

The Influence of pH and Sodium Hydroxide Exposure Time on Glucosamine and Acrylamide Levels in California-Style Black Ripe Olives

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Abstract: Acrylic acid, *N*-acetyl-glucosamine and glucosamine were investigated for their role in the formation of acrylamide in California-style black ripe olives [CBROs]. Levels of acrylic acid and glucosamine are reported for the first time in fresh (333.50 ± 21.88 and 243.59 ± 10.06 nmol/g, respectively) and in brine-stored olives (184.50 ± 6.02 and 165.88 ± 11.51 nmol/g, respectively). Acrylamide levels significantly increased when acrylic acid (35.2%), *N*-acetyl-glucosamine (29.9%), and glucosamine (124.0%) were added to olives prior to sterilization. However, isotope studies indicate these compounds do not contribute carbon and/or nitrogen atoms to acrylamide. The base-catalyzed degradation of glucosamine is demonstrated in olive pulp and a strong correlation ($r^2 = 0.9513$) between glucosamine in olives before sterilization and acrylamide formed in processed CBROs is observed. Treatment with sodium hydroxide (pH > 12) significantly reduces acrylamide levels over 1 to 5 d without impacting olive fruit texture.

Keywords: acrylamide, acrylic acid, glucosamine, olives, mass spectroscopy

Practical Application: Commercial California-style black ripe olive processing methods result in relatively high levels of acrylamide in the finished product. The mechanism for acrylamide formation in processed olives is not understood and remains elusive. Herein, we describe a method for measuring *N*-acetyl-glucosamine and glucosamine in olives and demonstrate that there is a relationship between glucosamine levels in raw olive fruit and acrylamide levels in processed olives. Additionally, we demonstrate that glucosamine undergoes base-catalyzed hydrolysis and that treatment of olives with sodium hydroxide (pH > 12) significantly reduces acrylamide levels over 1 to 5 d without impacting olive fruit texture.

Introduction

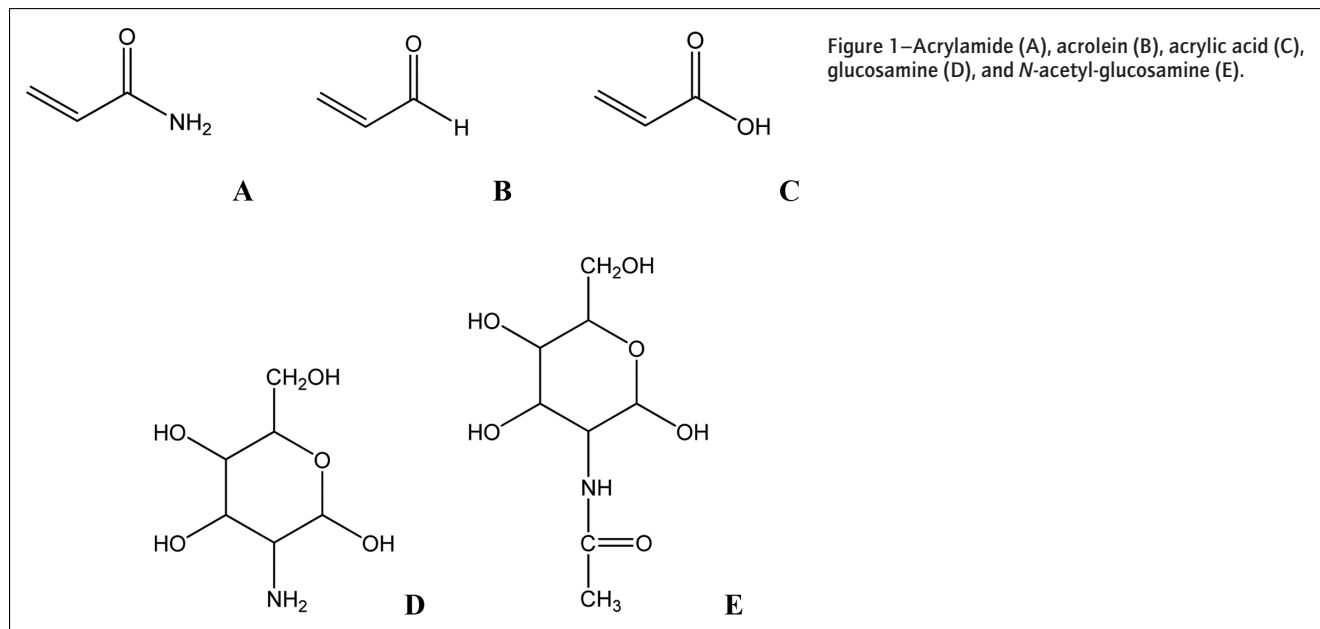
Olive fruit is bitter due to the presence of phenolic compounds and in particular the *ortho*-diphenol, oleuropein and its derivatives (Mateos and others 2004). California-style black ripe olive (CBRO) processing is one of the most widely used methods for removing the bitter compounds in olive fruit. CBRO processing involves harvesting olives before they are completely ripe. Olives not processed directly after harvesting, are preserved in a brine solution (5% to 10% sodium chloride) containing an acid to prevent the growth of spoilage organisms. The bitter compounds are removed via successive soaking in a lye solution (normally 1% to 2% sodium hydroxide, pH 13) for 2 to 24 h over 3 to 7 consecutive days. Lye treatment is considered complete when the sodium hydroxide reaches the level of the pit as determined with a pH indicator. During the intervals between lye treatments (lye-wash), the olives are suspended in water and air is bubbled through the tank to form the black color associated with these olives. The black pigments obtained through this process are not stable and are fixed with iron salts such as ferrous gluconate, ferrous sulfate or ferrous lactate. Olives are then neutralized with lactic acid or carbon dioxide and packed into cans in a sodium chloride solution. Finally,

canned olives are sterilized. In the United States, canned olives are sterilized at 115.6 to 121.1 °C for 50 to 60 min (Charoenprasert and Mitchell 2012).

Acrylamide (Figure 1A) is classified as a probably carcinogen to humans, and has been found in many foods (Friedman 2003). According to FDA survey data on acrylamide in foods, CBROs contain relatively high levels of acrylamide (226 to 1925 $\mu\text{g}/\text{kg}$) as compared to other foods including French fries (20 to 1325 $\mu\text{g}/\text{kg}$) and nut and nut butter products (<457 $\mu\text{g}/\text{kg}$) (U.S. Food and Drug Administration 2006). This is puzzling as unlike potatoes and other starch rich foods, olives contain relatively low levels of traditional precursors of acrylamide (for example, asparagine and glucose). Moreover, as olives are extensively rinsed with water prior to sterilization, the levels of low molecular weight amino acids and reducing sugars are low and do not correlate with acrylamide formation (Casado and Montaño 2008), suggesting that other mechanisms are involved in the formation of acrylamide in olives. Acrylamide is primarily formed during the sterilization of olives (Amrein 2007; Casado and Montaño 2008). Methods for reducing acrylamide using salts, amino acids, and antioxidants have been explored (Casado and others 2010) however the mechanism of its formation in olives remains elusive.

Olives are rich in fatty acids including oleic (80%), palmitic (16%), and stearic acid (3%) (Garrido Fernández and others 1997). Charoenprasert and Mitchell (2014) demonstrated that oxidation during lye-processing increases the formation of acrylamide in olives. During lye-processing, fatty acids are saponified and free fatty acids and glycerol are released. A thermal degradation

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product of glycerol and fatty acids is the simple aldehyde acrolein (Figure 1B) (Gerzt and Klostermann 2002; Mottram 2006; Shibamoto and Bjeldanes 2009). Acrolein (2-propenal) can be oxidized to form acrylic acid (Figure 1C) which has been shown to form in heated olive oil (Umano and Shibamoto 1987; Casella and Contursi 2004). Amino acids produce ammonia upon Strecker degradation, which has been shown to react with acrylic acid derived from lipids to form acrylamide (Yasuhara and others 2003). Ehling (2005) also demonstrated that acrylamide forms in model systems that contain acrylic acid and amino acids, and are heated at 170 °C for 30 min. Acrolein can also be formed from the degradation of amino acids, proteins, and of carbohydrates (Lingnert and others 2002). Based upon these observations we investigated the possibility that acrylamide forms as a reaction product between acrylic acid and amino acids in CBROs.

As the mechanism for the formation of acrylamide in CBRO is unknown, we also evaluated if amino sugars contribute to the formation of acrylamide in olives. Glucosamine and *N*-acetyl-glucosamine (Figure 1D and E) are the main amino sugars found in plant tissues (Priemet 1993; Gleason and Chollet 2011; Indorf and others 2011). These compounds contain carbon and nitrogen atoms and have several reactive functional groups including carbonyl, hydroxyl and amine, which can undergo intra- and inter-chemical reactions and that could participate in the formation of acrylamide in CBROs (Belitz 2004; BeMiller and Huber 2008). To date, the levels of glucosamine and *N*-acetyl-glucosamine have not been reported for olive fruit. To address this, an ultra-high pressure liquid chromatography mass spectrometry (UHPLC-(ESI)MS/MS) method was developed to measure glucosamine and *N*-acetyl-glucosamine in olive fruit and is reported herein. This method was used to measure the levels of these compounds in raw olive fruit and to systematically evaluate if glucosamine and/or *N*-acetyl-glucosamine are involved in the formation of acrylamide in CBROs.

As CBROs undergo extensive lye processing, we also investigated the effect of sodium hydroxide on glucosamine stability in olives and the relationship between glucosamine present in olive pulp and acrylamide formed in olive fruit after sterilization.

These studies provide new information on the content of *N*-acetyl-glucosamine and glucosamine in olive fruit and their role in the formation of acrylamide.

Materials and Methods

Reagents

All reagents were analytical grade unless otherwise stated. Acrylamide (99+%), acrylamide-2,3,3-d₃ (98.0%), acrylic-1-¹³C (99% ¹³C), acrolein-¹³C₃ (99% ¹³C), D-(+)-Glucose, *N*-acetyl-D-glucosamine (>99%), D-glucosamine-¹⁵N hydrochloride (98%) D-(+)-glucosamine hydrochloride (>99%), and formic acid (~98%) were purchased from Sigma-Aldrich (St. Louis, Mo., U.S.A.). Acrylic acid (99.5%), sodium hydroxide (NaOH) (>97%), hydrochloric acid (HCl) (36.5% to 38%), HPLC-grade methanol and hexane were obtained from Fisher Scientific (Fair Lawn, N.J., U.S.A.). D-glucosamine-¹³C₆ hydrochloride (99% ¹³C) was purchased from Omicron Biochemicals (South Bend, Ind., U.S.A.) and carbon dioxide liquefied gas (99.99%) was from Central Storehouse (Davis, Calif., U.S.A.).

Olive samples

A 15 kg sample of fresh and 30 kg sample of brine-stored olives were kindly supplied by Bell-Carter Foods Inc. (Corning, Calif., U.S.A.) and by Musco Family Olive CO. (Tracy, Calif., U.S.A.). These olives were graded as medium-sized Manzanilla olives harvested in 2011 and 2012. Each composite sample of olives was prepared from pooled samples representing numerous orchards; typical of commercial olive processing conditions. Directly after receipt of fresh olives, the processors prepared brine, and delivered both fresh and olives in brine (brined-stored) to the University of California (UC), Davis. Fresh olives were immediately frozen at -80 °C. Brine-stored olives were kept in brine at room temperature for 6 months, and then stored at -80 °C until they were processed.

CBRO processing

The olives used in this study were processed using a laboratory-scale CBRO processing model as described previously

(Charoenprasert and Mitchell 2014). The processing steps evaluated using this model reflect typical industry practices and include: lye-treatment, lye-wash with air-oxidation, ferrous gluconate treatment, and sterilization in a Steromaster MK II autoclave (Consolidated, USA) at 127 °C for 30 min. Fresh olives and brine stored olives were also evaluated. To determine the effect of air-oxidation, olives were processed with and without air introduction during the lye-wash steps (lye-wash with no air oxidation). All studies were performed and analyzed in triplicate or duplicate as stated below.

Formation of acrylamide from acrylic acid, glucosamine, and *N*-acetyl-glucosamine

After lye treatments the olive samples were neutralized and pitted. The pitted olives were freeze-dried and homogenized to a powder of controlled particle size. A 380 mg sample of olive powder was spiked with a 2.3 mL solution containing either: glucosamine, glucosamine-¹⁵N, glucosamine-¹³C₆, *N*-acetyl-glucosamine, glucose, acrylic acid, acrylic acid-1-¹³C, acrolein or acrolein-¹³C₃ or water for control. The pH of the solutions containing acrylic acid was controlled with 0.1 M of trisodium phosphate, while the pH of the glucosamine hydrochloride solution was adjusted with sodium hydroxide. The final pH of all test solutions was ~7.0. The concentration of the spiked test substances was 20 000 nmol/g of the olive mixture. The spiked test substance was allowed to react with the olive matrix at room temperature for 2 h prior to sterilization at 127 °C for 30 min. The studies were performed in triplicate and analyzed in duplicate.

The olive powder was also spiked with glucosamine at 500, 1000, 5000, 10 000 and 20 000 nmol/g mixture. The mixtures were sterilized at 127 °C for 30 min and levels of acrylamide were measured in the sterilized products. The study was performed in triplicate and analyzed in duplicate.

UHPLC-(ESI)MS/MS analysis of glucosamine

Glucosamine was measured using an Agilent 1290 Infinity ultra-high pressure liquid chromatography (UHPLC) interfaced to an Agilent 6460 triple quadrupole mass spectrometer equipped with electrospray ionization (ESI) via Jet Stream Technology (UHPLC-(ESI)MS/MS; Agilent Technologies, Santa Clara, Calif., U.S.A.). The UHPLC was equipped with a binary pump with integrated vacuum degasser (G4220A), an auto sampler (G4226A) and thermostatted column compartment (G1316C). The separation of glucosamine was performed on Zorbax 300-SCX column (2.1 × 150 mm, 5 μm, Agilent Technologies, Santa Clara, Calif., U.S.A.). The column temperature was controlled at 30 °C. The mobile phase was an isocratic mixture of 0.5% formic acid in water at a flow rate of 0.4 mL/min. The injection volume was 10 μL and the data were acquired for 11 min. The UHPLC-(ESI)MS/MS was optimized using a fragmentor voltage of 70 V and positive mode ionization. The drying gas temperature and flow rate were 300 °C and 5 L/min, respectively. The sheath gas temperature and flow rate were 400 °C and 11 L/min, respectively. The nebulizer gas pressure, nozzle voltage, and capillary voltage were 45 psi, 500 and 3000 V, respectively. The concentration of glucosamine was quantified using a transition ion of m/z 180 → 72 and qualified using a transition ion of m/z 180 → 162. Collision energies were 3 and 20 V for transition ions of m/z 180 → 162 and 180 → 72, respectively. Glucosamine-¹⁵N was quantified and identified with transition ions of m/z 181 → 73 and 181 → 163, respectively. The limit of detection (LOD) and the limit of quantification (LOQ)

of the instrument were 0.43 and 1.44 pmol/mL, respectively. Recoveries were determined at spiked levels of 210 nmol/g in fresh, 160 nmol/g in brine-stored and 90 nmol/g in CBROs. The recoveries of glucosamine were 102.8%, 90.4%, and 96.9%, respectively.

The effect of pH on glucosamine stability

To evaluate stability, a 100 μg mL⁻¹ glucosamine standard was prepared in water, 0.1 N of hydrochloric acid (pH 1) or in 0.25 N sodium hydroxide (pH 13). The solutions were heated at 95 °C for 20 min or kept at room temperature for 5 d. These solutions were analyzed for glucosamine content as described above. The experiment was performed in triplicate and samples analyzed in duplicate.

To determine stability in olive pulp, 60 mL of ultra-pure water was added to 30 g of fresh pitted olives (harvested in 2012). The mixture was homogenized and 25 g of the mixture was collected. A 15 mL aliquot of hexane was added to extract oil and other non-polar compounds (2×). The hexane was decanted and the sample was centrifuged at 4000 rpm to remove residual hexane. The homogenized olive (HO) sample was collected. A 1 g sample of HO was mixed with either 4 mL of water (HO:water), 0.1 N of HCl in water (pH~1) (HO:HCl) or 0.25 N NaOH (pH~13) (HO:NaOH). These mixtures were heated at 95 °C for 20 min and then filtered through 0.65 μm membrane. The filtered solutions (Filtrate 1 to 3) were collected and neutralized. The olive pulp left on the filter membrane was washed with 150 mL of ultra-pure water and dried under vacuum. The washed olive pulp was collected for analysis of bound glucosamine. The final weight of the pulp was approximately 0.35 g. To determine formation of acrylamide in the Pulp samples, a 4 mL aliquot of Filtrate 1 was added to three independent samples of each Pulp sample (1 to 3). This was to ensure that all precursors were present for acrylamide formation. The pH was approximately 7. These solutions were sterilized at 127 °C for 30 min, and analyzed for acrylamide levels. For clarity, a diagram of preparation methods for olive pulp samples is shown in Figure 2. The experiment was performed in triplicate. Samples were analyzed in duplicate.

Effect of pH and sodium hydroxide on acrylamide and glucosamine in CBROs

The effect of pH. Olives (harvested in 2012) were lye-processed but not sterilized. Fresh solutions of sodium hydroxide were prepared and adjusted (with HCL) to a final pH value of 9, 10, 11, 12, and 13. The processed olives were placed in one of the 5 sodium hydroxide solutions. For control samples, olives were placed in water. Olives (30) were immersed in the solutions for 5 d. The olive samples were then rinsed with water and treated with 0.15% ferrous gluconate solution for 4 h. During the ferrous gluconate treatment, carbon dioxide gas was bubbled into the solution to neutralize olives and phenolphthalein solution was used to verify the pH of olives. When a pH less than 8 was reached, the olives were rinsed and put into fresh water for 30 min to remove residual ferrous gluconate. Olives were then packed in a 250 mL Erlenmeyer flask, filled with water and sterilized at 127 °C for 30 min. Levels of acrylamide and glucosamine were measured by UHPLC-(ESI)MS/MS as described below. The experiment was performed in duplicated in olives from both processors. Samples were analyzed in duplicate.

Effect of sodium hydroxide treatment time. To determine the influence of sodium hydroxide exposure time, lye treated olives were rinsed with water and placed in fresh 0.25 N sodium

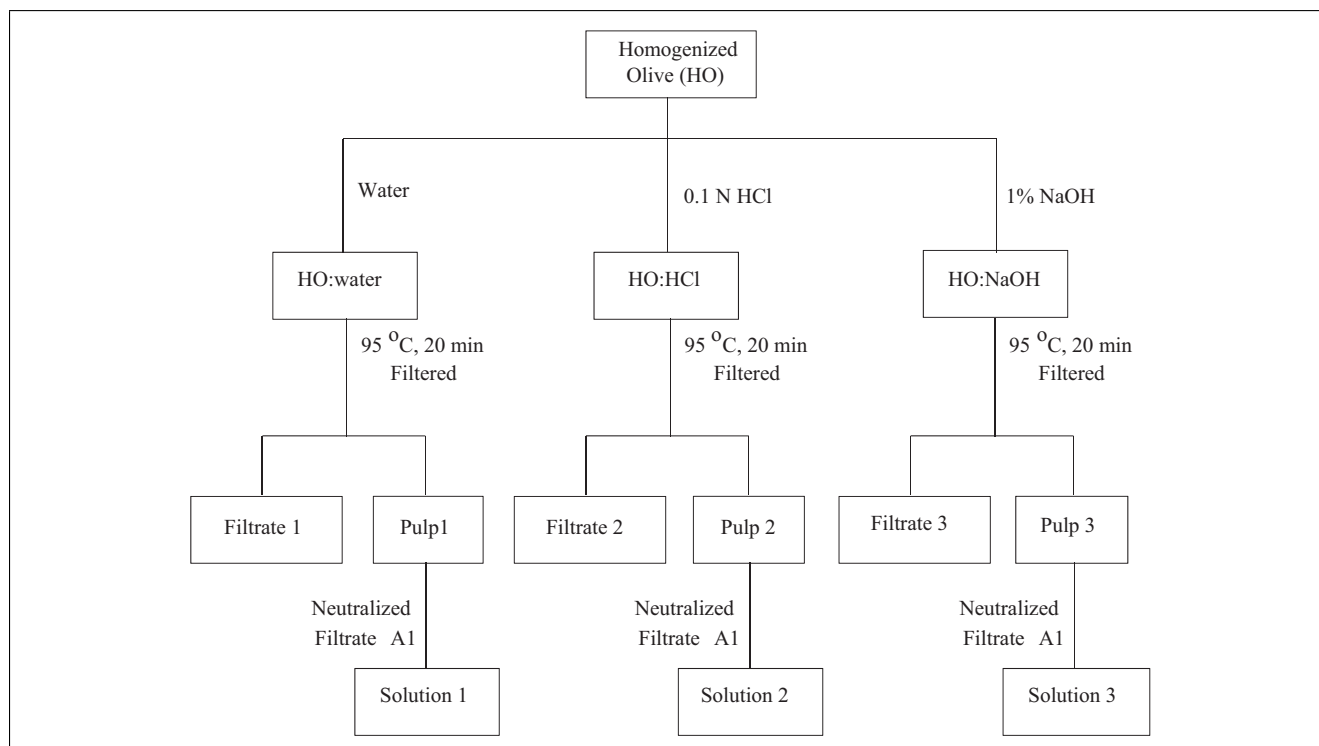


Figure 2—Diagram of the sample preparation scheme to determine the effect of pH on glucosamine stability under acidic and basic conditions, and acrylamide formation in olive pulp.

hydroxide solution (pH 13). Control samples were placed in fresh water. Olives were immersed for 1, 2, 3, 4, or 5 d. The olives were then rinsed with water, treated with ferrous gluconate solution and neutralized with carbon dioxide as described above. Residual ferrous gluconate was removed and approximately 14 olive fruit were collected for analysis of glucosamine. The rest of the olives were packed in a glass flask, filled with water and sterilized as described above. Approximately 14 olive fruit were collected for analysis of acrylamide using UHPLC-(ESI)MS/MS. Approximately 7 olives were used for analysis of texture (firmness). The experiment was performed in duplicate.

Analysis of acrylamide

Acrylamide levels were analyzed using ultra-high pressure liquid chromatography electrospray ionization tandem mass spectroscopy [UHPLC-(ESI)MS/MS] according to previously described methods (Charoenprasert and Mitchell 2014). To measure acrylamide levels in freeze-dried olive samples and Solutions 1 to 3, a 5-mL aliquot of ultra-pure water and 100 μ L of 4 ng/mL of D₃-acrylamide, an internal standard, was added (to identify acrylamide-¹³C₃ in the olive samples, no internal standard were used). A 5-mL aliquot of hexane was then added to remove oil and other non-polar compounds. The mixture was centrifuged at 4000 rpm for 30 minutes. A 3-mL aliquot of the aqueous layer was collected. Interfering compounds were removed and the sample concentrated using solid phase extraction (SPE) on a Strata-X-C cartridge (3 mL, 200 mg, Phenomenex, Torrance, Calif., U.S.A.). The SPE cartridges were preconditioned with the addition of two 1-mL aliquots of methanol, followed by two 1-mL aliquots of water at a flow rate of 2 mL/min. Acrylamide was eluted with 1 mL of 0.1% formic acid in water/methanol, 90:10, v/v. The eluted solution was collected and filtered through a 0.22 μ m membrane prior to UHPLC-(ESI)MS/MS. Quantification was achieved by mon-

itoring MS/MS transition ions of m/z 72 \rightarrow 55 for acrylamide; 73 \rightarrow 56 for acrylamide-¹³C₁; 74 \rightarrow 57 for acrylamide-¹³C₂; 75 \rightarrow 58 for acrylamide-¹³C₃. The limit of detection (LOD) and the limit of quantification (LOQ) of analysis of acrylamide was 0.01 and 0.02 nmol/g, respectively.

Analysis of acrylic acid

Acrylic acid was extracted from 30 g of pitted olives with 60 mL of ultra-pure water. The mixture was homogenized and 25 g of the mixture was collected. A 800- μ L aliquot of 40 μ g/g of acrylic acid-1-¹³C (internal standard) was added into the mixture. A 15-mL aliquot of hexane was added to remove oil and other nonpolar compounds. The mixture was centrifuged at 4000 rpm for 30 min. The aqueous layer was collected and adjusted to a pH of 2 with 6 N of hydrochloric acid. A 1-mL aliquot of the solution was loaded to a Strata-X (200 mg, 3 mL, Phenomenex, Torrance, Calif., U.S.A.) as described above. Interfering compounds were removed by washing the cartridge with 2 mL of 0.01 N of hydrochloric acid. The cartridge was dried under vacuum for 30 s. Acrylic acid was eluted with 1 mL of methanol and analyzed using a Hewlett Packard 6890 Series gas chromatograph equipped with a Hewlett Packard 5973 mass selective detector (Palo Alto, Calif., U.S.A.). Acrylic acid was separated on an Agilent DB-WAX capillary column, 30 m, 0.25 mm i.d., 0.25 μ m film thickness (Palo Alto, Calif., U.S.A.). The column was maintained at 60 $^{\circ}$ C for 1 min then programmed at 10 $^{\circ}$ C/min to the final temperature of 240 $^{\circ}$ C (total analysis time 19 min). Helium flow rate was 1 mL/min. Solvent delay was set at 8.5 min. Injection volume was 1 μ L and splitless mode was used. The ion source and quadrupole temperature were 230 and 150 $^{\circ}$ C, respectively. Scan cycle was 2.9 cycles/s. The molecular ion of m/z 72 was selectively monitored for quantification of acrylic acid and a fragment ion of m/z 55 was used for identification. For acrylic acid-1-¹³C, an

internal standard, and ions of m/z 73 and 56 were monitored for quantification and identification, respectively. The limit of detection (LOD) and the limit of quantification (LOQ) of the instrument were 0.04 and 0.13 nmol/mL, respectively. Recoveries were determined for spiked levels at 300 nmol/g in fresh, 160 nmol/g in brine-stored and 80 nmol/g in CBROs. The recoveries of acrylic acid were 93.5%, 98.1%, and 101.4%, respectively.

Analysis of glucosamine

For olive fruit, 30 g of pitted olive were homogenized with 60 mL of ultra-pure water and 3 g of the mixture was collected. A 100- μ L aliquot of 1000 μ g/g of glucosamine- 15 N hydrochloride (internal standard) and 1 mL of ultra-pure water were added. The pH of the mixture was adjusted with 6 N of hydrochloric acid to pH 1. For olive Pulp samples (1 to 3), a 4 mL of 1 N of hydrochloric acid in water was added to the sample and the mixture was spiked with 100 μ L of 1000 μ g/g of glucosamine- 15 N hydrochloride. To cleave bound glucosamine, the acidified mixture was heated at 105 °C for 6 h. Since *N*-acetyl glucosamine can be deacetylated to glucosamine by acid hydrolysis (Holan and others 1980; Chen and Robin, 1999), glucosamine concentration reported in this study represent the total concentration of glucosamine and/or *N*-acetyl glucosamine. After acid hydrolysis, 4 mL of ultra-pure water was added to the mixture and 5 mL of hexane was added to remove oil and other non-polar compounds. The mixture was then centrifuged at 4000 rpm for 30 min. The hexane layer was removed. A 0.1-mL aliquot of aqueous layer was diluted with 4.9 mL of water. The solution was passed through a 0.22- μ m filter and analyzed by UHPLC-(ESI)MS/MS as described above. For an authentic glucosamine standard, the standard was diluted 1:1000 with water. A 100 μ L aliquot of 1 μ g/mL of D-glucosamine- 15 N was added into 1 mL of the diluted solution prior or analysis by UHPLC-(ESI)MS/MS.

Analysis of texture

Sterilized olives were allowed to cool to room temperature. Each fruit was dried on a napkin prior to analysis with a TA.XT2 texture analyzer (Texture Technologies, Scarsdale, N.Y., U.S.A.) with a 75 mm compression platen (P75). The probe pressed olives parallel to the cross-section of the fruit. The maximum peak force was expressed as firmness (N). A compression depth of 3 mm and a compression rate of 2 mm/s were used. Firmness of each sample was calculated from mean values of approximately 7 olive fruits.

Three different commercial brands of pitted CBRO were purchased from local markets in 2014. The olives were dried on a napkin and a solid plastic tube was inserted into the pitted olive to prevent collapsing during texture analysis. Then the olives were analyzed with a TA.XT2 texture analyzer as described above. The experiment was performed in triplicate. Each sample consists of 7 olive fruits.

Statistical analysis

Difference between mean values was statistically analyzed with one-way ANOVA followed by multiple comparison test using Turkey (HSD). ANOVA was performed using XLSTAT version 2013.

Results and Discussion

Acrylic acid and glucosamine levels were measured in olives at various steps of the CBRO processing method and results are presented in Table 1. Fresh olives (Sample A) were found to have relatively high levels of acrylic acid (333.50 ± 21.88 nmol/g) and

Table 1—Effect of processing steps on concentrations of acrylic acid, glucosamine, and acrylamide in olives.

Olive sample*	Acrylic acid** (nmol/g)	Glucosamine (nmol/g)	Acrylamide (nmol/g)
Sample A	$333.50^a \pm 21.88$	$243.59^a \pm 10.06$	<0.01
Sample B	$184.50^b \pm 6.02$	$165.88^b \pm 11.51$	<0.01
Sample C	$66.47^d \pm 2.93$	$106.44^c \pm 2.02$	<0.01
Sample D	$92.90^c \pm 4.31$	$110.87^c \pm 7.48$	$11.52a \pm 0.58$
Sample E	$83.74^{cd} \pm 6.42$	$104.05^c \pm 3.76$	<0.01
Sample F	$94.29^c \pm 6.72$	$96.95^c \pm 9.13$	$6.27b \pm 0.11$
Sample G	NM***	NM	117.48 ± 0.71
Sample H	NM	NM	90.59 ± 0.62

*[Sample A] fresh olives; [Sample B] brine-stored olives; [Sample C] processed olives no sterilization (that is, lye, air-oxidation, ferrous gluconate); [Sample D] processed olives with sterilization (that is, lye, air-oxidation, ferrous gluconate, sterilization); [Sample E] processed olives no air-oxidation or sterilization (that is, lye, ferrous gluconate); [Sample F] processed olives no air-oxidation with sterilization (that is, lye, ferrous gluconate, sterilization); [Sample G] sterilized fresh olives; and [Sample H] sterilized brine stored olives.

**For comparing within the column, means with different letter are significantly different at 95% confident level.

***Not measured in these samples.

glucosamine (243.59 ± 10.06 nmol/g). To our knowledge, this is the first time that these compounds have been reported in olives. In olives that were brine stored for 6 months (Sample B), levels of acrylic acid and glucosamine were significantly reduced to 184.50 ± 6.02 and 165.88 ± 11.51 nmol/g, respectively. Decreases could reflect diffusion into the brining solution during brining. In olives that were subject to typical lye processing but were not sterilized (Sample C), the levels of acrylic acid and glucosamine decrease further to 66.47 ± 2.93 and 106.44 ± 2.02 nmol/g, respectively. Sterilization increased levels of acrylic acid by 39.8% to 92.90 ± 4.31 nmol/g, but has no significant effect on glucosamine levels (Sample D). In olives that underwent lye processing with no air-oxidation or sterilization (Sample E) the levels of acrylic acid and glucosamine did not significantly change from oxidized olives (Sample C) and sterilization had no significant impact on these levels (Sample F). However, eliminating the air-oxidation step did result in an approximate 50% decrease in acrylamide levels.

When fresh and brine-stored olives were directly sterilized (Table 1, Samples G and H), relatively high concentrations of acrylamide formed (117.48 ± 0.71 and 90.59 ± 0.62 nmol/g, respectively), and the levels were significantly higher than levels found in the lye-treated and sterilized olives (11.52 ± 0.58 nmol/g). The lower concentration of acrylamide in olives treated with lye correlate with the lower levels of acrylic acid and glucosamine found in these olives (Table 1). Purging with air during the lye-treatment step results in significantly higher (2 \times) levels of acrylamide in the olives (Table 1). However, concomitant increases in acrylic acid were not observed, and indicate that other compounds formed during air-oxidation contributed to the formation of acrylamide in olives.

To better understand if acrylic acid or the precursor acrolein (Figure 1) were directly involved in the formation of acrylamide in olives, stable isotope studies were performed using acrylic acid- 13 C and acrolein- 13 C₃. As shown in Table 2, the concentration of acrylamide increased by 35.2%, 33.8%, 35.6%, and 32.5% in the olive samples spiked with acrylic acid, acrolein, acrylic acid- 13 C, and acrolein- 13 C₃, respectively as compared to the non-spiked samples. Interestingly, only trace levels of the 13 C labeled acrylamide were found in the samples spiked with acrylic acid- 13 C and acrolein- 13 C₃ and they were not statistically significant from the non-spiked samples. The level of non-labeled acrylamide in olive samples (26.28 ± 0.33 to 26.89 ± 0.31 nmol/g) spiked

Table 2—Acrylamide formation in olives spiked with acrylic acid, acrolein, acrylic acid-1-¹³C, and acrolein-¹³C₃.

Compound tested	Acrylamide* (nmol/g)	Acrylamide- ¹³ C ₁ (nmol/g)	Acrylamide- ¹³ C ₂ (nmol/g)	Acrylamide- ¹³ C ₃ (nmol/g)
None	19.83b ± 0.46	0.51a ± 0.00	<0.01	<0.01
Acrylic acid	26.82a ± 0.91	0.52a ± 0.01	<0.01	<0.01
Acrolein	26.54a ± 0.50	0.52a ± 0.00	<0.01	<0.01
Acrylic acid-1- ¹³ C	26.89a ± 0.31	0.54a ± 0.00	<0.01	<0.01
Acrolein- ¹³ C ₃	26.28a ± 0.33	0.53a ± 0.01	<0.01	<0.01

*For comparing within the column, means with different letter are significantly different at 95% confident level.

Table 3—Acrylamide formation in olives spiked with glucosamine, N-acetyl-glucosamine, glucose, glucosamine-¹⁵N and glucosamine-¹³C₆.

Compound Tested	Acrylamide* (nmol/g)	Acrylamide- ¹³ C ₁ (nmol/g)	Acrylamide- ¹³ C ₂ (nmol/g)	Acrylamide- ¹³ C ₃ (nmol/g)
None	34.12c ± 2.57	1.05b ± 0.24	<0.01	<0.01
Glucosamine	76.42a ± 1.49	2.14a ± 0.04	<0.01	<0.01
N-acetyl-glucosamine	44.30b ± 1.40	1.23b ± 0.04	<0.01	<0.01
Glucose	34.73c ± 1.03	1.19b ± 0.06	<0.01	<0.01
Glucosamine- ¹⁵ N	74.16a ± 0.85	2.14a ± 0.11	<0.01	<0.01
Glucosamine- ¹³ C ₆	74.05a ± 1.76	2.13a ± 0.13	<0.01	<0.01

*For comparing within the column, means with different letter are significantly different at 95% confident level.

with acrylic acid, acrolein, acrylic acid-1-¹³C, and acrolein-¹³C₃ were not significantly different. These results suggest that although acrylic acid may be involved in enhancing the formation of acrylamide, it does not directly contribute to the carbon backbone of acrylamide. Our data suggests that the mechanism proposed for acrylamide formation through a reaction between acrylic acid and amino acids does not occur in olives at typical commercial sterilization temperatures and time profiles.

The main amino sugars in plants (for example, glucosamine and N-acetyl-glucosamine) could possibly contribute to the formation of acrylamide in olives. These compounds have several reactive functional groups (for example, carbonyl, hydroxyl, and amine) that could contribute to the acrylamide backbone. Normally, these compounds are bound to plant cell membrane (Chen and Chiou 1999; Indorf and others 2011) and our results indicate that only trace levels of glucosamine leach from olives during typical CBRO processing (Table 1). To determine if these amino sugars were involved in the formation of acrylamide, glucosamine, and N-acetyl-glucosamine were added to olive samples and samples were processed. The levels of acrylamide increased significantly in the olives samples spiked with glucosamine (124.0%) and N-acetyl-glucosamine (29.9%) as shown in Table 3. When increasing concentrations of glucosamine (0, 500, 1000, 5000, and 10 000 nmol/g) were added to olive samples, acrylamide levels increased by 0%, 33.2%, 44.3%, 92.2%, and 113.1%, respectively as compared to nonspiked samples (data not shown). When glucose was added to the olives prior to sterilization, the concentration of acrylamide did not differ significantly from the control (Table 3). These results indicate that glucose is not involved in the formation of acrylamide in processed olives and that the free amine group of glucosamine may promote the formation of acrylamide in olives.

To determine if glucosamine contributes carbon and/or nitrogen to the acrylamide structure, glucosamine-¹⁵N and glucosamine-¹³C₆ were evaluated (Table 3). Only trace levels of ¹⁵N or ¹³C labeled acrylamide were found in the samples spiked with the glucosamine-¹³C₆ or glucosamine-¹⁵N. However, the concentration of non-labeled acrylamide in samples spiked with glucosamine-¹³C₆ (74.05 ± 1.76 nmol g⁻¹) and glucosamine-¹⁵N (74.16 ± 0.85 nmol g⁻¹) increased significantly as compared to

the non-spiked samples (34.12 ± 2.57 nmol g⁻¹). These results indicate that although glucosamine does not directly contribute carbon or nitrogen atoms to acrylamide backbone, it promotes the formation of acrylamide in olives.

To further understand the role of glucosamine in the formation of acrylamide in olives, we investigated the effect of sodium hydroxide on glucosamine stability in olives and the relationship between glucosamine present in olive pulp and acrylamide formed after sterilization. Herein and in previous studies (Charoenpraser and Mitchell 2014), we demonstrated that acrylamide levels decreased by more than 85% in olives treated with 1% sodium hydroxide solution (0.25 N), used to remove olive bitterness, and sterilized as compared to sterilized fresh and brine-stored olives. The reduction of acrylamide in olives treated with sodium hydroxide may result from either the disruption of olive structure (that is, cell wall and cell membrane) leading to the release of acrylamide and its precursors during lye processing and/or degradation of the acrylamide precursors by sodium hydroxide. Therefore, the degradation of an authentic glucosamine standard was studied at (1) room temperature for 5 d and (2) at 90 °C for 20 min under neutral (water), acidic (0.1 N HCl) or basic conditions (0.25 N NaOH). The results (Table 4) indicate that the mean glucosamine

Table 4—Degradation of an authentic glucosamine standard in water, 0.1 N hydrochloric acid and 0.25 M sodium hydroxide solution.

Treatment	Solvent	Glucosamine concentration (µg/mL)
5 d, room temperature	H ₂ O	98.89a ± 4.13
	0.1 N hydrochloric acid	105.18a ± 3.23
	0.25 N sodium hydroxide solution	33.28b ± 2.92
20 min, 95 °C	H ₂ O	100.79a ± 1.65
	0.1 N hydrochloric acid	101.29a ± 5.10
	0.25 N sodium hydroxide solution	1.81c ± 0.18

*Means with different letter are significantly different at 95% confident level.

Table 5—Concentration of glucosamine and acrylamide in olive pulp previously treated with water (Pulp 1), 0.1 N hydrochloric acid (Pulp 2), and 0.25 M sodium hydroxide solution (Pulp 3).

Olive pulp treatment	Glucosamine* (nmol g ⁻¹ Pulp)	Acrylamide (nmol g ⁻¹ Pulp)
Pulp 1(H ₂ O)	1502.15a ± 173.09	365.16a ± 29.64
Pulp 2 (0.1 N HCl)	1395.73a ± 21.61	361.91a ± 2.64
Pulp 3 (0.25 N NaOH)	366.48b ± 52.99	10.48b ± 0.56

*For comparing within the column, means with different letter are significantly different at 95% confident level.

Table 6—Effect of pH values of sodium hydroxide solutions on concentration of acrylamide in California-style black ripe olives.

Treatment solutions	Acrylamide (nmol/g)*
Control (H ₂ O)	8.24a ± 0.28
Solution of sodium hydroxide, pH 9	7.79a ± 0.29
Solution of sodium hydroxide, pH 10	8.31a ± 0.62
Solution of sodium hydroxide, pH 11	7.93a ± 0.26
Solution of sodium hydroxide, pH 12	3.91b ± 0.40
Solution of sodium hydroxide, pH 13	2.17c ± 0.20

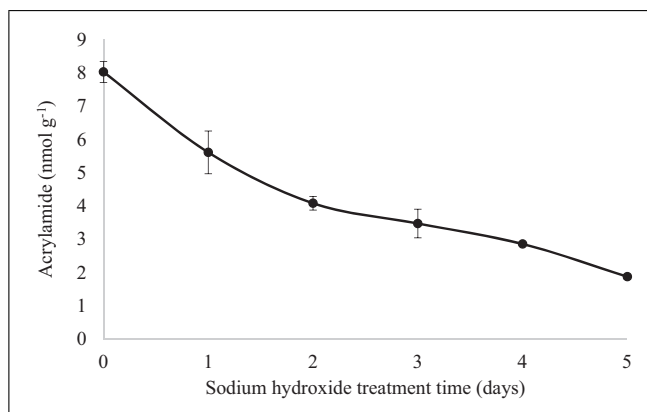
*Means with different letter are significantly different at 95% confident level.

content does not significantly change in water or in 0.1 N hydrochloric acid solutions over the 5 d or at elevated temperatures, whereas levels decreased significantly by 66.7% after 5 d at room temperature and 98.2% after 20 min at 95 °C, in the 0.25 N sodium hydroxide solution (pH 13).

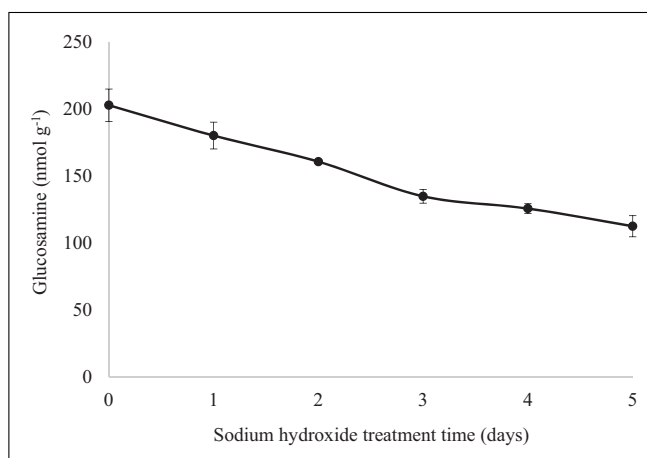
To determine if the same effect was seen in olives, glucosamine was measured in olives treated with either: water, 0.1 N hydrochloric acid, or 0.25 N sodium hydroxide (pH~13) as shown in Figure 2. Levels of glucosamine present in the pulp extracted from these treated olives (Pulp 1 to 3) is shown in Table 5. Results indicate that olives treated with sodium hydroxide (Pulp 3) contained significantly lower levels of glucosamine (366.48 ± 52.99 nmol/g) as compared to olives treated with either water (Pulp 1; 1502.15 ± 173.09 nmol/g) or acid (Pulp 2; 1395.73 ± 21.61 nmol/g). The levels of acrylamide were also evaluated in processed pulp samples (Table 5). Significantly lower levels of acrylamide were found in the processed pulp of the sodium hydroxide treated olives (Pulp 3; 10.48 ± 0.56 nmol/g) as compared to the processed pulp from the water (Pulp 1; 365.16 ± 29.64 nmol/g) or acid treated olives (Pulp 2; 361.91 ± 2.64 nmol/g). Taken together, these results indicate that sodium hydroxide (0.25 N) promotes the degradation of glucosamine in olives with a concomitant decrease in the levels of acrylamide formed upon the sterilization of these olives.

The lye-processed olives were further exposed to sodium hydroxide solutions of increasing pH values (that is, pH 9, 10, 11, 12, and 13) for 5 d and the olives were sterilized (Table 6). Acrylamide levels in olives exposed to solutions of pH of 9, 10, and 11, did not significantly differ from the control samples (8.24 ± 0.28 nmol/g). Olives treated with solutions of pH 12 and 13 demonstrated significantly lower acrylamide levels (3.91 ± 0.40 and 2.17 ± 0.20 nmol/g, respectively) as compared to the control samples. These results suggested that acrylamide levels in CBROs can be reduced using a sodium hydroxide solution with a pH higher than 12.

Typically, a 1% to 2% sodium hydroxide solution (pH ~13) is used to remove bitterness of olives and the lye treatment process is stopped when sodium hydroxide reaches the pit (usually 3 to 7 d). To evaluate if acrylamide levels could be reduced further by longer exposure times, lye-processed olives were exposed to a 0.25 N sodium hydroxide solution (pH~13) for 1 to 5 d. The olives were sterilized and acrylamide levels of the resulting products were

**Figure 3—Effect of sodium hydroxide treatment time on concentration of acrylamide in CBROs.**

quantified (Figure 3). Results indicate that longer sodium hydroxide treatment times result in lower acrylamide levels in the end product. Olives immersed in sodium hydroxide solution for 5 d, and sterilized, contained ~70% lower acrylamide levels (1.88 ± 0.08 nmol/g) than the control samples (8.04 ± 0.32 nmol/g). The levels of glucosamine in these olives also decreased with increasing exposure time to sodium hydroxide (Figure 4). The concentration of glucosamine reduced by approximately 44.6% after 5 d, as compared to levels in the control samples (202.98 ± 12.08 nmol/g). A strong correlation was found between glucosamine present in olives before sterilization, and acrylamide levels formed in the end products (correlation coefficient 0.9513) as shown in Figure 5. As the texture (firmness) of olives can change with extended exposure time to sodium hydroxide, the texture of these olives was monitored. Results indicate that the firmness of olives did not significantly change as compared to the control samples. Firmness of the control samples and olives treated with sodium hydroxide for 5 d were 35.67 ± 3.10 and 33.74 ± 4.50 N, respectively. For comparison, the firmness of several commercial CBRO samples (three independent labels) were evaluated. These values ranged between 14.58 ± 1.12 and 34.33 ± 7.41 N. The texture of commercial CBRO samples appears to vary significantly (likely due to different handling, processing treatments, brine time and shelf lives). However, our results suggest that the impact of a longer sodium hydroxide treatment time on texture may be negligible.

**Figure 4—Effect of sodium hydroxide treatment time on glucosamine levels present in olives before sterilization.**

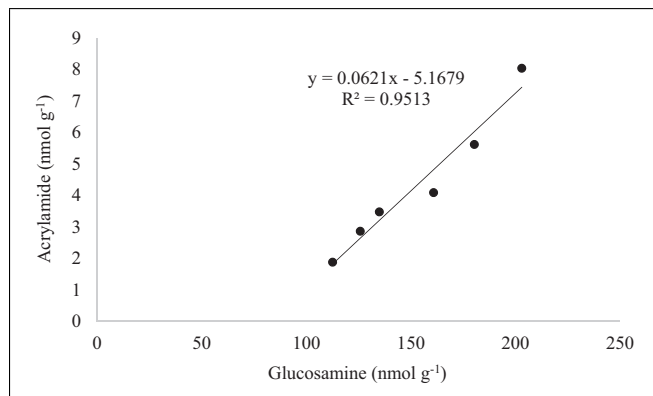


Figure 5—Correlation between glucosamine levels present in olives before sterilization and concentration of acrylamide formed in CBROs.

Conclusions

This study demonstrates that the mechanism of acrylamide formation in olives is different than the mechanism found in starch-rich foods. Glucosamine, and to a lesser extent acrylic acid and *N*-acetyl-glucosamine can increase levels of acrylamide when added to olives prior to sterilization but do not contribute carbon and/or nitrogen atoms to acrylamide. A strong correlation was found between glucosamine levels present in olives before sterilization and the concentration of acrylamide formed in sterilized olives. Exposure of olives to a 0.25 N sodium hydroxide solution (pH > 12) results in the degradation of glucosamine and lower concentrations of acrylamide in sterilized olives. Exposure to a 0.25 N sodium hydroxide solution for 5 d effectively reduces acrylamide levels in CBRO by more than 70% without impacting fruit texture in terms of firmness.

Acknowledgments

The authors would like to thank the California Olive Commission for kind financial support of this research. The authors would also like to thank Drs. Tomas Collins and Susan Ebeler of the UC Davis Food Safety and Measurement Facility for analytical and technical advice.

Author Contributions

Suthawan Charoenprasert, performed the experimental research, designed stable isotope studies, interpreted results, and drafted the first manuscript.

Jerry Zweigenbaum, provided technical support on method development for glucosamine and *N*-acetyl-glucosamine and provided editorial advice.

Gong Zhang, provided technical support and help analyzing the acrylic acid in olive samples.

Alyson Mitchell generated the research concept, designed the original study, interpreted results, edited manuscript and revisions.

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