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Release of angiotensin converting enzyme-inhibitory peptides during in vitro gastro-intestinal digestion of camel milk / Tagliazucchi, Davide; Shamsia, Sherif; Conte, Angela. - In: INTERNATIONAL DAIRY JOURNAL. - ISSN 0958-6946. - STAMPA. - 56:(2016), pp. 119-128. [10.1016/j.idairyj.2016.01.009]

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18/12/2025 11:10

# **Release of angiotensin converting enzyme-inhibitory peptides during *in vitro* gastro-intestinal digestion of camel milk**

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## 1   **Abstract**

2   The objective of the present study was to identify the angiotensin-converting enzyme (ACE)-  
3   inhibitory peptides released from camel milk after simulated gastro-intestinal digestion. The  
4   hydrolysis degree increased during digestion. The highest ACE-inhibitory activity was found in the  
5   post-pancreatic <3kDa fraction. Peptides responsible for the biological activity were isolated by  
6   reverse phase HPLC and identified by mass spectrometry. Among the identified sequences, 17 were  
7   identical to known bioactive peptides with ACE-inhibitory activity. Based on previous structure-  
8   activity relationship studies, the sequence of some peptides allowed us to anticipate the presence of  
9   biological activities. The anti-hypertensive tripeptide isoleucine-proline-proline (IPP) was identified  
10   and quantified in digested camel milk. The amount of released IPP was  $2.56 \pm 0.15 \text{ mg L}^{-1}$  of milk.  
11   For the first time, we showed that IPP is released during the gastro-intestinal digestion of camel  
12   milk  $\kappa$ -casein. This research provides the basis to increase the exploitation of the health benefits of  
13   camel milk.

14

## 15    **1. Introduction**

16    Previous research has recommended the potential use of camel milk as an alternative in case of cow  
17    milk allergy and in the manufacture of infant formula (El-Agamy, Nawar, Shamsia, Awad &  
18    Haenlein, 2009). In human milk as well as in camel milk,  $\alpha$ -lactalbumin is the main whey protein,  
19    whereas  $\beta$ -lactoglobulin is absent. The latter protein is the principal responsible for the allergic  
20    reactions to cow milk in adult and children (El-Agamy et al., 2009).

21    Milk proteins possess unique properties and contain in their sequences encrypted peptides that show  
22    potential physiological properties and health benefits. These peptides are inactive when included in  
23    the protein sequence but can be released after hydrolysis (by enzymes or microorganism) displaying  
24    their biological properties (Pihlanto, 2006). They can be released from both caseins (especially  $\beta$ -  
25    casein) and whey proteins and have been identified in milk of several species (Korhonen &  
26    Pihlanto, 2006).

27    The claimed biological activities of these peptides include antimicrobial, angiotensin-converting  
28    enzyme (ACE)-inhibitory, anti-hypertensive, antithrombotic and antioxidative activities (Nagpal et  
29    al., 2011). The main bioactive peptides studied are those with ACE-inhibitory activity (Phelan &  
30    Kerins, 2011). ACE is a dipeptidyl carboxypeptidase that catalyses, *in vivo*, the conversion of the  
31    plasmatic peptide angiotensin I into the potent vasoconstrictor angiotensin II. Inhibition of ACE  
32    plays an important role in blood pressure regulation and drugs that inhibit ACE are commonly  
33    prescribed for the treatment of hypertension or related cardiovascular diseases (Acharya, Sturrock,  
34    Riordan & Ehlers, 2003). Several peptides that inhibit ACE were reported after enzymatic  
35    hydrolysis of milk proteins and after milk fermentation with *Lactobacillus* (Hernández-Ledesma,  
36    García-Nebot, Fernández-Tomé, Amigo & Recio, 2014). Among ACE-inhibitory peptides derived  
37    from bovine caseins in milk fermented with *Lactobacillus*, valine-proline-proline [VPP;  $\beta$ -casein  
38    f(84–86)] and isoleucine-proline-proline [IPP;  $\beta$ -casein f(74–76)] have been the most studied  
39    (Solieri, Rutella & Tagliazucchi, 2015). Other ACE-inhibitory peptides have been discovered in  
40    enzymatic hydrolysates of milk caseins and whey proteins (Phelan & Kerins, 2011). Digestive

41 enzymes and combinations of different proteinases such as alcalase and thermolysin have been  
42 utilized to successfully generate bioactive peptides from various milk proteins. For example, two  
43 peptides derived from bovine  $\alpha$ S1-casein (RYLGY and AYFYPEL) after hydrolysis with pepsin,  
44 were able to reduce blood pressure *in vivo* when administered in spontaneously hypertensive rats  
45 (Contreras, Carrón, Montero, Ramos, & Recio, 2009). Three potent ACE-inhibitory peptides (LLF,  
46 LVRT and LQKW) were purified from caprine  $\beta$ -lactoglobulin hydrolysed with thermolysin  
47 (Hernández-Ledesma, Recio, Ramos & Amigo, 2002).

48 In a recent series of two papers, Picariello and co-workers (Picariello et al., 2010; Picariello et al.,  
49 2013) *in vitro* studied the release of bioactive peptides after digestion of bovine milk caseins and  
50 whey proteins. They found that some ACE-inhibitory peptides (such as the sequence HLPLP from  
51  $\beta$ -casein and the sequences GLDIQK and VLDTDYK from whey proteins) were released and stable  
52 under gastro-intestinal conditions and were able to across Caco-2 cells monolayers. ACE-inhibitory  
53 peptides (HLPLP and WSVPQPK) may be also released from human milk after *in vitro* gastro-  
54 intestinal digestion (Hernández-Ledesma, Quirós, Amigo & Recio, 2007). In addition, *in vitro*  
55 gastro-pancreatic digestion of donkey milk resulted in the release of a potent ACE-inhibitory  
56 peptide (VAPFPQPVPV) corresponding to the fragment f(176-185) of  $\beta$ -casein (Bidasolo, Ramos  
57 & Gomez-Ruiz, 2012).

58 Only a few studies on the antioxidant and ACE-inhibitory activities of camel milk protein-derived  
59 peptides have been performed. Salami et al. (2011) and Moslehishad et al (2013) found that camel  
60 milk proteins released ACE-inhibitory and antioxidant compounds after the treatment with digestive  
61 proteases or after fermentation with *Lactobacillus rhamnosus*. However, the sequence of bioactive  
62 compounds involved in the effect has not been revealed.

63 Caseins are, quantitatively, the most important proteins in camel milk. The sequence homology  
64 between bovine and camel milk caseins varies from 53% for  $\alpha$ S1-casein to 75% for  $\beta$ -casein.

65 Although many bioactive sequences are not conserved in camel milk caseins, some portions capable

66 of releasing peptides with ACE-inhibitory activities are present in the sequence of camel caseins  
67 (Kappeler, Farah & Puhan, 1998).  
68 In this study, therefore, we used an *in vitro* digestion procedure mimicking the chemical and  
69 physical condition of the gastro-intestinal tract to process skimmed camel milk. The digested  
70 sample was characterized for the ACE-inhibitory activity and then further separated with high  
71 performance liquid chromatography (HPLC) and the different fractions characterized for their  
72 ACE-inhibitory activity. The fractions with the highest activity were then analyzed with mass  
73 spectrometry (MS) with the aim to identify the bioactive peptides.

## 74    **2. Materials and methods**

### 75    *2.1. Materials*

76    Bile salts (mixture of sodium cholate and sodium deoxycholate), porcine  $\alpha$ -amylase, pepsin from  
77    porcine gastric mucosa, pancreatin from porcine pancreas (4xUSP), ACE from rabbit lung, mucin II  
78    and III, bovine serum albumin, 2,4,6-trinitrobenzenesulfonic acid (TNBS), sodium dodecyl sulphate  
79    (SDS), dithiothreitol (DTT), lysozyme and urea were supplied by Sigma (Milan, Italy). The  
80    tripeptide isoleucine-proline-proline (IPP; 95% purity) was synthesized by DBA (Milan, Italy).  
81    Amicon Ultra-4 regenerated cellulose 3 kDa were supplied by Millipore (Milan, Italy). The whole  
82    camel milk was obtained from farms at El-Alamin and Sidi-Barani areas around Alexandria  
83    (Egypt). All electrophoretic, HPLC and MS reagents were from Bio-Rad (Hercules CA, U.S.A.).  
84    All the other reagents were from Carlo Erba (Milan, Italy).

85

### 86    *2.2. Chemical analysis and camel skimmed milk preparation*

87    Whole camel milk was defatted, to obtain skimmed camel milk, by centrifugation at 2000g for 20  
88    min at room temperature. Skimmed milk sample was analyzed for pH, total solids, fat and ash  
89    according to Ling (1963), lactose by phenol-sulfuric acid method (Marier & Baulet, 1959), nitrogen  
90    fractions, *i.e.* the total nitrogen, non-casein nitrogen by micro-Kjeldahl (Rowland, 1938).

91

### 92    *2.3. In vitro gastro-intestinal digestion*

93    For the *in vitro* digestion, the protocol, developed within the COST Action FA1005 and further  
94    validated for milk (Kopf-Bolan et al., 2012), was followed.

95    Simulated salivary (SSF), simulated gastric (SGF), and simulated intestinal (SIF) fluids were  
96    prepared according to Kopf-Bolan et al. (2012). SIF was prepared by mixing pancreatic (PF) and  
97    bile (BF) fluids. Skimmed camel milk (9 mL) was added to 12 mL of SSF containing 150 U mL<sup>-1</sup> of  
98    porcine  $\alpha$ -amylase and incubated for 5 min (oral phase). Gastric digestion was performed by adding  
99    24 mL of SGF. The pH was adjusted to 2.0 with HCl 6 mol L<sup>-1</sup> and supplemented with porcine

100 pepsin (1115 U mL<sup>-1</sup> of SGF). The gastric bolus was further incubated for 120 min (gastric phase).  
101 The intestinal digestion was carried out by adding to the gastric bolus 36 mL of SIF (24 mL of PF  
102 and 12 mL of BF), adjusting the pH to 7.0 and supplemented with pancreatin. The chyme was  
103 further incubated for 120 min (pancreatic phase). All incubations were performed at 37°C on a  
104 rotating wheel (10 rpm). The digested samples were cooled on ice and immediately frozen at -80°C  
105 for further analysis. The digestions were performed in triplicate.

106 A control digestion, which included only the gastro-intestinal juices and enzymes and water in place  
107 of milk, was carried out to consider the possible impact of the digestive enzymes in the subsequent  
108 analysis.

109

#### 110 *2.4. Determination of protein hydrolysis during the digestion*

111 The determination of protein hydrolysis in the digested samples was carried out by measuring the  
112 peptide concentration by the TNBS method using leucine as standard (Adler-Nissen, 1979). The  
113 absorbance values at 340 nm were read using a Jasco V-550 UV/Vis spectrophotometer (Orlando  
114 FL, U.S.A.).

115 The hydrolysis degree (DH) was calculated as reported in equation (1):

$$116 \text{ DH} = (\mathbf{h}/\mathbf{h}_{\text{tot}}) \cdot 100 \quad (1)$$

117 where **h** is the hydrolysis equivalent, defined as the concentration in milliequivalents/g of protein of  
118  $\alpha$ -amino groups formed at the different stages of the simulated digestion, and **h<sub>tot</sub>** is the hydrolysis  
119 equivalent at complete hydrolysis to amino acids (calculated by summing the contents of the  
120 individual amino acids in 1 g of protein and considering caseins as the only proteins in milk).

121 According to Adler-Nissen (1979), the **h<sub>tot</sub>** value was fixed at 8, which is the value calculated for  
122 caseins.

123 Hydrolysis degree data were subtracted with the data obtained in the control digestion.

124



125    *2.5. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)*

126    Samples taken at different times of digestion were subjected to SDS-PAGE electrophoresis using  
127    17% polyacrylamide separating gel, to evaluate the changes in protein profile during the hydrolysis  
128    (Helal, Tagliazucchi, Verzelloni & Conte, 2014). Samples were diluted to similar end dilutions in  
129    Laemmli buffer (0.05 mol L<sup>-1</sup> Tris, pH 6.8, containing 2% SDS, 0.1 mol L<sup>-1</sup> DTT, and 0.025%  
130    Bromophenol Blue). Vials were heated in boiling water for 4 min, and 5 µL of each sample  
131    (corresponding to 5 µg of undigested milk proteins) were loaded into the gel. The running condition  
132    were 200 V, 60 mA and 1 hour. The gel was stained by incubation in Coomassie staining solution  
133    for 1 hour with gentle shaking. Coomassie staining solution was prepared by dissolving 0.05 g of  
134    Coomassie Brilliant Blue R-250 in 100 mL of a methanol/water/acetic acid solution (40/50/10).

135

136    *2.6. Fractionation of post-pancreatic digested samples*

137    Post-pancreatic digested samples (4 mL) were subjected to ultrafiltration with Amicon Ultra-4  
138    nominal cut-off 3 kDa, at 7500g for 120 min at 4°C. At the end of the separation the filtrate,  
139    containing low molecular weight peptides, were freeze dried and re-filled to 4 mL (original volume)  
140    with potassium phosphate buffer (0.1 mol L<sup>-1</sup>; pH 7). The peptide content of the fractionated  
141    samples was determined by using the TNBS method as described in paragraph 2.4 and expressing  
142    the results as mg of leucine equivalent mL<sup>-1</sup>.

143

144    *2.7. Measurements of ACE-inhibitory activity*

145    ACE-inhibitory activity was measured by the spectrophotometric assay of Ronca-Testoni (1983)  
146    using the tripeptide, N-[3-(2-furyl)acryloyl]-L-phenylalanyl-glycyl-glycine (FAPGG) as substrate.  
147    For the control assay, 350 µL of 1.6 mmol L<sup>-1</sup> FAPGG dissolved in the reaction buffer (Tris-Cl 100  
148    mmol L<sup>-1</sup> pH 8.2 and containing 0.6 mol L<sup>-1</sup> of NaCl) were mixed directly in cuvette with 330 µL of  
149    reaction buffer. The solution was kept at 37°C for 5 min before the addition of 20 µL of ACE  
150    solution (so that the final activity of the enzyme in the assay was 50 mU mL<sup>-1</sup>).

151 For the inhibition assay, variable amounts of sample were added in place of the buffer.  
152 The reaction was followed at 345 nm for 10 min using a Jasco V-550 UV/Vis spectrophotometer  
153 (Orlando FL, U.S.A.).  
154 Results are expressed as IC<sub>50</sub> that is defined as the concentration of peptides required to inhibit 50%  
155 of the enzymatic activity.

156

## 157 *2.8. HPLC analysis of peptides*

158 HPLC separation of the fractionated (<3 kDa) post-pancreatic digested camel milk was performed  
159 with a Jasco HPLC system equipped with a 250mm x 4.6mm reversed phase column Hamilton  
160 HxSil C18 (Hamilton, Reno, NV, USA) as described by Tagliazucchi, Martini, Bellesia & Conte  
161 (2015). A linear gradient of solvent B (acetonitrile-trifluoroacetic acid 0.027%) in A (water-  
162 trifluoroacetic acid 0.037%) ranging from 0% to 45% in 115 min with a flow rate of 0.5 mL min<sup>-1</sup>  
163 was used to separate the peptides contained in the low molecular weight fractions of digested milk.  
164 The PDA detector was set at 214 nm. Eight major fractions were collected. The volume of sample  
165 loaded into the HPLC system was 20 µL. To obtain enough amount of peptidic fractions for the  
166 assays, the injection was repeated 10 times. At the end of the collection procedure, the tubes  
167 corresponding at each specific fraction were mixed together, freeze-dried and re-filled to the  
168 original volume (200 µL) with a potassium phosphate buffer (0.1 mol L<sup>-1</sup>; pH 7).  
169 These fractions were characterized for their peptide content (paragraph 2.4) and ACE-inhibitory  
170 activity (paragraph 2.7).

171

## 172 *2.9. Peptide profile determination with nanoflow liquid chromatography accurate mass quadrupole* 173 *time-of-flight mass spectrometry with electrospray ionization (LC-ESI-QTOF MS)*

174 The fractions with the highest ACE-inhibitory activity were subjected to QTOF MS/MS analysis for  
175 peptide identification. Nano LC/MS and tandem MS experiments were performed on a 1200 Series  
176 Liquid Chromatographic two-dimensional system coupled to a 6520 Accurate-Mass Q-TOF LC/MS

177 via a Chip Cube Interface (Agilent Technologies, Santa Clara, CA, USA). Chromatographic  
178 separation was performed on a ProtID-Chip-43(II) including a 4mm 40 nL enrichment column and  
179 a 43mm  $\times$  75 $\mu$ m analytical column, both packed with a Zorbax 300SB 5  $\mu$ m C18 phase (Agilent  
180 Technologies). The mobile phases composition and the gradient were the same as reported by  
181 Tagliazucchi et al. (2015). The mass spectrometer was tuned, calibrated and set with the same  
182 parameters as reported by Dei Più et al. (2014).  
183 For peptide identification and sequencing, MS/MS spectra were converted to .mgf and *de novo*  
184 peptide sequencing was performed using Pepnovo software  
185 (<http://proteomics.ucsd.edu/ProteoSAFe/>). The following parameters were considered: enzyme,  
186 none; peptide mass tolerance,  $\pm$  40 ppm; fragment mass tolerance,  $\pm$  0.12 Da; variable modification,  
187 oxidation (M) and phosphorylation (ST); maximal number of PTMs permitted in a single peptide 3.  
188 A search for the biological activity of peptides identified was carried out through the BIOPEP  
189 database ([http://www.uwm.edu.pl/biochemia/biopep/start\\_biopep.php](http://www.uwm.edu.pl/biochemia/biopep/start_biopep.php)). Confirmation of peptides  
190 sequence in camel milk proteins was performed using Peptide Match  
191 (<http://research.bioinformatics.udel.edu/peptidematch/index.jsp>).

192

#### 193 2.10. Identification and quantification of IPP in fraction F4

194 IPP in fraction F4 was identified by comparing the retention time and fragmentation spectra of a  
195 synthetic standard tripeptide. IPP was quantified in fraction F4 using the method reported in Solieri  
196 et al. (2015). The synthetic tripeptide IPP was solubilized at 5 g L<sup>-1</sup> in 0.1 mmol L<sup>-1</sup> potassium  
197 phosphate buffer (pH 7.0), and then diluted 1:1000 with solvent A (H<sub>2</sub>O/acetonitrile/formic acid,  
198 96.9:3:0.1, v/v/v), to obtain the 5 mg L<sup>-1</sup> solution. Subsequent dilutions were made in the same  
199 solvent A to yield the following concentrations: 1, 5, 10, 20, 50 and 100  $\mu$ g L<sup>-1</sup>. Each solution  
200 contained the internal standard EGVNDNEEGFFSAR at the concentration of 50  $\mu$ g L<sup>-1</sup>. The  
201 calibration curve was constructed from the peak area of the peptide relative to the peak area of the

202 internal standard *versus* concentration. The concentrations of IPP was calculated using the  
203 following linear equation:

204  $y = 12585x - 3945$  ( $R^2 = 0.9940$ ).

205

206 *2.11. Statistical analysis*

207 All data are presented as mean  $\pm$  standard deviation (SD) for three replicates for each prepared  
208 sample. Univariate analysis of variance (ANOVA) with Tukey post-hoc test was applied using  
209 Graph Pad Prism 6.0 (GraphPad Software, San Diego, CA, USA). The differences were considered  
210 significant with  $P < 0.05$ .

## 211 3. Results and Discussion

212

### 213 3.1. Assessment of protein hydrolysis during simulated digestion

214 The chemical composition of skimmed camel milk expressed as percentage (w/w) was: total solids  
215 13%, total proteins 3.48%, caseins 2.68%, whey proteins 0.80%, lactose 4.87%, fat 0.05%. The pH  
216 was 6.61.

217 The hydrolysis of camel milk proteins during the *in vitro* digestion was characterized by assessing  
218 the DH value and through SDS-PAGE analysis.

219 As expected, simulated salivary digestion did not increase the hydrolysis degree. Un-digested camel  
220 milk showed a DH of  $2.7 \pm 0.1$ , which was  $2.9 \pm 0.1$  after 5 minutes of salivary digestion ( $P >$   
221  $0.05$ ). Instead, a significant increase ( $P < 0.0001$ ) in the DH was found already after 30 minutes of  
222 gastric digestion (**Figure 1**). After that, peptic activity resulted in a further but not significant  
223 increase in DH during the 120 minutes of gastric digestion. Pancreatic digestion had a deep effect  
224 on protein hydrolysis (**Figure 1**) which increased significantly ( $P < 0.0001$ ) from 20.5% (post-  
225 gastric sample) to 69.6% (post-pancreatic sample). The greatest increase was observed after 30  
226 minutes of pancreatic digestion ( $P < 0.0001$  respect to the post-gastric sample; 120 min of  
227 digestion). Subsequently, for the remaining 90 minutes of pancreatic digestion the value remained  
228 almost constant.

229 A comparison with the data reported by Kopf-Bolan et al. (2012) showed that camel milk proteins  
230 were more prone to hydrolysis by gastro-intestinal proteases respect to bovine milk. Complete  
231 digestion of bovine milk resulted in a DH of 54 respect to the value of 69.6 found for camel milk.

232 These results are also in agreement with Salami et al. (2008) who found that the extent of  
233 hydrolysis of camel caseins with pancreatic enzymes was greater than that of bovine caseins.

234 A comparison of the protein pattern of the digested samples revealed that salivary juice had no  
235 effect on protein (**Figure 2A, lane 2**) as compared to the un-digested camel milk (**Figure 2A, lane**  
236 **1**). The addition of gastric juice without pepsin caused a decrease in the intensity of the protein

bands probably related to precipitation at acidic pH values (**Figure 2A, lane 3**). All caseins and major camel milk proteins were completely digested within 5 minutes by the gastric juice with the exception of  $\alpha$ -lactalbumin (**Figure 2A, lanes 4-8**) suggesting that pepsin alone was not able to digest this whey protein. However, the addition of the intestinal fluid resulted in the hydrolysis of  $\alpha$ -lactalbumin (**Figure 2B, lanes 3-7**), indicating that the presence of pancreatic enzymes and bile salt is basic for  $\alpha$ -lactalbumin digestion. These results are in accordance with those of Salami et al. (2008) showing that camel  $\alpha$ -lactalbumin was slowly hydrolyzed by trypsin but it was more extensively hydrolyzed by chymotrypsin.

245

### 3.2. ACE-inhibitory activity and RP-HPLC fractionation of < 3 kDa fraction of digested camel milk

Peptidic fraction was further extracted from the post-pancreatic digested sample through ultrafiltration with a cut-off of 3 kDa. Hereinafter, we concentrated on the < 3 kDa fractions of digested camel milk because it is likely to contain peptides that are potentially less susceptible to further digestion by brush border proteases and may be absorbed at intestinal level reaching the blood stream. The total amount of peptides found in the < 3 kDa fraction was calculated resulting in a value of 21.74 mg mL<sup>-1</sup>.

The post-pancreatic peptidic fraction was used for the determination of the ACE-inhibitory activity of the peptides produced during the digestion. The IC<sub>50</sub> value was 1771.4 ± 3.1 µg of peptides mL<sup>-1</sup>. With the aim of identifying putative active peptides, the < 3 kDa fraction of the samples obtained during simulated digestion was loaded on the HPLC C18 column and peptides were detected at 214 and 280 nm with a photodiode array detector.

The chromatogram presented in **Figure 3** shows most peptides eluting between 6 and 45 min, whereas no additional peptides were found after 60 minutes of elution. As shown in **Figure 3**, eight fractions (F1–F8) were collected.

The peptide content of collected fractions ranged from 0.71 to 11.10 mg mL<sup>-1</sup> (**Table 1**). The yield of the eight collected fractions was estimated (**Table 1**). Fractions from F3 to F8 showed a similar

263 yield ranging from 2 and 5%. Fraction F1 showed the highest yield of approximately 51% (**Table**  
264 **1**). The sum of the peptide concentration in the collected fractions resulted in 18.69 mg mL<sup>-1</sup>, giving  
265 a total yield of 86%.

266 All fractions exerted some ACE-inhibitory activity. **Table 1** shows the ACE-inhibitory activity,  
267 expressed as IC<sub>50</sub> values, of the peptidic fractions isolated by RP-HPLC. Low IC<sub>50</sub> values, which  
268 means high ACE-inhibitory activity, were found in fractions F1, F2 and F4. The lowest IC<sub>50</sub> values  
269 of 37.2 ± 0.3 and 38.6 ± 0.4 µg peptides mL<sup>-1</sup> were found in fractions F4 and F1, respectively,  
270 which were about thirty times lower than the IC<sub>50</sub> value of the post-pancreatic < 3 kDa permeate.

### 271

### 272 3.3. NanoLC-ESI-QTOF-MS/MS analysis of the HPLC collected fractions

273 On the basis of ACE-inhibitory activities of the collected HPLC fractions, F1, F2 and F4 were  
274 selected and analyzed with nanoflow LC-ESI-QTOF MS to identify the peptides present in these  
275 fractions. **Figure 4** shows the full MS spectra of the fractions F1, F2 and F4. Each peak was  
276 selected for peptide identification by MS/MS ion scan using de novo sequencing software. Results  
277 from peptide identification were subjected to a manual evaluation, and the validated peptide  
278 sequences were responsibly for the majority of the most intense peaks in the MS spectra.

279 In the lowest part of the MS spectra of fraction F1 (**Figure 4A**) the most intense signals were  
280 identified as the amino acids T (m/z=120.0807), (iso)leucine (Lx; m/z=132.1014) and  
281 phenylalanine (F; m/z=166.0861). An additional signal at m/z of 182.0805 was assigned to the  
282 aromatic amino acid tyrosine (Y). In the peptidic part of the spectra the most intense signals  
283 corresponded to the dipeptide SLx (m/z 219.1330) and the tripeptides VPT (m/z= 316.2101) and  
284 GS(phospho)Q (m/z= 371.2290). The list of compounds identified in fraction F1 is shown in **Table**  
285 **2** together with the MS data, the protein precursor and the potential bioactivity.

286 In fraction F2 (**Figure 4B**), phenylalanine still represented the most intense signal (m/z=166.0854).  
287 An additional amino acid (threonine) was identified (m/z=120.0792). The peptides which gave the  
288 most intense signals in this fraction were the tripeptide NPT (m/z=331.1643) and the dipeptide PLx

289 (m/z=229.1554). The longest identified peptide was the  $\beta$ -casein-derived tetrapeptide LQPK  
 290 (m/z=485.3254). **Table 3** reports the complete list of identified compounds together with the MS  
 291 and bioactivity data.

292 In the fraction F4 (**Figure 4C**), the dominant signal was tryptophan (W; m/z=205.0942). The  
 293 peptides PLx and LxP (m/z= 229.1504), VPY (m/z=378.1953) and LQSP (m/z=444.2396) also  
 294 gave intense signals in the MS spectra. All the identified compounds are reported in **Table 4**.  
 295 Interestingly, all the amino acids identified in the three fractions (threonine, tyrosine, phenylalanine,  
 296 tryptophan and (iso)leucine) are essential for humans, suggesting that camel milk represents a good  
 297 source of essential amino acids.

298

299 *3.4. Identification of ACE-inhibitory peptides in HPLC fractions F1, F2 and F4*

300 A dipeptide with potent ACE-inhibitory activity (AI;  $IC_{50}= 3.4 \mu\text{mol L}^{-1}$ ) and several other  
 301 dipeptides (GLx, VP and DL) with higher  $IC_{50}$  values were isolated from fraction F1 (**Table 2**). The  
 302 dipeptide AI was previously isolated from soy sauce-like seasoning and pinto bean proteins after  
 303 gastro-intestinal digestion (Nakahara et al., 2010; Tagliazucchi et al., 2015).

304 In fraction F2 (**Table 3**), the most effective ACE-inhibitory peptide was VY ( $IC_{50}= 7.1 \mu\text{mol L}^{-1}$ )  
 305 followed by TF ( $IC_{50}= 18 \mu\text{mol L}^{-1}$ ). Three others peptides able to inhibit ACE activity were  
 306 detected in this fraction (VP, PLx and SF). In particular, the dipeptide VY, previously isolated from  
 307 brewed sake was effective also *in vivo* by decreasing systolic blood pressure of spontaneously  
 308 hypertensive rats of 31 mmHg (Saito, Wanezaki, Kawato & Imayasu, 1994). More interestingly,  
 309 VY was also found in human plasma after consumption of a peptide-enriched drink, indicating that  
 310 this peptide is bioavailable also in humans (Foltz et al., 2007). In addition, administration to mild  
 311 hypertensive subjects of hydrolysed sardine muscle proteins containing 6 mg of VY resulted in a  
 312 significant decrease of blood pressure (Kawasaki et al., 2000).

313 Four peptides in the fraction F4 showed very low  $IC_{50}$  values. The tripeptide LHP, previously  
 314 isolated from peptic hydrolysate of shrimp meat (Cao, Zhang, Hong, Ji & Hao, 2000) and the milk-



315 derived tripeptide IPP (Solieri et al., 2015) showed  $IC_{50}$  values of 1.6 and 5  $\mu\text{mol L}^{-1}$ , respectively.

316 The dipeptides LY and IY, which were previously isolated from sardine muscle, demonstrated  $IC_{50}$

317 values of 18 and 2.1  $\mu\text{mol L}^{-1}$ , respectively (Matsufuji et al., 1994). Two additional dipeptides (PL

318 and IP) and a tripeptide (VPY) with higher  $IC_{50}$  values were found in fraction F4 as reported in

319 **Table 4.**

320 Some other peptides may have the potential to exert inhibitory activity towards ACE. Structure-

321 activity relationships study (Wu, Aluko & Nakai, 2006) reported that for dipeptides, amino acid

322 residues at the C-terminal position with large bulk chains or hydrophobic side chains such as

323 aromatic amino acids (phenylalanine, tyrosine and tryptophan) and branched aliphatic side amino

324 acids (leucine, isoleucine and valine) are preferred for ACE-inhibitory activity (Wu et al., 2006).

325 Based on these considerations, two dipeptides in fraction F1 (VV, SLx), one dipeptide in fraction

326 F4 (PF) as well as one dipeptide found in both F1 and F2 fractions (TLx) and one found in both F2

327 and F4 fractions (VLx) could be active against ACE.

328 Several of these dipeptides share significant structural attributes with previously identified ACE-

329 inhibitory peptides. The dipeptide SL matches the last two C-terminal residues, which play a main

330 role in binding to the ACE active site, with the peptide ASL isolated from the silkworm *Bombyx*

331 *mori* (Wu, Feng, Lan, Xu & Liao, 2015). Similarly, the dipeptides VL and PF correspond to the C-

332 terminal residues of the ACE-inhibitory peptides LVL, isolated from porcine plasma (Hazato &

333 Kase, 1986) and DKIHPPF isolated from bovine  $\beta$ -casein (Gobetti, Ferranti, Smacchi, Goffredi &

334 Addeo, 2000).

335

### 336 3.5. Quantification of IPP in fraction F4

337 IPP was further quantified in the fraction F4. The amount of released IPP at the end of the digestion

338 procedure resulted in  $2.56 \pm 0.15 \text{ mg L}^{-1}$  of milk.

339 In camel milk, the tripeptide IPP is present only in  $\kappa$ -casein in position 99-101. According to

340 Kappeler, Farah, and Puhan (2003), the  $\kappa$ -casein component in camel milk represents 3.47% of the

total caseins. As the casein concentration was  $26.8 \text{ g L}^{-1}$ , the estimated  $\kappa$ -casein concentration was about  $930 \text{ mg L}^{-1}$  (approximately  $50 \text{ } \mu\text{mol L}^{-1}$ ). Considering that, the IPP concentration found at the end of the digestion was  $7.87 \text{ } \mu\text{mol L}^{-1}$  (IPP molecular weight 325.4182), the recovery yield of IPP was approximately 15.7%.

The tripeptide IPP exhibited low susceptibility *in vitro* to the brush border peptidases activity (Ohsawa et al., 2008), and was able to reach blood circulation un-degraded (Foltz et al., 2007). Several clinical studies on hypertensive subjects showed that the administration of daily doses of VPP/IPP in the range of 2-6 mg were associated with a decrease of the blood pressure between 1.5 and 10 mmHg (Cicero, Aubin, Azais-Braesco & Borghi, 2013).

In bovine milk, IPP is easily released from  $\beta$ - and  $\kappa$ -casein by the action of proteinases and peptidases from several strains of *Lactobacillus* during milk fermentation and cheese ageing (Solieri et al., 2015; Stuknite, Cattaneo, Masotti & De Noni, 2015). However, IPP was not released after hydrolysis of bovine  $\beta$ -casein using an *in vitro* gastro-intestinal digestion system suggesting that mammalian gastrointestinal proteolytic enzymes are not able to produce IPP itself (Ohsawa et al., 2008).

The analysis of the theoretical cleavage sites of the IPP-containing sequence in camel milk  $\kappa$ -casein (**Figure 5**), suggested that several digestive proteolytic enzymes might be involved in the release of IPP. Camel milk  $\kappa$ -casein contains the tripeptide in position 100-102. The peptide bond  $\text{A}_{99}\text{---I}_{100}$  can be easily hydrolyzed by pepsin, chymotrypsin, or elastase whereas the peptide bond  $\text{K}_{103}\text{---K}_{104}$  is a cleavage site for trypsin. The action of these enzymes should result in the release of the tetrapeptide IPPK (f100-103). The residue  $\text{K}_{103}$  can be removed by the action of the pancreatic carboxypeptidase B (C-terminal exopeptidase) which cleave specifically C-terminal lysine and arginine residues. As a confirmation of the suggested pathway of IPP formation, the tetrapeptide IPPK was found as double-charged ion in the fraction F4 (**Table 4**).

**Figure 5** also reports the theoretical cleavage sites by gastro-intestinal proteases of the sequences of  $\beta$ - and  $\kappa$ -caseins containing the tripeptide IPP. Bovine  $\kappa$ -casein contains IPP in position 108-110

and the amino acids close to IPP are the same as in camel  $\kappa$ -casein suggesting that the same pathway can be involved in the release of IPP. In bovine  $\beta$ -casein, IPP is located at position 74-76. As detailed in **Figure 5**, the peptide bonds N<sub>73</sub>—I<sub>74</sub> and L<sub>77</sub>—T<sub>78</sub> can be hydrolysed by chymotrypsin, releasing the tetrapeptide IPPL. The C-terminal L<sub>77</sub> residue can be subsequently removed by pancreatic carboxypeptidase A releasing the tripeptide IPP. On the other hand, as mentioned above, Ohsawa et al. (2008) failed to detect VPP and IPP after gastro-intestinal hydrolysis of bovine  $\beta$ -casein. This discrepancy can be ascribed to different causes. Firstly, the *in vitro* digestion system used in the Ohsawa study is different from that used in this study. The biggest difference is the amount of digestive enzyme added to the mixture, which was higher in our system. The digestion system used in this study has been validated for milk macronutrient digestion and closely mimics the digestive process in humans, especially for protein digestion, resulting in the formation of free amino acids and small peptides (2-6 amino acids) (Kopf-Bolanz et al., 2012). Secondly, for the identification of peptides, we used a *de novo* sequencing software, which is able to identify also shorter peptide such as di- or tri-peptides. Instead, the software commonly used for proteomic study and adapted for peptide identification, such as Mascot, have normally a minimum peptide length for identification of five residues (Koskinen, Emery, Creasy & Cottrell, 2011).

384

#### 385 **4. Conclusion**

This paper demonstrates for the first time the presence of ACE-inhibitory peptides in the low molecular weight (< 3kDa) fraction of digested camel milk. In addition, some of the identified peptides showed marked structural similarities with previously described ACE inhibitors. This work also provides evidence, for the first time, of the fact that IPP may be released during the gastrointestinal digestion of camel milk  $\kappa$ -casein. Basing on the protease specificity in the digestive system and on the sequence of bovine  $\beta$ - and  $\kappa$ -caseins it can be predicted that the anti-hypertensive tripeptide IPP (but also VPP) can be released during bovine milk digestion. Further experiment

393 using physiologically validated *in vitro* model of human digestion system and *de novo* sequencing  
394 software could confirm this hypothesis. Our results highlight the ability of gastro-intestinal  
395 proteases at physiological conditions to release ACE-inhibitory peptides. Likewise, based on the  
396 structure-activity relationship studies, several putative new bioactive peptides could be selected for  
397 further biological activities analysis. This research provides the basis to increase the exploitation of  
398 the health benefits of camel milk proteins.

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## Figure captions

**Figure 1.** Changes in hydrolysis degree (DH) of camel milk proteins during *in vitro* gastric and pancreatic digestion. Values represent means  $\pm$  SD of triplicate digestions. Different letters indicate that the values are significantly different ( $P < 0.05$ ).

**Figure 2.** Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of camel milk proteins. **(A)** Un-digested camel milk is shown in lane 1. Sample after salivary digestion is shown in lane 2. Sample in lane 3 is the camel milk protein profile after addition of simulated gastric fluid without pepsin. Samples after gastric digestion are in lanes 4 (5 minutes of gastric digestion), 5 (30 minutes), 6 (60 minutes), 7 (90 minutes) and 8 (120 minutes of gastric digestion). Samples after pancreatic digestion are reported in **(B)**. Lane 1 is un-digested camel milk. Sample in lane 2 is the camel milk protein profile after addition of simulated intestinal fluid without pancreatin. Samples after pancreatic digestion are in lanes 3 (5 minutes of intestinal digestion), 4 (30 minutes), 5 (60 minutes), 6 (90 minutes) and 7 (120 minutes of intestinal digestion). SDS-PAGE was carried in triplicate for each digestion. The showed electropherogram is representative of three independent experiments.

**Figure 3.** UV-chromatogram of the low molecular weight peptidic fraction ( $<3$  kDa) obtained from camel milk subjected to consecutive gastro-pancreatic digestion. 1-8 represent the collected fractions used for the identification of the angiotensin converting enzyme-inhibitory peptides. The black line represents the trace at 214 nm whereas the grey line represents the trace at 280 nm. The showed chromatogram is representative of three independent experiments.

**Figure 4.** Mass spectrum of peptide fractions F1 **(A)**, F2 **(B)** and F4 **(C)** from nanoflow LC-ESI-QTOF MS/MS analysis. Identified peptides are reported in tables 2, 3 and 4. The showed mass spectra are representative of three independent experiments.

**Figure 5.** Theoretical cleavage sites in the Ile-Pro-Pro (IPP)-containing sequences of camel and bovine  $\kappa$ -casein and bovine  $\beta$ -casein. CA: carboxypeptidase A; CB: carboxypeptidase B; Ch: chymotrypsin; E: elastase; P: pepsin; T: trypsin.

**Table 1.** Peptides concentration, yield, angiotensin-I converting enzyme (ACE)-inhibitory activity ( $IC_{50}$  values) of the post-pancreatic < 3 kDa permeate and peptide fractions obtained through reversed phase-high performance liquid chromatography purification of the post-pancreatic fraction. Values represent means  $\pm$  standard deviation of triplicate determination.

|                  | <i>Peptides concentration</i><br>(mg mL <sup>-1</sup> ) | <i>Estimated yield<sup>a</sup></i> (%) | <i>IC<sub>50</sub><sup>b</sup></i><br>( $\mu$ g peptides mL <sup>-1</sup> ) |
|------------------|---|--|---|
| < 3 kDa permeate | 21.74 $\pm$ 0.49 <sup>a</sup>                           | 100                                    | 1171.4 $\pm$ 3.1 <sup>a</sup>   |
| HPLC F1          | 11.10 $\pm$ 0.33 <sup>b</sup>                           | 51.1                                   | 38.6 $\pm$ 0.4 <sup>b</sup>   |
| HPLC F2          | 2.50 $\pm$ 0.11 <sup>c</sup>                            | 11.5                                   | 107.9 $\pm$ 1.2 <sup>c</sup>  |
| HPLC F3          | 1.07 $\pm$ 0.07 <sup>d</sup>                            | 4.9                                    | 720.3 $\pm$ 5.9 <sup>d</sup>  |
| HPLC F4          | 1.08 $\pm$ 0.09 <sup>d</sup>                            | 5.0                                    | 37.2 $\pm$ 0.3 <sup>b</sup>   |
| HPLC F5          | 0.44 $\pm$ 0.02 <sup>e</sup>                            | 2.0                                    | > 1000  |
| HPLC F6          | 0.79 $\pm$ 0.04 <sup>f</sup>                            | 3.6                                    | 927.4 $\pm$ 8.8 <sup>e</sup>  |
| HPLC F7          | 0.71 $\pm$ 0.02 <sup>f</sup>                            | 3.3                                    | > 1000  |
| HPLC F8          | 1.00 $\pm$ 0.02 <sup>d</sup>                            | 4.6                                    | > 1000  |

<sup>a</sup> yield was calculated as follows: (peptides concentration in the fraction)\*100/ (peptides concentration in the post-pancreatic < 3 kDa permeate).

<sup>b</sup>IC<sub>50</sub> is defined as the concentration of peptides needed to inhibit of 50% ACE activity.

Different letters within the same column indicate that the values are significantly different ( $P < 0.05$ ).

**Table 2.** Peptides and amino acids identified in the reversed phase-high performance liquid chromatography fraction F1 of < 3 kDa permeate obtained from camel milk after simulated gastro-intestinal digestion.

| <i>Observed mass (m/z)</i> | <i>Calculated mass<sup>a</sup></i> | <i>Peptide sequence<sup>b</sup></i> | <i>Protein precursor</i>   | <i>Bioactivity<sup>c</sup></i>   |
|----------------------------|------------------------------------|-------------------------------------|--|--|
| 120.0807                   | 122.0855                           | T                                   | Various proteins   | /  |
| 132.1014                   | 132.1019                           | Lx                                  | Various proteins   | /  |
| 166.0861                   | 166.0863                           | F                                   | Various proteins   | /  |
| 182.0805                   | 182.0812                           | Y                                   | Various proteins   | Antioxidant  |
| 189.1246                   | 189.1234                           | GLx                                 | $\beta$ -, $\alpha$ S1, $\kappa$ -casein, and $\alpha$ -lactalbumin    | ACE-inhibitor ( <u>GL/GI</u> IC <sub>50</sub> = >1000 $\mu$ mol L <sup>-1</sup> )<br>DPP IV inhibitor ( <u>GL/GI</u> ) |
| 203.1427                   | 203.1390                           | ALx                                 | $\beta$ -, $\alpha$ S1-, $\alpha$ S2-, $\kappa$ -casein                | ACE-inhibitor ( <u>AI</u> IC <sub>50</sub> = 3.4 $\mu$ mol L <sup>-1</sup> )<br>DPP IV inhibitor ( <u>AL</u> )         |
| 215.1413                   | 215.1390                           | VP                                  | $\beta$ -, $\alpha$ S2-, $\kappa$ -casein                              | ACE-inhibitor (IC <sub>50</sub> = 420 $\mu$ mol L <sup>-1</sup> )<br>DPP IV inhibitor                                  |
| 217.1561                   | 217.1547                           | VV                                  | $\beta$ -, $\alpha$ S1, $\kappa$ -casein, and $\alpha$ -lactalbumin    | DPP IV inhibitor   |
| 219.1330                   | 219.1339                           | SLx                                 | $\beta$ -, $\alpha$ S1-, $\alpha$ S2-casein                            | DPP IV inhibitor ( <u>SL</u> )   |
| 233.1496                   | 233.1496                           | TLx                                 | $\beta$ -, $\alpha$ S2-casein  | DPP IV inhibitor   |
| 233.1573                   | 233.1496                           | LxT                                 | $\beta$ -, $\alpha$ S2, $\kappa$ -casein, and $\alpha$ -lactalbumin    | DPP IV inhibitor ( <u>LT</u> )   |
| 246.1450                   | 246.1448                           | GGI                                 | $\alpha$ -lactalbumin f(19-21)   | /  |
| 247.1295                   | 247.1288                           | DLx                                 | $\beta$ -, $\alpha$ S1-, $\alpha$ S2-casein, and $\alpha$ -lactalbumin | ACE-inhibitor ( <u>DL</u> IC <sub>50</sub> = >1000 $\mu$ mol L <sup>-1</sup> )   |
| 253.1177                   | 253.1183                           | FS                                  | $\kappa$ -casein f(96-97)  | /  |
| 267.1349                   | 267.1339                           | FT                                  | $\beta$ -, $\alpha$ S2-casein  | /  |
| 269.1712                   | 269.1608                           | LH                                  | $\beta$ -, $\alpha$ S1-, $\alpha$ S2-, $\kappa$ -casein                | Inhibitor of lipid peroxidation<br>DPP IV inhibitor  |
| 276.1567                   | 276.1554                           | SLG                                 | Lactoferrin and lactotransferrin<br>Cytochrome b, peptidoglycan        | /  |
| 302.1971                   | 302.2074                           | LxLxG                               | recognition protein 1, milk fat globule EGF factor 8                   | ACE-inhibitor ( <u>LLG</u> IC <sub>50</sub> = >1000 $\mu$ mol L <sup>-1</sup> )  |

|          |          |              |                                  |                  |
|----------|----------|--------------|----------------------------------|------------------|
| 316.2101 | 316.2067 | VPT          | $\alpha$ S2-casein<br>f(116-118) | /                |
| 326.2049 | 326.2074 | PPL          | $\beta$ -casein<br>f(78-80)      | DPP IV inhibitor |
| 371.2290 | 371.1962 | GS(phospho)Q | Various proteins                 | /                |

<sup>a</sup>Monoisotopic mass

<sup>b</sup>Lx indicates leucine or isoleucine

<sup>c</sup>Potential bioactivities were achieved from the BIOPEP database; ACE: Angiotensin Converting Enzyme ; DPP IV: Dipeptidyl peptidase IV; IC<sub>50</sub> is defined as the concentration of peptides required to inhibit 50% of the enzymatic activity

**Table 3.** Peptides and amino acids identified in the reversed phase-high performance liquid chromatography fraction F2 of < 3 kDa permeate obtained from camel milk after simulated gastro-intestinal digestion

| <i>Observed mass (m/z)</i> | <i>Calculated mass<sup>a</sup></i> | <i>Peptide sequence<sup>b</sup></i> | <i>Protein precursor</i>             | <i>Bioactivity<sup>c</sup></i>   |
|----------------------------|------------------------------------|-------------------------------------|--------------------------------------|--|
| 120.0792                   | 122.0855                           | T                                   | Various proteins                     | /  |
| 166.0854                   | 166.0863                           | F                                   | Various proteins                     | /  |
| 215.1333                   | 215.1390                           | VP                                  | β-, αS2-, κ-casein                   | ACE-inhibitor (IC <sub>50</sub> 420 μmol L <sup>-1</sup> )<br>DPP IV inhibitor                               |
| 229.1554                   | 229.1547                           | PLx                                 | β-, αS1-, κ-casein and α-lactalbumin | ACE-inhibitor ( <u>PL</u> IC <sub>50</sub> = 330 μmol L <sup>-1</sup> )<br>DPP IV inhibitor ( <u>PL/PI</u> ) |
| 231.1706                   | 231.1703                           | VLx                                 | β-, αS1-, κ-casein                   | DPP IV inhibitor ( <u>VL/VI</u> )<br>Glucose uptake stimulating peptide ( <u>VL</u> )                        |
| 231.1764                   | 231.1703                           | LxV                                 | β-, αS2-casein                       | Glucose uptake stimulating peptide ( <u>LV/IV</u> )<br>DPP IV inhibitor ( <u>LV</u> )                        |
| 233.1453                   | 233.1496                           | TLx                                 | β-casein and α-lactalbumin           | DPP IV inhibitor ( <u>TL/TI</u> )  |
| 249.1273                   | 249.1267                           | VM                                  | β-, αS1-, αS2-casein                 | DPP IV inhibitor   |
| 253.1238                   | 253.1183                           | SF                                  | κ-casein f(96-97)                    | ACE-inhibitor (IC <sub>50</sub> 130 μmol L <sup>-1</sup> )<br>DPP IV inhibitor                               |
| 267.1293                   | 267.1339                           | TF                                  | β-, αS2-casein                       | ACE-inhibitor (IC <sub>50</sub> 18 μmol L <sup>-1</sup> )<br>DPP IV inhibitor                                |
| 281.1478                   | 281.1496                           | VY                                  | β-, αS2-casein                       | ACE-inhibitor (IC <sub>50</sub> 7.1 μmol L <sup>-1</sup> )<br>Antioxidant<br>DPP IV inhibitor                |
| 187.1329                   | 373.2445                           | LLQ                                 | β-, αS1-casein                       | /  |
| 272.1599                   | 272.1605                           | PGV                                 | Pancreatic lipase                    | /  |
| 290.1769                   | 290.1710                           | TVA                                 | κ-casein f(116-118)                  | /  |
| 316.2188                   | 316.2231                           | IAI                                 | κ-casein f(98-100)                   | /  |
| 318.1940                   | 318.2023                           | VVT                                 | αS1-casein f(151-53)                 | /  |
| 320.1868                   | 320.1816                           | SLxT                                | β-casein f(20-22) f(125-127)         | /  |
| 331.1643                   | 331.1612                           | NPT                                 | Cytochrome b f(207-209)              | /  |
| 344.1734                   | 344.1565                           | SHT                                 | β-casein                             | /  |

|          |          |      |   |   |
|----------|----------|------|---|---|
|          |          |      | f(62-64)                                  |   |
| 345.2180 | 345.2132 | VLN  | $\alpha$ S1-casein<br>f(22-24)            | / |
| 348.1860 | 348.1765 | DLT  | $\alpha$ -lactalbumin<br>f(84-86)         | / |
| 357.2130 | 35.2132  | PLxQ | $\beta$ -, $\alpha$ S1-, $\kappa$ -casein | / |
| 361.1988 | 361.2082 | QIT  | $\kappa$ -casein<br>f(156-158)            | / |
| 368.1748 | 368.1816 | VYS  | $\beta$ -casein<br>f(60-62)               | / |
| 400.2561 | 400.2554 | GVPK | Various proteins                          | / |
| 414.2329 | 414.2711 | KPVA | $\kappa$ -casein<br>f(63-66)              | / |
| 457.2305 | 457.2194 | TVTH | Various proteins                          | / |
| 485.3254 | 485.3082 | LQPK | $\beta$ -casein<br>f(89-92)               | / |

<sup>a</sup>Monoisotopic mass

<sup>b</sup>Lx indicates leucine or isoleucine

<sup>c</sup>Potential bioactivities were achieved from the BIOPEP database; ACE: Angiotensin Converting Enzyme ; DPP IV: Dipeptidyl peptidase IV; IC<sub>50</sub> is defined as the concentration of peptides required to inhibit 50% of the enzymatic activity

**Table 4.** Peptides and amino acids identified in the reversed phase-high performance liquid chromatography fraction F4 of < 3 kDa permeate obtained from camel milk after simulated gastro-intestinal digestion

| <i>Observed mass (m/z)</i> | <i>Calculated mass<sup>a</sup></i> | <i>Peptide sequence<sup>b</sup></i> | <i>Protein precursor</i>   | <i>Bioactivity<sup>c</sup></i>   |
|----------------------------|------------------------------------|-------------------------------------|--|--|
| 205.0942                   | 205.0972                           | W                                   | Various proteins   | Antioxidant  |
| 229.1504                   | 229.1547                           | PLx                                 | $\beta$ -, $\alpha$ S1, $\kappa$ -casein, and $\alpha$ -lactalbumin              | ACE-inhibitor ( <u>PL</u> IC <sub>50</sub> = 330 $\mu$ mol L <sup>-1</sup> )<br>DPP IV inhibitor ( <u>PL/PI</u> )  |
| 229.1504                   | 229.1547                           | LxP                                 | $\beta$ -, $\alpha$ S1, $\alpha$ S2-, $\kappa$ -casein                           | ACE-inhibitor ( <u>IP</u> IC <sub>50</sub> = 130 $\mu$ mol L <sup>-1</sup> )<br>DPP IV inhibitor ( <u>LP/IP</u> )  |
| 231.1675                   | 231.1703                           | VLx                                 | $\beta$ -, $\alpha$ S1, $\kappa$ -casein, and $\alpha$ -lactalbumin              | DPP IV inhibitor ( <u>VL/VI</u> )<br>Glucose uptake stimulating peptide ( <u>VL</u> )  |
| 245.1903                   | 245.1860                           | LxLx                                | $\beta$ -, $\alpha$ S1, $\kappa$ -casein, and $\alpha$ -lactalbumin              | ACE-inhibitor ( <u>LL</u> IC <sub>50</sub> = n.r.)<br>DPP IV inhibitor ( <u>LL/LI/IL/II</u> )<br>Glucose uptake stimulating peptide ( <u>LL/LI/IL/II</u> ) |
| 263.1351                   | 263.1390                           | PF                                  | $\beta$ -, $\alpha$ S1, $\alpha$ S2-casein                                       | DPP IV inhibitor   |
| 295.1626                   | 295.1652                           | LxY                                 | $\beta$ -, $\alpha$ S1, $\alpha$ S2-casein                                       | ACE-inhibitor ( <u>LY</u> IC <sub>50</sub> = 18 $\mu$ mol L <sup>-1</sup> ; <u>IY</u> IC <sub>50</sub> = 2.1 $\mu$ mol/L)<br>Antioxidant ( <u>LY/IY</u> )  |
| 196.1704                   | 391.1976                           | FPQ                                 | $\beta$ -, $\alpha$ S1-casein  | /  |
| 288.1856                   | 288.1918                           | GLxV                                | Lactoferrin, lactotransferrin, cytochrome b, porcine pepsin A, pancreatic lipase | /  |
| 314.2002                   | 314.2074                           | PVV                                 | $\beta$ -casein f(116-118)   | /  |
| 326.2048                   | 326.2074                           | IPP                                 | $\kappa$ -casein f(99-101)   | ACE-inhibitor (IC <sub>50</sub> = 5.0 $\mu$ mol L <sup>-1</sup> )  |
| 350.1644                   | 350.1710                           | AYP                                 | $\alpha$ S2-casein f(95-97)  | /  |
| 360.2050                   | 360.2129                           | ILD                                 | $\alpha$ -lactalbumin f(95-97)   | /  |
| 366.2057                   | 366.2136                           | LxHP                                | $\beta$ -, $\alpha$ S1-casein  | ACE-inhibitor ( <u>LHP</u> IC <sub>50</sub> = 1.6 $\mu$ mol L <sup>-1</sup> )  |
| 374.2358                   | 374.2286                           | ILE                                 | $\alpha$ S1-casein f(28-30)  | /  |
| 378.1953                   | 378.2023                           | VPY                                 | $\beta$ -casein f(178-180)   | ACE-inhibitor (IC <sub>50</sub> = 288 $\mu$ mol L <sup>-1</sup> )  |
| 400.1937                   | 400.2013                           | HIM                                 | $\alpha$ S1-casein f(58-60)  | /  |
| 219.1259                   | 437.2507                           | IAHP                                | $\alpha$ S1-casein f(188-191)  | /  |

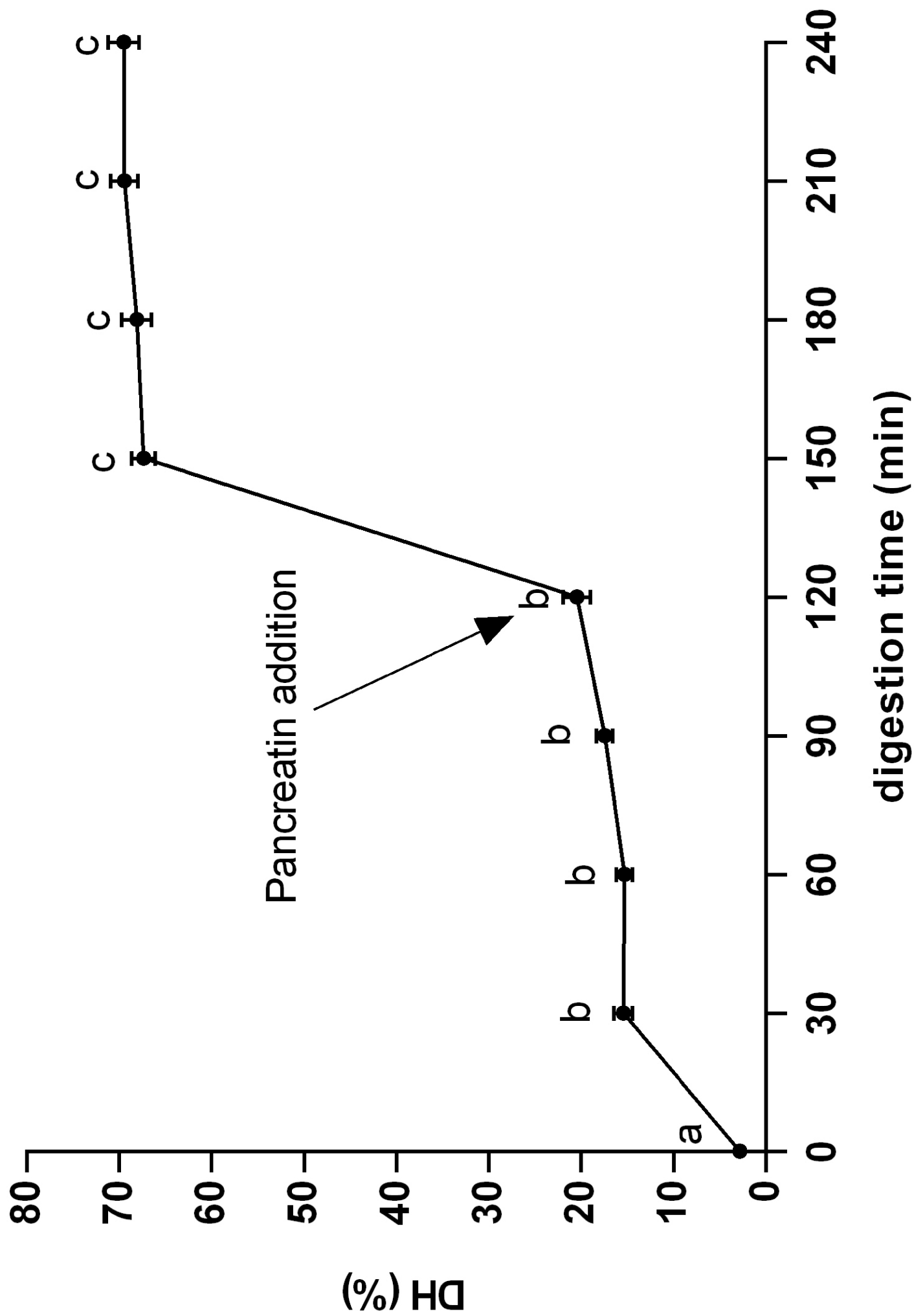


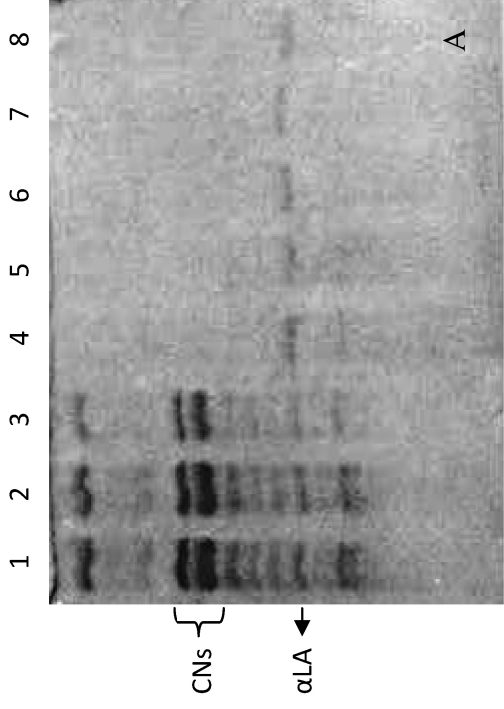
|          |          |        |                                  |   |
|----------|----------|--------|----------------------------------|---|
| 444.2396 | 444.2453 | LQSP   | $\beta$ -casein<br>f(113-116)    | / |
| 227.6459 | 454.3024 | IPPK   | $\kappa$ -casein<br>f(99-102)    | / |
| 459.2536 | 459.2449 | TEPI   | $\beta$ -casein<br>f(64-67)      | / |
| 235.6654 | 470.3337 | IIPK   | $\beta$ -casein<br>f(103-106)    | / |
| 504.2348 | 504.2453 | YPPQ   | $\alpha$ S1-casein<br>f(180-183) | / |
| 312.1753 | 623.3511 | VAHIPS | $\alpha$ S2-casein<br>f(27-32)   | / |
| 328.6844 | 656.3726 | TPVSPR | Serum albumin<br>f(313-318)      | / |
| 342.1603 | 683.3359 | SHTPEI | $\beta$ -casein<br>f(62-66)      | / |
| 344.1814 | 687.3762 | SLNEPK | Lactophorin<br>f(1-6)            | / |

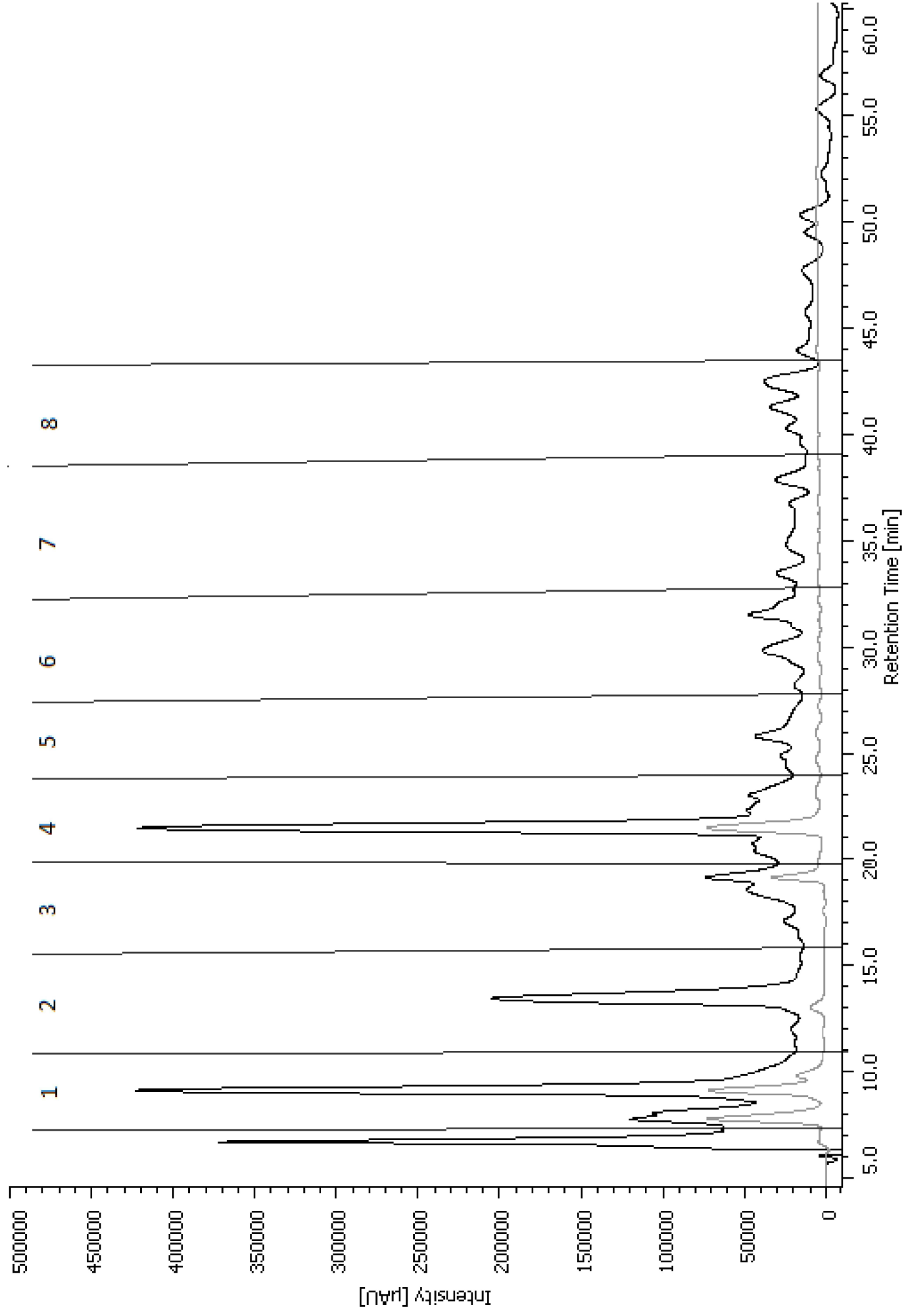
<sup>a</sup>Monoisotopic mass

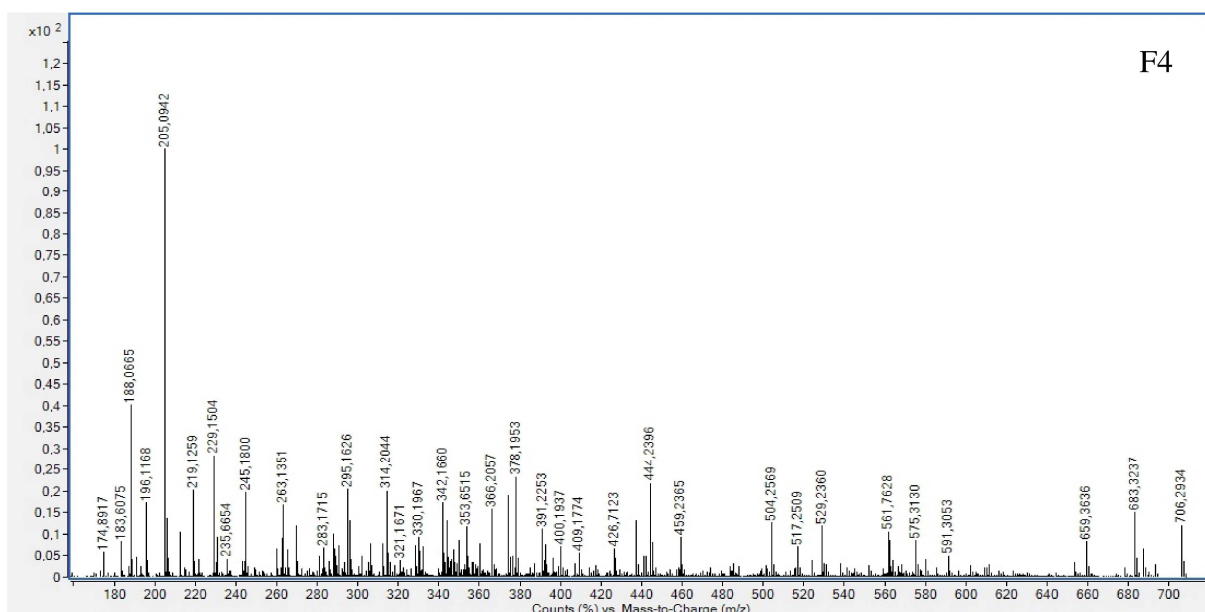
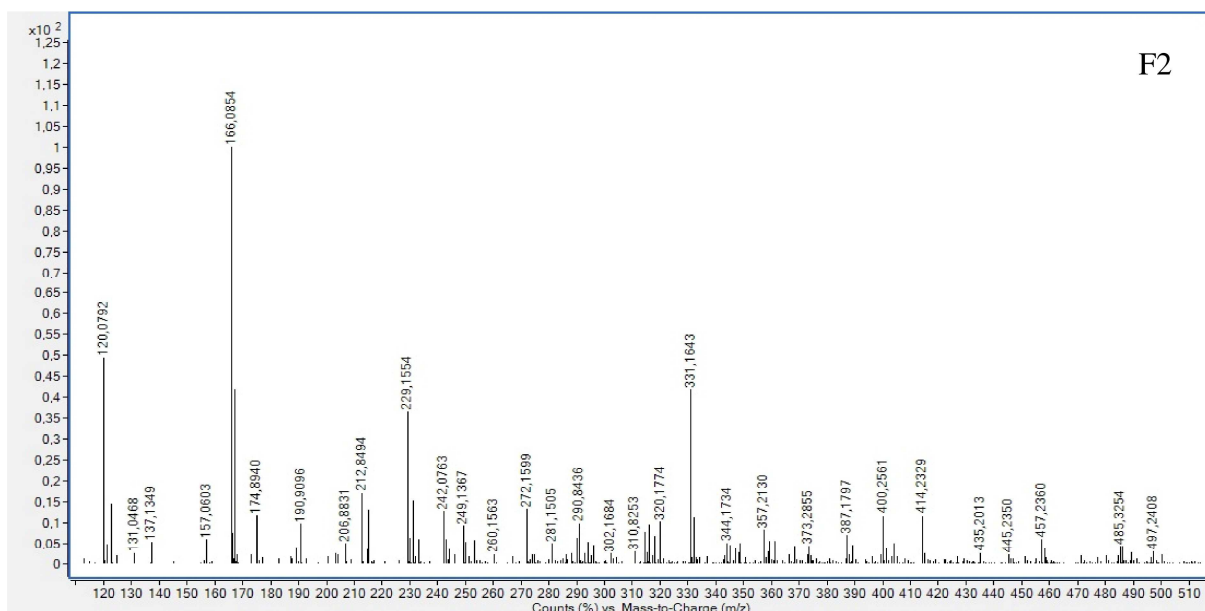
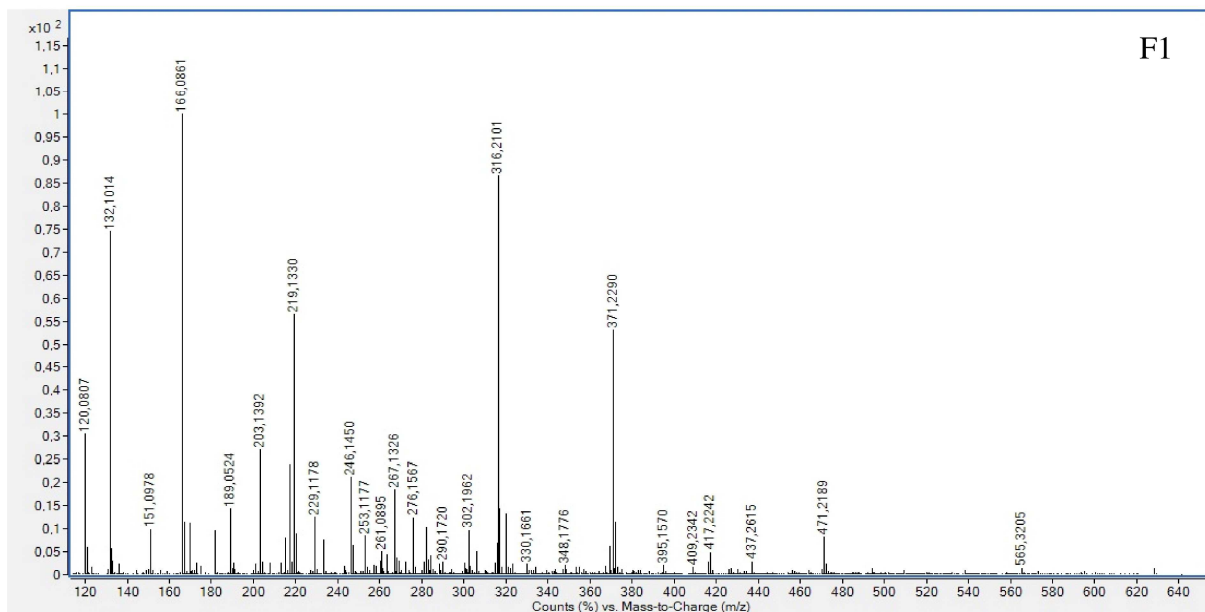
<sup>b</sup>Lx indicates leucine or isoleucine

<sup>c</sup>Potential bioactivities were achieved from the BIOPEP database; ACE: Angiotensin Converting Enzyme; DPP IV: Dipeptidyl peptidase IV; IC<sub>50</sub> is defined as the concentration of peptides required to inhibit 50% of the enzymatic activity









Camel  $\kappa$ -casein       $\begin{matrix} P_7\text{Ch}_7\text{E} & \text{CB} & \text{T} \\ \downarrow & \downarrow & \downarrow \end{matrix}$       **S F I A I P P K K T Q**      f(96-106)

Bovine  $\beta$ -casein       $\begin{matrix} \text{Ch} & \text{CA} & \text{Ch} \\ \downarrow & \downarrow & \downarrow \end{matrix}$       **L P Q N I P P L T Q T**      f(70-80)

Bovine  $\kappa$ -casein       $\begin{matrix} P_7\text{Ch}_7\text{E} & \text{CB} & \text{T} \\ \downarrow & \downarrow & \downarrow \end{matrix}$       **S F M A I P P K K N Q**      f(104-114)