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5 # Stability of ACE inhibitory ham peptides against heat treatment and *in*
6 # *vitro* digestion

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28 **Abstract**

29 Angiotensin I-converting enzyme (ACE) inhibitory peptides derived from Spanish dry-
30 cured ham have been examined for their stability during processing and after *in vitro*
31 digestion. Peptides preserved almost the same ACE inhibitory activity after applying
32 diverse heat tretaments (from 50 to 117 ºC), times of processing (from 3 to 60 min) and
33 simulated *in vitro* digestion with gastrointestinal proteases. Peptides KAAAAP,
34 AAPLAP, KPVAAP, IAGRP, and KAAAATP were the most potent peptides with IC_{50}
35 values ranging from 12.37 µM to 25.94 µM. Peptides IAGRP and PTPVP have also
36 been identified in the processed sample (6 min at 117 ºC), and in the *in vitro* digested
37 sample. This study has shown the high stability of ACE inhibitory peptides derived
38 from Spanish dry-cured ham against temperature of processing and gastrointestinal
39 digestion as well as the powerful ACE inhibitory activity of some of the peptides
40 identified in the Spanish dry-cured ham extract.

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43 *Keywords:* Dry-cured ham, ACE inhibitory peptides, processing, gastrointestinal
44 digestion, mass spectrometry.

45

46 **1. Introduction**

47 Many dietary proteins exert beneficial effects upon human health once released from
48 their parent protein either by digestive enzymes during gastrointestinal digestion or by
49 fermentation or ripening during food processing (Korhonen, Pihlanto-Leppala,
50 Rantamaki & Tupasela, 1998). Bioactive peptides usually range in size from 2 to 50
51 amino acid residues and can exhibit different activities, such as antimicrobial,
52 antioxidant, antithrombotic, antihipertensive, inmunomodulatory, and opioid, among

53 others (Meisel & Fitzgerald, 2003; Lopez-Fandino, Otte & Van Camp, 2006; Toldrá and
54 Reig, 2011). Peptides possessing specific biological properties make them potential
55 ingredients of functional or health-promoting foods since they could reduce the risk of
56 chronic diseases and promote human health (Hartmann & Meisel, 2007).
57 The industrial application of these peptides and their incorporation into foods may
58 affect the functional, nutritional, and biological properties of these peptides (Abdul-
59 Hamid, Bakar & Bee, 2002; Vaslin, Le Guillou, Hannoucene & Saint Denis, 2006; Paul
60 & Somkuti, 2009). On the other hand, when bioactive peptides are in the digestive tract
61 after consumption, the activity of gastric enzymes may also affect their biological
62 activities. In fact once ingested, proteins and peptides are subjected to hydrolysis by
63 different enzymes such as pepsin, trypsin, or chymotrypsin. Some of the released
64 peptides may exert a direct function at the gastrointestinal tract, whereas other peptides
65 can be absorbed and reach target organs and tissues through systemic circulation
66 (Shimizu, 2004). In previous studies, Escudero, Sentandreu and Toldrá (2010)
67 demonstrated the generation of ACE inhibitory peptides after gastrointestinal digestion
68 of pork meat. Moreover, in a recent study, Escudero, Toldrá, Sentandreu, Nishimura
69 and Arihara (2012) investigated the *in vivo* antihypertensive activity of three novel
70 peptides identified in the *in vitro* digest of pork meat, resulting in a significant decrease
71 in systolic blood pressure after oral administration to spontaneously hypertensive rats.
72 Our previous studies demonstrated that Spanish dry-cured ham is a natural source of
73 peptides that show ACE inhibitory and *in vivo* antihypertensive activity in
74 spontaneously hypertensive rats (Escudero, Aristoy, Nishimura, Ariahara & Toldrá,
75 2012; Escudero, Mora, Fraser, Aristoy & Toldrá, 2013). Since there is a lack of
76 information related to the influence of food processing and gastrointestinal digestion on
77 the bioactivity of ACE inhibitory and antihypertensive peptides, the study of the

78 bioactive peptides stability during processing and after gastrointestinal digestion as well
79 as the effects on their ACE inhibitory activity are of great importance. In this study, a
80 Spanish dry-cured ham extract rich in bioactive peptides showing ACE inhibitory
81 activity was used to study the impact of heat treatment and *in vitro* gastrointestinal
82 digestion on its stability.

83 **2. Materials and methods**

84 **2.1 Material and reagents**

85 Dry-cured ham used in this study was a Designation of Origin of Teruel ham (D.O.
86 Teruel, Spain), with a minimum time of ripening of fourteen months. The population
87 used for the production of D.O. Teruel are maternal line Landrace and Large White
88 crossbreds and paternal line purebred Duroc. Angiotensin-converting enzyme (from
89 rabbit lung) was purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A). Abz-
90 Gly-*p*-nitro-phe-pro-OH trifluoroacetate salt was obtained from Bachem AG.
91 (Bubendorf, Switzerland). Pepsin (from hog stomach) was purchased from Fluka
92 Chemie Gmbh (Buchs, Switzerland), and pancreatin (from porcine pancreas) was
93 purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A). Other chemicals and
94 reagents used were of analytical grade.

95 **2.2 Peptide extraction**

96 A sample of 100 g of Spanish dry-cured ham were minced and homogenized with 500
97 mL of 0.01N HCl in a Polytron® (Kinematica, Switzerland) for 5 min. The homogenate
98 was kept at 4 ºC overnight for decanting, and the supernatant was filtered through a
99 plastic mesh to retain the biggest pieces and then filtered again through a qualitative
100 filter paper (Whatman™, UK). After that, the sample was freeze-dried and further
101 submitted to solid phase extraction using an Oasis® HLB cartridge (35 cc, Waters,
102 Ireland) in which the peptides were retained and then eluted using methanol-distilled

103 water (95:5, v/v). The eluted sample was lyophilized, constituting the starting material
104 for the subsequent experiments.

105 **2.3 Assay of ACE Inhibitory Activity**

106 The ACE inhibitory activity of the Spanish dry-cured ham extract (control), processed
107 sample, processed and further digested sample, and the synthesized peptides was
108 measured according to the method developed by Sentandreu and Toldrá (2006). This
109 assay is based on the ability of ACE to hydrolyze the internally quenched fluorescent
110 substrate *o*-aminobenzoylglycyl-*p*-nitro-*L*-phenylalanyl-*L*-proline (Abz-Gly-Phe-(NO_2)-
111 Pro). A sample solution (50 µL dry-cured ham extract or synthesized peptide) was
112 mixed with 50 µL of 150 mM Tris-base buffer (pH 8.3) containing 3 mU/mL of ACE,
113 and the mixture was preincubated for 10 min at 37 ºC. The reaction was initiated by the
114 addition of 200 µL of 150 mM Tris-base buffer (pH 8.3) containing 1.125 M NaCl and
115 10 mM Abz-Gly-Phe- (NO_2)-Pro, which was preincubated again for 10 min at 37 ºC.
116 Finally, the reaction mixture was then incubated for 45 min at 37 ºC. The generation of
117 fluorescence due to the release of *o*-aminobenzoylglycine (Abz-gly) by the action of
118 ACE was measured using excitation and emission wavelengths of 355 and 405 nm,
119 respectively. ACE inhibition of control, processed sample, and processed and
120 subsequently digested sample is expressed as relative ACE inhibitory percentage and
121 the ACE inhibition of synthesized peptides is expressed as IC_{50}. The IC_{50} value is the
122 concentration associated with 50% ACE inhibition in the reaction mixture. The
123 experiments were performed by triplicate.

124 **2.4 Stability of dry-cured ham ACE inhibitory peptides**

125 Spanish dry-cured ham ACE inhibitory peptide solutions (5 mg/mL) were incubated at
126 various temperatures, 50, 72, 90 and 117 ºC for 6 min. On the other hand, the peptide
127 solutions were also incubated at 117ºC at different times 3, 6, 15, 30 and 60 min. All

128 solutions were taken to room temperature before the ACE inhibitory activity analysis,
129 which was determined as described above. Triplicate assays were performed for each
130 sample.

131 **2.5 *In vitro* Digestion of dry-cured ham peptides**

132 Stability of the peptides of Spanish dry-cured ham extract against *in vitro* gastric
133 proteases was assessed using pepsin and pancreatin according to the method of Laparra,
134 Vélez, Montoro, Barberá and Farré (2003) with some modifications. Pepsin solution in
135 6 M HCl (pH 2.0) was added to dry-cured ham extract at a 1:100 enzyme to substrate
136 ratio. After 2 h of digestion at 37 ºC and continuous stirring, the enzyme was inactivated
137 by adjusting the pH to 7.2 with 1 M $NaHCO_3$. Then, pancreatin was added at a 1:50
138 enzyme to substrate ratio. After 3 h of digestion at 37 ºC, enzyme activity was
139 terminated by heating for 10 min at 95 ºC. The reaction mixture was freeze-dried and
140 then reconstituted for ACE inhibitory activity determination.

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143 **2.6 Peptide identification by mass spectrometry in tandem (nESI-LC-MS/MS)**

144 The nanoLC-MS/MS analysis was performed using an Eksigent Nano-LC Ultra 1D Plus
145 system (Eksigent of AB Sciex, CA, USA) coupled to the quadrupole-time-of-flight (Q-
146 ToF) TripleTOF® 5600+ system from AB Sciex Instruments (Framingham, MA, USA)
147 that is equipped with a nanoelectrospray ionization source.

148 Desalted dry-cured ham extracts were resuspended in H_2O with 0.1% of trifluoroacetic
149 acid (TFA) to obtain a final concentration of 10 mg/mL. After centrifuge in cold for 3
150 min at 200xg, fifteen microlitres of each sample (control, processed dry-cured ham
151 extract, and processed and digested dry-cured ham extract) were cleaned and
152 concentrated using Zip-Tip C18 with standard bed format (Millipore Corporation,

153 Bedford, MA) according to manufacturer's instructions. Five microlitres of the
154 supernatant were injected into the LC-MS system through the autosampler.
155 Samples were then preconcentrated on an Eksigent C18 trap column (3µ, 350µm x
156 0.5mm) (Eksigent of AB Sciex, CA, USA), at a flow rate of 3 µL/min and using 0.1%
157 v/v TFA as mobile phase. After 5 min of preconcentration, the trap column was
158 automatically switched in-line onto a nano-HPLC capillary column (3µm, 75µm x 12.3
159 cm, C18) (Nikkyo Technos Co, Ltd. Japan). The mobile phases consisted of solvent A,
160 containing 0.1% v/v formic acid in water, and solvent B, containing 0.1% v/v FA in
161 100% acetonitrile. Chromatographic conditions were a linear gradient from 5% to 35%
162 of solvent B over 90 min, and 10 min from 35% to 65% of solvent B, at a flow rate of
163 0.3 µL/min and running temperature of 30 ºC.
164 The outlet of the capillary column was directly coupled to a nano-electrospray
165 ionisation system (nano-ESI). The Q/ToF was operated in positive polarity and
166 information-dependent acquisition mode, in which a 0.25-s ToF MS scan from *m/z* of
167 100 to 1200 was performed, followed by 0.05-s product ion scans from *m/z* of 100 to
168 1500 on the 50 most intense 1 to 5 charged ions.
169 **2.7 Data analysis**
170 Automated spectral processing, peak list generation, and database search were
171 performed using Mascot Distiller v2.4.2.0 software (Matrix Science, Inc., Boston, MA)
172 (hppt://www.matrixscience.com). The identification of protein origin of peptides was
173 done using UniProt protein database, with a significance threshold $p<0.1$ and a FDR of
174 1.5%. The tolerance on the mass measurement was 0.3 Da in MS mode and 0.3 Da for
175 MS/MS ions. BIOPEP database was used in the search of similar sequences previously
176 identified showing ACE inhibitory activity
177 (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep).

178 **2.8 Peptide Synthesis**

179 Considering the low molecular mass and the structural requirements for ACE inhibition,
180 some of the peptides identified from control sample, processed sample at 117 ºC during
181 6 min, and processed and further *in vitro* digested sample, were synthesized by
182 GenScript Corporation (Piscataway, NJ, USA) in order to assess their *in vitro* inhibition
183 of ACE. The purity of the synthesized peptides was certified by analytical LC-MS.

184 **3. Results and discussion**

185 **3.1 ACE inhibitory activity of Spanish dry-cured ham extract**

186 Bioactive peptides can be found in intact food molecule but they are generally inactive
187 within the sequence of protein molecule. During the processing of Spanish dry-cured
188 ham an intense proteolysis takes place resulting in an accumulation of peptides of
189 different sizes (mainly small peptides) (Mora, Sentandreu, Koistinen, Fraser, Toldrá &
190 Bramley, 2009; Mora, Sentandreu, Fraser, Toldrá & Bramley, 2009) and free amino
191 acids at the end of dry-curing (Toldrá, Aristoy & Flores, 2000). In previous studies it
192 was found that peptide fractions from dry-cured ham exhibited relevant ACE inhibitory
193 activity and *in vivo* antihypertensive activity (Escudero et al., 2012; Escudero et al.,
194 2013).

195 Spanish dry-cured ham extract (control sample) was analyzed at different concentrations
196 in order to get information about ACE inhibitory activity exerted (**Fig.1**) and select the
197 most convenient concentration for the stability assays. As can be observed in figure 1,
198 the extract showed inhibitory activity against ACE at all the assayed concentrations
199 having a maximum activity of 60.7% at 10 mg/mL. For subsequent experiments the
200 concentration of 5 mg/mL was chosen due to its proximity to the maximum ACE
201 inhibitory activity and thus better elucidate the effect of temperature, time, and *in vitro*
202 digestion on the bioactivity of the peptidic extract.

203 **3.2 Impact of temperature and time on the activity of ACE inhibitory peptides**

204 The bioactive peptides derived from Spanish dry-cured ham extract can be directly
205 ingested from dry-cured ham, either raw or heat treated, or incorporated into other meat
206 processed products. In this case, it is necessary to consider the stability of the ACE
207 inhibitory activity among the typical temperature and time processing conditions. The
208 temperature changes of the extracts were conducted by heating at 50, 72, 90 and 117 ºC
209 for 6 min (**Fig. 2a**) and the effect of time was assayed at 117 ºC during 3, 6, 15, 30 and
210 60 min (**Fig. 2b**). These temperatures and times are commonly used in the food industry
211 when processing meat products such as spreadable meat or ham-derived products. As
212 shown in **Fig. 2a** and **2b,** these peptides retained ACE inhibitory activity after applying
213 different combinations of temperature and time of processing, which indicates that dry-
214 cured ham ACE inhibitory peptides have good stability against heating. These results
215 are consistent with those found in ACE inhibitory peptides derived from tuna cooking
216 juice which exhibited good resistance to different temperatures (20-100ºC for 2 h)
217 reserving almost the same composition before and after treatments (Hwang, 2010).
218 Furthermore, peptides derived from soy-protein were also stable after incubating at
219 different temperatures (20- 100ºC for 2h) (Wu & Ding, 2002).

220 **3.3 Effect of gastric enzymes on ACE inhibitory activity of dry-cured ham peptides**

221 Digestion is one of the most important processes to release bioactive peptides which are
222 inactive within the intact protein. Proteolytic enzymes can generate bioactive peptides
223 during digestion (Hartman et al., 2007; Korhonen & Pihlanto, 2006; Escudero et al.,
224 2010; Escudero, Sentandreu, Arihara & Toldrá, 2010). ACE inhibitory peptides could
225 exert *in vivo* antihypertensive effect if they reach the cardiovascular system in an active
226 form (Vermeirsssen, Van der Bent, Van Camp, Van Amerongen & Verstraete, 2004).
227 So, after oral administration, peptides need to resist complete degradation by

228 gastrointestinal proteases and brush border peptidases, and they have to be absorbed
229 through the intestinal wall with preservation of their biological activity. During this
230 process, peptides can be degraded, resulting in an activation or inactivation of their
231 biological activity. The ACE inhibitory activity of processed sample (117 ºC during 6
232 min) and further digested by gastric proteases showed little change after *in vitro*
233 incubation (**Table 1**) suggesting that peptides present in processed Spanish dry-cured
234 ham extract may be resistant to digestion in the gastrointestinal tract or they may be
235 partially degraded into smaller peptides keeping the antihypertensive biological activity.
236 Previous reports have shown that small peptides still presented ACE inhibitory activity
237 after digestion (Hwang, 2010; Wu & Ding, 2002; Jang, Cheorun & Lee, 2007). These
238 results indicated that orally administered ACE inhibitory peptides could either keep
239 their sequence integrity in the stomach or break into new smaller bioactive peptides that
240 could reach the blood stream.

241 **3.4 Identification and synthesis of ACE inhibitory peptides by tandem mass**
242 **spectrometry**

243 The desalted dry-cured ham sample used as control, the processed sample (117 ºC
244 during 6 min), and the same processed and subsequently *in vitro* digested sample, were
245 analysed by nanoESI-LC-MS/MS mass spectrometry for the identification of their
246 peptides content. **Fig. 3** shows the total ion chromatograms (TICs) obtained after nano-
247 liquid chromatography in the mass spectrometry system for control sample, processed
248 sample at 117 ºC for 6 min, and processed and subsequently *in vitro* digested sample. In
249 the control and processed sample it can be observed an intensity of peaks very similar
250 and, as the time progresses, the intensity of peaks decreases. On the other hand, in the
251 processed and further digested sample it can be observed, in general, a decrease in the
252 intensity of the detected ions, possibly because of changes in the characteristics of

253 peptides after digestion. Biological activities of peptides are related to their amino acid
254 composition, sequence, size, and configuration (Matsui & Matsumoto, 2006). Most food
255 protein-derived peptides with ACE inhibitory activity have low molecular mass,
256 generally ranging from dipeptides to pentapeptides with molecular masses between 150
257 and 800 Da (Zhang, Wang & Wu, 2009). In this respect, the synthesised peptides had
258 molecular mass <600 Da and contained between 5 and 7 amino acids. Considering the
259 low molecular mass and structural requirements for ACE inhibition, some of the
260 identified peptides were synthesised and their IC_{50} calculated. **Table 2** shows the
261 sequence of the identified and synthesised peptides, the observed and calculated masses
262 together with the charge states, and the protein of origin. So, six and seven of the
263 peptides chosen for synthesis derive from myosin and titin proteins, respectively,
264 whereas only four come from other type of proteins. The abundance of peptides derived
265 from these proteins is due to the abundance of myosin and titin in skeletal muscle, and
266 proves the high level of hydrolysis occurred during the dry-cured processing (Mora,
267 Sentandreu, Koistinen, Fraser, Toldrá, and Bramley, 2009) as well as confirms that
268 these proteins constitute a good source of ACE inhibitory peptides (Escudero et al,
269 2010).
270 All peptides share sequence with previously identified protein fragments that have been
271 described as ACE inhibitors as shown in **Table 2**. In previous studies it has been
272 reported that binding to ACE was strongly influenced by the residues at the three
273 positions closest to the C-terminal site, especially hydrophobic amino acids as proline
274 (Rohrbach, Willians, Rolsad, 1981) which is the most favourable C-terminal amino acid
275 for binding to ACE. Also, the amino acid alanine close to the C-terminal position might
276 also positively influence the binding to ACE (Majumder & Wu, 2009). In fact, best
277 ACE inhibitory results in this study have been obtained for peptides KAAAAP,

278 AAPLAP, KPVAAP, IAGRP, and KAAAATP, with IC_{50} values of 19.79, 14.38, 12.37,
279 25.94, and 25.64 μM, respectively, with an alanine and proline residue close to C-
280 terminal and in the last C-terminal position, respectively. The activities of these novel
281 peptides were extraordinarily higher than those previously identified from pork meat
282 hydrolysates (Arihara, Nakashima, Mukai, Ishikawa & Itoh, 2001; Katayama et al.,
283 2008)
284 Peptide IAGRP has been identified by MS/MS in the control, the sample processed
285 during 6 min at 117 ºC, and in the processed extract after *in vitro* digestion with pepsin
286 and pancreatin enzymes, proving the stability of this antihypertensive peptide after
287 processing and digestion. Also the identity of peptide PTPVP has been confirmed in the
288 control, the processed sample, and in the sample after *in vitro* digestion. This peptide
289 has been previously identified and tested *in vitro* for its ACE inhibitory activity after a
290 simulated gastrointestinal digestion of pork meat, showing an IC_{50} of 256.41 μM (see
291 **Table 2**) (Escudero et al., 2010). More recently, the *in vivo* antihypertensive activity of
292 PTPVP has been tested in spontaneously hypertensive rats (SHRs), resulting in a
293 decrease of the systolic blood pressure of 24.52 mmHg ($p<0.01$) and 25.66 mmHg
294 ($p<0.01$) at 4 and 6 h after single oral administration, respectively (Escudero et al.,
295 2012). On the other hand, the synthesised peptide KAAAATP has shown an IC_{50} value of
296 25.64 μM in this study (see **Table 2**). The fragment AAATP was also previously
297 identified in a Spanish dry-cured ham and gave an IC_{50} value of 100 μM (Escudero et
298 al., 2013). These results are in agreement with the obtained results in this study.
299 The processing of dry-cured ham extract under extreme conditions of temperature and
300 time as well as the *in vitro* digestion with pepsin and pancreatin enzymes, could be the
301 responsible for the degradation of some of the ACE inhibitory peptides identified in the
302 control extract. Despite this situation, the ACE inhibitory activity remained constant

303 after processing and digestion, probably due to the presence of the tripeptide fragments
304 described in **Table 2**, which have been previously proved to be good antihypertensive
305 peptides. This fact could not be confirmed because the conditions of mass spectrometry
306 and data analysis tools used in this study does not allow the identification of di- and
307 tripeptides as their small size makes them difficult to be fragmented in the analysers,
308 and their short sequence decrease the possibility to find a specific origin protein as the
309 possibilities to be present in different sequences of *Sus scrofa* proteome increase with
310 the shortness of the peptide.

311 **4. Conclusion**

312 According to this study, the bioactivity of ACE inhibitory peptides was not affected by
313 heat treatments and they still showed relevant ACE inhibitory activity after *in vitro*
314 digestion by gastrointestinal proteases. A total of 16 peptides identified in the control
315 sample were synthesised and their IC_{50} calculated being KAAAAP, AAPLAP, KPVAAP,
316 IAGRP, and KAAAATP the most active peptides presenting IC_{50} values ranging from
317 12.37 µM to 25.94 µM. Peptide IAGRP has also been identified in the processed
318 sample during 6 min at 117 ºC, and in the processed and further *in vitro* digested sample.
319 Also, peptide PTPVP that has been previously described by Escudero et al. (2010;
320 2012) was identified in the control, processed sample, and processed and digested
321 sample. These findings prove the stability of ACE inhibitory activity of peptides
322 submitted to intense processing conditions and after digestion. ACE inhibitory activity
323 keeps constant probably due to the presence of the identified antihypertensive peptides
324 as well as small fragments resulting from their degradation occurred after processing
325 and *in vitro* digestion.

326 The knowledge of interactions of bioactive peptides with other food components during
327 processing and the evaluation of efficacy of bioactive peptides in animal model and

328 human clinical studies *per se* and in food systems will be crucial to ensure activity and
329 bioavailability of these bioactive peptides.

330 **Acknowledgements**
331
332 The research leading to these results received funding from the European Union 7th
333 Framework Programme (FP7/2007-2013) under Grant Agreement 312090 (BACCHUS).
334 This publication reflects only the authors´ views and the Community is not liable for
335 any use made of the information contained therein. The contract to E.E. within such
336 project is also acknowledged. JAEDOC-CSIC postdoctoral contract to L.M. is also
337 acknowledged. Mass spectrometry analysis was performed in the in the
338 SCSIE_University of Valencia Proteomics Unit, a member of ISCIII ProteoRed
339 Proteomics Platform.

340
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489 **LEGENDS FOR THE FIGURES**

490 **Figure 1.** ACE inhibitory (ACEI) activity of Spanish dry-cured ham extract (control) at
491 different concentrations.

492 **Figure 2.** Stability of Spanish dry-cured ham-derived ACE inhibitory peptides after: a)
493 6 min incubation at various temperatures, and b) 117ºC incubation at different times.
494 The relative ACE inhibitory percent was calculated as the ratio of ACE inhibitory
495 activity between the control and treatments. Bars represent means ± SD.

496 **Figure 3.** Total ion chromatograms (TICs) obtained after nano-liquid chromatography

497 in the mass spectrometry system for control sample, processed sample at 117 ºC for 6

498 min, and processed and subsequently *in vitro* digested sample.

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Responses to Technical Check Results

Lines were numbered 5 to 5 and are now numbered consecutively 1 by 1.

Table(s)

Table 1. Activity of the Spanish dry-cured ham extract following processing (117 ºC during 6 min), and processing and further *in vitro* digestion by gastrointestinal proteases.

Sample	ACE inhibition (%)	CV (%)
Control	45.9±1.115	2.42
Processed sample	40.42±0.54	1.33
Processed + digested sample	42.01±3.44	8.18

All values are mean±standard deviation for triplicate experiments.

Table 2. Peptides from the desalted dry-cured ham extract (control sample) identified by nanoESI-LC-MS/MS and synthesised to test the ACE inhibitory activity.

Peptide Number	P_0 [a]	Sequence	P_f [b]	Observed [c] (m/z)	Charge (H^+)	Calculated [d] Mr	Protein of origin
1	A	PAPPK [ф]	E	509.30	1	508.30	Myosin light chain 1/3
2	A	KAAAAP	A	528.27	1	527.31	Myosin light chain 3
3	Y	AMNPP	K	545.25	1	544.23	Myosin-3
4	N	IKLPP	G	567.59	1	566.38	Myosin-IXb
5	A	AAPLAP	I	539.34	1	538.31	Myosin-XV
6	I	KPVAAP	V	582.29	1	581.35	Myosin-XV
7	A	VPPAK	G	511.47	1	510.32	Titin
8	D	KPGRP	D	554.27	1	553.33	Titin
9	P	PSNPP	E	511.47	1	510.24	Titin
10	D	IAGRP*	L	257.18	2	512.31	Titin
11	P	EAPPK	R	541.27	1	540.29	Titin
12	R	PAAPPK	K	580.25	1	579.34	Titin
13	G	KVLPG	V	257.19	2	512.33	Phosphoglycerate kinase 1
14	F	TGLKP	E	515.26	1	514.31	Aspartate aminotransferase
15	P	AAATPL [¥]	A	272.22	2	542.31	Epithelial splicing regulatory protein 2
16	V	KAAAATP	F	629.30	1	628.35	PR domain zinc finger protein 2
17	K	PTPVP*	K	510.25	1	509.29	Titin

a. Amino acid residue preceding the peptide sequence; b. amino acid residue following the peptide sequence; c. Relation of mass/charge (m/z) observed in the nanoLC-MS/MS system; d. Calculated relative molecular mass in Daltons of the matched peptide; ф Peptide PAPPK has been also identified in the extract processed at 117°C during 5 min; * Peptides IAGRP and PTPVP have been identified in the processed extract at 117°C during 5 min and after digestion. PTPVP peptide was synthesized and tested *in vitro* and *in vivo* in a previous work as indicated in Table 2; ¥ Peptide AAATPL has been identified in the processed extract after digestion.

Table 3. ACE inhibitory activity (IC_{50}) of synthesised peptides and previously published ACE inhibitory fragments of these peptides.

Peptide Number	Sequence Identified	IC_{50} (μM)	Previously identified ACE inhibitory sequences[a]		
			Sequence	IC_{50}[b] (μM)	References[c]
1	PAPPK	199.58	PPK	>1000	Meisel H., Walsh D. J., Murray B. & Fitzgerald R. J. (2006);
				n.d.	Hayes M., Stanton C., Fitzgerald G.F. & Ross R.P. (2007)
2	KAAAAP	19.79	AAP	n.d.	Meisel H. (1993)
3	AMNPP	304.50	MNPP	945.5	Arihara K.,Nakashima Y., Mukai T., Ishikawa S. & Itoh M. (2001)
4	IKLPP	193.90	LPP	9.6	Maruyama S., Miyoshi S., Kaneko T. & Tanaka H. (1989)
5	AAPLAP	14.38	LAP	3.5	Fujita H., Yokoyama K. & Yoshikawa M. (2000)
6	KPVAAP	12.37	AAP	n.d.	Meisel H. (1993)
7	VPPAK	>1000	VPP	9	Nakamura Y., Yamamoto N., Sakai K., Yamazaki S. & Takano T. (1995)
8	KPGRP	67.08	GRP	19.9	Matsufuji H., Matsui T., Seki E., Osajima K., Nakashima M. & Osajima Y. (1994)
9	PSNPP	192.27	NPP	250.9	Arihara K., Nakashima Y., Mukai T., Ishikawa S. & Itoh M. (2001)
10	IAGRP	25.94	GRP	19.9	Matsufuji H., Matsui T., Seki E., Osajima K., Nakashima M. & Osajima Y. (1994)
11	EAPPK	>1000	PPK	>1000	Meisel H., Walsh D. J., Murray B. & Fitzgerald R. J. (2006);
				n.d.	Hayes M., Stanton C., Fitzgerald G.F. & Ross R.P. (2007)
12	PAAPPK	>1000	PPK	>1000	Meisel H., Walsh D. J., Murray B. & Fitzgerald R. J. (2006);
				n.d.	Hayes M., Stanton C., Fitzgerald G.F. & Ross R.P. (2007)
13	KVLPG	265.44	LPG	5.73	Byun H.-G. & Kim S.-K. (2002)
14	TGLKP	51.57	LKP	0.32	Fujita H., Yokoyama K. & Yoshikawa M. (2000)
15	AAATPL	n.d.	AAATP	100	Escudero E., Mora, L., Fraser P.D., Aristoy M.C., Arihara K. & Toldrá F. (2013)
16	KAAAATP	25.64	AAATP	100	Escudero E., Mora, L., Fraser P.D., Aristoy M.C., Arihara K. & Toldrá F. (2013)
17	PTPVP*		PTPVP	256.41	Escudero E., Sentandreu M. A., Arihara K. & Toldrá F. (2010)

a. ACE inhibitory sequences previously identified that share amino acid residues with the sequences identified in this work.; b. IC_{50} of the fragments previously published.; * PTPVP peptide was synthesized and tested *in vitro* and *in vivo* in a previous work.; n.d. means non-detected.

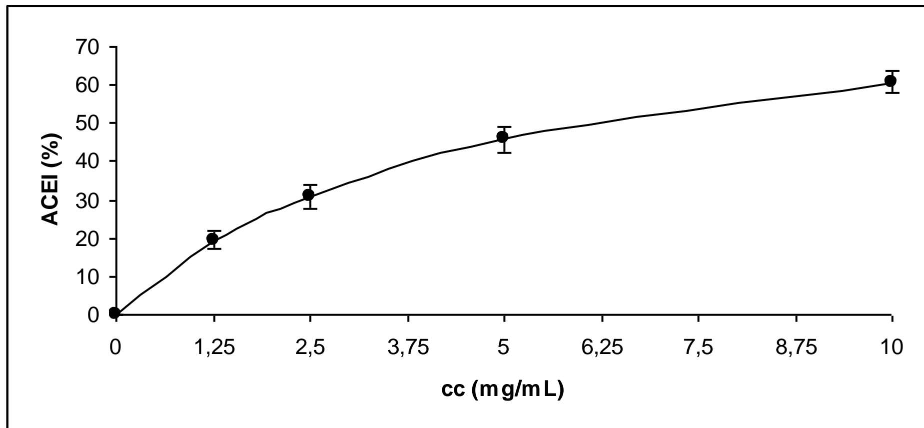
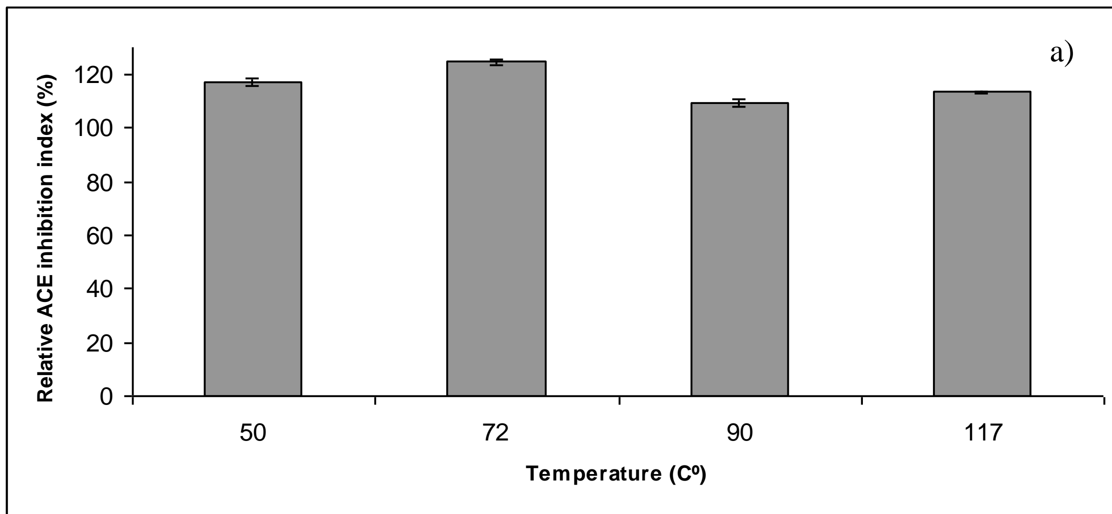


Figure 1

Figure 2



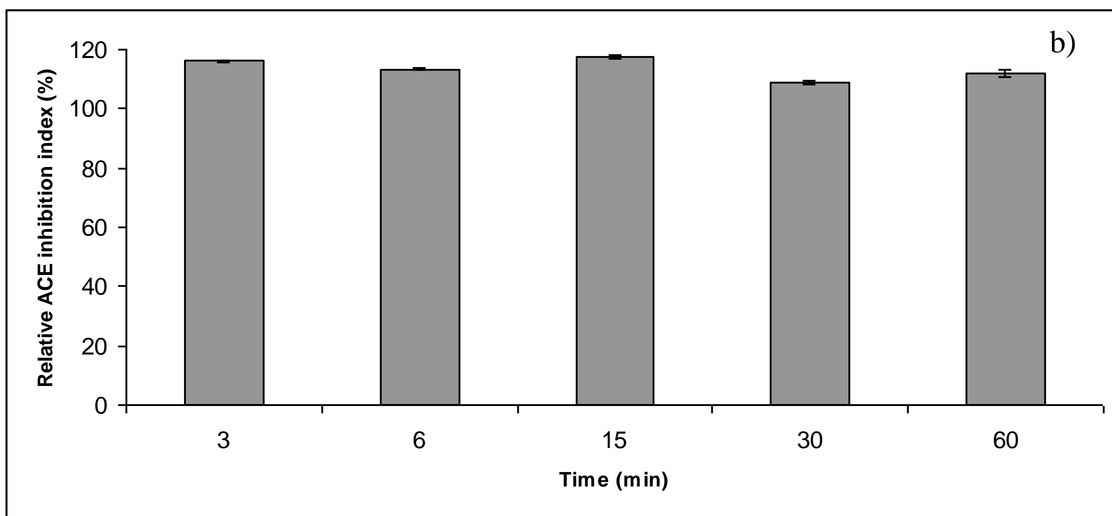


Figure. 2

Figure 3

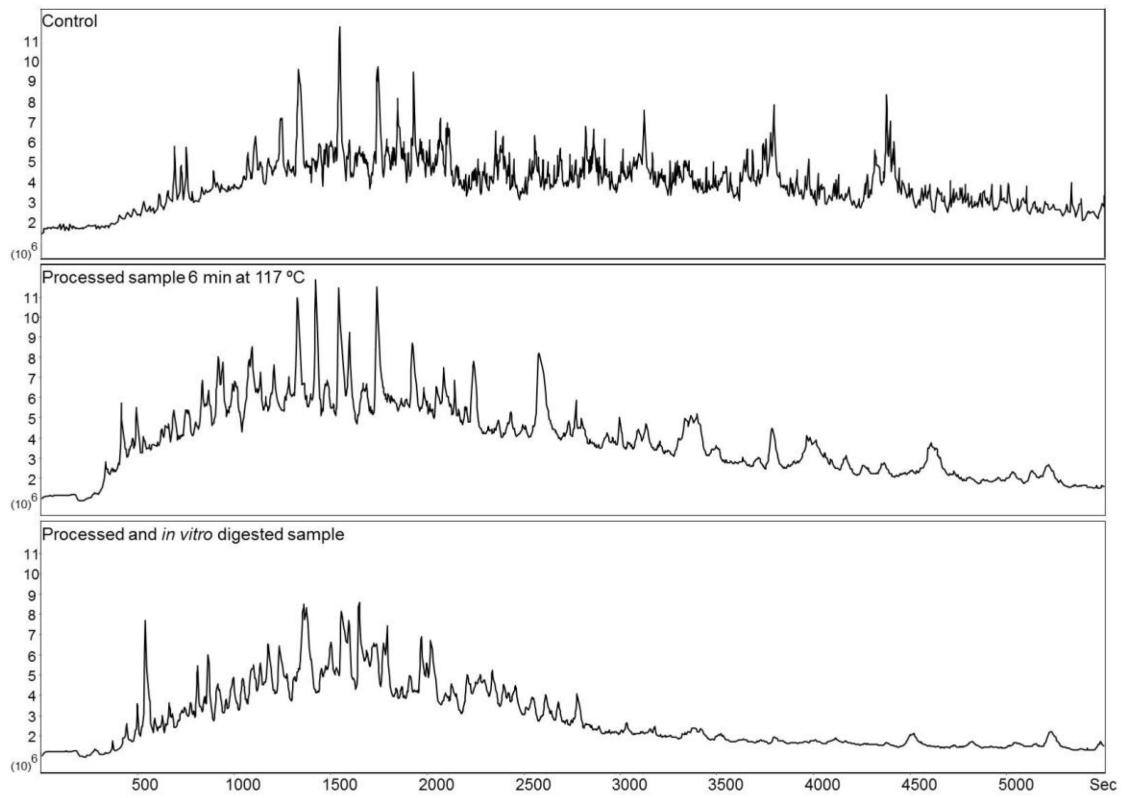


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Figure.3