Identification of mitigation strategies to reduce acrylamide levels during the production of black olives

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ABSTRACT

 Californian-style processes are widely applied to the elaboration of black ripe olives in the table olive industry. During this procedure, a carcinogenic contaminant known as acrylamide is generated in thermal oxidation. The content of this compound can be modified according to the stage of processing. The present study evaluates the conditions pertaining to different phases of the elaboration process with the aim of identifying optimal conditions for the production of table olives with the lowest acrylamide concentration possible. ‘Hojiblanca’ variety olives were used. Olives were harvested at two different maturation stages, specifically, the green and yellowgreen stages. Olives with yellow-green maturation indices had higher acrylamide concentrations than green table olives. Significant reductions in the contaminant were observed as storage time increased, with green olives stored for 21 months displaying the lowest acrylamide levels. Acrylamide was also decreased by spraying olives with water (18 % reduction) and washing them with water heated to 25ºC for 40 min (36 % reduction). Following treatment with lye during the packaging and preparation of olives, unpitted olives contained 12–31% more acrylamide than pitted olives and 42–62% more than sliced olives. Outcomes were the same for olives canned both with and without brine. The presence of CaCl₂ and the addition of greater NaCl concentrations (from 2% to 4% w/v), increased the acrylamide content in all olive formats. This increase was not observed in olives not stored in liquid. Outcomes reported here are valuable for redesigning the elaboration process of industrial black ripe olives and allowing producers to manufacture better-quality products with significantly reduced acrylamide concentrations.

1. INTRODUCTION

Acrylamide is a toxic compound that is present in a wide range of heat-processed foodstuffs such as fried potato, cereals and baked goods, toasted coffee and dark chocolate (Becalski et al., 2003; Kruszewski and Obiedzinski, 2020). It has been classified as a probable human carcinogen by the International Agency for Research on Cancer (IARC) (IARC, 1994). Exposure to this contaminant is a public health concern and a priority for the European Food Safety Authority (EFSA) (European Food Safety Authority (EFSA, 2015).

Acrylamide is not present in raw foods but is formed during the heating process when temperatures reach 120ºC or above (Rifai and Saleh, 2020). Although this toxic compound is primarily formed in food products derived from raw materials which are rich in carbohydrates and low in proteins, recently, acrylamide generation has also been associated with fat-rich foods such as table olives (Pan et al., 2020). Several studies have suggested that the high temperatures applied during the sterilization process promote acrylamide
formation (Casado and Montaño, 2008; Charoenprasert and Mitchell, 2014; Pérez-Nevado et al., 2018; Tang et al., 2016). This conclusion is supported by the fact that this compound is not detected prior to sterilization treatment (Casado and Montaño, 2008).

Olives can be processed to produce three main table products known as Spanish-style green olives, natural olives and ripe olives. The latter type is also known as Californian-style of which there are two different types, Californian-style green ripe olives and Californian-style black ripe olives (Charoenprasert and Mitchell, 2014). EFSA considers Californian-style table olives to be a potential source of acrylamide since these foods contain the same or, even, higher levels than other food products such as French fries, cereals and coffee (European Food Safety Authority (EFSA, 2015). For this reason, acrylamide determination in olives is of utmost importance to human safety. In this sense, the European Commission Recommendation (EU) 2019/1888, updated on 7 November 2019, added olives stored in brine solutions to the list of foods in which acrylamide levels must be controlled by the authorities of the member states of the European Union (EU) (EC, 2019).

Acrylamide in foods is mainly produced as a result of the reaction between asparagine amino acid and reducing sugars in the Maillard reaction, although different mechanisms appear to be involved in the formation of acrylamide in table olives (Casado et al., 2013; Charoenprasert and Mitchell, 2014). A wide range of acrylamide concentrations have been described in Californian ripe olives, ranging from 44 to 210 ng g⁻¹ in green ripe olives (Charoenprasert and Mitchell, 2014; Martín-Vertedor et al., 2020) to higher values in black olives or black ripe olives (30 to 1000 ng g⁻¹) (Charoenprasert and Mitchell, 2014; Martín-Vertedor et al., 2020; Pérez-Nevado et al., 2018). Until now, the aforementioned European regulation does not include benchmark levels for acrylamide in table olives (EC, 2017). However, the European Commission working group on industrial and environmental contaminants has proposed an initial reference level in table olives in the range of 300-400 ng g⁻¹. Charoenprasert and Mitchell (2014) have indicated that an acrylamide concentration of 250 ng g⁻¹ in olives does not present a serious threat to human health when the low consumption of these products is considered relative to that of fried potatoes, chips, cookies, cakes and bread. Nevertheless, the higher levels found in several Californian ripe olives mean that it is advisable to identify mitigation strategies which can be applied during the processing of this product (Table 1). Moreover, the production of black ripe olives has increased over recent decades because their shiny black colour makes them attractive to consumers (Romero et al., 2019). This reaffirms the need to control acrylamide formation in order to reduce exposure to it.

Some researchers have suggested that traditional table olive processing methods should be modified in order to ensure lower acrylamide levels. In a previous study, Martín-Vertedor et al. (2020) evaluated the effect of the ripeness stage, washing prior to lye treatment, presentation format and additives on acrylamide content in Californian-style green ripe olives. However, to our knowledge, such information does not exist for Californian-style black ripe olives. The influence of brine storage times and different preservation methods on acrylamide concentration in black ripe olives has been studied by Charoenprasert and Mitchell (2014) and Casado and Montaño (2008), respectively, but there is a lack of information about the effects of other pre-processing conditions on acrylamide formation in this foodstuff. Charoenprasert and Mitchell (2014) found acrylamide content in oxidized olives to decrease following storage for more than 30 days. In addition, olives processed using oxidation methods have greater acrylamide contents than olives processed without air oxidation (Martín-Vertedor et al., 2020). The type of cultivar also has a large influence on acrylamide, with ‘Manzanilla de Sevilla’ varieties presenting the highest concentrations (Casado and Montaño, 2008; Montaño et al., 2016; Martín-Vertedor et al., 2020). Other mitigation strategies have focused on the addition of certain additives such as amino acids, vitamins, phenols or sodium sulphite. Of these, sodium bisulphate was reported to be the most effective compound for inhibiting acrylamide synthesis. Despite this, its use is not permitted.
by the EU (Casado et al., 2010; López-López et al., 2014). Cysteine, proline and sarcosine (Casado et al., 2010; López-López et al., 2014), calcium chloride (Charoenprasert and Mitchell, 2014), and certain phenols such as hydroxytyrosol, tyrosol and oleuropein (Pérez-Nevado et al., 2018; Martín-Vertedor et al., 2019) have also been described as mitigators of acrylamide formation in olives.

The aim of the present work was to study the impact of different conditions prior to and during industrial processing on acrylamide synthesis in Californian-style black ripe olives. Aspects were considered such as the olive maturation index, length of olive storage, presterilization washing treatment, olive presentation format and use of additives such as CaCl$_2$ and NaCl during olive processing. In addition, the effect of packing olives in cans with and without brine on acrylamide content was studied. Results will enable critical points during processing to be identified and mitigation strategies to be designed. This will assist Californian-style black ripe olive producers in reducing acrylamide formation in this food.

2. MATERIALS AND METHODS

2.1. Reagents and solvents
Acetic acid solution was purchased from PANREAC APPLICHEM® (Darmstadt, Germany). Ferrous gluconate and sodium chloride were supplied by Sigma–Aldrich (St. Louis, MO). Calcium chloride was obtained by Tetra Chemicals (Helsingborg, Sweden). Acrylamide (>99 %) was purchased from Fluka (Buchs SG, Switzerland). 2,3,3-D$_3$-acrylamide (98 %) was obtained from Cambridge Isotope Laboratories (Andover, MA, USA). Analytical grade methanol was supplied by Merck (Darmstadt, Germany). A nylon syringe filter was obtained from the FILTER-LAB (Barcelona, Spain). ISOLUTE Multimode (300 mg, 6 mL) and ISOLUTE ENV+ (200 mg, 3 mL) solid phase extraction cartridges were obtained from International Sorbent Technology (Hengoed, Mid Glamorgan, UK). Nylon and nitrocellulose syringe filters (0.45 μm) were purchased from Tracer Analytical Technologies (Madrid, Spain). Water was purified with an Elix/Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Samples
Olives (Olea europaea L.) were obtained from olive groves linked to the "INTAEX-CICYTEX" research centre (Badajoz, Spain). The mentioned cultivars were located in the "Vegas Bajas del Guadiana" region and olives were collected during the 2017/18 crop season. ‘Hojiblanca’ variety olives were harvested for the different assays.

2.3. Experimental design
Different conditions were assayed prior to (assay 1) and during (assay 2) industrial processing of black ripe olives (Fig. 1).

2.3.1. Assay 1. Effect of different conditions prior to industrial processing: ripeness stage, storage time and washing
Table olives from the same crop years were harvested at different stages of ripeness in order to evaluate the influence of the maturation index (M.I.) on acrylamide formation in ‘Hojiblanca’ variety olives. Thus, samples were handpicked in perfect sanitary conditions at the beginning (green maturation stages, M.I. = 0) and end of October (yellow-green maturation stages, M.I. = 1). Olives were selected according to the colour of their skin and flesh evaluations, as proposed by Uceda and Frías (1975). After harvest, each batch was stored in triplicate at room temperature. Tanks had a capacity of 16,000 L and could store up to 10,000 kg of olives. Batches were stored in an acetic acid solution (3% v/v) under aerobic conditions for 4, 9 and 21 months, respectively. The temperature inside the tanks during storage varied by around 15°C.
At the next stage, only green table olives (M.I. = 0) stored for 4 months were selected. Samples were processed at a factory located in the northwest of Spain using Californian-style black ripe olives techniques: lye, oxidation in the presence of ferrous gluconate and washing (Martín-Vertedor et al., 2020). At this point, table olives were submitted to different washing treatments to remove excess acid: i) No washing treatment (unwashed olives), ii) olives sprayed with water (sprayed olives), and iii) olives washed with water heated to 25°C for 45 min (clean olives).

Following this, olives were treated with lye until the sodium hydroxide (NaOH) penetrated the olive seed. The NaOH solution was then removed and the olives were placed into fresh water. The pH of the samples was neutralized using lactic acid (80% v/v) and carbon dioxide gas. During the lye process, air-bubbling was employed to promote the oxidation process. Then, when olives were neutralized, a ferrous gluconate solution (0.15 %) was applied over 4 h to promote the black colour of the olive. Samples were washed to remove residual ferrous gluconate and introduced into tanks. A new brine solution containing sodium chloride (2% w/v) and ferrous gluconate (0.015 % w/v) was added.

2.3.2. Assay 2. Effect of different conditions during industrial processing: presentation formats, packaging and additives

At the next stage, only unwashed olives were selected. Samples were packaged in different presentation formats (unpitted, pitted and sliced) using a PSL-51 model olive pitting and slicing machine (OFM Food Machinery, Seville, Spain). Subsequently, olives were kept inside a densimeter, which contained 2% w/v NaCl, for 15 min to eliminate sharp or heavy objects. The pH value of both the final table olives and the brine solutions was 7.2. Olives were kept in the brine solution for 1 h. same brine solution and the remaining were packed without brine (olives stored without liquid).

Following this, the use of additives was studied. Firstly, the presence of CaCl₂ in the different presentation formats was examined: i) addition of CaCl₂ (2 g L⁻¹ w/v); ii) no addition of CaCl₂. Secondly, the influence of NaCl concentration in brine was examined: i) 2% NaCl w/v; ii) 4% NaCl w/v.

Finally, the different batches were sterilized in an autoclave at 120°C for 30 min (F₀ = 15 min) and acrylamide content was analysed in the olives and/or the brine solution.

2.4. Acrylamide analysis in olives and brine solutions

2.4.1. Acrylamide extraction

Acrylamide analysis was performed as described by Pérez-Nevado et al. (2018). In order to obtain a homogeneous olive paste, 2 g of fresh oxidized black olives was crushed with a thermobeater until a homogeneous mixture was obtained and stored at -80°C. Then, samples were homogenized with 10 mL of milli-Q water and shaken for 60 min. The mix was centrifuged at 1677 g and 4°C for 30 min to ease the separation of the liquid phase. The aqueous phase was filtered through a 0.45 μm nylon syringe filter.

Telos PCX (200 mg/3 mL) disposable extraction columns cartridges were used for the solid-phase extraction. The column was conditioned with 4 mL of methanol followed by 4 mL of Milli-Q water. Three mL of the sample was injected into the cartridge and eluted with 3 mL of Milli-Q water. The eluate was injected in another cartridge, Telos PRP (60 mg/3 mL), which had also been conditioned in the same way as the first cartridge, and eluted with 3 mL of Milli-Q water.

Standard addition method was used to quantify the concentration of acrylamide in olives. Samples were spiked with an acrylamide standard solution with concentrations in the range 50-150 ng mL⁻¹.
This procedure was performed on both the table olives and the brine, with a thermobeacon not being necessary for this second matrix. Acrylamide analysis was performed in all of the experiments. In assay 1, both the olives and the brine were analysed, whilst in assay 2, the olives, brine and non-liquid olives were analysed.

2.4.2. HPLC/MS-MS analysis of acrylamide

Samples were analysed using an Agilent 1290 Infinity II liquid chromatograph (Agilent Technologies), coupled to an Agilent 6460 triple quadrupole mass spectrometer, equipped with an electrospray ion source operating in positive ion mode. The chromatographic separation was achieved on a Zorbax Eclipse XDB-C18 column (150 mm × 2.1 mm, 3.5 μm) (Agilent technologies), which temperature was set at 30°C. The system was operated isocratically at a flow of 0.25 mL min⁻¹ with a mobile phase composed by 95 % of solvent A (0.1 % formic acid in Milli-Q water) and 5% of solvent B (0.1 % formic acid in methanol). The injection volume was 3 μL. The ion source parameters were set as follows: temp: 400°C, sheath gas flow: 12 L h⁻¹, capillary voltage: +2.5 kV, nozzle voltage: 300 V and delta EMV: 300.

Data acquisition handling and instrument control were performed by the MassHunter software version B.07.00 (Agilent). Multiple Reaction Monitoring (MRM) mode was used to collect mass spectral data of precursor and product ion transitions. The area of the chromatographic peaks of the extracted ion at m/z 55, due to the transition 72 → 55, were used for the quantitative analysis. The fragmentary voltage was 50 V and collision energy was 9 V and 20 V, respectively. Fig. 2 shows the extracted ion chromatogram (EIC) of an olive sample and its corresponding standard additions.

2.5. Statistical analysis

Statistical analyses were performed using SPSS 18.0 software (SPSS Inc. Chicago, IL, USA). Data were expressed as means and standard deviations (SD). One-way analysis of variance (ANOVA) was used, followed by Tukey’s multiple range test. Statistical significance was set at p < 0.05.

3. RESULTS

3.1. Assay 1. Effect of different conditions prior to industrial processing: ripeness stage, storage time and washing

In order to evaluate the effect of the olive ripeness stage and the length of storage on acrylamide formation in Californian-style black ripe olives, ‘Hojiblanca’ olives were submitted to the strategies proposed in assay 1 (maturation index variation and different storage periods). Acrylamide content was analysed in both the olives and the brine solutions. Acrylamide concentration in olives harvested during the green ripeness stage (M.I. = 0) ranged from 171.2 to 312.5 ng g⁻¹, whilst the brine displayed concentrations between 203.2 and 355.7 ng g⁻¹. These levels were significantly lower than those observed in olives harvested a month later during the same olive crop season. In this case, green-yellow olives (M.I. = 1) had acrylamide levels which ranged from 230.2 to 423.6 ng g⁻¹, whilst the brine displayed concentrations ranging from 283.4 to 371.9 ng g⁻¹ (Fig. 3). Overall, acrylamide content was 10–19% higher in brine than in olives at both maturation stages.

Significant differences in acrylamide concentration were observed in both the olives and brine of samples stored for different lengths of time. In this way, the acrylamide content of 312.5 ng g⁻¹ (M.I. = 0) and 423.6 ng g⁻¹ (M.I. = 1) in olives stored for 4 months decreased by about 27 % and 45 % following 9 months and 21 months of storage, respectively. The same trend was found in brine solutions, which decreased from 471.9 to 355.7 ng g⁻¹. Overall, the highest concentrations were found in the brine of table olives harvested with a
yellow-green maturation index and stored for 4 months (471.9 ng g⁻¹). On the other hand, the lowest concentrations were observed in olives at the green maturation stage stored for 21 months (171.2 ng g⁻¹) (Fig. 3).

Green olives (M.I. = 0) stored during 4 months were subjected to different treatments prior to the oxidation process (no washing, sprayed with water and washed with water). Acrylamide levels in unwashed olives decreased by around 18 % in sprayed olives and by around 36 % after washing with water heated to 25°C for 45 min (Fig. 4). A similar pattern was found in the brine, with acrylamide concentration being 12 % and 37 % lower in olives sprayed and washed with water, respectively, relative to unwashed samples. In this experiment, a higher acrylamide content was again recorded in the brine than the olives for all three washing conditions.

3.2. Assay 2. Effect of different conditions during industrial processing: presentation formats, packaging and additives

Table 2 presents acrylamide concentrations for all of the studied presentation formats (unpitted, pitted, and sliced) in unwashed olives packed both with and without brine. For samples packed with brine, unpitted olives presented the highest acrylamide levels (286 ng g⁻¹), followed by pitted olives (235 ng g⁻¹) and sliced olives (188 ng g⁻¹). This pertained to 18 and 34 % more acrylamide, respectively, in unpitted olives. A similar trend was observed in the acrylamide content of the brine solutions. Levels found in olives packed without brine ranged from 445.1 to 822.1 ng g⁻¹, this being significantly higher (around 65 %) than the concentration recorded in olives canned with brine, which ranged from 188.0 to 286.0 ng g⁻¹. Again, unpitted olives exhibited the greatest amount of this contaminant (822 ng g⁻¹), followed by pitted (661 ng g⁻¹) and sliced olives (445 ng g⁻¹). In this case, sliced olives had 33 % less acrylamide than pitted olives and 46 % less than unpitted olives.

The addition of CaCl₂ significantly affected the acrylamide concentration of olives packed with brine in all three presentation formats. It led to an increase of around 17 % in olives and a decrease of around 7.2 % in the brine (Table 2). In contrast to that observed in samples without CaCl₂, in this experiment brine presented lower acrylamide levels than olives. For olives not stored in liquid, CaCl₂ did not produce any significant effects, with olives elaborated with and without this additive exhibiting similar results.

Two different doses of NaCl (2 % and 4 % w/v) were added to the samples prior to sterilization. In olives packed with brine, higher salt concentrations led to higher acrylamide concentrations (around 9 % higher when the concentration of NaCl was doubled) (Fig. 5). The opposite was found in brine, with an acrylamide content decreasing from 307 to 286 ng g⁻¹ when NaCl concentration increased. In olives not stored with brine, varying additions of salt did not influence acrylamide content, however, acrylamide content was generally much higher in these olives than in those stored with brine.

4. DISCUSSION

In the present research, different strategies to mitigate acrylamide levels during black ripe olive industrial processing were evaluated. The aim of this was to identify critical points in the processing of Californian style green ripe olives and respond to the lack of information about how to reduce acrylamide formation in this foodstuff. With this in mind, different assays were performed prior to and during the industrial production of olives. Recently, the present research group reported mitigation strategies in Californian-style green ripe olives (Martín-Vertedor et al., 2020). The promising results obtained in relation to green olives led us to investigate similar proposals in black ripe olives. Previous studies have described higher acrylamide concentrations in black olives relative to green olives making it necessary to control acrylamide formation in this food matrix.
Traditionally, black ripe olives are packed in cans or jars and covered in brine. However, some consumers opt for purchasing black olives not stored in liquid given that removal of the liquid once the container is opened can be a problem. Nowadays, for ease of use, table olive companies make products available which do not come covered in brine so that they are more attractive to consumers (Romero et al., 2019, 2021). The effect of packing black olives in cans without brine on acrylamide formation has not previously been studied and is a novel aspect of the present work.

It should be mentioned that, according to previous studies, black table olives do not present microbiological activity following oxidation and sterilization treatment (Pérez-Nevado et al., 2018; Fernández et al., 2020). For this reason, microbiological analyses were not performed.

4.1. Assay 1. Effect of different conditions prior to industrial processing: ripeness stage, storage time and washing

The acrylamide levels observed in black ‘Hojiblanca’ variety olives subjected to standard conditions (M.I. = 0 and traditional elaboration process) were similar to those reported by Charoenprasert and Mitchell (2014). Results corroborate previous evidence that the highest content occurs in black olives relative to Californian-style green ripe olives (Montaño et al., 2016; Martín-Vertedor et al., 2020). This may be explained by the fact that black olives are subjected to oxidation processes during treatment with lye which promote the formation of acrylamide precursors and, consequently, acrylamide formation during the sterilization process (Montaño et al., 2016). The alkalization of raw materials may also affect the formation of acrylamide during the production process (Taeymans et al., 2005; Kruszewski and Obiedzinski, 2020).

As previously reported in green olives (Martín-Vertedor et al., 2020), fruits with a more advanced stage of ripeness exhibited higher acrylamide levels, as did the brine with which they were stored. In this case, olives harvested with a green-yellow colour (M.I. = 1) contained around 26% more acrylamide than those harvested at the green stage of ripeness (M.I. = 0) (Fig. 3). This may be explained by the composition and appearance alterations that olives undergo during ripening. At first, table olives have a deep green tone, which becomes less green and more yellow during maturation. Throughout this process, the olive tree generates different sugars and other compounds due to photosynthesis. These compounds include acrylamide precursors such as reducing sugars and asparagine which promote higher acrylamide concentrations during the sterilization process (Casado and Montaño, 2008; Gonzalves et al., 2020; Martín-Vertedor et al., 2020; Pérez-Nevado et al., 2018). Moreover, olives at the green stage contain higher amounts of phenolic compounds which could inhibit acrylamide formation (Martín-Vertedor et al., 2020). It can therefore be concluded that the state of maturity of table olives is an important variable to consider in the evaluation of acrylamide generation in Californian-style black ripe olives. Thus, olives should be harvested as early as possible in order to reduce formation of this contaminant.

Storage time prior to industrial elaboration is another important aspect to control. Once olives are harvested, they are usually selected and placed in storage tanks with salt and/or acid for at least 3 or 4 months (Fernández et al., 2020). However, sometimes due to market reasons, olives are stored for a longer period. During this period, olives acquire a more brownish hue which facilitates subsequent oxidation processes. Results from the present study show that greater storage times lead to lower acrylamide concentrations. Specifically, acrylamide reduced by 45.2% when storage time increased from 4 to 21 months. Similarly, Charoenprasert and Mitchell (2014) found that acrylamide levels in olives in stored in brine rose during the first month but then decreased as storage time increased. However, these authors only evaluated storage times of up to 8 months, making the present study the first to examine longer storage times (up to 21 months). According to
Casado and Montaño (2008), a greater diffusion of soluble precursors (free sugars and/or amino acids) to the surrounding medium occurs during storage, in this way, inhibiting acrylamide formation.

Once olives had been stored for the required time, they were transported to the production plant for washing prior to the oxidation process. Three washing treatments were trialled (no washing, sprayed with water and washed with water for 45 min). Outcomes demonstrated that washing treatments prior to oxidation significantly reduced acrylamide formation (Fig. 4). Whilst spraying the olives mitigated contaminant generation to some extent (18 %), the most effective treatment was to wash the olives with water for 45 min, with this reducing acrylamide by 36 %. This latter washing process involved greater contact between the olives and the water, allowing greater diffusion of the precursors into the water. The effectiveness of washing olives to control acrylamide formation was also observed in green olives, with levels of the contaminant being reduced by 25 % and 45 % when olives were washed with water heated to 25°C for 45 min and 2 h, respectively (Martín-Vertedor et al., 2020). This mitigation strategy has also been studied by other authors. Casado and Montaño (2008) found acrylamide concentration to be 80 % lower in 'Hojiblanca' variety olives subjected to a washing process relative to olives not cleaned to the same extent. The greater reduction reported by these authors may be explained by the longer washing time (24 h) employed and the fact that washing was performed after NaOH treatment. This process reduces the hardness of the fruit whilst also increasing porosity of the olive cell walls. This facilitates the diffusion of acrylamide precursors from the olives to the brine.

4.2. Assay 2. Effect of different conditions during industrial processing: presentation formats, packaging and additives

In order to meet different consumer demands, different presentation formats of black ripe olives are currently available on the market. The most commercial formats are unpitted, pitted and sliced. According to these different formats, variations are found in the contact surface between fruits and covering liquid. These aspects impact the rate of diffusion of acrylamide precursors surrounding the olives. As expected, the greatest losses occur in sliced olives, leading to reductions in the acrylamide content. In the present study, acrylamide concentrations in sliced olives were 20 % and 34 % lower relative to pitted and unpitted olives, respectively. The greater content of acrylamide in unpitted olives may be explained by the compact format and smaller contact surface of olives within the liquid which slows the diffusion of precursors into the liquid medium. In a recently published study of Californian-style green ripe olives conducted by the present research group, unpitted olives had 21–26 % higher acrylamide levels than pitted olives and 42–50 % higher levels than sliced olives (‘Manzanilla de Sevilla’, ‘Hojiblanca’ and ‘Manzanilla Cacereña’) (Martín-Vertedor et al., 2020). Similarly, Casado and Montaño (2008) found that oxidized sliced black olives had a significantly lower acrylamide concentration than pitted and whole ripe olives.

The addition of CaCl₂ to maintain olive firmness led to an increase in acrylamide concentration in olives packed in brine and a decrease in the concentration of the surrounding brine. This may be due to the effect of calcium ions. Calcium promotes the formation of cross-links between pectin molecules, strengthening plant cells and blocking the passage of acrylamide between olives and the brine despite the fact that this molecule is highly hydrophilic. In fact, the presence of calcium ions in the saline solution results in higher levels of acrylamide in the final foodstuff (Charoenprasert and Mitchell, 2014). Similar results were also found in previous studies conducted with black (Pérez-Nevado et al., 2018) and green ripe olives (Martín-Vertedor et al., 2020). Restraint with regards to applying CaCl₂ to olives should be considered by the industry in order to reduce acrylamide content given that this would modify the product’s sensory qualities and, consequently, gain consumers’ approval.
With regards to the addition of NaCl, Californian-style black ripe olives are sterilized in the final stage of production and so do not need large quantities of salt to be preserved. For this reason, this type of olive is usually low in salt, leading industries to add NaCl in order to enhance flavour. In order to assess the influence of added salt on the presence of acrylamide in olives, samples were treated with 2% (w/v) and 4% (w/v) NaCl. Outcomes showed that higher salt concentrations were linked with higher acrylamide contents in all presentation formats. As is the case with calcium, the application of salt also strengthens olive cellular membranes (Fadda et al., 2014), inhibiting the diffusion of precursors and increasing the acrylamide concentration. Similar outcomes have also been exhibited in Californian-style green ripe olives (Martín-Vertedor et al., 2020).

The effect of this additive on acrylamide concentration in olives not stored in liquid was the opposite of that observed in olives stored in brine. In general, when olives were not canned with brine, acrylamide content was around three times higher than in olives canned with brine. Outcomes were again independent of presentation format. No literature exists with which these outcomes can be compared. However, it could be suggested that the absence of brine in these samples prevents the migration of acrylamide precursors and the subsequent formation of acrylamide in the surrounding liquid. This leads to greater acrylamide levels in olives stored in this format. Due to the fact that no diffusion could take place in this format, the addition of additives to these olives did not affect acrylamide levels, with neither CaCl$_2$ nor NaCl producing any effects. Similar outcomes have been reported in Californian-style green ripe olives (non-oxidised olives) (Martín-Vertedor et al., 2020).

5. CONCLUSIONS

Californian-style black ripe olive manufacturing includes the sterilization of samples. This promotes acrylamide formation from the precursors found in the fruit. The shiny black colour of black ripe olives is highly attractive to consumers. This leads to a demand for this product which justifies the need to identify acrylamide mitigation strategies capable of reducing acrylamide exposure in the population. The present study suggests that fruits should be harvested as early as possible and stored in brine for at least 4 months. This will ensure that olives contain a lower number of precursors and promote their diffusion to the surrounding medium. Diffusion can also be increased by washing olives with water. A greater contact surface between packed fruit also promotes movement of acrylamide precursors from olives to the brine. As a result, sliced olives have lower acrylamide levels than pitted and unpitted olives. In contrast, the addition of CaCl$_2$ and resultant increase in NaCl concentration in the final brine strengthen olive cell membranes, resulting in a higher concentration of acrylamide in olives treated with these additives. Further, although olives canned without brine offer a novel and attractive format for consumers, the absence of liquid prevents the diffusion of both precursors and formed acrylamide into the liquid medium, leading to greater amounts of the contaminant. In conclusion, these findings will enable mitigation strategies to be designed for use by Californian-style black ripe olive producers to reduce acrylamide formation in this foodstuff. The results are also valuable for enabling regulatory bodies to conduct an accurate risk evaluation of dietary exposure to acrylamide through consumption of black ripe olives.

Author statement

Specifically the authors contributed to the following tasks:

- Conception and design the experiment: Daniel Martín-Vertedor, Antonio Fernández, Elisabet Martín-Tornero.
- Acquisition of data: Marta Mesías, Manuel Martínez, Daniel Martín-Vertedor, Antonio Fernández.
- Analysis and/or interpretation of data: All authors have contributed to this issue (Daniel Martín-Vertedor, Antonio Fernández, Marta Mesías, Manuel Martínez, Elisabet Martín-Tornero).
- Drafting the revised manuscript: Daniel Martín-Vertedor, Elisabet Martín-Tornero, Antonio Fernández.
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Author contributions
Formal analysis: D. Martín-Vertedor and A. Fernández; research: D. Martín-Vertedor; supervision: D. Martín-Vertedor; writing-original draft: D. Martín-Vertedor, A. Fernández and E. Martín-Tornero; writing-review and editing: D. Martín-Vertedor, A. Fernández, M. Mesías, M. Martínez, and E. Martín-Tornero; Funding acquisition: D. Martín-Vertedor, and M. Martínez. All authors read and approved the final manuscript.

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Declaration of Competing Interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References


Table 1. Studies evaluating acrylamide mitigation strategies during the processing of table olives. Ranges indicate minimum and maximum values reported in the referenced studies. Acrylamide concentration is expressed in ng g⁻¹.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Varieties</th>
<th>Mitigation Strategy</th>
<th>Acrylamide Concentration Range</th>
<th>Acrylamide analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Camilo and Montaño (2006)</td>
<td>‘Hojiblanca’</td>
<td>Lye treatment and water washings</td>
<td>237-1393 ng g⁻¹</td>
<td>OC-MS with ionization</td>
</tr>
<tr>
<td>Camilo et al. (2015)</td>
<td>‘Hojiblanca’</td>
<td>Additive inoculation</td>
<td>439-699 ng g⁻¹</td>
<td>OC-MS</td>
</tr>
<tr>
<td>Gaulco and Madinot (2017)</td>
<td>‘Manzanilla’</td>
<td>Composicion a: calcium chloride (0.2–0.4 %), sodium benzoate (0.2–0.3 %) and acidified to a pH of 3.7–4.0 with acetic acid; composicion b: olives stored in brine solutions without added acidic acid; composicion c: olives stored in brine solutions without sodium benzoate; composicion d: olives stored in brine solutions without calcium chloride. (nº): number of treatments performed in the mitigation strategies.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Composition a: calcium chloride (0.2–0.4 %), sodium benzoate (0.2–0.3 %) and acidified to a pH of 3.7–4.0 with acetic acid; composition b: olives stored in brine solutions without added acidic acid; composition c: olives stored in brine solutions without sodium benzoate; composition d: olives stored in brine solutions without added calcium chloride. (nº): number of treatments performed in the mitigation strategies.
Table 2. Acrylamide content (ng g⁻¹) in ‘Hojiblanca’ Californian-style black ripe olives with different presentation formats (Stone, Pitted and Sliced) subjected to CaCl₂ addition or not into the brine solution. Results are expressed as mean ± standard deviation of five sample replicates. Different capital letters indicate significant differences between formats of olives presentation. Different small letters indicate significant differences between the CaCl₂ addition or not (Tukey’s Test, p < 0.05).

<table>
<thead>
<tr>
<th>Samples</th>
<th>Acrylamide (ng g⁻¹)</th>
<th>Acrylamide (ng g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non CaCl₂</td>
<td>CaCl₂</td>
</tr>
<tr>
<td>Olives</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stone</td>
<td>286.0 ± 8.6 a C</td>
<td>347.3 ± 6.3 b C</td>
</tr>
<tr>
<td>Pitted</td>
<td>234.8 ± 9.6 a B</td>
<td>275.0 ± 7.1 b B</td>
</tr>
<tr>
<td>Sliced</td>
<td>188.0 ± 6.9 a A</td>
<td>233.5 ± 9.6 b A</td>
</tr>
<tr>
<td>Brine solution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stone</td>
<td>310.4 ± 7.3 b C</td>
<td>285.3 ± 8.9 a C</td>
</tr>
<tr>
<td>Pitted</td>
<td>267.3 ± 10.9 b B</td>
<td>245.0 ± 7.5 a B</td>
</tr>
<tr>
<td>Sliced</td>
<td>194.4 ± 8.0 b A</td>
<td>180.5 ± 8.2 a A</td>
</tr>
<tr>
<td>Non-liquid olives</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stone</td>
<td>822.1 ± 20.1 C</td>
<td>842.1 ± 20.2 C</td>
</tr>
<tr>
<td>Pitted</td>
<td>661.1 ± 22.2 B</td>
<td>675.9 ± 21.5 B</td>
</tr>
<tr>
<td>Sliced</td>
<td>445.1 ± 21.5 A</td>
<td>450.6 ± 23.3 A</td>
</tr>
</tbody>
</table>
Fig. 1. Experimental design of the present study.
**Fig. 2.** Extracted ion chromatogram (EIC) for the transition (72→55) of an olive sample unspiked and spiked with 50, 100 and 150 ng mL⁻¹ of acrylamide standard solution.
**Fig. 3.** Acrylamide content (ng g⁻¹) in ‘Hojiblanca’ olives harvested at two different stages of ripeness and stored for different periods. Olives and brine solutions were subjected to a conventional elaboration process to obtain Californian-style black olives. Results are expressed as mean ± standard deviation of five sample replicates. Different small letters indicate significant statistical differences according to the stage of maturation (Tukey’s Test, p < 0.05). Different capital letters indicate significant statistical differences according to the storage time (Tukey’s Test, p < 0.05).
Fig 4. Acrylamide content (ng g⁻¹) in olives and brine subjected to different washing processes. Results are expressed as mean ± standard deviation of five sample replicates. Different small letters indicate significant statistical differences between washing process (Tukey's Test, p < 0.05). Different capital letters indicate significant statistical differences between olives and brine in each washing treatment (Tukey's Test, p < 0.05).
Fig 5. Acrylamide content (ng g⁻¹) in olives with and without brine subjected to different concentrations of NaCl in brine. Results are expressed as mean ± standard deviation of the seven sample replicates. Different capital letters indicate significant differences between the presentation formats for a same NaCl treatment. Different small letters indicate significant differences between the NaCl treatments for a same sample (Tukey’s Test, p < 0.05).