Title: Evolution of endogenous enzyme activities and virgin olive oil characteristics during Chétoui and Chemlali olive ripening

Authors: Rim Hachicha Hbaieb[a], Faten Kotti[a], Stefania Vichi[b] and Mohamed Gargouri[a*].

Affiliations:

[a]*Biocatalysis and Industrial Enzymes Group, Laboratory of Microbial Ecology and Technology, Carthage University, National Institute of Applied Sciences and Technology (INSAT), BP 676, 1080 Tunis Cedex, Tunisia.*

[b]*Nutrition,Food Science and GastronomyDepartment, INSA-UB, XaRTA (Catalonian Reference Network on FoodTechnology), University of Barcelona, Food and Nutrition Torribera Campus, Av. Prat de la Riba, 171. 08921, Santata Coloma de Gramenet, Spain.*

***** Corresponding author. Tel.: +216 71 70 38 29; fax: +216 71 70 43 29; mobile phone: +216 98316764, E-mail addresses: mohamed.gargouri@insat.rnu.tn; mhdgargouri@yahoo.com (M. Gargouri).

Running title: Enzymes changes and VOO quality during olive ripening

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Abbreviations:

C5 ALC = total C5 alcohols

C5 ALD = total C5 aldehydes

C5 TOT = total C5 compound

C6 ALC = total C6 alcohols

C6 ALD = total C6 aldehydes

C6 EST = total C6 esters

C6 TOT = total C6 compound

DTT = Dithiothreitol

EDTA = Ethylene Diamine Tetraacetic Acid

LOX = lipoxygenase

PMSF= Phenyl Methyl Sulfonyl Fluoride

p-NPG = p-nitrophenyl- β,D-glucopiranoside

POX = Peroxidase

PPO = Polyphenoloxidase

RI = Ripening Index

SDS =Sodium Dodecyl Sulfate

TBC = Tert-butylcatechol

VOO = Virgin Olive Oil

Abstract:

The composition and biochemical characteristics of olive fruits, mainly olive enzymes system, are crucial in determining the final quality of virgin olive oil (VOO). Thus, olives endogenous olive enzyme activities were studied during Chétoui and Chemlali olive ripening. The compositional quality of the corresponding VOO was also studied. Peroxidase (POX) and β-glucosidase activities increased during olives ripening. However, polyphenoloxidase activity decreased slowly. Moreover,the POX enzyme appears to play an essential role in determining VOO total phenol amounts, as the decrease in phenol content registered during olive ripening coincided with the increase inPOX activity.

A positive correlation between oil antioxidant activity and the total phenol content was established for both the olive cultivars studied.

With regard to pigments, chlorophyll content wasmuch higher than that of carotenoids in both Chétoui and Chemlali oils. Moreover, different trends in chlorophyll and carotenoid contents were observed, depending on the olive cultivar.

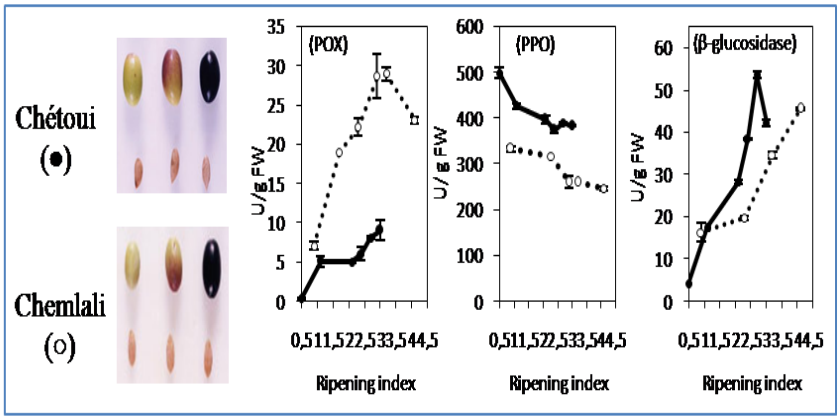
Concerning volatile compounds, our results showed that the highest content of total C6, C5 LOX compounds and pentene dimers was observed at a RI of approximately 3 for both cultivars. However, C6 alcohols and total C5 compounds decreased in Chétoui and Chemlali oils, respectively, during olive ripening.

Practical applications

Volatile and phenolic compounds play an important role in determining VOO quality. However, these compounds were affected by numerous factors, such as, endogenous olive enzyme activities. Monitoring these enzymes activities (peroxidase, polyphenoloxidase and β-glucosidase) during olive fruits ripening by a simple spectrophotometric assay could be

useful to establish an optimum harvesting date for each olive cultivar and their possible correlation with VOO total phenol amount.

Graphical abstract



Evolution of endogenous olive enzyme activities (peroxidase: POX; polyphenoloxidase: PPO and β-glucosidase) during Chétoui (●) and Chemlali (○) olive ripening.

1. Introduction

Olive oil is a fundamental component of the traditional Mediterranean diet. It is extracted by mechanical processes from and healthy olives with noinjuries or mechanical damages at an adequate point of ripening. It is highly appreciated by consumers and continuously attracts the interest of the scientific community for its sensory, nutritional and health-protecting properties. These are mainly due to its minor components, namely volatile and phenolic compounds [1].

In fact, volatile compounds are responsible for the aroma of VOO. They are formed during VOO extraction from free polyunsaturated fatty acids, especially linoleic and linolenic acids, via the lipoxygenase (LOX) pathway [2]. C6 and C5 compounds, in particular C6 aldehydes and alcohols such as hexanal, *E*-2-hexenal, 1-hexanol, *E*-2-hexenol and *Z*-3-hexenol, constitute the most important fraction of volatile compounds and contribute to the fruitiness, pungency and bitterness of VOO [3].

Nevertheless, it is the phenolic compounds that are responsible for the taste of VOO, in particular, its positive bitter and pungent sensory attributes. Moreover, they contribute to the oxidative stability, the shelf life, and some of the nutritional and bioactive properties of VOO [4].

The occurrence of these minor components in VOO depends essentially on agronomical and technological factors, such as the olive cultivar, degree of ripening, climatic conditions, and oil extraction process [5,6,7]. Indeed, the chemical and biochemical characteristics of the olive fruit, particularly the enzymes activities determined genetically in each olive cultivar, are crucial in determining the final quality of VOO.

Several studies have addressed the compositional quality of VOO according to olive cultivar [8, 9], stageof ripening [7, 8], pedoclimatic conditions[10], irrigation management [11], and olive oil extraction process [12]. Nevertheless, few studies have characterized

113 olive fruit enzymatic activities according to factors such as olive cultivar and ripening
114 degree, or their potential in determining VOO quality.
115 Given the increasing interest in the phenolic compound contents of VOO as a reliable
116 indication of its origin and quality, the present study takes into consideration only the
117 enzymes involved in shaping the phenolic profile of VOO.
118 Almost all the published studies of endogenous olive enzymes have focused on European
119 olive cultivars. In fact, García-Rodríguez*et al.* [13] studied the evolution of both PPO and
120 POX activities in Arbequina and Picual olives and they demonstrated the role they play in
121 promoting the oxidation of the main phenolic glycosides present in the fruit as well as those
122 phenolic compounds that arise during the olive oil extraction process. Moreover, Ramírez
123 *et al.* [14] studied the endogenous enzymes (β-glucosidase, esterase, POX and PPO)
124 involved in the transformation of oleuropein in table olive from Spanish cultivars such as
125 Gordal, Manzanilla, and Hojiblanca. Hachicha Hbaieb*et al.*[15] monitored the same
126 enzymes during Arbequina olive ripening and storage, and determined their impact on
127 VOO phenolic profile.
128 Nevertheless, there is a lack of studies of the evolution of endogenous enzyme activities
129 during the maturation of Tunisian olives cultivars. Jemai et al. [16] studied β-glucosidase
130 and esterase activities ofthe Tunisian olive cultivar Dhokar, with regard to fruit ripening.
131 Later, Hachicha Hbaieb et al. [17] studied the evolution of POX, PPO and β-glucosidase
132 during Chétoui and Arbequina olive ripening and storage, and the phenolic profile of the
133 corresponding VOOs, in order to determine the storage conditions that preserve the
134 phenolic fraction of the oils at similar levels than those extracted from freshly harvested
135 olives.
136 Therefore, the aim of this study was firstly to monitor these endogenous olive enzymes
137 from the two main Tunisian olive cultivars (Chemlali and Chétoui) cultivated in the same

olive grove during fruit ripening. Secondly, we aimed to determine the compositional quality of VOO in terms of the amount of phenolic compounds, antioxidant activity, pigments and the main volatile compounds in order to establish an optimum harvesting date for each olive cultivar. To the best of our knowledge, this paper reports the first research carried out to determine acorrelation between endogenous olive enzyme activities, the ripening index of olive cultivars and the phenolic compounds in Tunisian VOOs, as determined for the two main Tunisian cultivars at different stages of ripening.

2. Material and methods

2.1 Plant material

The study was carried out on monovarietal virgin olive oils from the two main Tunisian cultivars, namely Chétoui and Chemlali, planted in a non-irrigated orchard at the experimental field of the National Institute of Applied Sciences and Technology in Tunis during the crop season 2013-2014. Only healthy olive fruits, without any kind of infection or physical damage, were handpicked from October 2013 to January 2014. The harvesting dates and the corresponding ripening index (RI) for each cultivar are presented in Table 1. RI was determined for olive cultivar and sampling date, as described by Uceda and Frias [18]. Some 250g of olives was used to determine the enzymatic activities and 1 kg was used for olive oil extraction.

2.2 Chemicals

Reagents for enzymatic activity extraction and measurements were supplied by Sigma-Aldrich (St. Louis, MO). The phenolic compounds were dissolved in a mixture of methanol/water (80:20 v/v). Pure HPLC solvents were used in all cases (Sigma-Aldrich, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and Folin-Ciocalteu reagent were purchased from Sigma-Aldrich, USA and Carlo Erba, respectively.

2.3 Olive oil extraction

Olive oil was extracted using an Abencor analyzer (Comercial Abengoa, S.A., Seville, Spain). Approximately 1 kg of oliveswas crushed using a stainless steel hammer mill operating at 3000 rpm and fitted with a 5 mm sieve. The resulting olive paste was malaxed for 30 min at 30 °C. Paste was centrifuged by a basket centrifuge at 3500 rpm for 1 min, and the oils were decanted. Then, the oils were transferred into dark glass bottles and stored under nitrogen, in the dark and at -20 °C until analysis. For each date and cultivar, one sample of olive oil was obtained.

2.4 Enzyme extraction and activity assay

The acetonic powders were prepared from the pulp of fresh harvested olives [13]. PPO and β-glucosidase were extracted from acetone powders as previously described by Garcia-Rodriguez *et al.* [13] and Romero-Segura *et al.* [19], respectively. POX enzyme extracts were prepared from olives seeds, as previously reported by Garcia-Rodriguez *et al.*[13].

POX activity in the extracts was determined spectrophotometrically by measuring the rate of tetraguaiacol formation, due to guaiacol peroxidation, at 470 nm in the H_2O_2 detriment (ε = 26,600L/mol.cm). One unit of POX activity corresponds to the amount of enzyme oxidizing 1 μmol of guaiacol per min.

PPO activity was measured by monitoring the increase in absorbance at 400 nm corresponding to the quinone formation as a result of tert-butylcatechol (TBC) oxidation (ε = 1200 L/mol.cm) [13]. One unit of PPO activity was defined as the amount of enzyme forming 1 μmol of TBC-quinone per min [13].

The increase of theabsorbance at 405 nm, related to the generation of *p*-nitrophenol liberated from the synthetic glucoside 4-nitrophenyl-β,D-glucopiranoside (PNPG), was used to determine the β-glucosidase activity (ε= 552.8 L/mol.cm)[20]. One unit of β-glucosidase activity was defined as the amount of enzyme able to liberate 1 μmol of p-nitrophenol per min [20].

Enzyme extraction was performed in duplicate and all the enzymatic activity was determined in triplicate.

2.5 Colorimetric determination of total polyphenol

VOO phenolic fraction was extracted by liquid–liquid microextraction (LLME) according to the method described by Luz Pizarro*et al.* [21]. 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 obtained extracts were combined and used to determine the total phenols content by the colorimetric method.

Total phenolic compounds in olive oils were determined bythe Folin–Ciocalteu assay, as described by Gutfinger [22].The results were expressed as milligrams of caffeic acid per kilogram of oil, using a calibration curve of freshly prepared caffeic acid solutions within the range 0–0.5 mg/mL.

2.6 Antioxidant activity assay with DPPH

The radical-scavenging activity was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH, Carlo Erba, MI, Italy) radical cation decolorisation assay, following the methodology described by Brand-Williams *et al.* [23].

970 µL of 60 µmol/L methanolic DPPH was added to 30 µL of phenolic extract. An equal amount of methanol was used as a control. After 30 min of incubation in the dark at room temperature, the absorbance was measured spectrophotometrically at 517 nm.

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

$$\text{DPPH radical scavenging \%} = \frac{Ac - As}{Ac} \times 100$$

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

2.7 Chlorophylls and carotenoids content

213 The amount of pigments was determined using the specific extinction values, according to
214 Minguez-Mosquera *et al.* [24]. Briefly, 7.5 g of olive oil was dissolved in 25 mL of
215 cyclohexane. The absorbance of this solution was measures at 470 and 670 nm for
216 carotenoids and chlorophylls, respectively. The content of pigments was calculated as
217 follows:

218 Chlorophylls content (mg/kg) = $\frac{A670 \times 10\text{^}6}{613 \times 100 \times d}$

219 Carotenoid content (mg/kg) = $\frac{A470 \times 10\text{^}6}{2000 \times 100 \times d}$

220 Where *A* is the absorbance; E0 = 613 is the extinction coefficient of pheophytin 'a'; E_0 =
221 2000 is the extinction coefficient of lutein and *d* is the spectrophotometer cell thickness (1
222 cm).

223 ***2.8 Analysis of volatile compounds by GC-MS***

224 VOO volatile profile was analysed by solid phase microextraction, as described elsewhere
225 [25]. Briefly, 2 g of sample was placed into a 10 mL vial and spiked with 1 mg/kg (IS), by
226 adding 20 µL of 0.1 mg/mL solution of 4-methyl-2-pentanol in refined sunflower oil. The
227 vial was then fitted with a silicone septum and maintained under magnetic stirring (500
228 rpm) at 40°C. After 10 min of sample conditioning, a DVB/CAR/PDMS fiber was exposed
229 to the sample headspace for 30 min and immediately desorbed in the gas chromatograph
230 injector. The desorption temperature was 260 ºC. The extraction of each sample was
231 performed in duplicate.

232 Volatile compounds were identified by gas chromatography coupled to quadrupole mass
233 selective spectrometry using an Agilent 5973 Network detector (Agilent Technologies,
234 Palo Alto, CA, USA) [26]. Analytes were separated on a Supelcowax-10 (Supelco,
235 Bellefonte, PA) (30 m x 0.25 mm ID, 0.25 μm film thickness). Helium was the carrier gas
236 at a linear velocity of 2cm/s. The column temperature was held at 40°C for 5 min, increased
237 to 100°C at 3°C/min, 120°C at 6°C/min, 200°C at 15°C/min and then 250°C at 30°C/min.

The injector temperature was 265 °C and the time of desorption of the fiber into the injection port was fixed at 10 min. The temperature of the ion source was 175°C and the transfer line, 280°C. Electron ionization mass spectra (EIMS) were recorded at 70 eV ionization energy in the 15-250 u mass range, 2 scan/s.

Volatile compounds were identified by comparison of their mass spectra and retention times with those of standard compounds, or by comparison of the mass spectrum with those of the mass spectrum library Wiley6.

Quantitative determination was performed by the method of internal standards. Response factors were calculated by a calibration curve done by analyzing deodorized sunflower oil with different concentrations of the reference compounds. Standard solutions were prepared in the range 0.01–10 mg/kg and analyzed in duplicate under the same conditions described for the samples. For pentene dimers, a reference standard was not available, and their relative response factor was assumed to be 1. The amounts of volatile compounds identified in VOO headspace were expressed as mg/kg oil.

2.9 Statistics

The results were expressed as mean value (mv) ± Standard Deviation (SD). Statistical analysis was carried out using Statgraphics software. Significant differences between treatments were determined using one-way ANOVA.

3. Results and discussion

3.1 Monitoring olive POX, PPO and β-glucosidaseactivities during olive ripening

Figure 1A represents the variation of POX activity in Chétoui and Chemlali olive seeds during olives ripening. POX activity greatly increased in both thecultivars studied, but at different rates. In fact, it increased considerably in Chemlali olives from RI=1.2 to RI=2.9 and then remained constant until showing a slow decrease at RI=4.1. Meanwhile, in the case of Chétoui olives, POX activity continued to increase but at a slower rate than it did

in Chemlali olives. Generally, the trend in POX activity during Chemlali and Chétoui olive ripening is in agreement with previous studies of Spanish olive cultivars [13, 15].

Furthermore, POX activity was found to be lower in Chétoui olives than in Chemlali olives at the different sampling dates studied. In fact, it ranged from 0.348 to 9.01 U/g FW and from 7.06 to 28.99 U/g FW for Chétoui and Chemlali olives respectively.

The values of Chemlali POX activity are quite similar to those recently reported for Picual cultivars [13]. The maximum POX activity in Chétoui olives was9.01 U/g FW at RI=3 (3^{rd}December 2013), while for Chemlali olives it was approximately 28.68U/g FW at RI=2.9 (6^{th} November 2013). This observation could be a direct consequence of the difference in the maturation process between the two varieties studied. Indeed, Chétoui olives present a slower maturation process than Chemlali olives.

Consequently, these results show that POX activity depends markedly on olive cultivar and the degree of ripening.

PPO activity was measured in olive pulp during Chétoui and Chemlali olive ripening (Figure.1B). The first finding that must be noted is the decrease of PPO activity during the maturation processin both olive cultivars. In both the olive cultivars, PPO activity reduced by a total of approximately 25% on average, during the time of the study. In fact, it went from 498.7 to 382.6 U/gFW and from 332.5 to 245.2 U/g FW during Chétoui and Chemlali olive ripening, respectively. In contrast to our observations for POX activity, Chemlali olives presented the lower values of PPO activity over the whole period tested. This trend in PPO activity is similar to those reported previously for the Spanish (Gordal, Manzanilla and Picual) [13] and Italian (Taggiasca) cultivars [27].

These results show again that the cultivar and the degree of ripening of the olive significantly influence olive PPO activity.

The oliveβ-glucosidase (Fig.1C) showed an important increase of activity during ripening which was more important in Chétoui olives. In fact, the increase was some 12.5-fold in the case of Chétoui olives compared toonly 2.8-fold for Chemlali olives. This finding supports those reporting an increase of β-glucosidase activity during the initial ripening stage followed by a slight decrease later at advanced ripening stages. Several studies have related the increase of β-glucosidase activity to oleuropein degradation [28]. Moreover, higher β-glucosidase activity was observed in Chétoui olives, which could lead to a high content of total phenols in the corresponding VOO.

3.2 Total phenolic content

Table 1 shows the evolution in the content of phenolic compounds in Chétoui and Chemlali oils during olives ripening. The results show that the total phenol content ranged from 964 to 1302 mg/kg oil and from 242 to 373mg/kg during Chétoui and Chemlali ripening, respectively. Furthermore, a high variability in thetotal phenol content was observed, depending on the cultivar and harvesting date. In fact, the phenolic content decreased continuously with olive maturation, but differently from the two olive cultivars. This result is in good agreement with thereportsofothers authors [8, 15, 17]. In fact,Hachicha Hbaieb*et al.*[17] observed a drastic decrease in the content of VOO secoiridoids during Chétoui and Arbéquina olive ripening and storage. The decrease during ripening was more pronounced in Chétoui olives than in Chemlali olives.

Moreover, higher levels of total phenol content were observed in Chétoui oils at all ripening stages, in agreement with previous results [17]. This may be ascribed either to the higher β-glucosidase activity in the Chétoui cultivar or to its markedly later maturation than that of the Chemlali cultivar. Furthermore, as POX is involved in the oxidation of phenolic compounds, its lower activity in Chétoui than in Chemlali olives could explain the higher content of total phenols in the corresponding VOO.

3.3Correlations of total phenol content with endogenous olive enzyme activities for Chétoui and Chemlali cultivars

Recently, the role of endogenous olive enzymes (POX, PPO, β-glucosidase) in determining the phenolic profile of VOOs was studied [13, 15]. However, those studies were of Spanish olives cultivars. To the best of our knowledge, this is the first report of the correlation of endogenous Tunisian olive enzyme activities with total phenol content. Regression analysis

The regression analysis (Table 2) shows a statistically significant negative correlation between POX activity and total phenol amounts was obtained for both the Chétoui and Chemlali cultivars with R^2 = -0.932 and R^2 = -0.865, respectively. No statistically significant correlation was found between total phenol content, PPO and β-glucosidase activity. Therefore, the present results could suggest anessential role for the POX enzyme in determining VOO total phenolic amounts in both cultivars, as the decrease in VOO total phenol contentsolives ripening coincides with the increase in POX activity. However, Hachicha Hbaieb et al. [17] demonstrated that PPO activity and the availability of secoiridoid compounds could be the main responsible for the formation of VOO oxygenated secoiridoids.

3.4 Antioxidant activity

The evolution of radical scavenging activity (RSA) during Chétoui and Chemlali olives ripening is reportedin Table 1. RSA depends on the cultivar and the ripening index of each. Chétoui oil had higher RSA than Chemlali oil during all the studied period studied ($p < 0.05$), in agreement with the higher total phenol content of VOOs from this cultivar. Theseresults may be associated withthe advanced maturation of Chemlali compared to Chétoui olives, as the latter present a slower maturation process. Our results are in agreement with those obtained by Issaoui*et al.*[29] who also observed lower antioxidant activity in Chemlali than in Chétoui oils. Moreover, a decrease of VOO antioxidant activity

was observed during olive ripening;as expected, a positive correlation was found between the antioxidant activity and total phenols content (Table 2), confirming the contribution of VOO phenolic compounds to its antioxidant capacity, and consequently, that endogenous olive enzymes play also a crucial role in determining VOO antioxidant activity.

3.5 Chlorophylls and carotenoids contents

Different trends in chlorophyll and carotenoid contents were observed for the two olive cultivars during ripening. Generally, an increase of chlorophyll and carotenoid content was detected in Chétoui VOOs from RI=2.1 to RI=2.7, followed by a decrease which becomes apparent at RI=3 (Table1). Moreover, our results showed that the content of chlorophyll in Chétoui oils was higher than that ofcarotenoids.In the case of Chemlali VOOs, a continuous decrease in chlorophyll and carotenoid contents was observed. This decrease was more important forchlorophyll. The pigment change in Chétoui oils during olive ripening is in good agreement with reports by Mraicha *et al.* [30].

The loss of chlorophyll and carotenoid contents could be explained by the decrease in photosynthetic activity and the generation of other colored compounds, such as anthocyanins which are responsible for the violet or purple color of the ripe olive fruit. Thus, the earlier decrease in chlorophyll and carotenoid contents in Chemlali than in Chétoui VOOs could be considered normal, since Chétoui cultivar is characterized by a late maturation process than that of the Chemlali cultivar.

3.6 Volatile compounds

Besides phenolic compounds, the volatile compounds derived from the LOX pathway are crucial in determining VOO quality. Therefore, their quantification is essential to predict the optimal harvesting date for each olive cultivar. The analysis of volatile fractions from Chétoui and Chemlali VOOs during olive ripening (RI from 2.1 to 3 and from 2.3 to 4.1, respectively) showed that C6 and C5 volatile compounds represent the major aromatic

components detected in the oil samples. The content of these volatiles in oils obtained from Chétoui and Chemlali olives during olive ripening are reported in Table 3. The results show that the content of C6 and C5 LOX compounds varied greatly between the varieties studied.

C6 LOX compounds

As shown in Table 3, on all the harvest dates studied, Chemlali oils presented higher contents of total C6 LOX volatile compounds, which are responsible for the green and fruity attributes, than Chétoui oils. This result is in agreement with previous reports on Tunisian monovarietal VOOs [31]. In particular, the contents of C6 aldehydes and alcohols were in general higher in Chemlali oils, whilst C6 esters were present, in low amounts, only in Chétoui oils.

These results suggest that Chemlali olives possess higher activities of LOX, hydroperoxyde lyase and alcohol dehydrogenase and lower activity of alcohol acyl transferase than Chétoui olives.

Asignificant increase of total C6 aldehydes was observed during Chétoui olive ripening, with a maximum concentration (3.8 mg/kg) at RI = 3, in disagreement with our previous report[32], thus highlighting the strong effect of the harvest year on VOO characteristics. Similarly, in Chemlali oils, C6 aldehydes and alcohols reached their maximum amounts (19.07 mg/kg and 1.5 mg/kg, respectively) at RI = 3.2, while, the amounts of C6 alcohols in Chétoui olives decreased considerably during ripening, as previously reported [32], which could be explained by the increase of C6 esters observed in Chetoui olives (Table 3).

C5 LOX compounds and pentens dimers

Higher amounts of total C5 compounds, including C5 aldehydes and alcohols, were observed in Chemlali oils overall the period tested. Different trends in total C5 compounds were observed between the two cultivars during olive ripening:they increased significantly

during Chétoui olive ripening, and decreased during Chemlali olive ripening. The negative effect of the RI on total C5 compounds observed in Chemlali VOOs was also evidenced in other cultivars[32]. Moreover, a general increase of C5 aldehydes was observed until RI=3, while the amount of C5 alcohols presented different trends between the two cultivars studied. In fact, during olive ripening they decreased in Chemlali oils and increased notably in Chétoui oils.

Concerning pentenedimers, in both cultivars they reached a maximumconcentration at a RI of approximately 3.

These findings show that the main volatile compounds, particularly C6 and C5 LOX components, depend on the olive cultivar and ripening index. This variation can be explained by differences in enzymatic activities between the olive, cultivars which is genetically determined.

4. Conclusion

This is the first report to evaluate the change in endogenous olive enzyme activities and the VOO compositional quality of the main Tunisian olive cultivars (Chétoui and Chemlali) during olive ripening. The enzymatic data discussed in this report show different trends in endogenous olive enzyme during olives ripening. Moreover, the higher β-glucosidase and the lower POX activities in Chétoui olives, compared to Chemlali olives, seem to be responsible for the higher content of total phenols in the corresponding VOO. Furthermore, it appears that POX is the main enzyme that determines VOO phenolic content from Chétoui and Chemlali olives, as the decrease in its total phenol content coincides with the increase in POX activity. With regard to pigment, the chlorophyll content was much higher than that of carotenoids in both the varieties. Moreover, different trends of chlorophyll and carotenoid content were observed for the olive cultivars studied. Concerning the LOX

volatile compounds, total C6, C5 LOX compounds and pentene dimers, increased up to a maximum at a RI of approximately 3. Only C6 alcohols and total C5 compounds decreased in Chétoui and Chemlali oils, respectively, during olive ripening.

Regarding the optimal harvest period, VOOs obtained from olives with RI of approximately 2 presented higher amounts of phenols and pigments, and higher RSA, while volatile compounds from the LOX pathway were in general higher in oils extracted from olives with a RI close to 3.

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Conflict of interest statement

The authors have declared no conflict of interest.

References

[1] Fregapane, G., Salvador, M.D., Production of superior quality extra virgin olive oil modulating the content and profile of its minor components. *Food Res. Int*. 2013, *54*, 1907-1914.

[2] Sanchez, J., Harwood, J. L., Biosynthesis of triacylglycerols and volatiles in olives. *Eur. J. Lipid Sci. Tech*. 2002, *104*, 564-573.

[3] Kotti, F., Cerretani, L., Gargouri, M., Chiavaro, E., Bendini, A., Evaluation of the volatile fraction of commercial virgin olive oils from Tunisia and Italy: relation with olfactory attributes. *J. Food Biochem*. 2011, *35*, 681-698.

[4] Cicerale, S., Conlan, X.A., Sinclair, A.J., Keast, R.S., Chemistry and health of olive oil phenolics.*Crit. Rev. Food Sci.Nutr*. 2009, *49*, 218-236.

[5] Bakhouche, A., Lozano-Sánchez, J., Beltrán-Debón, R., Joven, J., Segura-Carretero, A., Fernández-Gutiérrez, A., Phenolic characterization and geographical classification of commercial Arbequina extra-virgin olive oils produced in southern Catalonia. *Food Res. Int*. 2013, *50*, 401-408.

[6] Clodoveo, M.L., Hachicha Hbaieb, R., Kotti, F., Mugnozza, G. S., Gargouri, M., Mechanical Strategies to Increase Nutritional and Sensory Quality of Virgin Olive Oil by Modulating the Endogenous Enzyme Activities. *Compr. Rev. Food Sci. Food Saf*. 2014, *13*, 135-154.

[7] Gouvinhas, I., de Almeida, J. M.M.M., Carvalho, T., Machado, N., Barros, A.I.R.N.A., Discrimination and characterisation of extra virgin olive oils from three cultivars in different maturation stages using Fourier transform infrared spectroscopy in tandem with chemometrics. *Food Chem.* 2015, *174*, 226-232.

[8] Gómez-Rico, A, Fregappane, G., Salvador, M.D., Effect of cultivar and ripening on minor components in Spanish olive fruits and their corresponding virgin olive oils. *Food Res. Int.* 2008, *41***,** 433-440.

[9] Ouni, Y., Flamini, G., Douja, D., Zarrouk, M., Effect of cultivar on minor components in Tunisia olive fruits cultivated in microclimate. *J. Hortic. For*. 2011, *3*, 13-20.

[10] Ouni, Y., Taamalli, A., Gómez-Caravaca, A. M., Segura-Carretero, A., Fernández-Gutiérrez, A., Zarrouk, M., Characterisation and quantification of phenolic compounds of extra-virgin olive oils according to their geographical origin by a rapid and resolutive LC-ESI-TOF MS method. *Food Chem.* 2011, *127*, 1263-1267.

[11] Ben Brahim, S., Gargouri, B., Marrakchi, F., Bouaziz, M., The Effects of Different Irrigation Treatments on Olive Oil Quality and Composition: A Comparative Study between Treated and Olive Mill Wastewater. *J. Agric. Food Chem*. 2016, *64*, 1223-1230.

[12] Ben Hassine, K., Taamalli, A., Ferchichi, S., Mlaouah, A., Benincasa, C., Romano, E. , Flamini, G., Lazzez, A., Grati-kamoun, N., Perri, E.,Malouche, D., Hammami, M., Physicochemical and sensory characteristics of virgin olive oils in relation to cultivar, extraction system and storage conditions. *Food Res. Int*. 2013, *54*, 1915-1925.

[13] García-Rodríguez, R., Romero-Segura, C., Sanz, C., Sánchez-Ortiz, A., Pérez, A.G., Role of polyphenol oxidase and peroxidase in shaping the phenolic profile of virgin olive oil. *Food Res. Int.* 2011, *44*, 629-635.

[14] Ramírez, E., Medina, E., Brenes, M., Romero, C., Endogenous Enzymes Involved in the Transformation of Oleuropeinin Spanish Table Olive Varieties. *J. Agric. Food Chem*. 2014, 62, 9569-9575.

[15] Hachicha Hbaieb, R.,Kotti, F.,García-Rodríguez, R.,Gargouri, M.,Sanz, C., Pérez, A.G., Monitoring endogenous enzymes during olive fruit ripening and storage: correlation with virginolive oil phenolic profiles. *Food Chem.* 2015, *174*, 240-247.

[16] Jemai, H., Bouaziz, M., Sayadi, S., Phenolic Composition, Sugar Contents and Antioxidant Activity of Tunisian Sweet Olive Cultivar with Regard to Fruit Ripening. *J. Agric. Food Chem.* 2009, *57*, 2961-2968.

[17] Hachicha Hbaieb, R., Kotti, F., Cortes-Francisco, N., Caixach, J., Gargouri, M., Vichi, S., Ripening and storage conditions of Chétoui and Arbequina olives: Part II. Effect on olive endogenous enzymes and virgin olive oil secoiridoid profile determined by high resolution mass spectrometry. *Food Chem*. 2016, *210*, 631-639.

[18] Uceda, M., Frias L., Trend of the quality and quantitative composition of olive fruit oil during ripening. Proceedings of the International Meeting on Olive Oil 25-46. Cordoba 1975, Spain.

[19] Romero-Segura, C., Sanz, C., Pérez, A.G., Purification and Characterization of an Olive Fruit beta-Glucosidase Involved in the Biosynthesis of Virgin Olive Oil Phenolics. *J. Agric. Food Chem.* 2009, *57*, 7983-7988.

[20] Romero-Segura, C., Garcia-Rodriguez, R., Sanchez-Ortiz, A., Sanz, C., Perez, A.G., The role of olive b-glucosidase in shaping the phenolic fraction of virgin olive oil. *Food Res. Int.* 2012, *45*, 191-196.

[21] Luz Pizarro, M., Becerra, M., Sayago, A., Beltrán, M., Beltrán, R., Comparison of Different Extraction Methods to Determine Phenolic Compounds in Virgin Olive Oil. *Food Anal.Meth*. 2013, *6*, 123-132.

[22] Gutfinger, T., Polyphenols in olive virgin oils. *J. Am. Oil Chem.Soc.* 1981, *58*, 966-968.

[23] Brand-Williams, W., Cuvelier, M. E., Berset, C., Use of free radical method to evaluate antioxidant activity. *Lebensm.Wiss.Technol*. 1995, *28*, 25-30.

[24] Mínguez-Mosquera, M.I., Gandul-Rojas, B., Garrido-Fernández, J., Gallardo-Guerrero, M.L., Pigments Present in Virgin Olive Oil. *J. Am. Oil Chem. Soc*. 1990, *67*, 192-196.

[25] Vichi, S., Pizzale, L., Conte, L.S., Buxaderas, S., Lopez-Tamames, E., Solid-phase microextraction in the analysis of virgin olive oil volatile fraction: modifications induced by oxidation and suitable markers of oxidative status. *J. Agric. Food Chem.*2003, *51*, 6564-6571.

[26] Vichi, S., Castellote, A.I., Pizzale, L., Conte, L.S., Buxaderas, S., López-Tamames, E., Analysis of virgin olive oil volatile compounds by headspace solid-phase microextraction coupled to gas chromatography with mass spectrometric and flame ionization detection. *J.Chromatogr. A* 2003, *983*, 19-33.

[27] Cardoso, S.M., Mafra, I., Reis, A., Nunes, C.,Saraiva, J.A., Coimbra, M.A., Naturally fermented black olives: Effect on cell wall polysaccharides and on enzyme activities of Taggiasca and Conservolea varieties. *Food Sci. Technol*. 2010, *43*, 153-160.

[28] Mazzuca, S., Spadafora, A., Innocenti, A.M., Cell and tissue localization of *β*-glucosidase during the ripening of olive fruit (Olea europaea) by in situ activity assay. *Plant Sci.* 2006, *171*, 726–33.

[29] Issaoui, M., Flamini, G., Brahmi, F., Dabbou, S., Ben Hassine, K., Taamali, A., Chehab, H., Ellouz, M., Zarrouk, M., Hammami, M., Effect of the growing area conditions on differentiation between Chemlali and Chétoui olive oils. *Food Chem*. 2010, *119*, 220-225.

[30] Mraicha, F., Ksantini, M., Zouch, O., Ayadi, M., Sayadi, S., Bouaziz, M., Effect of olive fruit fly infestation on the quality of olive oil from Chemlali cultivar during ripening. *Food Chem.Toxicol*. 2010, *48*, 3235-3241.

[31] Baccouri, O., Bendini, A., Cerretani, L., Guerfel, M., Baccouri, B., Lercker, G., Ben Miled, D.D., Comparative study on volatile compounds from Tunisianand Sicilian monovarietal virgin olive oils. *Food Chem*. 2008, *111*, 322-328.

[32] Hachicha Hbaieb, R., Kotti, F., Gargouri, M., Msallem, M., Vichi, S., Ripening and storage conditions of Chétoui and Arbequina olives: Part I. Effect on olive oils volatiles profile. *Food Chem*. 2016, *203*, 548-558.

[33] Aparicio, R., Luna, G., Characterisation of monovarietal virgin olive oils. *Eur. J. Lipid Sci. Technol.* 2002, *104,* 614-627.

FIGURE CAPTIONS

Figure 1. Evolution of olive POX (A), PPO (B) and β-glucosidase (C) activities (U/g FW, means of three replicates) duringChétoui (●) and Chemlali (○)olive ripening.

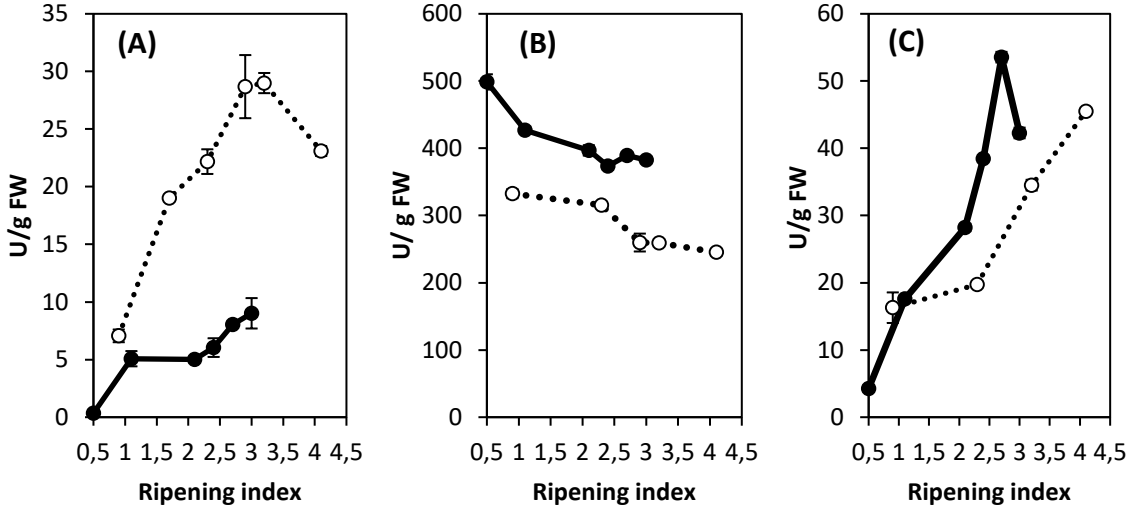

Figure 1.Evolution of olive POX (A), PPO (B) and β-glucosidase (C) activities (U/gFW, means of three replicates) during Chétoui (●) and Chemlali (○) olive ripening.

Table 1. Evolution of total phenol content, antioxidant activity and pigments content in Chétoui and Chemlali oils as function of olives ripening during the crop season 2013-2014.Different letters indicate statistically significant differences between means ($p< 0.05$).

Harvest Date	RI		Total phenol content		RSA (%)		Chlorophyll (mg/kg)		Carotenoids (mg/kg)	
	Chétoui	Chemlali	Chétoui	Chemlali	Chétoui	Chemlali	Chétoui	Chemlali	Chétoui	Chemlali
24/09/2013	0.5	0.9	-	-	-	-	-	-	-	-
08/10/2013	1.1	1.7	-	-	-	-	-	-	-	-
21/10/2013	2.1	2.3	1302^{c}	373^{d}	67.8^{b}	23.6^{c}	8.3^{b}	7.4^{b}	3.2^{b}	2.8^{b}
06/11/2013	2.4	2.9	1255^{c}	307^{b}	63.3^{b}	16.8^{b}	8.7^{b}	5.7^{a}	4.2^{d}	2.3^{a}
19/11/2013	2.7	3.2	1125^{b}	242^{a}	48.0^{a}	13.9^{a}	11.1^{c}	5.4^{a}	3.9^{c}	2.1^{a}
03/12/2013	3	4.1	964^{a}	347^{c}	47.9^{a}	20.9^{c}	7.2^{a}	5.3^{a}	2.4^{a}	2.3^{a}

$^{a-d}$ Different letters indicate significant differences between samples of the same cultivar at different RI ($p < 0.05$).

Table 2. Correlation of total phenol content with endogenous olive enzyme activities and antioxidant activity of Chétoui and Chemlali olive cultivars.

		Equation	R^2	Correlation coefficient	*p* value
Correlation of total phenol content with POX activity	Chétoui	y=-73.48x+1678.2	0.870	-0.932	<0.001
	Chemlali	y=-13.00x+74.85	0.748	-0.865	<0.01
Correlation of total phenol content with PPO activity	Chétoui	y=2.898x+43.90	0.041	0.203	NS
	Chemlali	y= 0.918x+69.10	0.256	0.506	NS
Correlation of total phenol content with β-glucosidase activity	Chétoui	y=-8.255x+1496.9	0.321	-0.567	NS
	Chemlali	y=-1.193x+381.8	0.214	-0.462	NS
Correlation of RSA with total phenol content	Chétoui	y=0.059x-12.68	0.759	0.871	<0.005
	Chemlali	y=0.070x-3.531	0.831	0.911	<0.005

Table 3. Concentration of C6 and C5 volatile compounds (mg/kg) in VOOs, during Chétoui and Chemlali olive ripening

Compounds		Concentration (mg/kg)							
		Chemlali				Chetoui			
	RI	2.3	2.9	3.2	4.1	2.1	2.4	2.7	3
sum PD		3.4a	3.6a	4.8b	3.7a	1.8a	2.7b	4.1c	4.8d
hexanal		1.4a	2.0b	2.9c	2.0b	0.45a	0.75b	1.5c	1.6c
E-2-pentenal		0.19ab	0.19ab	0.21b	0.15a	0.10a	0.15b	0.23c	0.24c
1-penten-3-ol		0.55b	0.47ab	0.44a	0.38a	0.29a	0.42b	0.64c	0.72d
Z-3-hexenal		0.38a	0.36a	0.47b	0.37a	0.14a	0.34b	0.44c	0.60d
E-2-hexenal		10.2a	10.6a	15.7b	12.0a	0.22a	0.59b	1.3c	1.6d
hexylacetate		Nd	nd	nd	nd	Nd	nd	0.04a	0.05b
E-2-pentenol		0.07b	0.06ab	0.08c	0.06a	0.04a	0.04a	0.06b	0.08c
Z-2-pentenol		0.54b	0.48ab	0.52ab	0.45a	nd	nd	Nd	nd
Z-3-hexenylacetate		nd	nd	nd	nd	0.45a	0.34a	0.41a	0.62b
1-hexanol		0.07a	0.10b	0.42d	0.17c	0.23b	0.04a	0.04a	0.04a
Z-3-hexenol		0.16b	0.11a	0.25c	0.32d	0.34b	0.09a	0.17a	0.20a
E-2-hexenol		0.22a	0.21a	0.80b	0.24a	0.03a	0.03a	0.03a	0.04b
C5 TOT		1.4b	1.2ab	1.3b	1.0a	0.43a	0.61b	0.93c	1.04d
C5ALD		0.19ab	0.19ab	0.21b	0.15a	0.10a	0.15b	0.23c	0.24c
C5 ALC		1.2b	1.0ab	1.0ab	0.89a	0.33a	0.46b	0.70c	0.80d
C6 TOT		12.4a	13.37ab	20.5c	15.0b	1.86a	2.18a	3.92b	4.72c
C6 ALD		12.0a	13.0ab	19.0c	14.3d	0.81a	1.7b	3.2c	3.8d
C6ALC		0.44a	0.42a	1.5c	0.73b	0.60c	0.16a	0.24b	0.28b
C6EST		nd	nd	nd	nd	0.45a	0.34a	0.45a	0.67b

[a-d] Different letters indicate significant differences between samples of the same cultivar at different RI ($p < 0.05$).