Title:

Ripening and storage conditions of Chétoui and Arbequina olives: Part II. Effect on olive endogenous enzymes and VOO secoiridoid profile determined by HRMS

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ABSTRACT:

Several factors affect virgin olive oil (VOO) phenolic profile. The aim of this study was to monitor olive hydrolytic (β-glucosidase) and oxidative (POX and PPO) enzymes during olive ripening and storage and to determine their capacity to shape VOO phenolic profile. To this end, olives from the cultivars Chétoui and Arbequina were stored at 4 °C or 25 °C for 4 weeks and their enzymatic activities and oil phenolic profiles were compared to those of ripening olives. We observed different trends in enzymes activities according to cultivar and storage temperature. Secoiridoid compounds, determined by HRMS, and their deacetoxylated, oxygenated, and deacetoxy-oxygenated derivatives were identified and their contents differed between the cultivars according to olive ripening degree and storage conditions. These differences could be due to β-glucosidase, POX and PPO activities changes during olive ripening and storage. Results also show that oxidised phenolic compounds could be a marker of VOO ‘‘freshness”.

KEYWORDS: Virgin Olive Oil; Olive Storage; Endogenous Enzymes; Secoiridoid Compounds; High Resolution Mass Spectrometry.

ABBREVIATIONS USED

3,4-DHPEA-EA = Oleuropein Aglycone

3,4-DHPEA-EDA = Deacetoxy Oleuropein Aglycone = Dialdehydic form of Oleuropein Aglycone

3,4-DHPEA-OH-EA = Oxygenated Oleuropein Aglycone

3,4-DHPEA-OH-EDA = Deacetoxy Oxygenated Oleuropein Aglycone

DTT = Dithiothreitol

EA = Elenolic Acid Aglycone

EA-OH = Oxygenated Elenolic Acid Aglycone

EDTA = Ethylene Diamine Tetraacetic Acid

p-HPEA-EDA = Deacetoxy Ligstroside Aglycone = Dialdehydic form of Ligstroside Aglycone

p-HPEA-EA = Ligstroside Aglycone

p-HPEA-OH-EA = Oxygenated Ligstroside Aglycone

PMSF = Phenyl Methyl Sulfonyl Fluoride

pNPG = *p*-Nitrophenyl β-D-glucopyranoside

POX = Peroxidase

PPO = Polyphenoloxidase

SDS =Sodium Dodecyl Sulfate

TBC = Tert-butylcatechol

VOO = Virgin Olive Oil

WAF = Weeks After Flowering

Chemical compounds studied in this article

Guaiacol (PubChem CID: 460); tert-butylcatechol (PubChem CID: 7381); 4-nitrophenyl-β, D-glucopiranoside (PubChem CID: 92930); Sodium Dodecyl Sulfate (PubChem CID: 3423265); Phenyl Methyl Sulfonyl Fluoride (PubChem CID: 4784); Ethylene Diamine Tetraacetic Acid (PubChem CID: 6049); Dithiothreitol (PubChem CID: 446094); Elenolic acid (PubChem CID: 169607); oleuropein aglycone (PubChem CID: 56842347); ligstroside aglycone (PubChem CID: 11652416).

Highlights

- Olive cultivar and storage conditions affect olive endogenous enzymes activities.
- Olive oil phenolic profile differed among olive cultivars during ripening and storage.
- Total secoiridoid compounds decreased during olive ripening and storage.
- Mainly PPO activity and precursor availability favour oxygenated secoiridoids in VOO.

1. Introduction

Virgin olive oil (VOO) extracted from healthy olives (*Olea europaea* L.) harvested at optimum maturity constitutes the main source of lipid in the Mediterranean diet. It can be consumed in its natural, unprocessed form or as a food ingredient. The increased interest in olive oil production and consumption in recent years is due to its nutritional, therapeutic and organoleptic properties (Fregapane & Salvador, 2013; Romero-Segura, García-Rodríguez, Sánchez-Ortiz, Sanz, & Pérez, 2012).

Recent studies show that minor components of VOO, especially phenolic compounds, help protect against several diseases due to their antioxidant activities (Cicerale, Conlan, Sinclair, & Keast, 2009; LeGendre, Breslin, & Foster, 2015; Parkinson & Keast, 2014). However, several factors, such as olive cultivar, the degree of ripening, environmental conditions, irrigation, and oil extraction techniques, give rise to considerable diversity in the phenolic profiles of olive oil (Dabbou et al., 2010). The phenolic profile of VOO is essentially determined by the amount of phenolic glycosides there are in the olive tissues and the activity of various endogenous enzymes that act on those glycosides (García-Rodríguez, Romero-Segura, Sanz, Sánchez-Ortiz, & Pérez, 2011; Hachicha Hbaieb, Kotti, García-Rodríguez, Gargouri, Sanz, & Pérez, 2015). Oleuropein, ligstroside, demethyloleuropein, verbascoside, elenolic acid glucoside, luteolin-7-glucoside, apigenin-7-glucoside, rutin, and quercetin-3-rutinoside constitute the major phenolic glycosides found in olive fruits from different varieties and at different stages of ripening (Gómez-Rico, Fregapane, & Salvador, 2008; Obied et al., 2008). During the olive oil extraction process, an endogenous β-glucosidase hydrolyses these compounds producing secoiridoid aglycons which are the most important phenolic fraction of VOO (Romero-Segura et al., 2012). Secoiridoid compounds are represented by the dialdehydic and aldehydic forms of the aglycons oleuropein (3,4-DHPEA-EDA and 3,4-DHPEA-EA, respectively) and

148 ligstroside (*p*-HPEA-EDA and *p*-HPEA-EA, respectively) (Montedoro, Servili, Baldioli,
149 Selvaggini, Miniati, & Macchioni, 1993). In addition, during the crushing and malaxation
150 steps, the oxidation of phenolic compounds is favoured by endogenous oxidoreductases, in
151 particular polyphenol oxidase (PPO) and peroxidase (POX) (Servili, Taticchi, Esposto,
152 Urbani, Selvaggini, & Montedoro, 2008). PPO (EC 1.14.18.1) is an enzyme that contains
153 copper and is widely distributed throughout nature. It catalyses two types of reactions: the
154 *o*-hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the
155 oxidation of *o*-diphenols to *o*-quinones (diphenolase activity). POX (EC 1.11.1.7) is a
156 glycoprotein that catalyses the oxidation of phenolic compounds to highly reactive and
157 easily polymerised free radical intermediates by means of either hydrogen peroxide (H_2O_2)
158 or organic peroxides as the oxidising agent (Gajhede, 2001).
159 As olive oil quality is directly related to the physiological conditions of the olives from
160 which the oil is extracted, and given the importance of the oil phenolic fraction in relation
161 to antioxidant activities, sensory properties, and health benefits, changes in the phenolic
162 profiles of VOO during ripening and storage of the olives may be important for quality
163 control.
164 Previous studies have focused on the role played by β-glucosidase, PPO, and POX in olive
165 phenolic metabolism during ripening (Montedoro, Baldioli, Selvaggini, Begliomini,
166 Taticchi, & Servili, 2002; García-Rodríguez et al., 2011; but none of those studies focused
167 on the changes in the activities of these enzymes or on their role in shaping the phenolic
168 profile of oil during storage of the olives. It was only recently that Hachicha Hbaieb et al.
169 (2015) studied the effect of storage of Arbequina olives on endogenous enzyme activities
170 and the correlations with the phenolic profile of VOO. However, no data are available on
171 the variation of the activities of these enzymes in Tunisian cultivars, either during ripening
172 or storage of olives. Additionally, several studies have focused on possible hydrolytic and

oxidative degradation of phenolic compounds present in extra VOO, during oil heating (Di Maio et al., 2011) and oil storage (Samaniego-Sánchez, Oliveras-López, Quesada-Granados, Villalón-Mir, & López-García de la Serrana, 2012). A decrease in the amount of VOO secoiridoids and an increase in simple phenols, elenolic acid, oxidised forms of elenolic acid, and oxidised forms of secoiridoids were observed during olive oil storage (Brenes, García, García, & Garrido, 2001; Lerma-García, Herrero-Martínez, Simó-Alfonso, Lercker, & Cerretani, 2009). Nevertheless, to date, no information is available on the evolution of these compounds in olive oils extracted from olives stored up to 4 weeks at different temperatures. Moreover, this is the first time that high-resolution mass spectrometry (MS) has been used to monitor the effect, during olive ripening and storage, of endogenous olive enzymes on the secoiridoid profile of VOO.

Therefore, the aim of this study was: firstly, to study the evolution of olive endogenous enzymes in Chétoui and Arbequina olives during ripening and storage; then to determine the phenolic profile of the resulting oils using high-resolution MS and finally, to determine adequate storage conditions that preserve the phenolic profile of the resulting oils in accordance with those extracted from freshly harvested olives. These storage conditions will be then compared to those identified in Part I, on the basis of VOO volatile compounds (Hachicha Hbaieb, Kotti, Gargouri, Msallem, & Vichi, 2016).

2. Materials and Methods

2.1. Chemicals

Reagents for enzymatic activity extraction and measurements were supplied by Sigma-Aldrich (St. Louis, MO, USA).

Mass spectrometry grade n-hexane and methanol (MS SupraSolv®) and formic acid (Suprapur®) were purchased by Merck (Darmstadt, Germany). Water was of ultrapure milli-Q grade. Nitrogen (Alphagaz N2, purity 99.999%, Air Liquide: España (Barcelona,

198 Spain)) was used in the Orbitrap-Exactive as nebulization and fragmentation gas. *o*-
199 Coumaric acid was from Sigma-Aldrich (St Louis, MO, USA).

200 ***2.2. Experimental materials and storage treatment***

201 Olives (*Olea europaea* L.) from Chétoui (native) and Arbequina (introduced) cultivars
202 were hand-harvested at the purple stage with reddish spots during the crop season
203 2012/2013 from Sadira orchad (Mornag, Tunisia). The harvest date and the corresponding
204 Maturity Index (MI) for both cultivars are shown in Table 1. The Maturity Index of olives
205 was determined visually as previously reported by Uceda & Frias (1975).
206 Healthy olives of each cultivar (10 kg) were dispersed randomly into 2 perforated plastic
207 boxes and stored in ambient and refrigerated room at 25 °C and 4 °C, respectively, during 4
208 weeks. Weekly, samples of each variety (1 kg) were taken for the oil extraction and about
209 250 g were used for the acetonic powder extraction.

210 ***2.3. Olive oil extraction***

211 After washing and leaf-removal, each sample was extracted using an Abencor analyser
212 (Comercial Abengoa, S.A., Seville, Spain) that simulates the industrial process of VOO
213 production at laboratory scale (Martínez, Muñoz, Alba, & Lanzón, 1975). Crushing of
214 samples (1 kg) was carried out using a stainless steel hammer mill operating at 3000 rpm
215 provided with a 5 mm sieve. After 30 min of mixing at 30 °C, olive paste was extracted by
216 means of a basket centrifuge at 3500 rpm during 1 min. Then all the oil samples were paper
217 filtered and stored in the dark in dark glass bottles at -20 °C until further analysis.

218 ***2.4. Enzyme extraction***

219 POX enzyme extracts were prepared from olives seeds as previously reported by García-
220 Rodríguez et al. (2011). However, PPO and β-glucosidase enzyme were extracted from
221 acetone powders prepared from mesocarp tissues of olives following a previously
222 described procedure (García-Rodríguez et al., 2011).

2.5. Enzyme activity assay

Peroxidase activity in the extracts was determined spectrophotometrically (25 °C) at 420 nm (ε = 26,600 M^{-1} cm^{-1}) corresponding to the formation of tetraguaiacol as a final product resulting from the peroxidation of guaiacol in the presence of hydrogen peroxide (Luaces, Romero, & Gutiérrez, 2007).

PPO activity was determined spectrophotmetrically (25 °C) at 400 nm related to the oxidation of tert-butylcatecholin corresponding quinine (ε = 1200 M^{-1} cm^{-1}) (García-Molina, Muñoz, Varón, Rodríguez-López, & García-Cánovas, 2007).

The increase in absorbance at 405 nm, related to the formation of *p*-nitrophenol (ε =552.8 M^{-1} cm^{-1}) released from the synthetic glucoside *p*-nitrophenyl β-D-glucopyranoside (*p*NPG), represents the β-glucosidase activity (Romero-Segura et al., 2012).

2.6. Peroxide value and extinction coefficient

Peroxide value (PV), expressed as milliequivalents of active oxygen per kilogram of oil (meq O_2/kg), and K_{232} and K_{270} extinction coefficients, calculated from absorption at 232 and 270 nm, respectively, were determined by the methods reported in Regulation EEC/2568/91 of the European Commission Regulation (EEC 1991).

2.7. Extraction of oil phenolic compounds

The phenolic compounds of VOO were extracted by adapting the procedure previously developed by Tsimidou, Papadopoulos, & Boskou (1992). An amount of 2 g of oil spiked with 0.5 mL of *o*-coumaric acid (0.05 mg mL^{-1} in methanol) was dissolved in 3 mL of n-hexane and then it was extracted twice with 0.5 mL of methanol:water 60:40 (v/v). The obtained extract was separated by centrifugation (2000 rpm), then washed with 2 mL of n-hexane and analyzed without any further concentration.

2.8. Analysis of secoiridoids by high performance liquid chromatography (HPLC) coupled to electrospray ionization-high resolution mass spectrometry (ESI-HRMS)

The phenolic extracts of VOO were analyzed by HPLC-ESI-HRMS according to Vichi, Cortés-Francisco, & Caixach. (2013). The LC system consisted of a Surveyor MS Plus pump coupled to an Accela Open automatic sampler (Thermo Fisher Scientific, San Jose, CA, USA) equipped with a 5 µL loop. A C18 Fused-Core column providing high column efficiency and short analysis time was used. Separation of phenolic compounds was then performed on Halo C18 Fused-Core column (2.1 x 100 mm, 2.7 µm particle size, Advanced Materials Technology, Wilmington, DE, USA). Elution was performed at a flow rate of 400 µL min^{-1}, using water/formic acid (99.9:0.1 v/v) (solvent A) and methanol/formic acid (99.9:0.1 v/v) (solvent B) as components of the mobile phase. The solvent gradient changed according to the following conditions: 95% (A)-5% (B) for 1 min, to 100% (B) in 21 min; 100% (B) for 5 min, then to 95% (A) in 1 min, and this percentage maintained for 5 min.

Mass spectrometric analysis was carried out with an Exactive-HCD Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with an electrospray source (H-ESI II). Analytical conditions in negative ionization mode were: spray voltage 2.5 kV, capillary voltage -25 V, skimmer voltage -18 V, tube lens voltage -110 V. Analytical conditions in positive mode were: spray voltage 3.00 kV, capillary voltage 32.5 V, skimmer voltage 18 V, tube lens voltage 110 V. In both cases, sheath gas flow rate was set at 55 arbitrary units (au), auxiliary gas flow rate was 25 au, capillary temperature was 250 ºC, and heater temperature was 25 ºC. The mass range was set to *m/z* 50-1000. The automatic gain control was used to fulfill the C-trap and gain accuracy in mass measurements (balanced mass accuracy mode, $5x10^6$ ions). Maximum injection time was 500 ms. High resolution defined as R: 50,000 (*m/z* 200, FWHM) was set, 2 Hz scan. High energy Collision Dissociation (HCD) voltage was 30 eV.

Elemental composition and structural information were obtained by Exactive-HCD in a single injection with the Orbitrap mass analyzer alternating full scan mode and all ions fragmentation (AIF) mode at a resolution of 50,000 (*m/z* 200, FWHM). The analyses have been carried out in negative and positive ionization modes. Mass accuracies better than 2 ppm and 5 ppm were achieved for precursor ions and fragment ions, respectively, always working with external calibration.

The molecular formulae calculation was performed with Xcalibur 2.1 software (Thermo Fisher Scientific, Bremen, Germany). Identification was based on molecular ion, RDB (rings plus double bonds equivalents), product ion spectra and mass error tolerance (2 or 5 ppm).

2.9. Statistics

Enzymatic extracts were obtained in duplicate and the enzymatic activity was analyzed in triplicate. The extraction of VOO phenolics and the determination of their concentrations were done in duplicate. The results were expressed as mean values (mv) ± Standard Deviations (SD). Statistical analysis was carried out using Microsoft Excel software. Analysis of variance (ANOVA) was applied, and comparison of means was done by the Fisher test at a significance level of 0.05.

3. Results and Discussion

The evolution of the activities of endogenous enzymes (POX, PPO, and β-glucosidase) in crude protein extracts obtained from Chétoui and Arbequina olives during ripening (from 21 November 2012 to 8 January 2013) and storage (4 weeks at 4 °C and 25 °C ± 2 °C) was studied in order to compare the enzymatic profiles of the two cultivars studied, and to determine whether the activities of these enzymes during olive ripening and storage could be related to changes in the phenolic compounds in the resulting oils.

3.1. Variation of enzymes activities during ripening and storage

297 Figure 1a illustrates the evolution of POX activities in Chétoui and Arbequina olive seeds
298 during olive ripening and storage at 4 °C and 25 °C. Chétoui olives had lower values of
299 POX activities during ripening and storage; and the activity levels increased slowly during
300 ripening. In fact, it went from 0.027 ± 0.0005 $Umg^{-1}Pr$ to 0.046 ± 0.003U $mg^{-1}Pr$ on the
301 third harvest date (MI=3.5). In contrast, POX activities in Arbequina cultivars showed
302 insignificant variations during ripening (MI from 2.5 to 4.9). In fact, the average POX
303 activity was approximately 0.11 U mg^{-1} Pr during the entire test period. These results are in
304 good agreement with previous studies for Arbequina and Picual olive seeds, which reported
305 constant POX activity after 28 WAF for both cultivars (García-Rodríguez et al., 2011).
306 Concerning variation in POX activity during olive storage, Figure 1a shows that the
307 temperature of storage had no significant effect on the level of this enzyme in Chétoui
308 cultivar olives during the whole period of storage; but a significant increase was noted for
309 Arbequina cultivar olives during the last period of storage at 25 °C. In this last case, POX
310 activities reached their highest levels. The increase of POX activity observed in Arbequina
311 olives stored at 25 °C compared to those stored at 4 °C is in agreement with results recently
312 reported by Hachicha Hbaieb et al. (2015) for green mature Arbequina olives (grown in
313 Sevilla, Spain) stored in similar conditions. In addition, an important decrease of POX
314 activities was observed during the first week of storage of Arbequina olives at 4 °C and 25
315 °C.
316 The evolution of PPO activities in the olive mesocarp during ripening and storage, for both
317 cultivars and at the two different temperatures, is shown in Figure 1b. Contrary to what was
318 observed for the POX activities, the highest values of PPO activities were detected in
319 Chétoui olives. In fact, the initial levels of PPO activities in Chétoui and Arbequina olives
320 were 54.374 ± 3.23 U mg^{-1} Pr and 34.804 ± 2.15 U mg^{-1} Pr, respectively. Also, PPO
321 activities of Chétoui olives maintained similar levels during ripening. This agrees with the

previous study by García-Rodríguez et al. (2011) which reports a non-significant modification in PPO activities for both Arbequina and Picual cultivars, after the color turning stage, 28 WAF. However, an important decrease of PPO activity was found in the case of Arbequina olives: only 27 % of the initial value was maintained on the last harvest date (MI = 4.7). This decrease of PPO activity during Arbequina ripening is in disagreement with a previous study on Spanish Arbequina olives (García-Rodríguez et al., 2011). Consequently, these results suggest a significant effect of cultivar and geographic growing area on the PPO enzymatic activity.

Regarding the evolution of PPO enzymatic activity during olive storage, an important decrease of PPO activities in olives stored at 25 °C was observed for both cultivars, in agreement with our previous work (Hachicha Hbaieb et al., 2015). Moreover, at 25 °C, this decrease was more significant in Chétoui than in Arbequina olives. In fact, 87% of PPO activity was lost after 4 weeks in Chétoui olives storage; compared to only 65% in the case of Arbequina olives. On the contrary, during storage at 4 °C the activity of PPO showed similar values than in ripening olives.

β-glucosidase activity in olive mesocarp was monitored during olive ripening and storage at two different temperatures: the results are shown in Figure 1c. As illustrated, the highest values of β-glucosidase activity were found in Chétoui olives during the whole experiment. During ripening, a gradual increase of β-glucosidase activity was observed in Chétoui olives over the period tested (MI from 2.1 to 3.9); from 0.517 ±0.046 to 1.640 ±0.072 U mg^{-1} Pr. Meanwhile, Arbequina olives reached maximal activity (0.852 ± 0.021 U mg^{-1} Pr) on the third harvest date (MI = 3.8) and then it decreased markedly to 0.448 ± 0.009 U mg^{-1} Pr at MI = 4.7. This same trend in β-glucosidase activity during fruit ripening was previously reported by Hachicha Hbaieb et al. (2015); Mazzuca, Spadafora, & Innocenti. (2006); Briante et al., (2000).

347 During storage of Arbequina olives, β-glucosidase activity decreased after 1 week of
348 storage at 25 °C or 4 °C. In contrast, β-glucosidase activity did not change significantly
349 during the first two weeks of storage for Chétoui olives, then it decreased sharply to 0.20 ±
350 0.02 U mg^{-1} Pr and 0.03 ± 0.001 U mg^{-1} Pr in olives stored at 25 ºC in weeks 3 and 4.
351 Meanwhile, at 4 °C, the activity continued to increase, reaching 1.56 ± 0.18 U mg^{-1} Pr in
352 the third week of storage, and then it started decreasing in the last week of storage, while
353 maintaining a relatively high value (0.66 ± 0.02 U mg^{-1} Pr). This trend in β-glucosidase
354 activity during the storage of Chétoui olives at the two different temperatures was similar
355 to that reported by Hachicha Hbaieb et al. (2015). The difference between the two studies
356 concerns the number of weeks after which the β-glucosidase activity of the olives stored at
357 25 °C decreased. This difference may be due either to the response of each olive cultivar to
358 storage conditions, or to the stage of ripeness. Consequently, monitoring of β-glucosidase
359 activity during olive storage revealed the interdependence between the effects of olive
360 storage conditions and olive cultivar.
361 In conclusion, results of monitoring POX, PPO, and β-glucosidase activities during the
362 ripening and storage of Chétoui and Arbequina olives revealed different trends of activities,
363 according to the olive cultivar and storage temperature. The main differences were
364 observed between olives stored at 25 °C and those freshly harvested or stored at 4 ºC.
365 Enzymes in Arbequina olives, which were slightly riper than Chétoui olives, were affected
366 less by the storage temperature.
367 ***3.2. Phenolic compound profiles during ripening and storage***
368 Secoiridoid compounds in VOO are strongly associated with VOO shelf life (Di Maio et
369 al., 2011). For this reason, their determination constitutes a key quality control parameter
370 for VOO.

The use of HRMS for the determination of secoiridoid profiles in oil extracted from Chétoui and Arbequina olives during ripening and storage allowed us to monitor the total isomers of oleuropein, ligstroside, and elenolic acid aglycons, their decarboxymethyl forms, as well as their oxygenated derivatives.

3.2.1. Variation of the content of total oleuropein, ligstroside, elenolic acid aglycons and their decarboxymetyl derivatives during ripening and storage

Oleuropein is a heterosidic ester of elenolic acid and 3,4-dihydroxyphenylethanol (3,4-DHPEA), whilst ligstroside is a heterosidic ester of elenolic acid and *p*-hydroxyphenylethanol (*p*-HPEA). These compounds represent the most abundant phenolic compounds in olives (Obied et al., 2008). Their aglycons, oleuropein aglycon (3,4-DHPEA-EA) and ligstroside aglycon (*p*-HPEA-EA), are produced during processing and oil extraction as a result of enzymatic hydrolysis of oleuropein and ligstroside, respectively. Therefore these aglycons are predominant in the hydrophilic extract of VOO (Servili & Montedoro, 2002).

Elenolic acid and its aglycon (EA), which represent non-phenolic secoiridoids, also characterise the polar fraction of olives and olive oil, respectively.

For both cultivars, HRMS data showed fifteen compounds with the molecular formula of 3,4-DHPEA-EA, fourteen compounds with the molecular formula of *p*-HPEA-EA and three with the molecular formula of EA (Figure 2a-c). Moreover, two not completely resolved isomers of 3,4-DHPEA-EDA and *p*-HPEA-EDA (Figure 2d-e) were detected. These deacetoxy secoiridoids, known also as oleacin and oleocanthal, respectively, are among the most abundant secoiridoids in VOO (Brenes et al., 2001).

The evolution of total isomers of 3,4-DHPEA-EA, *p*-HPEA-EA, and EA, and their decarboxymetyl derivatives 3,4-DHPEA-EDA and *p*-HPEA-EDA in oils extracted from Arbequina and Chétoui olives during ripening and storage at two different temperatures is

shown in Figures 3 and 4, respectively. During ripening and storage, Chétoui olive oil had the higher amounts of total 3,4-DHPEA-EA and *p*-HPEA-EA (compared to Arbequina oil) (Figure 3 a-c). For example, the initial amounts of total 3,4-DHPEA-EA and *p*-HPEA-EA in Chétoui oil (expressed as equivalents of *o*-coumaric acid) were approximately 846 mg kg^{-1} and 298 mg kg^{-1}, respectively, while only 517 mg kg^{-1} and 5.6 mg kg^{-1} of total 3,4-DHPEA-EA and *p*-HPEA-EA, respectively was found in Arbequina oil. Previous research found that the amounts of 3,4-DHPEA-EA and *p*-HPEA-EA were lower in Arbequina than in Chétoui oils (Bakhouche, Lozano-Sánchez, Beltrán-Debón, Joven, Segura-Carretero, & Fernández-Gutiérrez 2013). The higher amounts of total 3,4-DHPEA-EA and *p*-HPEA-EA in Chétoui oils could be related to either the higher β-glucosidase activity or the higher oleuropein and ligstroside content of Chétoui olives, compared to Arbequina olives. In contrast, the initial concentrations of decarboxymethyl secoiridoids were some nine times higher in Arbequina oils than in Chétoui oils (Figure 4a-b). For example, the initial amounts of total 3,4-DHPEA-EDA in Arbequina and Chétoui oils were 1521 and 165 mg kg^{-1}, respectively. This result could be due to higher esterase activity in Arbequina oils, which by subsequent decarboxylation (Obied , Karuso, Prenzler, & Robards, 2007) would lead to higher production of 3,4-DHPEA-EDA from 3,4-DHPEA-EA.

As expected, for both cultivars, the amount of total 3,4-DHPEA-EA and *p*-HPEA-EA decreased during the ripening of the olives. However, this decrease was more marked in Arbequina oils than in Chétoui oils. In fact, the total amount of 3,4-DHPEA-EA and *p*-HPEA-EA tended to zero in Arbequina oils; while total 3,4-DHPEA-EA and *p*-HPEA-EA was reduced by only 53% and 46%, respectively during Chétoui ripening. These trends in total 3,4-DHPEA-EA and *p*-HPEA-EA during ripening could be considered as normal, since a negative correlation between the secoiridoid levels in olive oil and MI of the olives was previously reported (Karkoula, Skantzari, Melliou, & Magiatis, 2014).

421 The decrease of total 3,4-DHPEA-EA and *p*-HPEA-EA may be due to the decrease of total
422 oleuropein and ligstroside during ripening (Mazzucca et al., 2006), even with high β-
423 glucosidase activity, or due to its rapid conversion to hydroxytyrosol and tyrosol,
424 respectively, by esterase activity (Obied et al., 2007). Therefore, by comparing the
425 evolution of total 3,4-DHPEA-EA and *p*-HPEA-EA with β-glucosidase activity, the present
426 data suggest that the amount of these secoiridoids during ripening could mostly be
427 regulated by the olive oleuropein and ligstroside contents, rather than by β-glucosidase
428 activity. This indicates that the enzyme is not a limiting factor, although in a previous
429 works (Hachicha Hbaiebet al., 2015) the sharpest decrease in total oleuropein concentration
430 during ripening coincided with the increase in β-glucosidase activity.
431 Arbequina oils had the highest initial amounts of EA, (Figure 3d-e) which decreased during
432 ripening; while during the ripening of Chétoui olives, an initial increase of EA was
433 observed, followed by a drastic decrease. The noticeable increment in the amount of EA
434 during the first three harvest dates of Chétoui olives may result from the lysis of
435 secoiridoid glucosides by esterase action. Moreover, the decline of total EA during the
436 ripening of Arbequina olives and during the last stage of ripening of Chétoui olives may be
437 due to the decrease in EA content in the olives. Consequently, EA behaviour during
438 ripening could be due to: hydrolysis of secoiridoid aglycons; degradation of free EA
439 glucoside; or other changes that EA may undergo during oil extraction.
440 Concerning the evolution of total 3,4-DHPEA-EA and *p*-HPEA-EA amounts during
441 storage, HRMS data showed a significant effect of the storage temperature (Figure 3a-c). In
442 fact, for both varieties, the decrease in these compounds was more important at 25 °C. For
443 example, the final concentration of total 3,4-DHPEA-EA after 4 weeks for Chétoui and
444 Arbequina olives stored at 4 °C represented 55% and 13% of the initial contents,
445 respectively. Nevertheless, only 0.8% and 0.7% of total 3,4-DHPEA-EA was maintained

when Chétoui and Arbequina olives, respectively, were stored at 25 °C. The results also showed an important reduction of total EA contents in Arbequina oils with respect to Chétoui oils (Figure 3d-2).

The decline in total 3,4-DHPEA-EA in stored Arbequina and Chétoui olives (Figure 3a) was parallel to the fall in β-glucosidase activity (Figure 1c). Additionally, the higher β-glucosidase activity in Chétoui olives stored at 4ºC was reflected by the higher levels of secoiridoid aglycons that were maintained in the oils extracted from olives stored at 4 °C.

A drastic decrease in total 3,4-DHPEA-EDA and *p*-HPEA-EDA was observed during olive ripening and storage, especially in olives stored at 25 °C (Figure 4a-b). This decrease could affect the biological activities of VOO. In fact, there is evidence that oleocanthal (p-HPEA-EDA) could be used as a therapeutic compound, due to its numerous biological effects, including anti-inflammatory (Lucas, Russell, & Keast, 2011) and neuroprotective activity (Abuznait, Qosa, Busnena el Sayed, & Kaddoumi, 2013). Moreover, oleacin (3,4-DHPEA-EDA), possesses a potent antioxidant activity due to its hydroxytyrosol group.

These results show that for both cultivars, the amounts of both 3,4-DHPEA-EA and *p*-HPEA-EA as well as the decarboxymetyl derivatives, 3,4-DHPEA-EDA and *p*-HPEA-EDA, depend closely on the olive variety and the experimental conditions (ripening and storage). Moreover, taking into account the content of VOO secoiridoids, it seem to be preferable to harvest Arbequina and Chétoui olives at an early maturation stage and to extract the oil after a short period of storage (up to one week for Arbequina olives and up to two weeks for Chetoui olives), at low temperature, to preserve the oil quality. These results confirm those reported in Part I of this study based on the volatile profile of the same oils (Hachicha Hbaieb et al., 2016).

3.2.2. Variation of the content of total oxygenated oleuropein, ligstroside, and deacetoxy-oleuropein aglycons during ripening and storage

VOO secoiridoids are subjected to enzymatic oxidation during the mechanical oil extraction process through the activation of oxidoreductase enzymes, such as PPO and POX. Oxidation products of secoiridoids could be used as molecular markers of VOO freshness to define the VOO autoxidation state (Di Maio et al., 2013).

The oxygenated secoiridoids observed in the present study (Figure 2) matched those reported by Vichi et al. (2013), and corresponded to 3,4-DHPEA-EA, *p*-HPEA-EA, and 3,4-DHPEA-EDA with an additional oxygen atom in the EA moiety. These secoiridoids should to be taken into consideration as VOO antioxidants, since their phenolic moiety structure is analogous to that of their non-oxygenated homologues.

The evolution of total 3,4-DHPEA-OH-EA, *p*-HPEA-OH-EA and 3,4-DHPEA-EDA in oils extracted from Arbequina and Chétoui olives during ripening and storage at 4 °C and 25 °C is presented in Figure 4c-e. During ripening, a decrease in total 3,4-DHPEA-OH-EA was noted in Arbequina oils; while a slight increase was observed in Chétoui oils (Figure 4c). However, during the first period of storage, a notable increase of 3,4-DHPEA-OH-EA was observed in Chétoui oils, and to a lesser extent, in Arbequina oils at 4ºC (Figure 4a). This is consistent with the amounts of 3,4-DHPEA-EA in oils (Figures 3a). Furthermore, a decrease in total oxygenated *p*-HPEA-EA in Arbéquina and Chétoui oils was observed during ripening (Figure 4d). This decrease was more pronounced in Chétoui oils than in Arbéquina oils. In fact, during ripening, 86% of total oxygenated *p*-HPEA-EA was reduced in Chétoui oils; while in Arbéquina oils, the reduction is only 76%.

Contrarily to what we found for 3,4-DHPEA-OH-EA, *p*-HPEA-OH-EA tended to decrease in the oils resulting from both Arbequina and Chétoui olives during storage of the olives, except for the initial increase during the first week of storage at 4° (Figure 4d). Higher formation of oxygenated 3,4-DHPEA derivatives than of oxygenated p-HPEA derivatives during some stages of storage could be explained by the fact that *o*-diphenols are stronger

antioxidants than tyrosol derivatives and as a consequence, it is more likely that they become involved in oxidative reactions.

For both cultivars, one isomer of deacetoxy-oxygenated oleuropein aglycone (3,4-DHPEA-OH-EDA) was detected (Figure2h). This compound followed a similar trend to that of 3,4-DHPEA-OH-EA: a decrease of 3,4-DHPEA-OH-EDA was observed during ripening of Arbequina olives (Figure 4e); but it increased markedly during the first period of storage of both varieties at 4ºC. In this case, the higher contents were observed in Arbequina oils, which is consistent with the higher amounts of 3,4-DHPEA-EDA in this variety (Figure 4b). The amounts of oxygenated derivatives were in general proportional to the amounts of the respective non-oxygenated compounds, suggesting that the availability of precursors drives the formation of oxidised derivatives. However, olive oxidoreductases are expected to influence the presence of oxygenated secoiridoids. POX, which increased in Chétoui olives during ripening, could explain the increasing trend of 3,4-DHPEA-OH-EA in these oils, but not the decrease of 3,4-DHPEA-OH-EDA, nor the lower 3,4-DHPEA-OH-EA content in Arbequina oils, which were extracted from the olives with the highest levels of this enzyme. Meanwhile, the higher PPO activity in Chétoui olives could explain, together with the higher abundance of precursors, the greater presence of 3,4-DHPEA-OH-EA and and *p*-HPEA-OH-EA in these oils.

On this basis, it seems that PPO activity and the availability of precursors could be mainly responsible for the formation of oxygenated secoiridoids. This is the first report on the behaviour of oxygenated secoiridoids during olive ripening and storage; and as far as we know, these are the first data to relate their presence in the oil with olive characteristics.

The phenolic compound profiles showed that during fruit ripening and storage, notable modifications take place. However, these variations are quantitative rather than qualitative.

520 Variation in the amounts of phenolic compounds seems to be essentially due to metabolic
521 processes: chemical and enzymatic reactions that occur during ripening and processing.

522 ***3.3. Variation of oil peroxide value and extinction coefficients during ripening and***
523 ***storage***

524 The peroxide value (PV) and extinction coefficients of the Arbequina and Chétoui oils
525 obtained from fruits stored at 4 °C and 25 °C are presented in Table2. The results show a
526 significant effect of olive cultivar and storage temperature on these parameters. For both
527 cultivars, the PV of oils extracted from olives stored at the same temperature followed
528 similar trends. In fact, when the olives were stored at 4 °C, PV increased sharply during the
529 first two weeks of storage and then decreased slowly and remained almost constant during
530 the last week of storage. However, when stored at 25 °C, the PV continued to increase
531 sharply during the whole storage period. This could be considered normal, as a high storage
532 temperature affects the physiological state of the olives and consequently promotes the
533 oxidation process.

534 A slightly higher PV was observed in Chétoui oils at both storage temperatures. The PV
535 values of the oils tested were below the limit accepted to certify “extra” VOO quality (20
536 mEq of oxygen kg^{-1} of oil).

537 K_{232} and K_{270} represent supplementary oil quality parameters. They are related to the
538 primary and secondary oxidation products, respectively.

539 The oils obtained from Arbequina and Chétoui olives stored at 4 °C and 25 °C showed
540 different trends in K_{232}. In fact, at 4 °C, K_{232} rose significantly over the first week of storage
541 and then decreased slightly and finally after remained constant. However, it continued to
542 increase when the olives were stored at 25 °C. The trend for K_{232} was similar to that of the
543 PV, as these two parameters reflect the primary oxidation products.

The limiting value of 2.50 for “extra” VOO was only exceeded by oils obtained from Arbequina olives stored for 3 or 4 weeks at 25 °C.

Our results show that the highest values of K_{270} were obtained at the highest storage temperature. Moreover, Chétoui oils had higher values of K_{270} at both storage temperatures. In fact, the Chétoui oils lost the “extra” VOO quality after the first week of storage at 25 °C. However, the limiting value of 0.2 to certify “extra” VOO quality was exceeded only by Arbequina oils extracted from olives stored for 4 weeks at 25 °C. The increase in K_{270} is probably related to the higher amounts of oxygenated 3,4-DHPEA-EA in Chétoui oils than in Arbequina oils.

The PV and extinction coefficients are insufficient to predict VOO quality. For this reason, oxidised secoiridoid compounds could indicate VOO ‘‘freshness”, as their occurrence was related to the oxidation and degradation of phenolic compounds.

4. Conclusions

HPLC coupled to HRMS revealed VOO secoiridoid compounds and their deacetoxylated, oxygenated, and deacetoxy-oxygenated derivatives. It resulted that the phenolic profiles of olive oils are highly dependent on the varieties studied (Arbequina and Chétoui) during olive ripening and storage. Chétoui oils have more total 3,4-DHPEA-EA and *p*-HPEA-EA and their oxygenated derivatives; while more deacetoxylated 3,4-DHPEA-EA and *p*-HPEA-EA, which are responsible for VOO antioxidant and therapeutic properties, respectively, were observed in the Arbequina oils. Moreover, for both varieties, a decrease of total secoiridoid compounds was noted during olive ripening and storage. However, this decrease was most important when olives were kept at 25 °C. Storing Arbequina and Chétoui olives at 4ºC up to one and two weeks, respectively, produced oils with similar or slightly higher amounts of secoiridoids than ripening olives.

The amount of VOO phenolic compounds depends both on the availability of secoiridoid glucosides and the hydrolytic (β-glucosidase) and oxidative (POX and PPO) enzymes activities during olive ripening and storage. Our results also show that the availability of secoiridoid aglycons and PPO activity could be the main determinants in the presence of oxygenated phenol derivatives in the oils.

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763

FIGURE CAPTIONS

Figure 1. Evolution of POX (a), PPO (b) and β-glucosidase (c) activities (U/mgPr, means of three replicates) during Chétoui (○) and Arbequina (♦) olives ripening (—) and storage at 4 (····) and 25 °C (– –), respectively.

Figure 2. Chromatograms obtained by selecting the exact masses of oleuropein aglycon (3,4-DHPEA-EA) (a); ligstroside aglycon (*p*-HPEA-EA) (b); elenolic acid aglycon (EA-A) (c); deacetoxy-oleuropein aglycon (3,4-DHPEA-EDA) (d); deacetoxy-ligstrosideaglycon (*p*-HPEA-EDA) (e); oxygenated oleuropein aglycon (3,4-DHPEA-OH-EA) (f); All Ions Fragmentation experiment (MS/MS) for oxygenated ligstroside aglycon (*p*-HPEA-OH-EA), extracted ion monitoring of characteristic product ion: *m/z* 333.1344 (g); and oxygenated deacetoxy-oleuropeinaglycon (3,4-DHPEA-OH-EDA) (h).

Figure 3. Evolution of the content of total oleuropein (3,4-DHPEA-EA) (a); ligstroside (*p*-HPEAEA) (b, c); and elenolic acid (EA) (d, e) aglycons during Chétoui (○) and Arbequina (♦) olives ripening (—) and storage at 4 (···)·and 25 °C (– –), respectively.

Figure 4. Evolution of the content of total deacetoxylated oleuropein (3,4-DHPEA-EDA) (a); deacetoxylated ligstroside (*p*-HPEA-EDA) (b); oxygenated oleuropein (3,4-DHPEA-OH-EA) (c); oxygenated ligstroside (*p*-HPEA-OH-EA) (d); and deacetoxy-oxygenated oleuropein (3,4-DHPEA-OH-EDA) (e) aglycons during Chétoui (○) and Arbequina (♦) olives ripening (—) and storage at 4 (···) and 25 °C (– –), respectively.

Table 1. Evolution of Maturity Index (MI) during Chétoui and Arbequina olives ripening during the crop season 2012-2013.

Table 2. Variation of Peroxide Values (PV) and Extinction Coefficients (*K232 and K270*) during Chétoui and Arbequina olives storage at 4 and 25 °C.

Table 1. Evolution of Maturity Index (MI) during Chétoui and Arbequina olives ripening during the crop season 2012-2013.

Harvest date	**WAF**	Chétoui	Arbequina
(D1) : 21/11/2012	**31**	**2.1**	**2.5**
(D2) : 08/12/2012	**33.5**	**3**	**3.2**
(D3) : 25/12/2012	**36**	**3.5**	**3.8**
(D4) : 08/01/203	**38**	**3.9**	**4.7**

Table 2. Variation of Peroxide Values (PV) and Extinction Coefficient (*K232 and K270*) during Chétoui and Arbequina olives storage at 4 and 25 °C.

			PV	K232	K270
Chétoui	On tree	0 (21 November)	4.34^{b}	$1.65b^{cde}$	0.19^{ef}
	Storage at 4 °C	1 Week	8.88^{n}	2.50^{i}	0.22^{gh}
		2 Weeks	8.49^{l}	1.59^{bc}	0.20^{f}
		3 Weeks	7.46^{j}	1.98^{fg}	0.22^{g}
		4 Weeks	8.08^{k}	1.64^{bcd}	0.18^{de}
	Storage at 25 °C	1 Week	5.16^{de}	1.81^{def}	0.29^{k}
		2 Weeks	4.92^{cd}	1.61^{bc}	0.25^{ij}
		3 Weeks	8.70^{lm}	2.04^{g}	0.24^{hi}
		4 Weeks	13.88^{p}	2.31^{h}	0.26^{j}
Arbequina	On tree	0 (21 November)	3.19^{a}	1.31^{a}	0.08^{a}
	Storage at 4 °C	1 Week	6.62^{h}	1.82^{ef}	0.12^{b}
		2 Weeks	7.04^{i}	1.60^{bc}	0.11^{b}
		3 Weeks	5.24^{e}	1.63^{bc}	0.12^{b}
		4 Weeks	5.59^{f}	1.69^{cde}	0.12^{b}
	Storage at 25 °C	1 Week	5.32^{e}	1.60^{bc}	0.15^{b}
		2 Weeks	4.82^{c}	1.49^{b}	0.17^{cd}
		3 Weeks	6.17^{g}	3.48^{j}	0.19^{ef}
		4 Weeks	9.70^{o}	5.89^{k}	0.31^{k}

$^{a\text{-}p}$ Mean values of each measured parameters with different letters within each week are significantly different ($P < 0.05$).

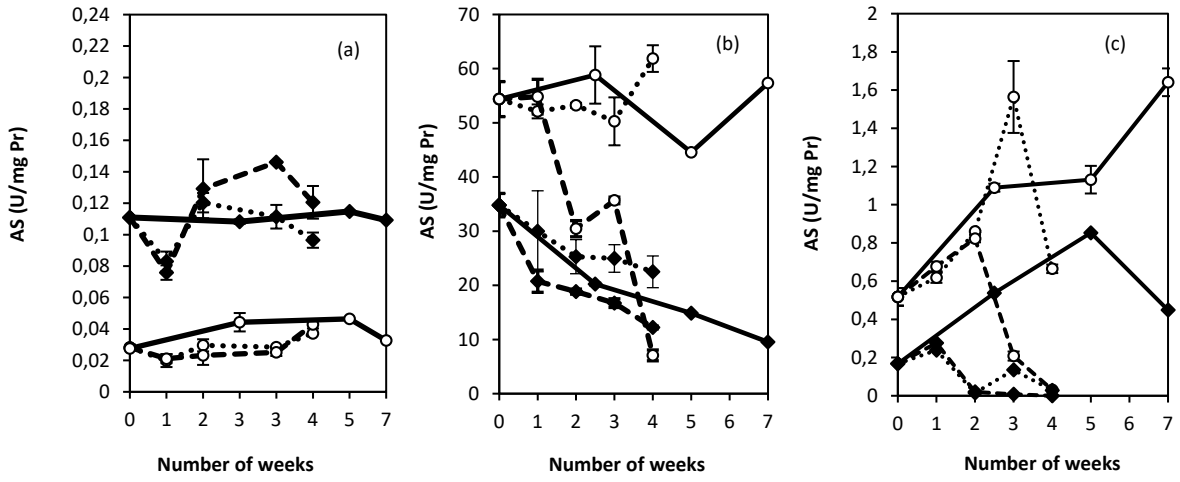
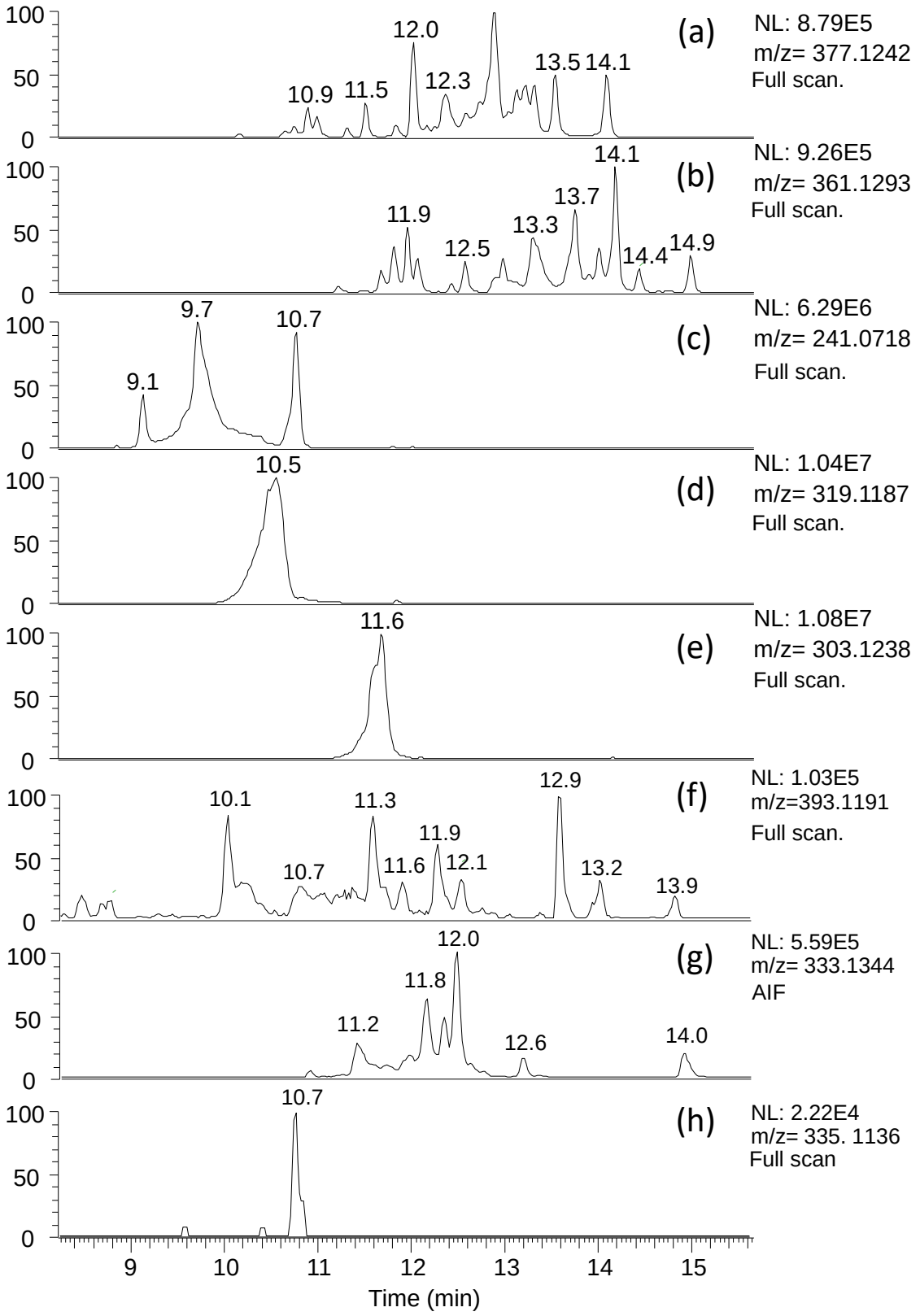


Figure 1. Evolution of POX (a), PPO (b) and β-glucosidase (c) activities (U/mg Pr, means of three replicates) during Chétoui (○) and Arbequina (♦) fruits ripening (—) and storage at 4 (·····) and 25°C (– –), respectively.



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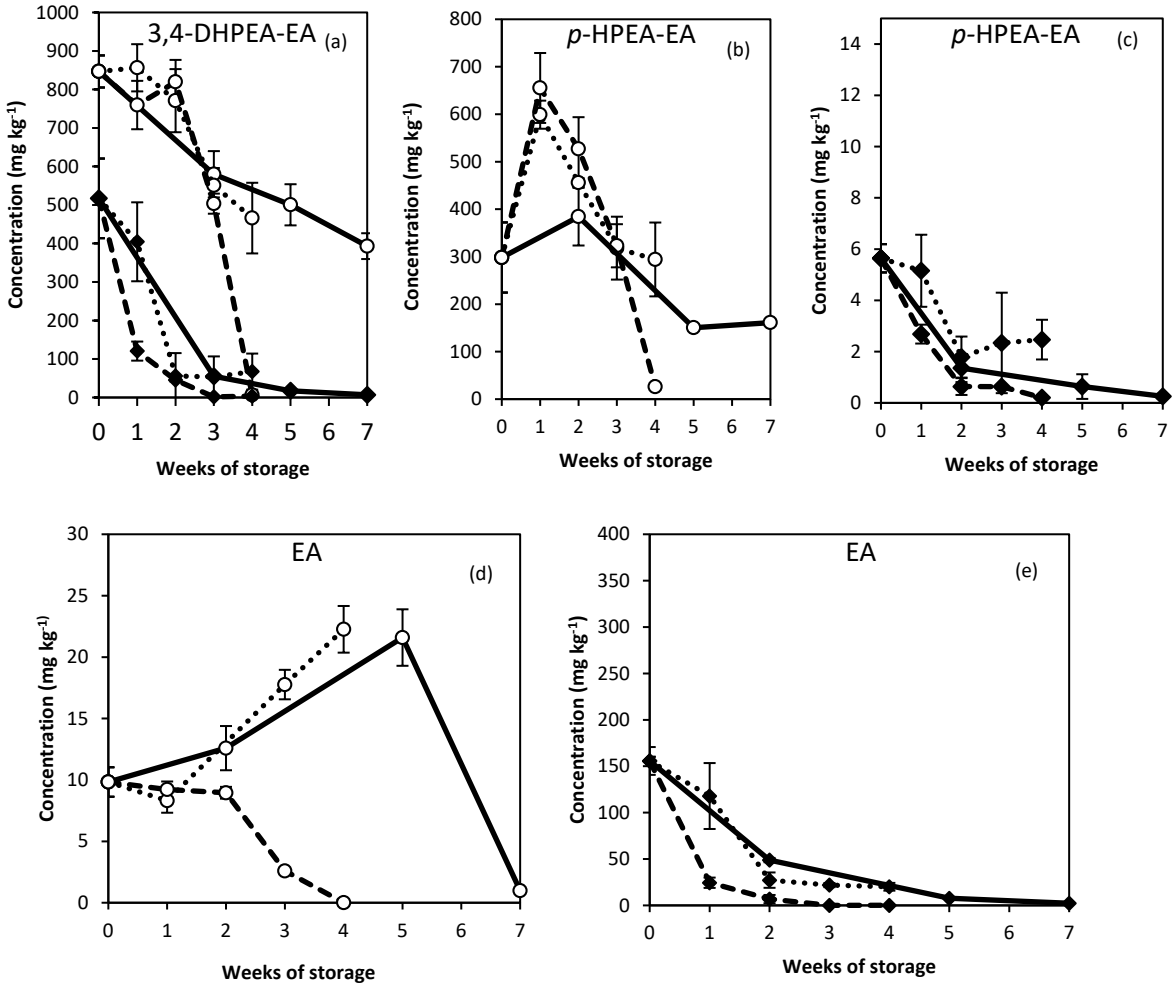

Figure 3. Evolution of the content of total oleuropein (3,4-DHPEA-EA) (a); ligstroside (*p*-HPEA-EA) (b, c); and elenolic acid (EA) (d, e) aglycons during Chétoui (○) and Arbequina (♦) fruits ripening ( ) and storage at 4 ( ) and 25°C ( ), respectively.

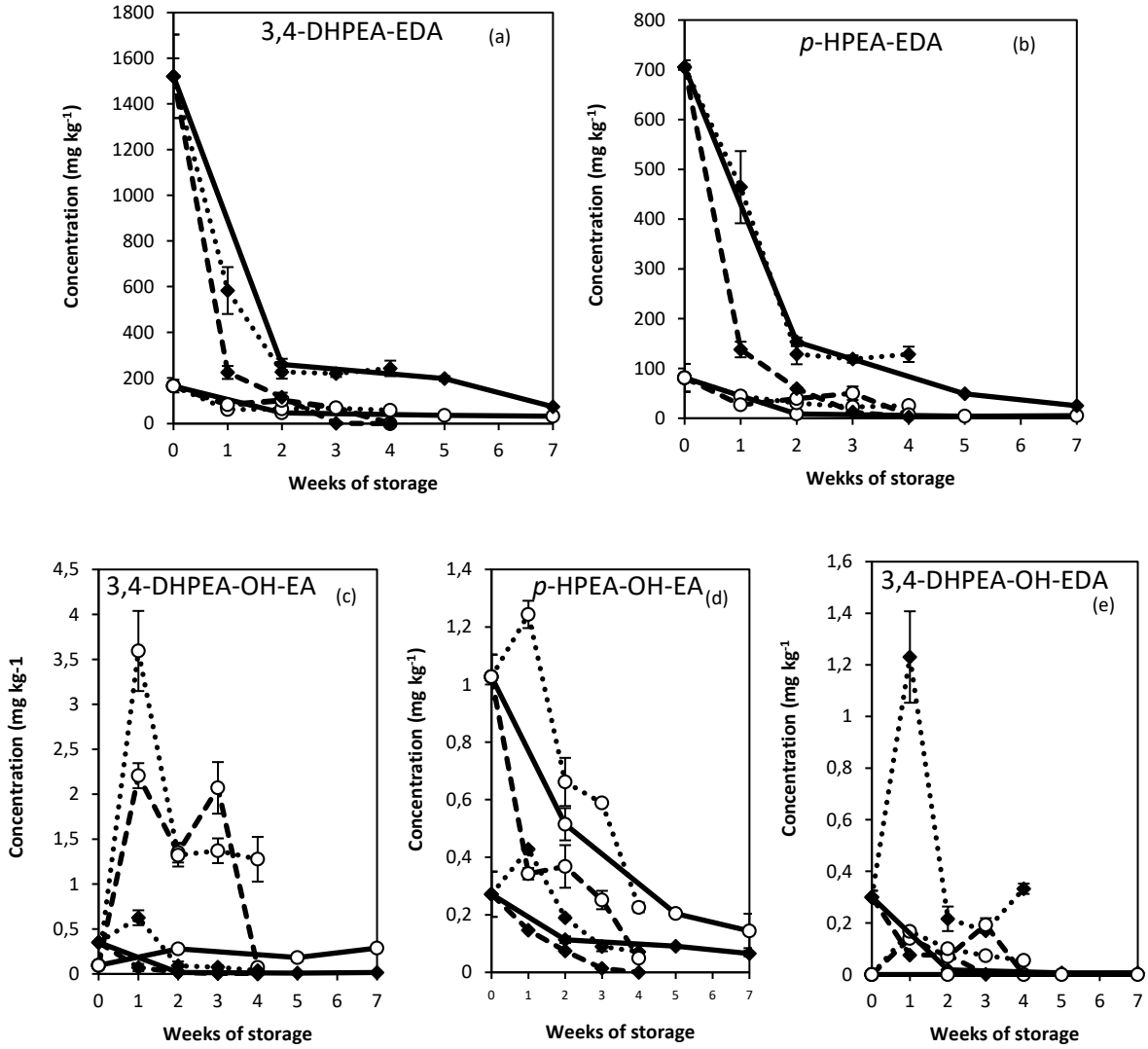

Figure 4. Evolution of the content of total deacetox oleuropein (3,4-DHPEA-EDA) (a); deacetoxy ligstroside (*p*-HPEA-EDA) (b); oxygenated oleuropein (3,4-DHPEA-OH-EA) (c); oxygenated ligstroside (*p*-HPEA-OH-EA) (d); and deacetoxy oxygenated oleuropein (3,4-DHPEA-OH-EDA) (e) aglycons during Chétoui (○) and Arbequina (♦) fruits ripening (—) and storage at 4 (·····) and 25°C (– –), respectively.