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ORIGINAL PAPER

Determination of apolar and minor polar compounds and other chemical parameters for the discrimination of six different varieties of Tunisian extra-virgin olive oil cultivated in their traditional growing area

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Abstract A study on the characterization of Tunisian extra-virgin olive oil varieties produced in their place of origin has been carried out. Due to the influence of the genotype and environmental, agronomic and technological factors on the chemical composition of olive oil and its quality, all the olives studied were collected on the same season, and the oil was obtained under the same processing technique. Several analyses were performed to characterize the different olive oils: free acidity, peroxide value, fatty acid composition, radical scavenging activity, Rancimat assay, pigments content and phenolic compounds by HPLC–MS. In order to evaluate all the results obtained (36 parameters for each variety), different statistical analyses were used to discriminate the extra-virgin olive oil varieties: one-way analysis of variance was performed to check significant differences among cultivars ($p \leq 0.05$); PCA was applied to the data showing that variables such as oleic, linoleic, quinic and vanillic acids, apigenin, luteolin, taxifolin, oleuropein aglycon, pinoresinol acetate, elenolic acid and oxidative stability allowed discriminating among the different varieties of extra-virgin olive oil studied. Besides, LDA model was able to classify the samples depending on their geographical origin in Tunisia (North, Centre and South).

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Introduction

The olive tree (*Olea europaea* L.) is one of the most important crops in Mediterranean countries. Agriculture is the mainstay of Tunisian economy, the olive tree being one of its principal agricultural and economical sectors. Tunisia represents one of the biggest producers of olive oil worldwide; today, it is the fourth largest exporter after Spain, Italy and Greece [1]. Its olive-growing areas are found all around the country, where a wide range of edapho-climatic conditions exists, from lower semi-arid to arid conditions. As it is widely known, the environmental factors, as well as genotype, agronomic and technological factors influence very much the chemical composition of olive oil, having a great impact on its quality [2–4].

Furthermore, virgin olive oil, due to its use without refining, shows very interesting nutritional and sensorial properties, being one of the pillars of the Mediterranean diet. Its fatty acid composition, monounsaturated, and its natural antioxidants provide numerous advantages for health [5–7].

The genetic diversity in the olive sector of Tunisia is very wide; there are many different olive varieties in the various regions of the country. However, *Chemlali* and *Chetoui* are the two main olive cultivars in Tunisia. *Chetoui* represents more than 20% of the national olive oil production [8], while *Chemlali* is the most abundant olive variety, which represents two-thirds of olive plantation, principally used for oil extraction, and it is the major contributor of the national olive oil production [9]. Despite

the fact that these two cultivars are the most important from the economical point of view, there are other cultivars that are grown in restricted geographical localizations and that have a limited diffusion outside these areas. The study of these less-common cultivars appears of particular interest because they may have agronomic characteristics which can influence the quality and oxidative stability of the olive oil obtained.

Many studies have been carried out about olive cultivars, and their olive oils in Tunisia: comparison of different cultivars and growing areas of autochthonous olive oils [10, 11] and comparison of Tunisian and European varieties cultivated in Tunisia [2, 12–14]. However, there is not much information about the characterization and comparison of the Tunisian autochthonous varieties of olive oil grown in their area of origin.

The present work was carried out on six monovarietal extra-virgin olive oils (EVOO) from the two main (*Chemlali* and *Chetoui*) and four minor Tunisian cultivars (*Oueslati, Jarboui, El Hor* and *Chemchali*) produced in their area of origin. Several analyses were performed to characterize the different olive oils: free acidity, peroxide value, fatty acid composition, radical scavenging activity, Rancimat assay, pigments content and phenolic compounds by HPLC–MS. This is a preliminary study with the aim of finding any variable able to discriminate among the monovarietal extra-virgin olive oils and evaluate the possibility to determine whether the parameters studied were able to classify the cultivars depending on their geographical origin.

The Tukey's honest-test was used to determine the level of significance of each evaluated parameter characterizing the varieties. Pearson test allowed checking the correlations between different analyses. The classification of EVOO samples according to their geographical origin was performed by linear discriminant analysis (LDA). Finally, principal component analysis and agglomerative hierarchical clustering were performed to check the usefulness of chemical parameters as a tool to discriminate among the monovarietal EVOO.

Materials and methods

Chemicals

All solvents used were analytical or HPLC grade (Panreac, Barcelona, Spain) and used as received. Double-deionized water was obtained with a Milli-Q water purification system (Millipore, Bedford, MA, USA). Standard compounds such as hydroxytyrosol, tyrosol, luteolin, apigenin, *o*-coumaric acid, ferulic acid and quinic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA), (+)-pinoresinol was acquired from Arbo Nova (Turku, Finland) and oleuropein from Extrasynthèse (Lyon, France). The stock solutions containing these analytes were prepared in methanol/water (50/50, v/v).

Samples

Oils used in this study were obtained from six Tunisian olive varieties cultivated in their origin regions: North (*Chetoui, Chemlali and Jarboui*), Centre (*Oueslati and El Hor*) and South (*Chemchali*). Only healthy fruits, without any kind of infection or physical damage, were processed. Olives were harvested in the same season in 2008. The maturity index of all the olives was of 3 and was based on the degree of skin and pulp pigmentation according to the method developed by the Agronomic Station of Jaén [15]. After harvesting, the olives were washed and deleafed. Then the fruits were crushed with a hammer crusher, and using an Abencor analyzer (MC2 Ingenierias y Sistemas, Sevilla, Spain) the paste was mixed at 25 °C for 30 min, centrifuged without addition of warm water and then transferred into dark glass bottles.

Oil yield

The determination of oil content was done as follows: 40 g of fruit samples was dried in an oven at 80 °C to constant weight. Then, the dry olives were extracted with petroleum ether using a Soxhlet apparatus according to Donaire et al. [16]. The results were expressed as percentage of dry matter (%DM).

Analytical indices

Determination of free acidity, peroxide value and specific ultraviolet absorbance were carried out following the analytical methods described in the EC Regulation [17, 18].

Fatty acid composition

The fatty acid composition of oil samples was determined as methyl esters by capillary gas chromatography analysis after alkaline treatment. The gas chromatograph (VARIAN CP-3800 Gas Chromatograph) was equipped with an autosampler (CP-8400), a capillary column HP Innowax (Agilent Technologies, USA) (30 m × 0.53 mm, 1 μm), a split–splitless injector and a flame ionization detector (FID). Alkaline treatment was carried out by mixing 0.1 g of oil dissolved in 3 mL of *n*-hexane with 0.5 mL of 0.2 N methanolic potassium hydroxide solution according to the method of Reg EC 2568/91 [19]. One microlitre of methyl esters was injected. Seven fatty acids including C16:0, C16:1, C18:0, C18:1, C18:2, C18:3 and C20:0 were

identified from their retention times compared to those of standard compounds.

Radical scavenging activity

The olive oil samples were examined for their capacity to scavenge the stable 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) according to Kalantzakis et al. [20]. Olive oil was solved in ethyl acetate (10%, w/v), 1 mL of this solution was added to 4 mL of a freshly prepared DPPH solution 10^{-4} M in a screw-capped 10-mL test tube. The reaction mixture was then shaken vigorously for 10 s in a Vortex apparatus, and the tube was maintained in the dark for 30 min, after which a steady state was reached. The absorbance of the mixture was measured at 515 nm against a blank solution using a UV–VIS dual beam spectrophotometer (UVS-2700 Labomed, Inc). A control sample was prepared and measured daily. The radical scavenging activity (RSA) towards DPPH was expressed as the % reduction in DPPH concentration by the constituents of the oils: %[DPPH]red = 100*(1-$[DPPH]_{30}$/$[DPPH]_0$), where $[DPPH]_0$ and $[DPPH]_{30}$ were the concentrations of DPPH in the control sample (t = 0) and in the test mixture after the 30-min reaction, respectively. The DPPH concentration in the reaction medium was calculated from the following calibration curve, determined by linear regression: $A_{515nm} = 12.024[DPPH]-0.0101$ ($r = 0.999$), where [DPPH] was the concentration of DPPH, expressed as mol/L.

Rancimat assay

Oxidative stability was evaluated by Rancimat method [21]. Stability was expressed as the oxidation induction time (h), measured with the Rancimat 743 apparatus (Metrohm, Switzerland), using an oil sample of 3.5 g warmed to 100 °C and an air flow of 10 L h^{-1}.

Pigment contents

Oil (7.5 g) was accurately weighted and dissolved in 25 mL of cyclohexane. Chlorophyll and carotenoid contents were determined from the absorption spectra of the oils. Absorption at 670 nm is usually considered to be related to the chlorophyll fraction (pheophytin 'a' as its major component) and 470 nm to the absorption of the carotenoid fraction (lutein). Chlorophyll and carotenoid amounts were calculated using the specific extinction values, by the method of Minguez-Mosquera et al. [22]. The extinction coefficients applied were $E_0 = 613$ for pheophytin 'a' and $E_0 = 2000$ for lutein. Thus, pigment contents were calculated as follows:

$$[\text{chlorophyll}]\,(\text{mg/kg}) = \left(A_{670} \times 10^6\right)/(613 \times 100 \times d)$$

$$[\text{carotenoid}](\text{mg/kg}) = \left(A_{470} \times 10^6\right)/(2,000 \times 100 \times d)$$

where A is the absorbance and d is the spectrophotometer cell thickness (1 cm). Chlorophyll and carotenoid contents were expressed as milligrams of pheophytin 'a' and lutein per kilogram of oil, respectively.

HPLC–MS analysis

Sample preparation

The polar fraction was extracted according to Tsimidou [23]. Of the oil sample, 2.5 g was weighted and dissolved in 5 mL of *n*-hexane. After that 5 mL of methanol/water (60/40) were added, the mixture was vortexed and then centrifuged at 2,275 g during 10 min. The polar extract was evaporated to dryness in a rotary evaporator under reduced pressure and at a temperature of 35 °C. The residue was dissolved in 0.25 mL of methanol/water (50:50 v/v) and finally filtered through a 0.45-μm filter before the HPLC analysis.

Chromatographic separation

An Agilent 1200-RRLC system (Agilent Technologies, Waldbronn, Germany) equipped with a vacuum degasser, autosampler, a binary pump and a UV–vis detector was used for the chromatographic determination. Polyphenolic compounds were separated by using a method previously described in bibliography [24]. The compounds separated were monitored with DAD (240 and 280 nm) and with a mass spectrometry detector.

Mass spectrometry

The RRLC system was coupled to a Bruker Daltonik microTOF (time of flight) mass spectrometer (Bruker Daltonik, Bremen, Germany) using an orthogonal electrospray interface (model G1607A from Agilent Technologies, Palo Alto, CA, USA) equipped with an ESI interface. Parameters for analysis were set using negative ion mode with spectra acquired over a mass range from *m/z* 50–1000. The optimum values of the ESI–MS parameters were as follows: capillary voltage, +4.5 kV; drying gas temperature, 190 °C; drying gas flow, 9 L/min; and nebulizing gas pressure, 2 bar. External calibration was performed using a sodium formiate solution injected at the beginning of the run, and all the spectra were calibrated prior to the polyphenol identification.

The accurate mass data for the molecular ions were processed using the software Data Analysis 3.4 (Bruker

Daltonik), which provided a list of possible elemental formulas by using the Generate Molecular Formula ™ editor. The Generate Molecular Formula™ editor uses the sigma Fit™ algorithm, a CHNO algorithm, which provides standard functionalities such as minimum/maximum elemental range, electron configuration and ring plus double-bond equivalents, as well as a sophisticated comparison of the theoretical with the measured isotope pattern (Sigma-Value™) for increased confidence in the suggested molecular formula.

Statistical analysis

One-way analysis of variance (ANOVA, Tukey's honest significant difference multiple comparison) and Pearson's linear correlations were evaluated using Statistica 6.0 software (2001, StatSoft, Tulsa, OK, USA).

Agglomerative hierarchical clustering (AHC) was performed using XLSTAT 2008.

The standardized variables were used to perform principal component analysis (PCA) and cluster analyses using XLSTAT software (v. 2010.4.01, Addinsoft, NY, USA). To group the accessions based on chemical dissimilarity, agglomerative hierarchical clustering (AHC) was conducted on the Euclidean distance matrix with the Ward method.

LDA statistical data treatment was performed using SPSS (v. 15.0, Statistical Package for the Social Sciences, Chicago, IL, USA).

Results and discussion

Oil yield of olives

As reported in Table 1, all the studied varieties are characterized by a high oil yield according to the classification of Tous et al. [25]. Expressed as percentage of dry matter, the oil yield presented significant differences between the six olive varieties; the content of oil in the samples was in the range of 48–57%, the *Chetoui* olives being the richest in oil with a mean value of 56.9%, and the *Oueslati* being the one with the lowest content in oil (47.7%).

VOOs samples included in our study were produced using Abencor system which is suitable for processing small quantities of olives, especially for typical productions such as monovarietal olive oils. However, it is

Table 1 Chemical characteristics of the different olive oil varieties

	Cultivars					
	Oueslati	*Chetoui*	*Chemlali*	*El Hor*	*Chemchali*	*Jarboui*
% Humidity	53.90 ± 0.14^{c}	50.65 ± 0.26^{e}	54.30 ± 0.04^{b}	60.60 ± 0.22^{a}	51.34 ± 0.01^{d}	47.28 ± 0.03^{f}
% Oil yield/dry matter	47.67 ± 0.21^{e}	56.87 ± 0.50^{a}	51.79 ± 0.42^{c}	49.09 ± 0.04^{d}	52.64 ± 0.41^{b}	51.41 ± 0.21^{c}
Free acidity (as %C18:1)	0.25 ± 0.01^{d}	$0.31 \pm 0.04^{c,d}$	$0.35 \pm 0.01^{b,c}$	0.42 ± 0.03^{b}	$0.30 \pm 0.03^{c,d}$	0.60 ± 0.04^{a}
Peroxide value ($meqO_2/kg$)	2.03 ± 0.20^{b}	3.16 ± 0.29^{a}	4.00 ± 0.11^{a}	3.66 ± 0.57^{a}	4.00 ± 0.01^{a}	3.66 ± 0.55^{a}
K_{232}	2.17 ± 0.33^{a}	$1.89 \pm 0.07^{a,b}$	$1.70 \pm 12^{a,b}$	$2.00 \pm 0.02^{a,b}$	1.70 ± 0.04^{b}	2.10 ± 0.12^{b}
K_{270}	0.20 ± 0.05^{a}	$0.17 \pm 0.01^{a-c}$	0.13 ± 0.01^{c}	$0.15 \pm 0.02^{a,b}$	$0.17 \pm 0.01^{a,c}$	$0.15 \pm 0.02^{b,c}$
% C16:0	13.60 ± 0.48^{b}	11.5 ± 0.90^{b}	17.8 ± 0.68^{a}	12.30 ± 0.78^{b}	15.90 ± 1.35^{a}	16.40 ± 0.61^{a}
% C16:1	1.30 ± 0.06^{b}	0.34 ± 0.15^{c}	2.60 ± 0.12^{a}	0.52 ± 0.14^{c}	1.33 ± 0.18^{b}	0.47 ± 0.09^{c}
% C18:0	1.90 ± 0.04^{c}	2.8 ± 0.039^{a}	2.00 ± 0.05^{b}	2.50 ± 0.06^{b}	2.40 ± 0.08^{c}	1.58 ± 0.09^{d}
% C18:1	69.90 ± 0.76^{a}	$63.27 \pm 0.94^{b,c}$	59.90 ± 0.59^{d}	$61.76 \pm 0.74^{c,d}$	65.02 ± 1.29^{b}	54.5 ± 0.33^{e}
% C18:2	12.14 ± 0.55^{e}	20.3 ± 0.03^{b}	16.00 ± 0.10^{c}	20.90 ± 0.76^{b}	13.70 ± 0.11^{d}	25.7 ± 0.159^{a}
% C18:3	0.69 ± 0.08^{b}	$0.62 \pm 0.02^{b,c}$	$0.64 \pm 0.01^{b,c}$	0.88 ± 0.03^{a}	0.58 ± 0.03^{c}	0.87 ± 0.038^{a}
% C20:0	0.40 ± 0.04^{a}	0.38 ± 0.10^{a}	0.40 ± 0.05^{a}	0.37 ± 0.02^{a}	0.47 ± 0.075^{a}	0.328 ± 0.007^{a}
% DPPH red	86.60 ± 6.79^{c}	97.10 ± 0.84^{a}	80.80 ± 0.70^{d}	$93.20 \pm 0.21^{a,b}$	$95.90 \pm 0.70^{a,b}$	$91.30 \pm 0.28^{b,c}$
Oxidative stability (h)	48.45 ± 0.13^{b}	49.48 ± 0.70^{b}	40.99 ± 0.13^{d}	46.87 ± 0.80^{c}	58.84 ± 1.36^{a}	46.31 ± 1.59^{c}
Chlorophyll (mg/kg)	$4.77 \pm 1.35^{a,b}$	$3.50 \pm 0.47^{b,c}$	2.79 ± 0.17^{c}	2.17 ± 0.07^{c}	2.42 ± 0.07^{c}	5.42 ± 0.52^{a}
Carotenoids (mg/kg)	1.57 ± 0.40^{b}	1.49 ± 0.18^{b}	1.55 ± 0.07^{b}	0.86 ± 0.05^{c}	1.50 ± 0.04^{b}	2.41 ± 0.05^{a}
$\sum$MUFAs	71.25 ± 0.79^{a}	63.59 ± 0.79^{b}	62.55 ± 0.48^{b}	62.29 ± 0.69^{b}	66.36 ± 1.11^{b}	54.98 ± 0.25^{c}
$\sum$PUFAs	12.83 ± 0.63^{f}	20.90 ± 0.03^{c}	16.65 ± 0.11^{d}	21.81 ± 1.05^{b}	14.24 ± 0.10^{e}	26.62 ± 0.12^{a}
MUFAs/PUFAs	5.55 ± 0.31^{a}	3.04 ± 0.04^{d}	3.76 ± 0.02^{c}	2.85 ± 0.02^{d}	4.65 ± 0.04^{b}	2.06 ± 0.17^{e}
C18:1/C18:2	5.76 ± 0.3^{a}	3.11 ± 0.05^{d}	3.74 ± 0.03^{c}	2.95 ± 0.02^{d}	4.75 ± 0.05^{b}	2.11 ± 0.02^{e}

Different letters in a row indicate significant differences among varieties ($p \leq 0.05$)

important to mention that the mini and microcondition of olive oil production could lead to obtain an oil different from that produced in industrial conditions as previously reported by Cerretani et al. [26] and Inarejos-García et al. [27].

Free acidity, peroxide value and specific ultraviolet absorbance

For all analysed oils, the mean values of studied quality parameters fell within the range allowed by the regulation EC [17, 18] for the extra-virgin olive oil category (free acidity $\leq 0.8\%$; peroxide value ≤ 20 Meq O_2 kg^{-1}; $K_{270} \leq 0.22$; $K_{232} \leq 2.5$) (Table 1).

Free acidity of the oils studied was in a range from 0.25 to 0.60%. This fact means that, taking into account the acidity of olive samples, all olive oils could be classified as "extra-virgin olive oils".

Concerning the peroxide values, samples ranged from 2.03 meqO_2/kg of *Oueslati* to 4.0 meqO_2/kg of *Chetoui* and *Chemchali*. These low values are a measure of the high freshness of the oils analysed [28, 29].

The specific ultraviolet absorbance K_{232} varied from 1.70 to 2.17 having the highest values in *Oueslati, Chetoui* and *El Hor* varieties, while K_{270} ranged from 0.20 of *Oueslati* to 0.13 of *Chemlali*.

Fatty acid composition

Methyl ester fatty acid composition and their levels in the analysed oils are shown in Table 1. As it can be observed, oleic (C18:1), linoleic (C18:2) and palmitic (C16:0) acids are the major fatty acids present in the studied samples. The fatty acid composition of olive oils varies widely depending on the cultivar. These findings are in good agreement with those of other authors working on Tunisian olive oil varieties [12, 30].

Among studied samples, the *Oueslati* olive oil showed the highest percentage of oleic acid (C18:1) and the lowest percentage of linoleic acid (C18:2) (69.9 and 12.1%, respectively), and the *Chetoui* olive oil showed the lowest percentage of palmitic acid which did not exceed 11.5%. Concerning palmitoleic (C16:1), stearic (C18:0), linolenic (C18:3) and arachidic (C20:0) acids, the studied olive oil varieties presented low amounts of all of them. The highest percentage of palmitoleic acid was found in *Chemlali* cultivar, stearic acid presented the highest amount in *Chetoui* variety, while *Jarboui* olive oil was the richest in linolenic acid.

Other interesting points for the chemical characterization of studied oils are the proportions of some classes of free fatty acids. The monounsaturated fatty acids have great importance because of their nutritional implication and effect on oxidative stability of oils [5]. Table 1 shows that the proportion of monounsaturated fatty acids also changed according to the cultivar. It reached a maximum value of 71.3% for the *Oueslati* olive oil, which was characterized, among the studied oils, by the highest MUFAs/PUFAs and C18:1/C18:2 ratios (5.6 and 5.8, respectively). The C18:1/C18:2 ratio has the most marked relationship with stability, and it is said that an oil presents a good stability index if this value is over 7. Nevertheless, Tunisian olive oils are described in bibliography to present lower C18:1/C18:2 ratios compared to most of the European ones [12, 31].

Pigment contents

The olive oil colour is directly related to the chlorophyll and carotenoid contents, and it has been proposed as a characterizing factor and as a quality index related to the oil extraction method and to the olive variety [22]. Besides, the colour is the first attribute of virgin olive oil evaluated by consumers. In analysed oils and according to the cultivar, chlorophyll and carotenoid contents ranged from 2.17 and 0.87 mg/kg to 5.42 and 2.41 mg/kg, respectively (Table 1), *Jarboui* olive oil being the richest in terms of both of them.

Identification and quantification of phenolic compounds

The identification of phenolic compounds was carried out comparing their migration times, UV data and mass spectra provided by TOF–MS with those of authentic standards when available. Remaining compounds were identified by the interpretation of their mass spectra obtained by the TOF–MS using Generate Molecular Formula Editor. In this way, 24 phenolic compounds could be identified. Table 2 includes the identified compounds, migration time, molecular formula, calculated and experimental *m/z*, sigma value and tolerance (ppm) in generated molecular formula. Major phenolic compounds previously observed in bibliography [32, 33] were also detected in the present study.

To build the calibration curves and carry out the quantification of the identified phenolic compounds, eleven standards were used: hydroxytyrosol (HYTY), tyrosol (TY), oleuropein (Ol), pinoresinol (Pin), luteolin (Lut), apigenin (Apig), taxifolin, vanillic acid, o-coumaric acid, quinic acid and ferulic acid. All of them presented good linearity between different concentrations, and regression coefficients were higher than 0.990 in most cases.

HYTY, TY, Pin, Lut, Apig, taxifolin, vanillic acid, ferulic acid, quinic acid, and o-coumaric acid were quantified using their own commercial standards. HYTY-acetate (HYTY-Ac) was quantified using HYTY calibration curve; elenolic acid (EA), H-elenolic acid (H-EA), Ol aglycon (Ol

Table 2 Phenolic compounds identified in an olive oil extract by HPLC–ESI-TOF–MS including retention time, *m/z* experimental and calculated, molecular formula and sigma value

Compounds	Retention time (min)	*m/z* experimental	*m/z* calculated	Molecular formula	Tolerance (ppm) in GMF*	Sigma
Quinic acid	2.1	191.0574	191.0561	C7H11O6	5	0.0010
HYTY	7.9	153.0582	153.0557	C8H9O3	10	0.0014
TY	9.7	137.0623	137.0608	C8H9O2	10	0.0040
Vanillic acid	11.1	167.0361	167.035	C8H7O4	5	0.0080
o-Coumaric acid	13.2	163.0424	163.0401	C9H7O3	10	0.0313
Taxifolin	13.6	303.0504	303.0561	C15H11O7	5	0.0270
Ferulic acid	13.7	193.0525	193.0506	C10H9O4	5	0.620
HYTY-Ac	13.8	195.0681	195.0663	C10H11O4	15	0.0107
EA	14.7	241.0755	241.0718	C11H13O6	5	0.0075
D-Ol Agl	15.9	319.1252	319.1187	C17H19O6	5	0.048
H-EA	15.3	257.0689	257.0667	C11H13O7	10	0.0089
H-D-Ol Agl	16.3	335.1148	335.1136	C17H19O7	5	0.0052
Syringaresinol	17.8	417.1547	417.1555	C22H25O8	5	0.0156
Pin	18.5	357.135	357.1344	C20H21O6	5	0.0048
D-Lig Agl	18.8	303.1258	303.1238	C17H19O5	5	0.0070
Ac Pin	19.0	415.1409	415.1398	C22H23O8	5	0.0031
Methyl D-Ol Agl	19.9	333.1355	333.1344	C18H21O6	10	0.0293
Sinapinic acid	21.3	223.0640	223.0612	C11H11O5	5	0.0107
10-H-Ol Agl	22.6	393.1188	393.1191	C19H21O9	5	0.0183
Ol Agl	22.8	377.1248	377.1242	C19H21O8	5	0.0002
Lut	23.3	285.0420	285.0405	C15H9O6	5	0.0026
H-Pin	24.4	373.1309	373.1293	C20H21O7	5	0.0258
Lig Agl	25.3	361.1308	361.1293	C19H21O7	5	0.0095
Apig	25.6	269.0481	269.0455	C15H9O5	10	0.0097

* *GMF* generated molecular formula

HYTY hydroxytyrosol, *TY* tyrosol, *Ol* oleuropein, *Pin* pinoresinol, *Lut* luteolin, *Apig* apigenin, *HYTY-Ac* HYTY-acetate, *EA* elenolic acid, *H-EA* H-elenolic acid, *Ol Agl* Ol aglycon, *D-Ol Agl* decarboxylated Ol Agl, *10-H-Ol Agl* 10 hydroxy Ol Agl, *Lig Agl* ligtroside aglycon, *D-Lig Agl* decarboxylated Lig Agl, *Ac-Pin* acetoxy pinoresinol

Agl), decarboxylated Ol Agl (D-Ol Agl), 10-H-OL Agl, ligtroside aglycon (Lig Agl), decarboxylated Lig Agl (D-Lig Agl), H-D-Ol Agl, methyl D-Ol Agl were quantified with oleuropein calibration curve; acetoxy Pin (Ac-Pin), H-Pin and syringaresinol with Pin calibration curve and sinapinic acid using Ferulic acid calibration curve.

Table 3 summarizes the concentrations of the phenolic compounds identified in the different olive oil varieties expressed as mg/kg of oil. It could be observed that the distribution of phenolic compounds varied significantly in the different cultivars ($p \leq 0.05$). Among major secoiridoids, Ol Agl, Lig Agl and D-Ol Agl were found in higher concentrations in *Chetoui/El Hor* (1839.9/1915.9 mg/kg), *Chemlali* (303.15 mg/kg) and *Chemchali* (2455.3 mg/kg), respectively. Meanwhile, *Oueslati* and *Jarboui* presented the lowest content in Ol Agl (222.6 mg/kg) and D-Ol Agl (146.1 mg/kg), respectively.

As regards simple phenols, *El Hor* cultivar was the richest in terms of HYTY and TY (10.9 mg/kg).

The rest of minor simple phenols identified were present in small amounts for all the samples. Five phenolic acids occurred in the studied samples. Quinic acid presented the highest levels (0.5–5.1 mg/kg), o-coumaric acid ranged from 0.1 up to 0.7 mg/kg, ferulic acid 0.0–0.1 mg/kg, sinapinic acid 0.1–0.6 mg/kg and vanillic acid which was present as traces in *Oueslati, Chemlali, El Hor* and *Jarboui* EVOOs was only quantified in *Chetoui* and *Chemchali* samples (0.9 and 1.5 mg/kg, respectively).

(+)-Ac-Pin was the lignan which presented higher amounts ranging from 0.46 to 4.45 mg/kg, followed by syringaresinol (0.5–2.3 mg/kg), (+)-Pin (0.3–1.8 mg/kg) and (+)-H-Pin (0.3–0.7 mg/kg), *Oueslati* olive oil being the richest in terms of lignans.

Concerning the flavonoid composition, luteolin was the most abundant flavonoid in studied samples, and the *Jarboui* EVOO was characterized by the highest amounts of both flavones Lut and Apig (10.20 and 2.86 mg/kg, respectively) and by the presence of the flavonol (+)-

Table 3 Quantification expressed as mg analyte kg-1 olive oil of the phenolic compounds by HPLC–ESI-TOF–MS (mean ± SD, $n = 7$)

Compounds	*Oueslati*	*Chetoui*	*Chemlali*	*El Hor*	*Chemchali*	*Jarboui*
Quinic acid	0.48 ± 0.02^{d}	2.35 ± 0.01^{b}	1.74 ± 0.01^{b}	5.05 ± 0.30^{a}	1.63 ± 0.13^{b}	0.87 ± 0.08^{c}
HYTY	7.18 ± 0.27^{a,b}	7.26 ± 0.60^{b}	6.80 ± 0.15^{b}	7.68 ± 0.31^{a}	4.32 ± 0.04^{c}	1.81 ± 0.23^{d}
TY	2.39 ± 0.04^{c}	3.53 ± 0.54^{a,b}	3.48 ± 0.05^{a}	3.25 ± 0.01^{a,b}	3.01 ± 0.17^{b}	2.27 ± 0.1^{c}
Vanillic acid	–	0.93 ± 0.34^{b}	–	–	1.51 ± 0.27^{a}	–
o-Coumaric acid	0.10 ± 0.03^{e}	0.41 ± 0.09^{c}	0.33 ± 0.00^{c}	0.15 ± 0.00^{e}	0.48 ± 0.03^{b}	0.66 ± 0.12^{a}
Taxifolin	–	–	–	–	–	0.147 ± 0.00
Ferulic acid	0.08 ± 0.01^{b}	0.11 ± 0.01^{a}	–	0.02 ± 0.00^{e}	0.03 ± 0.01^{d}	0.06 ± 0.00^{c}
HYTY-Ac	0.68 ± 0.04^{d}	1.68 ± 0.21^{c}	2.94 ± 0.26^{b}	2.67 ± 0.12^{b}	7.11 ± 0.06^{a}	1.05 ± 0.06^{d}
EA	638.61 ± 15.93^{c}	182.99 ± 44.57^{d}	806.28 ± 26.11^{a}	743.39 ± 20.88^{b}	12.13 ± 0.52^{e}	143.98 ± 32.38^{d}
D-Ol Agl	1834.12 ± 58.74^{b}	679.01 ± 75.93^{c,d}	808.01 ± 8.84^{c}	538.21 ± 37.87^{d}	2455.25 ± 102.46^{a}	146.07 ± 44.33^{e}
H-EA	0.58 ± 0.05^{c}	0.26 ± 0.05^{e}	2.44 ± 0.13^{a}	0.78 ± 0.06^{b}	0.47 ± 0.01^{c,d}	0.39 ± 0.08^{d}
H-D-Ol Agl	8.41 ± 0.20^{b}	0.55 ± 0.09^{d}	3.08 ± 0.03^{c}	0.23 ± 0.05^{d}	16.12 ± 1.36^{a}	1.02 ± 0.07^{d}
Syringaresinol	2.33 ± 0.02^{a}	0.53 ± 0.06^{e}	0.79 ± 0.03^{c}	0.47 ± 0.00^{e}	1.78 ± 0.01^{b}	0.67 ± 0.04^{d}
Pin	1.23 ± 0.02^{c}	1.34 ± 0.05^{b}	0.73 ± 0.02^{d}	1.79 ± 0.03^{a}	0.70 ± 0.02^{d}	0.25 ± 0.01^{e}
D-Lig Agl	16.11 ± 0.55^{b}	6.83 ± 0.46^{c,d}	12.67 ± 0.05^{b,c}	3.67 ± 0.16^{e}	60.77 ± 6.34^{a}	4.70 ± 0.32^{e}
Ac-Pin	4.45 ± 0.08^{a}	0.46 ± 0.02^{e}	2.51 ± 0.1^{c}	0.74 ± 0.01^{d}	0.87 ± 0.05^{d}	2.92 ± 0.18^{b}
Methyl D-Ol Agl	0.15 ± 0.04^{e}	2.54 ± 0.21^{b}	1.72 ± 0.06^{c}	1.22 ± 0.06^{d}	1.16 ± 0.06^{d}	3.11 ± 0.02^{a}
Sinapinic acid	0.59 ± 0.03^{a}	0.10 ± 0.01^{d}	0.21 ± 0.01^{b}	0.13 ± 0.01^{c}	0.05 ± 0.01^{e}	0.12 ± 0.00^{c,d}
10-H-Ol Agl	0.95 ± 0.02^{d}	2.27 ± 0.45^{c}	3.62 ± 0.05^{a}	2.40 ± 0.07^{b}	2.28 ± 0.17^{b}	3.61 ± 0.16^{a}
Ol Agl	222.62 ± 14.34^{d}	1839.87 ± 217.10^{a}	578.19 ± 101.26^{c}	1915.86 ± 81.74^{a}	971.91 ± 68.59^{b}	1042.85 ± 168.85^{b}
Lut	0.74 ± 0.04^{e}	4.18 ± 0.53^{b,c}	2.93 ± 0.09^{d}	4.38 ± 0.00^{b}	4.28 ± 0.33^{b}	10.20 ± 0.59^{a}
H-Pin	0.51 ± 0.01^{c}	0.56 ± 0.06^{b}	0.65 ± 0.02^{a}	0.35 ± 0.01^{e}	0.32 ± 0.01^{e}	0.44 ± 0.02^{d}
Lig Agl	9.89 ± 0.30^{c}	258.28 ± 59.10^{b}	303.15 ± 59.81^{a}	80.55 ± 13.15^{c}	17.36 ± 0.82^{c}	26.21 ± 6.97^{c}
Apig	0.09 ± 0.01^{b}	0.82 ± 0.22^{b}	0.52 ± 0.01^{b}	0.45 ± 0.00^{b}	0.53 ± 0.06^{b}	2.86 ± 0.82^{a}
Total	2752.29 ± 79.47^{b}	2996.86 ± 136.46^{b}	2542.79 ± 161.89^{b}	3312.58 ± 113.02^{a}	3564.07 ± 176.32^{a}	1396.27 ± 29.58^{c}

Different letters in a row for the same phenolic compound indicate significant differences among varieties ($p \leq 0.05$)

HYTY hydroxytyrosol, *TY* tyrosol, *Ol* oleuropein, *Pin* pinoresinol, *Lut* luteolin, *Apig* apigenin, *HYTY-Ac* HYTY-acetate, *EA* elenolic acid, *H-EA* H-elenolic acid, *Ol Agl* Ol aglycon, *D-Ol Agl* decarboxylated Ol Agl, *10-H-Ol Agl* 10 hydroxy Ol Agl, *Lig Agl* ligtroside aglycon, *D-Lig Agl* decarboxylated Lig Agl, *Ac-Pin* acetoxy pinoresinol

taxifolin which was not detected for the rest of studied oils.

It is known that the oxidative stability variations are affected by some minor compounds such as phenols and tocopherols [34] and as reported by other authors, oxidative stability was positively correlated to secoiridoids amounts ($r = 0.940$, $p < 0.001$) [35]. In general, our results agree with those previously reported. In fact, positive correlations were found between major secoiridoids and Ol Agl and DPPH radical scavenging capacity ($r = 0.640$ and $r = 0.715$; $p \leq 0.05$, respectively). The correlations were even higher between oxidative stability and D-Lig Agl and major secoiridoids ($r = 0.822$ and $r = 0.814$; $p \leq 0.05$, respectively).

Oxidative stability and radical scavenging activity

Oxidation stability is an important property of olive oil quality and is affected by lipid composition and different antioxidant compounds whose levels may be influenced by cultivar, year and place of production [3].

The oxidative stability of the extra-virgin olive oils was measured as the induction time determined using the Rancimat method. The highest oxidative stability was presented by *Chemchali* oil with a mean value of 58.8 h. This characteristic may be partially attributed to the low level of rainfall in the south region where it was cultivated as demonstrated by Tovar et al. [36], since oxidative stability is affected significantly by water regime that determines the phenol content of the olive fruit. Furthermore, the stability is the result of the oxidant–antioxidant balance. The weight of the antioxidants as phenolic compounds could be greater than this of fatty acid profile. In fact, *Chemchali* olive oil did not present the best MUFA/PUFA ratio (4.7 ± 0.0) however presented the highest content in phenolic compounds (3564.1 ± 176.3 mg/kg), and as the result it is the oil with the highest oxidative stability value. *Oueslati* was the oil with the highest

MUFA/PUFA ratio (5.5 ± 0.3); however, a lower concentration of phenolic compounds (2752.3 ± 79.5 mg/kg) provides a lower oxidative stability value (48.5 h).

It is known that especially antioxidant properties are very important due to the scavenging activity of free radicals in foods and in biological systems [37]. When an antioxidant reacts with a free radical, it yields an electron, is oxidized, and becomes a weak, non-toxic free radical that is stable and unable to propagate the reaction. An exogenous supply of antioxidants through the diet is necessary because of the incomplete efficiency of endogenous human antioxidant defences and the occurrence of situations in which excessive free radicals are produced [38].

Olive oils were examined for their radical scavenging activity towards the stable DPPH free radical. As reported in Table 1, the *Chetoui, El Horr* and *Chemchali* were the cultivars which showed higher percentage of DPPH reduced and thus presented higher antioxidant capacity to scavenge the free DPPH radical.

A positive correlation was found between the oxidative stability and the radical scavenging capacity ($r = 0.718$; $p \leq 0.05$).

Principal component analysis and cluster analysis

PCA was applied to all VOO samples. Except C20:0, which showed no significant variation among studied VOOs ($p = 0.117$, $\alpha = 0.05$), all variables were submitted to a PCA.

The first three PCs explained 73.9% of variance. The first PC accounted for 30.4% of total variance and was highly correlated with luteolin ($r = 0.919$ at $\alpha = 0.05$), apigenin ($r = 0.911$ at $\alpha = 0.05$), taxifolin ($r = 0.938$ at $\alpha = 0.05$), oleic ($r = -0.883$ at $\alpha = 0.05$) and linoleic ($r = 0.873$ at $\alpha = 0.05$) acids. The second PC (22.4% of variance) was correlated to OL Agl ($r = 0.888$ at $\alpha = 0.05$), quinic acid ($r = 0.844$ at $\alpha = 0.05$) and Ac Pin ($r = -0.814$ at $\alpha = 0.05$), while the third PC was correlated to oxidative stability ($r = 0.925$ at $\alpha = 0.05$), EA ($r = -0.898$ at $\alpha = 0.05$) and vanillic acid ($r = 0.850$ at $\alpha = 0.05$).

Figure 1 shows a projection of the six cultivars in the space defined by the first three principal components. We can distinguish the presence of four principal groups. Group 1 consists of *Chemlali* and *Oueslati* varieties which produce oils rich in EA and with fewer amounts of Ol Agl and Lut. Group 2 composed of the variety *Chemchali*, which is the one that presented the highest oxidative stability, was richer in D-Ol Agl, H-D-Ol Agl and presented low amounts of EA. Group 3 consists of the *Chetoui* and *El Hor* olive oil varieties that are characterized by higher amounts of Ol Agl and quinic acid and low amount of Ac-Pin. The last group was the *Jarboui* variety characterized by the presence of taxifolin, higher amounts of linoleic acid, Lut and Apig and lower amounts of oleic acid.

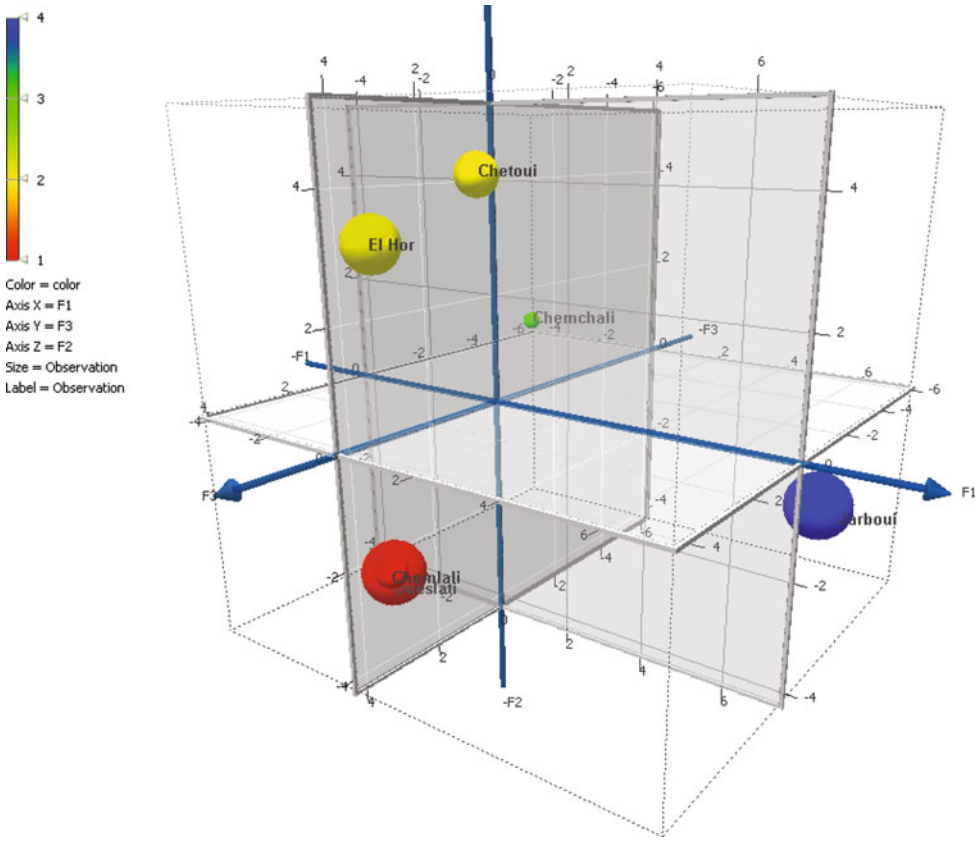

Fig. 1 Projection of the six cultivars in the space defined by the first three principal components

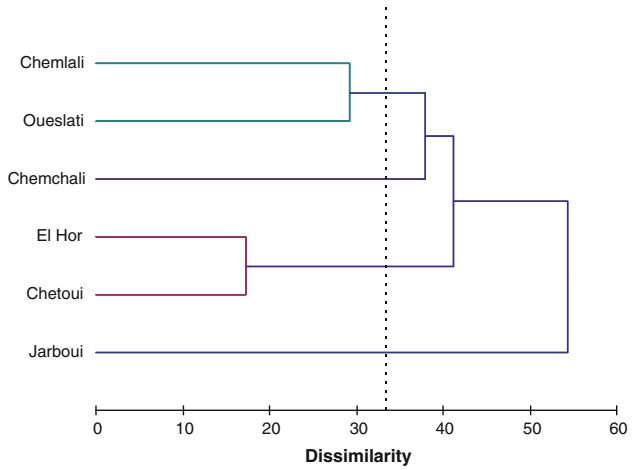

Fig. 2 Dendrogram of the Tunisian varieties

The results obtained with HCA are analogous to those from PCA: 'Jarboui' is the most dissimilar of the varieties, and low similarity exists between 'Chétoui, and 'El Hor' and the rest of varieties (Fig. 2).

LDA analysis

Linear discriminant analysis (LDA) is one of the most commonly used classification techniques. LDA is a supervised classificatory technique widely recognized as an excellent tool to obtain vectors showing the maximal resolution between a set of previously defined categories. In LDA, vectors minimizing the Wilks' lambda, λ_w, are obtained [39]. This parameter is calculated as the sum of squares of the distances between points belonging to the same category divided by the total sum of squares.

Using the normalized variables, an LDA model capable of classifying the EVOO samples according to their respective geographical origin was constructed (North, Centre and South).

When the LDA model was constructed, a good resolution between all the category pairs was achieved ($\lambda_w = 0.001$) as it can be seen in Fig. 3. For this model, and using leave-one-out validation, all the points of the training set were correctly classified. The corresponding evaluation set was then used to check the prediction capability of the model. Using a 95% probability, all the objects were correctly assigned.

Table 4 shows the variables selected by the SPSS stepwise algorithm (the predictors with large discriminant capabilities) and the corresponding standardized coefficients of this model. Variables such as oleic acid and EA were also among the variables that explained the largest portion of the variance in PCA. Besides, oleic acid is the most abundant fatty acid present in olive oil, and the rest of the variables constitute some of the most representative phenolic compounds of olive oil.

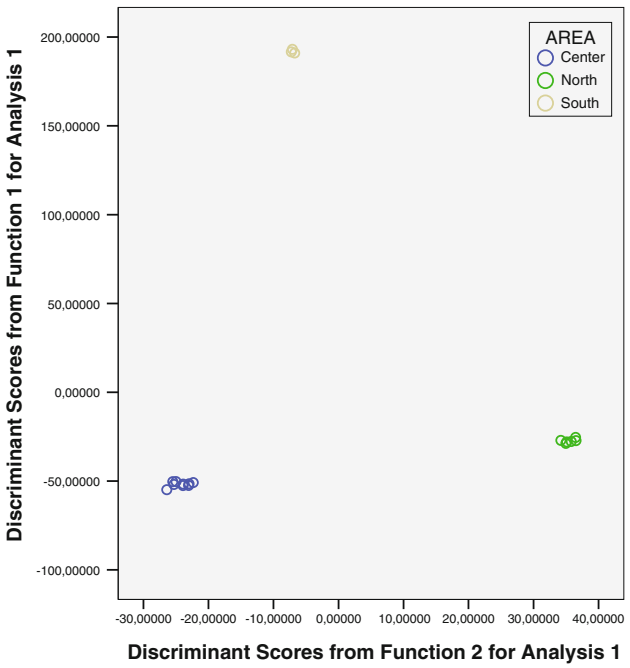

Fig. 3 Two-dimensional score plot of the two LDA discriminant functions obtained to predict the geographical origin of EVOOs

Table 4 Standardized canonical discriminant function coefficients

	Function	
	1	2
% C18:1	−0.325	5.106
Carotenoids	−3.703	−3.825
EA	−10.660	2.902
HYTY-Ac	15.136	3.928
D-Lig Agl	4.675	−2.358
H-Pin	−11.886	−15.269
Sinapinic acid	3.954	17.556
H-D-Ol Agl	12.519	−4.027

EA elenolic acid, *HYTY-Ac* HYTY-Acetate, *D-Lig Agl* decarboxylated Lig Ag, *H-Pin* hydroxypinoresinol, *H-D-Ol Agl* hydroxyl decarboxylated oleuropein aglicon

Conclusion

After the study of the phenolic profile and other chemical parameters for each of the six Tunisian extra-virgin olive oils and the use of specific statistical analysis, it could be observed that there were significant differences among studied varieties. Specifically, the statistical analysis allowed distinguishing the monovarietal olive oils depending on

their concentrations of oleic and linoleic acids, apigenin, luteolin, quinic acid, vanillic acid, taxifolin, oleuropein aglycone, pinoresinol acetate, elenolic acid and oxidative stability. Furthermore, it was possible to classify the varieties of olive oil depending on their geographical origin by using LDA analysis as long as each variety had been grown in its own place of origin. In the case of LDA analysis also variables as oleic acid and elenolic acid play an important role as discriminating functions in the differentiation of the geographical origin.

These preliminary findings would be hopefully confirmed by the analysis of a larger number of samples that take into consideration the variation of phenolic profile and chemical composition of EVOOs, which may also arise from agronomical practices, maturation index, harvesting years and processing technologies.

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