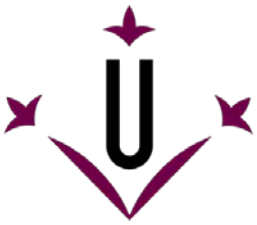

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Stability of alternariol and alternariol monomethyl ether during food processing of tomato products

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Abstract

The stability of two *Alternaria* mycotoxins, alternariol (AOH) and alternariol monomethyl ether (AME), has been investigated during the food processing of tomato products simulating commercial processing conditions. The production stages assessed were the storage of raw fruits, fruit washing, and thermal processing. It was observed that time of storage significantly reduced the initial concentration of AOH, but only if tomatoes were stored at 35 ºC. For AME, 12 h were sufficient to reduce the initial concentration, regardless of the temperature at which samples were stored (25, 30 and 35 ºC). The washing step achieved the highest reduction of AOH and AME. This reduction was even more efficient when using sodium hypochlorite solutions. Finally, during the

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heat treatment (80-110 ºC), results showed that heating tomato samples at 100 and 110 ºC, significantly affected AOH stability, though AME seemed to not be affected by these thermal processes.

Keywords: *Alternaria*; AOH; AME; Stability; Tomatoes

1 Introduction

Mycotoxins biosynthesized by *Alternaria* spp. are not regulated by any European Commission's legislation (EC, 2010). Two of the most frequent *Alternaria* mycotoxins found in food and feed commodities are alternariol (AOH) and alternariol monomethyl ether (AME) (Barkai-Golan, 2008; Logrieco, Moretti, & Solfrizzo, 2009; Ostry, 2008). Despite limited literature regarding the toxicity of *Alternaria* mycotoxins, it has been described that they are harmful for human and animals (Brugger, Wagner, Schumacher, Koch, Podlech, Metzler, et al., 2006; Pfeiffer, Schebb, Podlech, & Metzler, 2007; Pollock, Disabatino, Heimsch, & Hilblink, 1982).

Fruits may be contaminated by *Alternaria* spp. in the field and, once in the food industry, during the storage. When contamination has occurred, *Alternaria* spp. may begin the biosynthesis of mycotoxins. The spoiled products may pass through the culling step accidentally and enter into the food production chain. When this happens, fungi can still be destroyed during the heat treatment but it is uncertain what happens with mycotoxins themselves. In fact, there is few information on the stability and fate of *Alternaria* mycotoxins throughout the food processing operations and storage but there are studies that reveal *Alternaria* mycotoxins could remain quite stable, which consequently, may result in high levels of *Alternaria* mycotoxins in the finished products (Combina, Dalcero, Varsavsky, Torres, Etcheverry, Rodriguez, et al., 1999; Ozcelik, Ozcelik, & Beuchat, 1990; Scott & Kanhere, 2001; Siegel, Feist, Proske, Koch, & Nehls, 2010). Thus, the aim

of this work was to provide information about the stability of AOH and AME through the food processing of tomato products, since tomatoes are very susceptible to fungal decay, and *Alternaria* is the most common fungus on moldy tomatoes (Andersen & Frisvad, 2004). This study may help to identify which steps require more attention when aiming to decrease the initial concentration of toxin contaminants.

2 Material and methods

2.1 Chemicals

Standards of AOH (~94 %) and AME (~98 %) were supplied by Sigma–Aldrich (St. Louis, MO, USA). A stock solution was prepared for each standard by dissolving 5 mg of the purified mycotoxins in ethanol reaching a final concentration of 1000 µg/mL. From the stock standard solutions, working standard solutions at a concentration of 15 µg/mL were prepared. All standards were stored at -20 ºC in a sealed vial until use.

Acetonitrile (99.8 %) and methanol (99.9 %) were both HPLC (high-performance liquid chromatography) grade (Acros Organics, Morris Plains, NJ, USA). Pure water was obtained from a milli-Q apparatus (Millipore, Billerica, MA, USA).

2.2 Tomato sample preparation

Cherry tomatoes purchased from the supermarket were used as lower volume of mycotoxins was needed for fruit contamination. All tomato samples were surface disinfected with 70 % ethanol. For heat treatment tests, cherry tomatoes were blended (Turbo Habana, Palson, Spain) until getting a homogeneous tomato matrix and a tomato juice was prepared.

2.2.1 Spiking of samples

All tomatoes were spiked with 100 µL of an AOH and AME solution containing a known concentration of both mycotoxins (0.5 µg/g of tomato) dissolved in ethanol. Ethanol was

dried under a laminar flow hood at room temperature. All tomatoes were weighted individually, and all these data were used for the final result analysis. For heat treatments tests, 20 g of tomato juice were dispensed in glass tubes and then spiked with 100 µL of ethanol containing 0.5 µg of both AOH and AME per gram of tomato. Glass tubes were covered to avoid evaporation. AOH and AME extraction is described in section 2.4.

2.3 Food production chain analyzed

To study the stability of AOH and AME along the food processing of derived tomato products, those steps susceptible of causing any alteration or instability to both *Alternaria* mycotoxins were simulated on the laboratory (Fig. 1). All the experiments were performed in triplicate. A negative control test without spiked tomatoes was prepared to ensure no AOH or AME contamination on the raw fruit used.

2.3.1 Storage

Spiked cherry tomatoes were stored for different periods of time (12 h, 24 h, 2 days and 1 week) into different incubation chambers at various temperatures (25, 30 and 35 ºC) without any external light. Unstored spiked tomato samples were considered controls.

2.3.2 Washing

Five spiked cherry tomatoes were washed with 1 L of tap water or 1 L of a chlorinated water solution (150 or 250 mg/L of sodium hypochlorite, NaOCl). To prepare the chlorine water solution, a commercial sodium hypochlorite containing 8.25 % of NaOCl was used. Bleach volumes needed to prepare the desired concentration were dissolved in municipal water. To simulate the flow often used in the food industry, samples were stirred using a low homogenous magnetic field. Samples were collected from the beaker after 1, 2, 5 and 10 min. Fruit not dipped into water were considered controls.

2.3.3 Heat treatment

98 Glass tubes containing the spiked tomato juice samples were weighed before the assay
99 and then placed into an oil bath. Temperatures tested were 80, 90, 100 and 110 ºC.
100 Samples were taken from the oil bath after 30, 60 and 90 min and then they were stored
101 into the fridge (4 ºC). Before AOH and AME extraction, samples were weighed again and
102 milliQ water was added to compensate for evaporative losses. Unheated spiked tomato
103 juice samples were considered controls.

104 **2.4 AOH and AME extraction**

105 Mycotoxin extraction was developed as detailed in Estiarte et al. (2016). Separation,
106 detection and quantification of AOH and AME were performed on a HPLC system model
107 2510 HPLC pump (Varian, Inc., Palo Alto, CA) connected to one in-line Spectroflow
108 757 UV/Vis absorbance detector (Applied Biosystems, Foster City, CA). A reverse
109 phase Kinetex PFP column (5 µm, 4.6 × 150 mm, Phenomenex, Torrance, CA, USA)
110 preceded by a KrudKatcher classic HPLC in-line filter (0.5 µm depth filter, Phenomenex,
111 CA, USA) were used. Chromatographic and method performance characteristics for AOH
112 and AME detection and quantification are detailed in Estiarte et al. (2016).

113 **2.5 Statistical analysis**

114 All data were firstly analyzed using the multifactor ANOVA. When there was statistical
115 significance of any of the interactions assessed (p-value > 0.05), a One Way ANOVA test
116 was carried out. The Tukey-HSD test (Honest Significant Difference) was used to
117 compare means.

118

119 **3 Results and discussion**

120 **3.1 Stability of AOH and AME during the storage**

121 The harvest season of tomatoes usually extends from spring to summer and thus, it comes
122 with warm weather conditions. The effect of storing tomatoes for different periods of time

123 and at different temperatures has been analyzed on the stability of AOH and AME. It was
124 found that the initial concentration of AME was significantly reduced after 12 h of
125 storage, while remained constant later (Fig. 2). For AOH, storage at 35 ºC was necessary
126 to achieve a significant reduction of its initial concentration. Statistical analysis showed
127 that the temperature at which tomatoes were stored did not have any significant effect.
128 Nevertheless, as illustrated on Fig. 2, although the initial concentration of toxins
129 decreased when tomatoes were stored for any period of time, neither of the *Alternaria*
130 mycotoxins completely disappeared in this step, with any of the tested conditions. Here,
131 it was observed that after one week at 35 ºC, tomatoes were spoiled and were unacceptable
132 for human consumption. However, after 12 or 24 h they were still acceptable.
133 Results presented here support the findings of Ozcelick et al. (1990), who observed that
134 when tomatoes were stored at 25 ºC, AOH and AME decreased as storage time
135 progressed, though this decrease did not appear to be related to the temperature of storage.
136 It is important to notice that, in their assay, after 5 weeks of storage at 25 ºC, AOH and
137 AME were both present in tomato tissue. In another study, Dalcero et al. (1997) aimed to
138 evaluate the presence of *Alternaria* spp. and their mycotoxins in ensiled sunflower seeds.
139 Results from this study showed that the presence of *Alternaria* spp. and the levels of AOH
140 and TeA decreased as the time of ensiling increased. The ensiling process comprises
141 several variables that may have an effect on the stability of *Alternaria* mycotoxins, such
142 as changes in the pH or modification of the dry matter. Considering that AOH and AME
143 seem to not disappear during the storage, measures applied in this step that aim to
144 decrease the levels of toxins probably should be addressed to inhibit fungal mycotoxin
145 biosynthesis instead of altering the chemical structure of the mycotoxins produced. With
146 this purpose, it would be useful to find those conditions that are not favorable for fungal
147 development and mycotoxin production.

3.2 Stability of AOH and AME during the washing

An essential step of the food production chain in most of the food industries is the washing of raw fruits and vegetables that allows discarding contaminants coming from the field (soil, stones, insects or leaves) and, additionally, it may also help to reduce the microbial population present on the fruit surface. Tomatoes are very susceptible to *A. alternata* decay and the fungus just requires an injured or weakened tissue for penetrating. Hence, this step may be useful to remove mycotoxins from the more extern part of the fruit. To test the effectiveness of the washing step on removing AOH and AME, raw tomatoes, artificially spiked with both mycotoxins, were washed with water and two sodium hypochlorite solutions with different concentrations (150 ppm or 250 ppm). Results showed that the two factors assessed, both duration of the washing step and washing solution, and their interaction, were statistically significant in relation to the stability of AOH and AME (p-value < 0.05) (Fig. 3). According to multiple comparison tests (Tukey-HSD), for AOH, there were no significant differences between washing tomatoes with 150 or 250 ppm. However, washing tomatoes with water was significantly less efficient than washing them with a hypochlorite solution, at least if the washing was short (1 or 2 min). A One Way ANOVA test was carried out for each one of the washing solutions, and a Tukey-HSD test was used to compare means among different times of washing (Fig. 3). Dealing with tomatoes washed with water, it was shown that for AOH, a water bath of 1 minute was sufficient to significantly reduce the initial concentration of AOH. After 10 min of washing, the remaining percentage of AOH on tomatoes was 11.00 % (Fig. 3A). In contrast, AME was found to be more persistent on tomatoes washed with water, since 5 min were required to significantly reduce its initial concentration. After 10 min of washing, the initial concentration of AME decreased to 38.00 % (Fig. 3B). These findings may be linked to the solubility of the two mycotoxins. There is no experimental

data regarding the solubility in water of any of these compounds. However, there are software tools that predict the solubility for a given molecule. The Toxin and Toxin Target Database (T3DB - www.t3db.ca) is a resource that was specifically designed to capture information about the toxicity of all human environmental exposures from conception to death (Lim, Pon, Djoumbou, Knox, Shrivastava, Guo, et al., 2010; Wishart, Arndt, Pon, Sajed, Guo, Djoumbou, et al., 2015). From this resource, it was found the predicted solubility of AOH and AME in water (0.228 and 0.091 mg/mL, respectively). This indicates that AOH is more soluble in water than AME, and explains why AME is more persistent on the tomato surface. When fruit was washed with a sodium hypochlorite solution, both for AOH and AME, it was observed that 1 min was sufficient to significantly reduce the initial content of AOH about 87.52 and 88.14 %, respectively, and 66.66 and 54.78 % for AME, respectively. However, when using only water, the decrease was 56.75 % for AOH and only 20.84 % for AME. After 1 minute of washing, no significant reductions were observed for any other washing treatment. The higher reduction obtained with the sodium hypochlorite solutions may be explained by the pH of the chlorinated water, which is one of the parameters that may affect the properties of chlorine solutions. It was found that for the 250 ppm sodium hypochlorite solution the pH was 9.50, while for the 150 ppm one it was 9.09. For water, the pH was 7.85. There is scarce information regarding the stability of AOH and AME at different pH levels. Siegel et al. (2010) studied the chemical stability of AOH and AME by refluxing mycotoxins in aqueous solutions with different pH values. The two compounds were stable in an aqueous phosphate/citrate buffer (0.15 M, pH 5), but were completely degraded in a 0.18 M phosphate/citrate buffer at pH 7 and in a 0.1 M KOH solution (pH 13). The mechanism of degradation was suggested to involve the hydrolysis of the lactone group followed by decarboxylation, both steps favored by an elevated pH. Hence,

198 mycotoxins seem to be more stable at lower pH. As the pH of the solution increased, the
199 lower the AOH and AME stability may be.

200 **3.3 Stability of AOH and AME during the heat treatment**

201 Thermal processing is essential in many food production processes. However, most
202 mycotoxins are heat-resistant within the range of conventional food-processing
203 temperatures (80–121 °C), so little or no reduction in overall toxin levels occur as a result
204 of normal cooking conditions (Kabak, 2009). The aim of this work was to establish how
205 heat treatments affect AOH and AME. For this purpose, tomato juice samples were heated
206 at 80, 90, 100 and 110 ºC for different periods of time (30, 60 and 90 min). For AOH
207 (Fig. 4A), results showed that both factors tested (time and temperature of heating) and
208 their interaction were statistically significant in relation to its stability (p-value < 0.05).
209 No significant reduction in AOH was observed either at 80 ºC or 90 ºC, while significant
210 reductions occurred at 100 and 110 ºC, with little differences between them. Indeed, Scott
211 and Kanhere (2001) had previously studied the stability of AOH and AME in fruit juices,
212 and observed that there were no losses of *Alternaria* mycotoxins when fruit juices were
213 heated at 80 ºC during 20 min. In this study, it has been observed that at 100 ºC and 110
214 ºC, longer time treatments (over 30 min), did not lead to higher degradation levels. After
215 90 min of heating, the remaining AOH was 67.00 % for treatment at 100 ºC, and 56.00
216 % for treatment at 110 ºC. Heat treatment processes of up to 90 min, were shown to be
217 incapable of completely destroying AOH. Results for AME (Fig. 4B) were quite different,
218 as no significant differences were found with the temperature or the duration of the
219 treatment. From this result, it suggests that AME is stable when exposed to the heat
220 treatments used in this study. Only few studies have assessed the thermal stability of AOH
221 and AME. Siegel et al. (2010) designed a series of quantitative model experiments using
222 spiked wheat flour, and observed that *Alternaria* mycotoxins were minimally degraded

during wet baking. This study found that AOH and ALT were degraded slightly after 1 h at 230 °C, but AME was stable at all times and temperatures tested with wet baking. However, significant degradation occurred upon dry baking for all mycotoxins, though AME was observed to be the most stable, which is in accordance to results presented here. Combina et al. (1999) also evaluated the effect of heat treatment on the stability of AOH, AME and TeA in sunflower flour. They reported that concentrations of AOH and AME remained constant when heating samples at 100 ºC (humid heat) for up to 90 min, while TeA concentration decreased with time to 50 % after 90 min. In their study, the most effective treatment for reducing AOH and AME levels was heating samples at 121 ºC for 60 min combined with a pressure of 0.1 MPa.

Kabak (2009) described that there are several factors that may play a significant role in the stability of mycotoxins, such as the initial level of contamination, the type and concentration of the mycotoxin, the heating temperature together with the time employed, the degree of heat penetration, as well as the moisture content, pH and ionic strength of food, among other factors. The differences observed among the few studies available may be explained by some of the above mentioned factors.

The future research of *Alternaria* mycotoxin stability should be a deeper analysis on the novel degradation compounds formed product of their own degradation and their toxicity. In this sense, Siegel et al. (2010) found that two chemical compounds were formed, 6-methylbiphenyl-2,3′,4,5′-tetrol and 5′-methoxy-6-methylbiphenyl-2,3′,4-triol, from the degradation of AOH and AME under wet baking conditions, respectively. Nevertheless, the toxicological properties of the products of AOH and AME degradation are yet unknown, as they have been recently described. Thus, further studies on the toxic effects of the potential breakdown products of mycotoxins are necessary.

4 Conclusion

In this study, it has been demonstrated that AOH and AME are quite stable along the food processing chain. During the storage, neither AOH nor AME were completely destroyed. For AOH, 35 ºC were necessary to achieve a significant reduction of the initial concentration. Regarding AME stability, statistical analyses have shown that the temperature at which tomatoes are stored does not have any significant effect on its stability. Results showed that there were significant differences between the controls and the rest of treatments although, prolonging the period of storage did not have a major effect on its stability. Dealing with the heat treatment, temperatures of 100 or 110 ºC significantly affect the stability of AOH. Notwithstanding, AME appears to be stable when exposed to the different heat treatments. The greatest reduction of AOH and AME occurs at the washing step. Thus, to have a good control of *Alternaria* mycotoxins, it would be recommendable to reinforce this step in the food industry.

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Figure captions

Figure 1: Production flow chart of derived tomato products. Bold letters specify the assessed steps for AOH and AME stability.

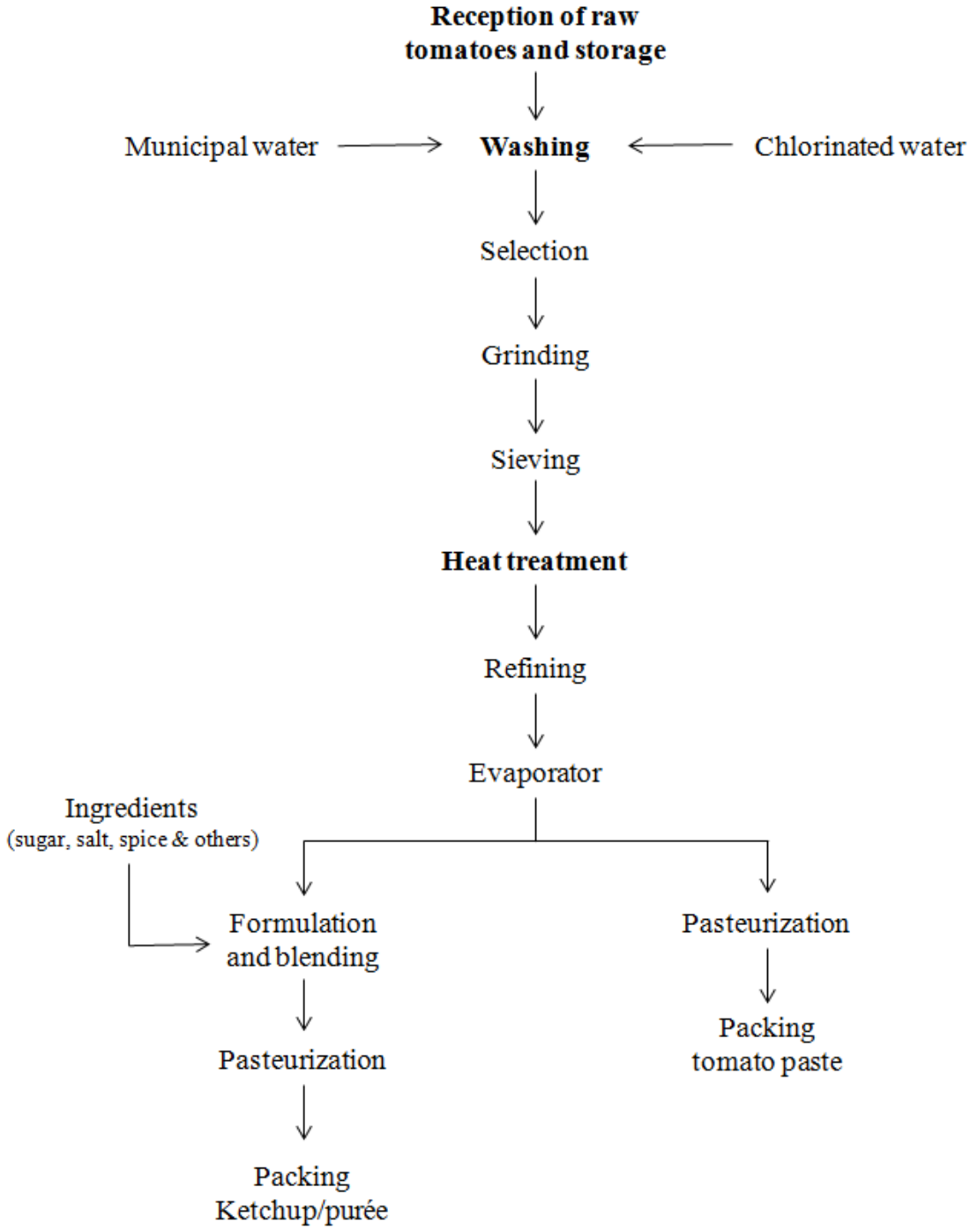

Figure 2: Stability of *Alternaria* mycotoxins during the storage of raw tomatoes. A. Remaining percentage of AOH. B. Remaining percentage of AME. Error bars indicate standard errors. All statistical data was analyzed by one-way ANOVA (p-value < 0.05). Tukey-HSD test was used to compare means between different stored temperatures tested. Capital letters indicate homogeneous groups.

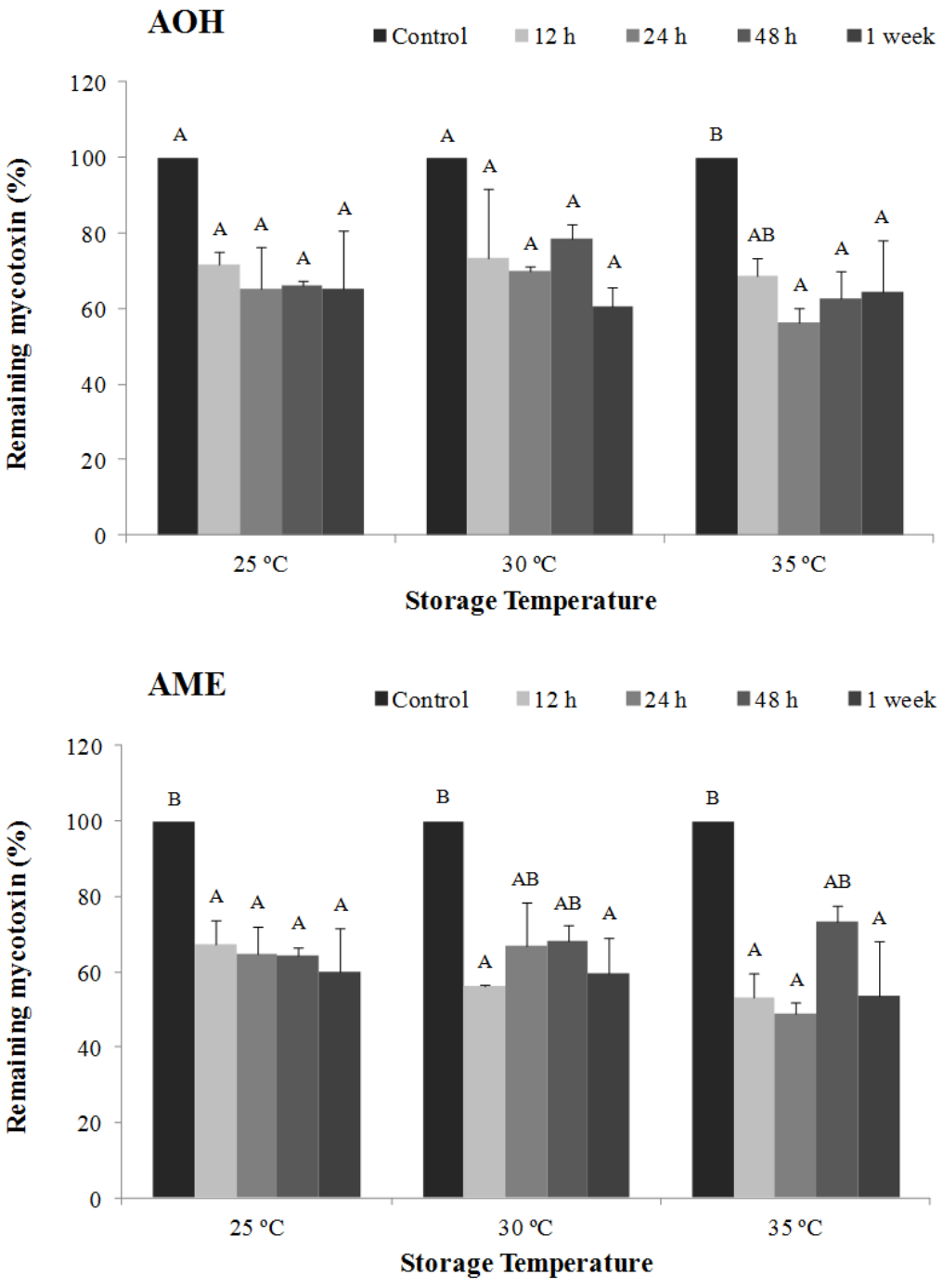

Figure 3: Stability of *Alternaria* mycotoxins during the washing of raw tomatoes. A. Remaining percentage of AOH. B. Remaining percentage of AME. Error bars indicate standard errors. All statistical data was analyzed by one-way ANOVA (p-value < 0.05). Tukey-HSD test was used to compare means between different types of washing, using just water or a concentrated sodium hypochlorite water solution (150 ppm or 250 ppm). Capital letters indicate homogeneous groups.

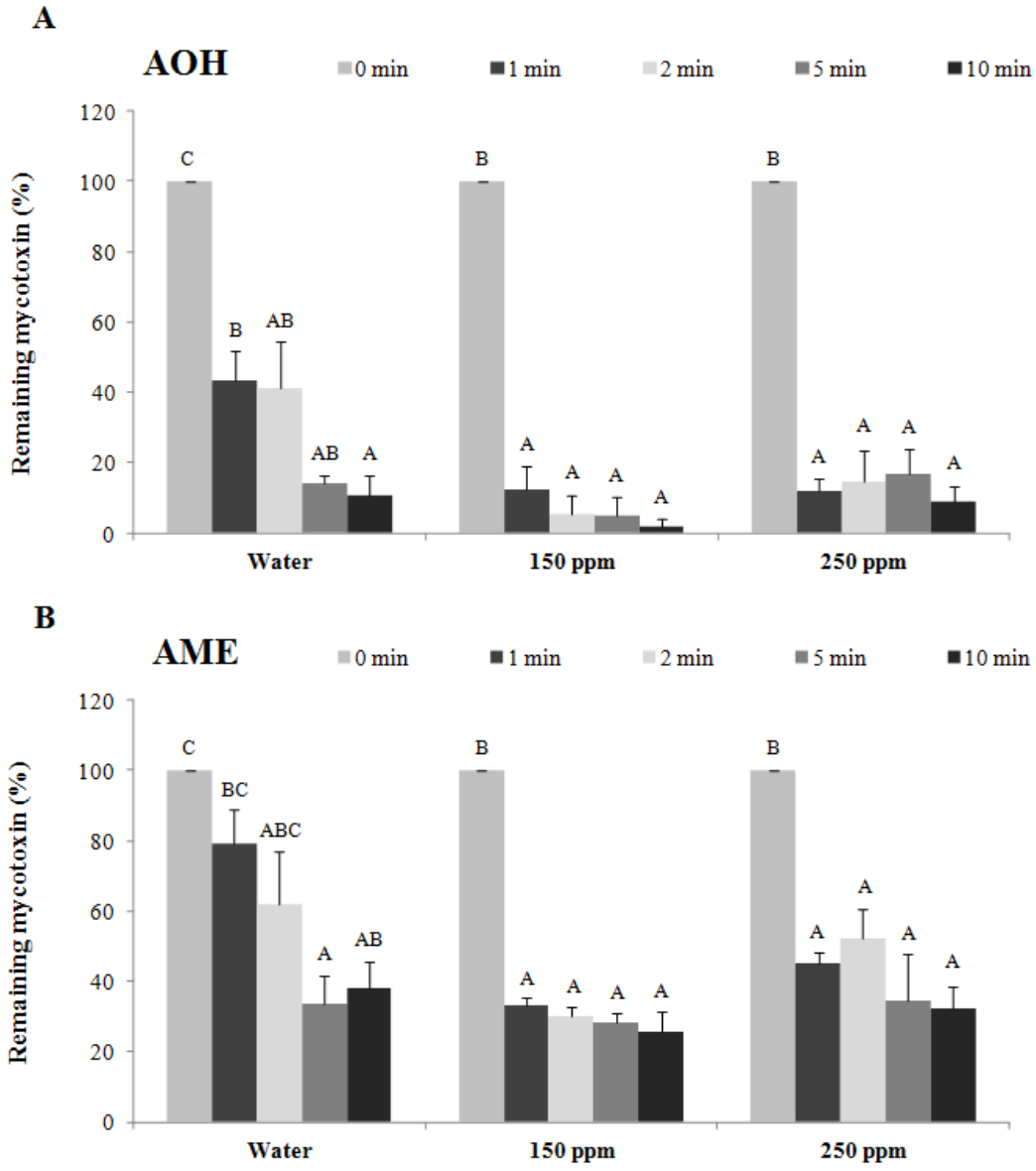

Figure 4: Stability of *Alternaria* mycotoxins during the heat treatment of tomato juice samples. A. Remaining percentage of AOH. B. Remaining percentage of AME. Error bars indicate standard errors. All statistical data was analyzed by one-way ANOVA (p-value < 0.05). Tukey-HSD test was used to compare means of the different temperatures used to assess the heat treatment effect on AOH and AME stability. Capital letters indicate homogeneous groups.