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Stability of the potent antioxidant peptide SNAAC derived from Spanish dry-cured ham

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Abstract

Antioxidant peptides positively regulate oxidative stress in the human body as well as delay, retard or prevent protein and lipid oxidation in food products. Spanish dry-cured ham has been reported as a good source of bioactive peptides, being SNAAC the most active antioxidant peptide identified to date in a meat product. In this work, the stability of this peptide against *in vitro* digestion, heat treatments and different salt concentrations was evaluated using three methods for measuring antioxidant activity: β-carotene bleaching assay, ABTS radical scavenging capacity and ORAC assay. In general, results evidenced a certain decrease in the antioxidant activity of SNAAC after gastrointestinal digestion, and the MALDI-ToF MS analysis revealed the degradation of the peptide after the process, the generation of the fragment SNAA, and the presence of a peptide dimer throughout the *in vitro* digestion. On the other hand, the peptide SNAAC showed good heat stability (50 ºC, 72 ºC, and 90 ºC), but its antioxidant activity evaluated by ORAC assay decreased substantially when exposed to 100 ºC. SNAAC remained stable in the presence of salt at concentrations ranging from 0 to 8% NaCl as well as it was able to inhibit about 40% of lipid oxidation in an emulsion system. These results reported the stability of the antioxidant peptide SNAAC to several conditions used in meat industry for the processing of dry-cured hams and ham-derived products and its effectiveness to partially prevent the lipid oxidation in these products.

Keywords: Peptides, antioxidant, mass spectrometry, gastrointestinal digestion, processing, oxidation.

1. Introduction

Spanish dry-cured ham is a high quality product resulting from a long and complex processing that involves an intense degradation of proteins into numerous peptides and amino acids (Toldrá & Flores, 1998). This product has been studied as a natural source of bioactive peptides due to the large amount of low molecular weight peptides naturally generated during its processing. So, several works have reported the identification of peptides derived from Spanish dry-cured ham showing *in vitro* bioactivities such as antihypertensive (Escudero, Mora, Fraser, Aristoy, Arihara, & Toldrá, 2013), antioxidant (Escudero, Mora, Fraser, Aristoy, & Toldrá, 2013), antidiabetic (Gallego, Aristoy, & Toldrá, 2014), and antimicrobial properties (Castellano et al., 2016).

Antioxidant activity is one of the most common studied bioactivity as antioxidant compounds positively regulate oxidative stress that benefit human health as well as delay, retard or prevent protein and lipid oxidation, which are processes related to quality deterioration of products (Liu, Xing, Fu, Zhou, & Zhang, 2016). Up to date, there is no standarised assay to characterise the overall antioxidant activity of a sample, thus it is recommended to use different antioxidant methods (Zulueta, Esteve, & Frígola, 2009). The methodologies typically used to assess the antioxidative potential of samples are classified into i) methods based on hydrogen atom transfer (HAT), where antioxidant and substrate compete for quenching free radicals, and ii) methods based on electron transfer (ET), where an antioxidant transfers one electron to reduce an oxidant. HAT-based assays include oxygen radical absorbance capacity (ORAC), total radical trapping antioxidant parameter (TRAP) and β-carotene bleaching assay, whereas ET-based assays comprise DPPH radical scavenging activity, ferric-reducing antioxidant power, and ABTS radical scavenging assay (Prior, Wu, & Schaich, 2005; Huang, Ou, &

Prior, 2005; MacDonald-Wicks, Wood, & Garg, 2006). Moreover, the antioxidant activity can be evaluated through the measurement of the capacity to inhibit lipid oxidation, using commonly the thiobarbituric acid reactive substances (TBARS) method. TBARS measures the concentration of malondialdehyde (MDA), which is one of the most abundant aldehydes generated during secondary lipid oxidation and frequently used as oxidation marker (Antolovich, Prenzler, Patsalides, McDonald, & Robards, 2002).

Several works have been focused on the identification of antioxidant peptides naturally generated in Spanish dry-cured hams (Escudero, Aristoy, Nishimura, Arihara, & Toldrá, 2012; Escudero, Mora, Fraser, Aristoy, & Toldrá, 2013; Mora, Escudero, Fraser, Aristoy, & Toldrá, 2014), being the peptide SNAAC, which is derived from the degradation of myosin heavy chain protein, the most active antioxidant identified to date. In the study done by Mora et al. (2014), SNAAC showed an IC_{50} value of 75.2 µM in DPPH radical scavenging assay, and 205 µM in ferric-reducing antioxidant power analysis, values similar to the positive control BHT (butylated hydroxytoluene). However, the peptide must resist the degradation by gastrointestinal proteases and be absorbed intact through the intestinal barrier to exert its physiological effect in the human system (Vercruysse, Van Camp, & Smagghe, 2005). The stability of SNAAC to temperature and processing conditions used for dry-cured hams and ham-derived products in meat industries should also be considered.

Thus, the aim of the present study was to evaluate the stability of the potent antioxidant peptide SNAAC to *in vitro* gastrointestinal digestion, heat treatments and different salt concentration as well as its effectiveness in retarding lipid oxidation in an emulsion system.

2. Materials and methods

2.1 Chemicals and reagents

Peptide SNAAC was synthesised by GenScript Corporation (Piscataway, NJ, USA) at the highest purity certified using liquid-chromatography mass spectrometry (LC-MS) analysis. Enzymes for *in vitro* digestion: salivary α-amylase, porcine pepsin, porcine pancreatic α-amylase, porcine pancreatic lipase, and porcine bile extract were purchased from Sigma-Aldrich, Co. (St. Louis, MO, USA), whereas trypsin and chymotrypsin were from Fluka (Sigma-Aldrich, Co., St. Louis, MO, USA). 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), fluorescein, 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH), β-carotene, linoleic acid, $FeCl_2$, 2-thiobarbituric acid (TBA), 1,1,3,3-tetramethoxypropan (TMP) were from Sigma-Aldrich, Co. (St. Louis, MO, USA). Potassium persulfate, butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), Triton X-100 were purchased from Panreac Quimica S.A.U. (Barcelona, Spain), whereas trichloroacetic acid (TCA) was from Sharlab, S.L. (Barcelona, Spain). All other chemicals and reagents were of analytical grade.

2.2 Stability of the peptide SNAAC

2.2.1 *In vitro* gastrointestinal digestion

The peptide SNAAC was subjected to *in vitro* digestion according to the method described by Minekus et al. (2014) with some modifications. Briefly, 6 mg of the peptide was suspended in 1.2 mL of 0.2 M $NaHCO_3$ (pH 7.0). The oral phase was simulated adding 17 U/mL of salivary α-amylase solution and 37 µL of 50 mM $CaCl_2$, maintaining the mixture at 37 ºC for 3 min. In the gastric phase, the pH was adjusted to 3.0, adding pepsin to achieve 2000 U/mL in the final mixture and 4 µL of 50 mM $CaCl_2$.

130 After 3 h of digestion at 37 ºC and continuous stirring, the enzyme was inactivated by
131 adjusting pH to 7.0. In the intestinal phase, enzymes were added to achieve the
132 following activities in the final mixture: 100 U/mL of trypsin, 25 U/mL of
133 chymotrypsin, 200 U/mL of pancreatic α-amylase, 2000 U/mL of pancreatic lipase and
134 10 mM of bile extract. A total of 16 µL of 50 mM $CaCl_2$ was also added and the mixture
135 was maintained at 37 ºC for 3h, finishing the process by heating for 2 min at 95 ºC. The
136 sample was deproteinised (3V ethanol, 4 °C, 20 h), centrifuged (12,000 *g*, 4 °C, 10 min),
137 and the resultant supernatant was dried in a rotatory evaporator and lyophilised. The
138 digestion was carried out in triplicate. For subsequent antioxidant assays, stock
139 solutions of 1 mg/mL in bidistilled water were prepared.

140 **2.2.2 Temperature and salt content**

141 Peptide SNAAC solutions (1 mg/mL) were treated at different temperatures: 37 ºC
142 (control), 50 ºC, 72 ºC, and 90 ºC for 10 min, as well as exposed to 100 ºC for 10 min at
143 different salt concentrations: 0 % (control), 2%, 4%, and 8% NaCl. Samples were then
144 cooled to room temperature in ice. Three replicates were done for each treatment.

145 **2.3 Antioxidant activity**

146 The antioxidant capacity of the peptide SNAAC subjected to *in vitro* digestion, heat
147 treatments and different salt contents was evaluated in triplicate using three different
148 methods.

149 **2.3.1 β-carotene bleaching assay**

150 The β-carotene bleaching assay was performed according to the method described by
151 Koleva, Van Beek, Linssen, De Groot, and Evstatieva (2002) with some modifications.
152 A total of 0.5 mg of β-carotene, 20 µL of linoleic acid and 200 µL of Tween 80 were
153 dissolved in 1 mL of chloroform to obtain the β-carotene/linoleic acid solution. Then,
154 chloroform was totally evaporated in a rotatory evaporator, and 100 mL of bidistilled

water was added and vigorously stirred. Finally, 50 µL of sample was mixed with 250 µL of the β-carotene solution and the absorbance was measured at 450 nm immediately (t_0) and after 180 min of incubation at 50 ºC in dark (t_f). Bidistilled water was used as blank and BHA as positive control. The antioxidant activity was calculated as: Antioxidant activity (%) = (1 - (Absorbance sample t_0 – Absorbance sample t_f) / (Absorbance blank t_0 – Absorbance blank t_f)) x 100.

2.3.2 Oxygen radical absorbance capacity assay (ORAC)

ORAC assay using fluorescein (FL) was performed as described by Dávalos, Gómez-Cordovés, and Bartolomé (2004) with some modifications. A total of 140 µL of sample at different concentrations was prepared in 75 mM phosphate buffer (pH 7.4), mixed with 70 µL of 200 nM fluorescein and incubated at 37 ºC in dark for 15 min. Then, 70 µL of 80 mM AAPH was added and the fluorescence was measured exactly every min for 100 min using excitation and emission wavelengths of 485 and 538 nm, respectively. Tryptophan was used as positive control and different concentrations of Trolox (2–16 µM) were used to obtain a standard curve. The integration of the relative fluorescence curve was used to calculate the area under curve (AUC) was calculated for each sample by integrating the relative fluorescence curve. The ORAC-FL values of samples were calculated and plotted against the concentration of trolox, expressing the results as nmol of TE (Trolox equivalents) per mg of sample.

2.3.3. ABTS radical scavenging capacity

The ABTS assay was carried out according to the methodology described by described by Re et al. (1999) with slight modifications. Briefly, 7 mM ABTS was dissolved in 2.45 mM potassium persulfate, and the mixture was kept in the dark at room temperature for 12-16 h to produce ABTS$^{\cdot+}$. The ABTS$^{\cdot+}$ solution was diluted with 50 mM phosphate buffer saline (PBS) (pH 7.4) in order to obtain an absorbance of 0.70 ±

180 0.02 at 734 nm. Then, 10 μL of sample at different concentrations was mixed with 990
181 μL of $ABTS^{\cdot+}$ solution, measuring the absorbance at 734 nm after 6 min of incubation
182 in dark. In the assays, ascorbic acid was used as positive control, PBS as negative
183 control, and different concentrations of Trolox (0.05–2 mM) were used to obtain a
184 calibration curve. The ABTS radical scavenging activity of samples was calculated and
185 plotted against the concentration of trolox, expressing the results as nmol of TEAC
186 (trolox equivalent antioxidant capacity) per mg of sample.

187 **2.4 Lipid oxidation in a linoleic acid emulsion**

188 The antioxidant capacity of the peptide SNAAC on lipid oxidation was evaluated in a
189 linoleic acid emulsion. The emulsion system was prepared with 10 mg of linoleic acid
190 and 4 mL of 0.1 M potassium phosphate buffer (pH 7) containing 0.5 % Triton X-100
191 and 0.05 mM $FeCl_2$, mixing by sonication at 40 ºC for 4 min.

192 Lipid oxidation was evaluated by the formation of thiobarbituric acid reactive
193 substances (TBARS) according to the method described by Buege and Aust (1978). For
194 that, 100 μL of the peptide SNAAC (1mg/mL) was added to 4 mL of linoleic acid
195 emulsion, maintaining the mixture at 80 ºC. TBARS assays were performed at different
196 time intervals by mixing 250 μL of the emulsion sample, 1 mL of TBA solution (0.375
197 % TBA, 15 % TCA, 0.04 % BHT in 0.25 M HCl), and 250 μL of 10 mM buffer sodium
198 phosphate (pH 7). The mixture was heated at 95 ºC for 15 min, and after cooling, it was
199 centrifuged at 10000 *g* for 5 min. Finally, the absorbance of the supernatant was
200 measured at 532 nm. BHT was used as positive control and different concentrations of
201 TMP (0.75–25 μM) were used as standard. The results were expressed as μM
202 malonaldehyde (MDA). The percentage of lipid oxidation inhibition measured after 9
203 hours incubation at 80 ºC was calculated as: Lipid oxidation (%) = (Absorbance blank –
204 Absorbance sample) x 100 / Absorbance blank.

205 **2.5 MALDI-ToF MS analysis**

206 In order to analyse the degradation of the peptide SNAAC after the *in vitro* digestion, 207 samples were analysed by matrix-assisted laser desorption/ionization time-of-flight 208 mass spectrometer (MALDI-ToF MS) using a 5800 MALDI-ToF/ToF (AB Sciex, MA, 209 USA). Samples before digestion (t0), after gastric phase (G) and after gastrointestinal 210 digestion (GI) were resuspended in 0.1 % trifluoroacetic acid (TFA) to prepare 1 211 mg/mL solutions. A total of 1 µL of each sample was spotted onto the MALDI target 212 plate, and the droplets were air-dried at room temperature. Then, 0.5 µL of matrix (5 213 mg/mL α-cyano-4-hydroxycinnamic acid (CHCA), Sigma-Aldrich, Co., St. Louis, MO, 214 USA) in acetonitrile:H_2O (7:3, v/v) with 0.1% TFA was added and air-dried at room 215 temperature. The plate was introduced in the MALDI-ToF/ToF instrument, which was 216 used in automatic positive-ion reflector mode for mass analysis between 150 and 600 217 Da. Previously, the plate and the acquisition method were calibrated with 1 µL of 218 TOF/TOF calibration mixture (AB Sciex, MA, USA) in 13 positions.

219 **2.6 Statistical analysis**

220 Statistical analysis was carried out using XLSTAT 2011 v5.01 (Addinsoft, Barcelona, 221 Spain). One-way analysis of variance (ANOVA) was performed for antioxidant and 222 TBARS assays. Fisher's multiple range test was used to evaluate significant differences 223 among mean values at $p < 0.05$.

224

225 **3. Results and discussion**

226 Most of antioxidant peptides from food sources have molecular weights from 400 to 227 2000 Da and they often include in their sequences amino acid residues such as Tyr, Trp, 228 Phe, Met, Cys, and His, which can act as free radicals scavengers, reducing agents, 229 metal chelators and inhibitors of lipid peroxidation (Samaranayaka, & Li-Chan, 2011;

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65

Power, Jakeman, & FitzGerald, 2013; Liu et al., 2016). Peptides are generally more effective as antioxidants than free amino acids due to their unique physico-chemical properties conferred by the composition and position of amino acids, structure, and hydrophobicity (Elias, Kellerby, & Decker, 2008; Sarmadi & Ismail, 2010). The peptide SNAAC, with a molecular weight of 464.17 Da, has been reported as a good antioxidant derived from Spanish dry-cured ham (Mora et al., 2014). Among the amino acid residues that this peptide contains in its sequence, Cys has been described as a potent antioxidant due to it is often involved in electron transfer and hydrogen donor reactions, having properties such as free radical scavenger, reducing agent and metal ion chelation (Atmaca, 2004; Piste, 2013). Additionally, the hydrophobic amino acid Ala could also act as free radical scavenger, although it is less effective (Liu et al., 2016).

3.1 Stability of the antioxidant peptide SNAAC

3.1.1 Effect of simulated gastrointestinal digestion

The peptide SNAAC was subjected to *in vitro* digestion, measuring its antioxidant activity before digestion (t0), after the gastric phase (G), and at the end of the gastrointestinal digestion (GI) (Figure 1). HAT-based methods (β-carotene bleaching assay and ORAC assay) showed that the peptide before digestion had the highest antioxidant activity, followed by the peptide after the complete GI digestion. On the other hand, results obtained from ABTS radical scavenging capacity indicated that there were not significant differences for the antioxidant activity prior and after to digestion (GI) (Figure 1C). In all used methods, the lowest antioxidant activity was obtained after the peptide was subjected to the gastric phase, where salivary α-amylase and pepsin were used. Many peptides are reduced to smaller fragments by pepsin, whereas they can be fully degraded by trypsin enzyme, with greater hydrolytic activity. So, enzymatic actions lead to expose internal groups and release free amino acids, which affect the

255 amount, size, structure, amino acid composition and hydrophobicity of peptides, and
256 thus their antioxidant capacity (Samaranayaka & Li-Chan, 2011; Zhu, Zhang, Kang,
257 Zhou, & Xu, 2014; Jang, Liceaga, & Yoon, 2016). In this work, the gastric phase seems
258 to alter the structure of the peptide, affecting negatively its antioxidant activity.
259 However, the active structure of SNAAC was not fully disrupted during the process as
260 high antioxidant activity was measured after the GI digestion.

261 In order to better understand the results obtained from antioxidant assays, a MALDI-
262 ToF MS analysis was done to study the degradation of the peptide SNAAC throughout
263 the *in vitro* gastrointestinal digestion (see Table 1). The intact peptide was detected
264 before digestion (t0) and after the G phase, but not after the GI digestion as it was
265 hydrolysed by the enzymes used during the process. Indeed, part of the peptide SNAAC
266 was already degraded during the G step, generating the fragment SNAA for releasing
267 the free amino acid Cys. This fragment was also detected at the end of the GI process.
268 Additionally, the analysis detected a peptide dimer structure during the entire digestion.
269 The amino acid Cys presented in the sequence of SNAAC can be oxidised and form a
270 disulfide bond derived from coupling of thiol groups (-SH) (Atmaca, 2004; Piste, 2013).
271 In fact, the mechanism for free-radical mediated oxidation by which cysteine can act as
272 antioxidant involves the formation of the dimer (Darkwa, Mundoma, & Simoyi, 1998;
273 Liu et al., 2009). This dimerization could increase the solubility of the peptide and its
274 resistance against proteases (Santos-Filho et al., 2015; Falciani et al., 2007), which
275 would explain the presence of the SNAAC dimer throughout the GI digestion.
276 Moreover, it has been described that the formation of a peptide dimer through a
277 disulfide bond could improve the activity of the peptide (Santos-Filho et al., 2015;
278 Thamri et al., 2017), although this effect is still unclear (Yang, Kim, & Shin, 2009;
279 Lorenzon et al., 2012).

3.1.2 Effect of temperature

The stability of the peptide SNAAC to different temperatures was evaluated by measuring the antioxidant activity. Figure 2 shows that there were no great differences between the control (37 ºC) and treatments at higher temperatures (52 ºC, 72 ºC, and 90 ºC). β-carotene bleaching assay revealed that the antioxidant activity at the highest concentration tested was slightly improved when the peptide was treated at 52 ºC and 90 ºC, whereas the ORAC assay showed a little decline as the temperature increased. So, the antioxidant activity of the peptide treated at 72 ºC and 90 ºC decreased about 8 % and 14 %, respectively, compared to the control. ABTS radical scavenging capacity did not show significant differences between the treatments, showing values around 2700 nmol TEAC/mg peptide (Figure 2C). Temperatures higher than 60 ºC could affect the secondary structure of the peptides, leading to a decrease in the antioxidant activity (Zhu et al., 2014; Wang et al., 2017). However, the peptide SNAAC showed good heat stability, maintaining a high antioxidant activity after exposure to different temperatures.

3.1.3 Effect of salt content

As it is shown in Figure 3, the antioxidant activity of the peptide SNAAC was not modified in the presence of NaCl when was measured by using ABTS radical scavenging capacity and ORAC assay, reaching values around 2600 and 1600 nmol TEAC/mg peptide, respectively, in all treatments. However, β-carotene bleaching assay indicated an increase of 20-30 % in the antioxidant activity of samples with salt compared to the control (0 % NaCl), which may result from some interferences during this assay due to the presence of salt (Figure 3A). Zhu et al (2014) reported that high salt contents (> 8 %) can disrupt the structure of peptides affecting the stability of the antioxidant activity, whereas Jang et al. (2016) did not found a predictive correlation between peptide stability and salt content. In the present work, the antioxidant peptide

SNAAC remained stable in the presence of NaCl at concentrations ranging from 0 to 8%.

Additionally, the ORAC assay showed a 40 % reduction in the antioxidant activity (Figure 3B) when these samples, which were exposed to 100 ºC for 10 min with different content of salt, were compared with the control peptide at 37 ºC (Figure 2B). These results could indicate a possible modification of the peptide structure due to extremely high temperatures rather than the presence of salt, which would lead to instability of the antioxidant activity of SNAAC.

3.2 Effect on lipid oxidation inhibition

A linoleic acid emulsion system was used to evaluate the effectiveness of the peptide SNAAC to inhibit lipid oxidation. As it is shown in Figure 4A, TBARS assay revealed a sharp increase of lipid oxidation in the emulsion system (control) up to 9 hours at 80 ºC, and then a gradual reduction until 40 hours. The formation of MDA reached a maximum value of 2.49 µM in the control, whereas it was 1.56 µM MDA in the presence of SNAAC. When the results were expressed as percentage of lipid oxidation (Figure 4B), SNAAC inhibited lipid oxidation to 61.5% while BHT (positive control) was able to reduce it to 6.7 % compared to the control. These results showed the effectiveness of SNAAC to prevent lipid oxidation, although it did not present as potent antioxidant activity as BHT.

Several amino acids such as Cys, Trp, Pro, Lys, and His display antioxidant activity in emulsion systems by inactivation of free radicals and metal chelation. Moreover, Cys and His have been reported to retard lipid oxidation by donating hydrogen from the thiol group and imidazole ring that contain, respectively, in their structure (Hernández-Ledesma, Dávalos, Bartolomé, & Amigo, 2005; Park, Nakamura, Sato, & Matsumura, 2012). Nevertheless, their effectiveness on lipid oxidation depends in both their

chemical structure and concentration, as some amino acids can act as pro-oxidants when they are present in high amount (Park et al., 2012)

4. Conclusions

The results of the present work evidenced that the antioxidant activity of the peptide SNAAC derived from Spanish dry-cured ham decreased after the simulated GI digestion, mainly after the G phase, due to the action of digestive enzymes that expose internal groups and release free amino acids which probably modifies the antioxidant capacity of the peptide. In fact, the analysis by MALDI-ToF MS revealed the degradation of the intact peptide at the end of the digestion as well as the generation of the fragment SNAA during the G and GI phases. Moreover, a peptide dimer structure was detected throughout the *in vitro* digestion, which could increase the activity, solubility and resistance of the peptide against proteases. On the other hand, the peptide SNAAC showed good heat stability after exposure to temperatures up to 90 ºC, remained stable in the presence of NaCl, and was effective to inhibit almost half of lipid oxidation in a linoleic acid emulsion. These results reported the stability of the antioxidant peptide SNAAC to several conditions used in meat industry for the processing of ham products and its effectiveness to prevent partially lipid oxidation. However, further studies are needed in order to study the bioavailability and physiologically effects of the peptide SNAAC in the human system.

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357

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465

466 **FIGURE CAPTIONS**

467 **Figure 1.** Antioxidant activity of the peptide SNAAC subjected to *in vitro*
468 gastrointestinal digestion (t0: before digestion, G: after gastric phase, GI: after
469 gastrointestinal digestion) measured by different methods: A) β-carotene bleaching
470 assay, B) ORAC assay, and C) ABTS radical scavenging capacity. The values represent
471 means of 3 replicates ± standard deviations, and bar letters indicate significant
472 differences among the values at $p < 0.05$.

473 **Figure 2.** Antioxidant activity of the peptide SNAAC treated at different temperatures
474 (37 ºC: control, 50 ºC, 72 ºC, and 90 ºC for 10 min) measured by different methods: A)
475 β-carotene bleaching assay, B) ORAC assay, and C) ABTS radical scavenging capacity.
476 The values represent means of 3 replicates ± standard deviations, and bar letters indicate
477 significant differences among the values at $p < 0.05$.

478 **Figure 3.** Antioxidant activity of the peptide SNAAC exposed to 100 ºC for 10 min at
479 different salt concentrations (0%: control, 2%, 4%, and 8% NaCl) measured by different
480 methods: A) β-carotene bleaching assay, B) ORAC assay, and C) ABTS radical
481 scavenging capacity. The values represent means of 3 replicates ± standard deviations,
482 and bar letters indicate significant differences among the values at $p < 0.05$.

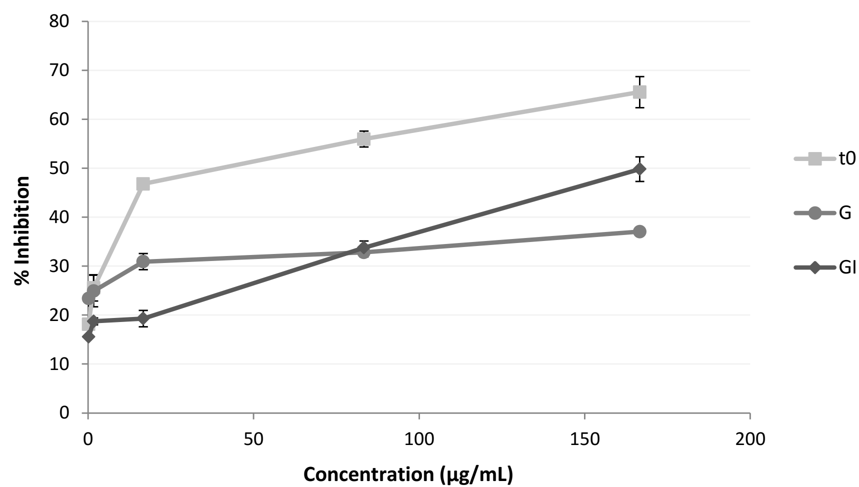
483 **Figure 4.** Effect of the peptide SNAAC on oxidation of a linoleic acid emulsion. A)
484 TBARS values expressed as μM of MDA. B) Percentage of lipid oxidation measured
485 after 9 hours incubation at 80 ºC. The values represent means of 3 replicates ± standard
486 deviations, and bar letters indicate significant differences among the values at $p < 0.05$.

487

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65

Figure 1

A)



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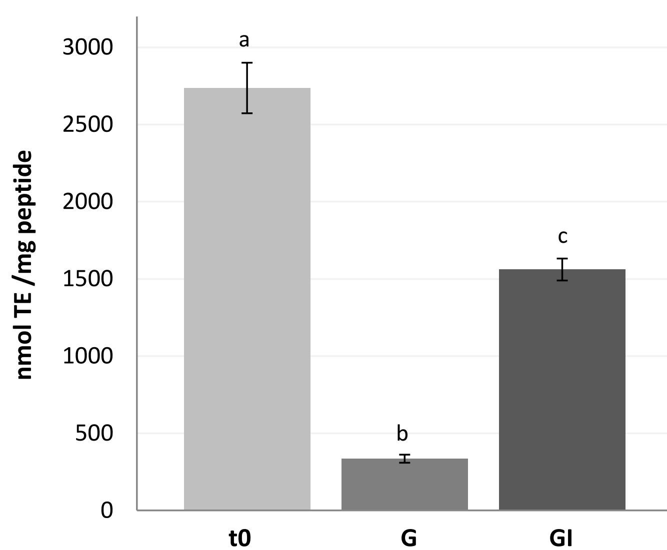

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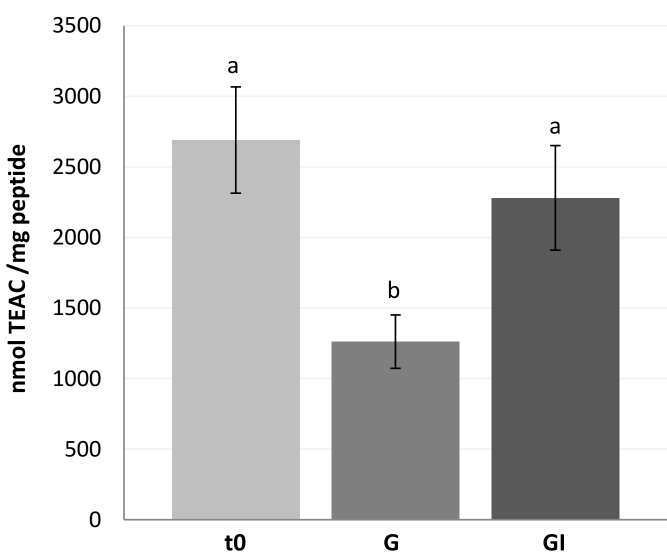

Figure 1.

Figure 2

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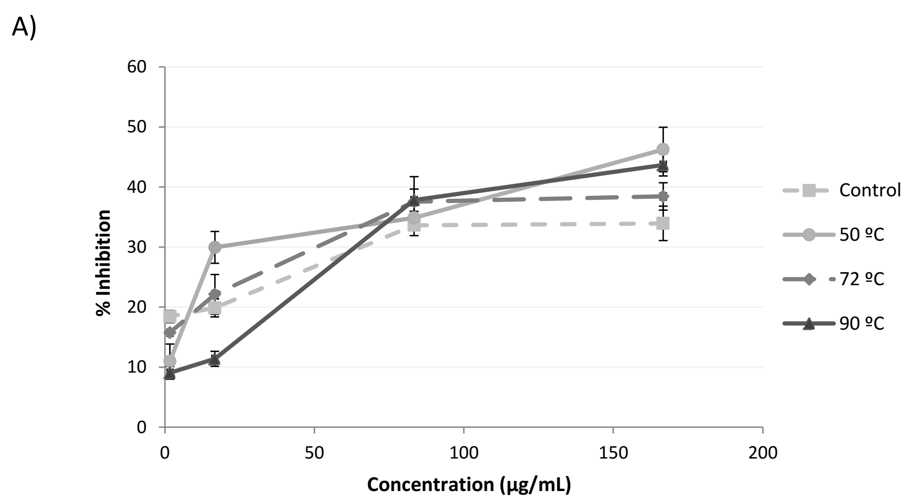

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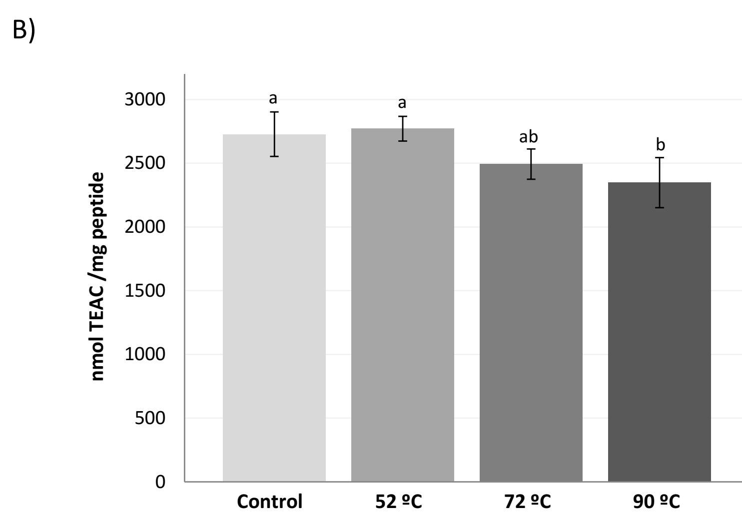

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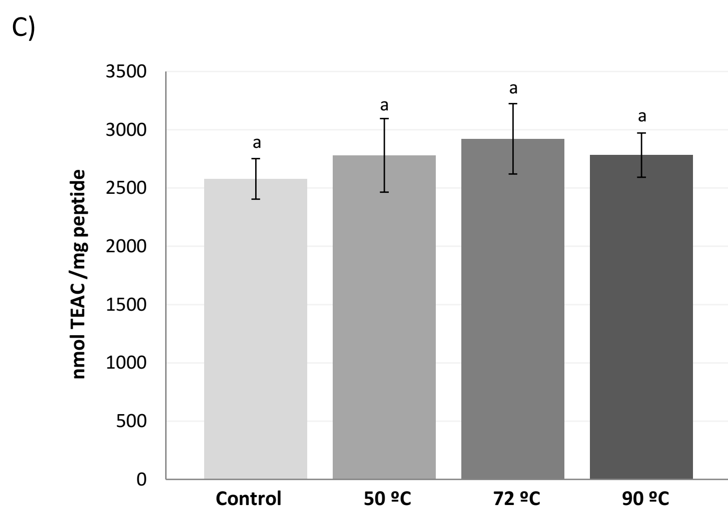
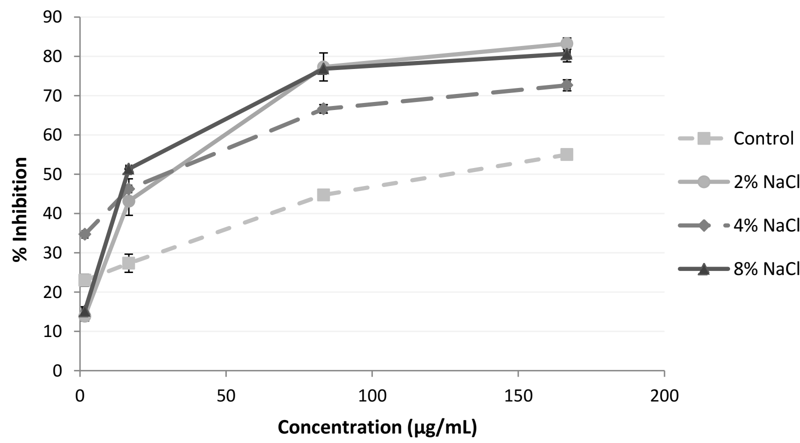


Figure 2.

Figure 3

A)



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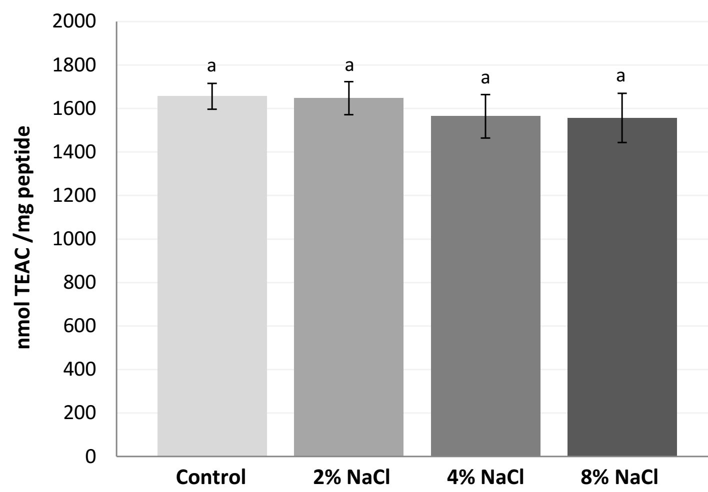

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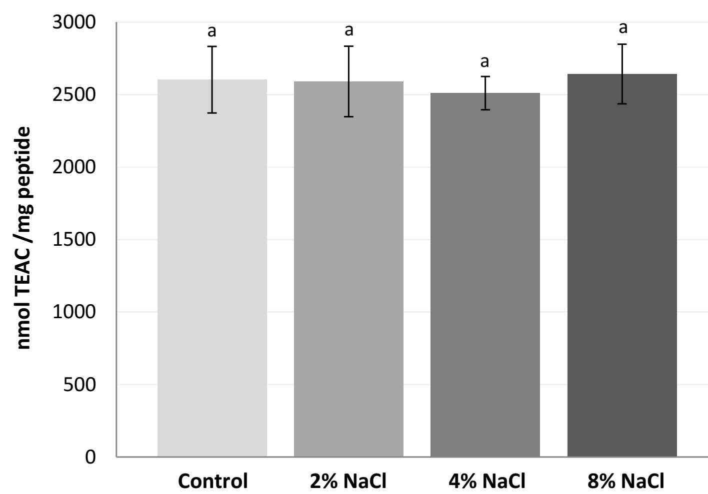
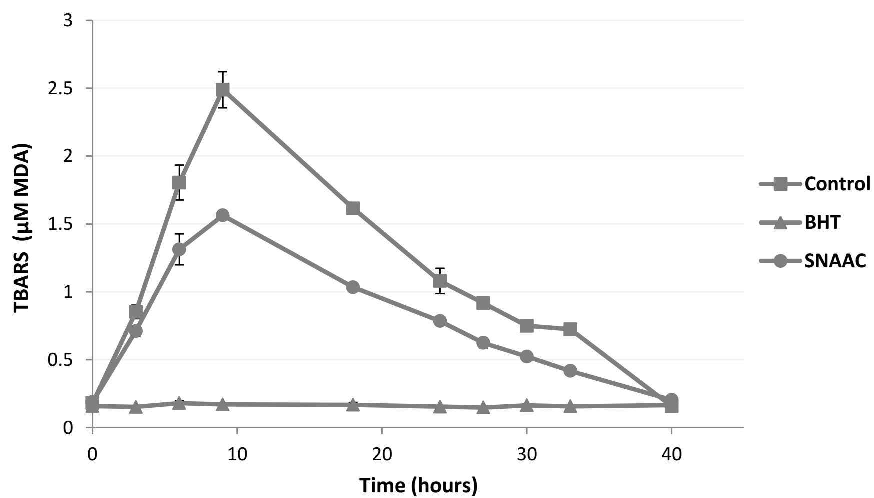


Figure 3.

Figure 4

A)



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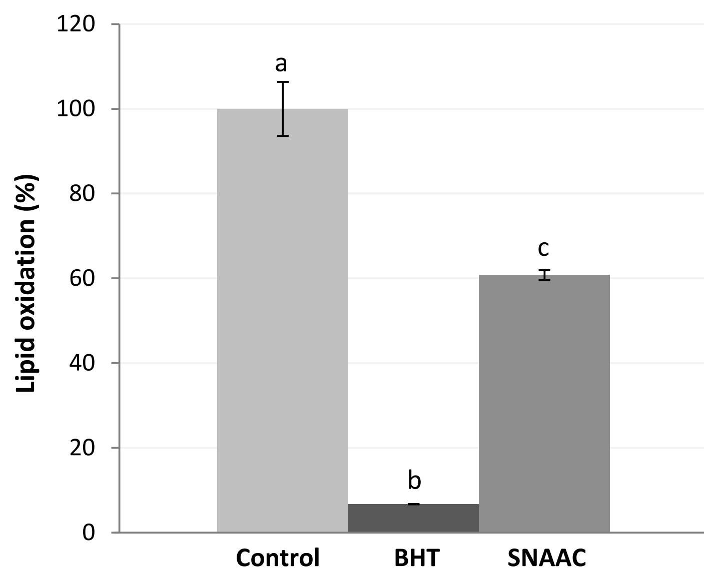

Figure 4.

Table 1

Table 1. Peptide sequences derived from SNAAC detected by using MALDI-ToF/ToF MS during the *in vitro* gastrointestinal digestion.

Precursor peptide	Peptide fragments[a]	Monoisotopic mass (Da)[b]	Samples t0[c]	G[d]	GI[e]
SNAAC		464.17	x	x	—
	NAAC	377.14	—	—	—
	SNAA	361.16	—	x	x
	SNA	290.12	—	—	—
	NAA	274.13	—	—	—
	AAC	263.09	—	—	—
	SN	219.09	—	—	—
	NA	203.09	—	—	—
	AC	192.06	—	—	—
	AA	160.08	—	—	—
SNAAC dimer		926.38	x	x	x

a Possible fragments derived from the degradation of the precursor peptide. b Monoisotopic molecular mass in Daltons of the matched peptide.c Peptides detected before the digestion. d Peptides detected after the gastric phase. e Peptides detected after the gastrointestinal digestion.x = detected; — = non-detected.