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3 **Effect of calcium on the preservation of green olives intended for black**
4 **ripe olive processing under free-sodium chloride conditions**

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Abstract

Black ripe olive processing produces large volumes of salty wastewater that may be reduced by using a salt-free solution during the preservation stage. In this study, the employment of calcium chloride and calcium lactate during this stage to improve the texture of olives of the Manzanilla, Hojiblanca and Cacereña cultivars was assessed. Neither calcium salt affected the preservation of the olives under aerobic conditions, with yeasts and acetic acid bacteria being the predominant microorganisms during this stage, whereas lactic acid bacteria were detected after several months of preservation with the increase of the ambient temperature, including *Oenococcus* sp., which has not been isolated from olives until now. Moreover, both calcium salts at 20 mM concentration were equally effective in delaying the softening of olives during all processing stages regardless of the olive cultivar. In addition, the color and flavor of the olives were not affected by either of the calcium salts. Hence, these results revealed that both calcium salts must be used for the preservation stage of black ripe olives in salt-free solution without any preference for either of them from a technical point of view.

Keywords: olive, calcium, *Oenococcus*, texture, fermentation

1. Introduction

Traditionally, olives, cucumbers, sauerkraut, capers, peppers, eggplants, kimchi and many other vegetables have been fermented and preserved in 5-15% NaCl. This high salt concentration helps to retain a firm texture of the fruit and to prevent the growth of undesirable microorganisms. However, regulatory agencies are implementing stricter limits on the permitted levels of conductivity in wastewaters, encouraging factories to reduce the level of salt. In addition, consumers are demanding food products with less sodium content, therefore new methods for vegetable preservation with low salt content or even without salt have been studied (Panagou, Hondrodinou, Mallouchos, & Nychas, 2011; Fleming, McDonald, McFeeters, Thompson, & Humphries, 1995; Wolkers-Rooijackers, Thomas, & Nout, 2013). A new salt-free method to ferment cucumbers based on the use of calcium chloride (0.1 M) instead of sodium chloride (1 M) has been developed (McFeeters, & Pérez-Díaz, 2010; Pérez-Díaz et al., 2015) although its implementation at industrial scale has presented some drawbacks such as (i) lower firmness in pickles fermented with CaCl₂ than control (NaCl); (ii) development of secondary fermentations that requires the addition of potassium sorbate; (iii) employment of initial acidification with acetic acid (25 mM) to inhibit growth of *Enterobacteriaceae* spp.; and (iv) controversy regarding the air-purging routine necessary to remove CO₂ (McMurtrie, Johanningsmeier, Breidt, & Price, 2019).

In the case of table olives, Vaughn, Martin, Stevenson, Johnson, & Crampton (1969) proposed a salt-free preservation method for green-yellow olives intended for processing as black ripe olives that has been adopted by American table olive factories with some adjustments. Olives are stored under anaerobic conditions in a solution containing a high concentration of acetic acid (> 2%, w/v), sodium benzoate (>0.2%, w/v) and calcium chloride (> 0.2%, w/v) (Luh, Ferguson, Kader, & Barret, 2005). However, this preservation method requires many washing steps during the darkening stage of olives to leach the sodium benzoate from fruits, so an alternative method was studied in Spain without the use of this preservative but with maintenance of the fruit under aerobic conditions in acetic acid solution (>1.5%, w/v) (de Castro, García, Romero, Brenes, & Garrido, 2007). The industrial application of this new method revealed the need to add calcium to the packing solution to reach a similar firmness in the final product to olives previously stored with NaCl, even in fruit of the Hojiblanca

cultivar which has a very strong texture (García, Romero, & Brenes, 2014). Hence, it is necessary to investigate the use of calcium salts during the preservation stage of black ripe olive processing not only for those of the Hojiblanca cultivar but also for Manzanilla and Cacereña cultivars that possess a softer texture than the former.

Among the calcium salts, calcium chloride is widely employed in food processing to maintain firmness, although calcium lactate has emerged as an alternative firming agent (Manganaris, Vasilakakis, Diamantidis, & Mignani, 2005). However, contradictory data have been reported on the effectiveness of these calcium salts to delay softening of fruits and vegetables; firmness was higher in lychee fruit and fresh-cut carrot and mango treated with calcium chloride than calcium lactate (Ngamchuachit, Sivertsen, Mitcham, & Barret, 2014; Phanumong, Sangsuwan, Kim, & Rattanapanone, 2016; Yu, Jiang, Ramaswamy, Zhu, & Li, 2018) but the opposite was found for fresh-cut melon (Luna-Guzmán, & Barret, 2000) and no differences were found for canned peaches and fresh-cut melon (Manganaris et al., 2005; Silveira, Aguayo, Chisari, & Artes, 2011). One of the main drawbacks of calcium salts is the bitterness that may be imparted to the food in high concentrations, particularly calcium chloride (Luna-Guzmán, & Barret, 2000). Increased bitterness has been found in black olives fermented with a higher concentration than 2.6% of CaCl_2 (Panagou et al., 2011) but this negative perception was not detected in olives processed with a mixture of calcium acetate and lactate tested at 32.3 mM each (Tataridou, & Kotzekidou, 2015). Consequently, the objectives of this research were (i) to assess the preservation of olives of the Hojiblanca, Cacereña and Manzanilla cultivars in a salt-free solution under aerobic conditions and (ii) to determine the effect of different calcium salts on the processing of these cultivars as black ripe olives.

2. Materials and methods

2.1. Processing of olives

2.1.1. Olive material

Olives from cvs. Manzanilla, Hojiblanca and Cacereña were purchased at the end of September during the 2017/2018 olive season from local farmers located in the Seville and Cáceres provinces (Spain). Fruits were harvested with green-yellow color

on the surface. On arrival at the laboratory, olives were sorted in order to use those with uniform size (280-320 fruit/kg).

2.1.2. Preservation stage

Olives were put into 35 L cylindrical polyethylene vessels which had a diameter of 38 cm with a cylindrical tube (15 cm diameter) in the center whereby fruits (21.3 kg) and preservation liquids (13.7 L) were introduced. Aerobic conditions were obtained by fitting an aeration column as described elsewhere and they were maintained throughout the preservation stage (de Castro et al., 2007). The level of CO₂ in the solutions was always maintained below 40 mg/100 mL to ensure good aerobic conditions.

Three different preservation solutions were evaluated for Manzanilla and Cacereña olives: (i) control (1.5% acetic acid, w/v); (ii) calcium chloride (1.5% acetic acid, and 20 mM calcium chloride); and (iii) calcium lactate (1.5% acetic acid and 20 mM calcium lactate). Calcium chloride was purchased from Sigma (St. Louis, MO, USA) and calcium lactate was supplied by Purac Bioquímica SA (Barcelona, Spain). The weight of fresh olives and olives preserved for eight months was recorded for each vessel although preservation lasted for one year. Experiments were run in duplicate at ambient temperature.

Hojiblanca olives were preserved only with 1.5% (w/v) acetic acid (control) or 1.5% (w/v) acetic acid + 20 mM calcium chloride (calcium chloride treatment).

2.1.3. Darkening

Olives stored for eight months were darkened following the recently developed one single lye treatment (Brenes, Romero, & García, 2017). Three kilograms of fruit were put into four methacrylate horizontal cylindrical containers and covered with 3 L of target solutions. Olives of the Manzanilla and Cacereña cvs. were submerged in 2.5% (w/v) NaOH solution (lye) and those of the Hojiblanca cv. in 2.8% (w/v) NaOH solution until the lye reached the pit. This alkaline treatment lasted for around 4.0 hours and 3.5 hours with olives preserved with and without calcium salt during the preservation step, respectively. After the lye treatment, olives were covered with their preservation solution diluted 1:1 with tap water, where they were aerated for 24 hours

with the pH controlled in the liquid below 8.0 units. Subsequently, the fruits were immersed in fresh tap water for another 24 hours under similar conditions that day, after which they were covered with 0.1% (w/v) ferrous gluconate solution for five hours to fix the black color formed. The olives were also weighed before and after the darkening stage.

2.1.4. Packing

The stones were removed from the olives before packing with an industrial pitting machine (OFM, Seville, Spain), and 145 mL of pitted olives were bottled in A314 jars (Juvasa, Dos Hermanas, Spain) and covered with 175 mL of a solution containing 32 g/L NaCl and 0.2 g/L ferrous gluconate. The jars were sterilized and maintained at ambient temperature for two months before physicochemical and organoleptic analyses.

2.2. Physicochemical analysis

The concentration of NaCl was analyzed by titration with a 0.1 N silver nitrate solution. The free acidity and pH of the brine were measured with a Metrohm 670 Titro processor (Herisau, Switzerland) (de Castro et al., 2007). Carbon dioxide was determined in the preservation liquids by the microdiffusion method adapted for olive brines by García, Durán & Garrido (1982).

2.3. Sugars and organic acids analyses

Sugars and organic acids in the preservation solutions were analyzed by HPLC, as described elsewhere (de Castro et al., 2007).

2.4. Phenolic compounds determination

Phenolic compounds were extracted from the olive pulp with dimethyl sulfoxide (DMSO) and analyzed by HPLC according to Ramírez, Gandul-Rojas, Romero, Brenes & Gallardo-Guerrero (2015). The analysis of the phenolic compounds in the brine was

carried out by mixing 0.25 mL of brine, 0.25 mL of internal standard (2 mM of syringic acid), and 0.5 mL of deionized water. After filtering through a 0.22 μ m pore size nylon filter, the mixture (20 μ L) was injected into the HPLC chromatograph.

2.5. Preparation of cell wall material as the alcohol insoluble residue (AIR)

Duplicate 50 g samples of the fruit flesh were homogenized in an Ultra-Turrax with 200 mL of 96% ethanol. After filtration, samples were washed again with 80% ethanol. The pellets were re-suspended several times in acetone until supernatants were colorless. The dried final cellulosic residue was hydrolyzed as described by Jimenez et al. (2001). Neutral sugars were quantified in the neutralized sample by reduction, acetylation, and gas chromatography measurements as described elsewhere (Jimenez et al., 2001).

2.6. Calcium analyses

Olives (1 g) and preservation liquids (1 mL) were put into a boron-silicate tube with 25 mL of HNO₃ and digested in a DigiPREP Jr. (SCP Science, Canada) at 120°C for eight hours. Subsequently, 5 ml of a solution HClO₄/HNO₃ (4:1) was added into the tube and HNO₃ was evaporated at 140°C. The solution was put into a graduated flask and filled up to 25 mL with deionized water. The sample was diluted with deionized water to have a concentration between 2-14 μ g/mL and lanthanum solution was added to reach 5000 μ g/mL. Solutions were introduced into a GBC model 932 AA atomic absorption spectrometer (Victoria, Australia) equipped with a hollow (Ca, Mg, Cu, and Zn) multi-element cathode lamp (Photometry, Victoria Australia).

2.7. Microbiological analysis

The liquids in each vessel and their decimal dilutions (in 9 g/L NaCl) were plated on to de Man, Rogosa, Sharpe MRS agar (Biokar diagnostics, Beauvais, France) with and without 0.2 g/L sodium azide (Sigma-Aldrich) to detect lactic acid bacteria (LAB). MRS plates were incubated under anaerobic conditions (AnaeroGen, Oxoid). Yeast and mold population were detected on glucose (20 g/L)-yeast extract (5 g/L) agar

with and without oxytetracycline (Oxoid Ltd., Basingstoke, England) incubated aerobically. For acetic acid bacteria (AAB), GYP agar was used with the following composition (g/L): glucose, 50; yeast extract, 10; neutralised bacteriological peptone, 5; agar 15; aerobic incubation (de Castro et al., 2007). GYP medium is not specific for acetic acid bacteria (AAB) but a good medium for AAB culture provided that a long time aerobic incubation is carried out. Yeasts and other microorganisms (LAB, for instance) can also grow on GYP. Plates with these media were kept at 30°C for up to eight days in some cases. Visual examination of colony morphologies, together with phase contrast microscopy of motility and cell morphologies enabled AAB to be distinguished from other microorganisms. Another medium was applied to culture microorganisms suspected to be *Oenococcus* sp. according to Hocine, Jamal, Riquier, Lonvaud-Funel, & Dols-Lafargue (2010). Phase-contrast microscopic observation of doubtful colonies was routinely carried out.

2.8. Molecular identification of microorganisms suspected to be *Oenococcus* sp.

As a consequence of lengthened incubation of GYP plates, unknown colonies developed and they were isolated and cultured for their identification. The total DNA used for molecular identification of bacterial isolates was extracted directly from pelleted cells in *Oenococcus* broth by the rapid chloroform method described by Ruiz-Barba, Maldonado, & Jiménez-Díaz (2005). The isolates were identified to species level by 16S rRNA gene sequence analysis of a PCR fragment (ca. 1400 bp) amplified using the primer pair 7for (5'-AGAGTTTGATYMTGGCTCAG-3') and 1510r (5'-TACGGYTACCTTGTTACGACTT-3') as previously described in Lucena-Padrós, & Ruiz-Barba (2016). The almost full-length 16S rRNA gene sequences were assembled using the SeqMan Pro software (v. 15.0, DNASTAR Inc., USA). The resulting assembled sequences were used to search for similarities using the BLASTN program against the database containing type strains with updated validly published prokaryotic names, by using the EzTaxon-e server (<http://eztaxon-e.ezbiocloud.net/>). For the identification of isolates from industrial samples, partial (ca. 1100 bp) 16S rRNA gene sequence was used with primer 7for as the sequencing primer.

2.9. *Assessment of olive quality*

The color of olives was measured using a BYK-Gardner model 9000 Color-view spectrophotometer. Reflectance at 700 nm was recorded (Ramírez et al., 2015).

The firmness of fruit was determined using a Kramer shear compression cell coupled to a Texture Analyzer TA.TX plus (Stable Microsystems, Godalming, UK). (de Castro et al., 2007).

2.10. *Sensory evaluation*

This was performed on packed olives (subsection 2.1.4) that were preserved at ambient temperature for two months. The olives were tested according to the “Method for sensory analysis of table olives” (IOC, 2011) by nine trained panelists among the staff of the Instituto de la Grasa (Seville, Spain) in a normalized testing room. A profile sheet with a continuous scale of 10 cm ranging from 1 (absence of perception) to 11 (strongest intensity perceived) was used.

2.11. *Statistical analyses*

Statistical comparisons of the mean values for each experiment were carried out by one-way analysis of variance (ANOVA), followed by the Duncan’s multiple range test ($p < 0.05$) using SPSS software v. 23.0 (IBM Corp., Armonk, NY).

3. Results and discussion

The presence of yeasts, LAB, and AAB throughout the preservation stage is depicted in Fig. 1. For reasons of clarity, only the results of cv. Cacereña are presented because those of Manzanilla and Hojiblanca were rather similar to the former. It can be seen that very few differences were detected among the treatments assayed. Yeast population was lower in the control vessels for the first 120 days compared to the calcium treatments, whereas the results of LAB and AAB were almost identical. AAB were present in high numbers from the beginning, staying ca 6 Log CFU/mL throughout all the time, irrespective of the treatment and cultivar, which was expected from previous works with olives and cucumbers preserved in a salt-free environment under

aerobic conditions (de Castro et al., 2007; Medina et al., 2016). On the contrary, LAB were not detected for the first four months although this group of microorganisms were eventually the most numerous once the antimicrobial olive compounds declined and the temperature increased (Fig. 2), which confirmed previous results with Hojiblanca olives (de Castro et al., 2007). Specifically, the analyses at 195 days revealed growth on MRS plates with and without sodium azide, with colony morphology typical of lactobacilli, which was microscopically confirmed. Besides this, pinpoint colonies were observed on GYP agar and microscopic observation of them showed ellipsoidal cocci in pairs and chains with a variable number of cells. Considering the acid environment, it was suspected that they could be *Oenococcus* sp., therefore a special medium was used to isolate and purify these bacteria. It has to be said that these colonies appeared firstly in both Cacereña and Manzanilla cultivars at 6.5 months, whereas they were not found in liquids from cv. Hojiblanca until 12 months, after the summer. In addition, samples from industrial tanks (Cooperativa San José, Badolatosa, Seville, Spain) with similar initial chemical conditions to treatments at pilot plant were also examined in order to know whether they also contained the same microorganisms, and they did. DNA from different isolates was extracted and when analyzed for homologies in the data banks, 16S rRNA gene sequence of the isolate C1a (from the pilot plant) showed 99.16% identity with *Oenococcus kitaharae* DSM 17330, the type strain of this species. When analyzed for homologies, partial 16S rRNA gene sequence of the isolates OE265, OE275BC, OE275PF, OE277 and OE291 (from industrial samples) showed 98.47, 98.64, 98.39, 98.65 and 98.65% identity, respectively, with *Oenococcus kitaharae* DSM 17330. This is the first time that viable *Oenococcus* sp. has been cultured from table olive samples, although *Oenococcus* DNA was recently found by high-throughput sequencing from process liquids of ripe olives darkened by oxidation (Medina, Brenes, García-García, Romero, & de Castro, 2018). Therefore, the influence of these microorganisms on the onset of lactic acid fermentation of black ripe olives must be studied in the future.

With regard to the chemical characteristics of the preservation solutions, the pH ranged between 3.75-4.11 units after 280 days (Table 1), which was below the limit of 4.3 units required for most of the factories to assure the shelf life of their olives. The free acidity, expressed as % acetic acid, was around 1.4% in most of the tanks containing Cacereña and Manzanilla olives whereas it was slightly lower for Hojiblanca

fruit (ca. 1.1%). These data are in agreement with those of the content of these solutions in lactic acid that reached around 0.4% in tanks of the former cultivars, but it was not detected at this time in tanks of the Hojiblanca cultivar. LAB grew in the tanks of the latter cultivar after summer and its substrate could be mannitol, because the concentration of other sugars (glucose, fructose) was very low at 280 days (Table 1). There is no explanation for the time delay of the LAB growth in the tanks of the Hojiblanca olives, the concentration of phenolic compounds was not much higher in the preservation solutions of this cultivar than in those of Manzanilla and Cacereña, except for hydroxytyrosol-4-glucoside and verbascoside (Table 2) but these compounds have not been reported as highly inhibitory against LAB growth (Medina, Brenes, Romero, García, & de Castro, 2007). It is also interesting to highlight the residual content of oleuropein in the preservation solutions of the Manzanilla cultivar at 280 days which must be related to the higher concentration of this secoiridoid in the raw material of this cultivar (20959 ± 2042 mg/kg fresh pulp) than Cacereña (9067 ± 679 mg/kg fresh pulp) and Hojiblanca (10791 ± 1091 mg/kg fresh pulp). In particular, the residual content of oleuropein was higher when calcium chloride was employed. Calcium delays the diffusion of substances from the olives to the surrounding liquid and vice versa (Brenes, García, Romero, & Garrido, 1993), so slower diffusion of the phenolic compounds in the presence of calcium was expected during the preservation stage of black ripe olives. However, it did not have any effect on the progress of the fermentation of the olives and secondary spoilage of the fruit with time was not detected at any time.

The effect of the different treatments assayed on the firmness of olives during their salt-free storage period is shown in Table 3. After 240 days of preservation, before summer, the addition of calcium in the initial cover solution gave rise to firmer texture of fruit, regardless of the type of calcium salt or olive cultivar. This effect was statistically significant for olives of the Manzanilla and Cacereña cultivars that possess a softer texture than Hojiblanca. However, the calcium firming effect was also significant for the latter cultivar after summer. The high ambient temperature during summer (Fig. 2) led to olive softening in all cases, although this action was partially inhibited by calcium, as has been previously reported for the softening rate of olives under acidic conditions (Brenes, García, & Garrido, 1994). At this point, it must be noted that calcium lactate led to higher texture in olives of the Manzanilla cultivar but not in Cacereña, whereas the concentration of calcium was rather similar in all treated

olives. The texture of Hojiblanca olives was higher than the other two cultivars (Table 3), as expected, but this cultivar also often needs calcium addition during the preservation stage or packing process in order to satisfy the required strong texture of black ripe olives (de Castro et al., 2007; García et al., 2014).

Fruit of all treatments were elaborated as black ripe olives after eight months of preservation and their physical and sensory characteristics are recorded in Table 4. As was observed for the texture of the fruit after the preservation stage (Table 3), olives with calcium added in the preservation liquor retained better firmness than those without calcium even after the darkening, packing and sterilization stages. Indeed, this firming effect was independent of the type of calcium salt and cultivar. An interesting finding was the lower content of calcium in processed Cacereña olives in comparison with the values of the other two cultivars, particularly with those of Manzanilla olives that were treated with the same alkaline strength during the darkening step (Table 4). Olives leached calcium from the pulp during the black ripe olive processing (Tables 3 and 4), and this was particularly intense for Cacereña fruit.

Among the different steps carried out during the elaboration of black ripe olives, the alkaline treatment is crucial for the firmness of the final product (Brenes et al., 2017). The alkali breaks cell walls and modifies composition. In Table 5, it is observed that the addition of calcium to the preservation liquor allowed neutral sugars to be retained in the cell wall for both Cacereña and Hojiblanca cultivars, which means less degradation of cell wall framework, and this was related to the firming effect found for calcium salts (Tables 3 and 4). These results are different from those reported for canned peaches treated with calcium chloride and lactate, which increased their content in calcium but the neutral sugars composition of the cell wall was not modified (Manganaris et al., 2005). In our case, olives were submitted to an alkaline treatment and this must be determinant for the different behaviour of olives in comparison with peaches.

A very important quality characteristic of black ripe olives is precisely their shiny black color. Neither calcium chloride nor calcium lactate exerted any influence on this parameter (Table 4). As commented before, calcium ions delay the diffusion of substances from the olives to the surrounding liquid and it could happen during the darkening stage. Hence, the diffusion of phenolic compounds responsible for the

formation of the black color could be affected by the presence of this ion, but this was not detected.

The sensory analysis carried out on processed olives indicated that none of the calcium salts employed during the preservation stage at 20 mM concentration gave rise to bitterness (Table 4), which could be expected because this concentration was much lower than employed for the fermentation of natural black olives (Panagou et al., 2011) and cucumbers (Pérez-Díaz et al., 2015). It is also noteworthy that tasters found stronger hardness in olives preserved with calcium than control, irrespective of the calcium salt tested, which is in accordance with objective measurements obtained by shear-compression (Table 4).

Finally, the weight of olives was also monitored during the different steps of the black ripe olive processing (preservation, darkening and packing), which has been previously analyzed for Hojiblanca olives preserved in brine (García et al., 2014). Olives of the three cultivars studied, Manzanilla, Cacereña and Hojiblanca, lost weight during their preservation stage in salt-free solutions and gained weight during the darkening stage (Fig. 3), but neither the calcium chloride or calcium lactate influenced these olive weight changes to a large extent. It must be stressed again that the diffusion of substances from the olive pulp to the liquid and vice versa is highly affected by the concentration of calcium in the olive pulp.

4. Conclusions

In this study, it has been demonstrated that the employment of calcium salts, chloride and lactate, in the salt-free preservation solutions of olives intended for processing as black ripe olives did not exert a great influence on the microbiota that grew in these solutions, and no secondary or spoilage fermentations were observed. In addition, this is the first time that viable *Oenococcus* sp. has been cultured from table olive samples, although the role of these bacteria in the olive fermentation must be studied in future. Regarding the firming effect of calcium salts, both chloride and lactate retained the olive texture to a similar percentage during the preservation and darkening stages without any significant effect on the flavor of the final product. Therefore, the use of any of these calcium salts for processing of black ripe olives would be an economic matter rather than the effect of them on the physico-chemical and sensory characteristics of the olives.

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485

486 **Figure captions**

487 **Fig. 1.** Evolution of yeast (A), acetic acid bacteria (B) and lactic acid bacteria (C)
488 during preservation of olives of the Cacereña cultivar for one year. Standard deviation
489 of duplicates is depicted for each point.

490 **Fig. 2.** Evolution of the ambient temperature during preservation of olives.

491 **Fig. 3.** Loss and gain of weight (%) of olives during the preservation (eight months) and
492 darkening steps respectively. M, Manzanilla; C, Cacereña; H, Hojiblanca. Standard
493 deviation of duplicates are depicted on the bars.

494

495

Table 1

Chemical characteristics of the preservation solutions of olives fermented under aerobic conditions with different calcium salts for 280 days.

	Manzanilla cultivar			Cacereña cultivar			Hojiblanca cultivar	
	Control	CaCl ₂	Ca Lactate	Control	CaCl ₂	Ca Lactate	Control	CaCl ₂
pH	3.97±0.08a ^x	4.11±0.01a	3.94±0.02a	4.03±0.01a	3.95±0.07a	4.06±0.01a	3.88±0.01a	3.75±0.02b
Free acidity (%) ^y	1.50±0.14a	1.55±0.14a	1.58±0.11a	1.57±0.02a	1.53±0.10a	1.55±0.08a	1.19±0.04a	1.04±0.05a
Lactic acid (%)	0.36±0.08a	0.41±0.04a	0.55±0.09a	0.34±0.05a	0.38±0.06a	0.46±0.09a	nd ^z	nd
Acetic acid (%)	1.30±0.02a	1.34±0.06a	1.51±0.15a	1.41±0.05a	1.40±0.06a	1.47±0.14a	1.17±0.05a	1.00±0.01b
Ethanol (%)	0.19±0.04a	0.27±0.01a	0.27±0.07a	0.16±0.04b	0.29±0.05a	0.27±0.05a	0.06±0.01a	0.07±0.03a
Glucose (%)	nd	nd	nd	nd	nd	nd	nd	nd
Fructose (%)	0.02±0.00a	0.03±0.00a	0.03±0.00a	0.03±0.00a	0.03±0.00a	0.03±0.00a	0.04±0.00a	0.03±0.00a
Mannitol (%)	0.04±0.03a	0.03±0.01a	0.05±0.02a	0.07±0.05a	0.02±0.01a	0.04±0.02a	1.33±0.06a	1.22±0.01b

^xMean value of duplicates±standard deviation. For each row and olive cultivar, values followed by the same letter do not differ (p<0.05) in significance according to Duncan´s multiple-range test. ^yExpressed as acetic acid. ^znd, not detected.

Table 2

Phenolic compounds (mg/kg) in the pulp of olives preserved under aerobic conditions in acidified solutions spiked with calcium salts for 280 days.

	Manzanilla cultivar			Cacereña cultivar			Hojiblanca cultivar	
	Control	CaCl ₂	Ca Lactate	Control	CaCl ₂	Ca Lactate	Control	CaCl ₂
Hydroxytyrosol	972±42a ^x	885±17a	926±11a	892±45a	803±32a	794±84a	663±17a	640±12a
Hy-4-glucoside ^y	nd ^z	nd	nd	17±14b	116±14a	118±36a	314±10a	273±14a
Tyrosol	120±8a	114±1a	104±1a	179±1a	172±6a	171±3a	62±1a	63±3a
Verbascoside	7±1a	40±28a	58±3a	47±25b	139±19a	185±8a	358±2a	307±21b
Oleuropein	241±1b	591±178ab	1058±182a	nd	nd	nd	nd	nd
Luteolin	69±10a	62±5ab	42±4b	56±3a	45±5a	49±5a	105±7a	95±7a
Others	250±10a	241±13a	160±2b	104±31a	140±3a	146±12a	176±3a	137±5b

^xMean value of duplicates±standard deviation. For each row and olive cultivar, values followed by the same letter do not differ (p<0.05) in significance according to Duncan's multiple-range test. ^yHydroxytyrosol-4-glucoside. ^znd, not detected.

Table 3

Firmness (N/100 g pitted olives) and calcium (mg/kg) in the pulp of olives preserved under aerobic conditions in acidified solutions spiked with calcium salts. Firmness was analyzed before (240 days) and after summer (350 days).

	Manzanilla cultivar			Cacereña cultivar			Hojiblanca cultivar	
	Control	CaCl ₂	Ca Lactate	Control	CaCl ₂	Ca Lactate	Control	CaCl ₂
<i>Firmness</i>								
t=240 days	5724±153b ^x	5959±38b	6575±255a	3526±183b	4529±63a	4391±175a	7836±513a	8189±425a
t=350 days	4775±7c	5578±72b	6155±159a	3061±337b	4038±153a	4202±524a	6462±29b	7116±392a
<i>Calcium</i>								
t=0 days	595±10	595±10	595±10	212±4	212±4	212±4	568±8	568±8
t=240 days	609±11b	927±63a	993±70a	490±56b	916±13a	969±55a	616±8b	975±83a

^xMean value of duplicates±standard deviation. For each row and olive cultivar, values followed by the same letter do not differ (p<0.05) in significance according to Duncan's multiple-range test.

Table 4

Influence of the addition of calcium salts during the preservation stage of olives on the color, calcium, texture and sensory characteristics of the final product processed as black ripe olives and stored for two months at ambient temperature.

Treatment	Color (R ₇₀₀)	Firmness (N/100 g pitted olives)	Calcium (mg/kg olive flesh)	Sensory attributes	
				Bitterness	Hardness
M-Control ^x	3.6±0.3a ^y	972±24b	603±60b	1.0±0.1a	3.0±0.5b
M-Ca chloride	3.2±0.2a	1240±155a	785±55a	1.1±0.2a	4.5±0.3a
M-Ca lactate	3.4±0.2a	1422±62a	779±135a	1.2±0.1a	5.2±0.6a
C-Control	3.0±0.1a	789±21b	381±2b	1.2±0.1a	3.0±0.4b
C-Ca chloride	2.8±0.2a	1128±82a	481±20ab	1.1±0.1a	5.0±0.4a
C-Ca lactate	3.0±0.1a	1162±100a	550±34a	1.2±0.2a	4.8±0.5a
H-Control	3.4±0.1a	1056±70b	676±12b	NA ^z	NA
H-Ca chloride	3.5±0.1a	1391±71a	845±1a	NA	NA

^xM, Manzanilla; C, Cacereña; H, Hojiblanca. ^yMean value of duplicate±standard deviation. For each column and olive cultivar, values followed by the same letter do not differ (p<0.05) in significance according to Duncan's multiple-range test. ^zNA, none analyzed.

Table 5

Glycosil composition (g/100 g) in the alcohol insoluble fraction of olive pulp samples of Cacereña and Hojiblanca cultivars preserved for eight months without calcium added (C-P and H-P), preserved without calcium and processed as black ripe olives (C-Black and H-Black), and preserved with calcium chloride and processed as black ripe olives (C-Ca-Black and H-Ca-Black).

	Rhamnose	Fucose	Arabinose	Xilose	Mannose	Galactose	Glucose	Total
C-P	0.039(0.027)a	0.015(0.003)a	0.636(0.002)b	0.041(0.018)b	0.066(0.007)a	0.196(0.008)a	1.490(0.087)a	2.484(0.115)a
C-Black	0.031(0.002)a	0.000(0.000)b	0.064(0.030)c	0.299(0.104)a	0.000(0.000)b	0.022(0.011)b	0.074(0.021)b	0.490(0.062)b
C-Ca-Black	0.019(0.002)a	0.000(0.000)b	0.779(0.024)a	0.066(0.000)b	0.074(0.003)a	0.217(0.015)a	1.672(0.213)a	2.827(0.256)a
H-P	0.019(0.001)a	0.001(0.000)b	0.547(0.023)a	0.051(0.003)b	0.065(0.004)a	0.211(0.008)a	1.392(0.028)a	2.285(0.052)a
H-Black	0.036(0.006)a	0.000(0.000)b	0.078(0.019)b	0.270(0.004)a	0.000(0.000)b	0.025(0.007)b	0.136(0.021)b	0.545(0.048)b
H-Ca-Black	0.020(0.005)a	0.008(0.012)ab	0.583(0.127)a	0.053(0.012)b	0.065(0.012)a	0.193(0.046)a	1.267(0.477)a	2.189(0.667)a

Standard deviation of duplicates is shown in parentheses, and column values with the same letter for each cultivar are not significantly different by Duncan's multiple-range test ($p < 0.05$).

Figure 1

Figure 2