cordoba	conv
91	2009	Spain	Andalusia	80% Hojiblanca, 20% Picudo	PDO Priego de Cordoba	conv
92	2009	Spain	Andalusia	70% Hojiblanca, 30% Picudo	PDO Priego de Cordoba	conv
93	2009	Spain	Aragon	100% Arbosana		conv
94	2009	Italy	Sicily	100% Nocellara Etnea		conv
95	2009	Spain	Andalusia	100% Vidueña		conv
96	2009	Italy	Apulia	70% Peranzana, 30% Leccino		conv
97	2009	Italy	Sicily	60% Nocellara del Belice, 20% Biancolilla, 20% Cerasuola	PDO Val di Mazara	conv
98	2009	Greek	Peloponnese	100% Koroneiki		org
99	2009	Italy	Sicily	60% Biancollila, 30% Cerasuola, 10% Nocellara del Belice		conv
100	2009	Turkey	Izmir	100% Memecik		conv
101	2009	Italy	Sicily	90% Cerasuola, 10% Biancolilla + Nocellara del Belice	PDO Valli Trapanesi	conv
102	2009	Greek	Kreta	100% Koroneiki	PDO Sitia	conv
103	2009	Italy	Sicily	33.3% Nocellara del Belice, 33.3% Biancolilla, 33.3% Cerasuola	PDO Valli Trapanesi	org
104	2009	Italy	Apulia	100% Ogliarola	PDO Dauno Gargano	conv
105	2009	Italy	Campania	50% Carpellese, 50% Frantoio		conv
106	2010	Spain	Catalonia	100% Arbequina		conv
107	2010	Italy	Sicily	100% Tonda Iblea		org
108	2010	Italy	Sicily	100% Tonda Iblea	PDO Monti Iblei	conv
109	2010	Italy	Sicily	100% Tonda Iblea		org
110	2010	Italy	Sicily	100% Nocellara del Belice		conv
111	2010	Spain	Andalusia	100% Vidueña		conv
112	2010	Italy	Sicily	100% Nocellara del Belice		conv
113	2010	Spain	Andalusia	70% Hojiblanca, 30% Picudo	PDO Priego de la	conv
114	2010	Italy	Marche	60% Leccino, 20% Frantoio, 20% Ascolana		conv
115	2010	Italy	Sicily	33.3% Nocellara del Belice, 33.3% Biancolilla, 33.3% Cerasuola		org
116	2010	Italy	Sicily	80% Cerasuola + Nocellara del Belice, 20% Biancolilla	PDO Valli Trapanesi	org
117	2010	Spain	Andalusia	100% Hojiblanca	PDO Priego de Cordoba	org
118	2010	Italy	Marche	100% Ascolana		conv
119	2010	Spain	Castile-La Mancha	50% Cornicabra, 50% Picual		org
120	2010	Spain	Extremadura	100% Arbequina		conv
121	2010	Spain	Andalusia	33.3% Picudo, 33.3% Picual, 33.3% Hojiblanca	PDO Baena	org
122	2010	Spain	Tarragona	100% Arbequina		conv
123	2010	Spain	Aragon	100% Empeltre		org
124	2010	Portugal	Ribatejo	Cobrançosa, Galega, Arbequina		conv
125	2010	Spain	Andalusia	100% Arbequina	PDO Mallorca	conv
126	2010	Spain	Catalonia	80% Arbequina, 10% Hojiblanca, 10% Koroneiki		conv
127	2010	Italy	Apulia	60% Olivastra tipica Martina Franca, 40% Ogliarola + Coratina + Picholine		conv
128	2010	Portugal	Centro	50% Cobrançosa, 20% Picual, 10% Arbequina, 10% Galega, 10% Cornicabra		conv
129	2010	Italy	Campania	60% Ravece, 30% Ogliarola, 10% Leccine		conv
130	2010	Italy	Campania	100% Peranzana		org

continue…

Sample	Crop year	Country	Region/Province	Cultivar	PDO	Farming system
131	2010	Spain	Andalusia	100% Picual	PDO Jaen	conv
132	2010	Italy	Tuscany	33.3% Frantoio, 33.3% Raggiaia, 33.3% Morrelino		conv
133	2010	Portugal	Nord	50% Madural, 30% Verdeal, 20% Negrinha	PDO Tràs-o-Montes	org
134	2010	Greek	Kreta	100% Koroneiki		org
135	2010	Italy	Sicily	100% Nocellara del Belice		conv
136	2010	Italy	Sicily	90% Cerasuola, 5% Nocellara, 5% Biancolilla	PDO Valli Trapanesi	conv
137	2010	Spain	Navarra	100% Arbequina	PDO Navarra	org
138	2010	Italy	Sicily	100% Cerasuola		conv
139	2010	Italy	Sicily	100% Biancolilla		conv
140	2010	Italy	Sicily	100% Nocellara del Belice		conv

2.2 The sensory evaluation by the Swiss Olive Oil Panel (SOP)

The sensory analysis was carried out by the SOP of ZHAW (Zurich University of Applied Sciences, Department of Life Sciences and Facility Management), from 2007 to 2010. The SOP, founded in 2002, was created primarily as a jury for the "International Olive Oil Award - Zurich" (IOOA), but then it was recognized by the IOC (International Olive Council) and accredited according to EN/ISO/IEC 17025. The Panel (SOP) consists of 40 panellists, regularly trained "in situ" as well as virtual (meaning by providing the panellists with training samples, collecting results and doing a feedback and moderation via Internet / e-mail). The evaluation of the samples was performed according to the rules established by Regulation (EC) 640/2008 and following the enhanced profile sheet reported and validated in the previous paper (Bongartz and Oberg 2011). Samples intended for tasting were kept in the standardised tasting glasses at 28° C ± 2° C throughout the test, as reported in IOC/T.20/Doc. No 15/Rev. 4. The test was conducted in the sensory laboratory of the Institute of Food and Beverage Innovation at the Department of Life Sciences and Facility Management in Wädenswil / Switzerland at room temperature (20° C ± 2° C) and 60% ± 5% relative air humidity. The profile sheet followed by the trained panellists is reported in **Fig. 1**. First of all, each assessor has to evaluate the presence and the intensity of the standard defects of the oils (rancid, fusty-muddy sediment, metallic, musty, winey, other defects), the fruitiness, the bitterness and the pungency, using the well-known 10 cm-scales (Regulation (EC) 640/2008). Also 17 aromatic descriptors (ortho- and retronasally perceived) were evaluated by the SOP as green fruity, or more precise: freshly cut grass, green nut-skin / -shell, green almond-skin / -shell, green pine-skin / -shell, green artichoke, green tomato, herbs, green apple, green banana and ripe fruity, with descriptors like dried nut kernel, dried almond kernel, ripe tomato, ripe apple, ripe banana and cassis. The gustatory descriptor sweet was also evaluated, meaning the absence of bitterness. A definition of most of them is reported in IOC T20 Doc. 22/ 2005, in IOC T20 Doc.15/Rev.2/ 2007 and in the sensory wheel described by Mojet and De Jons 1994. A 4-point scale was used to measure the intensity of each aromatic component and sweet as well, from 0 to 3 (Bongartz and Oberg 2011): 0 (zero) means that one component is "not detectable", 1 (one) means that it is "slightly detectable"; 2 (two) stands for a "noticeable" sensation and, finally, 3 (three) describes an "intense" sensation. The use of a more detailed scale would be possible, but would require even more training effort by each panellist. Aroma

descriptors were listed on the profile sheet, supporting tasters in finding and recognizing common aromas more easily. The list is divided into the sections “green” and “ripe”, which is helpful for the selection by the tasters and at the same time provides a hint to the decision on whether the fruitiness of an olive oil is more “green” or more “ripe” or both in equal parts. Panellists were also asked to evaluate two other objective sensory descriptors for each EVOO, such as harmony and persistency, on 10 cm bipolar scales (Bongartz and Oberg 2011): for this, intense training of all individual panellists as well as the whole panel is absolutely essential. The panel supervisor can either moderate the harmony value with single tasters or is allowed to eliminate single harmony results as outliers but the minimal number of valid results has to be at least 6 (Bongartz and Oberg 2011). The importance of these two parameters consists in discriminating the quality levels of EVOO, also within the group of excellent premium quality EVOOs. For considering the results as valid and acceptable, related to the intensities evaluated by the panellists on 10 cm scales (**Fig. 1**), their robust coefficient of variation (CVr) has to be below 10%. Single results that exceed a standard deviation of 1.5 in either direction have to be eliminated as outliers. Regarding the profiling of aromatic attributes, at least 33% of tasters have to recognize the same descriptor (e.g. green apple, banana or fresh almonds) in order to include it as part of the sensory description of the oil, within the allocation into the categories “green”, “ripe” or “green/ripe”. For the intensity of these selected attributes, the median values of each attribute were taken into account.

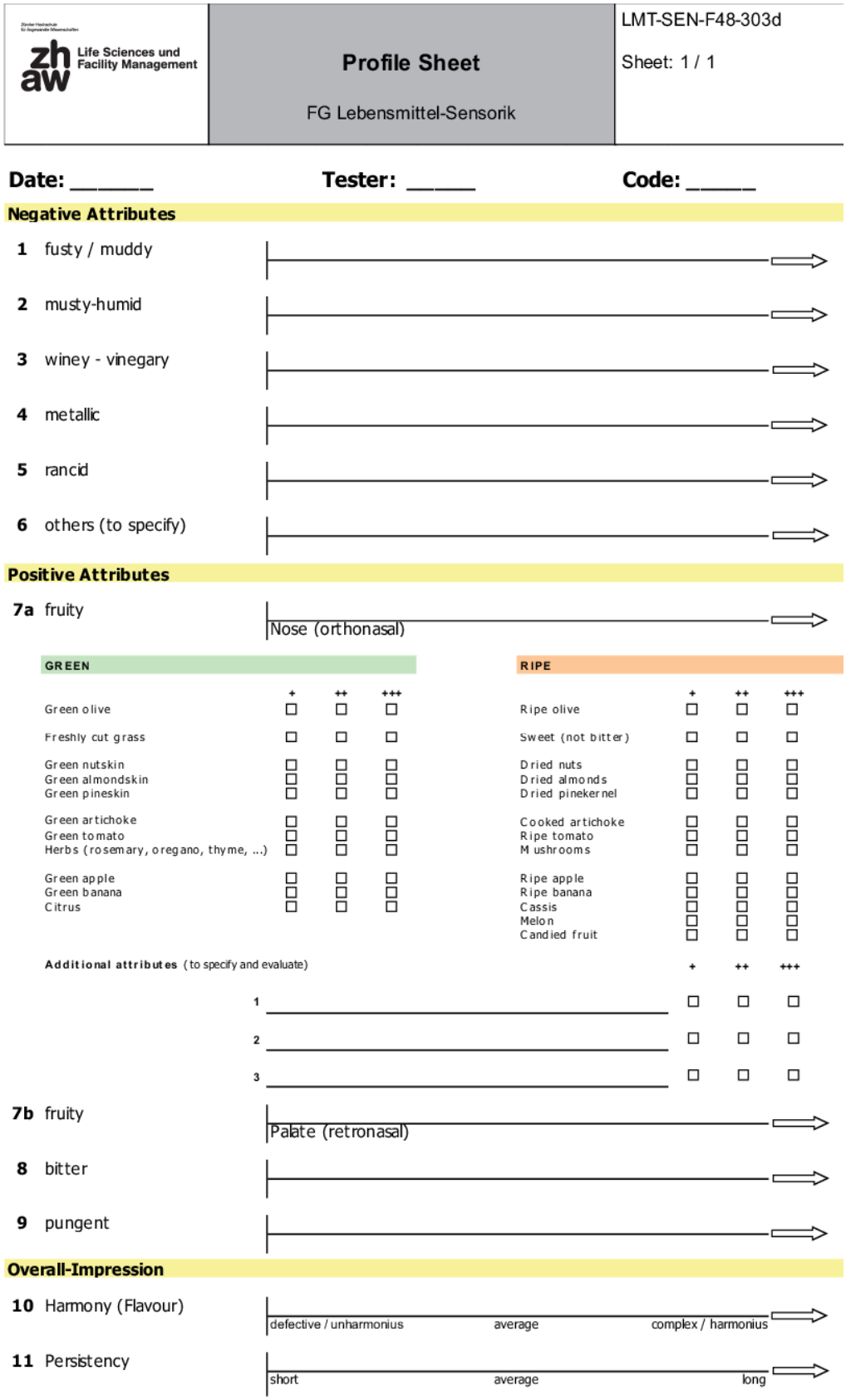

Fig. 1. profile sheet used for the objective evaluation by the Swiss Olive Oil Panel.

2.3 The "International Olive Oil Award –Zurich" (IOOA)

This event took place at the University of Applied Sciences Zurich (ZHAW) over the last decade from 2002 until now – and for the 11th time in the year 2012.. Every year the International Olive Oil Award - Zurich invites producers, importers and retailers to participate in this specific olive oil competition. Many of the best EVOOs on the Swiss market as well as many samples coming directly from producing countries, register for a sensory evaluation which includes - besides the classification of olive oil according to the Regulation (EC) 640/2008- the evaluation of harmony and persistency and the description of the aromatic components (see 3.2). The competition is organized in a three-step system. First step: a group of three SOP panellists check participating olive oils in order to find (and exclude) defective ones and in order to estimate the intensity of fruitiness to define the sample order for the upcoming tastings. Results of this screening have only orienting character. Second step: panel tests with a minimum of 8 tasters take place to evaluate the oils using the advanced methodology (see 3.2) and profile sheet (**Fig. 1**). All tests are blind tastings and the results are statistically relevant. Depending on the results and the quality of the oils round about 50% of these olive oils are selected for the third step. Third step: a second advanced panel test takes place with the aim to confirm the extraordinary quality of the best oils and thereby to define the winners of "Golden Olives", "Silver Olives" and "Awards". These tests are as well blind tastings and results are statistically relevant.

2.4 The "OLIO" – a consumer test at the trade fair "Gourmesse" (Zurich)

All 140 samples of this study were evaluated in different consumer tests sessions over the 4 years, on the occasion of the Gourmesse trade fair in Zurich, meaning that each year 35 oils were considered. All oils were tested for acceptance by altogether 68 consumers, who were distributed into 51% male and 49% female participants. The age of all the consumers involved during the four years was wide-distributed, from 0-10 years old (0.4%), 11-20 years old (6.2%), 21-30 years old (20.9%), 31-40 years old (22.2%), 41-50 years old (21.0%), 51-60 years old (18.0%), 61-70 years old (8.7%), 71-80 years old (2.6%). A 9 point-hedonic scale was used for evaluating the overall liking of each oil (1 = do not like at all, 9 = like very much).

1.5 Statistical analysis

The Analysis of Variance (ANOVA), the Correlation Matrix (Pearson's correlation, $p < 0.05$), the Principal Component Analysis (PCA), the Agglomerative Hierarchical Clustering (AHC) and the Preference Mapping were performed by the statistical software XLSTAT 2011 (Addinsoft, New York, USA).

2. RESULTS AND DISCUSSION

3.1 Calculation and evaluation of harmony and persistency

No defects were found for all the selected samples, according to the European official sensory evaluation (Regulation (EC) 640/08). All samples were characterized by a high level of harmony (above 5.2 and persistency (above 5.8), reflecting the strong degree of balance of all the positive characteristics of these EVOOs, from an aromatic, tactile and kinaesthetic point of view as well as their long length in the "aftertaste" phase of the degustation. As reported in a previous work (Bongartz and Oberg, 2011), the harmony and persistency attributes were joined together, calculating a new weighted one (called "H & P"), counting the harmony twice and persistency one time, as validated by Bongartz and Oberg 2011. Considering the "H & P" values of the 140 examined EVOOs, 130 were within the harmony category "very good / premium > 6.4", so they have a very complex aroma profile, a pronounced harmony and persistency and therefore a flavour which can be considered from "very good" up to "excellent" (Bongartz and Oberg, 2011). Considering these results, a further quality class could be introduced, which is "excellent > 7.4", representing oils with an excellent flavour profile, distinguishable from the very good / premium EVOOs for particular aromatic or gustatory notes and a complex equilibrium that take them to an "upper level": 63 samples (45% of all 140) could be classified in this class, confirming the excellent sensory quality of the oils object of this investigation (**Fig. 2**). With regard to this finding, it was surprising to see that only 27 samples (19.3%) were evaluated positively by the consumers (overall liking > or equal to 6.00) and even 33 EVOOs (23.6%) showed an overall liking below or equal to 5.00 (**Fig. 3**). This is a first hint to the fact that the behaviour of consumers and trained panellists might be very different (see 3.2 and 3.5).

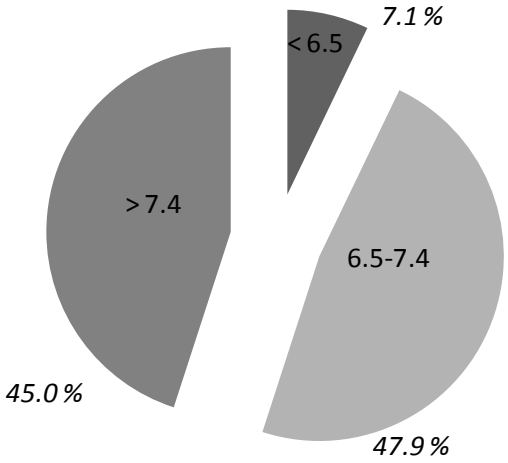

Fig. 2. Intensity of harmony and persistency (H&P) of the analysed samples, expressed as percentage on the total of samples.

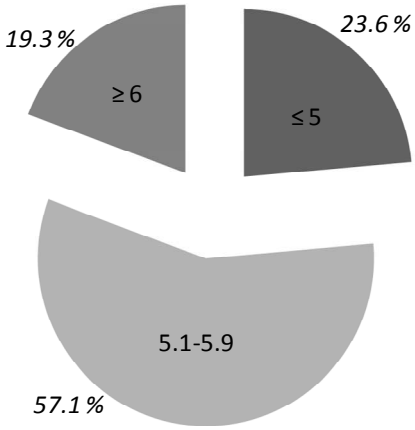

Fig. 3. Overall liking evaluated by consumers and expressed as percentage on the total of samples.

3.2 Principal Component Analysis (PCA): projection of the variables in the space

For the elaboration of the PCA (Principal Component Analysis), on the 23 positive sensory attributes considered in the extended profile sheet for each EVOO (**Fig.1**), it was decided to delete 11 aromatic attributes, that 1) showed very low correlations with overall liking scores of consumers, with harmony and as well the other main aromatic attributes and 2) were found very rarely in the analysed EVOOs. Therefore the sensory attributes considered for performing the PCA finally were: bitter, pungent, fruity (nose + palate, that means perceived both directly by smell and retronasally, as reported in Regulation (EC) 640/2008), green fruity, ripe fruity, sweet, freshly cut grass, green nut-skin / -shell, green

tomato, herbs, harmony and persistency. The consumers' overall liking was additionally considered in this statistical elaboration. The first two factors (F1 and F2) permitted to represent 61.58% of the initial variability of the considered data. The correlation circle (**Fig. 4**), which is a projection of the variables in the space, gave important information on how the sensory attributes are correlated. Looking at the correlation circle (**Fig. 4**), one can investigate the relationships among the variables that are confirmed in **Table 2**: on the one hand the overall liking vector is situated far from the centre, and directed opposite to the vectors for bitter and pungent, evidencing that it is negatively correlated with both. On the other hand the "overall liking" vector is situated near the vectors for ripe fruity and sweet, suggesting a positive correlation. It is interesting to observe the orthogonal position of the vector harmony, almost independent from the vectors for bitter, pungent and sweet on one side as well as the vectors for ripe fruity and overall liking on the other side. This means that the variables that were looked at are not directly correlated with harmony, confirming that EVOOs with high harmony scores cannot automatically be defined as having unique sensory characteristics, for example in terms of bitterness and pungency. In other words, the more bitter and the more pungent oils are, does not result in lower or a higher harmony scores. For defining it, the equilibrium among fruitiness, bitterness, pungency and all the olfactory notes, as well as their complexity, has to be evaluated by the trained panellists, as reported in the harmony scale (**Fig.1**) Nevertheless harmony strictly correlated with persistency, since both vectors are located far from the centre and one close to each other (**Fig. 4**).

3.3 Correlations among sensory attributes

As expected (**Fig. 4**), bitter and pungent were highly correlated (r = 0.808) (**Table 2**): this is proved by the fact that usually EVOOs that are very bitter are also very pungent, since these two sensory attributes share the same "chemical origin", that is phenolic compounds (Bendini et al. 2007). Bitter and green fruity were also positively correlated (r = 0.590), as reported in previous studies (Monteleone et al. 1996), supporting the statement that the green odour note has a positive significant effect on the perception of bitterness (Caporale et al. 2004).

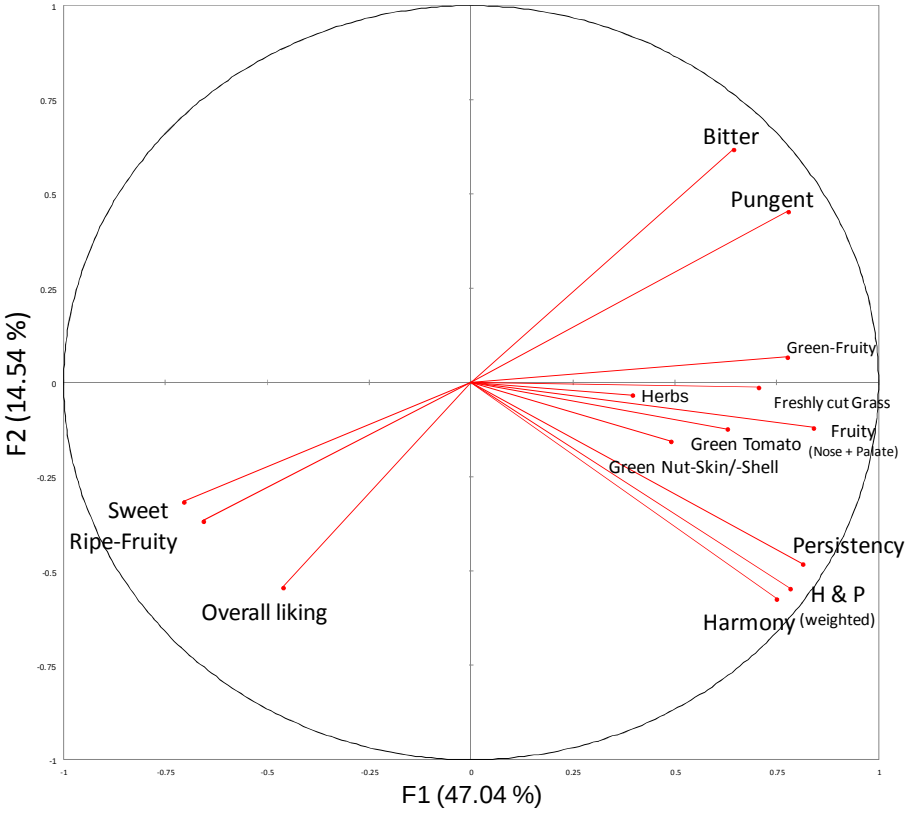

Fig. 4. Principal component analysis, correlation circle showing a projection of the selected variables in the factors plane.

Bitter is also positively correlated with the cut grass aromatic note (r = 0.406), as previously found (Mojet and de Jons 1994). It was also interesting to underline that bitter was negatively correlated with ripe fruity (r = -0.592), which is actually a more typical and common attribute for sweet EVOOs (see the positive correlation between ripe fruity and the attribute sweet (r = 0.574). At the same time, both ripe fruity and sweet were negatively correlated with pungent (respectively, r = -0.609 and r = -0.657). The relationships between attributes perceived during smell and taste phases were not easy to be explained. It seems highly probable that taste and aroma interact in a specific way, with synergetic, antagonistic or independent effects (Schifferstein and Verlegh 1996). Considering the descriptor harmony, first it was interesting to observe a very high correlation with persistency (r = 0.920), confirming that it was correct and useful to summarize them in only one weighted attribute as reported in Bongartz and Oberg, 2011 (see 3.2). High correlations were also found (**Table 2**) between harmony and fruity (r = 0.637). Harmony was not high correlated with bitter (r = 0.175) or pungent (r = 0.337),

confirming that a bitter and pungent oil is not always characterized by high harmony values (see 3.2). Interesting and significant (but not high) positive correlations were additionally found between harmony and green fruity (r = 0.495) as well as attributes like fresh cut grass (r = 0.462) and green tomato (r = 0.451), suggesting that the latter were perceived as important components for a complex and balanced (harmonious) olive oil by trained panellists. At the same time, ripe fruity and sweet were negatively correlated with harmony: this could be due to the fact that in the considered set of 140 EVOOs ripe fruity oils and sweet oils with high harmony were not widely represented. On the other side, it was interesting to underline that harmony and persistency were not linked to the overall liking perception of the consumers. This suggested that an evident discrepancy existed between the consumer perception and the attribute evaluated as harmony by the trained panellists. Overall liking was negatively correlated with bitter (r = -0.544) and pungent (r = -0.530), and positively with sweet (r = 0.420). This means that, as reported in previous investigations, consumers behaviour (acceptance) is rejecting very bitter oils (Garcìa et al. 2001) and, in general, considers the characteristic bitterness and pungency of EVOOs as rather negative attributes (Delgado and Guinard 2011). In particular, bitterness is a very common perception for EVOOs but, at the same time, can further be considered as a quite unusual, atypical and “different” sensation compared to pungent substances, since it is restricted to the throat and often leads to coughing and throat clearing (Peyrot de Gaschos et al. 2011). In our opinion, another relevant factor for the different consumer perspective, compared to the results of the trained panellists, was the way of testing the oils. Panellists and consumers always tested them “pure” and not combined with foods (salad, meat, vegetables, etc.). Previous works reported a methodological proposal for evaluating the harmony of EVOO and food pairing, by a trained sensory panel (Cerretani et al. 2007). Since it was clear that bitterness and pungency are characteristics that consumers are definitely not familiar with (and therefore react differently from trained and expert panellists), it could be interesting in future studies to verify and validate how consumers would react by comparing different EVOOs in combination with food. Maybe it will change their acceptance for the products, moving it to a more comprehensive way of using and tasting EVOOs.

3.4 Principal Component Analysis (PCA): projection of the samples in the factors plane and classification of EVOOs

The interpretation of the projection of the samples on the orthogonal plane was difficult, since a lot of samples were integrated into this evaluation, just forming a faint "cloud". Nevertheless the idea was to check if it possible to group the samples according to the cultivars and the growing regions (origins) of the olive oils, in order to verify if such a statistical approach could have (or not) a discriminant effect on their distribution. Looking at the cultivar, first one could see that the samples obtained by more than 50% olives from the cultivar Picudo were located in the first quadrant of the 2-D map (**Fig. 5A**), confirming their tendency to be very bitter and pungent oils (**Fig. 4**) and not so appreciated by the consumers. A similar situation was also found for samples obtained from Hojiblanca olives (considering the mono-cultivar and the blends with more than 50% from olives belonging to this cultivar), confirming that they shared basically the same sensory profiles and consumers' overall liking perception, except two of them (27 and 68). Similar grouping effects were also found for Tonda Iblea (**Fig. 5B**), Viduna and Cerasuola mono-cultivar EVOOs.

Another interesting effect is reported in **Fig. 6**: mixing of olives with other cultivars could influence the sensory characteristic of some mono-cultivar samples. In this particular case, only mono-cultivar 100% Cerasuola samples (full circles in **Fig. 6**) and blends (rhombs in **Fig. 6**) obtained from olives of the cultivar Cerasuola (more than 50%), Nocellara and Biancolilla were considered. The blends were characterized by more intense green aromatic attributes and a higher bitterness / pungency compared to mono-cultivar Cerasuola. This suggested that the adding of Nocellara and Biancolilla olives can influence the final sensory properties of oils produced also with Cerasuola olives.

Table 2. Correlation between the attributes. Values in bold are different from 0 with a significance level alpha = 0.05.

Attributes	Bitter	Pungent	Green Fruity	Ripe Fruity	Sweet	Freshly cut Grass	Green Nut-Skin / -Shell	Green Tomato	Herbs	Harmony	Persistency	Fruity (nose + palate)	H & P (weighted)	Overall liking
Bitter	**1**	**0.808**	**0.509**	**-0.592**	**-0.597**	**0.406**	**0.218**	**0.251**	**0.172**	**0.175**	**0.234**	**0.476**	**0.215**	**-0.544**
Pungent	**0.808**	**1**	**0.597**	**-0.609**	**-0.652**	**0.479**	**0.270**	**0.486**	**0.211**	**0.337**	**0.434**	**0.596**	**0.369**	**-0.530**
Green Fruity	**0.509**	**0.597**	**1**	**-0.456**	**-0.491**	**0.551**	**0.276**	**0.440**	**0.341**	**0.495**	**0.576**	**0.630**	**0.533**	**-0.373**
Ripe Fruity	**-0.592**	**-0.609**	**-0.456**	**1**	**0.574**	**-0.484**	**-0.251**	**-0.355**	**-0.188**	**-0.300**	**-0.359**	**-0.434**	**-0.330**	**0.359**
Sweet	**-0.597**	**-0.652**	**-0.491**	**0.574**	**1**	**-0.439**	**-0.260**	**-0.340**	-0.126	**-0.394**	**-0.443**	**-0.514**	**-0.404**	**0.420**
Freshly cut Grass	**0.406**	**0.479**	**0.551**	**-0.484**	**-0.439**	**1**	**0.261**	**0.463**	**0.262**	**0.462**	**0.524**	**0.534**	**0.506**	**-0.233**
Green Nut-Skin / -Shell	**0.218**	**0.270**	**0.276**	**-0.251**	**-0.260**	**0.261**	**1**	**0.313**	**0.406**	**0.349**	**0.380**	**0.449**	**0.348**	-0.129
Green Tomato	**0.251**	**0.486**	**0.440**	**-0.355**	**-0.340**	**0.463**	**0.313**	**1**	0.139	**0.451**	**0.484**	**0.521**	**0.469**	**-0.179**
Herbs	**0.172**	**0.211**	**0.341**	**-0.188**	-0.126	**0.262**	**0.406**	0.139	**1**	**0.204**	**0.248**	**0.435**	**0.209**	**-0.218**
Harmony	**0.175**	**0.337**	**0.495**	**-0.300**	**-0.394**	**0.462**	**0.349**	**0.451**	**0.204**	**1**	**0.920**	**0.637**	**0.975**	-0.089
Persistency	**0.234**	**0.434**	**0.576**	**-0.359**	**-0.443**	**0.524**	**0.380**	**0.484**	**0.248**	**0.920**	**1**	**0.682**	**0.953**	**-0.205**
Fruity (nose +palate)	**0.476**	**0.596**	**0.630**	**-0.434**	**-0.514**	**0.534**	**0.449**	**0.521**	**0.435**	**0.637**	**0.682**	**1**	**0.667**	**-0.288**
H & P (weighted)	**0.215**	**0.369**	**0.533**	**-0.330**	**-0.404**	**0.506**	**0.348**	**0.469**	**0.209**	**0.975**	**0.953**	**0.667**	**1**	-0.129
Overall liking	**-0.544**	**-0.530**	**-0.373**	**0.359**	**0.420**	**-0.233**	-0.129	**-0.179**	**-0.218**	-0.089	**-0.205**	**-0.288**	-0.129	**1**

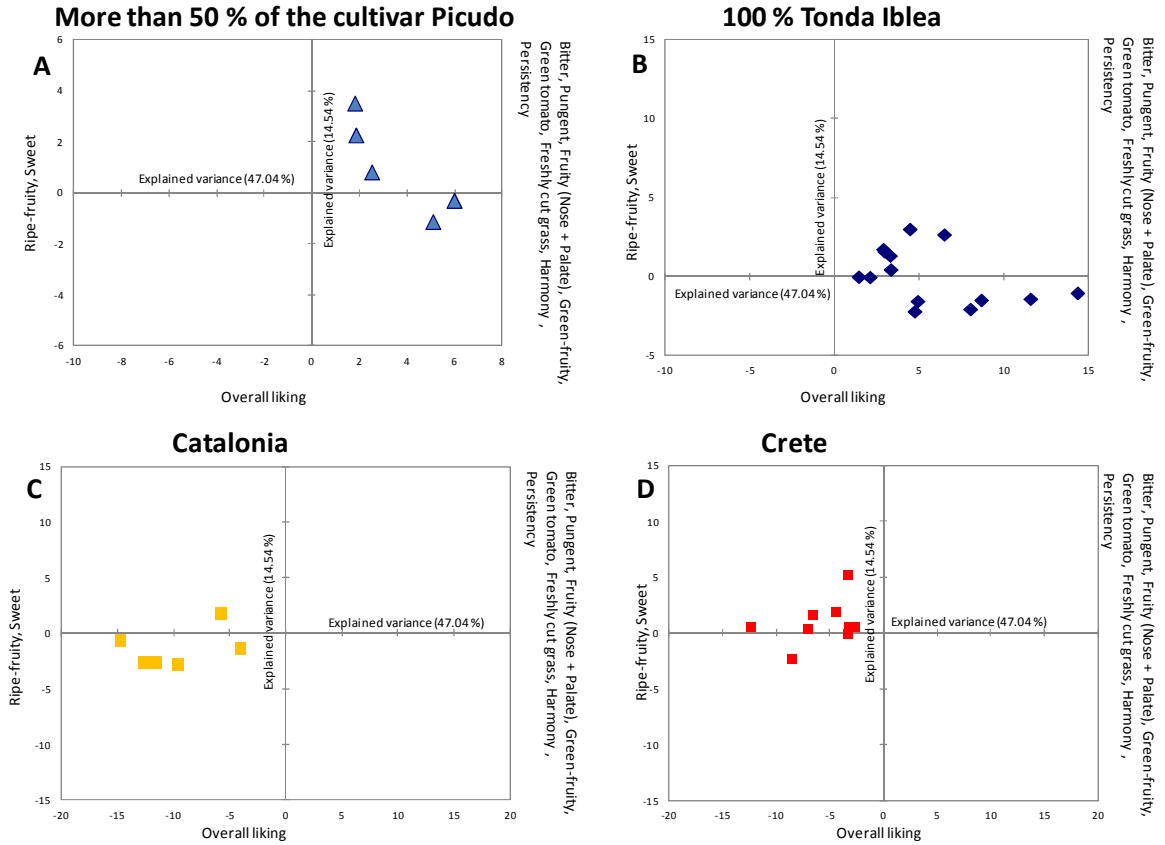

Fig. 5. Principal component analysis, projection of the samples in the factors plane. pca was elaborated considering all the sensory attributes as explained in paragraph 3.2 and each graph reported here show only the samples characterized by features reported above each one.

Considering the samples obtained from mono- cultivar Nocellara del Belice, except for two of them (30 and 110), they were located in the first and in the second quadrants (graph not shown), indicating similar sensory properties, such as high bitterness and pungency (mostly for samples 8 and 9), and also “green” attributes (green fruity, green tomato, freshly cut grass) combined with both a high harmony and persistency. Not expected positions were found for samples 30 and 110, which actually were very low in bitterness and pungency. It was also interesting to underline that the samples 8 and 9 were less appreciated by consumers than the other Nocellara del Belice mono-cultivar EVOOs, maybe because the first were too bitter and too pungent. Checking whether the samples can be grouped according to the growing region (origin) of olives, good results were found for the samples from Crete (Greece), since they were all grouped in the same region of the 2D-map (**Fig. 5C**). Interesting but not too surprising, was to find that samples coming from Andalusia (Spain) could not be grouped so well (graph not shown), because a very broad

harvest period (beginning in September and ending somewhere in January) and a large variety of different quality EVOOs exist is Andalusia, leading to a wide range of products. It was also interesting to observe the results for EVOOs coming from Catalonia, since they all shared the same region of the 2D-map (**Fig. 5D**). Moreover, it was evaluated if the samples could be grouped in the PCA on the basis of the presence or absence of a Denomination of Origin (PDO), the farming system (organic vs. conventional), or the employment of one or more cultivar of olives for obtaining the EVOOs (mono-cultivar vs. blends). For this purpose, it was decided to carry out two different tests, corresponding to two different approaches in building a PCA: *1)* considering the attributes: fruity (nose + palate), bitter, pungent, aromatic notes, harmony, persistency, overall liking, as variables and *2)* considering only the attributes: harmony, persistency and overall liking. For both elaborations, no discrimination in groups were found between mono-cultivar vs. blends, PDO vs. not PDO and organic vs. conventional EVOOs. This can be explained due to the fact that olive oils collected in the IOOA were all very high sensorial quality oils, regardless of the presence of a Designation of Origin or of the fact the oil was obtained by mono-cultivar olives or of the agronomic system (conventional vs. organic). Considering these latter results, it was not possible to say that both the consumers (overall liking) and the panellists (harmony) preferred one of the above mentioned categories of oils over another, since no discrimination in groups were found.

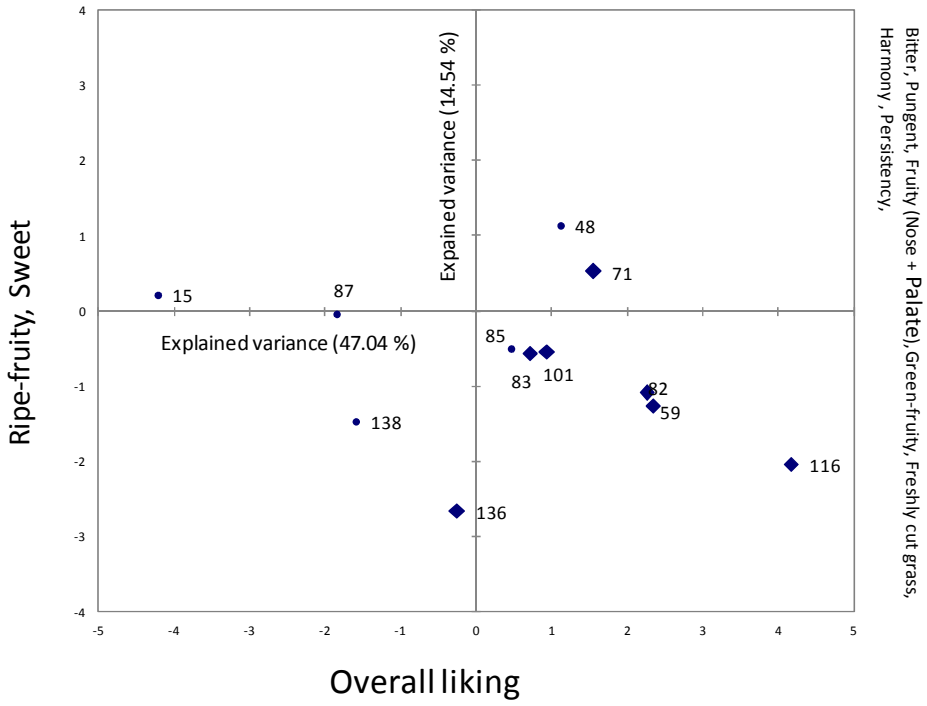

Fig. 6. Principal component analysis, projection of the samples in the factors plane. The graph show only the mono-cultivar 100% cerasuola samples (full circles) and blend samples (rhombs), obtained from olives of the cultivar Cerasuola (more than 50%), Nocellara and Biancolilla.

3.5 Building a PREFerence MAP

A PREFerence MAP was also built with XLSTAT, taking into account the results of the objective evaluation (first elaborated with the PCA, see 3.2 and 3.4) and the results of the consumer tests (overall liking). The PREFMAP was built using a quadratic complex model, which is the best and most complex one that takes into account the interactions between all the characteristic attributes as well as the overall liking. The ”heat-map” shows the areas in warm / hot colours (yellow, orange, red) where most of the consumers had a preference above average. Areas where only few consumers had a preference above average are shown in cold colours (blue). It was interesting to observe that the most appreciated samples (red zone) were in the direction of the axis that explains especially the ripe fruity and the sweet attributes (**Fig. 7**). Their position is opposite to the bitter axis, and to the attributes on the right hand side of the preference map, pungent, fruity (nose + palate), green fruity, freshly cut grass, green tomato, harmony, persistency, suggesting that these attributes were not much accepted by the consumers, as previously reported (Delgado and Guinard 2011). The most appreciated (accepted) samples came from different countries (mostly Spain, but also Greece and Italy) and they were both PDO and not PDO samples (see 3.2); moreover they were mono-cultivar EVOOs but also blends of different cultivars and organic or conventional EVOOs, which were equally appreciated by consumers.

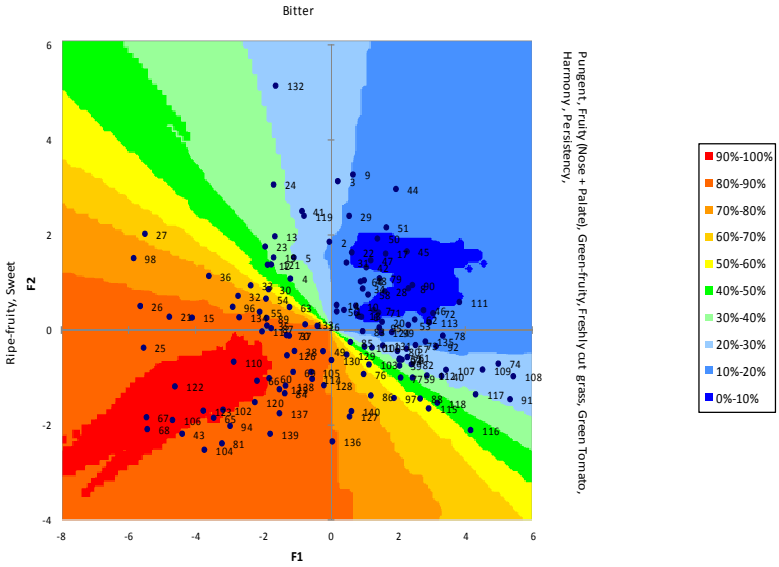

Fig. 7. Preference mapping, showing all the samples, built as described in the paragraph 3.5. the attributes reported in the axes are highly correlated with the factors 1 and 2.

3.6 Classification of consumers in clusters

As the number of consumers was significantly high, it was decided to try to classify them into homogeneous groups in order to make the PREFMAP results easier to interpret. In order to do this, the Agglomerative Hierarchical Clustering (AHC) was used. First an automatic truncation was performed, and thereby three groups of consumers were found. It was interesting to observe that the vectors explaining their preferences were in the same directions, suggesting a consumers' similar behaviour (in terms of acceptance). Increasing the number of consumer groups to 12, it was expected that some of the groups would orient into "another direction", for example preferring very bitter EVOOs; but actually, all consumers groups resulted oriented in the same direction. This consideration confirms two different but (maybe) complementary aspects: namely that the "rejecting" tendency is really "strong" and at the same time widespread in all the consumers. This finding is also linked to the problem of the "incomplete box" test design within the consumer test (not all consumers did evaluate all the 140 examined oils since there were 35 oils selected per year, so it was not possible for each consumer to taste such a large number of samples, and additionally the evaluation took place in four consecutive years of the OLIO (see 2.4). The different preferences between men and women were considered,

with regard to the overall liking (mean values per sample). Since the two different gender groups as well as all consumers (men and women joined together) showed a similar behaviour, being represented in the plane by vectors in the same direction, the interpretation of the consumers' acceptance of "premium-quality" EVOOs demonstrates a strong universality: in other words, the main trend for preference / acceptance, despite of gender aspects, is similar for all consumers.

3. CONCLUSIONS

The positive sensory attributes of EVOOs were not completely understood and appreciated (accepted) by consumers in this study. Consumers' behaviour was to reject the very bitter and pungent oils by trend and, in general, to consider the peculiar bitter and pungent of EVOOs as not pleasant and positive attributes. For this reason, consumers do not appear to practice a "well-informed" consumption of EVOOs. Actually, for a correct perception of the overall quality, including health aspects etc., the positive attributes should be accepted - or better perceived - by consumers as "healthy" indicators of quality and genuine taste, linked to its richness in pungent and bitter minor components (especially phenolic compounds). To achieve this purpose, dissemination of the importance of both pungency and bitterness of the EVOOs should be intensified in order to teach people about the importance of these attributes and their linkage to the beneficial effects towards the human health. Moreover, in this study the evaluation of harmony as objective sensory parameter confirms the capability to make differences among EVOOs of different quality levels, giving importers, traders and as well the consumer more insight to make the right purchase decisions (Bongartz and Oberg 2011). Discriminating effects according to the geographical origin of the EVOOs, the presence / absence of a Designation of Origin (P.D.O.), the different farming system (organic vs. conventional) and the aspect of mono-cultivar vs. blends of different cultivars were not proved in this investigation; only grouping effects were observed, considering some geographical areas and different cultivars of olives. This leads to the conclusion that the degree of quality of premium EVOOs object of this investigation was not dependent on the presence of a Denomination of Origin or on different farming systems (organic vs. conventional) or on the presence of one or more cultivars of olives.

4. CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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Chapter 5. Shelf-life of olive oils

Valli, E., Manzini, R., Accorsi, R., Bortolini, M., Gamberi, M., Bendini, A., Lercker, G. & Gallina Toschi, T. Quality at destination: simulating shipment of three bottled edible oils from Italy to Taiwan. Rivista Italiana delle Sostanze Grasse, in press.

ABSTRACT

The effects on quality and safety of foodstuffs after transportation process are important for both the producers and the consumers. Herein, a shipment of different bottled vegetable oils (olive oil, extra virgin olive oil and grape seed oils) from Italy to Taiwan, has been simulated within a climate-controlled chamber. The treated samples have been chemically and sensory analyzed, considering basic quality parameters; then, the results were compared with the non-simulated oils from the same production batches. The analyses demonstrate that there is a risk of oxidation due to the shipment to be taken into account.

INTRODUCTION

It is well-known that the shelf-life of a bottled vegetable oil is limited by two main processes, namely lipolysis and oxidation. Endogenous and exogenous lipases, responsible for initial degradation, act when the oil is still in the fruit, before extraction. This is especially true if olives are damaged, injured or not well-preserved, and gives rise to the formation of free fatty acids [1]. On the other hand, oxidation occurs mainly during extraction and storage [2]. The degree of unsaturation of fatty acids in a vegetable oil is directly proportional to the rate of oxidation. In fact, auto-oxidation and photo-oxidation of unsaturated fatty acids yield hydroperoxides (primary oxidation products), which are easily decomposed into different compounds (secondary oxidation compounds). Some of these are volatile and are responsible for the sensory degradation of oil, especially rancidity [3]. Extra virgin olive oil is renowned as an excellent foodstuff since it shows a high oxidative stability [4]. This particular behavior is strictly related to its high content of monounsaturated and saturated fatty acids and to the low amounts of polyunsaturated fatty acids, together with a high concentration of antioxidant compounds, especially phenols. In addition**,** olive oils are blends between refined and virgin olive oils [5], and for this reason, they generally show a lower overall quality than extra virgin olive oil. To date,

the impact of several factors such as temperature, light, pigments, oxygen availability, enzymes, metal contamination and microorganisms on oxidation have been studied. In particular, several investigations have assessed the effect of different storage conditions on the quality of olive oils [6, 7, 8, 9]. The effect of packaging material on the oxidation process of different vegetable oils, especially virgin olive oil, has also been assessed [10, 11], adopting also a predictive approach [12]. In this regard, several types of materials have been used for packaging vegetable oils, including glass, metals (tin-coated steel) and more recently plastics (PET, LDPE, PP), brick-type cartons, bag-in-box pouches and plastics coated paperboard/ alufoil laminates [13]. The main aim of the Food Supply Chain project at Bologna University is to trace and study the conditions of transportation of relevant foodstuffs. The project focuses on the analysis of the supply chain of wine, vegetable/olive oil and other foodstuffs to identify weaknesses and opportunities to improve the quality and safety of transportation processes. In particular, environmental factors of stress (i.e. temperature, humidity, vibrations, and light) are being analyzed during many shipments from Italy to generic consumers located in the E.U. or worldwide.

The project, for the first time in Italy, involves several food companies throughout the country with the aim to safeguard and promote exports of local products, and ensure quality and traceability to protect consumers. The main role of the project is to identify critical logistic nodes or activities that affect the quality and safety of food products.

In this report, the preliminary results obtained by monitoring the temperature of oil bottles during transportation from the producer to the consumer are inquired. The study herein simulates a journey from Italy to Taiwan (grape seed oils, olive oil and extravirgin olive oil of medium-low quality, different temperature changes).

MATERIALS AND METHODS

SAMPLES

One extra virgin (EV), one olive oil (OL) and two grape seed oils (GA and GB) were subjected to simulated shipping and were analyzed as described below. Samples were blinded as reported in Table I.

EV0	Extra virgin olive oil, 0.5 L dark glass bottle, before the simulation
EV1	Extra virgin olive oil, 0.5 L dark glass bottle, after the simulation
OL0	Olive oil, 1L transparent clear glass bottle, before the simulation
OL1	Olive oil, 1L transparent clear glass bottle, after the simulation
GA0	Grape seed oil, 0.5 L dark glass bottle, before the simulation
GA1	Grape seed oil, 0.5 L dark glass bottle, after the simulation
GB0	Grape seed oil, 0.5 L transparent clear glass bottle, before the simulation
GB1	Grape seed oil, 0.5 L transparent clear glass bottle, after the simulation

Table I. List of the samples analyzed and their description.

SIMULATION OF SHIPPING

This study is based on tracking and monitoring of the temperature profile of bottles of oil during shipment from Italy to consumers in Taiwan. Such a hypothetical profile has been simulated on "*time zero samples*" within a climate-controlled chamber.

The climate chamber was designed and developed to simulate heating and cooling stress cycles that foodstuffs can experience during handling and shipping activities. Particular attention was paid to transportation and storage conditions where high or low temperatures can be reached and maintained for long periods. Furthermore, the system approaches and realizes accelerated life thermal stress tests to study and assess the reliability and properties of particular property of products or packages, e.g. the maximum or minimum temperature where the packaging is not damaged and the quality/safety of the product is maintained. Consequently, the climate room was designed to: (1) heat or cool a sample of product to reach a specific temperature level, (2) automatically reproduce a given temperature profile measured during transportation or storage, (3) stress a product sample with accelerated thermal levels, i.e. accelerated life testing (ALT). The climate room can produce repeatable temperature cycles, with an average error of less than ±2°C, considering the air temperature inside the device, over any thermal profile between -10 and 65°C.

The integrated cooling system consists of an evaporator utilizing 21 g of R600a *iso*-butane as a refrigerant. The voltage was 220 V AC with a nominal input power of 90 W; the lowest temperature obtainable is -10°C. In order to increase the temperature in the climate chamber a heating system was added using two electrical resistors with 75 W power each. A closed-loop algorithm, developed with LabView National Instrument software, controlled the actuators so that the chamber temperature reached a defined setpoint.

INSTRUMENTATION

Determination of k_{232} and k_{270} were carried out using a UV-vis 1610 spectrophotometer (Shimadzu Co., Kyoto, Japan) with a six-slot shuttle and a temperature control system. Gas chromatography analyses for the determination of the fatty acid composition were performed using a Carlo Erba MFC 500 instrument (Carlo Erba, Milan, Italy).

REAGENTS AND STANDARDS

Gallic acid was acquired from Fluka (Buchs, Switzerland). All solvents were of analytical grade and obtained from Merck (Darmstadt, Germany).

ANALYSIS PLAN

Free acidity, peroxide value and determination of fatty acids were evaluated for all the analyzed samples. UV spectrophotometric indexes (k_{232}, k_{270}), extraction of phenolic compounds and determination of their total amount and that of *ortho*-diphenols were performed to evaluate the effect of the simulated journey on chemical properties.

BASIC QUALITY INDEXES

Free acidity, peroxide value and UV spectrophotometric indices (k_{232}, k_{270}) were measured according to the official methods described in EC Reg. 2568/91 and subsequent amendments of the European Union. All parameters were determined in duplicate for each sample.

FATTY ACID COMPOSITION

Fatty acid composition of oil samples was determined as fatty acid methyl esters (FAMEs) after alkaline treatment, obtained by mixing 0.05 g of oil dissolved in 2 mL of *n*-hexane with 1 mL of 2N potassium hydroxide in methanol, and subsequent gas chromatographic analysis, according to Bendini et al. [14], with slight modifications. The results were expressed as a percentage of fatty acid of the total fatty acid content.

EXTRACTION OF POLAR PHENOLIC EXTRACTS

The phenolic fraction was extracted from the oil by liquid/liquid extraction according to Pirisi et al. [15]. The dry extracts were redissolved in 5 mL of methanol/water (50:50, vol/vol) in a 5 mL flask. Extracts were stored at -43°C before spectrophotometric analysis.

SPECTROPHOTOMETRIC DETERMINATION OF TOTAL PHENOL AND ORTHO-DIPHENOL CONTENT

The total phenol (TP) content of extracts was measured spectrophotometrically using Folin-Ciocalteu reagent and absorbance determined at 750 nm [16]. The *ortho*-diphenol content was determined as described by Bendini et al. [17]. Total phenols and *ortho*-diphenols were both quantified using two different gallic acid calibration curves (TP: r^2 = 0.997; *ortho*-diphenols: r^2 = 0.994). The results were expressed as mg gallic acid kg^{-1} of oil.

SENSORY ANALYSIS

Sensory analysis was carried out only on the EV0 and EV1 samples in accordance with EC Reg. 2568/91 and subsequent amendments, since it is mandatory only for extra virgin olive oil. The other samples (refined oils) were not subjected to this analysis. The olive oils were tasted, in two replicates, by 8 trained and experts panelists, in the sensory room of the Campus of Food Science of the University of Bologna (Cesena, Italy).

STATISTICAL ANALYSIS

Means and standard deviations were calculated with Statistica 6.0 (2001, Starsoft, Tulsa, OK) software. Statistica was used to perform a one-way analysis of variance, and Tukey's honest significant difference test at a 95% confidence level (p<0.05) to identify differences between groups.

RESULTS AND DISCUSSIONS

RESULTS

The Food Supply Chain (FSC) is a research project to: 1) measure the effects of transportation and storage issues on quality and safety of food products, and 2) identify and develop effective solutions to control both quality and safety, especially in the presence of critical journeys from the place of origin and/or production to the consumer.

In particular, the FSC project involves the Department of Industrial Mechanical Plants at the Faculty of Engineering and the Department of Food Science at the Faculty of Agriculture. This is the first study on the chemical analysis of oil for food in relation to the journey and transportation/packaging.

The investigation simulated a shipment of oil bottles from Italy to Taiwan with the aid of a company that is a leader in the worldwide distribution of edible oils. The study is described below.

- *Monitoring* temperature during shipment to the consumer. This was performed using a data logger and tracking technologies (e.g. black box) in several shipments of goods from different starting points, e.g. sites of origin and/or production of food products, to different destinations worldwide.
- *Analysis* of data.
- *Simulation* of transport/shipment conditions in a laboratory using a simulator developed ad-hoc to simulate historical and monitored temperature profiles. The simulation was conducted on the so-called "zero-time" bottles of the same production lots of products shipped to consumer locations and whose temperature profile has been properly monitored. Only temperature values were collected and simulated.
- *Chemical and sensory analysis* in a food science laboratory was performed to measure the impact on product quality due to the simulated transport conditions.

A JOURNEY FROM ITALY TO TAIWAN

The study discussed in this paper refers to bottled edible oils shipped from Italy to Taiwan, and the temperature profile during this journey was tracked for the overall processes up to the final consumer. In particular, oil bottles were monitored after packing, sorting and loading at manufacturing facilities. Figure 1 shows the temperature profile during shipment.

The first critical step, as shown by the peaks in temperature, was the delay for loading at the departing port (Italy). The freight container was maintained at a fairly constant temperature, between 20-30°C, during ocean transhipment. The most critical process was waiting for delivery of the container at the arrival dock (Taiwan). It can be seen that the temperature increased up to 56°C, perhaps affecting the quality of oil and/or packaging.

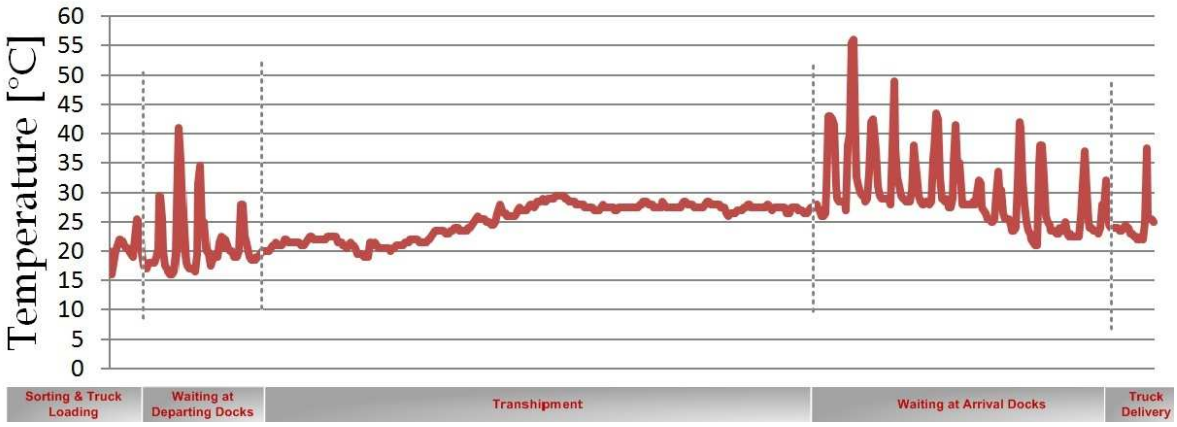

Figure 1. Temperature profile during shipment from Italy to Taiwan

The fatty acids compositions, link to the plant origin of the oils, are reported in Table II and III, for all the analyzed sample, before the simulation. Other chemical analyses were performed to determine the effects of shipment on quality and safety of oil (Table IV and V).

% Fatty acids	EV	OL
C16:0	10.53	11.99
C16:1 n-7	0.12	0.00
C16:1 n-5	0.75	0.16
C17:0	0.09	0.00
C17:1	0.14	0.15
C18:0	3.09	3.01
C18:1 n-9	73.95	70.82
C18:1 n-7	2.02	2.60
C18:2	7.76	9.74
C20:0	0.42	0.44
C18:3	0.70	0.67
C20:1	0.27	0.25
C22:0	0.11	0.18
C24:0	0.05	n.d.

Table II. Fatty acids composition of the extra virgin (EV) and olive oil (OL) samples. Data are expressed as percentage on the total fatty acid content.

% Fatty acids	GA	GB
C16:0	7.36	7.24
C16:1	0.09	0.09
C17:0	0.07	0.07
C18:0	3.63	3.80
C18:1 n-9	20.12	19.79
C18:1 n-7	0.90	0.76
C18:2 *trans*	0.83	0.96
C18:2	65.73	66.34
C20:0	0.31	0.27
C18:3	0.52	0.47
C20:2	0.18	n.d.
C20:1	0.15	0.21
C22:0	0.13	n.d.

Table III. Fatty acids composition of the grape seed oil samples (GA and GB). Data are expressed as percentage on the total fatty acid content.

Samples	Free acidity		PEROXIDE VALUE	
	Mean ± s.d.	*T*	*Mean ± s.d.*	*T*
EV0	0.5 ± 0.0	*A*	17.5 ± 0.0	*A*
EV1	0.5 ± 0.0	*A*	18.7 ± 1.8	*A*
OL0	0.3 ± 0.0	*A*	22.2 ± 0.4	*A*
OL1	0.3 ± 0.0	*A*	10.4 ± 0.2	*B*
GA0	0.2 ± 0.0	*A*	6.1 ± 0.9	*A*
GA1	0.2 ± 0.0	*B*	6.9 ± 0.9	*A*
GB0	0.2 ± 0.0	*A*	7.1 ± 0.2	*A*
GB1	0.1 ± 0.0	*A*	9.1 ± 1.2	*A*

Table IV. Free acidity and PEROXIDE VALUE of the samples. Free acidity is expressed as g oleic acid/100 g of oil for extravirgin (EV) and olive (OL) oil samples and as mg KOH/g of oil for grape seed oil samples (GA and GB), as reported in the Codex Alimentarius (CODEX-STAN 210, 2005). PEROXIDE VALUE is expressed as meq O_2 /kg oil. Means ± standard deviations are shown (n = 2). For each samples, within each column means followed by different letters are significantly different according to Tukey's test (p<0.05), before and after the simulation.

Samples	k_{232}		k_{270}		TP		*ortho*-diphenols	
	Mean ± s.d.	*T*	*Mean ± s.d.*	*T*	*Mean ± s.d.*	*T*	*Mean ± s.d.*	*T*
EV0	2.93 ± 0.04	*a*	0.18 ± 0.02	*A*	74.24 ± 4.29	*a*	31.83 ± 1.91	*A*
EV1	1.55 ± 0.02	*b*	0.19 ± 0.00	*A*	98.96 ± 7.30	*a*	35.94 ± 1.39	*A*
OL0	2.92 ± 0.02	*a*	0.71 ± 0.01	*A*	17.69 ± 1.82	*a*	10.89 ± 1.40	*A*
OL1	2.68 ± 0.02	*b*	0.70 ± 0.00	*A*	15.29 ± 2.22	*a*	7.26 ± 1.98	*A*

Table V. UV-spectrophotometric indexes, total phenols (TP, mg of gallic acid kg^{-1} of oil) and *ortho*-diphenols content (mg of gallic acid kg^{-1} of oil) in the extravirgin (EV) and olive (OL) oil samples. For each samples, within each column means followed by different letters are significantly different according to Tukey' s test (p<0.05), before and after the simulation.

DISCUSSIONS

The oil samples showed a typical fatty acid composition as expected (Table II and III), and were in the compositional range suggested by previous publications and the Codex Alimentarius [18]. For grape seed oil samples (GA and GB), the free acidity values obtained by the official method (and expressed as g oleic acid in 100 g of oil) were converted in mg KOH/g of oil, in order to standardize and compare the results with the limits reported by the Codex Alimentarius for vegetable oils [18] (Table IV). The acidity of the grape seed oil samples GA and GB before the journey were below the limits adopted by the Codex Alimentarius for vegetable oils, which is 0.6 mg KOH/g for refined oils [18]. For samples EV and OL, free acidity values were also below the legal limit for extra virgin and olive oils [5], respectively 0.8% and 1% (expressed as oleic acid). For all samples except for GA, no significant variation in free acidity was observed after the simulation of the journey, suggesting that substantial hydrolysis did not occur (Table IV).

Regarding oxidation, for grape seed oil samples (GA and GB) the peroxide value, an index of the primary oxidation products, was within the range adopted by the Codex Alimentarius [18], which is fixed at 10 meq O_2/kg oil for refined oils, both before (GA0 and GB0) and after (GA1 and GB1) simulation. EV0 showed a high peroxide value, which was however below the legal limit for extra virgin olive oil of 20 meq O_2/kg oil [5]. For samples EV, GA and GB, the simulation apparently caused a small increase (not significant) of the peroxide value; all remained below the legal limit after simulation of transport (EV1, GA1 and GB1). Before transport, olive oil sample OL0 showed a peroxide value that exceeded the legal limit for olive oil (15 meq O_2/kg oil) [5], indicating an advanced stage of oxidation; after simulation, a significant decrease in the peroxide value was observed. It should be mentioned that the peroxide value is an index measuring the rate of primary oxidation products; its reduction can be explained by following the Gaussian curve describing the trend of primary products during oxidation: when oxidation progresses, peroxides generate secondary degradation products [3]. Thus, taken alone the peroxide value does not reflect the oxidative quality of an oil. The advanced oxidation of OL0 and its "transport-simulated" counterpart OL1 was confirmed by the high values of k_{232} and k_{270} indices (Table V), even if k_{270} was below the legal limit for olive oils, which is 0.9 [5]. For both extra virgin olive oil samples EV1 and OL1, the k_{232} values decreased significantly after simulation, probably due to conversion of dienes into secondary products of

oxidation, such as aldehydes and ketones, as previously reported [19]. The sensory analysis required for classification of the oil as extra virgin classified the sample EV0 as extra virgin and the sample (transport-simulated) EV1 as virgin, with a rancid defect.

Total phenols and *ortho*-diphenols (Table V) were low in both olive oils (extra virgin and refined) from the beginning, especially for OL. During simulated transport, this small amount of antioxidants was not able to protect the oil and the simulation lead to measurable oxidation. No decreases in phenols were observed after transport simulation (Table V). In conclusion, this first simulation performed on different oils in terms of quality and saturation index (some of medium-low quality) did not result in a significant increase in the peroxide value, and did not show significant progression of the first phase of oxidation (from acylglycerols to peroxides). Nevertheless, it accelerated oxidation and produced (in one case) a slight defect of rancidity in oils that had already reached an advanced level of primary oxidation (high peroxide value before simulated transport).

When long and critical transport is planned, it is useful to protect the oil from light (visible, infrared and ultraviolet) to limit oxidation with the aim to commercialize oils possibly protected by phenols (virgin olive oils) as natural antioxidants. In fact, transport can accelerate oxidation, and give rise to a product that is no longer within legal limits. The grape seed oil GA0 and the corresponding "transport-simulated" GA1, which were respectively comparable to the samples of grape seed oil in samples GB0 and GB1, did not differ significantly before or after transport simulation. Moreover, samples GA1 and GB1 did not have significantly different peroxide values. Therefore, it would appear that, in the specific case of simulation of transport and considering the analytical tests performed, the different packaging used did not affect final quality.

Further research is warranted on the monitoring of shipments of different kinds of oils from Italy to destinations worldwide with attention given not only to temperature, but also vibrations, light exposure, and humidity. Towards this end, the research group at Bologna University is working on the development of a chamber that can simulate multiple stresses, including vibrations.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

ABBREVIATIONS

EV: Extra Virgin olive oil; OL: Olive oil; GA and GB: Grape seed oils; k_{232}, k_{270}: UV spectrophotometric indexes; FAMEs: Fatty Acid Methyl Esters; TP: Total Phenol content; FSC: Food Supply Chain.

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Chapter 6. Effect of thermal stresses on the quality of olive oils

Valli, E., Bendini, A., Cerretani, L., Fu, S., Segura-Carretero, A. & Cremonini, M.A. (2010). Effects of heating on virgin olive oils and their blends: focus on modifications of phenolic fraction. Journal of Agricultural and Food Chemistry, 58, 8158-8166.

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My contribution to these three papers was to review the literature, especially focusing on the most recent papers, to carry out the analytical plan, to interpret the results and to write some parts of the scientific manuscripts, under the supervision of the other co-authors.

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ARTICLE

Effects of Heating on Virgin Olive Oils and Their Blends: Focus on Modifications of Phenolic Fraction

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The phenolic profiles of two different virgin olive oils and their admixtures in different percentages have been analyzed after heating treatments by microwave or conventional oven. Changes in the phenolic profile upon heating were evaluated by chromatographic and spectroscopic methods, also monitoring the antioxidant activity by $ABTS^{\bullet+}$ test. 3,4-DHPEA-EA, *p*-HPEA-EA, and EA showed the highest decreases after thermal treatments. The only compounds that showed a clear increase with heating, in particular by conventional oven, were the dialdehydic form of elenolic acid (EDA) and *p*-hydroxyphenylethanol linked to the dialdehydic form of elenolic acid (*p*-HPEA-EDA). A comparison between the variations after heating of the sum of monoaldehydic and dialdehydic forms of phenolic compounds obtained by using different analytical approaches (HPLC-DAD/MSD and 1D and 2D NMR spectroscopy) was made. The results showed a good agreement of these two high-resolution techniques.

KEYWORDS: Virgin olive oil; phenols; heating; microwave oven; HPLC; NMR

INTRODUCTION

Extra virgin olive oil (EVOO) contains triglycerides, which represent >98% of the total oil weight and minor components, amounting to about 2% of the total oil weight. Minor components include more than 230 compounds, such as hydrocarbons, aliphatic and triterpenic alcohols, sterols, volatile compounds, and antioxidants (*1*). The main antioxidants of EVOO are carotenes, tocopherols, and polar phenols (*2*, *3*). Due to the presence of these compounds, EVOO is considered to be an excellent foodstuff compared to other vegetable oils, because of its excellent oxidative stability (*4*). Phenols are responsible for the organoleptic properties of EVOO (*5*,*6*); they also show beneficial biological activity, due to their anti-inflammatory (*7*), anticarcinogenic (*8*,*9*), and antioxidant (*10*−*12*) properties. The effects of thermal treatments on the phenolic pattern of EVOO have been well-studied in the literature. In a recent work (*13*) the authors found that the total phenol content, measured with Folin−Ciocalteu reagent, decreased by 55−60% after heat treatment at 100 °C for 142 h, with an air flow of 10 L/h. In particular, considering singular molecules, other researchers (*14*) showed that 3,4-dihydroxyphenylethanol (3,4-DHPEA), elenolic acid (EA), 3,4-dihydroxyphenylethanol linked to the dialdehydic form of elenolic acid (3,4-DHPEA-EDA), and 3,4-dihydroxyphenylethanol linked to elenolic acid (3,4-DHPEA-EA) reduced their concentrations more quickly, among antioxidant compounds present in EVOO, with thermal treatments at 180 °C in a conventional oven for 30 min. Such a trend for 3,4-DHPEA was confirmed by Nissiotis and Tasioula-Margari (*15*): during heating, the amount of this molecule decreased more rapdily than the amounts of other phenolic compounds. This agrees with the positive correlation between the degradation rate of phenols in EVOO and their antioxidant capacity (*16*), which is very high for 3,4-DHPEA (*17*). However, lignans show a weaker antioxidant capacity in comparison with other phenolic compounds, so they are the molecules most stable to thermal treatment (*14*). In two papers (*13*, *18*), an increase in lignans and *p*-hydroxyphenylethanol linked to the dialdehydic form of elenolic acid (*p*-HPEA-EDA) was observed after heating of EVOOs, but was explained by the authors as a probable coelution with oxidized compounds. After thermal treatment in a microwave oven for 10 min with a power of 0.5 kW, the amounts of 3,4-DHPEA, 3,4-DHPEA-EDA, *p*-hydroxyphenylethanol (*p*-HPEA), *p*-hydroxyphenylethanol linked to elenolic acid (*p*-HPEA-EA), and 3,4-DHPEA-EA decreased (*18*).

The aim of this study was to determine how the heat treatments by microwave or conventional oven under routine home-cooking conditions may affect the phenolic patterns of two samples of virgin olive oils (VOO) and their blends at different percentages, characterized by a predictable quali-quantitative phenolic composition. This study has been carried out using HPLC-DAD/MSD and 1D and 2DNMR spectroscopy to permit comparison of the results with these different analytical techniques.

MATERIALS AND METHODS

Apparatus. HPLC-DAD/MSD analyses of phenolic compounds were performed with a HP 1100 series instrument (Agilent Technologies, Palo Alto, CA) provided with a binary pump delivery system, degasser,

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autosampler, diode array UV−vis detector (DAD), and quadrupole mass spectrometer detector (MSD). The HPLC column was a C18 Luna column, 5 μm particle size, 250 mm × 3 mm i.d. (Phenomenex, Torrance, CA), with a C18 precolumn (Phenomenex) filter. The separation and identification of the phenolic compounds by HPLC-ESI-TOF-MS was also performed using an Agilent 1200 series Rapid Resolution LC with a vacuum degasser, an autosampler, and a binary pump equipped with a RP C18 analytical column (4.6 × 150 mm, 1.8 μm particle size, Agilent ZORBAX Eclipse plus). The measurement of antioxidant activity of phenolic extract by $ABTS^{\bullet+}$ assay was carried out using an UV−vis 1610 instrument (Shimadzu Co., Kyoto, Japan), which had a six-slot shuttle and a system for temperature control of working conditions. NMR spectra were obtained using a Varian Mercury Plus 400 MHz instrument (Varian NMR systems, Palo Alto, CA) using library sequences.

Reagents and Standards. All solvents used were of analytical or high-performance liquid chromatography (HPLC) grade and filtered through a 0.45 μm nylon filter disk (Lida Manufacturing Corp., Kenosha, WI) prior to use. The standard used for evaluation of antioxidant capacity of phenolic extracts (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Trolox), was purchased from Sigma-Aldrich (St. Louis, MO). 2,2′-Azinobis(3-ethylbenzothiazoline)-6-sulfonic acid, diammonium salt (ABTS), luteolin (LUT), apigenin (API), 3,4-dihydroxyphenylacetic acid (3,4-DHPAA), sodium hydroxide, formic acid, and isopropanol were acquired from Fluka (Buchs, Switzerland).

Methanol, *n*-hexane, acetonitrile, and formic acid (of HPLC grade) were obtained from Merck (Darmstadt, Germany). Deuterated dimethyl sulfoxide (DMSO-d_6) was obtained from Cambridge Isotopes Laboratories, Inc. (Andover, MA).

Samples and Thermal Treatment. Two samples of virgin olive oils (designated VOO1 and VOO2) were analyzed as well as two blends of these oils, prepared at 70−30 percent (70−30, v/v) and 50−50 percent (50−50, v/v) of VOO1 and VOO2, respectively. These blends were prepared with the purpose of obtaining a predictable phenolic profile from a qualitative and quantitative point of view. Unheated oils (termed TQ, "tal quale", meaning "as such" in Italian) were sampled as a control. For analytical purposes, 300 g of each kind of sample was inserted in an open glass container and subjected to microwave (MW) or conventional oven (CO) heating; the heated samples were respectively named MW and CO. The surface/volume ratio was constant for the samples (256 cm^2/330 mL, total capacity of glass container = 1.5 L, oil thickness = 1.6 cm). The amount of olive oil subjected to thermal treatment was sufficient to carry out all of the analyses in triplicate, especially for the extraction of phenolic compounds. The time−temperature conditions for both heating treatments were similar to home-cooking or food catering: all of the samples were heated for either 1 h in a conventional oven (type M20-VN, Instruments s.r.l, Bernareggio (MI), Italy) at 180 °C or for 9 min at 750 W of power in a microwave oven (model AMW214/WH, Whirlpool, Benton Harbor, MI), with a frequency of radiation of 2450 MHz. These combinations of time and temperature for each type of heating system were necessary to reach similar final temperatures. During heating, the temperature was registered at fixed intervals of thermal treatment by a thermocouple HI 98804 (Hanna Instrument, Woonsocket, RI) inserted at approximately the center of the samples. Both unheated and heated samples were stored at 12 °C in a thermostat and some aliquots in freezer at −43 °C prior to analyses. All analyses were performed in triplicate.

Extraction of Polar Phenolic Extracts. The liquid−liquid extraction (LLE) procedure was carried out according to the method of Carrasco-Pancorbo et al. (*19*). Briefly, 60 g of oil was dissolved in 60 mL of *n*-hexane, and the solution was extracted successively with four 20 mL portions of methanol/water (60:40, v/v). The combined extracts of the hydrophilic layer were brought to dryness in a rotary evaporator under reduced pressure at 40 °C. This extraction procedure was performed in triplicate, splitting each sample into two fractions of equal amount prior to the evaporation step (thus obtaining six dry extracts for each sample). Extracts were stored at −43 °C before analysis.

Measurement of Antioxidant Activity of Phenolic Extract by $ABTS^{\bullet+}$ Assay. The radical-scavenging capability of phenolic extracts was evaluated by $ABTS^{\bullet+}$ radical cation assay according to the procedure of Re et al. (*20*). ABTS was dissolved in H_2O at a concentration of 7 mM. The radical cation of ABTS was obtained by reaction with 2.45 mM potassium persulfate (final concentration) and allowing the stock solution to stand in the dark at room temperature for at least 12 h. Before use, the $ABTS^{\bullet+}$ solution was diluted with ethanol to reach an absorbance of 0.70 ± 0.02 at 734 nm at 30 °C. Next, 1 mL of this $ABTS^{\bullet+}$ solution was added to 0.01 mL of extract, and the decrease in absorbance was recorded for 10 min. Absorbance values were corrected for radical decay using a blank solution (0.01 mL of 50% aqueous methanol). Measurements were made in triplicate, and the antioxidant activity was calculated as the Trolox equivalent antioxidant capacity (TEAC, mmol of Trolox kg^{-1} of oil), using a calibration curve (equation: $y = 0.1304x - 0.0056$; $r^2 = 0.981$).

Determination of Phenolic Compounds by HPLC-DAD/MSD. The gradient elution was carried out using water/formic acid (99.5:0.5, v/v) as mobile phase A and acetonitrile as mobile phase B of the solvent system, according to the conditions described by Carrasco-Pancorbo et al. (*14*). The mobile phase flow rate was 0.5 mL min^{-1}, and the injection volume was 10 μL. UV−vis detection was set at 240, 280, and 330 nm. The detection was made using quadrupole MS as well, with an electrospray (ESI) interface operating in positive ion mode within the m/z 50−800 range and the following conditions: drying gas flow, 9 L min^{-1} at 350 °C; nebulizer gas pressure, 50 psi; capillary voltage, 3000 V. Nitrogen was used as nebulizer and drying gas. For HPLC analysis, the phenolic extracts were redissolved in 500 μL of methanol/water (1:1, v/v) and filtered through a 0.45 μm filter (VWR, West Chester, PA).

To carry out the quantification of phenolic compounds with HPLC-DAD, four standard calibration curves were made using three commercial reference compounds: 3,4-dihydroxyphenyl acetic acid (3,4-DHPAA), LUT, and API. EA and its dialdehydic form lacking a carboxymethyl group (EDA) were quantified using the calibration curve of 3,4-DHPAA at 240 nm (equation: $y = 11472x - 61846$; $r^2 = 0.999$); lignans, phenylethyl alcohols, and secoiridoids were quantified using the curve of 3,4-DHPAA at 280 nm (equation: $y = 14747x - 74555$; $r^2 = 0.999$); LUT and API were quantified with their respective calibration curves at 330 nm (for LUT, equation: $y = 57.22\,x$; $r^2 = 0.988$; for API, equation: $y = 108.18x$; $r^2 = 0.995$). All calibration curves show good linearity in the studied concentration range. The phenol content was expressed as milligrams of 3,4-DHPAA per kilogram of oil, except for LUT, which is expressed in milligrams of luteolin per kilogram of oil, and API, which is expressed in milligrams of apigenin per kilogram of oil.

Determination of Phenolic Compounds by HPLC-ESI-TOF-MS. Acidified water (0.5% acetic acid, v/v) and acetonitrile were used as mobile phases A and B, respectively. The mobile phase was programmed as follows: gradient from 5 to 30% B in 10 min, from 30 to 33% in 2 min, from 33 to 38% in 5 min, from 38 to 50% in 3 min, from 50 to 95% in 3 min, and from 95 to 5% in 2 min; an 8 min re-equilibration time was used after each analysis. The flow rate used was set at 0.80 mL min^{-1} throughout the gradient. The effluent from the HPLC column was split using a T-type phase separator before being introduced into the mass spectrometer (split ratio = 1:3). Thus, in this study the flow that arrived at the ESI-TOF-MS detector was 0.2 mL/min. The column temperature was maintained at 25 °C, and the injection volume was 10 μL.

The accurate mass data of the molecular ions were processed using the latest version of the Data Analysis 4.0 software (Bruker Daltonics, Bremen, Germany), which provided a list of possible elemental formulas by using the Smart Formula Editor. The Editor uses a CHNO algorithm, which provides standard functionalities such as minimum/maximum elemental range, electron configuration, and ring-plus double bonds equivalents, as well as a sophisticated comparison of the theoretical with the measured isotope pattern (sigma value) for increased confidence in the suggested molecular formula. The widely accepted accuracy threshold for confirmation of elemental compositions was established at 5 ppm.

External calibration was performed using sodium formate cluster by switching the sheath liquid to a solution containing 5 mM NaOH in the sheath liquid of 0.2% formic acid in water/isopropanol (1:1, v/v). Due to the compensation of temperature drift in the TOF, this external calibration provided accurate mass values (> 5 ppm) for a complete run without the need for a dual sprayer setup for internal mass calibration.

Determination of Phenolic Compounds by NMR Spectroscopy. For NMR spectroscopy, the phenolic extracts were redissolved in 750 μL of DMSO-d_6 and placed in 5 mm NMR tubes. After NMR spectroscopy, the same extracts were also analyzed by HPLC-DAD/MSD, for a comparative study. 1D and 2D NMR spectra of the phenolic fraction obtained as described above were recorded at 30 °C on a high-resolution spectrometer Varian Mercury Plus 400 MHz. The spectra were taken with

a 90° pulse angle of 6.3 μs at a power of 55 db, 10 s recovery delay, and 256−512 scans. All spectra were recorded without spinning the samples to avoid quantification problems due to the overlap of signals and spinning side bands. Heteronuclear multiple bond correlation (HMBC) spectra were recorded with the sequence provided by the Varian library of experiments, using standard parameters ($^1J_{CH}$ = 140 Hz and $^{2,3}J_{CH}$ = 8 Hz). NMR signals were assigned by comparison with Christophoridou et al. (*21*) and Montedoro et al. (*22*) and confirmed by following the connectivities provided by HMBC spectra. 2D NMR analysis was necessary as previous analytical work was carried out in a mixture of H_2O/CH_3CN/TFA (*21*) or in $CDCl_3$ (*22*) and the chemical shifts may differ from those recorded in DMSO. Next, the most representative signals for two classes of phenolic compounds (i.e., monoaldehydic and dialdehydic compounds) were integrated with the routine "fitspec" of the Varian "Vnmrj" software, in order to quantify them. ^{1}H NMR spectra were normalized with respect to the peak solvent area according to the literature (*23*).

Statistical Analysis. Means and standard deviations were calculated with Statistica 6.0 (2001, Starsoft, Tulsa, OK) statistical software. Statistica was used to perform one-way analysis of variance, and Tukey's honest significant difference test at a 95% confidence level ($p < 0.05$) to identify differences among groups.

RESULTS AND DISCUSSION

Measurement of Antioxidant Activity of Phenolic Extract by ABTS$^{\bullet+}$ Assay. To the best of our knowledge, there is only very limited information about the effects of heat treatments by microwave and conventional ovens on the phenolic profile of EVOO (*13*−*15*, *18*, *19*). As shown in **Table 1**, the antioxidant activity evaluated by ABTS$^{\bullet+}$ test, a parameter closely related to the total phenol content, was higher for VOO1 samples than VOO2 samples. Correspondingly, the total phenol amount was higher as well, according to Bendini et al. (*24*), in which EVOO samples (EVTQ, EVMW, EVCO) correspond to VOO1 samples (VOO1TQ, VOO1MW, VOO1CO, respectively), whereas DEO samples (DEOTQ, DEOMW, DEOCO) correspond to VOO2 samples (VOO2TQ, VOO2MW, VOO12CO, respectively). Their admixtures (70−30 and 50−50) showed a value of antioxidant activity near the mean value between the two whole samples (**Table 1**).

Table 1. Antioxidant Activity (ABTS) in Analyzed Samples before and after Thermal Treatment by Microwave (MW) or Conventional Oven (CO)[a]

	ABTS (mmol of Trolox kg^{-1} of oil)	
	mean	SD
VOO1TQ	0.935 a	0.032
VOO1MW	0.813 b	0.003
VOO1CO	0.793 bc	0.039
70−30TQ	0.749 c	0.023
70−30MW	0.684 d	0.015
70−30CO	0.659 d	0.017
50−50TQ	0.403 e	0.008
50−50MW	0.193 fg	0.014
50−50CO	0.162 g	0.019
VOO2TQ	0.222 f	0.006
VOO2MW	0.186 fg	0.006
VOO2CO	0.172 fg	0.006

[a] Data are expressed as mean of three determinations and standard deviation. The same letters within each column do not significantly differ ($p < 0.05$).

After thermal treatments, the total phenol content, determined with Folin−Ciocalteu reagent, significantly decreased ((*24*), the agreement of the codes of the samples is reported above), as reported in previous works (*13*−*15*, *25*). Upon heating, the same trend was seen for antioxidant activity, evaluated by ABTS$^{\bullet+}$ test. No significant variations were observed for the antioxidant activity between the two types of heating systems (**Table 1**).

Chromatographic Analysis of Phenolic Compounds by HPLC. On the basis of the study of UV and MSD spectra of phenolics carried out by HPLC-DAD/MSD, 12 different compounds were identified: 3,4-DHPEA, EDA, *p*-HPEA, EA, LUT, 3,4-DHPEA-EDA, PIN, AcPIN, *p*-HPEA-EDA, API, 3,4-DHPEA-EA, and *p*-HPEA-EA. The presence of these phenolic compounds was confirmed by micro-TOF-MS analysis, as reported in **Table 2**. A partial overlapping of (+)-1-acetoxypinoresinol (AcPin) and *p*-HPEA-EDA was noted. As reported in **Table 3**, 3,4-DHPEA, 3,4-DHPEA-EA, *p*-HPEA-EA, and EA showed the highest decrease with thermal treatment. Molecules with an *o*-dihydroxy structure (namely, *o*-diphenols), such as 3,4-DHPEA and 3,4-DHPEA-EA, are mainly responsible for the oxidative resistance of EVOO (*26*) and characterized by higher antioxidant activities; the ability to react rapidly with lipid radicals can partially explain their losses upon heating. On the other hand, EA, which is devoid of a phenolic ring and exhibits a weak antiradical capacity (*27*), decreased upon thermal treatment. This effect may be due to chemical conversion from its monoaldehydic to its dialdehydic form lacking a carboxymethyl group. This type of reaction was also described for oleuropein by Montedoro et al. (*28*, *29*) and Limiroli et al. (*30*) during crushing and malaxation of olives, and

Table 2. Phenolic Compounds Determined by HPLC-ESI-TOF-MS and Reported in Order of Elution

phenolic compound	time (min)	*m*/*z* exptl	*m*/*z* calcd	tolerance (ppm)	error (ppm)	σ value	formula	classification order
3,4-dihydroxyphenylethanol (3,4-DHPEA)	6.48	153.0565	153.0557	5	−4.8	4.2	$C_8H_{10}O_3$	1st (1)
dialdehydic form of elenolic acid lacking a carboxymethyl group (EDA)	7.69	183.0665	183.0663	5	−1.3	13.1	$C_9H_{12}O_4$	1st (1)
p-hydroxyphenylethanol (*p*-HPEA)	8.17	137.0613	137.0608	5	−3.5	11.4	$C_8H_{10}O_2$	1st (1)
elenolic acid (EA)	13.64	241.0700	241.0718	10	7.5	10.1	$C_{11}H_{14}O_6$	1st (3)
luteolin (LUT)	15.97	285.0387	285.0405	10	6.2	10.4	$C_{15}H_{10}O_6$	1st (4)
3,4-dihydroxyphenylethanol linked to the dialdehydic form of elenolic acid (3,4-DHPEA-EDA)	16.20	319.1186	319.1187	5	0.5	7.8	$C_{17}H_{20}O_6$	1st (2)
(+)-pinoresinol (PIN)	16.74	357.1353	357.1344	5	−2.6	19.0	$C_{20}H_{22}O_6$	1st (2)
(+)-1-acetoxypinoresinol (AcPIN)	17.37	415.1415	415.1398	10	−1.1	12.9	$C_{22}H_{24}O_8$	2nd (5)
p-hydroxyphenylethanol linked to the dialdehydic form of elenolic acid (*p*-HPEA-EDA)	17.81	303.1242	303.1238	5	−1.5	25.9	$C_{17}H_{20}O_5$	1st (2)
apigenin (API)	18.71	269.0445	269.0455	5	4.1	2.3	$C_{15}H_{10}O_5$	1st (1)
3,4-dihydroxyphenylethanol linked to elenolic acid (3,4-DHPEA-EA)	20.90	377.1262	377.1242	10	−5.4	5.9	$C_{19}H_{22}O_8$	1st (5)
p-hydroxyphenylethanol linked to elenolic acid (*p*-HPEA-EA)	23.28	361.1293	361.1293	5	−0.1	8.8	$C_{19}H_{22}O_7$	1st (3)

Table 3. Quantification of Phenolic Compounds (HPLC-DAD) before and after Thermal Treatments by Conventional Oven (CO) or Microwave Oven (MW)[a]

	EDA	EA	3,4-DHPEA	*p*-HPEA	3,4-DHPEA-EDA	PIN	*p*-HPEA-EDA + AcPIN	*p*-HPEA-EDA	3,4-DHPEA-EA	*p*-HPEA-EA	LUT	API
VOO1TQ	22.6 d	302.2 a	8.7 a	6.3 a	42.6 b	13.9 a	29.8 a	2.0 d	36.8 a	17.6 a	2.1 a	0.5 a
VOO1MW	23.9 c	276.4 a	9.3 ab	4.9 a	32.5 c	12.9 bc	27.5 b	3.8 c	19.4 cd	7.7 c	2.4 ab	0.5 ab
variation %	5.9	−8.5	7.1	−21.4	-23.6	−7.0	−7.9	90.8	−47.3	−56.3	17.8	7.8
VOO1CO	66.5 a	137.9 cd	5.2 abc	5.6 a	48.1 a	14.8 a	28.5 ab	7.9 a	12.5 e	5.5 cd	2.1 a	0.5 a
variation %	195.0	−54.4	−40.2	−10.9	12.9	6.8	−4.5	302.2	−66.0	−68.6	3.7	1.3
70−30TQ	19.8 cdef	198.3 b	8.7 ab	7.4 a	31.1 cd	10.6 de	21.4 c	2.0 d	25.9 b	10.9 b	1.3 ab	0.3 ab
70−30MW	17.7 cdef	176.1 bc	7.0 abc	7.0 a	22.3 e	10.3 de	18.5 de	2.2 d	19.3 cd	7.8 c	1.0 ab	0.3 abcd
variation %	−10.5	−11.2	−19.2	−4.7	−28.3	−2.3	−13.5	11.1	−25.6	−28.0	−19.6	−19.5
70−30CO	42.1 b	122.4 de	6.1 abcd	7.4 a	28.9 cd	11.1 cd	20.9 cd	4.5 cb	11.2 ef	6.8 cd	0.9 abc	0.3 abcd
variation %	113.0	−38.3	−30.3	−0.3	−7.3	4.7	−2.2	129.1	−56.9	−37.7	−29.8	−19.0
50−50TQ	21.1 cdef	171.4 bcd	8.3 ab	7.5 a	26.7 de	10.2 de	17.6 ef	2.3 d	24.3 b	10.4 b	1.2 abc	0.3 abc
50−50MW	14.5 ef	127.6 d	5.5 bcd	7.1 a	12.9 f	8.7 e	13.2 g	2.8 d	13.4 e	7.4 cd	0.8 bc	0.2 bcde
variation %	−31.1	−25.6	−34.5	−5.2	−51.5	−15.2	−24.9	21.6	−44.8	−28.2	−34.6	−28.9
50−50CO	44.8 b	65.4 f	3.8 cd	6.9 a	14.8 f	9.0 de	15.6 fg	4.8 bc	7.2 fg	5.8 cd	0.7 bc	0.3 bcde
variation %	112.2	−61.9	−54.5	−7.4	−44.5	−12.1	−11.6	110.6	−70.6	−43.9	−37.4	−22.8
VOO2TQ	16.4 def	74.1 ef	7.5 ab	7.9 a	6.3 g	5.3 f	4.6 h	2.1 d	14.1 de	7.8 c	0.3 c	0.1 de
VOO2MW	14.7 f	67.9 f	4.0 cd	8.4 a	4.5 g	4.7 f	4.1 h	2.2 d	8.0 g	7.3 cd	0.2 c	0.1 e
variation %	−10.4	−8.4	−47.3	5.6	−28.2	−11.1	−10.5	8.3	−43.7	−6.9	−28.2	−19.7
VOO2CO	22.6 cd	63.2 f	2.5 d	7.6 a	4.6 g	6.1 f	4.8 h	2.8 d	5.1 g	6.8 cd	0.2 c	0.1 cde
variation %	38.2	−14.7	−66.2	−4.5	−26.9	15.2	3.6	34.4	−63.6	−13.1	−35.3	0.0

[a] Expressed as mg kg^{-1} of oil. Data are related to phenolic extracts, redissolved in 500 μL of methanol/water (1:1, v/v). The abbreviations correspond to those in **Table 2**.

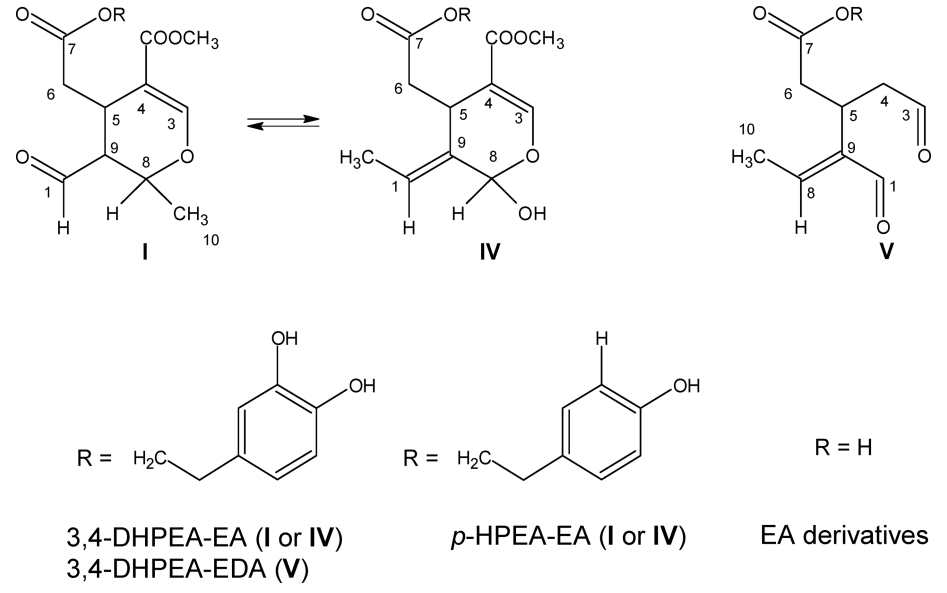

Figure 1. Type **I**, **IV**, and **V** structures. R = 2-(3,4-dihydroxyphenyl)ethyl, 2-(4-hydroxyphenyl)ethyl or H in 3,4-DHPEA-EA and 3,4-DHPEA-EDA, *p*-HPEA-EA, and EA derivatives, respectively.

it can be extended to EA, *p*-HPEA-EA, and 3,4-DHPEA-EA, which share the chemical moieties shown in **Figure 1**, distinguishing one from the other by a side group. EDA and *p*-HPEA-EDA were the only two compounds that clearly increased with heating, in particular by conventional oven (**Table 3**): thermal treatment by conventional oven led to a more intense oxidative and hydrolytic degradation of samples than microwave heating (*24*) and thus speeds the reaction pathway from monoaldehydic to dialdehydic forms of phenolic compounds. **Figure 2** shows the ratio between EA and EDA in all samples: the lowest values were reported after heating by conventional oven, suggesting a considerable decrease of the monoaldehydic structure and a concomitant increase of the dialdehydic form, both supported by strong thermal treatments. In fact, EDA and *p*-HPEA-EDA may originate from their respective monoaldehydic compounds (**Figure 3**). As seen in **Figure 3**, starting from a monoaldehydic structure (**I**), a retro-Michael equilibrium was proposed for structure **II**; the latter, via a keto−enol tautomerism, gives the dialdehydic form **III** that eventually yields **V** through loss of a carboxymethyl group, probably because of heating (*18*, *27*, *29*, *30*).

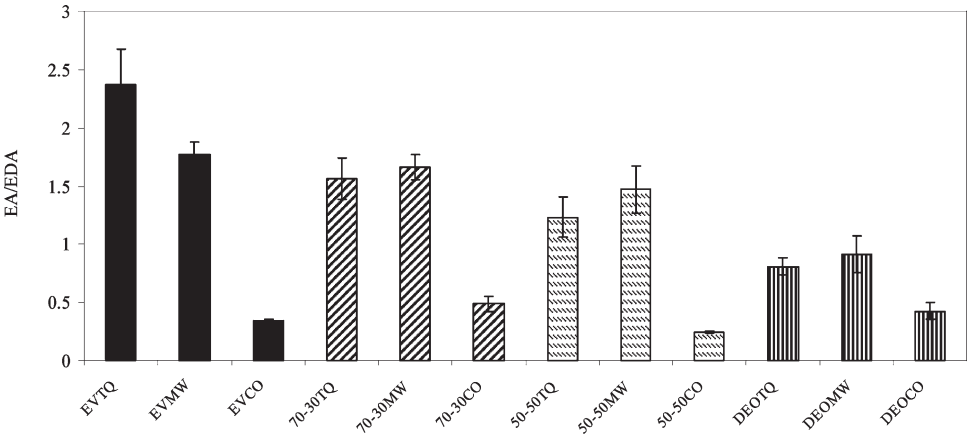

Figure 2. Ratio between EA and EDA in the samples. Data are referred to **Table 3**.

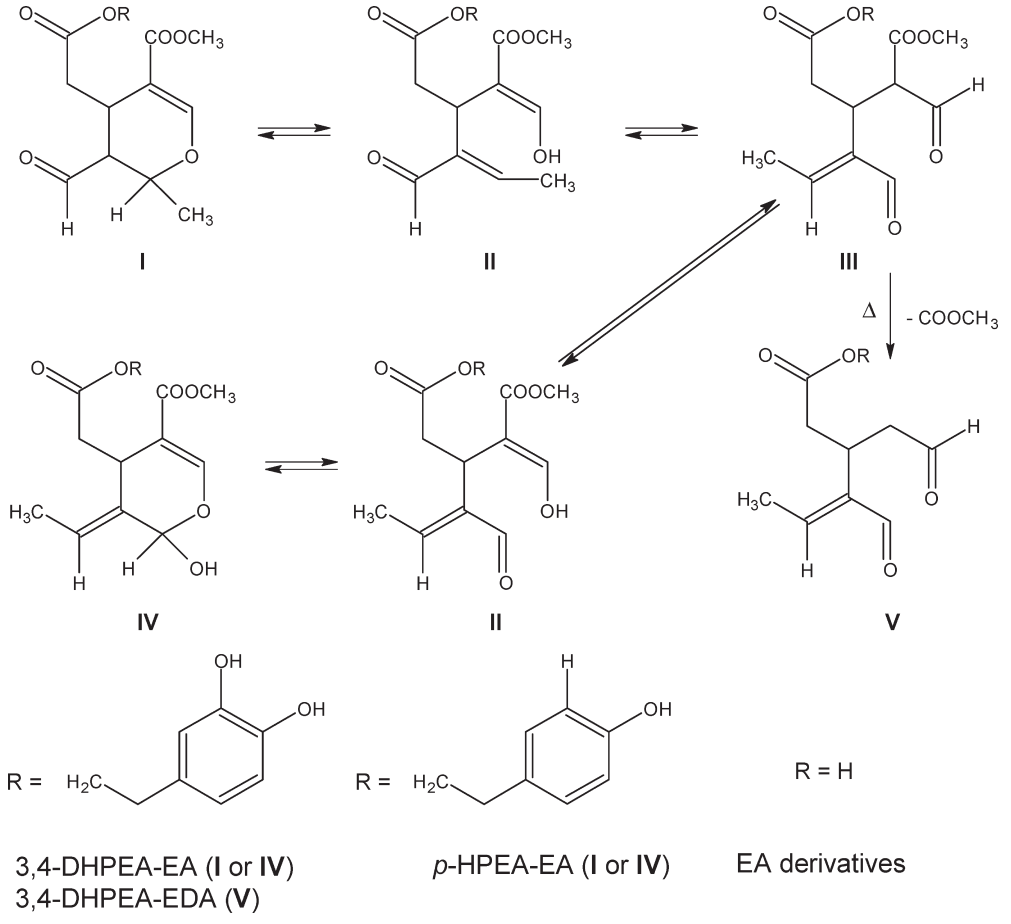

Figure 3. Chemical transformation induced by heating in type **I** and **IV** compounds to produce type **V** dialdehydic compounds.

This dialdehydic structure (**V**) may also originate from the corresponding aglycon **IV**, by a simple hemiacetal ring opening (**II**), followed by keto−enol tautomerism (**III**) and $-COOCH_3$ loss (**V**). It is worth noting that these chemical conversions do not modify the antiradical activity of 3,4-DHPEA-EDA because the catecholic ring of this molecule is unaffected by the reactions. No significant variations were seen for (+)-pinoresinol (PIN) or AcPIN upon heating (**Table 3**): in fact, lignans show a weaker antioxidant capacity in comparison with other phenolic compounds, and as suggested by several investigations (*14*, *18*), they are among the most stable compounds to thermal treatments. Finally, flavonoids (LUT, API) and *p*-HPEA also showed no significant variations after heating (**Table 3**).

Correlation between HPLC and NMR Analyses. To confirm the results for the variation of mono- and dialdehydic phenolic compounds with treatment (CO or MW), a comparison between the data obtained by HPLC and ^1H NMR spectroscopy was made for the VOO1 sample. In fact, provided that a spectrum has been recorded under proper conditions, the integrated areas of the NMR peaks are directly proportional to the relative concentrations of the compounds that yield the signals, and no calibration curve is necessary. A good correlation between NMR and HPLC results, therefore, helps strengthen the validity of the HPLC analysis if quantification of the analytes is obtained using a common calibration curve, as was done in our case, due to lack of commercial standards. Although it is true that the use of a

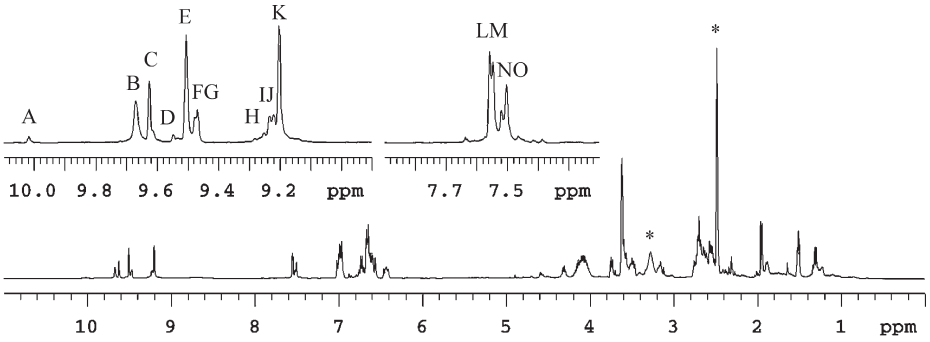

Figure 4. ^{1}H NMR spectrum of the phenolic fraction of a virgin olive oil (VOO1TQ). Close-up views of the aldehydic and enolethers regions are also shown.

Table 4. HPLC-DAD Quantification of Phenolic Compounds before (VOO1TQ) and after Thermal Treatments by Conventional Oven (VOO1CO) or Microwave Oven (VOO1MW)[A]

	VOO1TQ	VOO1MW	VOO1CO
EDA	24.4	31.5	43.4
EA	426.6	260	110
3,4-DHPEA	25.3	8.9	17.5
p-HPEA	16	8.6	12.9
3,4-DHPEA-EDA	51.5	51.9	34.1
PIN	17.1	19.6	15
p-HPEA-EDA + AcPIN	53.7	60.3	46.8
3,4-DHPEA-EA	45.3	28.1	10.5
p-HPEA-EA	19	5.2	2.3
LUT	3.8	3.8	2.1
API	0.8	0.8	0.5

[A] Expressed as mg g^{-1} of phenolic extract. These data are related to phenolic extracts redissolved in 750 μL of deuterated dimethyl sulfoxide (DMSO-d_6). The abbreviations correspond to those in **Table 2**.

common calibration curve does not impair the measure of the percentage variation of a *single* analyte along a series of experiments (it just multiplies the true concentration by an unknown coefficient that disappears in the calculation), it may give wrong results if percentage variations of classes of compounds (e.g., mono- vs dialdehydic) are to be measured (each true concentration, that is, summed to yield the total concentration of the class of compounds, is multiplied by a different unknown coefficient that does not disappear in the percentage calculation).

As the low sensitivity of NMR spectroscopy requires the use of concentrated solutions, the phenolic extracts were dissolved in DMSO-d_6, and the NMR results were compared to HPLC data obtained by specific experiments in the same solvent. **Table 4** shows the amount of phenolic compounds, determined by HPLC-DAD/MSD in DMSO-d_6 phenolic extracts of VOO1 samples, before and after the thermal treatments by microwave or conventional oven. EA was the most abundant compound, and in DMSO-d_6 extracts the increase in EDA with heating (especially by conventional oven) was quite evident.

The ^{1}H NMR spectrum of the phenolic fraction in DMSO-d_6 is quite complex (**Figure 4**), and the lines are broad, due to the superimpositions of signals from many different compounds. Due to high similarity of chemical shifts (*21*, *22*), it was almost impossible to discriminate among all of the signals of monoaldehydic (EA, 3,4-DHPEA-EA, and *p*-HPEA-EA) and dialdehydic compounds (EDA, 3,4-DHPEA-EDA, and *p*-HPEA-EDA) by NMR spectroscopy at 400 MHz. When assignment of a signal to a specific mono- or dialdehydic compound was not possible, the assignment was considered to a general "structure of type **I**" or "structure of type **V**" (**Figure 1**). Once identified, percentage variations of selected signals of these structures upon treatment will be compared to percentage variations of the pooled concentrations of the compounds in each class obtained by HPLC-DAD.

Two different parts of the ^{1}H NMR spectrum were considered for quantification (**Figure 4**): the aldehydic region (9−10 ppm), where 11 aldehydic signals appear, namely, A−K, which give information about the amount of compounds bearing one or two aldehyde groups and the enolethers region (around 7.5 ppm), which includes 4 main signals (L−O), typical of compounds containing the enolether moiety, such as the one present in compounds sharing structures such as **I** or **IV**.

A good starting point for the analysis of the HMBC spectrum is the group of signals around 7.55 ppm, typical of the enolether moiety (H-3 in **Figure 1**). This proton is connected to five carbons at 166.8, 106.8, 69.9, 27.2, and 156.0 ppm (peaks a, b, c, d, and e in **Figure 5B**) that can be assigned (*22*) to the carboxyl group of methyl ester (COOMe), C-4, C-8, C-5, and C-3, respectively (the latter via $^1J_{CH}$, yielding the doublet). It is worth noting that structures **I** and **IV** have different chemical shifts at C-8 (respectively, 70 and 95 ppm (*31*)). As no signals are present in the HMBC spectra around 95 ppm, it must be concluded that compounds containing structure **IV** were not present in the analyzed samples. Very similar connectivities were detected starting from a minor couple of enolether signals centered at about 7.50 ppm (peaks a′, b′, c′, d′, and e′ in **Figure 5B**), indicating presence of compounds containing the *R* epimer at C-8 of structure **I**, the chemical shift at H-3 of which is generally lower than in the *S* epimer (*21*).

To assign the aldehydic signals to aldehydic protons in compounds containing structure **I**, it is useful to start from the HMBC signal of C-5 of the major isomer (the one with *S* configuration at C-8) at 27.3 ppm, which is HMBC connected to two aldehydic protons at 9.68 and 9.62 ppm (peaks a and b in **Figure 5A**, corresponding to peaks B and C in **Figure 4**). These can be assigned (*21*) to the aldehydic protons of the *S* epimers of EA (peak B) and to the sum of the *S* epimers of 3,4-DHPEA-EA and *p*-HPEA-EA (peak C). Similarly, the C-5 of the *R* epimer at 26.1 ppm is connected to two aldehydic protons at 9.61 and 9.48 (peaks g and h in **Figure 5A**), which are assigned to the aldehydic protons of the *R* epimer of EA (peak C in **Figure 4**) and to the sum of the *R* epimers of 3,4-DHPEA-EA and *p*-HPEA-EA (peak F in **Figure 4**). The four assignments are corroborated by the presence of HMBC peaks between each of the aldehydic protons and their geminal carbons at C-9 (peaks c, d, i, and l in **Figure 5A**) with $^2J_{CH} \approx 23$ Hz (*32*) and by the presence of doublets due to residual $^1J_{CH}$ (peaks e, f, m, and n in **Figure 5A**). The nearly complete assignment of structure **I** in DMSO-d_6 is reported in **Table 5**.

The assignments of the signals of type **V** structure can be started by noting that H-5 is the only proton that can couple with two aldehydic carbons via HMBC; actually, the only proton

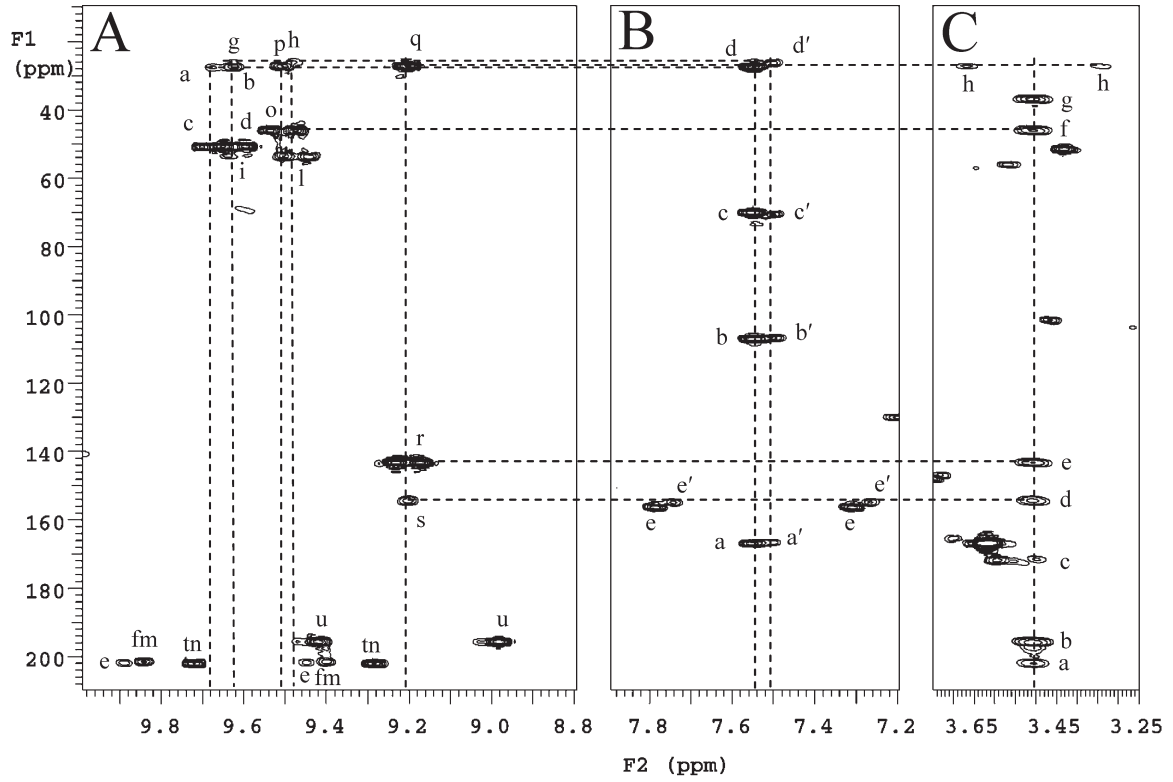

Figure 5. Portions of the HMBC 2D spectrum of the phenolic fraction of a VOO showing the relevant connections for the assignment of monoaldehydic and dialdehydic structures.

Table 5. NMR Assignments of Monoaldehydic Type **I** Structure (**Figure 1**) Contained in *p*-HPEA-EA, 3,4-DHPEA-EA, and EA

	chemical shift ^{1}H (^{13}C)	
atom no.	8*S* epimer	8*R* epimer
1	9.68[a] (201.7[a]) 9.62[b] (201.4[b])	9.61[a] (201.4[a]) 9.48[b] (201.5[b])
3	7.55 (156.0)	7.50 (154.5)
4	− (106.8)	− (106.6)
5	3.18 (27.2)	3.26 (26.1)
6	2.57, 2.64 (34.0[c])	2.50, nd[d] (36.7[c])
7	− (171.3)	− (nd)
8	4.31 (69.9)	4.57 (70.4)
9	2.64 (50.4)	2.61 (53.5)
10	1.51 (17.7)	1.30 (19.1)
$COOCH_3$	− (166.8)	− (166.4)
$COOCH_3$	3.62 (51.2)	3.57[c] (51.2[c])

[a] Values assigned to EA. [b] Values assigned to *p*-HPEA-EA and 3,4-DHPEA-EA. [c] Tentative assignment. [d] Not detected.

displaying the required connectivities is at 3.51 ppm, as it couples with two carbonyls at 201.8 and 195.6 ppm (peaks a and b in **Figure 5C**), corresponding via residual $^1J_{CH}$ doublets (peaks t and u, **Figure 5A**) to two aldehydic protons at 9.51 and 9.20 ppm (respectively, peaks E and K in **Figure 4**). Proton H-5 is also long-range connected to another five carbons at 171.7, 154.2, 143.2, 45.7, and 36.9 (peaks c−g in **Figure 4C**), assigned, respectively, to C-7, C-8, C-9, C-4, and C-6 both by comparison with the chemical shifts in $CDCl_3$ (*21*) and by following the key HMBC connectivities. In particular, the two olefinic carbons at 143.2 and 154.2 ppm are both coupled to the aldehydic protons at 9.20 ppm (respectively, peaks r and s in **Figure 5A**), only the first one showing a large $^2J_{CH} \approx 24$ Hz typical for a carbon geminal to a aldehydic proton (*32*). For the same reason, the carbon at 45.7 is assigned to C-4 (peak o in **Figure 5A**). Finally, C-5 (at 26.9 ppm) is HMBC

Table 6. NMR Assignments of Dialdehydic Type **V** Structures (**Figure 1**) Contained in *p*-HPEA-EDA, 3,4-DHPEA-EDA, and EDA

atom no.	chemical shift ^{1}H (^{13}C)
1	9.20 (195.6)
3	9.51 (201.8)
4	2.70, 2.70 (45.7)
5	3.51 (26.9)
6	2.53, 2.59 (36.6)
7	− (171.7)
8	6.74 (154.2)
9	− (143.2)
10	1.97 (14.9)

Table 7. Variations (Percent) of Monoaldehydic and Dialdehydic Forms of Phenolic Compounds after Thermal Treatment by Conventional (VOO1CO) or Microwave Oven (VOO1MW), Measured by NMR[a] and by HPLC-DAD/MSD [b]

	monoaldehydic forms			dialdehydic forms	
sample	B+C+F	L+M+N+O	HPLC	E+K	HPLC
VOO1CO	−69	−70	−75	−27	−4
VOO1MW	−57	−33	−40	14	11

[a] B+C+F corresponds to monoaldehydic signals; L+M+N+O corresponds to enolethers signals; E+K corresponds to dialdehydic signals. [b] Variations are calculated with respect to the untreated VOO1 (VOO1TQ).

connected with both aldehydic protons (peaks p and q in **Figure 5A**). The complete assignment is reported in **Table 6**.

From the assignments of the aldehydic (B+C+F) and enolether protons (L+M+N+O) to monoaldehydic compounds containing a structure of type **I** and aldehydic protons (E+K) to dialdehydic compounds containing a structure of type **V**, it is now possible to study the effect of microwave or conventional heating on aldehydic compounds by NMR spectroscopy and compare the results with the HPLC data (**Table 7**).

It is interesting to observe that the NMR results for the monoaldehydic compounds agree with HPLC data, although better agreement is found for CO than for MW treatment; however, even for the latter treatment, the HPLC value is close to the average variation measured by NMR spectroscopy (−40.3 vs −45%). Good agreement is also found for the variation of dialdehydic compound upon MW treatment, whereas the results for CO heating, while preserving the sign of the variation, are quite different. It is possible that this disagreement depends on the fact that *p*-HPEA-EDA and AcPIN coelute in a HPLC trace (see **Table 4**) so that the HPLC value may be biased.

In conclusion, an increase in dialdehydic forms of phenolic compounds (EDA, *p*-HPEA-EDA) has been observed after microwave and conventional heat treatments: NMR spectroscopy was able to confirm these results. It is likely that reasonable chemical conversions from EA, *p*-HPEA-EA, and 3,4-DHPEA-EA to their respective dialdehydic forms (EDA, *p*-HPEA-EDA, and 3,4-DHPEA-EDA) were induced by heating.

ABBREVIATIONS USED

1D NMR, monodimensional nuclear magnetic resonance; 2D NMR, bidimensional nuclear magnetic resonance; 3,4-DHPAA, 3,4-dihydroxyphenylacetic acid; 3,4-DHPEA, 3,4-dihydroxyphenylethanol; 3,4-DHPEA-EA, 3,4-dihydroxyphenylethanol linked to elenolic acid; 3,4-DHPEA-EDA, 3,4-dihydroxyphenylethanol linked to the dialdehydic form of elenolic acid; ABTS, 2,2′-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid, diammonium salt; API, apigenin; CO, conventional; COSY, correlation spectroscopy; DAD, diode array UV−vis detector; DMSO-d_6, deuterated dimethyl sulfoxide; EA, elenolic acid; EDA, dialdehydic form of elenolic acid lacking a carboxymethyl group; ESI, electrospray ionization; EVOO, extra virgin olive oil; HMBC, heteronuclear multiple bond correlation; HPLC, high-performance liquid chromatography; LLE, liquid−liquid extraction; LUT, luteolin; MSD, mass spectrometer detector; MW, microwave; *p*-HPEA, *p*-hydroxyphenylethanol; *p*-HPEA-EA, *p*-hydroxyphenylethanol linked to elenolic acid; *p*-HPEA-EDA, *p*-hydroxyphenylethanol linked to the dialdehydic form of elenolic acid; TOF, time of flight; TP, total phenol; VOO, virgin olive oil.

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Analytical Methods

A spectroscopic and chemometric study of virgin olive oils subjected to thermal stress

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ABSTRACT

The paper describes a study of thermal stress of three different samples of virgin olive oil in terms of oxidative stability. Fatty acid composition, evaluation of oxidative stability under forced conditions (OSI), determination of UV-spectrophotometric oxidation indexes (k_{232} and k_{270}) and spectral properties were explored along the thermal treatment. The samples were subjected to heating treatment at 180 °C and evaluated after 0, 30, 60, 90, 120, 150 and 180 min. Middle infrared (MIR) and visible–near infrared (Vis–NIR) spectra were elaborated by partial least squares modelling to individualise regions and bands where critical variations were present. Two bands were found as principal influential ones (1245–1180 cm^{-1} and 1150–1030 cm^{-1}) on MIR while one primary region was identified on Vis–NIR (2200–1325 cm^{-1}).

1. Introduction

Extra virgin olive oil (EVOO) is widely known because of its high health benefits and sensory quality in comparison to other oils and fats (Bendini et al., 2007), especially due to the presence of a high ratio between monounsaturated and polyunsaturated fatty acids and its antioxidant fraction (principally lipophilic and hydrophilic phenolic compounds) (Aparicio, Roda, Albi, & Gutiérrez, 1999).

Virgin olive oil is a principal ingredient in the Mediterranean diet and it is consumed in different ways: raw in salads, on traditional food (i.e. as breakfast in "tostada" in the South of Spain or as meal in pasta or "bruschetta" in the South of Italy), toasts and other foodstuffs (Cerretani, Biasini, Bonoli-Carbognin, & Bendini, 2007), but often it is also consumed after domestic heating, such as fried, boiled or after conventional and microwave heating (Brenes, García, Dobarganes, Velasco, & Romero, 2002). These thermal treatments are commonly utilised for home-cooking, food catering and industrial processes (Brenes et al., 2002; Carrasco-Pancorbo et al., 2007; Cheikhousman et al., 2005). Several studies published in literature up to now have compared the effects of conventional and microwave heating on the physical and chemical parameters of extra virgin olive oil (Albi, Lanzón, Guinda, Pérez-Camino, & León, 1997; Brenes et al., 2002; Caponio & Gomes, 2001; Vieira & Regitano-D'Arce, 1999; Valli et al., 2010).

It has been observed that heating can affect the phenolic fraction and the oxidation stability and degradability of oil. In fact, Cerretani, Bendini, Rodriguez-Estrada, Vittadini, and Chiavaro (2009) have studied the effect of microwave heating treatments on phenols compared to the effects produce by oxidation or heating by conventional oven; particularly lignans have shown the highest stability to the thermal treatments due to their strong antioxidant properties (Carrasco-Pancorbo et al., 2007). The parameters that have been proven to influence the extent of oxidation and the degradation of oils in a highest extension during heating are oil composition, time and temperature of heating, food (in the case that some food is in contact with the oil), ratio between surface and volume of the oil (Andrikopoulos, Kalogeropoulos, Falirea, & Barbagianni, 2002; Christie, Brechany, Sebedio, & Le Quere, 1993). However, strong interactions exist among these variables and because of that, they are difficult to control and define (Jorge, Márquez-Ruiz, Martín-Polvillo, Ruiz-Méndez, & Dobarganes, 1996).

More recently, Valli et al. (2010) have studied the phenolic fraction of two EVOO and their admixtures after thermal treatments using microwave and conventional oven by HPLC-DAD/MSD and NMR spectroscopy. In this work authors have observed an increase of the dialdehydic forms of secoiridoids (dialdehydic form of elenolic acid lacking a carboxymethyl group, EDA; *p*-hydroxyphenylethanol linked to the dialdehydic form of elenolic acid,

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p-HPEA-EDA) after microwave and conventional heat treatments. It is likely that reasonable chemical conversions from elenolic acid (EA), *p*-hydroxyphenylethanol linked to elenolic acid (*p*-HPEA-EA), and 3,4-dihydroxyphenylethanol linked to elenolic acid (3,4-DHPEA-EA) to their respective dialdehydic forms (EDA; *p*-HPEA-EDA; 3,4-dihydroxyphenylethanol linked to the dialdehydic form of elenolic acid, 3,4-DHPEA-EDA) were induced by heating.

Other analytical techniques as FT-IR have been recently applied to the analysis of phenolic fraction of EVOO (Cerretani et al., 2010). Spectroscopic FT-IR coupled with chemometrics methods have been successfully used to detect olive oil adulteration (Lerma-García, Ramis-Ramos, Herrero-Martínez, & Simó-Alfonso, 2009; Maggio, Cerretani, Chiavaro, Kaufman, & Bendini, 2010; Ozen & Mauer, 2002) and freshness (Sinelli, Cosio, Gigliotti, & Casiraghi, 2007). The chemometric algorithm partial least square (PLS) has been repeatedly and extensively used to obtain different quality parameters of edible oils (Al-Alawi, Van de Voort, & Sedman, 2004; Iñón, Garrigues, Garrigues, Molina, & De la Guardia, 2003; Li, Van de Voort, Ismail, & Cox, 2000b; Li et al., 2000a).

In this work three different EVOOs have been subjected to heating treatment by conventional oven with the following aims:

(1) to study the changes in the oxidative stability (k_{232}, k_{270} and OSI time) and in the spectroscopic properties (Vis–NIR and FT-MIR) occurring in this process;

(2) to find, by spectroscopic analysis, the compounds or the families of compounds that are most important in this oxidation pathway;

(3) to explore the opportunity of adopting some rapid spectroscopic methods (Vis–NIR and FT-MIR) for the control of cooked olive oils.

2. Materials and methods

2.1. Apparatus

Fatty acid (FA) analyses were performed using an Autosystem XL Perkin Elmer (Shelton, CT, USA) gas chromatograph equipped with a flame ionisation detector (FID). The determination of k_{232} and k_{270} was carried out using an UV–vis 1800 instrument (Shimadzu Co., Kyoto, Japan), which had a six slot shuttle and a system for temperature control of working conditions. The oxidative stability of samples was evaluated using an eight-channel oxidative stability instrument (Omnion, Decatur, IL, USA). The FT-MIR spectra were acquired on a Tensor 27™ FTIR spectrometer system (Bruker Optics, Milan, Italy), fitted with a Rocksolid™ interferometer and a DigiTect™ detector system coupled to an attenuated total reflectance (ATR) accessory. NIR analysis was carried out by NIR Lab Near Infra Red by transmittance instrument (SACMI Imola S.C., Imola, Bologna, Italy).

2.2. Materials, reagents and standards

Potassium hydroxide, methanol, *n*-hexane, isooctane and acetone were purchased from Merck (Darmstadt, Germany). The standard mixture of FA methyl esters (GLC 463) was supplied by Nu-Chek (Elysian, MN, USA).

2.3. Samples and thermal treatment

Three different samples of extra virgin olive oil (named A, B and C) from different Italian regions (Abruzzo, Marche and Puglia) harvested in the fall of 2009, were analysed in this experimental study. The oils were different in terms of cultivar and ripening degree.

For analytical purposes, six aliquots (50 g) of each sample were inserted in 250 mL opened glass beakers (7.2 cm i.d.) and subjected to conventional heating at 180 °C in a oven (model M20-VN, Instruments s.r.l, Bernareggio, Milan, Italy). The beakers were removed from the oven at fixed intervals of 30 min, obtaining samples with different heating treatments (30, 60, 90, 120, 150, 180 min) to be analysed. All heated samples were cooled at room temperature (23 ± 1 °C) for 30 min and stored in bottles without headspace at 12 °C before chemical analysis.

2.4. Fatty acid composition

The FA composition of oil samples was determined as fatty acid methyl esters (FAMEs) after alkaline treatment, obtained by mixing 0.05 g of oil dissolved in 2 mL of *n*-hexane with 1 mL of 2 N potassium hydroxide in methanol, and subsequent gas chromatographic analysis, according to Bendini, Cerretani, Vecchi, Carrasco-Pancorbo, and Lercker (2006), with slight modifications. Analytes were separated on a RTX-2330 capillary column (30 m × 0.25 mm i.d., 0.2 μm film thickness) from Restek (Bellefonte, PA, USA). Column temperature was held at 140 °C for 5 min and then it was increased at 2.5 °C min^{-1} until 240 °C. The FID and the injector temperatures were both set at 250 °C. Peak identification was accomplished by comparing the peak retention times with those of the GLC 463 FAME standard mixture, injected under the same gas chromatographic conditions. The GC response factor of each FA was also calculated by using the GLC 463 FAME standard mixture.

2.5. Determination of k_{232} and k_{270}

The UV-spectrophotometric indexes (k_{232} and k_{270}) were determined according to the European Communities official methods and the following amendments (European Union Commission, 1991). To calculate the k_{270} and k_{232} values, the oil samples were diluted in isooctane (1:100 v/v for k_{270} and 1:1000 v/v for k_{232}), placed into a 1 cm quartz cuvette, and analysed at the wavelengths of 270 and 232 nm, against a blank of isooctane. Three replicates were prepared and analysed per sample.

2.6. Evaluation of oxidative stability under forced conditions

An eight-channel oxidative stability instrument (OSI) (Omnion) was used. To obtain the OSI induction time, a stream of purified air

Table 1
FA composition, OSI values, k_{232} and k_{270} values of samples before the thermal treatment.[a]

	A	B	C
C16:0	15.43 ± 0.01	10.52 ± 0.02	12.79 ± 0.12
C16:1 A	0.13 ± 0.00	0.11 ± 0.00	0.12 ± 0.01
C16:1 B	1.27 ± 0.00	0.58 ± 0.00	1.07 ± 0.01
C17:0	0.06 ± 0.00	0.16 ± 0.00	0.04 ± 0.01
C17:1	0.10 ± 0.00	0.23 ± 0.00	0.08 ± 0.00
C18:0	2.01 ± 0.01	2.73 ± 0.02	1.78 ± 0.01
C18:1 *n*−9	65.64 ± 0.01	76.01 ± 0.07	72.81 ± 0.13
C18:1 *n*−7	3.26 ± 0.01	1.68 ± 0.03	2.79 ± 0.02
C18:2	10.34 ± 0.01	6.40 ± 0.01	7.10 ± 0.03
C20:0	0.38 ± 0.00	0.40 ± 0.00	0.30 ± 0.00
C18:3 *n*−3	0.95 ± 0.00	0.77 ± 0.00	0.76 ± 0.01
C20:1	0.29 ± 0.01	0.27 ± 0.00	0.26 ± 0.01
C22:0	0.12 ± 0.00	0.13 ± 0.00	0.09 ± 0.00
OSI time (h)	11.35	32.15	20.20
k_{232}	2.59 ± 0.10	2.29 ± 0.35	3.23 ± 0.40
k_{270}	0.10 ± 0.01	0.15 ± 0.01	0.26 ± 0.00

[a] Data are expressed as mean of three determinations, with the standard deviations (except for OSI time). Fatty acid composition, determined by gas chromatography analysis and expressed as percentages; OSI time, oxidative stability index expressed as hours and hundredths of hours; k_{232} and k_{270} values determined by spectroscopic analysis.

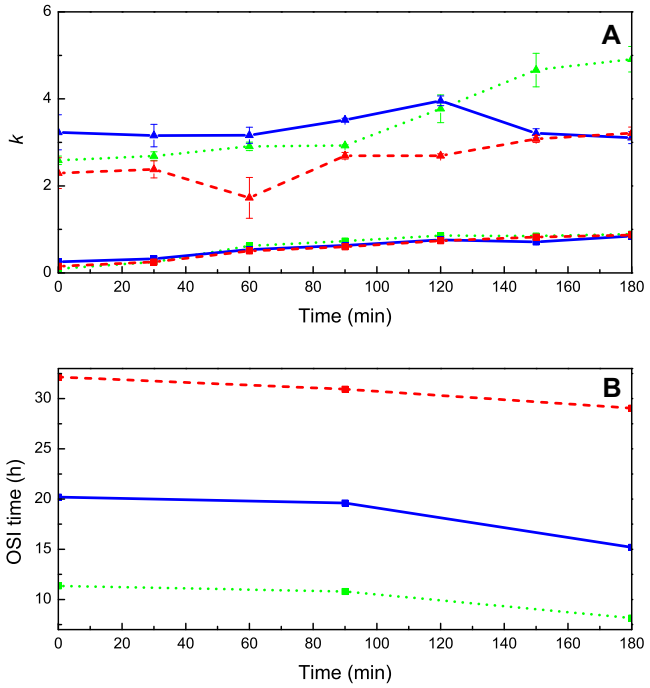

Fig. 1. (A) Plot of k_{232} (▲) and k_{270} (■) measured at heating times 0, 30, 60, 90, 120, 150 and 180 min for samples A (···), B (--) and C (—). (B) OSI time values (h), of samples A (···), B (--) and C (—) monitored at heating times 0, 90 and 180 min.

(120 mL min^{-1} air flow rate) was passed through 5.0 ± 0.1 g oil sample heated at 110 ± 0.1 °C, under atmospheric pressure. The effluent air contains especially short chain acids as formic acid and other volatile compounds formed during thermal oxidation of the oil; these substances were recovered and measured in deionised water, as an increase of conductivity. The OSI time was expressed in hours and hundredths of hours, which was defined as a measure of the oxidative stability of oil.

2.7. FT-MIR spectroscopy

A small amount of the oil samples (about 1 g) was uniformly deposited on the crystal surface of ATR accessory (Specac Inc., Woodstock, GA, USA), equipped with a ZnSe 11 reflection crystal. Analyses were carried out at room temperature. Spectra were acquired (32 scans/sample or background) in the wavenumber range of 4000–700 cm^{-1} at a resolution of 4 cm^{-1}, and the data exported by OPUS r. 6.0 (Bruker Optics) software in ASCII compatible format. For each sample, the absorbance spectrum was collected against a background, obtained with a dry and empty ATR cell. Three spectra per sample were recorded. After acquiring each spectrum, the ATR crystal was cleaned with a cellulose tissue soaked in *n*-hexane and then rinsed with acetone.

2.8. Vis–NIR spectroscopy

The samples were inserted in cuvettes (optical glass; light path: 20 mm; Hellma, Jena, Germany). Spectra were acquired using the halogen lamps as light source and an optical filter of 200 FN, a measuring time of 6 ms, within a wavenumber range of 33,000–9000 cm^{-1}. Three spectra per sample were recorded in ASCII compatible format. After acquiring each spectrum, the glass container was cleaned with *n*-hexane, soap and then rinsed with acetone.

2.9. Chemometrics methods

PLS regression aims to find the relationship between a set of predictor (independent) data, X $(m \times n)$, and a set of responses (dependent), Y $(m \times l)$. Here, n and l are the independent and dependent variables, respectively, and m is the observation vector.

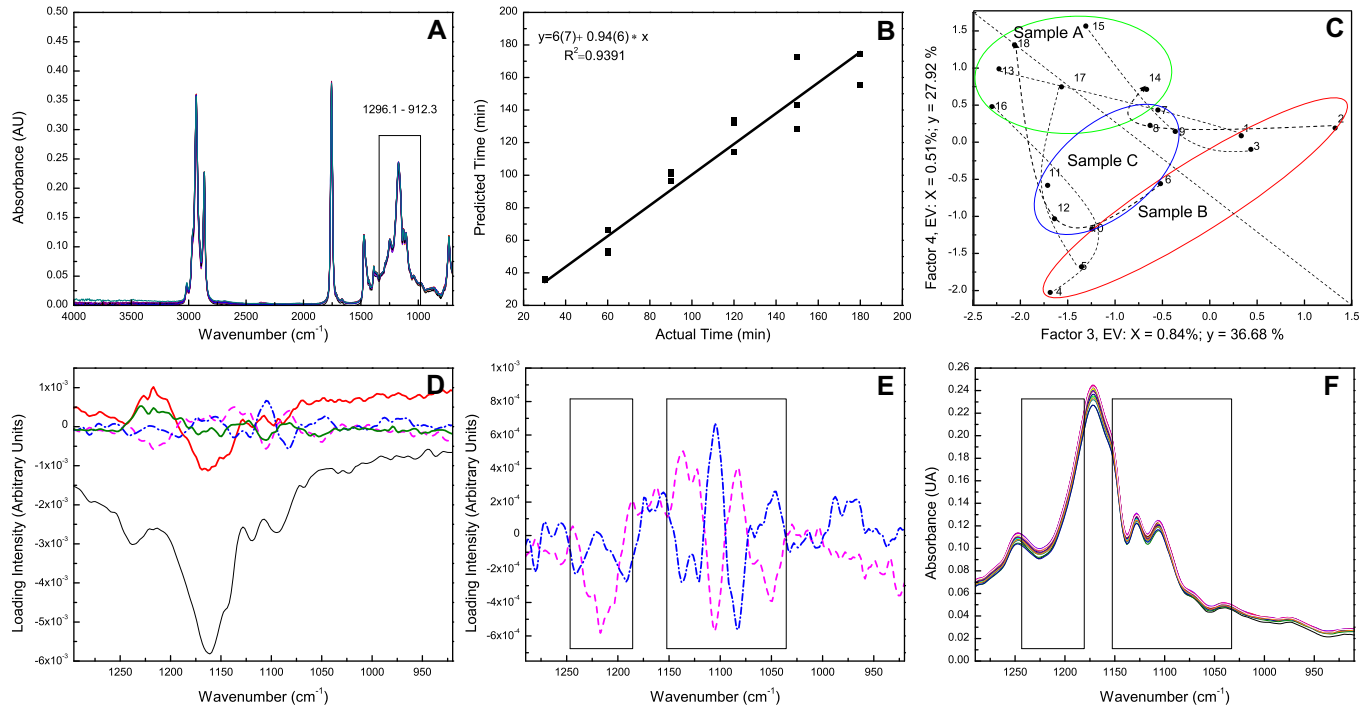

Fig. 2. (A) Full FT-MIR spectra (4000–700 cm^{-1}). (B) Actual vs. predicted heating time values in the calibration set in the selected spectral range (1296.1–912.3 cm^{-1}). (C) Third and 4th PLS factor score plot, see reference number in Table 2. (D) First 5 PLS loadings vectors. (E) Third and 4th PLS loadings vectors. (F) FT-MIR spectra in the selected wavenumber range (1296.1–912.3 cm^{-1}).

Table 2
Code Numbers used for each sample in PLS score plot of MIR and PLS score plot of NIR.

Code	PLS-FT-MIR	Time (min)	Code	PLS-NIR	Time (min)
1	B	120	1	B	120
2	B	150	2	B	150
3	B	180	3	B	180
4	B	30	4	B	30
5	B	60	5	B	60
6	B	90	6	B	90
7	C	120	7	C	120
8	C	150	–	–	–
9	C	180	8	C	180
10	C	30	9	C	30
11	C	60	10	C	60
12	C	90	11	C	90
13	A	120	12	A	120
14	A	150	13	A	150
15	A	180	14	A	180
16	A	30	15	A	30
17	A	60	16	A	60
18	A	90	17	A	90

However, it differs from the multiple linear regression technique (MLR) mainly that PLS is able to give stable predictions even when *X* contains highly correlated variables. Detailed description of PLS method and its algorithms could be found elsewhere (Haaland & Thomas, 1988).

The chemometrics computations were performed in Matlab 7.0 (Mathworks, Inc., Natwick, MA, USA). Variable selection was implemented using the graphical interface MVC1 provided by Olivieri, Goicoechea, and Iñón (2004). A PLS Score Plots was performed using Tomcat Toobox (Daszykowski et al., 2007). PLS was run on mean-centred data.

All programs were run on an ACER-Aspire 5050 computer with an AMD Turion™ 64 Mobile, 2.20 GHz microprocessor and 2.00 Gb of RAM.

3. Results and discussion

3.1. Compositional analysis

Table 1 groups the fatty acid composition, the OSI time, and the k_{232} and k_{270} coefficients for the three olive oils studied. Before thermal stress only sample B showed k_{232} and k_{270} values that were both below the limits established by the EC Regulation for EVOO category (European Union Commission, 2007), which corresponded to 2.50 and 0.22, respectively. Sample C showed both values over these limits, while sample A exceeded the limit only for k_{232} (Table 1).

Oxidative status of the samples were evaluated by monitoring the trend of conjugated dienes (k_{232}) and trienes (k_{270}) during the thermal stress (Hrncirik & Fritsche, 2005; Lerma-García, Simó-Alfonso, et al., 2009; Mancebo-Campos, Fregapane, & Desamparados Salvador, 2008) and analysing the samples heated at different times of treatment (Fig. 1, part A). The three samples underwent a significant increase of k_{270} with heating (Allouche, Jiménez, Gaforio, Uceda, & Beltràn, 2007; Bendini, Valli, Cerretani, Chiavaro, & Lercker, 2009), reaching values higher than the legal limit after only 30 min of thermal treatment (see Fig. 1, part A). During heating, a common trend for k_{232} among samples was not evidenced (Fig. 1, part A). As reported in Fig. 1, part B, the OSI time (h) was also checked halfway through the treatment (90 min) and at the end (180 min). Before the thermal treatment, the three samples showed high differences among OSI values: such a difference in oxidative stability could be easily related to the FA composition of the oils (Table 1), as previously reported in literature (Aparicio et al., 1999; Lerma-García, Simó-Alfonso, et al., 2009). In fact, sample B showed the highest OSI time value (32.15 h), the highest oleic acid content and the lowest amounts of polyunsaturated FA (linoleic and linolenic acids). Moreover, at the end of the thermal treatment sample B was the one that registered the lowest decrease (9.64%) in terms of OSI time, confirming its remarkable oxidative

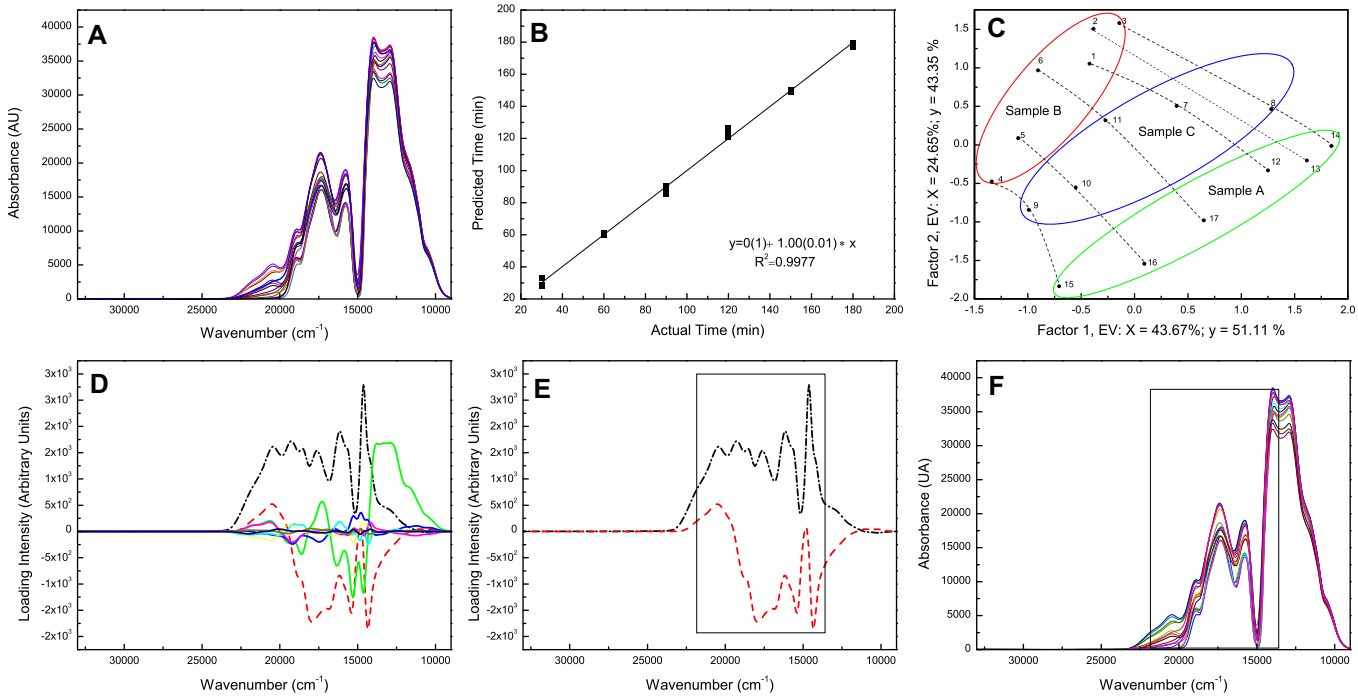

Fig. 3. (A) NIR spectra (33,000–9000 cm^{-1}). (B) Actual vs. predicted heating time values in the calibration set. (C) First and 2nd PLS factor score plot, see reference number in Table 2. (D) First 9 PLS loadings vectors. (E) First and 2nd PLS loadings vectors. (F) NIR spectra in the selected wavenumber range.

stability, while sample A and sample C presented a OSI time decrease of 28.19% and 24.75%, respectively (Fig. 1, part B).

3.2. Spectral analysis

The analysis of the variations in the FT-MIR spectra is not very easy, because these changes are very weak and there are so many peaks and shoulders without resolution (Fig. 2, part A). For a better visualisation of trends, the compression of the spectral information is needed. Principal component analysis (PCA) is one of the most used tools for this kind of works. Although, in this particular case it has not been possible to find any correlation between the PCA scores and the heating time.

Therefore, PLS was used, because it is a tool capable of addressing decomposition of the spectra to the dependent variable (heating time) (Carlson & Gautun, 2005). Since PLS was unable to find any acceptable correlation between oil spectra evolution and heating time using full spectrum FT-MIR, we used a wavelength selection algorithm (Xu & Schechter, 1996). The region 1296.1–912.3 cm^{-1} was selected as the optimum for regression (Fig. 2, parts A and B). Then we proceeded to analyse the score plots of significant PLS factors to see which of them was correlated with both the original composition of the oil and the evolution in time (Fig. 2, part C and Table 2). A segmentation was pointed out to 3rd and 4th PLS factor (Fig. 2, part C), which can distinguish among the different oils A, B and C. Moreover, the first (0–90 min) and the last (120–180 min) heating times into each kind of oil could be distinguish, as is showed with a dashed line. Once these factors were individualised as the most relevant ones, their loadings were evaluated (Fig. 2, parts D and E). Two regions were identified as principal influential ones: 1245–1180 cm^{-1} and 1150–1030 cm^{-1}, as shown in Fig. 2, part F. These regions comprise the bands belonging to $-CH_2-$ (1238), C–O (1238, 1138, 1118, 1097 and 1033 cm^{-1}), being C–O reported by Lerma-García, Simó-Alfonso, Bendini, and Cerretani (2011), as one of the regions more affected by oxidation.

In the case of the NIR spectra, shown in Fig. 3, part A, there were not many peaks but the analysis of the spectral variations was also complicated due to the low resolution among them.

Therefore, for better visualisation of trends, PLS and PCA were applied. UV–NIR spectra did not show any correlation between PCA scores and the heating times, but PLS was able to find an acceptable correlation (see Fig. 3, part B) with full spectrum NIR. Because of that, we proceeded to analyse score plots of significant PLS factors (Fig 3, part C and Table 2) to see which of them were correlated with both the original composition of the oils A, B and C and the evolution in time. A segmentation was evidenced to 1st and 2nd PLS factor, which distinguished among the different oils. In addition, the different heating times appear sorted in ascending way with the 2nd PLS-factor for A, B and C oils (Fig. 3, part C and reference number in Table 2). This fact shows that the 2nd PLS-factor is very important for the correlation. Once these PLS factors were detected as the most relevant, their loadings were evaluated (Fig 3, parts D and E). The major influence region was individualised in: 2200–1325 cm^{-1} (Fig. 3, part F).

4. Conclusions

This work describes a study of thermal stress on three virgin olive oils. Evaluation of oxidative stability under forced conditions, determination of k_{232} and k_{270} and spectral properties were explored along the thermal treatment. Fatty acid composition was explored at the beginning of the work. The three samples underwent a significant increase of k_{270} with heating, while a common trend for k_{232} among samples was not evidenced. Before the thermal treatment, the three samples showed high differences among OSI values. At the end of the thermal treatment sample B, with highest initial OSI time, highest oleic acid and lowest amounts of polyunsaturated FA content, registered the lowest decrease (9.64%) in terms of OSI time. The opportunity to adopt rapid spectroscopic methods (Vis–NIR and FT-MIR) in the control of cooked olive oils has been explored by using the chemometric analysis PLS. The most significant regions included the bands related to $-CH_2-$ (1238) and C–O (1238, 1138, 1118, 1097 and 1033 cm^{-1}) groups, concluding that C–O was one of bands more affected by oxidation. The spectroscopic data elaboration by PLS was able to distinguish among the different oils and the different heating times. This approach could be useful for monitoring the oxidative status of cooked oils, both in industrial scale or in food-catering field.

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PAPER

DSC EVALUATION OF OLIVE OIL DURING ACCELERATED OXIDATION

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ABSTRACT

Changes in differential scanning calorimetry cooling thermal properties of an extra virgin olive oil in the presence and absence of its phenolic fraction were evaluated at different times of accelerated storage treatment (up to 4 weeks at 60°C under air) and related to lipid oxidation molecules (measured with k_{232} and k_{270} indices) and total phenol content. Phenols did not appear to directly influence crystallization of extra virgin olive oil as neither cooling profiles nor thermal properties differed significantly between the two oils at the beginning of storage. However, oil samples deprived of phenols showed more significant changes at the longest storage time in comparison with untreated oil. Cooling transitions were all deconvoluted into three peaks. Changes in thermal properties were more evident for the two transition peaks at the highest temperature in both oil samples. Thus, a marked influence of lipid oxidation products on the crystallization pattern of these two peaks may be hypothesized.

- Key words: differential scanning calorimetry, extra virgin olive oil, lipid oxidation, phenols, thermal properties, storage -

INTRODUCTION

Extra virgin olive oil (EVOO) plays an important role in the Mediterranean diet as its consumption has been associated with beneficial effects on health and prevention against several diseases (BENDINI *et al.*, 2007). EVOO has a high resistance to oxidative deterioration that is related not only to its fatty acid composition (high monounsaturated to polyunsaturated fatty acid ratio), but also to the presence of minor compounds with powerful antioxidant activity. Lipid oxidation of EVOO occurs mainly during processing and storage when the oil is in contact with oxygen, and is recognized as the main cause of deterioration of the oil during its shelf-life (FRANKEL, 1985). The influence of several factors on the rate of deterioration as well as the importance of its prevention and its impact on sensory and olfactory attributes of the oil have been recently reviewed (BENDINI *et al.*, 2009). Among the minor components, phenolic compounds, which are responsible for sensorial properties of EVOO such as bitterness, pungency and astringency (CERRETANI *et al.*, 2008), also provide resistance to auto-oxidation (BALDIOLI *et al.*, 1996). At the same time, phenolic compounds appear to be only partially affected by heat treatment by microwave (particularly lignans) (BRENES *et al.*, 2002; CERRETANI *et al.*, 2009).

The evaluation of the oxidation process of EVOO under realistic storage conditions is quite slow, and complete oxidation occurs over a period of 12 to >18 months. On the other hand, high-stress oxidation conditions evaluated by oxidative stability tests performed at high temperature (e.g. Rancimat) hardly mimic realistic storage conditions due to the widely divergent kinetics of lipid oxidation at the high temperatures employed. Thus, accelerated storage conditions at a maximum of 60°C have also been evaluated as they do not alter the oxidation mechanism, and correlate well with experiments performed under normal storage conditions (FRANKEL, 1993).

Differential scanning calorimetry (DSC) is a calorimetric technique widely employed for the characterization of the thermal behaviour of oils and fats as it does not require sample preparation or the use of solvents, resulting in a reduced time analysis and low environmental impact. The use of DSC for assessment of oxidative deterioration of vegetable oils is well known (TAN and CHE MAN, 2002). In addition, thermal parameters obtained by cooling and heating thermograms have been found to be related to the chemical composition of vegetable oils (TAN and CHE MAN, 2000), and relationships have also been documented for both major and minor components of EVOO (JIMÉNEZ MÁRQUEZ and BELTRÁN MAZA, 2003 and 2007; CHIAVARO *et al.*, 2007, 2008a, and 2010).

Several papers have been recently published on the application of DSC for assessment of quality factors of EVOO, such as the discrimination of commercial categories of olive oil (CHIAVARO *et al.*, 2008b), detection of adulterations of EVOO with less expensive vegetable oils (CHIAVARO *et al.*, 2008c and 2009a) and discrimination of oil samples according to cultivar-environment effects (KOTTI *et al.*, 2009). However, few reports have examined the effects of storage and/or heating treatment on the DSC thermal properties of EVOO in relation to chemical oxidative changes. Auto- and thermo-oxidation of EVOO have been evaluated by DSC cooling thermograms (VITTADINI *et al.*, 2003), and the amount of oxidized volatile compounds was found to be correlated with such thermal parameters as crystallization enthalpy and on-set transition temperature. More recently, changes in DSC thermal parameters and transition profiles upon cooling and heating were evaluated on different commercial categories of olive oil after microwave heating in relation to chemical composition and stability indices (CHIAVARO *et al.*, 2009b).

The aim of the present investigation was to evaluate DSC thermal parameters upon cooling of an EVOO, in the presence and absence of phenolic fraction during an accelerated storage treatment of up to 4 weeks at 60°C, and to relate these changes to the state of lipid oxidation (UV absorbance at 232 and 270 nm) and phenolic content. The potential role of phenols in the crystallization of EVOO was also evaluated on a preliminary basis.

MATERIALS AND METHODS

Samples and storage

An Italian sample of extra virgin olive oil from Tuscany (blend of Leccino, Moraiolo and Frantoio cultivars) was employed in this study. The sample was divided into two aliquots: extra virgin olive oil with phenols ($EVOO_p$) and extra virgin olive oil without phenols ($EVOO_{p0}$). Phenolic compounds were removed from $EVOO_p$ according to the procedure described by BONOLI-CARBOGNIN *et al.* (2008). Briefly, 35 g of $EVOO_p$ were washed with several aliquots of 0.5M NaOH (4x15 mL). To eliminate the aqueous phase, the mixture was centrifuged (1,000 x g, 5 min) after each washing. Combined olive oil fractions were then washed with 0.5M HCl (2x10 mL) and saturated NaCl solution (5x10 mL), centrifuged at 1,000 x g for 5 min, dried with anhydrous sodium sulphate, and finally filtered under vacuum. Dried $EVOO_{p0}$ was then obtained.

Both samples ($EVOO_p$ and $EVOO_{p0}$) were divided in 8 aliquots each (250 mL, 228.8 g) and kept in the dark at 60°C for 4 weeks. Each aliquot was stored in an individual open glass bottle

of 300 mL (i.d. = 6 cm; surface area exposed to air 28.3 cm^2). Bottles of $EVOO_p$ and $EVOO_{p0}$ were removed each week from the oven and analyzed.

Chemical analysis

Free acidity (free fatty acid content of the oil expressed as the percentage of oleic acid), peroxide value (amount of hydroperoxides expressed as mequiv of O_2 kg^{-1}oil) and UV absorbance at 232 and 270 nm (k_{232} and k_{270} provide a measurement of the state of oxidation) were performed according to the official methods of the European Commission (EUROPEAN COMMUNITY, 2003).

Phenolic compounds were extracted from oil samples by a liquid-liquid extraction procedure using a modified version of the method by PIRISI *et al.* (2000). Briefly, 4 g of oil (± 0.001 g) were dissolved in 4 mL of *n*-hexane, and the solution was extracted twice with four 2 ml portions of methanol:water (60:40, v/v). The combined extracts of the hydrophilic layer were concentrated and dried by evaporative centrifuge (Mivac Duo of Genevac Inc., Valley Cottage, NY, USA) at a temperature of 40°C. Finally, the residue was redissolved in 0.5 ml methanol:water (50:50, v/v) and filtered through a 0.20 µm nylon filter (Whatman, Clifton, NJ, USA). HPLC analysis was carried out using a HP 1100 system (Agilent Technologies, Palo Alto, CA, USA) equipped with a binary pump delivery system, degasser, autosampler, diode array UV-vis detector (DAD) and mass spectrometer detector (MSD) using a reverse phase column C_{18} Luna 5 µm, 25 cm x 3.00 mm i.d. (Phenomenex, Torrance, CA, USA) according to ROTONDI *et al.* (2004). Each phenolic compound was expressed as mg 3,4-dihydroxyphenylacetic acid (3,4-DHPAA) kg^{-1} oil (calibration curve with r^2=0.9739).

Three replicates were analyzed per sample.

DSC analysis

Samples of oil (8-10 mg) were weighed in aluminium pans, covers were sealed into place and analyzed with a DSC Q100 (TA Instruments, New Castle, DE, USA). Indium (melting temperature 156.6°C, H_f = 28.45 J/g) and *n*-dodecane (melting temperature -9.65°C, H_f = 216.73 J/g) were used to calibrate the instrument and an empty pan was used as reference. Oil samples were equilibrated at 30°C for 3 min and then cooled at -80°C at a rate of 2°C/min. Dry nitrogen was purged in the DSC cell at a flow rate of 50 cm^3/min. Cooling thermograms were analyzed with Universal Analysis Software (Version 3.9A, TA Instruments) to obtain enthalpy (ΔH, J/g), onset temperature (T_{on}, °C) and offset temperature (T_{off}, °C) of the transitions. The range of the transitions was calculated as the temperature difference between T_{on} and T_{off}. Overlapping transitions of cooling thermograms were deconvoluted into individual constituent peaks using PeakFitTM software (Jandel Scientific, San Rafael, CA, USA). The following parameters were considered for each deconvoluted peak: T_{on}, T_{off} and peak temperatures (T_p), % peak area (percentage area of the total peak area) and range of the transitions. Three replicates were analyzed per sample.

Statistical analysis

Data were analysed using SPSS (Version 17.0, SPSS Inc., Chicago, IL, USA) statistical software. SPSS was used to perform one-way-analysis of variance (ANOVA) and least significant difference (LSD) test at a 95% confidence level ($p \leq 0.05$) to identify differences between storage times. A student *t* test ($p < 0.05$) was also used to identify differences between samples for the same parameter at each storage time.

RESULTS AND DISCUSSION

Chemical analysis

Free acidity and peroxide values were determined on both oil samples before storage to verify their conformity with legal limits established by the European Community (EUROPEAN COMMUNITY, 2003). The free acidity percentages of $EVOO_p$ and $EVOO_{p0}$ were largely under the limit established by the EC Regulation for EVOO (EUROPEAN COMMUNITY, 2003), and were 0.15 and 0.11%, respectively. Similarly, the peroxide values were below the legal limit (EUROPEAN COMMUNITY, 2003), ranging from 15.9 meq O_2 kg^{-1} oil to 13.6 meq O_2 kg^{-1} oil for $EVOO_p$ and $EVOO_{p0}$, respectively.

Phenol stripping was very efficient: after the removal process (BONOLI *et al.*, 2009) the total amount of phenolic compounds measured at storage time zero (t_0) decreased from 154.95 mg 3,4-DHPAA /kg^{-1} oil ($EVOO_p$) to a value lower than the limit of detection ($EVOO_{p0}$).

Oxidative status of $EVOO_p$ and $EVOO_{p0}$ was measured with conjugated diene (k_{232}) and triene (k_{270}) determination, as previously reported during an accelerated storage test carried out under the same experimental conditions (LERMA-GARCÍA *et al.*, 2009). In particular, k_{232} was previously reported to show a higher predictive value in the evaluation of the oxidative status of EVOO under accelerated storage test conditions (HRNCIRIK and FRITSCHE, 2005; MANCEBO-CAMPOS *et al.*, 2008). Before storage, both oil samples showed k_{232} and k_{270} values that were below the limits established by the EC Regulation for EVOO (EUROPEAN COMMUNITY, 2003), which corresponded to 2.50 and 0.22, respectively. $EVOO_p$ and $EVOO_{p0}$ also showed significant differences for k_{232} and k_{270} values at time 0, as previously observed (LERMA-GARCIA *et al.*,

2009), most likely because of the effects of phenol stripping on oxidation, as molecules with different polarity can be also extracted together with phenols.

Changes in oxidative indexes (k_{232} and k_{270}) and total phenol content were taken into account to evaluate the effects of storage on lipid oxidation and related to the DSC thermal properties. In general, k_{232} (conjugate dienes) and k_{270} (conjugate trienes) both increased with storage. After the first week, $EVOO_p$ and $EVOO_{p0}$ reached slightly higher k_{232} values than the legal limit (2.50), as shown in Table 1. After 2 weeks of storage, significantly higher values of k_{232} were also observed for $EVOO_{p0}$ in comparison with $EVOO_p$. This is probably due to very low content of phenolics in $EVOO_{p0}$ that was not able to inhibit oxidation, since the radical generation rate was too high for scavenging by antioxidants, as previously hypothesized under similar storage conditions (BENDINI *et al.*, 2006). However, lipid oxidation also occurred in $EVOO_p$. This is probably due to a partial oxidation of phenols, which was promoted by storage conditions applied (60°C under air), as previously reported (HRNCIRIK and FRITSCHE, 2005; LERMA-GARCIA *et al.*, 2009). In addition, k_{232} values appeared to reach a plateau after 3 weeks of storage for both samples. This is in accordance with a previous study where a stationary phase was observed for this index after a few weeks of storage at 60°C in EVOO samples with different phenol contents (MANCEBO-CAMPOS *et al.*, 2008).

The storage conditions used did not appear to prevent the increase of k_{270}, even in samples with phenol (Table 1). In addition, the $EVOO_p$ sample exceeded the legal value for k_{270} (0.22) after 3 weeks of storage, while $EVOO_{p0}$ samples exceeded this limit after 4 weeks (LERMA-GARCIA *et al.*, 2009), reaching a final value greater than $EVOO_p$. It can be hypothesized that hydroperoxide decomposition to hydroxy, keto and epoxy of fatty acids (FRANKEL, 1985) may be more marked in $EVOO_{p0}$ at the longest storage time due to the very low content of phenols that are well known to act as natural antioxidants (BALDIOLI *et al.*, 1996; CARRASCO-PANCORBO *et al.*, 2005).

Changes in phenolic content during storage for $EVOO_p$ are shown in Figure 1. At time 0, phenolic compounds were abundant in the $EVOO_p$ sample (154.95 mg 3,4-DHPAA/kg of oil). The total phenol content gradually and significantly decreased with increasing storage time (beginning from the first week of storage), reaching a value of 81.84 mg/kg^{-1} oil (-47.2%) after 4 weeks. Phenolic compounds have been previously found to decrease under the same storage conditions used in the present study (HRNCIRIK and FRITSCHE, 2005), and are transformed into oxidized molecules that have been tentatively identified (LERMA-GARCÍA *et al.*, 2009).

DSC analysis of cooling transition

Representative DSC cooling thermograms obtained for $EVOO_p$ and $EVOO_{p0}$ at 0 week and during accelerated storage are shown in Figg. 2A and 2B, respectively. Both samples showed curves similar to those previously reported by CHIAVARO *et al.*, (2007, 2008b and 2008c, 2009a and 2009b, 2010) with two well-defined exothermic events, namely a minor peak at the highest

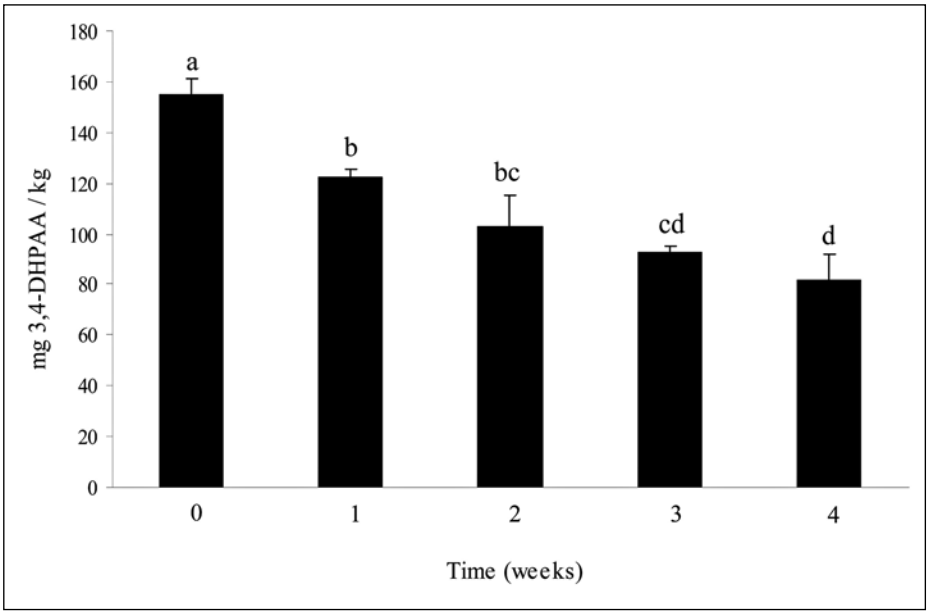

Fig. 1 - Changes in phenolic compounds in $EVOO_p$ at different storage times. Error bars represent +/- 1 standard deviation, (n = 3). Bars with the same letters are not significantly different ($p \leq 0.05$).

Table 1 - k_{232} and k_{270} values of $EVOO_p$ and $EVOO_{p0}$ samples at different storage times.

Storage time (weeks)	k_{232}		k_{270}	
	$EVOO_p$	$EVOO_{p0}$	$EVOO_p$	$EVOO_{p0}$
0	1.9 c	2.4 *c	0.125 *e	0.108 d
1	3.3 ab	3.2 bc	0.149 *d	0.108 d
2	3.9 b	4.5 *ab	0.191 *c	0.146 c
3	4.9 a	5.6 *a	0.250 *b	0.204 b
4	4.9 a	5.6 a	0.269 a	0.372 *a

a, b, c, d: The same letters within each column are not significantly different (n = 3, $p < 0.05$).
Means with an asterisk at the same storage time are significantly different (n = 3, $p < 0.05$).
RSD ≤ 3%.

and a major peak at lowest temperature. The major peak has been previously associated with crystallization of highly unsaturated triacylglycerols (TAG), in particular triolein (OOO), while the minor peak has been attributed to crystallization of more saturated TAG fractions, probably influenced by minor components (CHIAVARO *et al.*, 2007, 2010).

Oil samples with phenols ($EVOO_p$) did not exhibit changes upon storage except for a slight enlargement of the transition range, probably related to a shift in the onset temperature of crystallization towards a higher temperature at longer storage times. In contrast, the profile of $EVOO_{p0}$ showed more marked changes: in fact, the major peak height began to decrease after 3 weeks at 60°C, whereas the onset and offset temperatures of transition shifted towards higher and lower temperatures, respectively, which led to an increase of the crystallization range especially at the longest storage times. All these changes were most likely related to an increase in lipid oxidation products with storage, albeit less consistent for $EVOO_p$ (Table 1) where phenols have partially prevented oxidation. Molecules formed by hydrolysis and/or oxidation of lipids have been previously reported to interfere with TAG crystallization, hindering both contact and alignment of molecules under DSC experimental conditions in EVOO (VITTADINI *et al.*, 2003; CHIAVARO *et al.*, 2009b) and other vegetable oils (GLORIA and AGUILERA, 1998). A decrease of the height and a shift towards a lower temperature of the major exothermic transition was also observed by VITTADINI *et al.* (2003) for EVOO after 28 days of storage at 50°C when a 1.5% decrease of headspace oxygen content of the sample was measured. This different behaviour can be reasonably attributed to different experimental conditions of storage and/or initial lipid oxidation status of the oil.

Cooling thermal properties are reported in Table 2 for $EVOO_p$ and $EVOO_{p0}$ at different times of storage. At time 0, both oil samples did not show any significant differences in any thermal properties. Accordingly, it would appear that a different content of total phenol compounds did not have a direct influence on the DSC cooling thermal properties of EVOO. JIMÉNEZ MÁRQUEZ *et al.* (2007) found that the cooling thermal properties of an oil sample from the Arbequina variety with similar total phenol content than $EVOO_p$ did not significantly differ from those of the same oil sample deprived of phenolic compounds. However, phenolic compounds are partially dispersed in the water contained in olive oils (LERCKER *et al.*, 1994). Thus, their direct influence on crystallization transition cannot be excluded and requires further investigation.

Enthalpy did not exhibit significant changes for either sample (Table 2) during storage, except for $EVOO_{p0}$ at the longest storage time, which exhibited a significant decrease of the energy required for crystallization when the formation of conjugated dienes and trienes (increase of k_{232} and k_{270}, Table 1) from hydroperoxides became more pronounced. VITTADINI *et al.* (2003) also measured enthalpy among thermal properties and found a small decrease in this parameter for EVOO starting from about 14 days of storage at 50°C, when oxidation of the sample measured by depletion of headspace oxygen content in the dark slightly decreased.

Table 2 - DSC data from cooling thermograms of $EVOO_p$ and $EVOO_{p0}$ samples at different storage times.

Storage time (weeks)	ΔH (J/g)		T_{on} (°C)		T_{off} (°C)		Range[a] (°C)	
	$EVOO_p$	$EVOO_{p0}$	$EVOO_p$	$EVOO_{p0}$	$EVOO_p$	$EVOO_{p0}$	$EVOO_p$	$EVOO_{p0}$
0	56.2 a	56.4 a	-12.9 b	-13.0 b	-46.0 a	-44.8 a	33.3 b	31.9 c
1	55.5 a	55.6 a	-12.4 ab	-13.0 b	-45.9 a	-45.2 ab	33.5 b	32.2 bc
2	56.2 a	55.1 a	-11.8 a	-12.4 a	-45.9 a	-45.6 ab	34.1 ab	32.9 bc
3	55.4 a	54.8 a	-11.9 a	-12.5 a	-46.2 a	-46.2 b	34.5 a	33.4 b
4	56.4* a	53.5 b	-11.9 a	-12.3 a	-46.6 a	-51.1 *c	34.7 a	38.9 *a

a, b, c: The same letters within each column are not significantly different (n = 3, $p < 0.05$).
Means with an asterisk at the same storage time are significantly different (n = 3, $p < 0.05$). RSD ≤ 3%.
[a]Temperature difference between T_{on} and T_{off}.

Table 3 - Deconvolution parameters of cooling thermograms of $EVOO_p$ and $EVOO_{p0}$ samples at different storage times.

Storage time (weeks)	Area (%)		T_p (°C)		T_{on} (°C)		T_{off} (°C)		Range[a] (°C)	
	$EVOO_p$	$EVOO_{p0}$	$EVOO_p$	$EVOO_{p0}$	$EVOO_p$	$EVOO_{p0}$	$EVOO_p$	$EVOO_{p0}$	$EVOO_p$	$EVOO_{p0}$
Peak 1										
0	78.7 a	78.6 a	-39.2 a	-39.0 a	-33.0 a	-32.9 a	-45.1 a	-44.4 a	12.0 a	11.8 b
1	79.8 a	80.6 a	-39.3 a	-39.2 a	-33.5 a	-32.9 a	-45.4 a	-45.5 b	11.9 a	12.4 b
2	80.3 a	78.0 a	-39.1 a	-39.3 ab	-33.1 a	-33.0 ab	-44.6 a	-44.3 ab	11.7 a	11.8 b
3	80.4 a	79.8 a	-39.5 ab	-39.7 b	-33.3 a	-33.3 ab	-44.8 a	-45.2 ab	11.9 a	12.3 b
4	80.5 *a	73.4 b	-39.9 b	-42.3 *c	-33.1 a	-34.7 *b	-44.9 a	-50.1 *c	11.9 a	15.4 *a
Peak 2										
0	8.4 a	8.7 a	-33.0 a	-32.7 a	-27.0 a	-26.8 a	-36.5 a	-36.8 a	9.6 b	10.0 b
1	8.6 a	8.1 a	-33.4 a	-32.8 a	-27.6 a	-26.6 a	-37.0 a	-36.9 a	9.4 b	10.1 b
2	8.2 a	8.3 a	-33.1 a	-33.2 a	-27.4 a	-27.1 a	-36.5 a	-37.1 a	9.5 b	10.1 b
3	9.0 a	8.4 a	-33.4 a	-33.5 a	-27.0 a	-27.4 a	-37.4 a	-36.8 a	10.4 a	10.3 b
4	8.6 a	8.9 a	-33.3 a	-35.1 *b	-26.4 a	-27.9 *a	-36.9 a	-39.4 *b	10.5 a	11.5 *a
Peak 3										
0	11.8 a	11.7 a	-15.5 c	-15.3 b	-12.7 a	-11.3 b	-22.4 a	-22.7 a	10.2 b	11.3 b
1	11.6 a	11.3 a	-14.3 b	-15.3 b	-11.6 ab	-11.4 b	-21.9 a	-22.6 a	10.3 b	11.2 b
2	10.6 a	11.8 a	-13.2 a	-15.5 *b	-11.3 b	-11.7 b	-21.8 a	-22.9 a	10.5 b	11.2 b
3	10.6 a	11.8 a	-13.3 a	-14.4 *a	-11.2 b	-11.7 b	-22.5 a	-22.9 a	11.3 a	11.2 b
4	10.9 a	11.8 a	-13.4 a	-14.5 *a	-11.3 b	-10.3* a	-22.5 a	-24.2 *b	11.2 a	14.0 *a

a, b, c, d: The same letters within each column are not significantly different (n = 3, $p < 0.05$).
Means with an asterisk at the same storage time are significantly different (n = 3, $p < 0.05$). RSD ≤ 3%.
[a] Temperature difference between T_{on} and T_{off}.

The cooling thermal properties of $EVOO_p$ exhibited slight changes during storage. In particular, T_{on} shifted significantly towards a higher temperature after 2 weeks at 60°C, which also increased the range of transition. In contrast, $EVOO_{p0}$ exhibited more marked changes upon cooling, especially at the longest storage time, when oxidative changes were more evident (Table 1). In particular, T_{on} and T_{off} shifted towards higher and lower temperatures, respectively, and the range of transition significantly increased, becoming greater than in $EVOO_p$. These changes in thermal properties were related to the inhibition of TAG crystallization by molecules formed from lipid oxidation, and have already been observed in EVOO after thermal oxidation by microwave treatment (CHIAVARO *et al.*, 2009b).

Deconvolution analysis of cooling transition

Deconvolution of overlapping transitions has been previously applied to DSC cooling thermograms obtained for EVOO to better describe the complex nature of the crystallization process (CHIAVARO *et al.*, 2007) and to evaluate the ability of DSC to discriminate commercial categories of olive oil and/or identify oleic sunflower oil as an adulterant (CHIAVARO *et al.*, 2008b and 2009a). More recently, statistical correlations among the thermal properties of the deconvoluted peaks obtained by cooling thermograms and major and minor components such as diacylglycerols have been established (CHIAVARO *et al.*, 2010). In all these previous studies, cooling transitions of EVOO were deconvoluted into three peaks that were numbered starting from the lowest to the highest temperature, identified as peaks 1, 2, and 3. The predominant peak (peak 1) was an asymmetric double Gaussian function, with a rather symmetrical curve and a narrow profile, whilst peaks 2 and 3 were asymmetric double sigmoid functions and exhibited a more complex, asymmetrical shape.

In this study, deconvolution was applied for the first time to relate changes of thermal properties and cooling profiles to lipid oxidation. All cooling thermograms fit best with three peaks ($R^2 \geq 0.98$), as previously reported for EVOO (CHIAVARO *et al.*, 2007, 2008b, 2009a, and 2010), and the thermal properties are reported for $EVOO_p$ and $EVOO_{p0}$ at different storage times (Table 3). The thermal properties of peak 1 did not significantly change after up to 3 weeks of storage for either sample. In addition, no significant differences were found between $EVOO_p$ and $EVOO_{p0}$ up to the same storage period. However, at the end of storage, $EVOO_{p0}$ showed a significantly lower area% in comparison with $EVOO_p$, as well as a marked shift of peak 1 towards lower temperature, which also exhibited a larger range of transition. The thermal properties of peak 1, which accounted for the large

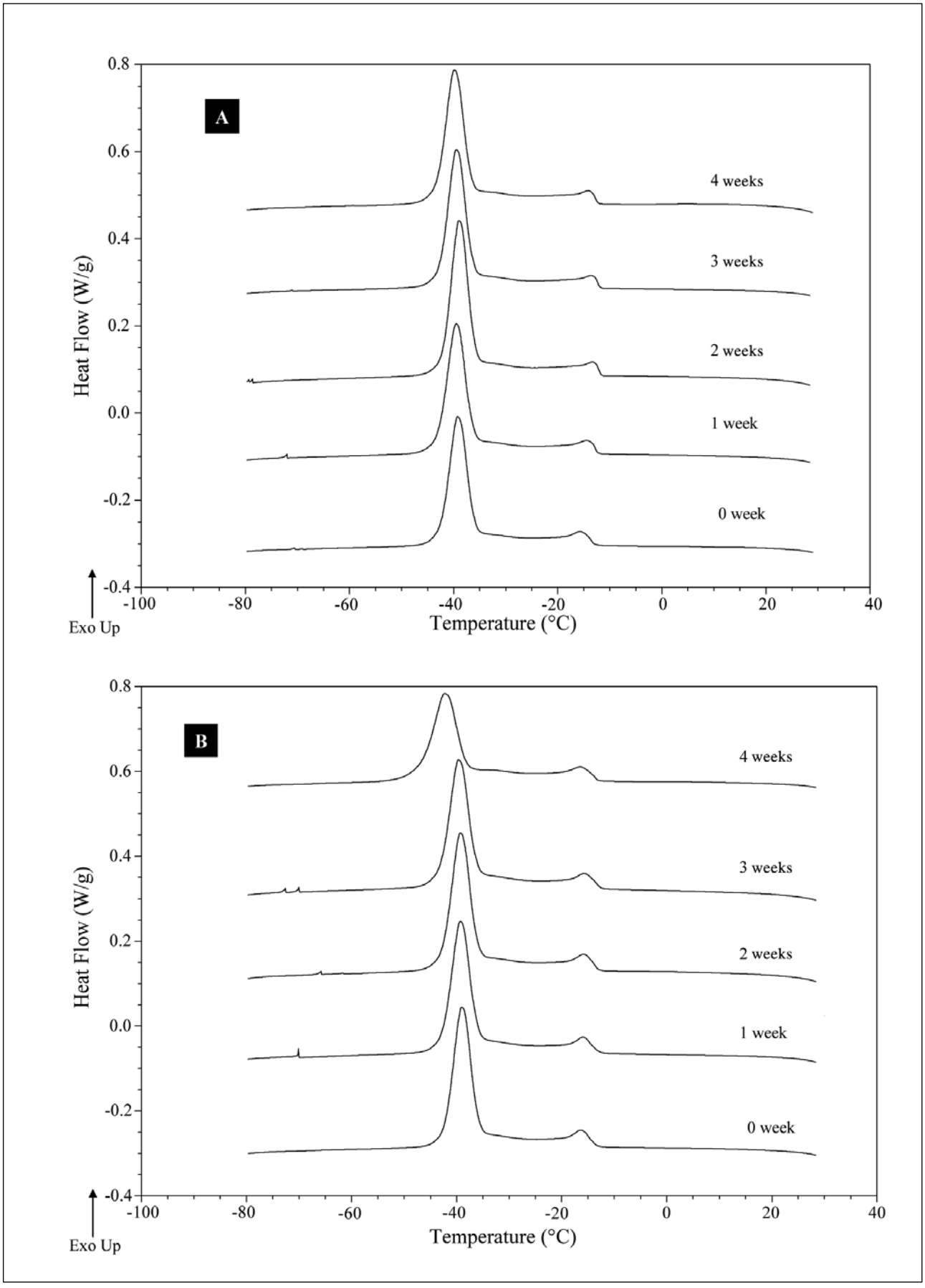

Fig. 2 - Representative DSC cooling thermograms of $EVOO_p$ (A) and $EVOO_{p0}$ (B) at different storage times.

fraction of crystallizing lipid, have been previously found to positively correlate with such major components of EVOO as OOO, oleic acid and monounsaturated fatty acid (MUFA), and negatively with linoleic acid and polyunsaturated fatty acid (PUFA) (CHIAVARO *et al.*, 2010). In addition, the thermal properties of peak 1 correlated well with the oxidative stability index (OSI) (CHIAVARO *et al.*, 2010). Thus, changes in thermal properties for peak 1 appeared to be strictly related to a larger extent of oxidation reached by the more unsaturated lipid fraction for $EVOO_{p0}$ at the end of storage (k_{232} and k_{270} values, Table 1), likely due to the absence of a protective effect by phenols. However, the thermal properties of peak 1 for $EVOO_{p}$ did not appear to be directly influenced by the decrease of phenolic compounds observed (Figure 1), and remained unchanged during storage.

The thermal properties of peak 2 did not show any significant changes during storage for $EVOO_{p}$. However, this deconvoluted peak clearly shifted towards a lower temperature for $EVOO_{p0}$ at the longest storage time compared with $EVOO_{p}$ (Table 3). A shift towards higher temperature was observed for peak 3 during storage for both samples, although more markedly for $EVOO_{p0}$ (Table 3). It has been previously hypothesized that the more complex crystallization pattern exhibited by these peaks in comparison with peak 1 may be related to the presence of minor chemical components like diacylglycerols and lipid oxidation products (CHIAVARO *et al.*, 2007). This hypothesis was recently confirmed by the high statistical correlation values found among these minor components and the thermal properties of the two peaks for EVOO (CHIAVARO *et al.*, 2010). The degree of lipid unsaturation (total fatty acid composition grouped as saturated, monounsaturated and polyunsaturated percentages) was found to clearly influence the thermal properties of these two peaks (CHIAVARO *et al.*, 2010).

Thus, it can be hypothesized that lipid oxidation products, derived from the reaction between hydroperoxides and unsaturated fatty acids (k_{232} and k_{270} values, Table 1), may have interfered with crystallization of the deconvoluted peaks 2 and 3 more markedly than for peak 1; in fact, some statistical differences were also found for $EVOO_{p}$ (where lipid oxidation was less pronounced) during longer storage times, especially for peak 3. On the other hand, the presence of phenols did not appear to directly influence the thermal properties of peaks 2 and 3, which were not significantly different between $EVOO_{p}$ and $EVOO_{p0}$ at time 0 of storage, as previously found for peak 1. Therefore, the thermal properties of peaks 2 and 3 appeared to be only indirectly influenced by phenols for $EVOO_{p}$, as their decrease probably led to a simultaneous increase in lipid oxidation products.

CONCLUSIONS

The results of this study confirm that cooling thermal properties and transition profiles of EVOO are influenced by lipid oxidation products formed by a relatively slow oxidation process that occurs during storage. Deconvolution analysis provided additional information about the relationship between lipid oxidation and cooling thermal properties. In particular, oxidized molecules appeared to influence the thermal properties of the two transitions, peaking at the highest temperatures independently of the extent of lipid oxidation related to the presence of antioxidant molecules such as phenols.

However, preliminary findings showed that the cooling thermal properties of EVOO did not seem to be influenced by phenols, although these results must be confirmed by the analysis of several oil samples with different phenolic content. Additionally, more information should be obtained to clarify the influence of both phenols and lipid oxidation products on EVOO crystallization by kinetic evaluation of this transition at different degrees of lipid oxidation and/or phenol depletion.

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Annex I. Other publications not related to the topic of this Ph.D. thesis

Here I reported the references of three papers not related to the topic of this Ph.D. thesis, but nevertheless realized during my 3-years-Ph.D. Course; they all focus on relations among objective sensory analysis, volatile compounds and consumers' preferences of different foodstuffs.

My specific contribution to these works was to review the literature, especially focusing on the most recent papers, to establish the analytical plans, to interpret the results and to write some parts of the papers, with the collaboration of the other co-authors. Since these are my first publications on sensory analysis, I would really to thank the co-authors for their supervisions and having supported and "trained" me about this new and interesting field.

Gallina Toschi, T., Barbieri, S., Valli, E., Bendini, A., Cezanne, M. L., Buchecker, K. & Canavari, M. Organic and conventional nonflavored yogurts from the Italian market: study on sensory profiles and consumer acceptability". Journal of the Science of Food and Agriculture, in press.

Bendini, A., Barbieri, S., Valli, E., Buchecker, K., Canavari, M. & Gallina Toschi, T. 2011. Quality evaluation of cold pressed sunflower oils by sensory and chemical analysis. European Journal of Lipid Science and Technology, 113, 1375-1384.

Vallverdú-Queralt, A., Bendini, A., Tesini, F., Valli, E., Lamuela- Raventós R.M. & Gallina Toschi, T. Chemical and sensory analysis of commercial tomato juices present on Italian and Spanish market. Journal of Agricultural and Food Chemistry, in press.

4. CONCLUSIONS

A full chemical and sensorial analytical plan, that has been carried out on different sets of samples, allowed me to have interesting information about the degree of quality of such a precious and healthy (*paragraph 2.8*) product as EVOO, mostly within the Italian market (*chapters 1* and *2*, see paper "Sensory and chemical quality..."). Here the range of quality of EVOOs was very wide, in terms of sensory attributes, price class and chemical parameters. Within this Ph.D. 3-year-project I studied and developed different analytical - both legal (according to European Union law) and so-called "unofficial" - parameters related to the quality and genuineness of the different commercial categories of oils obtained by olives, focusing especially on EVOOs (*chapters 1* and *2*).

In particular, I evaluated the content in fatty acid alkyl esters, a new official quality parameter recently adopted by the European Union, in those products that are sold and labelled as EVOOs, and also on other oils obtained by olives (*paragraph 3.1.13.2*). Moreover, I investigated the degree of freshness of many EVOOs, carrying out the determination of 1,2- and 1,3-diacylglycerols (*paragraph 3.1.3.2*). To ensure freshness, which is crucial for EVOOs, it seems important to have official (recognized by EU Regulations) analytical parameters (particularly the ratio between 1,2- and 1,3- DAG) that can define it (*chapter 2*, see paper "Sensory and chemical quality..."). Also the ratio between ethanol and (*E*)-2-hexenal was confirmed to be a valid analytical parameter, especially to distinguish genuine products (*paragraph 3.1.14*). I tested and applied innovative, faster, cheaper and more environmentally friendly analytical methods, in order to replace, confirm or simply add values to the official ones and/or to improve them (*paragraphs 3.1.10*, *3.1.15* and *chapter 2*, see paper "Detection of low-quality...").

I obtained interesting results regarding some new technological systems that are able to improve the effect of a particular process on the quality of the product, such as the clarification of olive oils by using inert gas (*chapter 3*) as an alternative approach with respect to traditional filtration.

Beside the production of olive oils, I also investigated the shelf-life of the product, after a simulation of its transport to a foreign country (*chapter 5*), up to its use for domestic/industrial cooking or, in general, when oil is subjected to thermal stresses (*chapter 6*). Such aspects are also very important, since the quality of a good product can be strongly (and negatively) affected by bad conditions of preservation before its

consumption by consumers. Considering the consumers' role as "main actors" of the food chain, I also investigated the sensorial perception of such a product, in terms of both objective sensory attributes and consumers' overall liking (*paragraph 2.8* and *chapter 4*). During this study the positive sensory attributes of EVOOs were not completely understood and appreciated (accepted) by consumers. Basically consumers rejected very bitter and pungent oils and, in general, they considered the peculiar bitter and pungent of EVOOs as unpleasant and not positive attributes. Actually, for a correct perception of the overall quality, including health aspects etc., positive attributes should be accepted - or better perceived - by consumers as "healthy" indicators of quality and genuine taste, linked to its richness in pungent and bitter minor components (especially phenolic compounds).

During the realization of this Ph.D. thesis I carried out other investigations focused on the relation among objective sensory analysis, volatile compounds and consumers' preferences, even if they were related to other foodstuffs different from olive oils (*annex I*): I was really interested in them, and I would like to improve my knowledge in this field with further investigations.

In general, the overall results indicate that progresses were done, but that there is still a lot to do to improve the quality of the oils obtained by olives that are sold in the market and to make consumers able to do a right and informed choice when buying and tasting them.

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Enrico