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OLIVE ZONING: ANALYSIS OF ENVIRONMENTAL INFLUENCES ON MONOVARIETAL OLIVE OILS (BOSANA CULTIVAR)

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Omnis tamen arboris cultus simplicior quam vinearum est longeque ex omnibus stirpibus minorem inpensam desiderat olea, quae prima omnium arborum est

De Re Rustica
Lucio Giunio Moderato Columella
libro V/8-1-3

SUMMARY

ABSTRACT

The characterization and differentiation of olive oil high-quality production play a key role in ensuring adequate levels of income for workers in the sector and to reward the merchandise, sensory, and health quality aspects. Furthermore, the development and implementation of new tools and methodologies is more and more required by the European Community in a framework of food security.

In this context, the main aim of the thesis is to explore the relationships between the environment factors and the virgin olive oils (EVOO) composition and microbiological quality in the double perspective of characterization and food safety, in order to increase and disseminate knowledge among consumers and operators in the olive oil chain.

The EVOO productions deriving from the most widespread cultivar in Sardinia (Italy) were characterized and differentiated in terms of chemical components on the basis of their growing area, and the influence of environmental factors on oil quality was explored through NMR spectroscopy integrated with multivariate statistic and Geographic Information System. Then, microbial species were isolated, selected, and identified in EVOO products.

The results (i) showed the potential of the proposed methodology for the construction of flexible and scalable chemometric models suitable to be applied for the zoning and protection of valuable products; and (ii) encouraged more studies and researches to clarify the role played by microbiological components.

INTRODUCTION

ECONOMIC FRAMEWORK AND STRATEGIES FOR THE ENHANCEMENT OF OLIVE OIL PRODUCTIONS

Internationally, the olive oil sector is going through a phase of expansion concerning both consumption and productions. This is clear observing the increasing trend in the world area under olive trees for the period 1967-2007, ranging from 3 to 9.2 million of hectares (FAO, 2015). The highest rates of increase were recorded until the mid-eighties when the surfaces under olive trees reached 7 million of hectares; subsequently, the increase rate was less marked (+ 1.5% *per annum* on average for the period 1997-2007) (Madau, 2010).

The production of virgin olive oils (hereafter EVOO), due to the expansion of areas planted with olive trees, increased considerably from 1.35 million tons recorded in 1967 to 3.5 million tons in 2012 (FAO, 2015). These figures indicate an increase in productivity in the sector, in particular since the mid-nineties onwards coinciding with the entry into full production of the new olive groves, planted with great pace until the mid-eighties in particular in Spain. An important feature of the produced quantities at the global level is the marked variability in volumes due to the still great importance of traditional plants, conducted without the use of irrigation, highly susceptible to fluctuations due to thermo-pluviometric patterns.

In this framework, since the mid-nineties, Italy represented the leading producing country, together with Spain. Then, Spain experienced an exponential growth of EVOO products going from 336,000 tons in 1995 to 1.56 million tons in 2012. In the same period Italy has held, in absolute terms, almost unchanged their production levels (630,000 and

570,000 tons respectively) (FAO, 2015), however seeing inevitably eroded its contribution to the world market.

Concerning the year 2012, Greece ranked as the third largest world producer after Spain and Italy, with 351,000 tons, followed by Turkey (206,000) and Syria (194,000). In the same period, it is noteworthy the growing contribution of non-Mediterranean countries, in particular Argentina with 30,000 tons but also Chile (9,000), USA (8,000) and Australia (7,500) (FAO, 2015).

As regard the period 2011-2012 in Italy, olive growing covers 1.1 million hectares that are the 58% of the total Italian area under permanent woody crops. The traditional growing areas are in southern and central Italy: Puglia (34 %), Calabria (16.5%), Sicily (12.5%), Tuscany (8.1%), Campania (6.5%), Lazio (6.1%), Abruzzo (3.7%), and Sardinia (3.3%) (ISTAT, 2014).

The important point of the Italian olive growing is the variability of the obtained product, due to the many available cultivar and different climatic conditions. From these factors, it follows the diversification of the products that allows different choices according to the tastes and job opportunities.

Given the market conditions and the aim of enhancing the sector reaching satisfactory levels of income for the various stakeholders along the production chain, different strategies are being consolidated. The first option tries to act on the qualitative differentiation of valuable product through their characterization, with the aim to increase the price of placement of the productions. A second one seeks to influence the market through the by promoting lower production costs while being attentive to cultivation models characterized by high mechanization to enhance and exploit the intensive cultivation system and/or introduce the super-intensive model. Both strategies have their validity concerning the production contexts, but it is also of utmost importance the choice of the various companies about the desired range of production and market placement

Although the present study does not address the issue of intensification of cultivations, it seems important to mention the super-intensive model. In the framework of the intensive cultivation, the primary objective to be achieved is the reduction of production costs and the enhancement of the income. The intensive plantings, based on 400 to 600 plants/ha with vase shape (classic, polyconic) in particular cultivation contexts allow to achieve positive economic results since it allows complete freedom of choice concerning the varieties to be planted. This is especially true when the aim is to get a “niche” product regarding sensory, nutritional and/or health qualitative characteristics and benefits. This market sector offers more profit compared to the traditional EVOO market (Caruso, 2012).

Furthermore, the super-intensive cultivation allows a substantial decrease of production costs. The super-intensive system is based on 1,600-2,000 plants/ha grown on a central stem that, due to the small distance among the trees (no more than 1.5 m along the row), gives rise to an espalier vegetation. It allows the harvesting in continuous modern processes using over-the-row harvesting machinery (Rallo and Muňoz-Diez, 2010; Caruso, 2012). One of the biggest limitation of the super-intensive model is that only a few varieties can be used (the Spanish Arbequina and Arbosana, and the Greek Koroneki, characterized by a low vigor). To overcome this limit, several research projects are ongoing to identify and describe other suitable varieties. For example, several programs are evaluating the adaptability of the olive varietal heritage in Italy (Camposeo and Godini, 2010), while Spanish research groups are focusing on the genetic enhancement and the development of suitable cultivars, as such Sikitita® variety, created and patented by the University of Cordoba (Romero et al., 2012).

Currently, given the lack of renewal of the olive tree plantation, the Italian strategy aims to focus and build on the relevant characteristics of olive oils. In this framework, continuous and substantial progress were made to improve the cultivation techniques, enhance the productions and reduce the costs. Particular attention is paid to the harvesting problems, also addressing the difficulties arising from uneven-aged, irregular planting distances, with trees morphologically heterogeneous, and with a mix of different cultivars. Until these kinds of olive groves can provide good incomes, relying on customers willing to pay a higher price, we can also enjoy the beauty that they give to particular landscapes. Although, very soon the Italian olive growers must deal with a market that tends to favor a competitive balance quality/price. Then Italy will have to ensure the essential, rigid protection from any fraud and unfair trade (Scaramuzzi, 2010).

COMPOSITION AND CONCEPTS OF QUALITY OF VIRGIN OLIVE OILS

The olive oil has an articulated chemical composition, due to the different nature of its constituents, from which the high nutritional value of the product depends on. The components consist of two components, saponifiable and unsaponifiable fractions.

Saponifiable fraction

The saponifiable fraction consists of the 98% of the oil chemical composition and comprises a mixture of triglycerides, partial glycerides, esters of fatty acids or free fatty acids and phosphatides. The fatty acids are divided into saturated and unsaturated depending to the double bonds in their chain. The fatty acids most represented are the monounsaturated, with a clear prevalence oleic acid (on average about the 75%); among the saturates fat, the palmitic and considerable quantity of the polyunsaturated (mainly linoleic and linolenic acid) (Aparicio and Aparicio-Ruiz, 2000) (Table 1).

Tab. 1: Fatty Acid Composition (Boskou et al., 2006)

Fatty Acid		Codex Alimentarius (2003)	IOOC*(2003)
lauric	C12:0	Not present in discernible amounts	Not specified
myristic	C14:0	< 0.1	< 0.05
palmitic	C16:0	7.5-20.0	7.5-20.0
palmitoleic	C16:1	0.3-3.5	0.3-3.5
heptadecanoic	C17:0	< 0.5	≤ 0.3
heptadecenoic	C17:1	< 0.6	≤ 0.3
stearic	C18:0	0.5-5.0	0.5-5.0
oleic	C18:1	55.0-83.0	55.0-83.0
linoleic	C18:2	3.5-21.0	3.5-21.0
linolenic	C18:3	**	≤ 1.0
arachidic	C20:0	0.8	≤ 0.6
eicosenoic	C20:1	Not specified	≤ 0.4
behenic	C22:0	< 0.3	≤ 0.2***
erucic	C22:1	Not present in discernible amounts	
lignoceric	C24:0	< 1.0	≤ 0.2

The glycerides are largely represented in the form of triglycerides and partial glycerides, such as mono- and diglycerides. These compounds represent the 90% of the total glycerides. The triacylglycerols found in significant proportions in olive oil are OOO (40-59%), POO (12-20%), OOL (12.5-20%), POL (5.5-7%) and SOO (3.7%) (Boskou, 1996). Other saponifiable minor components, such as waxes, phospholipids and sphingolipids are present in small quantities.

Unsaponifiable fraction

The unsaponifiable fraction is the component that distinguishes the virgin olive oil from other vegetable oils. There are approximately 230 substances, said minor compounds which, although they are present in minimal amounts (0.5-2%), affect in a decisive way the various quality aspects related to the olive oil composition. They represent a sort of fingerprint that could be used as a valuable analytical matrix for both the control of authenticity of the product from an anti-fraud standpoint and the characterizations of high-quality productions.

In the context of minor compounds, several chemical classes can be distinguished: hydrocarbons, higher and terpenic aliphatic alcohols, sterols, polyphenols, pigments, and volatile compounds.

Squalene is one of the most important hydrocarbons, is a polyunsaturated triterpene comprising of six isoprene units, and acts as a biochemical precursor of cholesterol and other steroids. There is another group of compounds present in the unsaponifiable fraction of olive oil called triterpene alcohols, which are co-chromatographed with 4-desmethylsterols (Boskou, 2006); among them, the cycloartenol and 24-metilencicloartenol are the most important. Plant sterols, also called phytosterols, include a major proportion of the unsaponifiables in vegetable oils. Total phytosterols content in olive oil varies between 1000 and 2300 ppm (Benitez-Sanchez et al., 2003), and according to IOOC (2011) the amount of desmethylsterol components such as apparent β-sitosterol in olive oil (% total sterols) is ≥ 93.0%. Boskou (2006) highlighted that factors such as cultivar, crop year, degree of fruit ripeness, as well as storage time of fruits before oil extraction and the extraction method itself can affect sterol composition and content of olive oil. Plant phenolic compounds are found exclusively in EVOO and are well known plant secondary metabolites belonging to the hydrophilic group (Tuck and Hayball, 2002). The phenolic composition of olive and olive oil and the average concentration of these compounds depends on several factors including maturation stage, part of the fruit, variety, season, and climatologic conditions (Boskou et al., 2005). In addition, these compounds are originated from phenol glucosides, oleuropein, dimetiloleuropein and ligstroside during the process of mechanical extraction.

Virgin oil contains phenyl acids, phenyl-alcohols, and various derivatives of secoiridoids originated in result of the activity of β-glucosidase of the fruit; in addition to the secoiridoids, the phenolic hydrophilic compounds more concentrated in EVOO are the lignans, pinoresinol, and acetoxy-pinoresinol (Servili et al., 2010).

Volatile compounds, retained by virgin olive oils during their extraction process, are responsible for the oil aroma. Approximately one hundred and eighty compounds, whose structure was assigned by means of gas chromatography-mass spectrometry, were found in virgin olive oil aromas (Angerosa, 2002). It is important to highlight that the Analysis of volatile components can be used as an indicator to check the quality of olive oil (Angerosa, 2002). Furthermore, the volatile composition of olive oil is influenced by cultivar, geographic region, fruit maturity, processing methods (Baccaouri et al., 2008).

Chemical composition and quality

The various aspects of merchandise, sensory and healthy quality that characterise virgin olive oils are closely linked to the chemical composition. The quality of the product is related to commercial classification aspects and is regulated by Reg. 2568/91 (as amended) guaranteeing about the genuineness of the product. The main parameters considered are acidity, number of peroxides, spectrophotometric parameters, Panel test.

An important factor within the sensory quality context is the visual part, linked to the color that affects the pigment content; on the other hand, the olfactory sensory component depends on the volatile compounds and many studies highlight the relationship between the different compounds of low molecular weight (C_5, C_6) and various hints (Kalua et al., 2007). From a taste point of view, despite the acidic composition can influence the flow of virgin olive oil, a major role is played by different hydrophilic phenolic compounds as responsible of spicy and bitter hints (Servili et al., 2004).

The healthy quality of virgin oils is widely attested by numerous researches, due both to the exceptional fatty acid composition with high content of oleic acid (18:1; ω9), linoleic (18:2; ω6) and linolenic acid (18:3; ω3) (Simopoulos, 1999), and to the healthy characteristics of the unsaponifiable fraction, in particular polyphenols (Visioli and Galli, 1998).

For example, Ghanbari et al. (2012) pointed out the potential antioxidants power of biophenol compounds that play an important role in the chemical, organoleptic and nutritional properties of the virgin olive oil (VOO) and the table olives.

Microbial activity

Despite the importance of the naturally occurring microorganisms in olive oil (Ciafardini et al., 2006), studies are limited and do not give a complete picture. Ciafardini and Zullo (2002a and 2002b) examined olive oils produced in central Italy and conducted microbiological analysis for aerobic and lactic acid bacteria, yeasts, and molds.

This is due both to oil physical state and because it is not a fermented food; on the other hand, olives are rich in microorganisms that, during the extraction process can get the oil and be persistent on it. Indeed, the freshly produced olive oil contains small amounts of water, which amount depends upon the processing methods, naturally emulsified in the oil. Koidis et al., (2008) observed that microorganisms were trapped in the water droplets in the freshly produced oils. In addition, the small size of the water droplet (from 1 to 5 μm) limits nutrient availability and “space” for microflora.

Among the positive impacts, it is possible to cite the enzymatic activities, particularly β-glucosidase and esterase. These activities allow hydrolyzing the glycoside oleuropein, which gives the bitter taste to olive oil. The activity of these two enzymes, hydrolyzing oleuropein, reduces the bitter taste of the oil and improves the sensory features (Ciafardini and Zullo, 2002).

UE, FOOD SECURITY AND OLIVE OILS

Ensuring the authenticity of food is a high-profile issue. Wherever there is a commodity that commands a premium in the market and has either high value or high-volume sales, such as the EVOOs, some people may be tempted to profit from illegal activity. On the other hand, offering EVOO to the consumers means to guarantee the EVOO characteristics, defending and protecting the consumers from frauds, defects and alterations of the product.

Food fraud usually involves misleading the consumer about the real nature, origin and provenance, substance and quality of the goods purchased; thus, food standards and labeling are infringed. The fraud can take the form of adulteration, counterfeiting, or sophistication.

• **Adulteration** is the EVOO component percentage variation without altering the nature of the product, by addition or by subtraction of an active substance. Adulteration happens, for example, deodorizing and deacidification oil to remove the taste and odor, and lowering the high acidity. The grinding processes stronger also change the composition of the oil, altering the general configuration.

• **Counterfeiting** consists in replacing the product entirely with another product of lesser value (seed oil sold for olive oil), or falsifying statements on the label of Appellations of Protected Origin (DOP) and Protected Geographical Indication (IPG) of olive oil.

• **Sophistication** consists in the addition of products of lesser quality in the olive oil. The addition of hazelnut, seeds, or synthesis oils is just an example.

The European Union is giving more and more attention to food security. Before the Mc Sharry reform (1992), this concept seemed inherently tied to the original objective of the Common Agricultural Policy (CAP) of supply security, meant as quantity. After this, it has extended to the qualitative protection of producers and consumers. In the framework of the EU programme for research and innovation Horizon 2020, the theme of Sustainable Food Security is promoted and supported as part of the Societal Challenge 2 on "Food security, sustainable agriculture and forestry, marine and maritime and inland water research and the Bioeconomy".

The EVOO sector appears to be a case in point in the context of food security, and it is one of the sectors in which the EU reserves increased efforts. This renewed interest is because, at the global level, the EU is at the same time the largest producer, consumer, and exporter of EVOO. EVOO authenticity problems can create harm in the marketplace. EVOO is commercialized at higher prices than other vegetal oils; thus it is exposed to numerous attempts of fraud, adulteration, and sophistications. Future sales of a product may be damaged if the consumers believe that the "quality" product is not worth the extra cost. So that, one of the EU priorities is the protection of the image of the product and to be able of ensuring quality and authenticity.

The EU has proposed measures to combat counterfeit oils under the Regulation n° 2568/91 of 1991, which focused on the characteristics of olive oil and the analysis methods. The regulation aims to guarantee the quality and authenticity of olive oil by describing the methods to be used to examine a number of physical, chemical and organoleptic characteristics.

The listed characteristics are updated according to advances in scientific knowledge. Despite these regular reviews, some issues have to find adequate solutions. In particular, it is necessary the development, validation and standardization of a method for the assessment of compositional characteristics and quality parameters addressing authenticity problems of EVOO. The specific challenges addressed by EU are, for example, the blend of extra-virgin olive oil or virgin olive oil with soft deodorized olive oil and the blend of extra-virgin olive oil or virgin olive oil with other vegetable oil.

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AIMS OF THE THESIS

The virgin olive oils represent an excellence at European, National and Regional level with high economic value due to their compositional characteristics, closely connected to the product's commercial, organoleptic and health-related aspects. The scientific community is called to provide tools to differentiate and distinguish valued productions in order to increase selling prices toward an adequate remuneration of the farmers. This can be achieved through the multidisciplinary works aimed to characterize qualitatively the varieties and the areas of production with respect to the chemical and microbiological composition.

Furthermore, the previously mentioned quality and value of the productions exposed the olive oils to numerous fraud attempts, adulteration, and sophistication. In this regards, and also from a food security point of view at European level, there is a high interest in promoting researches that may allow the development of methods and instruments for the protection of consumers.

The main aim of the present thesis is to explore the relationships between the environment factors and the virgin olive oils (EVOO) composition and microbiological quality in the double perspective of characterization and food safety, in order to increase and disseminate knowledge among consumers and among the operators in the olive oil chain. The thesis is structured in three chapters having specific objectives:

Experiment 1: to show that mono-cultivar EVOO deriving from different production areas in Sardinia (Italy) are differentiable from the composition point of view;

Experiment 2: to investigate and classify the environmental effects on oil quality in North-Central Sardinia (Italy), an ideal research context due to the presence of areas characterized by different soil types and a predominant olive cultivar (Bosana);

Experiment 3: to isolate, select, and identify microbial species present during the production process (olives, olive paste, and oils) of EVOO produced in Sardinia (Italy) from varieties belonging to Bosana, Semidana, Nera di Gonnos, and Nocellara del Belice.

EXPERIMENT 1 - CHARACTERIZATION OF SARDINIAN VIRGIN OLIVE OILS USING 1H NMR METABOLOMIC

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ABSTRACT

Extra Virgin Olive Oil (EVOO) composition is responsible for the sensory characteristic and quality of virgin olive oil. Fat composition, aldehydes, sterols and other compounds characterize cultivars and origin. The study has been carried out using Bosana cultivar extra-virgin olive oil samples collected from different areas of Sardinia (Italy) during the 2010-2011 harvest. High-resolution 1H NMR spectroscopy has been used to analyze 100 (82 training + 18 test sets) samples of extra virgin olive oil aiming to characterize the pattern components that define each oil and its relationship with growing area. The final goal is to obtain a protocol to recognize the composition of various Sardinian oils and correlate them with production areas. The data have been processed by multivariate statistical analysis, the results confirm that the evaluation of the profile of the "monovarietal olive oil" allows us to explain the influence of the complex environmental factors of every cultivation zone.

INTRODUCTION

Olive production is one of the main agricultural activities for countries of Mediterranean area, with important environmental, social and economic impacts. The product classification coded by international regulations that define olive oil genuineness and quality grade (extra virgin, virgin, lamp etc.) is based on free fatty acid content, aroma and taste, peroxide number and spectrophotometric constants (EC, Reg. 2568/1991 and subsequent).

The nature and quality of the EVOO, obtained only by mechanical press of the olive fruits at low temperature, results from the composite effect of a number of factors such as cultivars, environmental conditions and agricultural practice (El Riachy et al., 2011 and references therein). Nonetheless, it is now recognised that the identification of chemical components in natural foodstuff can provide markers for quality assessment and for the evaluation of its geographical origin (Mannina et al., 2001). Hence, a careful classification of quality, genuineness and geographical origin of food products must consider, as discriminating factors, not only major chemical parameters and sensory attributes but also their "minor components" unique pattern.

Several studies were directed to characterize extra virgin olive oils produced from cultivars of various geographical origins (Mincione et al., 1996). In a number of cases, the studies have been carried out using multivariate statistics applied to chemical parameters obtained by means of analytical techniques (e.g. Chromatography and Mass Spectrometry) (Armanino et al., 1989; Boschelle et al., 1994).

Following the approval of the EC directives concerning the Protected Denomination of Origin (PDO) of olive oils, priority was given in determining parameters able to discriminate olive oils originating from specific geographical areas in the Mediterranean basin (Aparicio et al., 1994; Aparicio et al., 1997; Dugo et al., 2000; Boccia et al., 2002).

High - resolution NMR spectroscopy has been successfully applied in several studies for different purposes (Sacchi et al., 1996; Scano et al., 1999; Bentivenga et al., 2003). Important results in geographical classification of Italian olive oils were obtained analysing the NMR data with chemometric techniques. In fact, it has been possible to discriminate between olive oils produced using various cultivars from different Italian regions (Tuscany, Sicily, Liguria, Apulia and Garda Lake area) (Sacco et al., 2000; Mannina et al., 2001; Del Coco et al., 2014; Karkoula et al., 2014; Popescu et al., 2015).

Promising results have been generated while attempting to identify the criteria to define the PDO for specific areas of Tuscany (Mannina et al., 2001). In another paper 13C NMR spectroscopy was combined with gas chromatography and multivariate statistical procedures succeeded to reveal the existence of a relationship between the fatty acid composition of olive oils obtained from several Sicilian cultivars and the site of production in Sicily (Mannina et al., 2003).

Composition of olive oil vary and mainly depends on the olive cultivar, stage of fruit maturation and the location in conjunction with agro-climatic conditions where olive trees are grown (Prenzler et al., 2002)

Finally, analysis has shown that the presence of the polyphenols in monovarietal olive oils may be a promising criterion to recognize the type of cultivar and the methodology (pressure or centrifugation) used for oil production (Vacca et al., 1993; Cecchi et al., 2011).

Overall, these studies have evidenced that each cultivar is characterized not only by a defined content of triacylglycerol and free fatty acids but also by a unique blend of compounds commonly identified as "minor components". As a result, a detailed knowledge of the modifications of the pattern of those compounds should allow to precisely certifying the geographical origin of monovarietal olive oils. It is evident that the use of this analytical protocol, if applied at industrial level, may produce a significant economic impact.

The study has been carried out on Sardinian monovarietal Bosana olive oils aiming at finding their origin, on the basis of fatty acids content (Scano et al., 2001). The combination of gas chromatography and statistical multivariate techniques allowed to discriminate between olive oils produced in the Northern and Central areas of Sardinia from the ones produced in the Southern and Eastern areas, but it failed to show any difference between the oils produced in the last two areas in spite of the fact these regions are quite different from a climatic and orographic point of view.

Based on these findings and recognizing that the quality of a genuine extra-virgin olive oil results not only from a balanced blend of triacylglycerols and free fatty acids, but also from the presence of a unique mix of minor and major components, that are responsible for its taste and flavour, a systematic study aimed at characterizing the distribution of those compounds in Sardinian cultivars was started.

In this paper, we extend this method to EVOO from Sardinia. The method allows environmental assessment and generates results at a sampling density sufficient to delineate detailed local variations in crop response.

The final goal of this study is to devise an experimental protocol based on high resolution 1H NMR, tuned for the analysis of the Sardinian extra virgin olive oils, and able to recognize the regional areas where they are cultivated. In fact, we feel this is the first crucial step for defying the Sardinian extra-virgin olive oil P.D.O. The described (Abeltino et al., 2004; Santona et al., 2014) special properties of Bosana olive oil stem directly from the morphological characteristics, soil and climate of the production areas.

MATERIAL AND METHODS

Sampling

100 samples of cultivar Bosana extra virgin olive oil, produced by local mills using olives from the harvest 2010-2011 and selected by the "AGRIS Sardegna" were collected (Table 1). In Fig. 1 the four growing areas in Sardinia, namely Sassarese (area 1), Nuorese-Baronia (2), Campidano (3) and Planargia-Montiferru (4) are shown. We added 18 samples (test set) from 2011 harvest for validation purposes. See table 1 for the distribution of samples.

Classification of growth areas

A general climatic classification of the areas was carried on by analyzing both an agroclimatic index (FAO-UNEP aridity index) and Rivas-Martinez bioclimatic classification. Climatic data series for indexes estimation were temperature and precipitation daily data of the reference period 1971-2000. Aridity index was calculated as the ratio between average climatic annual (or monthly) precipitation (P) and average potential evapotranspiration (ETo)*, and it explains for the demand of water from atmosphere and for the water available as rainfall. Based on aridity classes, an area is then classified as “arid” when average rainfall does not reach 50% of evapotranspiration from soil-plant systems (Motroni et al., 2008). Potential Evapotranspiration (ETo) was calculated with Hargreaves and Samani equation based on extraterrestrial Global Radiation These results are confirmed by the prevalent bioclimatic type of the study areas as classified by Rivas Martinez applied to Sardinian climatic conditions (Canu et al., 2015).

Tab. 1: Growth area distribution of samples.

Area/Samples	Group	Training Set	Test Set
SASSARESE	1	36	6
NUORESE-BARONIA	2	25	6
CAMPIDANO	3	9	2
PLANARGIA-MONTIFERRU	4	12	4

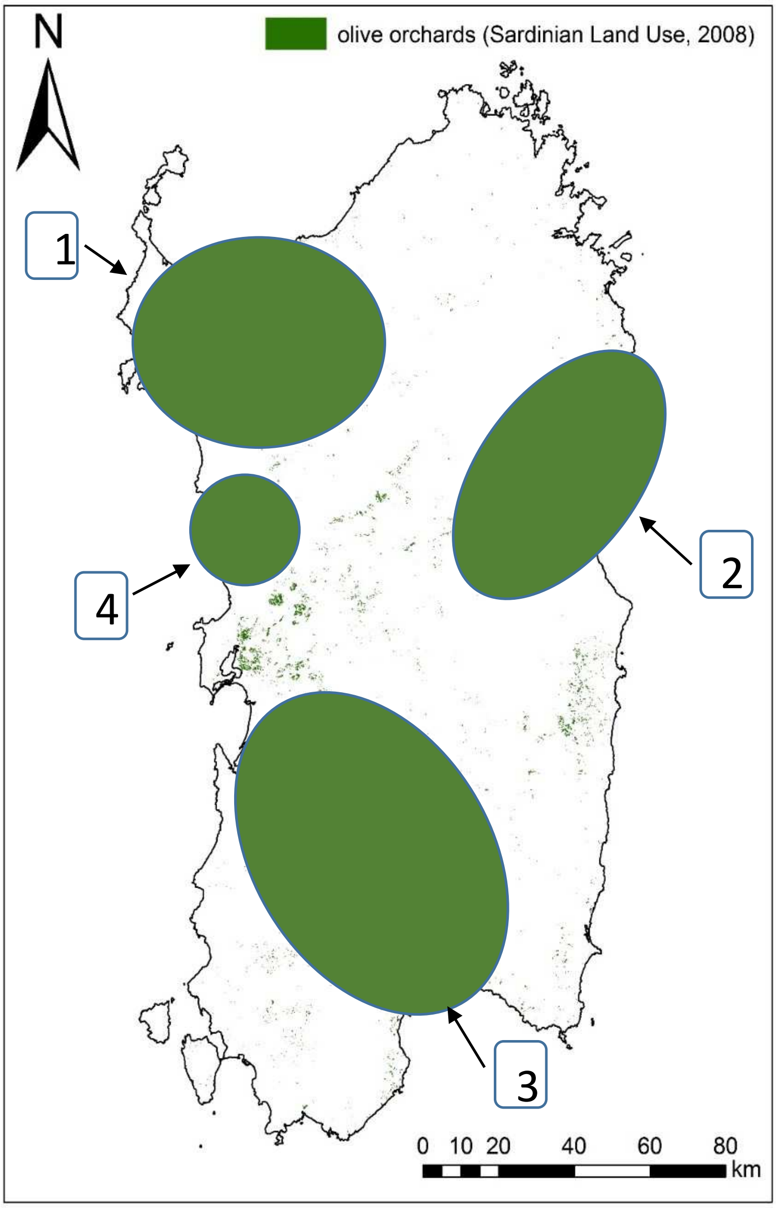

Fig. 1: Map of sampling areas and olive orchards distribution.

NMR analysis

For the NMR experiments each sample was prepared following a procedure previously published (Sacchi et al., 1996). Briefly, 20 μL of olive oil was placed into a 5mm NMR tube, mixed with 700μL of chloroform-*d1* (Aldrich, USA) and 20 μL of DMSO-*d6* (Aldrich, USA). 1H-NMR spectra were recorded on a Bruker AVANCE II 600 spectrometer (Bruker, Germany) operating at 600.13 MHz for 1H resonance. 1D 1H-NMR spectra was acquired using a standard protocol of 12 ppm spectral width and 128K data point for acquisition and processing were used. Prior to applying Fourier transform (FT), the FIDs were multiplied by an exponential function corresponding to a line broadening of 0.3Hz. All 1H NMR spectra were phased and baseline corrected within TOPSIN 2.0 (Bruker GmbH, Karlsruhe, Germany) and data reduced to 245 integrated regions corresponding to the chemical shift range of 10–0.5 with a region width of 0.02 ppm using Analysis of MIXtures (AMIX) (Bruker GmbH, Karlsruhe, Germany). 461 descriptors were obtained. The most important variable were assigned on the basis of literature data (Nieva-Echevarría et al., 2014) and by direct addition of standards.

Data analysis

Prior to chemometric analysis, the data were normalized by setting the total region of each spectrum to 100 and the generated ASCII file was imported into Microsoft EXCEL for the addition of labels. The bucket matrix was imported into SIMCA-P software version 12.0, (Umetrics AB, Umeå, Sweden) for statistical analysis. The subsequent analysis was based on a data matrix of 100 rows and 461 columns.

Statistical Methods

NMR data were subjected to multivariate statistics evaluation using the Simca-p software package (Umetrics Umeå, Sweden). In particular, the Principal Component Analysis (PCA) was used only for exploratory purposes and Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) (Trygg and Wold, 2002) was applied for model building.

To prove the reliability of our models and avoid over-fitting 7-fold full cross-validation was performed as well as a permutation test on the class response, in accordance with good practice for model validation (Westerhuis et al., 2008).

The classification of a test data set using the model-parameters based on the training data set provides information about robustness and generalization of models (Cloarec et al., 2005).

RESULTS AND DISCUSSION

Climatological classification of olive areas.

The map of Sardinia with sampling areas is reported in Figure 1. Areas are geographically distinctly well separated one from the other and homogeneous inside. In table 2 the FAO-UNEP Aridity Index results for the 4 areas of oil production are shown. Dry sub-humid class is dominant in zone 1 and 2 while zone 3 is characterized by most arid conditions. zone 4 is classified mainly as sub-humid, climatic conditions not very common in Sardinia, and particularly, for olive orchards, that usually are confined to marginal lands because of the plants capability to live and produce in very dry areas.

Tab. 2: FAO-UNEP Aridity Index results are shown for the four sampling areas

CLASS	ZONE 1	ZONE 2	ZONE 3	ZONE 4
ARID	4,9	3,6	60,9	0,6
DRY SUB-HUMID	75,4	62,3	22,5	38,3
SUB-HUMID	19,7	27,3	15,2	56,5
HUMID	0,0	6,7	1,4	4,7

While zone 1 is characterized by a "Lower Mesomediterranean, from Upper dry to Lower sub-humid, Weak euoceanic" bioclimatic type, in zone 2 both "Thermomediterranean and Mesomediterranean, upper and lower dry" bioclimatic types can be found. The isobioclimate of zone 3 is distinctly "Upper Thermomediterranean, Upper dry and weak oceanic" due to the lack of precipitation and averagely high temperature. Zone 4 is equally distributed between Mesomediterranean and Thermomediterranean bioclimatic types, depending on altitude and distance from the sea.

NMR spectra

NMR spectra of olive oil have been previously described in detail (Mannina et al., 2001). Resonances were assigned on the basis of the literature data and by direct addition of standards. Some of NMR signals refer to organic compounds such as aldehydes and terpenes, that are responsible for sensorial characteristics (almond flavour, fruity), polyphenols, often responsible for bitter and pungent taste, and other alcohols such as the β-sitosterol and cycloarthenol with nutritional interest. In figure 2 NMR spectra of EVOO are shown, arrows indicating relevant metabolites in our results.

Statistical analysis using Orthogonal Projections to Latent Structures (OPLS-DA).

This method was used because it simplifies model interpretation by excluding the variation of the input data not correlated to the response data. PLS-DA models were evaluated using the goodness-of-fit parameter (R2Y) and the predictive ability parameter (Q2Y), which was calculated by a seven-round internal cross-validation of R2Y. R2Y represents the proportion of variance explained by a given component in the model, whereas Q2Y is defined as the proportion of variance in the data predictable by the PLS-DA model under cross-validation.

As an additional measure of PLS-DA model validity, permutation testing was conducted to evaluate whether the specific classification of two classes in a model was significantly better than any other random classification in two arbitrary groups (Figure 5).

The discriminant functions have been derived using the stepwise method. In this case, the analysis has been carried out on all the oils available to find out if this method is more efficient than PCA to discriminate the area of oils origin. The resulting maximum number of discriminating functions that can be obtained after performing the canonical analysis is either equal to the number of variables or to the number of groups minus one, whichever number is smaller. In figure 3 we show OPLS-DA Scores plot of 4 class discrimination model.

The reference (the zero) in a 4 class model is the average of 4 classes; this assumption leads difficulties in a clear interpretation of differences. A series of 6 OPLS-DA pairwise models of NMR data were applied to maximize the discrimination of growing areas and to focus on metabolic variation between them. In Table 3 the results are reported and in Figure 3 the related O-PLSDA score plot are shown.

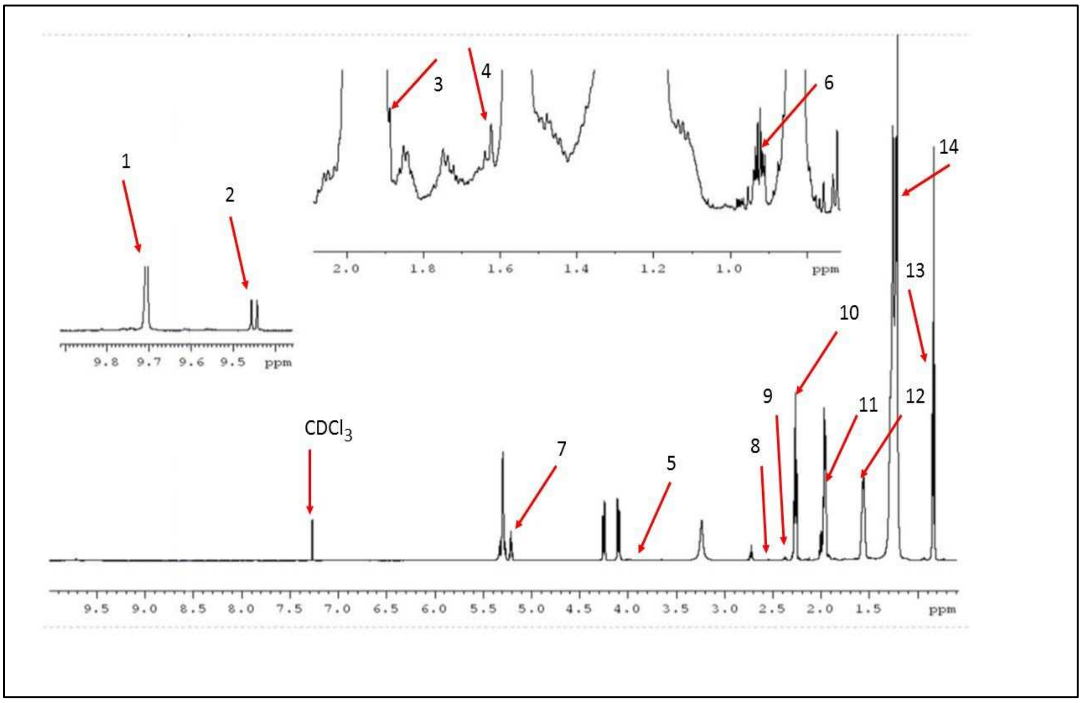

Fig. 2: NMR spectra of EVO 1 exanal; 2 exenal; 3 **CH2**-CH2-COOH; 4 Squalene; 5 1,3 DG; 6 Methyl of linolenic acid;7 CH-2 Glycerol; 8 CH2-**CH2**-COOH (free acids); 9 CH2-**CH2**-COOR (esterified); 10 **CH2**-COOH; 11 Allylic protons broad; 12 **CH2**-CH2-COOH;13 **CH3**-CH2; 14 CH3-**CH2**

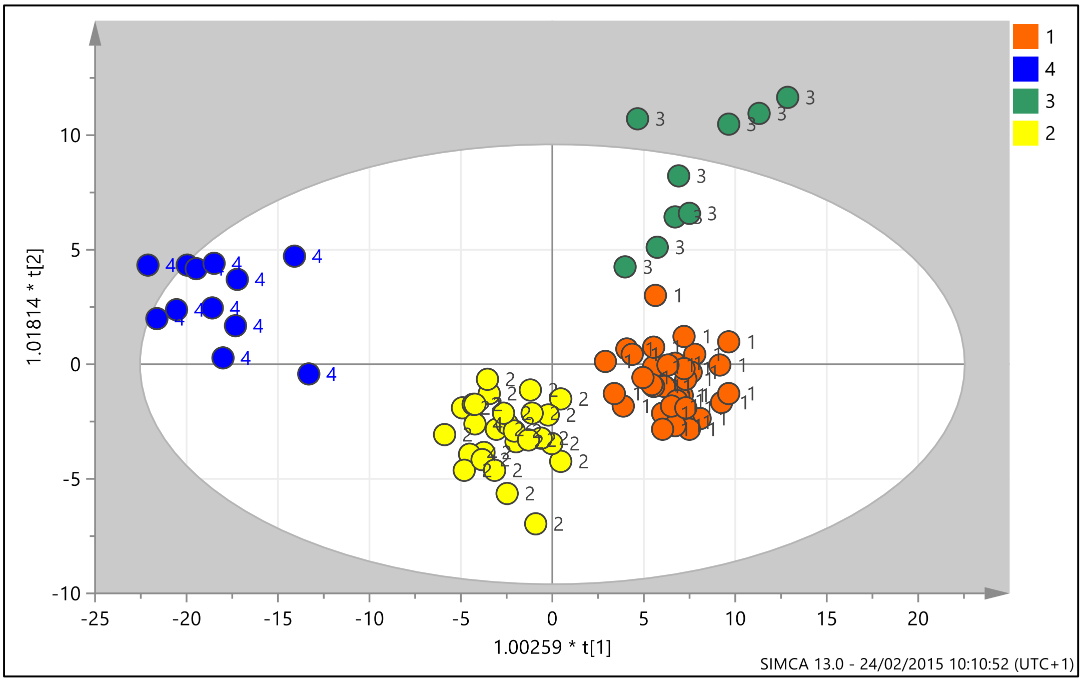

Fig. 3: OPLS-DA Plot of the Sardinian extra virgin olive oils from the 4 growing areas.

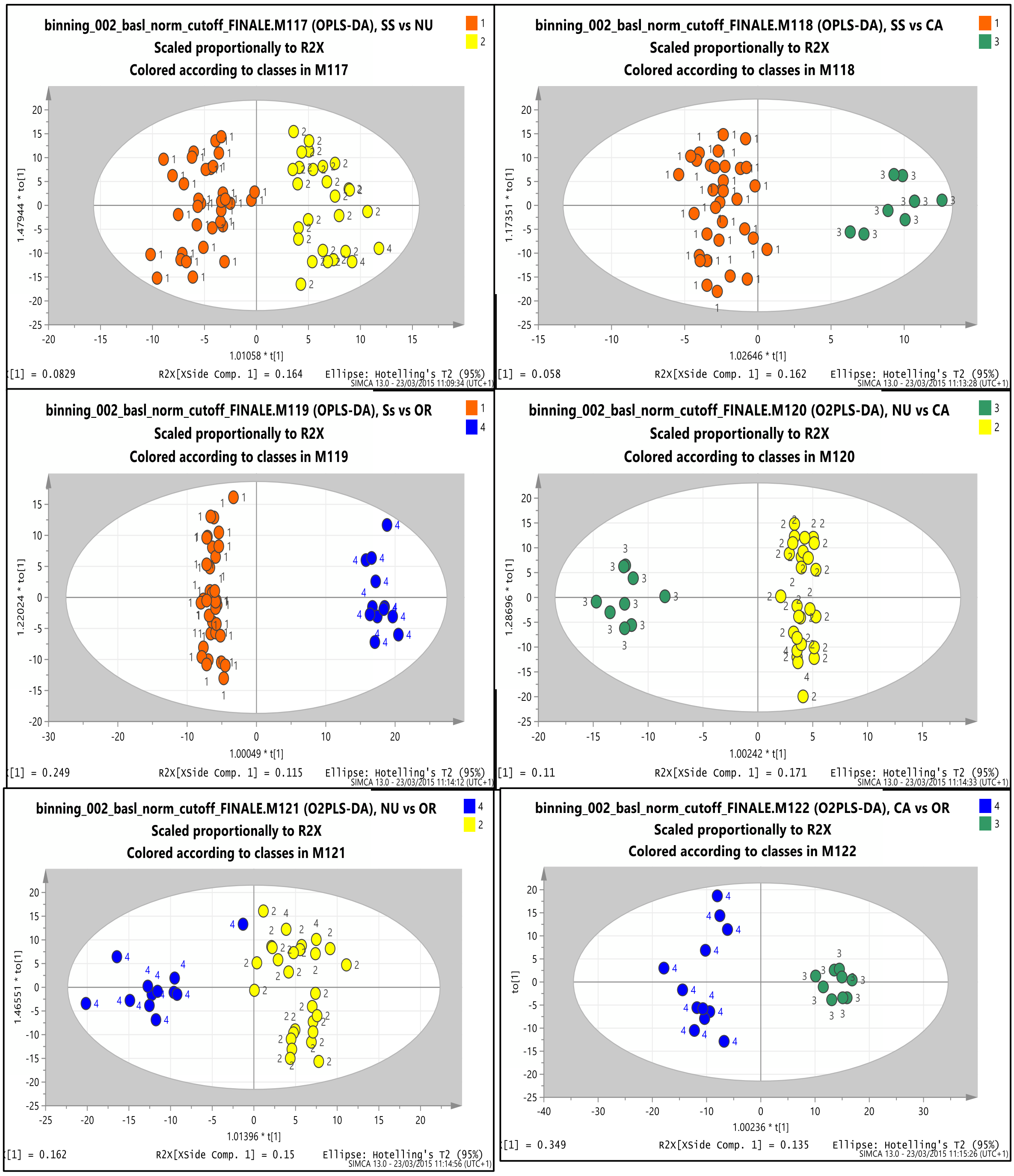

Fig. 4: Score plot of 6 pairwise models

In Table 4 a summary of the first 5 leading separation metabolites (in terms of Variable Importance) in pairwise models are presented. All models have been validated using permutation, cross validation, and external test-set.

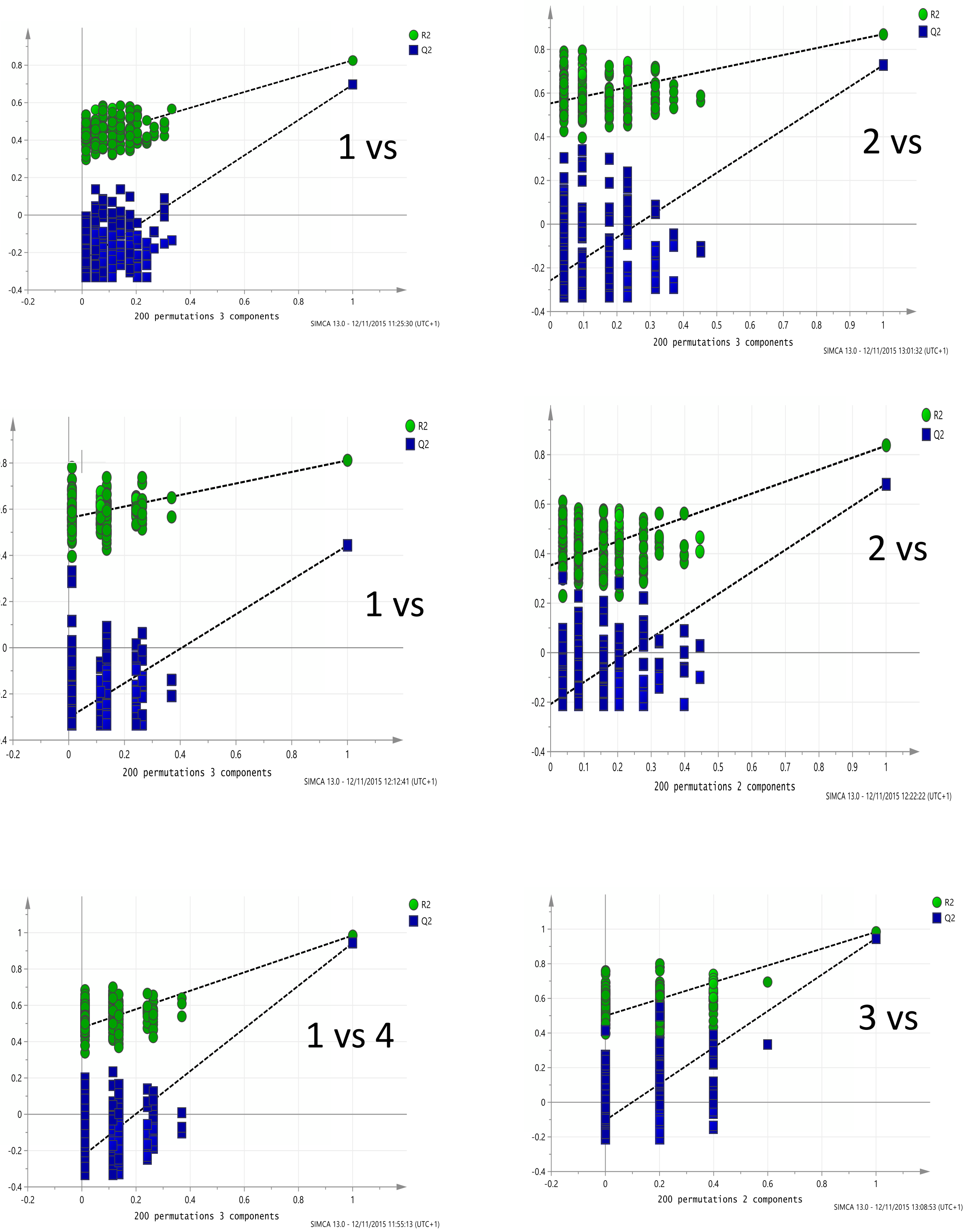

Fig. 5: Permutation test of pairwise models

Permutation test can evaluate whether the specific classification of the individuals in the designed groups is significantly better than any other random classification in arbitrary groups. In our cases, Q2 intercept became negative for each model and R2 decreased significantly (see Figure 5).

Cross validation were performed with 7-fold cross validation technique: 1/7th of original samples are kept out of model and predicted by the model, procedure are repeated until all samples are kept out once. A misclassification table was obtained for 4 class- and pairwise models (Table 3). Regarding external validation, although the levels classification are similar, test-set classifications are more variable compared to the original test data sets, due to the smaller size of the extra test data sets.

Tab. 3: Auto fit results for 4 class model and pairwise models. Cross validation and external validation results

Model	R2Y	Q2Y	Cross Valid.	Ext. Valid.
4 areas	0.834	0.567	89%	83%
1 vs 2	0.864	0.648	100%	67%
1 vs 3	0.917	0.568	100%	63%
1 vs 4	0.969	0.941	100%	80%
2 vs 3	0.978	0.692	100%	88%
2 vs 4	0.853	0.623	97%	70%
3 vs 4	0.951	0.916	100	83%

Tab. 4: **S**ummary (in terms of Variable Importance) of metabolites leading separation between 2 growth areas.

GROWTH AREA	2	3	4
1	1,3 DG; Saturated alcohols 13C satellite signal of Methylene protons of the Glyceryl group; 13C satellites of protons of Triacylglycerols ; Benzaldeide; CH2 of sn-1,3 diacylglycerols;	Formaldeide; Pentanal; (CH2)n; Exanal; CH2-O-C=O; CH3-CH2; Squalene; -(CH2)n- (acyl group); Exenal; CH2 of sn-1,2diacylglycerols;	CH3-CH2; CH3-CH2; CH2-CH2-COOH (free acids); Allylic protons broad; CH2-CH2-COOH; H2C-CH=CH-CH2; CH2-COOH; CH-2 Glycerol; CH2-CH2-COOR (esterified); Bis-allylicgroup;
2		CH3-CH2: CH3-CH2: Allylic protons (broad); CH2-COOH; Sugar moiety; CH2-CH2-COOH; CH2-CH2-COOR (esterified); CH-2 Glycerol;	CH3-CH2: CH3-CH2: H2C-CH=CH-CH2 Allylic protons (broad); CH2-COOH CH2-CH2-COOH; CH2-CH2-COOR (esterified); Sugar moiety; CH-2 Glycerol;
3			CH2 of sn-1,3 diacylglycerols; D-Glucose; H-C=C-H olefinic; CH2-O-C=O; 13C satellites of protons of triacylglycerols; Benzaldeide; saturated alcohols; Formate; Triacylglycerol; CH=HC-CH=CH;

CONCLUSIONS

Many adaptive growing and physiological mechanisms allow withstanding changes caused by environmental factors of every cultivation zone. Overall, main differences rise from Fatty Acids composition, and from sensory related molecules. These results are in accordance with the literature, where it is reported that the determination of FA and TAG compositions in combination with statistical methods of data processing, can lead to characterize EVOO oils according to their climatic classification (Longobardi et al., 2012).

Our findings indicate that differences in agro-climatic data contributes on the volatile composition of olive oil as presented in previous papers (Kiralan et al., 2012).

The data presented here are the first step towards the definition of more precise analytical profiles that can characterize the various monovarietal Sardinian oils.

It is worth mentioning that our final goal is not only to discriminate the oils produced in the Sardinian territory from all the others but also to identify the area in Sardinia where they come from.

Despite the limited number of samples, the lack of any previous knowledge about the pattern of components that characterizes each agro-climatic influence on it, results show that extra virgin olive oils produced in different growing areas of Sardinia can be recognized, simply on the base of the chemical information analytically determined by high resolution 1H NMR.

Work is now in progress to finely characterize the composition of some minor components (polyphenols) with respect to the production areas in order to define a sort of an ID card for each of them. Results already obtained evidence a good sensitivity of the method.

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EXPERIMENT 2 - NMR METABOLOMIC ANALYSIS OF ENVIRONMENTAL EFFECTS ON SARDINIAN OLIVE OIL COMPOSITION (CV. BOSANA)

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ABSTRACT

Virgin olive oil merceological, sensorial, and nutritional quality factors have been widely investigated in the last decades. While the effects of genetic, transformation related and agronomic factors on quality oil production seem clear, more uncertainties or discordant results show the influence of cultivation environment and, in particular, of soil characteristics and climatic conditions. North-central Sardinia is a monovarietal, somehow homogeneous study area where to investigate the above factors. Two-year present study was carried on traditional Bosana monovarietal olive groves, not irrigated and with tree older than 50 years, with 5 different soil types form different parent materials: limestone, basalts, trachyte, granites and alluvial. Climate classification of cultivation areas was completed by estimating agrometeorological indices. Olive ripening was monitored by applying Jaen Maturity Index; samples were taken at semi veraison maturity and transformed in a second phase mill following a standard protocol.

NMR (Nuclear Magnetic Resonance) spectral results were digitalized and O-PLS-DA (Orthogonal Projections to Latent Structures Discriminant Analysis) statistical analysis

processed showing that different types of soil significantly influence oil composition, particularly the polyphenol compounds responsible of aromatic and nutraceutical properties. NMR methodology was confirmed as an important tool for characterizing virgin olive oil and determining the influence of environmental factors on oil quality.

INTRODUCTION

The key factors influencing the composition and the commercial, sensorial, and nutritional quality of virgin olive oils have been widely studied (e.g., Romani et al., 1999; Kevin et al., 2003; Vinha et al., 2005). Scientific studies have investigated mostly genetic effects on cultivar, as well as technological and agronomic factors (e.g., Caponio et al., 2001; Patumi et al., 2002; Moriana et al., 2003). The influences of growing environment and pedoclimatic conditions are less clear and often ambiguous (Inglese et al., 2009; El Riachy et al., 2011). It is definitely recognized the influence of variety on olive oil and several researches highlighted the differences in composition between products from different cultivars (Esti et al., 1998; Romani et al., 1999; Rotondi et al., 2010), leading to a growing attention towards the mono-varietal sector. Also, the effects of olives crushing, time and temperature of malaxation, and water volumes added, are the main variables influencing the transformation phase (e.g., Di Giovacchino et al., 1994; Servili et al., 2003; Torres et al., 2006).

The ripeness degree of the olives plays an important role in determining the quality of oil and several studies (Amiot et al., 1986; Servili et al., 1999; Gomez-Rico et al., 2008) showed, for example, alteration of phenolic profile during fruit growth and maturation process. Furthermore, water availability has a considerable effect on phenolic composition of olive fruit. Major results support that increased amount of water produces oils with lower phenolic content (Gòmez-Rico et al., 2007; Patumi et al., 2002).

Environmental and meteorological factors related to the cultivation area have considerable influence on the chemical composition of the fruit and consequently of the oil, although the results of some studies on the influence of temperature (e.g., Ripa et al., 2008; Tura et al., 2008) appeared not completely homogeneous. Concerning altitude effects on oil composition, some studies (Osman et al., 1994 and Mousa et al., 1996) reported that fruits grown at low altitude have a greater quantity of phenols than those coming from higher altitude cultivation areas. On the other hand, Deidda et al. (1994) found a positive correlation between height and quality of the oil in Sardinia.

The effects of soil on olive oil quality have been less studied. Few Authors reported relationships between soil characteristics and composition (Ranalli et al., 1997) and between soil texture and level of polyphenols oils (Pannelli et al., 1990).

The Nuclear Magnetic Resonance (NMR) spectroscopy has been recognized as an effective method of analysis of virgin olive oils (Mannina et al., 2009), valuable tool for the olive oil characterization (Dais and Hatzakis, 2013), the evaluation of their quality (Alonso-Salces et al., 2010), and their authenticity (Mannina et al., 2009).

The present study aims at investigating the influence of one of the less studied factors contributing to olive oil quality, namely the pedoclimatic factor, on the qualitative characteristics of mono-varietal oil. The study area is represented by North-Central Sardinia, an ideal research context due to the presence of areas characterized by different soil types and a predominant olive cultivar, “Bosana”. Specifically, the calibration and validation of a methodology of olive zoning for the promotion of different *terroir* represents an ultimate goal of the present work. Furthermore, the developed approach could be an innovation opportunity for the certification brand rules of olive oil Protected Designation of Origin (PDO Sardinia).

MATERIALS AND METHODS

The study was conducted in North-Central Sardinia on fruits of the most common Sardinian cultivar Bosana during the 2012 and 2013 olive oil campaigns. This cultivar is well known for its great organoleptic and nutraceutical characteristics that result from the unique fatty acid composition (Filigheddu et al., 2013), the high polyphenol content (Vacca et al., 2003), and also nutraceutical properties of olive oils (Beaucamp et al., 2005). Several works, based on genetical (Erre et al., 2010) and morphological analyses (Bandino et al., 1999; Bandino et al., 2001) defined Bosana as synonym for Tondo Sassarese, Sassarese and Palma. Bosana population is estimated in about 3 million plants; one third are concentrated in Sassari area (Bandino and Dettori, 2001), the rest is spread in several cultivation areas of central and southern Sardinia.

Study area selection

A Geographical Information System was used to identify experimental areas). The Eco-Pedological map (1:250,000) (Madrau et al., 2006) was overlayed with the Land Use map of Sardinia (1:25,000) (RAS, 2008). Based on this analysis, several olive groves were mapped and geo-referenced with GPS (Global Position System) to define the experimental network consisting in 15 standard olive orchards, three for each soil types: granite, miocenic limestones, alluvial soils, trachytes, and basalts (Fig. 1).

The selected experimental areas are located in the olive-growing districts surrounding the towns of Sassari, Alghero, Ittiri, Berchidda, and Seneghe. Olive orchards were chosen considering several characteristics: absence of irrigation, trees older than 50 years, a small number of plants *per* hectare, mechanical or facilitated harvest, and homogeneous pest management.

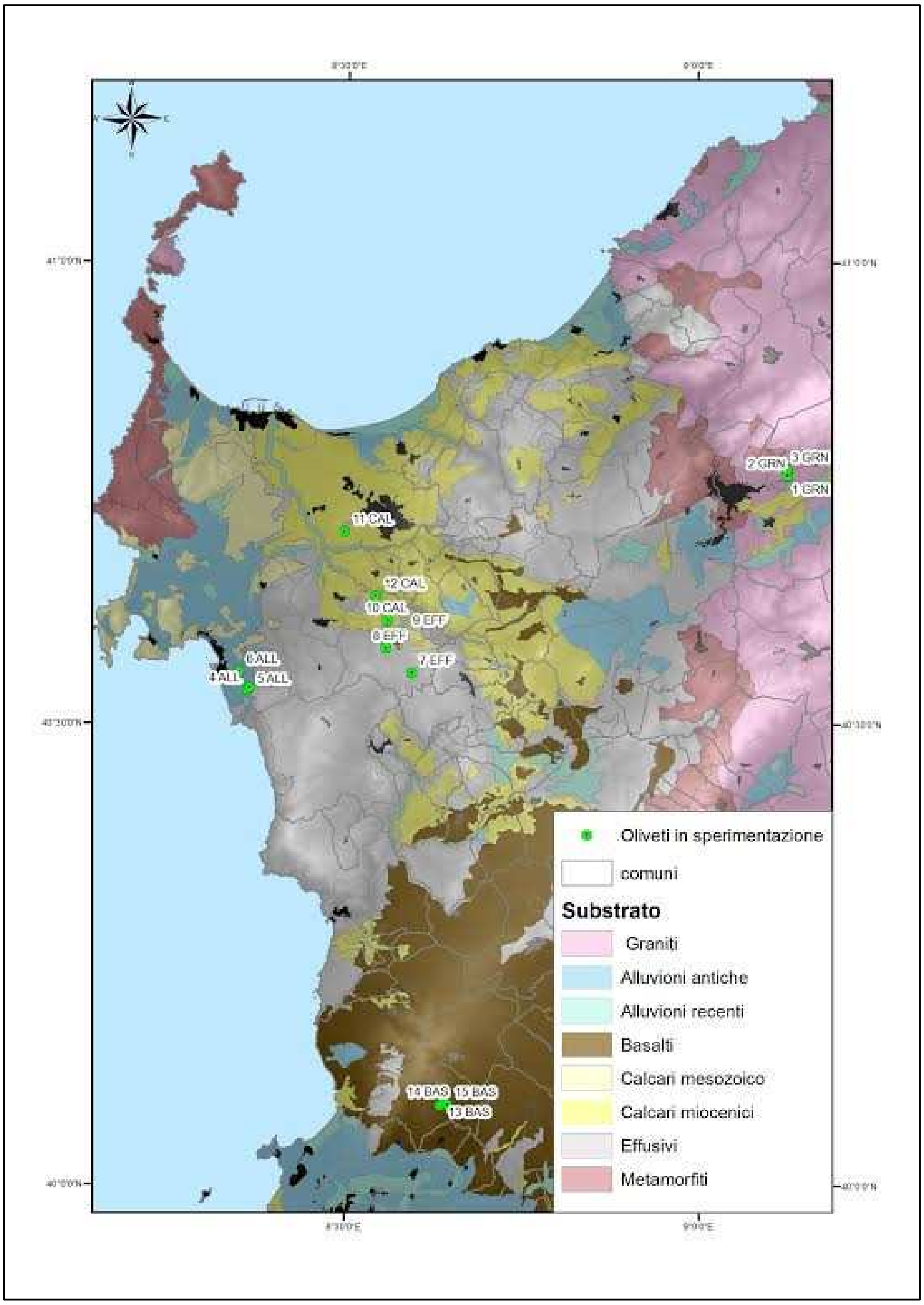

Fig. 1: Localization of olive orchards on Ecopedological map of Sardinia 1:250.000 (Madrau et al., 2006)

Climatic and agrometeorological characterization

A general climatic classification of the areas was carried on analyzing an agroclimatic index (FAO-UNEP aridity index). Climatic data series for indices estimation were temperature and precipitation daily data of the reference period 1971-2000 (Fig. 2).

The index is calculated as the ratio between average climatic annual (or monthly) precipitation (P) and average potential evapotranspiration (ETo), and it explains the demand of water from the atmosphere and the precipitation water availability. Potential Evapotranspiration ETo is calculated with Hargreaves and Samani (1985) equation based on extraterrestrial global radiation.

The P/ET0 parameter was also spatial interpolated for each of the 15 olive groves for 2012 and 2013 s as an agrometeorological index of drought conditions. Furthermore, the Degree Days (GDD_{10}) were calculated from January to December for both years.

Soil analysis

A soil sample was collected from each olive grove. Physical and chemical analyses of soils were performed according to the method described in the Official Gazette no. 248 of 21/10/1999, and particularly, texture, pH, carbon, organic matter, total nitrogen, available phosphorus, macro, and trace elements were determined (Tab. 1).

Transformation

Within each olive grove, fruit ripening was monitored using the Jaen maturation index (MI) applied to a sample of 100 kg. When the index reached the value of 2 (*semi veraison*) olives were collected from similar branches of the trees (same age, same vigor, same exposition, etc.) in order to minimize factors that might had an influence on ripening. All samples were processed by the same operator in a two-phases mini mill (TEM Oliomio) following a standard protocol. The temperature of the oil at the end of the process was up to 25°C; samples were subsequently filtered and stored in dark glass bottles.

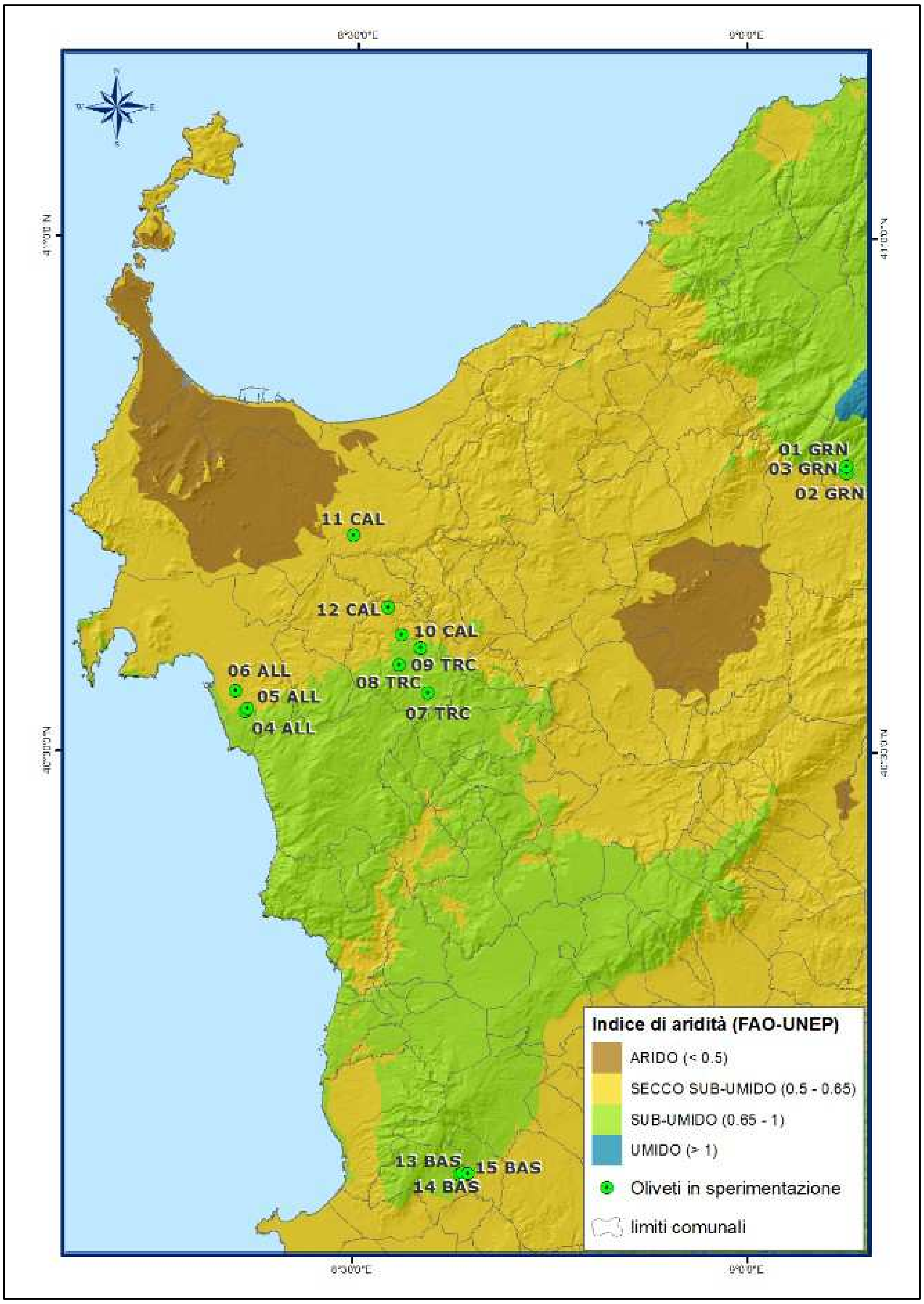

Fig. 2: Climatic characterization using FAO UNEP index.

Tab. 1: Average soil composition for each soil type

	Depth (cm)	Skeleton (%)	Sand Tot (%)	Silt, (%)	Clay (%)	Texture	pH	C (%)	O.M. (%)	N Tot (%)	P ass (ppm)	Ca (ppm)	Mg (ppm)	Na (ppm)	K (ppm)	Cu (ppm)	Zn (ppm)	Fe (ppm)	Mn (ppm)
Granits	49,4	29,6	86,3	6,5	7,1	1,6	6,2	2,0	3,5	0,2	6,7	1565,0	210,0	189,8	180	0,4	2,2	47,2	25,6
Alluvial	54,2	8,9	67,3	11,6	21,1	6,0	8,6	1,9	3,3	0,1	3,4	2286,7	186,7	410,0	165	5,9	0,7	15,7	16,8
Trachytes	51,3	23,5	63,4	21,7	15,0	5,0	7,2	0,9	1,9	0,1	4,3	2263,3	189,6	240,7	203	1,6	1,1	38,6	39,8
Limestone	28,8	12,6	63,8	24,2	12,1	4,0	8,6	2,2	3,9	0,2	4,6	1880,0	145,0	206,5	242	2,9	1,2	29,9	78,9
Basalts	30,0	11,5	79,8	8,8	11,4	2,3	6,6	2,2	3,8	0,7	8,9	2325,0	630,0	178,5	321	1,4	0,9	86,8	30,8

NMR analysis

For the NMR experiments each sample was prepared dissolving 50 mg of olive oil previously extracted (Montedoro et al., 1992) in 700µL of chloroform-d1 (Aldrich, USA). 1H-NMR spectra were recorded on a Bruker AVANCE II 600 spectrometer (Bruker, Germany) operating at 600.13 MHz for 1H resonance. 1D 1H-NMR spectra were acquired using standard protocol with a 12 ppm spectral width, 128K data point for acquisition. Prior to applying Fourier transform (FT), the FIDs were multiplied by an exponential function corresponding to a line broadening of 0.3Hz. All 1H NMR spectra were phased and baseline corrected within TOPSIN 2.0 (Bruker GmbH, Karlsruhe, Germany). Data were reduced to 245 integrated regions corresponding to the chemical shift range of 10–0.5 with a region width of 0.04 ppm using Analysis of MIXtures (AMIX) (Bruker GmbH, Karlsruhe, Germany). 240 descriptors were obtained. The most important variables were assigned on the basis of literature and by direct addition of standards.

Data analysis

Before chemometric analysis, the data were normalized by setting the total region of each spectrum to 100 and the generated ASCII file was imported into Microsoft EXCEL for the addition of labels. The bucket matrix was imported into SIMCA-P software version 12.0, (Umetrics AB, Umeå, Sweden) for statistical analysis. A 240 columns data matrix for subsequent analysis was set.

Statistical Methods

NMR data were submitted to multivariate statistical evaluation using the Simca-p software package (Umetrics Umeå, Sweden). In particular, the Principal Component

Analysis (PCA) and Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) were used. In order to avoid model overfitting, a 300 time permutation test validated the supervised models.

RESULTS AND DISCUSSION

According to the climatological FAO UNEP calculated for the reference period 1971-2000, the olive groves are placed in the classes "Dry sub-humid" and "sub humid", with values of P/ET0 ranging between 0.5 and 1 (Fig. 2).

Statistical OPLS-DA analysis that correlated elevation and agrometeorological indexes for 2012-2013 showed values below the significance thresholds (0.5 for the parameter R2, which represents the ability of classification, and 0.4 for Q2, which is the predictive efficiency of the models). In particular, elevation (Fig. 4) R2 value was 0.38 while Q2 was 0.14. The agrometeorological indexes analyzed were the degree days (GDD) calculated with a 10°C threshold for the period January-December and the P/ET0 annual ratio. Models related to both indexes (Fig. 5 and Fig. 6) were below the significance threshold (R2 values of 0.32 and 0.42, respectively; Q2 values of 0.06 and 0.25, respectively).

Interesting results were obtained when groups were analyzed based on soil types. All models showed R2 and Q2 values above the significance threshold (Fig. 7), except for the 1 to 1 Basalt vs Trachyte model. Being both Basalt and Trachyte magmatic extrusive type of rocks, they were grouped together and considered as one.

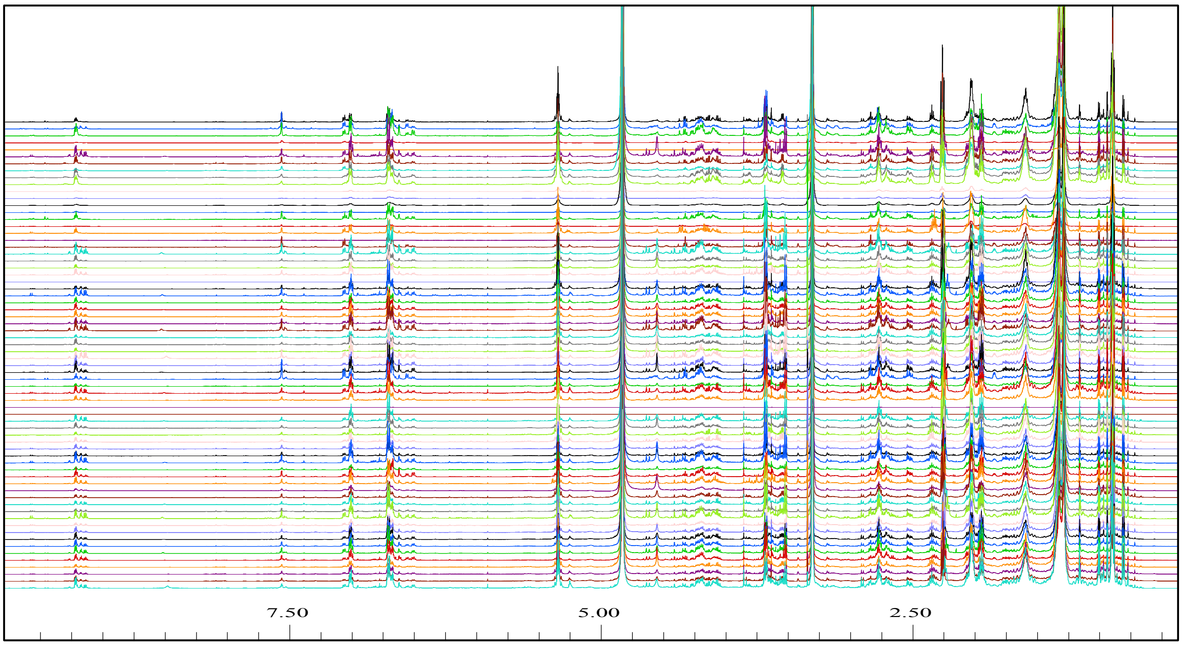

Fig. 3: NMR spectral firms of sampled oils.

The DA relative to all analyzed groups (Fig. 7) gave scores above the significance threshold. All 1 to 1 models, except for Limestone *vs* Trachyte (Fig. 8), showed statistically significant results: Limestone *vs* Granite (Fig. 9), Limestone *vs* Alluvial (Fig. 10), Limestone *vs* Basalt (Fig. 11), Granite *vs* Alluvial (Fig . 12), Granite *vs* Basalt (Fig. 13) and Limestone *vs* Granite (Fig 15).

S-Plot analysis related to each 1 to 1 model show the molecules having greater importance in discriminating oil parent material origin (Fig. 14 and Fig. 16 are two examples).

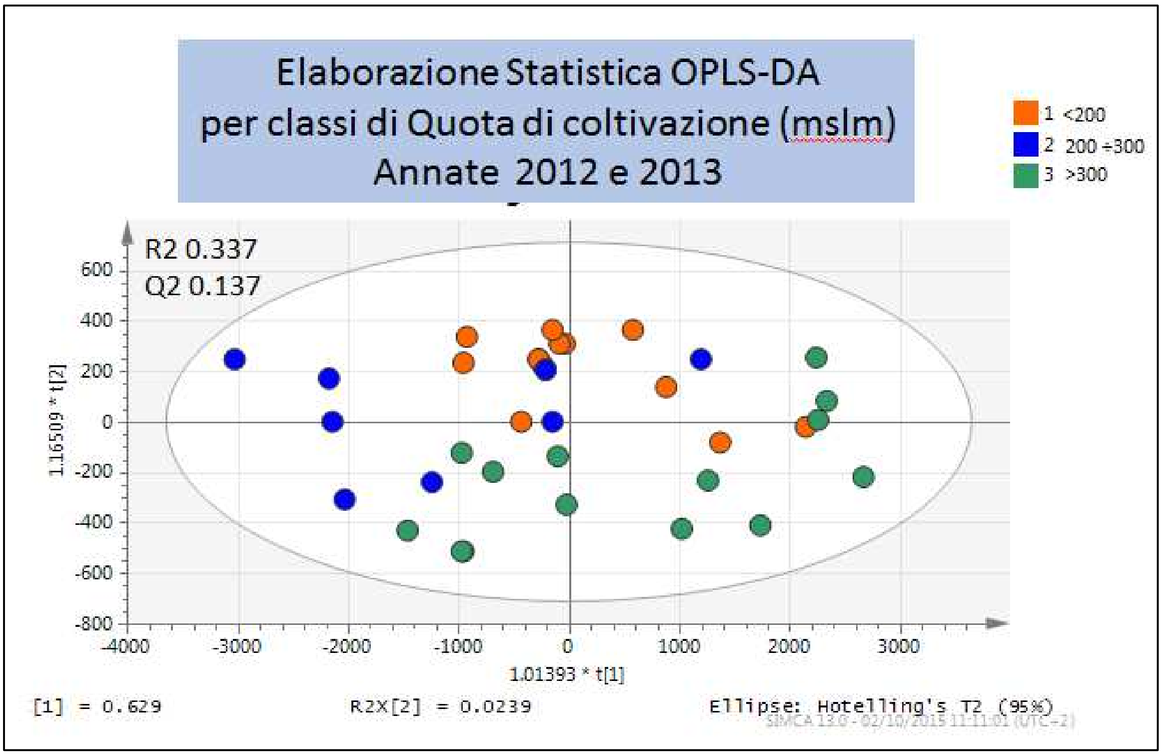

Fig.4: OPLS-DA model for cultivation altitude of experimental farms

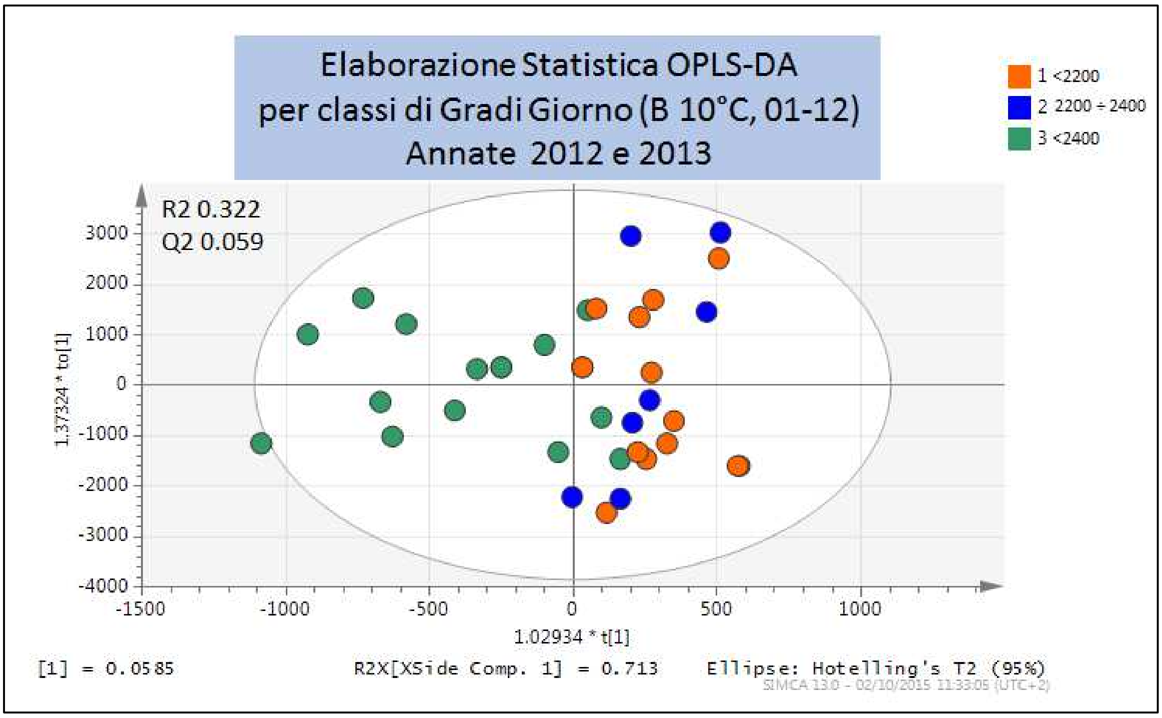

Fig.5: OPLS-DA model for cumulated Growing Degree Days.

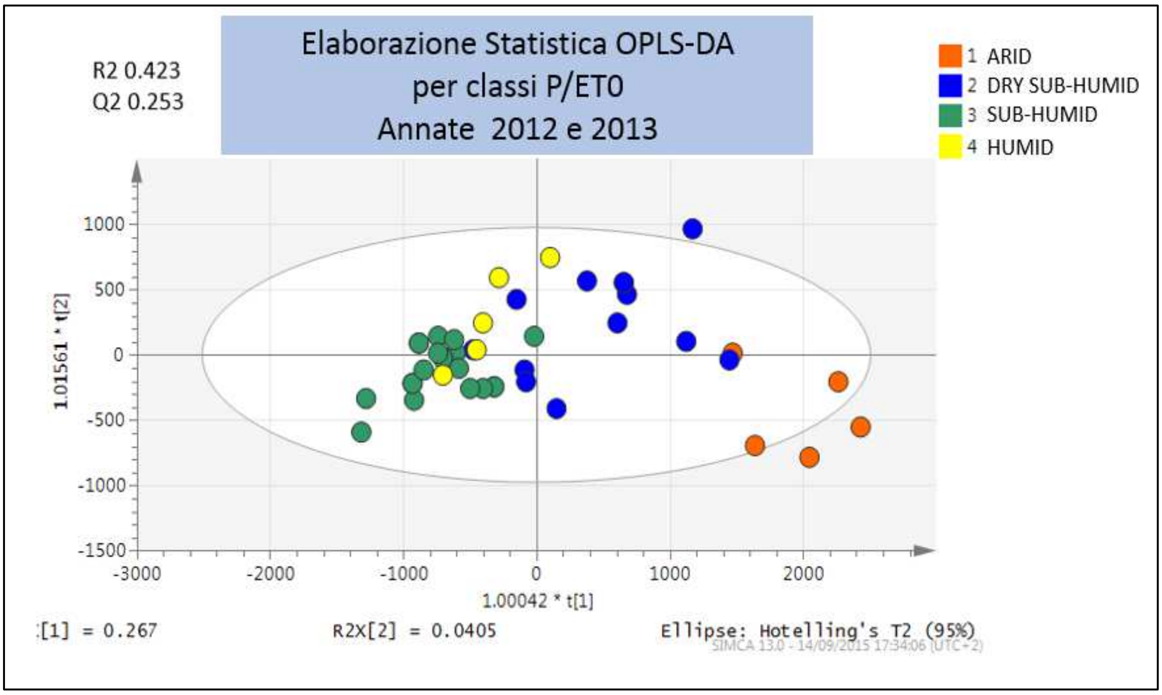

Fig. 6: OPLS-DA model for FAO UNEP index

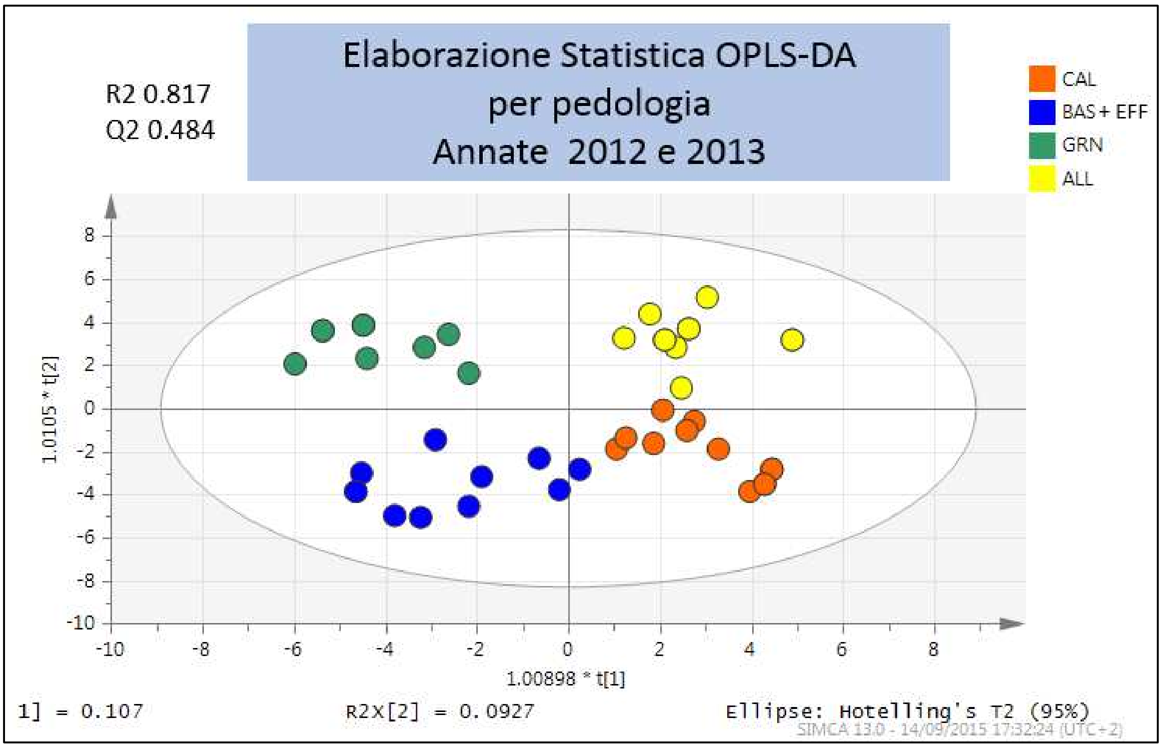

Fig. 7: OPLS-DA model for soil type

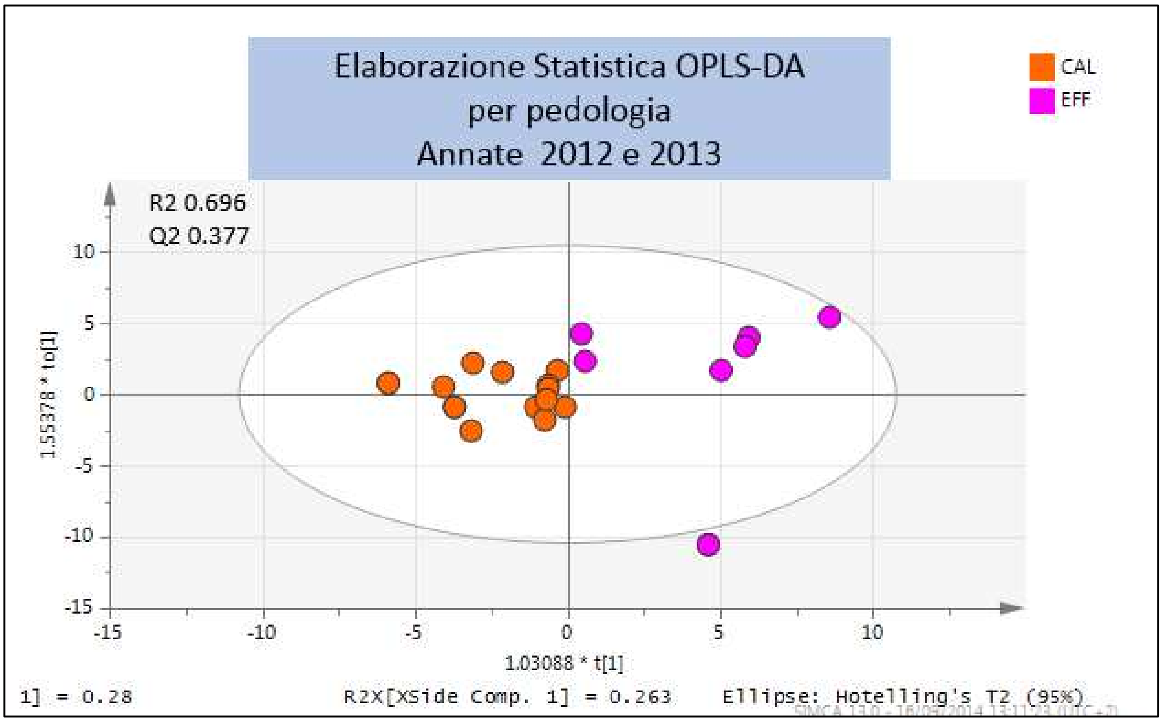

Fig. 8: OPLS-DA model limestones vs. trachytes

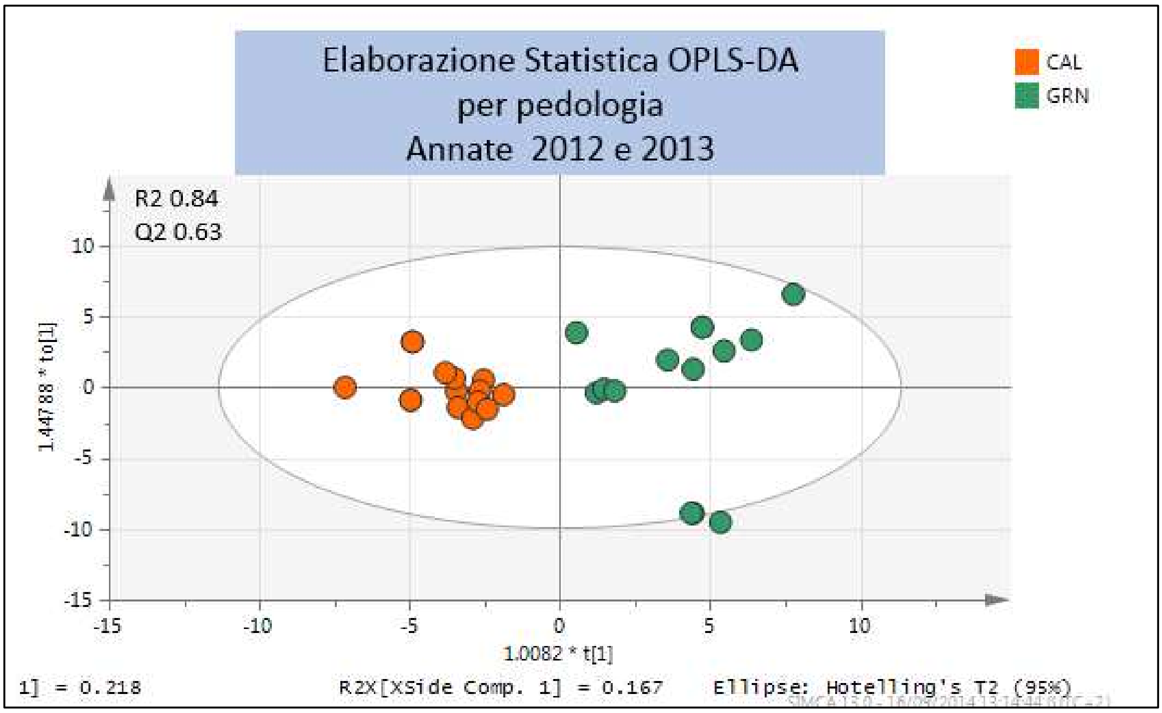

Fig. 9: OPLS-DA model limestones vs. granites

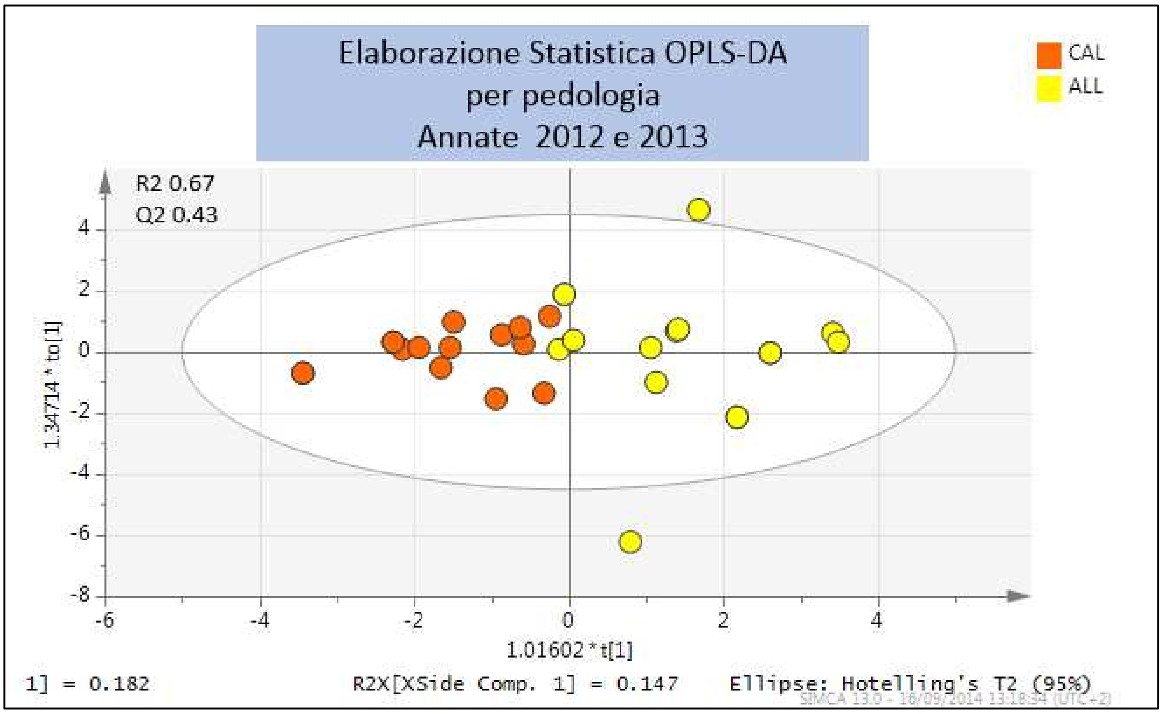

Fig. 10: OPLS-DA model limestones vs. alluvial

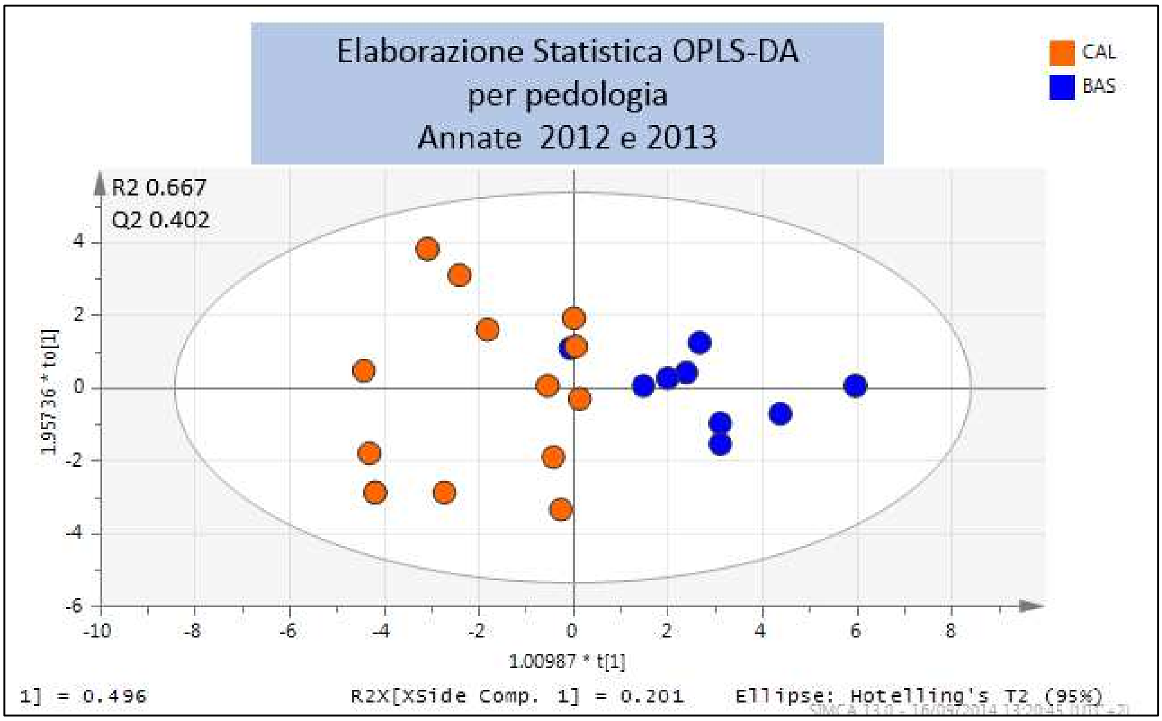

Fig. 11: OPLS-DA model limestones vs. basalts

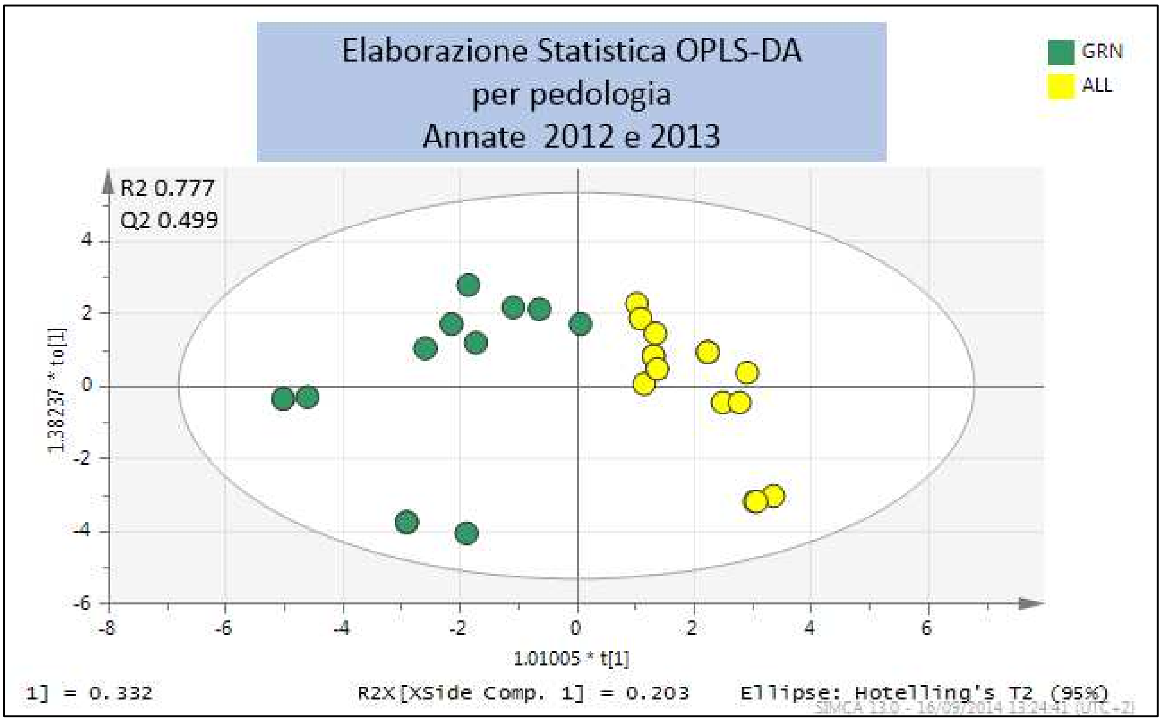

Fig. 12: OPLS-DA model granites vs. alluvials

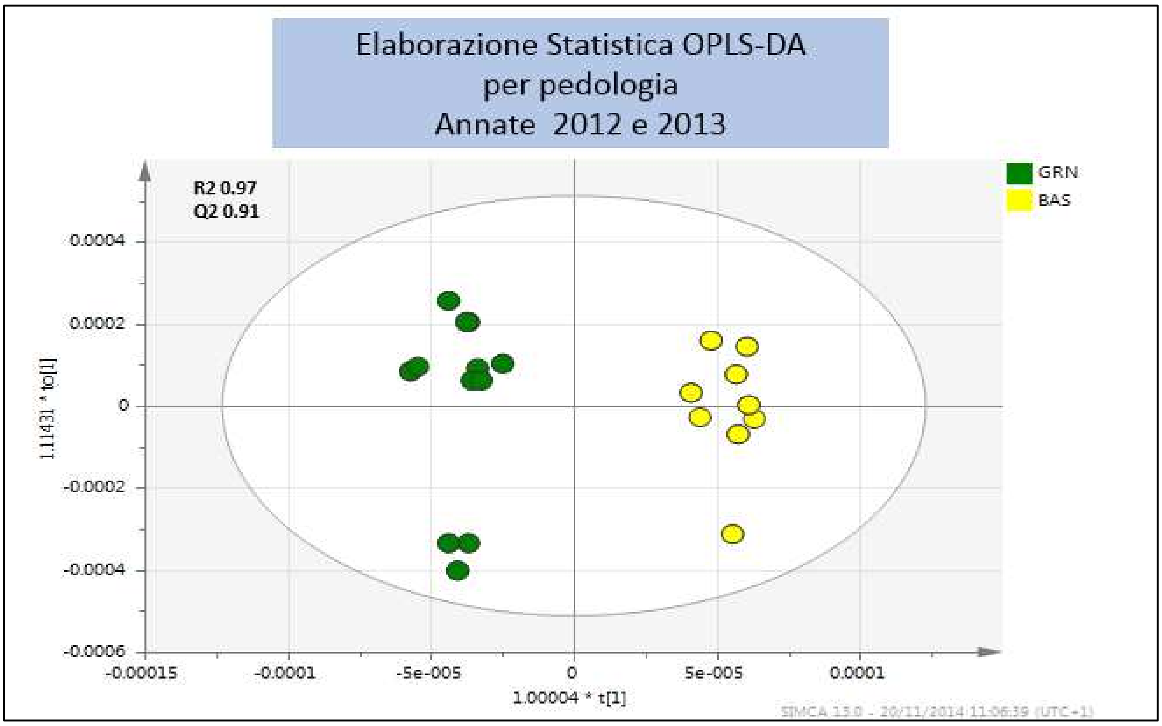

Fig. 13: OPLS-DA model granites vs. basalts

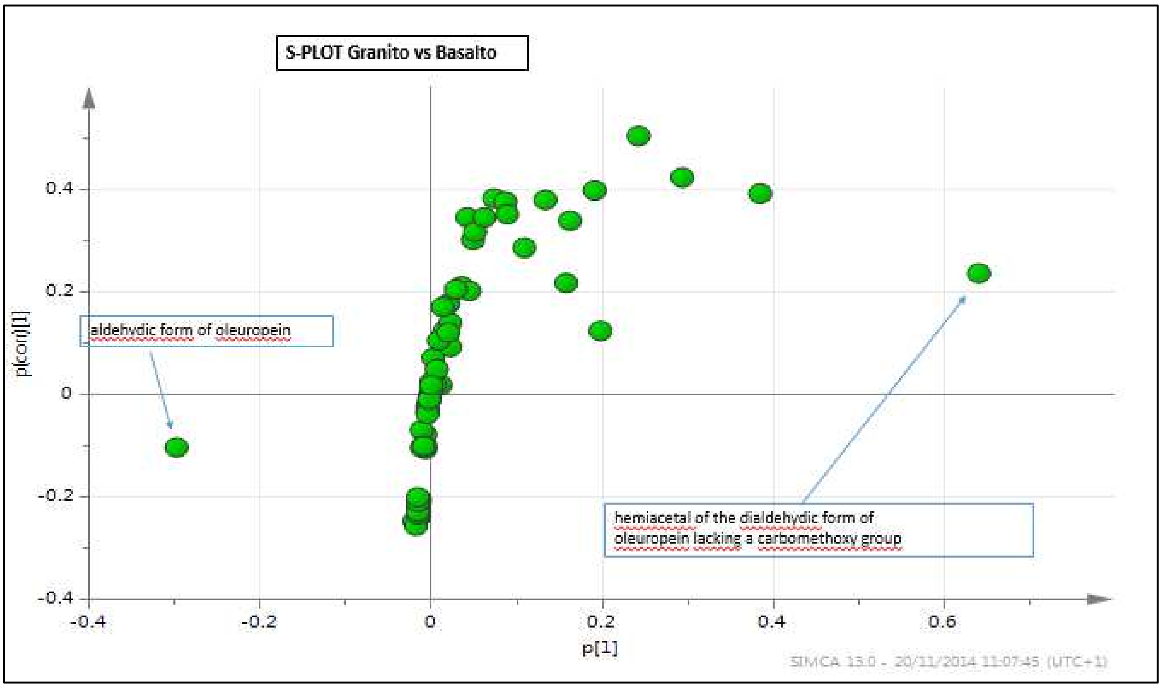

Fig. 14: S-Plot granites vs. basalts

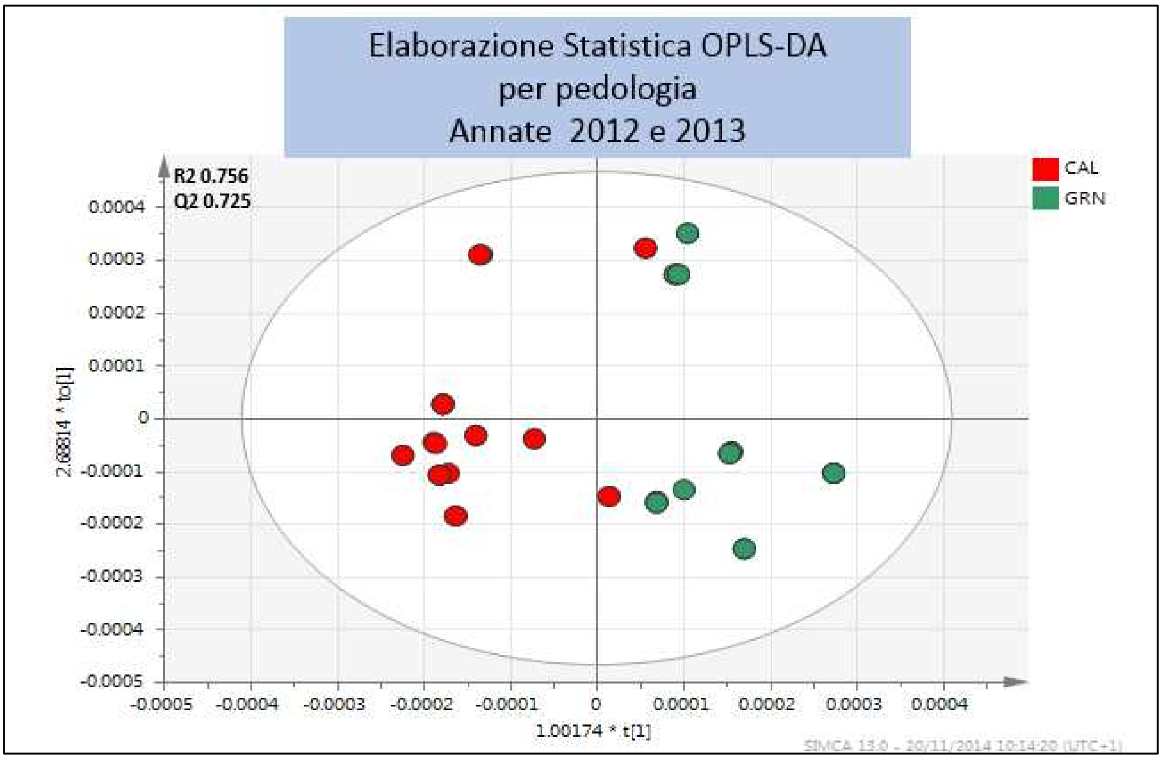

Fig. 15:OPLS-DA model limestones vs granites

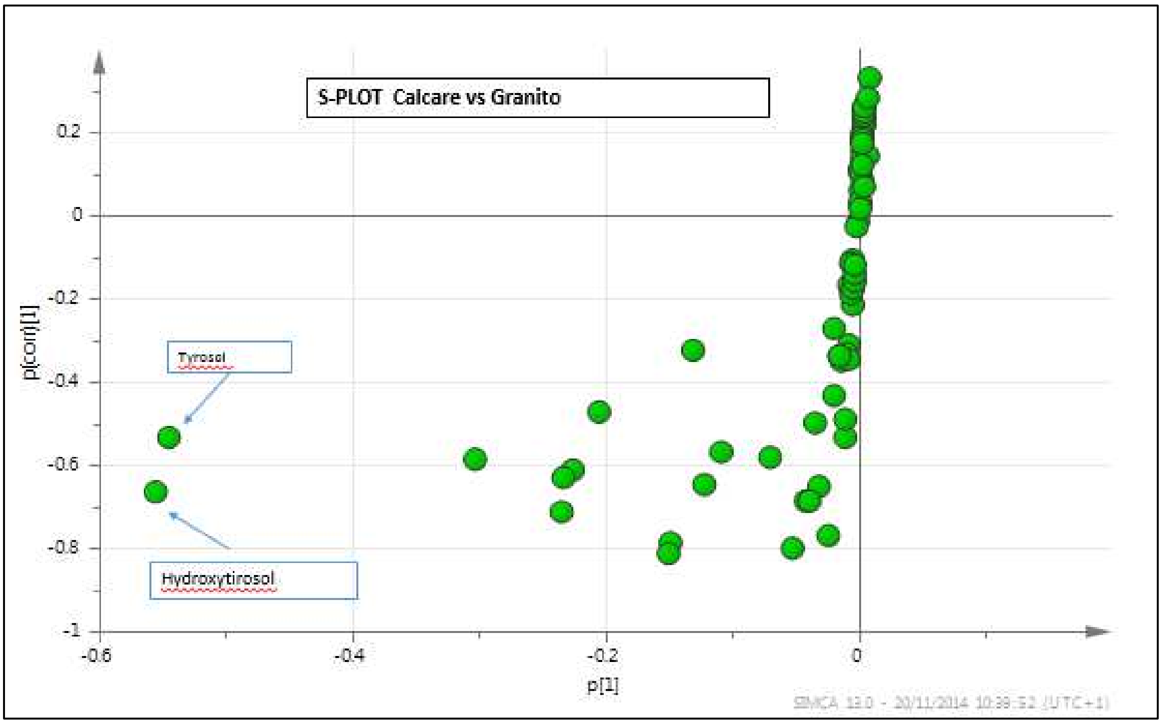

Fig. 16: S-plot limestones vs granites

CONCLUSIONS

NMR method together with multivariate statistics showed its capability to characterize the production of virgin olive oils. At the mesoscale, soil characteristics showed a strong influence on olive oil composition and quality. Statistical analysis showed a weak influence of elevation and agrometeorological indices (GDD10 and P/ET0) in determining oil quality, at least at this scale of study. This result is in agreement whit Tura et al., (2008), pointing out that this effect is more evident at 'macroclimate' scale than at 'mesoclimate' scale.

Chemical and statistical analyses, combined with GIS processing, seemed to be suited for the development and implementation of a chemometric model for the production identification, which would be an essential tool in the framework of Food Security and that could be usefully adopted in the production regulations with EU trademark.

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Experiment 3 - Yeast microflora in Sardinian extra virgin olive oils

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ABSTRACT

Olives are rich in microorganisms that, during the extraction process may be persistent on oil. Despite this, olive oil has been slightly analyzed from the microbiological point of view. Recently, it was observed that olive oil has a rich microflora that can influence positively or negatively on the physical-chemical and sensory quality. Once selected, the activity of the microbiota of the olives can greatly contribute to the flavor and overall quality of olive oil. For this reason, it is important to identify the microbiological activities that occur in them. The purpose of this preliminary study was to isolate, select and identify microbial genera and species present during the production process (olives, olive paste and oils) of extra virgin olive oils produced in Sardinia (Italy) from varieties belonging to Nera di Gonnos, Nocellara del Belice, Semidana and Bosana.

INTRODUCTION

Olives are rich in microorganisms that, during the extraction process can get the oil and be persistent on it. Despite this, olive oil, both for its physical state and because it is not a fermented food, has been slightly analyzed microbiologically. However, it was observed that olive oil has a rich microflora (Ciafardini and Zullo, 2002; Ciafardini et al., 2006; Zullo and Ciafardini, 2008; Romo-Sanchez et al., 2010; Zullo et al., 2010) that can influence positively or negatively on the physical-chemical and sensory quality of the oils. The presence of these microorganisms, particularly yeasts, is due to their passage from olives to olive oil during the extraction process. The above-mentioned authors have shown that the presence of microorganisms, in particular yeasts, may affect differently on the quality of the olive oils. The yeasts present in the fresh oils in fact may remain viable and metabolically active during the storage period.

Among the positive impacts, it is possible to cite the enzymatic activities, particularly β-glucosidase and esterase. These activities allow hydrolyzing the glycoside oleuropein, which gives the bitter taste to olive oil. The activity of these two enzymes, hydrolyzing oleuropein, reduces the bitter taste of the oil, and improves the sensory features (Ciafardini and Zullo, 2002). Among the factors that negatively affect there is the peroxidase activity, which adversely affects the composition of the oil as it has the oxidative degradation of phenolic compounds (Romo-Sanchez et al., 2010). It has also been detected lipase activity, which through the breakdown of triglycerides causes an increase of the acidity in the oil. This is also a negative parameter (Ciafardini et al., 2006).

The purpose of this study was to isolate, select and identify microbial species present during the production process (olives, olive paste and oils) of extra virgin olive oils produced in Sardinia (Italy) from varieties belonging to Bosana, Semidana, Nera di Gonnos and Nocellara del Belice.

MATERIALS AND METHODS

Isolation of yeasts

The microorganisms were isolated from olives, olive paste and oils. The sampling process was done during harvest time (2014/2015) from north Sardinia region. Samples were harvested or collected and transported to the laboratory in refrigeration under aseptic conditions. For olives, 10 grams were put under agitation for 1 h in 250 ml Erlenmeyer flasks filled with 100 ml of Ringer's solution (Oxoid). For the olive paste, aliquots of 10 g of olive paste were mixed with 90 ml of Ringer's solution in sterile bags and mix manually for 10 min. For oils, 10 ml of oil that was mixed with 90 ml of YEPD in 250 ml Erlenmeyer flanged flasks. The samples were then put under agitation for 48 h at 30 °C. For the subsequent isolation, from all samples (olives, olive paste and oils) 100 microliters of the solution were taken as it is and dilutions, and were plated onto YEPD and PCA plates. The plates were left to incubate at 30 and 37 °C for 48 h. The CFU obtained were picked up, re-streaked on selective media and stored at -80 °C.

Molecular characterization and identification of isolates

Molecular characterization of isolates was conducted by means of polymerase chain reaction restriction (PCR-RFLP) of two Internal Transcribed Spacers (ITS1 and ITS4) of 5.8 rRNA by using primers ITS1 (5’-TCCGTAGGTGAACCTGCGG-3’) ed ITS 4 (5’-TCCTCCGCTTATTGATATGC-3’), as described by Esteve-Zarzoso et al. (1999). PCR products were sequenced (BMR-Genomics, Università di Padova, Italy). A blast analysis of the DNA sequences was performed using the database present in National Center for Biotechnology Information (NCBI) in order to identify genera and species of the different isolates (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS AND DISCUSSION

Previous studies on the microflora of olive oils revealed that the oil microbiome may play an important role in the definition of the sensory characteristics of oils. In this work, through the isolation, and molecular identification of microorganisms obtained during the production process of different extra virgin olive oils produced in Sardinia (Italy) from different olive varieties it was possible to identify yeasts belonging to different genera. The yeast strains were isolated from olives, olive pastes and oils. Amplicons of around 500bp-800bp were obtained by PCR/RFLP and then sequenced. After screening for DNA homology using the NCBI Blast program, the yeast genera and/or species were identified. About 18 different species belonging to nine different genera of yeasts were identified. In particular, in the olives were found genera *Cryptococcus*, *Aureobasidium*, *Debaromyces*, *Metschnikowia*, *Rhodotorula*, *Pirula* and *Candida*, in the olive paste *Saccharomyces*, *Nakazawaea*, *Candida* and *Pichia*, and finally in the oils only *Candida* and *Pichia* (Tab. 1).

Wide biodiversity was observed for Nera di Gonnos variety (11 species), followed by Bosana (10 species) and Nocellara (7 species). For all analysed varieties, a broader biodiversity was found in the olive samples and it decrease from the olive paste during malaxation to olive oil. Some species were found only in the olives (the four species belonging to *Cryptococcus* genus, *Aureobasidium pullulans, Debaryomyces hansenei, Rhodotorula glutinis, Metschnikowia sp*.). Other species were found only in olive paste, such as *S. cerevisiae*, *C. diddensie*, *C. guilliermondi, C. wicherami e P. manshurica*. Only two species were found only in the olive oil, *C. temnochile* e *C. dendronema*. The species found in the olive oil samples are the following: *C. temnochile, C. molendinolei, C. adriatica, C. dendronema* e *P. mexicana*.

Several species were found in the olive oils from Nocellara and Nera di Gonnos varieties, while only *C. adriatica* was identified in Semidana olive oil and in four olive oil samples from Bosana although this variety showed a remarkable biodiversity on the olives and pastes.

Other authors in Central Italy (Molise and Umbria regions) and in Croatia (Cadez et al., 2012) also pointed out the presence of *C. adriatica* in EVOO. Furthermore, *C. molendinolei*s pecie was identified in olive oils produced in several countries such as Italy, Croatia, Slovenia and Israel.

The yeasts isolated so far from different olive oil types in Sardinia belong mainly to *Candida* genus, as already observed by Ciafardini et al. (2006). It is noteworthy to highlight the presence of the two species *C. adriatica* and *C. molendinolei* in the olive oils produced in Sardinia, as they were previously identified only in regions neighboring the Adriatic Sea (Cadez et al., 2012).

[redacted]

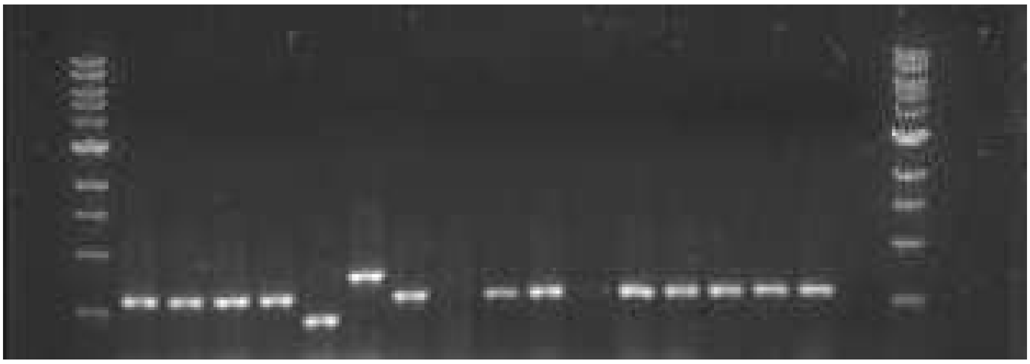

Fig. 1: Identified strain amplification profile, obtained through the analysis of ITS regions. M = marker, 1-6 strains isolated from Nera di Gonnos paste, 7-9 strains isolated from Nera di Gonnos olives, 10-13 strains isolated from Nera di Gonnos oil, 14-16 strains isolated from Nocellara oil.

Tab. 2: Yeast species isolated by olives, olive paste and olive oils

Species	Substrate	Cultivar	Notes
Cryptoccocus carnescens	Olives	Nocellara Bosana 1	
Cryptoccocus victoriae	Olives	Bosana1	
Cryptoccocus magnus	Olives	Bosana1	
Cryptoccocus JSKIM	Olives	Bosana 1	
Candida guilliermondi	Olives and olive paste	Nera di Gonnos	
Candida temnochile	Olives and **olive oil**	Nera di Gonnos	
Candida molendinolei	Olive paste and **olive oil**	Nocellara Nera di Gonnos Bosana1	Only olive oil on Nera di Gonnos
Candida wickerhamii	Olive paste	Nocellara	
Candida diddensiae	Olive paste	Nera di Gonnos	
Candida adriatica	Olive paste and **olive oil**	Semidana Bosana1,2,3,4	Only olive oil on Semidana
Candida dendronema	**Olive oil**	Nocellara Nera di Gonnos	
Aureobasidium pullulans	Olives	Nera di Gonnos Bosana1	
Debaryomyces hansenii	Olives	Nera di Gonnos	
Pichia manshurica	Olive paste	Nera di Gonnos	
Pichia mexicana	**Olive oil**	Nocellara Nera di Gonnos	
Saccharomyces cerevisiae	Olive paste	Nocellara Nera di Gonnos Bosana 1	
Rhodotorula glutinis	Olives	Bosana1	
Metschinowia sp	Olives	Bosana1	
Nakazawaea anatomiae	Olive paste	Nocellara	

CONCLUSIONS

The first observation is that there is a selection effect on the number of species, from olives to oils, and some genera are present in the next phase of the production process but not in the previous one. This phenomenon was observed for all cultivars under study. This fact could be due to the specific microflora present in every mill during olive paste production. The second observation is that most of the genera are typical of the oleic ecosystem like *Candida*, *Pichia*, *Saccharomyces* (Romo-Sanchez et al., 2010). Others are less common like *Cryptococcus*.

As stated before, the positive or negative action of these yeasts on olive oils depends on their enzymatic activities (Ciafardini and Zullo, 2002). For example, some species of the genus *Candida* has recently been shown to have negative effect on the oil, in particular *Candida adriatica* due to the enzymatic hydrolysis of the triacylglycerols (Ciafardini and Zullo, 2015). Obviously, it must be highlighted that there are also positive microbiological activity as already mentioned in the introduction.

Considering the results obtained here, the next step of this work will be to identify all different species isolated from different olive varieties, in order to investigate whether the microbioma may be associated to the olive varieties or to the geographic area. The ultimate aim is to analyze the main enzymatic parameters to evaluate whether the identified yeasts can impact positively or negatively on the physical-chemical and sensory quality of extra virgin olive oils.

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CONCLUSIONS AND FUTURE PERSPECTIVES

The virgin olive oil sector has been taken great attention at National, European, and even global level. In particular, at Italian level, the poorly renovated productive structure, which is still firmly tied to models of traditional olive groves, offers two possibilities. On the one hand, the Italian sector provides products identifiable with the territory of origin, especially in terms of the great varietal heritage. On the other hand, the olive-growing sector could seize the opportunities arising from intensive (which indeed allows to use the Italian varietal heritage) or super-intensive models, in a perspective of complementarity with other traditional models of olive growing to meet the EVOO growing demand at international level.

The aspects of differentiation of high-quality production will therefore continue to play a decisive role in order to ensure adequate levels of income for workers in the olive-growing sector and to reward the quality aspects in all the facets (merchandise, sensory, and health) of such a wide-ranging sector. Furthermore, in the framework of food security, the sector is urged to provide new tools and methodologies to protect consumers and the image of the EVOO products.

The experiments carried out in this thesis lie in this dual perspective, allowing to characterize the olive oil production on the basis of chemical and microbiological composition.

The olive oil productions deriving from the most widespread cultivar in Sardinia (Italy) were firstly characterized in terms of pattern components and differentiated on the basis of their growing area through high-resolution 1H NMR spectroscopy, with the final ail to obtain a protocol to recognize the composition of various Sardinian oils and correlate them with production areas. Then, the same methodology combined with multivariate statistic was applied to oils coming from different production areas with the aim to determining the influence of environmental factors on oil quality.

The results showed the potential of the proposed methodology for the construction of flexible and scalable chemometric models, based on the combination of NMR, multivariate statistics, and Geographic Information System, suitable to be applied for the zoning and protection of valuable products and to be usefully integrated in the specification requirements for the Register of protected designations of origin and protected geographical indications.

The enhancement and promotion of high-quality products also depends on a greater and deeper understanding of the role played by microbiological components, so far little investigated. The interesting findings of the third experiment carried out in this thesis pointed out the presence of microorganisms in the different phases of olive oil production, and encouraged more studies and researches to clarify the effects of enzyme activity. This has a very important role in particular during the phases of processing and storage of EVOO.

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