JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Kinetic Study of the Physicochemical and Microbiological Changes in "Seasoned" Olives during the Shelf-Life Period

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The changes that take place during the shelf life of "seasoned" olives (packing conditions: 4% NaCl, 0.1%citric acid, and 0.0175% sorbate) using fresh (FF) and stored (SF) cracked fruits were studied. Texture, *L**, *b**, NaCl, and pH experienced slight changes. Values of *a** and titratable and combined acidity increased following a first-order kinetic with higher constants for FF. Glucose was completely exhausted, and mannitol showed only a slight decline. Hydroxytyrosol and tyrosol contents in brines increased rapidly from the acidic hydrolysis of oleuropein, hydroxytyrosol-4-β-D-glucoside, and salidroside, the concentrations of which decreased. Sorbate content decreased with time and disappeared completely in SF. Yeasts grew rapidly in FF and were markedly inhibited in SF. There was a moderate growth of lactic acid bacteria in FF, whereas they grew markedly in SF during the first few days. Some of these changes limit the shelf life of the product.

KEYWORDS: "Seasoned" table olives; yeasts; lactic acid bacteria; brine; polyphenols; sorbic acid; combined acidity; titratable acidity; color; sugars

INTRODUCTION

Table olives are the main fermented vegetable in western countries. Their world production reaches ∼1 500 000 tonnes/year (*1*). Green Spanish style, naturally black Greek styles, and ripe Californian styles are well-established commercial presentations. Their elaboration processes and preservation systems have received wide research support (*2*).

Homemade "seasoned" olives are very common around the Mediterranean basin. According to tradition, they can be produced following different recipes and using diverse natural ingredients (peppers, lemon, thyme, etc.). Demand for this type of olive is increasing due to the progressive awareness of the consumer of traditional and natural products. Seasoned olives have a rather limited shelf life because a complete stabilization of the final product is usually not achieved. The length of this period is variable and depends on the raw material, seasoning products, preservatives used, etc., because the processing methods are not as standardized as those of green or ripe olives. Scientific information on these products is scarce. The effect of natural components can cause significant effects on texture. In preparations with lemon and garlic, a progressive increase in the cellulose and polygalacturonase activity was observed with shelf-life duration. These products can serve as a source of enzymes, which are able to solubilize into the brine and, then, act on the olives (*3*). In addition, endogenous enzymes are also able to act on the texture of the olives, modifying important polysaccharides of the cell wall. It was observed that the olives became more susceptible after the seasoning process. The enzymatic action is also stronger as the state of maturation progresses (*4*). Apparently, the concentration of NaCl and the values of pH used can favor the performance of these hydrolytic enzymes and, therefore, could exacerbate the detrimental changes that take place in the cell wall of the olive fruits (*3*). Turning-color olives had the highest concentration in polyphenols (∼1200 mg/kg). Therefore, seasoned olives, which are prepared from fruits at this maturation stage, might also be a good source of phenolic compounds for the human diet (*5*).

Polyphenols are inhibitory for lactic acid bacteria (LAB) (*6*). The presence of important concentrations of polyphenols may influence the microbial growth during olive storage (*7*). Thus, the growth of these bacteria may have some difficulties in seasoned, non-lye-treated olives.

Seasoned olives of the Aloreña cultivar have a general acceptance due to the special characteristics of the fruits and the spices and herbs used in them. Their production may reach ∼5000 tonnes/year. In this elaboration, olives are washed and cracked as they enter the factory. Cracked olives are then packed as seasoned olives or stored in brine and packed throughout the year according to demand. This product is destined for local markets. In general, its relatively low stability has prevented the implementation of industrial processes for seasoned olives without pasteurization. In part, this situation is due to the lack of scientific information on this type of olive.

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10.1021/jf050501+ CCC: $30.25
Published on Web 06/03/2005

The aim of this work was to investigate the effect of the raw material on the physicochemical and microbiological changes during the shelf-life period of seasoned packed olives of the Aloreña cultivar as a model for the different traditional elaborations of this type. The results of this study can be of interest for the design of new preservation systems that could improve the stability and safety of seasoned olives in general.

MATERIALS AND METHODS

Experimental Design. The experiment attempts to mimic the changes that occur in the product during the period elapsed from the time it reaches the shelf until the moment it is unmarketable (when it has suffered such a degradation that it must be removed). The study has been carried out in seasoned packed olives of the Aloreña cultivar, prepared using two different raw materials: fresh fruits (FF) and stored fruits (SF), with a period of ~1 month of previous storage in brine. Seasoning products consisted of a mixture of red pepper, garlic, thyme, and fennel and were added to the packages in the same proportion as in the industrial process. Samples (cracked olives) of either FF or SF were obtained directly from the packing line of a local producer (Aceitunas Bravo S.A., Alahurin el Grande, Málaga), introduced into the packages (together with the seasoning product), and covered with brines. Physicochemical conditions of packing were those used habitually by the industry in its normal production (4% NaCl, 0.1% citric acid, and 0.0175% sodium sorbate, expressed as w/v, including olives). Pet packages (Juvasa, Dos Hermanas, Seville, Spain) were of 1.6 L total capacity with 0.9 kg of fruits and 0.7 L of cover brine. Packages were maintained at room temperature (23 ± 2 °C) and analyzed periodically at 0, 3, 10, 15, 21, and 28 days (FF), except for microbial analysis, which was longer, or at 0, 3, 9, 16, 23, 28, 44, and 67 days (SF), in duplicate. The periods chosen for the study were based on maximum shelf lives for commercial preparations from FF and SF, respectively, according to the industry. Analyses at time 0 in fruits and brines were carried out just after the samples arrived at the laboratory, 3 h after packing. This period mimics transportation from factory to retailer. Opened packages were discarded.

Analysis of Physicochemical Characteristics. The analyses of olive brines for pH, titratable acidity (expressed as g of lactic acid/100 mL of brine), combined acidity (as milliequiv of HCl acid added to 1 L of brine to reach pH 2.6), and salt (g of NaCl/100 mL of brine) were carried out using the routine methods described by Garrido Fernández et al. (*2*).

Texture was measured using a Kramer shear compression cell coupled to an Instron Universal Testing Machine, model 1001. The cross head speed was 200 mm/min. The firmness of the olives was expressed as the mean of 8−10 replicated measurements, each of which was performed on 2−4 pitted olives. Shear compression force was expressed as newtons per 100 g of pitted olives.

Surface color analyses 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, which had a matt black interior. The data of each measurement are the mean of 20 olives.

The color of the brine was estimated as the difference of absorbance at 440 and 700 nm (*8*). Previously, liquids were centrifuged at 12000*g* for 10 min.

Moisture content was determined in duplicate in an oven-drying of 20 g of crushed olive flesh at 102 ± 2 °C until weight stabilization.

Analysis of Phenolic Compounds. Destoned olive fruits were crushed in a commercial mixer (Braun), the three phases (oil, olive juice, and pomace) were separated by centrifugation (6000 rpm for 8 min), and the water phase was separated. Olive juices (and brines) were then diluted 1:1 with distilled water and filtered through a 0.45 μm nylon filter. These samples were subsequently injected into the HPLC chromatograph system.

The system consisted of a Waters 2690 Alliance with pump, column, heater, and autosampler modules included, the detection being carried out with a Waters 996 photodiode array detector. The system was controlled with Millenium32 software (Waters Inc.). A 25 cm × 46 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 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 was increased for the last three steps 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.

The evaluation of each compound was performed using a regression curve in triplicate of three different concentrations ($r^2 > 0.99$). Oleuropein was purchased from Extrasynthese (Lyon Nord, Genay, France); tyrosol was provided by Sigma Chemical Co. (St. Louis, MO). Noncommercial phenolic compounds were obtained by preparative HPLC as described elsewhere (*9*).

Sugar Analysis. Twenty grams of crushed olive flesh and 5 mL of sorbitol internal standard solution (1 g/L) were extracted with 20 mL of hot (60−70 °C) deionized water for 30 min; agitation was always maintained during this operation. The mixture was then poured into a 50 mL volumetric flask and completed with deionized water until level. The mixture was poured into a bottle and centrifuged at 10 000 rpm for 10 min. The liquid was separated and filtered under vacuum. Then, 2 mL of the solution was put into contact with 1 g of IRA 120 (H^+-form, 16−45 mesh, Fluka) and 1 g of IRA 96 (free base, 20−50 mesh, Fluka) resins, previously washed with enough deionized water until a colorless solution was obtained, to remove salts. After 1 h of contact, 0.5−1.0 mL was introduced into an Eppendorf tube and centrifuged again. The clarified liquid was then used for the analysis. Sugar analysis in brine followed a similar procedure with the exception of extraction.

Sucrose, glucose, fructose, and mannitol in the purified solution (from flesh or brine) were analyzed by HPLC. The system consisted of equipment similar to that described for the analysis of phenols. In this case, an Aminex HPX-87C carbohydrate analysis column (Bio-Rad Laboratories) held at 65 °C was used. Deionized water was used as eluent at 0.7 mL/min. Detection was achieved by a Waters 410 differential refractometer. Quantification was estimated with respect to the internal standard.

Sorbic Acid Analysis. Sorbic acid in brine was measured directly from the liquid after a 1:100 dilution and centrifugation at 8000 rpm for 8 min. Subsequently, the mixture was passed through a 0.45 μm pore size nylon filter and injected into the chromatograph system.

Sorbic acid in flesh was quantified according to the method of Brenes et al. (*10*). In brief, 1 g of triturated olive flesh was mixed with 25 mL of 0.2 M NaOH solution, the mixture being agitated in a vortex for 2 min and centrifuged at 10 000 rpm for 5 min. Subsequently, it was diluted 1:1 with water and acidified with phosphoric acid to pH 3.5, centrifuged again, and filtered through a 0.45 μm nylon filter. Finally, 20 μL was injected into the chromatograph.

The analysis of sorbic acid was carried out using a HPLC apparatus similar to that described for phenols. The detection was performed with a Waters 996 photodiode array detector. A 25 cm × 4.5 mm i.d., 5 μm, Spherisorb ODS-2 (Waters) column was used. Separation was achieved by gradient elution using an initial composition of 90% water with 2% of 0.005 M ammonium acetate buffered at pH 4.2 with glacial acetic acid and 10% methanol. The concentration of the latter solvent was increased to 30% in 10 min and maintained for 10 min. Subsequently, the methanol percentage was increased to 40% in 5 min and to 100% in a further 5 min. After each analysis, the column was washed with methanol for 5 min, and the initial conditions were reached in 15 min. A flow rate of 1 mL/min and a temperature of 35 °C were used. Chromatograms were recorded at 260 nm.

Microbiological Analyses. They included yeasts, lactic acid bacteria, Enterobacteriacea, and *Bacillus*. Brine samples and appropriate decimal dilutions were plated using a Spiral System model DS (Interscience, Saint Nom La Breteche, France). Plates were counted using a CounterMat v. 3.10 (IUL, Barcelona, Spain). Aerobic mesophilic microorganisms were enumerated on plate count agar (PCA) (Oxoid, Basingstore, Hampshire, U.K.). Those catalase-positive colonies with

Table 1. Average [and Its Standard Error (SE)], Maximum, and Minimum Physicochemical Characteristics (Texture, Moisture, *L**, *b**, pH, NaCl, and Color) of Fruits and Brines in Seasoned Packed Olives from Fresh (FF) and Stored (SF) Aloreña Cultivar during the Shelf-Life Period

	FF				SF			
	mean	SE	max	min	mean	SE	max	min
fruits								
texture, N/100 g of flesh	71.2	5.4 (7)[a]	79.5 (3)[b]	63.3 (7)[b]	58.4	5.6 (8)[a]	65.7 (28)[b]	47.8 (67)[b]
moisture,[c] % (w/w)	66.2	0.3 (4)	66.6 (0)	65.9 (6)	64.2	0.5 (3)	65.1 (0)	63.8 (7)
*L**	53.7	1.2 (7)	55.8 (0)	52.7 (15)	54.0	1.4 (7)	55.7 (23)	52.2 (9)
*b**	34.8	1.8 (7)	38.0 (0)	32.8 (15)	34.8	1.7 (7)	36.9 (16)	33.0 (3)
brine								
pH	4.12	0.12 (7)	4.31 (0)	4.01 (15)	4.17	0.09 (8)	4.29 (67)	4.01 (3)
NaCl, g/100 mL of brine	7.58	0.58 (7)	8.3 (0)	6.8 (10)	6.0	0.20 (8)	6.2 (44)	5.8 (67)
color, $A_{440} - A_{700}$			2.41 (28)	0.85 (0)			1.80 (67)	0.28 (0)

[a] Number of means used for its estimation. [b] Period (days) after packing at which the value was observed. [c] Wet basis.

suspicious morphology (rough and diameter ≥ 5 mm) were viewed with a phase contrast microscope (Olympus BH) for spores. Those showing spores were considered to be *Bacillus* spp. (*11*). Enterobacteriaceae were counted on VRBD (crystal-violet neutral-red bile glucose) agar (Merck), LAB on MRS (de Man, Rogosa and Sharpe) agar (Oxoid) with 0.02% (w/v) azide (Sigma), and yeasts on oxytetracycline glucose yeast extract (OGYE) agar (Difco, Becton and Dickinson, Sparks, MD) with 0.005% gentamicin sulfate (*12*). Plates were incubated at 30 ± 2 °C for 48−72 h. The samples were also inoculated into differential reinforced clostridial broth (Merck) to detect spores of clostridia (*13*). Colony-forming units (cfu/mL) were calculated, and $\mathrm{Ln}(N/N_0)$ versus time was modeled according to a modification of the Gompertz equation proposed by Zwietering et al. (*14*):

$$y = A \exp\{-\exp[((\mu_{max} \times e)/A)(\lambda - t) + 1]\}$$

$y = \mathrm{Ln}(N/N_0)$, where N_0 is the initial microbial population and N is the microbial population at time t. $A = \ln(N_\infty/N_0)$ is the maximum value reached with N_∞ as the asymptotic maximum population, μ_{max} is the maximum specific growth rate, and λ is the lag phase period.

Statistical Data Analysis. A first-order kinetic model was considered to follow the equation $P = P_0 \times e^{-kt}$, in which P_0 is the value of a characteristic at time $t = 0$ and P that at time t. The model was applied as $\mathrm{Ln}\ P = \mathrm{Ln}\ P_0 - kt$, to permit a visualization of the (Ln) initial values in the graph. The reciprocal of k, in similitude with the D definition in sterilization calculus, is the time required to decrease (or increase, depending on the sign) by 2.718 (one ln cycle) the value of a specific characteristic.

Statistica software version 6.0 (StatSoft, Tulsa, OK) was used for data processing. Gompertz equation and first-order kinetic fitting were obtained using the nonlinear module and the simple regression option from the General Linear Model module, respectively. Parameters were considered to be significant when $p < 0.05$ and were expressed as estimates ± standard errors.

RESULTS AND DISCUSSION

Effect on Physicochemical Parameters. Firmness of the product prepared from FF was higher than that from SF, which had a slightly wider range (**Table 1**). Apparently, values obtained for this parameter did not steadily decrease during the shelf-life time. In FF, maximum and minimum values were observed at the beginning of the period, whereas in SF they were observed at 28 and 67 days. This behavior contrasts with other studies that found a steady decrease in this attribute with time due to the enzymes in endogenous and dressing products (*3*, *4*) or the effect of the low pH (*15*). In our case, successive periods of texture deterioration, partial recuperation, and later diminutions (which are not observed in FF, possibly because of its shorter shelf life period) seem to occur. The partial texture recuperation period might be due to calcium absorption from brine (*15*). After equilibrium is reached, deterioration continues again. Moisture changes were also minimal. Seasoned olives from SF had only 2% (w/w) less moisture than those from FF, with a very slight tendency to diminish during the first days, reaching their minimum at 6 days in FF and at 7 days in SF. Then, moisture increased slightly as the shelf-life period continued. With respect to color, changes in *L** and *b** were small, and no clear tendencies between (or within) FF or SF were observed (**Table 1**). Olives presented great luminosity (high *L**) and a yellow predominance (positive value for *b**). Values of pH were similar in preparations from FF and SF (**Table 1**), showing a slight decrease with time in FF and an increase in SF, with similar ranges (0.3 unit). The combined effect of pH (∼4.1) and NaCl (6−7%), apparently, makes this product safe. NaCl content was higher in the case of FF, which also showed a higher dispersion (SE of 0.58 in FF vs 0.2 in SF). However, no changes in SF and a slight decrease in FF during the shelf-life period were apparent, indicating a rapid equilibrium between flesh and brine (cracked fruits).

Initial brine color (**Table 1**) (0.85 and 0.28 for FF and SF, respectively) was due to the rapid diffusion into the brines of colored compounds from the cracked olive flesh during transportation to the laboratory. The color increased with time, becoming relatively dark at the end of the shelf-life period. This progressive darkening can be due to the presence of polyphenols and sorbic acid. At the end of the period, the brine from FF with higher polyphenol content was markedly darker than that prepared from SF. These changes may constitute an index of product deterioration, and a level of 2.0 units may be proposed as a tentative maximum acceptable limit for this parameter.

Changes in olive surface color, expressed as *a**, increased with time (**Figure 1**). Color changed from green to red as the shelf-life time was longer, following a pseudo-first-order (term applied to reactions that can be described mathematically as first order although the reaction is not mechanistically first order) kinetic reaction ($p < 0.05$; $R^2 = 0.83$ and 0.89 for FF and SF, respectively). As with the other characteristics, reactions of zero and second order were also checked, but their fittings were always poorer. Initial negative *a** values in FF indicate a higher green component than in SF (positive *a**), but changes in FF were more rapid ($k_{a,FF} = 0.0785 \pm 0.0021\ \mathrm{day}^{-1}$) than in SF ($k_{a,SF} = 0.017 \pm 0.003\ \mathrm{day}^{-1}$), taking 12.74 and 59 days, respectively, to increase 2.718-fold *a**. This parameter, together with the brine color, can be used for the establishment of shelf-life limits. In this case, a value of $a^* = \exp(1.2) = 3.32$ could also be proposed as a tentative maximum acceptable level, because it corresponds with the color reached at the end of the commercial shelf-life period.

The so-called combined acidity in table olives, directly related to the buffer capacity of brine, increased with time. It also followed a pseudo-first-order kinetic (**Figure 2**) ($p < 0.05$; R^2

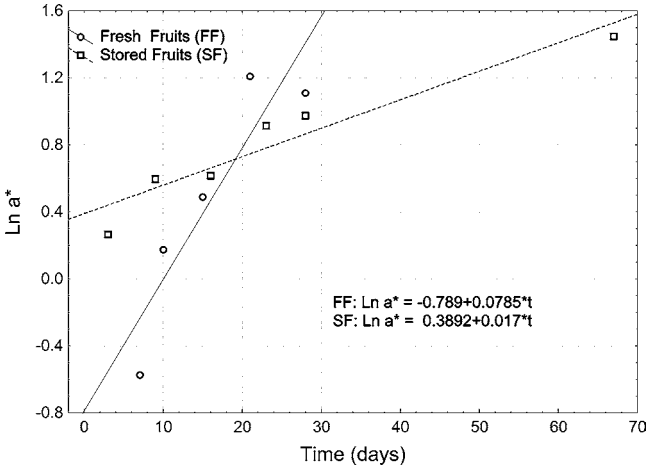

Figure 1. Changes in surface color of olives, expressed as *a**, with time for fresh (FF) and stored fruits (SF).

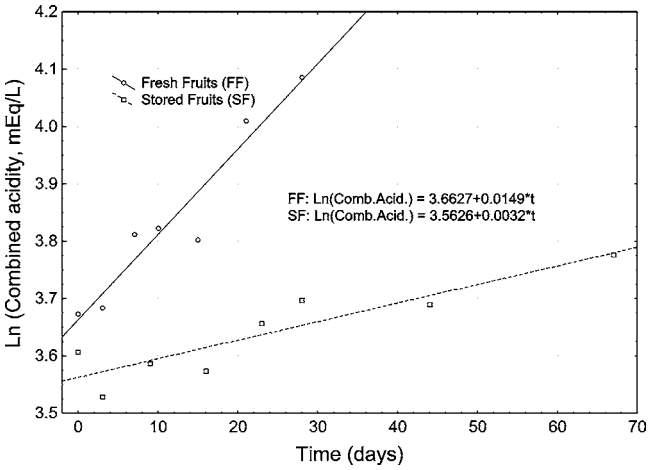

Figure 2. Changes in combined acidity of brine with time for fresh (FF) and stored fruits (SF).

= 0.93 and 0.83 for FF and SF, respectively). The initial values (due to the rapid diffusion into the brine of organic acids and salts from the cracked olives) were similar in products from FF and SF (~36 mequiv/L); however, the increase in FF was faster ($k_{ca,FF} = 0.0149 \pm 0.0019$ day^{-1}) than in SF ($k_{ca,SF} = 0.0032 \pm 0.0016$ day^{-1}). Theoretically, it will take 67 and 313 days, respectively, to increase the combined acidity 2.718-fold. The simultaneous increase in titratable acidity (see later) and the increase in combined acidity make it more probable that this was due to the production of acid by the microorganisms rather than to the leaching of organic salts from the flesh. This buffer capacity prevented an excessively low pH level as well as the deterioration of the fruit's color and texture (*15*). It may also contribute to a more standardized product flavor.

FF had a higher initial level of sugars than SF. However, sugar utilization followed a similar trend in both cases. Total sugar diminution in flesh and brine (expressed as 10 × concentration to separate lines) from FF followed a pseudo-first-order kinetic (**Figure 3**) ($p < 0.05$; $R^2 = 0.86$ and 0.99, respectively). Sugars were used in FF at an average rate of approximately $k = 0.060 \pm 0.007$ day^{-1} and were exhausted in <30 days in the case of SF (**Table 2**). The main substrate was glucose, followed by mannitol (**Table 2**). Lower contents of fructose and negligible concentrations of sucrose were also present (data not shown). Glucose was completely exhausted in SF, but a residual concentration of it was still observed at the end of the studied period in FF due to their higher initial

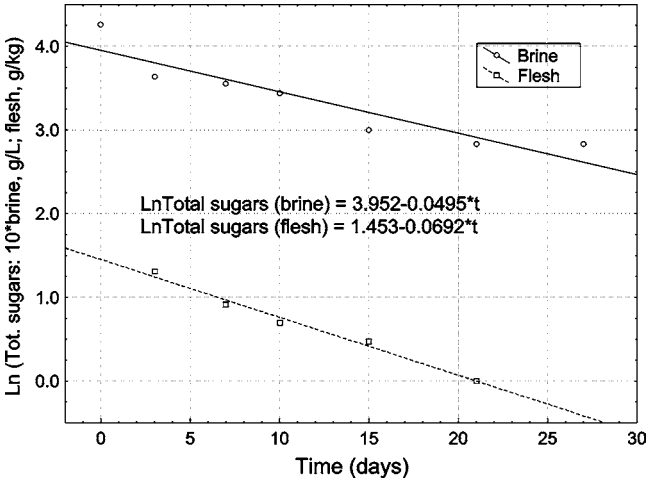

Figure 3. Changes in total sugar concentrations with time in the flesh and brine (concentration × 10) of fresh fruits (SF).

Table 2. Changes of Sugar Contents in Fruits and Brine of Seasoned Packed Olives from Fresh and Stored Aloreña Cultivar during the Shelf-Life Period

	flesh[a] (g/kg)		brine[b] (g/L)	
storage period (days)	glucose	mannitol	glucose	mannitol
fresh fruits				
0			4.3	1.0
3	1.3	1.3	0.8	1.8
7	0.6	1.4	0.6	2.3
10	0.4	1.3	0.5	2.2
15	0.6	0.8	0.3	1.4
21	0.4	0.6	0.2	1.4
27			0.1	1.5
stored fruits				
0	0.5	0.3	0.4	0.7
3	0.3	0.1	0.2	0.7
9	0.2	0.2	0.1	0.6
16	0.2	0.2	0.2	0.6
23	0.0	0.2	0.2	0.7
28	0.0	0.2	0.0	0.5
44	0.0	0.2	0.0	0.6
67	0.0	0.2	0.0	0.6

[a] SE < 10%. [b] SE < 5%.

content and shorter period of time. The presence of relatively high concentrations of mannitol must be emphasized. Apparently, this sugar is not metabolized (or it is used at a very slow rate) by the microflora present in seasoned olives. Concentrations in the flesh of FF were higher than in SF because of losses in SF during the previous storage in brine. In FF there was a slight decrease after 10 days. In brine, the concentration of mannitol remained constant in SF, whereas an increase followed by a slight decrease after 10 days (when it reached its maximum concentration) was observed in the case of FF. The presence of sugars in brine makes product stabilization difficult. Furthermore, their progressive diminution points to microbial activity during this period.

Increases in titratable acidity also followed a pseudo-first-order kinetic (**Figure 4**) ($p < 0.05$; $R^2 = 0.92$ and 0.73 for FF and SF, respectively). Changes were faster ($k_{ta,FF} = 0.031 \pm 0.004$ day^{-1}) in FF than in SF ($k_{ta,SF} = 0.022 \pm 0.011$ day^{-1}). Theoretically, it would take 32.3 and 45.5 days, respectively, to increase titratable acidity 2.7182-fold. FF reached a higher final titratable acidity (8 g/L) than SF (6 g/L). This acid production was due to the microbial activity (see later). The faster production and greater proportion in FF are a result of the higher sugar content in these olives. The production of acid

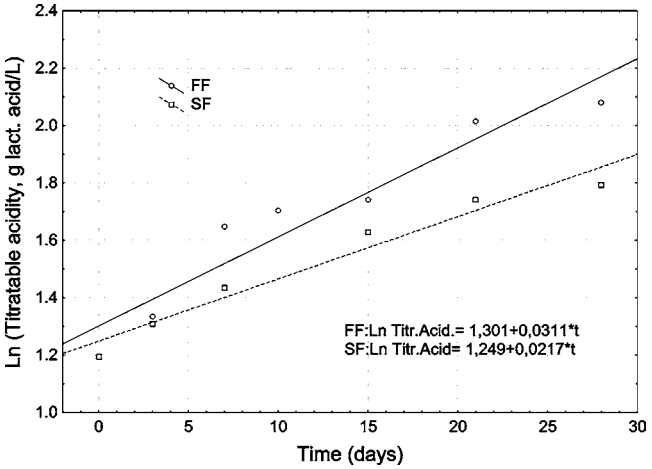

Figure 4. Changes in titratable acidity of brine with time for fresh (FF) and stored fruits (SF).

during shelf life is not a risk for consumers because it increases the added acid content and lowers the pH.

Seasoned olives are not lye-treated. Thus, the presence of phenolic compounds is an important factor from the organoleptic and microbiological points of view (*2*). Olives are bitter due to the presence of oleuropein. In lye-treated olives, oleuropein is hydrolyzed during the alkali treatment (*17*). However, in directly brined olives, debittering is, in principle, produced by a slow dissolution of oleuropein into the surrounding brine, where it later suffers an acidic hydrolysis (*9*). Concentration of this compound in fresh fruits was high (**Table 3**). However, hydroxytyrosol-4-β-D-glucoside was found in a higher proportion than oleuropein. Other phenolic compounds also present in marked proportions were salidroside, hydroxytyrosol, and tyrosol. Phenolic compound concentrations were higher in products from FF than in those prepared from SF because these have already lost part of their phenolic load during their previous storage in brine. In products obtained from both FF and SF, there was an evident decrease in the naturally occurring polyphenols (especially, oleuropein, and hydroxytyrosol-4-β-D-glucoside) with time, whereas those produced by their hydrolysis (hydroxytyrosol and tyrosol) increased progressively. Salidroside showed different patterns in FF and SF. Rutin was present in FF flesh for a short period ($\sim$10−15 days) and was absent in SF flesh. Data related to luteolin-7-glucoside were inconsistent. In general, concentrations of polyphenols in these preparations were lower than in green olives, higher than in ripe olives, and similar to those olives directly brined (*5*). The presence of oleuropein, which is absent in green and ripe olives (*5*), during the shelf-life period (FF) or part of it (SF) must be emphasized.

These olives are cracked as they are received in the factory, and diffusion of polyphenols from the flesh into the brine was very fast (**Table 4**). Consequently, the concentration of all phenols in brine was noticeable from the very beginning of packing and even higher than in the corresponding flesh due to the marked extraction during the 3 h period of transportation to the laboratory. Naturally occurring phenolics increased in brine during a certain period and then decreased progressively. Verbascoside, *p*-coumaric acid, and luteolin-7-glucoside followed a similar trend but with lower levels. Rutin was present in FF brine for only $\sim$10−15 days and was completely absent in SF brines. On the contrary, hydroxytyrosol concentration increased dramatically due to its formation by the hydrolysis of oleuropein and hydroxyrosol-4-β-D-glucoside. Tyrosol also increased, but its formation was slower due to the slower hydrolysis of salidroside. Its final content is also lower because its source is lower as well. This high concentration of phenols affects not only the organoleptic characteristics of the product but also its microbial population and its evolution.

The presence of high concentrations of polyphenols in flesh (**Table 3**) makes this elaboration a rich source of antioxidants and an interesting product to include in the habitual diet. In fact, due to its high consumption in the Mediterranean area, at least a part of the benefits attributable to the Mediterranean diet could come from seasoned olives, which have been the homemade elaboration traditionally consumed in the rural areas. In addition, the dietary fiber in table olives is also notable (*3*, *4*). Thus, seasoned olives can be considered as a good source of the recently defined antioxidant dietary fiber (*18*).

Effect on Microbial Population and Potassium Sorbate. The microbial population in seasoned olives is determined by the physiochemical characteristics of the fruit, packing conditions (type of acid, level of salt, pH, and preservative), and the natural herbs or spices added. Concentrations in nutrients and in inhibitory compounds in seasoned (non-lye-treated) are higher that in green olives (*2*). Thus, favorable and stressing factors to microbial growth are more relevant. Essential oils from herbs and spices can also affect the growth of yeasts related to the olive fermentation (*19*). The essential oils from Andalusian thyme, sage, winter and red thyme, lavender, rosemary, and fennel were, in the order mentioned, inhibitory for such yeasts in concentrations that ranged from 0.025−0.040 to 1.0% (*19*). A common drawback in all olive products is the relatively low proportion of proteins and available amino acids. In fact, Casolari et al. (*20*) found that *Clostridium botulinum*, *Satphylococcus aureus*, and *Staphylococcus typhimurium* were unable to spoil fermented green olives due to the low amino acid content ($<$0.1−0.5 mg/100 g) in olives. However, the product was able to support a previous fermentation process.

In seasoned olives of the Aloreña cultivar, regardless of the raw material, the initial population of Entrobacteriacea was $\sim$3 log cfu/mL, which disappeared in a 24−48 h period. *Bacillus* was never detected. The rapid elimination of Entrobacteriacea and the absence of *Bacillus* are mainly due to the acidic pH of the product and the appropriate olive and ingredient handling during processing. However, yeasts and LAB were present during the shelf-life period despite the use of sorbic acid as a preservative.

In the FF (**Figure 5**), the initial yeast population (4.7 log cfu/mL) progressively increased to 6.0 log cfu/mL. The growth could be described as a typical sigmoid curve, and their biological parameter obtained (**Table 5**). Values of λ and μ_{max} were 17 days and 0.167 day^{-1}, respectively. With respect to the LAB (4.8 log cfu/mL initial counts), after an initial delay/inhibition period, they experienced a limited growth (5.3 log cfu/mL final population). Changes in LAB were not consistent and could hardly be described in terms of biological parameters. However, this LAB population, despite its growth limitations in FF, was able to produce titratable acidity and at a higher rate than in SF (**Figure 4**). The microbiological behavior of fresh seasoned packed olives can be attributed to the physicochemical characteristic of the olives, mainly to the presence of high initial proportion of sugars and phenolic compounds, which are inhibitory for LAB. The presence of sorbic acid (**Table 6**) delayed the yeast growth for only 13 days. Later, the progressive decrease in sorbic acid content in both flesh and brine permitted the growth of yeasts. Apparently, the concentration of sorbic acid used in packing was not sufficient to ensure product stability because other studies (*21*) had shown that 0.13 and

Table 3. Changes of Polyphenol Contents in Fruits of Seasoned Packed Olives Prepared from Fresh and Stored Aloreña Cultivar during the Shelf-Life Period

shelf-life period (days)	polyphenol[a] (mg/kg)							
	Oleu	HyGlu	Hy	Sal	Ty	Verb	Lut7gluc	rutin
fresh fruits								
0	103.43	194.87	49.60	51.68	15.68	0.29	0.00	9.15
10	92.07	107.31	120.62	18.94	30.88	0.69	3.12	13.29
28	46.82	0.00	366.97	23.52	63.20	0.22	0.00	0.00
stored fruits								
0	34.68	29.25	93.90	26.89	23.85	0.28	6.26	0.00
16	14.63	0.00	168.88	32.48	26.25	0.11	0.00	0.00
67	0.00	0.00	299.31	42.01	45.81	0.00	0.00	0.00

[a] Oleu, oleuropein; HyGlu, hydroxytyrosol 4-β-D-glucoside; Hy, hydroxytyrosol; Sal, salidroside; Ty, tyrosol; Verb, verbascoside; Lut7Glu, luteolin-7-glucoside. Salidroside was quantified as tyrosol (using the calibration curve of tyrosol). SE < 15%.

Table 4. Changes of the Polyphenol Content in the Brine of Seasoned Packed Olives Prepared from Fresh and Stored Aloreña Cultivar during the Shelf-Life Period

shelf-life period (days)	polyphenol[a] (mg/L)								
	Oleu	HyGlu	Hy	Sal	Ty	Verb	Acpcum	Lut7gluc	rutin
fresh fruits									
0	131.14	151.20	85.46	29.75	19.53	1.88	5.14	18.04	2.27
3	160.36	223.16	185.78	49.36	45.93	-	12.00	16.01	44.58
7	213.23	222.12	251.35	54.92	54.04	3.47	9.63	15.31	41.42
10	246.56	177.82	296.52	45.34	67.06	4.02	9.18	18.34	46.76
15	98.54	15.42	400.83	68.64	87.30	3.72	4.66	8.94	0.00
21	119.75	10.13	500.71	165.88	99.00	2.22	11.13	12.31	0.00
28	24.63	0.00	567.67	42.51	106.69	0.00	3.25	8.33	0.00
stored fruits									
0	56.84	37.55	221.84	30.97	36.19	0.91	5.03	6.71	0.00
3	55.84	26.87	261.68	-	45.76	1.42	2.79	8.33	0.00
9	51.31	5.37	314.03	48.28	52.57	1.22	3.50	5.94	0.00
16	31.73	0.00	368.59	88.76	64.50	0.00	2.24	3.68	0.00
23	32.36	0.00	406.71	100.24	68.55	0.00	0.00	2.96	0.00
28	0.00	0.00	415.43	97.43	73.95	0.00	0.00	2.98	0.00
44	0.00	0.00	422.33	85.80	71.18	0.00	0.00	2.88	0.00
67	0.00	0.00	493.25	82.17	88.56	0.00	0.00	0.00	0.00

[a] Oleu, oleuropein; HyGlu, hydroxytyrosol 4-β-D-glucoside; Hy, hydroxytyrosol; Sal, salidroside; Ty, tyrosol; Verb, verbascoside; Acpcum, *p*-coumaric acid; Lut7Glu, luteolin-7-glucoside. Salidroside was quantified as tyrosol. SE < 10%.

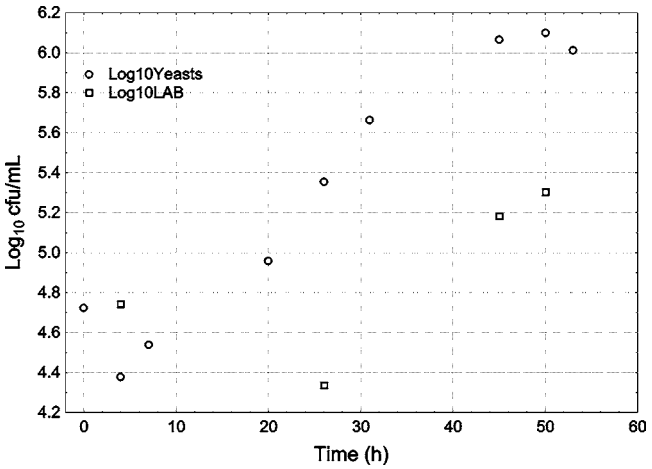

Figure 5. Changes in yeast and lactic acid bacteria (LAB) populations with time in the brine of fresh fruits (FF).

0.25% with respect to the brine volume led to an effective preservation period of at least 2 months. Longer periods required higher concentrations.

With respect to the presence of LAB, its limited growth in FF (**Figure 5**) was not due to the addition of sorbic acid since Turantas et al. (*22*) reported that LAB grew rapidly and produced a vigorous fermentation in naturally black olives in the presence of this preservative. In fact, some studies suggest that LAB generally tolerate sorbates and that sorbates exert a selective inhibition against all catalase-positive microorganisms and can be used as selective agents for catalase-negative LAB (*23*). However, sorbate concentrations of 1000−2000 ppm slightly inhibit the desired LAB population (*24*).

Table 5. Biological Parameters Related to the Evolution of the Microbial Population in the Brine of Seasoned Packed Olives Prepared from Fresh and Stored Aloreña Cultivar during the Shelf-Life Period

	A		μ_{max} (day^{-1})		λ (days)	
	estimate	SE	estimate	SE	estimate	SE
fresh fruits						
yeasts	3.16	0.30	0.167	0.065	17.22	3.46
stored olives						
yeasts	–4.37	0.80	–0.226	0.033	3.92	0.93
LAB	2.78	0.14	2.050	0.690	0.81	0.26

In seasoned olives prepared from SF (**Figure 6**), there was an inhibition of the initial yeast population (5.1 log cfu/mL) with time, but the curve showed a shoulder at the beginning and a tail, with a final asymptotic yeast population of 3.3 log cfu/mL remaining in the brine. The LAB population (5.6 log cfu/mL initial count) grew rapidly during the first days after packing and then stabilized at 6.9 log cfu/mL. Changes in the microbial population (**Figure 6**) could be expressed in terms of their biological parameters (**Table 5**). The lag phase for yeasts was ~4 days and their maximum specific dead rate 0.226 day^{-1}.

Table 6. Changes in the Sorbic Acid Content in the Flesh and Brine of Seasoned Packed Olives, Prepared from Fresh and Stored Aloreña Cultivar, during the Shelf Life Period

shelf-life period (days)	sorbic acid concn (mg/kg)	
	flesh[a] (mg/kg)	brine[b] (mg/L)
fresh fruits		
0	65.71	55.84
3		36.23
7		31.40
10		25.86
15		12.16
21		13.52
28	19.82	12.02
stored fruits		
0	68.35	22.82
3		19.17
9		14.26
16		9.72
23		1.75
28		0.00
44		0.00
67	0.00	0.00

[a] SE < 10%. [b] SE < 5%.

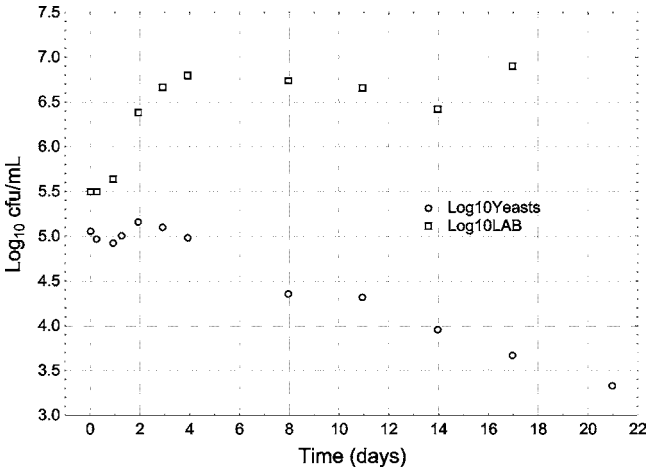

Figure 6. Changes in yeasts and lactic acid bacteria (LAB) populations with time in the brine of stored fruits (SF).

The residual presence of yeasts could be due to the progressive decrease in sorbic acid concentration, which was completely absent after ~25 days (**Table 6**), or the presence of some sorbic-resistant strains. On the contrary, LAB grew rapidly (μ_{max} = 2.05 day^{-1}) from the packing moment (λ = 0.81 days). This different behavior with respect to FF may be explained by the abundance of sugars in these fruits, which were able to support the yeast growth despite the presence of a moderate concentration of sorbic acid [half the maximum limit permitted by the Unified Qualitative Standard Applying to Table Olive in International Trade (*1*)]. However, LAB found a more stressing environment in such a product and experienced a limited growth. On the contrary, in SF, the lower sugar content, about half of that in FF, and the sorbic acid were more effective in inhibiting the yeast population; but the presence of polyphenols was lower, especially at the beginning of the shelf-life period, and permitted the rapid growth of LAB, which exhausted the remaining sugars. This lack of fermentable substrate in combination with the progressively higher phenol content resulted in the stabilizing of also the LAB population in SF.

Sorbic acid concentrations in FF and SF flesh were higher than in the brine due to the rapid absorption of this compound by the fat of cracked olives. The sorbic acid destruction in brine followed a pseudo-first-order kinetic ($p < 0.05$; $R^2 = 0.85$ and 0.86 for FF and SF, respectively) with constants $k_{s,FF} = -0.056 \pm 0.011$ day^{-1} and $k_{s,SF} = -0.102 \pm 0.024$ day^{-1}. Thus, the disappearance rate was higher in SF than in FF. Apparently, due to the lower sugars in SF, some of the microorganisms present in this product might eventually use sorbic acid as a carbon source. These results contrast with those found by Turantas et al. (*22*), who reported that, during the fermentation process of naturally black olives, the sum of the concentrations of sorbic and benzoic acids in the brine and flesh resembled the initial concentrations of preservatives added to the brine at the beginning and, in consequence, the sorbate and benzoate in brine were not metabolized by the microbial population present in such a process. The effect of sorbic acid on mold was more effective than on yeasts (*22*). Results of this study are also in agreement with such findings because no mold was detected in any treatment.

This work has shown that the habitual packing conditions used for preparing seasoned olives are good enough for the local markets, but they do not completely stabilize the product. Changes during the shelf-life period led to an increase in the titratable acidity, a decrease in sugar content, growth of microorganisms, and progressive destruction of sorbate. LAB were active and produced a marked titratable acidity in both FF and SF, although growth was more rapid at the initial period of SF. Yeast growth was evident in FF but was inhibited in SF, in which the yeast eventually died. These changes cannot be considered a risk for consumers. However, other changes such as fruit and brine darkening considerably shorten the commercial shelf life of the product.

ACKNOWLEDGMENT

We are grateful to Dr. Francisco Rodríguez Gómez for technical assistance.

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Received for review March 7, 2005. Revised manuscript received May 4, 2005. Accepted May 10, 2005. This work was supported by the Spanish government (AGL-2002−00779), EU (TDC-olive), and Junta de Andalucía (through financial support to group AGR-125).

JF050501+