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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 ± 0.15 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 κ-casein. This research provides the basis to increase the exploitation of the health benefits of

13 camel milk.

14

1. Introduction

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

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

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

enzymes and combinations of different proteinases such as alcalase and thermolysin have been

utilized to successfully generate bioactive peptides from various milk proteins. For example, two

peptides derived from bovine αS1-casein (RYLGY and AYFYPEL) after hydrolysis with pepsin,

were able to reduce blood pressure *in vivo* when administered in spontaneously hypertensive rats

(Contreras, Carrón, Montero, Ramos, & Recio, 2009). Three potent ACE-inhibitory peptides (LLF,

LVRT and LQKW) were purified from caprine β-lactoglobulin hydrolysed with thermolysin

(Hernández-Ledesma, Recio, Ramos & Amigo, 2002).

In a recent series of two papers, Picariello and co-workers (Picariello et al., 2010; Picariello et al.,

2013) *in vitro* studied the release of bioactive peptides after digestion of bovine milk caseins and

whey proteins. They found that some ACE-inhibitory peptides (such as the sequence HLPLP from

β-casein and the sequences GLDIQK and VLDTDYK from whey proteins) were released and stable

under gastro-intestinal conditions and were able to across Caco-2 cells monolayers. ACE-inhibitory

peptides (HLPLP and WSVPQPK) may be also released from human milk after *in vitro* gastro-

intestinal digestion (Hernández-Ledesma, Quirós, Amigo & Recio, 2007). In addition, *in vitro*

gastro-pancreatic digestion of donkey milk resulted in the release of a potent ACE-inhibitory

peptide (VAPFPQPVVP) corresponding to the fragment f(176-185) of β-casein (Bidasolo, Ramos

& Gomez-Ruiz, 2012).

Only a few studies on the antioxidant and ACE-inhibitory activities of camel milk protein-derived

peptides have been performed. Salami et al. (2011) and Moslehishad et al (2013) found that camel

milk proteins released ACE-inhibitory and antioxidant compounds after the treatment with digestive

proteases or after fermentation with *Lactobacillus rhamnosus*. However, the sequence of bioactive

compounds involved in the effect has not been revealed.

Caseins are, quantitatively, the most important proteins in camel milk. The sequence homology

between bovine and camel milk caseins varies from 53% for αS1-casein to 75% for β-casein.

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.

2. Materials and methods

2.1. Materials

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

2.2. Chemical analysis and camel skimmed milk preparation

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

2.3. In vitro gastro-intestinal digestion

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

Simulated salivary (SSF), simulated gastric (SGF), and simulated intestinal (SIF) fluids were prepared according to Kopf-Bolanz et al. (2012). SIF was prepared by mixing pancreatic (PF) and bile (BF) fluids. Skimmed camel milk (9 mL) was added to 12 mL of SSF containing 150 U mL^{-1} of porcine α-amylase and incubated for 5 min (oral phase). Gastric digestion was performed by adding 24 mL of SGF. The pH was adjusted to 2.0 with HCl 6 mol L^{-1} and supplemented with porcine

pepsin (1115 U mL^{-1} of SGF). The gastric bolus was further incubated for 120 min (gastric phase).

The intestinal digestion was carried out by adding to the gastric bolus 36 mL of SIF (24 mL of PF

and 12 mL of BF), adjusting the pH to 7.0 and supplemented with pancreatin. The chyme was

further incubated for 120 min (pancreatic phase). All incubations were performed at 37°C on a

rotating wheel (10 rpm). The digested samples were cooled on ice and immediately frozen at –80°C

for further analysis. The digestions were performed in triplicate.

A control digestion, which included only the gastro-intestinal juices and enzymes and water in place

of milk, was carried out to consider the possible impact of the digestive enzymes in the subsequent

analysis.

2.4. Determination of protein hydrolysis during the digestion

The determination of protein hydrolysis in the digested samples was carried out by measuring the

peptide concentration by the TNBS method using leucine as standard (Adler-Nissen, 1979). The

absorbance values at 340 nm were read using a Jasco V-550 UV/Vis spectrophotometer (Orlando

FL, U.S.A.).

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

DH= (h/h$_{tot}$) ·100   (1)

where **h** is the hydrolysis equivalent, defined as the concentration in milliequivalents/g of protein of

α-amino groups formed at the different stages of the simulated digestion, and **h$_{tot}$** is the hydrolysis

equivalent at complete hydrolysis to amino acids (calculated by summing the contents of the

individual amino acids in 1 g of protein and considering caseins as the only proteins in milk).

According to Adler-Nissen (1979), the **h$_{tot}$** value was fixed at 8, which is the value calculated for

caseins.

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

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

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

2.6. Fractionation of post-pancreatic digested samples

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

2.7. Measurements of ACE-inhibitory activity

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

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_{50} 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^{-1}

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^{-1}; 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

via a Chip Cube Interface (Agilent Technologies, Santa Clara, CA, USA). Chromatographic

separation was performed on a ProtID-Chip-43(II) including a 4mm 40 nL enrichment column and

a 43mm × 75μm analytical column, both packed with a Zorbax 300SB 5 μm C18 phase (Agilent

Technologies). The mobile phases composition and the gradient were the same as reported by

Tagliazucchi et al. (2015). The mass spectrometer was tuned, calibrated and set with the same

parameters as reported by Dei Più et al. (2014).

For peptide identification and sequencing, MS/MS spectra were converted to .mgf and *de novo*

peptide sequencing was performed using Pepnovo software

(http://proteomics.ucsd.edu/ProteoSAFe/). The following parameters were considered: enzyme,

none; peptide mass tolerance, ± 40 ppm; fragment mass tolerance, ± 0.12 Da; variable modification,

oxidation (M) and phosphorylation (ST); maximal number of PTMs permitted in a single peptide 3.

A search for the biological activity of peptides identified was carried out through the BIOPEP

database (http://www.uwm.edu.pl/biochemia/biopep/start_biopep.php). Confirmation of peptides

sequence in camel milk proteins was performed using Peptide Match

(http://research.bioinformatics.udel.edu/peptidematch/index.jsp).

2.10. Identification and quantification of IPP in fraction F4

IPP in fraction F4 was identified by comparing the retention time and fragmentation spectra of a

synthetic standard tripeptide. IPP was quantified in fraction F4 using the method reported in Solieri

et al. (2015). The synthetic tripeptide IPP was solubilized at 5 g L^{-1} in 0.1 mmol L^{-1} potassium

phosphate buffer (pH 7.0), and then diluted 1:1000 with solvent A (H_2O/acetonitrile/formic acid,

96.9:3:0.1, v/v/v), to obtain the 5 mg L^{-1} solution. Subsequent dilutions were made in the same

solvent A to yield the following concentrations: 1, 5, 10, 20, 50 and 100 µg L^{-1}. Each solution

contained the internal standard EGVNDNEEGFFSAR at the concentration of 50 µg L^{-1}. The

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 ± 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 $P < 0.05$.

3. Results and Discussion

3.1. Assessment of protein hydrolysis during simulated digestion

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

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

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

A comparison with the data reported by Kopf-Bolanz et al. (2012) showed that camel milk proteins were more prone to hydrolysis by gastro-intestinal proteases respect to bovine milk. Complete digestion of bovine milk resulted in a DH of 54 respect to the value of 69.6 found for camel milk. These results are also in agreement with Salami et al. (2008) who found that the extent of hydrolysis of camel caseins with pancreatic enzymes was greater than that of bovine caseins.

A comparison of the protein pattern of the digested samples revealed that salivary juice had no effect on protein (**Figure 2A, lane 2**) as compared to the un-digested camel milk (**Figure 2A, lane 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 α-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 α-lactalbumin (**Figure 2B, lanes 3-7**), indicating that the presence of pancreatic enzymes and bile salt is basic for α-lactalbumin digestion. These results are in accordance with those of Salami et al. (2008) showing that camel α-lactalbumin was slowly hydrolyzed by trypsin but it was more extensively hydrolyzed by chymotrypsin.

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^{-1}.

The post-pancreatic peptidic fraction was used for the determination of the ACE-inhibitory activity of the peptides produced during the digestion. The IC$_{50}$ value was 1771.4 ± 3.1 µg of peptides mL^{-1}. 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^{-1} (**Table 1**). The yield of the eight collected fractions was estimated (**Table 1**). Fractions from F3 to F8 showed a similar

13

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^{-1}, 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$_{50}$ values, of the peptidic fractions isolated by RP-HPLC. Low IC$_{50}$ values, which

268   means high ACE-inhibitory activity, were found in fractions F1, F2 and F4. The lowest IC$_{50}$ values

269   of 37.2 ± 0.3 and 38.6 ± 0.4 µg peptides mL^{-1} were found in fractions F4 and F1, respectively,

270   which were about thirty times lower than the IC$_{50}$ 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 β-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 µmol 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 µmol L^{-1})

305   followed by TF (IC_{50}= 18 µmol 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 µmol 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 µmol 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 DKIHPF isolated from bovine β-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 ± 0.15 mg L^{-1} of milk.

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

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

total caseins. As the casein concentration was 26.8 g L^{-1}, the estimated κ-casein concentration was

about 930 mg L^{-1} (approximately 50 µmol L^{-1}). Considering that, the IPP concentration found at the

end of the digestion was 7.87 µmol 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 β- and κ-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 β-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 κ-casein

(**Figure 5**), suggested that several digestive proteolytic enzymes might be involved in the release of

IPP. Camel milk κ-casein contains the tripeptide in position 100-102. The peptide bond A_{99}—I_{100}

can be easily hydrolyzed by pepsin, chymotrypsin, or elastase whereas the peptide bond K_{103}—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 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

β- and κ-caseins containing the tripeptide IPP. Bovine κ-casein contains IPP in position 108-110

367 and the amino acids close to IPP are the same as in camel κ-casein suggesting that the same

368 pathway can be involved in the release of IPP. In bovine β-casein, IPP is located at position 74-76.

369 As detailed in **Figure 5**, the peptide bonds N_{73}—I_{74} and L_{77}—T_{78} can be hydrolysed by

370 chymotrypsin, releasing the tetrapeptide IPPL. The C-terminal L_{77} residue can be subsequently

371 removed by pancreatic carboxypeptidase A releasing the tripeptide IPP.

372 On the other hand, as mentioned above, Ohsawa et al. (2008) failed to detect VPP and IPP after

373 gastro-intestinal hydrolysis of bovine β-casein. This discrepancy can be ascribed to different causes.

374 Firstly, the *in vitro* digestion system used in the Ohsawa study is different from that used in this

375 study. The biggest difference is the amount of digestive enzyme added to the mixture, which was

376 higher in our system. The digestion system used in this study has been validated for milk

377 macronutrient digestion and closely mimics the digestive process in humans, especially for protein

378 digestion, resulting in the formation of free amino acids and small peptides (2-6 amino acids)

379 (Kopf-Bolanz et al., 2012). Secondly, for the identification of peptides, we used a *de novo*

380 sequencing software, which is able to identify also shorter peptide such as di- or tri-peptides.

381 Instead, the software commonly used for proteomic study and adapted for peptide identification,

382 such as Mascot, have normally a minimum peptide length for identification of five residues

383 (Koskinen, Emery, Creasy & Cottrell, 2011).

384

385 **4. Conclusion**

386 This paper demonstrates for the first time the presence of ACE-inhibitory peptides in the low

387 molecular weight (< 3kDa) fraction of digested camel milk. In addition, some of the identified

388 peptides showed marked structural similarities with previously described ACE inhibitors.

389 This work also provides evidence, for the first time, of the fact that IPP may be released during the

390 gastrointestinal digestion of camel milk κ-casein. Basing on the protease specificity in the digestive

391 system and on the sequence of bovine β- and κ-caseins it can be predicted that the anti-hypertensive

392 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 1. Changes in hydrolysis degree (DH) of camel milk proteins during *in vitro* gastric and pancreatic digestion. Values represent means ± 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 κ-casein and bovine β-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 ± standard deviation of triplicate determination.

	Peptides concentration (mg mL^{-1})	Estimated yield[a] (%)	IC_{50}[b] (µg peptides mL^{-1})
< 3 kDa permeate	21.74 ± 0.49[a]	100	1171.4 ± 3.1[a]
HPLC F1	11.10 ± 0.33[b]	51.1	38.6 ± 0.4[b]
HPLC F2	2.50 ± 0.11[c]	11.5	107.9 ± 1.2[c]
HPLC F3	1.07 ± 0.07[d]	4.9	720.3 ± 5.9[d]
HPLC F4	1.08 ± 0.09[d]	5.0	37.2 ± 0.3[b]
HPLC F5	0.44 ± 0.02[e]	2.0	> 1000
HPLC F6	0.79 ± 0.04[f]	3.6	927.4 ± 8.8[e]
HPLC F7	0.71 ± 0.02[f]	3.3	> 1000
HPLC F8	1.00 ± 0.02[d]	4.6	> 1000

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

[b] IC_{50} 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.

Observed mass (m/z)	Calculated mass[a]	Peptide sequence[b]	Protein precursor	Bioactivity[c]
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	β-, αS1, κ-casein, and α-lactalbumin	ACE-inhibitor (GL/GI IC$_{50}$= >1000 µmol L^{-1}) DPP IV inhibitor (GL/GI)
203.1427	203.1390	ALx	β-, αS1-,αS2-, κ-casein	ACE-inhibitor (AI IC$_{50}$= 3.4 µmol L^{-1}) DPP IV inhibitor (AL)
215.1413	215.1390	VP	β-,αS2-, κ-casein	ACE-inhibitor (IC$_{50}$= 420 µmol L^{-1}) DPP IV inhibitor
217.1561	217.1547	VV	β-, αS1, κ-casein, and α-lactalbumin	DPP IV inhibitor
219.1330	219.1339	SLx	β-, αS1-,αS2-casein	DPP IV inhibitor (SL)
233.1496	233.1496	TLx	β-,αS2-casein	DPP IV inhibitor
233.1573	233.1496	LxT	β-, αS2, κ-casein, and α-lactalbumin	DPP IV inhibitor (LT)
246.1450	246.1448	GGI	α-lactalbumin f(19-21)	/
247.1295	247.1288	DLx	β-, αS1-,αS2-casein, and α-lactalbumin	ACE-inhibitor (DL IC$_{50}$= >1000 µmol L^{-1})
253.1177	253.1183	FS	κ-casein f(96-97)	/
267.1349	267.1339	FT	β-,αS2-casein	/
269.1712	269.1608	LH	β-, αS1-,αS2-, κ-casein	Inhibitor of lipid peroxidation DPP IV inhibitor
276.1567	276.1554	SLG	Lactoferrin and lactotrasferrin	/
302.1971	302.2074	LxLxG	Cytochrome b, peptidoglycan recognition protein 1, milk fat globule EGF factor 8	ACE-inhibitor (LLG IC$_{50}$= >1000 µmol L^{-1})

316.2101	316.2067	VPT	αS2-casein f(116-118)		/
326.2049	326.2074	PPL	β-casein f(78-80)	DPP IV inhibitor	
371.2290	371.1962	GS(phospho)Q	Various proteins		/

[a]Monoisotopic mass

[b]Lx indicates leucine or isoleucine

[c]Potential bioactivities were achieved from the BIOPEP database; ACE: Angiotensin Converting Enzyme ; DPP IV: Dipeptidyl peptidase IV; IC_{50} 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

Observed mass (m/z)	Calculated mass[a]	Peptide sequence[b]	Protein precursor	Bioactivity[c]
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$_{50}$ 420 µmol L^{-1}) DPP IV inhibitor
229.1554	229.1547	PLx	β-, αS1-, κ-casein and α-lactalbumin	ACE-inhibitor (PL IC$_{50}$= 330 µmol L^{-1}) DPP IV inhibitor (PL/PI)
231.1706	231.1703	VLx	β-, αS1-, κ-casein	DPP IV inhibitor (VL/VI) Glucose uptake stimulating peptide (VL)
231.1764	231.1703	LxV	β-, αS2-casein	Glucose uptake stimulating peptide (LV/IV) DPP IV inhibitor (LV)
233.1453	233.1496	TLx	β-casein and α-lactalbumin	DPP IV inhibitor (TL/TI)
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$_{50}$ 130 µmol L^{-1}) DPP IV inhibitor
267.1293	267.1339	TF	β-, αS2-casein	ACE-inhibitor (IC$_{50}$ 18 µmol L^{-1}) DPP IV inhibitor
281.1478	281.1496	VY	β-, αS2-casein	ACE-inhibitor (IC$_{50}$ 7.1 µmol L^{-1}) Antioxidant 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	αS1-casein f(22-24)	/
348.1860	348.1765	DLT	α-lactalbumin f(84-86)	/
357.2130	35.2132	PLxQ	β-, αS1-, κ-casein	/
361.1988	361.2082	QIT	κ-casein f(156-158)	/
368.1748	368.1816	VYS	β-casein f(60-62)	/
400.2561	400.2554	GVPK	Various proteins	/
414.2329	414.2711	KPVA	κ-casein f(63-66)	/
457.2305	457.2194	TVTH	Various proteins	/
485.3254	485.3082	LQPK	β-casein f(89-92)	/

[a]Monoisotopic mass

[b]Lx indicates leucine or isoleucine

[c]Potential bioactivities were achieved from the BIOPEP database; ACE: Angiotensin Converting Enzyme ; DPP IV: Dipeptidyl peptidase IV; IC_{50} 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

| Observed mass (m/z)	Calculated mass[a]	Peptide sequence[b]	Protein precursor	Bioactivity[c]
205.0942	205.0972	W	Various proteins	Antioxidant
229.1504	229.1547	PLx	β-, αS1, κ-casein, and α-lactalbumin	ACE-inhibitor (PL IC$_{50}$= 330 µmol L^{-1}) DPP IV inhibitor (PL/PI)
229.1504	229.1547	LxP	β-, αS1, αS2-, κ-casein	ACE-inhibitor (IP IC$_{50}$= 130 µmol L^{-1}) DPP IV inhibitor (LP/IP)
231.1675	231.1703	VLx	β-, αS1, κ-casein, and α-lactalbumin	DPP IV inhibitor (VL/VI) Glucose uptake stimulating peptide (VL)
245.1903	245.1860	LxLx	β-, αS1, κ-casein, and α-lactalbumin	ACE-inhibitor (LL IC$_{50}$= n.r.) DPP IV inhibitor (LL/LI/IL/II) Glucose uptake stimulating peptide (LL/LI/IL/II)
263.1351	263.1390	PF	β-, αS1, αS2-casein	DPP IV inhibitor
295.1626	295.1652	LxY	β-, αS1, αS2-casein	ACE-inhibitor (LY IC$_{50}$= 18 µmol L^{-1} ; IY IC$_{50}$= 2.1 µmol/L) Antioxidant (LY/IY)
196.1704	391.1976	FPQ	β-, αS1-casein	/
288.1856	288.1918	GLxV	Lactoferrin, lactotransferrin, cytochrome b, porcine pepsin A, pancreatic lipase	/
314.2002	314.2074	PVV	β-casein f(116-118)	/
326.2048	326.2074	IPP	κ-casein f(99-101)	ACE-inhibitor (IC$_{50}$= 5.0 µmol L^{-1})
350.1644	350.1710	AYP	αS2-casein f(95-97)	/
360.2050	360.2129	ILD	α-lactalbumin f(95-97)	/
366.2057	366.2136	LxHP	β-, αS1-casein	ACE-inhibitor (LHP IC$_{50}$= 1.6 µmol L^{-1})
374.2358	374.2286	ILE	αS1-casein f(28-30)	/
378.1953	378.2023	VPY	β-casein f(178-180)	ACE-inhibitor (IC$_{50}$= 288 µmol L^{-1})
400.1937	400.2013	HIM	αS1-casein f(58-60)	/
219.1259	437.2507	IAHP	αS1-casein f(188-191)	/

|				
444.2396	444.2453	LQSP	β-casein f(113-116)	/
227.6459	454.3024	IPPK	κ-casein f(99-102)	/
459.2536	459.2449	TEPI	β-casein f(64-67)	/
235.6654	470.3337	IIPK	β-casein f(103-106)	/
504.2348	504.2453	YPPQ	αS1-casein f(180-183)	/
312.1753	623.3511	VAHIPS	αS2-casein f(27-32)	/
328.6844	656.3726	TPVSPR	Serum albumin f(313-318)	/
342.1603	683.3359	SHTEPI	β-casein f(62-66)	/
344.1814	687.3762	SLNEPK	Lactophorin f(1-6)	/

[a]Monoisotopic mass

[b]Lx indicates leucine or isoleucine

[c]Potential bioactivities were achieved from the BIOPEP database; ACE: Angiotensin Converting Enzyme; DPP IV: Dipeptidyl peptidase IV; IC_{50} is defined as the concentration of peptides required to inhibit 50% of the enzymatic activity
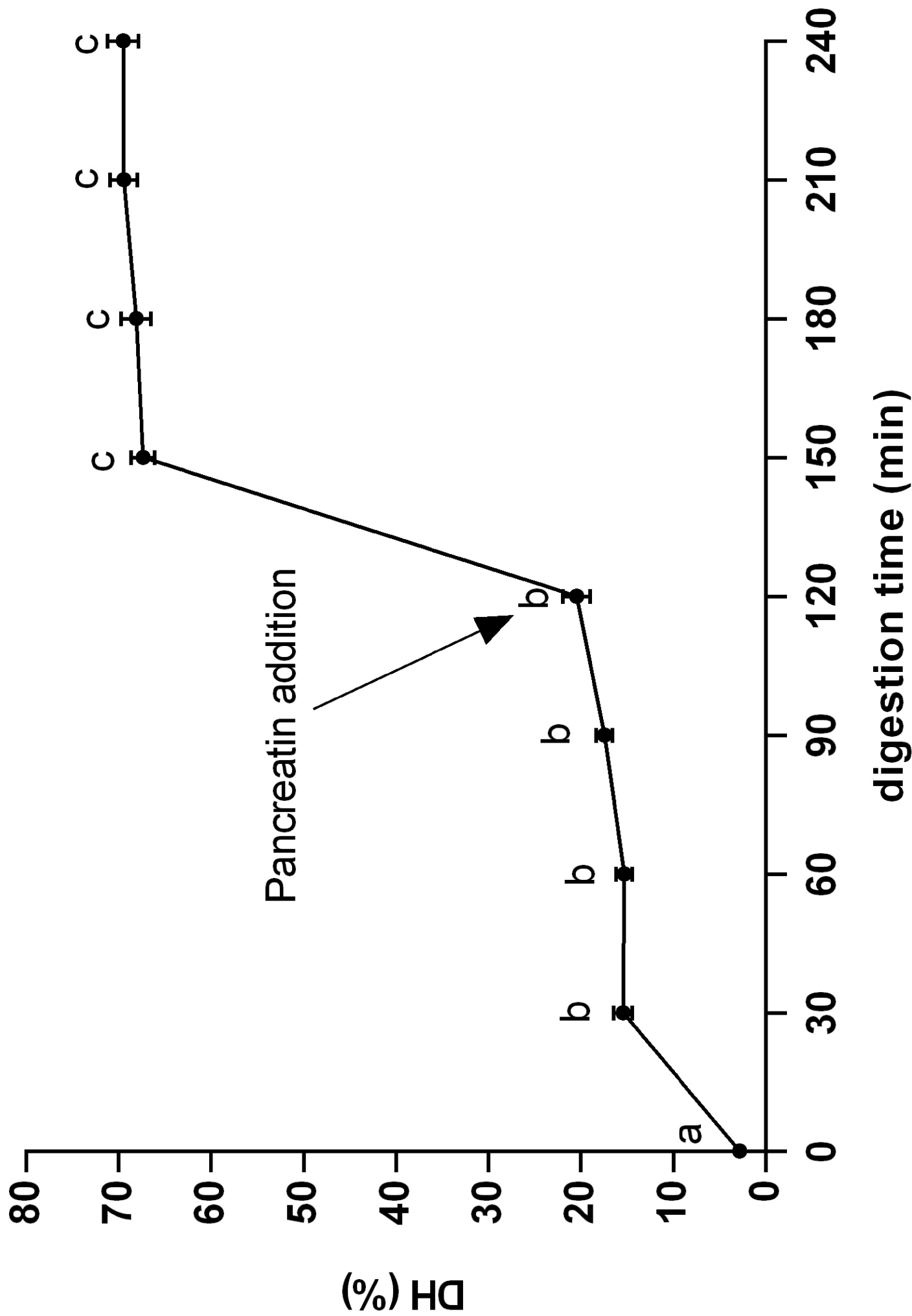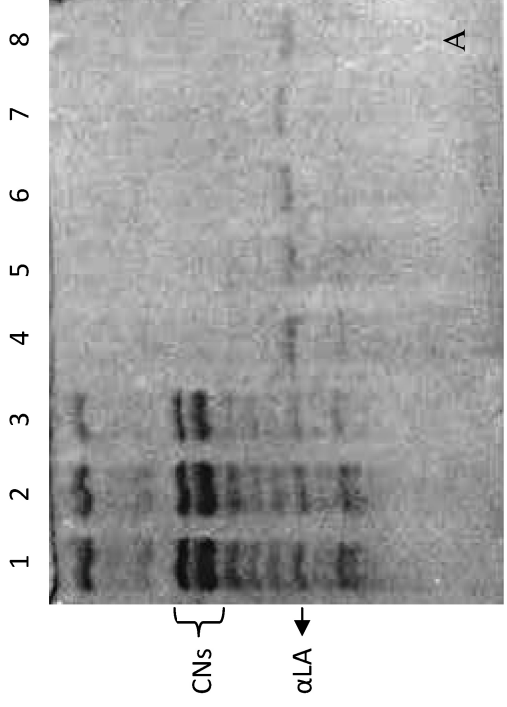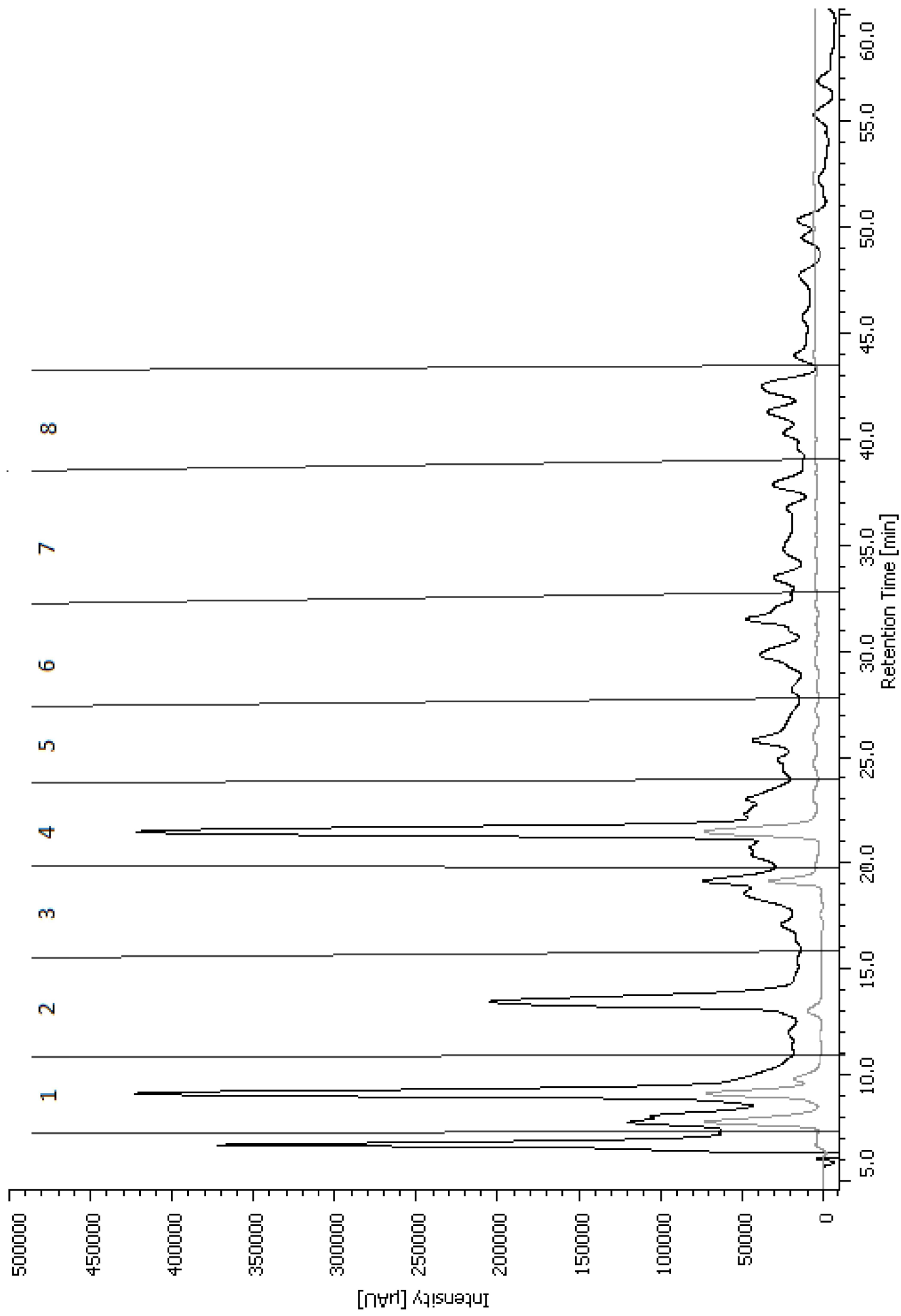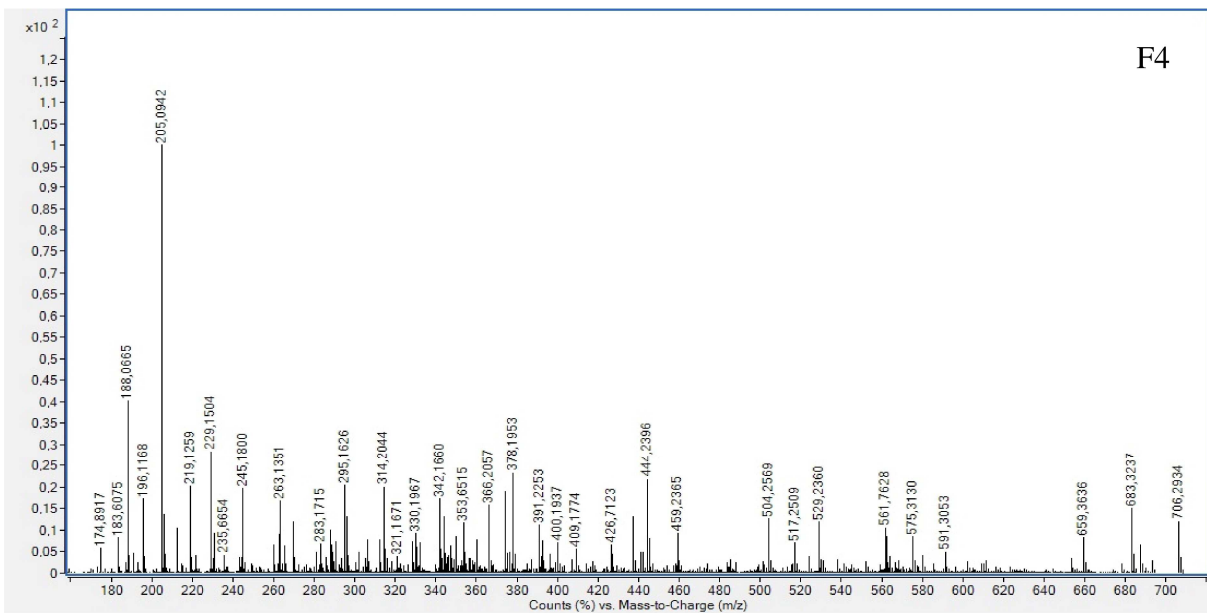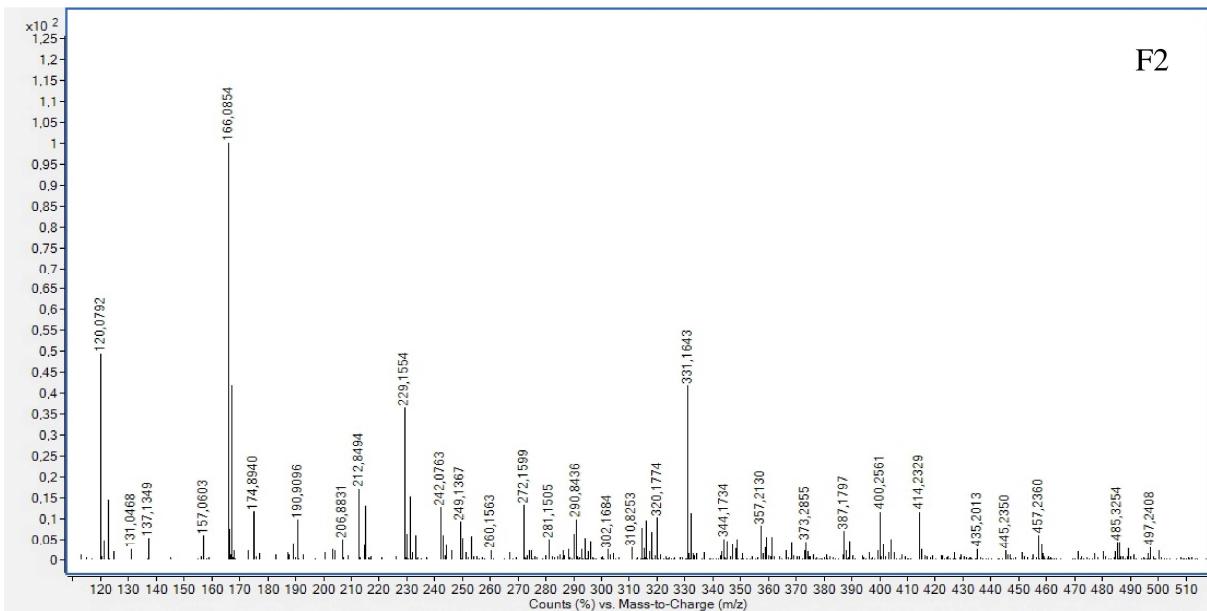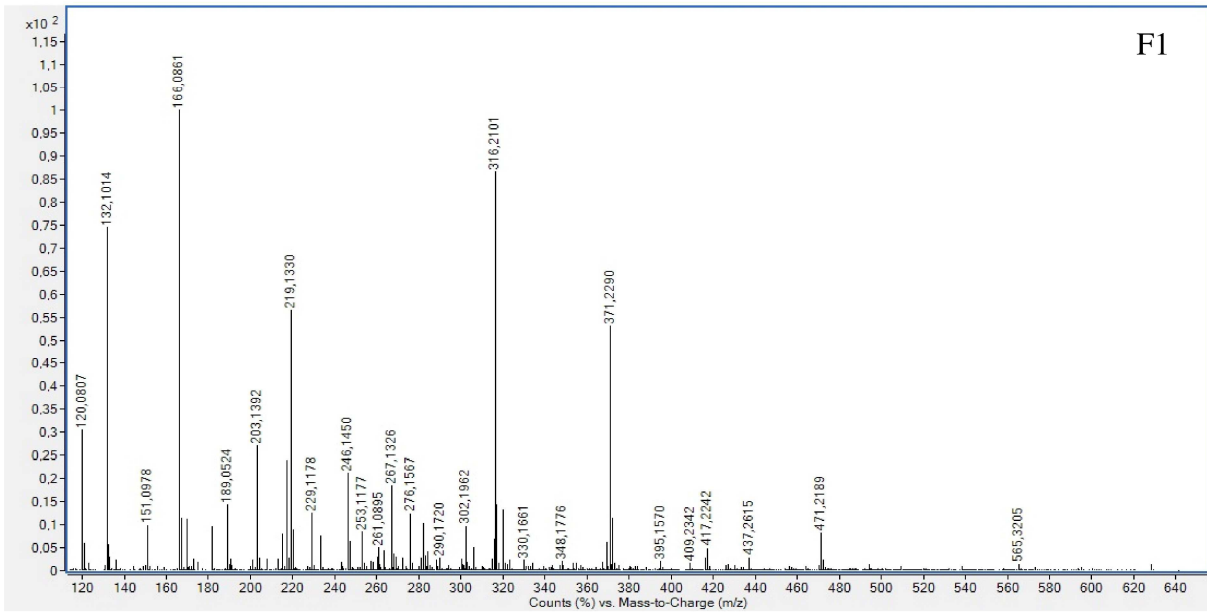
P;Ch;E   CB T

Camel κ-casein   S F I A I P P K K T Q   **f(96-106)**

Ch   CA Ch

Bovine β-casein   L P Q N I P P L T Q T   **f(70-80)**

P;Ch;E   CB T

Bovine κ-casein   S F M A I P P K K N Q   **f(104-114)**