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Influence of iron redox state on black ripe olive processing

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Running title: Iron in black olives

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Abstract

BACKGROUND: The oxidation and polymerization of the phenolic compounds present in black ripe olives give rise to their black shiny colour which is fixed by the addition of ferrous salts such as ferrous gluconate. Among the many variables that can affect the successful of this fixation step, the influence of the pH on this process was assessed on fruits of the Hojiblanca cultivar processed at pilot plant scale. Hence, the aim of this work was to gain knowledge on the development of this fixation step, in particular on olives that were oxidized with a single alkaline treatment.

RESULTS: It was found that the penetration of the ferric cation into the olive flesh was an extremely slow process and it led to less dark olives. Besides, at a pH higher than 6 the oxidation of the ferrous to the ferric cation was a very rapid reaction, and olives that were processed with initial pH adjusted to 4 units had darker colour than those without pH correction. Ultrafiltration/nanofiltration of oxidized olive solutions spiked with ferric ions or even pure oxidized ferric solutions through membranes of 10 kda, 3 kda and 1 kda revealed that ferric ions formed high molecular structures that inhibit the penetration of the ion into the olive flesh and therefore the fixation of the black colour.

CONCLUSION: The results presented in this study suggest that the formation of ferric ions during the colour fixation step of black ripe olives must be avoided to get darker olives. Consequently, the initial pH of the ferrous gluconate solution must be corrected to avoid oxidation of the ferrous to ferric cations during the fixation step. This study will contribute to the improvement of the colour fixation step of black ripe olive processing.

Keywords: Olive, Fe (II), Fe (III), colour, oxidation

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INTRODUCTION

According to the International Olive Council (IOC), global table olive production for the 2015/2016 season accounted for approximately 2.7 million tons, the main commercial types of table olives being the Spanish-style green, the California-style black and the Greek-style black olive. The method of producing black ripe olives was created at the end of the 19th century in the USA, and today they are widely consumed in pizzas and salads, in America and worldwide, due to their attractive black colour.^{1,2}

To produce black ripe olives, American processors store the Manzanilla olives in an acidic solution spiked with calcium chloride and sodium benzoate, and after several months, they are submitted to the oxidation process. The darkening step involves successive treatments (3-5) with a dilute solution of sodium hydroxide (lye); after each lye treatment, olives are put in water and air is bubbled through the tank.¹ The phenolic compounds present in the olive flesh, mainly the *o*-diphenols hydroxytyrosol and caffeic acid, are oxidized under the alkaline conditions to produce coloured polymers responsible for the appreciated black colour of these olives.² However, this process generates a high volume of wastewaters (lyes and washing waters),³ and a new method with a single lye treatment has recently been put forward.⁴

After the darkening step, olives are currently destoned, washed, canned and sterilized, and they can undergo discolouration. Hence, the use of ferrous gluconate and lactate is legally permitted to fix the colour.⁵ Both iron salts are effective in preventing colour deterioration although ferrous gluconate is most widely employed worldwide.^{6,7} The iron-phenol complexes formed are very stable and the black colour of the olives can be preserved even during their shelf-life of three years.⁸

The interaction between iron species and phenolic compounds has been extensively studied although results are sometimes contradictory. Chelation of simple phenols such as hydroxytyrosol and caffeic acid by Fe (II) has been documented,⁹ but also the interaction between Fe (III) and the flavonoids catechin and **quercetin**.¹⁰ Catrouillet et al proposed that the complexation of Fe (II) with humic acids is pH dependent,¹¹ the ion being mainly adsorbed onto carboxylic groups at acidic pH and phenolic groups at alkaline pH. Moreover, it has been reported that phenolic compounds

bind first to Fe (II) and then the Fe (II)-polyphenol complex autoxidizes to Fe (III) in the presence of oxygen,¹² and this has been the mechanism suggested for colour fixation in black ripe olives. After the darkening step, olives are covered with a 0.5-1.5 g L⁻¹ ferrous gluconate solution that allows the penetration of the ferrous ions into the olive flesh for the formation of the polyphenol-Fe (II) complexes that oxidizes to Fe (III) in the interior of the fruit.⁷ However, the oxidation of ferrous ions is a more rapid reaction than binding to phenolic compounds so the mechanism of colour fixation in black olives by iron species remains unclear.¹³

MATERIALS AND METHODS

Test to know the effect of iron on non-oxidized olives

Fruits of the Hojiblanca cultivar (*Olea europaea* L.) stored for six months in industrial tanks at the Agrosevilla SCA factory under acidic conditions were used for the assays.¹⁴ They had a pH of around 4 units and 3% salt concentration. One kilogram of the olives was covered directly with 1 g L⁻¹ ferrous gluconate solution (1 L). Subsequently, the mixture was aerated for 24 hours, and the colour (R₇₀₀) was measured.

Standard oxidation process of black ripe olives

Olives were oxidized in four methacrylate horizontal cylindrical containers where air was bubbled along the horizontal base of the cylinder. Temperature was controlled inside the oxidation tanks by a hot/cold thermostatic bath (Selecta, Spain), and the pH of the liquid by a pH meter (pH/mv Controller 252, Crison, Spain) connected to an automatic dosage system which bubbled CO₂ to neutralize the excess NaOH in the olive flesh.¹⁵

Assays were carried out on 3 kg of olives per chamber. Fruits were immersed in 3.2 L of a NaOH solution (28 g L⁻¹) until it penetrated to the stone. Then the lye was removed and the olives were placed in tap water for 20 hours. Air was continuously bubbled into the suspension water/liquid, and the pH of the liquid was maintained at 8.3 units by adding CO₂. Another 20 hour cycle with fresh tap water was repeated.

Effect of pH correction during the colour fixation step of black olives

Oxidized olives with a pH in the flesh of 8.3 units were placed in a 1 g L⁻¹ ferrous gluconate solution (Purac Bioquímica, S. A., Barcelona, Spain) without pH correction (5.5 units) or were pH corrected with acetic acid up to 4 units for six hours. Air was bubbled during the colour fixation step at 20°C and experiments were run in duplicate.

The pH of the olive flesh was analyzed with a pHmeter-Test 10BNC Spear (Eutech Instruments, Malaysia), simply by introducing the electrode tip into the olive.

Colour fixation of black olives with ferrous and ferric ions at pH 4.5

Oxidized fruits were covered with (i) a ferrous gluconate solution (1 g L⁻¹) with pH adjusted to 4.5 units with acetic acid, and (ii) a ferric gluconate solution (1 g L⁻¹) with the pH also adjusted to 4.5 units. The olives/colour mixture fixation solution was aerated for six hours and the pH maintained at 4.5 with occasional additions of acetic acid. To obtain the ferric gluconate solution, the pH of 1 g L⁻¹ ferrous gluconate solution was maintained at 8 units with the addition of 2M NaOH, and the liquid was aerated for 24 hours. Then the pH was dropped to 4.5 units with 2M acetic acid.

Influence of pH on the oxidation rate of ferrous ions in a pure ferrous gluconate solution

Three litres of 1 g L⁻¹ ferrous gluconate solution (Purac Bioquímica S. A., Barcelona, Spain) were put into each of the four oxidation chambers described above. Air was bubbled into these solutions for six hours, and their pH was maintained at 4, 6, 8 and 9 units by adding 2M acetic acid or 2M NaOH. Iron species were determined throughout the oxidation process.

Study of the colour fixation with ferrous and ferric ions in washing waters of black olives

Two litres of liquid at pH 8 from the second washing water of the black olive processing was obtained from an industrial oxidation tank (Agro Sevilla S.C.A, Seville, Spain) and transported immediately to the Instituto de la Grasa laboratory (Seville, Spain). Glacial acetic acid was added to 100 mL of this solution to drop the pH by up to 4.5 units and it was then mixed with 400 mL of a solution containing 1 g L⁻¹ ferrous gluconate with pH adjusted to 4.5 with 2M acetic acid. The mixture was left at 25°C for

five hours without aeration. At time 0 and five hours, 10 mL of the mixture was ultrafiltered through a Ultracel 3 kDa cut-off membrane (Millipore Corp., MA, USA) into an Amicon 50 mL stirred cell (Merck Group, Darmstadt, Germany) under nitrogen pressure. Iron species and colour of the liquid was analyzed. Assays were run in duplicate.

In parallel, 100 mL of the industrial solution with pH adjusted to 4.5 units were mixed with 400 mL of a ferric gluconate solution and left at 25°C for five hours without aeration. Colour and iron species were also measured in the non-ultrafiltered and ultrafiltered solutions obtained at time 0 and five hours, as explained above.

The ferric solution was also ultrafiltered through Ultracel 1000, 3000 and 10000 daltons cut-off membranes, and the total iron was analyzed in the ultrafiltered solutions. Assays were run in duplicate.

Analysis of iron species

The total iron in the olive flesh was extracted by wet digestion in triplicate. Two grams of exactly weighted triturated flesh were placed in 50 mL bore-silicate tubes, 25 mL of nitric acid (63% w/w) were added, and the tubes were covered with funnels. Subsequently, they were placed in a *DigiPREPJr* (SCP Science, Canada) and kept at 120°C for eight hours. Once at room temperature, the funnels were removed and 5 mL of a solution (1:4) of nitric acid (63% w/w) and perchloric acid (60% w/w) was added, with part of the liquid being evaporated by heating at 140°C for two hours. The concentrated solution was transferred to a 25 mL flask and filled with deionized water.

The analysis of total iron and Fe (II) in the liquids was carried out by the *o*-phenanthroline method described elsewhere with modifications.^{16,17} A mixture of 0.4-1 mL of sample, 2 mL of 0.5 M HCl, 1 mL of 2% fresh hydroquinone, 1 mL of 0.5% 1,10-phenanthroline chloride, and 0.6 mL of 25% sodium citrate was placed into a volumetric flask. Deionized water was added up to 25 mL, and the solution was left at room temperature for 30 minutes before measurement of the absorbance at 508 nm by using a Shimadzu UV-vis spectrophotometer. For the analysis of Fe (II), hydroquinone was not added into the mixture.

The concentration of Fe (III) was obtained from the difference between total iron and Fe (II).

Colour measurement of olives

The surface colour of the fruits was expressed as reflectance at 700 nm (R_{700}) using a BYK-Gardner Model 9000 Color view spectrophotometer (Silver Spring, Md., USA). The data of each measurement were the average of 20 olives. Lower reflectance values indicate darker fruit.

Colour measurement of liquids

Solutions were centrifuged at 10000 x g for five minutes, and their absorbance spectrum ($\lambda_{380}-\lambda_{740}$) was recorded using a Shimadzu UV-vis spectrophotometer, which was equipped with a computer software program to calculate the CIE L^* (lightness), a^* (redness) and b^* (yellowness) parameters.

RESULTS AND DISCUSSION

Effect of iron on non-oxidized olives

The additives ferrous gluconate (E-579) and ferrous lactate (E-585) are employed to fix the black colour formed in olives during the oxidation step but no data are available on the effect of iron on non-oxidized olives.⁵ The fruits submitted to aeration for 24 hours in a ferrous gluconate solution only diminished their colour parameter (R_{700}) from 41.1 to 35.1, which means that the olives had a light grey colour instead of the current black colour of oxidized fruit that have R_{700} values below 10-12 units. Therefore, it is necessary to oxidize the phenolic compounds present in the olive flesh, mainly the *o*-diphenols hydroxytyrosol and caffeic acid, to get a coloured polymer responsible for the black colour on this type of olive.^{1,2,18} Then, the ferrous salts are needed to fix the colour formed and to avoid discolouration during the further packing, sterilization and shelf-life steps.

The rest of the assays carried out in this study were aimed at gaining knowledge on this fixation step.

Effect of pH correction during the colour fixation step of black olives

The initial correction of the pH of the ferrous gluconate solution had a great effect on the diffusion rate of iron into the olive flesh (Figure 1). The concentration of total iron

196 in the liquid with initial pH correction diminished rapidly up to 60% during the first two
197 hours of aeration, whereas in the liquid without initial acidification, the total iron
198 content dropped only 30%, this decline being more marked during the first hour of
199 aeration. The rapid diffusion of total iron into the olive flesh during the assay with the
200 initial pH of the corrected liquid must be noted, since previous studies indicated a time
201 of more than five hours to stabilize the penetration of the total iron into the fruit.⁷ In our
202 experiments, olives were treated with a single lye of 28 g L⁻¹ strength, and previous
203 assays were run with weak lyes of 10-15 g L⁻¹, therefore the permeability of the former
204 olives was higher than the latter. At the end of the colour fixation step (six hours), the
205 difference in total iron concentration between the solutions with pH initially corrected
206 and non-corrected was around 35 mg L⁻¹.

207 The concentration of Fe (II) decreased in the liquids in a similar manner than
208 total iron (Figure 1). This process was quite fast during the first 1-2 hours, and low
209 differences of about 10 mg L⁻¹ between solutions with and without initial pH correction
210 were found after six hours of contact.

211 With regard to Fe (III), the content of this ion increased in the solution without
212 initial pH correction from 20 mg L⁻¹ to 40 mg L⁻¹, whereas it remained almost constant
213 during the fixation step in the solution with pH corrected to 4 units. This different
214 behaviour must be related to the evolution of the pH in the solutions during the fixation
215 process (Figure 2). When the pH of the ferrous gluconate solution was initially dropped
216 to 4 units, this parameter increased up to 5.5 units after six hours of contact. By
217 contrast, it increased from 5.5 units to 8 units in the solution without any initial
218 correction of the pH, and it probably accelerated the oxidation of the ferrous ions to
219 ferric.

220 To confirm this hypothesis, pure ferrous gluconate solutions were oxidized from
221 pH 4 to 9 at 20°C. Results are presented in Figure 3 and they revealed that oxidation of
222 iron at pH 4 is a very slow process and at pH 6 less than 30% of the initial ferrous ion
223 was converted to ferric after six hours of aeration. As the pH increased to 8 and 9 units,
224 the oxidation of ferrous ion was a very rapid reaction. Half of the ferrous ions were
225 converted to ferric in just a few minutes, and these findings can explain the formation of
226 ferric ions during the colour fixation step without any initial pH correction (Figure 1).

227 However, it remained unclear why the content of this ferric cation in the fixation

solutions did not decrease with time (Figure 1), in particular in the solutions without any initial pH correction. Indeed, this behaviour of Fe (III) in the fixation solution was correlated with the low absorption of total iron in the flesh of olives covered with ferrous gluconate without pH correction (Figure 4). In addition, the superficial colour of fruits submerged in the ferrous gluconate solution with the initial pH dropped to 4 units was darker, lower R_{700} , than those covered with the ferrous salt without pH correction (Figure 4). The R_{700} parameter decreased from 10.8 to 5.8 units in the former olives and to 7.4 units in the latter, therefore a darkening effect was observed during the colour fixation step, and it was more pronounced when the pH of the ferrous solution was adjusted to 4 units.

Colour fixation with ferrous and ferric ions at pH 4.5

There are no official or regulated conditions for the use of ferrous gluconate solution, therefore olive factories may add the salt directly to the aerated mixture of olives/water or may prepare the salt solution in advance with or without pH correction. In any case, the results provided in this work suggest that the oxidation of the iron salt must be reduced. In a new experiment, oxidized olives were put in both ferrous and ferric solutions for five hours under aeration and controlled pH at 4.5 units. As was expected, the R_{700} parameter of olives submerged in the ferrous solution decreased from 14.2 to 5.1 units (Figure 5), whereas it remained almost constant in olives covered with the ferric solution. The iron content diminished to a large extent in the ferrous solution but it only decreased by around 25% in the ferric solution.

These results (Figures 1, 4 and 5) pointed out that the colour of olives did not change with Fe (III) and this cation did not penetrate into the olive flesh. Two questions remained unanswered: (i) why does Fe (III) not penetrate into the olive flesh?; and (ii) does the Fe(III) form coloured complexes with oxidized polymers? An experiment with washing water as a model solution was designed to answer these questions. Results are presented in Table 1. Both ferrous and ferric ions darkened the washing water at time 0 of addition and more intensively after five hours of contact, the lightness parameter (L^*) being the more affected by the addition of the iron cations to the olive solution. This parameter decreased from 84.3 in a mixture of washing water/distilled water (1:4) to 63.3 and 55.4 in the ferrous and ferric solutions respectively after five hours of contact with the washing water (1:4), therefore these findings demonstrated that both iron

species were able to form coloured complexes in olive solutions. Besides, the nanofiltration of both ferrous and ferric solutions/washing water through 3000 daltons gave rise to an increase in brightness close to that of the washing water/distilled water mixture, which suggested that the increase in colour of the solution was mainly due to the formation of Fe-phenolic complexes of high molecular weight.

With regard to the concentration of iron in the spiked washing waters (Table 1), it must be highlighted that most of the Fe (II) passed through the 3000 daltons pore size membrane. By contrast, the membrane retained most of the Fe (III) and it could explain the low penetration of this cation into the olive flesh (Fig. 1 and 5) due to the formation of high molecular complexes with phenolic polymers. However, a pure Fe (III) solution was nanofiltered through membranes of 10 kda, 3 kda and 1 kda and it was found that 38%, 83% and 98% of the initial iron content was retained on the membrane respectively. Consequently, the polymeric structure formed during the oxidation of ferrous gluconate could inhibit the penetration of Fe (III) into olives, although the formation of polymeric Fe (III)-phenolic complexes must not be ruled out.

CONCLUSIONS

After the oxidation step, the pH of the olive flesh may range between 7.5 and 8.5 units and it allows for the increase of pH in the ferrous gluconate solution during the colour fixation step to up to 8-8.5 units. It has been found that at a pH higher than 6, the oxidation of the ferrous to ferric cation is a very rapid reaction, so the pH of the ferrous gluconate solution must be corrected at the beginning of the fixation step to reduce the oxidation of the metal. Surprisingly, the penetration of the ferric cation into the olive flesh was an extremely slow process and it led to less dark olives. Although the ferric ions were able to darken the colour of the washing waters, their high molecular structures formed during the oxidation of the ferrous gluconate solution to ferric state limited their penetration into the olive flesh, and thus the formation of coloured iron complexes. The results presented in this study suggest that the formation of ferric ions during the colour fixation step of black ripe olives must be avoided to get darker olives.

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Figure captions

Figure 1. Evolution of total iron, Fe (II) and Fe (III) in the ferrous gluconate solutions in contact with olives during the colour fixation step when the initial pH was corrected to 4 units or not corrected. Bars mean the standard deviation.

Figure 2. Influence of the initial pH correction up to 4 units on the evolution of this parameter in the ferrous gluconate solution used for the colour fixation of black ripe olives. Bars mean the standard deviation.

Figure 3. Influence of pH on the evolution of iron species during oxidation of a ferrous gluconate solution of 1 g L⁻¹. The solutions were aerated at 20°C for six hours.

Figure 4. Evolution of the colour and iron concentration of black ripe olives during the colour fixation step in a ferrous gluconate solution with and without initially corrected the pH. Bars mean the standard deviation.

Figure 5. Colour of olives and iron content in both ferrous and ferric solutions during the colour fixation step at pH 4.5. The ferrous solution had 92% Fe (II) and the ferric 75% Fe (III). Bars mean the standard deviation.

Table 1 Influence of iron oxidation status on the colour of a washing solution from black ripe olive processing. A mixture of this washing solution with a ferrous or ferric solution (1:4) was studied at pH 4.5 for five hours.

	L^*	a^*	b^*	Total iron (mg L ⁻¹)	Fe (II) (mg L ⁻¹)
<i>Ferrous solution</i>					
t = 0 hours, non-nanofiltered solution	74.3 (3.6) ^a	0.1 (0)	31.3 (0.3)	99.1 (1.9)	74.9 (4.1)
t = 5 hours, non-nanofiltered solution	63.3 (7.2)	-0.7 (0.4)	29.7 (0.3)	98.3 (1.1)	79.8 (3.4)
t = 0 hours, nanofiltered solution	81.3 (2.8)	-4.4 (0.2)	21.6 (1.7)	74.1 (4.1)	68.9 (1.3)
t = 5 hours, nanofiltered solution	79.5 (2.4)	-5.4 (0.7)	21.4 (0.6)	77.2 (6.4)	54.5 (2.8)
<i>Ferric solution</i>					
t = 0 hours, non-nanofiltered solution	66.0 (2.1)	2.5 (0.9)	44.8 (1.7)	79.1 (4.9)	14.8 (1.5)
t = 5 hours, non-nanofiltered solution	55.4 (6.5)	0.5 (0.2)	35.6 (0.8)	75.1 (2.3)	14.2 (1.3)
t = 0 hours, nanofiltered solution	84.6 (2.2)	-4.4 (0.3)	20.7 (1.8)	15.5 (1.0)	6.8 (3.8)
t = 5 hours, nanofiltered solution	83.3 (0.8)	-5.2 (0.1)	20.1 (1.2)	15.1 (1.8)	8.8 (1.2)

^aStandard deviation is shown between parentheses. Colour parameters of a mixture (1:4) of the washing solution and distilled water at pH 4.5 were the following: $L^* = 84.3$ (0.6), $a^* = 1.9$ (0.1), $b^* = 33.0$ (0.3).