1 **EFFECTS OF SELECTED AMINO ACIDS AND WATER-SOLUBLE**

2 **VITAMINS ON ACRYLAMIDE FORMATION IN A RIPE OLIVE MODEL**

3 **SYSTEM**

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1

13   **Abstract**

14   A ripe olive model system was used to evaluate the potential inhibiting effects on

15   acrylamide formation from a set of amino acids and water-soluble vitamins. The system

16   was based on ripe olive juice heated at 121 ºC for 30 min in a stainless steel tubular

17   reactor. The most potent acrylamide inhibitors were proline and sarcosine, both with

18   inhibition rates of ~75% at a 100 mM level. In addition, glycine, ornithine, taurine, and

19   γ-amino butyric acid were effective (50-65% inhibition) while the rest of the

20   compounds demonstrated weak or non-significant effects. Acrylamide contents in the

21   model system were found to be highly correlated with the corresponding contents in the

22   real product. The kinetic pattern for the formation of acrylamide in the absence and

23   presence of two selected amino acids, added separately or together, was well fitted

24   using a simple logistic function.

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26   **Keywords:** Acrylamide, Amino acids, Food model system, Kinetics, Mitigation,

27   Vitamins

28

1. Introduction

Acrylamide (AA) has been a focus of attention by the scientific community in recent years. This compound has been classified by the International Agency for Research on Cancer (IARC) as a probable human carcinogen (IARC, 1994). In early 2002, high AA levels were reported in potato products and cereals, such as fried potatoes, baked potatoes, bread, breakfast cereals, and biscuits (Tareke et al., 2002). Subsequent studies confirmed this finding (FDA, 2006) and also reported high AA levels in other products such as ripe olives (Casado and Montaño, 2008). Previous surveys also showed that AA was not detected in the olives of other processing types (e.g. Spanish-style green olives, directly brined olives, etc.), or in other low acid canned vegetables such as asparagus, green beans, and spinach (FDA, 2006).

Ripe olives (also called "Californian-style table olives") are one of the most important classes of table olive commercialized in the world. In this type of processing the olives are treated with a series of dilute NaOH solutions (lye) to remove their natural bitterness, which is caused by glucoside oleuropein. Between lye treatments, the fruits are aerated. During this operation, the fruits are progressively darkened due to polyphenol oxidation. After the lye treatments and oxidation, the olives are washed several times with water to remove most of the residual lye, reaching a final pH of around 7, and placed in 3-5% brine with ferrous gluconate or ferrous lactate to maintain their color (Sánchez et al., 2006). Finally, the olives are canned in mild salt brine, and heat sterilized (generally at 121-126 ºC).

The use of additives for the potential inhibition of AA formation is a strategy that has been investigated in different foods, including ripe olives. Of the additives tested in this product, only sodium bisulfite was able to totally eliminate AA with a

54   negligible repercussion on sensory quality (Casado et al., 2010). However, sodium

55   bisulfite is currently not permitted as an additive in table olives in accordance with

56   European regulation (Commission Regulation (EU) No 1129/2011). Therefore, studies

57   to find other additives to be used by the table olive industry with significant AA-

58   reducing effects and without any negative effects are necessary. Amino acids or

59   vitamins are especially attractive because their addition to food may also improve its

60   nutritional value. However, the results previously found with a few amino acids in ripe

61   olives were generally not satisfactory (Casado et al., 2010). Cysteine at 50 mM was

62   demonstrated to be a strong inhibitor of AA formation in ripe olives, but did generate

63   unpleasant off-flavors. In contrast, arginine and methionine at 50 mM had no negative

64   impact on the sensory quality of ripe olives, but their AA-reducing effects were little or

65   negligible. Protein amino acids such as tryptophan, proline, and histidine have

66   demonstrated significant AA-reducing effects in asparagine-glucose model systems

67   (Koutsidis et al., 2009). It has been suggested that these amino acids could form amino

68   acid-AA adducts through Michael addition type reactions thereby reducing the AA

69   content (Friedman and Levin, 2008; Koutsidis et al., 2009). Taurine, a non-protein

70   amino acid, has been reported to be an inhibitor of AA formation in aqueous and potato

71   chip model systems (Shin et al., 2010). Several water-soluble vitamins were reported to

72   significantly inhibit the formation of AA in model systems and in fried potatoes (Zeng

73   et al., 2009; Yuan et al., 2011). Although the action mechanism has not been

74   characterized, the presence of nucleophilic groups, in particular, an amino group in

75   some vitamins, might contribute to their inhibitory activity against AA formation.

76   The reduction of AA levels by using additives may significantly affect the

77   kinetic behavior of acrylamide formation. The kinetics of AA formation have been

78   previously studied in model systems of asparagine and glucose (Claeys et al., 2005;

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79   Knol et al., 2005; Zhang and Zhang, 2008), potato chips (Granda and Moreira, 2005),

80   potato crisps (Knol et al., 2009), and potato powder (Franke et al., 2009). Empirical

81   models have been proposed for modeling the formation of AA in foods and model

82   systems (Corradini and Peleg, 2006), In fact, the "Logistic-Fermi" and "Logistic-

83   Exponential" models have been used to fit the formation of AA in fried potato crisps

84   (Knol et al., 2009) and in an asparagine-glucose model system (Zhang and Zhang,

85   2008). Using these models that only give a mathematical description of the formation or

86   degradation of AA in food bypasses the problem of considering all the mechanisms that

87   occur during the processing of foods. Besides, AA precursors in olives are still

88   unknown. In the case of ripe olives the AA precursors appear to be different from those

89   in other heated foods. Thus, it is well-demonstrated that the Maillard reaction from

90   amino acids, mainly asparagine, along with reducing sugars, represents the main

91   formation route of AA in potatoes products (Amrein et al., 2004; Taubert et al., 2004),

92   roasted almonds (Amrein et al., 2005) and roasted tea (Mizukami et al., 2006).

93   However, previous studies in olives showed no correlation between their AA content

94   and any of the sugars or amino acids determined before sterilization, which appears to

95   indicate that these compounds are irrelevant as AA precursors in olives (Amrein et al.,

96   2007; Casado and Montaño, 2008). The aim of the present work was twofold. The first

97   aim was to assess the efficiency of selected amino acids and water-soluble vitamins to

98   eliminate or reduce AA in a ripe olive model system, which mimicked the chemical

99   composition and the heat treatment of ripe olives. For comparison purposes, a strong

100 inhibitor of AA formation in ripe olives, namely sodium sulfite, was also tested. To

101 evaluate the reliability of the model system, some results obtained from the model

102 system were compared with those obtained with the real product. The second aim was

103 to investigate the kinetic profile of AA formation in the ripe olive model both in the

104 absence and presence of selected additives.

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106 **2. Materials and methods**

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108 *2.1. Chemicals*

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110   Individual amino acids (L-arginine, glycine, L-tryptophan, L-proline, L-histidine,

111 DL-ornithine hydrochloride, taurine, sarcosine, and γ-amino-*n*-butyric acid), vitamins

112 (thiamine hydrochloride, VB1; nicotinic acid, VB3; pyridoxine, VB6; biotin, VB7; and

113 sodium- L -ascorbate, VC), and sodium sulfite were supplied by Sigma-Aldrich (St.

114 Louis, MO). Alliin (*S*-allyl-L-cysteine sulfoxide) was isolated from garlic powder by the

115 method of Mochizuki et al. (1997). The garlic powder was prepared as follows: fresh

116 garlic cloves were frozen in liquid nitrogen, immediately peeled, and lyophilized.

117 Methiin (*S*-methyl-L-cysteine sulfoxide) was synthesized as described by Shen and

118 Parkin (2000) using S-methyl-L-cysteine (Sigma) as the starting material. Deionized

119 water (Milli-Q; Millipore Corp.) was used throughout. All reagents and chemicals used

120 for the AA analysis were as described by Casado and Montaño (2008). All other

121 chemical and solvents were of analytical grade from various suppliers.

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123 *2.2. Preparation of ripe olive model systems*

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125   Green olives (Hojiblanca cultivar) were stored in 2.4% acetic acid for about

126 three months before processing, and were then subjected to darkening as follows: olives

127 (≈ 22 kg) were treated in a horizontal stainless steel cylindrical container (0.4 m

128 diameter x 0.7 m length) with a lye solution of 3%, which progressively penetrated the

129 flesh until the alkali reached the pit. Next, the lye was removed and the olives were

130 washed with water until the pH reached 8.0. During lye treatment and washing, air was

131 injected through the bottom of the container. Pressurized air is introduced through 15

132 spigots (0.5 mm diameter) uniformly located in the bottom of the container so that the

133 oxidation process is uniform. Then, a 0.1% ferrous gluconate solution with pH corrected

134 to 4.5 was added to fix the black color (López-López et al., 2009). One portion of ripe

135 olives (≈10 kg) was then subjected to the following operations: pitting, homogenization

136 using a mixer, filtration through cheesecloth, and centrifugation at $20,000g$ for 20 min.

137 After separation of the oil, the resulting juice was stored at -30 ºC until the moment of

138 performing the different tests. The selected compounds were added separately to juice

139 in known concentrations. The addition levels of the amino acids were 50, 100, and 200

140 mM whereas vitamins were assayed at 25, 50, and 100 mM. In all cases, prior to

141 heating, the pH of the mixture was adjusted to 7. Heat treatment was performed by

142 placing olive juice (1 mL) in a custom-made cylindrical stainless steel tubular reactor

143 (internal diameter 0.7 cm, length 3.0 cm) having one end closed. The reactor was sealed

144 with a stainless steel tube plug, and then heated in an oil bath at 121 ± 1 ºC for 30 min.

145 The bath was equipped with a stirrer to ensure a homogeneous temperature in the oil.

146 After heating, the sample was immediately cooled in ice water for 3 min to stop any

147 further reaction and analyzed for its AA content. The temperature profile inside the

148 reactor tube was obtained using a stainless steel temperature probe (Pt100 sensor)

149 coupled to a Crison thermometer model 620/3 (Crison Instruments, Barcelona, Spain).

150 The probe (3 mm diameter) was inserted through the hole of a rubber washer (14 mm

151 external diameter, 1 mm internal diameter, 3 mm thickness) placed under a nut

152 previously connected to the open end of the reactor. The temperature profile is shown in

153 Figure 1. The control was treated with the same experimental steps but without

154 additives. All heating experiments were performed in triplicate.

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156 *2.3. Preparation of packed ripe olives*

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158 Another portion of ripe olives was packed in "A314" glass bottles (145 g of

159 pitted oliveswith 170 mL of brine capacity) and covered with brine containing 3%

160 NaCl, 0.015% ferrous gluconate, and the corresponding compound (Na_2SO_3, Pro, Sar,

161 or Gly). Compounds were added to give fixed equilibrium values of 100 and 200 mM,

162 except for Na_2SO_3, which was added at 10 and 20 mM. A control product was prepared

163 using the same brine without additives. If necessary, before the olives were covered, the

164 pH of the packing brine was adjusted to 6.5-7.0 by adding NaOH or HCl. Before

165 sealing, the bottles were filled with hot brine (70 ºC). Two bottles from each treatment

166 were heated at 121 ºC for 15 min in a computer-controlled retort (Steriflow, SAS, Paris,

167 France). Before starting the retort cycle, the bottles were pre-heated at 50 ºC for 10 min.

168 In the heating phase or come-up time of the retort cycle, the water process was heated

169 by steam in the primary circuit of heat exchanger; in the holding phase, temperature

170 (121 ºC) and pressure (2.8 bar) were stabilized; and finally, in the cooling phase, cold

171 water was injected in the heat exchanger in order to cool down the process water. The

172 cycle period was 55 min. Sterilization treatment was performed in triplicate. After 3

173 months storage, AA analysis was carried out.

174 *2.4. Kinetic studies*

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176 Ripe olive juice without (control) and with selected additives at fixed

177 concentrations were heated at 121 ºC in tubular reactors for selected heating times (5,

178 15, 25, 35, 45, 55, and 65 min). Although heating times longer than 30 min are not of

179 practical significance in industrial ripe olive processing, prolonged heating times were

180 included in order to consider a possible degradation step of AA. Each heat treatment

181 was performed at least in duplicate. The obtained data were analyzed using different

182 kinetic models. The Logistic-Fermi model (Eq. 1) proposed by Corradini and Peleg

183 (2006) describes the formation of AA by a logistic function and the degradation by a

184 Fermi-type function:

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186 $$C(t) = \left[\frac{a(T)}{1+exp\{k_1(T)[t_{c1}(T)-t]\}} - \frac{a(T)}{1+exp[k_1(T)t_{c1}(T)]}\right] \cdot \frac{1}{1+exp\{k_2(T)[t-t_{c2}(T)]\}} \qquad (1)$$

187 where $C(t)$ is the concentration of AA, $t_{c1}(T)$ and $t_{c2}(T)$ are temperature-dependent time

188 characteristics for the inflection points in the formation (t_{c1}) and degradation (t_{c2}) of AA,

189 $k_1(T)$ and $k_2(T)$ are temperature-dependent steepness parameters around the inflection

190 points for the formation (k_1) and degradation (k_2) of AA and $a(T)$ serves as a

191 temperature-dependent "scale factor" for the AA concentration.

192   The Logistic-Exponential model (Eq. 2) differs from the above model in the

193 function that describes the degradation of AA:

194

195 $$C(t) = \left[\frac{a(T)}{1+exp\{k_1(T)[t_{c1}(T)-t]\}} - \frac{a(T)}{1+exp[k_1(T)t_{c1}(T)]}\right] \cdot exp\left(-\frac{t}{\tau(T)}\right) \qquad (2)$$

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197 where $\tau(T)$ is a temperature-dependent characteristic of time.

198   Since AA was apparently not degraded as a result of the applied heat treatments

199 the simple logistic function (Eq. 3) describing the AA formation was also studied:

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201 $$C(t) = \frac{a(T)}{1+exp\{k_1(T)[t_{c1}(T)-t]\}} \qquad (3)$$

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202

203 Since the R^2 value cannot be used to evaluate nonlinear models, the quality of fit was

204 expressed as the pseudo-R^2 value:

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206 $$\text{pseudo-}R^2 = 1 - (SS_{residual}/SS_{corrected\ total}) \tag{4}$$

207

208 where $SS_{residual}$ stands for the sum of squares of residuals and $SS_{corrected\ total}$ for the

209 corrected total sum of squares, that is, the squared difference of the observed value from

210 the mean summed over all observations (Motulsky and Christopoulos, 2003).

211 The adequacy of the kinetic model was also evaluated graphically by plotting the

212 predicted values against the experimental values.

213 It is known that that the coordinates of the inflexion point (P) of the above logistic

214 function are $(t_{c1}, a/2)$ and the curve´s slope (formation rate) at this point satisfies the

215 equation:

216 $$dP/dt = k_1P(1 - P/a) \tag{5}$$

217 Therefore,

218 $$dP/dt = k_1a/4 \tag{6}$$

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220 *2.5. Analysis of AA*

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222 Determination of AA in olive juice or ripe olives was carried out as described

223 previously (Casado and Montaño, 2008). AA was determined by gas chromatography-

224 mass spectrometry (GC-MS) after bromination and ethyl acetate extraction of the 2,3-

225 dibromopropionamide using $^{13}C_3$-AA as an internal standard.

226

227 *2.6. Statistical analysis*

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229   All experiments were performed in triplicate and statistically analyzed by means

230 of analysis of variance (ANOVA) on a significance level of $p=0.05$. The software

231 Statistica version 7.0 (Statsoft Inc., Tulsa, OK) was used.

232

233 **3. Results and discussion**

234

235 *3.1. Effect of additives on AA formation in ripe olive model system*

236

237   In control juice, 319 ± 50 μg/L (mean $\pm$ SD, n=3) of AA was formed after 30

238 min heating at 121 ºC. As expected, sodium sulfite was found to be a very effective AA-

239 reducing compound. The reduction rate reached 91% after adding 10 mM of sodium

240 bisulfite (data not shown). After the addition of 20 mM or more, the AA formation was

241 completely inhibited, which confirmed the effectiveness of bisulfite as a strong

242 inhibitor of AA formation in ripe olives as previously reported by Casado et al. (2010).

243 Of the tested protein amino acids (Figure 2), proline was the most potent inhibitor of

244 AA formation (reduction rates of 61%, 78%, and 91% at 50 mM, 100 mM, and 200

245 mM, respectively) followed by glycine (30%, 54%, and 64%). Tryptophan inhibited AA

246 formation (~36% reduction), but reduction was not dose-dependent. Arginine reduced

247 the formation of AA at concentrations of 100 and 200 mM (reductions of 24% and 42%,

248 respectively), but it was not effective at the 50 mM level. Finally, histidine was only

249 effective at the highest concentration assayed (39% reduction at 200 mM). It has been

250 demonstrated that the above-mentioned amino acids can form amino acid-AA adducts

251 through Michael addition type reactions thereby reducing the AA content, although a

252 clear identification in the specific condensation product was not obtained in the case of

253 arginine (Koutsidis et al., 2009; Adams et al., 2010).

254   Of the non-protein amino acids, sarcosine (*N*-methyl glycine) showed the

255 highest inhibition rates, similar to those of proline (Figure 3). The higher reactivity of

256 sarcosine in comparison with the corresponding primary amine containing amino acid

257 (i.e. glycine) is noteworthy. Ornithine, taurine, and γ-amino-*n*- butyric acid also gave

258 good inhibition rates (50-65% at 100 mM). Shin et al. (2010) reported significant

259 reductions of AA formation in a fried potato chip model when, prior to frying at 170 ºC

260 for 3 min, the potato slices were soaked in 0.1-2% taurine solution for 30 min.

261 However, to our best of knowledge, the potential use of other nonprotein amino acids

262 such as sarcosine, ornithine and γ-amino-*n*- butyric acid to decrease AA formation in

263 processed foods has not been previously suggested. In addition, all these nonprotein

264 amino acids could be particularly interesting from a healthy standpoint, as they are

265 widely used as components in nutritional supplements.

266   Natural compounds are attractive candidates to be added as inhibitors of AA

267 formation in processed foods. In the present study, the non-protein sulfur-containing

268 amino acids alliin and methiin were tested at concentrations between 25-100 mM

269 (Figure 3). These two *S*-alk(en)yl-L-cysteine sulfoxides are present in relatively high

270 concentrations in *Allium* vegetables (Horie and Yamashita, 2006) and they are thought

271 to be beneficial to health. Unfortunately, alliin did not show a significant AA-reducing

272 effect compared to the control juice in any case while methiin only reduced AA

273 formation at high concentrations with reduction rates rather modest(<40%). Moreover,

274 as a serious drawback, a distinct "garlic odor" after the heat treatment was noted in all

275 samples with added alliin or methiin. This odor can be attributed to thermal breakdown

276   products of these compounds (Kubec et al., 1997; Kubec et al., 1998). In our previous

277   study (Casado et al., 2010), the addition of minced blanched garlic at 15 g/kg of ripe

278   olives was reported to reduce the AA content in ripe olives by 23%, which was

279   hypothesized to be due to the presence of the cysteine sulfoxides in garlic, mainly alliin.

280   The above-mentioned amount of blanched garlic would correspond to alliin and methiin

281   concentrations of ~1 mM and ~0.1 mM, respectively, assuming that the levels of these

282   compounds in garlic are 35 and 3.5 mg/g of dm, respectively, and there are no losses

283   during the blanching treatment (Montaño et al. 2011; Beato et al., 2012). These

284   concentrations are 1-3 orders of magnitude lower than the concentrations tested in the

285   present study. Therefore, the above results appear not to confirm the hypothesis that

286   cysteine sulfoxides are involved in the AA reducing effect of blanched garlic,

287   suggesting that other unknown compounds would be responsible for this effect. It has

288   been reported that allicin (diallylthiosulfinate), which is formed from alliin by the action

289   of the enzyme alliinase when raw garlic is minced, effectively reduces AA formation

290   (>50% reduction) in an asparagine/fructose model system containing 0.0375% allicin

291   (Yuan et al., 2011). However, allicin is expected not to be present in blanched garlic, as

292   blanching treatment results in the deactivation of alliinase (Rejano et al., 2004).

293   Of the tested water-soluble vitamins, only VB1 significantly reduced the AA

294   content compared to the control juice (Figure 4). However, the addition of this vitamin

295   imparted a noticeable bad odor to heated juice. Presumably, this could be related to the

296   presence of a thiazole ring as a component of the VB1 molecule. The other water-

297   soluble vitamins had no significant AA-reducing effect in the olive model system in

298   general, which is in contrast with the significant AA reductions reported in other model

299   systems (Zeng et al., 2009; Yuan et al., 2011).

300

3.2. Comparison between the olive model system and the real product

Results obtained for a few additives in the model system were compared with those obtained for the same additives in the real product (packed ripe olives). AA formed in the latter product without additives was 598 µg/kg in olive pulp. A good correlation ($R^2 = 0.941$) between the AA contents obtained in the model system and those obtained in the real product was found (Figure 5), with a slope ±SE of 1.72 ±0.14. The reason for the higher AA contents in packed olives compared to model system could be related to the amount of AA precursors in each case. The model system based on olive juice is a simplified representation of the real product (packed olives). Recently, we have demonstrated that peptides and/or proteins are precursors of AA formation in sterilized olives (Casado et al., 2013). These compounds are not only in the olive juice (which more properly should be called olive water, i.e. the aqueous fraction of olive pulp) but also in the solid fraction linked to olive components (eg., fiber) and even in the olive oil (Hidalgo et al., 2001). Therefore, the amount of AA precursors in packed olives should be higher than in olive juice, which would explain the higher AA contents in packed olives.

The above correlation between the model system and the real product indicates that one could use the ripe olive model system as a simple and reliable tool for screening compounds with potential inhibitor effects on the AA formation in ripe olive processing. However, the obtained linear function could not be applicable under different conditions with respect to those used in the present study (i.e. olive juice and real product made with Hojiblanca olives processed by using the same darkening method). Changes in the darkening method or olive cultivar could affect the kinetic behavior of AA formation, which in turn would affect the final AA level in ripe olives (Casado and Montaño, 2008).

326

3.3. Kinetic studies

The kinetic profile of AA formation in the ripe olive model at 121 ºC for 5-65

min in the absence (control) and presence of selected additives is shown in Figure 6.

The additives selected were sarcosine at 100 mM and arginine at 200 mM, which are

examples of amino acids with strong and weak capacities, respectively, to inhibit AA

formation. After a "lag-phase" of at least 5 min, in which AA formation was negligible,

AA content increased and eventually reached an equilibrium value. This "lag-phase"

was expected taking into account that AA formation in foods appears to begin at

temperatures around 120 ºC (Stadler et al., 2002) and this temperature inside the tubular

reactor was just reached after 10 min heating (Figure 1). When the "Logistic-Fermi" and

"Logistic-Exponetial" models were applied, the fit of these empirical models to our data

did not give satisfactory results in any case (data not shown), which was probably due

to the absence of a degradation step of AA and the relatively low number of data points.

However, when a simple logistic pattern based on only three parameters was used (Eq.

3), the fit was quite good for the data set measured (Figure 6). Calculation results of the

kinetic parameters describing the formation of AA in each case along with the

corresponding pseudo-R^2 values are shown in Table 1. The goodness of fit of the model

on the data was confirmed graphically by the scatter plots shown in Figure 7, where a

high correlation was found between predicted and experimental values in all cases. The

parameters k_1 and t_{c1} in the juices with additives were not significantly different

compared to the control. In other words, the addition of the amino acids to the model

system did not appear to influence the nature of the kinetic model. Nevertheless, the

parameter a was significantly lower in the additive-containing samples compared to the

control. A consistently lower a value indicates a lower level of AA production. This

15

351 means that sarcosine and arginine at the concentrations tested had a significant

352 antagonist effect on the AA formation at 121 ºC. When a mixture of 100 mM Sar + 200

353 mM Arg was tested, the calculated parameter a was significantly lower than those

354 obtained for the amino acids separately, but again k_1 and t_{c1} did not significantly change.

355 This result demonstrates that the AA reducing effect of the above mixture was

356 significantly higher than the two amino acids added separately. It must be stressed that

357 the parameter k_1 does not actually represent a formation rate, but this can be calculated

358 from the curve´s slope at the inflexion point according to equation 6. The obtained

359 values of the formation rate (Table 1) were consistent with the differences observed in

360 the AA formation curves (Figure 6) where control system showed a much faster

361 formation compared to the systems with AA inhibitors.

362 The logistic model (or empirical models in general) as mentioned above only

363 describe the AA formation kinetics but they do not give much insight into the

364 mechanism behind the AA formation. A logistic function was previously used by

365 Granda and Moreira (2005) to model the kinetics of AA formation in traditional fried

366 potato chips. Knol et al. (2009) used the "Logistic- Exponential" model to make

367 predictions for the AA formation in potato crisps based on the strong correlation

368 between the parameter a obtained by the model and the reducing sugar content. Since

369 AA precursors in olives are still unknown, a similar correlation between the parameter a

370 derived from the logistic function and the content of AA precursor cannot be

371 determined as yet. Studies are in progress which use the tubular reactor methodology

372 described in this work to identify the main AA precursors in olives. Once these studies

373 are carried out and assuming that a good correlation between parameter a and precursor

374 content is found, the next step would be to investigate the kinetics of AA formation in

375 real food (i.e. ripe olives), under well-controlled conditions, and to apply the logistic

376 function to kinetic data. Models of the proposed kind might be used to simulate and

377 predict the generation of AA in ripe olives. In addition, the obtained information might

378 be useful for developing new strategies for the reduction or elimination of AA by

379 decreasing or eliminating precursors.

380

381 **4. Conclusion**

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383   The present study shows that our olive model system is a simple and reliable

384 tool for the screening of additives as potential AA inhibitors in ripe olive processing.

385 Excluding sulfite, the compounds which had the highest impact on the AA level were

386 proline and sarcosine. Further research is currently underway to determine whether

387 these compounds would be good candidates to decrease AA formation in ripe olive

388 processing taking into account their impact on the sensory characteristics of the product.

389 The ripe olive model system was useful in identifying the kinetics of AA formation at

390 121 ºC. This was modeled using a simple logistic model, characterized by an increase in

391 the AA content with heating time until an equilibrium value is eventually reached.

392 Hopefully, this type of model could be used in the future to predict the formation of AA

393 and to develop new strategies for its reduction or elimination in ripe olives.

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526

527 **Figure captions**

528

529 Figure 1. Temperature-time profile of samples heated in a custom-made cylindrical

530 stainless steel tubular reactor (internal diameter 0.7 cm, length 3.0 cm).

531 Figure 2. Dose-response effect of selected protein amino acids on the acrylamide

532 inhibition (percentage from control) in a ripe olive model system. Data values are mean

533 $\pm$ SD (n=3). Bars with an asterisk indicate significant difference from the control

534 ($p<0.05$). Abbreviations: Pro = proline, Gly = glycine, Trp = tryptophan, Arg =

535 arginine, and His = histidine.

536 Figure 3. Dose-response effect of selected non-protein amino acids on the acrylamide

537 inhibition (percentage from control) in a ripe olive model system. Data values are mean

538 $\pm$ SD (n=3). Bars with an asterisk indicate significant difference from the control

539 ($p<0.05$). Abbreviations: Sar = sarcosine, Orn = ornithine, Tau = taurine, and

540 GABA = γ-aminobutyric acid.

541 Figure 4. Dose-response effect of selected water-soluble vitamins on the acrylamide

542 inhibition (percentage from control) in a ripe olive model system. Data values are mean

543 $\pm$ SD (n=3). Bars with an asterisk indicate significant difference from the control

544 ($p<0.05$). Abbreviations: VB1 = thiamine hydrochloride, VB3 = nicotinic acid,

545 VB6 = pyridoxine, VB7 = biotin, and VC = sodium- L –ascorbate.

546 Figure 5. Relationship between the acrylamide contents in ripe olive model system and

547 those in real product (ripe olives).

548 Figure 6. Formation of acrylamide as function of heating time at 121 ºC in ripe olive

549 model system in the absence (control) and presence of two different acrylamide

550 inhibitors. The lines represent acrylamide predicted by the kinetic model (logistic

551 function with three parameters).

552 Figure 7. Plots of experimentally determined acrylamide levels and acrylamide levels

553 predicted by the kinetic model (logistic function with three parameters) in ripe olive

554 model system in the absence (control) and presence of two different acrylamide

555 inhibitors.

Table 1. Effect of additives on the kinetic parameters describing the formation of

acrylamide fitted by logistic function (Eq. 3 in text)

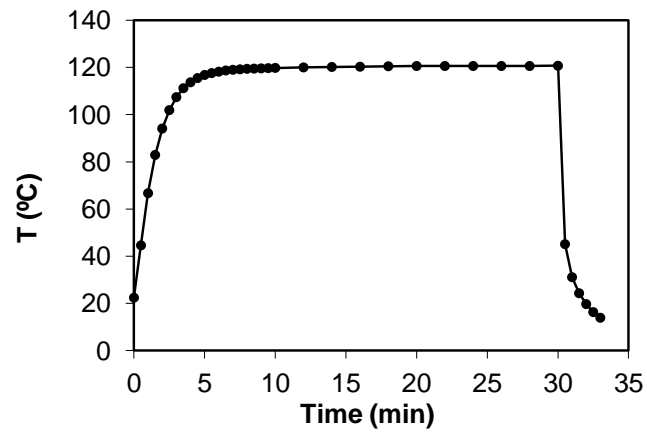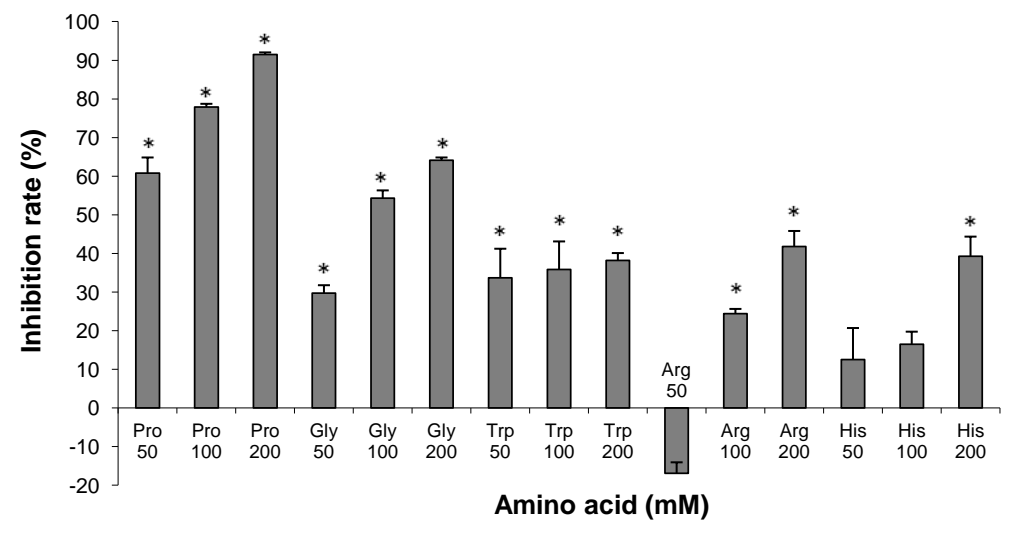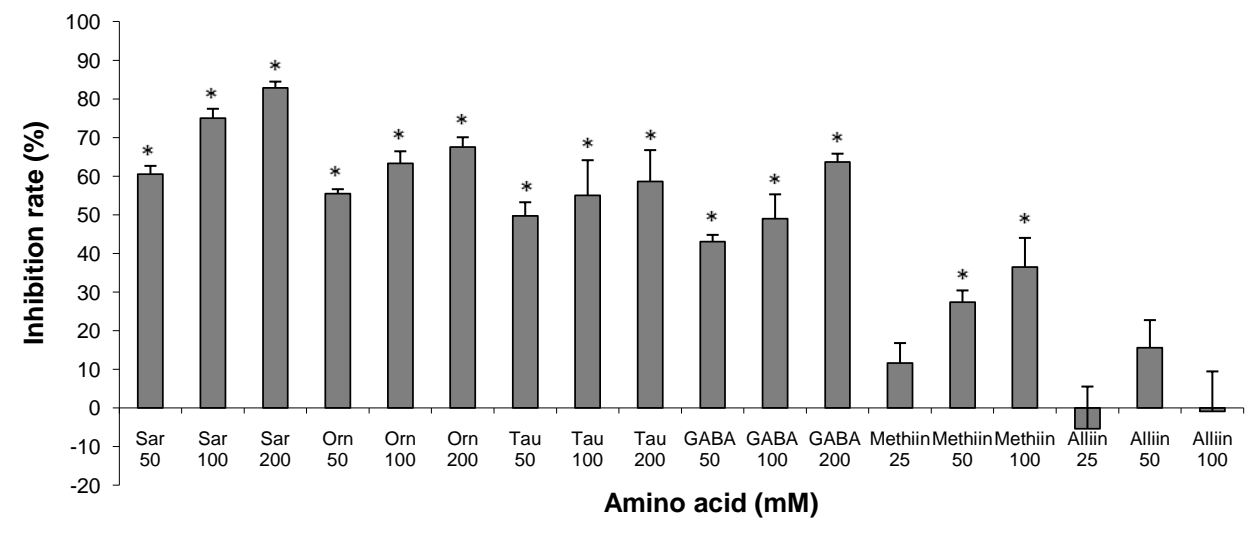| Sample	Logistic model[a]				
	a (µg/L)	k_1 (min^{-1})	t_{c1} (min)	Rate[b]	pseudo-R^2
Control	624 ± 43a	0.09 ± 0.02a	29 ± 3a	13.55	0.9590
+100 mM Sar (1)	196 ± 8c	0.12 ± 0.02a	22 ± 2a	6.14	0.9714
+200 mM Arg (2)	257 ± 13b	0.14 ± 0.03a	26 ± 2a	8.75	0.9454
(1) + (2)	134 ± 5d	0.20 ± 0.05a	20 ± 1a	6.68	0.9341
[a] Parameters are expressed as mean ± SE. Values of the same parameter with different letters are significantly different based on 95% confidence intervals. [b] Curve´s slope at the inflexion point (equation 6 in text).					

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 5 scatter plot: x-axis "Acrylamide in ripe olive model (µg/L juice)" ranging from -100 to 400; y-axis "Acrylamide in ripe olives (µg/kg pulp)" ranging from -100 to 700.

Regression equation: $y = 1.7211x + 38.343$, $R^2 = 0.9411$

Data point labels: Control, 100Arg, 200Arg, 100Gly, 200Gly, 100Pro, 100Sar, 200Pro, $10Na_2SO_3$, 200Sar, $20Na_2SO_3$

Figure 6

Figure 7