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Phenolic Composition, Sugar Contents and Antioxidant Activity of Tunisian Sweet Olive Cultivar with Regard to Fruit Ripening

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Dhokar olive cultivar growing in the south of Tunisia is an unusual olive tree which is characterized by the sweet taste of its fruit. We were particularly interested in the phenolic compounds of this cultivar in comparison to Chemlali cv. During the different maturation stages of olive fruits, the phenolic composition, sugar content, antioxidant activity and enzymatic change were examined. The phenolic composition was studied by using reverse-phase high-performance liquid chromatography followed by LC−MS and GC−MS analysis. Oleuropein, the major olive fruit biophenolic compound, decreased significantly during all the ripeness stages, and its level decreased from 3.29 g/kg fresh olive (July) to 0.16 g/kg (October) in Dhokar cv. and from 5.7 g/kg (July) to 3.75 g/kg (October) in Chemlali cv. This decrease inversely correlated with hydroxytyrosol concentrations until September. DPPH and ABTS assays show that the more important antioxidant capacity of olive extracts was found at the last stage of maturation. β-Glucosidase and esterase activities were evaluated during the ripening stage. The data obtained during the ripening indicate that polyphenol content and composition, in particular the oleuropein concentration, were in correlation with the measured enzymatic activities. Glucosidase and esterase showed their maximum values in September reaching 179.75 and 39.03 U/g of olive pulp, respectively. In addition, sugar content was quantified using colorimetric and chromatographic methods and was compared to its concentration in Chemlali olive fruit cultivar. Glucose and mannitol were the main sugars; they reached their highest level at the last stage of ripening: 8.3 and 79.8 g/kg respectively.

KEYWORDS: Dhokar cultivar; sweet olive fruits; ripening; total phenol content; oleuropein; hydroxytyrosol; sugar; enzymatic activities

INTRODUCTION

The olive tree is ubiquitous to countries in the Mediterranean basin and to a few other regions of the world with an appropriate climate. In Tunisia, olive trees cover an area of 1.6 million hectares and account for about 65 million trees which represent more than 4% of the olive oil produced in the world. Indeed, it is the second largest exporter of olive oil in the world. Tunisia is an important olive-producing country with a large variety of olive plantation systems. Because of the plasticity of the Tunisian olive oil varieties, the olive-growing areas spread from the northern to the southern regions, where a wide range of edapho-climatic conditions prevail, from lower semiarid to arid conditions. In the south of the country, tree density is generally very low (≤20 trees ha $^{-1}$). Olive trees make up at least 75% of the total number of trees, and olive there is known for its ability to exploit soil water at great depths (*1*). Chemlali and Chetoui are the two main olive cultivars in Tunisia, while Dhokar olive cultivar is one of the rare olive trees in Tataouine zone (South of Tunisia). The Dhokar variety is used as a pollinator for the Chemlali Tataouine variety and is grown as a very small number of trees because of the limited quantity of its oil. Varieties of that region, mainly Dhokar cultivar, are still commercially unexplored, and their chemical characteristics have not been characterized. Almost all studies on Tunisian olive varieties have focused on biochemical characterization of Chemlali and Chetoui cultivars (*2*−*5*).

The present study was motivated by the fact that a rise in temperature, low rainfall and soil erosion are becoming the principal environmental problems associated with olive cultivation in the arid Tataouine zone (*6*). Dhokar olive fruits are known by the absence of the bitter taste before the achievement of maturation. During the ripening process, several changes are noticed in the fruit: changes in weight, pulp/stone ratio and color as well as changes in chemical composition, oil accumulation and enzyme activity, which influence fruit firmness and olive oil chemical composition and sensory characteristics (*2, 7*).

Changes in phenol content during fruit development are important. In unripened olives, one of the main phenolic

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Table 1. Fruit Mass and Dominant Fruit Color at the Five Sampling Dates

harvest date	fruit mass (g)[a]	fruit color
July 05, 2006	1.15 ± 0.23 a	green
July 23, 2006	1.18 ± 0.12 a	green
Aug 14, 2006	1.38 ± 0.87 b	purple
Sept 15, 2006	1.48 ± 0.65 b	purple-black
Oct 23, 2006	1.56 ± 0.54 b	black

[a] The mass is the mean of 1 fruit derived from the weight of 10 fruits. Values given are the mean of three replicates ± standard deviation. Means with different letters are significantly different at $P \leq 0.05$.

compounds responsible for producing bitterness in fruits (*8*) is the secoiridoid oleuropein, a 3,4-dihydroxy-phenylethanol (hydroxytyrosol) ester with a β-glucosylated elenolic acid. This compound is known to be the most prominent and significant individual phenolic of olive pulp. The concentration of oleuropein varied with olive variety and declined with the fruit physiological development (*2, 5, 9*).

Several studies have focused on the phenolic content of olive. However, few works have been conducted on the sugar composition of olive fruit (*10*). There are some notable differences in sugar composition between the mature and immature fruit that are attributed to a series of chemical and enzymatic alterations of some polysaccharides during the maturation phase. These modifications include hydrolysis of polysaccharides by glycosidases and polymerization of free sugar (*10, 11*).

The aim of this study is to identify and quantify phenolic compounds and sugar in the sweet olive cultivar (Dhokar cv.) growing in the south Tunisia by using a reverse-phase high-performance liquid chromatography (HPLC) system and liquid and gas chromatography−mass spectrum (LC−MS and GC−MS). The evaluation of oleuropein, hydroxytyrosol, glucose and mannitol at different stages of maturation was compared to the main Tunisian variety, the well-known cultivar Chemlali. Moreover, an evaluation of enzymatic activities in the fruit was carried out to explain their effect on phenolic content. In addition, we studied the antioxidant potentials of olive extracts during all the maturation stages.

MATERIAL AND METHODS

Olive Samples. Olive fruits harvested from a Dhokar cultivar olive tree growing in Tataouine (Southern Tunisia) were picked by hand at five different ripening times from July to October in 2006. The samples analyzed are shown in **Table 1**. Olives (250 g in each harvest) were immediately frozen in liquid N_2 to block the enzymatic activities, and stored at −80 °C before analysis. In order to minimize the effect of the sun exposure, the collection was accomplished from different parts of the tree.

Extraction. The olive fruits were washed and crushed with a hammer crusher at 4 °C (using a bath of ice to avoid phenol oxidation). The obtained paste (100 g) was extracted according to the method reported earlier (*4*). A mixture of methanol and water (250 mL, 4:1 v/v) was added to the olive paste, and the mixture was left to stand under agitation for 24 h. Subsequently, the solution was filtered using GF/F filter paper and washed with hexane (100 mL) to remove oil. The extract was concentrated by Rotavapor to dryness at 40 °C, and the residue obtained was stored in glass vials, at 0 °C in the dark for HPLC analyses.

Reagents. Hydroxytyrosol and oleuropein were prepared as described previously (*12*). The phenolic compounds were dissolved in a mixture of methanol/water (8:2 v/v). Pure HPLC solvents were used in all cases. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,6-di-*tert*-butyl-4-hydroxytoluene (BHT), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and 2,2′-azinobis[3-ethylbenzothiazoline-6-sulfonate] (ABTS) were purchased from Sigma-Aldrich, USA.

HPLC Analysis. A reversed-phase high-performance liquid chromatographic technique was developed to identify and quantify the phenolic monomers. For this purpose, a standard mixture solution of phenolic compounds was analyzed. Sample concentrations were calculated, based on peak areas compared to those in external standards. The HPLC chromatograph was a Schimadzu apparatus equipped with a (LC-10ATvp) pump and a (SPD-10Avp) UV−vis detector. The column was (4.6 × 250 mm) (Shim-pack, VP-ODS), and the temperature was maintained at 40 °C. The flow rate was 0.5 mL/min. The mobile phase used was 0.1% phosphoric acid in water (A) against 70% acetonitrile in water (B) for a total running time of 40 min, and the gradient changed as follows: solvent B started at 20% and increased immediately to 50% in 30 min. After that, elution was conducted in the isocratic mode with 50% solvent B within 5 min. Finally, solvent B decreased to 20% until the end of running time.

LC−MS Analysis. The LC−MS/MS analyses were carried out with an Agilent 1100 LC system consisting of degasser, binary pump, auto sampler, and column heater. The column outlet was coupled to an Agilent MSD Ion Trap XCT mass spectrometer equipped with an ESI ion source. Data acquisition and mass spectrometric evaluation were carried out on a personal computer with Data Analysis software (Chemstations). For the chromatographic separation a Zorbax 300Å Extend-C-18 Column (2.1 × 150 mm) was used. The column was held at 95% solvent A (0.1% formic acid in water) and 5% solvent B (0.1% formic acid in acetonitrile) for 1 min, followed by an 11 min step gradient from 5% B to 100% B, then it was kept for 4 min with 100% B, and finally, the elution was achieved with a linear gradient from 100% B to 5% B in 2 min. The flow rate was 200 μL min^{-1} and the injection volume 5 μL. The following parameters were employed throughout all MS experiments: For electrospray ionization with positive ion polarity the capillary voltage was set to 3.5 kV, the temperature to 350 °C, the nebulizer pressure to 40 psi, and the drying gas flow to 10 L min^{-1}. The maximum accumulation time was 50 ms, the scan speed was 26,000 m/z s^{-1} (ultra scan mode) and the fragmentation time was 30 ms.

GC−MS Analysis. GC−MS analysis was performed with a HP model 5975B inert MSD, equipped with a capillary HP5MS column (30 m length, 0.25 mm internal diameter, 0.25 μm film thickness). The carrier gas was He used at 1 mL min^{-1} flow rate. The oven temperature program was as follows: 1 min at 100 °C, from 100 to 260 at 4 °C min^{-1} and 10 min at 260 °C. Olive paste was added to 40 mL of methanol/water (8:2), acidified at pH 2 by HCl (1 M) and then extracted with ethyl acetate (1:4 v/v). The organic layer was collected and reduced to 10 mL by rotary evaporation (37 °C) and then silylated. For the silylation procedure, a mixture of pyridine (40 μL) and BSTFA (200 μL) were added and vortexed in screw cap glass tubes and consecutively placed in a water bath at 80 °C for 45 min. From the silylated mixture 1 μL was directly analyzed by GC−MS.

Determination of Total Phenols. Total phenols were determined by using the Folin−Ciocalteu reagent according to the method of Singleton and Rossi (*13*). Appropriate dilutions are used for each extract sample. Briefly, a 50 μL aliquot of the extracts was assayed with 250 μL of Folin reagent and 500 μL of sodium carbonate (20%, w/v). The mixture was vortexed and diluted with water to a final volume of 5 mL. After incubation for 30 min at room temperature, the absorbance was read at 765 nm. Total phenols were expressed on a dry weight basis as gallic acid equivalents (GAE), using a calibration curve of a freshly prepared gallic acid solution. For the gallic acid, the curve absorbance versus concentration is described by the equation $y = 0.0012x - 0.0345$ ($R^2 = 0.9997$). The analyses were done in triplicate.

Determination of Total Flavonoids. Total flavonoids were measured by a colorimetric assay developed by Zhishen and al (*14*). A 1 mL aliquot of appropriately diluted sample (reconstituted in methanol/water 4:1 v/v) or standard solution of catechin (20, 40, 60, 80 and 100 mg/L) was added to a 10 mL volumetric flask containing 4 mL of double distillate H_2O. At zero time, 0.3 mL of 5% $NaNO_2$ was added to the flask. After 5 min, 0.3 mL 10% $AlCl_3$ was added. At 6 min, 2 mL of 1 M NaOH was added to the mixture. Immediately, the flask content was diluted filling to the mark with the addition of 2.4 mL of ddH_2O and thoroughly mixed. Absorbance of the mixture, pink in color, was

determined at 510 nm versus prepared water blank. Total flavonoids of fruits were expressed on a fresh weight basis as mg of catechin equivalents (CE)/100 g of fruit. Samples were analyzed in triplicate.

Free Radical Scavenging Activity. *1. DPPH Radical Scavenging Assay.* The hydrogen atom or electron donation ability of the corresponding extracts and some pure compounds was measured from the bleaching of purple colored methanol solution of DPPH. This spectrophotometer assay (UV-1650PC Shimadzu, Japan) uses stable radical diphenylpicrylhydrazyl (DPPH) (Sigma, Aldrich) as a reagent (*15*). Aliquots (50 μL) of various concentrations of the test compound in methanol were added to 5 mL of a 0.004% methanol solution of DPPH. After a 30 min incubation period at room temperature the absorbance was read against a blank at 517 nm. Inhibition free radical DPPH in percent (I %) was calculated in the following way: $I\ \% = [(A_{blank} - A_{sample})/A_{blank}] \times 100$, where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. Test compound concentration providing 50% inhibition (IC_{50}, expressed in μg mL^{-1}) was calculated from the graph plotted inhibition percentage against extract concentration. Synthetic antioxidant reagents hydroxytyrosol, pyrogallol, gallic acid, oleuropein, catechin, and butylated hyroxytoluene (BHT) were used as positive control, and all tests were carried out in triplicate.

2. ABTS Radical Scavenging Assay. The Trolox equivalent antioxidant capacity (TEAC) assay, which measures the reduction of the radical cation of ABTS by antioxidants, was performed as previously described (*16*). Briefly, ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and by allowing the mixture to stand in the dark at room temperature for 12−24 h before use. After incubation, the solution was diluted with ethanol or water (lipophilic and hydrophilic assay, respectively) to an absorbance of 0.70 (±0.02) at 734 nm. For the photometric assay 1 mL of the $ABTS^{\bullet+}$, the solution and 100 μL antioxidant solution were mixed for 45 s and measured immediately after 5 min at 734 nm (absorbance did not change significantly up to 10 min). Compounds were assayed at five different concentrations determined within the linear range of the dose−response curve. A calibration curve was prepared with different concentrations of Trolox (0−20 μM). Results were expressed in mM of Trolox.

Analysis of Sugars. A high-performance liquid chromatographic technique was developed to identify and quantify the sugars. The analysis was carried out by a Schimadzu apparatus equipped with a (LC-10 AT) pump and a (model RID-10Avp) detector. The column was (100 × 7.8 mm) fast carbohydrate, Biorad, and the temperature was maintained at 80 °C. The flow rate was 0.3 mL/min. The mobile phase used was water. A standard mixture solution of sugars was analyzed. Sample concentrations were calculated, based on peak areas compared to those of each of the external standards.

Reducing Sugar Assay. The amount of reducing sugars was measured by Wood and Bhat (*17*) assay against standard glucose solution at 550 nm. 100 μL of olive extract was incubated with 900 μL of citrate buffer (pH: 4.8; 50 mM) and 1.5 DNS (2,4-dinitrosalicylic acid). The reaction mixture was kept 10 min at 100 °C.

Glucosidase and Esterase Assays. *1. Extraction of Enzymes.* Fresh pulp (1.0 g) from fruits of both cultivars was extracted with 3.5 mL of 0.1 M borate buffer, pH 9.0, containing 5.0 mM EDTA, 1.0 mM PMSF (phenylmethanesulfonyl fluoride), 2-mercaptoethanol 1% (w/v) and PVP (polyvinylpolypyrrolidone) 10% (w/v). The resulting suspension was shaken gently for 1 h and centrifuged in a Sorvall at 27000*g* for 1 h. The aqueous phase was the enzymatic extract. All operations were carried out at 48 °C.

2. Enzyme Assay. β-Glucosidase activity was assayed according to the method of Wood and Bhat (*17*) toward pNPG substrate by measuring the increase in absorbance at 405 nm. Incubation of 100 μL enzyme with 900 μL substrate in 50 mM citrate buffer at pH 4.8 was carried out for 10 min at 50 °C. The esterase activity was evaluated according to Mackness et al. (*18*) using pNPA substrate. Enzymatic extract containing esterase was added to a solution of 2.9 mL of 9.2 mM Tris-HCl buffer. The pH value of the reaction medium was 7.5. The reaction mixture was incubated 4 min at 25 °C. The enzyme activities were calculated against a standard curve using pNP and expressed as international units (IU), and defined as the amount of enzyme required to produce 1 μmol of *p*-nitrophenol per minute.

Statistical Analysis. Results were expressed as mean ± standard deviation (SD). Statistical significance (*t* test: two-sample equal variance, using two-tailed distribution) was determined using Microsoft Excel statistical software (Microsoft Corporation, Microsoft Office Excel 2003, Redmond,WA). Differences at $P < 0.05$ were considered to be significant.

RESULTS AND DISCUSSION

The weight and the color (green, purple, purple-black and black) of Dhokar olive fruits harvested from July 5th to October 23rd are shown in **Table 1**. Each ripening stage was characterized by a coloration of the fruit skin varying from green, purple and finally black. The fruits are black early in the season (October) compared to Chemlali olive fruits, which became black at the end of November. This may be due to the desert climate where the olive tree is cultivated in shallow soils, at high temperature and low rainfall. The change in fruit color is related to the phenolic change and the increase in anthocyanin pigmentation with decrease of the chlorophyll concentrations (*4, 19*). Dhokar olive fruit weight increases significantly during the ripening stage and reaches its maximum at the end of olive ripening (1.56 ± 0.54 g). Its weight is higher than that of Chemlali fruit, whose weight per one fruit reaches 1.02 ± 0.09 g at the last stage of maturation (*4*).

Phenolic Identification. Dhokar olive fruit extracts were analyzed by a reverse-phase HPLC technique at 280 nm in order to identify the major phenolic compounds in the olive fruits (data not shown). The identification was based on comparisons of the chromatographic retention time and UV absorbance spectra of compounds in olive extracts with those of authentic standards. These chromatograms show that the olive extract is made up of hydroxytyrosol, tyrosol, *p*-coumaric acid, and oleuropein. In addition, another phenolic compound, 4-hydroxyl-3-methoxyphenylethylene glycol, was identified by GC−MS analysis (**Table 2**). The obtained mass fragments agreed with those described previously (*3*). Green and black olive extracts were also analyzed by LC−MS/MS using ESI in the negative ion mode. **Table 3** lists each of the identified phenolic compounds in an elution order. The structure assignment was based on a systematic search for molecular ions using extracted ion mass chromatograms and comparing those with data in the literature (*3, 5, 20*). For example, the spectra generated for compound **2**, hydroxytyrosol glucoside, in negative mode gave the deprotonated molecule $[M - H]^-$ at *m/z* 315. The ion fragment at *m/z* 153 is due to the loss of the glucose group. Three isomers of hydroxytyrosol glucoside were reported (*21, 22*): Romero et al. (*21*) found that hydroxytyrosol-4-β-D-glucoside was the most abundant isomer in olive fruits and derived products, while De Nino et al. (*23*) identified hydroxytyrosol-1′-β-glucoside in olive pulp and olive pomace. The fragmentation profile of the pseudomolecular ion at *m/z* 315 showed a great similarity to that reported by Romero et al. (*23*) for hydroxytyrosol-4-β-D-glucoside, allowing us to identify this compound as the former glucoside isomer.

Compound **7** exhibited a base peak $[M - H]^-$ at *m/z* 609 and also an intermediate ion at *m/z* 463 and an aglycon ion at *m/z* 301. The loss of 146 amu from the pseudomolecular ion indicates the sugar rhamnose and the loss of 162 amu from the intermediate ion is due to the loss of glucose. The obtained MS spectra suggested that compound **7** was rutin. Compound **8** had a deprotonated molecule $[M - H]^-$ at *m/z* 447, and a strong fragment at *m/z* 285 suggests that this compound was the luteolin 7-*O*-glucoside. The spectra generated for compound **10**, ver-

Table 2. Abbreviated Mass Spectra of Major Phenolic Monomers Trimethylsilyl Derivatives, Sugars and Polyol Identified in Dhokar Olive Extracts

	mass spectra (*m/z* and % of the base peak)
tyrosol	282 (M^+, 18); 267 (13); 193 (15); 179 (100); 103 (8); 73 (42)
hydroxytyrosol	370 (M^+, 39); 267 (100); 193 (20); 179 (12); 73 (35)
4-hydroxyl-3-methoxyphenyl ethylene glycol	385 (M^+, 5); 297 (100); 267 (7); 223 (5); 147 (15); 73 (98)
p-coumaric acid	308 (M^+, 81); 293 (100); 249 (50); 219 (92); 179 (16); 139 (10); 115 (6); 73 (60)
mannitol	146 (2); 133 (14); 115 (2); 103 (65); 85 (10); 73 (100); 61 (83); 43 (32); 31 (20); 19 (2)
glucose	180 (M^+, 1); 163 (2); 149 (6); 131 (6); 119 (8); 103 (14); 86 (14); 73 (100); 60 (62); 43 (84)
talose	435 (1); 305 (3); 231 (1); 204 (100); 147 (23); 103 (4); 73 (49)
mannose	435 (12); 345 (18); 305 (22); 270 (4); 243 (12); 204 (100); 147 (22); 103 (4); 73 (46)
sedoheptulose	319 (100); 205 (69); 147 (53); 103 (23); 73 (98)

Table 3. Phenolic Compounds Detected in Dhokar Olive Fruit Cultivar (Green and Black) with Their HPLC Retention Time and Mass Spectral Data

no.	compound	olive color[a]	retention time (min)	predominant negative ion $[M - H]^-$	main fragments by ESI-MS^2
1	hydroxytyrosol	b	1.9	153	123
2	hydroxytyrosol glucoside	a, b	2.15	315	153, 123
3	oleoside	b	2.3	389	371, 345, 209,165
4	nuzhenid	a, b	2.4	685	523, 453, 421, 299
5	11-methyloleoside	b	2.9	403	371, 342, 269, 223
6	oleuropein aglycon derivative	a, b	3.2	377	197, 153
7	rutin	a, b	4.1	609	463, 301, 271, 227
8	luteolin 7-*O*-glucoside	a, b	5.1	447	285, 327, 267
9	luteolin 7-*O*-rutinoside	a	5.2	593	447, 285
10	verbascoside	a	5.9	623	461, 315, 161
11	6′-β-hexopyranosyl-oleoside	a	7.6	551	533, 507, 389, 341, 281, 251, 221, 179
12	ligstroside	a, b	7.9	523	453, 421, 361, 291
13	6′-deoxyhexopyranosyl-oleoside	a, b	8.2	535	517, 491, 390 345, 325, 307, 265
14	oleuropein	a, b	8.3	539	377, 307, 275, 345, 275, 223, 153
15	oleuropein glucoside	a, b	10.8	701	507, 539, 443, 377, 307, 275

[a] a: Green olive. b: Black olive.

bascoside, in negative mode gave the deprotonated molecule $[M - H]^-$ at *m/z* 623. The ion fragment at *m/z* 461 is due to the loss of the glucose group or caffeic acid fragment. In the case of compound **11** the mass spectrum showed a strong peak at *m/z* 551 corresponding to the $[M - H]^-$ species. The principal fragment originating from the loss of 44 amu gives rise to the intense signal at *m/z* 507, the species at *m/z* 389 representing the oleoside structure. Its formation was accomplished by the loss of a hexose moiety (162 amu) suggesting that the compound was a hexoside derivative of oleoside. Moreover, the presence of a fragment at *m/z* 341, which corresponded to a disaccharide, indicated that this hexose molecule should be linked to the sugar moiety of oleoside. This suggested that **11** might be an oleoside derivative and the obtained scheme of fragmentation of compound **11** in negative mode agreed with those described previously (*20, 24*). According to the above results, compound **11** was a 6′-β-hexopyranosyl-oleoside. The mass spectrum of compound **13** showed a high-intensity ion at *m/z* 535. The ESI-MS^2 spectrum of that ion (**Table 3**) showed some similarities to the spectral profile of compound **8**. The main fragment was obtained by the loss of 44 amu (ion at 491), and an ionic species corresponding to the oleoside ion (*m/z* 390 and *m/z* 325) was also detected. The loss of 146 and 162 amu from the pseudomolecular ion can be accounted for the elimination of two sugars rhamnose and glucose, respectively. This compound was previously detected in olive leaves and fruit by the use of ESI-MS and was identified as 6′-deoxyhexopyranosyl-oleoside (*22*).

The comparison of the ESI-MS data with literature data was possible for compounds **1** (hydroxytyrosol), **3** (oleoside), **4** (nuzhenid), **5** (11-methyloleoside),**6** (oleuropein aglycon), **9** (luteolin 7-*O*-rutinoside), **12** (ligstroside), **14** (oleuropein) and **15** (oleuropein glucoside). Their fragmentation schemes were in agreement with those previously detected in *Olea europaea* leaves and fruits (*5, 20, 23−26*).

Changes of Specific Phenolic Compounds during Ripening. Oleuropein was the major phenolic compound identified in olive extracts in the early stage of fruit development. It was quantified during maturation as well as its degradation products such as hydroxytyrosol. A comparison of Dhokar values with the main Tunisian variety, Chemlali, was also carried out. **Figure 1** reveals a decrease in oleuropein content with the maturation process in the two cultivars. For the first harvest (July, green olive) oleuropein amount was 3.29 g/kg, then declined as the fruit began to mature, reaching to a negligible level in the fully mature black fruits (0.06 g/kg). Changes in oleuropein contents during fruit development and maturity in Dhokar cv. show a similar pattern reported for other cultivars (*27*). Compared to Chemlali olive cultivar, the value of oleuropein shows a different evolution with maturation. In fact, in Chemlali olive fruit, oleuropein concentration obtained during the green maturation attained its highest level at the end of August (6.75 g/kg), which is 2-fold higher than that of Dhokar cultivar, then it decreased to 3.75 g/kg of fresh fruit in October and reached its lower values in February when olive fruits were black (1.6 g/kg). Our results agreed with the findings of Amiot et al. (*9*), who observed a decline in oleuropein content with fruit maturity. Its levels vary from 45 to 3.3 g/kg and from 40 to 2.8 g/kg of olive fresh mass in cultivars Cailletier and Salonenque, respectively. Similarly, it was reported that oleuropein contents in the fruit of both cultivars Gentile (Larino) and Gentile (Colletorto) varied from 0.87 to 1.45 g/kg and from 1.5 to 2.08 g/kg, respectively (*8*). Bianco et al. (*28*) claimed

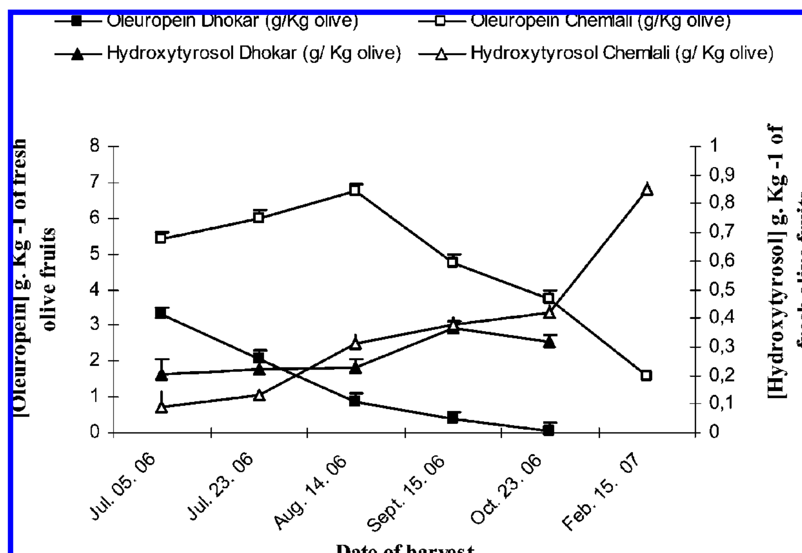

Figure 1. Evolution of oleuropein and hydroxytyrosol concentrations in Dhokar and Chemlali olive extracts during maturation. Data are expressed as the mean ($n = 3$) $\pm$ SD.

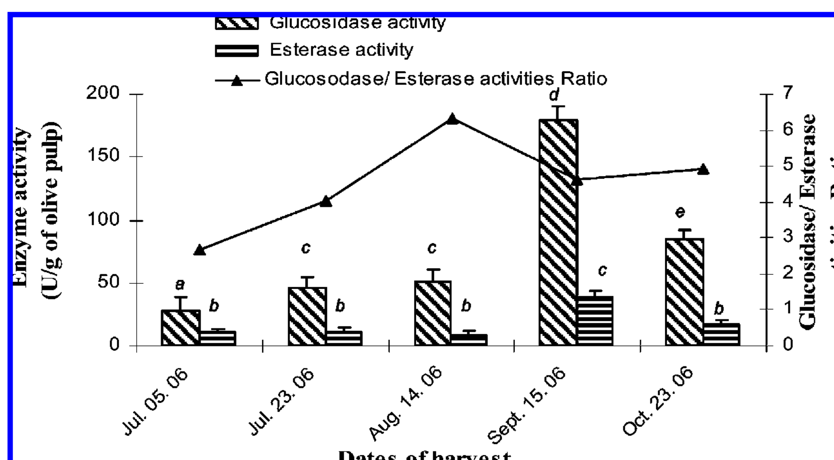

Figure 2. β-Glucosidase and esterase activities in Dhokar fruit extracts in different stages of maturation. Data are expressed as the mean ($n = 3$) $\pm$ SD. Means with different letters are significantly different at $P \leq 0.05$.

that the concentration of oleuropein fell to zero when olive fruits (*O. europaea* cv. Leccino) were completely black.

Hydroxytyrosol is one of the principal degradation products of oleuropein as previously described (*2*). It was found in much smaller quantities relative to oleuropein and showed an inverse evolution trend during ripening. Its concentration value was 204 mg/kg (all data are reported on a fresh mass basis) at the beginning of ripeness and increased to reach 320 mg/kg; then its level declined at the end of the harvest, in the fully mature black fruits. The enhancement of hydroxytyrosol concentrations could be attributed to could be attributed to the increased activity of the hydrolytic enzymes (*29*), in particular glycosidases and esterases which catalyze the hydrolysis of the oleuropein, with the production of oleuropein aglycon, elenolic acid and 3,4-dihydroxyphenylethanol (*30*). Compared to Chemlali olive fruits, we can conclude that hydroxytyrosol content in Dhokar olive fruit is lower. Indeed, hydroxytyrosol concentration in Chemlali olive fruit at the beginning of maturation (July) attains a value of 300 mg/kg and reaches its maximum in January (760 mg/kg). Our results confirm previous literature data that reported a decrease in oleuropein during maturation, paralleled by a contemporary increase in oleuropein derivatives and hydroxytyrosol (*2, 4, 27*). On the other hand, the hydroxytyrosol decline at the last stage of maturation could be explained as described in the previous study (*31*). These authors reported that the rise of verbascoside in fully developed fruits coincides with a decline in oleuropein in mature fruits, although not to the same extent, and that a part of oleuropein degradation during maturation might contribute to a rise in verbascoside (*31*). In other data, it has been noted that degradation of oleuropein could produce hydroxytyrosol (*2, 32*), which then can be converted into verbascoside (*33*). Tyrosol is the second phenolic monomer compound after hydroxytyrosol. It was also detected in olive fruits, but our extraction and separation techniques failed to reach enough quantitative measures of this monomer level.

Enzymatic Activities in Fruit during Ripening. β-Glucosidase and esterase activities were determined in the olive pulp. The two enzymes showed the same trend; they increase to reach a maximum in September followed by a slight decrease at the last harvest (**Figure 2**). The β-glucosidase activity of pulp extracts showed a variation, which can be related to a corresponding change in oleuropein concentration ($R^2 = 0.44$). The activity started at 28.31 U/g olive pulp in July, and then it increased considerably and reached its maximum in September (179.75 U/g olive pulp). In October, it declined to 84.24 U/g (**Figure 2**). The β-glucosidases include a family of enzymes that can hydrolyze a wide range of β-glucosides. The esterase activity measured in fruit extracts showed the maximal activity

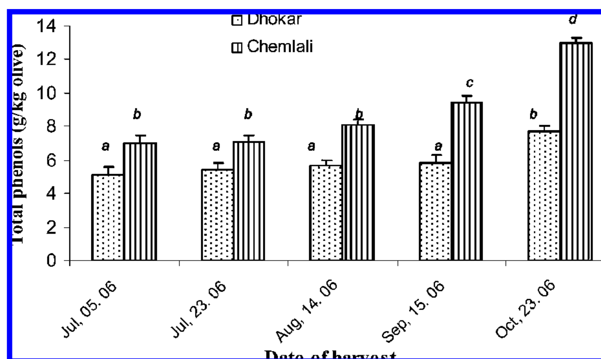

Figure 3. Total phenolic content (g/kg of equivalent gallic acid) in the olive fruits from Dhokar cultivar evaluated during ripening. Data are expressed as the mean ($n = 3$) ± SE. Means with different letters are significantly different at $P \leq 0.05$.

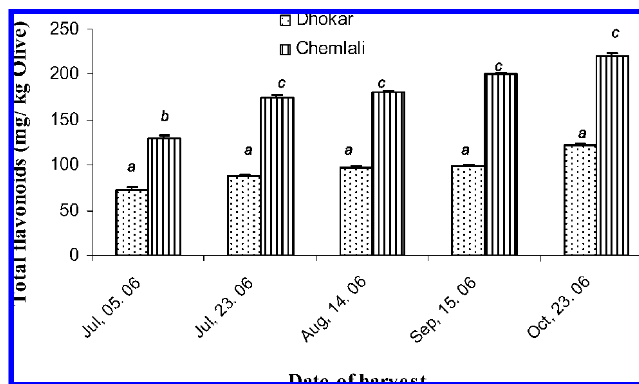

Figure 4. Total flavonoid content (mg/kg as catechin equivalents) in Dhokar olive extracts in different stages of maturation. Data are expressed as the mean ($n = 3$) ± SE. Means with different letters are significantly different at $P \leq 0.05$.

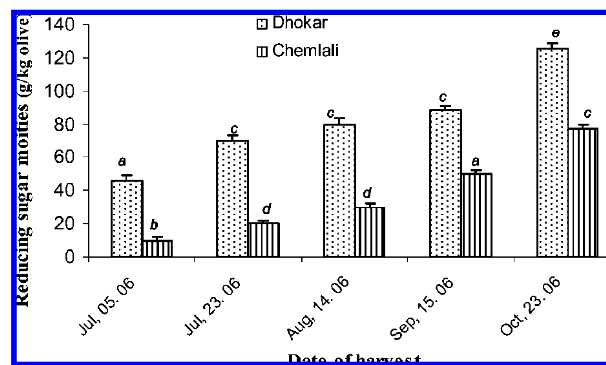

Figure 5. Reducing sugar content in Dhokar olive extracts. Data are expressed as the mean ($n = 3$) ± SE. Means with different letters are significantly different at $P \leq 0.05$.

in September (30.03 U/g) when the olive fruit were black, and then it decreased during the last maturation stage, reaching an activity with a value of 17.05 U/g (**Figure 2**). Similar results found by Amiot et al. (*9*) reported that esterase activity measured in olive fruit extracts increased considerably during the ripening stage, reaching its maximum during black maturation. Oleuropein seems to undergo two successive hydrolysis reactions. In the first step, we obtained oleuropein aglycon and glucose by β-glucosidase enzyme hydrolysis, and the second step leads to the formation of elenolic acid and hydroxytyrosol from aglycon by esterase activity (*34*). Indeed, in the Dhokar variety we noticed a significant reduction in oleuropein levels in the flesh of fully developed fruits (98%) compared to its level in the fruits in the first harvest. This result is consistent with earlier reports of increased hydrolytic enzymes activity during early maturation (*34*). Of course, the hydrolytic activity reaches a maximum during the ripening of black olives. We did not observe a linear correlation between oleuropein hydrolysis by endogenous β-glucosidase, esterase and hydroxytyrosol, which originate by enzymatic and/or chemical degradation of the oleuropein aglycon during fruit ripening ($R^2 = 0.62$). We also observed that an enzymatic activity is implicated in the loss of the bitter taste of ripe fruit pulp; thus we can conclude that the increased turnover of the β-glucosidase is widely related to the marked absence of the bitter taste in Dhokar olive fruit.

Phenolic and Flavonoid Content in Olive Drupae. Figure 3 shows that the total phenol concentration of olive extracts varied from 5.08 ± 0.49 to 7.68 ± 0.32 g/kg of equivalent gallic acid during maturation of the Dhokar cv. These total phenols values are lower compared to those found in Chemlali cv. harvested in the same date. Indeed in Chemlali cv. it varied from 6.98 to 13 g/kg (**Figure 3**). The low amount of the total phenols in Dhokar black olives (harvest at the end of October) could be related to the “sweet” taste of the fruit.

Flavonoids were also quantified, and their quantities were reported in milligrams per kilogram of fresh weight of olives (**Figure 4**). Their content expressed as catechin equivalents showed a slight increase with maturation. They varied from 73.2 ± 0.87 in July to 121.5 ± 0.91 mg/kg of fresh olive in October (**Figure 4**). Flavonoids in Dhokar showed a low concentration and variation compared to Chemlali cultivar in which flavonoid concentration was up to 400 mg/kg. In fact there is a significant difference between the flavonoid concentrations in Dhokkar and Chemlali fruit extracts in the different stages of maturation ($P \leq 0.05$). These results are supported by the finding of Bouaziz et al. (*2*), who reported the flavonoid content in Chemlali cv. during maturation which exceeded 400 mg/kg of fresh olive. In addition, it was found that the flavonoid glucoside increase during green maturation was followed by a decline of the latter and an increase of the level of flavonoid aglycons at the last stage of the fruits’ development (*2, 4*).

Sugar Content during Fruit Ripening. The reducing sugar moiety was quantified and showed a significant increase especially in the last stage of maturation (**Figure 5**). It reached 125.6 g/kg in October. This value is considered to be higher compared to sugar content in Chemlali olive fruits (77.5 g/kg). The GC−MS chromatograms show that the analyzed olive extracts are made mainly of mannitol, glucose, talose, mannose and sedoheptulose (**Table 2**).

Quantification of the major free sugar present in olive extracts during maturation using HPLC apparatus is given in **Figure 6**. Mannitol was the predominant sugar, followed by glucose. The concentration of mannitol increased with fruit ripeness and reached 79.8 g/kg in last harvest for Dhokar, whereas in the same stage of maturation it reaches 50.2 g/kg in Chemlali olive cultivar. Mannitol concentrations were increased significantly in Dhokar olive fruit extracts in the last stage of maturation compared with the earliest one. Furthermore, the values of mannitol concentrations in Dhokar olive extracts were significantly higher than those of Chemlali all along the ripening stages ($P \leq 0.05$).

Glucose content increased continuously and significantly from 4.6 g/kg in July to a final value of 8 g/kg when the fruits attain the complete fruit development (in October). This value is significantly higher ($P \leq 0.05$) compared to the glucose level found in Chemlali olive fruits (4.5 g/kg fresh olive fruit) harvested in the latter maturation stage. In fact, we have concluded that there are significant differences in the concentra-

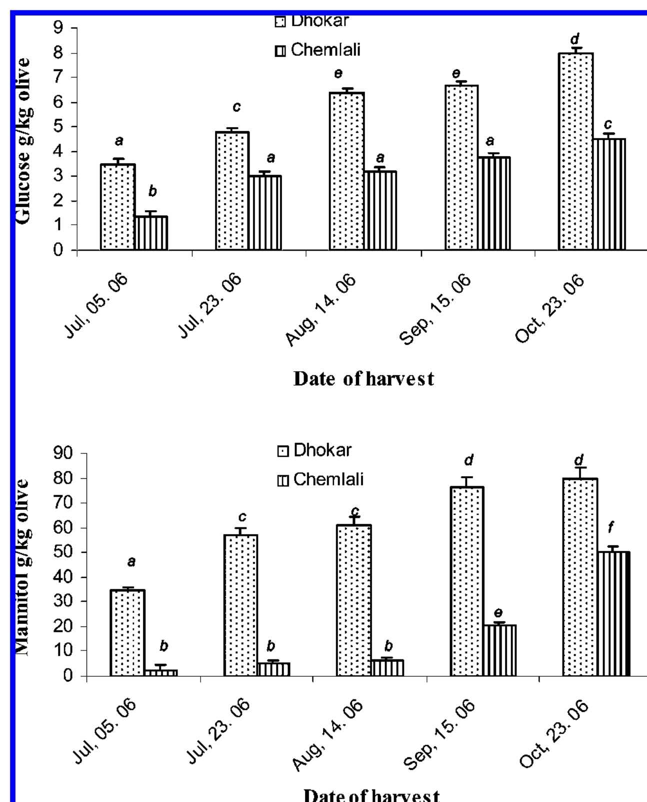

Figure 6. Amount of glucose and mannitol during the maturation period of Dhokar olive fruits. Data are expressed as the mean ($n = 3$) ± SE. Means with different letters are significantly different at $P \leq 0.05$.

tions of glucose in Dhokar extracts all along the ripening stages. Glucose concentrations increase significantly in the last stage compared with the first one. Indeed, glucose concentrations in Dhokar olive fruit extracts are significantly higher than those of Chemlali extract all along the ripening stages ($P \leq 0.05$).

Some sugar derivatives have also been described in other olive fruit cultivars, namely glucose, fructose and mannitol (*10, 11*). In addition, an associated rise in oil and sugar levels was noticed at the beginning of the fruit ripening period of 'Kadesh', while the sugar level of Manzanillo and Uovo de Piccione cultivars decreased during most of that period (*9*). Fernandez-Bolanos et al. (*35*) have found that glucose was the major sugar in olive fruit of 'Manzanillo', 'Gordal', 'Hojiblanca' and 'Verdial'. They noted also that fructose and mannitol were present in similar amounts (*36*). The enhancement of mannitol concentration in Dhokar cultivar may be related to the cultivar and the geographical origin where the olive grows. In fact, mannitol concentration progressively increases as the total salinity of the growth soil increases. Furthermore, mannitol production may confer several potential advantages including more efficient carbon use, resistance against oxidative stress, and salt tolerance (*37*).

Antioxidant Potential of Olive Extracts during Maturation. The DPPH radical scavenging effect for all of the methanol/water extracts is shown in **Table 4**. All extracts exhibited an antioxidant activity, which showed correlation between total phenol ($R^2 = 0.94$) and flavonoid ($R^2 = 0.92$) content and DPPH radical-scavenging activity over all the maturation stages. Olive extracts show similar trends of activity in both the ABTS assay and the DPPH radical scavenging assay (**Table 4**). The lower antioxidant potentials were found in the sample harvested at the beginning of maturation with high IC_{50} values (398.1 ± 3.32 μg/mL) and low TAC (0.83 ± 0.13 mM) values. This is probably due to the lower inhibition of the radical

Table 4. Radical-Scavenging Activities of Dhokar Olive Extracts in Different Stages of Maturation Compared to Those of Chemlali Olive Extracts[a]

harvest date	TEAC (mM) (ABTS assay)	$IC_{50(Dhokar)}$ (μg/mL) (DPPH assay)	$IC_{50(Chemlali)}$ (μg/mL) (DPPH assay)
July 05, 2006	0.83 ± 0.13	398.1 ± 3.32 a	151.8 ± 2.12 b
July 23, 2006	0.92 ± 0.12	158.48 ± 1.38 b	82.34 ± 1.15 c
Aug 14, 2006	0.99 ± 0.14	79.43 ± 1.32 c	38.1 ± 2.17 e
Sept 15, 2006	1.12 ± 0.14	50.11 ± 1.25 d	19.52 ± 2.62 f
Oct 23, 2006	1.65 ± 0.12	39.81 ± 1.41 e	15.89 ± 2.35 g
BHT	2.40 ± 0.12	8.13 ± 1.07	8.13 ± 1.07

[a] Values given are the mean of three replicates ± standard deviation. Means with different letters are significantly different at $P \leq 0.05$.

formed by a low phenolic content especially of *o*-diphenol such as hydroxytyrosol (*38*). The extract obtained from the last harvest showed the highest antioxidant activity. The comparison of IC_{50} values of the two cultivars olive fruit extracts shows significant differences. Indeed, Chemlali extracts were significantly more antioxidant all along the maturation stages ($P \leq 0.05$). The antioxidant activity and the level of the total phenols in the extracts suggest that the radical scavenging effect in the extract can be attributed to the structure of phenolic compounds and their content, in particular, the number of hydroxyl substituents in the aromatic ring (*3, 38*). On the other hand, the presence of a sugar as mannitol enhances the ability of the extract to act as an antioxidant because it is known to be a quencher of ROS and scavenger of hydroxyl radicals.

In conclusion, this work is the first report on the identification and characterization of phenolic, flavonoids and sugar in Dhokar sweet olive fruit cultivar from south Tunisia. Eighteen phenolic compounds have been identified. Phenolic and flavonoid content as well as sugar concentrations have been determined. The harvest at the last phase of maturation led to a significant decrease of the bitter taste of olive and oleuropein content and an increase in the level of flavonoid, mannitol, glucose and hydroxytyrosol. This evolution could be correlated with the recovered enzymatic activities especially β-glucosidase and esterase. The antioxidant potential of olive extract was found to increase during maturation in correlation with the total content of phenol, flavonoids and the high moieties of sugar mainly mannitol, which is known for its antioxidant activities. This data will be useful for a biochemical characterization of different *O. europaea* L. varieties as important parameters in testing the quality of the obtainable oils. In addition, Dhokar olive fruit is economically favorable because it could be eaten fresh as a table olive without previous debittering treatment. Further studies on the effects of environmental factors and growing conditions on the phenolic composition and oil of Dhokar olives are under investigation.

ABBREVIATIONS USED

p-NPA, *p*-nitrophenyl acetate; pNPG, 4-nitrophenyl-β-D-glucopyranoside; DNS, 3,5-dinitrosalicylic acid; DPPH, 2,2-diphenyl-1-picrylhydrazyl; BHT, 2,6-di-*tert*-butyl-4-hydroxytoluene; Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid); ABTS, 2,2′-azinobis[3-ethylbenzothiazoline-6-sulfonate].

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