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ORIGINAL PAPER

Biochemical and molecular profiling of unknown olive genotypes from central Italy: determination of major and minor components

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Abstract

The phenotypic and genetic variability of local olives in a perspective of diversity conservation should be maintained and preserved. Fourteen unknown olive genotypes were selected from abandoned and isolated plants in Umbria region and characterized by ten SSR markers. The molecular analysis led to identify ten previously unknown genotypes within the patrimony of the region, made up by about 10 main cultivars and more than 100 minor ones. Fruit samples were randomly collected from the selected plants during two growing seasons and oil was extracted from each sample. Analyses of fatty acid composition, phenolic profile (flavonoids, phenolic acids, phenolic alcohols and lignans), tocopherols, squalene and sterols were performed on these oils. Significant differences were observed in terms of main fatty acids (e.g. oleic acid from 71.83 to 76.73%), but not for the minor ones. The important differences were instead obtained on the amount of α-tocopherol, ranging from 149 to 583 mg kg^{-1}, and on squalene, spanning from 1059 to 5447 mg kg^{-1}. Evaluation of major and minor compounds with the principal component analysis of the main oil quality parameters revealed differentiation according to the genotypes. The analysis of neglected olive genotypes from a small area of cultivation has led to the identification of a promising source of variability for most sought-after traits, which could be exploited for new olive plantations and to magnify the spectrum of local olive oils.

Keywords Fatty acids · Food composition · Genetic diversity · Phenols · Tocopherols · Squalene

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Introduction

Olive oil is the most valuable vegetable oil for the population of the Mediterranean area, with a high potential for worldwide consumption. In fact, the use of olive oil has now extended to other parts of the world due to its unique flavor, the high content of healthy monounsaturated fatty acids (MUFA) [6] and the biologically valuable constituents, such as phenolic compounds and minor components, contributing to its health-promoting effects [26, 31, 42].

The spread of new intensive planting systems and the introduction of the olive cultivation into new areas with only a few cultivars have negatively impacted on the olive variability, making local types very rare or close to extinction [29]. The prospection and study of minor varieties, often represented by few or single abandoned trees, may thus be a way of preserving the genetic diversity and limiting its erosion [2, 33, 34]. On the other hand, the oil of local cultivars, when showing a high nutritional and health value, could increase the commercial value of regional olive oils, such as

those under PDO (Protected Denomination of Origin) and PGI (Protected Geographical Identification) denominations and the monovarietal olive oils [27].

Italy has 48% of the world's known germplasm (more than 600 cultivars), excluding numerous ancient and local olive trees still waiting to be identified. In Italy, olive oil production is also based mainly on the regional varieties that are cultivated on a family scale [30]. Among the Italian regions, Umbria region only contributes for 1% to the national production and almost 5% of the total PDO oils.

The main varieties cultivated in Umbria are Moraiolo, Frantoio and Leccino, distributed throughout the region, while Dolce Agogia, Nostrale di Rigali and Raio cultivars are found in the north, center and south of the region, respectively, and few others (Tendellone, Bianchella, Rosciola) are randomly distributed along the entire region. These cultivars cover more than 90% of Umbrian production, while minor cultivars are represented by a few trees distributed in limited areas or diffused as individuals or groups of trees [39]. These olives may include the following: seedlings derived from the spontaneous dissemination of most common varieties; survivors of ancient previously widespread varieties used for the production of traditional table olives; and trees acting as pollinators. As a consequence, there is a lack of information of these minor varieties, mainly for their chemical characteristics. The recovery and reuse of local olive varieties could represent a successful marketing strategy to differentiate niche olive oils from the mass and could help in the preservation of the local germplasm.

The phenolic compounds of extra virgin olive oil (EVOO) play a major role on its beneficial effects (lowering cholesterol, blood pressure and the risk of coronary disease [9]) and are responsible for its stability against oxidation and contribute to the organoleptic characteristics (bitter and pungent tastes). EVOOs contain five different classes of phenols clustered into: phenolic acids, phenolic alcohols, flavonoids, secoiridoids and lignans [20, 49]. The unsaponifiable fraction (UF) of EVOOs (about 1–2% of oil weight) is mainly made up of phenolic compounds [12]. The phenolic content and fatty acid (FAs) composition of EVOOs depend on several factors, such as genetic background, agroclimatic conditions and technological aspects of production and oil extraction [3, 17]. The unsaponifiable fraction also contains aliphatic and triterpenic alcohols, sterols, hydrocarbons (squalene), tocopherols, β-carotene, phytosterols, pigments and volatile compounds [1]. In recent years, the sterol fraction of olive oil has received attention owing to its nutritional and health benefits [25], such as tocopherols and aliphatic alcohols.

Among the four naturally occurring tocopherols, α-tocopherol has the maximum vitamin E activity and affects human nutrition and health aspects, whereas γ- and δ-tocopherol exert maximum in vitro thermo-oxidative protection [54] and show an intense activity in protecting seed compounds, such as fatty acids [53]. Average values of tocopherols in virgin olive oil are between 100 and 250 mg kg^{-1} oil, with around 90% of them being in the α-tocopherol form [8]. Squalene, one of the main components of the UF, is an essential element in the diet due to its chemo preventive potential against cancer. Furthermore, squalene has a protective effect on the oxidative stability of the oil under heating [43]. Its content in virgin olive oil is very wide, ranging from 200 to 7500 mg kg^{-1} [8]. Fruit pigments are responsible for the color of olives and oil and, among these, chlorophyll is considered as a predictor of the oil storage stability, due to the pro-oxidant action in the presence of light, while carotenoids, by quenching singlet oxygen, inhibit the photosensitized oxidation [23].

The aim of this research was to perform a complete characterization of local olive types from different areas of Umbria, based on a multidisciplinary approach, including the molecular characterization by using best ranked SSRs (Simple Sequence Repeats), widely applied to determine their genetic identity [4, 35, 51] and evaluating fatty acid, phenol, sterol, tocopherol and pigment content and composition. The work provides a useful contribution towards the differentiation of local, traditional and typical olive oils, from the perspective of diversity conservation and large-scale plantations of the most promising genotypes.

Materials and methods

Leaf sampling and SSR analysis

A total of 14 local olive trees were sampled from different areas of Umbria (Fig. 1). These trees were selected from small populations, isolated trees or abandoned olive orchards. The samples were labeled according to the collecting sites, and leaves were collected for DNA analysis.

Total DNA was extracted from leaves by GenElute Plant Genomic DNA Miniprep Kit (Sigma-Aldrich) and ten SSR markers were applied, representing the best ranking loci over numerous evaluated microsatellite regions [4]. The PCR amplification protocol was the same as Mousavi et al. [34]. The resulting PCR products were loaded onto an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), and output data were analyzed using GeneMapper 3.7 (Applied Biosystems). The SSR data results were compared with 16 Umbrian, 500 Italian and 300 cultivars from other countries, to evaluate their identity with known cultivars and to define their relationships with the most widely-cultivated olives [4, 34].

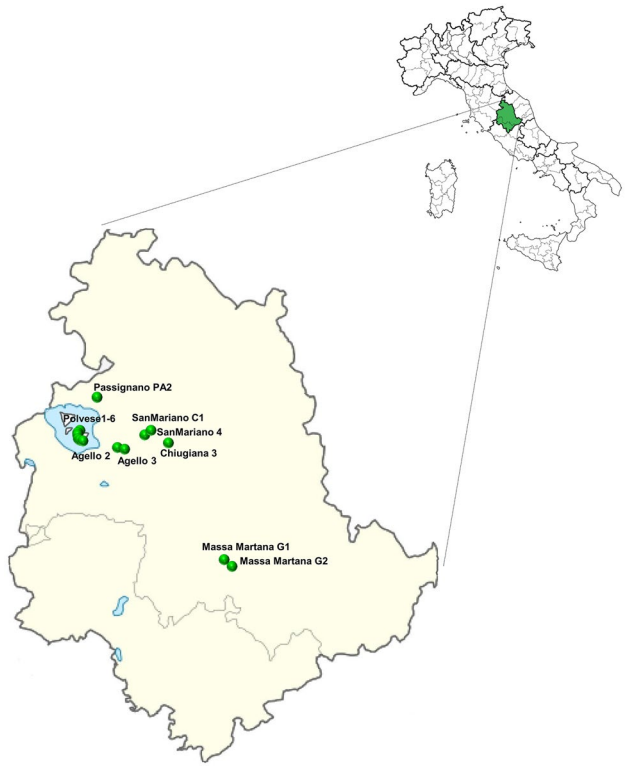


Fig. 1 Geographical location of 14 olive genotypes (Passignano PA2, Polvese from 1 to 6, Chiugiana 3, San Mariano C1 and 4, Agello 2 and 3, Massa Martana G1 and G2) in Umbria region (central Italy)

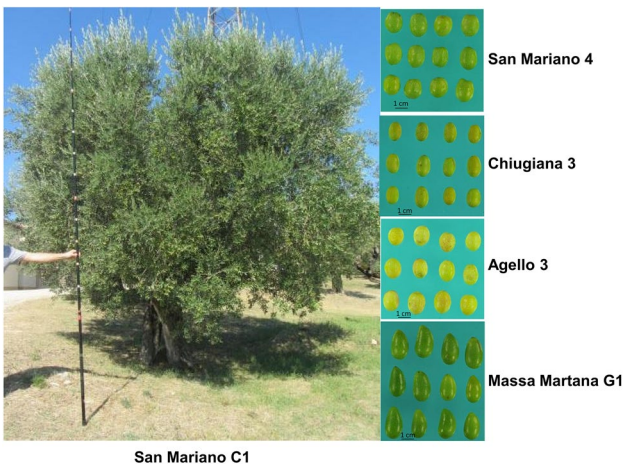


Fig. 2 Tree vigor and fruit shape of some studied olive genotypes

Fruit sampling and oil extraction

Two kilograms of undamaged, healthy fruits (Fig. 2) were randomly collected from a single tree of each genotype of interest in two consecutive growing seasons during November, the usual harvesting time in Umbria. In order to increase uniformity among fruit samples, they were harvested from different parts of each tree, to minimize the effect of watering and sun exposure. Since the studied genotypes were growing in semi-natural or abandoned conditions, without any agricultural care, their fruit yield was totally unpredictable. In fact, in November 2016 it was possible to harvest fruits from ten genotypes, showing unique genetic profiles according to the molecular results, while in 2017 only eight out of ten genotypes had fruit production.

To evaluate the geographical distribution and to keep information on the growing area of each sample, the following data were registered: latitude, longitude, altitude (m above sea level a.s.l.) and climatic data (average, minimum and maximum temperatures, based on the average values of coldest and hottest month and annual rainfall) (Supplementary material Table 1). Sampling site altitudes ranged from 331 to 483 m a.s.l. Rainfall ranged from 771 mm for Chiugiana area to 966 mm for San Mariano collection site in 2016, and from 441 mm for Polvese Island to 699 mm for Massa Martana in 2017. Lowest (3.32 °C) and highest (26.94 °C) average temperatures were related to Massa Martana area in 2016 and San Mariano area had the lowest (3.68 °C) and highest (25.33 °C) average temperature in 2017 (Supplementary material Table 1).

An Abencor olive oil mill was used to extract the oil from 1 kg of fruit per sample. This system reproduces the industrial process at a laboratory scale, through three basic elements: a hammer mill, a thermo-beater and a paste centrifuge. The olives were ground to a paste using the hammer mill, then the paste was placed in the thermo-beater and stirred for 30 min with the water bath set at 28 ± 1 °C, without adding warm water. Subsequently, vertical centrifugation for 2 min separated the oily phase, which was then collected and left to decant for 24 h. Finally, the oil was separated, placed in dark glass vials and stored at − 18 °C until analysis.

Analysis of pigments (chlorophylls and carotenoids)

Chlorophylls and carotenoids were determined at 670 and 470 nm, respectively, following Minguez-Mosquera et al. [32]. The oil samples were dissolved in cyclohexane (1.5:5 w/v) and absorbance was measured using a Perkin Helmer Lambda 10 UV–Vis spectrophotometer.

$$\text{Chlorophylls} = (A670 \times 106)/(613 \times 100 \times d)$$

$$\text{Carotenoids} = (A470 \times 106)/(2000 \times 100 \times d),$$

where A is the absorbance and d is the path length of the cell (1 cm).

Analysis of total phenolic compounds

The total phenolic content was determined by the Folin–Ciocalteu (FC) method according to the analytical protocol described by Singleton et al. [50]. The method was adapted for oils as follows: 5 g of EVOO was extracted with 5 mL of methanol/water (80:20 v/v) by 30 min shaking and 5 min of centrifugation (4500 rpm). A portion of 1 mL of the extract was added to 0.25 mL of FC reagent, 1.5 mL of Na_2CO_3 (20% w/v), in a 10-mL volumetric flask reaching the final volume with purified water. Each sample was stored for 90 min at the controlled temperature of 25 °C in dark conditions, and the spectrophotometric analysis was performed at $\lambda = 725$ nm. Results, expressed in mg kg^{-1} of gallic acid (GA), were obtained through a calibration curve with range from 1 to 15 µg mL^{-1} ($R^2 = 0.9985$).

Extraction of the phenolic fraction from olive oil

Following the method described by the International Olive Council [21], 5 g of olive oil was added to 5 mL methanol/water (80/20 v/v) and 100 µL of syringic acid as an internal standard (IS) was added to each sample. Samples were shaken for 1 min to homogenize the mixture and then centrifuged at 5000 rpm for 25 min at 4 °C. Finally, the supernatant was injected into the HPLC-DAD system.

Reverse-phase HPLC conditions for phenolic compounds

Individual minor polar compounds were identified by high-performance liquid chromatography with diode-array detection (HPLC-DAD, Varian ProStar-Diode Array Detector 330) using a 250 × 4.6 mm column 5 µm Kinetex EVO C18 100A (Phenomenex, Torrance CA, USA). Eluent "A" was made with water and phosphoric acid 0.2% (Carlo Erba) and Eluent "B" was methanol: acetonitrile (Carlo Erba) 50:50 (v/v). The elution gradient started from 4% eluent B and reached 100% B after 55 min for 15 min at a flow rate of 1.2 mL min^{-1}. Phenolic compounds were quantified at three wavelengths: 280, 310 and 360 nm using an authentic external standard.

Fatty acid profiles

Approximately 150 µL of olive oil in 2 mL of hexane was *trans*-methylated with 200 µL of a cold solution of KOH in methanol (2 M), according to the European Standard NF EN ISO 12966-2. Fatty acid methyl esters (FAMEs) were analyzed in accordance with the European Standard NF EN ISO 5508. Analyses were performed on a Varian gas chromatograph CP3800 equipped with the flame ionization detector (GC-FID) ($T = 320$ °C), using a capillary column (60 m × 0.25 mm i.d., 0.25 µm film thickness) coated with polyethylene glycol (Zebron, ZB-WAX, Phenomenex, Torrance CA, USA). The carrier gas was helium (column flow 1.5 mL/ min), and the split ratio was 1:100. The oven temperature was programmed as follows: 2 min at 140 °C, increased from 140 °C to 240 °C at 4°C/min, held for 15 min, then 42 min at 240 °C. FAMEs were identified by comparing the retention times with the standard solution of Supelco 37 Component FAME Mix (Sigma-Aldrich).

Analysis of tocopherols

Tocopherol composition was determined by modifying the HPLC procedure described in Tura et al. [52]; 0.15 g of olive oil was dissolved in 5 mL hexane and homogenized by stirring. Samples were analyzed using HPLC-DAD 330 and the same column as for the phenolic compounds. The calibration curve was obtained by injecting standard solutions of α-tocopherols at different concentrations. The HPLC analysis was performed using a mobile phase composed of Eluent "A" water with phosphoric acid 0.2% and eluent "B" was methanol, at a ratio A/B 10:90. The flow rate was 1.2 mL/min, the injection volume was 30 µL and the time of analysis was set for 20 min. Detection and quantification were performed at 290 nm.

Sterol and squalene analyses

Around 200 mg of extracted olive oil from each sample was placed in 10 mL propylene tubes. The analysis was performed by GC of the unsaponifiable fraction without a preliminary thin-layer chromatography fractionation. This approach has been used for the analysis of sterols and squalene on olive fruit and olive oil by many authors [15, 16, 47]. Alkaline hydrolysis was performed by adding 2 mL of KOH 2%; then the tubes were soaked in a water bath at 80 °C for 15 min, and the unsaponifiable fraction was extracted by vortexing with 1 mL hexane and 1.5 mL NaCl 1%. The upper hexane layer was transferred to 2 mL glass vials and dried under a nitrogen flow at 40 °C. Two hundred µL of an internal standard solution (5α-cholestan-3β-ol, Sigma-Aldrich) in hexane was added to the dried pellets, and vials were centrifuged for 10 min at 4000 rpm. The samples were conserved at − 20 °C until analysis, usually within 24 h from preparation.

Analyses were performed on a GC-FID, using a ZB-5HT INFERNO capillary column (15 m × 0.32 mm × 0.10 µm film thickness, Phenomenex, Torrance, CA, USA). The carrier gas was helium (column flow 1.5 mL/ min), and the split ratio was 1:100. The oven temperature was programmed as follows: 0.5 min at 150 °C, from 150 to 240 °C at 8 °C/min and from 240 to 370 °C at 25 °C/min held for 5 min, at 370 °C, followed by $T = 320$ °C for injector and $T = 350$ °C

for detector (FID). The quantification was performed by external standards.

Determination of quality indexes

Specific UV extinction coefficients K_{232} and K_{270} were calculated from absorption values at 232 and 270 nm, respectively (EU Regulation 299/2013), and measured with a UV–Vis spectrophotometer (Perkin Helmer Lambda 10 UV–Vis).

Statistical analysis

GenAlEx 6.501 software [40] was used to perform the genetic distance matrix and to create the input file for parentage analysis. A paternity analysis was conducted using CERVUS version 3.0.3, to determine the most probable parents for each genotype [22]. Chemical data were analyzed by DAASTAT [36] using one-way ANOVA. Duncan's test was used to compare mean values. A principal component analysis (PCA) was also applied for a total of 22 chemical variables using PAST software version 3.16, in order to verify which parameter could mainly influence the differentiation of olive genotypes [19]. The correlation among climate and chemical variables was evaluated by Pearson's coefficient using SPSS version 21.0 (IBM Corporation, New York, USA). All determinations were performed in triplicate; the results are presented as mean value with the relative standard deviation of 2 years' experiment except for Massa Martana G1 and Agello 3 genotypes due to the lack of fruits in 2017.

Results and discussion

Genetic profiling

The molecular characterization of the sampled local olives led to the identification of different genotypes and the estimation of their degree of similarity with the main cultivars. Three samples resulted identical to known varieties; in particular two trees from the Polvese island of Trasimeno Lake (Polvese 1 and 3) showed the same profile as cv. Dolce Agogia, the most common cultivar of that area, represented by thousands of centennial trees and Agello 2 which was identical to Frantoio cultivar. Two samples from the Massa Martana area (G1 and G2) showed the same profile, thus only one was selected for the biochemical analyses. Ten new genetic profiles were identified among the selected local varieties, not corresponding to any of the 816 matched cultivars, including Italian varieties and widespread international cultivars. Parentage analysis showed that the cultivar Dolce Agogia was one of the parents of Agello 3, Polvese 4 and Polvese 5, cv. Moraiolo was the parent of Polvese 6, and Correggiolo di Massa Martana was the parent of Massa Martana G1. In addition, Polvese 2 and San Mariano 4 were generated through a cross between Moraiolo and Dolce Agogia.

These results indicated that the cultivar Dolce Agogia, which has been cultivated in this area for centuries, has several locally spreading seedlings derived from crossing with other local cultivars such as Moraiolo. The practice of propagation by seedlings, instead of cutting or grafting, was also well documented by the "Olivetani" monks, a community established near the Trasimeno lake in the fifteenth century and active until last century [18]. They were actively involved in agricultural practices and used to propagate olives by seeds. The molecular profiling depicted the local origin and uniqueness of ten of total studied genotypes.

Chlorophylls, carotenoids and total phenols content

The chlorophyll and carotenoid pigment composition extracted from the olive fruits, strictly depended on their genetic profile, both quantitatively and qualitatively [11]. In fact, the chlorophyll and β-carotene contents showed significant differences among the ten genotypes analyzed. The lowest content of chlorophyll was found in the sample Agello 3 (0.86 mg kg^{-1}) and the highest was found in Chiugiana 3 (3.63 mg kg^{-1}), while the β-carotene content varied from 13.79 mg kg^{-1} in Passignano PA2 to 43.34 mg kg^{-1} in Polvese 4 (Table 1). The green color of the oil depends above all on the type and quantity of chlorophyll and carotenoid pigments in the fruit, which solubilize during the extraction procedures. In addition to the genetic effect, many other factors, such as agronomical and technological conditions, can affect the presence of green pigments in EVOO [7,

Table 1 Chlorophylls, β-carotene and total phenols evaluated in ten olive oil samples

Genotype	Chlorophylls	β-Carotene	Total phenols
Chiugiana 3	3.34 ± 0.96^{b}*	34.47 ± 10.97^{d}	715 ± 20^{c}
Passignano PA2	2.04 ± 0.39^{f}	13.79 ± 3.57^{j}	382 ± 207^{h}
Polvese 2	3.63 ± 0.48^{a}	17.86 ± 5.72^{i}	554 ± 133^{d}
Polvese 4	3.11 ± 0.59^{c}	43.34 ± 9.02^{a}	811 ± 31^{b}
Polvese 5	1.89 ± 0.14^{f}	24.37 ± 5.27^{f}	922 ± 28^{a}
Polvese 6	2.04 ± 0.10^{f}	25.28 ± 10.78^{e}	340 ± 110^{i}
San Mariano C1	2.43 ± 1.48^{e}	35.48 ± 11.06^{c}	500 ± 118^{e}
San Mariano 4	2.70 ± 1.29^{d}	39.08 ± 13.94^{b}	431 ± 326^{g}
Agello 3†	0.86 ± 0.00^{g}	20.55 ± 0.00^{g}	129 ± 0.00^{j}
Massa Martana G1†	1.95 ± 0.00^{f}	18.19 ± 0.00^{h}	500 ± 0.00^{f}

Concentration expressed as mg kg^{-1} of oil

†Data are related only to one-year analysis

*Mean ± SD. Significant differences in each single column are shown by different letters ($p < 0.01$)

11]. Both groups of compounds have functional properties because they affect the oxidative stability of the olive oil, and carotenoids are also vitamin-A precursors [10]. Thus, analyzing new olive genotypes for their pigment contents can help to predict their effect on oil conservation and nutraceutical properties by antioxidant activity.

Total phenol content, influencing antioxidant potential and sensorial properties, represents an important parameter to determine olive oil quality. Among the olive genotypes considered in the present study, significant differences were observed for total phenol content (Table 1). The analyzed samples can be categorized for their total phenol content into three levels [49]: Polvese 2, Polvese 4, Polvese 5, and Chiugiana 3 as high content (> 500 mg GA kg^{-1} oil), Massa Martana G1, Passignano PA2, Polvese 6, San Mariano C1, San Mariano 4 as medium content (from 250 to 500 mg GA kg^{-1} oil) and Agello 3 as low content (< 250 mg GA kg^{-1} oil), (Massa Martana G1 and Agello 3 categorized based on 1 year data).

Phenolic composition

The phenolic compounds which quantified for the studied genotypes are presented in Fig. 3. The highest content of oleuropein aglycon was found in Polvese 5 (58% of the total phenols in this genotype) and the lowest amount was

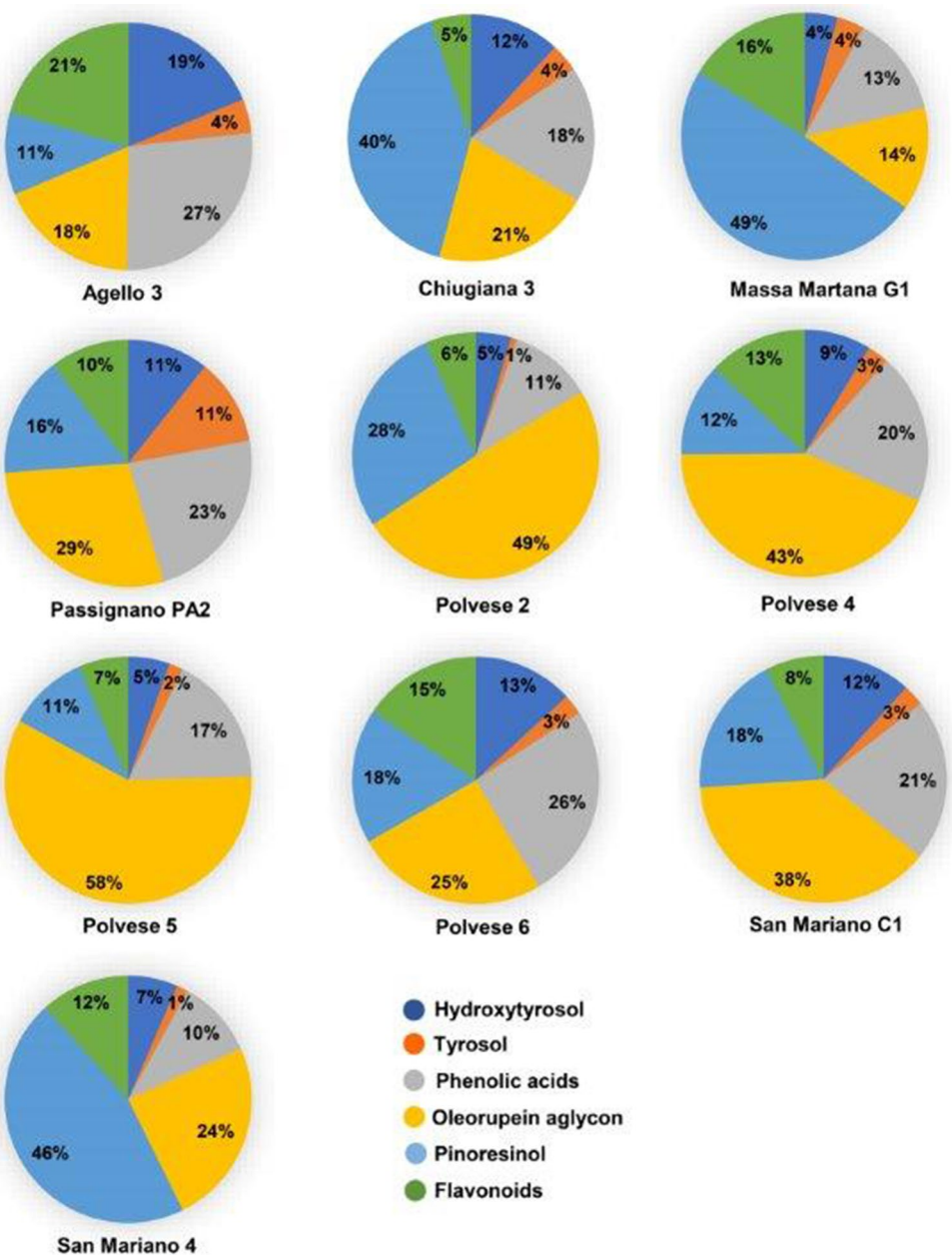


Fig. 3 The pie chart of phenolic fraction for each genotype based on the mean value of two growing season; results are expressed based on the relative percentage

measured in Massa Martana G1 (14%). The highest amount of hydroxytyrosol was in Agello 3 (19%), whereas in other olive genotypes was ranged from 4 to 13%. The tyrosol content showed a significant difference, especially for Passignano PA2 (11%), while the quantity of this phenol was approximately the same in other genotypes (Fig. 3).

The other important phenol identified in the olive oils obtained from the analyzed genotypes was pinoresinol, a lignan compound found for the first time in EVOOs in 2000 [38]. These compounds are present in the olive pulp and the woody portion of the seed and are released in the oil during the mechanical extraction without biochemical modifications during the extraction [48]. The highest content of pinoresinol was found in Massa Martana G1 (49%), followed by San Mariano 4 (46%) (Fig. 3). Lignans are one of the largest classes of chemical compounds in terms of phytoestrogens, structurally similar to estradiol, which is the primary estrogen hormone in humans. Research indicates that olive oil lignans are the best candidate to protect the body against breast cancer [41].

The other main group of phenols in olive oil is flavonoids. They are essential for human health because of the high pharmacological activities as radical scavengers [13]. Among the olive genotypes under observation, the highest content of flavonoids was measured in Agello 3 and Massa Martana G1 (21% and 16%, respectively, Massa Martana G1 and Agello 3 categorized based on 1-year data).

Fatty acids profile

The fatty acid composition has a relatively wide range due to genetic and environmental factors and can be used as a parameter for oil classification [7, 43]. Monounsaturated fatty acids have great importance because of their nutritional implications and effects on the oxidative stability of oils [45].

Table 2 presents the fatty acid profile, the sum of total saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids, MUFA/PUFA and PUFA/ SFA ratio. The oleic acid (C18:1) content ranged from 71.83% (San Mariano C1) to 76.73% (Agello 3), followed by palmitic acid (11.55–14.29%), linoleic acid (6.06–9.47%), linolenic acid (0.68–1.60%), stearic acid (1.25–2.91%) and palmitoleic acid (0.51–1.20%). Other fatty acids were found at a concentration lower than 1%. The Massa Martana G1 olive oil had the lowest SFAs (13.81%), mainly due to the lowest palmitic acid content, which represents the main fatty acid in the SFA fraction. Regarding total monounsaturated fatty acids (MUFAs), Massa Martana G1 and Agello 3 olive oil contained the highest percentage (77.92 and 77.8%, respectively) due to their high content in oleic acid (the results of 1-year analysis for the latter genotypes). The comparison among olive genotypes revealed significant differences for the main fatty acids but not for minor ones, such as arachidic and gondoic acids. The indexes and ratios calculated from the fatty acid profile, therefore, differ among the analyzed olive genotypes (Table 2).

These results are in accordance with previous findings showing that the fatty acid composition is predominantly characterized by genetic factors [43].

Tocopherols

In olive oil, vitamin E is represented by tocopherols, which have an inhibitory effect on LDL oxidation and several nutritional benefits [54]. Among the olive genotypes, Agello 3 had the highest amount of α-tocopherol (583 mg kg^{-1}) and Chiugiana 3 had the lowest amount (149 mg kg^{-1}). γ + β-tocopherol content varied from 9.65 mg kg^{-1} for Massa Martana G1 to 69.99 mg kg^{-1} in Agello 3. In addition to Agello 3, among studied olive genotypes, Polvese 5 and Polvese 2 had more tocopherols than the other genotypes, and Chiugiana 3, Massa Martana G1 and Passignano PA2 can be categorized as having a low tocopherol content (Table 3).

Sterols and squalene

Squalene, the primary hydrocarbon in olive oil, makes up more than 90% of the hydrocarbon fraction and its content is strictly related to the cultivar [5]. All analyzed samples contained high levels of squalene, as expected for good quality virgin olive oils (from 1059 to 5447 mg kg^{-1}) [37] (Table 3).

Sterols represent 20–23% of the unsaponifiable fraction of olive oil and their profile is highly species-specific [44]. *β*-sitosterol, the principal VOO sterol and one of the most studied due to its beneficial effects on health [25], ranged from 222 mg kg^{-1} for San Mariano C1 to 602 mg kg^{-1} for Agello 3 oil (Table 3). Campesterol and stigmasterol are also considered as major sterols and their ratio has been used as a parameter for oil quality and authenticity [24]. In the analyzed samples, the highest amount of campesterol (46.35 mg kg^{-1}) was found in Massa Martana G1, while the lowest one (12.33 mg kg^{-1}) was found in Passignano PA2. Furthermore, for this latter sample and for Polvese 2, the campesterol/stigmasterol ratio was lower than one (Table 3), suggesting that these two genotypes should be harvested at an earlier stage [28].

Quality parameters

Specific absorbance corresponding to the maximum absorption of the conjugated dienes and trienes must be lower than 2.50, 0.22 and 0.01 for K_{232}, K_{270} and ΔK, respectively [14]. The oxidation of unsaturated fatty acids and their fragmentation products results in the formation of hydroperoxides in

Table 2 Fatty acid profiles (%) of ten olive genotypes

Genotype	Chiugiana 3	Passignano PA2	Polvese 2	Polvese 4	Polvese 5	Polvese 6	San Mariano C1	San Mariano 4	Agello 3†	Massa Martana G1†
C16:0	13.92 ± 0.36^{b}*	13.74 ± 0.16^{bc}	12.53 ± 0.32^{f}	11.55 ± 0.47^{h}	13.45 ± 0.2^{d}	12.87 ± 0.35^{e}	14.29 ± 0.02^{a}	13.62 ± 0.48^{cd}	12.99^{e}	11.95^{g}
C16:1	0.84 ± 0.025^{bc}	0.59 ± 0.05^{de}	0.64 ± 0.045^{cd}	0.51 ± 0.09^{e}	1.18 ± 0.065^{a}	0.70 ± 0.035^{cd}	1.20 ± 0.002^{a}	0.81 ± 0.002^{bc}	0.97^{ab}	1.05^{ab}
C18:0	2.25 ± 0.03^{b}	2.91 ± 0.04^{a}	2.06 ± 0.26^{b}	2.24 ± 0.08^{b}	1.55 ± 0.30^{cd}	1.25 ± 1.07^{e}	2.02 ± 0.17^{b}	1.76 ± 0.08^{c}	2.06^{b}	1.51^{de}
C18:1	74.59 ± 1.04^{c}	73.68 ± 1.22^{e}	74.09 ± 0.95^{d}	74.79 ± 1.25^{c}	75.63 ± 1.04^{b}	73.56 ± 0.44^{e}	71.83 ± 1.14^{f}	73.76 ± 1.44^{e}	76.73^{a}	76.55^{a}
C18:2	6.85 ± 1.37^{e}	7.79 ± 1.06^{c}	9.38 ± 0.72^{a}	9.47 ± 0.74^{a}	6.54 ± 1.31^{f}	9.3 ± 0.70^{a}	9.24 ± 1.08^{a}	8.46 ± 1.02^{b}	6.06^{g}	7.41^{d}
C18:3	0.79 ± 0.02^{bc}	0.68 ± 0.00^{c}	0.83 ± 0.08^{bc}	0.71 ± 0.03^{bc}	0.96 ± 0.03^{b}	1.60 ± 0.53^{a}	0.79 ± 0.03^{bc}	0.88 ± 0.10^{bc}	0.76^{bc}	0.81^{bc}
C20:0	0.345 ± 0.045	0.48 ± 0.020	0.335 ± 0.015	0.38 ± 0.080	0.345 ± 0.045	0.375 ± 0.075	0.348 ± 0.012	0.356 ± 0.004	0.38	0.35
C20:1	0.28 ± 0.02	0.25 ± 0.005	0.28 ± 0.015	0.31 ± 0.00	0.30 ± 0.005	0.30 ± 0.005	0.24 ± 0.027	0.30 ± 0.010	0.1	0.32
MUFA	75.72 ± 0.99^{c}	74.52 ± 1.27^{e}	75.02 ± 0.98^{d}	75.61 ± 1.16^{c}	77.12 ± 1.10^{b}	74.57 ± 0.41^{e}	73.28 ± 1.17^{f}	74.88 ± 1.4^{6d}	77.8^{a}	77.92^{a}
PUFA	7.64 ± 1.36^{f}	8.47 ± 1.06^{d}	10.21 ± 0.64^{b}	10.18 ± 0.72^{b}	7.50 ± 1.29^{f}	10.90 ± 1.23^{a}	10.03 ± 1.05^{b}	9.34 ± 0.92^{c}	6.82^{g}	8.22^{e}
SFA	16.51 ± 0.43^{b}	17.13 ± 0.13^{a}	14.92 ± 0.57^{e}	14.17 ± 0.47^{g}	15.34 ± 0.15^{d}	14.49 ± 0.80^{f}	16.66 ± 0.14^{b}	15.73 ± 0.56^{c}	15.43^{d}	13.81^{h}
Index 1	10.25 ± 1.95^{c}	8.95 ± 1.26^{e}	7.38 ± 0.55^{g}	7.47 ± 0.64^{g}	10.61 ± 1.96^{b}	6.93 ± 0.82^{h}	7.39 ± 0.89^{g}	8.13 ± 0.94^{f}	11.41^{a}	9.48^{d}
Index 2	0.46 ± 0.09^{bc}	0.49 ± 0.06^{bc}	0.68 ± 0.02^{abc}	0.71 ± 0.03^{ab}	0.49 ± 0.09^{bc}	0.75 ± 0.13^{a}	0.60 ± 0.06^{abc}	0.59 ± 0.04^{abc}	0.44^{c}	0.59^{abc}

Index 1. MUFA/PUFA

Index 2. PUFA/SFA

†Data are related only to 1-year analysis

*Mean ± SD. Significant differences in each single row are shown by different letters ($p < 0.01$), P value for C20:0 and C20:1 were not significant

Table 3 Squalene, tocopherols and sterols composition in ten olive genotypes (concentration expressed as mg kg^{-1})

Genotypes	α-tocopherol	γ + β-tocopherols	Squalene	Campesterol	Stigmasterol	β-sitosterol	β-sitostanol
Chiugiana 3	149 ± 29^{j}	13.54 ± 0.34^{f}	1224 ± 124^{i}*	23.16 ± 0.76^{g}	17.22 ± 7.22^{cd}	359 ± 119^{d}	15.11 ± 5.24^{d}
Passignano PA2	170 ± 69^{i}	11.57 ± 1.57^{h}	5447 ± 557^{a}	12.33 ± 0.55^{i}	15.09 ± 6.29^{d}	343 ± 113^{d}	44.43 ± 19.38^{bc}
Polvese 2	450 ± 65^{c}	13.89 ± 0.61^{e}	2637 ± 579^{f}	15.96 ± 0.15^{h}	16.65 ± 7^{cd}	358 ± 112^{d}	44.63 ± 24.48^{bc}
Polvese 4	330 ± 90^{e}	20.3 ± 7.28^{d}	3497 ± 397^{d}	39.74 ± 1.2^{d}	19.34 ± 9.34^{bc}	374 ± 139^{d}	17.91 ± 6.81^{d}
Polvese 5	536 ± 236^{b}	31.86 ± 17.86^{c}	1059 ± 209^{j}	40.1 ± 1.45^{c}	21.44 ± 6.44^{b}	506 ± 186^{b}	45.64 ± 23.5^{bc}
Polvese 6	232 ± 68^{g}	12.88 ± 0.62^{g}	4116 ± 316^{c}	27.43 ± 0.33^{e}	16.05 ± 6.55^{d}	435 ± 161^{c}	51.13 ± 19.13^{b}
San Mariano C1	237 ± 69^{f}	10.89 ± 3.89^{i}	2751 ± 583^{e}	16.26 ± 1.31^{h}	14.54 ± 10.92^{d}	222 ± 68^{e}	16.04 ± 10.07^{d}
San Mariano 4	430 ± 200^{d}	32.23 ± 30.7^{b}	1954 ± 288^{h}	26.1 ± 2.59^{f}	15.39 ± 10.38^{d}	427 ± 154^{c}	40.27 ± 28.85^{c}
Agello 3†	583^{a}	69.99^{a}	2581^{g}	41.25^{b}	28.28^{a}	602^{a}	46.93^{bc}
Massa Martana G1†	176^{h}	9.65^{j}	4786^{b}	46.35^{a}	15.59^{d}	325^{d}	532^{a}

†Data are related only to 1-year analysis

*Mean ± SD. Significant differences in each single column are shown by different letters ($p < 0.01$)

which the double bands become conjugated. In the present study, the specific absorbance for all the extracted oils was below the standard limit (Supplementary material, Table 2).

Principal component analysis (PCA)

Twenty-two variables from the average of 2 years' data were used to perform the PCA analysis (Fig. 4). The first PC accounted for 30.29% of the total variance and had a positive correlation with ten variables, especially with α-tocopherol, total tocopherols, β-sitosterol, campesterol and stigmasterol and negative with 12 ones, especially with squalene and linoleic acid. In Fig. 4 it can be observed that the PC1–PC2 plot permits an excellent differentiation of two genotypes, Polvese 5 and San Mariano 4; both had positive correlations for α-tocopherol, total tocopherols, campesterol, β-sitosterol and luteolin while Chiugiana 3, Polvese 6, Passignano PA2 and San Mariano C1 had negative correlations for tyrosol and squalene. PCA allows us to observe groupings of samples, which define the structure of the data set. The interpretation of the results of PCA is usually carried out by visualization of the component scores in a biplot. In general, there was a high variability between the studied genotypes highlighting one more time the importance of local and undetermined olive genotypes. Moreover, considering these minor compounds are strictly related to human health [25, 46] it was demonstrated that they are widely present in our studied genotypes giving more importance to valorization of the abandoned olive germplasm.

Correlation between climatic and chemical data

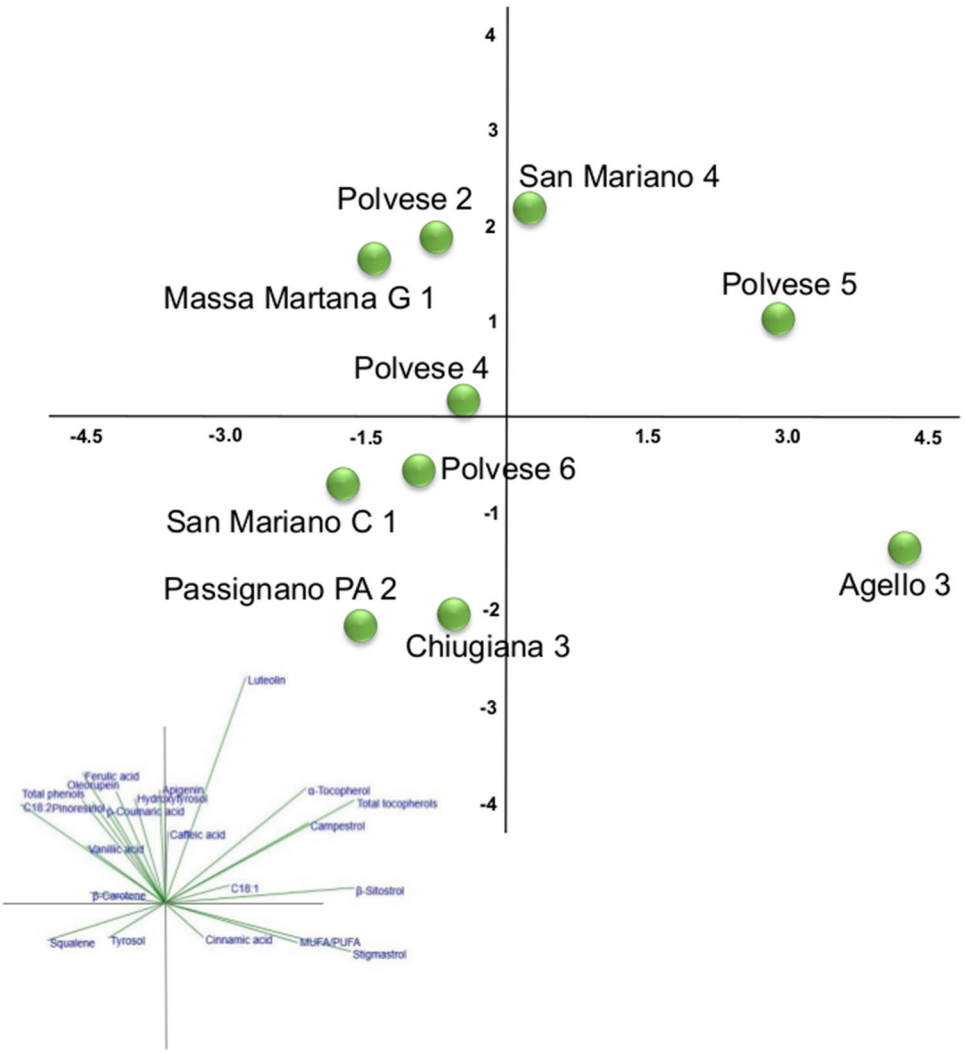
Chemical data from each growing year for the eight genotypes, which had fruits from two harvesting seasons, were compared with annual precipitation, average minimum and maximum temperature (Fig. 5). Results indicated that among all climate data, precipitation can positively affect the amount of Stigmasterol and β-sitosterol ($R^2 = 0.829$; $R^2 = 0.562$; respectively), while there was not any other correlation between chemical parameters and climate data These results could indicate that even with considerable variation of rain and temperatures, in these two seasons, the analyzed local genotypes are not significantly affected by climate variation (at least in 2 years of analysis) and maintain constant chemical values.

Conclusions

In the present work, ten olive genotypes collected from different sites in Umbria, in two consecutive growing seasons, were characterized at the molecular and biochemical levels. The aim was to verify their identity and evaluate their potential. The molecular analysis confirmed that these samples did not correspond to any known variety, but they are crosses of local cultivars. These plants were empirically selected, grown and maintained during centuries by monks and farmers, thus representing a local resource of autochthonous origin.

The chemical characterization of local olive oil types is mandatory for the selection of varieties that have adapted to the local climate and can produce virgin olive oil with desirable quality characteristics. Based on the biochemical analyses, Polvese 5 and San Mariano 4 genotypes can be classified as the best candidate in terms of oil quality and showed high values of major and minor components (for 22 out of 29 studied variables).

This study is the first screening performed in situ, on neglected local genotypes growing in different areas of Umbria, central Italy. In addition, for future experiments, we propagated the most promising genotypes to establish field trials for their agronomical evaluation. The potential of these



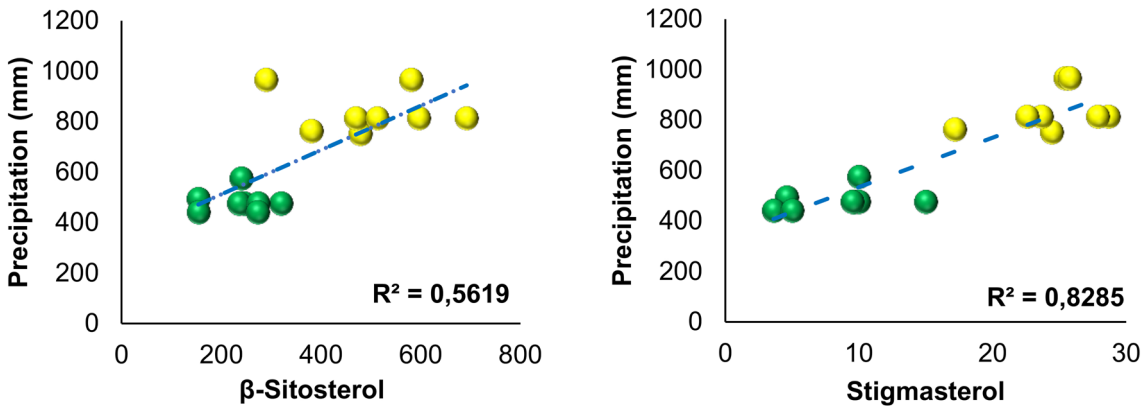


Fig. 4 PCA of ten olive genotypes by 13 oil quality parameters

Fig. 5 Pearson correlation between the annual precipitation and sterols (the concentration expressed as mg kg^{-1} of sterols in olive oil)

olive genotypes will be validated in different years under the same climatic conditions. These new varieties could be a novel source of variability for further scientific studies as a breeding program and to use them in large-scale plantations by olive growers after a deep study of various agronomical traits in different crop season.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Compliance with ethics requirements This article does not contain any studies with human or animal subjects.

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