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Extra Virgin Olive Oil Components and Oxidative Stability from Olives Grown in Tunisia

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Abstract The effects of the contents of lipids, pigments, α-tocopherol and phenols were studied in relation to the antioxidant capacity of five virgin olive oils obtained from five olive cultivars planted in Tunisia (Arbequina, Koroneiki, Leccino, Oueslati and Chemchali). The antioxidant capacities were evaluated by two different radical scavenging activities: radical scavenging activity by the DPPH assay (RSA-DPPH) and total antioxidant status by the ABTS test (TAA-ABTS). The highest contents of antioxidant compounds (75.96, 10.34, 6.32, 15.39 and 241.52 mg kg^{-1} for oleic acid, O/L ratio, carotenes, chlorophylls and total phenols, respectively) were found for the Koroneiki cultivar except for α-tocopherol and *o*-diphenols, which had the highest contents (369 and 160.7 mg kg^{-1}, respectively) in the Leccino and Chemchali cultivars (cvs). Furthermore, the highest antioxidant capacity in virgin olive oil was observed in the Koroneiki cultivar (0.24 mmol TE kg^{-1}) followed by the Chemchali and Leccino cvs (0.22 and 0.13 mmol TE kg^{-1}) for the TAA-ABTS test. However, the RSA-DPPH activity was higher for the Chemchali cultivar (19.9%) than for the Koroneiki and Leccino cvs (18.4 and 13.5%, respectively). Correlation between these capacities and the oil composition revealed that they were mainly influenced by the carotene content, followed by chlorophyll and phenolic contents where the ABTS test was more pronounced. Then, the antioxidant capacity of the virgin olive oils was correlated with polar components and the lipid profile which are important for its shelf life.

Keywords Olive oil · Antioxidant capacity · α-Tocopherol · Pigments · Fatty acids · Phenols · ABTS · DPPH

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Introduction

Olive oil, a food staple in the warmer regions around the Mediterranean Sea, is now becoming popular throughout Mediterranean and non-Mediterranean areas [1]. The excellent organoleptic and nutritive properties of the oil, together with the current tendency of consumers to select minimally processed foods, have prompted a re-assessment of its consumption in a regular diet [2]. Consumers are increasingly demanding that high food quality be maintained during the period between purchase and consumption. These expectations are a consequence not only of the primary requirement that the food should remain safe but also of the need to minimize unwanted changes in sensory quality. Virgin olive oil's characteristic aroma, taste, color and nutritive properties distinguish it from other edible vegetable oils [3]. It is therefore a matter of great concern for the olive oil industry to preserve its product without loss of these positive attributes [2]. The oxidative stability or the shelf life is a central parameter in the evaluation of extra virgin olive oil (EVOO) quality, as it gives a reliable evaluation of the susceptibility to oxidative degeneration,

which is the main cause of its adulteration [4]. It is usually evaluated by the induction period; the time period until a critical point of oxidation is reached, strictly related to a sensorial degradation of the oil [5]. The oxidative stability, evaluated by the Rancimat method, reveals the resistance to the development of rancidity. This phenomenon could depend on the autoxidation of fatty acids [6] and is combined with a vast array of flavor, color hues and distinct features. The combination depends on the cultivar of the olives from which the oil is extracted [1].

Oxidation takes place in the presence of oxygen, generating some unstable compounds that can modify the sensory and nutritional characteristics of the oil, thus leading to product spoilage. Autoxidation is a slow radical process, which proceeds via a chain reaction including induction, propagation and termination steps. During the induction period, alkyl radicals are formed and undergo a reaction with oxygen molecules to form hydroperoxides (ROOH) and peroxy radicals during the propagation phase. Termination proceeds via association of two radicals to form a stable adduct [7]. The whole sequence may result in variation in sensory characteristics and nutritional alterations due to the formation of off-flavor volatile compounds from degradation of ROOH and the disappearance of essential fatty acids [8]. Though unavoidable, the oxidation process can be delayed by endogenous antioxidants of the oils that enhance the oxidative stability by different mechanisms [9, 10], conferring an effective defense system against free radical attack, i.e. preventing first chain initiation by scavenging initiating radicals [11], metal chelating [12], decreasing localized oxygen concentration and decomposing peroxides [13].

The resistance to oxidative deterioration is usually attributed to two main reasons: (1) the fatty acid composition [8] and (2) the pool of minor compounds with powerful antioxidant activity. In fact, in the case of olive oil, the fatty acid composition is characterized by a high monounsaturated-to-polyunsaturated fatty acid ratio [8] whereas minor compounds consist mainly of tocopherols and polyphenols (both simple and aglycons) but also by chlorophylls and carotenes. Some authors have estimated the contribution of these antioxidants to oil stability, that of phenolic compounds being around 30%, fatty acids 27%, α-tocopherol 11%, and carotenes 6% [9, 14]. Carotenes, and in particular β-carotene, are efficient antioxidants due to their ability to quench oxygen radical species, and they also can act as light filters [15]. Tocopherols are considered as the most important lipid phase natural antioxidants, which prevent lipid peroxidation by scavenging radicals in membranes and lipoprotein particles [16]. They act as antioxidants by two primary mechanisms, a chain-breaking electron donor mechanism, in which they donate their phenolic hydrogen atom to lipid free-radicals and a chain-breaking acceptor mechanism, which includes singlet oxygen scavenging or quenching [17].

Various authors have demonstrated a positive linear relationship between oil stability and the total content of polyphenols [18, 19]. Polyphenols are able to donate a hydrogen atom to the lipid radical formed during the propagation phase of lipid oxidation [11] and then radicals obtained are stable species and will stop the oxidation chain reaction [8]. Many studies have indicated their antioxidant capacity with respect to oxidative alterations due to free radicals and other reactive species [20] and the oxidation of low-density lipoproteins [21]. It is well-known that the antioxidant properties of *o*-diphenols are related to hydrogen-donation, i.e., their ability to improve radical stability by forming an intramolecular hydrogen bond between the free hydrogens of their hydroxyl group and their phenoxyl radicals [22]. In fact, although specific investigations on the structure–activity relationship of olive oil phenols are yet to be carried out, similar studies have been performed on flavonoids and have indicated that the degree of antioxidant activity is correlated with the number of hydroxyl substitutions [23]. Particularly, the *o*-diphenol substitution confers a high antioxidant capacity, whereas single hydroxyl substitutions, as in the case of tyrosol, provide no activity [24].

An important aspect of the study of antioxidants is the assessment of antioxidant activity. Various methods have been introduced to test antioxidant activity of olive oil where most of them investigate the ability of oil to scavenge free radicals [14, 25, 26]. The objective of this study was to analyze phenolics, *o*-diphenolics, fatty acids, α-tocopherol and pigments in five virgin olive oils and determine both their ability to scavenge free radicals and their induction period. Correlations among these parameters were studied and discussed in the hope of evaluating their possible significance for the quality and stability of virgin olive oil obtained from olives planted in new growing areas.

Materials and Methods

Plant Material and Growing Areas Selected

Olive fruits (*Olea europaea* L.) of the cultivars Arbequina, Koroneiki, Leccino, Oueslati and Chemchali were collected in the 2007–2008 season at approximately the same ripeness index [27] varied between 2 and 3 from trees in the same orchard, Boughrara Conservatoire [a national Collection, 35 km from Sfax (34°44′N; 10°46′E)—the south of Tunisia) that received the same cultural practices. Fifteen-year-old olive trees (*Olea europaea* L.) were used. The experimental field Boughrara Conservatoire is characterized by a rainfall of 220 mm/year, an average temperature of 20 °C, an average humidity of 60% and a deep and sandy ground. Trees were planted at a layout of 12 × 8 m (density of 104 plant/ha, no irrigation).

Oil Extraction Process

The olives were picked manually using rakes. After harvesting, the olive fruit samples were immediately transported on the same day, to the laboratory where they were transformed into oil within 24 h. Only undamaged and uninfected fruits were processed at laboratory scale system (Abencor analyzer). All the olive fruits were washed and deleafed, 1.5–2 kg of olives were crushed with a hammer mill and slowly mixed for about 30 min at 27 °C. Then, the paste obtained was centrifuged at 3,500 rpm for 3 min to extract the oil. Finally, the oils were decanted and immediately stored in dark bottles at 4 °C until analysis.

Analytical Methods

Quality Parameters

Determination of free fatty acids (FFA), peroxide value (PV) and UV absorption characteristics at 232 and 270 nm were carried out following the analytical methods described in the European Union Regulations EEC 2568/91 and EEC 1429/92 [28]. The *p*-anisidine value (*p*-AV) was determined following the AOCS official method (Cd 18-90) [29], using a UV–vis spectrophotometer. The TOTOX index was derived from the PV and the *p*-AV, according to the following equation:

$$\text{TOTOX} = (2\text{PV}) + p - \text{AV}$$

Fatty Acid Methyl Esters (FAMEs) Analysis

The FAMEs were prepared from olive oil by vigorously shaking a solution of the oil in hexane (0.1 g in 2 ml) with 0.2 ml of 2N methanolic sodium as described in the Regulations EEC/2568/91, EEC/1429/92 of the European Union Commission [28]. The individual FAMEs were separated and quantified by gas chromatography by using a model 5890 series II instrument (Hewlett-Packard Ca Palo Alto, Calif) equipped with a flame ionisation detector, and a fused silica capillary column (HP-INNOWAX, 30 m × 0.25 mm × 0.25 μm) according to the method described by Dabbou et al. [30]. Results of FAMEs were expressed as a relative area percentage of the total.

Iodine values (IV) were calculated according to Maestri et al. [31] from fatty acid percentages whereas the oxidative susceptibility (OS) of oils was estimated according to Cert et al. [32].

Total Phenols and o-Diphenols

The phenolic extract was obtained as previously reported [33]. Briefly, 10 ml of a methanolic solution [methanol/water (80:20, v/v) and 20 mg of Tween 20 (2%, v/w)] were homogenized with 10 g of olive oil, using an Ultra-Turrax T25 (IKA Labortechnik, Janke & Kunkel, Staufen, Germany), for 1 min at 15,000×*g* and then centrifuged at 5,000×*g* for 10 min at 4 °C; the extraction was repeated twice. To eliminate the oil droplets, the methanolic extract was kept in a freezer for 24 h. Then, total phenols and *o*-diphenols were determined colorimetrically [33] and the results are expressed as hydroxytyrosol equivalents.

Pigments

Carotenes and chlorophylls were determined as described by Minguez-Mosquera et al. [34] using 7.5 g of oil dissolved in cyclohexane. The carotene and chlorophyll pigments were determined by measuring the absorbance at 470 and 670 nm, respectively. The results were expressed as mg of pheophytin "a" and lutein per kg of oil, respectively.

α-Tocopherol

α-Tocopherol was measured by the method reported by Gimeno et al. [35]; 200 μl of a solution of oil was transferred to a screw capped tube, where 600 μl of methanol and 200 μl of the internal standard solution (300 mg ml^{-1} of α-tocopherol acetate in ethanol) were added. The chromatographic separation was performed using a liquid chromatography system with an HP-1100 pump system and a photodiode array detection system (HP1100). The column was Tracer Extrasil ODS-2 (150 × 4.6 mm × 5 μm). The mobile phase was methanol–water (96:4, v/v) and the elution was performed at a flow-rate of 2 ml min^{-1}. The analytical column was kept at room temperature. Detection was performed at 292 nm and each run lasted 6 min [36].

Radical Scavenging Activity (RSA) Using DPPH Assay (DPPH)

The effect of the methanolic extracts obtained previously from the different virgin olive oils, on DPPH* (1,1-diphenyl-2-picrylhydrazyl radical) was estimated according to the procedure described by Brand Williams et al. [7]. DPPH* is a stable radical in methanol solution. Extracts can scavenge the DPPH* and the reduction of DPPH* is monitored by the decrease of the absorbance at 515 nm at 25 °C. The color from purple, in the initial solution, turns to yellow when all the free radicals have been blocked by the antioxidants. An aliquot of methanol solution (0.1 ml) containing the methanolic extracts, was added to 3.9 ml of DPPH solution (6 × 10^{-5} M in methanol), mixed well and the absorbance was measured after 60 min ($sample_{60}$). The absorbance of the DPPH* without any antioxidant in methanol ($control_0$) was measured daily and kept in the dark. The radical scavenging activity (RSA) toward DPPH*

was expressed as the % of scavenging effect = 100 × (1 − absorbance of sample$_{60}$/absorbance of control$_0$). The concentration of the sample required to scavenge 50% of the DPPH free radical, was determined from the plot between %inhibition and concentration and labelled as EC50. The experiments were performed in triplicate.

Total Antioxidant Status (TAA Test with ABTS)

The free radical scavenging activity of the phenolic fraction of olive oil obtained previously was also determined by an ABTS radical cation discoloration assay. A stock solution of 7 mM of 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) aqueous solution was prepared. ABTS radical cations (ABTS•+) were produced by the reaction of the ABTS stock solution with 2.45 mM potassium persulfate (final concentration) [23, 37]. The stock solution was kept in the dark at room temperature for 16 h, allowing it to form the ABTS radicals (ABTS*$^+$). The radicals were stable in this form for more than 2 days when stored under these conditions. Finally, the stock solution was diluted with ethanol (approx. 1/88) to obtain an absorbance of 0.7 ± 0.02 at 734 nm and equilibrated at 30 °C. A reagent blank reading was taken (A0). For the spectrophotometric assay, 3.9 ml of the ABTS*$^+$ diluted solution was mixed with 100 µl of phenolic fraction or trolox. Mixtures were mixed vigorously for 30 s and allowed to stand for 6 min in the dark at room temperature. Then, the absorbance for each sample (ABTS*$^+$ solution plus compound, At) was measured at 734 nm and corrected for the absorbance of a control (ABTS*$^+$ solution without test sample, A0). The absorbance reading was taken at 30 °C exactly 6 min after the initial mixing. The radical-scavenging activity of the samples was expressed as mmol Trolox equivalent (TE) per kg.

Accelerated Oxidation Tests

Measurement of the induction period (IP) was performed using the well-established Rancimat method. A Rancimat apparatus, model 734 (Metrohm, Herisau, Switzerland) was operated at 120 °C [38]. A dry air flow of 20 l h^{-1} was passed through the oil sample (3 ± 0.001 g). The volatile oxidation products, arising from the oxidation of the oil, were dissolved in cold milliQ water (60 ml) causing an increase in the electrical conductivity parameter value. This test is based on the change of conductivity of the distillate collected from oil subjected to an accelerated oxidation at a prefixed temperature. The change of conductivity is due to the production of formic and other carboxylic acids because of the oxidation of secondary products during the forced oxidation. All tests were performed in triplicate. The time taken to reach an inflection point at the induction curve was considered as the IP.

Statistical Analysis

All parameters analyzed were carried out in triplicate. The results were reported as mean values of three repetitions and the standard deviation. Significant differences among cultivars studied were determined by analysis of variance which applied a Duncan's multiple range test with a 95% significant level ($p = 0.05$), using the SPSS program, release 11.0 for Windows (SPSS, Chicago, IL, USA). To point out the correlation between the analyzed parameters, Pearson's test was carried out.

Results and Discussion

Analytical Parameters of Olive Oils

Table 1 lists the values of several parameters of the virgin olive oils obtained from different olive cultivars. Oils are grouped according to their area of origin where they were grown. Quality and authenticity criteria for various olive oil types are described in detail in the EEC/2568/91 [28]. Physico-chemical characteristics of all the oils produced and analyzed had values (Table 1) completely within the limits required by the IOC [39] as required for the 'extra virgin' category.

Free Fatty Acids

In the samples studied, the FFA values remained below the limits reported by the IOC [39], which prescribes a value below 0.8 g of oleic acid per 100 g for a virgin olive oil, these values ranged from the minimum for the oil obtained from the Arbequina cultivar (0.2%) to the maximum for the oil obtained from the Koroneiki cultivar (0.62%).

Peroxide, *p*-Anisidine and Totox Values

The content of hydroperoxides in the oil under examination is expressed by the PV. PV varied from 3.92 to 11.50 meq O_2 kg^{-1} which are values lower than that described in the EU Regulations (20 mequiv O_2 kg^{-1}). This parameter is influenced by natural circumstances (e.g. temperatures below freezing, dacic infestations, drought, etc.), olives incorrectly harvested, bad processing during milling, incorrect hygiene in the olive-press and/or of the vessels and finally storage conditions as prolonged exposure of the oil to light or heat sources [40]. Determination of α and β-alkenals content are based on the measurement of the *p*-AV, which is another possibility for assessing advanced oxidative rancidity [41]. The studied oils had *p*-AV valued that ranged from the minimum for Leccino (1.81) to the

Table 1 Quality indices of virgin olive oils from studied varieties

Parameters	Introduced cultivars			Autochthonous cultivars	
	Arbequina	Koroneiki	Leccino	Oueslati	Chemchali
FFA (% of Oleic acid)	0.20 ± 0.03^{b}	0.62 ± 0.23^{a}	0.48 ± 0.04^{a}	0.27 ± 0.02^{b}	0.24 ± 0.01^{b}
PV (meq O_2 kg^{-1})	$6.67 \pm 0.67^{b,c}$	$5.92 \pm 1.08^{b,c}$	3.92 ± 0.92^{c}	11.50 ± 3.83^{a}	$8.84 \pm 1.50^{a,b}$
p-AV	2.23 ± 1.25^{a}	2.08 ± 1.32^{a}	1.81 ± 0.65^{a}	2.29 ± 1.13^{a}	2.95 ± 0.68^{a}
Totox	15.56 ± 2.58^{b}	13.90 ± 0.85^{c}	9.64 ± 1.18^{c}	25.29 ± 6.54^{a}	$20.62 \pm 2.32^{a,b}$
K232	2.47 ± 0.05^{a}	1.81 ± 0.00^{b}	1.89 ± 0.02^{b}	2.46 ± 0.10^{a}	2.33 ± 0.38^{a}
K270	0.21 ± 0.00^{a}	0.22 ± 0.01^{a}	0.19 ± 0.00^{b}	0.21 ± 0.01^{a}	0.21 ± 0.01^{a}

Values are the means of the three different VOO samples ($n = 3$) ± standard deviations

FFA free fatty acids, *PV* peroxide value, *p-AV* *p*-anisidine value

Different letters indicate significant differences ($p < 0.05$) between cultivars

maximum for Chemchali (2.95). Total oxidation of the studied oils, evaluated as TOTOX (2PV + *p*-AV), was more than 2 times higher in Chemchali and Oueslati oils (20.62 and 25.29, respectively) than in Leccino oil. Statistical analysis of olive oils showed that cultivars had no significant influence on these analytical parameters (Table 1), which confirms a previous study [42].

Spectrophotometric Investigation in Ultraviolet

It is known that the "ageing" of the oil, with its phenomena of oxidation, increases the value of the spectrophotometric indices which are the essential parameters for the determination of olive oil quality [40]. This test consists of measuring K232 and K270 parameters determined during the same analytic procedure. The greater the value of K232 is, the greater the concentration of conjugated dienes is, whereas K270 is proportional to the concentration of conjugated trienes. However, compounds of oxidation of the conjugated dienes contribute to K232 while compounds of secondary oxidation (aldehydes, ketones, etc.) contribute to K270 which measures, consequently, the conjugated ketodienes produced from polyunsaturated lipids [43]. Extra virgin olive oil is required to have an extinction coefficients of less than 2.50 and 0.22 at 232 and 270 nm, respectively. The five oil samples studied had K232 absorbance values below the limit allowed by the EEC Regulations for classification of olive oil. However, the K270 values were slightly higher in the oils studied except for Leccino oils (0.19).

Furthermore, it can be concluded from Table 1 that, contrary to the other parameters, there are no significant differences among *p*-AV and K270 values in the different olive oils samples. This result could be explained by the fact that both *p*-AV and K270 estimates the secondary oxidation products of unsaturated fatty acids, principally conjugated dienals and 2-alkenals [42].

Fatty Acids

The distribution of fatty acids, from all tested samples, covered the normal expected range for olive oil (Table 2) except for margaroleic and gadoleic acids. Fatty acid compositions of the oils differ according to the cultivars since the olives were from the same zone of production, latitude, climate and harvested at the same ripeness index. This result is in accordance with previous findings showing that the fatty acid composition is one of the characteristics predominantly genetically determined, with a lesser impact of the environmental factors [44]. However, the higher levels of margaroleic and gadoleic acids could be the result of pedoclimatic conditions since being cultivated in the north of Tunisia [30], these cultivars had shown levels within the range of extra virgin olive oil. Furthermore, Greek (Koroneiki) and Italian (Leccino) olive oils were low in linoleic and palmitic acids and had a higher percentage of oleic acid (>71%). Tunisian olive oils were high in linoleic acid but had moderate levels of palmitic acid. Oleic acid content was higher in Oueslati olive oil than in Chemchali oil. In fact, the low oleic acid level of Chemchali oil can be attributed to a genetic factor [45] whereas the low oleic acid level in the Arbequina cultivar to its low adaptation to Tunisian geographic conditions [30]. The Oleic/Linoleic acid ratios (O/L) of Leccino and Koroneiki (8.9 and 10.34, respectively) were quite higher than those of Arbequina, Oueslati and Chemchali oils (<4.5).

Pigments

The green-yellowish color of virgin olive oil, which is considered a quality parameter and an influential factor in consumer's preferences, is due to the presence of various pigments, i.e. chlorophylls and carotenes [5]. In fact, depending on their types (chlorophyll a or b; pheophytin a or b) and on the presence of light, these pigments can act

Table 2 Fatty acids composition (%) evaluated in the virgin olive oils samples from studied varieties

	Introduced cultivars			Autochthonous cultivars	
	Arbequina	Koroneiki	Leccino	Oueslati	Chemchali
Palmitic	15.37 ± 0.02^{a}	10.24 ± 0.03^{c}	11.73 ± 0.20^{b}	10.09 ± 0.05^{c}	11.81 ± 1.28^{b}
Palmitoleic	2.72 ± 0.13^{a}	0.85 ± 0.00^{b,c}	1.11 ± 0.04^{b}	0.70 ± 0.00^{c}	0.89 ± 0.32^{b,c}
Margaric	0.13 ± 0.01^{a}	0.06 ± 0.00^{b}	0.06 ± 0.00^{b}	0.05 ± 0.00^{b}	0.06 ± 0.01^{b}
Margaroleic	0.31 ± 0.02^{a}	0.12 ± 0.02^{b}	0.12 ± 0.06^{b}	0.10 ± 0.02^{c}	0.10 ± 0.00^{b,c}
Stearic	1.53 ± 0.04^{d}	2.91 ± 0.01^{a}	2.81 ± 0.09^{a}	2.20 ± 0.23^{c}	2.56 ± 0.06^{b}
Oleic	61.42 ± 0.25^{d}	75.96 ± 1.08^{a}	71.08 ± 0.45^{b}	69.26 ± 0.87^{c}	61.31 ± 1.33^{d}
Linoleic	16.42 ± 0.58^{b}	7.49 ± 1.23^{c}	8.41 ± 2.29^{c}	15.33 ± 0.84^{b}	20.10 ± 0.57^{a}
Linolenic	0.71 ± 0.03^{b,c}	0.87 ± 0.01^{a}	0.63 ± 0.11^{c}	0.80 ± 0.01^{a,b}	0.70 ± 0.13^{b,c}
Arachidic	0.24 ± 0.00^{b}	0.35 ± 0.01^{a,b}	0.39 ± 0.13^{a}	0.32 ± 0.01^{a,b}	0.34 ± 0.00^{a,b}
Gadoleic	1.01 ± 0.14^{b}	1.01 ± 0.13^{b}	0.60 ± 0.00^{c}	1.12 ± 0.25^{b}	1.90 ± 0.06^{a}
Lignoceric	0.13 ± 0.13^{a}	0.13 ± 0.05^{a}	0.19 ± 0.01^{a}	0.04 ± 0.04^{a}	0.20 ± 0.03^{a}
Oleic/Linoleic ratio	3.74 ± 0.15^{b}	10.34 ± 1.86^{a}	8.90 ± 2.46^{a}	4.53 ± 0.31^{b}	3.05 ± 0.02^{b}
Iodine value	89.67 ± 0.79^{a,b}	85.13 ± 1.28^{b,c}	81.98 ± 4.92^{c}	92.97 ± 0.76^{a}	94.40 ± 3.48^{a}
Oxidative susceptibility	874.33 ± 29.02^{b}	501.46 ± 54.88^{c}	513.40 ± 115.09^{c}	839.99 ± 37.62^{b}	1037.19 ± 126.31^{a}

Values are the means of the three different VOO samples ($n = 3$) ± standard deviations

Different letters indicate significant differences ($p < 0.05$) between cultivars

as prooxidants in the presence of the light (photooxidation process) and as antioxidants in darkness (autoxidation), which is of concern in this study.

Significant differences in pigment levels between cultivars ($p < 0.05$) were observed (Table 3). The levels of 7.51–15.39 mg kg^{-1} and 3–6.32 mg kg^{-1} for chlorophylls and carotenes were observed, respectively. These levels of green–yellow colors of virgin olive oil are influenced by the olive cultivar, maturation index, as a quality index though no standardized method exists for its measurement [34, 46], and handling and duration of storage. Pigments undergo a natural breakdown during fruit ripening and oil storage [47] and could be considered as an indicator of product freshness. Furthermore, pigments contents were similar in autochthonous oils (Oueslati and Chemchali) and Spanish and Italian oils (Arbequina and Leccino), whereas Koroneiki oil had the highest levels.

Total Phenols and *o*-Diphenols

Evidence suggests that phenolic compounds influence the sensory quality [48] and oxidative stability [19] of olive oil and have beneficial biological activity [49]. The total phenols and *o*-diphenols results expressed as hydroxytyrosol equivalents are given in Table 3. Significant differences ($p < 0.05$) were observed between different cultivars studied where the ranges for samples of olive oils were wide (i.e. 175.75–241.52 mg kg^{-1} for total phenol concentration and 93.61–160.71 mg kg^{-1} for *o*-diphenols). However, the highest amount of the total phenols was present in Koroneiki Greek olive oil (241.52 mg kg^{-1}) followed by Chemchali Tunisian oil (207.7 mg kg^{-1}). Thus, our samples were relatively poor in total phenols since the average reported contents varied from 200 to 500 mg kg^{-1} [1]. *o*-Diphenols concentrations were also studied and differences were found in their content in the analyzed samples. Contrary to phenol concentrations, the highest content of *o*-diphenols was observed in Chemchali oil (160.71 mg kg^{-1}), followed by Koroneiki (142.38 mg kg^{-1}), Oueslati and Arbequina (136 and 134 mg kg^{-1}), while Leccino oil showed the lowest value (93.61 mg kg^{-1}). These observations suggest the existence of a genotype effect since the extraction system, the olive ripeness, pedoclimatic and agronomic conditions were the same for the cultivars studied.

α-Tocopherol

α-Tocopherol is the predominant tocopherol in olive oils [44]. The α-tocopherol content between cultivars was significantly different ($p < 0.05$). In fact, α-tocopherol amounts ranged from 152.9 mg kg^{-1} (Arbequina cultivar) to 369 mg kg^{-1} (Leccino cultivar) (Table 1). These results are in agreement with previous reports found suggesting that tocopherol content is highly variety-dependent [50].

Accelerated Oxidation Tests

The accelerated oxidation test (Table 3) is useful for evaluating the effects of antioxidants on olive oil resistance to oxidation, and to compare the storage stability of different oils. Results showed that the induction time varied according to the cultivar. In the Koroneiki and Oueslati

Table 3 Stability parameters of virgin olive oils from studied varieties

Parameters	Introduced cultivars			Autochthonous cultivars	
	Arbequina	Koroneiki	Leccino	Oueslati	Chemchali
Carotenes (mg kg^{-1} of oil)	3.02 ± 0.37^{d}	6.32 ± 0.60^{a}	3.82 ± 0.12^{c}	4.41 ± 0.06^{b}	4.95 ± 0.00^{b}
Chlorophylls (mg kg^{-1} of oil)	7.51 ± 1.40^{c}	15.39 ± 3.28^{a}	7.89 ± 1.29^{c}	10.25 ± 0.08^{b,c}	12.09 ± 0.50^{b}
α-Tocopherol (mg kg^{-1} of oil)	152.93 ± 7.24^{d}	251.44 ± 5.27^{b}	369.04 ± 22.38^{a}	191.65 ± 6.70^{c,d}	213.24 ± 43.00^{b,c}
o-Diphenols (mg hydroxytyrosol kg^{-1} of oil)	134.78 ± 44.20^{a}	142.38 ± 15.57^{a}	93.61 ± 14.98^{b}	136.82 ± 3.83^{a}	160.71 ± 6.01^{a}
Total phenols (mg hydroxytyrosol kg^{-1} of oil)	189.11 ± 39.87^{b}	241.52 ± 1.72^{a}	176.16 ± 11.38^{b}	175.75 ± 16.52^{b}	207.70 ± 0.70^{a,b}
IP (h)	0.68 ± 0.50^{c}	12.66 ± 1.38^{a}	5.57 ± 2.08^{b}	12.11 ± 1.08^{a}	2.28 ± 0.08^{c}
EC50 (60 min)	7.37 ± 0.85^{e}	18.41 ± 0.37^{b}	13.48 ± 0.34^{c}	10.13 ± 1.16^{d}	19.90 ± 0.67^{a}
TAA-ABTS (mM trolox kg^{-1})	0.05 ± 0.00^{e}	0.24 ± 0.01^{a}	0.13 ± 0.01^{c}	0.09 ± 0.00^{d}	0.22 ± 0.01^{b}

Values are the means of three different VOO samples ($n = 3$) ± standard deviations

IP induction period, *EC50* the concentration of sample required for 50% scavenging of the DPPH free radical, *TAA-ABTS* total antioxidant status test with ABTS

Different letters indicate significant differences ($p < 0.05$) between cultivars

cultivars, olive oil had a long induction time (>12 h) followed by Leccino olive oil (5.57 h) whereas the other cultivars (Arbequina and Chemchali) showed very low values (<2 h). The different oxidative stabilities of the five samples are probably due to the combination of fatty acid composition and the effect of various pro/antioxidants present in the oils [44]. The higher stability of Koroneiki, Oueslati and Leccino oils is probably due to a relatively low content of polyunsaturated fatty acids and higher contents of antioxidants. The fatty acid composition was similar in the Chemchali and Arbequina oils, thus the higher concentrations of total phenols, *o*-diphenols, pigments and α-tocopherol may explain the higher stability of Chemchali oil compared to Arbequina oil. The contribution of fatty acids to stability seems to be less pronounced.

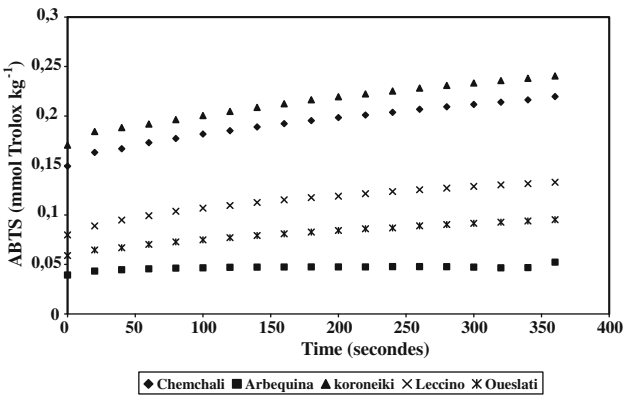

Fig. 1 The radical scavenging activities of phenolic extracts from olive oils as measured by ABTS method. Data are expressed in mmol Trolox kg^{-1} after the reaction between the radical and the extract during 6 min

Antioxidant Capacities

The results of the free radical scavenging properties of all olive oils studied evaluated by the two different methods are summarized in Table 3 and Fig. 1. The radical scavenging capacity of the phenolic antioxidants in the study is shown in Fig. 1. Standard deviation values for all compounds were below 6%. All samples tested were able to scavenge ABTS* radical cations due to their antioxidant compounds (Table 2). Arbequina olive oils had the lowest value (0.05 mM), while oils from Koroneiki and Chemchali cvs had the highest values (0.24 and 0.22 mM, respectively). No significant differences were observed in the values at 1 and 6 min for the oils from Arbequina (Fig. 1). Differences in ABTS* scavenging were more obvious for the oils from the other cultivars where they reached two times the values in Leccino and Oueslati oils.

Chemchali and Koroneiki oils had the highest EC50 values (19.90 and 18.41%) and Arbequina olive oils had the lowest value (7.37%). Consequently, the antiradical activities tested showed that Chemchali oils had the highest capacity followed by Koroneiki, Leccino, Oueslati and Arbequina. The higher stability of Koroneiki, Oueslati and Leccino oils can be explained by the higher contents of total phenols or *o*-diphenols.

Stability and Antioxidants Compounds

In the present work, we determined the antioxidant capacity of the different compounds by means of a number of procedures that measure the induction period of lipid oxidation by the Rancimat method to test oxidative stability in oils [19] and the disappearance of free radicals (ABTS*, DPPH*, etc.) by the effect of radical scavengers [7, 51] in the hope of evaluating the role of the endogenous compounds. The correlation between the antioxidant compounds and the antioxidant capacities of all studied

olive oils is given in Table 4. Statistical results indicate that the major factor affecting radical-scavenging activity was the type of the pigments (carotenes or chlorophylls with positive correlations $r = 0.77$**, $p = 0.001$ and $r = 0.69$**, $p = 0.04$, respectively) followed by total phenols ($r = 0.554$*, $p = 0.032$). Although the correlation was significant, the level of the correlation was rather moderate. This result can be attributed to the double role of the pigments as prooxidants in photooxidation and antioxidants in darkness. Phenolic content is an important parameter in the evaluation of the extra virgin olive oil quality because phenols protect the triacylglycerols from oxidation and contributes to oil flavor and aroma [52]. A high total phenolic content appears to be beneficial for the shelf life of the oil [53] where a good correlation of stability (expressed as TAA or RSA) and total phenol content was observed (Table 4). Our study, based on TAA-ABTS test and RSA-DPPH assay (Table 4), revealed that the best correlation between total phenol contents and antioxidant capacity was given by the ABTS method ($r = 0.641$*, $p = 0.01$) which is in accordance with the findings of Samaniego Sánchez et al. [54]. These conclusions seem to be the same for the contents of carotenes and chlorophylls ($r = 0.85$**, $p = 0.00$ and $r = 0.76$**, $p = 0.001$ respectively) and FFA ($r = 0.54$*, $p = 0.036$).

Results presented in Table 4 show a negative correlation between the PV and lipid profile, especially the O/L ratio ($r = -0.57$*, $p = 0.026$). This correlation was low for *p*-AV. Antioxidants present in oils are important for the stabilization of FFA [19]. FFA as a measure of hydrolytic rancidity and their determination is a general indicator of the palatability and edibility of the oil [55]. Therefore, our results (Table 4) show a good positive correlation between FFA and the shelf life measured by Rancimat (IP) ($r = 0.58$*, $p = 0.023$). Negative correlations between FFA and OS and *p*-AV were established ($r = -0.708$**, $p = 0.003$ and $r = -0.519$*, $p = 0.047$, respectively). Tocopherols are known as powerful lipid radical scavengers. Their antioxidative effect, however, strongly depends on various parameters such as concentration, degree of unsaturation of the lipid substrate, and presence of other antioxidants [55]. No significant correlation was observed between α-tocopherol and IP (Table 4) whereas there is a good correlation between IP and carotenes ($r = 0.609$*, $p = 0.016$) and oleic acid ($r = 0.830$*, $p = 0.00$). These data are in agreement with the findings of Baldioli et al. [19], which showed that the contribution of α-tocopherol to VOO oxidative stability, although its impact, evaluated by the Rancimat test, was smaller compared to those of polar phenolic [9, 56], fatty acid composition [9] and total chlorophylls [56]. At high temperature, tocopherols are unstable, so they contribute little to defining the IP value [19]. The phenol fraction had a more powerful action against radicals ($r = 0.64$*, $p = 0.01$ and $r = 0.554$*, $p = 0.032$) when ABTS* and DPPH* were added to the sample (Table 4). Results showed that the correlation with lipophilic phenols (α-tocopherol) was more important than hydrophilic phenols (total phenols and *o*-diphenols). In fact, previous research on the radical scavenging activity or Rancimat measurement have shown that phenolics are generally considered to be the most effective antioxidants in VOO [44], which is not in agreement with the results of the present study. However, the measurement of the total phenolics in these studies was carried out by the Folin–Ciocalteu colorimetric assay which does not take into account the phenolic profile. Indeed, the phenolic profile varies considerably between various VOO samples which influence their antioxidant activity [57]. As for phenols, *o*-diphenols have not shown a good ability to scavenge free radicals. This data is not in accordance with Lavelli [57] indicating that the *o*-diphenols family can be identified as the main source of the overall antioxidant activities of EVOOs also compared to tocopherol [44]. When, the two parameters (ABTS and DPPH) were correlated with each other, their correlation coefficients were very high and significant ($r = 0.96$**; $p = 0.00$). In fact, this high correlation of ABTS and DPPH assays is based on the scavenging of radical cations. The presence of antioxidant species leads to the disappearance of these radical chromogens which can be followed by spectrophotometric methods. Moreover, these electrons transfer mechanism and the hydrogen atom abstraction are a marginal reaction pathway because they occur slowly in strong hydrogen-bond accepting solvents [58, 59]. Therefore, the two assays may be considered as parameters of stability against oxidation. In addition, both IP values and tests able to scavenge a free radical (ABTS and DPPH) depend on the phenolic pool, even though the Rancimat value takes into account the whole oil moiety [10] whereas ABTS and DPPH values consider any amphipathic antioxidant extracted with 80% methanol. Then, this methanolic extract did not contain lipophilic compounds such as tocopherol and fatty acids [33]; which confirms our findings in the non significant correlation between IP and ABTS and DPPH values. Data from oxidative susceptibility and the O/L ratio (Table 4) showed a significant negative correlation ($r = -0.95$**, $p = 0.000$). Furthermore, the Rancimat test only gives a total estimation of the antioxidant potential of the oil, without information on the possible contribution of single compounds and their positive or negative interactions. Then, oxidative stability is not dependent on a single parameter, but is rather affected by the fatty acid composition and a complex pool of antioxidants and prooxidants [60].

Table 4 Matrix of correlation of selected parameters of virgin olive oils from the studied varieties

Correlation matrix	Phenols	*o*-Diphenols	α-Tocopherol	Carotenes	Chlorophylls	TAA-ABTS	EC50	C18:1	O/L ratio	IP	FFA	PV	*p*-AV	OS
Phenols	1													
o-Diphenols	0.631*	1												
α-Tocopherol	ns	−0.543*	1											
Carotenes	0.726**	ns	ns	1										
Chlorophylls	0.735**	ns	ns	0.956**	1									
TAA-ABTS	0.641*	ns	ns	0.855**	0.756**	1								
EC50	0.554*	ns	ns	0.771**	0.689**	0.965**	1							
C18:1	ns	ns	0.527*	0.592*	ns	ns	ns	1						
O/L ratio	ns	ns	0.610*	ns	ns	ns	ns		1					
IP	ns	ns	ns	0.609*	ns	ns	ns	0.830*	ns	1				
FFA	ns	ns	0.574*	ns	ns	0.544*	ns	0.764**	0.684**	0.582*	1			
PV	ns	ns	ns	ns	ns	ns	ns	ns	−0.572*	ns	ns	1		
p-AV	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	−0.519*	ns	1	
OS	ns	0.583*	−0.607*	ns	ns	ns	ns	−0.858**	−0.950**	ns	−0.708**	0.592*	ns	1

EC50 the concentration of sample required for 50% scavenging of the DPPH free radicals, *TAA-ABTS* total antioxidant status test with ABTS, *C18:1* Oleic acid, *O/L ratio* oleic/linoleic ratio, *OS* oxidative susceptibility, *IP* induction period, *FFA* free fatty acids, *PV* peroxide value, *p*-AV *p*-anisidine value

* Significant correlation (0.05)

** Significant correlation (0.01)

Conclusion

European Extra virgin olive oils produced under Tunisian growing conditions were evaluated for the first time to identify their oxidative stability in relation to their endogenous compounds. Firstly, the different cultivars analyzed produced oils with different composition where Koroneiki, Leccino and Oueslati oils showed the best fatty acid and phenolic compositions. Secondly, VOOs studied varied greatly in oxidative stability, which depends on many intrinsic factors such as fatty acid composition and natural levels of pro/antioxidants. These natural antioxidants, despite their low concentrations, prevent oil oxidation. As a matter of fact, European oils showed better antioxidant activity except for Arbequina oil. In addition to the individual role of each antioxidant compound to monitor the oils stability, there may also be the effect of synergism of all the antioxidants present such as phenols, lipidic profile, α-tocopherol, and pigments which can contribute in such monitoring.

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