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REGIONE AUTÒNOMA DE SARDIGNA
REGIONE AUTONOMA DELLA SARDEGNA

FSE 2007-2013
POR SARDEGNA
obiettivo competitività regionale e occupazione

Fondazione di Sardegna

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Ciclo XXX

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Dr. Pierfrancesco Deiana

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La presente tesi è stata prodotta durante la frequenza del corso di dottorato in Scienze Agrarie dell'Università degli Studi di Sassari, a.a. 2016/2017 - XXX ciclo, con il sostegno di una borsa di studio cofinanziata con le risorse del P.O.R. SARDEGNA F.S.E. 2007-2013 - Obiettivo competitività regionale e occupazione, Asse IV Capitale umano, Linea di Attività l.3.1 "Finanziamento di corsi di dottorato finalizzati alla formazione di capitale umano altamente specializzato, in particolare per i settori dell'ICT, delle nanotecnologie e delle biotecnologie, dell'energia e dello sviluppo sostenibile, dell'agroalimentare e dei materiali tradizionali".

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ABSTRACT

Global virgin olive oil (VOO) production and consumption constantly increased during last 20 years. The expansion of growing areas in southern Mediterranean and in new countries, together with the enhancement of growing techniques, has led a reduction of production costs, maximizing productivity. Interest on the high quality and the nutraceutic properties of VOO is rising.

The wide biodiversity of Italian olive germplasm is an important resource for improve quality, differentiate and promote specific productions.

The aim of this thesis is to improve the knowledge of the Sardinian olive germplasm, with a particular focus on those varieties that characterize PDO "Sardegna", trying to identify the ones that might contribute better to improve label's quality.

In a three years field test, phenological behavior and interaction with meteorological conditions of 26 local and national varieties were evaluated. Corresponding olives samples were processed and monovarietal VOO were analyzed. The influence of harvest period on the VOO quality of 3 Sardinian varieties was studied. Similar environmental, agronomic and extraction conditions were maintained.

The study of phenological behavior and VOO composition revealed some peculiar qualitative aspects, specific of some varieties. Moreover, genetic factor affected fruit ripening process. Further studies might help to evaluate better the potentialities and valorization of minor varieties.

INTRODUCTION

VIRGIN OLIVE OIL, COMPOSITION AND NUTRACEUTICAL PROPERTIES

Consumption of VOO has steadily increased by 3% annually between 1990/91 and 2011/12; on 2012, countries of European Union (EU), which first Italy (23.5% of global), were the principal consumers (63% of global) (Lynch and Rozema, 2013). However, the great increase of VOO demand became, during last years, from not traditional consumer countries, like United States, Germany, France, Japan, China, Canada, Russia and Brazil, which greater interest is addressed to a functional food product, with high quality and nutraceutical properties, authenticity and provenience.

Virgin olive oil (VOO) is the principal lipidic source of Mediterranean diet, which, associated with a balanced lifestyle, it has shown many health benefits, such as lower incidence of cardiovascular, cancer, inflammatory and neurodegenerative diseases (Beauchamp et al, 2005; Martin-Pelaez et al, 2013). Numerous studies claimed its nutritional and nutraceutical properties that differentiate it from other vegetable oils (Waterman and Lockwood, 2007).

Its acidic composition is characterized by high content in mono and polyunsaturated acids. The most representative is oleic acid (55-83%). Furthermore, the presence of essential fatty acids ω6 (linoleic acid, 18: 2) and ω3 (linolenic acid, 18: 3) is worth noting. The latter are present in lower quantities than other vegetable oils, but in an optimal ratio (Aparicio et al, 1999). Olive oil differs from other vegetable oils because of the higher content of monounsaturated fatty acids than polyunsaturated, which makes it more resistant to oxidation phenomena (Aparicio et al, 1999; Waterman and Lockwood, 2007). Traditionally, the nutraceutical properties of olive oil were attributed to the high content of oleic acid that evidenced beneficial effects at gastrointestinal and cardiovascular level (Piroddi et al, 2017).

Currently scientific research has extended these properties to its smaller fraction (about 2%) called not-saponificable, characterized by numerous bioactive molecules. The most representative compound in this fraction is squalene, accounting within the range 200 – 12,000 mg/kg (Lanzòn et al, 1994), for which olive oil is the main vegetal and edible source (Naziri et al, 2011; Spanova and Daum, 2011). It is present in lower amount in other plant

species such as pumpkin oil, amaranth seed oil, soybean oil, sunflower oil, palm and rice bran oil (Naziri et al, 2011; Spanova and Daum, 2011). It is an acyclic triterpenoid hydrocarbon (2,6,10,15,19,23-hexamethyl-6,6,10,14,18,20-tetracosahexane), a lipidic molecule composed by 30 carbon atoms, six double bonds and four possible conformations (Naziri et al, 2011). Different benefits to human health have been attributed to squalene. It is present in skin surface lipids and, in in a mutually reinforcing way with other substances like vitamin E, plays an important role to protect skin to UV rays due to its capacity to act as a singlet oxygen scavenger (Visioli and Galli, 2002); to squalene have been attributed chemo-preventive effects on colon and breast cancer (Owen et al, 2000). Due to its nutritional benefits and chemical properties, nowadays squalene has found various applications in diseases management, pharmaceutical and cosmetic applications. It is easily administered orally or intravenously in the way of emulsion. It has been adopted as vaccine delivery or adjuvant. Moreover, squalene has been demonstrated to ameliorate the effect of anticancer drugs if used as co-formulated (Reddy and Couvreur, 2009). Squalene has been utilized also as adjuvant in antiviral drugs (Desmaële et al, 2012). Recently, squalene has been introduced as a component of functional food (Naziri and Tsimidou, 2013). Squalene plays a little role on VOO stability, acting as antioxidant when present in appropriate amounts (Psomiadou and Tsimidou, 1999; Psomiadou and Tsimidou, 2002a).

Squalene acts as intermediate in the phytosterols biosynthetic pathway (Spanova and Daum, 2011). In VOO they account usually between 1,000 and 2,000 mg/kg, mainly represented by β-sitosterol (>93% of total sterols), together with Δ-5-avenasterol, stigmasterol and campesterol (Boskou, 2008). Phytosterols are considered important for human nutrition; studies demonstrated their activity on the reduction of serum cholesterol levels (Piironen et al, 2000). Phytosterols, particularly β-Sitosterol, have antitumoral properties, mainly in prostate, colon and breast cancer (Boskou, 2008).

Other important molecules that characterize the unsaponificable fraction of VOO are tocopherols or Vitamin E, which includes two groups of compounds, tocopherols and tocotrienols, which have both four isoforms named: α- β- γ- δ-tocopherol and α- β- γ- δ-tocotrienol. The isoform α of tocopherols is the most representative in VOO (95 -99%). In some olive varieties small quantities of isomers γ and β have been also found, thus detection of these two minor isomers has proven to be a useful tool for varietal characterization (Beltran

et al, 2010). It is present also in other olive tree products such as table olives and leaves (Tsimidou, 2012). Tocopherols are bioactive molecules that play an important role in protection of VOO from autoxidation and photooxidation processes, acting as radical chain breaker and physical and chemical quencher of singlet oxygen during photooxidation (Okogeri and Tasioula-Margari, 2002; Psomiadou et al, 2003; Del Caro et al, 2006). Because of its antioxidant activity several beneficial health properties have been attributed to tocopherols (Schneider, 2005). Tocopherols content in VOOs could vary from values close to 100 mg/kg of oil to values over 600 mg/kg (Gomez-Rico et al, 2006; Beltran et al, 2010).

Phenolic compounds, which concentration may range in VOOs from values lower than 100 mg/kg to more than 1000 mg/kg (Servili et al, 2004), are the molecules with the highest antioxidant power; they influence positively VOO stability against autoxidation processes improving the product shelf life (Blekas et al, 2002; Psomiadou et al, 2003). Phenolic compounds are responsible of pungent and bitter sensations of VOO, and may affect also the awareness and the persistence during storage of some volatile molecules responsible of fruity and off flavors by hyding them (Genovese et al, 2015a, b). Nowadays more than 30 phenolic molecules have been described pertaining to five classes: flavonoids, lignans, phenolic acids derivatives, phenolic alcohols, and secoiridoids; the latter are exclusive for VOOs (Servili et al, 2004; Kalogeropoulos and Tsimidou, 2014). Interest of scientific communities has been recently addressed principally to secoiridoids health properties, mainly for the most representative: oleacein (dialdehydic form of decarboxymethyl oleuropein aglycon), oleocanthal (dialdehydic form of decarboxymethyl ligstroside aglycon) and oleuropein aglycon (Servili et al, 2004; Bendini et al, 2007). To the secoiridoids are attributed important anti-inflammatory activities (similar to ibuprofen) and antidegenerative anti-inflammatory activities, with prospects for use in the treatment of neurodegenerative diseases such as Alzheimer's (Beauchamp et al, 2005). To the same molecules has recently been attributed antimicrobial activity against *Helicobacter pylori*, responsible for stomach cancer (Romero et al, 2007). Nutraceutical properties of biophenols of VOO have been recently recognized by the European Food Safety Authority (EFSA) that in 2012 authorized a health claim on VOO labels declaring that a daily intake of “5mg of hydroxytyrosol and its derivatives (e.g. oleuropein complex and tyrosol) contained in 20g of olive oil may protect blood lipids from the oxidative stress” (EC Reg. No 432/2012). However this health claim, according to

Tsimidou and Boskou (2015), is still inaccurate both for the definition of the phenolic compounds included and for the lack of a clear and unique analytical protocol for quantify them.

Other important minor constituents of VOO that have a role for determining VOO quality are chlorophylls and carotenoids. Their occurrence may vary between 1 and 70 mg/kg (Psomiadou and Tsimidou, 2001). Chlorophyllic compounds (chlorophylls *a* and *b*, pheophytins *a* and *b*) are responsible for green color of VOO, *a* forms are the main components. Lutein and β-carotene are the components that confer yellow color (Psomiadou and Tsimidou, 2002). Several authors described a protection activity from autoxidation reactions with the interaction of tocopherols (Psomiadou and Tsimidou, 2001); on the other hand, chlorophylls have shown contrasting behavior acting as antioxidants at dark conditions whilst as pro-oxidants under light exposure (Psomiadou and Tsimidou, 2002b). Widely known are the positive effects of carotenoids on human health, particularly important are the preventive effects on skin and eye disorders (Piroddi et al, 2017).

Composition and quality of VOO depends on several biotic and abiotic factors, among which the most important is the genetic factor. Several studies described the variability on fatty acid, sterolic, phenolic and volatile composition, squalene, tocopherol and pigments content related to genetic factor (Angerosa et al, 2004; Casas et al, 2004; Zarrouk et al, 2009; Beltran et al, 2010; Alagna et al, 2012; Giuffrida et al, 2011; Beltran et al, 2015). Because of its strong dependence on genetic factor, and its relative stability among other factors, for fatty acid and sterolic composition of VOO have been defined by EU a range of values that characterize it and differentiate it among other vegetable oils (Reg EC No 1151/2012); it has a found also wide applications on varietal and quality label characterization (Bronzini de Caraffa et al, 2008; Ripa et al, 2008).

On the basis of varietal choice there is the knowledge of the interaction between genotype and environmental conditions, agronomical management and extraction technologies (Inglese et al, 2011).

GLOBAL AND ITALIAN VIRGIN OLIVE OIL PRODUCTION, ISSUES AND PERSPECTIVES

During the last decades the average world production of virgin olive oil increased considerably. From 1991 to 2012 global production increased more than 100%, from 1.45 to

3.40 million tons (Lynch and Rozema, 2013). During last decades of twentieth century, production growth was mainly driven by traditional EU countries: Spain, Italy and Greece. Whereas more recently, rise of production became from southern and eastern Mediterranean countries, such as Tunisia, Morocco, Algeria, Syria and Turkey. In addition, new areas of cultivation are entering the olive oil market with new surfaces under olive tree growing, mainly in South America (Chile, Argentina), United States (California), Australia and South-Africa, where climate is similar to Mediterranean regions (Lynch and Rozema, 2013). Particularly in Chile and Australia production increased rapidly during the last ten years.

Recent improve on mechanization and technological innovations on several aspects of production chain lead to increase productions and minimize costs. The impact of costs of production is different according to countries, grove density, mechanization level, labor costs, farm specialization and grove productivity itself (Lynch and Rozema, 2013).

Italian production, after a great increase until 2000s, stabilized around 400,000 tons. It covers nowadays around 16% of global olive oil production and is losing an important market share during last decades (Mattas and Tsakiridou, 2017). The causes of the lower growth and loss of competitiveness of Italian olive oil prduction are multiple. The International Olive Council, in a recent study indicated that production costs in Italy are among the highest registered in IOC production countries and reported values always over the average in all cultivation systems analyzed (IOC, 2016). High fragmentation of farms, with low dimension (1.32 ha on average), in many cases not specialized and managed at familiar level, marginality of traditional olive groves and the morphological heterogeneity of the territory slow down the spreading of mechanization and technological innovations (Deidda et al, 2006).

Traditional and marginal systems are still the major component of Italian olive groves, 50 and 20% of total acreage respectively (Santilli et al, 2011). They play an important role in terms of landscape, economic and cultural characterization, as well as environmental protection of soil and biodiversity of the ecosystem. Moreover, when managed with agronomical practices aimed to preserve soil structure, they stock carbon in soil and plants, subtracting high amounts of CO_2 from the atmosphere and promote development of soil microflora (Palese et al, 2013; Pascazzi et al, 2018). However, due to the difficult economical sustainability, most of traditional olive groves are being abandoned. It would be necessary to

be aware of the important environmental and social benefits that traditional olive systems offer, recognizing reasonable values to the deriving productions (Palese et al, 2013).

Diffusion in Italy of new growing systems like super-high-intensity (SHD) for new plantations rise slowly because of the little number of typical varieties adaptable to SHD. Another aspect that reflects the fragmentation and high costs of olive-growing sector in Italy is the high number and low dimension of mills (about 5,000) that generally operate at local scale (Lynch and Rozema, 2013).

Although, some described aspects of Italian olive sector that present weaknesses might also considered as strengths and opportunities of enhancement. The local scale of Italian milling sector allows differentiating and rising quality of productions. Interaction and information exchange with producers leads to reduce times between harvest and milling and leads to adapt better transformation of olives to producer's objectives, olive maturation stage and to olive varieties (Roselli et al, 2017).

Another important resource of Italian olive sector is the richness of varietal germplasm: 631 varieties and 827 accessions (Muzzalupo, 2012). In addition to representing an important source of biodiversity, the richness of Italian olive oil germplasm gives the possibility of differentiating productions, making it an expression of a specific territory and tradition. Moreover, valorization of VOO from specific cultivars or well defined varietal blends reflects a marketing policy aimed at offer consumers' high quality products differentiated for sensory profiles, attributing higher values and price to the products (Ilarioni and Proietti, 2014).

Most of the Italian varieties are typical of a specific cultivation area and adapted to specific pedo-climatic conditions. This important resource of variability could be exploited to develop studies aimed to face further issues like climate change and resistance to several biotic and abiotic stresses, or to identify varieties suitable for new cultivation systems. Moreover, this genetic resource leads to select new genotipes promoting breeding programs. Recent studies, aimed to identify some possible tool facing the destructive disease named "Olive Quick Decline Syndrome" (OQDS) caused by the bacteria *Xylella fastidiosa* subs *pauca*, observed that some Italian cultivars (e.g. Leccino) showed some tolerance or resistance traits to this bacteria, which dramatically affects southern region of Apulia (Giampetruzzi et al, 2016; Baù et al, 2017). Preliminary studies performed in several

important olive producing areas indicated the potential suitability to SHD systems of some Italian varieties interesting for low vigor, early production and crop efficiency: new ones derived by breeding programs like Urano®, FS 17® and KALAT (Camposeo and Godini, 2010; Marino et al, 2017) and some traditional like the Tuscan Maurino (Proietti et al, 2015; Farinelli and Tombesi, 2015) and the Sicilian Abunara and Cerasuola (Marino et al, 2017).

As well as for many others agro-food products, in the global olive market, Italian brand is still recognized as synonym of best quality in terms of both sensorial and nutritional characteristics (Lynch and Rozema, 2013), allowing Italian VOOs to differentiate among others and obtaining higher prices.

Important tools for valorize quality and typicality of food products are the geographical denominations instituted since 1992 by the European Union (Reg. EC No 2081/1992): Protected Designation of Origin (PDO), the Protected Geographical Indication (PGI) and the Traditional Specialty Guaranteed (TSG).

Italy has the highest number of quality brands: 814 names registered in the EU register, of which 291 agro-food products and 523 wines. The market of geographical indication (GI) products has been growing steadily over the last few years (ISMEA, 2017). Concerning the olive oil sector, nowadays 126 denominations (110 PDO and 16 PGI) have been registered. Italy is the country with more denominations: 42 PDOs and 4 PGI, followed by Spain (31 PDO), Greece (19 PDO and 11 PGI), France (7 PDO), Portugal (6 PDO), Croatia (4 PDO) Slovenia (1 PDO and 1 PGI) (DOOR). Requests for 7 denominations have been recently presented (4 PDO and 3 PGI).

GI oils are present in all the Italian regions except for Valle d'Aosta and Piedmont. The regions with the highest number of denominations are Tuscany and Sicily (5 PDO and 1 PGI), followed by Puglia and Campania with 5 denominations. On respect of other food sectors, in VOO segment the opportunities of product valorization offered by the geographical denominations are still not exploited at best. The production covers the 4% of total olive oil production and the 7.7% of economical value at production phase. The channels of sale are characterized by a particularly high fraction directly sold by the producer (10%). More than the half of production is destined to foreign trade, reflecting the rising global interest of higher quality of extra virgin olive oils (EVOO). The regions where PDO have a major economic

impact are Tuscany, Umbria, Apulia, Sicily, Liguria and Lombardy (ISMEA, 2017). Is interesting to notice that during the last two years the marketing policy on quality VOO is changed to a rising interest on PGI ("Olio di Calabria", "Sicilia" and "Marche") referred to a single region, as the Tuscany model. Strategy probably aimed to attract a larger number of potential consumers exploiting the better known Region's name.

OVERVIEW ON SARDINIAN OLIVE CULTURE

Cultivation of olive tree in Sardinia dates back to Phoenicians and Greek trades and developed during roman ages (Milella, 1957). Along centuries developed under Genoese, Pisan and Spanish influences (Mulas et al, 1994). Since roman ages, olive cultivation spread around peri-urban areas becoming a fundamental contribution of inhabitants' economy. Still existing examples could be found in the north-western area of the Island, around the royal cities of Sassari, Alghero and Bosa, characterized by century's old plants of Bosana variety (Dettori and Filigheddu, 2008; Dettori, 2013). Another important example of typical historical rural landscape, characterized by the presence of monumental olive trees, is the case of "S'Ortu Mannu" (dating from the 13th century: Pisan period) sited in the south-west of Sardinia (Villamassargia, Sulcis), and recently indicated as a site with national interest (Dettori et al, 2016).

The growth of municipalities and the peri-urban olive groves gave shape to bigger and complex areas of cultivation that most of them coincide with the current principal areas: Sassarese, Romangia, Coros and Algherese (Nord-West), Baronia and Nuorese, between the municipalities of Oliena and Orosei (Nord-East), Bosa, Montiferru and Oristanese area (West), Medio Campidano (South-West), Parteolla and Marmilla (South), Ogliastra (East).

The Sardinian olive and oil chain plays a little role on national olive sector: during the last years it covered around 3% of dedicated surface and 1.5% of total national oil production.

In line with national framework, Sardinian olive sector is fragmented: dimension of farms do not achieve 1 ha, 120 active mills are present along the Island. It presents several points of weakness, in terms of productivity, mechanization, costs and professional skills (Bandino and Dettori, 2001).

Sardinian olive germplasm is rich and diverse. Bandino et al (2001), utilizing morphological and structural parameters, described 28 varieties and 62 accessions. They are principally spread in traditionally growing areas. Genetic studies grouped some of the 28 typical local varieties into groups of synonymies (Erre et al, 2010).

The most widespread Sardinian cultivar is Bosana, including about the 65% of the cultivated trees (Bandino et al, 2011). It is mainly diffused in north-western areas. Due to its low resistance to *Spilocaea oleagina* does not adapt well to the warmer and more humid climate of south Sardinia (Bandino and Dettori, 2001). Its main use is oil production, well known for the relatively high content in phenolic compounds (Rotondi et al, 2010; Campus et al, 2013). Oils have particularly bitter and pungent sensations accompanied by characteristic flavors of artichoke, fresh almond and grass/leaf (www.olimonovarietali.it).

Tonda di Cagliari (also known as Nera di Gonnos) is the second common cultivar, diffused mainly at south (3,000 ha). It is also known as Sivigliana da Mensa in the northern areas and Majorca or Confetto in the Baronia and Ogliastra areas. It is mainly grown for olive table production; it can also be used for oil extraction thanks for the good oil yield (Bandino and Dettori, 2001).

Semidana, principally diffused in the Oristanese and Montiferru, recently has trigged interest for high and constant yields coupled with low vigor. Its main use is for oil extraction. Semidana VOOs show medium-high phenolic contents (Fadda et al, 2012; Campus et al, 2013) and are characterized by good sensorial balance and typical grass, tomato leaf, artichoke flavors (www.olimonovarietali.it).

Tonda di Villacidro (also known as Nera di Oliena) is widespread principally in the Medio Campidano region and in Nuoro Province. It can be found also in the rest of the territory known with diverse synonymies, it is used both for oil and table olives production (Bandino and Dettori, 2001). VOOs of Tonda di Villacidro have recently caught broad appreciation thanks to the peculiar high fruity sensations, grass/leaf and tomato flavors (www.olimonovarietali.it).

Less widespread varieties are Pizz'e Carroga, Pezz'e Guaddu, Cariasina, Corsicana da Olio and Sivigliana da Olio. Generally present as single plants in traditional secular olive groves.

Sardinian germplasm have been studied under several points of view, regarding mainly the principal and most widespread varieties. Some studies aimed to evaluate productivity and some agronomical parameters have been carried out (Bandino et al, 1999; Bandino et al, 2002; Mulas et al, 2010). Moreover, phenological behavior and interaction with weather conditions of Bosana and Nera di Gonnos were described together with preliminary predictive models (Nieddu et al, 1997; Nieddu et al, 2002; Cesaraccio et al, 2006).

VOOs from the principal four Sardinian varieties was described by some authors on regard to its chemical composition, principally fatty acids composition, triacil glicerols, unsaponificable fraction and volatiles compounds (Angerosa and Basti, 2003; Giansante et al, 2003; Rotondi et al, 2004; Gallina-Toschi et al, 2005; Cerretani et al, 2006; Del Caro et al, 2006; Fadda et al, 2012; Campus et al, 2013; Beltran et al, 2015; Tuberoso et al, 2016). Moreover, recent studies on Bosana, Semidana and Tonda di Cagliari described the influence of genetic factor on the presence of microorganism in olives paste during extraction process and VOO produced (Santona et al, 2018).

Some recent studies investigated on the influence of geographical origin on VOOs from Bosana, identifying some biomarkers typically influenced by environmental conditions (Culeddu et al, 2017).

The only PDO for VOO registered in Sardinian territory is named “Sardegna” (2007). It plays a little role on the total regional production (around 2.5%) as well as within national PDO production: 1.8% (ISMEA, 2017). PDO Sardegna can be produced with olive trees cultivated along the whole regional territory. According to the application for registration submitted on March 18, 2003, it must be characterized at list for the 80% by the four principal Sardinian varieties: Bosana, Tonda di Cagliari, Tonda di Villacidro, Semidana and the respective synonymies. In the remaining 20% could be included oils from other varieties grown in the regional territory. Quality standards such as low free acidity (< 0.50%) and peroxide content (< 15 meq of O_2/kg of oil), total phenolic and tocophenol content (≥ 100 mg/kg of oil) are indicated in the label regulation, together with the general sensorial evaluation (median ≥ 7).

Compared to the other Italian VOO PDO, “Sardegna” is the one that covers the biggest portion of national territory; furthermore, four varieties are admitted to make part of the 80%

of total composition that, considered the synonymies, became 17 varieties. The size of the territory and the high number of possible varietal blends make difficult the characterization, in terms of chemical composition and sensorial profile, of this label.

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AIMS OF THE DOCTORAL THESIS

As well as Italy, Sardinian olive-oil sector presents wide margin of improvements regarding production, new technologies and product valorization.

There is a lack of information on regard to minor local varieties. Within an international framework characterized by an increasing interest on higher quality, provenience and typicality of virgin olive oils, the reconsideration and valorization of specific values of local varieties might by a successful marketing strategy for both Italian and Sardinian productions. Indeed, detailed information on regard to the minor varieties and synonyms of the principal, can lead to improve the quality of actual productions and of the PDO Sardegna label, selecting the varieties that can contribute better to the production objective.

Moreover, the knowledge of local germplasm will be an important tool for adapt regional olive sector to innovative technologies both for growing systems and extraction processes.

Local varieties are well adapted to specific microclimates. They might be a resource on facing climate changes that indicate Mediterranean basin as one of the areas that might be strongly involved. A general increase of temperatures and extreme weather events are expected. Climate changes might affect the selection of varieties with specific chill or heat requirements; moreover, interaction between traditional pests and olive tree might change. On this regard is fundamental to study deeply the phenological behavior of varieties in order to be able to forecast with relative accuracy specific vegetative and reproductive phases, for instance the onset of vegetative development, flowering period and fruit maturation. These are useful tools for support producer's decisions, optimizing several key field operations such as pest management, fertilization, pruning or varietal selection. As well as olive phenology, climate change may affect chemical VOO quality, it is important to evaluate varietal adaptability also under this point of view.

The research carried out during the three years of PhD was focused on the overall evaluation, at same environmental and agronomic conditions, of most of the Sardinian varieties admitted to PDO Sardegna: Bosana, Semidana, Tonda di Cagliari, Tonda of Villacidro and their synonyms, compared to local varieties of lesser diffusion, national and international varieties.

Particularly, on regard to some varieties considered synonyms of the principal Sardinian ones and to minor local varieties, this is the first time that information on tree phenology and chemical composition of respective VOO have been provided.

Research has been divided into three main parts written as paper format to be submitted as peer review journal:

1. EXPERIMENT 1: phenological study of Sardinian and national olive varieties aimed at identifying the various varietal responses to the meteorological conditions during early vegetative and reproductive phenological stages, with particular attention to the differences between the groups of synonymic varieties present in the field.

2. EXPERIMENT 2: potential variability and quality on VOO composition of PDO Sardegna connected to the high number of varieties that might characterize it, compared to national and international varieties.

3. EXPERIMENT 3: evaluation of the influence of harvest period of fatty acid and phenolic composition of VOOs obtained from Bosana, Semidana, Tonda di Cagliari and Coratina.

EXPERIMENT 1: PHENOLOGICAL BEHAVIOR OF LOCAL (SARDINIA) AND NATIONAL VARIETIES

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ABSTRACT

Olive tree (*Olea europaea* L.) is considered an indicator species for Mediterranean climate changes, due to its sensitivity to seasonal weather variations. A detailed description of first vegetative and reproductive phenology (from the onset of vegetative growth to fruit set) of 20 typical Sardinian olive varieties, compared to 6 varieties widespread at national level, was carried out during a 3 year study (2015 – 2017). Plants were cultivated at same environmental and agronomical conditions in order to evaluate the influence of genetic factor, growing year and interaction on phenological behavior without any other source of variability. Moreover, the effect of canopy sun exposition was taken into account. Average monthly and seasonal temperatures were calculated and correlated with the onset of vegetative growth, onset of pre-flowering, onset of flowering, full flowering and respective length of pre-flowering and flowering phases. Heat requirements (growing degree days, GDD) necessary to achieve full flowering phase was calculated for the four principal Sardinian varietal groups (Bosana, Manna, Semidana and Terze). Two GDD calculation methods and three accumulation starting dates were tested. Finally a large range of temperatures was tested in order to find the most suitable lower threshold temperature for estimating full flowering occurrence days. Results showed that the trend of the main meteorological parameters in the growth year is the factor that mainly affects phenology of olive tree. Differences between varieties were observed, both for occurring of phases and interannual variability. A clear effect of canopy sun exposition was observed only for flowering phase. Correlation analyses with weather related variables revealed a positive influence of November temperatures on the onset of both vegetative and reproductive phases. Increase of average temperatures of the period February-April seems to cause an advance of onset of flowering and full flowering. Optimum lower threshold

temperatures obtained (13 – 17 °C) showed slight differences between groups and tested methods. In conclusion, knowledge of early vegetative and reproductive phenology can be a useful tool to identify difference or similarities between varieties and support producer's decisions on key field operations like harvest time, pruning, fertilizing and pest management.

INTRODUCTION

Mediterranean basin is the principal area of olive cultivation and plays an important socioeconomic and traditional role in countries of major production. Spain, Italy, Greece, Turkey, Tunisia and Portugal cover together more than 95% of worldwide oil production (IOC).

Mediterranean basin is considered as a hotspot of climatic changes. Recent projections indicate increase of temperatures of about 1.5°-2.0°C and a substantial decrease of precipitations of about 5% (Gualdi et al, 2013). Phenology and productivity of olive are strongly influenced by climatic conditions; this sensitivity to seasonal weather variations led olive phenology to be considered as a good indicator of Mediterranean climate changes (Osborne et al, 2000). Several studies that investigated long temporal climatic trends reported a gradual anticipation on flowering and emission of pollen (Orlandi et al, 2014; Aguilera et al, 2015a). Moreover, the same authors observed in different areas of Mediterranean basin a decreasing trend in air pollen concentration that might affect negatively subsequent olive production. Orlandi et al (2014) evidenced that increasing temperatures may affect olive mainly in terms of water consumption during late spring and summer, while changes in winter weather related variables might not affect particularly olive production. Climate change may affect also biological behavior of pest insects related to olive tree, such as olive fly [*Bactrocera oleae* (Rossi)], whose population is expected to increase in inland areas at higher elevation, where upper temperature limits might be not achieved (Ponti et al, 2014).

Seasonal temperature is the weather related variable that mostly affects olive phenology (Spano et al, 1999). Several authors observed that negative correlations between average temperatures of the months preceding flowering period are able to predict the appearing of the phase with good accuracy (Bonfiglio et al, 2008; Perez-Lopez et al, 2008; Orlandi et al, 2009, 2010; Agilera et al, 2015a; Rojo and Pérez-Badia, 2015), and that the average temperatures of the period March-April-May were the more effective variables. According to Spano et al (1999), native species of Mediterranean basin, included olive tree, do not show great response to water availability on regard to flowering date, whereas other authors indicated that precipitation events during the month preceding flowering may delay the reproductive process (Bonfiglio et al, 2008). Nevertheless, have been demonstrated that water availability during fruit growth period have an important role in modulating the production of the next year

(Deidda et al, 2003). Some authors recognized that inclusion of photoperiod variable in flowering prediction models might improve their accuracy (De Melo-Abreu et al, 2004; Garcia-Mozo et al, 2009). Other variables directly connected to temperature conditions, such as sun exposition, and solar radiation have demonstrated to play a role in determining timing of reproductive phases (Cesaraccio et al, 2006; Rojo and Pérez-Badia, 2014).

Biological developing rate of plants is strictly connected to air temperature. Predicting models expressed this relationship with the concept of degree days (DD), this relationship is assumed to be positive and linear within determinate lower and upper temperature thresholds (Cesaraccio et al, 2001). In order to achieve a determinate phenological phase, plants need to accumulate heat units, i.e. growing degree days (GDD). Several methods for the GDD calculation based on daily or hourly temperatures have been proposed (Arnold, 1960; Zalom et al, 1983; Yang et al, 1995; Cesaraccio et al, 2001). An important issue, largely discussed during the last years, is the determination of the time when heat accumulation begins. Usually, both for crop and forest plants, the starting date of heat accumulation, to predict the occurring of a specific phase (e.g. flowering or maturation), correspond to the end or the onset of a preceding phase or to an established conventional date (Spano et al, 1999; Pérez-Lopez et al, 2008; Galán et al, 2005). On regard to early vegetative and reproductive stages of olive tree, some authors stressed on the importance to consider the time of the fulfillment of chilling requirements necessary to break dormancy as the onset of heat accumulation (De Melo-Abreu et al, 2004; Galán et al, 2005; Aguilera et al, 2014). During winter season, olive tree enter a period of dormancy in which physiological activity is reduced or stopped, a determinate quantity of "chill units" during this period are necessary to release initiated floral buds' dormancy (Rallo and Martin, 1991).

Mediterranean climate is characterized by high year to year variability and heterogeneity in microclimate, landforms, soils and cover crop (Spano et al, 2013). Due to climate variability, a large range of GDD values and different optimum temperature thresholds (TT) have been reported in literature for olive flowering, among a wide range of typical growing areas of Spain, Italy and Tunisia. According to several authors, bioclimatic factors such as latitude, altitude and distance from the sea can affect the phenological behavior of olive tree, indicating a positive correlation with latitude and date of flowering (Galán et al, 2005; Orlandi et al, 2005; Orlandi et al, 2009; Aguilera et al, 2014; Rojo and Pérez- Badia, 2014).

On regard to onset of flowering, Rojo and Pérez-Badia (2014) observed a delay of 2.5 days each 100m increment in altitude, attributing this trend to the lower heat accumulation occurring at higher altitudes. According to Aguilera et al (2014), olive varieties grown in southern Mediterranean regions (Tunisia) achieve flowering period earlier in respect to northern regions, as a defense mechanism to avoid temperatures above 30-35°C occurring on late spring months, that are detrimental for a correct flowers development and fertility (Barranco, 2007). Garcia-Mozo et al (2009) stressed on the importance to consider varieties adapted to specific local meteorological conditions when a predictive phenological model is developed. Differences in varietal phenological behavior have been also recognized to several fruit crops (Spano et al, 1999; Ruml et al, 2010).

Knowledge of the dynamics of olive phenology has several practical applications: for instance, it could help for choosing varieties according to their adaptation to spring frosts or late spring high temperatures. Predict flowering period may help producers on agricultural practices applications, such as ripening time, fertilization, pruning or pest control (Rojo and Pérez-Badia, 2015). Moreover, some models aimed to predict olive production according to pollen intensity have been proposed (Galan et al, 2004).

Despite a favorable climate for olive growing, Sardinian olive production plays a little role in Italian olive market, around 1.5% of total national olive production (ISMEA, 2017). - Olive cultivation spreads along mesomediterranean – mainly in northern Island, inland and hilly areas – and thermomediterranean – southern lowlands and coastal areas – climates and is concentrated in traditional areas of cultivation (Bandino and Dettori, 2001; Canu et al, 2015). As well as Italy, Sardinian germplasm is rich and diverse, 28 varieties have been described by morphological and structural parameters (Bandino et al, 2001). Recently, similarities and synonymies within Sardinian germplasm and between Sardinian and Italian varieties have been discovered throughout genetic studies; varietal groups referred to some of the principal Sardinian varieties were identified (Erre et al, 2010).

Bosana, Tonda di Cagliari, Semidana and Tonda di Villacidro are the local varieties most widespread in Sardinia (Bandino and Dettori, 2001). Some studies were carried out on Bosana and Nera di Gonnos, synonym of Tonda di Cagliari, describing phenological behavior in the northern region of Sardinia (Nieddu et al, 1997; Canu et al, 1998; Nieddu et al, 2002;

Cesaraccio et al, 2006). Less information is available on regard to the phenology of other minor local varieties (Chessa et al, 2000).

The aim of this study is to describe the phenological behavior, during early vegetative and reproductive development, in a Mid-West site of Sardinia, of 20 local Sardinian varieties, compared with 6 of the most widespread varieties in the Italian peninsula, in order to evaluate the differences or similarities within the local germplasm and evaluate the behavior of some minor local varieties. A further objective was to give a preliminary analysis of the relationships occurring between the analyzed varieties and weather related variables. A deeper study was carried out on the principal Sardinian varietal groups, evaluating phenological differences and heat requirements for flowering phase.

MATERIALS AND METHODS

Study area

The area of the study is placed in the Experimental Station "A. Milella" of the Department of Science for Nature and Environmental Resources, located at San Quirico – Fenosu, Oristano, Italy (39°54'12" N, 8°37'19" E, 13m a.s.l).

The bioclimate of the area has been classified as "Mediterranean Pluviseasonal-Oceanic; isobioclimate 6: Upper Thermo Mediterranean, Lower Dry, Euoceanic Weak" (Canu et al, 2015). This bioclimate is typical for the biggest Sardinian lowland called "Campidano" that spreads from Oristano province to the southern Cagliari region. The Thermo Mediterranean thermobioclimatic belt comprises also the coastal and other lowlands areas of Sardinia (Canu et al, 2015). Soils are alluvial deposits classified as Typic, Aquic, Utic Palexeralfs, Xerofluvent, Xerofluvent, Ochraqualfs (Aru and Baldaccini, 1992).

Weather data (2006-2017) from the Oristano weather station, daily maximum (Tmax), minimum (Tmin) and average temperatures (Tmean) and precipitations (Prec), were kindly provided by the Department of Meteorology and Climatology Environmental Protection Agency of Sardinia (ARPAS). The annual mean rainfall is 580 mm, precipitation are mainly concentrated in autumn and winter months (i.e. November and January are the rainiest months, 105 mm and 80 mm respectively); the annual average temperature is 17.1 °C, with an average maximum at 23.9 °C and minimum at 11.3 °C. Winters are mild (February is the

coldest month) whereas summers are hot and dry, with July and August as the warmest months (Figure 1). The intermediate season is characterized by high variability of temperatures and precipitations and by a constant high humidity. The area of study is characterized by western dominant winds (Spano et al, 1999) that might mitigate air temperatures or bring humidity and saline air from the close sea.

Plant material

The trees analyzed were from a collection field established in 1998 at a space of 6 x 6 m and drip irrigated with ca. 2500 m^3/ha during the period June-October.

In the experimental field were present 28 varieties, 21 of which autochthonous Sardinian varieties and 7 from Italian germplasm. Three trees per cultivar are present. The presence in the same field of all the varieties gave the possibility to focus the study on the differences related to the cultivar.

The varieties (26) objects of this study were grouped as follows:

1. **Bosana**: Bosana, Palma, Sassarese, Olieddu.
2. **Manna**: Tonda di Cagliari, Nera di Gonnos, Confetto, Sivigliana da Mensa, Maiorca.
3. **Terze**: Tonda di Villacidro, Nera di Oliena, Terza Grande, Terza Piccola, Paschixedda, Corsicana da Mensa, Itrana.
4. **Semidana**: Semidana, Bianca di Villacidro.
5. **MinorSardinia**: Corsicana da Olio, Sivigliana da Olio, Pizz'e Carroga.
6. **SouthItaly**: Coratina, Carolea.
7. **Tuscany**: Frantoio, Leccino, Santa Caterina.

The first four groups were proposed on the basis of (1) our preceding field observations, (2) genetic similarities (Erre et al, 2010) and (3) morphological characteristics (Bandino et al, 2001). Varieties included in groups "MinorSardinia", "SouthItaly" and "Tuscany" does not show strong genetic or morphological similarities. So, they were grouped according to their geographical origin (Muzzalupo, 2012) in order to simplify the further results and discussion.

Data collection

Phenological data were collected following the BBCH scale modified for olive trees by Sanz-Cortés et al (2002) as reported in Table 1. Moreover, in our analysis, we proposed

phenological periods in according with those described by other authors (Rojo and Pérez-Badia, 2015).

Three plants per variety were monitored. Four branches (50 – 100 cm length) per tree were selected, oriented by the cardinal points and positioned at a height between 1.5 and 2.0 m. Twelve (3 trees x 4 cardinal points) observations per cultivar were made weekly during the earlier vegetative growth and pre-flowering phases, and twice a week during the period from flowering until fruit set. The phenological phases described on the following principal growth stages were monitored: bud and leaf development, inflorescence emergence, flowering. Phase BBCH 07, considered in this work the onset of vegetative development, was reported as: onVEG. Phase BBCH 51 was indicated as "onPRE" (onset of pre-flowering). The pre-flowering period (PRE) was calculated as the number of days occurring between onPRE and the opening of the first flowers (BBCH 60). Phase 61 was indicated as "onFLO". The flowering period (FLO) was calculated as the number of days occurring between phase 61 and 69 (fruit set). Finally, phase 65 was indicated as full flowering (FF).

Observation data were linearly interpolated in order to obtain a date expressed as *days of year* (DOY). A database containing twelve observations for phenological BBCH phase per variety, for the three years of study, was obtained.

Data analysis

Dataset general analysis

At first a preliminary analysis of dataset was performed with the use of ANOVA analysis.

The influence of field position on the occurring of phenological phases during growing season was verified. On this purpose were utilized data from Manna and Terze groups because the plants were uniformly distributed in the field (Figure 2). Analysis was performed utilizing two variables: plant position in north-south oriented rows– explaining the influence of proximity to western or eastern borders – (NorthSouth) and plant position in east-west oriented rows – explaining the influence of proximity to northern or southern borders – (EastWest). Two linear mixed models (lme) were performed as follows: DOY as response variable, BBCH phase and NorthSouth or EastWest position as fixed factors, plant and sun exposition (Exp) as random effects. Models were run year by year, for vegetative (BBCH 07-19) and reproductive (51-69) phases separately.

Lme analysis was performed also to verify if the relationship between phenological phases (BBCH) and DOY – as expected positive – was influenced by variety, varietal group or exposition (Exp). Interaction between aforementioned factors and BBCH was included in the model. Lme test was considered the most proper to evaluate these factors because of the presence of repeated measurement of the same plant, exposition and cultivar. Two models were performed: 1) in *model1* DOY was the response variable, BBCH, cultivar and Exp were the fixed factors while plant and Exp were considered in the model as random effects; 2) in *model2* the cultivar factor was substituted by varietal group. Models were run year by year, for vegetative and reproductive phases separately.

Furthermore, it was evaluated whether the difference of BBCH phases occurring between years was significant and if this difference was influenced by cultivar, varietal group or Exp. Two lme models were performed: 1) in *model3* DOY was the response variable, BBCH, year and cultivar were the fixed factors while plant and Exp were random effects; 2) in *model4* group was the third factor instead of cultivar.

Once checked the effectiveness of cultivar and varietal group, a more thorough analysis was performed on Bosana, Manna, Semidana and Terze. A linear discriminant analysis (LDA) was performed in order to evaluate the capability of phenological phases, utilized as single variable, to discriminate genetic varietal groups. In addition, a canonical discriminant analysis (CDA) was carried out in order to verify the presence of phenological phases with more discriminant power.

Statistical analysis was performed by the use of R Studio software (2017).

Weather data analysis

The following weather related variables were calculated: monthly average values of Tmax, Tmin and Tmean from the previous November until May. Moreover, average values for the periods: November-December-January (NDJ), February-March (FM), and February-April (FMA) were also calculated. The calculated variables were correlated (Pearson test) with the following phenological data: onVEG, onPRE, onFLO, FF, length of PRE and FLO periods, separately for each cultivar.

GDD calculation and minimum threshold temperature determination

Single triangle method and single sine method (Zalom et al, 1983) were used to calculate daily degree days (DD). The heat requirement, expressed as growing degree days (GDD), of onPRE and FF was calculated testing different starting dates: 1st January and the occurring date of onVEG; for FF was also tested the respective occurring date of onPRE. Different threshold temperatures (TT), from 0 to 20°C, were tested to find the most suitable threshold value able for estimating the days of selected phases. A specific optimum TT was calculated for the groups Bosana, Manna, Terze, Semidana and a reference group that included Frantoio and Leccino. The optimum TT and GDD calculation method was selected calculating the RMSE between observed and predicted days (Snyder et al, 1999).

RESULTS

Phenological data analysis

The influence of cultivar, group and canopy sun exposition was first verified throughout analysis of variance. One way ANOVA was performed for single phenological phases, from phase 07 until phase 69. Significant differences for cultivars and groups factors were observed (P-value < 0.001) in all analyzed phases during the three years of study; lower significances were observed (P-value < 0.01) during first vegetative growth and inflorescence development, mainly in 2017. Sun exposition seemed to be an influent factor for the duration of flowering process in all three years, while less significant differences between cardinal points were observed during leaf and inflorescence development, particularly in 2015 and for all phases in 2016. Phenological phases, in most of the cases, appeared earlier in southern and eastern sides of the tree.

The effect of dominant winds was evaluated together with the effect of the position of plants in the field, as the effect of NorthSouth row position (see materials and methods). Vegetative and reproductive development of Manna and Terze groups was analyzed singularly, uniformly distributed within the field (Table 2). In group Manna was not observed any influence of field position, neither for NorthSouth nor for EastWest rows, while in some cases was observed a significant interaction between field position and phenological phases. A different behavior was registered in Terze varieties which showed no influence of western dominant winds exposition (NorthSouth) but a recurrent influence of position in EastWest rows; in this case was observed an advance in phase occurring in plants close to the southern

side of the field (Figure 3). The interaction between BBCH and field position, was significant in 5 cases over the 12 considered in Manna, while in 7 cases for Terze, indicating a different "border effect" throughout phenological behavior.

Lme analysis confirmed the significant positive relationship between time and phenology for all growing seasons, both for vegetative and reproductive phases. In all the analyzed cases, (tables 3 and 4) phenology was significantly influenced by cultivar and varietal group. Interaction between phenology and both factors was significant, indicating that varieties and groups have different phenological behavior mainly due to the different duration of processes. On regard to Exp effect, was observed that southern and eastern face were generally anticipated than northern and western ones. As ANOVA analysis revealed, this factor affected less the phenological behavior, not significant in 2015 and 2016 during vegetative development. In addiction, was not observed significant interaction CV:Exp and VG:Exp indicating that the slight effect observed did not change according to variety or varietal group; on the other hand, the interaction BBCH:Exp was significant for vegetative phases of 2015 (model1) and during 2017 for both vegetative and reproductive phases (model1 and model2). The latter result indicates that some specific phenological phases might be more sensitive to the slight differences of temperature caused by different exposition to the sun of the canopy.

Finally, the effect of year for both vegetative and reproductive phases was significant, as well as for the interaction between growing season and cultivar or varietal groups. In 2016 vegetative phases compared with the other two growing seasons appeared earlier (Figure 4). Clear differences between 2015 and the other two growing seasons were observed during reproductive phases (Figure 5); 2015 started on average more than 10 days later than 2016. Anyway, the gap between growing seasons diminished gradually until fruit set. 2017 was an intermediate year, more similarly to 2015 during early vegetative phases, while comparable to 2016 during reproductive phases. Varieties responded differently to the growing season conditions, mainly as far as concern 2016 and 2017.

As previously mentioned, a clear difference between groups and varieties was observed. The four proposed groups of Sardinian varieties (Bosana, Manna, Terze and Semidana) were deeper analyzed with the use of CDA, cross-validated with "leave-one-out" cross validation technique, showing a good but not complete separation. The first two canonical functions did not separate well Semidana from Terze and Bosana groups (Figure 6). In Figure 7 we can

observe that the introduction of the third canonical function improved the separation between Terze and Semidana. Throughout the same graphs is possible to observe that the effect of variables representing vegetative and reproductive phases was different (figure 6). Moreover, vegetative phases and earlier stages of flowering seem to have more discriminant power. In Table 5 the LDA results, cross-validated with "leave-one-out" cross validation technique, are reported. In 2015 and 2016 was achieved an accuracy, expressed as the ratio between reference and predicted groups, of 0.89, and 0.87 in 2017 (table 5). Due to the marked difference between the three growing seasons, a lower discrimination among groups was observed when a dataset including the three years was used (accuracy = 0.73). The separation by year was correct at 95% (table 6). Moreover the CDA graph in Figure 8 represents the observations grouped by year, indicating a clear difference between 2015 and 2016, while an intermediate position of 2017. These results suggest a stronger influence of the growing season if compared to genetic factor.

Bud and leaf development (BBCH 07-19)

Focusing on single varieties, we can observe that Leccino was the first variety that expressed a clear restart of vegetative growth (Table 7), followed by Sivigliana da Mensa, Sassarese, and the Semidana group varieties, Bianca di Villacidro and Semidana, together with the other two varieties from Tuscany, Frantoio and Santa Caterina. However, Tuscany varieties were the ones that showed the highest variability between years for this specific phase (CV 31.1-35.1%), while Bosana and Manna groups showed the lowest variability (CV 12.5 – 27.5%). Last varieties for which was observed the restart of vegetative growth belonged all to Terze group (55 – 59), with an average delay of 11 days.

Leccino and Frantoio were also the ones that achieved first the typical leaves dimensions, BBCH 19 (Table 7), together with Coratina from Apulia, and the minor Sardinian Corsicana da Olio. Also for phase 19 the latest varieties belonged to Terze group, even if three of them (Itrana, Paschixedda and Nera di Oliena) manifested 3-5 days earlier the phase.

Interannual variability decreased during vegetative development (5 – 12.8%) and was lower within Sardinian varieties. Different variability was observed between varieties within the same group, suggesting that, during first growing stages, varieties react differently to seasonal conditions even within genetic groups.

Inflorescence emergence and flowering (BBCH 51-69)

Phase 51 was first observed in varieties belonging to Manna group, mainly in Confetto and Sivigliana da Mensa (Table 7), as well as Pizz'e Carroga that showed a constant increasing advance until fruit set (BBCH 69). As for vegetative development, the varieties of group Terze (DOY 78 – 87) were the latest on starting the reproductive phase, similarly to Frantoio, Leccino, Carolea and Semidana group. These varieties maintained similar timing along the whole inflorescence development. During the first reproductive phases interannual variability ranged from 8.6% (Terza Grande) to 26.6% (Sivigliana da Mensa). Then, variability decreased gradually until fruit set, both within and between varieties. The average duration of inflorescence development ranged from 30 days (Carolea, 2015) to 66 days (Sivigliana da Mensa, 2016). Similar behavior was observed between the varieties belonging to the same groups, the group Terze showed the lowest lengths, while Manna and Semidana the largest.

Behavior and trends observed among cultivars on phase occurring and length was generally confirmed also during flowering phases. Flowering phases occurred in a time period between DOY 127 and 148 during 2015, 111-144 in 2016, and 119-146 in 2017. As for macrophase 5, during flowering process, the differences between the three growing seasons decreased until fruit set. The flowering process lasted between 11 days (Palma, 2015) and 26 days (Confetto, 2016). In 2016 phase 6 lasted longer for almost all the cultivars. Bosana and Semidana groups stood out from the other groups for a short flowering period, in contrast to Manna and Terze, which maintained open flowers for a larger period. In addition, in Terze group was particularly visible the effect of sun exposition.

Pizz'e Carroga, together with Manna group were the earliest and more variable cultivars, while Terze, Semidana and the Tuscany Leccino and Frantoio confirmed their behavior as least cultivars. Flowering period, and even more fruit set timing, of these last cultivars seemed to be less affected to seasonal weather conditions.

Meteorological data

Figure 9 shows monthly temperatures variations from the average year (2006-2017) during the three studied growing seasons from November to May. A particularly warm November was observed in 2014, while in 2015-2016 and in 2016-2017 seasons were

registered higher temperatures in December and February. Tmax recorded in the studied period were almost constantly higher during 2016 and 2017, while a general decrease of Tmin was observed mainly in 2014-2015 and 2016-2017.

In table 8 a summary of the results of the Pearson correlation analysis is reported, indicating the number of varieties that showed a significant correlation (P-value < 0.05) with weather related variables and the sign of correlation in brakets. Probably due to the low number of years analyzed, few varieties showed significant correlations between phenological phases and weather related variables, nevertheless indicating which variables might affect stronger the phenological behavior in the studied area.

Temperatures of November seem to be an influent variable as well as Tmax and Tmean of December. The onset of vegetative development, onVEG, seemed to be influenced mainly by minimum temperatures of December and January; higher temperatures during December might cause a delay of the phenological phase occurrence, while opposite effect seem to have Tmin of January. An opposite effect of December temperatures was observed for the occurring of onPRE, onFLO and FF; in these cases significant correlations were observed also for Tmax and Tmean. February temperatures seem to be an influent factor on determining onPRE, showing a negative correlation, as well as the average Tmin of February and March (FM). An interesting positive correlation was found with Tmin of March, as observed also in the case of FF.

Apart from November and December temperatures, with respectively positive and negative correlations, the onset of flowering phase (onFLO) was negatively affected by the average temperature of the period February-April (TmeanFMA), as well as the occurring date of FF. The latter was also affected by Tmax of November and December, Tmin of February and March.

A negative relationship was observed between onPRE and PRE, as well as for onFLO and FLO, indicating that when the phase begins earlier lasts more time. Both period analyzed, PRE and FLO, gave more significant correlations than the respective onset dates. November, December, February and March, together with TmeanFMA seem to be the periods that influence more pre-flowering length. On regard to FLO, significant correlations for all months taken into account were observed. May was the month with the highest number of significant

correlations, a negative correlation of Tmax whereas positive of Tmin was observed. A contrasting effect between Tmax and Tmin was also observed in March, with an opposite behavior on respect to May temperatures.

GDD and lower temperature thresholds calculations

Results of GDD and temperature thresholds calculations are reported in Table 9. Little differences were found between the two calculation methods; anyway, at the same starting date, single sine method was more precise for all varietal groups. Different optimum thresholds (TT) were observed between groups. Optimum TT varied from 13 °C in Bosana and Manna, using single triangle method and 1st January as starting date (T-J), to 17 °C obtained for the other three groups, using both calculation methods and using both onVEG (S-07 and T-07) and onPRE (S-51 and T-51) dates as the beginning of heat accumulation. In general, GDD calculations from these two starting times gave higher TT. For methods T-51 of Bosana and Manna and S-51 for Manna (Table 9) the best TT was observed at 0°C. Nonetheless, the curve of the same methods was comparable to the curves obtained for the other groups because showed other minimum around 15 and 16 °C. The lowest RMSE values, for all varietal groups analyzed, were obtained adopting S-J method. The five varietal groups, on average achieved full flowering (FF) at similar dates, between 9 and 14 May (DOY 129-134). Varietal groups that achieved earlier the phase (Bosana and Manna), together with Terze, were the ones that showed the lowest TT and the highest heat requirements.

DISCUSSION

Canopy exposure was the less effective factor affecting olive phenology evaluated in this study. Its effect was observed mainly during flowering phases. In a study carried out in northern Sardinia (cultivar Bosana), pre flowering and flowering phases were different in terms of timing and lasting of phases according to canopy exposure (Cesaraccio et al, 2006). The same authors attributed the recurrent advance of southern and eastern sides to the indirect influence of temperatures, supposedly higher in the sides of trees better exposed to the sun, determining differences in heat accumulation. The same trend between north and south face of the tree was reported by Rojo and Badia-Perez (2014).

Our findings highlighted that genetic factor have an effect on phenological behavior, both for time of occurring and duration of phases. Similarities between varieties belonging to the

same groups were confirmed, particularly for those grouped mainly on the base of genetic evidences. Moreover, we observed that varieties with an earlier development, e.g. Pizz'e Carroga and varieties of the Manna group, showed a higher variability between lengths of growing seasons on respect to other varieties, e.g. Terze, characterized by a later development. Differences between varieties were widely described in literature, in terms of adaptation to determinate environmental conditions such as chill requirements (Rallo and Martin, 1991; Aguilera et al, 2014), resistance to frost (Orlandi et al, 2002), and heat requirements for reproductive development (Reale et al, 2006; Garcia-Mozo et al, 2009).

Timing of flowering phase in Oristano was similar to those described by other authors for southern Italian regions, mainly Sicily and southern coastal areas of Apulia (Orlandi et al, 2005, 2009, 2010); similar dates were also found for some growing areas of Andalusia, Spain (De Melo-Abreu et al, 2004; Aguilera et al, 2015a). On the contrary, our results differed from those obtained in the northern area of Sardinia, a typical area of olive cultivation where the presence of Bosana trees is predominant (Nieddu et al, 1997, 2002; Cesaraccio et al, 2006). The authors showed a later occurrence of reproductive phases, up to two weeks when compared to our results regarding the different phenological development of Bosana and Nera di Gonnos, that showed an anticipate behavior, according to those reported in previous studies (Nieddu et al, 2002). The similarities and differences described could be attributed to the bioclimate that characterizes the area of Oristano. The upper termo-mediterranean thermotype, similarly to Oristano, is typical of several areas of Sicily and southern Apulia (Pesaresi et al, 2014), whilst the area around Sassari falls within the lower meso-mediterranean belt (Canu et al, 2015). Our phenological data felt in the range of dates described by Spano et al (1999) for floral bud burst and full flowering of olive trees, obtained during a study performed in the same area of study.

In any case, as revealed also by the results of discriminant analyses, the factor that most affected the phenological behavior of olive trees was the growing year and connected weather conditions. Particularly, the varieties that begin first inflorescence development and flowering exhibited higher variability between years.

According to our findings, higher temperatures during winter season, particularly during November and December, might cause a delay on the onset of both reproductive and vegetative development, as observed also in Cornicabra olive groves growing in Toledo

(Spain) (Rojo and Pérez-Badia, 2015). The positive correlation found between winter temperatures and both vegetative and reproductive budburst might be attributed to a delay in the achievement of chilling requirements (Rojo and Pérez-Badia, 2014).

Onset of pre-flowering and flowering were principally affected by the temperatures of the months immediately preceding the phase, February and March for pre-flowering, also April for flowering. Bonfiglio et al (2008), observed that flowering, in southern Italy, was negatively correlated with mean temperatures preceding the phase from 1st January, indicating a phase advance of 1 day each 2.5°C increase of air temperature. Perez-Lopez et al (2008) observed that mean temperatures of April and May were the most effective for predicting flowering in the region of Ciudad Real (Spain). We also observed that the onset of pre-flowering and flowering was negatively correlated with the duration of respective phases, a delay on the phase onset corresponded to a shorter phase duration, as already reported in literature (Rojo and Pérez-Badia, 2015). Many other researchers indicated the influence of March, April and May temperatures on olive flowering (De Melo-Abreu et al, 2004; Avolio et al, 2008, 2012).

Values of TT obtained for the five varietal groups analyzed, are placed between those described for southern Italy and coastal areas of Spain (Galán et al, 2005; Orlandi et al, 2005; Aguilera et al, 2014), indicating that the optimum lower threshold temperature may be affected by the bioclimates of the growing area and by the varieties grown in the same area. Different optimums TT were observed between varietal groups. Lowest values were found for the two most widespread Sardinian varietal groups, Bosana and Manna. For the other three groups higher values up to 17°C were found. It is interesting to observe that cultivars like Frantoio and Leccino showed the highest TT; they are cultivars typical of colder areas, where lower optimum TT have been detected for heat accumulation until flowering (Orlandi et al, 2009; Moriondo et al, 2001; Orlandi et al, 2006). Moreover, Frantoio and Leccino achieved onPRE and FF slightly later on respect to most of Sardinian varieties. This behavior could be probably explained as an adaptation mechanism apt to avoid spring frosts, more frequent in the area of origin.

Optimum lower threshold temperatures for estimation of heat requirements may vary according to the calculation method adopted (Yang et al, 1995; Spano et al, 1999; Ruml et al, 2010; Aguilera et al, 2014). In this work the two compared methods, single triangle and single

sine method gave similar results. However single sine method was always more precise, in line with other authors (Orlandi et al, 2005). The better performance of single sine method could be attributed to the sine wave curve that the equation draws between Tmax and Tmin that better approximate daily temperature curve.

Some authors set starting dates for calculation heat accumulation according to the timing of fulfillment of chilling requirements in order to predict flowering phases (De Melo-Abreu et al, 2004; Galán et al, 2005; Aguilera et al, 2014). More frequently, probably to overcome a lack of information, 1st January was adopted as starting date in GDD calculations for flowering period (Snyder et al, 1999; Spano et al, 1999; Galán et al, 2005; Orlandi et al, 2005; Bonfiglio et al, 2008; Perez-Lopez et al, 2008). In this study we used the onset of vegetative development and the onset of pre-flowering in order to consider as starting dates two physiological well defined periods. We observed that both estimated well the FF days. However better results were obtained by the conventional date (1st January). The higher precision of this approach could be attributed to the longer period of accumulation considered and the lower variability of daily amount included in the calculation. Moreover, it is possible to notice as TT increased when the starting date for heat accumulation was delayed, probably due to the higher average temperature of the considered period.

CONCLUSIONS

Knowledge of the specific varietal phenological behavior at the beginning of growing season is a useful tool for the producer's decision making for several agronomical practices. With this preliminary phenological study focused on early vegetative and reproductive phases, functional information applicable for the optimization of pruning, fertilization or irrigation periods apt to improve fruit set were provided.

Most of the similarities and differences between local Sardinian varieties described in literature through genetic and morphological methods were confirmed.

The use of phenological phases as singular variables for discriminant analysis seems to be a useful tool for the identification of varietal groups; the model might be improved with the inclusion of variables describing phenological phases of fruit development and maturation, where probably varietal differences are more evident.

Further studies involving more years of study will help to understand better the relationships occurring between these Sardinian local varieties and weather related variables.

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TABLES

Table 1. Principal growth stages and phases description as reported by Sanz-Cortés et al (2002) for BBCH scale adapted to olive.

Principal growth stage	Phase	Description
0: *Bud development*	00	Foliar buds at the apex of shoots grown the previous crop-year are cmpletely closed, sharp-pointed, stemless and ochre-coloured
	01	Foliar buds start to swell and open. Showing the new foliar primordia
	03	Foliar buds lengthen and separate from the base
	07	External small leaves open, not completely separated, remaining joined at apices
	09	External small leaves opening further wit their tips inter-crossing
1: *Leaf development*	11	First leaves completely separated. Greenish-grey colour
	15	The leaves are longer without reaching their final length. First leaves turn greenish on the upperside
	19	Leaves achieve the typical cultivar lenght and shape
3: *Shoot development*	31	Shoots reach 10% of final length
	33	Shoots reach 30% of final length
	37	Shoots reach 70% of final length
5: *Inflorescence emergence*	50	Inflorescence buds in leaf axils are completely closed. They are sharp-pointed, stemless and ochre-coloured
	51	Inflorescence buds start to swell
	53	Inflorescence buds open. Flower cluster development starts
	54	Flower cluster growing
	55	Flower cluster totally expanded. Floral buds start to open
	57	Corolla green coloured, longer than calyx
	59	Corolla changes colour from green to white
6: *Flowering*	60	First flowers open
	61	Beginning of flowering: 10% of flowers open
	65	Full flowering: at least 50% of flowers open
	67	First petals falling
	68	Majority of petals fallen or faded
	69	End of flowering, fruit set, not-fertilised ovaries fallen
7: *Fruit development*	71	Fruit about 10% of final size
	75	Fruit about 50% of final size. Stone becomes lignified (shows cutting resistance)
	79	Fruit about 90% of final size. Fruit suitable for picking green
8: *Maturity of fruit*	80	Fruit a deep green colour becoming light green or yellowish
	81	Beginning of fruit colouring
	85	Increasing specific fruit colouring
	89	Harvest maturity: fruits achieve the typical cultivar colour, remain turgid and are suitable for oil extraction
9: *Senescence*	92	Overripe: fruits lose turgidity and start to fall

Table 2. P-values of lme analysis on the effects of field position.

Cultivar	Phase[1]	Year	p-values			
			NorthSouth[2]	EastWest[3]	BBCH: NorthSouth[4]	BBCH: EastWest[4]
Manna	Repr	2015	N.S.	N.S.	N.S.	N.S.
		2016	N.S.	N.S.	<0.01	N.S.
		2017	N.S.	N.S.	<0.01	<0.05
	Veg	2015	N.S.	N.S.	<0.05	<0.05
		2016	N.S.	N.S.	N.S.	N.S.
		2017	N.S.	N.S.	N.S.	N.S.
Terze	Repr	2015	N.S.	<0.05	<0.05	<0.001
		2016	N.S.	N.S.	N.S.	N.S.
		2017	N.S.	<0.01	<0.01	<0.001
	Veg	2015	N.S.	N.S.	N.S.	<0.05
		2016	N.S.	N.S.	N.S.	N.S.
		2017	N.S.	<0.001	<0.05	<0.001

[1]Repr = reproductive phases from 51 to 69, Veg = vegetative phases from 07 to 19. [2]NorthSouth indicates the plant field position in the row oriented from north to south. [3]EastWest indicates the plant field position in the row oriented from east to west. [4]Interaction between phenological phases and respective field position.

Table 3. Results of lme model 1 analysis expressed with respective p-values.

Phase[1]	Year	BBCH	Variety	Exp	BBCH:Variety[2]	BBCH:Exp[2]	Variety:Exp[2]
Repr	2015	<0.001	<0.001	<0.001	<0.001	N.S.	N.S.
	2016	<0.001	<0.001	<0.001	<0.001	N.S.	N.S.
	2017	<0.001	<0.001	<0.001	<0.001	<0.001	N.S.
Veg	2015	<0.001	<0.001	N.S.	<0.001	<0.01	N.S.
	2016	<0.001	<0.001	N.S.	<0.001	N.S.	N.S.
	2017	<0.001	<0.001	<0.001	<0.001	<0.001	N.S.

[1]Repr = reproductive phases from 51 to 69, Veg = vegetative phases from 07 to 19. [2]Interaction between phenological phases and variety, phenological phases and exposition, variety and exposition

Table 4. Results of lme model 2 analyses expressed with respective p-values.

Phase[1]	Year	BBCH	Group	Exp	BBCH:Variety[2]	BBCH:Exp[2]	Variety:Exp[2]
Repr	2015	<0.001	<0.001	<0.001	<0.001	N.S.	N.S.
	2016	<0.001	<0.001	<0.01	<0.001	N.S.	N.S.
	2017	<0.001	<0.001	<0.001	<0.001	<0.001	N.S.
Veg	2015	<0.001	<0.001	N.S.	<0.001	N.S.	N.S.
	2016	<0.001	<0.001	N.S.	<0.001	N.S.	N.S.
	2017	<0.001	<0.001	<0.001	<0.001	<0.05	N.S.

[1]Repr = reproductive phases from 51 to 69, Veg = vegetative phases from 07 to 19. [2]Interaction between phenological phases and variety, phenological phases and exposition, variety and exposition

Table 5. Classification matrix, according to LDA cross validated, for Bosana, Manna, Semidana and Terze groups, performed separately for each year and performed with the whole 3 years dataset.

Year	Prediction ↓	Reference →				Accuracy[1]
		Bosana	Manna	Semidana	Terze	
2015	Bosana	44	1	2	0	0.89
	Manna	1	57	0	2	
	Semidana	3	0	15	6	
	Terze	2	4	3	71	
2016	Bosana	37	6	4	1	0.89
	Manna	4	55	0	1	
	Semidana	0	0	21	2	
	Terze	2	2	1	75	
2017	Bosana	36	5	1	6	0.87
	Manna	1	55	0	4	
	Semidana	1	1	18	4	
	Terze	3	1	1	75	
3 years data	Bosana	92	22	10	19	0.73
	Manna	10	129	0	41	
	Semidana	19	1	44	7	
	Terze	14	24	2	200	

[1]Accuracy is expressed as the ratio between reference groups and predicted groups

Table 6. Classification matrix, according to LDA cross validated, for growing seasons.

Prediction ↓	Reference →			Accuracy[1]
	2015	2016	2017	
2015	210	0	1	0.95
2016	3	198	10	
2017	16	2	194	

[1]Accuracy is expressed as the ratio between reference groups and predicted groups

Table 7. Average date (DOY) and coefficient of variation (CV), expressed as percetange (%), of 26 cultivars for some main phenological phases.

Group	Cultivar	07		19		51		60		65		69	
		DOY	CV	DOY	CV	DOY	CV	DOY	CV	DOY	CV	DOY	CV
Bosana	Bosana	51	16.9	98	10.4	74	23.1	128	4.5	133	3.3	144	2.1
	Olieddu	51	13.8	99	5.8	70	23.1	125	5.1	130	3.8	141	3.1
	Palma	54	12.5	106	5,0	75	21.8	128	4.6	133	3.7	143	2,0
	Sassarese	49	22.4	100	9.5	76	16.8	130	2.9	133	2.7	144	2.6
Manna	Confetto	51	27.5	102	11.6	65	26.6	122	5.4	128	4.2	143	2.8
	Maiorca	52	25.6	105	8.3	69	23,0	125	3.8	129	3.2	143	3.4
	Nera di Gonnos	53	23.1	104	8.5	70	23,0	125	5.3	130	4.1	145	2.7
	Sivigliana da Mensa	48	26.7	104	6.1	65	26.6	123	4.6	128	3.2	141	3.2
	Tonda di Cagliari	54	26.5	105	9.9	70	21.4	123	4.7	128	3.3	142	3.4
Semidana	Bianca di Villacidro	50	25.8	102	5.9	71	21,0	129	3.3	134	2.5	145	2.1
	Semidana	50	31.1	106	9.2	77	13,0	131	2.4	135	2.5	147	2.3
Terze	Corsicana da Mensa	58	30,0	110	10.7	78	12.4	128	3,0	133	2.8	147	2.1
	Itrana	58	17.3	103	7.4	85	9.2	129	2.8	133	2.4	146	2.6
	Nera di Oliena	58	20.9	104	7.7	81	10.6	128	3.6	132	3,0	146	2.5
	Paschixedda	55	19.5	103	7.8	77	16,0	127	4.3	132	3.4	145	2.5
	Terza Grande	58	24.2	109	9.2	83	8.6	129	2.6	134	2.2	147	2,0
	Terza Piccola	58	21.6	107	7.9	84	9.8	129	2.9	133	2.3	147	2.7
	Tonda di Villacidro	59	21.7	108	8.2	86	11.6	130	3.1	135	2.6	149	2.1
MinorSardinia	Corsicana da Olio	56	16.2	95	9.8	79	16.8	128	3.8	132	3.4	145	3.3
	Pizz'e Carroga	50	24.2	100	9,0	70	26.5	119	5.5	124	5.5	138	3.5
	Sivigliana da Olio	54	18,0	101	6.9	74	16.7	128	3.5	132	3.3	145	3,0
SouthItaly	Carolea	57	23.1	108	6.6	81	16,0	126	3.9	130	3.4	142	3.8
	Coratina	55	21.5	92	11.2	73	20.4	126	4.7	131	4.4	144	3.6
Tuscany	Frantoio	50	31.1	88	12.8	80	18.2	128	4.3	133	3.6	146	2.8
	Leccino	47	33.3	92	11.6	81	20.1	130	3.5	134	3.2	147	2.7
	Santa Caterina	50	35.1	100	11.5	69	22.8	126	4,0	130	3.4	143	2.6

Table 8. Number of varieties that showed a significant Pearson correlation (P-value < 0.05) between phenological phases and weather related variables. Symbol in brackets indicate the sign of the correlation.

Weather variable		onVEG	onPRE	PRE	onFLO	FLO	FF
Tmax	Nov		2 (+)	4 (–)	1 (+)	1 (–)	2 (+)
	Dec		1 (–)	3 (+)		2 (+)	1 (–)
	Feb		3 (–)	1 (-)		1 (+)	
	Mar			1 (+)		1 (+)	
	Apr					2 (+)	
	May					4 (–)	
	FM		1 (–)	1 (+)			
Tmin	Nov		2 (+)	3 (–)	1 (+)	1 (–)	
	Dec	11 (+)	1 (–)		2 (–)	4 (+)	
	Jan	2 (–)		1 (–)		4 (+)	
	Feb			3 (+)		1 (+)	1 (–)
	Mar		2 (+)	2 (–)		2 (–)	1 (+)
	May					5 (+)	
	NDJ[1]			2 (–)			
	FM[2]		3 (–)	1 (+)			
	FMA[3]					4 (+)	
Tmean	Nov		1 (+)	2 (–)	1 (+)	1 (–)	
	Dec				1 (–)		
	Jan		1 (+)				
	Feb		1 (–)	3 (+)		1 (+)	
	Mar			1 (+)		4 (–)	
	May					3 (–)	
	NDJ[1]					1 (–)	
	FM[2]			1 (+)			
	FMA			7 (+)	6 (–)	1 (+)	9 (–)
onPRE vs PRE[4]			8 (–)				
onFLO vs FLO[5]					2 (–)		

[1]NDJ = average value of November, December and January; [2]FM = average value of February and March; [3]FMA = average value of February, March and April. [4]Correlation between the onset of pre-flowering and the length of pre-flowering period. [5]Correlation between the onset of -flowering and length of flowering period.

Table 9. Optimum threshold temperatures for predicting full flowering (FF), corresponding GDD and RMSE, obtained by single sine and single triangle methods, starting at three different periods of heat accumulation, for the four Sardinian groups proposed and the reference group Frantoio + Leccino (Fra-Lec)

Group	Method[1]	FF Day	Threshold (°C)	GDD	Heat Acc Period[2] (day)	RMSE (day)
Bosana	S - J	132 ± 5	14	252 ± 24	132 ± 5	0.3
	S - 07		14	220 ± 29	81 ± 5	0.82
	S - 51		15	161 ± 18	58 ± 11	1.31
	T - J		13	273 ± 26	132 ± 5	0.9
	T - 07		14	191 ± 31	81 ± 5	1.35
	T - 51		0	894 ± 98	58 ± 11	1.74
Manna	S - J	129 ± 4	14	234 ± 20	129 ± 4	0.33
	S - 07		16	132 ± 21	77 ± 9	1.76
	S - 51		0	907 ± 111	61 ± 12	1.67
	T - J		13	253 ± 20	129 ± 4	1.31
	T - 07		16	107 ± 22	77 ± 9	2.25
	T - 51		0	907 ± 111	61 ± 12	1.67
Semidana	S - J	134 ± 3	16	167 ± 19	134 ± 3	0.26
	S - 07		17	123 ± 21	85 ± 11	0.89
	S - 51		16	142 ± 18	60 ± 10	1.09
	T - J		14	226 ± 19	134 ± 3	0.68
	T - 07		17	98 ± 21	85 ± 11	1.22
	T - 51		17	94 ± 20	60 ± 10	1.27
Terze	S - J	133 ± 3	14	257 ± 16	133 ± 3	0.17
	S - 07		17	117 ± 20	75 ± 9	1.05
	S - 51		16	132 ± 19	51 ± 6	1.09
	T - J		15	167 ± 18	133 ± 3	0.79
	T - 07		17	93 ± 21	75 ± 9	1.25
	T - 51		17	88 ± 20	51 ± 6	1.25
Fra - Lec	S - J	133 ± 4	16	163 ± 23	133 ± 4	0.14
	S - 07		16	152 ± 24	85 ± 11	0.83
	S - 51		17	109 ± 18	53 ± 11	1.04
	T - J		14	221 ± 24	133 ± 4	0.79
	T - 07		17	96 ± 24	85 ± 11	1.23
	T - 51		17	88 ± 20	53 ± 11	1.36

[1]S-J = single sine method – starting day 1st January, S – 07 = single sine method – starting day onVEG, S – 51 = single sine method – starting day onPRE, T – J = single triangle method – starting day 1st January, T – 07 = single triangle method – starting day onVEG, T – 51 = single triangle method – starting day onPRE; [2]Heat Acc Period = heat accumulation period from onVEG to onPRE.

FIGURES

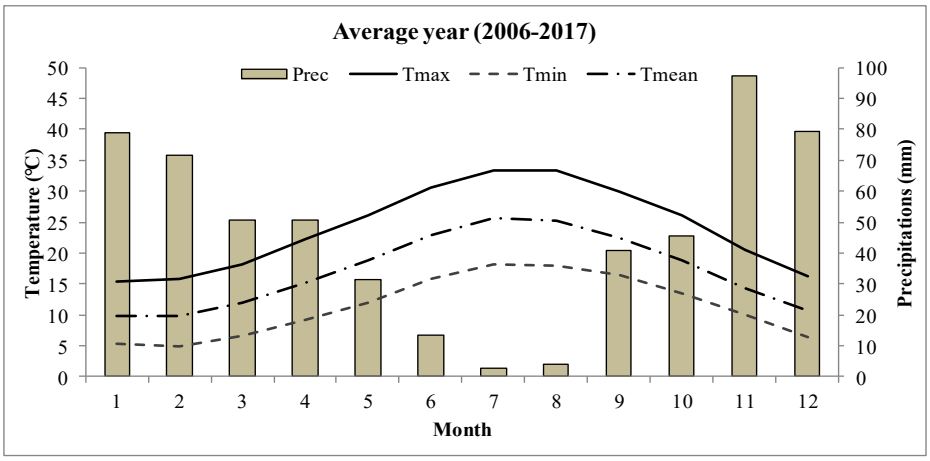

Figure 1. Average weather conditions of the last ten years for Oristano station (ARPAS).

		North						
		NS row 1	NS row 2	NS row 3	NS row 4	NS row 5	NS row 6	NS row 7
West	EW row 1			Manna	Terze		Manna	
	EW row 2			Manna	Terze		Manna	
	EW row 3			Manna	Terze		Manna	
	EW row 4	Manna			Manna	Terze		Manna
	EW row 5	Manna			Manna	Terze	Terze	Manna
	EW row 6	Manna			Manna	Terze	Terze	Manna
	EW row 7	Terze		Terze		Terze		
	EW row 8	Terze		Terze		Terze		
	EW row 9	Terze		Terze		Terze		
	EW row 10				Terze			
	EW row 11				Terze			
	EW row 12				Terze			
		South						

East

Figure 2. Representation of the experimental field with plant position of Manna and Terze groups. "NS row" indicate rows oriented from north to south, "EW" row indicate rows oriented from west to east.

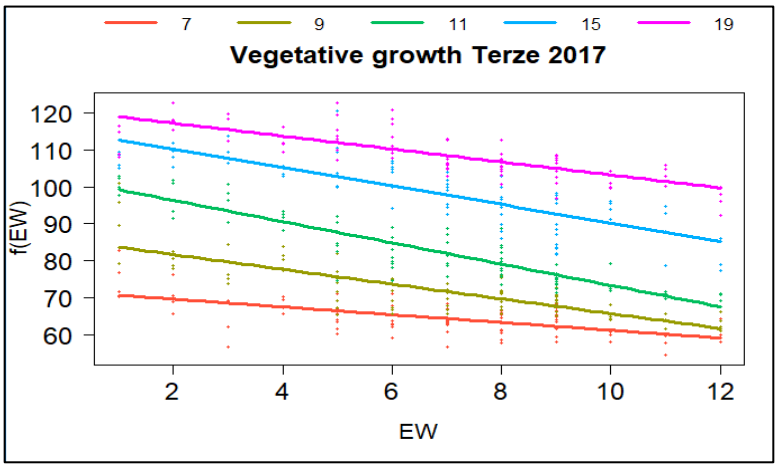

Figure 3. Effect of EastWest (EW) row position on vegetative phases dates, in group Terze during 2017 growing season, fitted with linear mixed-effect models.

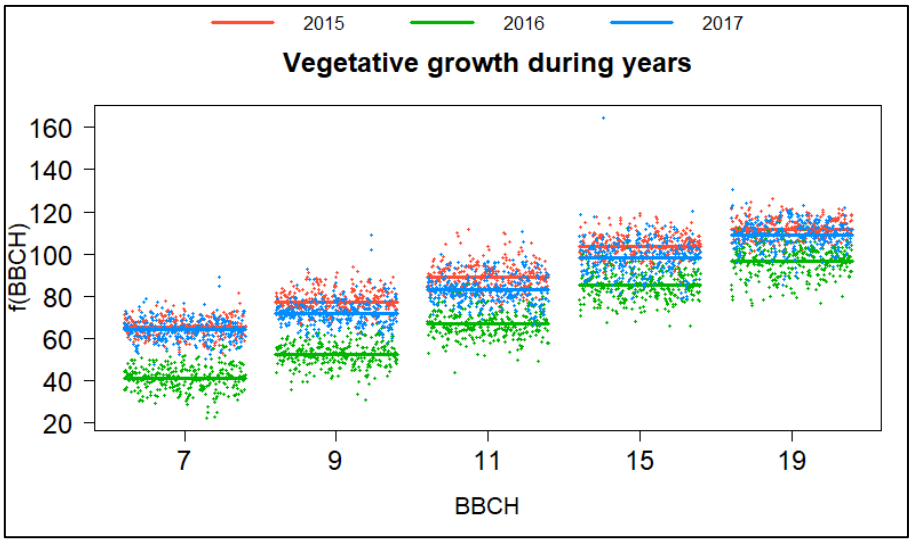

Figure 4. Occurring dates (DOY) of vegetative development from BBCH 07 to 19 during the three years of study.

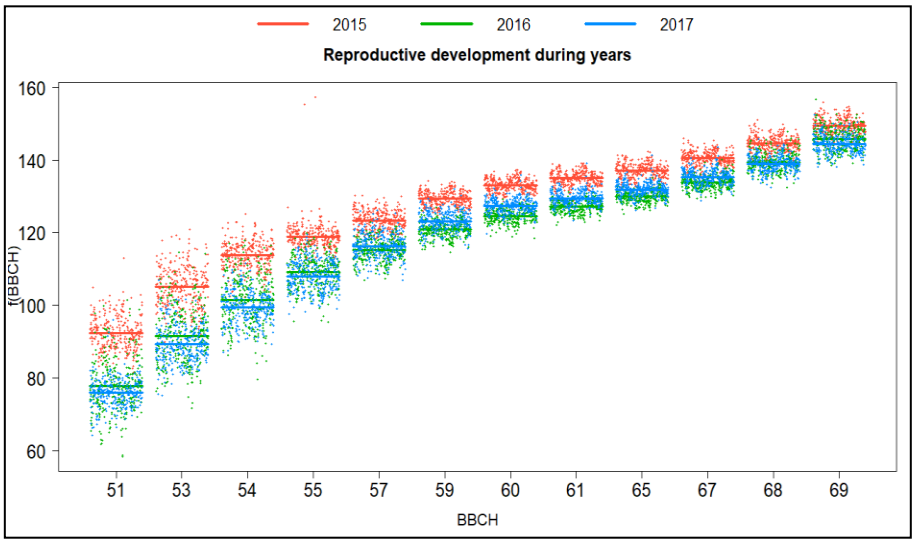

Figure 5. Occurring dates (DOY) of reproductive development from BBCH 51 to 69 during the three years of study.

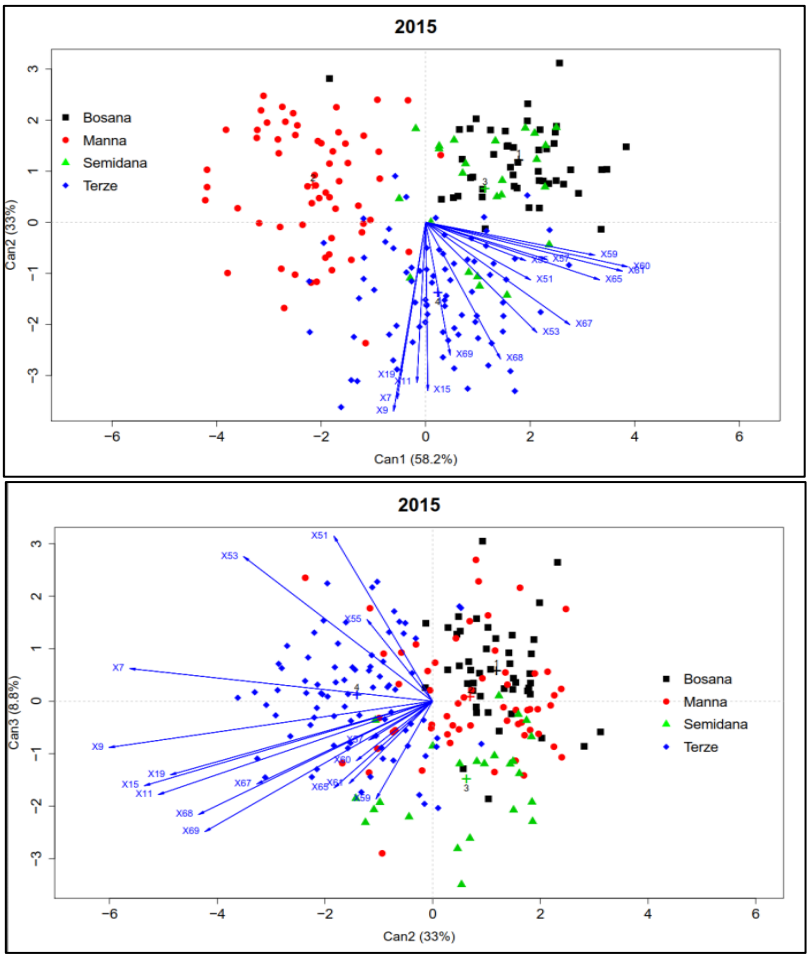

Figure 6 and 7. Similarity map of canonical discriminant analysis (CDA) obtained for classification by the four Sardinian genetic groups in 2015 growing season, determined by discriminant factors 1 and 2 (figure 5) and discriminant factors 2 and 3 (figure 6).

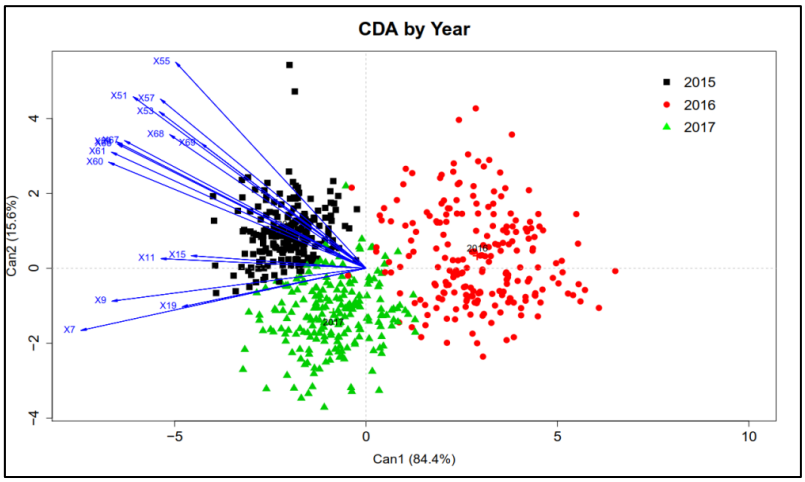

Figure 8. Similarity map of canonical discriminant analysis (CDA) obtained for classification by the three years of study, determined by the two canonical functions.

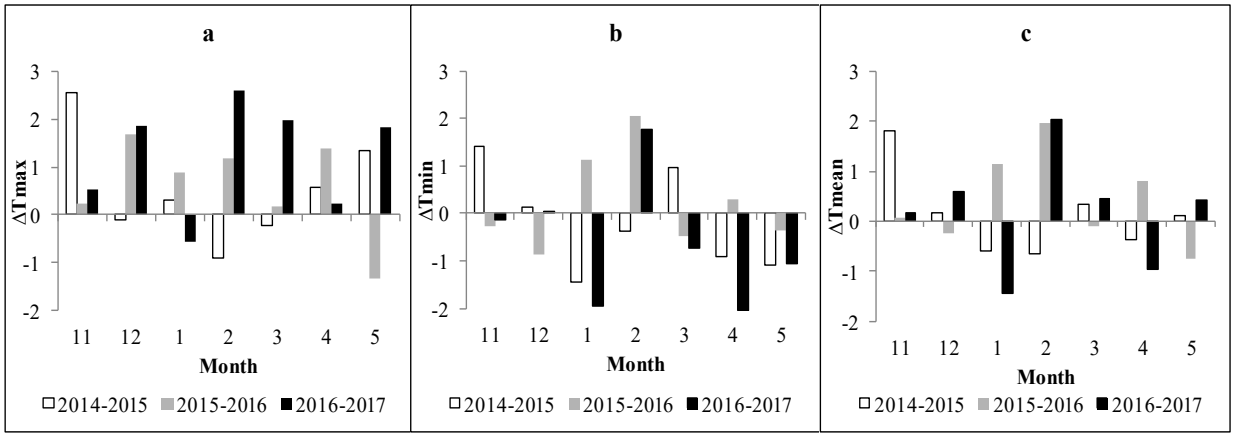

Figure 9. Variation of monthly Tmax (a), Tmin (b) and Tmean (c) from the average year, during the three monitored growing seasons.

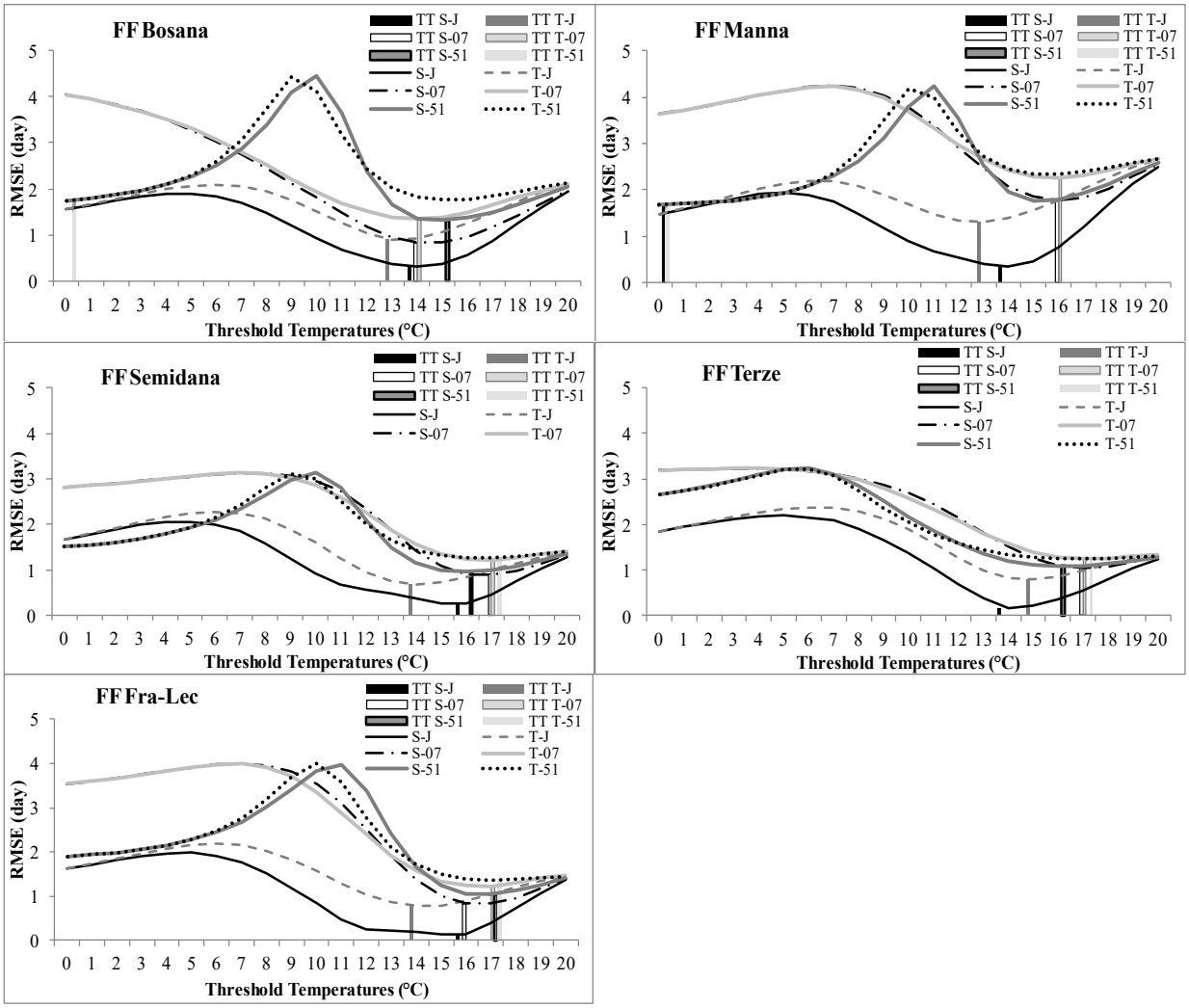

Figure 10. Root mean square error (RMSE) for the different heat accumulation starting dates and the different threshold temperatures calculated through two different GDD calculation methods for each varietal group proposed. Least RMSE values are indicated as TT.

EXPERIMENT 2: CAN ALL THE SARDINIAN VARIETIES SUPPORT THE PDO "SARDEGNA" VIRGIN OLIVE OIL?

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ABSTRACT

Protected Designation of Origin (PDO) labels are important tools to promote high quality virgin olive oils (VOO). To better valorize and differentiate among others these labeled products is necessary a deep knowledge of characteristics and features of monovarietal VOO admitted to characterize them. In Sardinia, only one PDO, named "Sardegna", is present. It covers the whole regional territory and several local varieties are admitted to its characterization, making difficult to define precisely the product. The aim of the study was to examine in depth chemical and nutritional characteristics of some monovarietal VOO included in the PDO Sardegna, with the purpose of identify the varieties and specific characteristics that might contribute to improve the quality of the label. PDO Sardegna VOO were compared with some minor Sardinia cultivars, Italian and Greek varieties, all grown with the same agronomic and environmental conditions. Fatty acid, phenolic and sterol composition from thirty-five VOO representative samples were determined. Moreover, α-tocopherol, squalene and apparent chlorophylls contents were determined. Results confirmed the high potential quality and variability of VOO produced by varieties affering to PDO Sardenga, proving to be good sources of bioactive molecules such as squalene, α-tocopherol and phenolic compounds. Some similarities and synonymies were observed whithin local varieties in accordance to genetic similarities. Moreover, genetic groups highlighted some distinctive features that might be useful for a further characterization of the label.

INTRODUCTION

Protected Designation of Origin (PDO) and Protected Geographical Indication (PGI) are quality and geographic labels introduced by European Union (EU) to promote and protect the quality names of food and wine products. They are connected to a territory as well as to support food safety and quality through the development of sustainable agriculture (Reg. EC No 2081/92, 1151/2012). According to the Database Of Origin and Registration (DOOR) concerning the olive oil sector, over the past 20 years EU has registered 124 extra virgin olive oil (EVOO) denominations (109 PDO and 15 PGI). Italy is the country with the highest number of denominations, 42 PDO and 4 PGI, followed by Greece (19 PDO and 11 PGI) and Spain with 30 PDO. The Italian EVOO quality labels usually are referred to a limited territory and a selected number of varieties, reflecting the peculiar characteristic of Italian olive growing: most of the 800 autochthonous documented cultivars are well adapted to a specific microclimatic area and are cultivated only in their area of origin (Muzzalupo, 2012). During the last period the Italian strategy regarding designation of origin turned towards PGI labels referring to a larger territory: Regions (e.g. PGI "Sicilia", "Olio di Calabria" and "Marche") (DOOR), names able to attract a larger number of potential consumers.

Similar policy probably guided in 2003 the drawing of the application for registration for the only virgin olive oil PDO, named "Sardegna", subsequently registered in Sardinia in 2007. Nowadays this label holds a small percentage of the regional olive oil market, less than 3% (ISMEA, 2016). PDO "Sardegna" can be produced along the whole territory and must be characterized at least for the 80% by the four principal autochthonous Sardinian varieties: Bosana, Tonda di Cagliari, Tonda di Villacidro, Semidana and respective synonymies. The remaining 20% may include virgin olive oils obtained from other varieties grown in the island. Growing areas are concentrated in five main zones: Sassarese and Alghero, Oristanese and Montiferru, Nuorese and Ogliastra, Medio Campidano and Marmilla, Parteolla and Trexenta (Bandino and Dettori, 2001). The Sardinian olive germplasm is rich and strongly linked to the traditions of the territory. Bandino et al (2001), utilizing morphological and structural parameters, described 28 autochthonous varieties. The principal are mainly diffused in the respective areas of origin and rarely cultivated out of such areas. The most widespread Sardinian cultivar is Bosana, including about the 65% of the cultivated trees (Bandino et al, 2011). Minor varieties (e.g. Pizz'e Carroga, Pezz'e Guaddu, Cariasina, Corsicana da Olio and

Sivigliana da Olio) are usually present as scattered trees inside secular olive orchards. Several studies have been carried out on Sardinian varieties regarding genetic relationships between Mediterranean germplasm and wild forms (Baldoni et al, 2006, 2009) and within Sardinian germplasm and between Sardinian and Italian varieties (Erre et al, 2010). Chemical composition and sensorial profile of some Sardinian virgin olive oil (VOO) have been widely studied under several aspects (Vacca et al, 2001; Angerosa and Basti, 2003; Giansante et al, 2003; Gallina-Toschi et al, 2005; Cerretani et al, 2006; Filigheddu et al, 2012; Bandino et al, 2011, 2012; Campus et al, 2013; Beltran et al, 2015; Tuberoso et al, 2016). Some authors investigated the effect of storage and extraction technology on the Sardinian VOO's quality (Del Caro et al, 2006, 2012; Vacca et al, 2006; Fadda et al, 2012). Moreover, Sardinian VOOs have been involved in chemometric studies aimed to discriminate cultivars and geographical origin (Giansante et al, 2003; Bianchi et al, 2001; Culeddu et al, 2017). The role of microorganisms in Sardinian oleic ecosystems, and in particular the potential effect of the enzymatic activity of bacteria and yeasts on the sensory and physico-chemical properties of oil, has been recently described (Santona et al, 2018).

The size of the territory and the high number of possible varietal blends of PDO "Sardegna" strongly influence chemical composition and sensorial profile of EVOO, and makes it difficult to define with precision this label. A first step towards a deeper label characterization is a detailed study of the monovarietal VOO of all the varieties included in the label, particularly the ones considered synonyms, which have been investigated only under some agronomical aspects (Bandino et al, 1999).

The aim of this work is to describe the principal chemical and nutritional properties of the monovarietal VOO included in the PDO "Sardegna", compared with some minor Sardinian cultivars, Italian and Greek varieties grown with the same agronomical conditions. Moreover, the quality evaluation of monovarietal Sardinian oils compared to national and international cultivars could be a useful tool to increase the label value with higher quality olive oils. Moreover, knowledge of this monovarietal VOO could provide good guidelines to producers in the case of the formulation of new blends.

MATERIALS AND METHODS

Experimental design

Area of study: pedoclimatic conditions

The study was carried out at the olive varietal collection field of the Experimental Station "A. Millela" of the University of Sassari, located in San Quirico - Fenosu, Oristano, Sardinia (39°54'12" N, 8°37'19" E), sited at 13m above sea level.

The bioclimate of the area is classified as "*Mediterranean Pluviseasonal-Oceanic; isobioclimate 6: Upper Thermo Mediterranean, Lower Dry, Euoceanic Weak*" (Canu et al, 2015). This bioclimate is typical for the biggest Sardinian lowland called "Campidano" that spreads from Oristano province to the southern Cagliari province. The thermo Mediterranean thermobioclimatic belt comprises also the coastal and other lowlands areas of Sardinia. Similar climatic conditions could be found in some other areas of southern Italy (i.e. south Apulia, some coastal areas of Campania and Calabria and some areas of Sicily) (Pesaresi et al, 2014). At European level, it could be found in the eastern and southern coastal areas of Spain, a large part of Andalusia, southern Portugal and coastal areas of southern Greece (Rivas-Martinez et al, 2013 a and b).

According to the data provided for Oristano by the Department of Meteorology and Climatology Environmental Protection Agency of Sardinia (ARPAS), the annual mean rainfall is 580 mm, mainly concentrated in autumn and winter months; the annual average temperature is 17.1 °C, with an average maximum at 23.9 °C and minimum at 11.3 °C. Winters are mild (February is the coldest month) whereas summers are hot and dry. The intermediate seasons are characterized by a constant high humidity, variability of temperatures and precipitations.

Soils are alluvial deposits classified as *Typic, Aquic, Utic Palexeralfs* (Aru and Baldaccini, 1992).

Plant and olive samples

Olive orchard was implanted in 1998 at a space of 6 x 6 m and drip irrigated with ca. 2500 m^3/ha during the period June-October. The collection field was considered appropriate for the present study because it provided genetically certified plant material. The presence in the same field of almost all the candidate Sardinian varieties for the aim of this study gave the

possibility to evaluate the genetic influences on the VOO chemical composition. Varieties in the field were represented by three trees.

Olive samples were collected during 2015 growing season (the fruit harvest started on 16th November and ended on 2nd December). Olives were mechanically harvested and weighted separately from each tree. Olive fruits from trees that produced at least 17 – 20 kg were processed separately in order to obtain two or three samples per variety; otherwise the whole olive production of the same variety was utilized to obtain one sample.

35 olive samples coming from 23 varieties were processed (Table 1). The 23 varieties were divided into two groups, in order to simplify the discussion of results:

- **Group A:** comprised 14 of the Sardinian varieties (comprised synonyms) indicated in PDO "Sardegna": Bosana; Tonda di Cagliari (Nera di Gonnos, Maiorca, Sivigliana da Mensa, Confetto); Tonda di Villacidro (Nera di Oliena, Paschixedda, Terza Grande, Terza Piccola, Corsicana da Mensa); Semidana (Bianca di Villacidro); synonymies (in brakets) have been described under a genetic point of view in 2010 (Erre et al, 2010).
- **Group B:** comprised 3 minor Sardinian varieties not included in the 80% of PDO composition (Corsicana da Olio, Pizz'e Carroga and Sivigliana da Olio), 4 Italian (Coratina, Frantoio, Itrana and Leccino) and 2 Greek varieties (Kalamata and Koroneiki).

Maturation Index

Maturation Index (MI) was determined for each olive sample throughout the procedure described by the Agronomic Station of Jaén, Spain (Uceda and Frias, 1975). Samples were harvested at an average MI of 3.0 (sd ± 0.7) except for Semidana and Bosana olives that where harvested at different MI: 1.1; 1.4; 3.0; 4.0 Semidana and 2.2; 3.8 Bosana (Table 2).

Oil extraction

Olives (20 – 25 kg), were processed within 18h after harvest using a small scale industrial mill "Sintesi 80" Mori TEM (Tavernelle Val di Pesa, Italy), equipped with a blade crusher, 40 kg vertical malaxator working under reduced pressure and two phase decanter. The extraction was performed maintaining the same parameters for all the samples: temperature of olives was 20 °C (± 2.5); temperature of olive paste after crushing (3000 rpm) was 24°C (± 2.5); olive paste was kneaded for 15 min at 25 °C (± 2.5); average decanter temperature was 29°C

(± 2.5) (3500 - 3700 rpm). The oil samples obtained were filtered and stored in 100mL sealed and filled dark glass bottles, away from the light at 11°C until further analysis.

Standards and solvents

Folin Ciocalteu phenol reagent, hydroxytyrosol (≥98%) and tyrosol (≥98%), oleuropein (≥98%), vanillin (≥99%), vanillic acid (≥97%), caffeic acid (≥98%), p-coumaric acid (≥98%), o-coumaric acid (≥98%), ferulic acid (≥98%), pinoresinol (≥95%), cinnamic acid (≥99%), luteolin (≥98%), apigenin (≥95%), FAME mixture and squalene (≥ 98%), were all purchased from Sigma–Aldrich (Milano, Italy and St. Louis, MO, USA). α-Tocopherol (α-T) (> 96%) and pyrogallol (> 98%) were from Fluka Chemie GmbH (Buchs, Switzerland). HPLC grade solvents were used without further purification. 2-Propanol (Chromasolv®), acetone (HPLC 95%) and acetonitrile were all provided from ChemLab (Zedelgen, Belgium). Methanol for HPLC (≥99.9%) and *n*-hexane Chromasolv ®, for HPLC, ≥97.0% (GC) were purchased from Sigma Co (St. Louis, MO, USA). Ultrapure water (H_2O) was prepared using a Milli-Q system (Millipore Corporation, Billerica, MA, USA).

Under the law quality of olive oil samples

Free acidity (% of oleic acid), peroxide value (meq O_2/kg olive oil) and K values were determined according to European Union standard methods on olive oils (Reg. EC No 2568/91).

Determination of fatty acid methyl esters (FAME)

Analysis of FAME was performed by gas chromatography according to cold transesterification method (Reg. EC No 2568/91). Instrumental analysis was executed on an Agilent gas chromatograph 6890N equipped with a mass spectrometer (5973N) (Agilent Technologies, Palo Alto, CA, USA) and capillary column DB-23 (30 m x 0.25 mm x 0.25 μm) (Agilent Technologies). The operating conditions were: carrier gas, helium (2.1 mL/min); oven temperature, 150°C (1 min), 150-200°C at 3°C/min, 200-250°C at 20°C/min (2 min); transfer line temperature, 230°C. The injection volume was 1 μL (splitless mode).

Percent of the individual FAME was calculated on the basis of the total area of the peaks present. Repeatability of the method was found satisfactory for all the fatty acid methyl esters

used as standards (CV = 0.23 – 7.76%, *n* = 5). Samples were analyzed in triplicate. Compounds were identified using their retention time with those of standards.

Analysis of phenolic compounds

Sample preparation

The phenolic extracts were obtained following International Olive Council method (IOC, 2009) with slight modifications. Olive oil sample (4 g) was dissolved in 5 mL of a mixture of methanol/water (80:20, v/v). The mixture was shaken (30 min) and then centrifuged (5 min, 5000 rpm). The polar extract was removed with a glass pipette. The extraction process was repeated twice and the extracts were combined and filtered through a 0.45 μm PVDV filters. Samples were stored at -78°C until further analysis.

Total phenolic content determination

Total phenolic content was determined on methanolic extracts using the Folin-Ciocalteu assay (Bazzu et al, 2017). Results were expressed as mg of gallic acid equivalents (GAE) per 1 kg of oil by means of a calibration curve of gallic acid (10-40 mg L-1, R2= 0.996). Samples were analyzed in triplicate.

RP-HPLC analysis

Analysis of phenolic compounds was performed on an Agilent 1100 LC System (Agilent Technologies, Palo Alto, CA, USA) consisted of a quaternary pump (G1311A), degasser, column thermostat, auto-sampler (G1313A), diode array detector (G1315 B, DAD) and a Luna C18 column (250 x 4.6 mm, 5 μm) from Phenomenex (Torrance, CA, USA) with a security guard cartridge (4 × 2 mm). The flow rate was set at 1 ml/min and the column temperature at 30 °C. Elution was carried out with a ternary mobile phase of solvent A (water and 0.1% trifluoracetic acid), solvent B (methanol) and solvent C (acetonitrile). The following gradient program was performed: initial percentage eluent composition was 96:2:2 (A:B:C); 50:25:25 from 0 to 40 min; 40:30:30 from 40 to 45 min; 0:50:50 from 45 to 60 min. This composition was maintained for 10 min, then returned to initial conditions and left to equilibrate for 12 min. Total run time was 82 min. The injection volume was 20 μL. Phenolic compounds were detected at 280 and 320 nm. Phenols were identified and quantified using the calibration curves of a mixture of 13 standards (1.5, 3, 4.5, 6, 7.5 mg/kg). Results were

expressed as mg of phenols per kg of oil. Identification of the phenols which were not referred by standards was based on comparison of their HPLC retention times with mass spectra and data reported in literature (IOC, 2009; Daskalaki et al, 2009; Tasioula-Margari and Tsabolatidou, 2015; Kotsiou and Tasioula-Margari, 2016). Repeatability of the method was found satisfactory for all the identified compounds (CV = 1.42 – 9.14%, n = 5).

Mass spectroscopy

An Agilent Technologies (Palo Alto, CA, USA) 1200 series LC equipped with a Q-Exactive Orbitrap (Thermo Fisher Scientific, Bremen, Germany) mass spectrometer was used for LC MS analysis. Chromatographic separation was achieved with a Gemini C18 column (100mm × 4.6 mm, 3µm, 110 A°, (Phenomenex, Torrance, CA, USA) using a mobile phase consisting of 0.2% acetic acid in water (A) and acetonitrile (B) at a flow rate of 500 µL/min. Phenolic compounds separation was obtained using the following linear gradient: A/B (v/v): 0 min 90/10, 0.1-20 min 70/30, and 20.1-40 min 50/50, 40.1-50 min 30/70 and 50.1-60 min 30/70. Mass detection was carried out after electrospray ionization in both Positive and Negative scan ion mode (HESI+ and HESI-). The source voltage was 3.5 kV and 3.2 kV respectively for negative and positive ion mode. Nitrogen was used as the sheath and aux gas, with flow rates of 30 and 5 arbitrary units, respectively. The aux gas heater was set at 280 ºC, and the capillary temperature was 300 ºC. HRMS mode operations in Full Scan was: resolution (FWHM) 70000; AGC target, 10^6. Injection time: 250 ms; scan range, 130–1000 m/z. Parallel-reaction-monitoring (PRM) and Target selected ion monitoring data dependent (tSIM-dd MS/MS) were also tested as MS/MS mode of acquisition: Data-dependent scanning was carried out without the use of a parent ion list. Operation parameters were as follows: (FWHM) resolution 70000 for precursor ions and 35000 for product ions; AGC target, 10^6 (precursor ions), $2 \cdot 10^5$ (product ions); injection time, 250 ms (precursor ions), 120 ms (product ions). An external calibration for mass accuracy was carried out the day before the analysis according to the manufacturer's guidelines. Data were analyzed using XCalibur software v. 3.0.63 (Thermo Fisher Scientific).

Analysis of α-tocopherol

HPLC analysis of tocopherols was performed on a system consisted of a pump, model P4000 (Thermo Separation Products, San Jose, CA), a Midas autosampler (Spark, Emmen,

The Netherlands) and a UV 6000 LP diode array detector (DAD) (Thermo Separation Products) in series with an SSI 502 fluorescence detector (FLD) (Scientific Systems Inc., State College, PA, USA). The data were processed with the aid of Chrom Quest software (version 3.0, Thermo Separation Products).

Analysis of α-tocopherol (α-T) was performed following the method proposed by Psomiadou and Tsimidou (1998). Oil sample (0.16g ± 0.01) was dissolved in a mixture of *n*-hexane/2-propanol (99/1, v/v) (2 mL). Separation was achieved on a LiChrospher-Si column (250 x 4 mm i.d., 5 μm) (MZ Analyzentechnik, Mainz, Germany). The flow rate was 1.2 mL/min and the injection volume was 20 μL. A gradient elution was used with *n*-hexane/2-propanol (99:1 v/v) (A) and 2-propanol (B) as eluents. The gradient for A was as follows: 100% (10 min); 100 – 95% (10 – 14 min); 95% (16 – 20 min); 95-100% (20 – 24 min); 100% (24 – 30 min). Detection of α-T was performed at 294 nm by DAD and by fluorescence at 294 nm (ex) and 330 nm (em). α-T was identified and quantified using the calibration curve (y = 1E + 10^6 x - 15486, R^2 = 0.997) of a standard solution at five different concentrations (7.5, 15, 30, 60, 80 mg/kg). Repeatability of method was found satisfactory (CV% = 1, *n* = 5 for a mean value of 219.1 mg of α-T/kg oil). Samples were run in duplicate and periodically in triplicate in order to verify the repeatability of measurement.

Analysis of squalene

RP-HPLC analysis of squalene was performed on a solvent delivery system consisted of an LC 20AD liquid chromatograph pump (Shimadzu Corporation, Kyoto, Japan) and a SPD-10AV UV-VIS detector (Shimadzu, Corporation). The data were processed with the aid of the software Clarity Data Apex (Prague Czech Republic). Analysis of squalene was carried out following the saponification method described by Grigoriadou et al. (2007). Specifically, 0.1g ± 0.02 of oil was added in a 25mL glass stopped tube followed by the addition of 3mL KOH (600g/L), 2mL ethanol and 5mL ethanolic pyrogallol solution (60g/L). The tube was flushed with nitrogen, closed and vortexed (5 sec). Alkaline saponification was carried out in 75°C water bath for 30 min. After that, 15mL of NaCl solution (10g/L) was added and the mixture was extracted twice (5 min) with 15 mL of *n*-hexane/ethyl acetate (9:1, v/v). Saponification was performed in triplicate and the unsaponified extracts were collected together. After evaporation of the solvent, the dry matter was diluted in acetone. Repeatability of the method was verified (CV% = 6.89, *n* = 7 for a mean value of 4216 mg squalene/kg oil) and found

satisfactory. Squalene content in the samples was determined on a reversed phase LiChroCART column (125 × 4.0 mm i.d.; 4 m). The elution solvent was acetonitrile (100%); the volume of the injection was 10µL; the flow rate was fixed at 1.2mL/min. Detection and quantification of squalene was performed at 208 nm. Squalene was identified and quantified using the calibration curve ($y = 4.1181\ x - 339.63$, $R^2 = 0.997$) of a standard solution at 5 different concentrations (25, 50, 100, 150, 250 mg/kg). Results were expressed as mg of squalene per kg of oil. Analyses were performed in duplicate, periodically were verified the repeatability of method in triplicate.

"Apparent" chlorophyll content and photometric color index (PCI) determination

"Apparent" chlorophyll content (Psomiadou and Tsimidou, 1998) was estimated using the following equation (1):

$$C_{(\text{pheophytin a, mg/kg})} = 345.3[A_{670}-(A_{630}+A_{710})/2]/L$$

A_λ is the absorbance of the oil at the respective wavelength and L is the cell thickness (mm).

The photometric color index (PCI) was determined according to the work of Giacomelli et al. (2006). This color parameter was calculated from the visible spectrum of the oil samples using the following equation (2):

$$PCI = 1.29 \times A_{460} + 69.7 \times A_{550} + 41.2 \times A_{620} - 56.4 \times A_{670}$$

A_λ is the absorbance at the respective wavelength (nm).

Sterol composition and triterpene dialcohols analysis

Analysis of sterolic composition and triterpene dialcohols was carried out following the method described by the European Economic Community (Reg. EC No 2568/91).

RESULTS AND DISCUSSION

Meteoclimatic conditions

According to ARPAS data, mean temperatures (Tmax, Tmin and Tmean) of 2015 were in line with the average period (2006-2017). At monthly detail (Figure 1a, 1b), is possible to observe lower temperature values than average during autumn (October) and winter months (January and February) – except for December –, while opposite trends have been observed during spring and summer with peaks of +1.33 (Tmax) and +1.42 (Tmin) during July.

Different trends between Tmax and Tmin were observed during winter and intermediate seasons, showing a general increase of temperature range. Precipitations concentrated during winter months, reaching a peak of +92 mm above the average in February. On the contrary from April to December were observed values below the average, except for October.

Under the law quality indices

Quality indices showed a good quality of all the samples, classifiable as VOOs (data not shown).

Fatty acid composition

The fatty acid composition of the 23 varieties analyzed in this study is reported in Table 3. All the values fell within the ranges proposed by FAO (Codex Alimentarius, 2003) for VOO. The monounsatured fatty acids (MUFA) content ranged between 80.55% (Koroneiki) and 64.26% (Confetto). The same varieties showed respectively the lowest (5.77%) and highest (18.32%) values for poliunsatured fatty acids (PUFAs). MUFA/PUFA ratio ranged between 13.99 (Koroneiki) and 3.51 (Confetto), showing a decreasing trend from the Greek and Italian varieties to the Sardinian one. Tonda di Cagliari and synonyms were the varieties characterized by the lowest ratio values within the PDO varieties, as well as Corsicana da Olio and Sivigliana da Olio within group B. Bosana, Tonda di Villacidro and synonyms, and Semidana at earlier MI reported higher MUFA/PUFA ratio, close to those of Frantoio and Itrana varieties. Pizz'e Carroga sample showed a good MUFA/PUFA ratio (7.20), in contrast to what reported previously in literature (Cerretani et al, 2006). A high MUFA/PUFA ratio gives more stability to oxidation process and consequently a longer shelf life (Paz-Aguilera et al, 2005). Palmitic acid ranged from 9.74% (Kalamata) to 16.51% (Tonda di Cagliari), Tonda di Cagliari and synonyms, Semidana samples and Sivigliana da Olio showed the highest concentrations.

Rotondi et al. (2010, 2013) described the fatty acid profile of a high number of monovarietal Italian VOO; our findings were in line with the reported profiles of Bosana and Leccino while Coratina, Frantoio and Itrana profiles showed some slight differences principally in oleic, linoleic and stearic acids. Several studies revealed that the fatty acid composition is mainly influenced by temperatures occurring during oil accumulation in the fruit: the areas characterized by higher temperatures during fruit maturation, reported lower

quantities of oleic acid and higher percentages of linoleic, palmitic and linolenic acids; the amplitude of these changes is specific for each variety (Paz Aguilera et al, 2005; Lombardo et al, 2008; Mailer et al, 2010). The area of study is characterized by a warmer and dryer climate compared to traditional growing areas of Leccino, Frantoio and Itrana (central Italy) (Pesaresi et al, 2014), this could explain the differences occurred with our findings for Frantoio and Itrana, mainly for linoleic acid. Our results of fatty acid profile of Koroneiki and Kalamata were in agreement to previous literature (Patumi et al, 2002; Vekiari et al, 2010; Dabbou et al, 2011), whereas we observed slight differences for Bosana, Semidana, Tonda di Cagliari, Tonda di Villacidro and Nera di Oliena grown in different areas of Sardinia (Campus et al, 2013; Tuberoso et al, 2016).

Semidana VOOs, at different ripening stages, showed a slight decreasing trend in oleic margaric, linolenic, arachidic and eicosenoic acids and opposite behaviour in linoleic acid. An increasing trend of oleic acid and decreasing for palmitic, palmitoleic and linoleic acids during maturation was observed in Bosana. Changes in the fatty acid profile during maturation seem to be strictly dependent to the genetic factor (Baccouri et al, 2008; Vekiari et al, 2010).

Phenolic composition

Total phenolic content

The data of total phenolic content are reported in Table 2. Results showed a wide range of values, from 155.1 mg/kg (Nera di Oliena) up to 746.3 mg/kg (Bosana 1). Half of the values attested within the range 300-500 mg/kg, while the 32% between 100 mg/kg and 300 mg/kg (Figure 2a).

Our findings of total phenolic content with regard to the Sardinian varieties, both principal and minor ones, satisfied the minimum level requested by the application for registration document for PDO Sardegna for this quality parameter (100 mg/kg) and indicated the good potential of these varieties if good practices are adopted throughout the processing and storage conditions. Moreover, 12 of the 17 Sardinian varieties analyzed reported values higher than 300 mg/kg, Bosana 1 stand out for total phenolic content above 700 mg/kg, while Bosana 2 and Semidana 2 showed values between 500 and 700 mg/kg.

The effect of fruit ripening on total phenolic content was observed in Semidana and Bosana oils. In both cases, the first harvests reported the highest values, as well as reported previously in literature (Boskou, 2008; Gambacorta et al, 2010). Values observed in these two cultivars were generally in the upper limits reported in literature, the same occurred with regard to Pizz'e Carroga (Gallina-Toschi et al, 2005; Cerretani et al, 2006; Campus et al, 2013; Del Caro et al, 2006; Fadda et al, 2012). Our results on Tonda di Villacidro agreed with literature but not for Nera di Oliena, in our case, unusually poor in phenols (Campus et al, 2013).

Frantoio, Leccino, Koroneiki and Coratina are some of the most common varieties in the world and consequently extensively studied. With regard to total phenolic content, a wide range of values have been reported in literature, our findings placed within these reference ranges (Servili et al, 2007; Gambacorta et al, 2010; Anastasopoulos et al, 2011; Dabbou et al, 2011; Stefanoudaki et al, 2011; Caruso et al, 2014).

The high variability assessed in literature, within the same cultivar, might be attributed to the wide number of factors that influence the content of these kinds of molecules: geographical origin (Bajoub et al, 2016), meteoclimatic conditions during growing season, irrigation (Stefanoudaki et al, 2011; Caruso et al, 2014), extraction technology (Boskou, 2008). As observed in Figure 1b for 2015 growing season, the particular conditions of stress occurred during fruit growth and pit hardening (July – August) might have contributed to the high phenolic concentration observed in our samples.

Phenolic composition

The Figure 3 shows a representative HPLC chromatogram of phenolic profile from Tonda di Villacidro sample. Twenty-four phenolic compounds were identified, 18 of them were quantified. All VOOs analyzed showed a qualitatively similar phenolic profile, except for elenolic acid, the second isomer of dialdehydic form of decarboxymethyl elenolic acid linked to tyrosol (p-HPEA-EDA II), o-coumaric acid, ferulic acid and two isomers of oleuropein aglycon (peaks IV and V) that were not detected in all samples. Traces of siringaresinol, overlapped with dialdehydic form of oleuropein aglycon (peak III), were detected in Sivigliana da Olio and Bosana, while traces of pinoresinol, overlapped with p-HPEA-EDA, were detected in Corsicana da olio, Leccino, Coratina and Frantoio. The Peaks VI were

characterized by the presence of two molecules co-eluted: isomers of oleuropein aglycon and ligstroside aglycon, finding already reported in literature (Jerman Klen et al, 2015).

Table 5 reports the phenolic compounds concentration (mg/kg of oil) for the 23 varieties analyzed. Secoiridoids were the main phenols accounting for 63 - 94% of the total amount, followed by lignans (0.5 - 27.3%) and phenolic alcohols (0.8 - 18.4%), this wide variability in phenolic composition highlights the genetic biodiversity among the cultivars (El Riachy, 2011).

Dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol (3,4-DHPEA-EDA), p-HPEA-EDA and oleuropein aglycon (peak 7, 10 and 15) were the most abundant molecules, reporting a wide range of values, 4.0 – 196.0 mg/kg, 18.0 - 246.7 mg/kg, 8.1 - 102.5 mg/kg respectively. The highest were observed in Bosana, Maiorca and Semidana at earlier stages of maturation (group A), and in Sivigliana da Olio and Coratina (group B). Two isomers of a dialdehydic form of ligstroside aglycon were quantified (peaks 8, 9), Tonda di Villacidro, and similar varieties, showed the highest concentrations, as well as for elenolic acid (peak 6). Itrana showed similar values, in accordance to genetic studies where these varieties are linked together (Erre et al, 2010). Bosana stood out from the other varieties for the relatively high concentration of the two isomers of p-HPEA-EDA (peaks 13, 14), while the two isomers of ligstroside aglycon were abundant in Bosana and Coratina (peak 17) and in Kalamata (peak 18). To our knowledge, minor secoiridoids described in this study were not previously quantified in Sardinian VOOs.

Sum of hydroxytyrosol, tyrosol and derivatives (peaks 7, 15 and 8, 9, 10, 13, 14, 17, 18 respectively) was found to be higher than 250 mg/kg in 8 varieties (Bosana, Semidana, Maiorca, Tonda di Villacidro, Terza Piccola, from group A; Sivigliana da Olio, Coratina, Itrana from group B), limit required by EFSA (Reg EC No 432/2012) for the only health claim approved for olive oil. Tonda di Villacidro and similar varieties distinguished for the relatively high content in vanillic acid and vanillin. Acetoxypinoresinol was the only lignan quantified, and the highest concentrations were observed in Corsicana da Olio, Semidana 1 and Sivigliana da olio. The abundance of both luteolin and apigenin characterized Tonda di Cagliari and similar varieties.

Our findings concerning Sardinian, Italian and Greek varieties, compared to literature, as well as for total phenolic content, showed some quantitative differences, principally between the main secoiridoids (Cerretani et al, 2006; Del Caro et al, 2006; Baiano et al, 2009; Anastasopoulos et al, 2011; Stefanoudaki et al, 2009, 2011).

In Semidana samples, during maturation process, were observed several changes in phenolic composition, but without a clear trend, only luteolin and ligstroside aglycon isomer 2 reported a constant increase. In any case, it might be necessary a further detailed study on this topic, also why literature reports contrasting results (Gambacorta et al, 2010; Vekiari et al, 2010; Dabbou et al, 2011).

α-tocopherol content

In table 2 are reported α-tocopherol concentrations. The lowest and the highest values were reported in Nera di Oliena (198.2 mg/kg) and Leccino (506.0 mg/kg) respectively. Half of the samples contained 200-300 mg α-tocopherol/kg, followed by a 42% of samples ranging from 300 to 400 mg/kg (Figure 1b). None of the olive oil samples of group A had >400 mg α-T/kg in contrast to group B in which a 20% of the samples (Leccino and Koroneiki) overcame 400 mg/kg, among the highest values reported for European oils (Tsimidou, 2012). Such values for Leccino had also been mentioned in extra virgin olive oil extracted from unripe fruit (Lavelli and Bondesan, 2005) while Koroneiki has also been found to yield oils with sometimes high values of α-tocopherol (Tsimidou, 2012). Moreover, the α-tocopherol content in Leccino and Koroneiki VOOs grown in different regions of Australia showed the same high levels for the first one but a much lower content in the Greek variety (Mailer et al, 2010). This result strengthens the fact that the content of VOO in bioactive compounds depends, among other factors, also on the growing region. As well as for phenolic content, stress conditions caused by high temperatures occurred during fruit development might have contributed to increase α-tocopherol concentration (Mailer et al, 2010).

Regarding PDO samples, α-tocopherol content in VOO from Bosana, Semidana, Tonda di Cagliari and Tonda di Villacidro were in the upper levels reported from previous results (Cerretani et al, 2006; Tuberoso et al, 2016).

Semidana VOO, at different maturation degree, showed a reduction of a-tocopherol content, as reported in literature (Boskou, 2008), but this was not the case for Bosana.

However, in our case due to an insufficient number of samples, we could not end up in a safe conclusion.

In the other Sardinian, Italian and Greek cultivars, the ranges of α-tocopherol levels were generally higher than those reported in the literature (Tsimidou, 2012; Psomiadou et al, 2000).

All the PDO Sardegna varieties (group A) achieved broadly the minimum limit requested by the label regulation (100mg/kg). High quality virgin olive oils contain >250 mg α-tocopherol/kg just after production whereas even higher levels (>350 mg/kg) has been mentioned in certain monovarietal products derived from healthy olives from different cultivars or regions or under controlled laboratory extraction conditions (Tsimidou, 2012).

Squalene content

Squalene content of the 23 varieties analyzed (Table 2) showed a wide range of values from 3423.9 mg/kg (Kalamata) to 9384.5 mg/kg (Confetto). More than 55% of samples hovered within the range 4000 – 6000 mg/kg, both for group A and B (Fig. 1c). Despite that, group A showed higher squalene concentrations (always above 5000 mg/kg) than group B, in which Koroneiki and Itrana showed the highest values. According to squalene content categories described by Beltran et al (2015), eight cultivars from Group A can be defined as varieties with "medium squalene content" (4000-6000 mg/kg), four as "high squalene content" (6000-7500 mg/kg), two as "very high squalene content" (>7500 mg/kg), bringing to light that the Sardinian cultivars of PDO Sardegna might be a good potential source of squalene; mainly Tonda di Cagliari and similar varieties. Semidana and Bosana VOOs showed a highest squalene content at earlier stages of maturation. Decrease of squalene content in olive oil during fruit ripening has been already reported in literature, attributed to the effect of two biosynthetic pathways, which develop concurrently: the sterols and triterpenoid acids biosynthesis, in which squalene is involved as precursor, and the oil accumulation (Fernandez-Cuesta et al, 2013).

As far as we know, this is the first time that squalene content in VOOs in less known Sardinian varieties has been studied, and no previous published data have been found for VOOs from Itrana and Kalamata. Our findings on regard to Bosana, Semidana, Tonda di Cagliari and Tonda di Villacidro felt within the ranges described in literature (Beltran et al, 2015; Tuberoso et al, 2016), as well as Coratina, Frantoio and Leccino (Manzi et al, 1998;

Hrncirik and Fritsche, 2005), while the squalene content in Koroneiki VOO was found at higher levels than what reported previously (Anastasopoulos et al, 2011; Kalogeropoulos and Tsimidou, 2014). Squalene content in VOOs seems to be mainly related to the genetic factor (Beltran et al, 2015).

Apparent total chlorophylls content

Apparent total chlorophylls content (Table 2) ranged between 1.5 mg/kg (Coratina) and 16.8 mg/kg (Itrana). The distribution frequency histogram (Fig. 1d) shows that, for group A, values are uniformly distributed between the first three categories identified.

Within group A, Tonda di Villacidro and synonyms, at similar M.I., showed higher mean values (11.1 mg/kg) than Tonda di Cagliari and synonyms (5.0 mg/kg), suggesting that total chlorophyll content might be strongly influenced by the genetic factor related to fruits characteristics (Psomiadou and Tsimidou, 2001). In fact, the two genetic groups show different behavior in fruit changing color: when the skin of Tonda di Villacidro drupes turn color to violet (MI > 3), the pulp still remains green, instead, when drupe skin of Tonda di Cagliari starts turning violet, the same process starts in pulp (data not shown). Moreover, different total chlorophyll amount at similar MI was observed within group B. During ripening, in Semidana and Bosana VOO were observed a total chlorophyll decreasing trend, in line with literature (Aparicio-Ruiz et al, 1999; Psomiadou and Tsimidou, 1998). Literature reported wide ranges of total chlorophylls content in VOOs from Bosana, Semidana, Tonda di Cagliari, Tonda di Villacidro and Pizz'e Carroga, our results fell within or above such ranges (Cerretani et al, 2006; Del Caro et al, 2006; Fadda et al, 2012; Tuberoso et al, 2016;), differences also were found for Italian and Greek varieties (Psomiado and Tsimidou, 2001; Giuffrida et al, 2011; Condelli et al, 2015) probably due to different stages of ripening or different oil extraction conditions.

Throughout PCI index was measured the color of VOO samples based on chlorophylls and carotenoids UV-absorption wavelengths intensity. Values were negatively correlated with total apparent chlorophylls content ($R^2 = 0.835$). In group A the lowest values were found in Semidana 1, Bianca di Villacidro and Tonda di Villacidro synonyms while the highest in Semidana at later stages of ripening. In group B Itrana and Coratina reported the lowest and

the highest values respectively, corroborating the strict correlation with total chlorophylls content.

Sterols and triterpenic dialcohols composition

Table 6 reported sterolic composition, uvaol and erythrodiol content of the 23 varieties analyzed. Total sterols content ranged between 696 mg/kg (Coratina) and 1637 mg/kg (Leccino). Within PDO varieties, Tonda di Cagliari and synonyms expressed the highest values (1202-1624 mg/kg), while Semidana the lowest (1077-1001 mg/kg). High values were also registered in Frantoio and Leccino, both settling around the upper levels previously reported (Mailer et al, 2010). European Union defined the content of sterol in olive oils in order to detect contamination with other vegetable oils (EEC, 1991). Coratina, Terza Piccola, Sivigliana da olio and Itrana did not achieve the minimum limit of total sterols indicated by EU (1000 mg/kg). In Coratina and Koroneiki VOO were previously detected low amounts of sterols (Ranalli and Angerosa, 1996; Mailer et al, 2010). Sivigliana da Olio was the only variety that exceeded the EU limit of 4% of campesterol, the high amount of this molecule might be a characteristic related to the variety or to the growing season as well as for the total sterolic content (Koutsaftakis et al, 1999; Mailer et al, 2010; Giuffrè and Louadj, 2013). The other parameters indicated by EU complied with the limits.

In accordance to literature (Boskou, 2008), the most abundant sterols in VOO were β-sitosterol (77.0-88.6%), Δ5-avenasterol (4.9-15.8%) and campesterol (2.2-4.6%). Tonda di Villacidro and similar varieties, showed generally the highest relative amounts in β-sitosterol, while Bosana the lowest amounts. Reversely Bosana reported the highest relative amount of Δ5-avenasterol (14.6-15.8%). High quantities of Δ5-avenasterol were also registered in Koroneiki, Leccino and Corsicana da Olio. Low variability was observed in Tonda di Cagliari and synonyms (5.3-5.9). This molecule was strictly negatively correlated (R^2=0.968) to β-sitosterol, as reported in literature (Temime et al, 2008). During ripening process, β-sitosterol usually decreases while Δ5-avenasterol increases (Vekiari et al, 2010; Lukić et al, 2013), as found in Semidana and Bosana samples harvested at different maturation index. The other sterols detected seemed to be not influenced by fruit ripening, however, due to the low number of samples, we cannot end up with a safe conclusion on regard to these varieties. High campesterol/stigmasterol ratio and low presence of the second one are believed to be indicators of VOOs good quality (Koutsaftakis et al, 1999; Temime et al, 2008): Sivigliana da

Olio showed the highest ratio while Kalamata the lowest. Campesterol/stigmasterol ratio in PDO varieties ranged between 3.2 and 4.3. Low quantities of cholesterol, 24-Metilencholesterol, campestanol, chlerosterol, sitostanol, Δ-5,24-Stigmastadienol, Δ-7-Stigmastenol and Δ-7-Avenasterol were registered, only Sivigliana da Olio stood out for high values of sitostanol (1.3%) and Leccino, for Δ-7-Avenasterol (1.6%). High amounts of erythrodiol + uvaol in VOOs may indicate contamination with pomace oil, thus EU fixed a maximum limit of 4% of total sterols (Reg EC No 2568/91); more than 80% of varieties analyzed felt within 1.0 and 1.9%. The highest relative amounts of erythrodiol + uvaol were registered in Bosana (both ripening stages), Bianca di Villacidro and Koroneiki (2.2-3.2%). The sterols present in VOO and respective ratios have been widely proposed as indicators useful to distinguish varieties (Marini et al, 2004; Lukić et al, 2013). As long as we know, this is the first time that a complete sterolic profile of VOOs from 16 Sardinian varieties, grown at similar pedo-climatic and agronomical conditions, has been described.

Some differences were found between our results and literature concerning Bosana, Frantoio, Leccino, Coratina and Koroneiki VOOs, probably due to the influence of several factors such as growing area, extraction conditions or fruit ripening stage (Del Rio et al, 1995; Stefanoudaki et al, 2001; Marini et al, 2004; Mailer et al, 2010; Anastasopoulos et al, 2011).

CONCLUSIONS

The VOOs of 14 varieties included in PDO Sardegna (group A), obtained under same processing and storage conditions have shown good chemical quality indexes and high concentrations of bioactive compounds. Minimum levels of total phenolic and tocopherols content (both 100 mg/kg) requested by the PDO application for registration document were always achieved with a large margin, suggesting the possibility to raise the quality increasing the actual minimum levels. These varieties may contribute in different way to the quality label: Bosana and unripe Semidana seem to be the highest sources of polar phenols, particularly secoiridoids. Tonda di Villacidro and synonymies showed a higher content in oleic acid and good MUFA/PUFA ratio, on the other hand, a medium – high phenolic and tocopherols content. Tonda di Cagliari and synonymies have proved to be a good source of squalene and sterols with relatively low levels of antioxidants.

Due to the large number of varieties included in PDO Sardegna, a proper characterization of VOO is very difficult. Genetic groups expressed some specific characteristics within phenolic profile, fatty acid or sterolic composition. Specifically the varietal groups better represented were those belonging to Tonda di Cagliari and Tonda di Villacidro. Varieties within both groups shared clear characterizing features and homogeneity. An exception was represented by the cultivar Maiorca that slightly differed from its genetic group, showing higher concentrations of antioxidant compounds and lower in squalene and sterols. Nevertheless further additional studies will be needed to define appropriate descriptors for groups.

Minor Sardinian varieties showed characteristics in line with the others but without significant peculiarities. Sivigliana da olio showed very interesting concentration of secoiridoids in relation to total phenols, but conversely, it presented low MUFA/PUFA ratio and sterol values out of EU limits for extra VOO.

Preliminary investigation on the influence of fruit ripening on the chemical composition of Sardinian monovarietal VOO was presented and set the basis for further research on this specific topic, still never studied for these varieties and very important for producer's decisions. Moreover, for a more complete monovarietal characterization would be appropriate further years of study in order to take into account interannual variability and studies in different growing areas of the island in order to investigate the varietal interaction with different pedoclimatic condions.

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TABLES

Table 1. List of 23 the varieties studied and respective number of samples (N).

Sardinian Varieties	N	Italian Varieties	N	Greek Varieties	N
Bosana	2	Coratina	1	Kalamata	2
Semidana	4	Frantoio	1	Koroneiki	2
Bianca di Villacidro	1	Itrana	2		
Tonda di Cagliari	1	Leccino	1		
Confetto	1				
Maiorca	1				
Nera di Gonnos	1				
Sivigliana da Mensa	1				
Tonda di Villacidro	2				
Corsicana da Mensa	2				
Nera di Oliena	1				
Paschixedda	1				
Terza Grande	3				
Terza Piccola	2				
Corsicana da Olio	1				
Pizz 'e Carroga	1				
Sivigliana da Olio	1				

Table 2. Maturation Index (MI), α-Tocopherol, squalene, total chlorophylls, PCI and total phenols content of the 23 varieties analyzed.

Group	Variety	MI	α-Tocopherol	Squalene	Tot chlorophylls	PCI	Tot phenols
A	Bosana 1	2.2	340.7	7050.5	7.4	-11.2	746.9
A	Bosana 2	3.8	353.8	5887.9	6.0	-8.1	604.7
A	Semidana 1	1.1	291.7	8256.5	13.6	-18.2	467.4
A	Semidana 2	1.4	297.9	5837.3	9.6	-11.4	563.7
A	Semidana 3	3.0	219.1	6585.9	3.6	0.6	327.1
A	Semidana 4	4.0	227.4	6654.4	3.3	0.3	359.9
A	Bianca di Villacidro	3.5	327.9	5565.6	10.9	-14.1	413.4
A	Tonda di Cagliari	3.3	246.6	9220.8	4.5	-6.1	366.6
A	Confetto	3.3	248.7	9384.5	4.3	6.3	308.9
A	Maiorca	2.9	307.7	5726.1	4.5	-6.9	468.0
A	Nera di Gonnos	3.1	210.4	5831.4	3.9	-5.3	387.9
A	Sivigliana da Mensa	3.8	257.6	6897.6	8.0	-9.7	270.4
A	Tonda di Villacidro	3.2	289.5	5336.3	7.9	-9.5	430.6
A	Corsicana da Mensa	2.6	314.7	5401.8	11.4	-13.7	374.5
A	Nera di Oliena	3.1	198.9	6824.0	5.5	-6.3	155.2
A	Paschixedda	3.4	377.5	5112.3	13.2	-14.8	285.2
A	Terza Grande	3.0	306.9	5106.7	14.8	-16.3	342.7
A	Terza Piccola	3.3	313.1	5397.0	13.5	-15.8	427.0
B	Corsicana da Olio	3.9	291.8	3682.1	1.9	-2.2	290.7
B	Sivigliana da Olio	2.6	337.2	4950.5	8.4	-6.7	465.2
B	Pizz 'e Carroga	3.6	216.7	4062.2	5.8	-7.3	236.2
B	Kalamata	2.9	328.2	3423.9	2.5	0.7	205.6
B	Koroneiki	2.9	409.3	6532.8	7.8	-10.9	546.2
B	Coratina	3.0	300.5	4658.9	1.5	3.8	659.6
B	Frantoio	2.4	249.0	3619.5	7.7	-10.3	202.1
B	Itrana	2.9	344.6	5705.1	16.8	-20.3	426.2
B	Leccino	4.2	505.9	4369.2	4.1	-2.6	220.6

Table 3. Fatty acid profile of the 23 varieties analyzed.

Group	Variety	C16:0	C16:1	C17:0	C18:0	C18:1	C18:2	C18:3	C20:0	C20:1	[1]MUFA/ PUFA
A	Bosana 1	13.34	0.78	n.d.	1.88	72.16	11.18	0.36	0.21	0.10	6.33
A	Bosana 2	12.90	0.76	n.d.	1.83	74.34	9.50	0.36	0.21	0.10	7.63
A	Semidana 1	14.74	0.91	0.09	2.07	70.60	10.75	0.48	0.24	0.07	6.37
A	Semidana 2	13.55	1.02	0.03	1.73	73.45	9.59	0.37	0.18	0.09	7.48
A	Semidana 3	14.62	0.84	0.02	1.74	70.51	11.71	0.35	0.18	0.02	5.92
A	Semidana 4	14.91	0.82	0.04	1.74	69.44	12.55	0.33	0.17	n.d	5.46
A	Bianca di Villacidro	13.75	0.93	n.d.	1.58	71.84	11.43	0.33	0.13	0.02	6.19
A	Tonda di Cagliari	16.51	1.58	0.08	1.59	63.19	16.44	0.37	0.17	0.09	3.86
A	Confetto	15.79	1.38	0.04	1.44	62.86	17.98	0.34	0.14	0.02	3.51
A	Maiorca	13.11	0.90	0.03	1.71	70.94	12.72	0.32	0.18	0.10	5.52
A	Nera di Gonnos	16.09	1.40	0.05	1.46	63.16	17.31	0.32	0.16	0.06	3.67
A	Sivigliana da Mensa	14.56	1.28	0.03	1.48	67.20	14.91	0.36	0.14	0.05	4.49
A	Tonda di Villacidro	13.29	0.94	n.d.	1.42	74.32	9.62	0.28	0.12	0.02	7.62
A	Corsicana da Mensa	13.03	0.98	n.d.	1.43	74.48	9.53	0.36	0.13	0.06	7.63
A	Nera di Oliena	13.89	1.15	n.d.	1.35	71.25	11.85	0.35	0.13	0.03	5.94
A	Paschixedda	13.23	1.04	n.d.	1.50	73.38	10.23	0.40	0.15	0.08	7.01
A	Terza Grande	13.09	0.88	n.d.	1.52	74.02	9.86	0.40	0.15	0.08	7.30
A	Terza Piccola	13.26	0.98	0.02	1.67	73.21	10.25	0.37	0.17	0.09	7.01
B	Corsicana da Olio	14.27	1.13	n.d.	2.32	69.78	11.87	0.33	0.20	0.10	5.82
B	Pizz 'e Carroga	14.24	1.03	0.01	1.81	72.43	9.90	0.31	0.18	0.10	7.20
B	Sivigliana da Olio	16.38	1.35	0.12	1.81	66.36	13.30	0.41	0.20	0.06	4.94
B	Kalamata	9.74	0.58	0.10	1.59	78.11	9.19	0.41	0.16	0.10	8.23
B	Koroneiki	11.63	0.73	0.02	1.82	79.71	5.39	0.38	0.21	0.11	13.99
B	Coratina	9.95	0.35	n.d.	1.69	79.13	8.26	0.29	0.18	0.16	9.32
B	Frantoio	12.78	0.70	n.d.	1.33	75.34	9.41	0.27	0.10	0.07	7.86
B	Itrana	12.97	0.95	n.d.	1.62	74.59	9.32	0.34	0.14	0.07	7.86
B	Leccino	13.30	1.18	n.d.	1.65	75.93	7.41	0.30	0.14	0.08	10.02

Values are expressed as percentage of total amount [1]The ratio of sum of monounsaturated fatty acid (%)/sum of polyunsaturated fatty acids (%).

Table 4. Phenolic compounds identified in VOO samples by HPLC-MS.

Peak n	Retention Time (min)	Phenolic Compound	Wavelenght (nm)	Molecular Weight	M-H	Fragments
1	12.40	Hydroxytyrosol	280	154.17	153.054	
2	16.60	Tyrosol	280	138.17	137.06	
3	19.90	Vanillic acid	280	168.14	167.054	
4	23.56	Vanillin	280	152.15	151.054	
5	25.48	p-Coumaric acid	280	164.16	163.038	
I	26.98	Ferulic acid	280	194.19	193.049	
6	28.51	Elenolic acid	280	241.07		
II	32.08	o-Coumaric acid	280	164.16	163.038	
7	33.21	3.4-DHPEA-EDA	280	320.00	319.118	195.065; 337.124; 639.245
III	33.60	Oleuropein aglycon dialdehydic form	280	378.13	377.240	307.082; 755.255
8	37.20	Ligstroside aglycon dialdehydic form 1	280	362.14	361.129	291.087; 723.266
9	37.60	Ligstroside aglycon dialdehydic form 2	280	362.14	361.129	291.087; 723.266
10	38.17	p-HPEA-EDA	280	304.13	303.123	165.054; 285.113; 607.255
11	39.08	Acetoxypinoresinol	280	416.426	415.15	
12	39.70	Luteolin	280 - 320	286.05	285.050	
13	40.14	p-HPEA-EDA Isomer 1	280	304.13	303.123	165.054; 285.113; 607.255
14	40.50	p-HPEA-EDA Isomer 2	280	304.13	303.123	165.054; 285.113; 607.255
IV	41.78	Oleuropein aglycon Isomer 1	280	378.13	377.240	307.082; 755.255
V	42.23	Oleuropein aglycon Isomer 2	280	378.13	377.240	307.082; 755.255
15	43.77	Oleuropein aglycon	280	378.13	377.240	307.082; 755.255
16	44.10	Apigenin	280 - 320	270.06	269.040	
VI	44.79	Ol aglycon + Lig aglycon	280	378.13 + 362.14		
17	47.56	Ligustroside aglycon 1	280	362.14	361.129	291.087; 723.266
18	48.22	Ligustroside aglycon 2	280	362.14	361.129	291.087; 723.266

Table 5. Phenolic composition of the 23 varieties analyzed.

Group	Variety	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
A	Bosana 1	1.6	5.6	0.5	0.2	0.1	2.3	132.6	0.7	0.4	79.6	7.5	5.7	19.9	9.4	85.2	3.1	14.7	1.4
A	Bosana 2	1.2	6.2	0.4	0.3	0.2	2.8	128.3	0.9	0.5	82.0	14.8	5.1	12.2	6.4	63.3	2.7	9.6	3.3
A	Semidana 1	1.1	4.0	0.7	0.6	0.2	1.6	58.9	0.7	0.3	48.1	49.4	1.9	8.3	4.5	34.4	1.0	6.8	1.1
A	Semidana 2	2.1	4.7	1.5	1.0	0.5	3.3	100.8	2.7	2.0	54.8	10.9	3.3	9.0	6.5	102.5	1.9	7.2	1.4
A	Semidana 3	7.8	13.5	1.1	0.4	0.3	1.4	9.5	1.6	0.7	16.9	13.9	3.6	6.7	2.6	30.8	1.7	1.9	1.5
A	Semidana 4	4.1	8.0	0.9	0.4	0.3	1.5	22.2	1.7	0.6	17.9	13.6	4.4	5.1	4.5	50.8	1.9	1.8	2.3
A	Bianca di Villacidro	2.6	6.1	1.5	1.0	0.3	5.4	81.4	2.9	1.9	48.2	9.4	3.4	4.6	5.2	66.7	1.6	4.5	1.5
A	Tonda di Cagliari	1.2	5.6	0.3	0.4	0.3	2.5	88.4	3.6	0.8	66.0	1.1	9.5	3.7	2.5	27.3	6.3	2.5	2.5
A	Confetto	1.2	5.6	0.4	0.6	0.4	2.5	71.3	3.0	0.9	52.9	1.1	6.6	2.0	2.5	26.0	3.9	2.1	3.0
A	Maiorca	1.0	5.1	0.7	0.8	0.3	2.3	128.0	1.8	1.2	124.7	19.9	3.4	3.7	2.7	41.8	2.1	7.1	1.6
A	Nera di Gonnos	1.5	5.1	0.3	0.4	0.3	3.4	75.2	3.7	0.8	53.5	1.4	9.2	4.8	3.4	35.1	5.6	2.1	2.4
A	Sivigliana da Mensa	1.3	3.6	0.7	0.8	0.5	3.2	61.5	3.3	1.9	48.7	3.0	4.4	1.3	3.2	25.3	2.4	2.6	3.9
A	Tonda di Villacidro	2.0	3.7	1.9	1.2	0.8	7.7	115.5	3.9	3.4	65.8	7.3	3.9	4.	5.7	60.4	2.3	5.0	1.8
A	Corsicana da Mensa	2.5	4.0	2.5	1.7	0.4	10.1	97.0	3.7	3.0	57.9	7.4	3.1	3.6	5.3	46.7	1.8	3.5	1.0
A	Nera di Oliena	0.5	4.1	2.4	0.5	0.7	3.3	17.7	3.6	3.1	48.3	4.1	3.4	1.6	3.3	14.1	2.6	2.7	1.3
A	Paschixedda	0.8	2.6	1.8	1.5	0.6	11.7	75.6	3.5	2.7	65.3	21.5	3.0	n.d.	4.4	28.4	1.8	3.4	1.1
A	Terza Grande	0.9	2.6	1.8	1.4	0.3	9.2	70.2	3.3	2.7	62.1	23.3	2.4	n.d.	4.8	35.1	1.6	3.8	1.0
A	Terza Piccola	1.6	3.8	1.6	1.5	0.4	8.3	131.3	3.0	2.5	83.6	22.5	3.0	n.d.	3.6	52.2	2.1	6.9	4.6
B	Corsicana da Olio	0.5	5.2	0.4	0.5	0.1	2.1	17.1	1.0	0.4	70.5	56.9	3.1	3.6	0.7	35.1	1.8	8.2	1.1
B	Pizz 'e Carroga	1.0	6.8	1.4	0.7	0.1	4.9	21.9	2.1	1.0	29.1	17.5	5.6	3.5	1.8	24.7	1.5	3.6	2.1
B	Sivigliana da Olio	1.1	2.7	1.1	0.6	0.2	4.1	196.4	2.3	4.0	187.7	36.5	3.8	n.d.	3.0	17.3	3.0	3.1	0.9
B	Kalamata	0.8	19.4	0.8	0.4	0.7	0.5	21.3	2.8	2.2	84.0	10.1	5.2	1.3	1.9	8.5	1.5	1.6	8.0
B	Koroneiki	2.0	11.0	0.6	0.2	0.7	2.1	51.2	1.6	1.2	52.0	31.0	4.6	12.3	5.7	53.8	2.9	7.2	6.9
B	Coratina	1.9	10.0	0.4	0.5	0.2	n.d.	178.7	0.4	0.2	246.7	33.9	2.3	6.0	2.6	62.4	1.2	20.5	2.3
B	Frantoio	1.2	7.8	1.5	0.7	0.1	3.1	11.5	1.2	1.0	31.8	23.1	2.8	4.3	1.6	20.0	1.1	3.8	2.1
B	Itrana	1.2	2.5	1.6	1.1	0.4	7.2	131.6	3.3	3.0	75.6	6.5	3.0	3.5	4.8	51.3	1.8	3.7	1.2
B	Leccino	1.5	8.1	0.9	0.7	0.1	3.3	58.1	2.2	0.5	65.3	8.4	2.17	2.3	2.0	10.9	1.6	1.3	2.0

Heading numbers corresponds to the compounds described in table 4. Values are expressed as mg of the corresponding standards/kg oil; compounds n. 6-10, 13-15 and 17-18 as mg of oleuropein/kg oil.

Table 6. Sterolic composition of the 23 varieties analyzed.

Group	Variety	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
A	Bosana 1	0.1	0.2	2.6	0.1	0.8	1.1	78.1	0.3	14.6	1,0	0.3	0.8	95.1	1068	3.2
A	Bosana 2	0.1	0.3	3,0	0.1	0.7	1.1	77,0	0.2	15.8	0.9	0.2	0.7	95,0	1018	2.8
A	Semidana 1	0.1	0.1	3.3	0.1	0.9	1.1	87.6	0.5	4.9	0.5	0.2	0.6	94.6	1015	1,0
A	Semidana 2	0.1	0.1	2.8	0.1	0.7	1.1	87.9	0.5	5.3	0.5	0.3	0.6	95.3	1010	1.3
A	Semidana 3	0.2	0.1	3.1	0.1	0.9	1.1	85.1	0.4	7.6	0.5	0.2	0.7	94.7	1001	1.1
A	Semidana 4	0.1	0.2	3,0	0.1	0.8	1.1	83.3	0.3	9.7	0.5	0.2	0.7	94.9	1004	1.1
A	Bianca di Villacidro	0.1	0.1	2.7	0.1	0.7	1.1	86.4	0.4	6.8	0.5	0.3	0.8	95.2	1077	2.2
A	Tonda di Cagliari	0.1	0.1	2.5	0.1	0.7	1,0	87.3	0.4	5.6	0.8	0.5	1.1	95.1	1529	1.2
A	Confetto	0.1	0.1	2.6	0.1	0.8	1,0	87.1	0.4	5.4	0.9	0.5	1,0	94.8	1624	1.4
A	Maiorca	0.1	0.1	2.6	0.1	0.7	1.1	86.8	0.5	5.9	0.7	0.5	0.9	95,0	1202	1.5
A	Nera di Gonnos	0.1	0.1	2.5	0.1	0.7	1,0	87,0	0.4	5.6	1,0	0.5	1,0	95,0	1581	1.3
A	Sivigliana da Mensa	0.1	0.1	2.6	0.1	0.8	1,0	87.6	0.4	5.3	0.8	0.4	0.8	95.1	1340	1,0
A	Tonda di Villacidro	0.1	0.1	2.6	0.1	0.6	1,0	86.8	0.4	6.7	0.5	0.4	0.8	95.4	1014	1.6
A	Corsicana da Mensa	0.1	0.1	2.6	0.1	0.6	1,0	88,0	0.4	5.7	0.6	0.3	0.6	95.7	1191	1.6
A	Nera di Oliena	0.1	0.1	2.6	0.1	0.7	1,0	87.3	0.4	6,0	0.6	0.3	0.8	95.3	1292	1.3
A	Paschixedda	0.1	0.1	2.7	0.1	0.7	1.1	88.1	0.5	5.3	0.4	0.3	0.6	95.4	1072	1.5
A	Terza Grande	0.1	0.1	2.6	0.1	0.6	1.1	88.6	0.5	4.9	0.5	0.3	0.5	95.6	1109	1.5
A	Terza Piccola	0.1	0.1	2.6	0.1	0.6	1.1	87.8	0.6	5.4	0.6	0.4	0.9	95.3	934	1.5
B	Corsicana da Olio	0.1	0.1	2.6	0.1	0.6	1,0	80.7	0.5	11.6	1.3	0.5	0.9	95.1	1500	1.3
B	Pizz 'e Carroga	0.1	0.1	3,0	0.1	0.7	1,0	83.7	0.4	8.9	1.1	0.3	0.7	95.1	1302	1.4
B	Sivigliana da Olio	0.2	0.4	4.6	0.1	0.4	1.1	81.1	1.3	9,0	0.8	0.2	0.8	93.3	844	1.2
B	Kalamata	0.1	0.1	2.8	0.1	1.1	1.2	85.5	0.3	7.3	1.1	0.2	0.5	95.3	1447	1.5
B	Koroneiki	0.2	0.4	3.7	0.1	0.6	1.1	77.4	0.3	14.8	1,0	0.2	0.5	94.4	1022	2.3
B	Coratina	0.2	0.3	3.1	0.1	0.8	1.1	84.5	0.8	7.5	1,0	0.2	0.4	94.9	696	1.9
B	Frantoio	0.1	0.1	2.8	0.1	0.6	1,0	83.4	0.4	9.6	0.9	0.3	0.7	95.3	1612	1,0
B	Itrana	0.1	0.1	2.6	0.1	0.7	1.1	88,0	0.6	5.6	0.5	0.4	0.5	95.7	993	1.8
B	Leccino	0.1	0.1	2.2	0.1	0.9	1,0	78.6	0.6	13.3	1,0	0.5	1.6	94.5	1637	0.7

Values are expressed as percentage of total sterols. Total sterols are expressed as mg/kg of oil.
1 = Cholesterol; 2 = 24-Metilencholesterol; 3 = Campesterol; 4 = Campestanol; 5 = Stigmasterol; 6 = Chlerosterol; 7 = β-Sitosterol; 8 = Sitostanol; 9 = Δ-5-Avenasterol; 10 = Δ-5,24- Stigmastadienol; 11 = Δ-7-Stigmastenol; 12 = Δ-7-Avenasterol; 13 = Total β-Sitosterol; 14 = Total sterols (mg/kg); 15 = Erythrodiol + Uvaol.

FIGURES

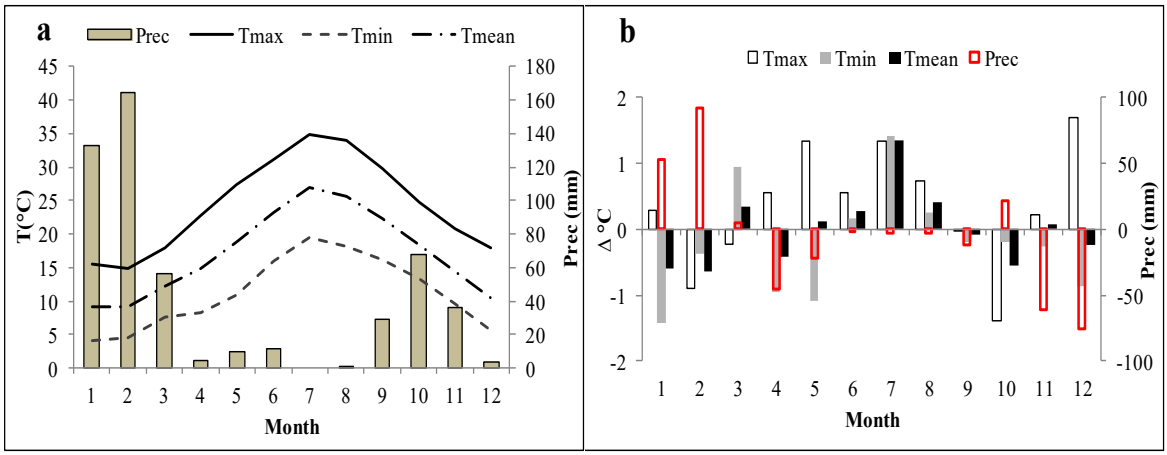

Figure 1. Meteorological conditions (Tmax, Tmin, Tmean, Precipitations) of 2015 growing season (a) and monthly variations from the average year (b).

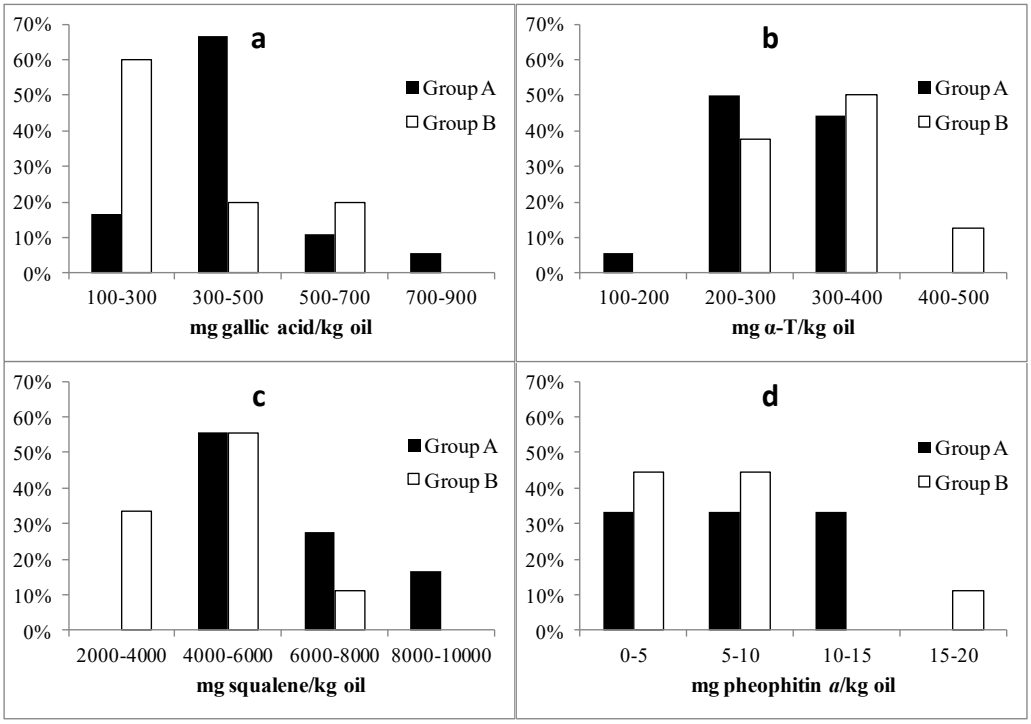

Figure 2. Distribution frequency histogram of total phenolic content (a), α-tocopherol content (b), squalene content (c) and total apparent chlorophylls (d), in group A and B samples

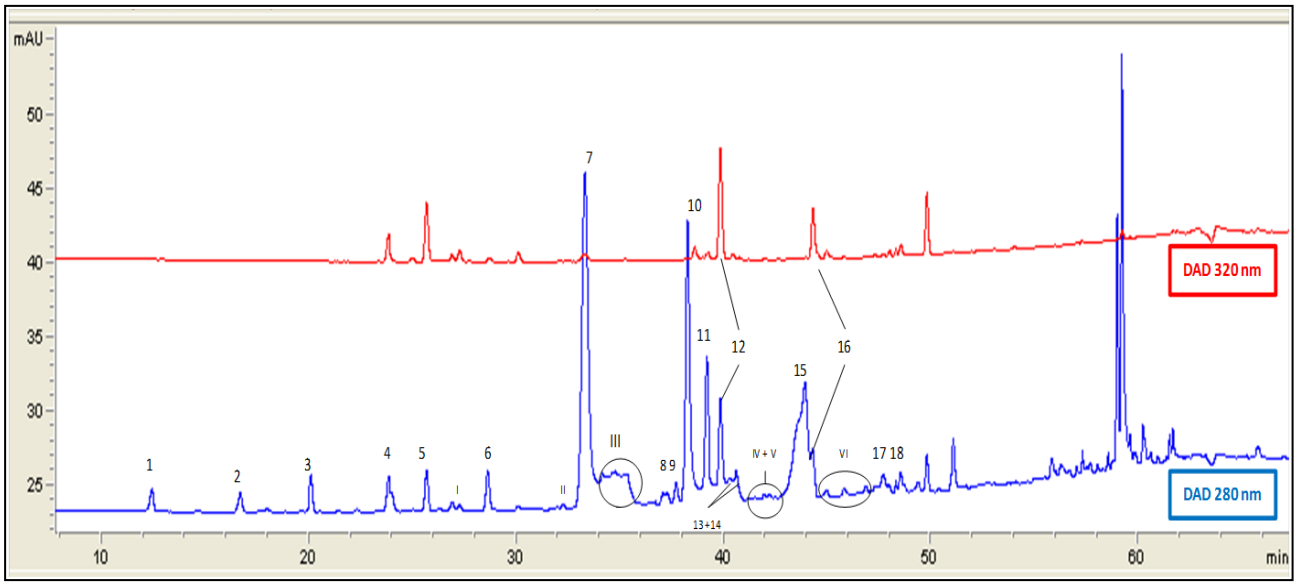

Figure 3. Representative HPLC chromatogram (280 and 320nm) of sample 28 (Tonda di Villacidro). Peak numbers correspond to compounds described in table 4.

EXPERIMENT 3: PHENOLIC AND FATTY ACID COMPOSITION RELATED TO HARVEST PERIOD IN SARDINIAN EXTRA VIRGIN OLIVE OILS

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ABSTRACT

Virgin olive oil (VOO) is known for its high quality and health properties determined by its specific chemical composition. Changes occurring in olive fruits during ripening process are cultivar specific and affect quality of the final product. The choice of the most appropriate harvest period depends on the production objective, variety, agronomic management and seasonal climate. Fatty acid and phenolic composition of Bosana, Semidana, Tonda di Cagliari and Coratina VOO, grown at similar environmental and agronomic conditions, was determined at several harvest dates. Determination of compounds was performed on GC-MS and RP-HPLC-DAD, respectively. Data were processed through one-way and two-way ANOVA. Moreover, Canonical Discriminant Analysis (CDA) and Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) were used to evaluate the discriminant power of the analyzed compounds. Results indicated that genetic factor influence strongly VOO fatty acid and phenolic profile, as well as maturation process. Multivariate analysis revealed that fatty acids and some minor phenolic compounds such as isomers of oleuropein and ligstroside aglycon were strongly influenced by genetic factor. Ripening effect was more evident on phenols, showing generally a decreasing trend, on respect to fatty acid composition.

INTRODUCTION

During the last years, request of high quality virgin olive oils (VOO) is increased, especially within not traditional consumers. The interest on this vegetable oil typical of Mediterranean culture is globally rising due to numerous recent studies that claimed diverse beneficial effects on human health of Mediterranean diet and its major lipidic source (Waterman and Lockwood, 2007; Perona and Botham, 2013). Healthy properties of VOO are connected to the specific chemical composition characterized by high concentration in bioactive molecules: phenolic compounds, tocopherols, carotenoids, triterpenic acids and squalene. The initial interest on the nutritional value of VOO is owned to its content in monounsaturated fatty acids (MUFA), particularly in oleic acid, and in low quantity of saturated and polyunsaturated fatty acids (SFA and PUFA). To this fatty acid (FA) composition have been attributed several benefits to human health such as reduced blood pressure and cholesterol concentration in blood serum (Terés et al, 2008). A high MUFA/PUFA ratio confers to VOO more resistance to oxidation phenomena elongating its shelf-life (Paz Aguilera et al, 2005; Zarrouk et al, 2009).

FA composition has been widely studied as an effective tool for varietal and geographical characterization and quality evaluation (Tsimidou and Karakostas, 1993; Ripa et al, 2008; Zarrouk et al, 2009). The genetic factor plays an important role on regard to the relationship between environmental conditions and FA composition (Mannina et al, 2001; Paz Aguilera et al, 2005; Mailer et al, 2010; Di Vaio et al, 2013). Temperatures occurring during oil accumulation process affect negatively oleic acid content conversely to linoleic, linolenic or palmitic acids (Lombardo et al, 2008); as temperatures depend on latitude, altitude and distance from the sea, also these factors contribute to determine its composition.

More recently, numerous nutraceutical properties have been attributed to polar phenols (Martin-Pelaez et al, 2013; Rodriguez-Moratò et al, 2015). They are the most important components that influence VOO stability against autoxidation processes (Blekas et al, 2002; Kalogeropoulos and Tsimidou, 2014). It has been proved that secoiridoids and phenolic alcohols have the highest antioxidant activity, mainly oleacein (dialdehydic form of decarboxymethyl oleuropein aglycon), oleocanthal (dialdehydic form of decarboxymethyl ligstroside aglycon) and oleuropein aglycon (Beauchamp et al, 2005; Di Maio et al, 2013). Secoiridoids are the most representative class of VOO phenolic compounds, followed by

lignans, phenolic alcohols, flavonoids and phenolic acids. Secoiridoids are also responsible of the sensorial attributes of bitterness, pungency and astringency (Bendini et al, 2007). Since phenolic compounds are the result of secondary metabolism, they are produced as a defense from environmental stress such as high temperatures, UV rays, wounding and water stress (Ryan et al, 2002). Indeed, a negative correlation between irrigation and total phenolic content has been largely reported (Patumi et al, 2002; Servili et al, 2007; Caruso et al, 2014). Although not all the phenolic classes are affected equally, secoiridoids decrease as more water is available, while lignans seem to be not affected. Other phenolic compounds have reported contrasting effects (Tognetti et al, 2007; Dabbou et al, 2011). The importance of environmental factors on determining phenolic composition was confirmed by several authors that, adopting multivariate analyses, were able to distinguish VOOs from different areas of origin (Bakhouche et al, 2013; Bajoub et al, 2015; Culeddu et al, 2017). Also oil extraction process cause several changes in phenolic profile due to the activity of endogenous enzymes that are influenced by temperatures, time and oxygen concentration, activated mainly during malaxation process (Taticchi et al, 2013). In addiction, the type of crusher, the presence or not of the stone, as well as the use of two or three phase decanter, influence phenolic concentration (Del Caro et al, 2006; Stefanoudaki et al, 2011; Fadda et al, 2012; Ranalli and Contento, 2013). Over all, is the genetic factor the one that influences principally composition and amount of phenolic compounds (Alagna et al, 2012)**.**

Besides genetic and environmental factors, VOO quality parameters are influenced by changes occurring on fruits during ripening that involve FA, phenolic and volatile composition, tocopherols, squalene and pigments concentration (Angerosa et al, 2004; Baccouri et al, 2008; Boskou, 2008; Inglese et al, 2011).

Variations in FA composition during maturation are strictly dependent to the genetic factor; oleic, palmitic and linoleic acid can increase or decrease, or in some cases any changes have been observed; generally changes in oleic acid were correlated to changes in linoleic acids presenting opposite trends (Baccouri et al, 2008; Vekiari et al, 2010). During fruit ripening, there is a general decrease of total phenolic concentration, mainly due to the hydrolysis of secoiridoids (Gambacorta et al, 2010) that may also affect oxidative stability of VOO and consequently decrease the potential shelf life (Rotondi et al, 2004; Jimenez et al, 2013). In contrast, some authors observed distinct trends on secoiridoids and other less

representative phenolic classes (Artajo et al, 2006; Vekiari et al, 2010; Dabbou et al, 2011).

Knowledge of the maturation pattern of olive varieties allows producers to identify the optimal harvest period in accordance to the production objective. An early harvest can lead to obtain oils characterized by strong bitter, pungent, green-leaf and artichoke attributes, whereas a later harvest can lead to obtain more delicate sensations, less bitter and pungent, with hints of ripe fruit, tomato and dried fruits (Angerosa et al, 2004; Rotondi et al, 2004). In general, the choice of the optimum technological maturation takes place when drupes achieve a satisfactory oil yield with a good amount of antioxidant compounds (Inglese et al, 2011). Moreover, harvest time depends on the technologies adopted (Camposeo et al, 2013).

Usually in Sardinia, harvest begins on the second half of October, in the warmer areas, and lasts until the end of January. Harvest period is principally affected by the maturation trend of Bosana variety, the most widespread variety in Sardinia (Bandino and Dettori, 2001), which optimum ripening stage is considered to be achieved when 50% of drupes peel turned color to violet (Fadda et al, 2012). Nowadays, to our knowledge, changes occurring on VOO composition during ripening stage of Sardinian varieties have not been yet investigated.

The aim of the present study was to determine the changes occurring on fatty acid and phenolic composition of three of the most important Sardinian cultivars, Bosana, Semidana and Tonda di Cagliari compared with the Apulian Coratina at several harvest times, with the aim to identify the most appropriate harvest period, for the first time for the Sardinian varieties, in accordance to the commercial purpose designed.

MATERIALS AND METHODS

Study area

The study was carried out in the Experimental Station “A. Millela” of the University of Sassari, located in San Quirico - Fenosu, Oristano, Sardinia (39°54’12” N, 8°37’19” E), at 13m a.s.l.

Trees were planted at a space of 6x6m and conducted with the following agronomic practices: soil managed with mowing of the spontaneous vegetation, drip irrigation (ca. 2000-2500 mc per year), yearly pruning according to the “polyconic vase”. The area is characterized by a Thermo-Mediterranean bioclimate (Canu et al, 2015). According to the

Department of Meteorology and Climatology, Environmental Protection Agency of Sardinia (ARPAS), annual average temperature of the area is 17.1°C, with an average maximum at 23.9°C and a minimum at 11.3°C. Winters are mild and summers hot and dry; intermediate season is characterized by high variability of temperatures and a constant high humidity. Precipitations are concentrated mainly during autumn and winter months, annual mean rainfall is around 580 mm.

Olive and oil samples

Two representative olive samples per four variety (Bosana, Semidana, Tonda di Cagliari and Coratina) were collected mechanically at five (Bosana) or four (the other varieties) harvest dates, from 15 October to 15 December 2016, with an interval of 15 days. Semidana was collected first on 30 October due to its later maturation. For each sample was determined the maturation index (MI) according to the method described by the Agronomic Station of Jaén, Spain, based on changes occurring on pulp and skin color of drupes (Uceda and Frias, 1975).

Olive samples, 25 – 30 kg, were processed soon after collection throughout the use of a small scale industrial mill "Sintesi 80" Mori TEM (Tavernelle Val di Pesa, Italy) equipped with a blade crusher, 40 kg malaxator and two phase decanter. The same parameters of extraction process were maintained for all the samples: environmental and olive temperatures was kept at 20°C, olive paste was malaxed for 15 minutes at 25°C, decanter temperature was set at 28°C.

Oil samples obtained were filtered and stored in dark glass bottles at 11°C and protected from any source of light. Analyses were carried out within one month after storage.

Standards and solvents

Folin Ciocalteu phenol reagent, hydroxytyrosol (≥98%), tyrosol (≥98%), oleuropein (≥98%), vanillin (≥99%), vanillic acid (≥97%), p-coumaric acid (≥98%), pinoresinol (≥95%), luteolin (≥98%), apigenin (≥95%), FAME mixture, methanol for HPLC (≥99.9%) and *n*-hexane Chromasolv ® for HPLC (≥97.0% GC) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Determination of fatty acid methyl esters (FAME)

FAME profile was determined by gas chromatography according to the method described by EU based on cold transesterification (Reg. EC No 2568/91). Analysis was carried out on an Agilent gas chromatograph 6890N equipped with a mass spectrometer (5973N) (Agilent Technologies, Palo Alto, CA, USA) and capillary column DB-23 (30 m x 0.25 mm x 0.25 μm) (Agilent Technologies). The operating conditions were: carrier gas helium (2.1 ml/min); oven temperature 150°C (1 min), 150-200°C at 3°C/min, 200-250°C at 20°C/min (2 min); transfer line temperature 230°C. The injection volume was 1 μl (splitless mode).

Fatty acids methyl esters were identified comparing their retention time with those of standard and content was calculated as percentage of peak area. Repeatability of the method was found satisfactory (CV = 0.72 – 6.45%, n = 5). Samples were analyzed in duplicate.

Determination of phenolic composition

Extraction

Phenolic compounds were extracted in accordance with the method described by the International Olive Council (IOC, 2009) modified as follows: 4g of oil were dissolved in 5 ml of methanol/water solution (80:20, v/v). The mixture was stirred for 30 min and, after 15 min decantation, was centrifuged for 5 min at 5000 rpm. The surnatant was removed with a glass pipette and the extraction process was repeated a second time. Extracts were joined and filtered through a 0.45 μm PVDV filters. Samples were stored at -78°C until further analysis.

Total phenolic content

Total phenolic content was determined throughout Folin-Ciocalteu assay method (Bazzu et al, 2017). Quantification was obtained by means of a calibration curve of gallic acid (10-40 $mg*kg^{-1}$, R^2= 0.996). Results were expressed as mg of gallic acid equivalents (GAE) per kg of oil. Samples were analyzed in duplicate.

Determination of phenolic composition

An Agilent 1100 LC System (Agilent Technologies, Palo Alto, CA, USA) equipped with a quaternary pump (G1311A), degasser, column thermostat, auto-sampler (G1313A), diode array detector (G1315 B, DAD) was used for RP-HPLC analysis of phenolic compounds. Chromatographic separation was achieved with a Luna C18 column (250 x 4.6 mm, 5 μm)

from Phenomenex (Torrance, CA, USA) with a security guard cartridge (4 × 2 mm). Flow rate was set at 1 ml/min, column temperature at 30°C. The gradient elution consisted on a ternary mobile phase of water and 0.1% trifluoracetic acid (A), methanol (B) and acetonitrile (C). Gradient program was set as follows: initial percentage eluent composition was 96:2:2 (A:B:C); 50:25:25 from 0 to 40 min; 40:30:30 from 40 to 45 min; 0:50:50 from 45 to 60 min; 0:50:50 for 10 min; 96:2:2 12 min. Injection volume was 20 µl. Detection was performed at 280 and 320 nm. Phenols were identified according to retention time of a mixture of standards and quantified using respective calibration curves (1.5, 3, 4.5, 6, 7.5 mg/kg). Phenolic compounds which were not referred by standards were identified with mass spectra and data reported in literature (IOC, 2009; Tasioula-Margari and Tsabolatidou, 2015; Kotsiou and Tasioula-Margari, 2016), as described in Experiment 2. Repeatability of the method was considered satisfactory for all the quantified compounds (CV= 0.53-7.65%, n=5).

Statistical analyses

Two-way ANOVA was used to evaluate the influence of the harvest time and the genetic factor. In addition, significance of interaction between the two factors was evaluated. When appropriate, one-way analysis of variance (ANOVA) was performed on some of the analyzed parameters to assess separately the influence of the two factors. Significance of differences between means was determined with the use of Tukey's test ($\alpha < 0.05$).

A chemometric approach was adopted to evaluate whether phenolic and fatty acid composition was able to discriminate among analyzed cultivars and harvest dates. The average values of 8 samples collected for Coratina, Semidana and Tonda di Cagliari and Bosana were used to perform the analysis. Bosana 5th harvest was not included in the models in order to have a balanced scheme. Discriminant capacity of variables was evaluated with the use of canonical discriminant analysis (CDA) and Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA). In CDA models, correlated data variations are distributed in a multidimensional space. In OPLS-DA the correlated data variations are included only in the first latent variable, y axis in bidimensional representations indicate the inter group variability. Variables were scaled before analysis.

Data were processed with the use of R Studio statistical software (2017) and SIMCA-P software version 13.0 (Umetrics AB, Umea, Sweden).

RESULTS AND DISCUSSION

Weather conditions of the studied year

The meteoclimatic conditions of the year are described in Figure 1a, while monthly deviations from the average values (ten years period: 2006 – 2017) are reported in Figure 1b. 2016 was characterized by yearly average temperatures in line with the average year, whilst a lower amount of precipitations was registred (456 mm). First months of the year was characterized by higher values above the average of Tmax and Tmin and lower precipitations. On the contrary values below the average year were observed along the warm season, from May to August. The months usually involved on olive fruit ripening showed values of Tmax above the average mean, whereas Tmin values kept in line with average; during this period were registered relative high precipitation events during September followed by values below the average during later months.

Maturation Index

During harvest period MI showed a slow constant increase (Table 2) from values around 1 to values around 2.5. Only in Bosana was observed a fluctuating behavior, to be attributed to the particularly high crop load. Indeed it is known that a high quantity of fruits in plants can delay fruit maturation in terms of skin and pulp color (Barone et al, 1994). Moreover, lower temperatures during fruit development and and the precipitations above the average of September might have delayed the fruit colouring process, expressed as MI. Similar weather effects were observed also by other authors (Berenguer et al, 2006; Gucci et al, 2007).

Fatty acid composition

Fatty acid composition of all the samples analyzed fell within the ranges indicated by FAO (Codex Alimentarius, 2003) for extra virgin olive oil classification. Seven fatty acids were quantified: oleic, linoleic and palmitic acids were the most representative. Stearic, vaccenic and palmitoleic were found in smaller amounts. Other fatty acids, mainly linolenic, arachidic and behenic acids, identified in VOO were found only small quantities (less than 0.40%) or in traces. Two-way ANOVA (table 3) showed a strong influence of the genetic factor with significant differences on all the fatty acids quantified except for linolenic acid, due to its sporadic presence in samples. Both qualitative and quantitative differences between

cultivars were observed. Linolenic acid was observed at all harvests in Tonda di Cagliari, whereas in Semidana was not found in the first harvest, and only in traces in Bosana and Coratina. Palmitoleic acid was not detected in Coratina, while was found in traces at 3rd and 4th harvest of Bosana. In Coratina VOOs were also registered low quantities of vaccenic acid, whilst in Sardinian varieties values ranged between 1.71% (Bosana) and 2.93% (Tonda di Cagliari). Coratina was the variety with the highest content in oleic acid and the lowest content in linoleic, palmitic and stearic. Similar fatty acid profile of Coratina VOO, grown in different growing areas, was already described in literature (Stefanoudaki et al, 2000; Bianchi et al, 2001; Mailer et al, 2010; Rotondi et al, 2010). On the contrary, Tonda di Cagliari showed the lowest concentrations of oleic acid at all harvest dates and the highest values of linoleic and palmitic acids. Bosana and Semidana reported a similar fatty acid composition, the former with a slightly higher MUFA/PUFA ratio. Fatty acid profiles obtained for the Sardinian varieties were in line with those described previously in literature (Bianchi et al, 2001; Rotondi et al, 2010 ; Campus et al, 2013; Tuberoso et al, 2016), confirming that FAME profile is a very useful component for monovarietal VOO characterization.

Harvest period did not affect palmitoleic, linolenic and stearic acids. In addition, interaction was not significant for these fatty acids (table 3). Palmitic, oleic, vaccenic and linoleic acids were significantly influenced by harvest time. For the same variables, interaction between cultivar and harvest date showed lower P-values (Table 3). Indeed, fatty acids composition, during harvest period, varied similarly among varieties (Table 4). A clear trend was observed for oleic and linoleic acids in all analyzed cultivars, decreasing and increasing respectively. Bosana showed a higher stability on regard to maturation process: it maintained a constant percentage of oleic acid, around 70%, until the 4th harvest, as well as for linolenic acid (9.35 – 11.77%). On the other hand, in Semidana and Tonda di Cagliari VOO was observed an average decrease of 10% of oleic acid with a strong increase in linoleic acid (around 40%). A slight increasing trend was also reported by palmitic acid in Semidana VOO, from 13.65% to 15.58%, even if only the first date was significantly different from the others. Different changing patterns during ripening process have been previously described, mostly depending by genetic factor (Di Vaio et al, 2013; Baccouri et al, 2007 and 2008). Several authors described the same behavior observed in this study for oleic and linoleic acids in Turkish and Italian varieties (Negriz and Engez, 2000; Scamoci et al, 2011). These trends

might be connected to the fatty acids biosynthetic pathway that involves first the biosynthesis of SFA (e.g. stearic and palmitic acids), then the biosynthesis of oleic and, subsequently, linoleic acid by enzymatic activity of oleate desaturase (Hernandez et al, 2011). The increase of palmitic acid observed in Semidana might mean that oil biosynthesis process was still ongoing. Similar patterns were also described previously in Sardinian varieties (Solinas, 1990). Changes in fatty acid profile of Semidana were in line with those observed for the same variety during Experiment 2.

Phenolic composition

Total phenolic content

Total phenolic content, as widely reported in literature (Baccouri et al, 2008; Gambacorta et al, 2010; Dabbou et al, 2011; Alagna et al, 2012), decrease with ripening process, because of the occurring of enzymatic hydrolysis, in drupes, of compounds like oleuropein and derivatives (Ryan and Robards, 1998). Degradation of oleuropein was followed by the increase of other phenols such as demethyloleuropein and elenolic acid glucosides, in turn hydrolyzed subsequently (Ryan and Robards, 1998; Alagna et al, 2012). Clear differences were observed between cultivars, both for phenolic amount and for decreasing trend. Values (figure 4) ranged between 134.5 mg/kg, (Semidana on 15th December) and 1039.2 mg/kg (Coratina on 15th October). Coratina and Bosana showed the highest values of phenolic content. The two varieties showed a good stability, in terms of total phenolic amount, until the 3rd harvest; at last harvest loosed respectively around 45% and 33%. A high total phenolic content was observed also in Semidana and Tonda di Cagliari VOO at first harvests (528.8 and 507.3 mg/kg respectively) which however decreased linearly until 134.5 and 142.1 mg/kg respectively. Our findings were among the highest values reported in literature for these cultivars (Del Caro et al, 2006; Gambacorta et al, 2010; Stefanoudaki et al, 2011; Fadda et al, 2012; Campus et al, 2013; Taticchi et al, 2013; Tuberoso et al, 2016).

Phenolic composition

Changes on the phenolic profile occurring during harvest period of Bosana, Coratina, Semidana and Tonda di Cagliari VOOs are reported in table 5. Eighteen chromatographic peaks were identified and quantified. Molecules identified corresponded to six classes, phenolic alcohols, phenolic acids, aldehydes, secoiridoids, lignans and flavonoids (table 5).

Two-way ANOVA revealed that all the phenolic compounds quantified were significantly affected by genetic factor and harvest period (table 3). Interaction was found significant for all compounds except for the tyrosol. Chromatographic analysis showed qualitative and quantitative differences among varieties, highlighting some cultivar-specific characters.

Secoiridoids was the most represented phenolic class. Bosana and Coratina were the varieties with the highest content in secoiridoids, on average 92% with points of 96% (Bosana at 1st harvest); relative content of secoiridoids in Coratina was stable over harvest dates. Semidana was the variety that showed the lowest percentage (77%) and also the highest decrease during ripening. Oleocanthal (oleoc), oleacein (oleac) and aglycones of oleuropein (Ol agl) and ligstroside (Lig agl) were the most abundant molecules, as reported by other authors that also described these molecules as the ones with the highest antioxidant power (Bendini et al, 2007; Servili et al, 2009). Bosana was the variety with the highest content of oleacein (237 mg/kg) and oleocanthal (156 mg/kg), while Coratina showed the highest concentrations of oleuropein (269 mg/kg) and ligstroside aglycon (121 mg/kg). These four molecules showed a general decrease with ripening process, except for Coratina, where oleacein and oleocanthal raised during later harvests. Contrasting performances related to cultivar were already reported in literature (Baccouri et al, 2008; Gomez-Rico et al, 2008; Vekiari et al, 2010). Other secoiridoids derivatives were quantified at lower concentrations, some of them seemed to be cultivar specific. A second isomer of ligstroside aglycon reported values in the range of 5.71 – 17.70 mg/kg. It was present in higher amounts in Tonda di Cagliari, as well as for a dialdehydic form of Lig agl identified (isomer 1). Differently to Sardinian varieties, in Coratina was not detected the isomer 2 of the dialdehydic form of Lig agl, neither the elenolic acid at later harvests. A second isomer of Ol agl was detected in all cultivars, mainly in Bosana, wherease only at last harvest in Coratina. Between Ol agl and Lig agl isomers, a further peak, characterized by the two isomers co-eluted, was quantified. Co-elution of the two molecules was already reported in literature: some authors attributed the presence of several aglycon isomers to the reactions occurring during analytical phenolic extractions (Karkoula et al, 2014; Jerman Klen et al, 2015). Minor secoiridoids generally reported a decreasing trend.

Phenolic alcohols showed a decreasing trend in all varieties, except for tyrosol in Bosana, which however did not result statistically different from the other varieties. Coratina showed

the highest values both for hydroxytyrosol (5.71 mg/kg) and tyrosol (11.21 mg/kg).

Lignans were the second phenolic class in terms of concentration. Pinoresinol and 1-acetoxypinpresinol (AcPin) are the principal lignans described in VOO, their amount and ratio is cultivar specific (Brenes et al, 2000). In this study only AcPin was quantified, ranging between 2.58 mg/kg (Tonda di Cagliari) and 32.34 mg/kg (Coratina); Semidana was the variety with the highest relative amount, increasing from 6% to 17%. In the other varieties never achieved 7%, where absolute content increased until 3rd sampling, then suffered a drastic reduction. Similar trends were observed also for luteolin and apigenin in Bosana variety, while a constant increase was observed in the other three cultivars. Flavonoids and lignans behavior is in accordance to what described previously for other varieties (Artajo et al, 2006; Bengana et al, 2013). Semidana and Tonda di Cagliari registered the highest relative amounts of flavonoids, increasing during ripening, above 5%. Also in Bosana and Coratina was observed a constant increase of flavonoids, however in Coratina VOO never achieved 1% of total phenolic content. Relative increase of lignans and flavonoids is due mainly to the stronger decrease of secoiridoids; moreover, as reported by Alagna et al (2012), these phenolic classes follow different biosynthetic pathways.

Phenolic acids, together with vanillin were the compounds that reported the lowest values. Moreover, it seems that ripening process does not affect concentration of these molecules. Semidana showed the highest content.

Multivariate analyses

Analysis by cultivar

A complete separation between cultivars was achieved with the use of CDA analysis. Results represented in Figure 2 give an idea of the clear differences between Sardinian varieties and Coratina. In addition, the model suggested a possible similarity between Bosana and Semidana VOO, already described also on a genetic point of view (Erre et al, 2010). The first two canonical functions explained the 94.7% of total variability; first function (71.3% of variability explained) was able to separate Coratina, Semidana and Bosana, whereas second function was useful to separate Tonda di Cagliari and Bosana. It is important to take into account that intensity and direction of vectors are influenced by all categorical groups in the model, thus were not identified univocal cultivar biomarkers, but it was possible to identify

variables useful to discriminate among the studied varieties (Culeddu et al, 2017). Variables representation highlighted the predominant role of fatty acids as varietal descriptors. Coratina stood out for the higher content in oleic acid in contrast to the lower content in palmitic and vaccenic. Stearic acid seems to be a determining variable for Semidana and Bosana. Moreover, some phenolic compounds seem to have an influent role on varietal characterization. Ol agl 2 seem to be specific of Bosana and Semidana, while Lig agl 2, apigenin and Lig agl diald 1 seems to be characterizing factors for Tonda di Cagliari. Finally, Coratina differed from Sardinian varieties for the higher content of AcPin, Ol agl and Lig agl principal isomers, as well as for the lower content of elenolic acid and luteolin. CDA results were confirmed by OPLS-DA model. A complete separation between cultivars was achieved (Figure 3a). The model showed a good reliability, indicated by the goodness of fit parameters R^2x (0.846) and R^2y (0.896) and by Q^2y (0.833) parameter that indicates goodness of predictability. Variable influence on projection (VIP) values (Figure 3b) indicated 9 phenolic compounds (Ol agl 2, Lig agl 2, Van ac, Oleac, Api, AcPin, Ol+Lig agl, Vanil, Oleoc) and 2 fatty acids (Stearic and Vaccenic) as the variables with higher discriminant power (VIP $\geq$ 1), results quite in line with what observed on CDA. Importance of fatty acid composition for varietal characterization achieved by multivariate analysis was also reported by other authors (D'Imperio et al, 2007; Genovese et al, 2015).

Analysis by harvest date

CDA analysis showed also a good separation between the four harvest dates, indicating a significant gradual change in oil composition during time. As possible to observe in Figure 4, the distance between groups of samples increased according to harvest dates, which were separated by 1st canonical function that explained the 88.8% model variability. As observed with ANOVA analysis, fatty acids were less influenced by ripening process. Worse performance gave O2PLS-DA analysis according to harvest period (Figure 5). Low values of Q^2y (0.156) and R^2y (0.239) were obtained. Indeed, the last two harvest period did not achieve a complete separation. Despite that is possible to observe a coherent group distribution from the right side of the graph (1st harvest) to the left one (4th harvest), observing arrows, colored according to cultivar (Figure 5a), became clear the cultivar-specific maturation pattern. Moreover, 3rd and 4th harvest did not separate well due to Bosana and Coratina behavior. Similar discriminant power (Figures 4 and 5b) for variables in both

multivariate methods was observed. The four more representative phenolic compounds (Oleac, Oleoc, Ol agl and Lig agl), together with flavonoids, hydroxytyrosol and elenolic acid were the molecules most affected by harvest period. Moreover, oleic and linoleic were the most effective fatty acids, as also observed previously.

CONCLUSIONS

Conversely to a low or irregular fruit coloring trend, were observed significant changes in VOO characteristics that seem more influenced by maturation process. For this reason it would be appropriate to not rely exclusively on MI for estimating the most opportune harvest period. The choice of the most appropriate ripening index derives from the commercial and qualitative objectives and the technologies adopted, for instance, for harvests and oil extraction. Thus, in order to obtain a more accurate process monitoring, determination of MI should be coupled with further indexes such as drupe firmness or drupe detachment resitance. Anyway the decision of harvest period could not be addressed without the knowledge of the ripening pattern of the variety adopted, under a qualitative point of view.

Fatty acid and phenolic compounds are some of the most important components determining VOO quality. During ripening process they evolve with different manner according to the specific physiology of varieties. Even if varieties like Bosana and Semidana show a relatively similar chemical composition, they have demonstrated a different behavior. VOO quality of Semidana, as well as Tonda di Cagliari, decreased rapidly during harvest period, both in terms of MUFA/PUFA ratio and phenolic content. On regard to these varieties, it would be appropriate to start harvest when drupes start to change color. On the other hand, Bosana and Coratina showed a higher stability, keeping important phenolic amounts for a longer period. This aspect might give to producers the possibility of a better harvesting processes management and a wider range of products. Changes of enzymatic activities, due to physiological maturation process and different environmental conditions, might modify final product, mainly on regard to sensorial attributes. Moreover, phenolic components are responsible to pungent and bitter sensations that might hide or decrease some volatile attributes negatively affecting sensorial balance of VOO (Genovese et al, 2015). Product differentiation might be also a possibility to attract a wider number of potential consumers.

Further years of study on interaction between maturation process, cultivar and different environmental conditions might be useful in order to better understand the specific varietal physiology and improve quality of products.

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TABLES

Table 19. Abbreviations

Name	Code
1-acetoxypinoresinol	Ac-Pin
apigenin	Api
arachidic acid	C20:0
behenic acid	C22:0
dialdehydic form of ligstroside aglycon isomer 1	Lig agl diald 1
dialdehydic form of ligstroside aglycon isomer 2	Lig agl diald 2
elenolic acid	Ele ac
hydroxytyrosol	Hyd
isomers of oleuropein and ligstroside aglycon co-eluted	Ol+Lig agl
ligstroside aglycon isomer 1	Lig agl 1
ligstroside aglycon isomer 2	Lig agl 2
linoleic acid	C18:2
linolenic acid	C18:3
luteolin	Lut
monounsaturated - polyunsaturated fatty acids ratio	MUFA/PUFA
monounsaturated fatty acids	MUFA
oleacein	Oleac
oleic acid	C18:1Ω9
oleocanthal	Oleoc
oleuropein aglycon isomer 1	Ol agl 1
oleuropein aglycon isomer 2	Ol agl 2
palmitic acid	C16:0
palmitoleic acid	C16:1
para coumaric acid	p-coum ac
polyunsaturated fatty acids	PUFA
saturated fatty acids	SFA
stearic acid	C18:0
tyrosol	Tyr
vaccenic acid	C18:1Ω11
vanillic acid	Van ac
vanillin	Vanil
virgin olive oil	VOO

Table 210. Evolution of Maturation Index during harvests for the four analyzed varieties

Variety	1st harvest 15 ott	2nd harvest 30 ott	3rd harvest 15 nov	4th harvest 30 nov	5th harvest 15 dic
Bosana	1.42±0.16	1.51±0.04	2.19±0.07	1.42±0.05	2.02±0.12
Coratina	0.95±0.02	1.17±0.05	2.25±0.1	2.60±0.15	
Semidana		0.87±0.12	1.13±0.14	1.98±0.21	2.75±0.34
Tonda di Cagliari	1.03±0.14	1.70±0.03	1.67±0.02	2.14±0.04	

Values are the mean of two independent measurements ± standard deviation

Table 3. Significance values for the two-ways ANOVA for all the variables analyzed.

Variables		Cultivar	Harvest	Interaction
Fatty acids	C16:0	***	***	*
	C16:1	***	n.s.	n.s.
	C18:0	***	n.s.	n.s.
	C18:1Ω9	***	***	**
	C18:1Ω11	***	**	**
	C18:2	***	***	**
	C18:3	n.s.	n.s.	n.s.
	SFA	***	***	*
	MUFA	***	***	**
	PUFA	***	***	*
	MUFA/PUFA	***	***	***
Phenolic compounds	Hyd	***	***	**
	Tyr	***	*	n.s.
	Van ac	***	***	***
	p-coum ac	***	***	***
	Vanil	***	***	***
	Ele ac	***	***	***
	Oleac	***	***	***
	Lig agl diald 1	***	**	***
	Lig agl diald 2	***	**	**
	Oleoc	***	***	***
	Ol agl 1	***	***	***
	Ol agl 2	***	***	***
	Ol+Lig agl	***	***	**
	Lig agl 1	***	***	***
	Lig agl 2	***	***	**
	Ac-Pin	***	***	***
	Lut	***	***	***
	Api	***	***	**
	Total phenols	***	***	***

*** = p-value < 0.001; ** = p-value < 0.01; * = p-value < 0.05; n.s. = not significant difference.

Table 4. Changes in the fatty acid profile of Bosana, Coratina, Semidana and Tonda di Cagliari related to harvest period.

Fatty acid	Bosana					Coratina				Semidana				Tonda di Cagliari			
	1	2	3	4	5	1	2	3	4	2	3	4	5	1	2	3	4
C16:0	13.93^{ab,y}	14.52^{ab,x}	13.38^{b,x}	13.57^{ab,y}	14.89^{a}	11.27^{z}	11.04^{y}	10.06^{y}	10.55^{z}	13.95^{b,x}	14.78^{ab,x}	15.33^{a,x}	15.58^{a}	15.30^{x}	14.84^{x}	14.98^{x}	16.25^{x}
C16:1	0.58	0.22	tr.	tr.	0.26	n.d.	n.d.	n.d.	n.d.	0.15	0.36	0.22^{y}	0.30	0.46	0.36	0.51	0.83^{x}
C18:0	2.24^{a,x}	1.99^{ab}	1.79^{b,xy}	1.76^{b,x}	1.78^{ab}	1.63^{y}	1.71	1.44^{z}	1.63^{xy}	2.03	2.06^{x}	1.84^{x}	1.66	1.53^{y}	1.58	1.54^{yz}	1.44^{y}
C18:1Ω9	71.24^{a,y}	69.22^{ab,y}	71.41^{a,y}	70.95^{a,y}	66.37^{b,x}	81.42^{a,x}	79.64^{ab,x}	80.54^{ab,x}	77.86^{b,x}	70.52^{a,y}	66.32^{b,z}	64.46^{bc,z}	63.63^{c,y}	66.25^{a,z}	64.30^{ab,z}	63.59^{ab,z}	59.32^{b,w}
C18:1Ω11	2.44	2.44^{x}	1.71^{y}	1.95^{y}	2.25	tr.	0.38^{y}	tr.	0.94^{z}	2.20^{b,x}	2.78^{a,x}	2.44^{ab,xy}	2.43^{ab}	2.54	2.43^{x}	2.59^{x}	2.93^{x}
C18:2	9.35^{c,y}	11.60^{b,y}	11.71^{b,y}	11.77^{b,z}	14.46^{a,y}	5.67^{c,z}	7.09^{b,z}	7.97^{ab,z}	9.02^{a,w}	11.15^{c,y}	13.50^{b,y}	15.60^{a,y}	16.31^{a,x}	13.79^{b,x}	16.37^{ab,x}	16.42^{ab,x}	19.15^{a,x}
C18:3	0.21	tr.	tr.	tr.	tr.	tr.	0.14	tr.	tr.	tr.	0.20	0.10	0.08	0.09	0.11	0.37	0.07
C20:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0,04	tr.	tr.	tr.
C22:0	n.d.	tr.	tr.	tr.	n.d.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.
SFA	16.18^{ab,x}	16.51^{a,x}	15.17^{b,x}	15.33^{ab,z}	16.67^{ab}	12.91^{a,y}	12.75^{ab,y}	11.50^{b,y}	12.17^{ab,z}	15.98^{x}	16.84^{x}	17.17^{x}	17.25	16.87^{x}	16.42^{x}	16.52^{x}	17.69^{x}
MUFA	74.27^{a,y}	71.89^{bc,y}	73.12^{ab,y}	72.91^{ab,y}	68,87^{c,x}	81.42^{a,x}	80.02^{ab,x}	80,54^{ab,x}	78.80^{b,x}	72.87^{a,y}	69.46^{b,yz}	67.12^{c,z}	66.36^{c,y}	69.25^{a,z}	67.09^{ab,z}	66.69^{ab,z}	63.08^{b,w}
PUFA	9.56^{c,y}	11.60^{b,y}	11.71^{b,y}	11.77^{b,z}	14.46^{a,y}	5.67^{c,z}	7.23^{b,z}	7.97^{ab,z}	9.02^{a,w}	11.15^{c,y}	13.70^{b,y}	15.71^{a,y}	16.39^{a,x}	13.88^{b,x}	16.49^{ab,x}	16.79^{ab,x}	19.22^{a,x}
MUFA/PUFA	7.79^{a,y}	6.21^{b,y}	6.28^{b,y}	6.20^{b,y}	4.76^{c,x}	14.52^{a,x}	11.21^{b,x}	10.13^{b,x}	8.77^{b,x}	6.55^{a,y}	5.08^{b,z}	4.30^{c,z}	4.05^{c,y}	5.07^{a,z}	4.12^{ab,z}	4.03^{ab,z}	3.28^{b,w}

Values, expressed as % of total composition, are the average of four independent measurements. Letters "a, b, c, d" indicates significant differences (Tukey test, $p < 0.05$) among harvest dates for the same cultivar. Letters "x, y, z, w" indicates significant differences (Tukey test, $p < 0.05$) between cultivars at the same harvest date.

Table 5. Changes in the phenolic profile of Bosana, Coratina, Semidana and Tonda di Cagliari related to harvest period.

Phenolic compounds		Bosana					Coratina				Semidana				Tonda di Cagliari			
Class	Code	1	2	3	4	5	1	2	3	4	2	3	4	5	1	2	3	4
Phenolic alcools	Hyd	2.56^{a}	2.44^{ab,xy}	1.99^{b,z}	2.67^{a,x}	2.61^{ab,x}	5.71^{a}	2.71^{b,xy}	2.69^{b,y}	1.97^{b,y}	3.37^{a,x}	3.19^{a,x}	1.35^{b,z}	1.43^{b,y}	3.10	1.29^{y}	1.38^{w}	1.15^{z}
	Tyr	4.79^{c}	4.13^{d}	4.97^{bc,yz}	5.41^{b,yz}	6.60^{a,x}	11.21^{a}	5.93^{b}	6.58^{b,xy}	5.81^{b,xy}	5.76	7.67^{x}	6.70^{x}	5.19^{y}	6.34	4.59	4.04^{z}	4.49^{z}
Phenolic acids	Van ac	0.62^{b,x}	0.47^{c,w}	0.67^{b,z}	0.67^{b,z}	0.94^{a,y}	0.40^{c,y}	0.64^{ab,z}	0.69^{a,z}	0.56^{b,w}	0.83^{b,y}	1.53^{a,x}	1.56^{a,x}	1.51^{a,x}	0.64^{c,x}	1.04^{b,x}	1.17^{a,y}	1.11^{a,y}
	p-coum ac	0.56^{a,x}	0.44^{bc,y}	0.39^{c,z}	0.47^{b,x}	0.49^{b,y}	0.40^{a,z}	0.37^{ab,z}	0.35^{b,w}	0.33^{b,z}	0.58^{b,x}	0.52^{b,x}	0.40^{c,y}	0.89^{a,x}	0.45^{b,y}	0.57^{a,x}	0.46^{b,y}	0.45^{b,x}
Aldehyds	Vanil	0.89^{a,x}	0.50^{b,y}	0.63^{b,yz}	0.42^{b,z}	0.53^{b,y}	0.62^{y}	0.67^{y}	0.59^{z}	0.64^{y}	0.98^{a,x}	0.95^{a,x}	1.02^{a,x}	0.72^{b,x}	0.96^{a,x}	0.88^{a,x}	0.84^{a,xy}	0.59^{b,y}
Secoiridoids	Ele ac	6.21^{a,x}	4.34^{ab,x}	2.28^{c,x}	2.51^{bc}	1.93^{c}	1.14^{y}	0.90^{z}	n.d.	n.d.	3.31^{a,y}	2.13^{b,x}	1.67^{bc}	0.90^{c}	5.87^{a,x}	3.58^{b,xy}	2.60^{bc,x}	1.33^{c}
	Oleac	221.96^{a,x}	237.22^{a,x}	137.14^{b,x}	71.76^{c,y}	69.69^{c,x}	111.96^{a,z}	65.09^{b,y}	129.95^{a,x}	147.69^{a,x}	90.76^{a,y}	32.55^{b,y}	20.43^{bc,z}	4.45^{c,y}	138.76^{a,y}	59.58^{b,y}	44.47^{c,y}	13.53^{d,z}
	Lig agl diald 1	0.61	1.74^{x}	1.10^{z}	1.50^{xy}	0.84^{y}	n.d.	0.33^{y}	1.50^{-x}	1.00^{y}	0.48^{c,y}	1.02^{bc,z}	1.31^{ab,y}	1.78^{a,x}	2.02^{b}	2.35^{ab,x}	2.42^{a,x}	2.33^{ab,x}
	Lig agl diald 2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.69	n.d.	1.04	n.d.	1.03	n.d.	1.01	1.57
	Oleoc	155.63^{a,x}	126.91^{b,x}	104.97^{b,y}	70.79^{c,y}	49.21^{c,x}	116.89^{bc,y}	89.35^{c,y}	153.73^{a,x}	141.02^{ab,x}	57.47^{a,z}	31.98^{b,z}	26.86^{bc,z}	16.99^{c,y}	107.96^{a,z}	65.45^{b,yz}	52.05^{c,z}	23.17^{d,z}
	Ol agl 2	3.77^{ab}	4.24^{a}	3.77^{ab}	2.65^{bc}	1.33^{c}	n.d.	n.d.	n.d.	3.03	1.49	1.26	1.04	1.11	n.d.	1.59	1.43	1.17
	Ol agl 1	94.53^{b,y}	116.38^{a,y}	118.65^{a,y}	53.4^{c,y}	32.95^{d,x}	268.91^{a,x}	153.18^{c,x}	181.02^{b,x}	94.62^{d,x}	66.23^{a,z}	39.92^{b,z}	37.70^{b,z}	11.61^{c,y}	59.9^{a,y}	37.57^{ab,w}	21.94^{ab,w}	13.72^{b,w}
	Ol+Lig agl	4.17^{xy}	3.72^{xy}	3.92^{y}	3.38^{y}	2.34	5.77^{a,x}	4.59^{ab,xy}	5.28^{ab,x}	3.59^{b,xy}	2.69^{a,y}	1.92^{b,z}	1.45^{b,z}	n.d.	3.30^{b,y}	4.83^{a,x}	3.39^{b,y}	4.22^{ab,x}
	Lig agl 1	31.46^{ab,y}	26.68^{b,y}	34.71^{a,y}	16.13^{c,y}	9.49^{c,x}	120.75^{a,x}	68.26^{b,x}	60.43^{b,x}	25.40^{c,x}	17.22^{a,y}	10.77^{b,z}	8.97^{bc,z}	5.52^{c,y}	26.25^{y}	20.15^{y}	12.98^{z}	8.39^{z}
	Lig agl 2	8.89^{a,y}	10.18^{a,y}	6.92^{b,y}	5.71^{b,z}	6.98^{b}	10.7^{a,y}	8.68^{b,z}	6.32^{c,y}	9.93^{ab,y}	8.91^{a,z}	10.77^{b,y}	7.34^{ab,z}	6.10^{bc}	17.70^{x}	15.63^{x}	14.69^{x}	15.89^{x}
Lignans	Ac-Pin	12.07^{y}	13.44^{y}	14.10^{z}	12.32^{y}	12.25	26.89^{b,x}	29.32^{b,x}	32.34^{a,x}	17.08^{c,x}	17.36^{a,y}	18.35^{a,y}	16.33^{a,x}	13.57^{b}	6.91^{z}	7.91^{z}	8.09^{w}	2.58^{z}
Flavonoids	Lut	1.49^{c,y}	2.42^{b,x}	3.56^{a,x}	2.93^{b,xy}	2.67^{b}	0.95^{c,z}	0.97^{c,z}	1.59^{b,w}	2.20^{a,z}	1.77^{b,y}	2.12^{b,z}	2.68^{a,y}	2.78^{a}	2.37^{b,x}	2.75^{ab,x}	2.82^{a,y}	3.05^{a,x}
	Api	0.99^{c,y}	1.38^{b,xy}	2.04^{a,y}	1.46^{b,y}	1.43^{b}	0.58^{c,y}	0.79^{bc,y}	0.90^{b,z}	1.20^{a,z}	0.93^{d,y}	1.11^{c,z}	1.35^{b,yz}	1.53^{a}	2.27^{x}	1.96^{x}	2.50^{x}	2.61^{x}
Total phenols		788.9^{a,y}	817.3^{a,x}	775.6^{a,y}	592.3^{b,y}	433.6^{c,x}	1039.2^{a,x}	844.8^{c,x}	934.6^{b,x}	688.5^{d,x}	528.8^{a,y}	397.6^{b,z}	310.7^{b,z}	134.5^{c,y}	507.3^{a,z}	357.5^{b,z}	265.2^{bc,w}	142.1^{c,w}

Values, expressed as mg/Kg of olive oil, are the average of four independent measurements. Letters “a, b, c, d” indicates significant differences (Tukey test, $p < 0.05$) among harvest dates for the same cultivar. Letters “x, y, z, w” indicates significant differences (Tukey test, $p < 0.05$) between cultivars at the same harvest date.

FIGURES

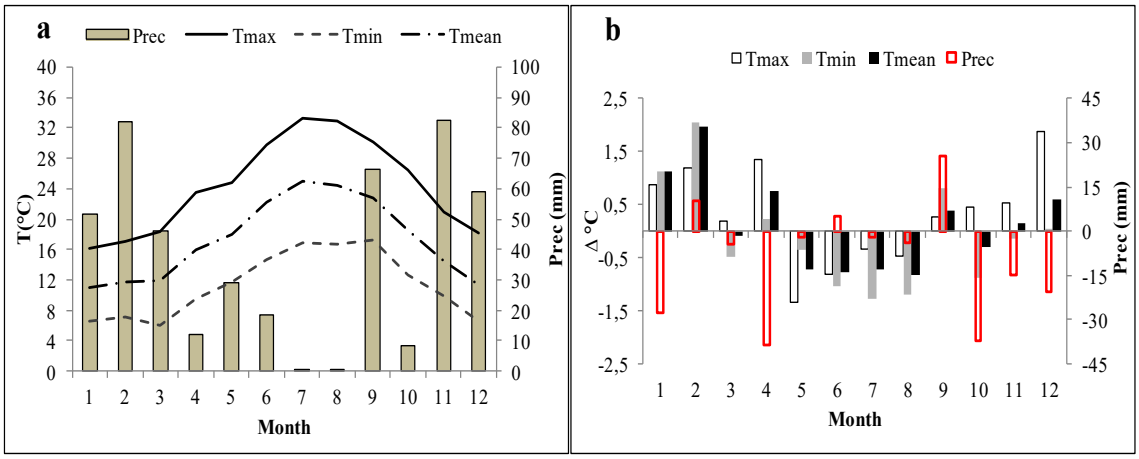

Figure 1. Meteorological conditions (Tmax, Tmin, Tmean, Precipitations) of 2016 growing season (a) and monthly variations from the average year (b).

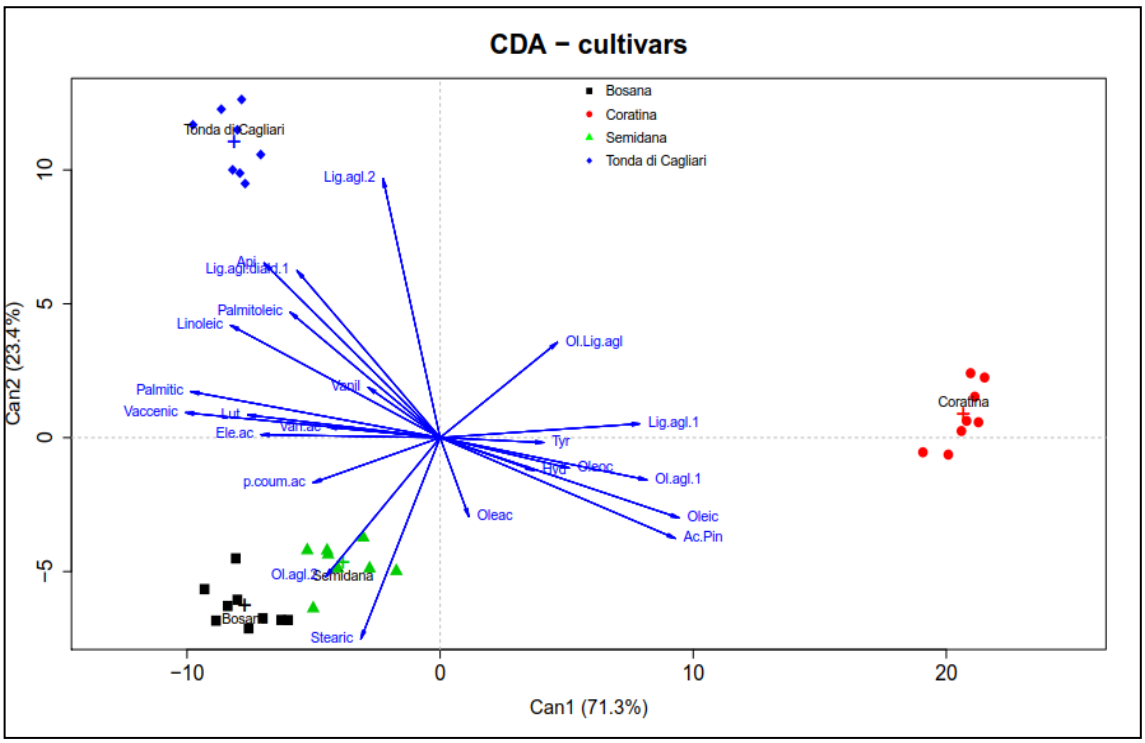

Figure 2. Similarity map of Canonical Discriminant Analysis (CDA) obtained for classitication by cultivar, determined by 1st and 2nd canonical functions

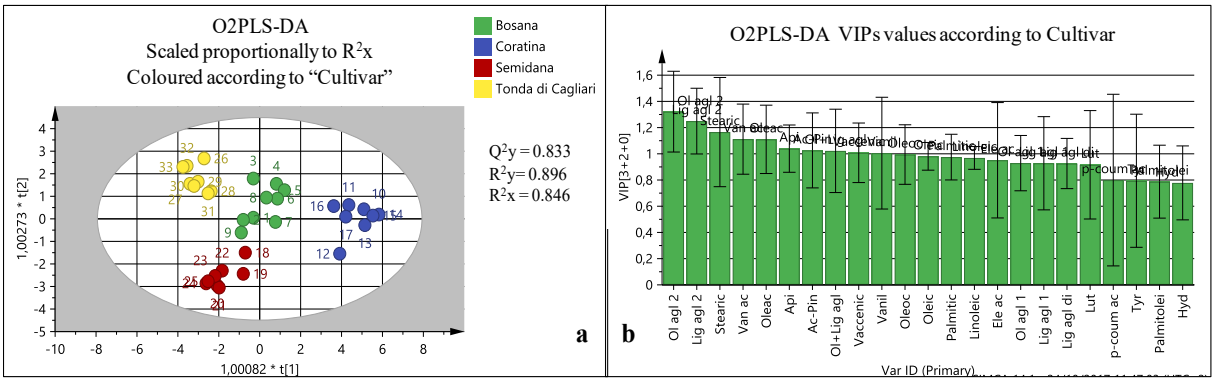

Figure 3. O2PLS-DA scoreplot for Bosana, Coratina, Semidana and Tonda di Cagliari VOO (a) and Variable Influence on Projection (VIP) values (b) for all the 23 variables used in the model.

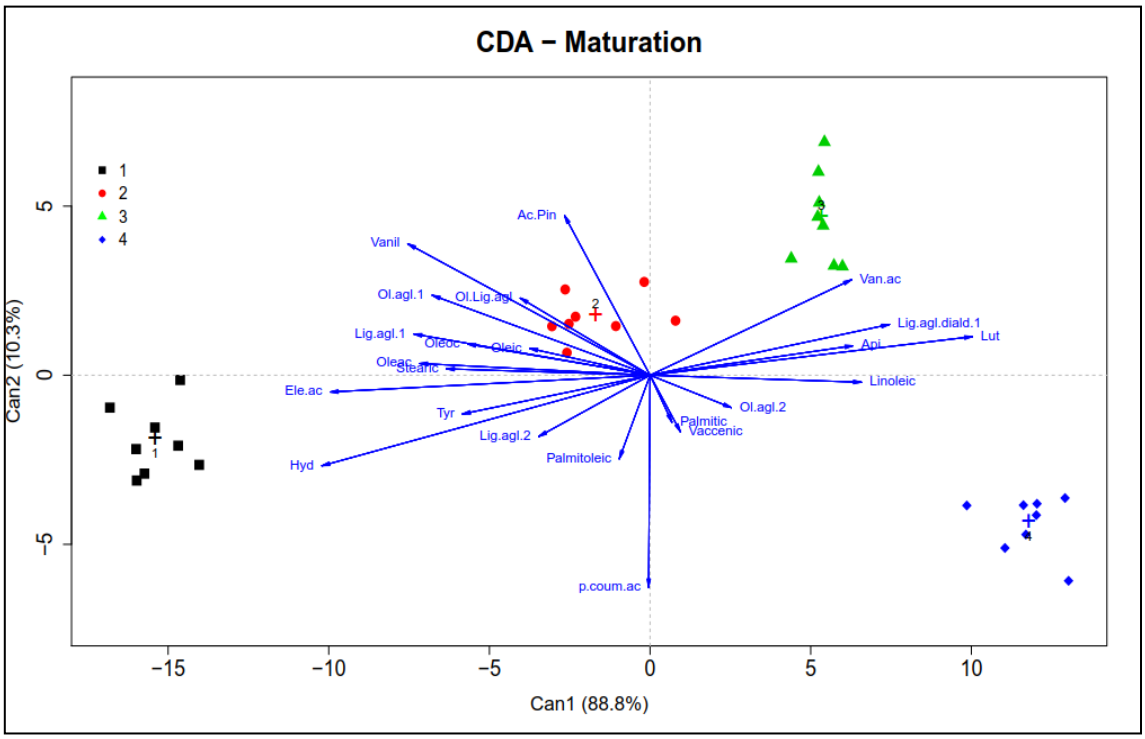

Figure 4. Similarity map of Canonical Discriminant Analysis (CDA) obtained for classification by harvest succession, determined by 1st and 2nd canonical functions.

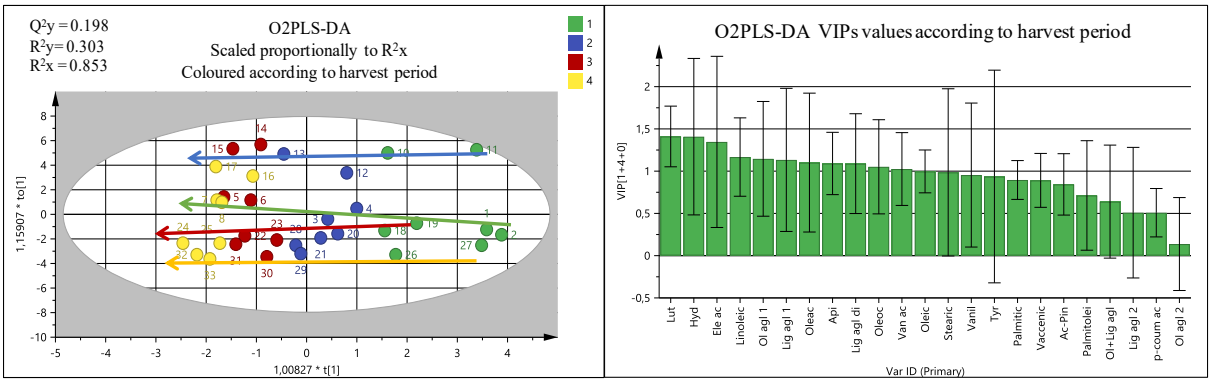

Figure 5. O2PLS-DA scoreplot for the five harvest dates (a) and Variable Influence on Projection (VIP) values (b) for all the 23 variables used in the model.

GENERAL CONCLUSIONS AND PERSPECTIVES

The recent trends in the olive oil market denoted an increase of olive oil production and an even more competitiveness of the southern Mediterranean countries vs. the new areas of cultivation. Moreover, also the interest on nutraceutical properties and specific geographical origin of VOO with higher quality is in expansion.

Due to the higher costs of production characterizing traditional olive grove systems and the scarce inclination to invest in new areas of cultivation, Italian olive sector loosed competitiveness during last years. Despite that, Italian extra VOO is still synonym of high quality.

If on one hand, the fragmented structure of Italian olive sector – characterized by low dimension of farms, high number and small dimension of mills and marginality of traditional olive groves – is a constraint for innovation and development; on the other hand, the specificity of regional productions identified by well adapted local varieties is an opportunity for differentiate production according to the increasing demand of higher quality of VOO.

In this context, a deep knowledge of the high Italian varietal heritage is an important instrument for valorization of productions and for the identification of specific characters for the adaptability to both new technologies and environmental conditions in view of expected climate changes.

In this general framework, this study aimed to support the Sardinian olive sector, with a particular focus on PDO "Sardegna", giving a detailed description of the performances of a large number of Sardinian varieties, both principal and lesser widespread, for which few information is available.

Phenological behavior, fruit and oil composition changes during harvest period were investigated under the same environmental and agronomical conditions, as well as VOOs were obtained with the same extraction process. These aspects led to evaluate clearly the influence of genetic factor. Moreover, the selected area of study, Oristano, is characterized by higher summer temperatures and lower amount of precipitations if compared with other

typical Sardinian areas of cultivation (e.g. North-West); thus it might be considered an interesting case of study in view of further climate changes.

In general, our findings confirmed varietal differences and similarities previously described by genetic studies (Erre et al, 2010).

During the three years of phenological monitoring, differences between varieties were observed, both in terms of the occurrence of phenological stage and variability during years. Multivariate analysis revealed to be a useful tool for discriminate among varietal groups, on the basis of their phenological behavior. Earlier varieties, like Pizz'e Carroga and Manna group (Tonda di Cagliari and similar), generally showed higher interannual variability indicating a stronger relationship with annual and seasonal temperature regime. Anyway, was observed that phenological behavior of olive trees is more affected by interannual variability than genotype. The positive correlation between November temperatures and the onset of vegetative and reproductive phases indicate the importance of the winter frosts for the release of dormancy period. The aspect of chill requirements is an important current topic in view of climate change; our preliminary findings suggest further investigations on regard to this issue. Further years of study might be useful to define better the relationships between the single varieties and weather related variables.

For the first time, the chemical profile of VOO from some minor Sardinian varieties was described. According to the chemical composition of VOOs, Sardinian varieties seem to be good sources of bioactive molecules, contributing differently for specific characters. Comprehensively Sardinian varieties stood the comparison with international ones. Even if compositional variability was high, it was possible to partially resume it into varietal groups: Bosana and Semidana were the varieties with the highest phenolic content, Tonda di Villacidro and similar showed the highest MUFA/PUFA ratio, while Tonda di Cagliari and similar varieties where the major sources of squalene.

Quality parameters indicated in PDO Sardegna regulation were largely achieved, suggesting the possibility to raise the minimum limits enhancing the quality of the label. The road to achieve an adequate label characterization is still long. Further studies are necessary to define better volatile and sensorial profiles of minor varieties. Other varieties admitted to PDO Sardegna are still almost unknown (e.g. synonyms of Bosana). Results on the variety

Maiorca, which stood out from his varietal group for a comprehensive higher VOO quality, are interesting because suggest a final goal that might be the selection of the varieties within varietal groups that better contribute to the quality of the label.

Finally, evolution on VOO fatty acid and phenolic composition, during the harvest period investigated in this study, differed according to the variety, particularly the latter. It was interesting to observe the high level of VOO quality of Bosana, keeping high amounts of phenolic compounds for a relatively long period.

This research gave some useful practical tools for producer's decisions on regard to agronomic practices occurring during early phases of growing season, aimed to improve reproductive process; moreover useful indications were provided to support the choice of variety and harvest period most suitable in relation to the production objective.

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