Quality parameters, chemical compositions and antioxidant activities of Calabrian (Italy) monovarietal extra virgin olive oils from autochthonous (Ottobratica) and allochthonous (Coratina, Leccino, and Nocellara Del Belice) varieties

AUTHENTICATION OF CORATINA, OTTOBRATICA, LECCINO AND NOCELLARA DEL BELICE MONOVARIETAL EXTRA VIRGIN OLIVE OILS: QUALITY AND CIELAB PARAMETERS, CHEMICAL COMPOSITION AND ANTIOXIDANT ACTIVITY

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ABSTRACT

Keywords: Extra virgin olive oil; chemical profile; colorimetric parameters; oxidative stability

Introduction

Olea europaea L. (Oleaceae) is one of the most socio-economically important cultivated fruit tree in the Mediterranean region [Dias et al., 2020]. Extra virgin olive oil (EVOO), mechanically obtained from the fruits of *Olea europea* L., represented a central foods and principal fat of Mediterranean diet and Italy is the first EVOO world consumer [Lanza & Ninfali, 2020]. Additionally, Italy produces 15% of the world EVOO market and this production is based on autochthonous cultivars (cv) that contributes to maintain large part of EVOO ancient diversity [Loizzo et al., 2012; Marra et al., 2006]. In particular, more than 80 % of EVOO is produced in the regions of Southern Italy, including Calabria, Sicily and Campania [Global olive oil market 2019]. In Calabria, both autochthonous and allochthonous olive varieties are largely cultivated along the region, with identification of 33 different cultivars [Giuffrè et al., 2010; Giuffrè et al., 2007].

Among them Coratina cv represents one of the most widespread and appreciated varieties of southern Italy. The plant has a high oil yield, up to 25%, about twice compared to other varieties grown in Italy. The extra virgin olive oil obtained from Coratina cv is intensely fruity, with a strong flavor and bitter notes and accompanied by a good spicy (Perri et al., 1995). Also, Leccino cv, is recognized for its widespread presence. The plant showed a oil yield of 18-21%. The oil presents a medium intensity of olive fruity, bitter and pungent accompanied by a higher intensity of green almond and, in a smaller measure, of herb and artichoke (Rotondi et al., 2010). Ottobratica of the Calabrian region is an autochthonous variety of *Olea europaea* L. The oil yield is on average 18% and reaches 20% in late November-December (Giuffrè et al., 2006). Its extra virgin olive oil is on average bitter and spicy and slightly sweet, with sensations of dried fruit and ripe fruit (Sicari et al., 2009). Oil produced by 'Nocellara del Belice', (average yield of 18%) showed an intense olive fruity, bitter and pungent sensory profile. The pleasant notes were mainly ascribable to herb and

green tomato accompanied by smooth artichoke and green almond notes (Rotondi et al., 2010).

Recent investigation underline that Italian consumers are attracted to the increase EVOO quality as well as their chemical compositions correlated to health benefits [Lanza & Ninfali, 2020]. EVOO quality is dependent upon both olive variety (cultivar), health condition, degree of ripening, and operating conditions of the extraction process (time, temperature and exposure to oxygen) [Migliorini et al., 2011].

The beneficial health properties of EVOOs have been known for centuries, and more recently its consumption with the diet has been correlated to a decreased incidence of certain disease correlated to its antioxidant preventive effects, including coronary heart disease, obesity, and cancer [Arslan & Ok, 2019]. The health characteristics can be attributed both a high content of monounsaturated fatty acids (MUFAs), with particular reference to oleic acid (OA, ω-9), and to essential fatty acids, including linoleic acid (LA, ω-6) and α-linolenic acid (ALA, ω-3). Additionally, minor components such as phenolic compounds and tocopherols are recognized for their antioxidant activity, contribute to EVOO positive sensory notes and the shelf life as well as nutritional quality [Giuffrè et al., 2013; Serrelli et al., 2018].

For this purpose, the goals of this study were as follows: *i*) quality parameters including CIELAB, *ii*) oxidative stability, *iii*) total phenolic, flavonoid, carotenoid and tocopherol contents, *iv*) fatty acids methyl esters, fatty alcohol, sterols and waxes composition, and *v*) *in vitro* antioxidant activity of *Olea europea* L. cv Coratina, Ottobratica, Leccino and Nocellara del Belice extra virgin olive oils.

Materials and Methods

Chemicals and reagents

All reagents were purchased from Sigma-Aldrich S.p.a. (Milano, Italy), whereas analytical-grade solvents were obtained from VWR International s.r.l. (Milan, Italy).

Samples

Olives from Coratina, Ottobratica, Leccino and Nocellara del Belice cultivated in different area of Calabria (Italy) harvested in autumn 2019 were collected and used to prepare extra virgin olive oils (EVOO) by Frantoio Meringolo, Corigliano Calabro (Cosenza, Italy). EVOO samples were accomplished the UNI10939, 2001 certification. EVOOs were stored at 10 °C in the dark using green glass bottles without headspace before analysis. EVOOs were analysed 1 month after production.

Quality and CIELAB parameters

Peroxide index, free acidity value, UV light absorption (K232 and K270) and ΔK, sterol, and fatty acid profiles of EVOOS were analysed according to the methods described by EC Regulation (EU, 2011). Chromatic coordinates were measured at 25 °C using a PCE CSM-4 colorimeter (PCE, Lucca, Italy to obtain the colour according to the CIEL a* b*method (Moyano et al., 2008). Data were expressed as higher saturation of color or chroma (C*). C* considered as the quantitative indicator of colorfulness, is used to determine the degree of difference in a hue in comparison to a grey color with the same lightness. The higher the chroma values, the higher color intensity of samples is perceived by humans. Hue angle (h*), considered the qualitative indicator of color, is an attribute according to which colors have been traditionally defined as reddish, greenish, and is used to define the difference of a certain color with the reference to grey color of the same lightness. This attribute is related to the differences in absorbance at different wavelengths. A higher hue angle represents a lesser yellow character in the assays. An angle of 0° or 360° represents red hue, whilst angles of 90°, 180° and 270° represent yellow, green and blue hues, respectively (Pathare et al., 2013).

Determining of oxidative stability of EVOOs

The analysis with OXITEST (VELP, Scientifica) allowed to determine the oxidative stability of EVOO samples under accelerated condition. Following AOCS International Standard Procedure (Cd 12c-16), 5 g of sample were evenly distributed in hermetically sealed titanium chambers setting the reactor temperature at 90° and 6 bar of pressure. The OXITEST response is the induction period (IP), which is automatically calculated from oxidation curve by graphical method (two tangent methods) through the OXISoft™ software.

EVOO phenolic extract

The EVOO phenolic extract was obtained following proposed by Montedoro et al. (1992). In brief, EVOOs (5 g) were extracted with 2 mL of MeOH/H_2O (7:3, *v*/*v*), then 1 mL of *n*-hexane was added. The mixture was centrifuged at 12000 rpm for 10 min at 4 °C. The extracts were pooled and evaporated at low temperature. The dry extracts were re-suspended in 1 mL of methanol.

Total phenolic, flavonoid, carotenoid and tocopherols contents

TPC was determined as previously described (Gao et al., 2000). The absorbance was read at 765 nm. TPC was determined in triplicate and expressed as ppm. TFC was determined using a previously reported method (Yoo et al., 2008). Absorbance was read at 510 nm. TFC was determined in triplicate being expressed as ppm. TCC was determined following a previously reported procedure (Minguez-Mosquera et al., 1990). Briefly, 5 mL of EVOO were mixed with *n*-hexane (1:1, *v*/*v*) and the absorbance was measured at 470 nm. Three separate analyses of the EVOO extract were done and data are expressed as mean ± S.D in ppm.

For identification and quantification of alpha tocopherol, the oil samples were extracted following the method described by Bakre et al. 2015. After the preparation of the oils, direct

injection of the sample was performed for the determination of the alpha tocopherol through Utra High Performance Liquid Chromatography (UHPLC). For this purpose, 5µL was applied in the UHPLC PLATINblue (Knauer, Germany) equipped with a binary pump system using a Knauer column C18 (1.8 µm, 150 × 3 mm) coupled with a Schimadzu RF-20Axs Fluorescence Detector. As isocratic elution methanol and acetonitrile (1:1) were used with a flow rate of 4 ml/min. The analytical column was kept at 30°C and fhe fluorescence detector was set at 295 nm excitation wavelength and 325 nm emission wavelength. The mobile phase was methanol at 100%, in an isocratic system with 1.0 mL/min flow and wavelength of 292 nmI. The identification and quantification of the alpha tocopherol had been established by external standard and the chromatograms elaborated through Clarity 6.2.software.

Antioxidant activity

DPPH and ABTS tests

DPPH test was applied to investigate the radical scavenging activity [Leporini et al., 2020]. An ethanolic solution of DPPH at concentration of 1.0 × 10−4 M was mixed with the extracts (concentrations in the range 1-1000 µg/mL). The reaction mixtures left in the dark for 30 min. The absorbance was measured at 517 nm. The DPPH radical scavenging activity (%) was calculated according the following equation:

$$DPPH\ radical\ scavenging\ activity\ (\%) = [(A0 - A1/A0) \times 100]$$

where A0 is the absorbance of the control and A1 is the absorbance in the presence of the extract. Ascorbic acid was used as the positive control.

The ABTS assay was applied by using the previously published methodology [Leporini et al., 2020]. ABTS solution was mixed with potassium persulphate and left in the dark before use for 12 h. The ABTS solution was diluted with ethanol to an absorbance of 0.70 at 734

nm. A mixture of extracts (at concentrations in the range 1-400 µg/mL) and diluted ABTS solution was prepared and after 6 min the absorbance was read at 734 nm. The ABTS radical scavenging activity of the samples was calculated according to the following equation:

$$ABTS\ radical\ scavenging\ activity\ (\%) = [(A0 - A)/A0] \times 100$$

where A0 is the absorbance of the control reaction and A is the absorbance in the presence of samples.

β-carotene bleaching test

The protection of lipid peroxidation was tested by *β*-carotene bleaching test [Loizzo et al., 2019]. In this assay Tween 20, linoleic acid, and *β*-carotene were mixed. After evaporation of the solvent and dilution, the emulsion was added into the 96-well microplate containing samples at different concentrations and incubated at 45 °C for 30 min then the absorbance was measured (λ= 470 nm). Propyl gallate was used as a positive control.

Ferric Reducing Activity Power (FRAP) Assay

FRAP reagent was prepared by mixing 2.5 mL of 10 mM tripyridyltriazine (TPTZ) solution, 40 mM HCl, 2.5 mL of 20 mM $FeCl_3$ and 25 mL of 0.3 M acetate buffer (pH 3.6) [Loizzo et al., 2019]. Sample at concentration of 2.5 mg/mL was mixed with FRAP reagent and water and incubated for 30 minutes at 25 °C. The absorbance was read at 595 nm. Ethanolic solutions of known Fe (II) concentration, in the range of 50–500 µM ($FeSO_4$), were used for obtaining the calibration curve. FRAP value was expressed as mM Fe(II)/g. Butylated hydroxytoluene (BHT) was used as a positive control.

Statistical analysis

Data are expressed as means of three different experiments ± standard deviation (S.D.).

Analysis of variance (ANOVA) was performed to determine statistically significant differences between the samples. Variable means were compared by using SPSS 17.0 (Chicago, IL, USA),. statistics software. Analysis of variance (ANOVA) was done by Turkey's test to determine any significant difference among all treatments at different levels: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Principal Component Analysis (PCA) was applied by SPSS software for Windows, version 17.0 (Chicago, IL, USA).

Relative Antioxidant Capacity Index (RACI) is an integrated statistical application to evaluate the antioxidant capacity values generated by different *in vitro* methods (Sun & Tanumihardjo, 2007). Data obtained from ABTS, DPPH, β-carotene, FRAP were used to calculate the RACI value for the extracts by using the following equation:

$$RACI = (x - \mu)/\sigma$$

where x is the raw data, μ is the mean, and σ is the standard deviation.

GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA, USA) was used to calculate the concentration giving 50% inhibition (IC_{50}) by plotting the percentage inhibition versus concentration. *Pearson's* correlation coefficient (*r*) was calculated by using Microsoft Excel 2010 software.

Results and Discussion

EVOO quality parameters

The highest quality EVOOs must feature a free acidity lower than 0.8%. Free acidity values ranging from 0.2 to 0.8% for Nocellara del Belice and Coratina EVOOs, respectively whereas peroxide levels ranging from 8.4 to 12.7 meq O_2/kg of oil for Ottobratica and Leccino, respectively. All samples exhibited a ΔK value of 0.0001 (Table 1). These data indicated that both investigated oils could be classified as "extra virgin" according to the EC 2568/91

225 regulation [European Union Commission 1991 and 2003]. Our values are in agreement with
226 those reported by Lavelli et al. (2005) for Leccino and by Giuffré (2006) for Ottobratica.
227 Previously, Gambacorta et al., (2010) found peroxide level and free acidity values of 4.93
228 meq O_2/kg and 0.25% for Coratina EVOO from Puglia (Italy). A lower free acidity value of
229 0.26 was found for Ottobratica by Piscopo et al. (2016) from Calabria.
230 EVOO phenolic compounds are responsible for the pungent perception as well as for the
231 resistance to the oxidative process that reduce EVOO quality. A significant difference ($P <$
232 0.01) was observed between the two EVOOs regarding total phenolic content (TPC), total
233 flavonoid content (TFC) and total carotenoid content (TCC) (Table 1). 3-Times lower TPC
234 content was recorded in Nocellara del Belice sample. In particular, Ottobratica EVOO
235 showed the highest TPC with value of 452.4 ppm followed Coratina EVOO (392.9 ppm). It
236 is interesting to note that TPC of 286.73 ppm was found for Ottobratica by Piscopo et al.,
237 (2016). TPC values of 322.18 and 228.58 ppm were reported for Coratina and Leccino
238 EVOOs, from Puglia (Italy), respectively (Baiano et al. 2009). A TPC value of 246 ppm was
239 discovered in Leccino from Abruzzo (Italy). A great variability in TPC values was recorded
240 by Leporini et al., (2018) for Frantoio monovarietal EVOOs derived from olive fruits
241 collected in different area of Calabria. TPC values in the range 367-530 ppm have been
242 reported for other two Calabrian monovarietal EVOO Sinopolese and Roggianella [Sicari et
243 al., 2017]. A statistically significant difference was observed also in TFC with values ranging
244 from 13.8 to 24.5 for Nocellara del Belice and Ottobratica EVOOs, respectively. A great
245 difference in TPC was observed in Nocellara del Belice EVOO phenolic fraction by Tripoli
246 et al. (2009) with mean value of 187.51 ppm. The EVOO color depends on the wavelengths
247 of transmitted visible light, and is influenced by two groups of minor oil constituents such as
248 carotenoids and chlorophylls. The high content of these natural pigments is relevant to EVOO
249 stability and quality. Often the consumer chooses the oil based on its color [Gargouri et al.,
250 2013]. For this reason in this work, we have decided to monitor the TCC, chlorophylls and

the CIELAB colorimetric parameters of Calabrian monovarietal EVOOs. A great variability was observed with regard to TCC with values ranging from 3.1 to 23.5 ppm for Nocellara del Belice and Ottobratica, respectively. Carotenoids are considered the most important EVOO pigments after chlorophyll due they strong protective effect against EVOO oxidation induced by light exposure. This variability was observed, also in chlorophyll content (2.16-50.6 ppm). These values are in line with those reported for monovarietal Tuscan EVOOs from Frantoio, Leccino, Moraiolo, and Pendolino cultivars [Borrello& Domenici, 2019] whereas values in the range from 20.9 to 47.6 ppm with respect to TCC and from 6.5 to 10.5 ppm with respect to chlorophyll were recorded by Tuberoso et al., (2016) for Sardinian monovarietal EVOO from Semidana and Tonda di Cagliari cultivar, respectively. Lower quality parameters of Sardinian EVOOs were found in the range values, in the range 0.84-2.89 ppm, and 0.67-1.70 ppm for chlorophylls and TCC, respectively, were observed in Algerian EVOOs [Zegane et al., 2015]. Table 1 reported the chromatic data CIELab color parameters (L*, a*, b*, C and H) for all analysed EVOOs. The parameter L was highest in Leccino whereas C* in Ottobratica EVOO. No correlations were found between CIELab parameters and pigments (TCC and chlorophyll). In each case high L index indicating the rise in oil brightness which could be due to the autoxidation and subsequently the degradation of chlorophylls. Previously Piscopo et al., (2018) investigated the effect of storage on Ottobratica EVOO and found CIELAB colorimetric parameters in line with our data. Different CIELAB parameters were recorded in EVOOs from Spain. In particular, L was found in the range 91.5–97.2 CIELAB units from the PDOs Sierra de Segura, Poniente de Granada (except the Lucio cultivar), Siurana, Flor de Espadán and Estepa. These EVOOs are lighter than our samples (Becerra-Herrera et al. 2018).

In recent years, researchers addressed their attention to EVOO minor components such tocopherols due their contribution to EVOO sensory notes, nutritional quality and health properties [Lavelli et al., 2006]. The following TTC trend was observed: Coratina > Leccino

277 > Ottobratica > Nocellara del Belice. Our data on TTC are in disagreement with those
278 reported for Ottobratica EVOO by Piscopo et al., (2016) that found a TTC value of 334.80
279 ppm. Among tocopherols, α-tocopherol represent the dominant compound. Ottobratica
280 EVOO showed the highest value with 175.1 ppm. Previously, Ambra et al., (2016)
281 investigated the α-tocopherol content in seven Italian monovarietal EVOO and found the
282 highest value in Leccino (266 ppm). This data is 2-time higher than that found for our sample
283 Calabrian Leccino EVOO (113.4 ppm). Previously, Fuentes et al. (2018) reported the α-
284 tocopherol content of Coratina, Nocellara del Belice and Leccino EVOOs from germoplasm
285 bank in Chile and found mean values of 116, 56 and 172 ppm, respectively. Values ranging
286 between 98 and 370 ppm were found in Greek EVOOs (Psomiadou et al., 2000).
287
288 *EVOO fatty acids, tocopherols, fatty alchool and waxes profile*
289 Table 2 reports the fatty acids (FA) composition of all analysed EVOOs. As expected the
290 differences among the different olive cultivars were slight, however, these differences
291 resulted statistically significant for all fatty acids excepting linolenic and behenic acid. OA
292 with values ranging from 69.6 to 76.3 was the dominant fatty acids of 79 and 72% for
293 Ottobratica and Nocellara del Belice, respectively that contributes to most of the total
294 concentration of MUFAs (Table 2). Among saturated fatty acids (SFAs), palmitic acid (PA)
295 was detected in great amount (values ranging from 11.1 and 15.1% for Nocellara del Belice
296 and Ottobratica, respectively). A statistically significant difference was observed in OA/LA
297 ratio ($P< 0.01$), an indicator of the stability of the oil [Alvarruiz et al., 2003]. In particular,
298 Nocellara del Belice EVOO showed an OA/LA value of 9.42. Our data on fatty acid
299 composition are in line with those reported in literature for different monovarietal EVOO
300 from Calabria (South of Italy) (Patumi et al., 2003; Sicari et al., 2010; Leporini et al., 2018;
301 Piscopo et al 2016). In particular, Ottobratica from Reggio Calabria province showed an OA
302 content of 71.82% followed by PA content of 14.90% (Piscopo et al., 2016). Values of 76.2

and 13.2 for OA and PA were found for Leccino obtained from olive fruits cultivated in Central Italy [Blasi et al., 2019]. Previously, Tripoli et al. (2009) reported the fatty acid composition of Nocellara del Belice from wester Sicily (Italy) and found mean values of 72.60 and 12.83% for OA and PA, respectively. A LA content of 8.45% was, also, observed.

Sterols are constituents of the unsaponifiable fraction and are related to the quality and the authenticity of an EVO (EU, 2016; COI, 2018). The habitual consumption of plant sterols in the human diet is related to blood lipid concentration (Anderson et al., 2004) and reduces intestinal cholesterol absorption (Ostlund et al., 2002), in addition phytosterol consumption is inversely related to cholesterol content in serum (Anderson et al., 2004; Klinberg et al., 2008). The data of sterols are listed in the table 3. Values of the four studied cultivars showed very high significant differences (p<0.001). Nocellara del Belice oil showed the highest total sterol content (1710.0 mg/kg), followed by Leccino (1610.5 mg/kg), by Coratina (1530.5 mg/kg) and lastly by Ottobratica (1396.5 mg/kg), leading to the conclusion that from the sterolic point of view all cultivars produced an EVOO because both the European regulation (EU, 2016) and the Trade Standard of the International Olive Council (COI, 2018) state 1000 mg/kg as minimum total sterol content for an EVOO. Also all other parameters were closed in the values proposed by the International regulations for an EVOO. No cholesterol content occurred to as much as 0.5%, campesterol was less than 4%, stigmasterol was lower than campesterol, apparent β-sitosterol was more than 93%, Δ^7-stigmastenol was less than 0.5% and the sum of erythrodiol and uvaol was less than 4.5% of the total sterol content. In detail the maximum cholesterol content was found in the Leccino oil (0.22%) and the highest campesterol content was in Nocellara del Belice (3.31%). The minimum apparent β-sitosterol was detected in

325 Nocellara del Belice (94.53%), finally the highest sum of erythrodiol and uvaol was 1.64%
326 found in Coratina.
327 Long chain fatty alcohols from pomace olive oil may confer a protective role against
328 inflammatory damage in different pathologies, including atherosclerosis (Fernández-Arche et
329 al., 2009). Docosanol was found to be enhancing the antiviral activity against herpes viruses
330 (Marcelletti, 2002). Octacosanol administred to humans was found to decrease neutral sterol
331 and bile acid concentration in feces (Keller et al., 2008), besides it was found to be very
332 efficacious to low LDL and to increase HDL and also gives cytoprottive effects (Taylor,
333 2003). The data of the present work are summarised in the table 4. Seven compounds were
334 detected: four with an even chain carbon number (docosanol, tetracosanol, hexacosanol and
335 octacosanol) and three with an odd chain carbon number (tricosanol, pentacosanol and
336 eptacosanol). The total fatty alcohol content varied from a low 45.50 mg/kg in Nocellara del
337 Belice and a high 134.53 mg/kg in Ottobratica, i.e. 296% more than in Nocellara del Belice.
338 However the behaviour of Ottobratica allows the oil of this cultivar to remain well within 350
339 mg/kg namely the maximum limit exceeding which an oil can be considered as a pomace oil
340 (EU, 2016). In all cultivars, the sum of the even chain carbon fatty alcohols was always in
341 higher quantity with respect the sum of odd chain carbon fatty alcohols. The ratio Σ ECFA /
342 Σ OCFA was closed betwwen 13.63 (Ottobratica oil) and 9.86 (Nocellara del Belice oil).
343 Hexacosanol predominated in the oil of all cultivars ranging between 27.64% (Ottobratica)
344 and 34.38% (Nocellara del Belice). The second and the third major fatty alcohols were always
345 tetracosanol and octacosanol. Tricosanol was the lowest fatty alcohols ranging between 1.44%

346 (Leccino) and 2.31% (Nocellara del Belice). No odd chain carbon fatty alcohols occurred to
347 as much as 5.41% of pentacosanol found in Leccino.

348 Cuticular wax plays an important role because protects the aerial surfaces of the land
349 plants from biotic and abiotic stresses. It is mainly composed of long-chain aliphatic
350 compounds derived from very long chain fatty acids. However in olive oil, waxes are
351 considered negatively because they are largely contained in pomace olive oil being easily
352 dissolved in the hexane used to extract oil from pomace. Oils with a total wax content of
353 between 300 mg/kg and 350 mg/kg (calculated as the sum of C40 - C42 - C44 -C46) are
354 considered to be crude olive-pomace oil if the total aliphatic alcohol content is above 350
355 mg/kg and if the erythrodiol and uvaol content is greater than 3.5 % (EU, 2016). Waxes values
356 are listed in table 5. The wax content ranged between 47.51 mg/kg (Coratina) and 111.29
357 mg/kg (Ottobratica), namely less than 300 or 350 mg/kg stated by the European Union (EU,
358 2016) to consider an oil as a pomace one. If only the sum of C42, C44 and C46 waxes is
359 considered, the highest value was 88.78 mg/kg of Ottobratica oil, i.e. 1.68 times less than 150
360 mg/kg stated by the Euopean Union and by the Olive International Council for an EVO (EU,
361 2016; COI, 2018). Coratina oil contained mainly C42 (32.37%) and C40 in low amount
362 (13.62%). Leccino and Ottobratica oils contained mainly C42 (31.86% and 36.92%
363 respectively), whereas Nocellara del Belice oil showed an high content of C40 (32.48%).
364

365 *EVOOs oxidative stability*

366 As illustrated in Figure 2, the oxidative stability of olive oils analysed is influenced by
367 varietal factor. IP ranged between 29:5 and 16:4 hours. Greater stability was observed for

Ottobratica cv whose α- tocopherol content was highter that other oils. While Leccino and Nocellara del Belice showed an induction time of 27.52 and 25.37 respectively.

The lowest induction time was that of Coratina cv equal to 16:5 hours, although coratina oil had a tocopherol content equal to 162.9 hours.

According to reports by Baccouri et al. 2008 the effect of α tocopherol on oxidative stability depends on the concentration of phenolic compounds. In addition, as reported Piscopo et al. 2016, the highest stability of Agristignana cv could be related to a synergistic effect between o- diphenol and MUFA/PUFA ratio.

Antioxidant activity

In order to estimate the antioxidant potential of EVOOs phenolic extracts were tested by using different *in vitro* assay (Table 6). An approach based on the use of different assays is necessary due the facts that antioxidants can exert their ability trough different mechanisms of inactivation of the radicals [Antolovich et al., 2002]. This scientific evidence is applied and justifies the diversity of results between the samples in relation to the test applied. Generally, ABTS radical was more sensible to the radical scavenging activity of Calabrian EVOOs. In particular Coratina EVOO showed the highest potency with IC_{50} value of 21.3 μg/mL followed by Ottobratica phenolic extract (IC_{50} value of 26.3 μg/mL). This trend was observed also in DPPH assay were IC_{50} values of 29.2 and 35.8 μg/mL for Ottobratica and Coratina EVOO phenolic extracts were found. Correlation coefficient calculation showed that both even and odd chain fatty alcohols are positive correlated with radical scavenging assays DPPH and ABTS. A statistically significant difference was observed in β-carotene bleaching test, which investigate the ability of EVOO to protect from lipid peroxidation. In this case, Ottobratica EVOO showed the highest protective effect with IC_{50} value of 176.3 μg/mL. *Pearson's* correlation coefficient evidenced a strong positive correlation between β-

carotene bleaching and DPPH test and EVOO's total sterol content with *r* values of 0.99 and 0.93, respectively.

All EVOOs phenolic extracts showed a greater ferric reducing power with FRAP values higher than that reported for the positive control BHT. Among them the following trend was observed Ottobratica > Coratina > Nocellara del Belice > Leccino. *Pearson's* correlation coefficient evidenced a positive correlation between FRAP and EVOO's α-tocopherol, TCC and TPC content (r =0.96). Additionally, FRAP was positively correlated with odd carbon chain fatty alcohols whit r =1. The statistical approach RACI allowed us to identify the following trend in the antioxidant potential of the samples: Ottobratica > Coratina > Leccino > Nocellara del Belice.

Previously, Sicari et al. (2017) reported the radical scavenging potential of Ottobratica EVOO from Reggio Calabria (Italy) and found a percentage of inhibition of 27.37 and 2.52% for DPPH and ABTS test, respectively. Successively a percentage of inhibition of 33.09 and 41.75% against ABTS and DPPH, respectively for Ottobratica EVOO (Piscopo et al., 2018). Mean IC_{50} values of 186, 218, and 171.5 mg oil were recorded for Coratina, Leccino, and Nocellara del Belice, respectively (Fuentes et al., 2018). Leporini et al. [2016] investigated the radical scavenging activity of monovarietal Frantoio EVOO produced in four different area of Calabria and found an average value of 117.2 and 131.9 μg/mL for ABTS and DPPH, respectively. The effect of whole and destonedtechnology on antioxidant activity of Coratina EVOO was investigated by Gambacorta et al. (2010) that found how stoning increase the antioxidant potential of the phenolic extracts with percentage of inhibition of 53.14 and 31.54% for ABTS and β-carotene bleaching test, respectively.

Pearson's correlation coefficient evidenced a positive correlation between antioxidant activity and EVOO's phenolic extract TFC and TCC (r =1).

Principal component analysis

In the present study, PCA was performed to group and separate the variables analysed in the virgin olive oil cultivars. Principal Component Analysis (PCA) is an unsupervised linear transformation technique that is widely used across different fields, most prominently for feature extraction and dimensionality reduction (D'Agostino et al., 2014).

PCA analysis shows the results obtained by chemical-physical analysis Fig. 4 reports the three-dimensional score plot in which separation among clusters is not complete. As one can see, the variances explained by the first two principal components are 43.41 % and 39.12 % for the first and the second, respectively.

The first component (PC1) was highly positively correlated with 24-methylene-cholesterol, Δ^7-campesterol, sitostanol, uvaol, docosanol, tetracosanol, SECFA/ SOCFA, total fatty alcohols, C42, linoleic acid, PUFA, free acidity, peroxide value and FRAP. PC2 was positively correlated with cholesterol, stigmastero, clerosterol, Δ^5-Avenasterol, apparent ß-sitosterol, pentacosanol, arachidic acid, TPC1, TFC2, TCC3, TTC4, α-Tocopherol and Chlorophyll.

The scores plot was used to gain an overview of the similarities or differences among the EVOO analysed. Fig. 4 reports a projection of the different olive oil samples in the space defined by the first two principal components. We can distinguish the samples of Leccino, Coratina, Ottobratica and Nocellara del Belice cultivars that are well discriminated. In fact, the analysis demonstrated that among the oils analysed, Coratina cultivar was located in the top right quadrant and shows a positive correlation with PC1 variables. In particular, it has high correlations with free acidity, peroxide value, TCC, TTC, α-tocopherol, chlorophyll, linoleic acid, α-linolenic acid, erythrodiol, uvaol, docosanol and total fatty alcohols.

Ottobratica cultivar, was located in the lower right quadrant and shows a positive correlation with PC1 variables. Leccino cultivar, unlike the Ottobratica and Coratina cultivars, was located in the top left quadrant these and was positive correlations in particular, with the variables of PC2, such as TPC, TFC, TCC, cholesterol, stigmasterol, clerosterol, Δ^5-avenasterol, apparent ß-sitosterol, pentacosanol, α-tocopherol and chlorophyll. Finally, Nocellara del Belice cultivar was located in the lower left quadrant and shows negative correlation with variables PC1 and PC2.

The PC1 allow us to identify two major groups. In fact, the group 1 includes Coratina and Ottobratica that is distinguished to the others by having the highest levels of free acidity, peroxide value, TTC, linoleic acid, ∑PUFA and tetracosanol.

Conclusions

Author Contributions: Conceptualization, M.R.L. and A.M.G; methodology, V.S., M.T.P. and R.T.; software, A.M.G. and V.S.; validation, M.L. and R.T.; formal analysis, M.L., T.F. and A.R.; data curation, A.M.G. and V.S; writing-review and editing, M.R.L.; project administration, M.R.L.; funding acquisition, M.R.L. and A.M.G.

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Abbreviations

ABTS: 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

ALA: α-linolenic acid

βC: β-carotene

BHT: Butylated hydroxytoluene

C*: Chroma

CA: chlorogenic acid

DDPH: 2,2-Diphenyl-1-picrylhydrazyl

DW: dry weight

EVOO: extra virgin olive oil

FRAP: Ferric Reducing Activity Power

GC: gas chromatography

H*: Hue

LA: linoleic acid

MS: mass spectrometry

MUFA: monounsaturated fatty acid

OA: oleic acid

PA: palmitic acid

PCA: Principal Component Analysis

PUFA: polyunsaturated fatty acid

RACI: Relative Antioxidant Capacity Index

QE: quercetin equivalents

SA: stearic acid

SFA: saturated fatty acid

TCC: total carotenoid content

TFC: total flavonoid content

TPC: total phenolic content

TTC: Total tocopherol content

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Table 1. Chemical qualitative and CIELAB parameters of Coratina, Leccino, Ottobratica and Nocellara del Belice monovarietal EVOOs produced in Calabria (Italy) during season 2019-2020.

Quality parameter	EVOO				Sign.
	Coratina	**Leccino**	**Ottobratica**	**Nocellara del Belice**	
Free acidity (%)	0.8 ± 0.0[a]	0.5 ± 0.0b	0.4 ± 0.0[c]	0.2 ± 0.0[d]	***
Peroxide (meqO_2/kg)	11.4 ± 1.2[b]	12.7 ± 1.7[a]	8.4 ± 1.2[c]	8.6 ± 1.1[c]	***
K232	2.0 ± 0.9[b]	2.3 ± 0.9[a]	2.0 ± 0.9[b]	1.8 ± 0.6[c]	***
K270	0.1 ± 0.02[a]	0.1 ± 0.02[a]	0.1 ± 0.02[a]	0.1 ± 0.02[a]	n.s.
ΔK	0.001 ± 0.00	0.001 ± 0.00	0.002 ± 0.00	0.001 ± 0.00	n.s.
TPC[1]	392.9 ± 7.9[b]	220.9 ± 3.1[c]	452.4 ± 4.6[a]	135.8 ± 2.5[d]	***
TFC[2]	20.7± 1.3[b]	18.3± 1.1[c]	24.5± 1.7[a]	13.8± 1.0[d]	***
TCC[3]	16.4± 1.2[b]	8.0 ± 0.6[c]	23.5 ± 1.6[a]	3.1 ± 0.3[d]	***
TTC[4]	272.1 ± 5.3[a]	221.7 ± 2.3[b]	205.7 ± 3.5[d]	212.8 ± 2.0[c]	***
α-Tocopherol[5]	162.9 ± 3.7[b]	113.4 ± 3.2[c]	175.1 ± 2.8[a]	100.1 ± 2.7[d]	***
Chlorophyll[5]	31.2 ± 1.0[b]	10.6 ± 0.8[c]	50.6 ± 1.7[a]	2.16 ± 0.3[d]	***
L*	31.9 ± 1.0[c]	36.3 ± 1.7[a]	30.5 ± 1.2[d]	34.0 ± 1.5[b]	***
a*	-1.1 ± 0.0[a]	-1.8 ± 0.1[b]	-1.9 ± 0.1[c]	-3.1 ± 0.4[d]	***
b*	24 ± 0.8[c]	23.4 ± 0.7[c]	34.1 ± 1.5[a]	28.9 ± 1.0[b]	***
C*	24 ± 0.7[c]	26.5 ± 0.8[b]	34.2 ± 1.6[a]	23.4 ± 0.8[c]	***
H*	87.3 ± 2.4[a]	85.5 ± 2.2[b]	86.8 ± 2.3[a]	84.0 ± 2.2[c]	***

[1] TPC: Total Phenolic Content, expressed as ppm; [2] TFC: Total Flavonoid Content, expressed as ppm; [3] TCC: Total Carotenoid Content, expressed as ppm. [4] TTC: Total Tocopherol Content expressed as ppm. [5]:as ppm. L*: lightness; a*: red/green coordinate; b*:yellow/blue coordinate; C*: chroma; H*: hue. Data are reported as mean ± Standard Deviation (SD) (n = 3). *** Significance at $P < 0.001$; ** Significance at $P < 0.05$; n.s. not significant. Results followed by different letters in a same row are significantly different by Tukey's multiple range test.

Table 2. Coratina, Leccino, Ottobratica and Nocellara del Belice monovarietal EVOOs produced in Calabria (Italy) during season 2019-2020 fatty acid composition.

Fatty acid	EVOO				Sign.
	Coratina	**Leccino**	**Ottobratica**	**Nocellara del Belice**	
Myristic acid (C14:0)	0.01 ± 0.00	n.d.	0.01 ± 0.00	0.01 ± 0.00	n.s.
Palmitic acid (PA, C16:0)	12.6± 1.3[c]	14.2± 1.2[b]	15.1 ± 2.2[a]	11.1± 1.0d	***
Palmitoleic acid (C16:1)	0.9 ± 0.03[b]	1.3 ± 0.04[a]	1.4 ± 0.1[a]	0.6 ± 0.01[c]	***
Margaric acid (C17:0)	0.02 ± 0.00[b]	0.02 ± 0.00[b]	0.02 ± 0.00[b]	0.08 ± 0.00[a]	***
Heptadecenoic acid (C17:1)	0.03 ± 0.00[c]	0.09 ± 0.00b	0.04 ± 0.00c	0.17 ± 0.01a	***
Stearic acid (SA, C18:0)	1.7 ± 0.2[b]	2.5 ± 0.8[a]	2.2 ± 0.4[a]	2.2 ± 0.4[a]	***
Oleic acid (OA, C18:1)	71.7 ± 4.5[b]	70.5 ± 3.9[c]	69.6 ± 6.2[c]	76.3 ± 4.5[a]	***
Linoleic acid (LA, C18:2)	10.4 ± 0.9[ab]	10.4 ± 0.6[a]	9.9 ± 0.7[b]	8.1 ± 0.7[c]	***
α-Linolenic acid (ALA, C18:3)	0.80 ± 0.02[b]	0.67 ± 0.04[c]	0.90 ± 0.02[a]	0.58 ± 0.03[d]	***
Arachidic acid (C20:0)	0.40 ± 0.03[b]	0.40 ± 0.02[b]	0.50 ± 0.03[a]	0.44 ± 0.03[ab]	**
Gadoleic acid (C20:1)	0.20 ± 0.01[b]	0.21 ± 0.02[b]	0.30 ± 0.01[a]	0.32 ± 0.03[a]	***
Behenic acid (C22:0)	0.10 ± 0.01[a]	0.10 ± 0.01[b]	0.10 ± 0.01[b]	0.10 ± 0.01[b]	***
OA/LA	6.89[c]	6.78[d]	7.03[b]	9.42[a]	***
∑SFA	14.83[c]	17.22[b]	17.93[a]	13.83[d]	***
∑MUFA	72.83[b]	72.10[c]	71.34[d]	77.39[a]	***
∑PUFA	11.20[a]	11.07[a]	10.80[b]	8.68[c]	***
MUFA/PUFA	6.50[b]	6.51[b]	6.60[b]	8.92[a]	***

Data are reported as the mean ± Standard Deviation (SD) (n = 3). ND: not detected. ***Significance at $P < 0.001$; ** Significance at $P < 0.05$; n.s. not significant. Results followed by different letters in a same row are significantly different by Tukey's multiple range test.

Table 3. Sterol composition of Coratina, Leccino, Ottobratica and Nocellara del Belice monovarietal EVOOs produced in Calabria (Italy) during season 2019-2020.

Sterol	EVOO				Sign.
	Coratina	**Leccino**	**Ottobratica**	**Nocellara del Belice**	
Cholesterol (%)	0.17 ± 0.04^{b}	0.22 ± 0.01^{a}	0.15 ± 0.02^{b}	0.15 ± 0.01^{b}	***
24-Methylene-Cholesterol (%)	0.08 ± 0.01^{b}	0.11 ± 0.01^{a}	0.10 ± 0.01^{ab}	0.06 ± 0.01^{c}	***
Campesterol (%)	2.81 ± 0.04^{c}	2.54 ± 0.03^{d}	3.17 ± 0.03^{b}	3.31 ± 0.04^{a}	***
Campestanol (%)	0.12 ± 0.01^{c}	0.18 ± 0.01^{b}	0.20 ± 0.02^{b}	0.24 ± 0.02^{a}	***
Stigmasterol (%)	1.34 ± 0.04^{b}	1.50 ± 0.02^{a}	1.24 ± 0.02^{c}	0.97 ± 0.03^{c}	***
Δ^{7}-Campesterol (%)	0.08 ± 0.01^{a}	0.08 ± 0.01^{a}	0.09 ± 0.01^{a}	0.02 ± 0.01^{b}	***
Clerosterol (%)	1.13 ± 0.04^{b}	1.33 ± 0.02^{a}	0.92 ± 0.02 c	0.89 ± 0.01^{c}	***
β-Sitosterol (%)	83.87 ± 0.12 ^{c}	81.01 ± 0.8^{d}	86.85 ± 0.12^{a}	86.03 ± 0.08^{b}	***
Sitostanol (%)	1.19 ± 0.04^{a}	0.94 ± 0.02^{c}	1.08 ± 0.04^{b}	0.55 ± 0.02^{d}	***
Δ^{5}-Avenasterol (%)	8.17 ± 0.01^{b}	11.05 ± 0.03^{a}	5.41 ± 0.07^{d}	6.64 ± 0.04^{c}	***
$\Delta^{5,24}$-Stigmastadienol (%)	0.40 ± 0.03^{b}	0.39 ± 0.01^{b}	0.29 ± 0.02^{c}	0.43 ± 0.01^{a}	***
Δ^{7}-Stigmastenol (%)	0.31 ± 0.01^{a}	0.26 ± 0.01^{b}	0.21 ± 0.02^{c}	0.30 ± 0.02^{a}	***
Δ^{7}-Avenasterol (%)	0.37 ± 0.02^{b}	0.43 ± 0.01^{a}	0.33 ± 0.01^{c}	0.45 ± 0.01^{a}	***
Apparent β-Sitosterol (%)	94.74 ± 0.01^{a}	94.71 ± 1.5^{a}	94.53 ± 0.01^{b}	94.53 ± 0.08^{b}	***
Campesterol/Stigmasterol	2.10 ± 0.08^{c}	1.70 ± 0.04^{d}	2.57 ± 0.07^{b}	3.41 ± 0.14^{a}	***
β-Sitosterol/Δ^{5}-Avenasterol	10.27 ± 0.15^{c}	7.33 ± 0.01^{d}	16.05 ± 0.23^{a}	12.96 ± 0.10^{b}	***
Erythrodiol (%)	1.39 ± 0.06^{a}	0.80 ± 0.03^{b}	0.82 ± 0.03^{b}	0.66 ± 0.01^{c}	***
Uvaol (%)	0.25 ± 0.04^{a}	0.12 ± 0.01^{b}	0.25 ± 0.03^{a}	0.08 ± 0.01^{c}	***
Total sterols (mg/kg)	1530.5 ± 7.78^{c}	1610.5 ± 8.49^{b}	1396.5 ± 6.36^{d}	1717.0 ± 4.24^{a}	***

Data are reported as the mean ± Standard Deviation (SD) (n = 3). All the sterols as well as erythrodiol and uvaol are expressed as percentage of the total sterol content. *** Significance at $P < 0.001$. Results followed by different letters in a same row are significantly different by Tukey's multiple range test.

Table 4. Fatty alcohol composition of Coratina, Leccino, Ottobratica and Nocellara del Belice monovarietal EVOOs in Calabria (Italy) during season 2019-2020.

Fatty alcohol	EVOO				Sign.
	Coratina	**Leccino**	**Ottobratica**	**Nocellara del Belice**	
Docosanol	16.88 ± 0.19^{a}	11.60 ± 0.03^{c}	15.87 ± 0.10^{b}	12.26 ± 0.01^{d}	***
Tricosanol	1.54 ± 0.05^{c}	1.44 ± 0.01^{d}	1.88 ± 0.02^{b}	2.31 ± 0.03^{a}	***
Tetracosanol	25.08 ± 0.09^{b}	23.03 ± 0.01^{c}	26.53 ± 0.06^{a}	17.31 ± 0.03^{d}	***
Pentacosanol	3.90 ± 0.11^{c}	5.41 ± 0.02^{a}	2.65 ± 0.08^{d}	4.30 ± 0.06^{b}	***
Hexacosanol	30.57 ± 0.20^{c}	33.44 ± 0.04^{b}	27.64 ± 0.41^{d}	34.38 ± 0.04^{a}	***
Heptacosanol	2.79 ± 0.07^{a}	1.64 ± 0.03^{c}	2.30 ± 0.02^{b}	2.60 ± 0.04^{b}	***
Octacosanol	19.23 ± 0.25^{c}	23.45 ± 0.04^{b}	23.12 ± 0.29^{b}	26.84 ± 0.04^{a}	***
Σ Even Carbon Chain Fatty Alc.	91.76 ± 0.22^{b}	91.52 ± 0.06^{c}	93.17 ± 0.04^{a}	90.79 ± 0.01^{d}	***
Σ Odd Carbon Chain Fatty Alc.	8.24 ± 0.22^{c}	8.49 ± 0.06^{b}	6.83 ± 0.04^{d}	9.21 ± 0.01^{a}	***
Σ ECFA/Σ OCFA	11.14 ± 0.32^{b}	10.79 ± 0.09^{c}	13.63 ± 0.08^{a}	9.86 ± 0.02^{d}	***
Total Fatty Alcohols (mg/kg)	90.68 ± 0.58^{c}	96.38 ± 0.24^{b}	134.53 ± 1.01^{a}	45.50 ± 0.08^{d}	***

Data are reported as the mean ± Standard Deviation (SD) (n = 3). fatty alcohols are expressed as percentage of the total alcohol content. *** Significance at $P < 0.001$. Results followed by different letters in a same row are significantly different by Tukey's multiple range test.

Table 5. Waxes composition of Coratina, Leccino, Ottobratica and Nocellara del Belice monovarietal EVOOs produced in Calabria (Italy) during season 2019-2020.

Waxes (%)	EVOO				Sign.
	Coratina	**Leccino**	**Ottobratica**	**Nocellara del Belice**	
C40 (%)	13.62 ± 0.23^{d}	31.95 ± 0.04^{c}	32.79 ± 0.18^{a}	32.48 ± 0.08^{b}	***
C42 (%)	32.37 ± 0.19^{b}	31.86 ± 0.03^{c}	36.92 ± 0.20^{a}	13.14 ± 0.02^{d}	***
C44 (%)	30.67 ± 0.26^{a}	17.39 ± 0.05^{c}	6.37 ± 0.56^{d}	25.17 ± 0.02^{b}	***
C46 (%)	23.34 ± 0.17^{b}	18.81 ± 0.04^{c}	16.12 ± 0.20^{d}	29.23 ± 0.04^{a}	***
mg/kg (Total) 40+42+44+46	47.51 ± 0.25^{d}	65.94 ± 2.61^{c}	111.29 ± 2.61^{a}	88.78 ± 0.76^{b}	***
mg/kg (EVOO) 42+44+46	41.04 ± 0.11^{d}	44.87 ± 0.02^{c}	74.80 ± 1.56^{a}	59.95 ± 0.44^{b}	***

Data are reported as the mean ± Standard Deviation (SD) (n = 3). *** Significance at $P < 0.001$. Results followed by different letters in a same row are significantly different by Tukey's multiple range test.

Table 6. Antioxidant activities of Coratina, Leccino, Ottobratica and Nocellara del Belice monovarietal EVOOs produced in Calabria (Italy) during season 2019-2020.

Sample	DPPH[1]	ABTS[1]	β-carotene bleaching[1]	FRAP (μM Fe(II)/g)
~~*EVOO phenolic extracts*~~				
Coratina	35.8 ± 2.7****	21.3 ± 2.0****	256.8 ± 9.5****	82.3 ± 2.7^{ns}
Leccino	85.9 ± 2.2****	57.8 ± 2.6****	273.2 ± 7.4****	65.8 ± 2.2*
Ottobratica	29.2 ± 2.6****	26.3 ± 1.8****	176.3 ± 4.6****	90.6 ± 2.8^{ns}
Nocellara del Belice	103.1 ± 5.3****	82.1 ± 4.3****	322.5 ± 9.8****	67.8 ± 2.0*
Positive controls				
Ascorbic acid	5.0 ± 0.8	1.71 ± 0.03		
Propyl gallate			1.00 ± 0.01	
BHT				63 ± 4

[1] IC_{50} value (μg/mL). Data are expressed as means ± S.D. (n= 3). DPPH Radical Scavenging Activity Assay; Antioxidant Capacity Determined by Radical Cation ($ABTS^{+}$); β-carotene bleaching test; Ferric Reducing Antioxidant Power (FRAP). Ascorbic acid, BHT and Propyl gallate were used as positive control in antioxidant tests. Differences within and between groups were evaluated by one-way ANOVA followed by a multicomparison Dunnett's test (α= 0.05): ****$P < 0.0001$, *$P < 0.1$, compared with the positive controls. n.s. not significant

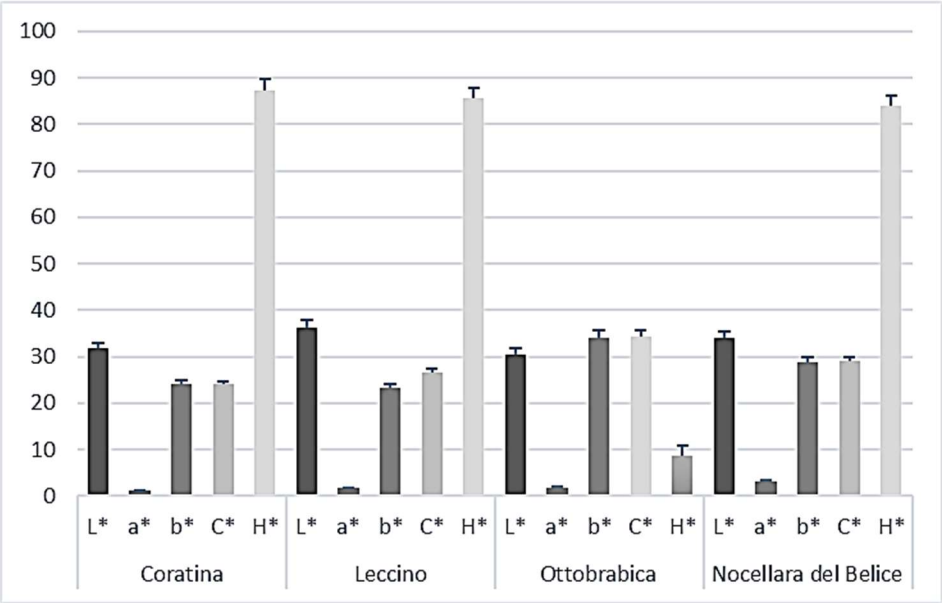

Fig. 1. CIELAB colorimetric parameters of Calabrian monovarietal EVOOs.

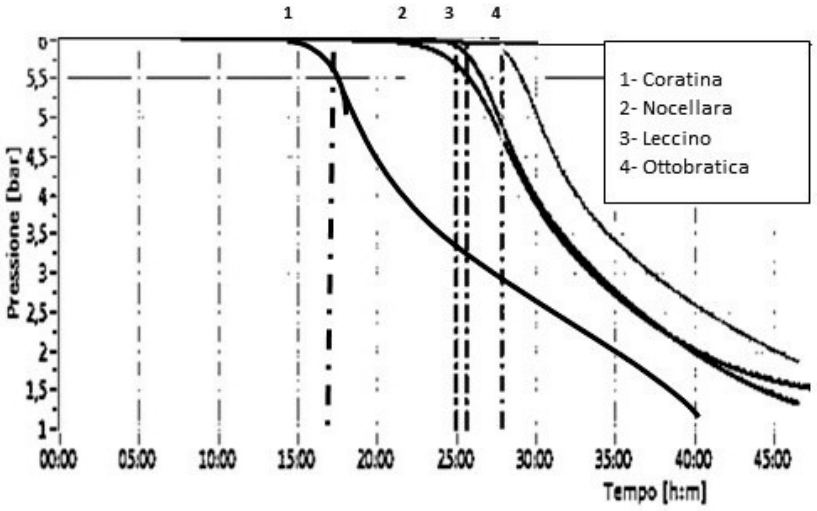

Fig. 2. Oxidative stability of Coratina, Leccino, Ottobratica and Nocellara del Belice monovarietal EVOOs produced in Calabria (Italy) during season 2019-2020.

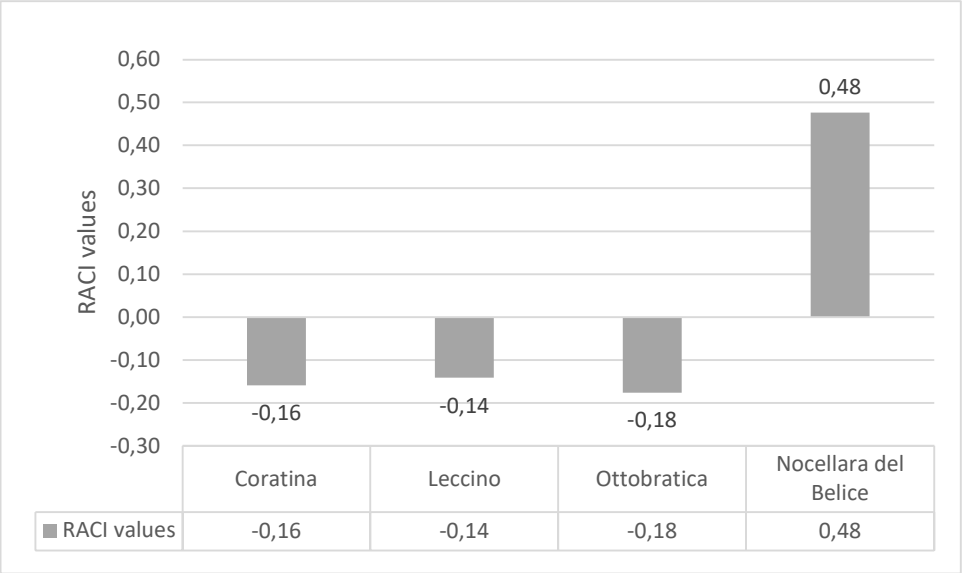

Fig. 3. RACI values of Calabrian monovarietal EVOOs

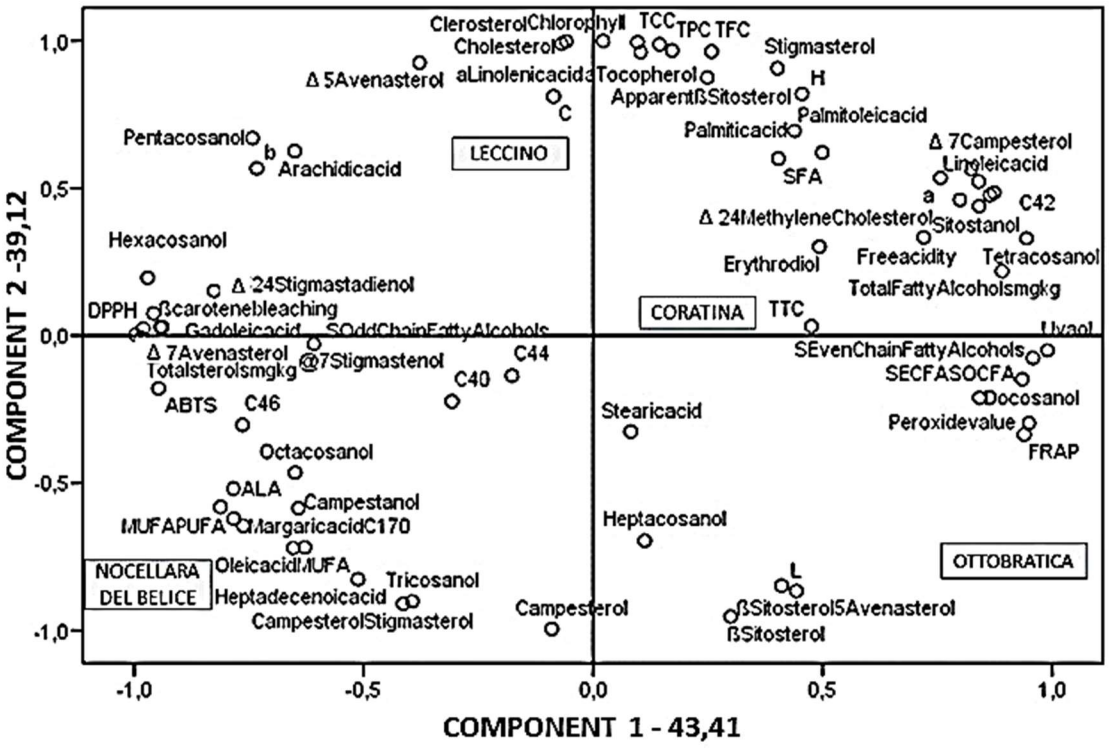

Fig. 4. Two-dimensional principal component analysis (PCA) plot of the studied virgin olive oils from different cultivars based on fatty acid, fatty alcohol, sterolic, waxes profile, total phenolic, flavonoid, carotenoid and tocopherols contents, antioxidant activity and physical-chemical parameters