Effect of Storage Process on the Sugars, Polyphenols, Color and Microbiological Changes in Cracked Manzanilla-Aloreña Table Olives

F. N. Arroyo-López,* M. C. Durán-Quintana, C. Romero,
F. Rodríguez-Gómez, and A. Garrido-Fernández

Departamento de Biotecnología de Alimentos, Instituto de la Grasa (C.S.I.C.); Avenida Padre García
Tejero Number 4, 41012, Seville, Spain

The green cracked "seasoned" Manzanilla-Aloreña table olive is a specialty with a high demand when prepared from fresh fruits; however, when stored fruits are used, the product loses its green color, presents a brownish tone, and loses demand. Different alternative storage systems for preventing such changes and preserving the freshness of the fruits were studied, and their effects on sugar, polyphenol, color, and microbiological changes were analyzed. The application of two washing waters in the presence of different compounds before brining markedly decreased the sugar and polyphenol contents in the flesh, without negatively influencing the color; it also caused the inhibition of yeasts and lactic acid bacteria (except in treatments using sodium metabisulfite and saturated carbon dioxide (CO_2) in the storage olive brines. Salicylic acid inhibited microbial growth during washings and storage. The best long-term color was achieved in the presence of sodium metabisulfite. A combination of two washing waters (containing 5% sodium chloride (NaCl) and 0.1% sodium metabisulfite or saturated CO_2), followed by immersion of the fruits in 15% NaCl brine with 0.1% sodium metabisulfite or brine under saturated CO_2 added, led to the best storage conditions.

KEYWORDS: Table olives; storage; sodium metabisulfite; ascorbic acid; oxalic acid; salicylic acid; carbon dioxide; color of fruits; sugars; polyphenols

INTRODUCTION

Current worldwide table olive production is about 1 700 000 tons/year. Most of the olives belong to the so-called green Spanish-style, natural black (Greek-style), and ripe (Californianstyle) olives (*1*). However, there are many other traditional table olive elaboration recipes. They are usually prepared with the addition of some natural products like garlic, fennel, thyme, etc., at the very beginning of their elaboration and are eaten as soon as they have reduced their bitterness. Some of these specialties have progressively gained the favor of consumers and are increasing their productions. The International Olive Oil Council (IOOC) was sensible to their progressive importance by including them under the heading "Specialties" in the current "Trade Standard Applying to Table Olives" (*2*). "Seasoned" Manzanilla-Aloreña table olives, which are picked when they reach the green maturation stage, may be included in this group. Its production reaches about 7 000 000 kg/season and plays a marked role in the economy of northern Malaga (Spain). In addition, they may also represent a model for similar products elaborated in any other table olive producing country. During processing, the fresh fruits are cracked, "seasoned", and commercialized on a local scale in plastic containers containing garlic, fennel, thyme, and pimento strips. The freshness of the product (related to the retention of the natural green color of the fresh fruits) is highly appreciated by consumers (*3*). However, the entire production cannot be sold fresh and part of it must be stored (and partially fermented) while awaiting demand. Initially, these fruits were preserved in a similar way as other directly brined olives (*4*), but they were prone to softening. To improve storage, a process using about 1% acetic acid and a low initial sodium chloride (NaCl) concentration to facilitate lactic acid fermentation was developed (*5*), but olives rapidly lost their green color and became brownish. Packed "seasoned" olives made from these stored fruits then have a more limited demand and reach sensibly lower prices in the market.

Storage of the cracked olives in a cold room, using 11% NaCl (wt/vol) brine, was recently applied to maintain the esteemed green color and the product's freshness longer. The procedure is becoming more and more common but the low storage temperature prevents the consumption of sugars that pass into the final "seasoned" packed product and may then facilitate microbial growth and the subsequent product spoilage. In addition, the process is expensive. So research for the improvement of storage at room temperature is necessary since no

* Corresponding author. Dr. Francisco Noé Arroyo López, Phone: 34-954-692516. Fax: 34-954-691262. E-mail: fnarroyo@cica.es

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Table 1. Changes in Sugar and Polyphenol Contents during Washings of Cracked Manzanilla-Aloreña Olives[a]

		olive flesh (mg/kg)		exhausted washing waters (mg/L)	
parameter	raw material	after 1st washing	after 2nd washing	1st washing (24 h)	2nd washing (24 h)
sugars					
sucrose	1496(74)	1159(460)	906 (532)	699 (362)	33 (89)
glucose	23040 (706)	15207 (3247)	14067 (4363)	5004 (2236)	2994 (945)
fructose	4799 (219)	3642 (780)	3279 (948)	1639 (507)	1176 (174)
mannitol	3184 (165)	2024 (472)	1917 (653)	1014 (333)	702 (45)
total	32519 (380)	22032 (380)	20169 (6032)	8356 (3360)	4905 (1086)
polyphenols					
HxGluco		425 (31)	376 (36)	135 (17)	104 (24)
hydroxytyrosol		76 (15)	75 (21)	55 (5)	64 (8)
tyrosol		52 (24)	48 (26)	13 (1)	14 (2)
L-7-Glucoside		55 (18)	52 (18)	11 (10)	6 (5)
rutin		43 (13)	47 (18)	18 (8)	12 (7)
oleuropein		127 (73)	238 (149)	190 (154)	127 (84)
verbascoside		1.0 (0.5)	1.0 (0.5)	2 (1)	2.0 (0.5)
tyrosol-glucoside		124 (8)	113 (108)	17 (6)	22 (5)

[a] Degree of freedom in data from treatments was 6, whereas data for the raw material comes from two replicates. Standard deviation in parentheses. Abbreviations used: HxGluco, hydroxytyrosol glucoside; L-7-Glucoside, luteolin-7-glucoside.

Table 2. Microbial Growth and Mean Physicochemical Characteristics of Washing Waters of Cracked Manzanilla-Aloreña Olives[a]

treatment	yeast log cfu/mL	LAB log cfu/mL	*Enterobacteriaceae* log cfu/mL	pH	color absorption 400–700 nm	total sugars mg/L	total polyphenols mg/L
ascorbic acid							
1st wash	4.94	nd	2.9		0.443	8721	481
2nd wash	5.06	3.2	4.3	3.60	0.083	5044	454
sodium metabisulfite							
1st wash	4.6	1.3	4.1		0.523	8363	287
2nd wash	4.8	4.1	5.3	4.60	0.259	5070	234
oxalic acid							
1st wash	4.9	1.6	2.5		0.529	7977	702
2nd wash	5.6	3.5	3.1	3.18	0.123	5479	370
salicylic acid							
1st wash	1.6	nd	nd		0.645	9118	570
2nd wash	nd	nd	nd	3.72	0.231	6259	498
saturated CO_2							
1st wash	5.0	nd	3.6		0.669	8797	304
2nd wash	7.7	3.9	5.0	4.56	0.213	1076	295
control							
1st wash	5.4	1.6	4.5		0.717	7163	305
2nd wash	6.3	4.1	5.4	4.54	0.362	3144	262

[a] NaCl in all treatments was 5% (wt/vol). Data are average from two replicates. Relative standard deviation was always below 5%. nd, not detected.

physicochemical or microbiological study of stored cracked olives has yet been carried out.

The fermentation process that takes place during the storage of Manzanilla-Aloreña cracked fruits is thought to be responsible for some of the negative effects on the quality of the fruits and, especially, the loss in their fresh appearance (*3*). Therefore, a decrease in the available sugar content or a partial or total microbial inhibition might prevent such changes. The use of antioxidants during storage could delay browning. The anti-browning effect of ascorbic acid in apples and peas is well documented (*6, 7*). The use of ascorbic acid in table olives is permitted by the "Trade Standard Applying to Table Olives" (*2*).

Sulfites have antioxidant and antimicrobial properties (*8*). Sulfites have been used as alternative microbial preservatives in the storage of red bell peppers in a salt-free acidified cover solution (*9*). Oxalic acid inhibits polyphenol oxidase (PPO) activity, due to its binding with copper to form an inactive complex which diminishes catechol−quinone product formation (*10*). Salicylic acid has also been reported to inhibit the browning of the collision spots on green olives (*11*). The presence of carbon dioxide (CO_2) apparently also decreases PPO activity

(*12*). A detailed study of these compounds on microbial growth can be found in *Antimicrobial Food Additives* (*8*).

The aim of this work was to investigate the effect of alternative storage systems for the cracked Manzanilla-Aloreña cultivar, using potential antibrowning/antioxidant or preservative agents, and assess their effects on (i) the kinetics of sugars, polyphenols, and microbiological changes, (ii) the kinetics of parameters related to freshness, and (iii) the selection, based on these changes, of the most effective previous storage systems for fruits which could later be used for preparing "seasoned" cracked olives.

MATERIALS AND METHODS

Samples and Experimental Design. Fresh cracked Manzanilla-Aloreña olives (a Manzanilla cultivar especially adapted to the Álora area (Málaga, Spain)) were obtained directly from the industry (Aceitunas Bravo S.A., Alhaurín el Grande, Málaga, Spain). Usually, cracked fruits are obtained by passing the olives between two stainless plates in which the separation is controlled by an eccentric device which is fitted to the upper plate. Olives go over the fixed plate (base) while the plate above it falls on the olives. The lowest distance between plates is graduated according to olive size so that the pressure cracks the olive flesh while the pit remains intact. For each treatment, 6 kg of cracked

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Table 3. Kinetic Parameters and Final Residues of Sugar in the Flesh of Stored Cracked "Manzanilla-Aloreña" Olives with and without Previous Washings

treatment/sugar	kinetic parameters			residual content (mg/kg)
	S_0 (mg/kg)	k (h^{-1})	t_{50} (h)	
Washed Olives				
all treatments (except salicylic acid)				
sucrose	1158 (79)[a]	0.004 (0.00063)	173	69 (64)[d]
glucose	12428 (737)	0.030 (0.0070)	23	335 (205)
fructose	2819 (149)	0.032 (0.0064)	22	264 (50)
mannitol	1687 (119)	0.031 (0.0084)	22	34 (18)
total	17655 (1040)	0.030 (0.006)	23	702 (247)
salicylic acid				
sucrose	1454 (271)[b]	0.002 (0.0009)	347	129 (7)[e]
glucose	11945 (806)	0.060 (0.0163)	12	3641 (170)
fructose	2440 (189)	0.058 (0.0175)	12	1304 (58)
mannitol	1646 (141)	0.051 (0.0160)	12	288 (79)
total	15570 (1436)	0.050 (0.019)	14	5362 (313)
Unwashed Olives				
all treatments				
sucrose	1501 (337)[c]	0.001 (0.0007)	693	56 (20)[f]
glucose	18649 (759)	0.010 (0.0008)	69	1006 (394)
fructose	3810 (173)	0.007 (0.0011)	99	164 (39)
mannitol	2626 (141)	0.005 (0.0009)	138	12 (6)
total	26159 (1046)	0.010 (0.001)	96	1238 (451)

[a] Degrees of freedom for average below heading is 51. Standard error in parentheses. [b] Degrees of freedom for average below heading is 8. Standard error in parentheses. [c] Degrees of freedom for average below heading is 36. Standard error in parentheses. [d] Degrees of freedom for average below heading is 7. Standard error in parentheses. [e] Degrees of freedom for average below heading is 1. Standard error in parentheses. [f] Degrees of freedom for average below heading is 4. Standard error in parentheses.
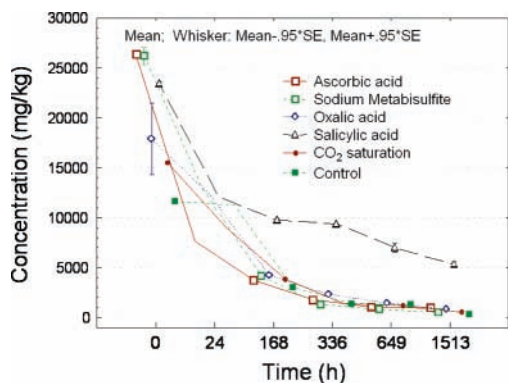
Figure 1. Sugar content changes in the flesh of washed cracked Manzanilla-Aloreña olives. When error bars are not visible, determinations were within the range of the symbols on the graph.

olives were put into 10 L plastic containers and subjected to two washings (24 + 24 h) with 5% NaCl (wt/vol) brine added separately with each of the following compounds: 1% ascorbic acid, 0.1% sodium metabisulfite, 0.25% oxalic acid, 0.25% salicylic acid, and carbon dioxide (CO_2) to saturation, making thus a total of five different treatments. Concentrations of the chemicals were adapted from previous values used in the literature, while saturated CO_2 was selected to remove as much oxygen as possible from the brine and was chosen because of its easy achievement in the current olive storage fermenters. A control was washed with only 5% NaCl brine. Then, 15% NaCl (wt/vol) brines, containing the same compounds and concentrations, were added to the appropriate treatment. A brine of only 15% NaCl was added to the control. The experiment was repeated with unwashed olives 15 days later (to facilitate their physicochemical and microbiological control and to compare the effect of the washing treatments on the further storage process), except for those using oxalic and salicylic acid treatments due to their observed unfavorable effects on the surface color

in the first experiment. All treatments were carried out in duplicate and their physicochemical and microbiological characteristics monitored for 3 months.

Physicochemical Analysis. The analyses of olive brines for pH, titratable acidity (expressed as g lactic acid/100 mL brine), combined acidity (as milliequivalents of HCl acid added to 1 L of brine to reach pH 2.6, mEq/L), and salt (g NaCl/100 mL of brine) were carried out as described by Garrido Fernández et al. (*4*).

Texture was measured using a Kramer shear compression cell coupled to an Instron universal testing machine model 1001 as described by Arroyo López et al. (*13*). The cross head speed was 200 mm/min. The firmness of the olives was expressed as the mean of 8−10 measurements, each of which was performed on 2−4 pitted olives. Shear compression force was expressed as N/100 g pitted olives.

Superficial color analyses on olives (to evaluate the changes in freshness, related to the retention of the natural green color of the fruits) were performed using a BYK-Gardner model 9000 Color-view spectrophotometer, equipped with computer software to calculate the CIE coordinates $L*$ (lightness), $a*$ (red-green), and $b*$ (yellow-blue) values. Interference by stray light was minimized by covering samples with a box, which had a matte black interior. The data of each measurement are the mean of 20 olives. Changes in the $a*$ parameter was modeled according to a pseudo-second-order kinetic model that gave the following equation:

$$a* = a_0^* + \frac{dt}{c + t} \qquad (1)$$

were a_0^* is the constant (interception), d is a specific value for each fit, and c is the time required to reach a 50% decrease in the green (or 50% formation red) color. The kinetic constant was estimated from these two parameters as $k = 1/(dc)$ with the units h^{-1} (*14*).

The color of the brine was estimated as the difference in absorbance at 400 and 700 nm (*15*). Previously, liquids were centrifuged at 10 000 rpm for 10 min.

Polyphenols in fruits and brines were analyzed by high performance liquid chromatography (HPLC) as described Romero et al. (*16*), using a Waters 2695 Alliance chromatograph and a 25 cm × 46 mm i.d. 5 μm Lichrospher 100 (Merck, Darmstard, Germany) column. Sucrose, glucose, fructose, and mannitol in fruits and brines were analyzed by HPLC as described Sánchez et al. (*17*), using an Aminex HPX-87C carbohydrate analysis column (Bio Rad Labs) held at 85 °C. Sugar decrease in flesh was considered to follow a first-order decay kinetic model according to the equation:

$$S_{(t)} = S_0 \, e^{-kt} + S \qquad (2)$$

where the term S_0 is an estimated value for the initial sugar concentration at time $t = 0$, k is a kinetic parameter (unit is t^{-1}) which is a measurement of the rate of change, S the residual sugar content, and t_{50} is the time needed to decrease the sugar concentration to half (ln 2/k).

Microbiological Analyses. Brine samples and appropriate decimal dilutions were plated using a Spiral System model DW Scientific (Don Whitley Scientific Limited, England) on appropriate medium. Plates were counted using a CounterMat v.3.10 (IUL, Barcelona, Spain) image analysis system. *Enterobacteriaceae* were counted on VRBD (crystal-violet neutral-red bile glucose) agar (Merck, Darmstadt, Germany) after incubation at 37 °C for 24 h. Lactic acid bacteria (LAB), specifically *Lactobacillus* spp and mesophilic lactobacilli, were counted on MRS (de Man, Rogosa and Sharpe) agar (Oxoid Ltd, Basingstoke, Hampshire, England) with 0.02% (wt/vol) sodium azide (Sigma, St. Louis, MO) and yeasts on yeast−malt−peptone−glucose medium (YM agar, Difco, Becton and Dickinson Company, Sparks, MD) with oxytetracycline and gentamicin sulfate as selective agents for yeasts; plates were incubated at 30 °C for 48−72 h. Colony forming units (CFU/mL) were calculated. Growth was modeled according to a modification of the Gompertz equation proposed by Zwietering et al. (*18*):

$$y = A \exp\{-\exp[((\mu_m e)/A)(\lambda - t) + 1]\} \qquad (3)$$

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where $y = \ln(N/N_0)$, N_0 is the initial microbial population, N 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_m is the maximum specific growth rate, and λ the lag phase period.

Microbial inactivation during storage was modeled according to the Weibull distribution using the survival function:

$$\log S_{(t)} = \log(N/N_0) = -(t/D_\beta)^\beta \qquad (4)$$

where $S_{(t)}$ is the fractional count (N/N_0), D_β is a transformed scale parameter ($D_\beta = \alpha(\ln(10))^{1/\beta}$), usually referred to as the scale parameter, and β is the shape parameter. The parameter D_β represents the time taken to achieve the first decimal reduction ($S_{(t)} = 0.1$). When $\beta = 1$, D_β in eq 4 is equal to the decimal reduction time (D-value). When $\beta \ne 1$, parameters D_β and β are needed to predict the reduction level of the microbial population (*19*).

The partial inactivation and later growth observed in some treatments was modeled according to the following equation, proposed by Pruitt and Kamau (*20*):

$$N = N_{max}/(1 + \exp(-\mu(t - \tau))) + N_{dying} \exp(-\alpha t) \qquad (5)$$

where N is the population size at time t, N_{max} is the maximum population reached after growth, μ is the growth rate, and τ is the time at which the growth reaches $N_{max}/2$. N_{dying} is the part of the subpopulation fatally damaged by the effect of the applied treatment, and α is a dead rate constant in the inactivation period. N_{max} was considered a parameter, making a total of five estimates.

Statistical Data Analysis. Statistica software version 6.0 (StatSoft, Tulsa, OK) was used for data processing. Microbial modeling equations and first-order kinetic fitting were obtained using the nonlinear and the simple regression options of the software. Parameters were considered significant when $p < 0.05$ and expressed as estimates of $\pm$ standard errors.

RESULTS AND DISCUSSION

Effect of Washing on Cracked Fruits. The total amount of sugars in fresh olives was about 32 500 mg/kg. The application of the first washing removed about 10 000 mg/kg and left the olives with 22 000 mg/kg, which still decreased to $\approx$20 000 mg/kg at the end of the second washing (**Table 1**). This rapid elimination of sugars by the washing waters was possible because cracked olives permitted an easy exchange between the water solutions and the flesh. On average, the exhausted washing waters contained about 8000 and 5000 mg/L in the first and second washing waters, respectively. So both washing waters removed about 13 000 mg/L sugars from the olive flesh, an elimination that markedly reduced the available fermentation substrates during the subsequent storage phase. In whole fruits, the exchange between olives and the surrounding solutions was very slow because of the barrier of the olive skin and was practically unappreciable after 24 or 48 h (*4*).

The only debittering mechanism applied to these olives (which are not lye treated) is dilution and subsequent hydrolysis of oleuropein. Washing waters removed a significant proportion of olive polyphenols (**Table 1**), mainly hydroxytyrosol glucoside (first washing, 135 mg/L, second washing, 104 mg/L) and oleuropein (first washing, 190 mg/L; second washing, 127 mg/L). The proportion of polyphenols removed decreased with the successive washings. After the washings, the most prominent polyphenols in the olive flesh were hydroxytyrosol glucoside (376 mg/kg) and oleuropein (238 mg/kg). As a result, the olives became sweeter (due to the direct relationship between polyphenol content and bitterness) and their storage period could be shortened. A reduced content in polyphenols could also lead to a lower inhibitory effect on LAB in the subsequent storage

Table 4. Kinetic Parameters and Final Residues of Sugar in the Brines Storage of Cracked Manzanilla-Aloreña Olives with and without Previous Washings[a]

treatment/sugar	kinetic parameters			residual content (mg/kg)
	S_0 (mg/kg)	k (h^{-1})	t_{50} (h)	
Washed Olives				
ascorbic acid				
sucrose	1214 (266)[b]	0.003 (0.0012)	231	112 (0.53)[c]
glucose	3764 (787)	0.002 (0.0012)	347	1279 (40)
fructose				215 (5)
mannitol	233 (37)	0.0018 (0.00062)	385	243 (33)
total	5234 (722)	0.002 (0.0008)	347	1850 (77)
sodium metabisulfite				
sucrose				nd
glucose	2609 (410)[b]	0.003 (0.0009)	231	nd
fructose				290 (7)[c]
mannitol	501 (75)	0.0009 (0.00032)	770	168 (15)
total	3699 (218)	0.003 (0.0003)	231	457 (21)
oxalic acid				
sucrose				122 (5)[c]
glucose	7062 (3098)[b]	0.009 (0.003)	77	nd
fructose	116 (44)	0.0021 (0.00214)	330	330 (13)
mannitol	193 (55)	0.0021 (0.00164)	330	368 (9)
total	6324 (2084)	0.007 (0.002)	99	819 (9)
saturated CO$_2$				
sucrose	686 (194)[b]	0.0038 (0.0016)	182	nd
glucose	3431 (740)	0.005 (0.0012)	140	nd
fructose				286 (4)[c]
mannitol				nd
total	3823 (500)	0.003 (0.007)	231	286 (4)
Unwashed Olives				
all treatments				
sucrose	1500 (340)[d]	0.001 (0.0007)	693	42 (57)[e]
glucose	18649 (759)	0.010 (0.0008)	69	630 (184)
fructose	3810 (173)	0.007 (0.0011)	77	482 (70)
mannitol	2626 (141)	0.005 (0.0009)	139	230 (151)
total	26159 (1046)	0.010 (0.001)	69	1385 (335)

[a] Residual sugar in brines of treatments not subjected to kinetic studies: salicylic acid (126 (24), 5299 (180), 1336 (0.1), 618 (1.20); control, nd (not detected), 324 (2), 124 (25), 448 (23) for sucrose, glucose, fructose, and mannitol, respectively. Standard errors are in parentheses. Sugar content in brine of the control treatment was always low, and the changes during the storage period were negligible. [b] Degrees of freedom for average below heading is 9. [c] Degrees of freedom for average below heading is 1. [d] Degrees of freedom for average below heading is 36. [e] Degrees of freedom for average below heading is 7.

period, depending on the balance between polyphenols and nutrients (*21*).

The final physicochemical and microbiological characteristics in the washing waters depended on treatment conditions (**Table 2**). Microbial growth (composed of yeast, LAB, and *Enterobacteriaceae*) was always observed, except in the presence of salicylic acid in which no microorganisms were detected. The average population was systematically higher (but not significantly different at $p < 0.05$) in the second washing than in the first one. Apparently, the second washing water was inoculated by drops of the previous solution which remained adhered to the olive surface. The average population levels in the second washing water was 5.9 ± 1.2 log cfu/mL for yeasts, 4.0 ± 0.6 log cfu/mL for LAB, and 3.8 ± 0.4 log cfu/mL for *Enterobacteriaceae* (**Table 2**). There was a tendency for high yeast and *Enterobacteriaceae* populations in treatments using saturated CO$_2$ and the control. *Enterobacteriaceae* were also observed in the treatment using sodium metabisulfite (**Table 2**), possibly due to the relatively high pH of these solutions at the end of the washing phase, which were pH 4.56 and 4.60, respectively.

The solution color followed the same trend (**Table 2**) as the sugars and polyphenols: the first ones always had more color. Interestingly, the color of the second washing water with
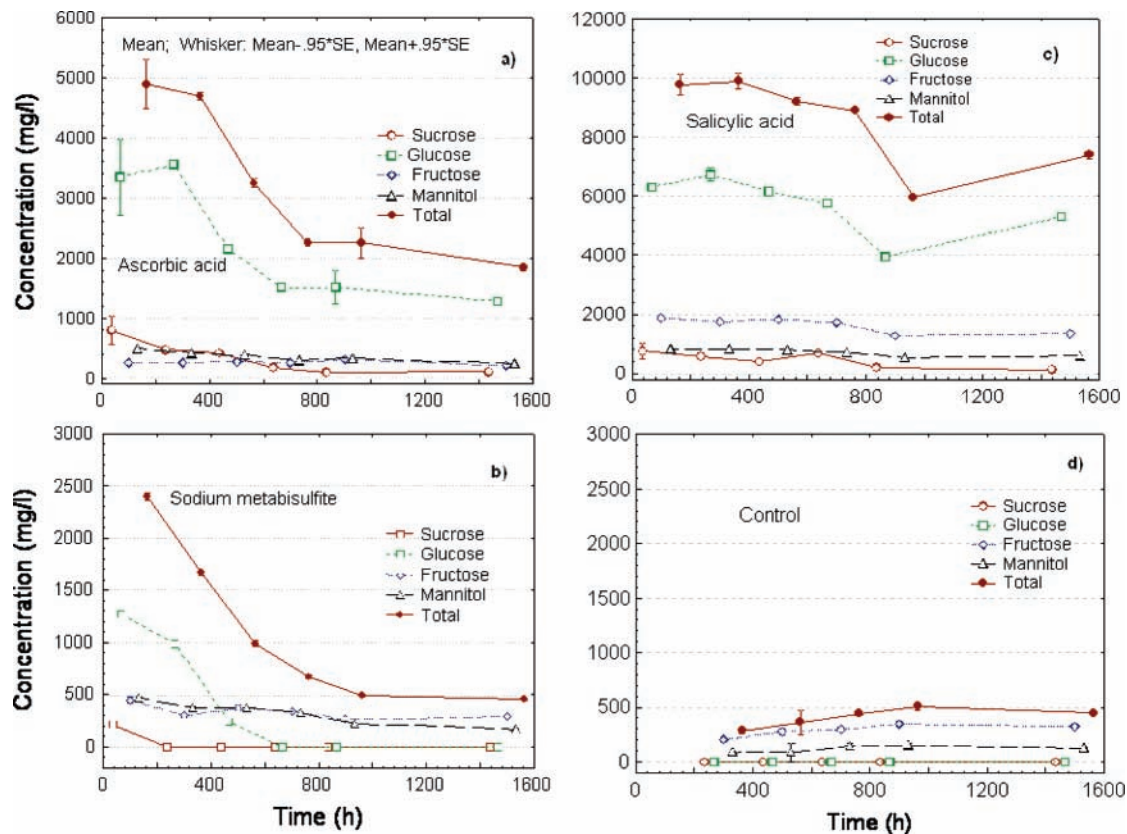
Figure 2. Sugar content changes in the brines of washed cracked Manzanilla-Aloreña olives, according to selected alternative storage systems. When error bars are not visible, determinations were within the range of the symbols on the graph.

ascorbic acid was very light due to the possible antioxidant activity of this compound. Globally, the total amount of sugars and polyphenols removal were markedly high (**Table 2**); differences in sugar extraction due to treatments were not significant but washing waters containing sodium metabisulfite, saturated CO_2, or just brine showed slightly lower polyphenol removal, although their effects were also outstanding ($\approx 500-600$ mg/L) . The washing waters from green Spanish-style olives (*22*) also removed significant amounts of sugars and polyphenols in a short period of time, but the skin permeability was achieved in this case by the lye treatment.

From the above results, it can be deduced that the application of a previous washing stage to cracked Manzanilla-Aloreña olives may reduce their fermentable substrate and polyphenol (related to the bitter taste) contents, effects that can be favorable for an earlier commercialization as "seasoned" table olives (*4*).

Changes in pH, NaCl Content, Combined Acidity of Brines and Texture of Olive Fruits According to Storage Systems. These parameters are usual to control any table olive processing. Proper values are essential for an adequate storage process (*4*). In washed olives, the average or pH ranges for the different treatments were about 3.6 for ascorbic acid; from 5.0 (initial) to 4.0 (final) for sodium metabisulfite; about 3.2 for oxalic acid; about 3.7 for salicylic acid; from 4.6 to 4.3 for saturated CO_2; and from 4.5 to 4.1 for the control. In unwashed fruits, results were fairly similar except for ascorbic acid for which the pH was higher and ranged from 4.4 to 4.0. NaCl content in the brine of the different treatments decreased during storage from the initial 15% to 7.8–10.7% (washed olives) or to 6.0–7.8% (unwashed fruits). Apparently, the 5% of NaCl in the washing waters (first case) caused an initial increase in the NaCl absorption in the washed olive flesh which equilibrated

at higher levels during storage than in the unwashed ones. Titratable acidity was 0.2–0.5% and 0.3–0.7% in washed and unwashed fruits, respectively. Combined acidity was higher in unwashed fruits (41–60 mequiv/L) than in washed (20–30 mequiv/L). García García et al. (*5*) mentioned a decrease from 4.5 to 3.9 units in pH during the fermentation of unwashed whole Manzanilla-Aloreña olives and an increase of titratable acidity from 0.15 to 0.50% at 9% NaCl (wt/vol).

Texture (initially 68–71 N/100 g pitted olives) decreased slightly with storage, except when using oxalic and salicylic acids for which a slight increase was observed.

Changes in Sugars in Washed or Unwashed Olives and Their Storage Brines. The fermentation process during storage depended, among other nutrients, on the concentration of sugars in both olive flesh and brine. There was a leaching of the olives' compounds from the flesh into the brine. In washed olives, there was a similar progressive decrease in all the sugar compounds regardless of treatments, which then were grouped for modeling, except for that using salicylic acid for which reduction was slower and was modeled separately (**Figure 1**). The contents of sugars in unwashed fruits were always above those from washed but followed a similar trend in all treatments, which were also grouped for modeling. Several kinetic models were checked to express sugar evolution in the flesh. The best fit for sugar changes in flesh was obtained with a first-order decay with intercept. The values obtained for the different parameters of the model and the residual sugar concentrations in the flesh of olives after storage are shown in **Table 3**.

In washed fruits, sucrose was the sugar most slowly removed from olive flesh ($k = 0.004 \pm 0.000\,63$ h^{-1} for treatments with ascorbic acid, sodium metabisulfite, saturated CO_2, control, and oxalic acid of washed olives) and $k = 0.001 \pm 0.0007$ h^{-1} for
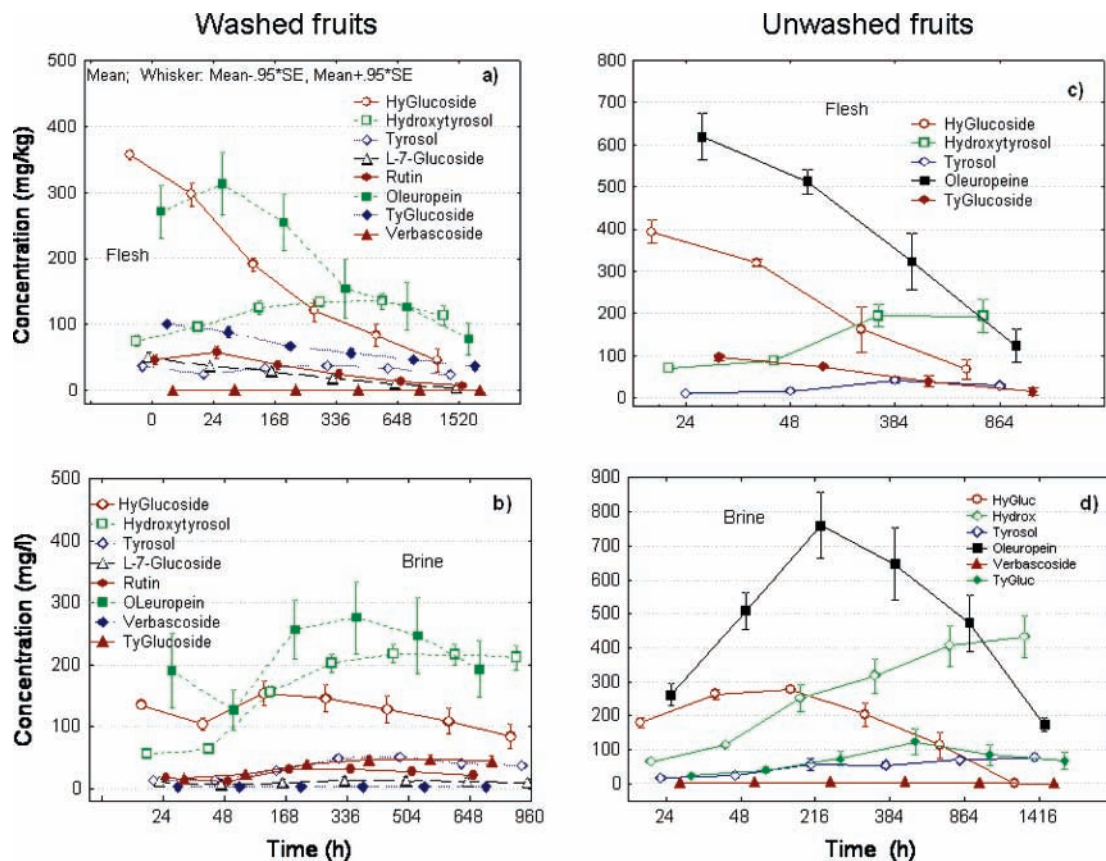
Figure 3. Polyphenol changes in the flesh and brine of washed and unwashed cracked Manzanilla-Aloreña olives during storage. The following abbreviations are used: HyGlucoside, hydroxytyrosol glucoside; TyGlucoside, tyrosol glucoside; L-7-Glucos, luteolin-7-glucoside. When error bars are not visible, determinations were within the range of the symbols on the graph.

all treatments of unwashed fruits. As result, the t_{50} values of this sugar, which may also depend on the magnitude of the decrease (initial and final contents), were always fairly high (173, 347, and 693 h, respectively, for the same groups of treatment mentioned above).

The k values for the rest of the individual and total sugars were fairly similar within the diverse groups. In washed fruits, the decay rates of glucose, fructose, mannitol, and total sugar in sodium metabisulfite, oxalic acid, and saturated CO_2 treatments was ≈ 0.03 h^{-1} (**Table 3**). In unwashed olives, for the same sugars, the decay rates ranged from 0.005 to 0.010 h^{-1}. As result, t_{50} in sodium metabisulfite, oxalic acid, and saturated CO_2 for the such sugars had intermediate values (22−23 h) while the same parameter in unwashed olives ranged from 69 to 138 h. Final residual contents were markedly higher in unwashed olives (≈ 1300 mg/kg total sugars) than in washed fruits (≈ 700 mg/kg total sugars). Then, elimination of sugar during storage was more rapid in washed than in unwashed olives and also led to fruits with lower residual contents, a condition which might be favorable for a better preservation of the final product after packing (*4*). García García et al. (*5*) showed that the concentration in the unwashed flesh of Manzanilla-Aloreña olives was approximately 17 000 mg/kg after 1440 h storage, while in this study the concentration for unwashed fruits was 1238 mg/kg after approximately the same period.

Sugar behavior in the treatment with salicylic acid was different; it had the highest decrease rates (and t_{50} values), except for sucrose, but due to the low use of sugar in brines during storage, the final concentration at the end of the period was fairly high. This circumstance, together with its negative effect

on the fruits' color makes the use of this acid in the storage phase difficult and its treatment was not applied in unwashed fruits.

Sugar contents increased rapidly in the storage solutions from the beginning of brining (**Figure 2**) due to the rapid exchange between the cracked olives and the solutions. The proportion of each individual sugar with respect to the total was similar to that observed in the flesh. Their concentrations in brines were decreasing with storage time according to treatments, except for those using salicylic acid which showed only a very slight decrease (possibly caused by a sporadic or undetected microbial growth). Sugar changes in this medium could not be adapted to any model. The sugar content in the brine using salicylic acid was fairly high as was the residual sugar content in olive flesh (**Table 3**). Olives preserved in this medium should be subjected to a strong washing phase to remove both sugars and salicylic acid before packing.

Changes in sugar concentration in brines were affected by treatments. In washed olives, those using ascorbic acid showed remarkably high concentrations of sugars and the residual level in the solution at the end of the storage period was high (close to 2000 mg/L) (**Figure 2a**, **Table 4**). The control had the lowest concentration of sugars in brine throughout the storage period and showed a fairly low final residual level (below 500 mg/L) (**Figure 2d**); apparently, in this treatment, sugars were used by the microbial population as they reached the solution. In treatments with ascorbic acid (**Figure 2a**), sodium metabisulfite (**Figure 2b**), oxalic acid, and saturated CO_2, sugars showed a progressive decay with different final residues for each and total sugar content. Because of the varying behavior, changes were not grouped for the kinetic study. Glucose and total sugar
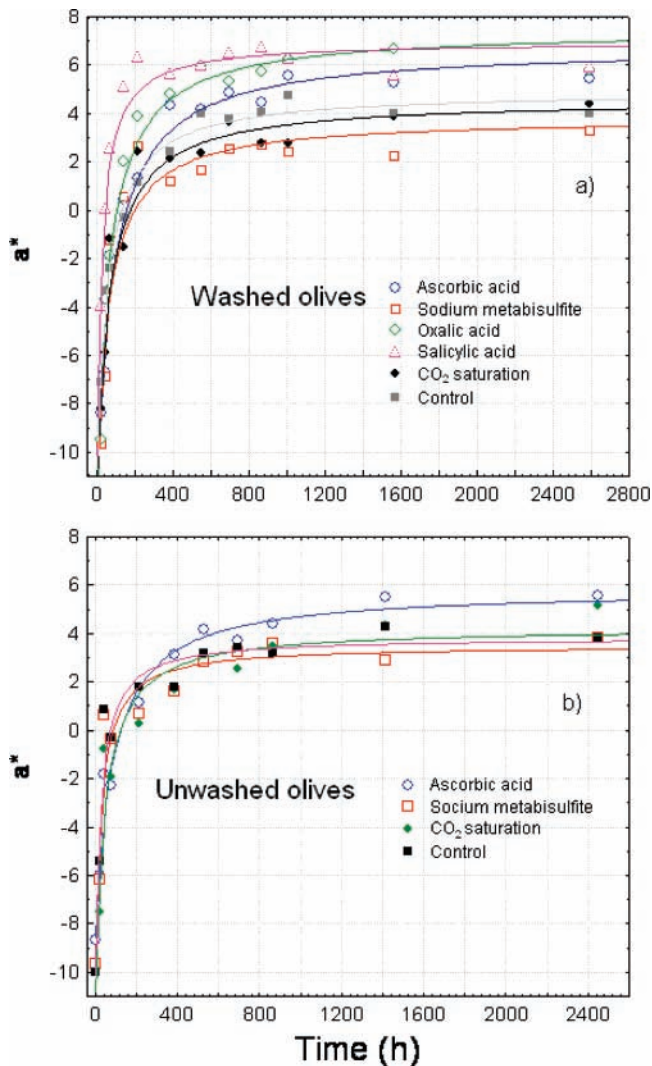
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Figure 4. Changes in CIE *a** parameter for washed and unwashed alternative storage systems. Values are the average of duplicates from 20 olive measurements each.

Figure 5. Microbial evolution (LAB, yeast, and *Enterobacteriaceas*) in the brines of washed cracked Manzanilla-Aloreña olives, according to alternative storage systems. Values are the average of duplicates. Symbols mean growth of yeast, lactic acid bacteria (LAB), and *Enterobacteriaceae* (Ent) in ascorbic acid (Asc), sodium metabisulfite (Meta), oxalic acid (Oxal), CO_2 saturation (CO$_2$), and control (Contr).

changes always followed pseudo-first-order kinetics as in the olive flesh, but sucrose, fructose, or mannitol disappeared rapidly in some treatments and their changes were unable to be fitted to any kinetics (**Table 4**). The lowest residual sugar content was found in the treatment using saturated CO_2 ($\approx$286 mg/L), followed by the control. Fruits from these two treatments could then, apparently, show the most favorable conditions (lowest residual fermentable substrates) for their further packing as "seasoned" olives (*4*). Storage in the presence of sodium metabisulfite and oxalic acid produced higher values, $\approx$300–800 mg/L, respectively. Ascorbic acid had a remarkably high residual concentration (1850 mg/L) which is not acceptable for fruits which must be packed.

In unwashed fruits, great amounts of sugars passed very rapidly from flesh to brines as a consequence of the absence of washings and the easy exchange between cracked fruits and their surrounding solutions (data not shown). This led to marked high initial sugar concentrations in all treatments (ascorbic acid, metabisulfite, and saturated CO_2) followed by a very rapid consumption, although the final residual concentrations of all sugars were higher than in those prepared from washed fruits (**Table 4**). No significant differences among treatments were observed, so data from all of them were grouped for the kinetic analysis. In unwashed olive brines, the lowest
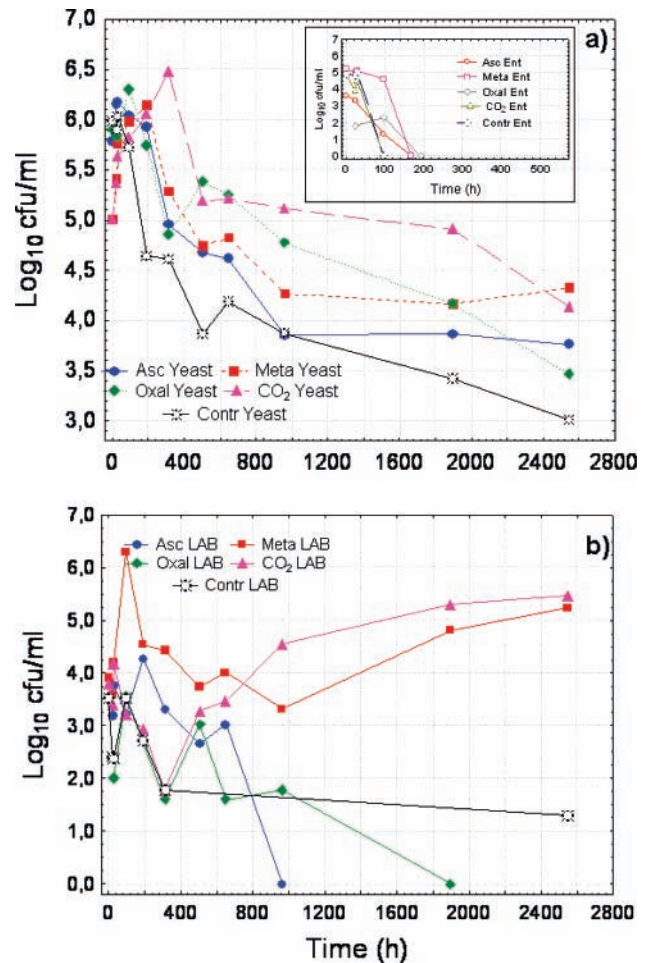
Table 5. Biological Kinetic Parameters ($\pm$Standard Error) of Yeast Evolution in the Brines of Washed Cracked Olives[a]

treatment	growth (Gompertz)		inactivation (Weibull)	
	μ_m (h^{-1})	λ (h)	D_β	β
ascorbic acid			341 ± 110	0.5 ± 0.1
sodium metabisulfite	0.07 ± 0.01	18 ± 1	414 ± 205	0.4 ± 0.2
oxalic acid			903 ± 146	0.8 ± 0.2
salicylic acid				
CO_2 atmosphere	0.05 ± 0.01	16.0 ± 0.1	649 ± 248	0.6 ± 0.2
control			187 ± 62	0.4 ± 0.07

[a] μ_m, growth rate; λ, lag phase; D_β, first decimal reduction time; β, shape parameter.

rate was observed for sucrose (0.001 h^{-1}), which t_{50} was the longest (693 h). Mannitol also had a low decay constant (0.005 h^{-1}) and required a long t_{50} (139 h). Decay of glucose had the highest k (0.01 h^{-1}). Total sugar decay was similar to glucose because this was the main sugar component. The total residual sugars were fairly high (1385 mg/L in brine and 1238 mg/kg in the flesh) (**Table 3**). These olives may provide enough sugars to the packed "seasoned" olives so as to support microbial growth which may, in turn, contribute to spoilage in the commercial product.

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Table 6. Yeast Kinetic Parameters (±Standard Error) in Brines of Unwashed Olives, Obtained by Fitting the Pruitt and Kamau Model[20,a]

treatment	μm (h^{-1})	τ (h)	Dp (log cfu/mL)	γ (h^{-1})	N_{max} (log cfu/mL)
ascorbic acid	0.061 ± 0.008	26 ± 2	1.8 ± 0.3	0.010 ± 0.001	6.5 ± 0.5
sodium metabisulfite	0.063 ± 0.006	34 ± 3	2.1 ± 0.3	0.100 ± 0.040	6.1 ± 0.5
CO$_2$ atmosphere	0.032 ± 0.002	30 ± 2	1.6 ± 0.2	0.150 ± 0.040	6.3 ± 0.4
control	0.041 ± 0.008	32 ± 2	2.1 ± 0.9	0.107 ± 0.030	6.2 ± 0.5

[a] μ_m, growth rate; τ, time to reach N_{max}/2; Dp fatally damaged population; γ, death rate.

As a result, stored washed olives are, apparently, more suitable (contain less redidual sugars) than unwashed ones for their further used as packed "seasoned" Manzanilla-Aloreña products (*4*).

Polyphenol Changes in the Flesh and Brines of Washed and Unwashed Olives. No significant differences in the changes of polyphenol contents between treatments were observed; so the results from all of them were combined. The most abundant polyphenols in the flesh of washed and unwashed cracked olives were oleuropein and hydroxytyrosol glucoside, which decreased with time to reduce their final contents to levels around 100 mg/kg (**Figure 3**a,c). This decrease in the concentrations of both polyphenols was due to their hydrolysis by the acidic pH of the medium and/or the possible effect of microorganisms. Tyrosol-glucoside, luteolin-7-glucoside, tyrosol, rutin, and verbascoside showed the same trend but their changes were less marked, since they were originally in sensibly lower proportions. They had thus low residual concentrations after storage (values around or below 50 mg/kg). The only polyphenol that showed a marked increase with time in the flesh of both types of fruits was hydroxytyrosol due to its progressive formation through the hydrolysis of hydroxytyrosol glucoside and oleuropein. Oleuropein always showed the highest standard error due to the effect of pH differences among treatments on its hydrolysis rates. A slight increase in tyrosol content, due to the hydrolysis of tyrosol glucoside, was observed in the flesh of unwashed olives but it was negligible in washed ones because of the lower content of these in the precursor. The progressive and rapid solubilization of polyphenols (related to bitterness) in these cracked olives may favor their sweetening and facilitate their commercialization in a shorter period of time.

Oleuropein was the polyphenol that showed the highest concentration in the brines of washed and unwashed olives from the very beginning of brining (**Figure 3b,d**). It was followed by hydroxytyrosol glucoside. The concentration of both increased for a period (in which their solubilization rates were higher than the hydrolysis rates) and then decreased progressively. Brines from unwashed olives had higher concentrations than those from washed ones, with the concentration of oleuropein in the second markedly higher than in first. Because of oleuropein and hydroxytyrosol glucoside hydrolysis, hydroxytyrosol steadily increased throughout the storage process and reached fairly high final concentrations, which were around 2-fold higher in the brines of unwashed fruit (≈400 mg/kg) than in those from washed fruit. Tyrosol also showed a slight increase due to the slow hydrolysis of tyrosol glucoside. The rest of the polyphenols analyzed were in low concentrations and showed only a limited increase with time. The solubilization of polyphenol (especially oleuropein and hydroxytyrosol glucoside) was slower than sugars. This behavior may be due to their more difficult diffusion because of the size of their molecules, although the phenomenon overlapped with their partial hydrolysis as they passed into the brine. At the end of the storage period,

a very slight decrease in all the polyphenols may be observed except for hydroxytyrosol, which apparently counteracted such an effect with its formation from their precursors (**Figure 3b,d**).

Changes in the Surface Color of Fruits. The surface green color of fruits is one of the attributes that gives the freshness appearance to "seasoned" Manzanilla-Aloreña table olives. Thus, the green color should be maintained as much as possible. Its evolution is related to the presence and degradation of chlorophylls, and its retention has been used as a measure of quality in green vegetables (*23*). Gold and Weckel (*24*) related the green color changes with parameter CIE *a**.

Storage produced a decrease in luminance (*L**) with time which depended on treatments (the average: 60, initial, to 47−58, final). Higher changes were observed in *b**. The initial value was around 43 whereas final values were about 28−29 and 37−40 for washed and unwashed olives, respectively. The parameter most related to the changes observed in the surface color was *a** which moved from the green (negative values) to the red region (positive values). Therefore, the surface color changes were mainly followed as a function of the *a** parameter. The evolution was rapid from the very beginning of brining, with a gradation related to the different antioxidants used, and the *a** values moved quickly from the green region (negative values) to the red region (positive values) (**Figure 4**). Ascorbic acid showed a slightly more protective effect during the first days in brine but was less effective in the long term. López Nicolás et al. (*25*) reported that once the ascorbic acid has been completely oxidized to dehydroascorbic acid, browning occurs. At the end of the storage period the lowest line (best color) in the equilibrium corresponded to fruits treated with sodium metabisulfite. The color behavior in treatments using unwashed olives was fairly similar to that in washed ones (as deduced from the situation of their respective curves) (**Figure 4**, lower vs upper graphs). The color changes in all treatments followed pseudo-second-order hyperbolic kinetics, for which equations and kinetic constants were the following:

ascorbic acid:

$$a^* = -11.7 + \frac{18.4t}{90.6 + t}; \quad k = 6.0 \times 10^{-4}\,\text{h}^{-1}$$

sodium metabisulfite:

$$a^* = -12.1 + \frac{16t}{63 + t}; \quad k = 10.0 \times 10^{-4}\,\text{h}^{-1}$$

oxalic acid:

$$a^* = -11.9 + \frac{19t}{67.8 + t}; \quad k = 7.6 \times 10^{-4}\,\text{h}^{-1}$$

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salicylic acid:

$$a* = -11.1 + \frac{18t}{28 + t}; \quad k = 16.6 \times 10^{-4}\,h^{-1}$$

$$CO_2: \quad a* = -11.4 + \frac{16t}{71 + t}; \quad k = 10.0 \times 10^{-4}\,h^{-1}$$

control:

$$a* = -10.8 + \frac{18t}{61 + t}; \quad k = 10 \times 10^{-4}\,h^{-1}$$

The final lowest $a*$ parameter value corresponded to olives treated with sodium metabisulfite ($a* \approx 3.5$) (**Figure 4**). Thus, washed cracked olives immersed in this treatment had a less pronounced red tone than the control and, in consequence, this compound may be useful to preserve color. The worst final color was observed in treatments using salicylic and oxalic acids (final $a* \approx 7$).

In unwashed olives, the following kinetic equations were obtained:

ascorbic acid:

$$a* = -8.5 + \frac{14.4t}{84 + t}; \quad k = 8.0 \times 10^{-4}\,h^{-1}$$

sodium metabisulfite:

$$a* = -9.9 + \frac{13.4t}{32 + t}; \quad k = 23 \times 10^{-4}\,h^{-1}$$

$$CO_2: \quad a* = -11.1 + \frac{15.3t}{47 + t}; \quad k = 14.0 \times 10^{-4}\,h^{-1}$$

control:

$$a* = -10.2 + \frac{14.0t}{28 + t}; \quad k = 25 \times 10^{-4}\,h^{-1}$$

The final $a*$ values for the treatment with sodium metabisulfite were the lowest, followed by the control, while the treatment with ascorbic acid was (as in washed fruits) more effective during the first period of storage (**Figure 4**).

Final evolution and values of $a*$ in the same treatments with washed and unwashed fruits were very similar, thus leading to the conclusion that the use of the two washing waters had negligible effect on the final color of stored fruits. Treatments using salicylic and oxalic acids, in the studied conditions, had a markedly negative effect which advises against their use in the storage phase. The rest of the treatments did not have significant effects on color, except ascorbic acid which produced a slight improvement during the first days and sodium metabisulfite which effect was observed at longer periods of storage time. The favorable effect of ascorbic acid on the green color surface of apples, alone or combined with different types of cyclodextrins, was also reported by López Nicolás et al. (*25*).

Kinetics of the Microbial Evolution in Brines of Washed and Unwashed Olives during Storage. A population of yeasts, LAB, and *Enterobacteriaceae* was observed immediately after brining washed olives. Fernández Diez et al. (*26*) mentioned the presence of yeasts and LAB in directly brined green table olives. This population was a consequence of the inoculation of the brine with the drops of washing waters adhered to the olives. *Enterobacteriaceae* was rapidly inhibited and disappeared from all treatments after about 200 h (5−6 days) (**Figure 5a**, detail). In treatments with sodium metabisulfite and saturated

Table 7. Lactic Acid Bacteria Kinetic Parameters (±Standard Error) in Brines of Unwashed Olives, Obtained by Fitting the Reparametized Gompertz Equation[a]

treatment	μ_m (h^{-1})	λ (h)	N_{max} (log cfu/mL)
ascorbic acid	0.103 ± 0.010	19 ± 5	6.7 ± 0.6
sodium metabisulfite	0.005 ± 0.001	101 ± 18	6.5 ± 0.3
CO$_2$ atmosphere	0.010 ± 0.007	438 ± 91	5.5 ± 0.2
control	0.009 ± 0.002	387 ± 72	6.3 ± 0.2

[a] μ_m, growth rate; λ, lag phase; N_{max}, maximum population reached.

CO_2 there was an initial growth of yeasts followed by a later inactivation phase. The kinetic parameters of this initial growth phase are shown in **Table 5**. The maximum specific growth rate and the lag phase were very similar in both treatments (about 0.06 h^{-1} and 17 h, respectively). In the long term, the general trend was of inactivation, including treatments with sodium metabisulfite and CO_2. This inactivation trend in all treatments could be fitted by the model based on the Weibull distribution. The strongest inactivation (**Table 5**) was observed in the control with the lowest first decimal reduction time (187 ± 62 h). Inactivation was also strong in treatment with ascorbic acid (341 ± 110 h). They were followed by the treatments with sodium metabisulfite, saturated CO_2, and oxalic acid. The shape was always upward concave (**Figure 5a**), corresponding to values of $\beta < 1$. Then, it was apparent that the washings had led to a decrease in nutrients which was sufficient not only to prevent the growth of yeast but also to inactivate a part of the initial population. However, extrapolation of the inhibitory effect observed in this storage period to the final product packing is not straightforward and requires further experimentation.

The evolution of LAB in the brines of washed olives was diverse and depended on the treatment (**Figure 5b**). The initial LAB population supported the high initial NaCl level fairly well (15%) and only a part of the initial population was damaged. Furthermore, in the presence of sodium metabisulfite, a clear increase was observed just after brining. However, there was no general behavior, and their changes did not follow any model. In some treatments, LAB was progressively inactivated to a final complete disappearance (ascorbic and oxalic acids) or at very low levels (control), but in others, with sodium metabisulfite and saturated CO_2, a moderate growth was observed after NaCl equilibrium ($\approx$600 h) at NaCl concentrations between 7.7 and 10.7%; however, the production of titratable acidity was low, even in these cases, when compared with a typical green olive fermentation (*4*), possibly because most of the LAB energy consumption was used for maintenance functions. The behavior of LAB in these two treatments was unexpected since LAB are usually more sensitive to NaCl concentration and lack of nutrients than yeasts (*27*), but the yeast decline, together with other slightly more favorable environmental conditions, might have facilitated the moderate increase observed in the presence of metabisulfite and saturated CO_2.

The microbial trend in the brines of unwashed olives was different from that observed in washed ones. No *Enterobacteriaceae* was observed at the beginning of the storage process of unwashed olives, and yeasts showed an initial phase of inactivation followed by another of growth and a stabilization of the population. LAB were inhibited at the beginning of the process (although they were always present), but after the NaCl content stabilization (at 6.0−7.6%, slightly lower level than that reached in washed olives due to the absence of the initial contribution of washings), they initiated a vigorous growth and reached final populations similar to those of yeasts. The kinetic

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parameters of the inactivation and growth phase of yeasts, which were fit by the Pruitt and Kamau model (*20*), are shown in **Table 6**. The initial population fatally damaged at the beginning was lower in the treatment subjected to saturated CO_2, followed by that with ascorbic acid and the rest of the treatments but the differences were not significant at $p < 0.05$. The lowest death rate was observed in the treatment with ascorbic acid (0.01 h^{-1}) and the highest in the treatment with saturated CO_2 (0.15 h^{-1}), although the differences between this and the rest of treatments were insignificant. The lowest growth rate value was observed in saturated CO_2 (0.032 h^{-1}) followed by the control (0.041 h^{-1}) with no significant differences between them. The other two treatments with antioxidants (ascorbic acid and sodium metabisulfite) showed rates relatively high and similar (0.061 and 0.063 h^{-1}, respectively). No significant differences among the sizes of the final populations were observed.

The kinetic parameters of LAB changes in unwashed olive treatments are shown in **Table 7**. The highest growth rate (0.103 h^{-1}) and lowest lag phase (19 h) were observed in the treatment containing ascorbic acid, in which the growth began practically from the very beginning of brining; however, in the other treatments there were markedly higher lag phases (101, 438, and 387 h for sodium metabisulfite, saturated CO_2, and the control, respectively) circumstance that can hardly be related to only the NaCl levels in these treatments. Regardless of the general LAB growth, a noticeable increase in titratable acidity was observed only in ascorbic acid and sodium metabisulfite treatments. Final LAB population was significantly lower in the saturated CO_2 treatment than in any other. García García et al. (*5*) also reported the growth of yeasts and LAB during the storage of unwashed whole Manzanilla-Aloreña olives at 9% NaCl.

Results from the kinetic changes of sugars, polyphenols, color, and microbial population as well as the evolution of other physicochemical characteristics of the different studied systems lead to the conclusion that a process that includes two washing waters (using 5% NaCl brine added with 0.1% sodium metabisulfite or saturated CO_2), followed by storage in 15% NaCl brine under saturated CO_2 or 0.1% sodium metabisulfite, can be recommended due to the fact that the resulting fruits had the overall best conditions demanded for their further use as a "seasoned" packed product.

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