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# Hydroxytyrosol fatty acid esters as new candidate markers for detecting olive oil inadequate storage conditions by UHPLC-QqQ-MS/MS

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## ABSTRACT

Recently, lipophenols (or phenolipids), which are composed of a fatty acid (FA) and a phenolic core such as hydroxytyrosol (HT), have been detected in extra virgin olive oils (EVOO), promoted by the esterification reaction between FA and HT in a unique lipophenolic molecule (HT-FAs). However, the levels of HT-FAs, as well as potential alterations in their concentrations during the storage of EVOO, are still unexplored. Thus, the present study uncovers, by UHPLC-MS/MS triple quadrupole analyzer, the changes of HT-FA levels in extra virgin olive oil (EVOO) during 12 months of storage, mimicking both commercial temperatures (room temperature (RT)), as well as inappropriate conditions of storage at high temperatures (40 °C). In addition, levels of HT-FAs in olive oils (blends with refined olive oil) compared to EVOO were assessed to investigate the effect of the refining process on lipophenol generation. Free HT concentration in EVOO was increased, after 12 months, by 121.31% and 236.80% at RT and 40 °C, respectively. Additionally, HT-FAs in EVOO samples, mainly, esterified with linoleic acid and oleic acid, enhanced up to 2.6-fold and 5.5-fold, respectively after 12 months at 40 °C. Nonetheless, in blends with refined oils, the levels of free HT decreased by 80.2%, whereas esterified HT augmented significantly. The type of FA linked to HT core, as well as the degree of unsaturation of FA seem to influence the lipophenolic derivative generation. Significant concentration differences found during storage time supported their potential use as candidate markers, but these findings so require further assessment to establish the HT-FAs concentration range for authentication purposes. Likewise, the ratio between esterified/free HT could be employed as freshness or aging indices of the EVOO during its shelf life, when used in conjunction with validated olive quality parameters.

## 1. Introduction

Extra virgin olive oil (EVOO) is obtained from the fruit of *Olea europaea* L. by mechanical or physical procedures exclusively [1]. Unfortunately, there are malpractices in olive oil production, transportation, and storage that may modify its quality generating not only threats and challenges to the suppliers, but also affect health benefits linked to its health-promoting compounds. In this regard, the factors

that promote the degradation of its components and alter olive oil quality can be divided into intrinsic factors, such as the variety of olive fruits and external factors, which include exposure to light, oxygen, temperature, time or the type of material used for packaging, and storing [2]. Regarding the storage time, International Olive Council (IOC) limited the best before the date to 24 months after bottling, respecting the “first in first out principle” (FIFO); items stored first are retrieved first. Based on the best practice guidelines for the storage of olive oils for

*Abbreviations:* ALA,  $\alpha$ -linolenic acid; BHA, butylated hydroxyanisole; DHA, docosahexaenoic acid; DMSO, dimethyl sulfoxide; EPA, eicosapentaenoic acid; ESI, electrospray ionization; EVOO, extra virgin olive oil; FAs, fatty acids; FAAEs, fatty acid alkyl esters; HT, hydroxytyrosol; IOC, International Olive Council; LA, linoleic acid; MRM, multiple reaction monitoring; OA, oleic acid; OO, olive oil; ROC, receive operating characteristic; RT, room temperature; UHPLC-QqQ-MS/MS, ultra-high pressure liquid chromatography coupled to triple quadrupole mass spectrometry.

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human consumption by IOC, recommended keeping pre-distribution storage rooms at a light intensity lower than 500 lx (on average) and at temperatures between 13 and 25 °C (COI/BPS/Doc. No 1. 2018). Nevertheless, during the storage and transport, EVOOs are affected by unstable temperatures over time, which modify the oil quality as a consequence of the degradation of some components like phenolic compounds, affecting its natural antioxidant content and therefore, its biological activity [3]. In this context, several studies have reported these effects of storage conditions in a decrease of secoiridoid derivatives and the subsequent increase of hydroxytyrosol (HT), tyrosol, and, oleocanthalic acid [4–7]. Apart from the storage conditions, the refining process is another factor that also affects the olive oil quality, producing the loss of the quantity profile of antioxidants and hence, affecting its oxidative stability [8]. Particularly, HT is one of the phenolic compounds that is firstly eliminated during the refining step of alkaline treatment [9].

Previous researches have informed about the formation of esterified molecules, particularly fatty acid alkyl esters (FAAEs), in aged olive oils, changing their content during the shelf life and during the processing and considering them as parameters for assessing the quality of olive oils by IOC [10,11]. In addition, hydroxytyrosyl esters bearing fatty acid acyl chain (HT-FAs) as “natural” lipophenols, particularly HT linked to  $\alpha$ -linolenic acid (HT-ALA), HT-linoleic acid (HT-LA), and HT-oleic acid (HT-OA), have been identified in several edible vegetable oils and their by-products [12–15]. Interestingly, an increase of molecules of tyrosol esterified with OA in low-quality oils in greater exerts than EVOO has been published recently [16]. The generation of HT-FAs during *in vitro* intestinal digestion, pinpointing the enzymes responsible for their synthesis has also been reported [17]. In addition, olive oil lipophenols have been extensively studied recently not only from the chemical but also from the biological point of view due to multiple activities like antioxidant, anti-inflammatory, anti-diabetes, and skin regenerative properties [13,14,18–20]. According to this background information, and considering that the content of HT-FAs during storage of EVOO is still unexplored, the main aim of the current study was to investigate the alterations of HT-FA molecules after storage of olive oils at inappropriate conditions, by simulation of high temperatures (40 °C) compared to commercial temperature (room temperature (RT)). Additionally, the blends with refined olive oil were analyzed to obtain the quantitative profile of HT-FAs in these mixtures, which could provide useful information for olive oil authentication. In this context, hydroxytyrosyl ester derivatives could be considered as new potential markers, which in conjunction with classical parameters of EVOO quality, could allow detecting EVOO aging, poorly storage conditions, or blends prepared with refined oils and aged EVOO.

## 2. Materials and methods

### 2.1. Chemical and reagents

Hydroxytyrosol, with the following specifications: purity 99.6%, moisture 3.7%, and pH 4.26, was provided by Seprox BIOTECH S.L. (Murcia, Spain). The authentic standards of high-purity HT-FA esters (HT-ALA, HT-LA, and HT-OA) were synthesized and fully characterized by the Institut des Biomolécules Max Mousseron (IBMM) (Montpellier, France) according to previously published procedures [12]. All lipophenols were dissolved in dimethyl sulfoxide (DMSO) from Sigma Aldrich (St. Louis, MO, USA) to obtain a stock solution, and then successive dilutions were prepared in methanol/Milli-Q water (50:50, v/v). Acetone and butylated hydroxyanisole (BHA) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and all LC – MS-grade solvents (deionized water, methanol, and acetonitrile) were from J.T. Baker (Phillipsburg, NJ, USA). All other reagents were of analytical grade.

### 2.2. Olive oil samples and storage conditions

Extra virgin olive oil samples (Picual monovarietal; *Olimendros* S.L. (Murcia, Spain), acidity 0.13 g/100 g) and olive oil (OO) samples from blending of the virgin olive oils (not lampante oil) with refined olive oil with an acidity of 0.4 g/100 g, belong to OO according to EU Council Regulation (EC) No 1234/2007 (*La Española*, Acesur Group (Seville, Spain)) were selected in this research due to their dissimilar concentration of HT [21] and because of being the most consumed edible vegetable oils in the Mediterranean basin. Both olive oils (EVOO and OO) were produced in the 2019–2020 campaign. All olive oil samples were kept in dark glass bottles and closed with screw caps with 8% headspace. All samples were kept in darkness and the bottles were not opened until analysis to prevent oxidation. All EVOO samples were analyzed just after production in the 2019/2020 crop season (time 0) and then EVOO samples were divided into two batches, the first batch was stored at room RT ( $23 \pm 2$  °C) like common commercial conditions and the second one was maintained at  $40 \pm 2$  °C in an oven to simulate inadequate temperature storage conditions. Storage conditions were chosen to emulate Southern Spain temperatures and areas with similar climatology, the temperatures in summer seasons are very high (35–40 °C). Two batches were analyzed every 30 days for the following 360 days of storage after production. The lack of 210 days' samples was due to the COVID-19 pandemic confinement and the impossibility of collecting these specific samples on schedule.

### 2.3. Extraction of hydroxytyrosol fatty acid esters

The HT-FAs were extracted from olive oils according to the methodology validated previously by our research team for oily matrices [15]. Briefly, for the extraction of HT and its lipophenolic derivatives (HT-ALA, HT-LA, and HT-OA), olive oil samples were dissolved in 1 mL of acetone with BHA (0.005%) solution, vortexed for 1 min, sonicated for 30 min at 40 kHz, and centrifuged at 8750g for 5 min at 4 °C according to the procedure described [15]. Then, the samples were dried using a SpeedVac concentrator, and the dry extracts were reconstituted with 200  $\mu$ L of MeOH, sonicated for 10 min, centrifuged at 8750g for 5 min, and filtered through a 0.45  $\mu$ m filter (Millipore, Burlington, MA, USA). The samples were extracted and analyzed in triplicate using the analytical method described below.

### 2.4. UHPLC-QqQ-MS/MS analysis

The separation of HT and HT-FAs as shown Fig. 1A was performed using a UHPLC coupled with a 6460 triple quadrupole-MS/MS (Agilent Technologies, Waldbronn, Germany), using the chromatographic column BEH C18 1.7  $\mu$ m (2.1  $\times$  50 mm) (Waters, Milford, M.A.), according to the previous methodology validated by our research team [15]. The column temperature was 30 °C and the mobile phases consisted of 0.1% formic acid in water (LC – MS grade) (A) and acetonitrile (B). The injection volume and flow rate were 15  $\mu$ L and 0.3 mL/min, respectively. The chromatographic separation of HT-FAs was achieved upon the following linear-gradient (time (min), %B): (0.0, 30%); (3.0, 98%); (6.0, 100%), and (6.1, 30%). An additional post-run period of 1.5 min at 30% solvent B was considered for column equilibration. Analyses were performed by multiple reaction monitoring (MRM) in the negative mode, assigning preferential MRM transitions (quantification and confirmation) for the corresponding analytes: HT (153>123; 153>95), HT-ALA (413>277; 413>260), HT-LA (415>279; 415>262), and HT-OA (417>281; 417>264). The ionization and fragmentation conditions were as follows: fragmentor 90 V, collision energy 9 eV, gas temperature 325 °C, gas flow 8 L/min, nebulizer 30 psi, sheath gas temperature 350 °C, jetstream gas flow 12 L/min, capillary voltage 4000 V, and nozzle voltage 1000 V, according to the most abundant product ions. Data acquisition and processing were performed using Mass Hunter software version B.08.00 (Agilent Technologies, Waldbronn, Germany).

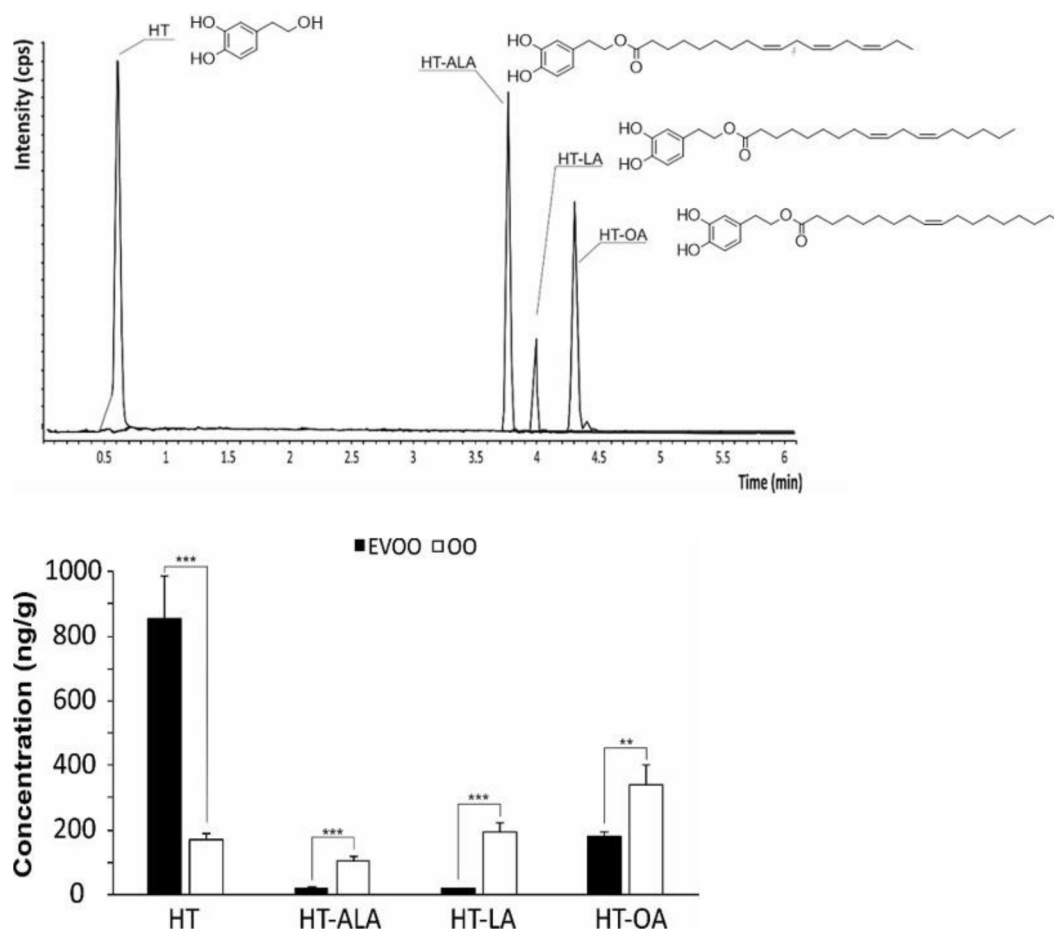


Fig. 1. UHPLC-QqQ-MS/MS-extracted ion chromatograms corresponding to free HT and HT esters derivatives (HT-ALA, hydroxytyrosol- $\alpha$ -linolenic acid; HT-LA, hydroxytyrosol-linoleic acid; and HT-OA, hydroxytyrosol-oleic acid) present in extra virgin olive oil using the quantification MRM transitions (A) and concentration (ng/g) of HT and HT-ALA, HT-LA, and HT-OA in EVOO and OO samples evaluated (B). T-test differences: \*\*\* $p$ -value < 0.001 and \*\* $p$ -value < 0.01. CPS, charges per second.

### 2.5. Statistical analysis

All experiments were carried out in triplicate, and the HT and HT-FAs concentrations are presented as mean  $\pm$  standard deviation (SD). Significant differences were set at  $p$ -value < 0.05 using the SPSS v. 28.0 software package (LEAD Technologies, Inc., Chicago, USA) by which one-way analysis of variance (ANOVA) and the multiple-range (Tukey's) test were performed to identify significant differences. The normal distribution of the residuals and the homogeneity of variance were tested with the Kolmogorov-Smirnov and Levene's tests, respectively. A Spearman analysis was performed to evaluate correlations between variables. A paired Student's  $t$ -test was carried out to detect significant alterations in the content of HT and its lipophenolic derivatives at time 0 and after 12 months of storage at different temperatures. Finally, univariate receive operating characteristic (ROC) analysis was carried out to evaluate the ability of HT and HT-FAs to classify oils according to storage time and temperature using the web-based application MetaboAnalyst v. 5.0, created at the University of Alberta, Canada [22].

## 3. Results and discussion

### 3.1. Identification and quantification of hydroxytyrosol fatty acid esters in extra virgin and olive oils

The qualitative profile of unesterified and esterified HT molecules in olive oils (EVOO and OO) was set up by chromatography and mass spectrometry with triple quadrupole technology (Fig. 1A). As expected,

the retention time of HT lipophenolic derivatives showed a reverse correlation with the degree of unsaturation of its lipid part. Thus, HT-ALA, HT-LA, and HT-OA with respectively three, two, and one unsaturation in the carbon chain, were eluted at 3.8, 4.0, and 4.4 min (Fig. 1A), which are consistent with previous experimental results for lipophenols, particularly esterified tyrosol molecules [23]. Related to quantitative assessment, the free HT (unesterified) was presented at 853.15 and 168.79 ng/g in EVOO and OO, respectively (Fig. 1B). These concentrations were in line with those registered in previous reports, which ranged from 870 to 2600 ng/g for EVOO samples [5,17] and 230–2300 ng/g for OO samples [24]. In the current study, lipophenolic derivatives of HT (HT-ALA, HT-LA, and HT-OA) were found at respectively 18.82, 17.96, and 179.50 ng/g, in EVOO samples (Fig. 1B), findings that are in concordance with previous results on HT-FAs in edible oils [15,17]. However, in OO samples 103.95, 195.21, and 341.03 ng/g were found, increasing respectively 5.5, 10.9, and 1.9-fold for HT-ALA, HT-LA, and HT-OA, compared to EVOO samples (Fig. 1B). The discrepancy with the data reported by Lee and colleagues [12], which found lower levels of HT-FAs in OO than EVOO samples, could be related to multiple factors, including differences in varieties, ripening stage, geographical origin, proportion of refined oil in OO samples, and production procedures. On the contrary, a recent work carried out by Benincasa and colleagues [16] reported that tyrosol lipophenolic derivatives, specifically tyrosol esterified with OA was found in higher concentration in defective or low-quality olive oils ( $\sim$ 4.33 mg/kg) in comparison with EVOO samples ( $\sim$ 0.68 mg/kg), increasing 6.4 fold in olive oils with low quality, being in accordance with our findings. These authors also mentioned that the

reaction of tyrosol with OA, leading to the formation of lipophenol molecules may occur during crushing, kneading processes when suitable enzymes are activated [16]. Hence, the increase of lipophenolic molecules of HT, esterified with FAs found in OO, may be due to the refining process owing to a heat-promoted esterification reaction between free HT and FAs, as previously described for other phytochemical sterols [25]. This study displayed that phytosterols were progressively lost during refining, altering the ratio of esterified/free sterols. The deodorization conditions induced an esterification reaction between free sterols and FA, resulting in an increase in the content of steryl esters [25]. As proof of concept, this study may provide preliminary findings of new paths in the authenticity of olive oil and HT ester derivatives could be used as candidate markers for blending olive oil. However, further assays with multiple mixtures (EVOO spiked with different concentrations of refined olive oil and/or other cheaper edible oils used in EVOO adulteration) should be incorporated in forthcoming studies to establish the HT-FAs concentration range into the blended EVOO matrix.

On the other hand, and as mentioned earlier, commercial OO is a mixture of refined olive oil with virgin olive oil, and the percentage of virgin olive oil in the mixture may be known because of the content in phenyl ethyl alcohol like HT, has been previously described [26]. In the current study, the content of HT in OO samples in comparison with EVOO was low, which was reduced by 80.20%. Those results are in line with previous work that reported up to ~ 80% less, usually lost during the refining process [21,27]. Nevertheless, the concentration of phenolic compounds and their lipophenolic derivatives present in edible oils may

greatly vary depending on variety, degree of maturation of olive fruit, climate, altitude, cultivation practice, and manufacturing process, as well as the type and time of storage before its consumption [28].

### 3.2. Hydroxytyrosol fatty acid esters as markers for detection of extra virgin olive oil under inadequate storage conditions

The content of free HT and HT esterified with fatty acids (HT-ALA, HT-LA, and HT-OA) in EVOO samples stored for 12 months at different temperatures (RT and 40 °C) was investigated. Regarding the storage of EVOO samples mimicking commercial conditions at RT, the free HT experienced an increase of 2.2-fold after 12 months of storage as shown in Fig. 2A. However, at RT, the differences in the content of HT were not significant until the 150 days of storage, in which the rise was 2.1-fold compared to time 0. In our present work, a rise of 121.31% of HT after 12 months of storage at RT was detected, in concordance with Castillo-Luna 2021 and colleagues [5] that estimated an increase of 126.90% for HT in the EVOO samples analyzed after storage for 12 months. In this regard, many additional studies have reported an increase in HT (~40–130%) after 12–18 months of storage [4,5,29–32]. Our findings reinforce the evidence that HT may be a reliable marker to detect aged EVOO samples and blends prepared with aged EVOO as recently reported [5]. In this context, the increase of HT content has been associated with the hydrolysis of the olive oil secoiridoid aglycons during storage, which are transformed into hydrophilic molecules such as HT and consequently, enhancing its content [33,34]; therefore, the

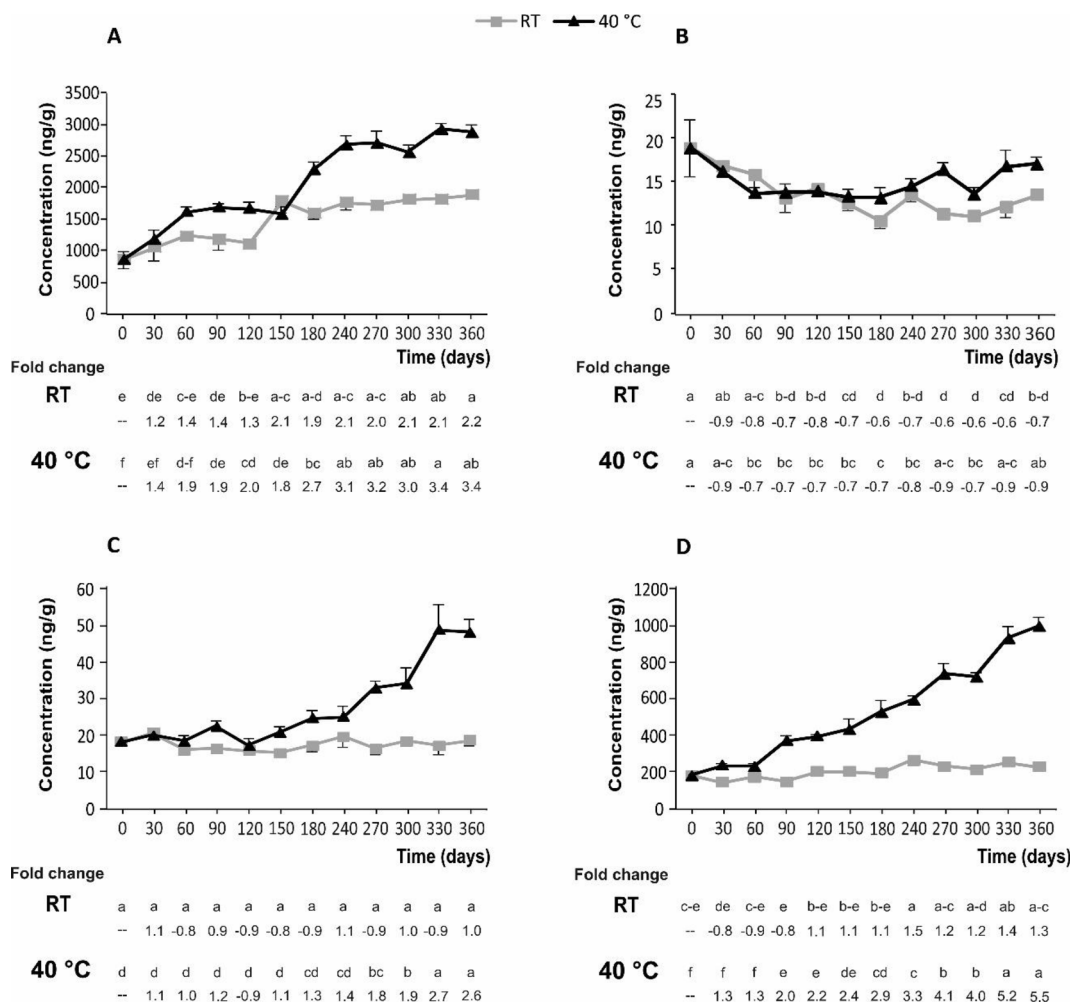


Fig. 2. Concentration (ng/g) of HT (A) and HT-FAs (HT-ALA (B), HT-LA (C), and HT-OA (D)) during 360 days of storage at room temperature (RT) and at 40 °C. Different lowercase letters are significantly different at  $p$ -value < 0.05 according to the analysis of variance (ANOVA) and Tukey post-hoc test.

EVOO freshness should be correlated to a lower and not to the higher value of HT [30]. Nevertheless, other potential variations in the free HT and HT-FAs content of olive oil may be associated with other parameters such as the cultivar, geographic origin, altitude, edaphoclimatic conditions, time of harvest, degree of maturation of the olive fruits, as well as, aging with different storage conditions (exposure to oxygen, light, and temperature) and research effort should be performed to establish the range of HT-FAs concentration that is present in fresh and old olive oil samples for authentication purposes.

Regarding the content of HT ester derivatives (HT-FAs) in EVOO samples stored at RT, no differences ( $p$ -value  $> 0.05$ ) were found for HT-LA (Fig. 2C), while the concentration of HT-ALA decreased during the storage, with significant differences ( $p < 0.05$ ) from the third month (Fig. 2B). These findings may be due, on the one hand to the instability of ALA or a decrease of ALA content during storage as described previously [35]. And secondly, the reduction of ALA levels could hypothetically be related to its oxidation non-enzymatically in oils and generate phytoprostanes (PhytoPs; plant oxylipins) [36], what could occur the subsequent formation of HT-PhytoP molecules. However, more research is necessary to test this hypothesis. Apart from that, at RT, the concentration of HT-OA enhanced significantly ( $p < 0.05$ ) from the 240 days of storage (Fig. 2D), probably because of the increase of HT and OA percentage after 12 months of storage as was previously reported [37].

Related to the storage of EVOO samples under inadequate storage condition at high temperatures (40 °C), the mean concentration of HT increased from 853.15 to 2873.42 ng/g (~236.80%, on average) after 12 months (Fig. 2A) due to probably the degradation of secoiridoids compounds. As stated by Olmo-Cunillera and colleagues [38] and Marx and colleagues [39], higher temperatures decreased the levels of the secoiridoids, being the phenolic group the most affected by temperature [38,39] and its depletion has been attributed to the action of enzymes oxidoreductases in a greater extent in secoiridoids derivatives from HT than derived from tyrosol [38,40]. In this work, the fold-change of HT after 12 months of storage was 3.4 at 40 °C in comparison with 2.2 at RT and these alterations were statistically significant from the third month of storage at high temperature in contrast to the fifth month of storage at RT (Fig. 2A). In the case of lipophenols, HT-ALA was the only esterified molecule that decreased its concentration after storage at high temperature from 18.83 at time 0 to 16.98 after 360 days of storage, reducing 9.83% on average (Fig. 2B). This loss may be the consequence of the higher unsaturation number of ALA, making it prone to oxidation more easily than other FAs such as LA or OA. Regarding the molecule of HT esterified with LA, a 2.6 fold-change was detected after 360 days of storage, from 17.96 ng/g on average at time 0 to 47.56 ng/g on average after 12 months at 40 °C (Fig. 2C), but the alterations in HT-LA content was significant from the 270 days (9 months) of storage with respect to the starting point (time 0). On the other hand, the rise of HT-OA concentration at 40 °C ranged from 179.51 on average to 994.15 ng/g on average at time 0 and after 12 months of storage, respectively as shown in Fig. 2D, displayed a 5.5 fold-change among these two points of storage (0 and 360 days). It is worth pointing out that LA is more unstable than OA, having one bis-allylic position, it could also be more easily oxidized than OA, and this might explain lower quantities of HT-LA than HT-OA. Furthermore, the changes in HT-OA content were significantly earlier than other HT-FAs studied, from the third month of storage at 40 °C, just like the rise of unesterified HT, therefore both compounds (HT and HT-OA) might be considered as suitable markers for detecting extra virgin olive oil inadequate storage conditions and blends with aged EVOO, but the different sources of variability in lipophenol content should be assessed to establish the concentration range in fresh and aged olive oils for authentication purpose. In fact, we found a significant linear correlation between the content of free HT and esterified HT during the storage at 40 °C ( $R = 0.845$ ;  $p < 0.01$  and  $R = 0.926$ ;  $p < 0.001$  for HT-ALA and HT-OA, respectively). But, at RT, only HT-OA showed a significant linear correlation with HT ( $R = 0.721$ ,  $p$ -value  $< 0.01$ ) (Data not shown). These results are in line with recent work, which

reported the molecule of tyrosol esterified with OA increased for the long-time storage under inappropriate conditions when the concentration of the free precursors (tyrosol and OA) were augmented [16]. In our study, HT-OA was the lipophenol with higher content in olive oil samples analyzed, which may be largely due to OA being the most abundant FA in olive oils, in greater amount (78.23 g/100 g fw) than LA or ALA (6.67 and 0.50 g/100 g fw, respectively) as described by our research team in a previous report [15]. In this regard, the formation of lipophenols may be explained as a result of the lipase activity coupling FA and HT in a unique lipophenolic molecule. The enzyme lipase may catalyze not only the hydrolysis of ester bonds but also their synthesis in nonaqueous media, which explains esterification reactions [41]. This enzyme might come from yeasts (lipase-positive) present in both olive fruits and EVOO as previously reported [42]. In fact, Plastina and colleagues [14] already informed that HT-OA was formed in olive by-products such as olive pomace as a result of esterase activity coupling OA and HT present in high amounts in those matrices [14]. In addition, the formation of lipophenols was a greater exert at 40 °C compared to RT, probably be due to the fact that the catalytic activity of olive lipase can be encouraged by high temperatures (30–40 °C) as previously described [43], although further research on yield of lipophenols formation at different temperatures should be addressed. Also, the acidic features of olive oils could promote the esterification reactions and affect the generation of lipophenols, as it was the case for other reactions between phenolic alcohols and acylglycerols, also present in the oily matrices [44] and as recently reported for the generation of quercetin-docosahexaenoic acid (DHA) and quercetin-eicosapentaenoic acid (EPA) lipophenols, during the storage of fish oil supplemented with 10 mg/g of quercetin after heating at 60 °C for 5 days [45].

As mentioned above, comparing the two temperatures of storage, after 12 months of EVOO samples storage, the content of HT raised significantly 1.52-fold at 40 °C (2873.42 ng/g on average) compared to RT (1888.10 ng/g on average) ( $p$ -value  $< 0.001$ ) (data not shown). This result is in agreement with a recent article, which investigated the content of phenolic compounds after 12 months of storage under different temperatures, and the storage at 40 °C increased the concentration of HT 1.72-fold as compared to RT storage [4], given that the high-temperature conditions (40–60 °C) have been associated with increased levels over time of compounds derived from the hydrolysis and oxidation of secoiridoid derivatives like HT [4] and oleocanthalic acid [7]. At this juncture, previous work also reported that the initial degradation rate of secoiridoids during long-term storage (18 months) was similar at 5 and 15 °C, but increased considerably at 25 °C and was even faster at 50 °C [30]. So, recent studies reported that the behaviour of HT was closely related not only to the initial concentration of phenolic compounds in matrices [5] but also to the storage temperature of these matrices [30]. These findings are consistent with the results found in the present work, and it might explain the high increase in the HT concentration observed in this study at 40 °C in comparison with RT (Fig. 2A). In addition, the molecules of HT esterified with FAs showed the same rising trend than unesterified HT. So, the storage during 12 months at 40 °C caused a significantly enhanced ( $p$ -value  $< 0.001$ ) compared to RT, the concentration of HT-ALA, HT-LA, and HT-OA respectively increased by 1.25, 2.59, and 4.40-fold on average (Fig. 2B-D).

In a previous report, the ratio of simple phenolic like HT to secoiridoid phenolics has been proposed as indices of freshness or aging indices of EVOO during its shelf life [30]. Likewise, the ratio of free HT to esterified HT might be used as the suitable index to identify inappropriate storage conditions or to detect blends with aged EVOOs stored at high temperatures. For example, regarding the HT-OA, at 40 °C during 90 days was 368.78 ng/g on average and free HT content in the same conditions of storage was 1681.60 ng/g on average, the ratio between esterified/free HT was 0.22. This index enhanced up to 0.34 after 360 days of storage. However, this ratio was 0.12 at RT both 90 days and 360 days. Thus, the esterification reaction was sped up by high

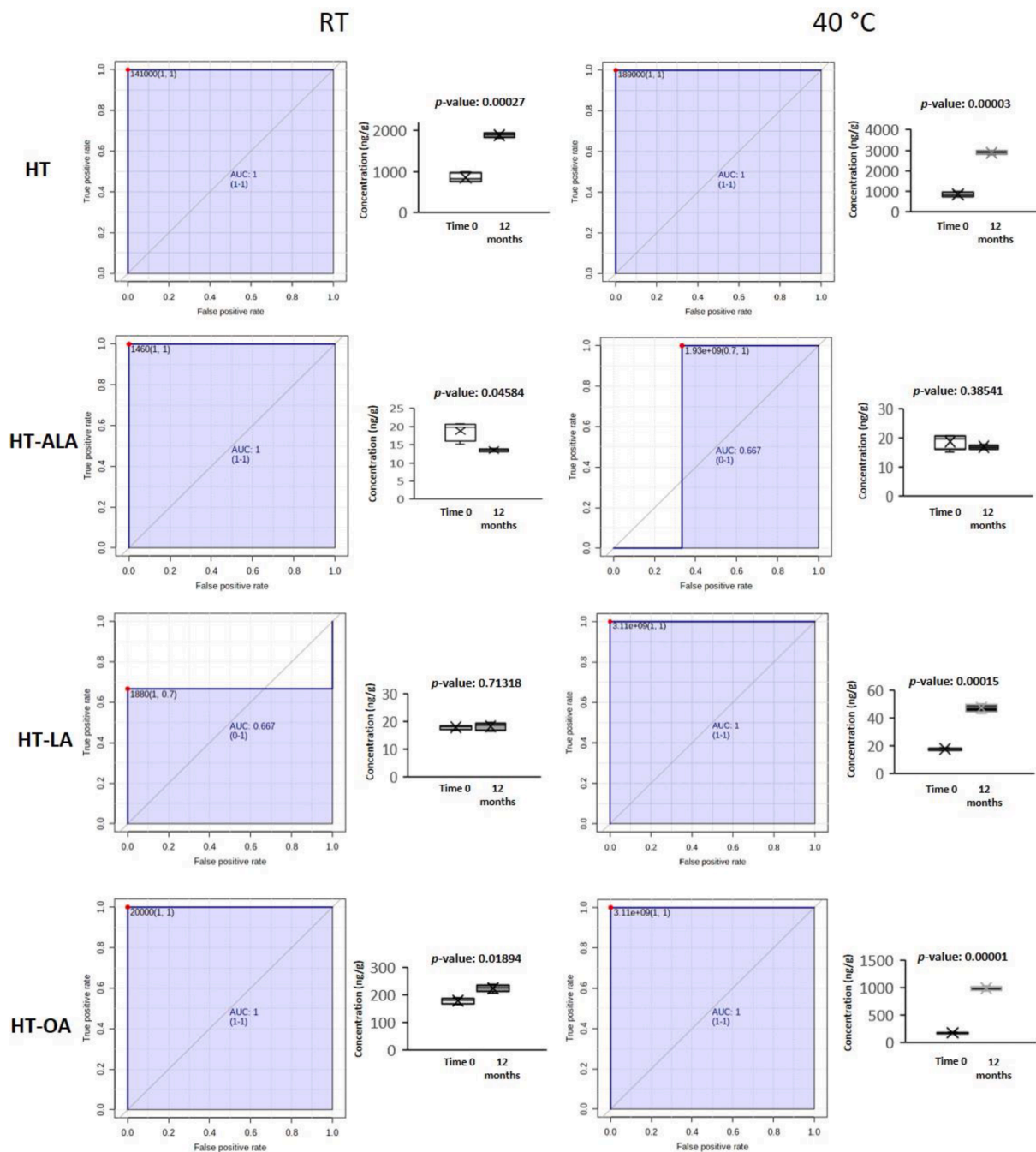
temperatures (40 °C) as mentioned above.

In order to investigate the classification performance, a ROC analysis of both free HT and esterified HT molecules (HT-ALA, HT-LA, and HT-OA) was performed to distinguish EVOO samples at time 0 and after 12 months of storage, at RT and at 40 °C. In this context, Fig. 3 displays the individual ROC curve for HT and HT-FAs, as well as box-and-whisker plots, comparing the content of free HT and lipphenols at these two points of storage, revealed accurate classification of the data, as well as high significance in changes of the concentration of analytes. With the exception of the HT-LA at RT and HT-ALA at 40 °C, in which no significant differences were found ( $p = 0.713$  and  $p = 0.385$ , respectively), both molecules were characterized by a low classification capability at these particular temperatures (Fig. 3). However, both free HT and

esterified with OA seem to present the best classification ability of EVOO samples, therefore, both molecules could be used as candidate markers to discriminate fresh and aged EVOO samples, as well as identify undeclared blends.

#### 4. Conclusions

In the current work, the levels of HT ester derivatives (HT-ALA, HT-LA, and HT-OA) have increased with storage time at RT and 40 °C, but to a larger extent at high temperatures (40 °C) and in blends with refined olive oils. These alterations might be associated on one hand with the degradation of secoiridoids compounds with a subsequent rise of HT, and on the other hand to the esterification reaction between FA and HT



**Fig. 3.** Receiver operating characteristic (ROC) analysis to assess the availability of free and esterified HT (HT-ALA, HT-LA, and HT-OA) to distinguish EVOO samples at time 0 (0 days) and after 360 days of storage at room temperature (RT) and at 40 °C. Box and Whisker plots to compare HT and their esters derivatives concentrations (ng/g) at time 0 and after 12 months of storage at different temperatures (RT and 40 °C).

yielding lipophenolic molecules, enhanced by high temperature. However, the types of FA linked to HT core, as well as the degree of unsaturation of FA seem to influence the lipophenol generation. Additionally, the degradation of more oxidizable molecules such as ALA and LA could explain the less amount of HT-ALA and HT-LA lipophenol derivatives recovered.

From an analytical perspective, this study reinforces the previous results about HT as a marker for detection of EVOO aging, contributing with new knowledge on HT esters derivatives as new candidate markers of EVOO quality which, in combination with classical parameters, might be very useful for monitoring EVOO aging and EVOO aging in inappropriate conditions. In this context, HT-FAs can be potentially used as markers of olive oil exposure to high temperatures. Likewise, the ratio between esterified/free HT could be used as freshness or aging indices of the EVOO during its shelf life. Nevertheless, new research is needed to investigate the mechanisms underlying the formation of HT-FAs during olive oil storage, in consideration of the newly formed HT-FAs esters may provide improved biological activities compared to unesterified HT.

#### CRedit authorship contribution statement

**Sonia Medina:** Conceptualization, Methodology, Investigation, Formal analysis, Writing – review & editing, Project administration, Funding acquisition. **David Auñón:** Conceptualization, Validation, Writing – original draft. **Jordan Lehoux:** Validation, Visualization. **Thierry Durand:** Writing – review & editing, Visualization, Supervision. **Céline Crauste:** Validation, Visualization. **Ángel Gil-Izquierdo:** Conceptualization, Investigation, Writing – review & editing, Supervision, Funding acquisition.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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