This is the peer reviewed version of the following article: Sánchez, A. H., Romero, C., de Castro, A., Rejano, L. and Brenes, M. (2016), Assays to control the development of the green staining alteration in Spanish-style green olives of the Gordal variety. J. Sci. Food Agric., 96: 4032–4036, which has been published in final form at: http://doi.org/ 10.1002/jsfa.7598. This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Self-Archiving.

Assays to control the development of the green staining alteration in Spanish-style green olives of the Gordal variety

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Running title: Green staining alteration in Gordal olives

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Abstract

BACKGROUND: Olives of the Gordal variety processed according to the Spanish-style sometimes develop an alteration in color known as green staining (GS) due to the formation of harmless copper-chlorophyll complexes that make the product less valuable. The aim of this study was to investigate methods to minimize the impact that this alteration supposes for the table olive industry.

RESULTS: Calcium chloride, sorbic, benzoic and ascorbic acids and SO_2 did not inhibit the development of the alteration in olives packed under their own fermentation brine or new fresh brine. It was also discovered that the incubation of olive samples at 45 ºC for 20 days accelerates the formation of GS, and it can be a very useful tool to predict the incidence of the alteration in advance. By applying this test to numerous industrial tanks for four consecutive seasons, it was found that GS was mainly present in olives harvested at the beginning of the season.

CONCLUSION: The formation of GS in olives of the Gordal variety is time and temperature dependent, and none of the additives tested avoided or retarded the development of the alteration. However, an accelerated test to predict the development of the GS formation has been proposed that could contribute to minimize the effects of the alteration.

Keywords: table olives; copper-chlorophyll; color; alteration; Gordal

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INTRODUCTION

Among the olive varieties intended for table olives, Gordal is one of the most appreciated by consumers due to its large size and high flesh/pit.[1] This variety is also known as Sevillana, and is usually processed according to the Spanish-style. The olives are picked during September and October in Spain when they have a green-yellow color on their surface. Upon arrival to the olive factories, the fruit is treated first with a dilute NaOH solution to hydrolyze the bitter glucoside oleuropein. Subsequently, the olives are washed with tap water to remove excess alkali and covered with a NaCl solution in which spontaneous lactic acid fermentation takes place for months.[2] At the end of the process, the olives have a yellow color that makes them very attractive for consumers. However, the Gordal olives sometimes develop an alteration in color known as "green staining" (GS). This is seen as bluish-green zones distributed over the skin that are initially small spots but with time can cover a great part of the fruit surface (Figure 1).

The formation of Cu-chlorophyll complexes with the endogenous copper of the fruit has been associated with the appearance of GS in Gordal olives fermented following the Spanish-style.[3] Nevertheless, these metal-chlorophyll complexes have also been detected in olives elaborated following the Castelvetrano method,[4] and in several samples of commercial table olives.[5]

It has been proposed that the pectin chain acts as a reservoir of the Cu which forms a complex with chlorophyll derivatives,[6] but the involvement of oxidative disintegration of the fruit chloroplasts is also necessary to allow contact between the chlorophyll pigments and the copper.[7] However, the fact that this alteration is mainly expressed in fruit of the Gordal variety raises the question of specific characteristics of this variety.

Gordal olives are amongst those of the biggest size employed for table olive processing,[1] and they show a high respiration rate during the post-harvest period.[8] It is considered a sweet variety due to its low content in phenolic compounds, particularly the bitter glucoside oleuropein,[9] and it favors lactic acid fermentation.[10] Gordal olives also possess higher polyphenoloxidase activity than Hojiblanca but similar to Manzanilla, which are the two most employed Spanish varieties for processing as table olives.[9] PPO is a metallo enzyme with Cu in its prosthetic group that could be involved

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in the formation of the Cu-chlorophyll complexes although this relationship has not been found.[11] Besides, none of these two varieties usually develops GS.

Although the Cu-chlorophyll complexes possess inhibitory activity against mutagenesis and high antioxidant activity,[12,13] color is important for consumer acceptance and GS alteration is of great concern for olive factories. Hence, methods to inhibit or retard the development of GS in fermented Gordal olives are required. The aim of this work was to gain knowledge about the variables that affect the formation of GS in this particular olive variety, ways and methods to prevent the formation of the alteration, and to develop an accelerated test to predict the formation of GS.

MATERIALS AND METHODS

Assessment of GS

An accelerated test was developed to predict the formation of GS with time. To this aim, around 580 g of olives obtained from industrial tanks were packed in quarter-liter jars and covered with 350 mL of fermentation brine. Subsequently, they were sealed and incubated in a thermostatic chamber at 45 ºC for 20 days. Finally, five trained experts visually assessed the appearance of bluish-green zones on the surface of every olive. The effectiveness of the method was checked during the season 2006/2007 by comparing the percentage of olives with GS predicted by the accelerated test in January, 2007 and the presence of GS in fruit of the same batch stored at ambient temperature until September, 2007.

The GS test was carried out on olives of the Gordal variety processed according to the Spanish-style from the 2006/2007 to 2009/2010 season. The fruits were elaborated in three olive factories located in the province of Seville (Spain), and the method consisted of placing the fruits in a NaOH solution of 15-20 g L^{-1} for 8-10 h until the alkaline solution reached two-thirds of the way to the pit of the olives. Subsequently, the fruit were washed with tap water for 5-10 h and then covered with a 100 g L^{-1} NaCl solution where spontaneous lactic acid fermentation took place for months. The fermentation step was carried out in tanks containing around 10000 kg of olives and 5000 L of brine. The number of tanks analyzed was 146, 223, 364 and 233 during the 2006/2007, 2007/2008, 2008/2009 and 2009/2010 seasons, respectively.

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Packing experiments to prevent the development of GS

Gordal olives processed according to the Spanish-style were obtained from 10 industrial tanks in June 2006 during the 2005/2006 season (samples A-J). Subsequently, the fruits were packed in the jars, and covered with i) the same fermentation brine, ii) a new brine of 60 g L^{-1} NaCl, iii) this new brine spiked with 3 g L^{-1} calcium chloride. The jars were incubated at 45 ºC until September, except a lot with fermentation brine that was stored at 7 ºC. Finally, the presence of GS was assessed by the trained olive experts.

Olives from only one tank of the 2005/2006 season with symptoms of GS were packed in June in the jars, and covered with the fermentation brine alone or the brine spiked with the preservatives potassium sorbate (1 g L^{-1} sorbic acid), sodium benzoate (2 m L^{-1} benzoic acid) or citric acid (6 g L^{-1}).The jars were incubated until September at 45 ºC. Olives packed with the fermentation brine alone were also incubated at 7 ºC, 25 ºC and 65 ºC until September for the assessment of GS.

Fruits from the season 2006/2007 with symptoms of GS were obtained in February 2007 from an industrial tank of Gordal olives processed according to the Spanish-style. The olives were packed in the jars and covered with the fermentation brine or this brine spiked with ascorbic acid (1 g L^{-1}) or SO_2 (0.2 g L^{-1}). The jars were incubated at 45 ºC until June.

Fermentation experiments to prevent the development of GS

At the beginning of the 2009/2010 season (second week of September 2009) an experiment was performed to study the influence of the processing conditions on the development of GS in olives of the Gordal variety. The fruits were processed during three consecutive days in 15 industrial tanks made of fiberglass and buried into the ground. Around 10000 kg of olives were covered in each tank with 5000 L of NaOH 17 g L^{-1} until the alkaline solution penetrated two thirds of the way to the pit of the fruit. Subsequently, the NaOH solution was discharged and tap water was added. After 4-8 h, the washing water was eliminated, and the olives were immersed in a 100 g L^{-1} NaCl solution for fermentation. A batch of 15 kg of debittered olives immersed in their own

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brine was taken out from each tank and transported to the Instituto de la Grasa facilities. Three kilograms of olives from each industrial tank were put into four polyethylene recipients, covered with 1.5 L of the brines and left for fermentation at ambient temperature (Treatment A). Two of the recipients were inoculated with the strain *Lactobacillus pentosus* LP99 from the Instituto de la Grasa's own collection (Treatment B). The strain was grown in flasks with MRS broth + 40 g L^{-1} NaCl and incubated overnight at 32 ºC. The cultures were centrifuged and the pellets washed with saline. The washed cells were centrifuged again, re-suspended in saline and added to the fermenters 48 h after brining. In order to overcome the unfavorably high pH values, the inocula were calculated to achieve an initial population of ca. of 10^8 CFU mL^{-1}. The population of lactic acid bacteria in each fermenter before and after inoculation was checked by seeding their brines onto MRS agar plates with a spiral plater, and counting the colonies after 72 h of anaerobic incubation at 32 ºC.

At the same time, a batch of 12 kg of raw olives was obtained from each industrial tank before processing and transported to the Instituto de la Grasa facilities. Three kilograms of fruit from each lot were put into four polyethylene recipients, covered with a NaOH solution, washed with tap water and put into brine in a similar manner as that done at industrial scale. Two of the recipients were left at ambient temperature in the Instituto de la Grasa (Treatment C), and the olives from the other two recipients were transported to the olive factory and immersed inside a plastic net into each industrial tank for fermentation (Treatment D).

The accelerated test was performed on olives of all treatment in March, 2010.

Chemical analyses

The titratable acidity, pH and combined acidity of the olive solutions were measured using a Metrohm 670 Titro-processor (Herisau, Switzerland). Titratable acidity was determined by titrating up to pH 8.3 with NaOH 0.2 M and expressed as percent (w/v) lactic acid.

RESULTS AND DISCUSSION

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The results from the packing experiments carried out to assess the influence of calcium, temperature and type of brine on GS development are presented in Table 1. Packing with fresh brine did not show a lower incidence of GS that using the same fermentation brine, particularly when focusing on the three samples with higher percentages of altered fruit (E, H and J). It must be noted that the pH was rather similar for olives packed in both types of brine after equilibrium. Moreover, the addition of calcium into the brines did not affect the percentage of the alteration despite the fact that a higher Ca content has been reported in altered fruit than unaltered, and higher in the altered zones than in the unaltered zones of the same fruit.[6] Pectin chains have been proposed as a storage place for Cu, and displacement of this cation by calcium from the galacturonic units could occur. Besides, the addition of calcium to olives preserved under acidic conditions inhibits cell wall degradation thereby lessening the softening rate of the fruit with time.[14] In fact, the formation of Cu-complexes has been associated with cell degradation, particularly chloroplasts.[7] Likewise, this phenomenon seems to be temperature dependent, which can be observed in Table 1. Olives preserved at 7 ºC had a lower incidence of GS than those at 45 ºC regardless of the type of brine or calcium addition.

A new packing assay was performed with olives obtained from industrial tanks in June. They were packed in their own fermentation brine, and stored until September. Again, GS percentage was higher with increasing storage temperature from 7 ºC to 45 ºC (Table 2). Surprisingly, the alteration was lower when olives were kept at 65 ºC, which could suggest the involvement of enzymatic reactions during the formation of GS. However, it must be noted that the olives suffered a severe alkaline treatment at the beginning of the Spanish-style processing that probably inactivated most olive enzymes.[15] Moreover, the influence of the preservatives sorbic and benzoic acids as well as the metal-chelating citric acid was also tested during this season 2005/2206 but none of them prevented the formation of GS during summer storage of the fruit at 45 ºC.

Because of the suspicion about enzymatic involvement in the formation of GS, a new assay was carried out during the next 2006/2007 season with olives that were pasteurized after packing in their own fermentation brine with and without the addition of the antioxidants ascorbic acid and SO_2. The results presented in Table 3 reveal a continuous increase in the GS alteration from February to June regardless of the

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addition of antioxidants. Also, the formation of GS occurred in these pasteurized olives and therefore the involvement of enzymatic reactions must be ruled out.

From the foregoing it is deduced that none of the additives studied and with permitted use in table olives inhibited or retarded the formation of GS in Gordal olives.[16] Hence, an accelerated test was performed to predict the development of the alteration as soon as possible after primary fermentation, which may last from September to December. With this aim, samples of 1-2 kg of olives from 33 industrial tanks from the 2006/2007 season were packed in jars and covered with their fermentation brine in the second fortnight of December. One jar was incubated at 45 ºC and another left at ambient temperature. In January, after 20-25 days of incubation at 45 ºC the jars were opened and the percentage of GS alteration assessed. Likewise, the alteration was checked in the olives left at ambient temperature in September. The results are presented in Table 4. First, it is noteworthy that it was predicted in January that olives from 24 tanks would not develop the alteration, which was confirmed in September with the olives stored at ambient temperature, none of them had GS. There were 9 tanks with symptoms of the alteration, and particularly three of them contained fruit with a high percentage of GS, which was clearly predicted in January. Therefore, the accelerated test is a simple and reliable method to predict the appearance of GS in olives of the Gordal variety as soon as 2-3 months from brining.

Once the accelerated test was developed, it was employed for four consecutive seasons during the months of December-January. Figure 2 shows the percentage of tanks liable to be affected by the alteration with olives processed from the beginning to the end of the 2006/2007 and 2007/2008 seasons. It is clear from the data of both seasons that the alteration occurs mainly in olives picked at the beginning of the harvesting period. About half of the tanks containing olives harvested from 13 to 21 of September presented the alteration while olives harvested in October did not develop GS. Changes in sugars, fatty acids and phenolic compounds during the maturation of Gordal olives have been reported,[1] and their behavior was rather similar to that found for other olive varieties non-prone to GS. The evolution of the superoxide dismutase and polyphenoloxidase activities, which are related with the protection of the chloroplast pigments against oxidative species, during the growth of Gordal was similar to that observed for the Manzanilla variety.[11] To our knowledge, there is no explanation for the higher formation of GS in olives early harvesting.

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In addition, the intensity of the alteration depended on the olive season. As can be seen in Figure 3, the percentage of tanks with olives showing GS was around 30% for the 2006/2007 and 2007/2008 seasons whereas it was lower than 5% for the 2008/2009 season. In the following 2009/2010 season this percentage raised again to up to 23%. These figures are very reliable since a very high number of tanks were analyzed each season and it means that just lower than 30% of olive tanks may present olives with GS during fermentation. Of course, not all the olives in the tanks possess the alteration, though consumers may reject a commercial sample with just a few altered olives in it.

Gallardo-Guerrero *et al*.,[7] reported that the alkaline treatment causes chlorophyll oxidation as well as cell deterioration, and both phenomena are related with the formation of GS. They also suggested that the reduction in pH of the brine below 4.5 units is also necessary to allow the insertion of Cu into the chlorophyll molecule. We carried out an experiment at laboratory and industrial scale to assess the influence of the debittering and fermentation steps on the development of the alteration. Table 5 shows the results obtained with olives from 5 different tanks although the assay was performed with another 10 tanks. Unfortunately, the olives from the latter 10 tanks did not present the alteration thereby the data are not shown. The most surprising result from these experiments was the absence of GS in the olives which were debittered and fermented at the laboratory (treatment C). Likewise, the olives debittered at the laboratory but fermented at the olive factory showed a low incidence of the alteration, this was particularly observed in the olives of tank nº 5. By contrast, fruit debittered at the olive factory developed a high percentage of GS regardless of whether they were fermented at the factory or laboratory, even in inoculated batches. As it was explained in the materials and methods section, the debittering conditions were rather similar at both laboratory and industrial scale. However, the size of the batches was very different, around 3 kg in the laboratory fermenters and 10000 kg in the factory tanks. It has been indicated that during the alkaline treatment an increase in temperature takes place inside the industrial tanks.[17] The intensity of the alkaline treatment has been associated with the appearance of GS in Gordal olives due to its effect on chloroplast disintegration and could be an explanation for the different results obtained at laboratory and industrial scale.[7]

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CONCLUSIONS

The formation of GS in olives of the Gordal variety is time and temperature dependent, and none of the additives tested avoided or retarded the development of the alteration. Moreover, it has been seen that the elaboration of the fruit at small scale reduced the appearance of the alteration to a large extent, although further assays are necessary to confirm these data.

An accelerated test to predict the development of the GS formation has been proposed, which is based on incubation of the olive sample at 45 ºC for 20 days. This test was performed on olives from numerous industrial tanks for 4 consecutive seasons and it was found that the incidence of the alteration was higher in fruit elaborated at the beginning of the season. In addition, the percentage of tanks affected by the alteration depended on the season but it was lower than 30 % of the total. Therefore, processors can use this new accelerated test to manage the alteration and minimize the effects of the alteration.

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Table 1

Influence of calcium, type of brine and temperature on the development of green staining alteration. Gordal olives of the 2005/2006 season processed according to the Spanish-style at industrial scale were packed in glass containers in June and stored for analysis. The percentage of fruit with alteration (%) was evaluated in September.

% olives with green staining					
	Treatment				
Samples	FB-7[a]	FB-45	FB-45-Ca	NB-45	NB-45-Ca
A	0	2	2	0	2
B	2	0	0	0	0
C	0	0	0	0	0
D	2	0	0	0	0
E	16	21	13	16	31
F	2	2	5	7	2
G	0	0	0	0	5
H	17	35	28	39	31
I	0	5	5	9	7
J	13	28	32	12	28

[a]FB-7, fermentation brine and 7º C; FB-45, fermentation brine and 45 ºC; FB-45-Ca, fermentation brine, calcium and 45 ºC; NB-45, new brine and 45 ºC; NB-45-Ca, new brine, calcium and 45 ºC.

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Table 2

Influence of temperature and several food additives on the development of green staining alteration. Gordal olives of the 2005/2006 season were taken from an industrial tank, put into glass containers, and covered with the fermentation brine and additives in June. The percentage of fruit with alteration (%) was evaluated in September.

Treatment	% olives with green staining
7º C	23
25 ºC	28
45 ºC	33
65 ºC	21
45 ºC + sorbic acid (1 g L^{-1})	35
45 ºC + benzoic acid (2 g L^{-1})	25
45 ºC + citric acid (6 g L^{-1})	34

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Table 3

Influence of ascorbic acid and SO_2 on the development of green staining alteration in Gordal olives of the 2006/2007 season. Fruits were put into jars, covered with the fermentation brine and pasteurized in February. The jars were incubated at 45 ºC until June and the presence of GS was evaluated.

	% olives with green staining		
Treatment	February	March	June
Control	5.5 (0.7)[a]	18.0 (2.8)	33.0 (1.4)
Ascorbic acid (1 g L^{-1})	5.0 (1.4)	18.0 (5.6)	32.0 (5.6)
SO_2 (0.2 g L^{-1})	5.0 (1.4)	18.0 (1.4)	41.0 (2.8)

[a]Standard deviation of duplicates

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Table 4

Green staining formation predicted with the accelerated test in January and development of the alteration in olives stored at ambient temperature (23±4 ºC) up to September. Gordal fruits were obtained from fermentation tanks of the 2006/2007 season.

	Percentage of green staining (%)	
Tanks (nº)	Predicted in January	Developed in September
24	0	0
3	1	0
2	2	0
1	2	1
1	36	44
1	52	34
1	81	61

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Table 5

Percentage of olives affected with green staining alteration (%) from fruit processed following different processing methods during the season 2009/2010.

	Treatment[a]			
Sample	A	B	C	D
1	5.7	2.8	0	5.1
2	0	1.0	0	3.6
3	0.8	1.8	0	0
4	0	6.6	0	0
5	21.9	32.2	0	6.2

[a]A, olives debittered at the olive factory and fermented at the laboratory; B, olives debittered at the olive factory and fermented with inoculation at the laboratory; C, olives debittered and fermented at the laboratory; D, olives debittered at the laboratory and femented at the olive factory.

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Figure legends

Figure 1. Photograph of the green staining alteration.

Figure 2. Percentage of industrial tanks liable to be affected with the green staining alteration during the seasons 2006/2007 and 2007/2008. The number of tanks analyzed with the accelerated test performed in January was 146 and 223 for the seasons 2006/2007 and 2007/2008, respectively.

Figure 3. Percentage of industrial tanks liable to be affected with the green staining alteration from the seasons 2006/2007 to 2009/2010. The number of tanks analyzed is indicated on the top of the bars. The accelerated test was performed in January.

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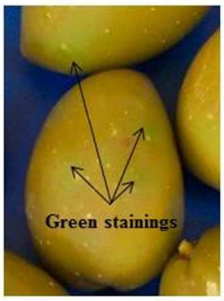

Figure 1
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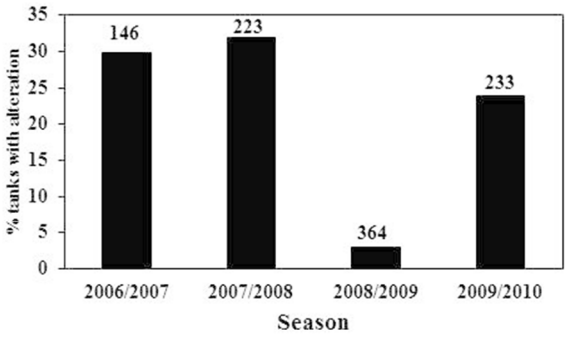

Figure 3
190x254mm (96 x 96 DPI)