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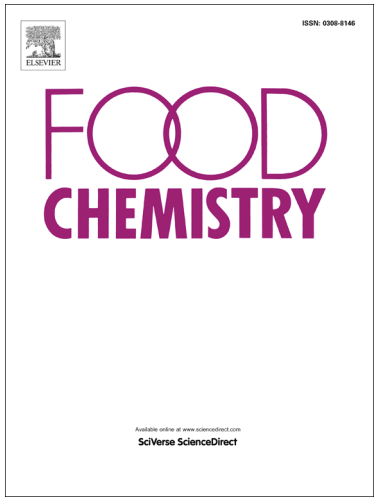

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Multivariate approach to assess the chemical composition of Italian virgin olive oils as a function of variety and harvest period

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ABSTRACT

Fatty acids, phenolic compounds, and tocopherols of Coratina, Bosana, Semidana, and Tonda di Cagliari virgin olive oils, were measured over a 45-day harvest period. Phenolic composition was the primary factor distinguishing Bosana, Tonda di Cagliari, and Semidana, whereas fatty acids differentiated Coratina and the other cultivars. Harvest period principally influenced oleacein, oleocanthal, oleuropein and ligstroside aglycones, and flavonoids. High phenolic content was observed for Coratina (1039 – 688 mg/kg) and Bosana (788 – 592 mg/kg). A drastic decrease in phenolic content was observed in Semidana (529 – 134 mg/kg) and Tonda di Cagliari (507 – 142 mg/kg) during the harvest period. These two cultivars also had low MUFA/PUFA (6.0 – 4.0 and 4.9 – 3.2 respectively), suggesting that these varieties should be harvested earlier in the season. These results provide information to producers for improved management of the harvesting process, which is strongly affected by varietal factors.

Keywords: Virgin olive oil health properties; fatty acids; polar phenolic composition; α-tocopherol; Orthogonal Projection to Latent Structures; chemometrics; Sardinian virgin olive oil; fruit ripening

1. Introduction

Over the last few years, several studies have confirmed the beneficial effects of the Mediterranean diet on human health (Perona & Botham, 2013), thanks in part to its major lipidic source, virgin olive oil (VOO). VOO is characterized by high concentrations of monounsaturated fatty acids (MUFA) and bioactive molecules such as polar phenolic compounds, tocopherols, carotenoids, triterpenic acids, and squalene (Perona & Botham, 2013). A recent study (Santona et al., 2018) found that the health benefits of the aforementioned chemical compounds in VOOs are potentiated by a microflora with a probiotic environment.

The composition and concentration of bioactive molecules, fatty acid (FA) composition, sensorial profile, and associated volatile compounds, define the quality of an extra VOO, which is

influenced by genetics (i.e. variety), seasonal conditions (i.e. weather condition), environmental, agronomic and technological factors (i.e. extraction) (Inglese, Famiani, Galvano, Servili, Esposto & Urbani, 2011).

A crucial issue in obtaining high quality products is the choice of optimal harvest time, which is dependent on commercial objectives, expected quality (e.g. FA composition, concentration of bioactive compounds, and sensory profile) and the adopted technologies (e.g. for harvest and oil extraction). For this reason, several indirect indicators of olive ripeness are linked to fruit physical parameters (e.g. drupe weight, moisture, color, and firmness), fruit chemical composition (e.g. polar phenolic concentration and composition, and total sugars), and harvest technology (e.g. resistance to detachment), have been proposed as new markers for optimal harvest time that find a compromise between VOO yield and quality (Camposeo, Vivaldi & Gattullo, 2013; Cecchi et al., 2013; Dag, Harlev, Lavee, Zipori, & Kerem, 2014).

Changes in the composition of FA, phenolics and volatiles, tocopherols, squalene, and pigments have been observed in VOO during ripening (Angerosa, Servili, Selvaggini, Taticchi, Esposto, & Montedoro, 2004; Beltrán, Aguilera, Del Rio, Sanchez, & Martinez, 2005; Baccouri et al., 2008; Kalogeropoulos & Tsimidou, 2014).

Variations in FA composition during ripening is closely associated with genetic factors and seasonal weather conditions (Dag et al., 2014), which determine positive and negative trends in oleic, palmitic, and linoleic acids concentrations. Generally, an increase in oleic acid (the principal monounsaturated FA of VOO) concentration has been shown to be linked to a decrease in linoleic acid (the principal polyunsaturated FA of VOO), or vice versa (Poiana & Mincione, 2004; Baccouri et al., 2008, Dag et al., 2014). Variations in FA profiles during fruit ripening affect the monounsaturated/polyunsaturated FA ratio (MUFA/PUFA), an important indicator of VOO quality which is directly linked to oxidative stability and, therefore, to the potential shelf-life of VOO (Beltrán et al., 2005; Zarrouk, Baccouri, Taamalli, Trigui, Daoud, & Zarrouk, 2009).

In general, during fruit ripening, there is a decrease in the amount of polar phenolic

compounds, and α-tocopherol (α-T) (Beltrán et al., 2005; Gambacorta, Faccia, Previtali, Pati, Notte, & Baiano, 2010). The phenolic class of secoiridoids includes the most important chemical components protecting VOO against autoxidation processes, these include oleacein (dialdehydic form of decarboxymethyl oleuropein aglycon), oleocanthal (dialdehydic form of decarboxymethyl ligstroside aglycon), and oleuropein aglycon (Servili et al., 2009). Moreover, secoiridoids are responsible for the sensorial attributes of bitterness, pungency, and astringency (Servili et al., 2009). Tocopherols, as well as polar phenols, play an important role in protection of VOO from autoxidation and photo-oxidation processes (Tsimidou, 2012). The major isoform of vitamin E found in VOOs is α-T. Levels of α-T in VOO depend principally on genetic factors (Tsimidou, 2012).

In this study, FAs, phenolic composition and the α-T content of VOOs from Coratina, Bosana, Tonda di Cagliari, and Semidana were examined for four harvest periods and then cross-compared. Coratina is an olive variety cultivated all over the world (Zarrouk et al., 2009; Mailer, Ayton, & Graham, 2010; Rondanini, Castro, Searles & Rousseaux, 2014; Dabbou, Dabbou, Chehab, Taticchi, Servili & Hammami, 2015) owing to its resilience to a wide range of bioclimatic conditions. Several studies performed in southern regions of Italy (Apulia, Calabria, and Molise), described Coratina as a mid-late variety based on drupe pigmentation (Cinquanta, Esti & Di Matteo, 2001; Poiana & Mincione, 2004; Camposeo et al., 2013). Some studies have reported that ripening has little effect on Coratina FA composition (Cinquanta et al., 2001; Poiana & Mincione, 2004; Rondanini et al., 2014). Semidana and Tonda di Cagliari are varieties which are receiving increased interest in the Sardinian olive oil production sector. In fact, their extra VOOs, and that of Bosana, are guaranteed by the Protected Designation of Origin (PDO) "Sardegna" certification. Therefore, to guarantee high quality PDO products, knowledge of the changes in VOO chemical composition during harvest period is essential. Today, only preliminary information regarding Semidana VOO composition during ripening is available (Deiana et al., 2019), while no studies for Tonda di Cagliari are available.

Bosana olives, the most common olive variety in Sardinia and one of the most important on a National level (Culeddu et al., 2017), are considered matured when 50% of the drupes skin change their color to violet (Deiana et al., 2019). For this variety, a recent study described a slow rate of ripening and low changes of chemical composition in secoiridoids, saturated FA (SFA) and pigments (Morrone, Neri, Cantini, Alfei, & Rotondi, 2018).

The purpose of this study was to evaluate VOO chemical composition as a function of harvest period and cultivar. VOO from Coratina, Bosana, Tonda di Cagliari, and Semidana grown under Sardinian (Italy) bioclimatic conditions were investigated. Environmental, agronomic, and technological conditions were kept the same for all of the varieties. Since their active role in determining the nutraceutical properties and chemical quality of extra VOOs (EVOO's) (Dag et al., 2014; Kalogeropoulos & Tsimidou, 2014), fatty acid profile, tocopherol content and phenolic composition were the variables we investigated. Experimental data were subjected to Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) to evaluate the effect of genetic factors and the OPLS regression model was used to assess the relationship between ripeness and VOO chemical composition. To our knowledge this is the first paper where the OPLS regression model has been applied for this purpose. This work provides general guidelines for olive oil producers on timing optimization for the harvesting processes according to some cultivar-specific VOO's qualitative and nutraceutical aspects. To our knowledge, there are no studies on the influence of the ripening process on chemical composition and quality of Coratina and Tonda di Cagliari VOOs grown under Sardinian bioclimatic conditions.

2. Materials and methods

2.1. Area of study

This study was carried out in the San Quirico - Fenosu (Sardinia, 39°54'12" N, 8°37'19" E) experimental station of the University of Sassari (Italy) which is 13 m above sea level. Olive trees were planted in 1998 with a density of 6 x 6 m and conducted with the following agronomical practices: mowing of the spontaneous vegetation, drip irrigation (ca. 2000 – 2500 mc per year) and annual pruning according to the "polyconic vase" model. The study area bioclimate is classified as

Thermo-Mediterranean (Canu, Rosati, Fiori, Motroni, Filigheddu, & Farris, 2015), with annual mean, maximum, and minimum average temperatures of 17.1°C, 25.4°C (July), and 9.6°C (February), respectively. Precipitation is concentrated in the autumn and winter seasons, and the annual mean rainfall is 581 mm (Environmental Protection Agency of Sardinia, ARPAS, average year 2006 – 2017). However, in 2016 the area experienced a year characterized by draught with average precipitation of 456 mm, and while the average temperatures were normal, the maximum and minimum temperatures in the warm season were below average and the maximum temperature for late summer and autumn was above the average mean (SM1). During the same period, higher than average precipitation during September was followed by lower than average precipitation during later months.

2.2. Olive sampling and processing

Two separate olive batches for each variety were handpicked at four harvest dates (2 week intervals from one to the other): Coratina, Bosana and Tonda di Cagliari from 15 October 2016 to 30 November 2016; Semidana, due to its later ripening, from 30 October 2016 to 15 December 2016. Therefore, for each variety 8 separate oil samples were obtained (4 harvest dates per 2 separate olive batches). A 45-day harvest period is typical of a harvest campaign in Sardinian olive oil farms as characterized by similar bioclimatic conditions of our area of study. In fact, in Sardinia in warm areas, the earliest start of the harvest is usually around the second half of October, and in colder areas lasts until the end of January.

For each sample, the maturation index (MI) was determined following the method described by the International Olive Council (IOC) which is based on changes occurring on the drupes pulp and skin color (IOC, 2011).

Samples of 25 – 30 kg of olive were processed soon after harvest using a small-scale industrial mill “Sintesi 80” Mori TEM (Tavernelle Val di Pesa, Italy) equipped with a blade crusher, 40 kg capability vertical malaxator, and two-phase decanter. The same procedure was

replicated for all the samples: laboratory temperature was kept at 20°C, 30 minutes malaxation time at 25°C, and decanter temperature at 28°C. The obtained oil samples were filtered and stored in dark glass bottles at -18°C and protected from any source of light until analyses.

2.3. Standards and reagents

Folin Ciocalteu phenol reagent, hydroxytyrosol (≥98%) and tyrosol (≥98%), oleuropein (≥98%), vanillin (≥99%), vanillic acid (≥97%), caffeic acid (≥98%), p-coumaric acid (≥98%), pinoresinol (≥95%), luteolin (≥98%), apigenin (≥95%), and fatty acid methyl ester (FAME) mixture (≥ 98%), were all purchased from Sigma–Aldrich (Milano, Italy and St. Louis, MO, USA). We purchased α-T (>96%) from Fluka Chemie GmbH (Buchs, Switzerland). Acetonitrile and 2-propanol (Chromasolv®) were purchased from ChemLab (Zedelgen, Belgium). Methanol (≥99.9%) and n-hexane Chromasolv® (≥97.0%; GC) for high performance liquid chromatography (HPLC) were purchased from Sigma–Aldrich (Milano, Italy and St. Louis, MO, USA). Ultrapure water (H_2O) was obtained from a Milli-Q system (Millipore Corporation, Billerica, MA, USA).

2.4. Determination of FAME profiles

FAME profiles were determined by gas chromatography according to the cold transesterification method as regulated by the European Union (Reg. EC No 2568/91 and subsequent amendments, Reg. EC No 2015/1833), with slight modifications on data detection. 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 following operating conditions were used: carrier gas helium (2.1 ml/min); oven temperature of 150 °C (1 min), 150 – 200 °C 3 °C/min, 200 – 250 °C at 20 °C/min (2 min); transfer line temperature of 230 °C. The injection volume was 1 μl (splitless mode) and runs were carried out in scan mode. FAMEs were identified by comparing their retention time with those of standards, and content was calculated as the percentage of the total ion

current peak area. We found the repeatability of this method to be satisfactory (Coefficient of Variation, CV = 0.72 – 6.45%, *n* = 5). Samples were analyzed in duplicate.

2.5. Determination of phenolic composition

Phenolic compounds were extracted according to the method described by IOC (IOC, 2009) and modified as follow. We dissolved 4 g of oil sample in 5 ml of methanol/water (80:20, v/v). The mixture was shaken for 30 min and then centrifuged for 5 min at 5000 rpm. The polar supernatant was separated. The extraction process was performed twice and the extracted polar fraction filtered through 0.45 μm PVDV filters. An Agilent 1100 Liquid Chromatography (LC) System (Agilent Technologies, Palo Alto, CA, USA) equipped with a quaternary pump (G1311A), degasser, column thermostat, auto-sampler (G1313A), and a diode array detector (G1315 B, DAD) was used for reverse-phase HPLC (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). Chromatographic conditions were the same as in Deiana et al. (2019). Detection was performed at 280 nm 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/L). Samples were appropriately diluted before injection. The phenolic compounds, secoiridoids (oleacein, oleocanthal, oleuropein and ligstroside aglycon, dialdehydic form of ligstroside aglycon, and respective isomers) and acetoxypinoresinol were identified by liquid chromatography–mass spectrometry (LC-MS) analysis, using an Agilent Technologies (Palo Alto, CA, USA) 1200 series LC system equipped with a Q-Exactive Orbitrap (Thermo Fisher Scientific, Bremen, Germany) mass spectrometer. Chromatographic separation, the elution program and the operation program followed Deiana et al. (2019). Phenolic compounds were identified according to their molecular weights and respective fragments. Quantification of secoiridoids and acetoxypinoresinol was achieved using an HPLC with a diode-array detector (HPLC-DAD) (Deiana et al., 2019). A calibration curve of oleuropein was used to quantify secoiridoids, while a calibration

curve of pinoresinol was used for acetoxypinoresinol quantification. Results were expressed as mg of phenolic compounds per kg of oil. The repeatability of the method was considered satisfactory for all the quantified compounds (CV = 0.53 – 7.65%, *n* = 5).

Total phenolic content was determined using the Folin-Ciocalteu assay method. Briefly, 1 ml of methanol extract was mixed in a 25 ml volumetric flask with the Folin-Ciocalteu reagent (1:1) and with 10 ml of sodium carbonate (7.5%). The reaction mixture was incubated in the dark at room temperature for 120 min. The absorbance was measured at 750 nm. Quantification was obtained by means of a calibration curve of gallic acid (10 40 mg/L, R^2 = 0.996). Results were expressed as mg of gallic acid equivalents (GAE) per kg of oil. All samples were analyzed in duplicate.

2.6. Determination of tocopherols content

Extraction of α-T was performed according to the method described by Tasioula-Margari and Okogeri (2001) with the following modifications: a 0.5 g olive oil sample was dissolved in 5 ml of methanol-isopropanol solution (75%/25% v/v). The mixture was vortexed for two minutes at room temperature, then, the separated alcoholic phase was analyzed by HPLC without further purification. Analysis of tocopherols was performed on an Agilent 1100 LC System (Agilent Technologies, Palo Alto, CA, USA) equipped with a quaternary pump (G1311A), degasser, column thermostat, auto-sampler (G1313A) and a fluorescence detector (HP 1064 A). Chromatographic separation was achieved using a Zorbax eclipse XDB-C18 column (4.6 x 150 mm x 5 µm) connected to a security guard cartridge (4 × 2 mm). Instrument settings were as follows: injection volume 20 µl; temperature 30 °C; flow rate 1 ml/min; finally, the elution solvent was methanol (100%). Detection and quantification were performed at λ 290 nm (extinction) and λ 330 nm (emission). We used a calibration curve (R^2 = 0,998) of a standard solution at five different concentrations (5, 10 15, 20 e 30 mg/kg) to identify and quantify α-T. The repeatability of method was satisfactory (CV% = 0.59, *n* = 5). Samples were analyzed in duplicate.

2.7. Statistical analyses

A two-way analysis of variance (ANOVA) was employed to evaluate the influence of the genetic factors (although genetics embrace different factors, here we consider only variety, hereafter referred to as a genetic factor), harvest time, and their interaction, on VOO FA profile, polar phenolic composition and α-T content. The effect of harvest time on each individual variety was estimated by one-way ANOVA.

A multivariate analysis approach was adopted to evaluate the effect of cultivar and harvest date on the chemical composition of VOO samples. OPLS-DA was performed to distinguish varieties from one another and to select the most important variables in the discriminatory space. In addition, an OPLS regression model was performed to identify the molecules most affected by the harvest period. Due to the different variable scales and units, a pareto block-scale was applied to re-scale each variable variance to its initial standard deviation. Model fitting was evaluated by the parameters R^2Y and Q^2. The first parameter indicates the percentage of variation explained by the model in Y (response variable), and the latter represents the proportion of variance in the data predictable by the model (Culeddu et al., 2017). According to good practices for model validation, the corresponding PLS-DA (for the OPLS-DA model) and PLS (for OPLS model), a 7-fold cross-validation and permutation tests were performed (Culeddu et al., 2017). OPLS analysis is an extension of PLS which can give a simplified interpretation of multivariate data. In the OPLS method, the variation from X matrix (variables used to describe the model) not correlated to the response variable Y (in our case, the varieties or harvest dates) is separated (Trygg and Wold, 2002), and the predictive information is focused in one component.

Data were processed within the R Studio statistical software (one-way and two-way ANOVA analyses) and SIMCA-P software version 13.0 (Umetrics AB, Umea, Sweden) for OPLS and OPLS-DA analysis.

3. Results and discussion

3.1. Maturation Index

During ripening MI showed a slow, but constant, increase (Table 1) from values around 1 to around 2.5. An end of harvest fluctuation behavior was only observed in Bosana with a slight decrease (from 2.2 to 2.0), probably due to the crop load. In fact, fruit ripening, as defined by skin and pulp color, can be slower in plants characterized by high yield (Barone, Gullo, Zappia, & Inglese, 1994). Moreover, weather conditions, temperatures below and precipitation above the annual averages, as experienced during September, might have delayed the fruit coloring process.

3.2. FA composition

Out of the seven quantified FAs, oleic (C18:1ω9), linoleic (C18:2), and palmitic acids (C16:0) were the most abundant (Table 1). The FA values of all the analyzed samples complied with the limits set by the European Union for extra VOOs (Reg. EC. No 2016/2095). Both qualitative and quantitative differences between cultivars were observed. Between the 4 varieties, Coratina had the highest proportion of oleic acid and the lowest percentage of linoleic, palmitic, palmitoleic (C16:1) and *cis*-vaccenic (C18:1ω7). Oppositely, Tonda di Cagliari showed the lowest values of oleic acid and the highest percentage of linoleic, palmitic, and *cis*-vaccenic acids in all harvest periods. Bosana and Semidana had a similar FA composition, with the former containing a slightly larger MUFA/PUFA ratio. FA profiles obtained for these 4 varieties are consistent with those described elsewhere for the same and different growing areas (Poiana and Mincione, 2004; Mailer et al., 2010; Tuberoso, Jerković, Maldini, & Serreli, 2016; Morrone et al., 2018; Deiana et al., 2019), verifying that FAME profiles are a very useful parameter for monovarietal VOO characterization. The two-way ANOVA showed that genetic factors had a strong influence on all FAs (see supplementary material, SM 2). In addition, two-way ANOVA also showed that only stearic (C18:0), oleic, and linoleic acids were significantly influenced by harvest time. All the cultivars presented a similar pattern in oleic and linoleic acids, with percentages decreasing and

increasing, respectively, along the harvesting period (Table 1). An exception was observed in Bosana VOOs, which maintained a constant percentage of oleic acid (~70%) during the ripening process, until the 4th harvest. The same pattern was found for linoleic acid but slightly delayed (from the 2nd harvest date). A recent paper discussed the influence of the ripening process on the composition of VOO from Bosana trees grown in three different areas in North Sardinia (Morrone et al., 2018). Morrone et al. (2018) found only a significant decreasing trend in the percentage of palmitic acid but they did not identify any significant differences in FA composition. Here, we observed a similar behavior for the same variety, with slight differences in palmitic, stearic, oleic, and linolenic acid dynamics. We observed significant changes in the percentage of stearic and linoleic acids instead of palmitic and oleic acids. Such minor discrepancies can be attributed to different pedo-climatic conditions and to the different extension of the area of study considered.

Like Bosana, Coratina showed a small decrease in oleic acid content (from 79.8% to 77.0%) and, consequently, an increase in linoleic acid (from 5.6% to 8.9%). Coratina's FA profile changed confirming what was reported by Dag et al. (2014), but contradicting previous findings for the same variety grown in other regions of Italy (Cinquanta et al., 2001; Poiana & Mincione, 2004). Warmer meteoclimatic conditions in our study area may have been responsible for these differences (Pesaresi, Galdenzi, Biondi, & Casavecchia, 2014). In fact, warmer temperatures during fruit maturation can accelerate ripening and the associated biosynthetic processes (Hernandez, Padilla, Sircado, Mancha, & Martinez-Rivas, 2011; Dag et al., 2014; Rondanini et al., 2014).

In samples from Semidana and Tonda di Cagliari, an average 10% decrease in oleic acid with along with a strong increase (~40%) in linoleic acid was observed during the harvest campaign. These two varieties suffered a rapid decrease of MUFA/PUFA ratio, suggesting that an earlier harvest could reduce losses of the product shelf life because of the high percentage of linoleic acid (11.0 – 16.1% and 13.7 – 19.1%, respectively). Moreover, the presence of linolenic acid (C18:3ω3) negatively affects shelf life (Zarrouk et al., 2009; Jolayemi, Tokatli, & Ozen, 2016). A cultivar-specific behavior during harvest period was observed for linolenic acid that increased in

Coratina and Tonda di Cagliari while it decreased in Bosana and Semidana. A contrasting pattern between the cultivar were also observed for palmitoleic and *cis*-vaccenic acids as shown elsewhere (e.g. for some Tunisian varieties by Baccouri et al., 2008).

The FA biosynthetic pathway first involves SFA biosynthesis (e.g. stearic and palmitic acids), then the biosynthesis of oleic and, followed by biosynthesis of linoleic acid by the enzymatic activity of oleate desaturase (Hernandez et al., 2011). The increase in palmitic acid (from 13.8% to 15.3%) observed in Semidana could be due to ongoing oil biosynthetic processes during the ripening stage. Variations in the FAME profile in Semidana were similar to those reported by Deiana et al. (2019).

3.3. Phenolic composition

Eighteen phenolic compounds were identified and quantified by LC (Figure 1 reports the dynamics of 16 molecules; complete dataset is available in supplementary material, SM 3). Two-way ANOVA results revealed that all the phenolic compounds were significantly affected by genetic factors and harvest period (SM 2). Interaction between these two factors was found to be significant for all compounds except tyrosol.

The chromatographic analysis (representative chromatograms at 280 nm and 320 nm are reported in supplementary materials, SM 4) showed qualitative and quantitative differences among varieties, and emphasize some cultivar-specific characteristics. Servili et al. (2009) reported that oleocanthal, oleacein and aglycon of oleuropein, and ligstroside were the most abundant molecules in all varieties. Bosana had the highest concentration of oleacein (237.2 mg/kg) and oleocanthal (155.6 mg/kg), while Coratina had the highest concentrations of aglycon of oleuropein (268.9 mg/kg) and ligstroside (120.8 mg/kg). The content of these four molecules decreased during the ripening process in all varieties, except for Coratina (oleacein and oleocanthal increased during later harvests) and for Bosana (oleuropein aglycon increased until the 3rd harvest and then drastically decreased at the 4th harvest). Contrasting results concerning the relationship between the harvest

period and the content of these secoiridoids in VOO have been previously reported (Morellò, Romero & Moltiva, 2004; Baccouri et al., 2008), suggesting the involvement of some cultivar-specific traits. Our results are in contrast to what has been previously reported for Bosana (Morrone et al., 2018) and Coratina (Gambacorta et al., 2010). Such differences within the same varieties can be attributed to different bioclimatic and seasonal conditions. Indeed, we know that phenolic composition and amounts, both in fruits and in corresponding VOOs, are strongly affected by environmental and agronomical conditions, which in turn affect the stress status in olive trees (Ryan & Robards, 1998; Inglese et al., 2011).

In general, minor secoiridoids content decreased during ripening (Figure 1), except for the dialdehydic form of ligstroside aglycon. The second isomer of oleuropein aglycon was detected in all cultivars and with higher prevalence in Bosana. The second isomer of ligstroside aglycon had concentration values in the range of 17.7 – 5.7 mg/kg. Tonda di Cagliari had the greatest amounts of the second isomer of ligstroside aglycon as well as the dialdehydic form of ligstroside aglycon. Between oleuropein and ligstroside aglycon isomers an additional peak, characterized by co-elution of the two isomers, was quantified in all cultivars, as found by Deiana et al. (2019). In general, Bosana and Coratina were the varieties with the highest secoiridoid content (92% on average). The relative content of secoiridoids in Coratina was stable across the harvest period. Semidana had the lowest percentage of secoiridoids (77%) and also the largest decrease during the harvest period.

Phenolic alcohols were characterized by a decreasing pattern in all varieties, except for tyrosol in Bosana. Coratina had the highest values of both hydroxytyrosol (5.7 mg/kg) and tyrosol (11.2 mg/kg). A review of the literature reveals contrasting results regarding phenolic alcohol behavior during the ripening process which can be attributed to varietal factors (Morellò et al., 2004; Baccouri et al., 2008; Kalogeropoulos & Tsimidou, 2014; Jolayemi et al., 2016; Trombetta et al., 2017).

In our study, lignans were the second most represented phenolic class. Semidana was the variety with the highest relative amount, with respect to total phenolic compounds, increasing from

6% to 17% during the harvest period. Acetoxypinoresinol content varied from 2.6 mg/kg (Tonda di Cagliari) and 32.3 mg/kg (Coratina). This increased until the 3rd sampling after which it drastically reduced. A similar trend was observed for luteolin and apigenin in the Bosana variety, while an increase across all the 4th sampling was observed in the other three cultivars. For Bosana, this behavior is in contrast with what has been found by Morrone et al. (2018), and may be due to different meteoclimatic conditions which may have affected the maturation process. Increases in flavonoid content throughout ripening were also observed in Arbequina VOOs by Artajo, Romero & Moltiva (2006), and by Deiana et al. (2019) in Semidana VOOs. In contrast, opposite and cultivar-specific trends were described in the literature for Turkish (Ayvalic and Memecik) and other Italian varieties (Casaliva and Frantoio) (Jolayemi et al., 2016; Trombetta et al., 2017). Semidana and Tonda di Cagliari registered the highest proportion of flavonoids, which increased above 5% during ripening. Furthermore, a constant increase in flavonoid content was observed in Bosana and Coratina, although Coratina VOO did not reach 1% of the total phenolic content. The relative increase in lignans content and flavonoids was often linked to a decrease in secoiridoids. Semidana had the highest concentrations of phenolic acids and vanillin, which was unaffected by the ripening process; only vanillic acid increased slightly (SM 3). Similar results were reported in previous studies (Jolayemi et al., 2016; Morrone et al., 2018; Deiana et al., 2019) but not in Morellò et al. (2004) which reported a negative influence of the harvest period length on vanillic acid and vanillin content in Arbequina and Farga VOOs.

Total polar phenolic (TPP; Figure 1) content decreased during the ripening process confirming results from previous findings (Baccouri et al., 2008; Gambacorta et al., 2010; Alagna et al., 2012). Ryan and Robards (1998) attributed the loss of polar phenolic compound in drupe's pulp to enzymatic hydrolysis which occurs against molecules like oleuropein and its derivatives. Degradation of oleuropein was followed by an increase of other phenols such as demethyloleuropein and elenolic acid glucosides, followed by hydrolysis (Alagna et al., 2012). Coratina and Bosana VOOs showed the highest concentrations of TPP, losing around 33% and 25%

at the last harvest date, respectively. High TPP concentrations were observed in Semidana and Tonda di Cagliari VOOs at early harvests (528.8 mg/kg and 507.3 mg/kg respectively) followed by a drastic decrease (134.5 mg/kg and 142.2 mg/kg, respectively) in subsequent harvests. Our findings were among the highest TPP values reported in literature for these cultivars (Gambacorta et al., 2010; Dabbou et al., 2015; Tuberoso et al., 2016).

3.4. α-T content

We found that α-T content decreased in all varieties with the ripening process (Figure 1 and SM 3), as already reported by Beltrán et al. (2005). Coratina was the variety with the lowest loss of α-T (17.5%), whereas Sardinian varieties' loss was around 30%. These results confirm that the ripening process is not a critical issue for α-T content as it is for polar phenolic compounds (Cinquanta et al., 2001; Kalogeropoulos and Tsimidou, 2014). Bosana VOOs had the highest α-T content, while Semidana the lowest (Figure 1). Values of α-T were within the ranges known for these varieties (Gambacorta et al., 2010; Tuberoso et al., 2016) or lower (Mailer et al., 2010; Deiana et al., 2019). Several studies agreed that tocopherol content decreases with increasing latitude, suggesting that the vitamin E concentration in VOO is much higher when the temperature increases during fruit ripening (Mailer et al, 2010; Kalogeropoulos & Tsimidou, 2014). Such a theory could also explain the differences within VOOs of the same varieties, obtained during years characterized by higher summer temperatures (Deiana et al., 2019).

3.5. Multivariate analyses

A complete separation of the four cultivars was obtained by the use of OPLS-DA model (SM 5). The model was developed for the whole dataset: 32 observations (8 oil samples per variety) and 26 variables (7 FAs, 17 phenolic compounds, TPP, α-T). The model showed good reliability, $R^2Y = 0.870$, and predictive ability, $Q^2 = 0.815$ (Table 2). In order to identify the most important variables for each variety, a model "single variety vs others" was performed for each (Figure 2).

Results show that both FAs and phenolic compounds are recurring important variables in all varieties. Model fitting statistics R^2Y and Q^2 were consistently above 0.85 and 0.75, respectively (Table 2). The predictive accuracy of the models was also confirmed by permutation analyses (see supplementary material, SM 6). FA composition was the main factor that distinguished Coratina VOOs from the VOOs of the other three varieties (Table 3). Coratina VOOs were characterized by the highest relative content of oleic acid and acetoxypinoresinol, and lowest amounts of palmitic, palmitoleic and *cis*-vaccenic acids. Bosana samples clustered for a higher content of oleacein (Figure 1), second isomer of oleuropein aglycon, α-T and relative content of stearic acid (Table 3). The high content of some minor secoiridoids (e.g. the second isomer of ligstroside aglycon and the first isomer of its dialdehydic form), apigenin and linoleic acid (C 18:2), together with a low acetoxypinoresinol content distinguished Tonda di Cagliari VOOs. Semidana was characterized by the highest vanillic and linolenic acid content, together with a low secoiridoid content (oleacein, oleocanthal and the peak with oleuropein and ligstroside aglycon co-eluted).

During the 45-day harvest period, even when the drupes color changed slowly, from green/yellowish (MI ≈ 1) to red spotted (between MI between 2 and 3) (Table 1), corresponding to the first steps of the coloring process, it was still possible to observe marked changes on VOO composition in all varieties, particularly in Semidana and Tonda di Cagliari. This suggests that metabolic processes occur in drupes independently to drupe color changes. As ripening is a continuous metabolic process that occurs over time, we considered the OPLS regression model appropriate to verify which metabolites were most influenced during the harvest period studied. The day of year (DOY) values corresponding to four harvest dates (15 October, 30 October, 15 November and 30 November) were set as Y continuous variable; the harvest of 15 December was not included in the model because was represented only by Semidana samples. The accuracy of the model was good as proven by goodness of fit (R^2Y = 0.930), predictive ability (Q^2 = 0.838) parameters (Table 2), and by permutation analysis on the corresponding PLS model (see supplementary material, SM 7). S-plot data (Figure 3) shows the variables mostly affected by

harvest time. The most represented secoiridoids (i.e. oleacein, oleocanthal, oleuropein and ligstroside aglycon) and hydroxytyrosol were the molecules mostly negatively affected by harvest time. As well as flavonoids, vanillic acid, linoleic and linolenic acids were the molecules mostly positively affected.

4. Conclusions

FA and phenolic compounds are some of the most important components determining VOO quality. During ripening they evolve differently according to the specific physiology of the varieties. The changes we observed in VOO composition seem to be more influenced by ripening than fruit coloring variations, suggesting that it would be appropriate to not rely exclusively on MI for estimating the optimal harvest period.

This study was performed in the same experimental olive grove, thus the environmental conditions and agronomic management were the same, as well as the technology and transformation protocols. Controlling the external conditions allowed the multivariate analyses to identify the metabolites that discriminate between the studied varieties. Moreover, OPLS regression model was effective in assessing which were the molecules most affected by the ripening process. OPLS might be a very useful tool for future studies especially with more VOO qualitative components (e.g. volatile compounds, squalene, sterols, oil content). In time, this will allow for a more accurate forecast of the optimal varietal-specific harvest period.

Coratina was able to adapt to the bioclimatic conditions of the area of study. Bosana and Coratina VOOs showed high phenolic content through the harvest period, especially secoiridoids like oleacein and oleocanthal, and a higher MUFA/PUFA ratio than the other studied varieties. These findings indicate that these two varieties tolerate a wider harvest period, thereby preserving the qualitative properties of the VOOs. On the other hand, Semidana and Tonda di Cagliari VOO quality reduced rapidly during harvest period due to MUFA/PUFA and phenolic concentration. Therefore, in this case, in order to avoid risks of low oxidative stability and short VOO shelf life, it

would be appropriate to start the harvest earlier, before drupes start to change color from green to purple or violet. Taken together, our results about these two local varieties and the rising interest in the Sardinian olive oil production sector can help to improve the production of high quality VOOs guaranteed by PDO Sardegna certification, blended with Tonda di Cagliari or Semidana VOOs. Finally, these results provide information to producers for a better management of the harvesting process and to develop a wider range of products that might attract a wider number of potential consumers.

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Conflict of interest

The authors declare no conflict of interest.

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Figure 1

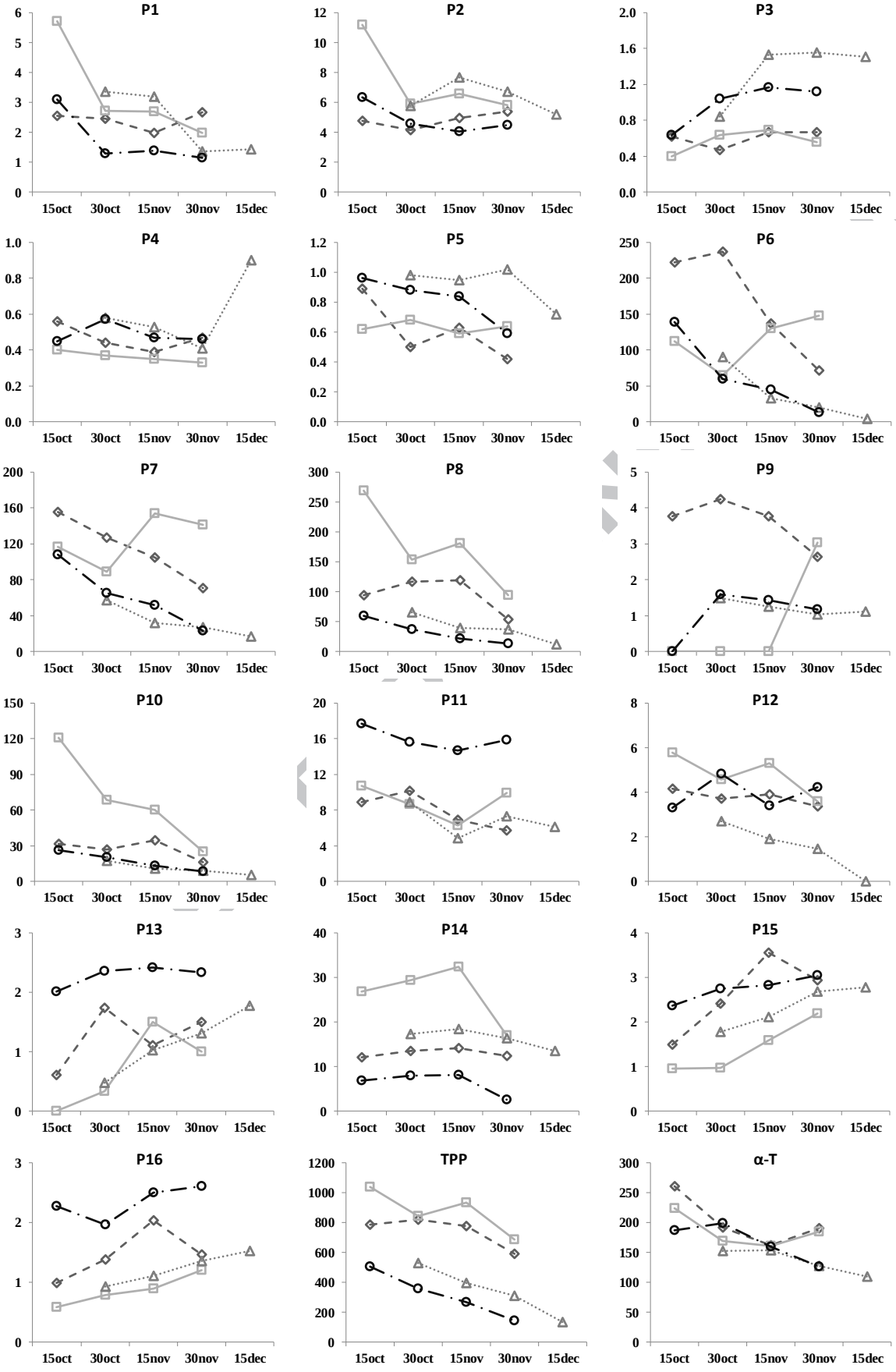

Figure 1. Concentration of phenolic compounds[1], total phenolic content and α-tocopherol content (expressed as mg/kg of olive oil, vertical axis) of Coratina (continuous line, □), Bosana (dashed line, ◊), Semidana (round dotted line, Δ) and Tonda di Cagliari (dashed/dotted line ○) VOOs related to harvest period (horizontal axis).

[1]P1 = hydroxytyrosol; P2 = tyrosol; P3 = vanillic acid; P4 = p-coumaric acid; P5 = vanillin; P6 = oleacein; P7 = oleocanthal; P8 = oleuropein aglycon 1; P9 = oleuropein aglycon 2; P10 = ligstroside aglycon 1; P11 = ligstroside aglycon 2; P12 = oleuropein co-eluted with ligstroside aglycon; P13 = dialdehydic form of ligstroside aglycon; P14 = acetoxypinoresinol; P15 = luteolin; P16 = apigenin; TPP = total phenolic content; α-T = α-tocopherol. Values are the average of 4 measurements (2 independent VOO samples, per harvest date, for each variety, analyzed twice).

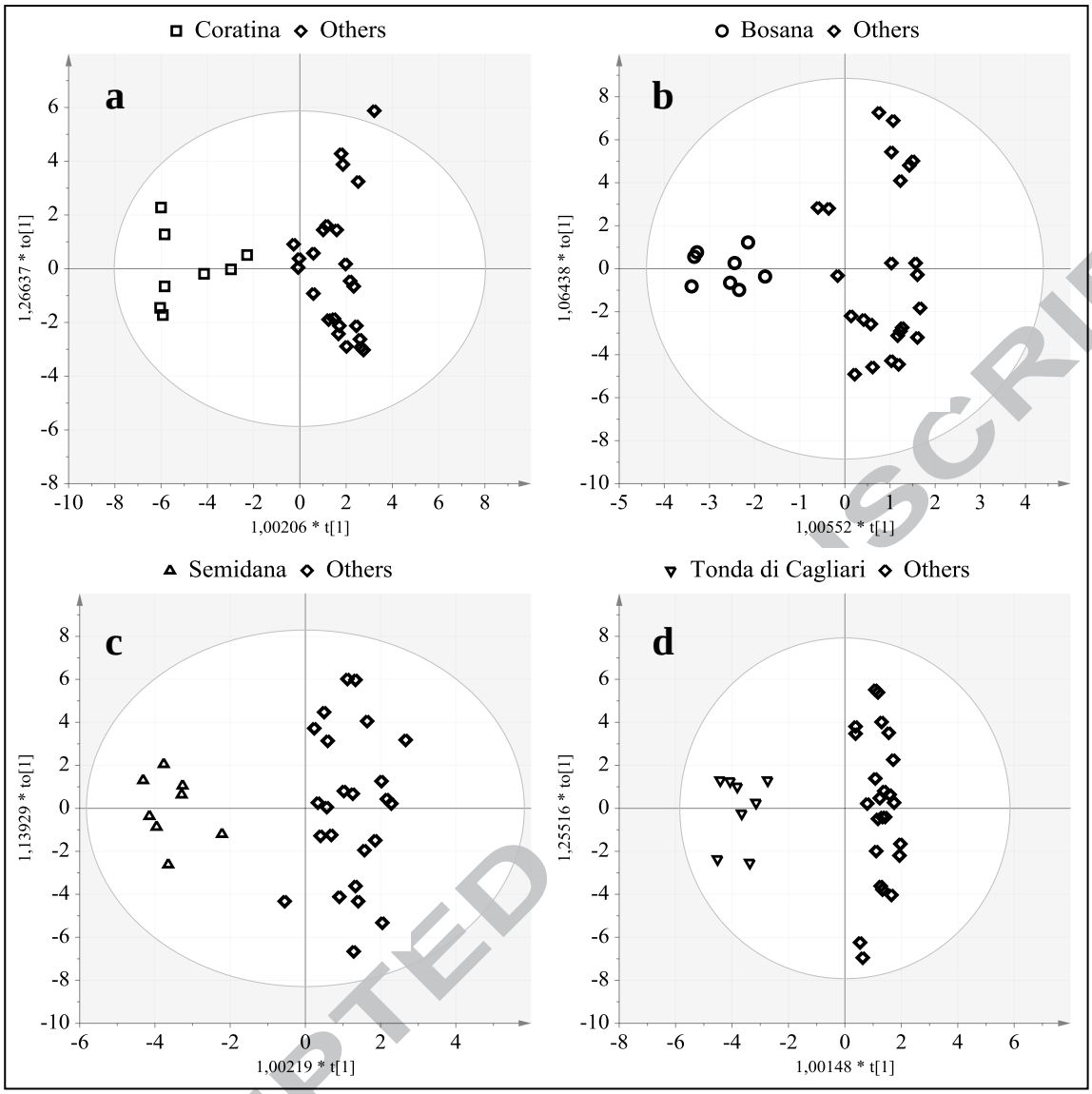

Figure 2. Score plots of Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) models: Coratina VOOs vs other VOOs (a), Bosana VOOs vs others (b), Semidana VOOs vs others (c), Tonda di Cagliari VOOs vs others (d). Horizontal axis indicates variability between groups, whilst vertical axis indicates variability within groups.

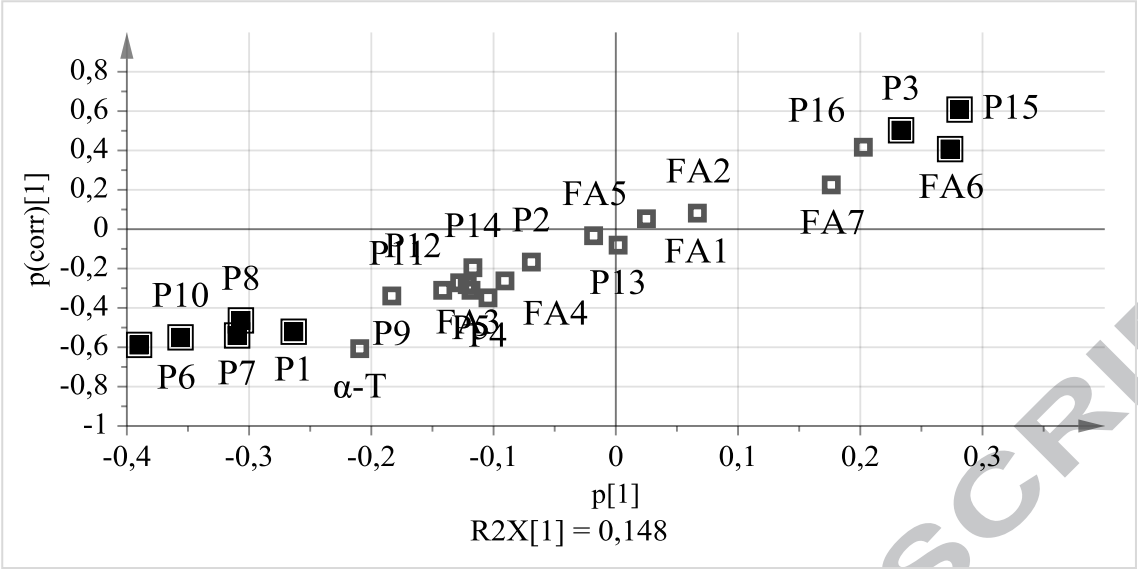

Figure 3. S-Plot provides the visualization of OPLS predictive component[1]. The 24 variables[2] (fatty acids, phenolic compounds and α-tocopherol of VOOs) used to build the model are represented.

[1]Variables indicated with a black box are the 8 most influenced by harvest date (presenting Variable Influence on Projection (VIP) values ≥1), the ones with the highest predictive power.

[2]FA1 = palmitic acid (C16:0); FA2 = palmitoleic acid (C16:1); FA3 = stearic acid (C18:0); FA4 = oleic acid (C18:1ω9); FA5 = *cis*-vaccenic acid (C18:1ω7); FA6 = linoleic acid (C18:2); FA7 = linolenic acid (C18:3); P1 = hydroxytyrosol; P2 = tyrosol; P3 = vanillic acid; P4 = p-coumaric acid; P5 = vanillin; P6 = oleacein; P7 = oleocanthal; P8 = oleuropein aglycon 1; P9 = oleuropein aglycon 2; P10 = ligstroside aglycon 1; P11 = ligstroside aglycon 2; P12 = oleuropein co-eluted with ligstroside aglycon; P13 = dialdehydic form of ligstroside aglycon; P14 = acetoxypinoresinol; P15 = luteolin; P16 = apigenin; TPP = total phenolic content; α-T = α-tocopherol.

Table 1 Fatty Acid (FA) composition of VOOs and Maturity Index (MI) of respective fruits of Coratina, Bosana, Semidana and Tonda di Cagliari related to harvest period.

Fatty acids[1]	Harvest period[2]							
	1	2	3	4	1	2	3	4
	Coratina virgin olive oils				***Bosana virgin olive oils***			
Palmitic	11.0±0.4[3]	10.8±0.7	9.9±0.2	10.4±1.0	13.9±0.1	14.4±0.5	13.2±0.9	13.4±0.3
Palmitoleic	0.3±0.1	0.3±0.1	0.2±0.0	0.3±0.1	0.7±0.2	0.7±0.1	0.5±0.2	0.5±0.1
Stearic	1.6±0.4	1.7±0.2	1.4±0.1	1.6±0.3	2.2±0.2^{a}	2.0±0.2^{ab}	1.7±0.2^{b}	1.7±0.1^{b}
Oleic	79.8±0.7^{a}	78.3±1.5^{ab}	78.9±0.4^{ab}	77.0±1.9^{b}	70.9±1.2	68.5±1.2	70.6±2.5	70.3±0.3
cis-Vaccenic	1.6±0.4	1.6±0.2	1.4±0.1	1.3±0.4	2.4±0.5	2.4±0.3	2.0±0.5	1.9±0.2
Linoleic	5.6±0.6^{c}	7.0±0.7^{b}	7.8±0.4^{ab}	8.9±0.6^{a}	9.3±0.2^{b}	11.5±0.5^{a}	11.5±0.8^{a}	11.7±0.5^{a}
Linolenic	0.2±0.2^{b}	0.3±0.2^{ab}	0.4±0.1^{a}	0.5±0.1^{a}	0.6±0.2	0.5±0.2	0.4±0.2	0.4±0.1
ΣSFA[4]	12.6±0.3	12.5±0.9	11.3±0.2	12.0±0.9	16.1±0.2^{ab}	16.3±0.4^{a}	15.0±1.0^{b}	15.2±0.3^{ab}
ΣMUFA	81.6±0.9^{a}	80.2±1.3^{ab}	80.5±0.6^{ab}	78.6±1.5^{b}	74.0±0.6	71.7±0.8	73.1±1.9	72.8±0.2
ΣPUFA	5.7±0.8^{c}	7.3±0.9^{b}	8.2±0.5^{ab}	9.4±0.6^{a}	9.9±0.4^{b}	12.0±0.5^{a}	11.9±1.0^{a}	12.0±0.5^{a}
MUFA/PUFA	14.2±2.2^{a}	11.0±1.3^{b}	9.8±0.6^{b}	8.4±0.7^{b}	7.5±0.3^{a}	6.0±0.3^{b}	6.1±0.7^{b}	6.0±0.3^{b}
MI	1.0±0.02^{c}	1.2±0.1^{c}	2.3±0.1^{b}	2.6±0.2^{a}	1.4±0.2^{c}	1.5±0.04^{c}	2.2±0.1^{a}	2.0±0.1^{b}
	Semidana virgin olive oils				***Tonda di Cagliari virgin olive oils***			
Palmitic	13.8±0.8^{b}	14.7±0.7^{ab}	15.3±0.4^{a}	15.3±0.4^{a}	15.2±0.8	14.7±1.1	14.9±1.4	16.2±0.2
Palmitoleic	0.4±0.2	0.8±0.1	0.6±0.2	0.6±0.2	0.5±0.1	0.6±0.2	0.6±0.1	0.8±0.2
Stearic	2.0±0.3	2.0±0.2	1.8±0.1	1.7±0.2	1.5±0.1	1.6±0.3	1.5±0.1	1.4±0.1
Oleic	69.7±1.1^{a}	65.7±0.6^{b}	63.7±1.3^{c}	63.2±0.7^{c}	66.0±3.4^{a}	63.8±2.9^{ab}	63.3±3.2^{ab}	59.1±0.5^{ab}
cis-Vaccenic	2.2±0.2^{b}	2.7±0.1^{a}	2.5±0.3^{ab}	2.5±0.4^{ab}	2.5±0.5	2.4±0.7	2.6±0.3	2.9±0.1
Linoleic	11.0±0.7^{c}	13.3±0.4^{b}	15.4±1.3^{a}	16.1±0.5^{a}	13.7±1.9^{b}	16.3±1.7^{ab}	16.3±1.6^{ab}	19.1±0.3^{a}
Linolenic	0.9±0.3^{a}	0.8±0.2^{a}	0.7±0.2^{a}	0.7±0.2^{a}	0.4±0.1	0.5±0.3	0.7±0.2	0.5±0.2
ΣSFA	15.8±1.1^{b}	16.7±0.7^{ab}	17.1±0.5^{b}	17.0±0.4^{ab}	16.8±0.8	16.3±0.8	16.4±1.4	17.6±0.2
ΣMUFA	72.3±1.3^{a}	69.2±0.6^{b}	66.8±1.2^{c}	66.3±0.7^{c}	69.1±2.8^{a}	66.9±2.2^{ab}	66.5±3.0^{ab}	62.8±0.5^{b}
ΣPUFA	12.0±0.6^{c}	14.1±0.4^{b}	16.0±1.4^{a}	16.7±0.4^{a}	14.1±2.0^{b}	16.8±1.5^{ab}	17.1±1.7^{ab}	19.6±0.4^{b}
MUFA/PUFA	6.0±0.3^{a}	4.9±0.1^{b}	4.2±0.4^{c}	4.0±0.1^{c}	4.9±0.9^{a}	4.0±0.5^{ab}	3.9±0.6^{ab}	3.2±0.1^{b}
MI	0.9±0.1^{c}	1.1±0.1^{c}	2.0±0.2^{b}	2.8±0.3^{a}	1.0±0.1^{c}	1.7±0.03^{b}	1.7±0.02^{b}	2.1±0.04^{a}

[1]Values, expressed as % of total composition, are the average of four measurements (two independent VOO samples analyzed twice).

[2]Harvest period: numbers from 1 to 4 indicate the four harvest dates (two weeks interval): from 15 October to 30 November for Coratina, Bosana and Tonda di Cagliari whereas from 30 October to 15 December for Semidana.

[3]Letters "a, b, c, d" indicates significant differences of 1-way ANOVA analysis (Tukey test, $p < 0.05$) between harvest dates, within the same cultivar.

[4]ΣSFA = sum of saturated fatty acids; ΣMUFA = sum of monounsatured fatty acids; ΣPUFA = poliunsatured fatty acids.

Table 2 Autofit results for the four class model (Orthogonal Projections to Latent Structures Discriminant Analysis, OPLS-DA) "single olive variety vs others" models and harvest dates models (OPLS), according to VOO chemical composition.

Model	OPLS-DA			PLS-DA		
	R^2Y	Q^2	Comp	R^2Y	Q^2	Comp
4 cultivars	0.870	0.815	3+1	0.900	0.824	5
Coratina vs Others	0.872	0.829	1+1	0.872	0.829	2
Bosana vs Others	0.860	0.761	1+2	0.788	0.718	2
Semidana vs Others	0.892	0.832	1+1	0.892	0.843	2
Tonda di Cagliari vs Others	0.953	0.886	1+2	0.935	0.886	3
Harvest date[1]	0.930	0.838	1+4	0.895	0.792	3

[1] Harvest date models are OPLS and PLS respectively. Comp = number of latent components.

Table 3 Summary (in terms of variable importance) of the first five metabolites (fatty acids, phenolic compounds, and α-tocopherol of VOOs) per each of the four OPLS-DA1 models leading separation between single varieties and the others.

Model	**Variables**[2]
Coratina vs others	Palmitic acid (-); Oleic acid (+); Palmitoleic acid (-); *cis*-Vaccenic acid (-); Acetoxypinoresinol (+);
Bosana vs others	Oleuropein aglycon 2 (+); Oleacein (+); Stearic acid (+); Vanillin (-); α-Tocopherol (+);
Semidana vs others	Vanillic acid (+); Oleuropein +Ligstroside aglycones (-); Oleocanthal (-); Linolenic acid (+); Oleacein (-);
Tonda di Cagliari vs others	Ligstroside aglycon 2 (+); Apigenin (+); Ligstroside agycon dialdehydic form 1 (+); Linoleic acid (+); Acetoxypinoresinol (-);

[1]Orthogonal Projections to Latent Structures Discriminant Analysis

[2]Simbols in brackets indicates if the molecule characterized the variety for a higher (+) or a lower (-) amount.

Highlights

- Coratina and Bosana VOOs have high phenolic content along the whole harvest period
- Tonda di Cagliari and Semidana VOOs have high nutraceutical content at first harvest
- Oleacein and oleocanthal increase during ripening only in Coratina variety
- OPLS shows with high accuracy changes in oleacein and ligstroside aglycon content