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Polyphenol Changes during Fermentation of Naturally Black Olives

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The individual evolution of phenolic compounds has been studied during the natural fermentation of black olives for the first time. Cyanidin 3-rutinoside and cyanidin 3-glucoside were the main anthocyanins identified in fresh olives, and they were not detected after 1 month of storage either in brine or in olive. The fruit colors were different when aerobic or anaerobic conditions were used and as a consequence of the different anthocyanin polymerizations that took place. At time zero, the polyphenols observed in the olive juice were hydroxytyrosol-4-β-glucoside, oleuropein, hydroxytyrosol, tyrosol, salidroside, and verbascoside and, after 12 months, the main phenol was hydroxytyrosol. The polyphenol content in the oil phase of olives was also analyzed. The dialdehydic form of elenolic acid linked to hydroxytyrosol and tyrosol, oleuropein aglycon, and ligstroside aglycon were the main compounds found at the beginning of fermentation but were not detected after 3 months. In contrast, hydroxytyrosol, hydroxytyrosol acetate, tyrosol, and tyrosol acetate were the main polyphenols detected in the oil phase of the final product. The acid hydrolysis of the initial glucosides (in olive juice) and the aglycons (in oil phase) was, therefore, the main reaction that took place during fermentation.

KEYWORDS: Naturally black olives; polyphenols; fermentation; oil

INTRODUCTION

Naturally black olives are obtained from fruits harvested when fully ripe or just before reaching full ripeness. They have a fruity flavor, and the product usually retains a slight bitter taste. They are quickly washed with water to remove superficial dirt, placed under anaerobic conditions into a brine with a salt concentration ranging from 6 to 8% NaCl, and spontaneously fermented for months (*1*). The Spanish Hojiblanca cultivar is very sensitive to fish-eye or gas pocket spoilage; most effective in overcoming this problem has been fermentation under aerobic conditions. In essence, the process consists of the introduction of air into the fermentation brine at a sufficient rate to induce growth of facultative or oxidative microorganisms (*2*).

As do other commodities, naturally black olives contain a high proportion of phenolic compounds. Oleuropein, the major component producing bitterness in olives, decreases in concentration with fruit maturation (*3*), while demethyloleuropein increases (*4*). However, hydroxytyrosol-4-β-glucoside has recently been proposed as the main polyphenol compound in naturally black olives (*5*, *6*). Other natural polyphenols have been identified in olive pulps such as hydroxytyrosol, salidroside, tyrosol, vanillic acid, verbascoside, rutin, and luteolin 7-glucoside (*6*−*8*).

The olive color changes with fruit maturation from green to purplish black, which is a consequence of the increase of some monomeric anthocyanins, mainly cyanidin 3-glucoside and cyanidin 3-rutinoside in fruits (*9*−*12*). Polymerization of anthocyanins during maturation (*13*) and fermentation of naturally black olives also contributes to color stabilization (*14*, *15*).

Recently, we have reported the importance of polyphenols present in the lipid phase of packed table olives to the total polyphenol content of the product (*15*). The compounds identified have been hydroxytyrosol, tyrosol, vanillic acid, vanillin, hydroxytyrosol acetate, tyrosol acetate, catechol, 1-acetoxypinoresinol, and pinoresinol, lignans very representative of olive oil (*16*), but the aglycons of oleuropein and ligstroside, the main polyphenols in virgin olive oil (*17*, *18*), have not been found.

Lately, special attention has been paid to the polyphenolic compounds of olives, particularly of olive oil, because of their beneficial properties for human health. Hydroxytyrosol has shown hypoglycemic and hypocholesterol properties (*19*, *20*); and hydroxytyrosol acetate (*21*) and lignans (*22*) also possess antioxidant properties.

The aim of the present study is to investigate the polyphenol changes during fermentation of naturally black olives. This knowledge will allow us to obtain a better understanding of the relationships that exist between these substances and the nutritional and organoleptic properties of the fruit. In particular, the phenolic composition of these olives and their evolution with fermentation have been studied for the first time.

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MATERIALS AND METHODS

Fermentation Procedure. Olive fruits of the Hojiblanca cultivar (Casariche, Sevilla) with a ripeness index of 3.5 units were hand collected in the 2000 crop year (*23*). Olives (16.5 kg) were placed in cylindrical PVC vessels and covered with 12 L of brine (8% NaCl, 0.4% acetic acid) for spontaneous anaerobic and aerobic fermentation. Application of the anaerobic and aerobic conditions have been described elsewhere (*2*). The experiment was run in duplicate at ambient temperature (20−25 °C) for a period of 12 months.

Chemical Analyses. Sugars (glucose, fructose, sucrose), mannitol, ethanol, and acetic acid were analyzed by HPLC as described elsewhere (*24*). The pH of the brine was measured in a Beckman model 45 pH meter.

Superficial Color Analyses. Colorimetric measurements on olives were performed using a BYK-Gardner model 9000 Color-view spectrophotometer, equipped with computer software to calculate the CIE L^* (lightness), a^* (redness), and b^* (yellowness) parameters. Interference by stray light was minimized by covering samples with a box that had a matte black interior. The data of each measurement are the average of 20 olives.

Brine Color Analyses. Absorption spectra were obtained by scanning solutions in 1 mm path length quartz cells from 400 to 700 nm in a Cary 1E UV−vis spectrophotometer (Varian, Mulgrave, Australia), which was equipped with a computer software program (Varian) to calculate the L^*, a^*, and b^* values.

Total Anthocyanin Analyses. The total anthocyanin content was determined according to the bisulfite procedure (*25*) on pigment solution (brine and olive juice). The concentration, expressed as millimolars of cyanidin 3-rutinoside (molecular weight = 594), was calculated using a molar absorptivity value of 28840 (*26*). The calibration curve for cyanidin 3-rutinoside was defined by the equation $y = -0.0035 + 2.0532x$ with a correlation coefficient of $r^2 = 0.99$.

Analysis of Phenolic Compounds. The olive fruits were crushed in a commercial mixer (Braun) after removal of the stone, the three phases (oil, olive juice, and pomace) were separated by centrifugation (8 min, 6000 rpm), and the oil phase was filtered by gravity.

Olive juices and brines were diluted 1:1 and 1:4, respectively, with distilled water, filtered through a 0.45 μm nylon filter, and injected into the chromatograph system.

The phenolic extract of olive oil was obtained following the procedure described elsewhere (*27*). Briefly, a 0.6 mL sample of olive oil was extracted using 3 × 0.6 mL of *N*,*N*-dimethylformamide (DMF). The extract was then washed with hexane, and nitrogen was bubbled into the DMF extract to eliminate residual hexane. Finally, the extract was filtered through a 0.45 μm nylon filter and injected into the chromatograph system.

HPLC Analysis of Anthocyanin Compounds. The HPLC method used was based on the same method proposed elsewhere (*6*). The HPLC system consisted of a Waters 717 plus autosampler, a Waters 600E pump, a Waters column heater module (40 °C), and a Waters 996 photodiode array detector operated with Millenium 2010 software (Waters Inc., Milford, MA). An Extrasil ODS-2 (5 μm, 25 cm × 4.6 mm i.d., Technokroma, Barcelona, Spain) column was used, and the elution conditions were as follows: flow rate = 1 mL/min; volume injected = 20 μL; solvent A, water 1% perchloric acid; solvent B, methanol. The mobile phase consisted initially of 80% of A; using a linear gradient, the concentration of methanol was increased to 50% over 35 min and to 98% at 40 min, held for 2 min at 98% of B to wash the column, and then returned to the initial conditions (20% of B) for 10 min. Chromatograms were recorded at 520 nm.

HPLC Analysis of Non-anthocyanin Compounds in Brine and Olive Juice. The HPLC system consisted of a Waters 2690 Alliance with a pump, column heater, and autosampler modules included, the detection being carried out with a Waters 996 photodiode array detector. The system was controlled with Millennium^{32} software (Waters Inc.). A 25 cm × 4.6 mm i.d., 5 μm Lichrospher 100 (Merck, Darmstadt, Germany) column was used. Separation was achieved by gradient elution using an initial composition of 90% water (pH 2.5 adjusted with 0.15% phosphoric 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 and maintained for 5 min. Finally, the methanol percentage for the last three steps was increased to 60, 70, and 100% in 5 min periods. Initial conditions were reached in 15 min. An injection volume of 20 μL, a flow rate of 1 mL/min, and a temperature of 35 °C were used. Chromatograms were recorded at 280 nm.

HPLC Analysis of Non-anthocyanin Compounds in Olive Oil. The HPLC system consisted of a Waters 717 plus autosampler, a Waters 600E pump, and a Waters column heater module (Waters Inc.). A Sherisorb ODS-2 (5 μm, 25 cm × 4.6 mm i.d., Technokroma) column was used. Separation was carried out under conditions similar to those given above for brine and olive juice analyses. A Waters 996 diode array detector and a Jasco FP-920 fluorescence detector (Jasco Corp., Tokyo, Japan) were connected in series. Vanillin and vanillic acid were monitored by UV at 280 nm and the rest of the phenolic compounds by fluorescence with an excitation wavelength of 280 nm and an emission wavelength of 320 nm. Both detectors were operated with Millennium^{32} software (Waters Inc.).

Reference Compounds. The evaluation of each compound was performed using a regression curve in triplicate of three different concentrations ($r^2 \geq 0.99$). Oleuropein was purchased from Extrasynthese S.A. (Lyon Nord, Genay, France); catechol, tyrosol, vanillic acid, and vanillin were provided by Sigma Chemical Co. (St. Louis, MO); verbascoside was quantified as described elsewhere (*6*). No commercial phenolic compounds were obtained by preparative HPLC as described elsewhere (*5*, *16*, *18*) except salidroside and tyrosol acetate, which were quantified as tyrosol.

Table 1. Changes of Some Chemical Parameters during Fermentation of Naturally Black Olives

storage process	time (months)	pH	ethanol (g/100 mL of brine)	acetic acid (g/100 mL of brine)
aerobic	0	3.09	0	0.38 (0.00)
	1	4.17	0.32 (0.01)[a]	0.22 (0.02)
	1.5	4.35	0.48 (0.17)	0.16 (0.04)
	3	4.46	0.80 (0.05)	0.18 (0.04)
	12	4.42	0.50 (0.30)	0.13 (0.07)
anaerobic	0	3.09	0	0.38 (0.00)
	1	4.06	0.26 (0.06)	0.23 (0.03)
	1.5	4.26	0.36 (0.04)	0.24 (0.01)
	3	4.36	0.57 (0.06)	0.24 (0.01)
	12	4.25	1.43 (0.15)	0.26 (0.03)

[a] Standard deviation is given in parentheses ($n = 4$).

RESULTS AND DISCUSSION

Olives underwent a spontaneous fermentation, which can be considered as normal for this type of olive processing. A slight increase in pH was observed with time for both the aerobic and anaerobic fermentations, although in the latter it was lower (**Table 1**). This effect can be attributed to the diffusion of organic salts from fruits into the surrounding brines and, therefore, the increase in buffer capacity of solutions (*1*). Likewise, differences in storage methods can be related to the final amount of acetic acid in each type of fermentation. It must be noted that lactic acid was not detected through any fermentation.

Glucose and mannitol accumulated in brines during the first 3 months up to 80 mM. Glucose concentration declined with time to <2 mM and mannitol up to 40 mM. Apparently, mannitol was not well assimilated by the yeasts usually found in these media (*28*).

Ethanol is volatile product of yeast metabolism found during the fermentation of naturally black table olives (*29*). Its concentration increased with time during fermentation (**Table 1**).

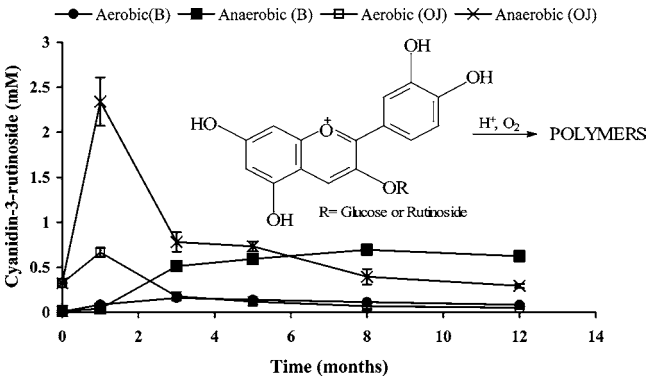

Figure 1. Evolution of the total anthocyanins in brine (B) and olive juice (OJ) of naturally black olives fermented for 12 months. Each point is the average of four measurements, and where error bars are not visible, determinations are within the size of the symbols on the graph.

Under aerobic conditions, it rose to 0.80 g/100 mL of brine after 3 months, but at the end of the storage period its value was 0.50 g/100 mL of brine. In contrast, under anaerobic condition, there was an accumulation of ethanol in the medium, reaching 1.43 g/100 mL of brine at the end of storage period.

Anthocyanins are the components responsible for the color of naturally black olives (*9*−*12*). Initially, only two anthocyanins were found in the fresh pulp, cyanidin 3-glucoside (0.66 mmol/kg of dry weight) and cyanidin 3-rutinoside (1.27 mmol/kg of dry weight). Monomeric anthocyanins were not detected by HPLC in the olives after 1 month of fermentation, presumably due to the dilution and polymerization reactions during this period. A similar occurrence can be seen with red wine, in which monomeric pigments decrease during the fermentation and aging period while polymeric anthocyanins increase (*30*−*33*). Other authors have reported a similar behavior during the ripening of olive fruits (*13*) and their processing by the Greek-style method (*14*). The content of total anthocyanins in olive juice and brine was estimated by a colorimetric method (*25*).

There was an increase in total anthocyanins in the brine (**Figure 1**) during the first month, being higher under anaerobic than aerobic conditions, as a consequence of a diffusion process from the pulp. A steady state was reached at the third month, and an equilibrium between the amount in olive juice and brine was observed. A value of 0.5 mM of cyanindin 3-rutinoside was found under anaerobic conditions, whereas lower concentrations were detected under aerobic conditions (0.1−0.2 mM cyanidin 3-rutinoside). Air injection was probably the cause of a higher oxidation and polymerization of anthocyanins.

These differences in total anthocyanins influenced, to a large extent, the color developed in olives during the fermentation process. As one might expect, brines decreased in lightness with time (**Figure 2**), being darker (lower *L** values) under aerobic than anaerobic conditions. On the other hand, *a** and *b** parameters continuously increased, with the aerobic brines resulting in more redness and yellowness than the anaerobic brines.

Fruit color evolution varied (**Figure 3**). Aerobic fruits maintain the same lightness (*L**) through the fermentation period, ∼22 units, but *a** and *b** parameters initially changed and, after 1 month, the color was stable. However, anaerobic fruits increased in lightness and showed more redness and yellowness than aerobic fruits. In conclusion, the evolution of color was different under aerobic and anaerobic conditions, and differences between fruit and brine color were also found. Such differences can be due to numerous reactions that can take place

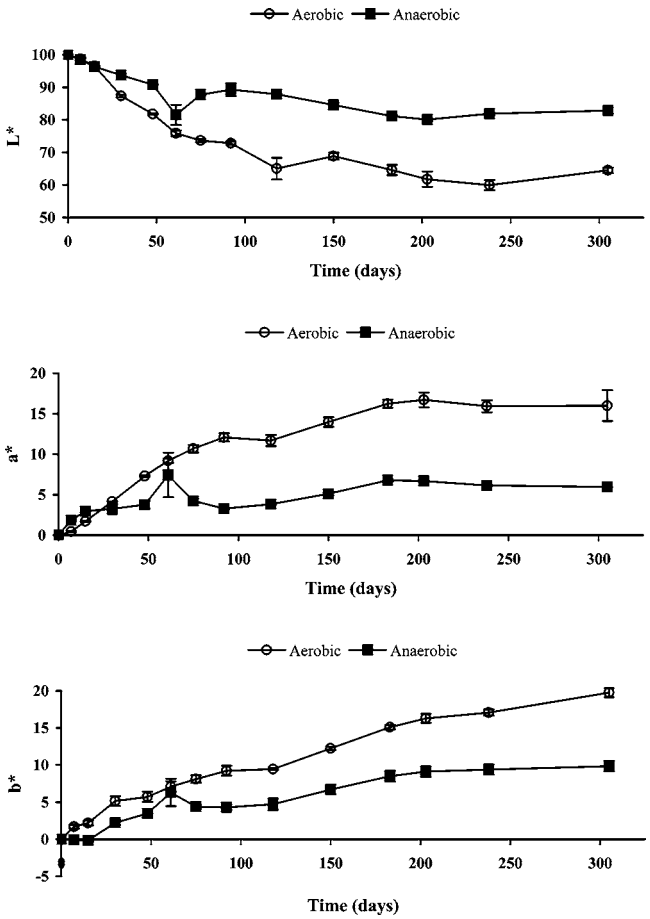

Figure 2. Color evolution (expressed as CIE *L**, *a**, *b** parameters) in the fermentation brine. Each point is the average of two measurements, and where error bars are not visible, determinations are within the size of the symbols on the graph.

during the fermentation−storage process, hydrolysis, oxidation, polymerization, etc. Therefore, the developed polymers, which are responsible for the color, will be very different in any conditions.

Likewise, the evolution of phenolic compounds in olive juice and brine was also studied during fermentation. The main phenolic compound detected in olive flesh by HPLC was hydroxytyrosol-4-β-glucoside. In fact, this phenol has recently been proposed as a very important phenolic compound in natural black olives and derived products (*5*, *6*). Its concentration (32 mM) declined with time in olive juice (**Figure 4**), reaching only 5 mM hydroxytyrosol-4-β-glucoside after 12 months. Along with this decline in flesh, the compound increased in brine for the first 3 months, mainly in anaerobic conditions. Then, its concentration decreased until the end of the storage period, reaching 7 and 2.5 mM hydroxytyrosol-4-β-glucoside in anaerobic and aerobic conditions, respectively. The loss of this phenol in olive juice was mainly due to its diffusion into the brine, but an acid hydrolysis of this glucoside, forming hydroxytyrosol, occurred in acidic juices and brines, which can explain their losses with fermentation. An extra loss of hydroxytyrosol-4-β-glucoside was observed in aerobic brine, which must be related to the oxidation reaction of orthodiphenols.

As might be expected, the amount of oleuropein in fresh pulp (**Figure 5**) was not as high at time zero (1.4 mM). This phenol, just like hydroxytyrosol-4-β-glucoside, diminished in olive juice, and a maximum concentration was found after ∼80−90 days in brine. This phenol decreased with time, as the hydroxytyrosol-

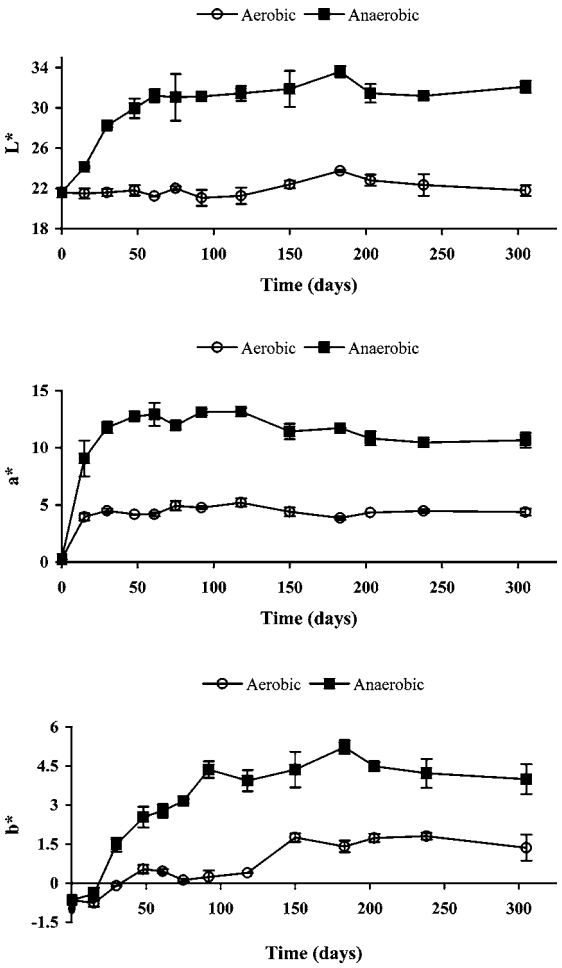

Figure 3. Evolution of the surface color of olives (expressed as CIE *L**, *a**, *b** parameters) during the fermentation process. Each point is the average of four measurements, and where error bars are not visible, determinations are within the size of the symbols on the graph.

4-β-glucoside, because of its acid hydrolysis and the formation of hydroxytyrosol. Similar behavior in storage brines of ripe olives has been reported by other authors (*34*).

Hydroxytyrosol was the second phenol in importance. The change observed in its concentration during fermentation is given in **Table 2**. Apparently, during the first 3 months of fermentation this phenol decreased in olive juice and then started to increase, rising to a higher amount in anaerobic than in aerobic conditions. At the same time, there was an accumulation of this compound in brines during the first few months of brining due to its diffusion from the olives into the surrounding liquids. This accumulation of hydroxytyrosol in olive juice and brines came from the acid hydrolysis of oleuropein and hydroxytyrosol-4-β-glucoside, phenols that decreased during the storage process as mentioned above (**Figures 4** and **5**). Therefore, at the end of fermentation storage, hydroxytyrosol was the main phenol in olive juice and brine.

The other phenolic compounds identified in olive juices were salidroside, tyrosol, and verbascoside, whereas tyrosol and verbascoside were found in brines. Salidroside is a glucoside of tyrosol, and it has recently been identified in the flesh of naturally black olives (*6*), although long recognized in olive seeds (*35*). This phenol increased for the first month and then started to decrease; tyrosol initially decreased in olive juice, but after the eighth month began to increase. On the other hand, salidroside was not detected in brine, but tyrosol continuously

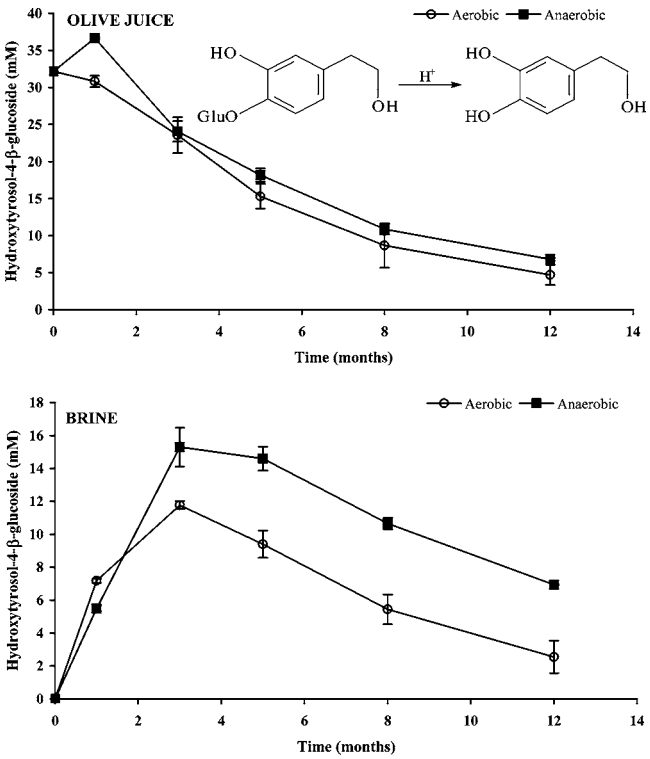

Figure 4. Evolution of hydroxytyrosol-4-β-glucoside concentration in olive juice and brine of naturally black olives during 12 months. Each point is the average of four measurements, and where error bars are not visible, determinations are within the size of the symbols on the graph.

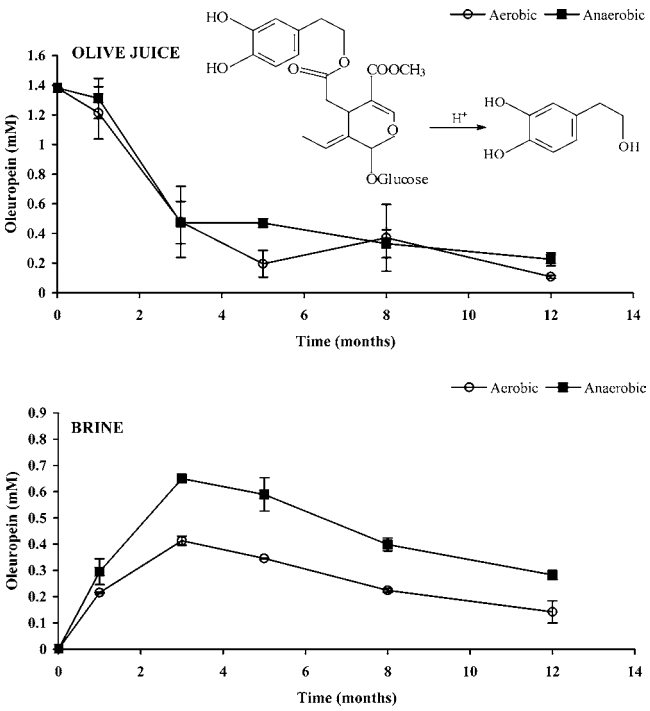

Figure 5. Evolution of oleuropein concentration in olive juice and brine of naturally black olives during 12 months. Each point is the average of four measurements, and where error bars are not visible, determinations are within the size of the symbols on the graph.

increased. Obviously, salidroside, under the acid conditions, was hydrolyzed in the flesh, and then its derived phenol (tyrosol) was diffused from the pulp into the brine.

Verbascoside was very stable during fermentation, and most of its initial concentration remained in the flesh.

Table 2. Changes of Phenolic Compounds (Millimolar) in Olive Juice and Brine during Fermentation of Naturally Black Olives

	time	olive juice				brine		
storage process	(months)	hydroxytyrosol	salidroside[b]	tyrosol	verbascoside	hydroxytyrosol	tyrosol	verbascoside
aerobic	0	13.01 (0.00)[a]	0.96 (0.00)	1.78 (0.00)	0.03 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
	1	9.72 (0.27)	1.63 (0.21)	0.35 (0.13)	0.04 (0.00)	3.00 (0.11)	0.28 (0.01)	0.01 (0.00)
	3	7.74 (0.92)	1.56 (0.52)	0.25 (0.09)	0.02 (0.00)	8.77 (0.10)	0.39 (0.06)	0.02 (0.00)
	5	7.98 (1.84)	1.01 (0.01)	0.30 (0.01)	0.01 (0.01)	10.36 (0.01)	0.52 (0.01)	0.02 (0.00)
	8	10.69 (1.56)	0.98 (0.43)	0.78 (0.06)	0.01 (0.01)	13.50 (0.59)	0.80 (0.05)	0.02 (0.00)
	12	12.07 (0.91)	0.65 (0.20)	1.08 (0.11)	0.01 (0.00)	14.36 (0.57)	1.09 (0.10)	0.02 (0.00)
anaerobic	0	13.01 (0.00)	0.96 (0.00)	1.78 (0.00)	0.03 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
	1	15.04 (0.92)	2.00 (0.03)	0.36 (0.10)	0.08 (0.01)	2.72 (0.06)	0.19 (0.01)	0.01 (0.00)
	3	10.69 (0.48)	1.44 (0.09)	0.20 (0.01)	0.06 (0.00)	9.07 (1.30)	0.42 (0.10)	0.03 (0.00)
	5	11.17 (0.37)	1.23 (0.03)	0.24 (0.04)	0.04 (0.01)	11.82 (0.02)	0.43 (0.02)	0.04 (0.00)
	8	12.13 (1.20)	0.72 (0.55)	0.54 (0.00)	0.03 (0.00)	14.52 (0.82)	0.66 (0.03)	0.04 (0.00)
	12	12.67 (0.42)	0.89 (0.01)	0.66 (0.08)	0.03 (0.00)	15.28 (1.20)	0.79 (0.03)	0.04 (0.00)

[a] Standard deviation is given in parentheses ($n = 4$). [b] Salidroside has been quantified as tyrosol.

Table 3. Changes of Phenolic Compounds (Micromoles per Kilogram of Oil) in the Oil Phase during Fermentation of Naturally Black Olives

storage process	time (months)	hydroxytyrosol	catechol	tyrosol	vanillic acid	vanillin	1-acetoxypinoresinol	pinoresinol
aerobic	0	45.27 (6.68)[a]	0.42 (0.00)	20.84 (0.43)	0.00 (0.00)	0.00 (0.00)	18.84 (1.37)	9.00 (0.23)
	1	61.99 (0.51)	1.09 (0.29)	29.88 (5.62)	0.00 (0.00)	2.60 (0.58)	20.20 (1.85)	7.20 (0.65)
	3	2.61 (0.12)	0.63 (0.04)	23.12 (2.92)	0.00 (0.00)	3.33 (0.99)	22.04 (2.22)	9.39 (1.14)
	5	4.34 (1.86)	0.19 (0.09)	32.28 (5.62)	4.01 (0.45)	0.62 (0.32)	24.63 (3.52)	9.51 (2.88)
	8	19.38 (25.09)	0.22 (0.16)	51.54 (1.47)	11.37 (5.93)	5.42 (3.22)	34.58 (8.50)	19.36 (0.90)
	12	57.95 (23.76)	0.00 (0.00)	76.80 (8.39)	12.27 (1.25)	0.84 (0.22)	42.60 (5.59)	19.70 (8.15)
anaerobic	0	45.27 (6.68)	0.42 (0.00)	20.84 (0.43)	0.00 (0.00)	0.00 (0.00)	18.84 (1.37)	9.00 (0.23)
	1	122.98 (25.75)	1.45 (0.29)	30.18 (1.62)	0.00 (0.00)	2.73 (0.18)	22.97 (0.76)	7.72 (0.20)
	3	4.05 (0.57)	0.55 (0.08)	24.50 (1.28)	0.00 (0.00)	3.16 (1.60)	24.01 (5.68)	9.03 (1.45)
	5	11.15 (5.66)	0.43 (0.04)	31.78 (0.14)	3.26 (1.31)	2.12 (0.63)	20.63 (1.59)	10.41 (0.53)
	8	19.21 (5.33)	0.19 (0.11)	44.01 (4.58)	2.81 (0.94)	1.98 (0.50)	24.15 (1.95)	15.80 (1.29)
	12	47.68 (28.01)	0.00 (0.00)	53.99 (2.31)	5.73 (0.09)	1.54 (0.28)	28.03 (0.01)	20.74 (0.05)

[a] Standard deviation is given in parentheses ($n = 4$).

So far, we have evaluated the phenolic content in olive juice, but, as is well-known, that there are two well-differentiated phases in the olive flesh, juice and oil, and the phenolic content of each phase is different. Consequently, the composition in the oil phase has also been studied.

At time zero, the main phenols found in the oil phase were the dialdehydic form of elenolic acid linked to hydroxytyrosol (HyEDA) and tyrosol (TyEDA), oleuropein aglycon (HyEA), and ligstroside aglycon (TyEA). Specifically, these compounds are the most important in virgin olive oil of Spanish olive cultivars (*18*, *36*). However, in this oil phase, these phenols quickly decreased, from 335.54 μmol/kg of oil of HyEA, 188.18 μmol/kg of oil of HyEDA, 129.03 μmol/kg of oil of TyEA, and 134.75 μmol/kg of oil of TyEDA at time zero to 240, 45.03, 80, and 50.55 μmol/kg of oil, respectively, after 1 month; they were not detected at 3 months. Thus, these components were hydrolyzed during the fermentation process, and consequently the concentrations of their hydrolysis products, hydroxytyrosol and tyrosol, increased with time (**Table 3**).

Other phenolic compounds were also found in the oil phase of table olives (**Table 3**)—catechol, vanillic acid, vanillin, and the lignans 1-acetoxipinoresinol and pinoresinol; they were also present in olive oil (*16*, *18*) and in packed table olives (*15*). Although catechol decreased during storage, vanillin remained stable and vanillic acid markedly increased under aerobic conditions. Pinoresinol increased under either condition, but 1-acetoxypinoresinol only increased twice under aerobic conditions and remained quite constant under anaerobic conditions. We have no explanation for this phenomenon.

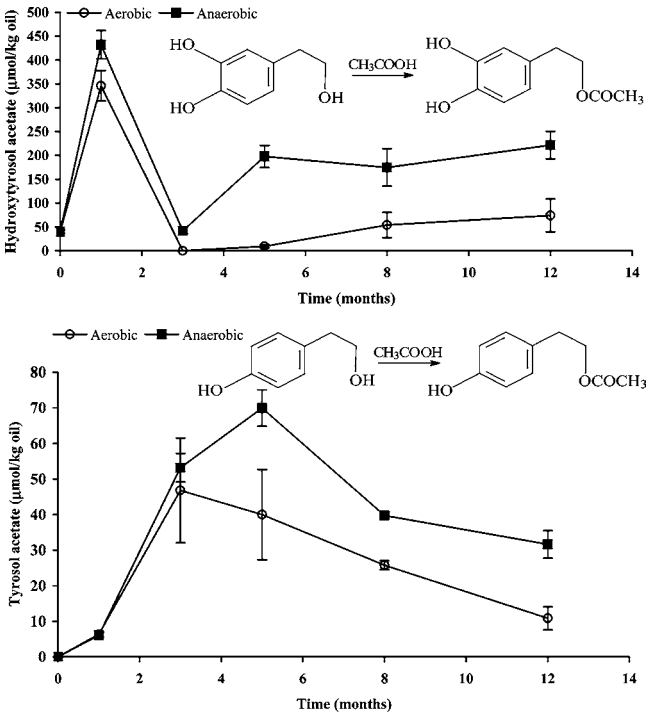

Figure 6. Evolution of hydroxytyrosol acetate and tyrosol acetate concentration in the oil phase of naturally black olives during 12 months. Tyrosol acetate has been quantified as tyrosol. Each point is the average of four measurements, and where error bars are not visible, determinations are within the size of the symbols on the graph.

Finally, tyrosol acetate (TyAc) and hydroxytyrosol acetate (HyAc) were also phenols present in this oil phase, both of which have been previously reported in virgin olive oil (*18*, *37*) and in packed table olives (*15*). During the first months, there was an acid hydrolysis of HyEA, HyEDA, TyEA, and TyEDA as a consequence of the acetic acid concentration in the medium (added in the initial brine). As would be expected, an initial increase of both derived acetates was observed (**Figure 6**) and, after the fermentation period, HyAc concentration rose at a steady state but TyAc decreased continuously until the end of storage.

In conclusion, a great difference between the phenol composition of fresh and processed fruits as a consequence of the reactions occurring during the fermentation period has been observed. The acid hydrolysis of the initial glucosides (in olive juice) and the aglycons (in oil phase) was the main reaction that took place during fermentation; therefore, hydroxytyrosol was the most important phenol detected in the final product (12 mM). As a consequence of the presence of acetic acid in the medium, acetylation reactions were also observed, and marked concentrations of hydroxytyrosol acetate and tyrosol acetate were detected. Polymerization of anthocyanin compounds was the cause of the final color developed in olives. All of these reactions are of great importance for the nutritional and organoleptic properties of the fruit.

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