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Influence of Processing Conditions on Acrylamide Content in Black Ripe Olives

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The presence of acrylamide was investigated in different presentations of commercial black ripe olives, a well-known sterilized alkali-treated product. The analysis was performed by gas chromatography–mass spectrometry (GC-MS) after bromination of acrylamide, using ($^{13}C_3$)acrylamide as internal standard. In-house validation data for commercial ripe olives showed good precision and accuracy of the method, with repeatability below 3% and recoveries between 94 and 105%. Acrylamide was detected in all samples, but its concentration varied significantly from 176 to 1578 μg/kg of pulp. The effects of different processing conditions (two preservation methods and three darkening methods), cultivar (Hojiblanca or Manzanilla), and presentation form (pitted or sliced olives) on acrylamide content were evaluated in experiments performed in an olive-processing plant. All canned samples were sterilized at 121 °C for 30 min. Statistical analysis of the data indicated that the effects of darkening method and olive cultivar were the most pronounced. Acrylamide contents did not significantly differ after 6 months of storage. The small amounts of free amino acids and reducing sugars found in olives before sterilization did not significantly correlate with the acrylamide formed.

KEYWORDS: Acrylamide; black ripe olives; processing; olive cultivar; sterilization

INTRODUCTION

Acrylamide is classified as a probable carcinogen by the International Agency for Research on Cancer (*1*). The discovery by Swedish scientists of acrylamide in starch-rich foods (*2*) has created much concern among regulating authorities, the food industry, and the public. Widely consumed processed foods with high amounts of acrylamide (>1000 μg/kg) included French fries, potato chips, crispbreads, biscuits, and breakfast cereals (*3*). Particular attention has been paid to potato products because of the high acrylamide concentrations and the rate of consumption as a staple food. Comparatively little information is available on acrylamide in black ripe olives (also called "Californian-style table olives"), although this product presents high levels of acrylamide (200–2000 μg/kg) according to earlier exploratory surveys (*4*). These surveys also showed that acrylamide was not detected in other processing types of table olive. The black ripe olive has been included among the top 20 foods by mean acrylamide intake (micrograms per kilogram of body weight per day) and among the top 8 foods by acrylamide per portion (*5*).

Black ripe olives are one of the most important classes of table olive commercialized in the world. In the United States, >99% of table olive production is destined for canning as black ripe olives (*6*). This processing style accounted for 42% of total table olives exported in 2005 from Spain, the world's main producer and exporter of table olives (*7*). In this type of processing (*8*), the olives, mostly in the green and cherry stages of ripening, are stored in brine with 4–6% NaCl from 2 to 6 months, depending on the need of production. The brine may be acidified to pH 4 with acetic acid and kept in aerobic conditions to prevent spoilage. Alternatively, salt-free preservation, combining acidulated water (acetic acid) in anaerobic/aerobic conditions, may be used (*9*). Once the stored fruits are sorted and graded, they are treated with a series of dilute NaOH solutions (lye). Between lye treatments, the fruits are suspended in water through which air is bubbled. During this operation, the fruits darken progressively. After the lye treatments and oxidation, the olives are washed several times with water to remove most of the residual lye, reaching a final pH of around 7, and placed in 3–5% brine with ferrous gluconate or ferrous lactate to maintain the black color. Finally, the olives are canned in a mild salt brine and heat sterilized (generally at 121–126 °C).

From the above-mentioned reported surveys (*4*), it appears that acrylamide levels in black ripe olives are affected by variations in processing conditions at the different manufacturers, as well as in different forms of olive presentation (sliced, pitted, chopped) by the same manufacturer. However, because it is not known whether acrylamide is stable in the final product, the storage time must not be ruled out as another source of variation. Significant decreases of acrylamide concentrations during storage have been found in some foods, particularly in coffee and cacao powder (*10*). The acrylamide content in black ripe olives can be assumed to be formed during the sterilization treatment, but this needs confirmation. It is well-established that

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amino acids, mainly asparagine, in the presence of reducing sugars or a suitable carbonyl source are important precursors for acrylamide in heated foods (*11–14*). Therefore, the relationship between the formation of acrylamide and the amounts of amino acids and/or sugars before sterilization should be elucidated in black ripe olives. Sugars have been quantified during the preservation stage of black ripe olive processing (*9, 15*), but no data are available on the sugar and free amino acid contents of black ripe olives just before sterilization.

The objectives of the present work were to examine the amounts of acrylamide in different Spanish brands of black ripe olives and to study the effect of different factors on acrylamide formation during the industrial processing of black ripe olives. The effects of preservation method (traditional or salt-free) and darkening process (three different lye treatments), as well as those of olive cultivar (Hojiblanca or Manzanilla) and form of presentation (pitted or sliced), were considered. In addition, the stability of acrylamide during storage time was studied. The amounts of free amino acids and sugars before heat treatment were also determined to study their correlation with acrylamide levels. Our aim is for this study to contribute to the improvement of the process for the reduction of acrylamide in black ripe olives.

MATERIALS AND METHODS

Reagents. A solution of [1,2,3-$^{13}C_3$]acrylamide (1 mg/mL) in methanol (99% ^{13}C) was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). Deionized water was obtained from a Milli-Q system (Millipore, Bedford, MA). Bromination reagent was prepared according to the method of Castle (*16*) by dissolving 20 g of potassium bromide (Sigma, St. Louis, MO) in 16 mL of saturated bromine solution and adding 1 mL of 48% hydrobromic acid (Fluka, Buchs, Switzerland); a total volume of 100 mL was obtained by adding deionized water. The saturated bromine solution was prepared by adding bromine (3 mL, Fluka) to deionized water (80 mL) and stirring in an ice–water bath for 1 h (*17*). Amino acids, both the D- and L-enantiomers, were of the highest available purity from Sigma and included aspartic acid (Asp), glutamic acid (Glu), asparagine (Asn), serine (Ser), glutamine (Gln), histidine (His), glycine (Gly), threonine (Thr), arginine (Arg), alanine (Ala), tyrosine (Tyr), valine (Val), phenylalanine (Phe), isoleucine (Ile), leucine (Leu), and lysine (Lys). The internal standard, D-α-amino-*n*-butyric acid (D-Abu), was obtained from Aldrich (Milwaukee, MI). *o*-Phthaldialdehyde (OPA), ethyl acetate (Pestanal jf072960bjf-2007-02960b grade), and sugars (fructose, glucose, sucrose, and sorbitol; all 99+% purity) were also supplied by Sigma. *N*-Isobutyryl-L-cysteine (IBLC, optical purity ≥ 99.5%) was obtained from Fluka. All other chemicals and solvents were of analytical or chromatographic grade from various suppliers.

Samples. Different brands of commercial packed black ripe olives (identified by letters A−J, **Table 1**) were obtained locally. Duplicate samples of each lot number were analyzed for acrylamide content. Sample J*, which was identical to sample J but with a different lot number, was also analyzed.

Experiments to analyze the effects of different processing conditions (two preservation methods, three darkening steps, two forms of presentation, two olive cultivars) on the formation of acrylamide and its precursors were carried out in an olive-processing plant in Seville province. The experimental design for this study is shown in **Figure 1**. Olives (Hojiblanca or Manzanilla cultivar), prior to the darkening process and canning, were preserved in fiberglass tanks, which contained about 10000 kg of olives and 5000 L of cover solution. For Hojiblanca olives, two different preservation methods (PM-T, PM-A) were used: PM-T, the traditional method using an acidified brine, 3.5% NaCl (w/v), and 1.2% acetic acid (w/v); and PM-A, a method using acidified water, 1.6% (w/v) acetic acid. In both cases, aerobic conditions were obtained by bubbling a flow of air for 4 h a day and 3 days a week, throughout preservation time. Manzanilla olives were preserved only by method PM-T. After preservation for about 6 months, olives were sorted by size (the size 280/300 was selected—these numbers representing the number of olives contained in a kilogram) and placed in individual industrial tanks, in which the darkening process was carried out. For each olive cultivar and preservation method, three darkening methods (DM-1, DM-2, DM-3), having different numbers of lye solutions and water washings, were evaluated: DM-1, one lye solution (3% NaOH, penetration to reach the stone, 4.5 h duration) plus one water washing (24 h duration); DM-2, one lye solution as for the above method plus two water washings (both 24 h duration); and DM-3, two lye solutions plus three water washings, according to the sequence (1) lye treatment with 3% NaOH for 2 h to just penetrate the skin, (2) water washing for 18 h, (3) lye treatment with 2.5% NaOH for 3 h to reach the stone, and (4) two water washings, each of 24 h duration. During all of these treatments, air was bubbled and, after oxidation, carbon dioxide was bubbled to neutralize the excess NaOH. Subsequently, in all three cases, the olives were suspended in a 0.1% (w/v) ferrous gluconate solution for 18–24 h to fix the black color. Finally, the olives were pitted or pitted and sliced, canned, and sterilized at 121 °C for 30 min. Sampling was performed before canning (after treatment with ferrous gluconate solution) and after canning (after sterilization treatment). The samples before canning (whole olives and the corresponding cover solution) were collected in glass bottles and maintained at 4 °C until being sent to our laboratories within 48 h; on arrival, the bottles were immediately opened and drained, and the olives were pitted, homogenized, and frozen at −30 °C until analysis. The canned samples were divided into two lots. One lot was stored at 4 °C and analyzed for acrylamide after 20 days, and the other was stored at room temperature and analyzed after 6 months.

Analysis of Acrylamide. Acrylamide was analyzed according to the method described by Pittet et al. (*18*), in which the analyte was

Table 1. Acrylamide Content in Different Brands of Commercial Black Ripe Olives

sample[a]	presentation form	olive cultivar	acrylamide[b] (μg/kg of fresh wt)
A	pitted	Cacereña	224 ± 19
B	pitted	Cacereña	202 ± 23
C	pitted	Manzanilla	1578 ± 122
D	whole	*c*	364 ± 32
E	whole	Cacereña	629 ± 59
F	whole	Manzanilla	1058 ± 239
G	whole	*c*	433 ± 37
H	whole	Cacereña	223 ± 20
I	sliced	Hojiblanca	176 ± 20
J	sliced	*c*	1221 ± 21
J*	sliced	*c*	726 ± 61

[a] A−H, olives packed in tin cans (350 g net weight, 240/300 olive size); I−J*, olives packed in glass bottles (240 g net weight); J and J*, same brand, different lot. [b] Values are means ± standard deviation of duplicate bottles, each analyzed in duplicate ($n = 4$). [c] Olive cultivar not declared on label.

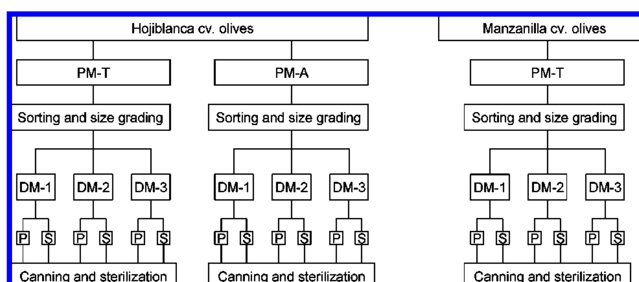

Figure 1. Experimental design for the study of factors that affect acrylamide formation in black ripe olive processing: PM-T, traditional preservation in brine; PM-A, preservation in acid solution; DM-1, darkening method with one lye treatment and one water washing; DM-2, darkening method with one lye treatment and two water washings; DM-3, darkening method with two lye treatments and three water washings; P, pitting; S, slicing. Sterilization was conducted at 121 °C for 30 min.

detected as 2-bromopropenamide by GC-MS, employing ($^{13}C_3$) acrylamide as internal standard, with several modifications (inclusion of a purification step using SPE instead of Carrez reagents before bromination, elimination of the Florisil cleanup step after bromination, and use of a more polar capillary column for GC separation). Olives, which were separated from cover brine and dried with a tissue, were pitted (if appropriate) and then homogenized in a blender. A portion of slurry (10 g) was weighed into a centrifuge bottle and spiked with 5 mL of ($^{13}C_3$)acrylamide (1 μg/mL). The sample was mixed with 95 mL of water and homogenized for 30 s with an Ultra Turrax homogenizer. The mixture was centrifuged at 21000*g* (4 °C) for 30 min. Then, the aqueous supernatant (1 mL) was applied to an SPE cartridge (Discovery DSC-MCAX, 300 mg; Supelco, Bellefonte, PA) that had been conditioned with methanol (1 mL) and water (2 mL). The SPE eluate was collected into a test tube, then water (2 mL) was applied to the cartridge, and the eluate was collected into the same test tube. For bromination, 6 mL of bromination reagent was added and the mixture was left overnight at 4 °C in a refrigerator. Then, the excess bromine was reduced to a colorless solution by adding 1 M sodium thiosulfate solution (60 μL). The mixture was transferred to a 250 mL separating funnel and extracted twice with 20 mL of ethyl acetate. The extract was dried over anhydrous sodium sulfate, and the solvent was evaporated to ~2 mL on a rotary evaporator at 30 °C and then to dryness under a stream of nitrogen. The residue was redissolved in 100 μL of ethyl acetate, and 10 μL of triethylamine was added to convert 2,3-dibromopropionamide to 2-bromopropenamide. The extract (2 μL) was injected into the GC-MS system, using the splitless injection method. The GC-MS system used was an HP5890 series II gas chromatograph coupled to an HP5972 benchtop mass selective detector operated in selected ion monitoring (SIM) mode with positive electron impact ionization. The GC column was a BPX70 capillary column (30 m × 0.22 mm i.d., 0.25 μm film thickness; SGE, Ringwood, Australia), and the carrier gas was helium at a column head pressure of 65 kPa. The injector temperature was 200 °C, and the temperature program used was as follows: isothermal for 1 min at 65 °C, temperature increased by 15 °C/min to 170 °C, by 5 °C/min to 200 °C, followed by 40 °C/min to 250 °C, and isothermal for 15 min. The GC-MS interface transfer line was held at 280 °C. Under these conditions, the retention time of acrylamide and ($^{13}C_3$)acrylamide derivatives was 10.6 min. Ions monitored were m/z 70, 149, and 151 for 2-bromopropenamide and m/z 110 and 154 for 2-bromo($^{13}C_3$)propenamide. Acrylamide in samples was quantified using the ion at m/z 151 for 2-bromopropenamide and the ion at m/z 154 for 2-bromo($^{13}C_3$)propenamide. The other ions at m/z 70, 110, and 149 were considered only for confirmation purposes.

Determination of Free Amino Acids. Free amino acids in olive pulp were analyzed by HPLC according to a previous method (*19*), with slight modifications in sample preparation. A homogenized olive pulp sample weighing 10 g was spiked with 1 mL of 125 μM D-α-amino-*n*-butyric acid (internal standard), and the extraction was performed using 25 mL of 70% (v/v) ethanol and swirling the contents with a magnetic stir bar for 15 min. An aliquot was filtered through filter paper and then through a 0.45 μm membrane filter prior to HPLC analysis that employed precolumn derivatization using OPA in combination with IBLC. The system consisted of a Jasco model PU-2089 pump (Jasco Corp., Tokyo, Japan), a Jasco model AS-2057 autosampler, a Jasco model FP-920 fluorescence detector (λex = 230 nm, λem = 445 nm), and a computer with Jasco-Borwin chromatography software version 1.5. A Luna 5μ C18 (2) (250 × 4.6 mm) column (Phenomenex, Torrance, CA) with a C18 guard column (Phenomenex) and a linear gradient of methanol/acetonitrile (92:8) in acetate buffer were used for separation of amino acids. The column was kept at 30 °C in a Gecko-2000 column heater. The amino acid contents were analyzed in triplicate.

Determination of Sugars. Sugars (glucose, fructose, and sucrose) in olive pulp were determined by HPLC following a previous method (*20*) with slight modifications. The HPLC system used was a Waters 2690 separations module connected to a Perkin-Elmer LC-25 refractive index detector. The separation was performed on a Rezex RCM Monosaccharide column (300 × 7.8 mm i.d., Phenomenex) held at 60 °C, using deionized water as the mobile phase at a flow rate of 0.5 mL/min. A mixture of 10 g of homogenized olive pulp and 60 mL of hot (≈70 °C) water was homogenized with an Ultra Turrax homogenizer. The mixture was quantitatively transferred to a volumetric flask (100 mL) and, after the contents had been swirled with a magnetic stir bar for 10 min, water was added to the mark. An aliquot was filtered through filter paper and then centrifuged at 11600*g* for 5 min. The extract (1.5 mL) was then desalted by adding 1 g of a strongly acidic resin (Amberlite IR-120, Fluka) plus 1 g of a weakly basic resin (Amberlite IRA-93, Fluka). An internal standard (0.5 mL of 0.3% sorbitol) was also added for quantification by the internal standard method. Samples were shaken occasionally during a 60 min desalting period. An aliquot of the solution was filtered through a 0.45 μm membrane filter, and 50 μL of the filtrate was injected into the chromatograph. The sugar contents were analyzed in triplicate.

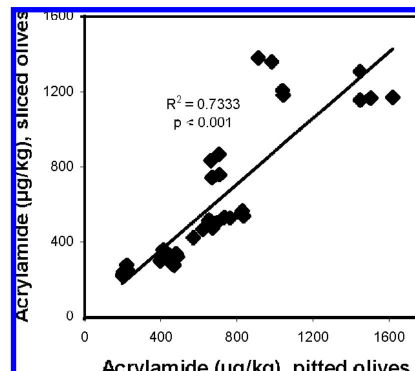

Figure 2. Correlation between acrylamide content in pitted ripe olives and acrylamide content in sliced ripe olives. Values are given in olive pulp (micrograms per kilogram of fresh weight).

Statistical Analysis. Statistica software version 6.0 (StatSoft Inc., Tulsa, OK) was used for data processing. The General Linear Model (GLM) was used to test the effects of presentation, cultivar, preservation method, oxidation treatment, and sterilization temperature. Differences were considered to be significant at $p < 0.05$. Wherever F values were significant, Duncan's multiple-range test was used to separate the means of mean effects.

RESULTS AND DISCUSSION

Validation of the Method Used for the Acrylamide Analysis in Ripe Olives. A purification step before bromination using an SPE cartridge was used in the present work instead of the Carrez reagents used by Pittet et al. (*18*). Most interferences of the matrix were retained in the Discovery DSC-MCAX SPE cartridge, which uses two retention mechanisms (reversed phase and cation exchange). The chromatographic conditions were the same as those of Pittet et al. (*18*), but a more polar capillary column was used in the present work. For the calibration test, the measurements were performed in triplicate, using seven standard solutions with different concentrations of acrylamide (5, 10, 20, 40, 60, 100, 200 ng/mL) and each solution containing 100 ng/mL ($^{13}C_3$)acrylamide. The calibration curve, when the areas of the peaks at m/z 151 and 154 were used, produced a correlation coefficient of 0.997. The detection and quantification limits were evaluated according to the ICH guidelines (*21*). These values were calculated to be 6 and 20 ng/mL, respectively, from the values obtained using standard solutions from 5 to 20 ng/mL in triplicate. Replicate (n = 6) analysis of a commercial sample of black ripe olives with an acrylamide concentration of 580 μg/kg gave a relative standard deviation of 2.9%. To verify the accuracy, we carried out a recovery test by adding known amounts of standard acrylamide (100, 200, and 1100 μg/kg) to a commercial sample of ripe olives that had been shown to contain 108 μg/kg of acrylamide in pulp. The overall

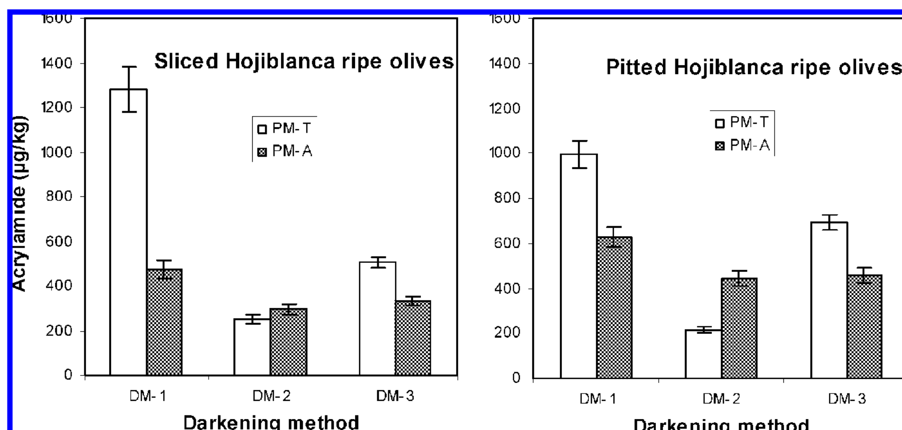

Figure 3. Acrylamide content (micrograms per kilogram of fresh weight) in canned Hojiblanca ripe olives according to form of presentation (sliced versus pitted), preservation method (PM-T versus PM-A), and darkening method (DM-1, DM-2, and DM-3). Data points represent means ± SD of duplicate bottles, each analyzed in duplicate ($n = 4$).

Table 2. Main Effects of Preservation Method, Darkening Method, and Form of Olive Presentation on Acrylamide Content of Black Ripe Olives (Hojiblanca Cultivar)

main effect	acrylamide[a]	n[b]	F value[c]
preservation			297***
PM-T	656 a	24	
PM-A	439 b	24	
darkening			636***
DM-1	844 a	16	
DM-2	300 c	16	
DM-3	496 b	16	
presentation			14***
pitted	571 a	24	
sliced	523 b	24	

[a] Micrograms per kilogram of fresh weight. Means with different letters for each effect are significantly different ($p < 0.05$). [b] Number of experimental data. [c] F value, assessment of overall differences obtained from analysis de variance; ***, $p < 0.001$.

Table 3. Main Effects of Olive Cultivar, Darkening Method, and Form of Olive Presentation on Acrylamide Content of Black Ripe Olives (Preserved by PM-T)

main effect	acrylamide[a]	n[b]	F value[c]
cultivar			310***
Manzanilla	925 a	24	
Hojiblanca	656 b	24	
darkening			949***
DM-1	1245 a	16	
DM-2	455 c	16	
DM-3	671 b	16	
presentation			12**
pitted	817 a	24	
sliced	764 b	24	

[a] Micrograms per kilogram of fresh weight. Means with different letters for each effect are significantly different ($p < 0.05$). [b] Number of experimental data. [c] F value, assessment of overall differences obtained from analysis de variance; ***, $p < 0.001$; **, $p < 0.01$.

recovery rates, evaluated by triplicate analysis of fortified sample, ranged from 94 to 105%. The results thus obtained confirmed the validity of the analytical method.

Acrylamide Levels in Commercial Black Ripe Olives. The content of acrylamide in different samples of commercial black ripe olives was investigated (**Table 1**). The acrylamide level in olive pulp ranged from 176 to 1578 μg/kg of fresh weight. Although the data come from a very limited survey, some interesting observations can be made. Data variability (which included bottle-to-bottle variation) was, on average, 9.8%. Lot-to-lot variation appeared to be considerably higher, as deduced from comparison of acrylamide levels in samples J and J*. Acrylamide values in black ripe olives reported by the FDA (*4*) fall inside the above-mentioned range. Analysis of variance (ANOVA) showed that the mean value of acrylamide in whole ripe olives was not significantly ($p < 0.05$) different from that of pitted or sliced ripe olives. The mean content of acrylamide in ripe olives of the Manzanilla cultivar was significantly ($p < 0.05$) higher than those in Cacereña and Hojiblanca cultivars.

It must be pointed out that the production date of samples analyzed was unknown. The wide range of acrylamide levels found could be partly explained by assuming that acrylamide was not stable during storage. If acrylamide is stable, the above results suggest that processing conditions, which usually vary from one olive-processing plant to another, and olive cultivar are important factors for acrylamide formation in black ripe olives, whereas the form of presentation is less important. This was clarified in experiments performed in a selected olive-processing plant, as discussed in the next section.

Effect of Different Factors in Black Ripe Olive Processing on Acrylamide Formation. The whole set of data on acrylamide content in pitted ripe olives correlated well ($R^2 = 0.733$, $p < 0.001$) with acrylamide content in sliced ripe olives (**Figure 2**). The mean ± SE of acrylamide content in pitted ripe olives was 714 ± 60 μg/kg of fresh weight, which was not significantly different from that in sliced ripe olives (632 ± 61 μg/kg of fresh weight). This finding is in agreement with the above-mentioned result in commercial ripe olives.

Values of acrylamide content in all canned samples of Hojiblanca cultivar were subjected to factorial (2 × 3 × 2) ANOVA to test the effects of preservation method (PM-T versus PM-A), darkening method (DM-1, DM-2, and DM-3), and form of presentation (pitted versus sliced olives). The ANOVA revealed highly significant ($p < 0.001$) effects for all three factors and their interactions. The interaction between preservation method, darkening method, and form of presentation is shown in **Figure 3**. The effect of darkening method was the most pronounced, as revealed by its highest F value ($F = 636$; **Table 2**). On the other hand, the difference between the Hojiblanca pitted olives and Hojiblanca sliced olives, although significant, was practically negligible. Of the three darkening methods studied, the method DM-1 generated the highest acrylamide content in the canned product. Also, it appears that more than two washings did not result in substantial reductions in the acrylamide content. In fact, as shown above, the darkening

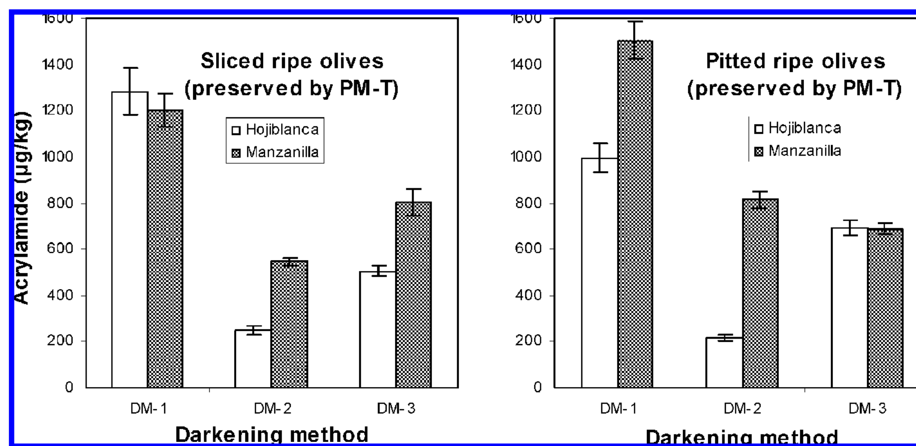

Figure 4. Acrylamide content (micrograms per kilogram of fresh weight) in canned ripe olives, preserved by the traditional method, according to form of presentation (sliced versus pitted), olive cultivar (Hojiblanca versus Manzanilla), and darkening method (DM-1, DM-2, and DM-3). Data points represent means ± SD of duplicate bottles, each analyzed in duplicate ($n = 4$).

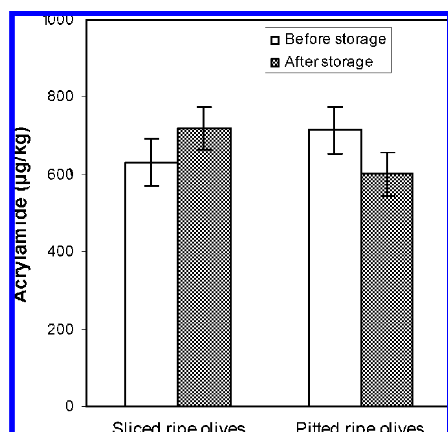

Figure 5. Average acrylamide levels in sliced ripe olives and pitted ripe olives before and after 6 months storage at room temperature. Bars represent standard errors ($n = 36$).

method with three water washings (DM-3) yielded a slightly higher mean content of acrylamide than that with two washings (DM-2).

Data of acrylamide content of canned Manzanilla olives were compared with those of canned Hojiblanca olives (only those preserved by PM-T) to test the effects of olive cultivar (Hojiblanca versus Manzanilla), darkening method (DM-1, DM-2, and DM-3), and form of presentation (pitted versus sliced olives). The ANOVA revealed highly significant ($p < 0.001$) effects for cultivar and darkening method (**Table 3**). Again, the influence of form of presentation was minor ($p < 0.01$). All of the interactive effects were highly significant ($p < 0.001$), except for the interaction presentation × darkening method ($p < 0.05$). The interaction between olive cultivar, darkening method, and form of presentation is shown in **Figure 4**.

It must be pointed out that, because the experiments were performed only once, the above results can be considered as preliminary data. To provide more conclusive results, a greater number of identically processed samples will need to be analyzed. Besides, although the sterilization treatment applied (121 °C for 30 min) was the same in all cases, the repeatability of this treatment was not tested. Bearing this in mind, it is possible to speculate on the variations of acrylamide due to differences in the processing conditions and olive cultivar. Acrylamide is a rather reactive component, so it could be expected to react rather rapidly with various other components present or formed in the food (*22*). Therefore, the concentration of acrylamide in food depends on the interplay of formation and elimination. The highest content of acrylamide in olives darkened by DM-1 in comparison with those darkened by DM-2 or DM-3 (**Tables 2** and **3**) might be explained by the presence of acrylamide precursor(s) in a higher concentration as a consequence of a smaller number of washings. Despite different number of washings, the washing efficiency appeared to be similar or slightly decreased in samples darkened by DM-3 compared to those darkened by DM-2, explaining the slightly higher contents of acrylamide in the former. In this connection, the values of combined acidity (also called "residual lye") of olives before sterilization treatment followed a tendency similar to that of acrylamide content, that is, DM-1 > DM-3 ≥ DM-2 (data not shown). The higher concentration of acrylamide in Manzanilla in comparison with Hojiblanca olives was in agreement with the above-mentioned results in commercial black ripe olives. This could be attributed to a higher concentration of precursor components and/or to a slower elimination rate in case of Manzanilla olives. Differences between olives preserved by PM-T and PM-A might be due to differences in content of acrylamide precursors, which may be affected by microbial growth during the preservation step. In this connection, de Castro et al. (*9*) found that, in addition to yeasts, acetic acid bacteria grew in olives preserved by different methods (one of them being PM-A), but these bacteria were not detected in olives preserved by PM-T.

The influence of time and/or temperature of sterilization on acrylamide formation was not studied in the present work. This subject warrants a separate study. Preliminary experiments (data not published) performed in our laboratory with Hojiblanca ripe olives, packed into glass bottles (240 g net weight), showed that acrylamide formation increased with increasing sterilization time (at 121 °C) (average values of 58, 134, and 283 μg/kg for 15, 27, and 35 min, respectively). Therefore, the conditions used in the present work (i.e., 121 °C, 30 min), although more than sufficient to reach an accumulated lethality of 15Fo, which is the minimum reported value to ensure proper preservation (*8*), appear not to be the optimum to minimize the acrylamide level in ripe olives. This means that by strict attention to sterilization conditions, the content of acrylamide could be reduced to <100 μg/kg, at least in the case of Hojiblanca ripe olives.

Approximately 6 months after quantitating acrylamide in the canned ripe olives, samples were reanalyzed to determine whether there was any detectable change in the level of acrylamide during storage at room temperature. For both sliced

Table 4. Sugar and Free Amino Acid Compositions in Olive Pulp before Sterilization and Acrylamide Content before and after Sterilization of Ripe Olives

compound[b]	sample[a]									
	HT1	HT2	HT3	MT1	MT2	MT3	HA1	HA2	HA3	av[c]
glucose	1726	1719	1299	2434	nd[d]	3177	1791	2593	2110	2106 (29)
aspartic acid	1.1	3.6	2.5	3.5	4.1	5.1	3.4	2.0	2.2	3.1 (40)
glutamic acid	1.0	4.1	2.4	8.5	10.7	7.9	2.7	1.0	1.6	4.4 (82)
asparagine	0.5	1.5	1.5	0.9	0.8	1.0	0.4	0.9	0.7	0.9 (44)
serine	2.1	4.0	2.9	6.6	6.9	10.2	5.9	5.8	4.8	5.5 (44)
glutamina	0.6	1.5	1.0	9.1	8.9	4.5	0.8	0.8	1.0	3.1 (113)
arginine	0.9	1.3	1.2	4.8	4.7	3.5	1.1	1.0	1.1	2.2 (76)
alanine	1.5	3.1	2.1	4.7	8.6	8.4	3.5	2.7	2.7	4.1 (63)
tyrosine	nd	0.9	0.9	1.5	2.1	2.6	1.3	0.5	0.5	1.3 (58)
valine	0.9	3.2	2.4	2.6	4.2	4.9	2.7	1.7	2.0	2.7 (45)
total free amino acids	8.6	23.4	16.9	42.3	51.0	48.1	21.9	16.3	16.4	27.2 (58)
acrylamide										
before sterilization	1.9	tr[e]	tr	1.9	tr	tr	nd	tr	nd	
after sterilization	16.0	3.3	8.4	19.0	9.6	10.5	7.8	5.2	5.6	9.5 (54)

[a] In acronyms, the first and second letter indicate olive cultivar and preservation method, respectively, and the number represents the darkening method. Values in micromoles per kilogram of fresh weight. [b] Values for glucose and amino acids are means of triplicate determinations (method precision for glucose was 5.9%, whereas for amino acids it varied from 4 to 13%). Values for acrylamide before sterilization are means of triplicate determinations, whereas after sterilization values are means of eight determinations (two bottles, each analyzed in duplicate, of pitted sample plus two bottles, each analyzed in duplicate, of sliced sample). [c] Coefficient of variation (percentage) in parentheses. [d] nd, not detected. [e] tr, trace amounts of acrylamide, <1 μmol/kg of fresh weight.

and pitted black ripe olives, acrylamide content did not significantly vary after this period of storage (**Figure 5**). This suggests that acrylamide is stable in this product. The same result has been reported for other food matrices such as cookies, cornflakes, crispbreads, raw sugar, potato crisps, and peanuts (*10*). On the other hand, significant decreases of acrylamide concentration during storage have been found in foods such as coffee, cacao, and roasted almonds, which is attributed to the reaction of acrylamide with special food components and/or reaction products (*10, 23*).

Correlation between Sugar and Amino Acid Contents in Olives before Sterilization and the Acrylamide Content after Sterilization. For practical reasons in the olive-processing plant where our experiments were performed, samples could not be taken immediately after pitting or slicing operations. Instead, samples analyzed before sterilization were of whole olives taken after the darkening process. Because pitting or slicing operations could contribute to additional losses, the actual amounts of sugars and free amino acids in the olive pulp at the moment of sterilization could be somewhat lower than those measured, which are shown in **Table 4**. In addition to sugar and free amino acid compositions, samples were analyzed for acrylamide to determine whether this compound was present before heat treatment. Except for two samples in which the acrylamide content was 1.9 μmol/kg of olive pulp, in general only trace or undetectable amounts of acrylamide were present before heat treatment. Therefore, it was demonstrated that the acrylamide in black ripe olives was mainly formed during the sterilization treatment (**Table 4**).

Of the reducing sugars, only residual glucose (range = 1299–3177 μmol/kg of olive pulp) was found in all samples, with the exception of one sample (MT2) in which sugars were not detected (**Table 4**). The presence of residual glucose is surprising, considering the rather severe treatments (aerobic preservation for 6 months, lye treatment, water washings) applied to olives during ripe olive processing. It has been reported that due to microbial growth, mainly of yeasts, sugars are consumed during the preservation stage of ripe olive processing, practically disappearing after 7 months (*15*). With regard to free amino acids, low levels of Asp, Glu, Asn, Ser, Gln, Arg, Ala, Tyr, and Val were found in olive pulp, with Asn being the minor one (on average, 0.9 μmol/kg of olive pulp). In general, the molar concentration of Asn was lower than that of acrylamide formed. In addition, multiple regression analysis showed that the concentration of glucose and free amino acids (both total and individual amino acids) before heat treatment had no significant correlation with the level of acrylamide formed. Therefore, it appears that free amino acids and reducing sugars do not play an important role in acrylamide formation in black ripe olives. In contrast, strong correlations between acrylamide and asparagine have been found in roasted almonds (*23*), roasted tea (*24*), and bread (*25*) and between acrylamide and reducing sugars in potatoes (*26, 27*). Dehydroalanine (formed, for example, from serine or cysteine) has been suggested as a possible acrylamide precursor in the case of lye-treated olives (*3*). Acrylic acid (formed from acrolein, which in turn is formed from lipids) and ammonia (from the amino acid upon heating) could be important precursors in the case of black ripe olives, as suggested for lipid-rich foods (*28*). The formation of acrylic acid from sugar degradation products has also been suggested (*29*). However, this cannot explain the formation of acrylamide in the absence of glucose, as in the case of sample MT2. Additional studies are needed to confirm or reject the above-mentioned hypotheses. Such studies are currently being carried out in this laboratory.

To our knowledge, this is the first study in which influences of processing conditions were evaluated for the formation of acrylamide in black ripe olives. On the basis of this study's findings, variability in acrylamide levels in this food was found to be dependent on a number of factors, mainly darkening method and olive cultivar. The lowest levels of acrylamide were found in Hojiblanca ripe olives processed using the traditional preservation in brine, penetration of lye to reach the stone, and two water washings. Olives processed using one lye treatment and one water washing showed the highest acrylamide contents. The effects of presentation form and storage time were minor or not significant. Black ripe olives showed no correlation between the acrylamide content and any of the sugars and amino acids determined before sterilization, which appears to indicate that these compounds, in contrast to the case of other cooked foods, are not important acrylamide precursors in this product. However, this result needs to be corroborated, along with other possible hypotheses on acrylamide formation in ripe olives. The possibility of reducing the acrylamide content by modifying the sterilization conditions must be also considered in more detail. Together, these studies would be helpful for the improvement

of processing conditions aimed at reducing acrylamide formation without having a negative impact on sensory properties and product preservation.

LITERATURE CITED

(1) IARC. Acrylamide. *Monographs on the Evaluation of Carcinogenic Risks to Humans: Some Industrial Chemicals*; International Agency for Research on Cancer: Lyon, France, 1994; Vol. 60, pp 389–433.

(2) Tareke, E.; Rydberg, P.; Karlsson, P.; Eriksson, S.; Tornqvist, M. Analysis of acrylamide, a carcinogen formed in heated foodstuffs. *J. Agric. Food Chem.* **2002**, *50*, 4998–5006.

(3) Friedman, M. Chemistry, biochemistry, and safety of acrylamide. A review. *J. Agric. Food Chem.* **2003**, *51*, 4504–4526.

(4) U.S. Food and Drug Administration, 2006. Survey Data on Acrylamide in Food: Individual Food Products. U.S. Department of Health and Human Services, Center for Food Safety and Nutrition; http://www.cfsan.fda.gov/~dms/acrydata.html.

(5) DiNovi, M.; Howard, D. The updated exposure assessment for acrylamide. JIFSAN, 2004, Acrylamide in Food Workshop: Update—Scientific Issues, Uncertainties, and Research Strategies; http://www.jifsan.umd.edu/acrylamide2004.htm.

(6) Boriss, H. Commodity profile: olives; Agricultural Issues Center, University of CA, 2006; http://aic.ucdavis.edu/profiles/Olives_2006.pdf.

(7) ASEMESA, Asociación de Exportadores e Industriales de Aceitunas de Mesa. Análisis del sector de la aceituna de mesa. Período 2001–2005; http://www.alimentalia.com/encuentro/documentos/aceitunas_2001_2005.pdf.

(8) Sánchez, A. H.; García, P.; Rejano, L. Elaboration of table olives. *Grasas Aceites* **2006**, *57*, 86–94.

(9) de Castro, A.; García, P.; Romero, C.; Brenes, M.; Garrido, A. Industrial implementation of black ripe olive storage under acid conditions. *J. Food Eng.* **2007**, *80*, 1206–1212.

(10) Hoenicke, K.; Gatermann, R. Studies on the stability of acrylamide in food during storage. *J. AOAC Int.* **2005**, *88*, 268–273.

(11) Mottram, D. S.; Wedzicha, B. L.; Dodson, A. T. Acrylamide is formed in the Maillard reaction. *Nature* **2002**, *419*, 448–449.

(12) Stadler, R. H.; Blank, I.; Varga, N.; Robert, F.; Hau, J.; Guy, P. A.; Robert, M.-C.; Riediker, S. Acrylamide from Maillard reaction products. *Nature* **2002**, *419*, 449–450.

(13) Becalski, A.; Lau, B. P.-Y.; Lewis, D.; Seaman, S. Acrylamide in foods: occurrence and sources. *Abstracts*,116th Annual AOAC International Meeting, Los Angeles, CA, Sept 22–26, 2002; AOAC: Gaithersburg, MD, 2002; pp 125–126.

(14) Sanders, R. A.; Zyzak, D. V.; Stojanovic, M.; Tallmadge, D. H.; Eberhart, B. L.; Ewald; D. K. An LC/MS acrylamide method and its use in investigating the role of asparagine. *Acrylamide Symposium*,116th Annual AOAC International Meeting, Los Angeles, CA, Sept 26, 2002; AOAC; Gaithersburg, MD, 2002.

(15) Garrido, A.; García, P.; Montaño, A.; Brenes, M.; Durán, M. C. Biochemical changes during the preservation stage of ripe olive processing. *Nahrung* **1993**, *6*, 583–591.

(16) Castle, L. Determination of acrylamide monomer in mushrooms grown on polyacrylamide gel. *J. Agric. Food Chem.* **1993**, *41*, 1261–1263.

(17) Cheng, W. C.; Hsiao, S. W.; Chou, S. S.; Sun-Hwang, L.; Lu, T. J.; Yeh, A. I. Determination of acrylamide in Chinese foods by GC-ion trap MS using 2-bromopropenamide and 2-bromopropenamide-$^{13}C_3$. *J. Food Drug Anal.* **2006**, *14*, 207–214.

(18) Pittet, A.; Périsset, A.; Oberson, J. M. Trace level determination of acrylamide in cereal-based foods by gas chromatography—mass spectrometry. *J. Chromatogr., A* **2004**, *1035*, 123–130.

(19) Casado, F. J.; Sánchez, A. H.; Rejano, L.; Montaño, A. D-amino acid formation in sterilized alkali-treated olives. *J. Agric. Food Chem.* **2007**, *55*, 3503–3507.

(20) Sánchez, A. H.; de Castro, A.; Rejano, L.; Montaño, A. Comparative study on chemical changes in olive juice and brine during green olive fermentation. *J. Agric. Food Chem.* **2000**, *48*, 5975–5980.

(21) ICH Steering Committee. ICH Harmonized Tripartite Guideline: Validation of Analytical Procedures: Methology; recommended for adoption at step 4 of the ICH process on Nov 6, 1996.

(22) Claeys, W. L.; De Vleeschouwer, K.; Hendrickx, M. E. Kinetics of acrylamide formation and elimination during heating of an asparagine—sugar model system. *J. Agric. Food Chem.* **2005**, *53*, 9999–10005.

(23) Amrein, T. M.; Lukac, H.; Andres, L.; Perren, R.; Escher, F.; Amadò, R. Acrylamide in roasted almonds and hazelnuts. *J. Agric. Food Chem.* **2005**, *53*, 7819–7825.

(24) Mizukami, Y.; Kohata, K.; Yamaguchi, Y.; Hayashi, N.; Sawai, Y.; Chuda, Y.; Ono, H.; Yada, H.; Yoshida, M. Analysis of acrylamide in green tea by gas chromatography—mass spectrometry. *J. Agric. Food Chem.* **2006**, *54*, 7370–7377.

(25) Surdyk, N.; Rosén, J.; Andersson, R.; Aman, P. Effects of asparagine, fructose, and baking conditions on acrylamide content in yeast-leavened wheat bread. *J. Agric. Food Chem.* **2004**, *52*, 2047–2051.

(26) Becalski, A.; Lau, B. P. Y.; Lewis, D.; Seaman, S. W.; Hayward, S.; Sahagian, M. R.; Ramesh, M.; Leclerc, Y. Acrylamide in French fries: influence of free amino acids and sugars. *J. Agric. Food Chem.* **2004**, *52*, 3801–3806.

(27) Amrein, T. M.; Bachman, S.; Noti, A.; Biedermann, M.; Barbosa, M. F.; Biedermann-Brem, S.; Grob, K.; Keiser, A.; Realini, P.; Escher, F.; Amadò, R. Potential of acrylamide formation, sugars, and free asparagine in potatoes: a comparison of cultivars and farming systems. *J. Agric. Food Chem.* **2003**, *51*, 5556–5560.

(28) Yasuhara, A.; Tanaka, Y.; Hengel, M.; Shibamoto, T. Gas chromatographic investigation of acrylamide formation in browning model systems. *J. Agric. Food Chem.* **2003**, *51*, 3999–4003.

(29) Vattem, D. A.; Shetty, K. Acrylamide in food: a model for mechanism of formation and its reduction. *Innovative Food Sci. Emerging Technol.* **2003**, *4*, 331–338.

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