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ORIGINAL ARTICLE

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Profile of enzyme in drupe of oueslati's cv. olives during ripening phases: A support method implementation in the production of extra virgin olive oil

Rim Hachicha Hbaieb[1] | Faten Kotti[1] | Antonello Paduano[2] | Pasquale Crupi[3] | Maria Lisa Clodoveo[3] | Raffaele Sacchi[4] | Mohamed Gargouri[1]

[1]Laboratory of Microbial Ecology and Technology, Biocatalysis and Industrial Enzymes Group, Carthage University, National Institute of Applied Sciences and Technology (INSAT), Tunis Cedex, Tunisia

[2]Department of Agricultural and Environmental Science, University of Bari, Bari, Italy

[3]Interdisciplinary Department of Medicine, University of Bari, Bari, Italy

[4]Department of Agricultural Sciences, University of Naples Federico II, Portici, Italy

Correspondence
Mohamed Gargouri, Laboratory of Microbial Ecology and Technology, Biocatalysis and Industrial Enzymes Group, Carthage University, National Institute of Applied Sciences and Technology (INSAT), BP 676, 1080 Tunis Cedex, Tunisia.
Email: mhmdgargouri@gmail.com

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Abstract

Quality of virgin olive oil (VOO) depends on phenolic molecules content, which depends on the biochemical characteristics of olive fruits, namely endogenous enzymes. In order to ascertain the influence of olive fruit ripening degree on the phenol content, enzyme activities in olive fruits, and the quality of the corresponding oils were studied during Oueslati olive ripening. In fact, three enzymes were studied: peroxidase (POX) in olive seeds, polyphenoloxidase (PPO), and β-glucosidase (β-GL) in olive fruits mesocarp. Each enzyme showed specific trend: POX activity increased gradually until reaching a maximum (17.061 ± 0.101 U g^{-1} FW) at ripening index (RI) 3.6 and then decreased slowly at advanced ripening stage. However, the maximum of PPO activity (240.421 ± 0.949 U g^{-1} FW) was observed earlier at RI of 0.7. Concerning β-glucosidase activity, its maximal was 60.857 ± 1.105 U g^{-1} FW at RI 2.8, then, it decreased sharply to reach 17.096 ± 0.865 U g^{-1} FW at RI 3.9. A significant increase of total phenol content as well as the antioxidant activity were observed during Oueslati olive ripening. Moreover, phenolic profile indicated that appropriate harvesting date of Oueslati olives coincided with RI 3.9 given that highest content of most important individuals phenolic compounds responsible for the main VOO biological properties achieved on this date. Furthermore, phenols amount of Oueslati VOO was principally due to PPO enzyme activity as the increase in total phenols coincides with the decrease in PPO activity.

KEYWORDS
olive endogenous enzymes, Oueslati olives, phenolic compounds, ripening, virgin olive oil

INTRODUCTION

Extra virgin olive oil (EVOO) is considered worldwide as a crucial commodity, in other words, an undifferentiated product in the market where competition is only based on price strategies, founded on favoring information asymmetry and reducing profits for producers. The health claims approved by European Food Safety Authority (polyphenols, tocopherols, oleic acid, and unsaturated fatty acids) allow the consideration of EVOO not only as functional foods capable of earning premium price but also capable of increasing consumers' willingness. For a specific cultivar in its typical environment, olives' biochemical characterization and phenolic profile identification in corresponding VOO, are useful to support the VOO producers by choosing the optimal ripening period (Roselli et al., 2017).

In fact, the olive tree (*Olea europaea* L.) is the most important fruit tree in Mediterranean countries. Tunisian olive grove extends from north to south, thereby,

Abbreviations: β-GL, β-glucosidase; 3,4-DHPEA, hydroxytyrosol; 3,4-DHPEA-EA, oleuropein aglycone; 3,4-DHPEA-EDA, decarboxymethyl oleuropein aglycone; EFSA, European Food Safety Authority; *p*-HPEA, tyrosol; *p*-HPEA-EA, ligstroside aglycone; *p*-HPEA-EDA, decarboxymethyl ligstroside aglycone; POX, peroxidase; PPO, polyphenoloxidase; RI, ripening index; VOO, virgin olive oil; WAF, weeks after flowering.

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indicating great wealth. Olive cultivar in Tunisia account for more than 50 namely Chétoui and Chemlali are considered the two dominant cultivars (Dabbou et al., 2009). In fact, they represent 95% of the total olive tree orchards and they contribute with more than 90% of the national olive oil production. Recently, characterization of minor Tunisian cultivars has been conducted (Manai-Djebali et al., 2012). In particular, Oueslati cultivar, cultivated in smaller areas, essentially in Kairouan and Siliana regions. It is known with its fruity, little bitter, and almondy flavor typical of fresh almond oil. Moreover, Oueslati oil is resistant to oxidation thanks to its high content of total phenols and tocopherols (Ouni et al., 2011). Phenolics compounds in olive oils are characterized by a powerful antioxidant activity (Cicerale et al., 2009). Phenolic compounds in VOO include phenolic alcohols, phenolic acids, secoiridoid derivatives, lignans, and flavonoids. Phenolic contents could be affected by endogenous (olive cultivar and ripening degree) and exogenous factors (agronomic, environment, and technology) (Bakhouche et al., 2013; Gouvinhas et al., 2015; Manai-Djebali et al., 2012; Romero-Segura et al., 2012). Additionally, phenolic profile of VOO can be determined by phenolic glycosides content in olive tissues and endogenous enzymes activities specific for these glycosides (García-Rodríguez et al., 2011; Hachicha Hbaieb et al., 2015). These glycosides are oleuropein, ligstroside, demethyloleuropein, verbascoside, elenolic acid glucoside, luteolin-7-glucoside, apigenin-7-glucoside, rutin, and quercetin-3-rutinoside (Gómez-Rico et al., 2008). Moreover, β-glucosidase (β-GL) and oxidoreductases (polyphenoloxidase [PPO] and peroxidase [POX]) which contribute considerably in the phenolic profile of VOO during oil production. Olive β-GL (E.C.3.21.1.21) generates secoiridoid compounds through the hydrolysis of the phenolic glycosides during oil production (Romero-Segura et al., 2009). However, the oxidation of phenolic compounds was essentially determined by PPO and POX during the milling and kneading steps, respectively (García-Rodríguez et al., 2015; Segovia-Bravo et al., 2009; Servili et al., 2008).

Several studies focused on VOO quality. However, studies on endogenous enzyme activities and its effect on VOO phenolic profiles, particularly for minor Tunisian cultivars were limited.

The enzymatic characterization of olive and its effect on VOO quality were studied both on Spanish cultivars (Arbequina and Picual) (García-Rodríguez et al., 2011) and on the main Tunisian ones (Chétoui and Chemlali) (Hachicha Hbaieb et al., 2017; Hachicha Hbaieb, Kotti, Cortes-Francisco, et al., 2016b; Hachicha Hbaieb, Kotti, Vichi, & Gargouri, 2016a). Consequently, we are interested in this study in monitoring endogenous enzymes in olive fruit from the Tunisian olive cultivar (Oueslati) during fruit ripening as well as the contribution of these enzymes in the phenolic composition in VOO.

TABLE 1 Evolution of ripening index (RI) during Oueslati olives ripening during the crop season 2014–2015

Oueslati		
Harvest date	**Weeks after flowering**	**Ripening index**
September 24, 2014	23.5	0.4
October 2, 2014	24.5	0.7
October 21, 2014	27	1.2
November 3, 2014	29	1.8
November 13, 2014	30.5	2.4
November 24, 2014	32	2.8
December 3, 2014	33.5	3.1
December 16, 2014	35.5	3.3
December 30, 2014	37.5	3.6
January 16, 2015	40	3.9

MATERIALS AND METHODS

Plant material

The study was carried out on monovarietal virgin olive oils from the minor Tunisian cultivar, namely Oueslati. Only undamaged and healthy olive fruits, from young olive trees, were handpicked from different positions from the same tree in an irrigated orchard Mabrouka nursery (Naassen 13 km from Tunis center) during the crop season 2014–2015.

Endogenous enzyme activities in olive fruits were monitored during the ripening period from September 24, 2014 to January 16, 2015. Approximately, 250 g of olives were used for the determination of enzymatic activities. The harvesting dates, the number of weeks after flowering (WAF) and the corresponding ripening index (RI) are presented in Table 1. RI was determined on each sampling date as described by Hermoso et al. (2001). One kilogram of olives was used for olive oil extraction.

Chemicals

Reagents for enzymatic activity measurements (ethylenediaminetetraacetic acid, phenylmethylsulfonyl fluoride, benzamidine hydrochloride, α-aminocaproic acid, Triton X-100, sodium dodecyl sulfate [SDS], dithiothreitol, hydrogen peroxide [H_2O_2], guaiacol, tert-butylcatechol [TBC] and 4-nitrophenyl-β, D-glucopyranoside [*p*-NPG]) were supplied by Sigma–Aldrich (St. Louis, MO).

Pure HPLC solvents were used in all cases (Sigma–Aldrich). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and Folin–Ciocalteu reagent were purchased from Sigma–Aldrich and Carlo Erba, respectively.

Olive oil extraction

Abencor analyzer (Comercial Abengoa, S.A., Seville, Spain) was used to extract olive oil. About 1 kg of healthy olive fruits was crushed then malaxed for 30 min at 22°C. After kneading, olive paste was centrifuged in a centrifuge basket at 3500 × *g* for 1 min, and later the oils were decanted and transferred into dark glass bottles, dabbled with nitrogen gas (N_2), and stored in the dark at −20°C until analysis.

Enzyme extraction and activity assay

Fresh harvested olive fruits mesocarp were used to prepare acetone powders as previously described by García-Rodríguez et al. (2011). Briefly, 10 g of tissue was homogenized with 150 ml of cold acetone (−20°C) by a warning blender. After filtration, the residue, was re-extracted twice with 20 ml of cold acetone (−20°C) until the residue became a whitish powder, which was finally rinsed with diethylether, dried and stored at −20°C.

Peroxidase

POX enzyme extracts were prepared as described earlier by García-Rodríguez et al. (2011). Briefly, olive seeds were homogenized five times in sodium phosphate buffer (100 mM, pH 6.7) using an Ultra-turrax, then centrifuged (15,000 × *g*, 20 min at 4°C).

POX activity in the extracts (supernatant) was determined spectrophotometrically by measuring the rate of tetraguaiacol formation due to guaiacol peroxidation at 470 nm and 25°C in the detriment of H_2O_2 ($\varepsilon = 26{,}600\ M^{-1}\ cm^{-1}$). The reaction mixture consisted of 1.5 ml of 25 mM sodium phosphate buffer pH 6.5, 5.5 mM guaiacol, 2 mM H_2O_2, and the appropriate amount of enzyme (5–30 μl). Enzyme's quantity necessary to oxidize 1 μmol of guaiacol per min corresponds to one unit of POX activity.

Polyphenoloxidase

PPO enzyme extracts were prepared by homogenizing acetonic powder in sodium phosphate buffer (100 mM, pH 6.7) and centrifuging the homogenate at 15,000 × *g* for 20 min at 4°C (García-Rodríguez et al., 2011).

The determination of PPO activity consisted in measuring the increase in absorbance at 400 nm corresponding to quinone formation via TBC oxidation ($\varepsilon = 1200\ M^{-1}\ cm^{-1}$). The reaction medium consisted of 1.5 ml of 25 mM sodium phosphate buffer pH 6.5, 1 mM SDS, 7 mM TBC, and the appropriate amount of enzyme (1–5 μl). One unit of PPO activity was defined as the amount of enzyme forming 1 μmol of TBC-quinone per min.

β-Glucosidase

β-GL enzyme extracts were prepared as described previously by Romero-Segura et al. (2009). In general, acetone powder was homogenized in 100 mM borate buffer (pH 9.5) using an Ultra-turrax homogenizer. The clear supernatant, used as crude extract, was obtained after centrifugation at 27,000 × *g* for 20 min at 4°C.

β-GL activity was determined by measuring the rise in absorbance at 405 nm corresponding to *p*-nitrophenol released from the synthetic glucoside *p*-NPG. Hydrolytic reaction quantification was carried out considering an extinction coefficient of 552.8 $M^{-1}\ cm^{-1}$. The reaction medium consisted of 1.5 ml of 50 mM sodium acetate buffer pH 5.5, 15 mM *p*-NPG, and the appropriate amount of enzyme (5–20 μl) One unit of β-GL activity corresponded to the quantity of enzyme generating 1 μmol of *p*-nitrophenol per min at 40°C (Romero-Segura et al., 2012).

Enzymatic extraction was performed in duplicate while the whole enzymatic activity was performed in triplicate.

Colorimetric determination of total phenol

Phenolic fraction of olive oil was extracted using the Liquid–Liquid Micro Extraction according to the method described by Luz Pizarro et al. (2013). Briefly, 0.5 g of olive oil was extracted with 1 ml of a methanol/water (80:20 v/v) in 2 ml Eppendorf tubes. This mixture was vortex-stirred for 1 min, then centrifuged at 13,400 rpm for 5 min at room temperature. This process was performed three times and the resulted extract were combined and used to determine the total phenols content colorimetrically at 765 nm using Folin–Ciocalteu reagent. Results were expressed as milligrams caffeic acid per kilogram oil (Gutfinger, 1981).

The colorimetric determination of total phenol was performed in triplicate.

Antioxidant activity assay with DPPH

Radical-scavenging activity (RSA) was measured using DPPH radical (Carlo Erba, Cornaredo, Italy) as described by Brand-Williams et al. (1995).

Scavenging activity (%) was calculated using the following formula:

$$\text{DPPH radical scavenging}\% = \frac{A_c - A_s}{A_c} \times 100.$$

A_c and A_s represent the absorbance at 517 nm of control solution and samples extracts, respectively.

The antioxidant activity determination of phenol extracts was performed in triplicate.

Phenols chromatographic analysis using HPLC-DAD

Phenolic compounds in EVOO were analyzed as described by COI/T.20/Doc n. 29 method, with some modifications (Sacchi et al., 2015). Phenolic compounds from olive oil were extracted by a hydro-alcoholic solution and subsequently quantified by HPLC-DAD at 279 nm.

Briefly, 5 g of oil was dissolved in 10 ml of hexane, added of 1 ml of the internal standard solution (syringic acid, 0.015 mg ml^{-1}) and extracted three times in a separating funnel with 7 ml of a mixture of methanol/water (60/40 v/v). The hydro-alcoholic extract phase was collected, washed with hexane and centrifuged 5 min at 3000 rpm. Then it was evaporated at 40°C and the residue was collected using 1 ml of methanol for the HPLC injection.

Phenolic compounds were identified by comparing retention time, relative elution order and UV absorbance spectra with those of authentic standards, when available, or with those reported in the literature (Brenes et al., 2000; Mateos et al., 2001; Met. COI/T.20/Doc. n. 29, 2009; Montedoro et al., 1993; Rovellini & Cortesi, 2002). Phenolic compound was measured in triplicate with internal standard method using five concentration points calibration curves; the detection limit (LOD) and quantification limit (LOQ) were calculated on the basis of chromatograms and defined as signal-to-noise (six times SD of baseline) ratio of 3 and 10, respectively. The content of natural and oxidized oleuropein and ligstroside derivatives, lignans, flavonoids, and phenolic acids was expressed in milligrams of tyrosol equivalent per kg of olive oil ($R^2 = 0.9989$; LOD = 0.052 mg kg^{-1}; LOQ = 0.133 mg kg^{-1}).

Statistics

The results were expressed as mean value ± SD. Statistical analysis was carried out using Statgraphics Centurion XVI software. Significant differences between treatments were determined using one-way analysis of variance.

RESULTS AND DISCUSSION

Olive endogenous enzymes activities (POX, PPO, and β-GL) monitoring during ripening

Evolution of the activities of endogenous enzyme in olive fruits during Oueslati fruits ripening was studied in order to determine whether these activities could affect VOO phenolic profile or not.

POX activity in Oueslati olive seeds during fruits ripening was monitored and results were presented in Figure 1a. As illustrated, POX activity in Oueslati olive seeds was about 6.67 ± 0.08 U g^{-1} FW at RI = 0.4 corresponding to 23.5 WAF. Moreover, a gradual increase of POX activity was observed during fruit ripening to reach 17.061 ± 0.101 U g^{-1} FW at RI = 3.6 corresponding to 37.5 WAF. Then, it decreased slowly to 14.111 ± 0.434 U g^{-1} FW at RI = 3.9 corresponding to 40 WAF. The highest value of POX activity in Oueslati cultivar was quite similar to that obtained in Picual fruits (20.8 U g^{-1} FW) (García-Rodríguez et al., 2011). Similar trends of POX activity were also observed during Chétoui and Chemlali olive ripening (Hachicha Hbaieb et al., 2017). For example, in the case of Chétoui olives, POX activity increased from

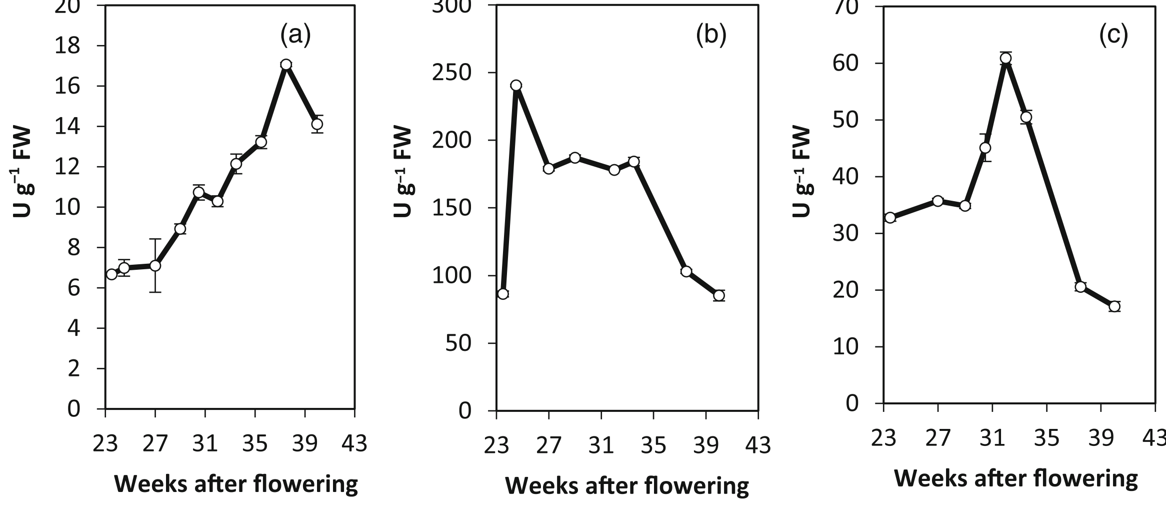

FIGURE 1 Evolution of olive endogenous enzymatic activities (U g^{-1} fresh weight, means of three replicates) during Oueslati (◦) olives ripening: (a) Peroxidase activity in olive seeds; (b) Polyphenoloxidase activity and (c) β-glucosidase activity in olive fruits mesocarp

TABLE 2 Comparison of peroxidase (POX), polyphenoloxidase (PPO) and β-glucosidase (β-GL) activities changes during Oueslati and others olive cultivars ripening

	POX	PPO	β-GL
Oueslati	POX activity increased until reaching a maximum (17.061 ± 0.101 U g^{-1} FW) at RI = 3.6; WAF = 37.5	PPO activity increased then decreased at advanced ripening stages with a maximum activity (240.421 ± 0.949 U g^{-1} FW) attained at RI = 0.7; WAF = 24.5	β-GL activity increased until reaching a maximum (60.85 ± 1.10 U g^{-1} FW) at RI = 2.8; WAF = 32 and it decreased later
Chétoui (Hachicha Hbaieb et al., 2017)	A significant increase on POX activity followed by a slight decrease was observed during Chétoui olive ripening with a maximum (24.3 ± 0.23 U g^{-1} FW) at RI = 4	Two peaks of PPO activity (533 ± 9.17 U g^{-1} FW at RI = 1.2 and 500 ± 14.5 U g^{-1} FW at RI = 3)	An important increment of β-GL activity during Chétoui fruit ripening until reaching a maximum 44.71 ± 0.79 U g^{-1} FW at RI = 3. Then, it decreased sharply at advanced ripening stage.
Chemlali (Hachicha Hbaieb et al., 2017)	POX activity increased until reaching a maximum (31 ± 1.27 U g^{-1} FW) at RI = 2.5. Then it decreased slightly.	Two peaks of PPO activity (265 ± 3.24 U g^{-1} FW at RI = 2.5 and 304 ± 3.69 U g^{-1} FW at RI = 4)	An important increment of β-GL activity followed by a sharp decrease during Chemlali fruit ripening with a maximum (61.54 ± 0.89 U g^{-1} FW) at RI = 2.5
Arbequina and Picual	POX activity was maximum at WAF = 28 (15.21 U g^{-1} FW and 20.8 U g^{-1} for Arbequina and Picual olives, respectively). Then it remained constant. (García-Rodríguez et al., 2011).	Both cultivars showed nonsignificant differences in PPO activity after turning stage (28 WAF) (García-Rodríguez et al., 2011).	The activity values found in both cultivars were significantly different only at 20 and 35 WAF. At the latest ripening stage Picual fruits displayed twice as much β-GL activity as Arbequina fruits (Romero-Segura et al., 2012)

Abbreviations: β-GL, β-glucosidase; POX, peroxidase; PPO, polyphenoloxidase; RI, ripening index; WAF, weeks after flowering.

0.57 U g^{-1} FW at RI = 0.3 to 24.3 ± 0.23 U g^{-1} FW at RI = 4 (Table 2). Then it decreased to reach 13.86 ± 0.20 U g^{-1} FW at RI = 5.

However, García-Rodríguez et al. (2011) and Hachicha Hbaieb et al. (2015) observed a constant POX activity after an important increase during the first ripening stage of Arbequina and Picual cultivars.

Consequently, both olive cultivar and ripening degree affect considerably POX activity.

PPO activity during olive ripening was also monitored (Figure 1b). It was approximately similar to that obtained in Picual fruits (García-Rodríguez et al., 2011). An increase of PPO activity was observed from RI = 0.4 to RI = 0.7 (from 86.381 ± 2.159 to 240.421 ± 0.949 U g^{-1} FW). Then, the activity decreased at RI = 1.2 (178.873 ± 1.582 U g^{-1} FW) and remained approximately constant until RI = 3.1. PPO activity decreased noticeably at the advanced stage of ripening reaching 85.173 ± 1.494 U g^{-1} FW at RI = 3.9. This result is in good agreement with those previously reported for Spanish cultivars Gordal, Manzanilla and Picual olive cultivar fruits (García-Rodríguez et al., 2011; Hornero-Mendez et al., 2002). Nevertheless, two peaks of PPO activity were detected during Chétoui and Chemlali olive ripening (Hachicha Hbaieb et al., 2017). In fact, the first peak was attained at RI = 1.2 and 2.5 of Chétoui (533 ± 9.17 U g^{-1} FW) and Chemlali (265 ± 3.24 U g^{-1} FW) olives, respectively, while, the second peak was observed at RI = 3 and 4 of Chétoui (500 ± 14.5 U g^{-1} FW) and Chemlali (304 ± 3.69 U g^{-1} FW) olives, respectively (Table 2).

Mean values of PPO activity are in good agreement with those recently reported for Chemlali (Hachicha Hbaieb et al., 2017), Taggiasca (Cardoso et al., 2010), Arbequina and Picual (García-Rodríguez et al., 2011) cultivars.

Once again, results demonstrated that olive cultivar and ripening degree act on olive PPO activity.

Concerning β-GL enzyme, a significant increase on its activity during fruit maturation was observed (until turning stage RI = 3.9) (Figure 1c). Then, it decreased sharply at advanced ripening stage. The activity of β-GL was 17.096 ± 0.865 U g^{-1} FW at RI = 3.9 (40 WAF). β-GL activity which proved to be high in green fruit and low in black fruit was in agreement with previous studies (Hachicha Hbaieb et al., 2015, 2017; Mazzuca et al., 2006). For example, in the case of Chétoui olives, β-GL activity increased during fruit ripening until reaching 44.7 ± 0.79 U g^{-1} FW at RI = 3. Then, it decreased sharply at the advanced ripening stage to reach 11.4 ± 0.15 U g^{-1} FW at RI = 5 (Table 2) (Hachicha Hbaieb et al., 2017).

TABLE 3 Phenolic composition (expressed as milligrams of tyrosol equivalent per kg of oil) total phenol content and antioxidant activity (means of three replicates) of Oueslati virgin olive oils at different ripening degrees

Samples	November 3, 2014 (RI = 1.8)	November 13, 2014 (RI = 2.4)	November 24, 2014 (RI = 2.8)	December 03, 2014 (RI = 3.1)	December 16, 2014 (RI = 3.3)	December 30, 2014 (RI = 3.6)	January 16, 2015 (RI = 3.9)
3,4-DHPEA	0.73 ± 0.03a	3.09 ± 0.09e	1.16 ± 0.07b	2.77 ± 0.06d	0.90 ± 0.05a	2.54 ± 0.06c	3.02 ± 0.11e
p-HPEA	1.88 ± 0.03a	6.42 ± 0.15e	3.91 ± 0.04d	3.47 ± 0.04c	2.94 ± 0.07b	3.87 ± 0.04d	2.87 ± 0.07b
3,4-DHPEA-EDA	1.84 ± 0.03a	5.78 ± 0.08d	2.82 ± 0.05b	3.70 ± 0.10c	10.22 ± 0.08f	6.32 ± 0.04e	21.18 ± 0.17g
p-HPEA-EDA	10.30 ± 0.11d	11.4 ± 0.4d	12.63 ± 0.05e	10.04 ± 0.14bc	9.18 ± 0.12a	9.5 ± 0.2ab	18.12 ± 0.13f
Lignans	25.70 ± 0.07b	30.82 ± 0.19f	30.33 ± 0.06e	29.7 ± 0.03d	21.49 ± 0.08a	27.91 ± 0.04c	33.23 ± 0.10g
3,4-DHPEA-EA	4.12 ± 0.04a	8.18 ± 0.06d	5.39 ± 0.13bc	5.07 ± 0.07b	5.09 ± 0.09b	5.69 ± 0.08c	9.7 ± 0.2e
3,4-DHPEA DER	6.68 ± 0.03a	17.0 ± 0.2f	9.37 ± 0.03b	11.55 ± 0.10c	16.20 ± 0.08e	14.55 ± 0.08d	33.9 ± 0.2g
p-HPEA DER	12.18 ± 0.13a	17.8 ± 0.5d	16.54 ± 0.09c	13.51 ± 0.17b	12.12 ± 0.06a	13.4 ± 0.2b	20.99 ± 0.07e
SEC	18.86 ± 0.13a	34.9 ± 0.7d	25.91 ± 0.08b	25.1 ± 0.3b	28.32 ± 0.06c	27.9 ± 0.3c	54.9 ± 0.3e
RSA (%)	18.33 ± 1.63ab	22.30 ± 0.36c	26.45 ± 1.17d	20.20 ± 1.76bc	15.25 ± 2.35a	22.94 ± 0.90c	21.66 ± 0.18bc
Total phenols content[a]	106.4 ± 4.75a	114.5 ± 1.95ab	171.5 ± 8.36b	114.3 ± 4.14ab	112.4 ± 4.18c	122.8 ± 4.13a	240.0 ± 2.13d

Note: For each compound, values (mean ± SD) with different letters are significantly different ($p < 0.05$).
Abbreviations: 3,4-DHPEA, hydroxytyrosol; 3,4-DHPEA-EDA, di-aldehydic form of the oleuropein aglycone; 3,4-DHPEA DER, hydroxytyrosol derivatives is the sum of 3,4-DHPEA, 3,4-DHPEA–EDA, and 3,4-DHPEA–EA; *p*-HPEA, tyrosol; *p*-HPEA-EDA, di-aldehydic form of the ligstroside aglycone; *p*-HPEA DER, tyrosol derivatives is the sum of *p*-HPEA and *p*-HPEA–EDA; RSA (%), antioxidant activity; SEC, secoiridoid derivatives is the sum of *p*-HPEA DER and 3,4-DHPEA DER.
[a]Total phenols content determined colorimetrically.

This β-GL trend was predictable, as β-GL activity was correlated to oleuropein degradation (Mazzuca et al., 2006). In fact, β-GL activity was higher in green fruit because of high oleuropein amount, while, in black fruit, it was lower due to oleuropein concentration.

Total phenolic content and antioxidant activity

Quantification of phenolic compounds is an important criterion to evaluate VOO quality since they are implicated in oxidation resistance, biological properties and oil bitterness. Table 3 represents the contents of phenolic compounds (milligrams of caffeic acid equivalent per kilogram oil) in Oueslati VOO during ripening. A high variability of total phenol content depending on harvesting date was revealed. Moreover, a significant increase of total phenol content in Oueslati olive oil during ripening was observed. In fact, it passes from 106.4 ± 4.75 mg caffeic acid kg^{-1} oil at RI = 0.4 (green stage) to 240.0 ± 2.13 mg caffeic acid kg^{-1} oil at RI = 3.9 (black olives). This result was in agreement with that previously reported by Vekiari et al. (2010) on Koroneiki and Throumbolia olive cultivars. Nevertheless, the phenolic content decreased continuously but differently with Chétoui and Chemlali olive ripening (Hachicha Hbaieb, Kotti, Vichi, & Gargouri, 2016a). In fact, in the case of Chétoui olives, it passed from 1302 mg caffeic acid kg^{-1} oil at RI = 2.1 to 964 mg caffeic acid kg^{-1} oil at RI = 3. However, phenolic content varied between 373 and 242 mg caffeic acid kg^{-1} oil during Chemlali olive ripening (RI from 2.3 to 3.2).

Total phenol content obtained in Oueslati oil was lower compared to that obtained by Ouni et al. (2011). In fact, at RI = 2.8, the content of total phenols in the studied sample was about 171.5 ± 8.36 mg caffeic acid kg^{-1} oil. However, at ripening degree around 3, the content of total phenols was about 859.81 ± 0.20 mg caffeic acid kg^{-1} in Oueslati oil from Jbel Rihan (Ouni et al., 2011).

This result could be explained by the negative impact of irrigation on the amounts of totals phenols as reported by Gómez-Rico et al. (2007). In fact, L-phenylalanine ammonia-lyase activity, which is the main enzyme involved in phenolic compound synthesis, is lower when the water supply increases in olive tree (Morellóm et al., 2005). Additionally, previous studies showed that olive tree age affect the physicochemical characteristics of EVOO (Chtourou Bouchaalaa et al., 2014). In fact, adult trees produce oil with higher phenolic content compared to younger ones.

Different methods were used to evaluate VOO antioxidant activity. In this work, radical scavenging activity (RSA) percentage method was used with DPPH radical as reagent.

Antioxidant activity varied from 15.25% to 26.45% during ripening process (Table 3). These values were also low compared to those obtained by Ouni et al. (2011). In fact, antioxidant activity was equal to 26.45 ± 1.17% in studied Oueslati oil at RI = 2.8. Nevertheless, at ripening degree around 3, the antioxidant activity was about 38.81 ± 0.52% and 95.05 ± 0.06% in Oueslati oil from Menzel Rais and Jbel Rihan, respectively (Ouni et al., 2011). The lowest antioxidant activity of the studied Oueslati oils could be related to its low content on total phenols given that antioxidant activity was generally positively correlated with the content of phenolic compounds (Hachicha Hbaieb et al., 2017; Sicari, 2017).

Identification and quantification of phenolic compounds

As phenolic compounds are responsible for the bitterness and oxidation resistance, its content and composition are important parameters of quality (Bendini et al., 2007). In agreement with previous reports (Sacchi et al., 2015), the principal phenolic compounds detected in VOO samples were phenolic alcohols presented by hydroxytyrosol (3,4-DHPEA) and tyrosol (*p*-HPEA) in their free forms, oleuropein aglycone (3,4-DHPEA-EA), decarboxymethyl oleuropein aglycone (3,4-DHPEA-EDA), decarboxymethyl ligstroside aglycone (*p*-HPEA-EDA) and lignans as acetoxy pinoresinol (Figure 2, Table S1). Moreover, phenolic compounds amount in Oueslati VOOs during olive maturation was quantified using HPLC-DAD (Table 3). Results showed an important variation in the phenolic profile with olive ripening degree. For example, the

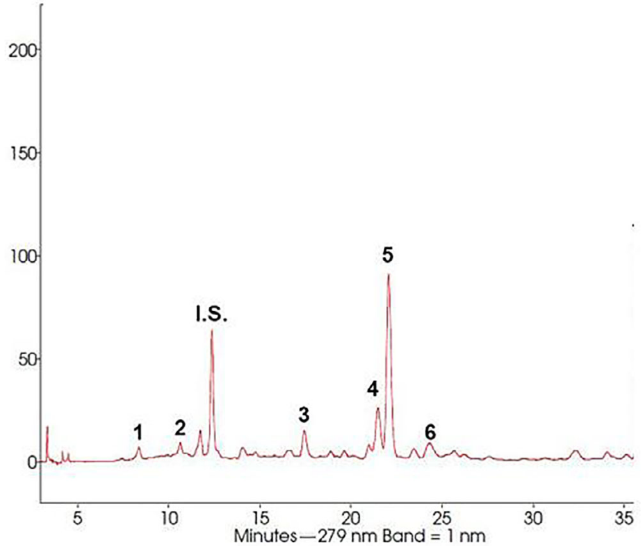

FIGURE 2 Example of HPLC chromatogram (sample ripening index = 3.6)

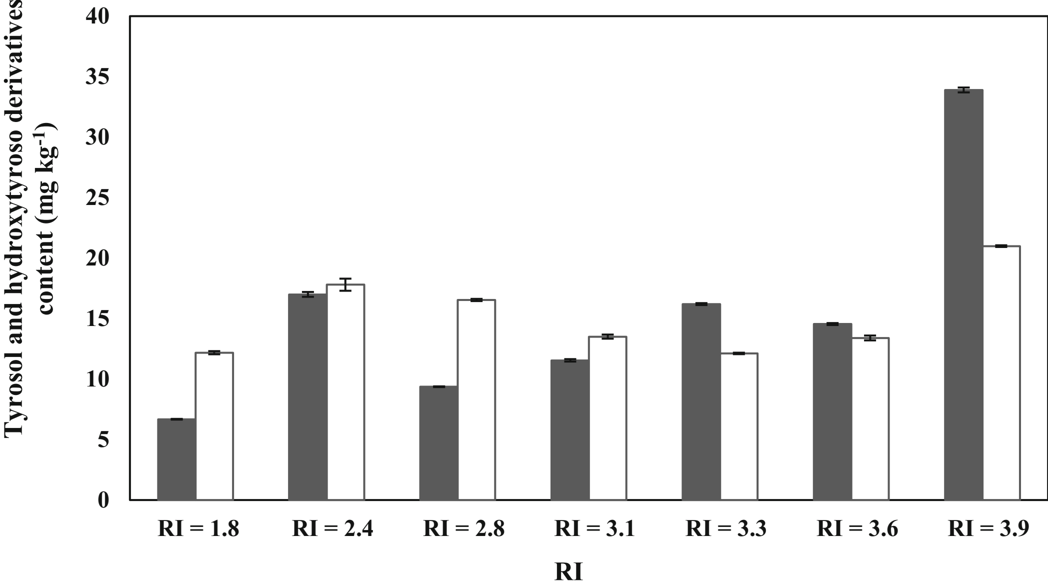

FIGURE 3 Evolution of tyrosol (□) and hydroxytyrosol (■) derivatives content (mg kg^{-1}) in olive oils during Oueslati olives ripening. RI, riping index

content of decarboxymethyl oleuropein aglycone (3,4-DHPEA-EDA) varied from 1.84 ± 0.03 mg kg^{-1} at RI = 1.8 to 21.18 ± 0.17 mg kg^{-1} at RI = 3.9.

Generally, highest content of each phenolic compounds identified was observed at RI = 3.9 except for tyrosol whose highest amount was obtained at RI = 2.4. Moreover, results showed that tyrosol was the principal phenolic alcohols in tested VOOs.

In fact, its content ranged from 1.88 ± 0.03 to 6.42 ± 0.15 mg kg^{-1}. However, hydroxytyrosol content did not surpass 3.09 ± 0.09 mg kg^{-1}. This result was in disagreement with that obtained by Ouni et al. (2011) who reported that Oueslati VOO extracted from Ain Jlola farm (Kairouan region) had the highest hydroxytyrosol concentration (6.45 mg kg^{-1}) whereas, highest content of tyrosol (3.12 mg kg^{-1}) was obtained in Oueslati VOO extracted from Menzel Rais farm (Kairouan region). The highest content of tyrosol in the studied samples coincided with those obtained in Chemlali VOOs as previously reported by Hachicha Hbaieb et al. (2017).

Concerning secoiridoids (3,4-DHPEA-EA; 3,4-DHPEA-EDA; *p*-HPEA-EA and *p*-HPEA-EDA), Oueslati VOOs are generally richer in tyrosol derivatives than hydroxytyrosol except at RI = 3.9 (Figure 3). In this stage, tyrosol and hydroxytyrosol derivatives were 20.99 ± 0.07 and 33.9 ± 0.2 mg kg^{-1}, respectively.

Among secoiridoids, *p*-HPEA-EDA was the main phenolic compounds in Oueslati VOOs oils. In fact, its concentration varied from 9.18 ± 0.12 to 18.12 ± 0.13 mg kg^{-1}. However, 3,4-HPEA-EA reached maximum concentration (9.7 ± 0.2 mg kg^{-1}) at RI = 3.9.

A highest content of lignans was also observed in Oueslati VOOs. In fact, it varied between 21.49 ± 0.08 and 33.23 ± 0.10 mg kg^{-1}. However, Ouni et al. (2011) reported that lignans content did not exceed 5.68 mg kg^{-1} in Oueslati VOO from Ala farm (Kairouan region). The highest content of lignans in the studied samples agreed with those obtained in Chétoui and Chemlali VOOs (92.2 and 39.5 mg kg^{-1}, respectively) as previously reported by Hachicha Hbaieb et al. (2017).

These results confirmed that the content of each phenolic compounds identified in Oueslati VOOs varied widely according to olive ripening degree. According to the previous studies mentioned (Ouni et al., 2011), Oueslati phenolic profile depends on geographic location (Tunis and Kairouan regions).

Correlation analysis

The evolution of total phenol content and the antioxidant activity with the RI of olives were presented in Figure 4. As shown, a positive correlation between total phenol content in olive oils on the one hand and antioxidant activity on the other hand with the RI was observed. This means that total phenolic compounds in oils and antioxidant activity increased with the RI.

A positive correlation was also observed between antioxidant activity and total phenols amount. This result was reported by previous studies (Condelli et al., 2015; Gargouri et al., 2013). Individual phenolic compounds amount as well as fatty acid composition contribute to VOO antioxidant (Hachicha Hbaieb et al., 2017; Ozkan Yorulmaz & Bozdogan Konuskan, 2017).

The evolution of olive oils phenols content with Oueslati endogenous enzymes activities was studied

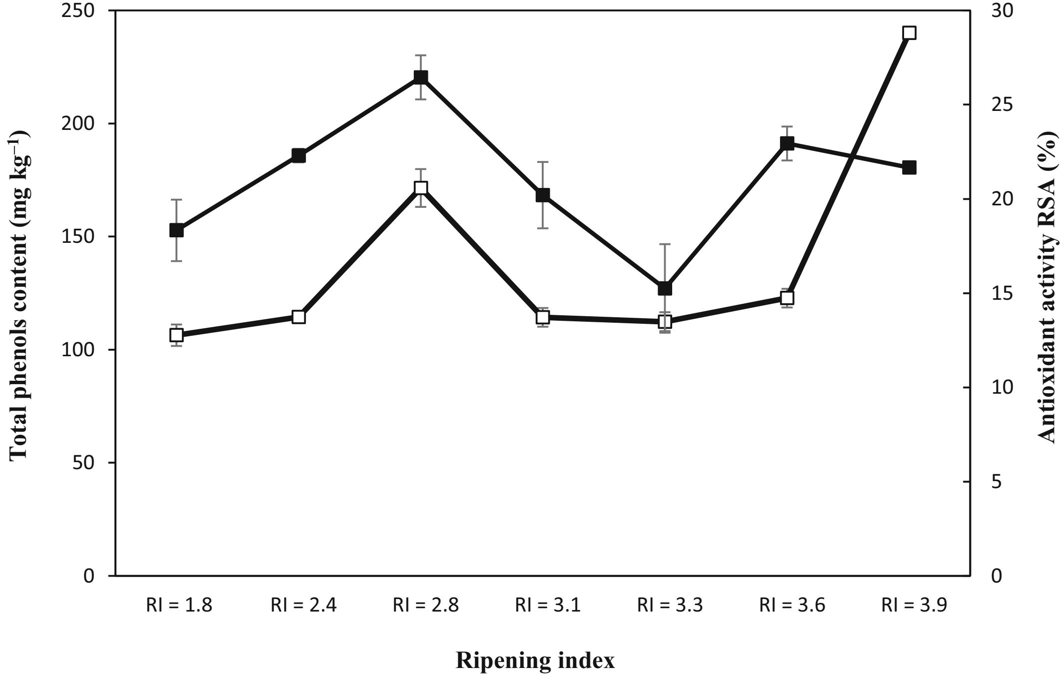

FIGURE 4 Evolution of total phenols content (□) and antioxidant activity (■) in olive oils during Oueslati olives ripening. RI, riping index; RSA, radical-scavenging activity

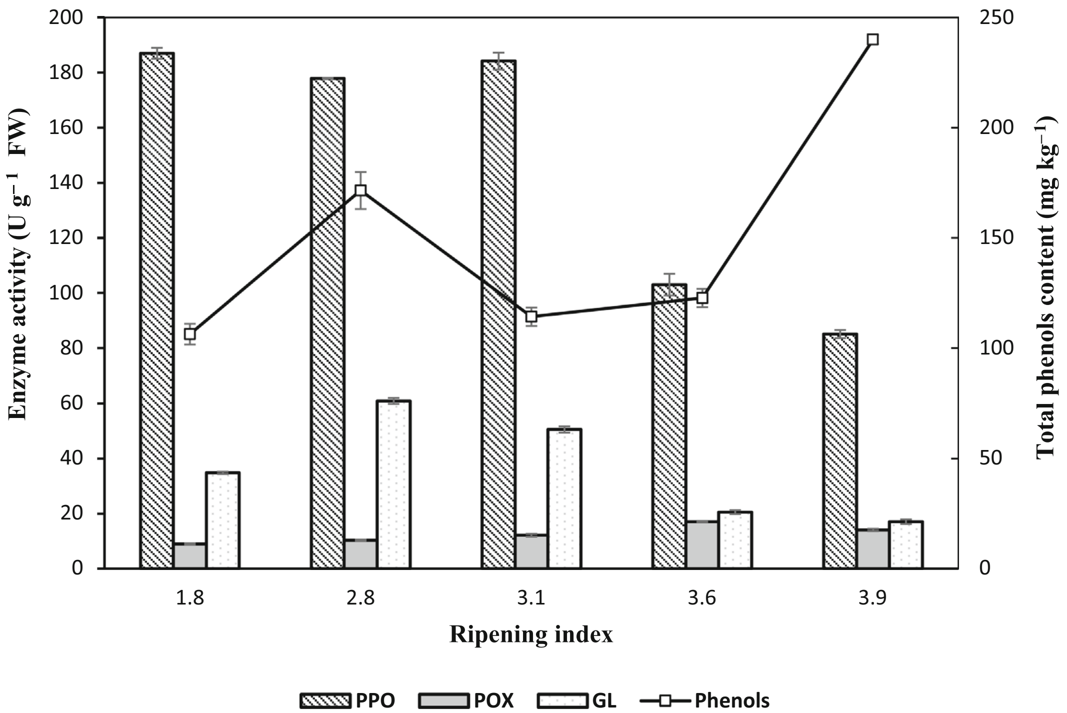

FIGURE 5 Evolution of total phenols content (□) in olive oils and enzyme activities in olive (peroxidase [POX] activity in olive seeds; polyphenoloxidase [PPO] and β-glucosidase [β-GL] activities in olive fruits mesocarp) with the ripening index of Oueslati cultivar

for the first time in order to determine which enzyme was responsible for shaping olive oil phenolic profile (Figure 5). The results obtained showed that total phenols increase with POX activity increasing and vice versa. In fact, it varied from 106.4 $\pm$ 4.75 to 240 $\pm$ 2.13 mg kg^{-1} when POX activity passed from 8.922 $\pm$ 0.24 to 14.111 $\pm$ 0.434 U g^{-1} FW.

However, total phenols content progresses inversely with PPO and β-GL activities during olive ripening. In fact, the content of total phenols increased

from 106.4 ± 4.75 to 240 ± 2.13 mg kg^{-1} when PPO and β-GL activities decreased from 187.102 ± 1.987 to 85.173 ± 1.494 U g^{-1} FW and from 34.819 ± 0.460 FW to 17.096 ± 0.865 U g^{-1} FW, respectively.

García-Rodríguez et al. (2015) demonstrated a negative correlation between oxidoreductases (POX and PPO) activities and VOO phenolic compound content during kneading and milling steps, respectively. The actual study showed that the increase in total phenols coincides with the decrease in PPO activity. Therefore, PPO could be considered as the main enzyme defining Oueslati VOO phenols content.

However, Hachicha Hbaieb et al. (2015, 2017) reported that the key endogenous enzyme determining Arbequina, Chétoui and Chemlali VOOs total phenols amount was β-GL.

Therefore, the three enzymes (POX, PPO, and β-GL) act in a different way in shaping VOO phenolic profile depending both on olive cultivars and ripening degree.

CONCLUSION

This study was the first enzymatic characterization of Oueslati olive and their effect on determining VOO phenolic profile during maturation. Each enzyme (POX, PPO, and β-glucosidase) showed specific trend. Generally, an increase of these enzyme activities followed by a decrease was observed during fruits ripening. The maximum of PPO (240.421 ± 0.949 U g^{-1} FW), β-glucosidase (60.857 ± 1.105 U g^{-1} FW) and POX (17.061 ± 0.101 U g^{-1} FW) activities was observed at RI 0.7, 2.8, and 3.6, respectively.

Concerning the compositional quality of VOO, the content of total phenol increased during maturation of olive fruits. Moreover, 3.9 was considered as the appropriate RI for Oueslati olives since the highest content of most important individuals' phenolic compounds (3,4-DHPEA-EDA, 3,4-DHPEA-EA, and *p*-HPEA-EDA) responsible for main VOO biological properties was reached on this date. Furthermore, results showed that phenols content in Oueslati VOO was principally determined by PPO enzyme as the increase in total phenols coincides with the decrease in PPO activity. It was thus indicated that Oueslati cultivar has its own enzymatic characteristics that are reflected in its phenolic profile which distinguishes it from main Tunisian cultivars Chétoui and Chemlali. Consequently, Oueslati VOO deserves to be valued and may contribute to Tunisian olive-growing heritage wealth. This study on biochemical factors is able to modify the phenol content of Tunisian Oueslati VOOs and to support producers to be up to their main international competitors.

EVOO quality is a combination of good agronomic and technological practices, starting from the choice of olives harvesting time. It is the result of the combination of genotype, environment, and extraction process. Therefore, enzymes characterization and phenolic profile identification in VOO from a specific cultivar/area have led to the development of expert systems that will assist both, olive growers and oil millers not only in their operational choices, but also in a quantitative–qualitative result. This ambitious project will help agriculture and millers to have detailed information about the fruits still on the tree. Furthermore, it allows to assess degradation products by different endogenous enzymes during VOO extraction that will be carried in future studies to identify phenolic compounds existing in the matrix and to propose mechanisms of complex phenolic compounds degradation by enzymes.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

Rim Hachicha Hbaieb, Faten Kotti, and Mohamed Gargouri conceived and designed the study. Rim Hachicha Hbaieb carried out the research and wrote the first draft of the manuscript. Antonello Paduano and Pasquale Crupi realized phenols chromatographic analysis. Maria Lisa Clodoveo contributed to the conclusion writing. Rim Hachicha Hbaieb and Faten Kotti analyzed the data. Mohamed Gargouri, Faten Kotti, and Maria Lisa Clodoveo contributed to the draft revision. All authors contributed to the final draft of the manuscript and approved it.

ORCID

Antonello Paduano https://orcid.org/0000-0002-6623-8631
Mohamed Gargouri https://orcid.org/0000-0002-6924-2230

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