Phenolic Compounds in Spanish Olive Oils

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Phenolic compounds in Spanish virgin olive oils were characterized by HPLC. Simple phenols such as hydroxytyrosol, tyrosol, vanillic acid, *p*-coumaric acid, ferulic acid, and vanillin were found in most of the oils. The flavonoids apigenin and luteolin were also found in most of the oils. The dialdehydic form of elenolic acid linked to tyrosol and hydroxytyrosol was also detected, as were oleuropein and ligstroside aglycons. The structure of a new compound was elucidated by MS and NMR as being that of 4-(acetoxyethyl)-1,2-dihydroxybenzene. Changes of phenolic compounds in virgin olive oils with maturation of fruits were also studied. Hydroxytyrosol, tyrosol, and luteolin increased their concentration in oils with maturation of fruits. On the contrary, glucoside aglycons diminished their concentration with maturation. No clear tendency was observed for the rest of the phenolic compounds identified.

Keywords: *Olive oil; phenolic; 4-(acetoxyethyl)-1,2-dihydroxybenzene; maturation*

INTRODUCTION

The world production of olive oil is ~2.5 million tons, and the major producers are Spain, Italy, Greece, and Maghrebian countries. Half of the Spanish production is obtained from the Picual cultivar and the rest from Hojiblanca, Picudo, Cornicabra, Arbequina, Lechín, etc.

Phenolic compounds constitute an important group of naturally occurring compounds in plants. In contrast to other crude oils, virgin olive oil produced from olives of good quality is consumed unrefined. Thus, virgin olive oils contain phenolic compounds that are usually removed from other edible oils in various refining stages.

The amount of phenolic compounds in virgin olive oil is an important factor to bear in mind when the quality of virgin olive oil is evaluated. The natural phenols improve its resistance to oxidation (Vázquez et al., 1975; Baldioli et al., 1996) and are responsible for its sharp bitter taste (Gutiérrez et al., 1977, 1989). The pharmacological interest in olive phenolic compounds is also well-known (Berra et al., 1995; Manna et al., 1997; Visioli and Galli, 1998).

Most of the phenol determinations reported in virgin olive oils before the 1990s were carried out by using the Folin−Ciocalteu colorimetric method. Several simple phenols were identified in virgin olive oils in the 1970s and 1980s, but the most important phenolic compounds, such as oleuropein and ligstroside aglycons, were discovered in the 1990s (Montedoro et al., 1993; Cortesi et al., 1995). However, the phenolic fraction of olive oils is complex, and some peaks remain unidentified (Angerosa et al., 1996; Pirisi et al., 1997), and new compounds may appear in oils of unstudied cultivars or because of analytical improvements. Thus, the presence of certain flavonoids in virgin olive oils (Rovellini et al., 1997) and glucosides of 3,4-dihydroxyphenylethanol (hydroxytyrosol) have recently been reported (Bianco et al., 1998).

Some of the most representative phenolic compounds in olive oil are the oleuropein and ligstroside aglycons and the dialdehydic form of elenolic acid linked to hydroxytyrosol or tyrosol. Aglycons arise from glucosides present in olive fruits that may be hydrolyzed by endogenous β-glucosidases, possibly activated during the crushing and malaxation processes.

The phenolic composition of olive oils is the result of a complex interaction between several factors, including cultivar, degree of maturation, and climate (Uceda and Hermoso, 1997) as well as type of crushing machine, conditions during malaxation, etc. (Catalano and Caponio, 1996; Frega et al., 1997).

The purpose of this work was to characterize the phenolic fraction of Spanish olive oils and to elucidate the structure of some new compounds. Changes of these compounds with maturation in the Spanish virgin olive oils were also followed.

MATERIALS AND METHODS

Virgin Olive Oil. Olive fruits from the tree *Olea europaea* collected by hand in Cabra (CIFA, Córdoba, Spain), Nueva Carteya (Córdoba, Spain), and Arahal (Sevilla, Spain) were used. The index of ripeness (ir) of olives was calculated using a subjective evaluation color of the skin and flesh proposed by Uceda and Frías (1975). The procedure consists of distributing 100 olives in 8 groups, according to the following characteristics: group 0, skin bright green; group 1, skin green-yellowish; group 2, skin green with reddish spots; group 3, skin reddish brown; group 4, skin black with white flesh; group 5, skin black with <50% purple flesh; group 6, skin black with ≥50% purple felsh; and group 7, skin black with 100% purple flesh. The ripeness index is determined by the equation

$$\text{ripeness index} = 3(in_i)/100$$

where i is the number of the group and n_i the number of olives in it.

Olives of the cultivars Picual (ir = 1.6, 2.4, 4.8), Picudo (ir = 1.0, 3.1, 5.0), Hojiblanca (ir = 1.2, 3.1, 4.9), Arbequina (ir = 3.1), Jardúo (ir = 2.9), Blanqueta (Iir = 3.3), Empeltre (ir = 3.1), Villalonga (ir = 4.0), Cornicabra (ir = 5.6), Cornezuelo (ir = 2.0), Lechín (ir = 3.2), Manzanilla (ir = 3.0), and Verdial (ir = 2.6) were used. The oil was obtained by an Abencor

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Table 1. Spectroscopic Data for 4-(Acetoxyethyl)-1,2-dihydroxybenzene

^{1}H NMR (DMSO-d_6) (δ)[a]	^{13}C NMR (DMSO-d_6) (δ)	MS (%, ion structure)
1.98s (3H, CH_3CO)	20.7 (CH_3CO)	197 (10, $M^+ + 1$)
2.68t (2H, J = 7.1 Hz, H1′)	33.6 (C1′)	196 (90, M^+)
4.10t (2H, J = 7.1 Hz, H2′)	64.6 (C2′)	153 (12, $M^+ - COCH_3$)
5.42 br (1H, OH)	115.5, 116.1, 119.3, and 128.3 (C3−C6)	137 (100, $M^+ - OCOCH_3$)
6.5 m (3H, phenyl)	143.7 and 145.1 (C1−C2)	123 (67, $M^+ - CH_2OCOCH_3$)
	170.2(CO)	77 (89, phenyl)
		68 (18, phenyl)
		51 (51, phenyl)

[a] The signal of the second OH was hidden in the spectrum.

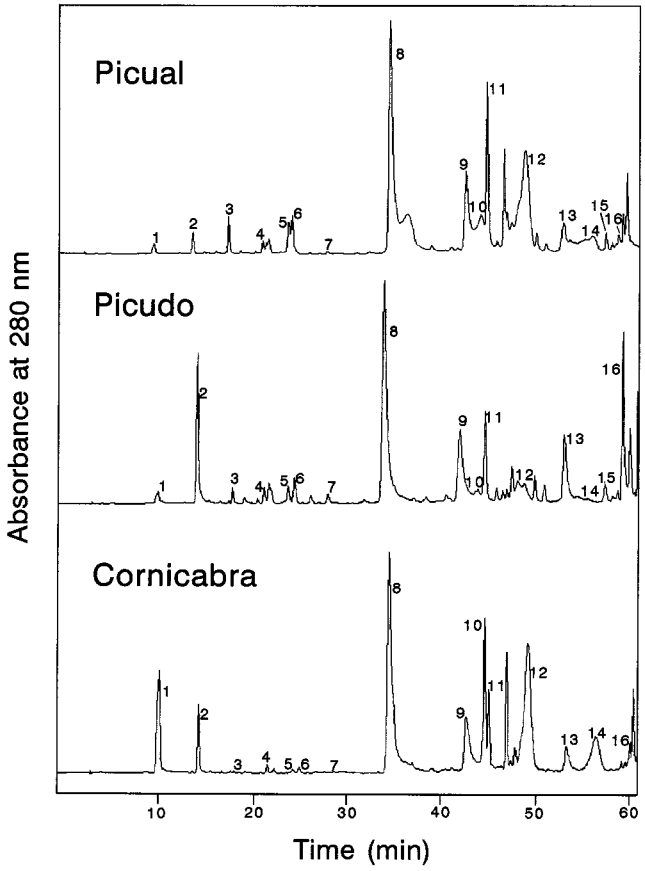

Figure 1. HPLC chromatogram of phenolic compounds in virgin olive oils of the Picual, Picudo, and Cornicabra cultivars at 280 nm. Indices of ripeness of Picual, Picudo, and Cornicabra olives used were 1.6, 3.1, and 5.6, respectively. Peaks correspond to (1) hydroxytyrosol, (2) tyrosol, (3) vanillic acid, (4) vanillin, (5) 4-(acetoxyethyl)-1,2-dihydroxybenzene, (6) *p*-coumaric acid, (7) ferulic acid, (8) dialdehydic form of elenolic acid linked to hydroxytyrosol, (9) dialdehydic form of elenolic acid linked to tyrosol, (10) unidentified, (11) unidentified, (12) oleuropein aglycon, (13) luteolin, (14) ligstroside aglycon, (15) unidentified, and (16) apigenin.

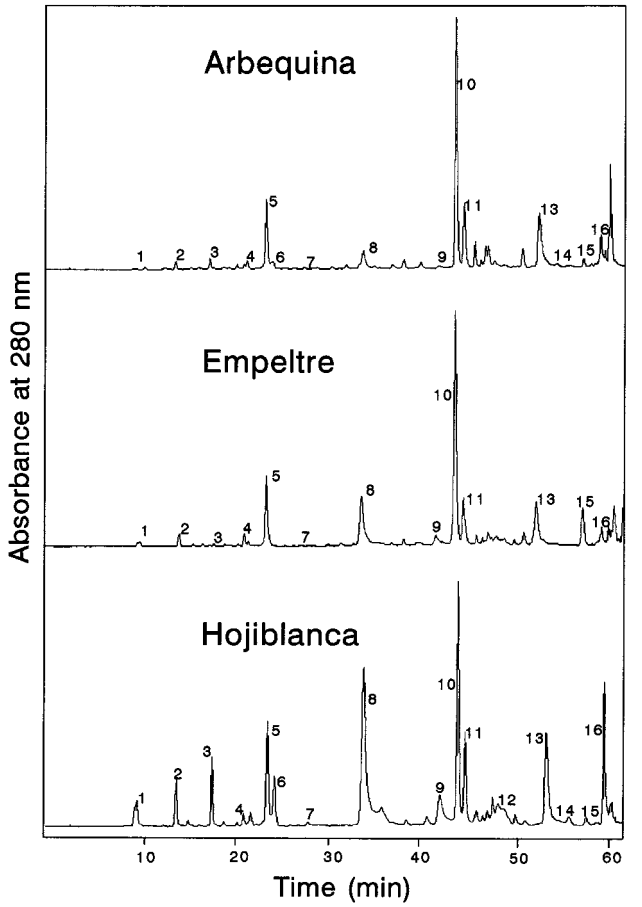

Figure 2. HPLC chromatograms of phenolic compounds in virgin olive oils of the Arbequina, Empeltre, and Hojiblanca cultivars at 280 nm. Index of ripeness of olives used was 3.1. See Figure 1 for identification of peaks.

analyzer (Comercial Abengoa S.A., Sevilla, Spain), consisting of three basic elements: a mill, a thermobeater, and a pulp centrifuge (Martínez et al., 1975).

Analysis of Phenolic Compounds. *Phenolic Extraction.* The phenolic extract of virgin olive oil was obtained following the procedure of Montedoro et al. (1992). Briefly, 14 g of virgin olive oil was extracted using 4 × 14 mL of methanol/water (80:20 v/v). After the methanol had been removed, the residue was taken up to 15 mL of acetonitrile. Washings with hexane (3 × 20 mL) were performed, and the resulting acetonitrile solution was evaporated under vacuum, giving a residue that was dissolved in 1 mL of methanol.

HPLC Analysis of Phenolic Compounds. The HPLC system consisted of a Waters 717 plus autosampler, a Waters 600 E pump, a Waters column heater module, and a Waters 996 photodiode array detector operated with Millenium 2010 software (Waters Inc., Milford, MA). A Spherisorb ODS-2 (5 μm, 25 cm by 4.6 mm i.d., Technokroma, Barcelona, Spain) column was used. Separation was achieved by elution gradient using an initial composition of 90% water (pH adjusted to 3.1 with 0.2% acetic acid) and 10% methanol. The concentration of the latter solvent was increased to 30% in 10 min and maintained for 20 min. Subsequently, the methanol percentage was raised to 40% in 10 min, maintained for 5 min, increased to 50% in 5 min, and maintained for another 5 min. Finally, the methanol percentage was increased to 60, 70, and 100% in 5 min periods. Initial conditions were reached in 15 min. A flux of 1 mL/min and a temperature of 35 °C were also used.

Reference Compounds. Oleuropein, luteolin, and apigenin were obtained from Extrasynthèse Co. (Genay, France). Tyrosol, vanillic acid, vanillin, *p*-coumaric acid, and ferulic acid were obtained from Sigma Chemical Co. (St. Louis, MO). The rest of the phenolic compounds were obtained using a semipreparative HPLC column (Spherisorb ODS-2, 5 μm, 25 cm by 10 mm i.d.) and a flow rate of 4 mL/min. The mobile phases were water and methanol, and the gradient was the same as

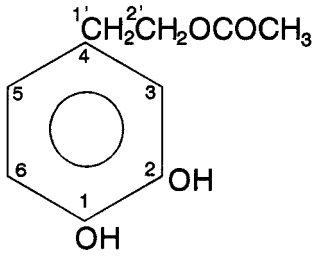

Figure 3. Chemical structure of 4-(acetoxyethyl)-1,2-dihydroxybenzene.

described above. The collected fractions were frozen and freeze-dried (Alpha 1−4, Martin Christ, Germany).

MS and NMR Analyses. ^{1}H and ^{13}C NMR spectra at 300 and 75.4 MHz, respectively, were determined on a Bruker AC-300P instrument (Karlsruhe, Germany) using tetramethylsilane as an internal standard. Mass spectra were obtained in a Finnigan MAT95s (Bremen, Germany) at 70 eV. The accelerating voltage was 4 kV, and the trap current was 100 μA.

RESULTS AND DISCUSSION

Figures 1 and 2 show the HPLC chromatograms of the phenolic extracts from virgin olive oils from 6 of the 13 Spanish olive cultivars studied. These chromatograms were obtained with the same eluents widely used by other researchers (Montedoro et al., 1992), although the gradient was modified to obtain a better discrimination between peaks. An outstanding improvement in the procedure consisted in controlling the oven temperature (35 °C) during the analysis, which facilitated the reproducibility of the retention times and quantification of peaks.

A series of simple phenols already described in the literature such as hydroxytyrosol, tyrosol, vanillic acid, *p*-coumaric acid, and ferulic acid (Montedoro et al., 1993; Pirisi et al., 1997) were found in the phenolic extracts from the oil of all the Spanish cultivars studied. On the contrary, other simple phenols such as caffeic acid, syringic acid, and homovanillic acid determined by some authors in certain oils have not been found in this study. Additionally, we have found in most of the Spanish virgin olive oils analyzed the presence of vanillin, which is the first time that it has been described in this product. Vanillin was identified by means of its retention time as well as by comparison of the characteristic spectrum of a standard with that obtained from peak 4.

Specific chromatographic methods for flavonoid analyses have demonstrated the presence of some of these substances in olive leafs, fruits, and oils. Luteolin and apigenin have been described in some Italian virgin olive oils (Rovellini et al., 1997). Luteolin may originate from rutin or luteolin 7-glucoside and apigenin from apigenin glucosides. All of these compounds have been found in the flesh of olive fruits (Amiot et al., 1986; Rovellini et al., 1997). Luteolin and apigenin were characterized in most of the Spanish virgin olive oils studied. They were identified by comparison of their retention time and spectra with those obtained from the corresponding standards. These compounds are mentioned in the literature, but their positions in the complete chromatogram of olive oil phenolic extracts have never been described and their contents have not been quantified. Luteolin (retention time = 53 min) and apigenin (retention time = 60 min) were present in remarkable quantities in most of the Spanish virgin olive oils.

Aglycons of oleuropein and ligstroside, peaks 12 and 14, were confirmed by mass spectrometry because they have been described previously (Montedoro et al., 1993; Cortesi et al., 1995). Peaks 8 and 9 were identified by mass spectrometry as the dialdehydic form of elenolic acid linked to hydroxytyrosol or tyrosol, respectively (Montedoro et al., 1993). However, it must be stressed that these peaks have also been described as the

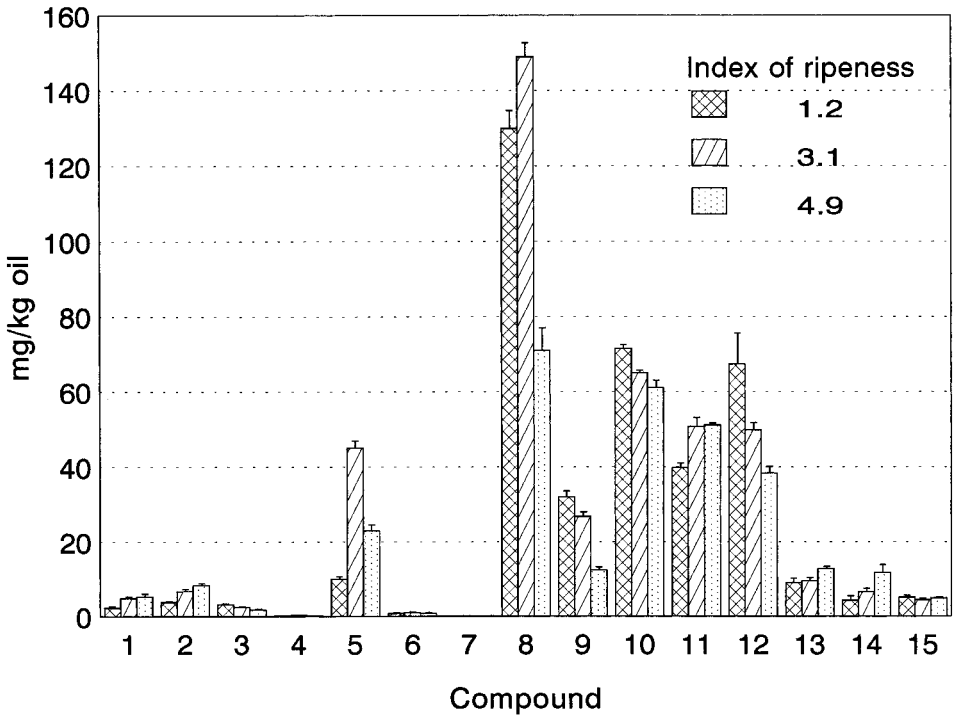

Figure 4. Phenolic compound changes with maturation in virgin olive oils of Hojiblanca cultivar. See Figure 1 for identification of compounds.

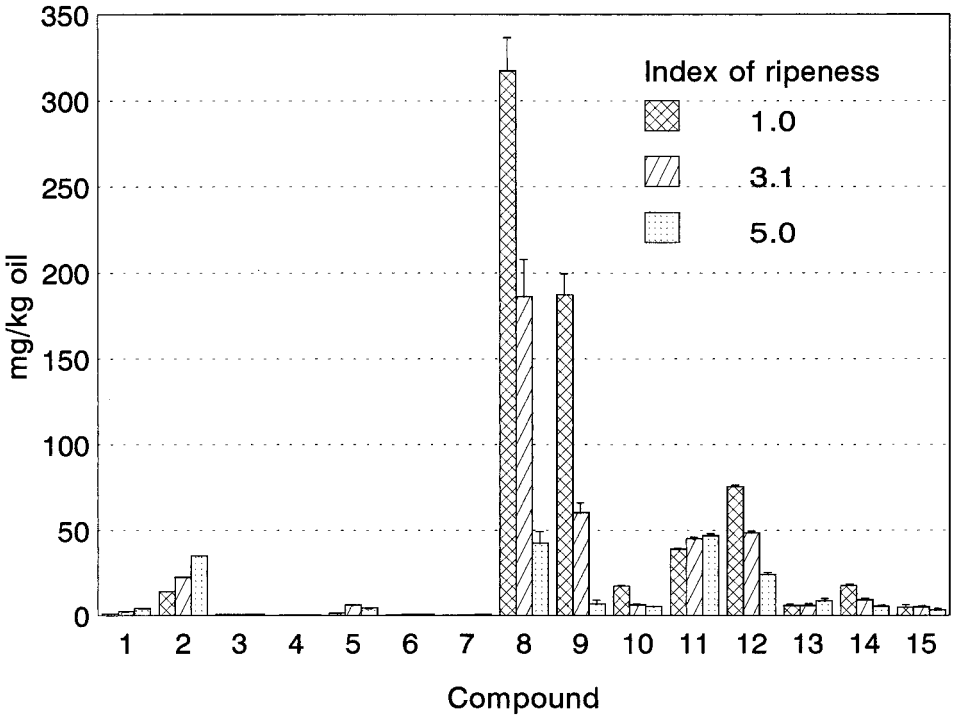

Figure 5. Phenolic compound changes with maturation in virgin olive oil of Picudo cultivar. See Figure 1 for identification of compounds.

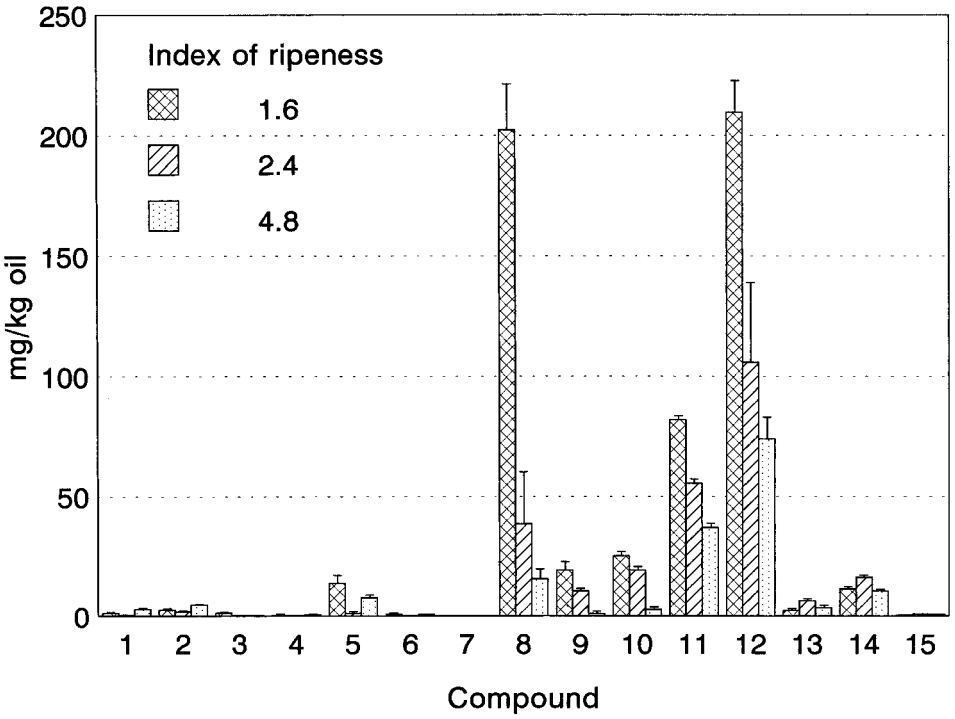

Figure 6. Phenolic compound changes with maturation in virgin olive oil of Picual cultivar. See Figure 1 for identification of compounds.

deacetoxy oleuropein or ligstroside aglycons (Cortesi et al., 1995). In fact, an isomerization reaction between the deacetoxy and the dialdehydic forms has been reported (Limiroli et al., 1995; Pirisi et al., 1997).

The chemical structure of isolated peak 5 was determined on the basis of its spectroscopical data (Table 1). Thus, it had ^{1}H and ^{13}C NMR spectra similar to those of hydroxytyrosol but with an acetyl group linked to a methylene group, according to the assignation of H2′ (Figure 3). Expected δ values were calculated by using the tables collected by Pretsch et al. (1976). Mass spectrometry confirmed the proposed structure. This compound, 4-(acetoxyethyl)-1,2-dihydroxybenzene had a UV spectrum similar to that of hydroxytyrosol with a maximum at 277.8 nm. This is the first time that this compound has been described in olive oils, and it was found in most of the Spanish virgin olive oils. Besides, in some oils the peak area was significant, as in Arbequina, Empeltre, or Hojiblanca oils (Figure 2).

Peaks 10 and 11 correspond to unknown phenolic substances. Peak 11 was found in all olive oils analyzed, and its UV spectrum was similar to that of hydroxy-

tyrosol, with a maximum at 277.8 nm. Elucidation of the structure of this compound as well as that of peak 10 is in progress. As can be seen in Figures 1 and 2, peak 10 may be the one with the biggest peak area in some olive oils. The UV spectrum of peak 10 was similar to that of tyrosol, with maxima at 228.2 and 277.8 nm. The structure of this compound has not been elucidated, although it has been suggested that it could be a tyrosol derivative compound (Montedoro et al., 1993; Cortesi et al., 1995). Peak 10 has been observed in all of the olive oil chromatograms, although in a very low peak area in some virgin oils such as those of the Picual, Picudo, and Jarduo cultivars. In contrast, this is the biggest peak in olive oils of Arbequina, Empeltre, and Villalonga cultivars. Besides, in the oils in which peak 10 was the biggest one, the peaks corresponding to glucoside aglycons (8, 9, 12, and 14) were small. Olive oils from other cultivars such as Cornicabra, Hojiblanca, Cornezuelo, Lechín, Manzanilla, Verdial, and Blanqueta showed balanced peak 10 and glucoside aglycon peaks.

Figures 4−6 show the phenolic changes in virgin olive oils of Hojiblanca, Picudo, and Picual cultivars with maturation. Oleuropein is the main phenolic compound in olive fruits, and its content diminishes with maturation (Amiot et al., 1986). It is also well-known that the total phenol concentration in oils, determined by colorimetric methods, decreases with maturation (Uceda and Hermoso, 1997). This may indicate an eventual relationship between changes of phenolic substances in fruits and those found later in the oil obtained from them.

Two tendencies were found in the simple phenol evolution with maturation. Vanillic acid, vanillin, *p*-coumaric acid, and ferulic acid practically remained constant, and their concentrations were <2−4 ppm. However, an increase in maturation always caused higher concentrations of tyrosol and hydroxytyrosol in the oils, the concentration of tyrosol always being greater than that of hydroxytyrosol. These compounds are not usually found free in nonmature fruits, although, with maturation, some chemical or enzymatic degradations of their corresponding glucosides can be produced and, consequently, an increase of their contents in oils with maturation may be observed. In fact, an increase in the enzymatic esterase activity in olives as maturation progresses has been reported (Amiot et al., 1989).

The two flavonoid compounds, luteolin and apigenin, showed opposite evolutions. Luteolin concentration increased with maturation in the virgin olive oils while apigenin did not show any defined tendency with maturation. Usually, the glucosides of flavonoids decrease with maturation (Amiot et al., 1986). However, the production of free aglycons could increase during maturation, and thus higher concentrations of aglycon flavonoids in oils may be found. The amount of luteolin was found in some oils to be >10 ppm, which is high enough to be considered of interest from a health and nutrition viewpoint because of the numerous useful effects attributed to flavonoids (Hertog et al., 1995).

Concentration of peak 10 diminished with maturation in Hojiblanca, Picual, and Picudo oils. Peak 11 content slightly increased with maturation in Picudo and Hojiblanca and decreased in Picual oils.

In general, both the dialdehydic forms of elenolic acid linked to tyrosol or hydrosytyrosol and the oleuropein or ligstroside aglycons diminished their concentration with olive maturation in the Spanish olive oils studied. This is in agreement with the decrease in the content of glucosides with maturation (Amiot et al., 1986). However, this transformation needs an enzymatic activity to break the glycosidic linkages because the chemical hydrolysis requires acidic conditions that are uncommon in olives (Brenes and de Castro, 1998). The enzymatic activity must occur during milling or malaxation, because the presence of aglycons in fresh fruits has not been reported (Amiot et al., 1986, 1989).

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