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Characterization of Phenolic Profiles of Italian Single Cultivar Olive Leaves (*Olea europaea* L.) by Mass Spectrometry

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Abstract

The crude methanol extracts of ten of the most common variety of Calabrian single cultivar olive leaves (*Olea europaea* L.), Carolea, Cassanese, Ciciariello, Dolce di Rossano, Grossa di Gerace, Ottobratica, Pennulara, Tondina, Sinopolese and Tonda di Strongoli were analyzed by HPLC-MS/MS in order to study their phenolic profiles. The data obtained showed a different distribution of these compounds and, among the cultivars, these differences were mainly quantitative. Samples collected during the hottest months have been shown to have the richest phenolic profiles.

Keywords: Olive leaves; Phenols extraction; HPLC; Mass spectrometry; Statistical analysis

Introduction

The ability of food to maintain health and prevent disease is a proven scientific fact. Research shows health and nutritional claims have become a significant contributor to the customer's brand choice [1-3] and the new research terms and disciplines emerged, such as "nutrapharmacology", "medical food", "nutrigenomics", "nutriproteomics", "nutraceuticals", "medical nutrition", "functional food" are a strong indication of it, as well as the increasing variety of foods enriched with beneficial molecules available on the market. In many experimental studies, phenols, very highly prized compounds, have demonstrated a wide spectrum of pharmacological activities beyond their antioxidant properties [4]. A potential source of bioactive compounds are leaves: phenols in leaves are numerous and of diverse nature. They are grouped with regard to major molecular characteristics as simple phenols and acids, lignans, and flavonoids [5]; including flavones (luteolin-7-glucoside, apigenin-7-glucoside, diosmetin-7-glucoside, luteolin, and diosmetin), flavonols (rutin), flavan-3-ols (catechin), substituted phenols (tyrosol, hydroxytyrosol, vanillin, vanillic acid, and caffeic acid), [6] oleuropein and secoiridoids. These last are exclusive to the Oleaceae family; in fact, oleuropein related secoiridoids and other derivatives are the principal compounds of olive leaves [7] among which the major compound frequently reported is oleuropein. Flavonoids may occur in appreciable amounts [8] while simple phenols and acids are present in lower amounts. Olive cultivation is very important in Calabria, a southern region of Italy, and the extensive olive groves show it (200,000 ha). The oil production (192,625 tons, 10.3 q per hectare) represents the 33% of the Italian production. Moreover, the isolation and structural characterization of secondary metabolites from plant matrices is a very interesting topic, as the recovery of bio active molecules from plant extracts. In this contest, foliar waste, from pruning, or just because this plant component is available all year long, represents a good resource. The interest in the use of olive leaves as a potential source of phenol compounds for the production of functional food, nutraceuticals and for their use in the pharmaceutical industry is highlighted by the fact that olive leaf extracts have been marketed as dietary product [9] and commercial products in the form of herbal teas or food supplements are available all over the world, as complete dried leaves, powder, extracts or tablets [7]. However, several factors may influence the qualitative and quantitative phenols composition of leaves [10]. It is known that genetics, environment and agricultural practices [11-15] greatly affect the phytochemical profiles of plants; informations of the impact of these factors on the bioactivities of olive leaves it would help in the selection of cultivar(s) to obtain the greatest levels of samples with antioxidant, anti-inflammatory and anti-diabetic activities. Therefore, in this article, in order to determine the influence of variety and harvest month on the phenolic compounds, ten of the most common Calabrian single cultivar olive leaves were collected in four consecutive months.

Materials and Methods

Olive leaves sampling

Four different periods were chosen for the collection of leaf samples, March, July, August and November. Ten single cultivar olive leaves (Carolea, Cassanese, Ciciariello, Dolce di Rossano, Grossa di Gerace, Ottobratica, Pennulara, Roggianella, Sinopolese and Tonda di Strongoli) were harvested in the crop year 2016 in their areals of origin located in the Calabria region (Italy). The leaf collection was carried out at man height across the entire circumference of the plant. The samples were freeze dried, grounded and stored in light protected plastic flasks until analysis.

Extraction of phenolic compounds

In order to perform the extraction of the phenolic compounds, 1 g of homogenized olive leaves dried powder was weighed in a 50 mL volume test tube and 20 mL of methanol added. The mixture was homogenized by means of ultra-turrax system at 8000 rpm for 1 min. To maximize the extraction process, the solution was kept under shaking in an ultrasonic bath in the darkness for 20 min. After this

period, a centrifugation at 5000 rpm for 25 min at 8°C allowed the recovery of the supernatant. Subsequently, the solvent was removed under vacuum by means of a rotary evaporator set at 40°C and 60 rpm. Solvent free extracts were recovered with 2 mL of a solution of water/methanol (v/v 80:20), filtered through a 0.45-µm PVDF filter and analysed by HPLC-MS/MS.

HPLC–MS/MS analysis

The chromatographic separation, achieved by using an Eclipse XDB-C8-A HPLC column [(5 µm particle size, 150 mm length and 4.6 mm i.d.), was performed by means of an HPLC 1200 series instrument (Agilent Technologies, Santa Clara, California). A MSD Sciex Applied Biosystem API 4000 Q-Trap mass spectrometer was used to analyze the samples in negative ion mode using multiple reactions monitoring (MRM). The LC–MS experimental conditions were optimized for each transition monitored [16].

Quantitative analysis

The standards used for the analyses were purchased from Sigma–Aldrich (Riedel-de Haën, Laborchemikalien, Seelze) and Extrasynthese (Nord B.P 62 69726 Genay Cedex, France). Methanol and formic acid were LC/MS grade; aqueous solutions were prepared using ultrapure water (Millipore, Bedford, MA, USA).

Quantitative analysis was performed by external calibration curves built using a least-squares linear regression analysis. For this purpose, standard stock solutions of homovanillic acid (Hom), caffeic acid (Caf), vanillin (Van), vanillic acid (Vco), o-cumaric acid (Cum), ferulic acid (Fer), apigenin (Ap), apigenin-7-O-glucoside (Ap7), diosmetin (Dio), hydroxytyrosol (HyTyr), tyrosol (Tyr), oleuropein (Olp), luteolin (Lut), verbascoside (Ver), luteolin-7-O-glucoside (Lu7), luteolin-4-O-glucoside (Lu4) and rutin (Rut) were dissolved in methanol and further diluted with water/0.1% formic acid to obtain six calibration standards at concentrations in the range between 100 and 2000 µg ml^{-1}. The correlation coefficients of the calibration curve ranged between 0.9994 and 0.9997. Each compound was monitored by MRM mode which scans, on the third quadrupole, the main fragments of the deprotonated molecular ion $[M-H]^{-1}$ produced in the first quadrupole.

The analysis parameters, such as the equation for external calibration curve, the correlation coefficient R^2, the molecolar ion $[M-H]^{-1}$ monitored on the first quadrupole and the major fragments monitored on the third quadrupole, for each phenolic compound analysed by LC-MS/MS, are summarized in Table 1.

Phenolic compound	Equation for external calibration curve	Correlation coefficient	Molecolar ion [M-H]-1 on the first quadrupole	Fragments on the third quadrupole
		R^2	m/z	m/z
Homovanillic acid	Y=575.8X+240	0.999	181	-
Caffeic acid	Y=2880.4X+32229	0.999	179	135
Vanillin	Y=1250.2X+22400	0.999	151	135
Vanillic acid	Y=740.95X+5685.7	0.998	167	151
O-coumaric acid	Y=726.89X+523.2	0.999	163	119
Ferulic acid	Y=2580.3X+108229	0.999	193	-
Apigenin	Y=1834.8X+76029	0.999	269	117 - 151
Apigenin-7-O-Glucoside	Y=4536.8X+82000	0.999	431	267
Diosmetin	Y=570.91X+13286	0.999	299	-
Hydroxytyros	Y=8934.3X+438286	0.999	153	123
Tyrosol	Y=55.832X+2640	0.999	137	-
Oleuropein	Y=599.57X+10029	0.999	539	275 - 307
Luteolin	Y=416.87X+10571	0.999	285	133 - 151
Verbascoside	Y=758.32X+18200	0.999	623	161 - 461
Luteolin-7-O-Glucoside	Y=3014.3X+61600	0.999	447	285
Luteolin-4-O-Glucoside	Y=5681.8X+3142.9	0.999	447	285
Rutin	Y=617.69X+17743	0.999	609	301

Table 1: HPLC-MS/MS analysis parameters: equation for external calibration curve, correlation coefficient R^2, molecolar ion $[M-H]^{-1}$ monitored on the first quadrupole and major fragments monitored on the third quadrupole for each phenolic compound analysed by LC-MS/MS.

Statistical analyses

The statistical treatment was performed by using the statistics program STATGRAPHICS Plus Version 5.1 (Statistical Graphics Corporation, Professional Edition - Copyrigth 1994-2001).

In particular, to determine whether the concentration of phenolic compounds of the samples were specific to cultivar and collection period one-way analysis of-variance (ANOVA) and post-hoc Fisher comparison test were performed. To explore relationships between dependent variables and to graphically display the relative positions of data points in fewer dimensions while retaining as much information as possible, Principal Component Analysis (PCA) and Linear Discriminant Analysis (LDA) were carried out.

At first, PCA and LDA were performed to all the data produced during the entire period of sampling, from March to November; subsequently, as in this study the differences observed between the cultivars were mainly quantitative with a higher concentration of phenolic compounds in the samples collected in the warmer month, PCA was performed only for the data obtained from olive leaves collected in August.

	Carolea	Cassanese	Ciciariello	Dolce di Rossano	Grossa di Gerace	Ottobratica	Pennulara	Tondina	Sinopolese	Tonda di Strongoli
Homovanillic acid	56.126 ± 7.361 ^{c}	55.127 ± 3.232 ^{c}	60.257 ± 1.111 ^{b}	30.157 ± 2.365 ^{fg}	47.181 ± 3.256 ^{de}	33.261 ± 1.259 ^{f}	69.217 ± 3.215 ^{a}	26.558 ± 2.555 ^{cde}	49.377 ± 6.969 ^{cd}	52.892 ± 5.588 ^{bc}
Caffeic acid	1.218 ± 0.161 ^{bc}	1.851 ± 0.169 ^{ab}	1.976 ± 0.164 ^{a}	1.962 ± 0.111 ^{a}	1.207 ± 0.589 ^{bc}	1.074 ± 0.201 ^{bc}	1.856 ± 0.589 ^{ab}	1.074 ± 0.07 ^{bc}	0.960 ± 0.151 ^{bc}	1.417 ± 0.251 ^{bc}
Vanillin	2.59 ± 0.182 ^{d}	1.012 ± 0.161 ^{g}	1.874 ± 0.161 ^{ef}	0.522 ± 0.161 ^{g}	1.574 ± 0.106 ^{ef}	7.226 ± 1.202 ^{a}	4.586 ± 1.287 ^{b}	0.766 ± 0.052 ^{c}	3.426 ± 0.235 ^{g}	0.766 ± 0.111 ^{g}
Vanillic acid	25.368 ± 1.259 ^{e}	70.128 ± 4.473 ^{a}	46.585 ± 20.12 ^{c}	23.150 ± 1.255 ^{e}	10.248 ± 1.547 ^{g}	19.127 ± 1.254 ^{f}	64.550 ± 5.269 ^{b}	39.335 ± 3.369 ^{d}	41.115 ± 4.147 ^{c}	46.328 ± 2.369 ^{d}
o-cumaric acid	14.213 ± 0.215 ^{h}	19.086 ± 2.258 ^{f}	29.098 ± 0.152 ^{e}	43.142 ± 0.162 ^{c}	11.176 ± 1.291 ^{i}	54.193 ± 0.524 ^{b}	31.481 ± 3.256 ^{d}	16.137 ± 1.258 ^{b}	54.191 ± 5.501 ^{a}	71.675 ± 4.289 ^{g}
Ferulic acid	72.572 ± 8.263 ^{f}	63.257 ± 2.210 ^{h}	125.459 ± 1.259 ^{b}	104.028 ± 1.259 ^{d}	64.374 ± 3.258 ^{h}	89.115 ± 3.298 ^{e}	67.263 ± 7.214 ^{g}	165.237 ± 5.784 ^{i}	45.619 ± 4.014 ^{c}	119.455 ± 5.887 ^{a}
Apigenin	11.216 ± 1.255 ^{f}	15.418 ± 1.101 ^{i}	7.087 ± 0.161 ^{d}	5.419 ± 0.160 ^{h}	5.620 ± 1.27 ^{i}	42.085 ± 3.32 a	12.086 ± 0.987 ^{e}	22.276 ± 4.231 ^{c}	20.187 ± 2.225 ^{g}	8.927 ± 1.012 ^{b}
Apigenin-7-O-Glucoside	37.126 ± 2.121 ^{c}	33.148 ± 2.356 ^{d}	37.257 ± 1.258 ^{c}	19.249 ± 1.257 ^{f}	6.485 ± 1.232 ^{g}	50.157 ± 8.125 ^{a}	22.128 ± 2.014 ^{f}	45.217 ± 2.201 ^{e}	27.151 ± 1.259 ^{b}	45.225 ± 5.023 ^{b}
Diosmetin	57.127 ± 2.577 ^{d}	17.238 ± 1.246 ^{h}	39.217 ± 1.259 ^{e}	36.968 ± 1.222 ^{ef}	29.216 ± 2.111 ^{g}	28.113 ± 2.444 ^{g}	162.127 ± 11.025 ^{a}	101.234 ± 4.444 ^{c}	72.234 ± 1.987 ^{efg}	33.214 ± 2.501 ^{b}
Hydroxytyrosol	1091 ± 30 ^{d}	935 ± 42 ^{de}	602 ± 52 ^{g}	760 ± 39 ^{f}	1329 ± 21 ^{c}	1016 ± 25 ^{de}	1329 ± 24 ^{c}	2238 ± 54 ^{f}	698 ± 28 ^{b}	1804 ± 62 ^{a}
Tyrosol	2202 ± 87 ^{d}	1733 ± 52 ^{e}	2408 ± 87 ^{c}	1683 ± 102 ^{e}	2802 ± 53 ^{a}	1733 ± 78 ^{e}	2528 ± 84 ^{b}	2185 ± 81 ^{d}	2188 ± 92 ^{d}	2134 ± 102 ^{d}
Oleuropein and derivates	33247 ± 126 ^{d}	29245 ± 123 ^{g}	45754 ± 858 ^{a}	37500 ± 221 ^{b}	30497 ± 152 ^{e}	27069 ± 184 ^{h}	29497 ± 147 ^{f}	22866 ± 177 ^{i}	24693 ± 128 ^{c}	34751 ± 245 ^{l}
Luteolin	1527 ± 42 ^{b}	1003 ± 62 ^{d}	656 ± 957 ^{f}	625 ± 54 ^{f}	563 ± 82 ^{fgh}	2000 ± 107 ^{a}	956 ± 52 ^{d}	747 ± 29 ^{c}	1300 ± 45 ^{gh}	550 ± 37 ^{e}
Verbascoside	5776 ± 97 ^{c}	1646 ± 28 ^{i}	2888 ± 101 ^{f}	1903 ± 28 ^{h}	7547 ± 136 ^{a}	1996 ± 100 ^{g}	4934 ± 35 ^{d}	4000 ± 102 ^{l}	679 ± 53 ^{b}	6972 ± 54 ^{e}
Luteolin-4-O-Glucoside	86.423 ± 14.362 ^{e}	102.423 ± 5.236 ^{d}	129.423 ± 23.587 ^{b}	142.423 ± 1.247 ^{a}	86.223 ± 2.878 ^{e}	130.423 ± 24.123 ^{b}	75.923 ± 5.214 ^{f}	122.923 ± 5.697 ^{e}	95.923 ± 1.369 ^{b}	130.423 ± 10.257 ^{c}
Luteolin-7-O-Glucoside	87,675 ± 12.012 ^{c}	88.175 ± 4.263 ^{c}	59.175 ± 2.258 ^{f}	78.425 ± 2.584 ^{d}	49.175 ± 5.214 ^{g}	102.675 ± 5.879 ^{b}	69.925 ± 6.333 ^{e}	71.925 ± 4.478 ^{f}	46.675 ± 4.217 ^{a}	113.925 ± 11.577 ^{e}
Rutin	4.323 ± 1.159 ^{e}	32.550 ± 2.367 ^{a}	17.328 ± 1.369 ^{bc}	19.331 ± 2.695 ^{bc}	11.532 ± 3.669 ^{d}	5.109 ± 1.295 ^{e}	8.105 ± 1.547 ^{de}	31.894 ± 2.546 ^{d}	15.701 ± 2.202 ^{bc}	21.518 ± 5.555 ^{a}

Table 2: Concentrations of phenols in olive leaves collected in August. The data expressed in mg/kg, represent the mean values of tree replications with their relative standard deviation (RSD). For each measurement, the data marked by different letters in a row indicate significant difference ($p<0.05$).

Results

Phenolic profile

The data showed that differences among cultivars were mainly quantitative and the concentration of the phenolic compounds in olive leaves was higher in the hottest month (Table 2).

Oleuropein and secoiridoids

As expected, oleuropein is one of the major components of olive leaves. Oleuropein constituents are best known for their blood pressure-lowering effects, but the latest studies reveal their health benefits extend well beyond that. Additional anti-inflammatory and antioxidant properties offer promise in fighting atherosclerosis, diabetes, cancer, neurodegenerative diseases, and even arthritis [17-22]. Among the ten analyzed varieties, the amount of oleuropein in olive leaves was higher in August and lower in November. More specifically, Ciciariello was the cultivar richest in oleuropein (45753 kg/mg) while Tondina (17118 mg/kg) the poorest one.

Substituted phenols

Hydroxytyrosol is found in olive leaf in the form of its elenolic acid ester oleuropein and, especially after degradation, in its plain form. The importance of hydroxytyrosol in protection of low-density lipoproteins and consequently its implication in the reduction of cardiovascular disease risk has been highlighted by the European Food Safety Authority, concluding that 5 mg of hydroxytyrosol and its derivatives should be consumed daily to reach this effect at physiological level [23]. Among the ten analyzed varieties, leaves collected in August showed a higher content in hydroxytyrosol while leaves collected in March gave the lowest amount. More specifically, Tondina (2238 mg/kg) was the cultivar richest in hydroxytyrosol while Ciciariello (602 mg/kg) the poorest one. Tyrosol, although there are some very minor differences, is considered almost identical to hydroxytyrosol. In fact, the two antioxidants are often considered interchangeable. It is classified as a phenolic antioxidant and anything in the phenolic family has great antiseptic value [24]. Among the ten analyzed varieties, leaves collected in August showed a higher content in tyrosol while leaves collected in March gave the lowest amount. More specifically, Grossa di Gerace was the cultivar richest in this substituted phenol (2802 mg/kg) while Dolce di Rossano (1683 mg/kg) the poorest one. Also, leaves collected in August showed a higher content in homovanillic acid (Pennulara 69 mg/kg), caffeic acid (Ciciariello 1.98 mg/kg), vanillin (Ottobratica 7.23 mg/kg), vanillic acid (Cassanese 70 mg/kg), ferulic acid (Tondina 165 mg/kg) and o-cumaric acid (Tonda di Strongoli 72 mg/kg) in respect of leaves collected in March.

Flavonoids

Many biological effects such as anti-oxidant, anti-inflammatory, anti-thrombotic, cytoprotective, vasoprotective and anti-microbial activity have been associated to this class of compounds. Rutin in one of the most important flavonoid in olive leaves [25-27]. Among the ten analyzed varieties, leaves collected in August showed a higher content in rutin while leaves collected in March gave the lowest amount of this flavonoid. More specifically, Cassanese was the cultivar richest in rutin (32.55 mg/kg) while Carolea (4.32 mg/kg) the poorest one. Luteolin, luteolin-7-O-glucoside and luteolin-4-O-glucoside in several studies have been detected in olive fruits of different cultivars and they are responsible, along with other carotenoid compounds, for the colour of the drupes. Luteolin has proved to possess important biological properties, such as anti-oxidant, anti-inflammatory, anti-microbial and cardio-tonic activity, ability to scavenge free radicals and to inhibit low-density lipoprotein oxidation [28-31]. Among the ten analyzed varieties, leaves collected in August showed a higher content in luteolin, luteolin-4-O-glucoside and luteolin-7-O-glucoside while leaves collected in March gave the lowest amount of these flavonoids. More specifically, Ottobratica was the cultivar richest in luteolin (2000 mg/kg) while Grossa di Gerace (563 mg/kg) the poorest one. Tonda di Strongoli (114 mg/kg) and Dolce di Rossano (142 mg/kg) were the cultivars richest in luteolin-4-O-glucoside and luteolin-7-Oglucoside, respectively, while Sinopolese (47 mg/kg) and Pennulara (76 mg/kg) the poorest ones. Among the ten analyzed varieties, leaves collected in August showed a higher content in verbascoside while leaves collected in November gave the lowest amount. More specifically, Grossa di Gerace (7547 mg/kg) was the cultivar richest in verbascoside while Sinopolese (679 mg/kg) the poorest one. Moreover, leaves collected in August showed a higher content in apigenin (Ottobratica 42 mg/kg), apigenin-7-O-glucoside (Ottobratica 50 mg/kg) and diosmetin (Pennulara 162 mg/kg).

Statistical analyses

Principal Component Analysis (PCA)

From the first PCA, six components have been extracted, having Eigenvalues greater or equal to 1.0, and together they account for 77.50% of the variability in the original data (Figure 1). The active compounds that mainly contributed to the highest absolute loading values on PC1, all of them being positive, were caffeic acid, ferulic acid, luteolin-7-O-glucoside, hydroxytyrosol, tyrosol, rutin and oleuropein. Luteolin-4-O-glucoside, apigenin, apigenin-7-O-glucoside, vanillin and o-cumaric acid contributed to the highest absolute loading values on PC2.

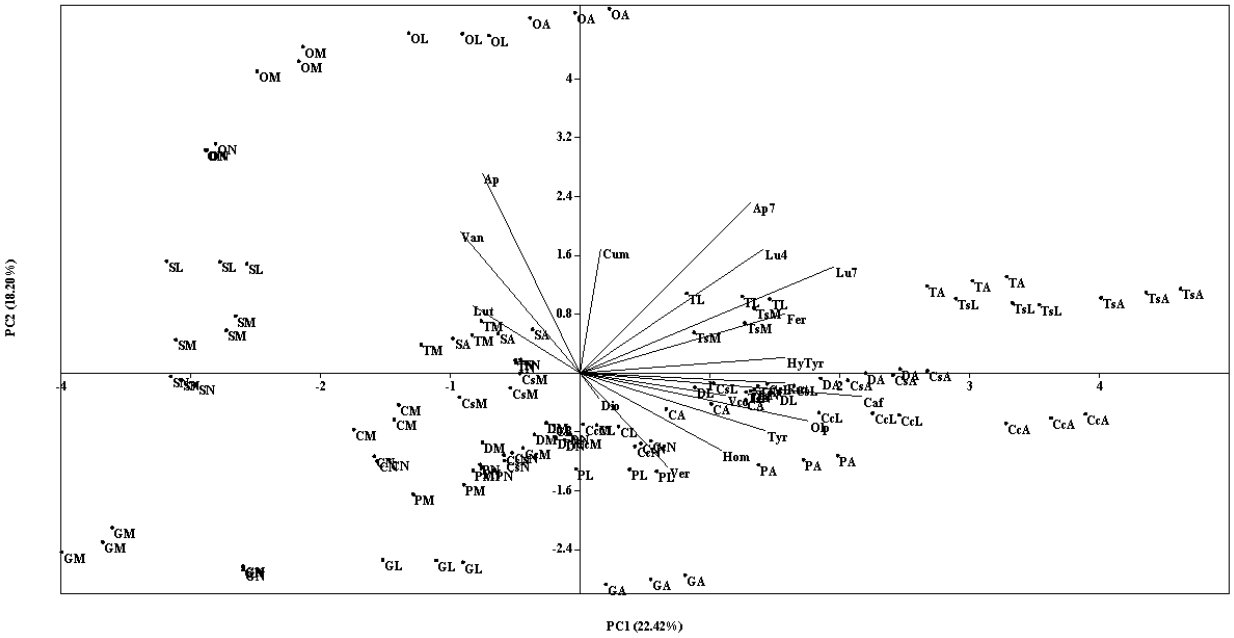

Figure 1: Score and loading bi-plot obtained with Principal Component Analysis of the phenolic compounds found in olive leaf samples collected in March (M), July (J), August (A) and November (N) (cultivar: C=Carolea, Cs=Cassanese, Cc=Ciciariello, D=Dolce di Rossano, G=Grossa di Gerace, O=Ottobratica, P=Pennulara, T=Tondina, S=Sinopolese and Ts=Tonda di Strongoli. Phenols: Hom=homovanillic acid, Caf=caffeic acid, Van=vanillin, Vco=vanillic acid, Cum=o-cumaric acid, Fer=ferulic acid, Ap=apigenin, Ap7= pigenin-7-O-glucoside, Dio=diosmetin, HyTyr=hydroxytyrosol, Tyr=tyrosol, Olp=oleuropein and derivates, Lut=luteolin, Ver=verbascoside, Lu7=luteolin-7-O-glucoside, Lu4=luteolin-4-O-glucoside and Rut=rutin).

From the bi-plot it is evident the concentration trend of the phenolic compounds in the olive leaves throughout the entire period of sampling. In fact, on the abscissa, going from negative to positive values, it can be noticed that the samples are distributed depending on the month of collection, from the coldest to the hottest month. In particular, on PC2 it can be highlighted the cultivar Ottobratica for having the highest absolute loading values on PC2 for apigenin, luteolin, vanillin, luteolin-7-O-glucoside and luteolin-4-O-glucoside followed by the cluster formed by Sinopolese Tondina and Tonda di Strongoli. On the contrary, Grossa di Gerace, Dolce di Rossano, Pennulara, Carolea, Cassanese and Ciciariello were negatively related to PC2 for having the highest absolute loading values for diosmetin, tyrosol, homovanillic acid, caffeic acid, verbascoside and oleuropein. From the second PCA, applied only to the concentration of phenolic compounds in the olive leaves collected in August, five components have been extracted, having Eigenvalues greater or equal to 1.0, and together they account for 84.59% of the variability in the original data (Figure 2). The active compounds that mainly contributed to the discrimination of the cultivars on PC1 are: apigenin, apigenin-7-O-glucoside, lutein, luteolin-4-O-glucoside, luteolin-7-O-glucoside and vanillin for positive values; tyrosol, oleuropein, caffeic acid and diosmetin for negative values. The phenolic compounds that mainly contributed to the discrimination of the cultivars on PC2 are: rutin, ferulic acid, luteolin-4-O-glucoside, luteolin-7-O-glucoside and oleuropein for positive values and lutein, vanillin, apigenin, tyrosol for negative values.

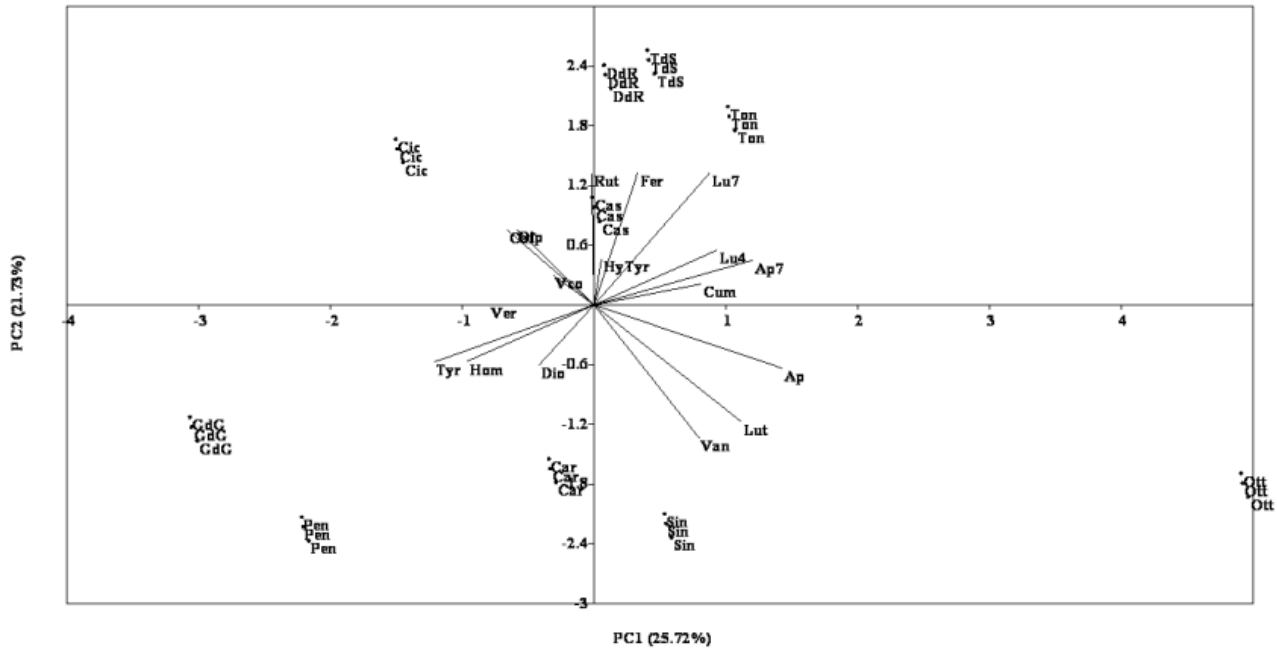

Figure 2: Score and loading bi-plot obtained with Principal Component Analysis of the phenolic compounds found in olive leaf samples collected in August (cultivar: Car=Carolea, Cas=Cassanese, Cic=Ciciariello, DdR=Dolce di Rossano, GdG=Grossa di Gerace, Ott=Ottobratica, Pen=Pennulara, Ton=Tondina, Sin=Sinopolese and TdS=Tonda di Strongoli. Phenols: Hom=homovanillic acid, Caf=caffeic acid, Van=vanillin, Vco=vanillic acid, Cum=o-cumaric acid, Fer=ferulic acid, Ap=apigenin, Ap7= pigenin-7-O-glucoside, Dio=diosmetin, HyTyr=hydroxytyrosol, Tyr=tyrosol, Olp=oleuropein and derivates, Lut=luteolin, Ver=verbascoside, Lu7=luteolin-7-O-glucoside, Lu4=luteolin-4-O-glucoside and Rut=rutin).

From the bi-plot, a differentiation could be observed among the cultivars. In particular, it can be highlighted the cultivar Ottobratica for having the highest absolute loading values on PC1 for apigenin, luteolin, vanillin, luteolin-7-O-glucoside and luteolin-4-O-glucoside followed by the cluster formed by Dolce di Rossano, Tonda di Strongoli, Tondina, Cassanese e Sinopolese. Conversely, Carolea, Ciciariello, Pennulara and Grossa di Gerace were negatively related to PC1 for having the highest absolute loading values for diosmetin, tyrosol, homovanillic acid and oleuropein. On PC2 three clusters can be highlighted: Ciciariello, Dolce di Rossano, Tondina and Tonda di Strongoli followed by Cassanese for positive values and Ottobratica, Sinopolese, Pennulara, Grossa di Gerace and Carolea for negative ones.

Linear Discriminant Analysis (LDA)

LDA analysis was considered in order to develop a model to discriminate among the 4 months (March, July, August and November). To build that model all the data were used and 7 predictor variables that mainly contributed to the highest absolute loading values on PCA (diosmetin, apigenin, luteolin-4-O-glucoside, vanillic acid, caffeic acid, hydroxytyrosol and oleuropein) entered. Three discriminating functions had P-values less than 0.05 and statistically significant at the 95% confidence level. The resulting LDA plot is showed in Figure 3. Amongst the 120 observations (10 single cultivar olive leaf in triplicate each month) used to fit the model, 100% were correctly classified. The scores of the first two functions produced from LDA showed a separation into 4 groups: each group contains the olive leaves collected during the four months of the trial, without considering the belonging cultivars. In particular, it can be noted that the olive leaves sampled in March and November occupy the negative part of the graph, whilst, the olive leaves sampled in August the positive one. Olive leaves sampled in July are in the middle. The graph, in a simple straightforward way, shows the variation of the concentration of the phenolic compounds in the olive leaves over the period of sample collection. The quantity of phenolic compounds increases as the temperature rises, in particular, it is higher in olive leaves collected in the warmer months and lower in olive leaves collected in the colder ones.

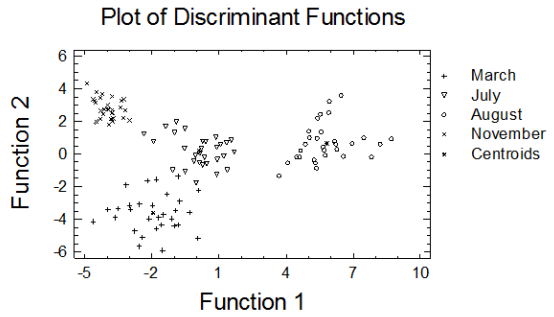

Figure 3: Plot of Discriminant Functions of the phenolic compounds found in olive leaf samples collected in March, July, August and November.

Discussion

The data obtained in this study have shown that the concentrations of phenolic compounds in olive leaves do not depend on cultivar, but on the particular sampling time. The environment and climate in which the plants were located seems to be the main factor effecting the phenols concentrations in leaves. More specifically, the results have pointed out an increase of phenols in spring/summer period and this could be correlated with the general increase of biological activities at the renewal of the leaves vegetative cycle but, also, to abiotic factors such as hydric deficiency and light exposition. All phenols investigated in olive leaves increased from March to August to decrease in November. This highlights the importance of phenols in the olive leaves antioxidant defence mechanism. Oleuropein and other secondary metabolites such as hydroxytyrosol and verbascoside possess an ideal chemistry for free radical scavenging actively acting as plant antioxidants. In a very sunny climate the plant is directly exposed to light and phenols, especially flavonoids, are affected by it. Protection against ultraviolet B (UV-B) radiation may be afforded by these compounds acting as a barrier against damaging UV radiation owing to their adsorption maxima in the UV region [32]. On the other hand, in winter, a significant decrease in temperature may possibly lead to inactivation of metabolic enzymes of the plant, thereby hampering the cascade of production of secondary metabolites in leaves. Rainfall, as well, imparts two-way effect on the secondary metabolite production. A high rainfall helps in increasing the water content of the soil, thereby enhancing the related edaphic factors, which further will aid in better growth of plant as well as elevated production of phyto-constituents. But, if the rainfall levels crosses threshold, then it poses a contradictory effect i.e., further diluting the already produced phyto-moeities. It is of great importance to take into account these factors when leaves are used as a source of phenolic compounds, because they can predict which family or compounds are available in the moment of sampling. Indeed, the huge number of researches related to the valuable effect of olives leaves phenolic compounds on health in last decade should encourage the industry to the valorization of olive leaves as a source of antioxidant to produce medicines, cosmetics, nutraceuticals and to develop functional foods.

Conclusion

Olive leaves have always played an important role in Mediterranean medicine and culture: they were first used in ancient Egypt, where it was believed having divine power. Olive leaves, available throughout all the year, are a big source of antioxidant and anti-inflammatory compounds. In this work, ten of the most important Calabrian varieties of olive leaves were studied: Carolea, Cassanese, Ciciariello, Dolce di Rossano, Grossa di Gerace, Ottobratica, Pennulara, Roggianella, Sinopolese and Tonda di Strongoli. The quantitative analyses were achieved by HPLC-MS/MS. Secoiridoids and flavonoids were the principal molecules found in the leaf samples. The data obtained pointed out that climate and plant variety play an important role in the production and quantity of these important compounds. In particular, in winter, the decrease in temperature hampers the cascade of production of secondary metabolites in leaves, whereas in summer, certain components of sunlight directly affect the production of phenols.

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Conflict of Interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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