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1 **COMPOSITIONAL PROPERTIES AND BIOACTIVE POTENTIAL OF WASTE MATERIAL FROM**
2 **SHRIMP COOKING JUICE**

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12 requirements of the following standard: ISO 9001:2008.

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ABSTRACT

A functional concentrate rich in proteins and lipids was obtained from an industrial shrimp cooking juice by using a centrifugal separator, and it was characterized in terms of chemical composition parameters and functional or biological activity (antioxidant and ACE-inhibitory capacities). The concentrate presented 116 g/kg protein, 135 g/kg crude fat and ash content less than 10 g/kg, with predominance of Na ions, followed by K, Ca, Mg, Cu, Fe, Zn and Mn. A relative abundance of glucose, glycerol, polyalcohols, acetate and phosphate was found. The antioxidant (as determined by ABTS, FRAP, chelating and photoluminescence assays) and ACE-inhibitory capacities of the material recovered from the shrimp cooking juice were strongly related to the presence of small peptides (1355-502 Da), with a clear predominance of Gly, Pro, Glu, Asp and Arg. Other antioxidants, such as free astaxanthin (*cis* and *trans* isomers) and derived esters, were also detected.

Keywords: shrimp, cooking juice, concentrate, antioxidant, ACE-inhibitory peptides

28 **1. Introduction**

29 The process of cooking shellfish generates a large amount of effluent with a high organic load
30 and generally also high salt contents, which represents an environmental hazard and is subject
31 to wastewater treatment regulations (Cros, Lignot, Jaouen, & Bourseau, 2006). Thus, the
32 recovery of the residual organic material present in crustacean cooking water could reduce the
33 cost of depolluting treatments, and it would also involve a valorization of waste and a search
34 for potentially bioactive molecules. Crustacean species such as shrimp, crab, lobster, etc. are
35 rich sources of amino acids, peptides, protein and other useful biochemicals, such as sugars,
36 organic acids, carotenoids, etc., which may be recovered for utilization as ingredients in
37 various food applications (Simpson, Nayeri, Yaylayan, & Ashie, 1998). The resulting protein
38 powders can also be used in feed formulations for farm animals and also for fabricated
39 seafoods such as shrimp analogue or shrimp crackers in order to provide essential amino acids
40 as well as carotenoids. Astaxanthin is the major naturally occurring carotenoid pigment in
41 marine crustaceans and is the main compound responsible for their typical orange-pink
42 colouration. Carotenoids are normally found forming strong associations with proteins and
43 fatty acids, therefore cooking treatments and enzymatic hydrolysis have been shown to be
44 successful pre-treatments to recover free carotenoids from crustaceans (Simpson et al., 1998;
45 Mezzomo, Maestri, Dos Santos, Maraschin, & Ferreira, 2011; Sowmya, Rathinaraj, & Sachindra,
46 2011).

47 The process of cooking shrimp and oyster has been shown to generate a large amount of
48 effluent containing appreciable amounts of soluble components, such as peptides, amino
49 acids, glycogen, and other organic compounds (Cambero, Jaramillo, Ordoñez, Cobos, Pereira-
50 Lima, & García de Fernando, 1998; Kim et al., 2000). These effluents are also a source of
51 seafood flavouring compounds (Cambero et al., 1998), owing to the presence of certain amino
52 acids, nucleotide-related compounds, organic acids and Maillard reactions between free amino

53 acids and sugars present in the cooking juice as a result of thermal hydrolysis (Mandeville,
54 Yaylayan, & Simpson, 1992), or else favoured by enzymatic hydrolysis (Kim et al., 2000).
55 Cooking effluents, however, may also contain high amounts of salts, and therefore a two-step
56 membrane process, combining desalination by electrodialysis and concentration by reverse
57 osmosis, has been reported to be technically feasible to produce aroma concentrates from
58 shrimp cooking juices (Cros et al., 2006).

59 Shrimp wastes subjected to enzymatic hydrolysis have been shown to be an important source
60 of bioactive peptides with antioxidant (Guerard, Sumaya-Martínez, Laroque, Chabeaud, &
61 Dufossé, 2007) and antihypertensive activity (inhibiting angiotensin I-converting enzyme, ACE)
62 (Cheung, & Li-Chan, 2010). Antioxidant peptide sequences obtained from *Penaeus japonicus*
63 (Suetsuna, Ukeda & Ochi, 2000) and ACE-inhibitory peptides obtained from *Acetes chinensis*
64 (He, Chen, Sun, Zhang, & Zhou, 2006) have been identified. However, to our knowledge,
65 bioactive peptides have not been yet reported from shrimp cooking effluents.

66 The aim of the present work is to obtain a functional concentrate from industrial shrimp
67 (*Penaeus* spp.) cooking juice and to explore its bioactive potential by determining different
68 antioxidative mechanisms and antihypertensive (ACE-inhibitory) capacity.

69 **2. Materials and methods**

70 **2.1. Materials**

71 Shrimp (*Penaeus* spp.) cooking juice (CJ) (90 L) was obtained from a local crustacean
72 processing factory (Gambastar, Burgos, Spain) operating under industrial conditions,
73 immediately before being sent to the water treatment plant. The recovered cooking juice was
74 tempered and frozen at –20 °C until use. Nitric acid and hydrogen peroxide were purchased
75 from Panreac (Moncada i Reixac, Barcelona, Spain). HPLC grade acetonitrile, methanol and
76 formic acid were from VWR international Inc. (Barcelona, Spain). HPLC grade water was

77 prepared from distilled water using a Milli-Q system (Millipore Laboratory, Bedford, MA).
78 Methyl *t*-butyl ether (MTBE) was obtained from Labscan Ltd. (Dublin, Ireland). Vitamin C, all-
79 trans astaxanthin, ABTS radical [2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)],
80 potassium persulphate, $FeCl_3$, $FeSO_4$, ascorbic acid, Trolox and 2,4,6-tripyridyl-s-triazine were
81 from Sigma-Aldrich (St. Louis, MO, USA). ACW and ACL kits, reagent 2 and reagent 3 for the
82 photochemiluminescence assay (Photochem®) were purchased from Analytik Jena AG (Jena,
83 Germany).

84 **2.2. Sample preparation**

85 The thawed cooking juice (CJ) was passed through a centrifugal separator (GEA Westfalia
86 Separator, OKA 2-06-566, Düsseldorf, Germany) at 9560 rpm and constant flow rate (50l/h),
87 resulting in a liquid supernatant (CJ-L) and a pasty-looking concentrate (CJ-S). The CJ-L fraction
88 was repeatedly extracted using a solvent mixture of ethyl acetate and water (1:1) until no
89 colour in the organic phase was observed; the CJ-L/solvent ratio was 1:10. Extraction was
90 performed in a separating funnel submitted to vigorous agitation for two minutes, and the
91 aqueous (CJ-L-AP) and organic phases (CJ-L-OP) were collected. The CJ-L-AP was frozen and the
92 CJ-L-OP was cold stored under nitrogen atmosphere and in darkness until analysis.

93 **2.3. Proximate analysis and pH**

94 Moisture and ash contents were determined in triplicate following AOAC (2000). The protein
95 content of each sample was determined by a LECO FP-2000 nitrogen/protein analyser (Leco
96 Corp., St. Joseph, MI, USA), using a nitrogen-to-protein conversion factor of 6.25. Fat content
97 was evaluated (in triplicate) according to Bligh and Dyer (1959). The pH was determined using
98 a MeterLab pHM 93 (Radiometer Analytical, Denmark) on a homogenate of 1 ml of sample in
99 10 ml of distilled water.

100 **2.4. Total Volatile Basic Nitrogen**

101 The total volatile basic nitrogen (TVB-N) determination was effected according to the method
102 of Antonacopoulos and Vyncke (1989). The results were expressed as g of TVB-N per l or kg of
103 sample. Determinations were carried out at least in triplicate.

104 **2.5. Mineral content**

105 Samples (in triplicate) were prepared by acid digestion, in the presence of nitric acid and
106 hydrogen peroxide (Panreac), in closed vessels, with a microwave furnace (Microwave
107 Digestion LabStation, Milestone Inc., Shelton, USA). The minerals were quantified on an atomic
108 absorption spectrophotometer (Perkin-Elmer, model 5100 PC, Norwalk, Connecticut, USA)
109 with an air- and acetylene-oxidizing flame, which was used to determine calcium, magnesium,
110 sodium, potassium, zinc, manganese, iron and copper cations.

111 **2.6. Ion chromatography**

112 For the analysis of inorganic ions (Fluoride, Chloride, Nitrate, Phosphate, Sulphate), organic
113 acids (Citrate, Malate, Lactate, Formate, Acetate) and sugars and polyalcohols (Myo-inositol,
114 Glycerol, Mannitol, Glucose, Sucrose), CJ and CJ-L samples were diluted in water 1:40 v/v,
115 gently stirred for 30 min and filtered through Whatman no. 1 filter paper. The dilution of CJ-S
116 was 1:100 w/v. The CJ-L-AP sample was diluted 7-fold in water. Diluted samples were filtered
117 through 0.45 µm pore size filters.

118 Analysis of anions and organic acids was carried out in triplicate on a Metrohm Advanced
119 Compact Ion Chromatograph (model IC-861, Metrohm AG, Switzerland) equipped with an
120 Advanced Conductivity Detector (IC-819, Metrohm AG). The columns employed were a
121 Metrosep A Supp 5–250 column (250 × 4 mm, 5 µm particle size) for anion determination and
122 Metrosep organic acids (250 × 7.8 mm, 8–10 µm particle size) for organic acids determination
123 (Metrohm AG), using the following elution conditions: 3.2 m mol/l sodium carbonate/1 m
124 mol/l sodium hydrogen carbonate at 0.70 ml/min as mobile phase.

125 Analysis of sugar and polyalcohols was carried out in triplicate on an ion chromatography 817
126 Bioscan (Metrohm AG, Switzerland) equipped with a pulsed amperometric detector (PAD) and
127 a gold electrode. The column employed was Metrosep Carb (1–250/250 × 4.6mm, 5 µm
128 particle size). Samples were injected using an autosampler (model 838 Advanced Sample
129 Processor, Metrohm AG) with a flow rate of 1 ml/min, using 150 m mol/l NaOH and 500 m
130 mol/l acetate–Na in 150 m mol/l NaOH as mobile phase. Compounds were identified by
131 comparing their retention times with those of standards (Sigma-Aldrich, St Louis, MO) and
132 quantified by measuring the normalized peak areas.

133 **2.7. Reverse phase HPLC chromatography**

134 Carotenoid determination was performed by RP-HPLC analysis of the CJ-L-OP fraction. Analysis
135 was performed on a Beckman System Gold binary delivery system (module 126) equipped with
136 a UV–vis photodiode array detector (model 168, Beckman Instruments, Fullerton, CA).
137 Analytical separations were carried out on a stainless steel (250 x 4.6 mm i.d.) Develosil UG C_{30}
138 (5 µm particle size) column (Nomura Chemical, Sojo, Japan) with a guard cartridge
139 (Phenomenex, Macclesfield, U.K.) packed with ODS C_{18}. Sample injection was performed by
140 means of a valve (Rheodyne, Cotati, CA) with a 20 µl peek loop. Elution was performed
141 following a linear mobile phase gradient using methanol (4 % H_2O)/MTBE from 83/17 to 33/67
142 over 60 min at a flow rate of 1 ml/min. The column was thermostated at 22 °C on a Shimadzu
143 CTO-10AS (Columbia, MD) column oven. The Gold Nouveau software data system was used.

144 Samples to be injected were dried in a rotary evaporator and dissolved in MTBE, 50 µl was
145 filtered through a 0.45 µm filter and a volume of 20 µl was injected. Commercial astaxanthin
146 and spectral data were used to identify carotenoid peaks.

147 **2.8. Antioxidant activity**

148 CJ and CJ-L samples were diluted in water 1:40 v/v, gently stirred for 30 min and filtered
149 through Whatman no. 1 filter paper. The dilution of CJ-S was 1:100 w/v. To measure the
150 chelating activity, CJ, CJ-L and CJ-S samples were diluted 1:40. In the case of CJ-L-AP no further
151 dilution was done.

152 **2.8.1. ABTS assay**

153 The ABTS radical [2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)] scavenging capacity
154 was determined as described by Alemán, Giménez, Pérez-Santín, Gómez-Guillén and Montero
155 (2011). Results were expressed as mg Vitamin C Equivalent Antioxidant Capacity (VCEAC)/ml of
156 sample based on a standard curve of vitamin C. All determinations were performed at least in
157 triplicate.

158 **2.8.2. FRAP assay**

159 The ferric reducing/antioxidant power (FRAP) assay was carried out as described previously
160 (Alemán et al., 2011). Results were expressed as µmol Fe^{2+} equivalents/ml of sample based on
161 a standard curve of $FeSO_4 \bullet 7H_2O$. All determinations were performed at least in triplicate.

162 **2.8.3. Chelating activity on Fe^{2+}**

163 The chelating of Fe^{2+} was measured by the method of Boyer and McCleary (1987) with a slight
164 modification. Diluted sample (4.7 ml) was mixed with 0.1 ml of 2 m mol/l $FeCl_2$ and 0.2 ml of 5
165 m mol/l ferrozine. The reaction mixture was allowed to stand for 20 min at room temperature.
166 The absorbance was then read at 562 nm; the blank was prepared in the same manner except
167 that distilled water was used instead of the sample. The chelating activity was calculated as
168 follows:

169 Chelating activity (%) = [(B – A)/B] x 100, where A is A_{562} of sample and B is A_{562} of the blank.

170 **2.8.4. Photochemiluminescence assay**

171 This assay involves the photochemical generation of superoxide ($O_2^{\bullet -}$) free radical combined
172 with chemiluminescence detection of luminol, which acts as a photosensitizer as well as an
173 oxygen radical detection reagent. This reaction takes place in the PHOTOCHEM® (Analytik Jena
174 AG). The ACW and ACL kits provided by the manufacturer were used to measure hydrophilic
175 and lipophilic antioxidant capacity, respectively. Ascorbic acid and Trolox were used as the
176 calibration reagents for ACW and ACL, respectively, at measuring concentration ranges of 1–3
177 n mol. The results were expressed as µg ascorbic acid equivalent/ml or µg Trolox/ml of sample.
178 All samples were measured in duplicate.

179 **2.9. Angiotensin-converting-enzyme (ACE) inhibition**

180 For the analysis of ACE inhibition activity, CJ and CJ-L samples were diluted in water 1:40 v/v,
181 gently stirred for 30 min and filtered through Whatman no. 1 filter paper. The dilution in the
182 case of CJ-S was 1:100 w/v. In the case of CJ-L-AP no further dilution was done. Reverse phase
183 high performance liquid chromatography (RP-HPLC) was used to determine ACE inhibition as
184 described previously (Alemán et al., 2011). All determinations were carried out at least in
185 triplicate. The IC_{50} value was defined as the concentration of sample (mg or µg/ml) required to
186 reduce the HA peak by 50 %.

187 **2.10. Amino acid composition**

188 The amino acid contents in the CJ-L-AP and CJ-L-OP samples were determined by direct
189 hydrolysis in vacuum-sealed glass tubes at 110 °C for 24 h, with 6 mol/l HCl and 0.1 % phenol
190 with norleucine (Sigma-Aldrich, Inc.) as the internal standard, as reported earlier (Alemán et
191 al., 2011). Determinations were performed in triplicate and the data correspond to mean
192 values. Standard deviations were in all cases lower than 6 %.

193 **2.11. Molecular weight distribution**

194 The molecular weight distribution of CJ-L-AP was evaluated by size-exclusion HPLC (model SPE-
195 MA10AVP, Shimadzu, Kyoto, Japan) on a Superdex Peptide PC 3.2/30 column (GE Healthcare
196 Bio-Sciences, Barcelona, Spain), with a fractionation range between 7000 and 100 Da. The
197 injection volume was 10 µL and the flow rate 0.1 ml/min using Milli-Q water as mobile phase.
198 Vitamin B12 (1355 Da) and HHL) 502 Da), both from Sigma-Aldrich (Inc., St. Louis, MO, USA),
199 were used as molecular weight standards.

200 **2.12. Statistical analysis**

201 Statistical tests were performed using the SPSS® computer program (SPSS Statistical Software,
202 Inc., Chicago, IL, USA). One-way analysis of variance was carried out. The difference of means
203 between pairs was resolved by means of confidence intervals using a Tukey-b test at a level of
204 significance of $p < 0.05$.

205 **3. Results and discussion**

206 **3.1. Compositional properties**

207 The cooking juice had a cloudy yellowish appearance with orange particles in suspension. A
208 pilot plant centrifugal separator that allowed a continuous flow was used for the concentration
209 of the solid matter contained in the cooking juice (CJ) in order to simulate a feasible,
210 economically profitable, non-contaminating industrial procedure. The centrifugation produced
211 a translucent liquid supernatant (CJ-L) with a slightly yellowish orange colour and a solid
212 precipitate (CJ-S) with a more intense orange colour, shiny appearance and creamy
213 consistency. Table 1 shows the proximate analysis, pH and total basic volatile nitrogen (TBV-N)
214 of the original juice (CJ), and of CJ-L and CJ-S. The protein fraction was the major component of
215 the cooking juice (26.2 g/l), representing ≈53 % of the dry matter (d.m.), while the juice also
216 contained 9.5 g/l of fat (≈19 % d.m) and 10.8 g/l of ash (≈22 % d.m). In the protein fraction,
217 however, the possible contribution of small peptides, free amino acids and non-protein

218 nitrogen must be taken into account. In this regard, Cambero et al. (1998) reported the total
219 nitrogen in shrimp cooking broth to be mainly composed of peptides of less than 600 Da
220 (around 50 % of the water-soluble nitrogen), followed by the amino acid fraction, peptides of
221 molecular weight greater than 600 Da and protein nitrogen in variable amounts depending on
222 the cooking temperature.

223 The fat content of CJ (9.5 g/l) was slightly higher than that reported by Cambero et al. (1998),
224 which ranged between 3.0 and 6.5 g/l. The fat content of the shrimp edible part is low (1–3
225 g/kg), and can vary slightly depending on species and time of capture (Rosa & Nunes, 2003).
226 The fat content in shrimp waste, however, has been reported to be higher owing to the lipid
227 content of the viscera located in the head (Mandeville et al., 1992).

228 The mineral content of crustacean cooking juices is a parameter that can vary considerably,
229 depending on the process, in relation to the quantity of salt (normally NaCl) added to the
230 cooking water. Cros et al. (2006) reported a mineral content ranging from 15 to 40 g/l in
231 shrimp cooking juices collected in a seafood processing factory, values higher than the 10.8 g/l
232 found in the present study.

233 After processing with the centrifugal separator, the concentrate (CJ-S) consisted mainly of a
234 lipid component (135.2 g/kg), followed by protein (115.8 g/kg), which, expressed in terms of
235 dry matter, represented ≈48 % and ≈41 %, respectively. However, the percentage of ash in the
236 concentrate (CJ-S) tended to reduce significantly with respect to the original juice,
237 representing ≈3 % of the dry matter. Kim et al. (2000) reported a total nitrogen content of 66.9
238 g/kg, 84.8 g/kg of ash and 803 g/kg of moisture in a concentrate obtained from industrial
239 oyster cooker effluent, finding lipids as trace amounts. They found that nucleotides and their
240 related compounds comprised around 7 % of the total extractable nitrogen, whereas
241 trimethylamine (TMA), trimethylamine oxide (TMAO) and total creatinine comprised only
242 around 1 % of the extractable nitrogen.

243 The pH of CJ, CJ-L and CJ-S ranged between 6.23 and 6.38, and the TVB-N content remained at
244 low levels (0.10 g/kg) in CJ-S and increased slightly to 0.14 g/l in CJ-L, indicative of an
245 acceptable microbiological quality in all cases. The low TVB-N content suggests only a small
246 contribution of non-protein nitrogen to the total nitrogen fraction.

247 The presence of Na clearly predominates in the ash content (Table 2) of CJ, attributed mainly
248 to the incorporation of salts (principally NaCl) into the cooking water. Also notable, in order of
249 importance, were K, Ca and Mg, and appreciable amounts of Cu, Fe, Zn and Mn were detected.
250 The results expressed in terms of dry matter content reveal a notable decrease in Na, K, Ca and
251 Mg in the solid concentrate (CJ-S). Kim et al. (2000) reported a high NaCl content (76 g/l) as the
252 main component of the ash fraction of an oyster cooker effluent concentrate.

253 The relatively high concentration of chloride ions, followed by sulphate ions (Table 3) in the
254 cooking juice was attributed basically to the addition of salt (NaCl) and to supplementation of
255 the shrimp with sulphites to prevent melanosis. Phosphate, fluoride and nitrate ions were also
256 identified. Except for nitrates, the ions content tended to diminish ($p<0.05$) in both CJ-S and CJ-
257 L as compared to the original cooking juice. Moreover, when the values are expressed on a dry
258 weight basis the content in CJ-S was considerably lower than in CJ-L. Appreciable levels of
259 some organic acids (acetate, citrate and, to a lesser extent, formate), mono- and disaccharides
260 (glucose, sucrose) and other polyalcohols (glycerol, myo-inositol and mannitol) were also
261 found (Table 4), and in terms of dry matter, they tended also to exhibit lower concentrations in
262 CJ-S. A relative abundance of glucose and other sugars and polyalcohols has also been
263 reported in other studies on recoveries from shellfish broth, as products of glycolysis and
264 nucleotide degradation (Cambero et al., 1998). The presence of appreciable amounts of
265 glycogen in oyster cooker effluent has been reported (Kim et al., 2000). Both glucose and
266 glycogen are essential energy sources in crustaceans in response to stressors (Zhou, Wang &
267 Xian, 2011). As a consequence of cooking, free sugars are exposed to thermal decomposition.

268 Moreover, depending on the intensity and duration of the heat treatment, interactions with
269 free amino acids or small peptides through the Maillard reaction may also take place,
270 producing compounds responsible for the characteristic flavour and aroma of cooked shellfish
271 (Mandeville et al., 1992; Cambero et al., 1998; Kim et al., 2000).

272 On the other hand, the presence of appreciable amounts of glycerol and other polyalcohols,
273 acetate, phosphate, etc. may be a result of thermal hydrolysis of lipids such as triglycerides,
274 fatty acid esters, phospholipids, etc. Cambero et al. (1998) reported the fat components in
275 shrimp cooking broth to be highly sensitive to thermal degradation, since the fat content
276 gradually diminished with increases in temperature.

277 In general, there was a relative decrease in the concentrations of minor soluble compounds in
278 the concentrated product, since they tended to remain in the aqueous phase. In the case of
279 glucose, sucrose and mannitol, it was surprising that the concentrations in CJ-L were
280 significantly greater than in CJ, bearing in mind that the proximate composition was very
281 similar. A possible explanation might be that during the separation process there was strong
282 homogenisation of the cooking juice, facilitating the breaking up of the suspended particles
283 and the consequent release of certain hydrophilic compounds which could accumulate in CJ-L.

284 **3.2. Biological properties**

285 The antioxidant properties of CJ, CJ-L and CJ-S were evaluated in terms of their free radical
286 scavenging capacity (ABTS, PCL), ferric ion reducing capacity (FRAP) and metal chelating
287 capacity (Table 5). Unlike the metal chelating capacity, the radical scavenging capacity (ABTS
288 and PCL) and the reducing capacity are concentrated in the solid fraction, CJ-S. The notable
289 antioxidant activity can be attributed to the presence of carotenoids (Liñán-Cabello, Paniagua-
290 Michel & Hopkins, 2002). They are largely associated with proteins, in the form of
291 carotenoproteins, which are stable complexes of carotenoids bound to a high-density
292 lipoprotein (Shahidi, Metusalach & Brown, 1998). Other antioxidant compounds might be

293 principally peptides, reducing sugars, citrates or phenolic compounds. The presence of small
294 peptides in the shrimp cooking juice can be attributed to products of endogenous proteolysis
295 of the visceral content of the heads, and also to products of protein thermal degradation. In
296 this regard, Sowmya et al. (2011) reported the hydrolysed protein component in a shrimp head
297 carotenoprotein isolate to be mainly responsible for its antioxidant activity. Phenolic
298 compounds with antioxidant capacity have also been reported to occur in shrimp shell wastes
299 (Seymour, Li & Morrisey, 1996).

300 The cooking juice (CJ) presented measurable ACE-inhibitory capacity, with an IC_{50} value of 3.81
301 mg/ml (Table 6). When the CJ-L and CJ-S fractions were separated, the ACE-inhibitory activity
302 increased considerably in the concentrate, revealed by a lower IC_{50} value (1.70 mg/ml). The
303 ACE-inhibitory IC_{50} value of an enzymatic hydrolysate of the shrimp *A. chinensis* was 0.96 ±
304 0.03 mg/ml (He et al., 2008).

305 The biological activity of the less concentrated CJ-L fraction was further analysed, in order to
306 elucidate the nature of the main active compounds present in the cooking juice waste
307 material. For that purpose, a separation of the aqueous (CJ-L-AP) and organic (CJ-L-OP) phases
308 was performed. CJ-L-OP had strong orange colour and CJ-L-AP a slightly yellow-green colour.
309 The orange colour of the organic phase might be due to carotenoids. To confirm the presence
310 of these compounds the CJ-L-OP fraction was analysed by RP-HPLC, and the chromatogram
311 obtained is shown in Figure 1. On the basis of the retention behaviour and absorption
312 spectrum, peak 1 was identified as *trans*-astaxanthin; peaks 2 and 3 were tentatively identified
313 as *cis*-astaxanthin. Most of the other peaks showed an absorption spectrum similar to
314 astaxanthin and could be astaxanthin mono- or diesters. Astaxanthin in shrimp *Litopenaeus*
315 *vannamei* has been reported to occur as approximately 50, 30 and 20 % in the form of diester,
316 monoester and free, respectively (Armenta, & Guerrero-Legarreta, 2009).

317 Biological properties of the CJ-L aqueous (CJ-L-AP) and organic (CJ-L-OP) phases are shown in
318 Table 7. The PCL method revealed a notably high value in the organic phase; this activity might
319 be largely due to lipid soluble antioxidants, presumably carotenoids, which still remained in
320 the CJ-L fraction. Astaxanthin has been referred as a potent antioxidant and attenuates
321 oxidative stress, DNA damage and cell death (Shimidzu, Goto & Miki, 1996). On the contrary,
322 the chelating activity was predominant in CJ-L-AP, suggesting a more prominent role of
323 hydrophilic compounds, presumably peptides, in this antioxidative mechanism.

324 The ACE-inhibitory activity in CJ-L-AP might be also largely associated with the presence of
325 hydrophilic compounds, among them small peptides are thought to play a key role (He, Chen,
326 Wu, Sun, Zhang, & Zhou, 2007). This activity was found with a relatively high intensity, allowing
327 the calculation of an IC_{50} value of 89.47 μg/ml, which was comparable to the activity reported
328 for two isolated peptides from *Acetes chinensis* (IC_{50} = 3.4 μ mol/l and 12.3 μ mol/l) (He et al.,
329 2006).

330 To elucidate the possible contribution of small peptides to the antioxidant capacity and,
331 especially, the noticeable ACE-inhibitory capacity found in CJ-L-AP, this fraction was further
332 characterized in terms of amino acid composition and molecular weight distribution. The total
333 amount of amino acids present in the aqueous phase was found to be 2.35 g/l. It is worth
334 noting that no appreciable presence of amino acids was detected in the organic phase (CJ-L-
335 OP) (data not shown), which corroborates that neither free amino acids nor peptides were
336 extracted in this phase. Figure 2 shows the composition of the amino acids present in the CJ-L-
337 AP fraction. The most abundant amino acids were, in descending order, Glu, Gly, Pro, Asp and
338 Arg, and there were also significant amounts of Ala, Lys and Leu. Other amino acids, such as
339 Val, Ser, Thr, Phe, Tyr and Ile, were found in concentrations between 50 and 100 mg/l in the
340 aqueous phase studied, whereas His, Met and Cys were the amino acids with the lowest
341 concentrations. The dominant amino acids found by Cambero et al. (1998) in shrimp cooking

342 broths were Arg and Gly, followed in order of quantitative importance by Ser, Ala, Pro, Leu,
343 Lys, Phe and Glu. In an oyster cooker effluent, Gly, Ala, Glu and Arg were reported to comprise
344 around 40 % of total free amino acids (Kim et al., 2000). The most abundant amino acids
345 present in cooked shrimp waste were found to be Pro, Arg and Phe, accounting for 84.2 % of
346 the total free amino acids in this material (Mandeville et al., 1992). Those authors reported
347 that the amino acid most affected by cooking was Asp, followed by Glu, Pro, Ala, Ser and Phe.

348 The high antihypertensive activity found in the present study seems to be related with the
349 abundance of certain amino acids, especially Gly and Pro, forming part of small peptides. In
350 fact, if the amino acid composition is expressed in relative terms (number residues/1000
351 residues), Gly (198 ‰) and Pro (122 ‰), followed by Glu (121 ‰), were the most abundant
352 amino acid residues. Interestingly, Pro seems to be one of the most effective amino acids in
353 increasing ACE-inhibitory activity and it has been described in many naturally occurring ACE
354 peptide inhibitors, including shrimp hydrolysates (He et al., 2006). In particular, the dipeptide
355 Gly-Pro has been reported to present an antihypertensive effect in spontaneously
356 hypertensive rats (Ichimura, Yamanaka, Otsuka, Yamashita, & Maruyama, 2009).

357 The molecular exclusion profile of CJ-L-AP (Figure 3) reveals the presence of various peaks
358 corresponding to peptides with a molecular weight less than 7 kDa, which is the molecular
359 exclusion volume of the column. Most of the material is comprised in a molecular weight range
360 between 1355 and 502 Da, with the most abundant peak corresponding to peptides with a
361 molecular weight of around 1 kDa. ACE-inhibitory peptides are relatively short sequences with
362 low molecular mass, since the active site of ACE cannot accommodate large peptide
363 molecules. To this regard, He et al. (2006) reported that most of the ACE-inhibitory peptides
364 present in hydrolysates from *A. chinensis* were short peptides with molecular mass lower than
365 3 kDa.

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367

368 **4. Conclusions**

369 A concentrate rich in lipids and protein with low ash content was obtained from industrial
370 shrimp cooking juice using a centrifugal separator. The crustacean aroma, attractive orange
371 colouring and noticeable antioxidant and ACE-inhibitory capacities, make this concentrate
372 appropriate to be used for the formulation of functional foods. The high ACE-inhibitory activity
373 found in the cooking waste material is largely attributed to the presence of Gly and Pro-rich
374 peptides of around 1 kDa.

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378

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451

452 FIGURE CAPTIONS

453 Figure 1. RP-HPLC chromatogram of carotenoids from the organic phase (CJ-L-OP). Peak
454 identification: (1) all-trans-astaxanthin, (2) tentatively identified as cis-astaxanthin, (3)
455 tentatively identified as cis-astaxanthin. Most of the other peaks were tentatively identified as
456 astaxanthin mono- or diesters.

457 Figure 2. Amino acid composition of the CJ-L-AP aqueous fraction.

458 Figure 3. Molecular weight distribution of the CJ-L-AP aqueous fraction

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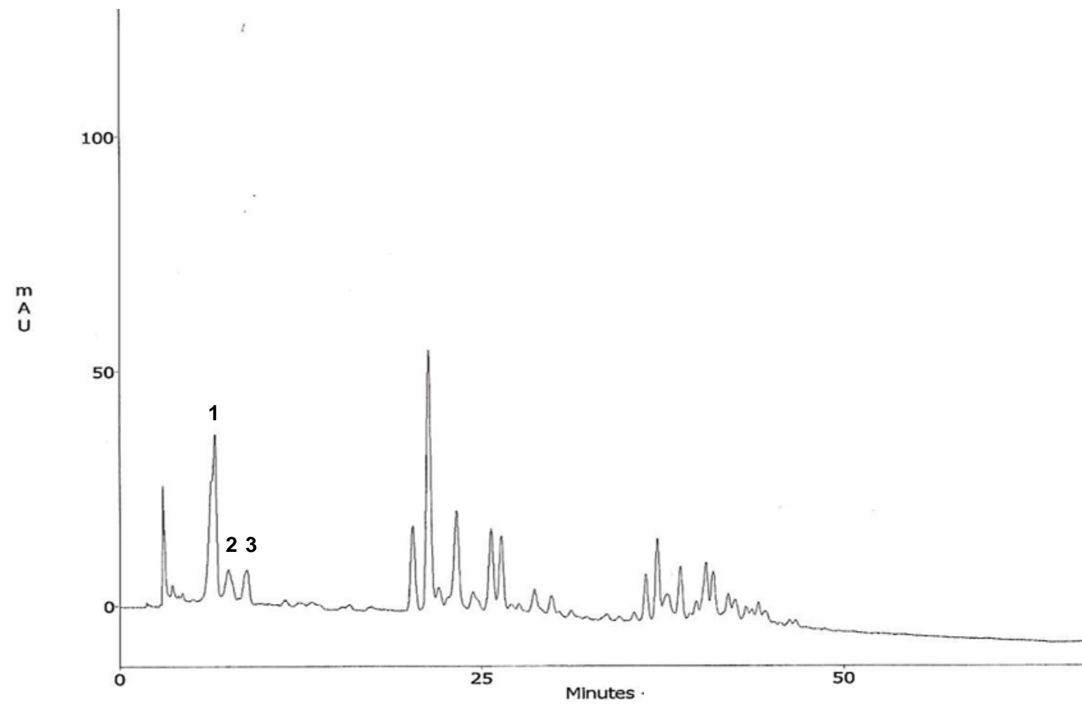
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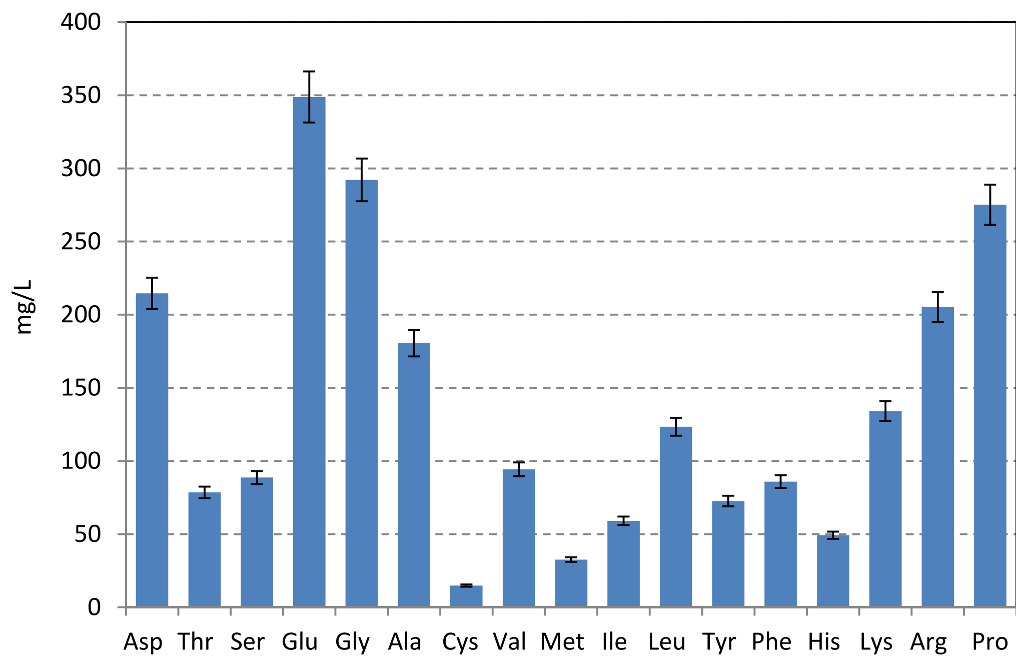

FIG. 2

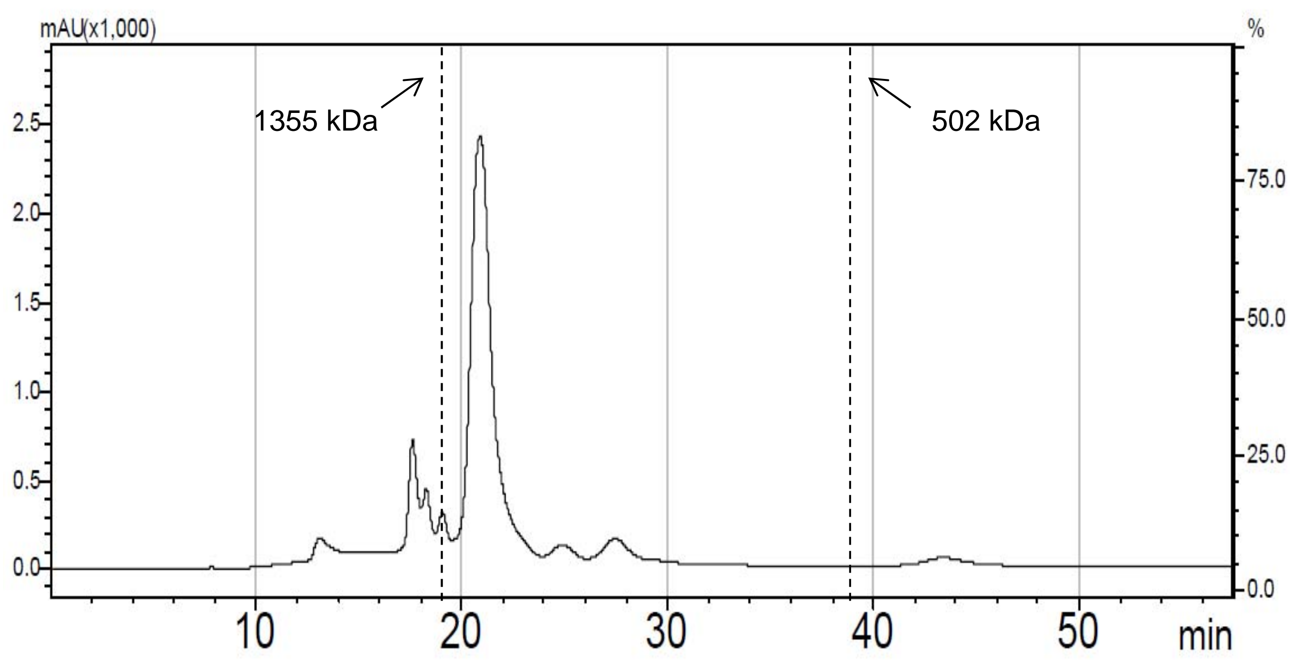

FIG. 3

Table 1. Proximate analysis, pH and total volatile basic nitrogen (TVB-N)

	Moisture g/l	Protein g/l	Fat g/l	Ash g/l	pH	TVB-N g/l
CJ	950.2 ± 0.2^{c}	26.2 ± 0.1^{de} (52.61)	9.5 ± 1.7^{abc} (19.08)	10.8 ± 0.3^{e} (21.69)	6.24 ± 0.01^{b}	0.10 ± 0.0^{bc}
CJ-L	949.3 ± 0.4^{c}	25.0 ± 0.1^{d} (49.31)	12.2 ± 0.2^{bc} (24.06)	10.7 ± 0.3^{de} (21.10)	6.38 ± 0.01^{e}	0.14 ± 0.0^{d}
CJ-S*	720.3 ± 4.5^{a}	115.8 ± 1.3^{g} (41.40)	135.2 ± 1.5^{f} (48.34)	9.2 ± 0.3^{c} (3.29)	6.23 ± 0.01^{b}	0.10 ± 0.0^{bc}

Means with different letters in the same column are significantly different ($P < 0.05$).
Data in ( ) express the results as percentage of dry matter.
* expressed in g/kg

Table 2. Mineral content expressed as g/l of sample.

	Ca	Mg	Na	K	Zn	Fe	Cu	Mn
CJ	0.253 ± 0.003^{cd}	0.122 ± 0.001^{f}	3.290 ± 0.075^{d}	1.175 ± 0.019^{e}	0.003 ± 0.001^{abc}	0.005 ± 0.001^{a}	0.019 ± 0.001^{c}	0.002 ± 0.001^{a}
	(5.08)	(2.45)	(66.06)	(23.59)	(0.060)	(0.100)	(0.381)	(0.040)
CJ-L	0.248 ± 0.005^{c}	0.114 ± 0.001^{cd}	2.860 ± 0.130^{c}	1.188 ± 0.070^{e}	0.003 ± 0.001^{c}	0.005 ± 0.001^{a}	0.018 ± 0.001^{c}	0.002 ± 0.001^{a}
	(4.89)	(2.25)	(56.41)	(23.43)	(0.059)	(0.098)	(0.355)	(0.039)
CJ-S*	0.261 ± 0.005^{e}	0.116 ± 0.001^{e}	2.430 ± 0.075^{b}	0.897 ± 0.011^{b}	0.026 ± 0.001^{e}	0.016 ± 0.001^{c}	0.096 ± 0.004^{d}	0.006 ± 0.001^{b}
	(0.93)	(0.41)	(8.69)	(3.21)	(0.009)	(0.057)	(0.343)	(0.021)

Means with different letters in the same column are significantly different ($P < 0.05$).

Data in ( ) express the results as g per kg of dry matter.

* expressed in g/kg

Table 3. Ionic content expressed as mg/l of sample.

	Fluoride	Chloride	Nitrate	Phosphate	Sulphate
CJ	194.1 ± 4.9^{e} (3.9)	2820 ± 20^{e} (56.6)	78.6 ± 0.5^{e} (1.6)	574.6 ± 2.0^{c} (11.5)	1930 ± 129^{e} (38.8)
CJ-L	105.2 ± 6.5^{b} (2.1)	1598 ± 51^{c} (31.5)	45.4 ± 5.4^{b} (0.9)	305.3 ± 24.6^{b} (6.0)	1023 ± 128^{c} (20.2)
CJ-S*	124.0 ± 6.2^{bc} (0.4)	1724 ± 82^{c} (6.2)	98.3 ± 5.2^{f} (0.3)	309.2 ± 44.3^{b} (1.1)	1204 ± 60^{bc} (4.3)

Means with different letters in the same column are significantly different ($P < 0.05$).
Data in ( ) express the results as g per kg of dry matter.
* expressed in mg/kg

Table 4. Organic Acids and Sugar content expressed as mg/l of sample.

	Citrate	Formate	Acetate	Myo-inositol	Glycerol	Mannitol	Glucose	Sucrose
CJ	1880 ± 17^{e}	43.5 ± 4.5^{b}	3081 ± 145^{e}	19.3 ± 1.5^{c}	256.3 ± 27.6^{e}	587.3 ± 34.8^{d}	296.0 ± 14.1^{b}	18.6 ± 1.5^{b}
	(37.75)	(0.88)	(61.87)	(0.39)	(5.1)	(11.8)	(5.9)	(0.4)
CJ-L	1759 ± 31^{d}	37.9 ± 0.6^{ab}	2562 ± 31^{d}	21.1 ± 0.5^{c}	182.7 ± 5.9^{c}	693.0 ± 22.9^{e}	729.6 ± 1.5^{f}	48.8 ± 0.8^{d}
	(34.69)	(0.75)	(50.53)	(0.42)	(3.6)	(13.7)	(14.4)	(0.9)
CJ-S*	1468 ± 26^{b}	102.4 ± 2.5^{c}	1892 ± 26^{a}	13.2 ± 0.4^{b}	117.9 ± 3.3^{b}	509.2 ± 10.3^{c}	543.0 ± 30.8^{d}	13.8 ± 0.8^{a}
	(5.25)	(0.36)	(6.76)	(0.05)	(0.4)	(1.8)	(1.9)	(0.1)

Means with different letters in the same column are significantly different ($P < 0.05$).

Data in ( ) express the results as g per kg of dry matter.

* expressed in mg/kg

Malate and Lactate were not detected.

Table 5. Antioxidant activity.

	ABTS VCEAC/mL	FRAP µmol Fe^{2+} eq/mL	Chelating %	Photochem µg ascorbic acid/mL
CJ	2.01 ± 0.05^{c}	6.64 ± 0.03^{c}	68.7 ± 1.4^{e}	518.4 ± 19.7^{d}
CJ-L	1.91 ± 0.07^{c}	6.67 ± 0.13^{c}	65.0 ± 1.4^{e}	462.4 ± 26.3^{c}
CJ-S	3.23 ± 0.04^{d}	15.89 ± 0.36^{f}	42.0 ± 11.6^{d}	636.9 ± 17.9^{e}

Means with different letters in the same column are significantly different ($P < 0.05$).
VCEAC: mg Vitamin C Equivalent Antioxidant Capacity.

Table 6. ACE-inhibitory activity.

	IC_{50} ACE mg/mL
CJ	3.813 ± 0.004 [a]
CJ-L	4.004 ± 0.047 [a]
CJ-S	1.700 ± 0.004 [b]

Means with different letters in the same column are significantly different ($P < 0.05$).

The IC_{50} value was defined as the concentration in mg of sample per mL of enzyme system solution required to reduce the HA peak by 50%.

Table 7. Biological activity of CJ-L aqueous (CJ-L-AP) and organic (CJ-L-OP) phases.

	Chelating %	Photochem	IC_{50} ACE µg/mL
CJ-L-AP	19±7.1	1.7±0.1[1]	89.47±4.43
CJ-L-OP	8±4.8	82.1±7.9[2]	n.d

[1] µg ascorbic acid/mL
[2] µg trolox/mL
The IC_{50} value was defined as the concentration in µg of sample per mL of solution enzymatic system required to reduce the HA peak by 50%.
n.d= not determined