Total Phenolic Compounds and Tocopherols Profiles of Seven Olive Oil Varieties Grown in the South-West of Spain

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Abstract: This article reports about the presence of some of the components of minor fraction of virgin olive oils, polyphenols and tocopherols, in several of the VOO varieties from Extremadura. The relationship between both classes of compounds and the oxidative stability of the oils is also examined. The levels of total phenols, α , β , and γ tocopherols showed significant differences ($p < 0.05$) among the different varieties. The concentration of total phenolic compounds varied from 130 to 1203 mg/kg. The α -tocopherol was the most representative in the seven varieties (95.97 %) and ranged from (288 - 170) to (485 - 244) mg/kg in the *Morisca* and *Carrasqueña* varieties respectively. On the other hand, a positive high lineal correlation was observed between oxidative stability and studied along the maturity of the fruit and the total phenolic compounds (natural antioxidants) ($r^2 > 0.90$; $p < 0.05$), α -tocopherol ($r^2 > 0.85$; $p < 0.05$), β -tocopherol ($r^2 > 0.70$; $p < 0.05$) and total-tocopherols ($r^2 > 0.87$; $p < 0.05$), in all the olive oils obtained from the seven varieties of olive from Extremadura. It is noticeable that α -tocopherol fraction contributed equally to the oxidative stability of all the VOO whereas the largest contribution was provided by the oil phenolic fraction, as it was the case of the *Carrasqueña* variety.

Key words: monovarietal virgin olive oil, stage of maturation, phenolic compounds, tocopherols, oxidative stability

1 INTRODUCTION

Virgin olive oil is one of the main fatty foods in the countries of the Mediterranean basin where production the olive oil is concentrated. Its regular consumption is a characteristic of the so called "Mediterranean diet" based also on the consumption of great amounts of vegetables, cereals and fish, and whose beneficial effects on the health have been well established¹⁾. These seem to be primarily related to its balanced fatty acid composition, in particular, to the high monounsaturated-to-polyunsaturated ratio, and the presence of considerable amounts of minor compounds that play a major role in preventing oxidation^{2, 3)}. In the study of the VOO composition they have been classically distinguished two fractions: saponifiable, which is the major part (98.5-99.5%) and it is mainly constituted by triacylglycerols, free fatty acids and phospholipids; and the unsaponifiable (0.5-1.5%), minor fraction that can be divided into two groups; the first group consists of fatty acids de-

rivatives and the second group includes compounds that are not directly related with the fatty acids, such as hydrocarbons, aliphatic alcohols, free sterols, tocopherols, chlorophylls, carotenoids, and phenolic compounds⁴⁾. Within the phenolic compounds present in VOO, both hydrophilic and lipophilic phenols, as tocopherols, are included. In VOO, the last, are mainly represented by the α -tocopherol (95%), whereas there are only small amounts of β and γ -tocopherols. In the rest of vegetable oils they can be found in different proportions⁵⁾. Regarding to the hydrophilic phenols, referred as phenolic compounds hereinafter, they are not present in the other vegetable oils, because they are lost during the different stages of the refining process⁶⁾.

Depending on the minor components that are present and their proportion, a wide variety of VOO can be found, with different biological value. This way, these minor compounds are important because of their nutritional and sen-

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social interest and their ability to prevent diseases that may be related to oxidative damages such as coronary heart diseases, stroke, and certain types of cancers^{7, 8)}. They protect the low density proteins (LDL) against oxidation⁹⁾ because they act as antioxidants against reactive species¹⁰⁾ at different levels in the oxidative sequence involving lipid molecules. Therefore, the biology and therapeutic value of olive oil is directly related to its chemical composition. In most cases, health and dietary benefits induced by consumption of VOO has been related to its several minor constituents such as vitamins, phytosterols, pigments, terpenic acids, squalene and phenolic compounds^{11, 12)}.

Although these compounds are found at levels of mg/kg, and even lowers, in the minority fraction of VOO, many of them are greatly responsible not only for its healthy properties but also for the high oxidative stability of this oil and its desirable organoleptic characteristics and nutritional qualities in comparison with the rest of edible vegetable oils^{10, 13, 14)}. It must be taken into consideration that the distinctive aroma of virgin olive oil is attributed to a large number of chemical compounds of different chemical classes, i.e., aldehydes, alcohols, esters, hydrocarbons, ketones and probably, to other unidentified volatile compounds^{15, 16)}, but phenolic compounds in the AOV greatly influence also the sensory properties of the same. In particular, these compounds have been associated with the bitter and astringent taste^{8, 17)}. On the other hand, and in spite of its high stability, virgin olive oil is also susceptible to lipid oxidative processes, enzymatic oxidation, photo-oxidation and autoxidation. During these reactions, a series of compounds are formed in virgin olive oil (VOO), while minor components are degraded. The secondary oxidation products have an unpleasant flavor and odor and may adversely affect the nutritional value of the oil, causing finally consumer rejection. It has been found that phenolic compounds and carotenoids decrease autoxidation in oil, while tocopherols, chlorophylls and phospholipids demonstrate both antioxidant and prooxidant activity depending on the oil system and storage conditions¹⁸⁾. However, the vitamers of vitamin E are in general important natural antioxidants in foods because they have the ability to block the propagation of radical reactions although the different vitamers vary widely in activity. The oil antioxidant content in VOO depends on the cultivar, fruit ripening stage, agroclimatic conditions and olive growing techniques^{19, 20)}, and provides information about the oil's oxidative status^{21, 22)}.

According to the above, it is clear the interest in the better knowledge of phenolics and tocopherols profiles of the different VOOs, since it could increase their competitiveness in the sector. For that reason, the aim of this work has been to the characterization of seven varieties of olive most representative of the southwest of Spain (*Arbequina*, *Carrasqueña*, *Corniche*, *Manzanilla Cacereña*, *Morisca*, *Picual*, and *Verdial de Badajoz*), with regards to its phe-

nolic compounds and tocopherols profiles at different stage of maturation.

2 MATERIALS AND METHODS

2.1 Samples

The study was carried out in an experimental olive (*Olea europaea* L.) cultivar maintained by the Researcher Center "Finca La Orden" (Badajoz, Spain) within the limits of the olive-growing area "Tierra de Barros" during the olive season 2011/12. The UTM coordinates of cultivar were x = 702192.29, y = 4303406.71. The climate of the area is Mediterranean; the average annual rainfall was 404 mm, mostly distributed outside of a 4-month summer drought period. The olive orchard was composed of fifteen-years-old olive trees (plantation frame 6 × 6 m²) of seven varieties predominate in the southwest of Spain: *Arbequina*, *Carrasqueña*, *Corniche*, *Manzanilla Cacereña*, *Manzanilla Sevillana*, *Picual*, and *Verdial de Badajoz*. The soil at the experimental orchard was a sandy loam (depth 2 m). The orchard was managed under drip irrigation with linear irrigation scheduling, of 3582 cm³ water/ha (15th May to 18th November) and no tillage conditions; weeds were controlled with post-emergence herbicides. Note that the fact of getting all the test samples from the same experimental olive grow, allowed to study the effect of variables "variety" and "maturity stage" on the minor components of fruits developed in the same geographic area, under the same agronomic and pedoclimatic conditions. In our experiments we used a statistical design which is based in randomized blocks with three replicates for each variety. Each elementary block consisted on three olive trees. We studied the effect of different olive varieties in analytical parameters, and also the evolution of these parameters along the stage of maturation. A total of 63 samples of olives were handpicked, in perfect sanitary conditions. This samples were collected from November to January in three stages of maturation, ripeness index of (0-2), (2-3) and (4-7), (green, spotted and ripe respectively), using the subjective evaluation of color of the skin and flesh, as proposed by Uceda and Frías²³⁾. The olive samplings were carried out in the morning, taking samples randomly in different parts of the central area of the olive tree, assuming a total of 6 kg per variety and maturity. After harvesting, the olive fruit samples were immediately transported to the laboratory in ventilated storage trays to avoid compositional changes. The oil was extracted within 24 h.

Oil extraction was carried out within 24 h from harvest in similar industrial extraction conditions using an Abencor analyzer (MC2 Ingeniery systems, Sevilla, Spain)²⁴⁾. Olives were crushed with a hammer mill and were slowly mixed for 30 min at 25°C. Then, the obtained paste was centrifuged at 3500 rpm over 3 min. All the oil samples were sep-

arated by decantation and were stored away from the light in amber-colored glass bottles at 4°C until analysis (within 1 month).

2.2 Chemicals and Reagents

Folin-Ciocalteu reagent, caffeic acid, and sodium carbonate used for polyphenolic content determination were provided by Panreac (Barcelona, Spain). Standards of α , β , γ and δ tocopherol, and HPLC mobile phase (98.5% hexane + 1% isopropanol + 0.5% ethanol) were provided by Calbiochem (U.S. and Canada) and Panreac respectively.

2.3 Instrumental

An UV-Vis spectrophotometer model HP8453 (Agilent, Madrid, Spain) was used for total polyphenols. Tocopherols contents were determined with an HPLC system (Agilent 1100 series, Madrid, Spain) equipped with a fluorescence detector and a Lichrosorb Si60 analytical column (25 cm $\times$ 4.6 mm, 5 μ m) (Merck).

2.4 Analytical methods

2.4.1 Determination of total polyphenol content

Total polyphenol content in virgin olive oil was determined according to the Folin-Ciocalteu colorimetric method, using caffeic acid as a standard²⁵. Briefly, extraction of polyphenols from olive oil samples was achieved by dissolving 10 g of oil in 75 mL of hexane, followed by liquid-liquid extraction using a methanol/water (40:60 v/v) mixture completed to 100 mL with distilled deionized water. A 2 mL aliquot of the methanolic extract was mixed with 1 mL Folin-Ciocalteu reagent, 2 mL saturated sodium carbonate and 15 mL of distilled water. After 1 h, absorbance at 725 nm was read against a blank similarly prepared. Each sample was prepared in duplicate and quantification was carried out on the basis of the external standard calibration curve of caffeic acid whose linearity ranged from 5 to 75 mg/L. Results of polyphenol content in the olive virgin oil samples are expressed as caffeic acid equivalent (CAE), in mg/kg oil.

2.4.2 Determination of tocopherols

Tocopherols were analysed by the IUPAC 2.432 standard method, slightly modified²⁶. The identification and quantification were carried out with Agilent 1200 liquid chromatograph, equipped with fluorescence detector with the excitation wavelength set at 290 nm and the emission wavelength at 330 nm was used. The analytical column was a Lichrosorb Si60 (250 $\times$ 4.60 mm i.d., 5 μ m). 2.5 g of oil were dissolved in 5 mL of n-hexane. The chromatographic separation was carried out for isocratic form using a mobile phase composed of a mixture of n-hexane: isopropanol: ethanol (98.5:1.0:0.5, v/v) at a flow rate of 1 mL/min. The volume of injection and the temperature of the column were of 10 μ L and 30°C respectively. The different tocopherols were identified by comparing retention times with

those of commercial standards (Merck, Spain). Results are expressed as mg/kg of oil for each tocopherols.

2.4.3 Oxidative stability

The oxidative stability of the examined oils was assessed in a 743 Rancimat (Metrohm, Herisau, Switzerland) eight-channel oxidative stability instrument. Briefly, 2.5 g of oil were placed in each of the Rancimat channels. A continuous air stream was forced through the oil solution to 10 L/h, and the system temperature was established at 100°C²⁷. Changes in conductivity caused by lost of volatile organic acids, mainly formic acid, were measured automatically and continuously. The peroxidation curve was recorded, and the inflection point was selected as the induction time (IT, expressed in hours). The higher the IT value, the higher the oxidative stability of the sample is.

2.4.4 Statistical analysis

The results are expressed as mean values and standard deviations. The data were statistically analyzed by ANOVA and Duncan's multiple range tests. Statistical significance was accepted at a level of $p < 0.05$. For variance analysis (ANOVA) and linear regression analyses the SPSS 18.0 software (SPSS Inc., Chicago, IL, USA) was used.

3 RESULTS AND DISCUSSION

3.1 Total phenol compounds in VOOs

The results obtained in the evaluation of the total phenolic content, according the above described procedure, of the oils obtained from olives of the various varieties, and picked up at different stages of maturation, are shown in **Table 2**. In general terms, the amount of total phenol contents showed significant differences ($p < 0.05$) among the different varieties. The range of values of total phenolic compounds obtained in all varieties from Extremadura varied from 130 to 1203 mg/kg. The lowest values found corresponds to Morisca variety which concentration was from 120 to 537 mg/kg, while Carrasqueña variety showed the greater concentration in phenolic compounds with values from 461 to 1203 mg/kg. The highest contents in phenolic compounds were presented in Carrasqueña variety at green stage of ripeness while the lowest contents were observed in Arbequina, Manzanilla Cacereña, Morisca, and Verdial de Badajoz varieties. Intermediate contents were observed in Picual and Corniche varieties.

If these results are observed, according to the classification established by Tous *et al.*²⁸ on the basis of the total polyphenol contents, it can be deduced that the oils obtained from Arbequina and Verdial de Badajoz varieties at the ripe stage of maturation are within the category of "low content in polyphenols", because they showed concentrations lower than 200 mg/kg; a great proportion of oils from the various varieties at the ripe or spotted stage corresponds to the category of "intermediate content in poly-

phenols” because they presented concentration between 200-350 mg/kg. However, oils obtained with olives at the green stage are mostly in the “high content in polyphenols” category because they presented concentration between 350-550 mg/kg or even higher. It is noticeable the high polyphenol content of oils from *Carrasqueña* olives and, to a less extent, in *Corniche*, and *Manzanilla Cacereña*.

Aparicio *et al.*²⁹⁾ and Pardo *et al.*³⁰⁾ reported that the phenolic content in VOOs of *Cornicabra*, and *Picual* varieties were the highest of all Spanish varieties. From our results, it can be deduced that *Picual* can indeed be one of the varieties with higher polyphenols content when comparing oils resulting of spotted olives. Thus, Sánchez *et al.*³¹⁾ and Fuentes *et al.*³²⁾ found significant differences in oils from the same varieties of olives processed through Abencor system. On the other hand, reported results for other varieties from Spain or other countries show considerably lower polyphenols content. Pérez-Arquillué *et al.*³³⁾ found values between 160-169 mg/kg for the Empeltre variety in the “bajo Aragón”. Gutiérrez *et al.*³⁴⁾ studied Andalusian oils and they showed a mean value of polyphenols of 181mg/kg with a great variability depending on the year and the olive variety. Dugo *et al.*³⁵⁾ studied Italian varieties and they showed that oils with highest contents in phenolic compounds were from *Nocellara del Belice* and *Cerasuola* varieties with values of 277 and 328 mg/kg. However, *Biancolilla* and *Tonda Oblea* varieties showed an intermediate values in phenolic compounds, with values of 133mg/kg and 137mg/kg respectively.

The amount of phenolic compounds in VOOs is an important factor when we assess its quality, informing that natural phenolic compounds are able to improve the oxidation resistance⁸⁾. In this sense, the analysed oils can be similar to some Tunisian varieties like *Betsijina*, *Chétoui*, and *Oueslali* that showed a high content in total phenolic compound (>1000mg/kg)^{36, 37)}. This is a desirable charac-

teristic because of the beneficial effects of these components on human health³⁷⁾.

On the other hand, a decrease of phenolic contents, expressed as milligrams of caffeic acid per kilogram of oil, during ripening was observed in general for all varieties analyzed (Table 2). This decreasing trend was more pronounced in *Carrasqueña* and *Manzanilla Cacereña* varieties because they decreased significantly from green to spotted. These varieties lost almost 50% of phenolic compounds when were obtained from oils in the spotted stage of ripeness. Nevertheless, the phenolic compounds content was constant from spotted to ripe stage. *Arbequina* variety showed the same evolution but with less marked decrease in concentration of phenolic compounds. *Corniche* and *Picual* varieties phenolic contents appreciably decreased in all stages of maturation, while for *Morisca* variety significantly increased from spotted to ripe stages. Previous studies have described a general decrease of total phenolic compounds as fruit ripening advances, while different trends of specific compounds were observed³⁵⁾. In the opposite sense, Baccouri *et al.*³⁸⁾ showed that total phenolic compounds analysed by HPLC increased progressively throughout the ripening period until reaching a maximum in the ripening index between 3 and 4, subsequently decreasing. These results are in agreement with those observed in *Cornicabra* variety³⁹⁾. However, Oueslali *et al.*⁴⁰⁾ did not find clear relation between the content of phenolic compounds and the ripening index.

3.2 Tocopherols fraction in VOOs

A calibration with external standard method was used for the quantification of the tocopherols. For this purpose, calibration curves were obtained from standard solutions α , β , γ , and δ tocopherols in n-hexane of accurately known concentrations. The quantification of each tocopherol was obtained by plotting the peak area vs concentration for each standard tocopherols tocopherols. In Fig. 1 the chro-

Fig. 1 Chromatograms of the standards and one of the analyses samples. (I) α -tocopherol; (II) β -tocopherol; (III) γ -tocopherol and (IV) sample.

Table 1 Analytical figures of merit.

Parameters	α -Tocopherol	β -Tocopherol	γ -Tocopherol	δ -Tocopherol
Linear regression	$y=11.4x+91$	$y=17.5x+1.3$	$y=17.4x-2.61$	$y=21.5x-6.7$
Determination coefficient (r^2)	0.995	0.995	0.985	0.999
% Linearity	96.8	96.3	97.6	98.6
Sy/x	48.8	2.4	5.5	5.6
LOD ^a (mg/L _{injected sol} & mg/kg _{oil})	12 & 24	0.29 & 0.58	0.70 & 1.4	0.35 & 0.70
LOD ^b (mg/L _{injected sol} & mg/kg _{oil})	18 & 36	0.51 & 1.0	1.2 & 2.4	0.72 & 1.4
LOQ ^c (mg/L _{injected sol} & mg/kg _{oil})	39 & 78	0.97 & 1.9	2.3 & 4.6	1.2 & 2.4

^a Limit of detection, Long and Winefordner method³²⁾^b Limit of detection, Clayton *et al.* method³³⁾ ($\alpha = \beta = 0.05$)^c Limit of quantification, from LOD (Long and Winefordner method) $\times 3.33$ **Table 2** Total phenolic compounds, total tocopherols and oxidative stability of seven varieties of VOOs in three stages of ripening.

Ripening index	Variety	Total phenolic compound (mg CAE/kg)•10	Tocopherols				Total Tocopherol (mg/kg)•10	Oxidative Stability (hours)
			α -Tocopherol (mg/kg)•10	β -Tocopherol (mg/kg)	γ -Tocopherol (mg/kg)	δ -Tocopherol (mg/kg)		
Green	Arbequina	40.9 $\pm$ 5.4 ^{aB}	37.1 $\pm$ 1.6 ^{bB}	2.10 $\pm$ 0.10 ^{ab}	nq	nd	37.3 $\pm$ 1.6 ^{bB}	68.5 $\pm$ 4.4 ^{abB}
	Carrasqueña	120.3 $\pm$ 15.3 ^{dB}	28.8 $\pm$ 5.6 ^{aB}	1.96 $\pm$ 0.21 ^a	19.9 $\pm$ 1.76 ^{dB}	nd	31.0 $\pm$ 5.8 ^{aB}	163 $\pm$ 25 ^{dB}
	Corniche	70.8 $\pm$ 9.6 ^{cC}	36.3 $\pm$ 3.0 ^{bC}	3.07 $\pm$ 0.34 ^{dNS}	nq	nd	36.6 $\pm$ 3.1 ^{abC}	171 $\pm$ 13 ^{dC}
	Manzanilla Cacereña	70.7 $\pm$ 7.3 ^{cB}	33.3 $\pm$ 3.1 ^{abB}	2.79 $\pm$ 0.29 ^{cdB}	nq	nd	33.6 $\pm$ 3.1 ^{abB}	140.0 $\pm$ 7.7 ^{cB}
	Morisca	53.7 $\pm$ 7.7 ^{abC}	48.5 $\pm$ 2.7 ^{cC}	3.80 $\pm$ 0.15 ^{eC}	12.3 $\pm$ 1.58 ^{bA}	nd	50.1 $\pm$ 2.6 ^{cC}	54.7 $\pm$ 7.2 ^{aC}
	Picual	67.1 $\pm$ 3.4 ^{bcC}	34.6 $\pm$ 2.0 ^{abC}	3.62 $\pm$ 0.21 ^{eB}	5.31 $\pm$ 1.61 ^{aA}	nd	35.5 $\pm$ 1.9 ^{abC}	151.9 $\pm$ 4.8 ^{cdB}
	Verdial de Badajoz	55.9 $\pm$ 7.9 ^{abcB}	29.2 $\pm$ 2.3 ^{aB}	2.51 $\pm$ 0.12 ^{bcB}	16.2 $\pm$ 1.54 ^{cB}	nd	31.1 $\pm$ 2.2 ^{aB}	47.6 $\pm$ 2.1 ^{aB}
Spotted	Arbequina	23.4 $\pm$ 2.4 ^{aA}	21.4 $\pm$ 0.6 ^{aA}	nq	nq	nd	21.40 $\pm$ 0.55 ^{aA}	49.0 $\pm$ 1.0 ^{aA}
	Carrasqueña	62.9 $\pm$ 16.2 ^{bA}	19.4 $\pm$ 2.6 ^{aA}	nq	8.59 $\pm$ 2.28 ^{bA}	nd	20.3 $\pm$ 2.8 ^{aA}	94 $\pm$ 2.5 ^{bA}
	Corniche	49.7 $\pm$ 5.5 ^{bB}	30.2 $\pm$ 3.6 ^{cB}	2.76 $\pm$ 0.33 ^b	10.6 $\pm$ 1.38 ^{bcB}	nd	31.5 $\pm$ 3.5 ^{bB}	150 $\pm$ 10 ^{cB}
	Manzanilla Cacereña	21.7 $\pm$ 13.2 ^{aA}	23.6 $\pm$ 4.1 ^{abA}	2.06 $\pm$ 0.33 ^{aA}	4.67 $\pm$ 1.17 ^{aA}	nd	24.3 $\pm$ 4.1 ^{aA}	80 $\pm$ 10 ^{bA}
	Morisca	13.0 $\pm$ 3.9 ^{aA}	30.7 $\pm$ 0.4 ^{cB}	2.43 $\pm$ 0.21 ^{abA}	13.6 $\pm$ 1.95 ^{cdA}	nd	32.30 $\pm$ 0.21 ^{bB}	28.3 $\pm$ 4.3 ^{aA}
	Picual	59.3 $\pm$ 2.2 ^{bB}	27.7 $\pm$ 0.8 ^{bcB}	2.37 $\pm$ 0.03 ^{aA}	15.5 $\pm$ 1.96 ^{dC}	nd	29.50 $\pm$ 0.65 ^{bB}	148.3 $\pm$ 2.8 ^{cB}
	Verdial de Badajoz	24.5 $\pm$ 5.2 ^{aA}	21.7 $\pm$ 0.9 ^{aA}	2.18 $\pm$ 0.05 ^{aA}	15.3 $\pm$ 2.27 ^{dB}	nd	23.5 $\pm$ 1.0 ^{aA}	27.9 $\pm$ 5.8 ^{aA}
Ripe	Arbequina	16.0 $\pm$ 3.2 ^{aA}	21.3 $\pm$ 1.3 ^{bcA}	nq	nq	nd	21.3 $\pm$ 1.3 ^{bA}	45.5 $\pm$ 3.9 ^{bcA}
	Carrasqueña	46.1 $\pm$ 19.6 ^{cA}	17.0 $\pm$ 1.9 ^{aA}	nq	4.72 $\pm$ 3.03 ^{aA}	nd	17.5 $\pm$ 2.1 ^{aA}	84 $\pm$ 17 ^{eA}
	Corniche	29.9 $\pm$ 3.8 ^{abA}	20.5 $\pm$ 0.6 ^{bA}	nq	7.14 $\pm$ 1.22 ^{abA}	nd	21.21 $\pm$ 0.59 ^{bA}	113.7 $\pm$ 3.2 ^{fA}
	Manzanilla Cacereña	20.1 $\pm$ 8.6 ^{aA}	24.3 $\pm$ 2.0 ^{dA}	2.23 $\pm$ 0.14 ^{aA}	7.62 $\pm$ 0.63 ^{abB}	nd	25.3 $\pm$ 1.9 ^{cA}	70 $\pm$ 13 ^{deA}
	Morisca	23.2 $\pm$ 0.7 ^{aB}	24.4 $\pm$ 1.5 ^{dA}	2.93 $\pm$ 0.21 ^{bB}	26.9 $\pm$ 2.84 ^{dB}	nd	27.4 $\pm$ 1.8 ^{cA}	30.6 $\pm$ 2.7 ^{abB}
	Picual	41.9 $\pm$ 0.5 ^{bcA}	14.7 $\pm$ 1.6 ^{aA}	nq	12.4 $\pm$ 0.70 ^{cB}	nd	15.9 $\pm$ 1.5 ^{aA}	123.4 $\pm$ 4.7 ^{fA}
	Verdial de Badajoz	18.3 $\pm$ 4.9 ^{aA}	19.6 $\pm$ 1.0 ^{bA}	nq	9.8 $\pm$ 1.26 ^{bcA}	nd	20.58 $\pm$ 0.84 ^{bA}	24.4 $\pm$ 5.1 ^{aA}

CAE, caffeic acid equivalents. Results are expressed as mean $\pm$ SD of three sample replicates. Different small letters in the same row indicate significant statistical differences (Duncan's Test, $p < 0.05$) among varieties. Different capital letters in the same column indicate significant statistical differences (Duncan's Test, $p < 0.05$) during the stage of maturation. nd, not detected. nq, unquantifiable.

matograms of one of the standards and one of the analyses samples are shown as example. The analytical figures of merit corresponding to this method are in **Table 1**. A linear trend was observed between the concentrations of these compounds and the analytical signals.

The limits of detection (LODs) obtained ranged between 0.26 and 12 mg/L (0.52 and 24 mg/kg oil, according the procedure of sample preparation) by Long-Winefordner method⁴¹⁾ and between 0.51 and 18 mg/L (1.0 and 36 mg/kg oil) by Clayton method⁴²⁾. The limits of quantification (LOQs), calculated as LOD (Long and Winefordner) $\times 3.33$, ranged from 0.98 to 39 mg/L (1.7 and 79 mg/kg oil). The LODs were calculated by the ACOC software package⁴³⁾.

The tocopherols found in the analyzed VOOs were α , β , and γ tocopherols (**Table 2**). Even though these oils come from different cultivars, the results herein reported are in good agreement with other results previously reported for tocopherol contents in VOOs^{38, 44–46)}. Thus, α -tocopherol was the most abundant in the seven examined varieties, representing around 96.0% of the total while γ -tocopherol content was always greater than β -tocopherol. There are several references about the presence of different levels of δ -tocopherol in olive oils from various countries^{47, 48, 59)}. However, the oils analysed in this study did not content δ -tocopherol above the LOD (0.35 mg/L) (0.7 mg/kg oil).

As shown in **Table 2**, α -tocopherol shows significant dif-

ferences between cultivars ($p < 0.05$). In fact, α -tocopherol concentration ranges (485 - 244) mg/kg in *Morisca* variety to (288 - 170) mg/kg in *Carrasqueña* variety. Therefore, these results suggest that tocopherol contents are highly variety-dependent, accordingly with others referenced^{37, 49, 60}. In literature different values of α -tocopherol level, in general in the order of some tens to three hundred mg/kg can be found^{45, 49, 60, 65}. For example, compared to Italian olive oils, Spanish oils have α -tocopherol levels that are among the highest reported (160-253 mg/kg)^{50, 61}.

In terms of content in β -tocopherol, we found concentrations ranged from 1.96 to 3.80 mg/kg in all varieties, while levels of γ -tocopherol ranged from 4.7 -19.9 mg/kg in *Carrasqueña* variety and from 12.3 to 26.9 mg/kg in *Morisca* variety. García *et al.*⁵¹ found β and γ tocopherols levels similar to those described herein in olive oils from different Spanish cultivars (*Picual*, *Hojiblanca*, *Arbequina*, and *Cornicabra* varieties, all acquired in Spanish markets) and others reported slightly higher values⁴⁶. The quantitative differences verified by these researcher can be explained by several reasons such as cultivar employed, ripening stage, agroclimatic conditions, olive-growing techniques, and storage conditions⁴⁶. In this sense, Beltran *et al.* have found variability in the tocopherol contents in oils from 29 olive cultivars, related to crop year, due to the rainfall levels, being oils from the drier crop year which showed the highest tocopherol content. This effect was also cultivar-dependent, and, on the other hand, tocopherols generally decreased during the ripening process although γ -tocopherol showed an increase for the last harvesting dates that was related to the chlorophyll losses in the oil⁶³.

Regarding to the influence of the stage of maturation of fruit over the tocopherols content of oil, α -tocopherol contents presented a declining trend, in general, as the olive ripening advances (Table 2), as described by others⁶². As it was already mentioned, according these results α -tocopherol contents showed significant differences among cultivars. However, all the varieties were above 280, 190, and 140 mg/kg of α -tocopherol in green, spotted and ripe stage of maturation respectively, presenting concentrations greater than oils from Algerian⁵² but with similar values found from Tunisian oils^{36, 64} or European oils⁵⁰. Deiana *et al.*⁴⁹ indicated, in agreement with our results, that α -tocopherol contents varied from 139.4 (*Sredki* variety) to 402.2 mg/kg (*Chladmi* variety). Giacometti and Milin⁵³ reported contents of α -tocopherol from 1.49 to 33.7 mg/100g in VOOs from Northern Adriatic region of Croatia. It is interesting to emphasize the high level of α -tocopherol in oils from *Morisca* variety in the three stages of maturation. Besides, the highest loss of α -tocopherol contents (about 40 %) was observed in *Morisca* variety from green to spotted stage followed by the decrease observed in *Picual* variety (about 15%). This last variety was one with lower α -tocopherol content in

ripe stage of ripening, and the same happened with total tocopherols content. Similar concentrations in this stage were found in *Carrasqueña* variety which presented lower concentration at the three stages of maturation. In *Manzanilla Cacereña* variety α -tocopherol content decreased from green to spotted stage and, besides, *Manzanilla Cacereña* variety, together with *Morisca* variety, presented the greatest concentration of α -tocopherol in ripe stage of maturation.

The total tocopherols contents presented the same trend as the olive ripening advances that α -tocopherol content from all studied varieties from Extremadura. It is obvious because, as we indicated previously, α -tocopherol represented almost the 95% of these compounds and their contribution to the total of tocopherols is greater than for the rest of tocopherols. We can highlight contents of (501 - 274) mg/kg and (310 - 175) mg/kg in *Morisca* and *Carrasqueña* varieties respectively. In the literature there are also referenced different tocopherols levels for other oil varieties⁴⁵. All the varieties were significantly decreasing in tocopherols content from green to spotted stage. The values were constant in *Arbequina*, *Carrasqueña*, *Manzanilla Cacereña*, and *Verdial de Badajoz* varieties from spotted to ripe stage of ripening. The rest of varieties were continuously decreasing until ripe stage of ripening. Finally, we can observe that *Morisca* variety presented the highest concentration of total tocopherols in the three stage of maturation.

Relating to the other tocopherols, a similar behaviour happened for β -tocopherol (Table 2). In terms of the decrease in concentration of β -tocopherol with the maturation of the fruit, we can observe that this decrease in *Corniche* variety was no significant of green to spotted stage. From the spotted stage until ripe, the results did not show a defined clear trend because the content in β -tocopherol was constant in *Manzanilla Cacereña* variety, while increased for *Morisca* variety. We can observe that *Morisca* variety decreased more sharply from green to spotted stage than the rest of varieties. In terms of the concentration of β -tocopherol, the examined varieties can be classified into three groups in green stage of maturation. The first, with the highest concentration of this compound, would correspond to *Morisca* and *Picual* varieties with 3.80 and 3.62 mg/kg respectively. The second group would correspond to *Corniche*, *Manzanilla Cacereña*, and *Verdial de Badajoz* varieties with intermediate contents (2.79 mg/kg average value) that they are significantly different from the rest of varieties. Finally, the third group would be *Arbequina* and *Carrasqueña* varieties in the green stage of maturation with significantly lower contents in β -tocopherol of 2.10 and 1.96 mg/kg respectively. On the other hand, the contents in β -tocopherol in oils from fruits at the spotted stage of maturation were similar between varieties. We can note that β -tocopherol in *Arbequina* and

Carrasqueña varieties are below LOQs ($< 0.97 \text{ mg/l}$). In this sense, it has not been possible to quantify the β -tocopherol in ripe stage of maturation in *Arbequina*, *Carrasqueña*, *Corniche*, *Picual*, and *Verdial de Badajoz* varieties. Previous referenced data confirmed that the olive ripening stage influence on the content of tocopherols, being those obtained from green olives richest in this type of compounds than those obtained from ripe olives^{48, 62}. On the other hand, the values of γ -tocopherol were below LOQ in *Arbequina* variety in all stage of maturation, while in *Corniche* and *Manzanilla Cacereña* varieties it has not been quantified in green stage of maturation. However, γ -tocopherol did not show a definite trend as the olive ripening advances. In this sense, the reported results about content of each one of the tocopherols are diverse. Gutiérrez *et al.*⁵⁴ showed a clear decrease of the content in α -tocopherol during the ripening process in *Picual*, *Verdial*, and *Villalonga* varieties. Baccouri *et al.*³⁸ observed that the content of α -tocopherol remained virtually constant until ripeness index of 3.5, decreasing until the last stage of maturity in *Chemlali* variety. These same authors observed, in oils of *Chitoui* variety without any application of irrigation, that α -tocopherol levels did not show a clear trend during the maturation of the olives. However with the same variety, these authors observed that with irrigation, the content of α -tocopherol decrease slightly throughout the ripening. Gutiérrez *et al.*⁵⁴ observed similar behaviour in *Hojiblanca* variety. Salvador *et al.*³⁹ found that α -tocopherol content varied slightly during the ripening in *Cornicabra* variety, but it did not show a clear trend. On the contrary, several researchers were unable to show a clear relationship between the content in α -tocopherol and the degree of ripeness of the olives⁴⁰. In this sense, Baccouri *et al.*³⁸ observed that the content of β and γ tocopherol did not show a clear trend as the olive ripening advances. This behaviour has been found by other cultivars⁵⁴.

3.3 Contribution of phenolic compound and tocopherol to oxidative stability in VOOs

It is interesting to evaluate the relationship between the two examined fractions of phenolic compounds with the oxidative stability of these VOO varieties since previous literature supports that α -tocopherol content together with the phenolic compounds contributes to the stability of the oil^{38, 44}.

In relation with the total phenolic content, it correlated well with the oxidative stability (oxidation induction period with Rancimat equipment) of the oils obtained using Abencor system from the seven Extremadura varieties, being $r^2 > 0.90$ ($p < 0.05$) between both parameters over the maturity of the olives.

This agrees with other reported data. Hence, some researchers⁵⁵ found a high correlation between total pheno-

lic content and oxidative stability in *Arbequina*, *Arbosana*, and *Koreneiki* varieties ($r^2 = 0.80$, $p < 0.05$). Therefore, the olive oils obtained with high content phenolic compounds presented a greater oxidative stability. It is important to evaluate the stability of the oil during the maturation. In the case of the oils examined here, the highest correlation between these two parameters was observed in *Carrasqueña* and *Verdial de Badajoz* varieties ($r^2 > 0.95$; $p < 0.05$). Other authors^{30, 32, 33, 40} observed a good correlation (0.78–0.90) between oxidative stability and phenolic compound in VOO from different varieties and places. Besides, these researchers suggested that higher degrees of correlation could be obtained if the oils were from homogeneous groups, either by their origin or extraction method, and probably, if instead of globally assess all polyphenols, only those that present activity as antioxidants were evaluated.

About the relationship between tocopherols and oxidative stability, the results of this study confirmed that α , β , and total tocopherols presented a high positive correlation with oxidative stability in the seven studies varieties ($r^2 > 0.85$, $r^2 > 0.70$, $r^2 > 0.87$ respectively; $p < 0.05$). A clear relation between γ -tocopherol content and oxidative stability could not be established because while *Carrasqueña* and *Verdial de Badajoz* varieties showed a positive correlation between both, the rest of varieties show a negative correlation, being the higher values for *Carrasqueña* ($r^2 = 0.92$; $p < 0.05$), and *Manzanilla Cacereña* ($r^2 = 0.81$; $p < 0.05$) varieties, and low for the rest of varieties ($r^2 < 0.30$; $p < 0.05$).

Again, these results agree with other previous findings. In this sense, Allalout *et al.*⁵⁵ indicated that α -tocopherol presented a high correlation with oxidative stability ($r^2 = 0.90$; $p < 0.001$). Baccouri *et al.*³⁸ showed the contribution of content in α -tocopherol to oxidative stability for *Chétoui* and *Chemlali* tunisian varieties with correlation values ($r^2 = 0.76$ and 0.78 ; $p < 0.05$), although less important than for phenolic compounds. Similar results have been found for other oils of different countries²⁹, but not for all of them³⁷. Deiana *et al.*⁴⁹ and Mateos *et al.*⁴⁴ indicated that tocopherols seem to have a synergic effect in association with some phenolic compounds with significant antioxidant activity and α -tocopherol content becomes effective when the activity of the fraction of polar compounds is reduced and the primary products of autooxidation reach the critical concentration. According to this suggestion, the evaluation of the correlation between oxidative stability and α -tocopherol plus total phenolic compounds content seemed of interest. The results obtained are shown in Fig. 2. It can be observed a high positive linear correlation for the seven varieties studied ($r^2 > 0.87$ $p < 0.05$). It must be emphasized that those varieties that had higher total polyphenol contents are not those that have higher concentration of α -tocopherol. Hence, the *Carrasqueña* variety which presented the highest concentration of total phenolic compound in green stage of ripening, 1203 mg/kg, also it

Fig. 2 Linear correlation between oxidative stability and total phenolic compounds in seven Extremadura varieties in VOOs produced with Abencor system.

had the lowest concentration of α -tocopherol, 288 mg/kg (80 and 20% the phenolic fraction respectively). However, this variety is one of the seven varieties with higher oxidative stability (163 hours Rancimat). It is obvious to think that the greatest contribution to the oxidative stability of VOO was provided by phenolic fraction and although α -tocopherol fraction contributes also to this stability it is not the primarily responsible for oxidative stability. In this sense, recently, some authors⁵⁶⁾ studied the levels of contribution of different olive oil constituents on stability and they reported that phenols appear to have the highest influence. Similar results were obtained by Manai-Djebali *et al.*³⁷⁾. They analysed five tunisian varieties and they observed that Betsijina variety which the highest oxidative stability (110 hours Rancimat) was that presented the greatest concentration of phenolic compounds (1400 ppm) and the greatest concentration of α -tocopherol (238 ppm). Several researchers⁵⁷⁾ indicated that part of the phenolic fraction exercised greater protective activity in the early stages of oxidation of the olive oil, against the content of α -tocopherol, overall when the content in primary products of oxidation were relatively low. In the same line, other authors⁵⁸⁾ have shown that phenolic compounds are more effective than tocopherols in the improvement of stability of the olive oil towards oxidation.

We conclude that the elevated presence of polyphenols and tocopherols in some of the VOO varieties from Extremadura suggests that these compounds are highly variety-dependent and allows the characterization of olive oils from the seven varieties in terms of the concentration of

these compounds. The data could be used to define the ideal ripening degree to confer a good balance between chemical profiles, together with the best oxidative shelf-life of the extra virgin olive oils. Also, it is noticeable that *Carrasqueña* variety has highest mean values of total phenols and oxidative stability that can be used to improve the shelf life of other varieties by blending oils. A good correlation was found between total phenolics content, α -tocopherols, and total tocopherols, and the oxidative stability in the oils obtained from the seven Extremadura varieties, studied along the maturity of the fruit, indicating that phenolic compounds contribute significantly to the inhibition of the lipidic oxidation.

ABBREVIATIONS

VOO, virgin olive oil; EVOO, extra virgin olive oil; LOD, limit of detection; LOQ, limit of quantification; CAE, caffeic acid equivalent; IT, induction time.

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