Milk versus caseinophosphopeptides added to fruit beverage. Resistance and release from simulated gastrointestinal digestion

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ABSTRACT

The influence of simulated gastrointestinal digestion on caseinophosphopeptides (CPPs) formation in milk based fruit beverage was evaluated, together with resistance of a pool of CPPs added to fruit beverage. In milk based fruit beverage, four CPPs were identified that can be justified by their presence in raw milk or due to processing. When it was subjected to simulated gastrointestinal digestion, 10 CPPs were identified, and only one presented the cluster (SpSpSpEE) (three phosphoseryl group followed by two glutamic acid residues), which corresponded to α_{s2}-CN(1-19)4P. CPPs added to fruit beverage are resistant to simulated gastrointestinal digestion, and 16 CPPs were identified originating from the fragmentation of added CPPs, and with a greater presence of the cluster compared with CPPs originating from milk based fruit beverage. This could justify the use of CPPs as functional ingredients, and offer a good alternative to milk based fruit beverage for improving mineral bioavailability.

KEYWORDS: caseinophosphopeptides; simulated gastrointestinal digestion; fruit beverage; high-performance liquid chromatography; mass spectrometry.

1. Introduction

The caseinophosphopeptides (CPPs) are bioactive peptides that are released by enzymatic hydrolysis, gastrointestinal digestion of casein (CN), or during food processing. It is accepted that CPPs from CN offer different applications due to their antioxidant [25] and cytomodulatory potential, and to their capacity to form complexes with mineral elements through the clusters (SpSpSpEE) (three phosphoseryl groups followed by two glutamic acid residues), which can play an important role in mineral bioavailability. This fact has been reviewed for calcium [19, 30], zinc [36] and iron [3]. These properties imply that CPPs can be used as functional food ingredients in preventing the risk of diseases such as osteoporosis, dental caries and anemia [14].

The formation of CPPs has been evidenced after the ingestion of β-CN [40] or CN in rats [40, 46], as well as of CN in minipigs [31, 32]. Accordingly, they have been identified in the faeces of rats fed CN [22]. In humans, CPPs also have been identified after the ingestion of milk [8, 29] and yogurt [8].Various in vitro studies identified CPPs after the hydrolysis of CN, β-CN or α_s-CN via the action of trypsin [2, 7, 26, 44, 51], pancreatin [1, 50], and trypsin and chymotrypsin [13, 27]. CPPs also have been identified using simulated gastrointestinal digestion of CN with pepsin and trypsin [41] or of β-CN under gastro-analogous conditions using pepsin [47, 48]. Previous studies by our group have identified and characterized CPPs from CN [37, 38], β-CN [38], α_{s2}-CN [34] and infant formulas [33, 35, 39] subjected to simulated gastrointestinal digestion with pepsin and pancreatin. CPPs can also be released during food processing. In this sense, the only studies that evaluate the formation of CPPs during manufacture have been conducted in processes such as ripening and fermentation [4, 28, 43, 49].

52 The resistance of CPPs to gastrointestinal digestion has been demonstrated in
53 humans [29] and rats [6, 20, 23] following the ingestion of CPP preparations, and in
54 rats, after duodenal perfusion with β-CN(1-25) [5]. This also has been shown in vitro
55 (ileal extract of rats) by Brommage et al. [6]. Of all these studies, only Brommage et al.
56 [6], Hirayama et al. [20] and Bouhallab et al. [5] have identified and characterized the
57 structure of CPPs resistant to gastrointestinal digestion.
58 The consumption of fruit beverages has increased in the last decade. Fruit
59 beverages are often commercially supplemented with vitamins and/or minerals to
60 increase their nutritional value. The addition of milk or CPP preparations could improve
61 mineral bioavailability, thereby increasing the functional character of these foods. To
62 the best of our knowledge, there are no studies of this kind of matrix associated to CPP
63 formation from the dairy fraction present in milk based fruit beverage versus the
64 addition of CPPs and the influence of gastrointestinal digestion upon the formation of
65 these peptides.
66 The objective of this study was to evaluate the influence of simulated
67 gastrointestinal digestion in milk based fruit beverages upon the release of CPPs, and to
68 evaluate the resistance of CPPs added to fruit beverages and subjected to simulated
69 gastrointestinal digestion. The results obtained will allow evaluation of the potential use
70 of CPPs as functional ingredients versus CPP released from milk based fruit beverages.
71
72 **2. Materials and methods**
73 2.1 Chemicals and reagents
74 Enzymes and bile salts were purchased from Sigma Chemical Co. (St. Louis,
75 MO, USA): pepsin (E.C. 3.4.23.1; 1:10.000, 975 U/mg protein), pancreatin and bile
76 extract (porcine). Water of cellular grade was used for the preparation of reagents and

77 throughout the in vitro digestion assay (Aqua B Braun, Braun Medical, Barcelona,
78 Spain), while Milli-Q water (18.2 MΩ cm resistivity) was used for all solvents.
79 Trifluoroacetic acid (TFA), absolute ethanol, and acetonitrile were purchased from
80 Merck (Darmstadt, Germany) and all these reagents were of analytical grade. $CaCl_2$ was
81 purchased from Panreac (Barcelona, Spain).
82
83 2.2 Obtainment of the pool of CPPs
84 The pool of CPPs was prepared by simulated gastrointestinal digestion of whole CN
85 according to the method of Miquel et al. [37] with demineralized enzymatic
86 preparations [42]. Briefly, high temperature short time pasteurized skimmed milk
87 purchased from a local certified farm was subjected to isoelectric precipitation with 1 M
88 HCl at pH 4.6 and centrifuged at 4500g for 20 min at 10 ºC. The precipitate was freeze-
89 dried and kept at -20 ºC until use. CN (1.2 g) was digested by simulated gastrointestinal
90 digestion using two enzymatic solutions (solution of pepsin at pH 2 with 6 M HCl for 2
91 h at 37 ºC and pancreatin-bile solution at pH 5.2 with 1 M $NaHCO_3$ for 2 h at 37 ºC).
92 Finally, the pH was adjusted to 7.2 with 0.5 M NaOH, and the preparation was
93 centrifuged at 3500g for 60 min at 4 ºC. The CN not digested was precipitated at pH 4.6
94 as describe above, and then the supernatants were selectively precipitated according to
95 the method of Miquel et al. [37] with $CaCl_2$ (10%, w/v) and an equal amount of
96 absolute ethanol at pH 8 with 1 M NaOH. The preparation was then centrifuged for 10
97 min at 12000g at 10 ºC. The precipitate was washed with 50% (v/v) ethanol, lyophilized
98 and stored at -20 ºC until further analysis.
99 Repeatability was estimated by the relative standard deviation (RSD) of the peak
100 areas for two consecutive analyses of three different preparations (simulated
101 gastrointestinal digestion and selective precipitation) of the pool of CPPs (sample A).

HPLC-MS quantification was performed by extracting the most abundant ion for each peptide and its sodium and potassium adducts. Peak area was calculated by using QuantAnalysis 1.3 software from Bruker Daltonik.

2.3. Evaluation of processing and simulated gastrointestinal digestion in CPP formation from milk based fruit beverage (FbM)

Milk based fruit beverage (FbM) containing grape, orange and apricot was used for the evaluation of CPP formation by processing. Eighty grams of FbM were subjected to centrifugation (4500g, 20 min, 10 ºC), and the supernatants were selectively precipitated (sample B) as described above.

Eighty grams of FbM were subjected to simulated gastrointestinal digestion and selective precipitation (sample C) as described above, for the evaluation of CPP formation.

2.4. Evaluation of the resistance of CPPs to simulated gastrointestinal digestion

Eighty grams of fruit beverage (Fb) were mixed with 0.287 g of the pool of CPPs (sample A), and the mixture was subjected to simulated gastrointestinal digestion and selective precipitation as described above (sample D). The amount of CPPs added to Fb was the same as the amount of CPPs proceeding from FbM, taking into account that the latter contains 11% (v/v) of skimmed milk. Besides, in our laboratory the yield in obtaining CPPs (simulated gastrointestinal digestion and selective precipitation) was 15.49% (w/w) from CN. This CN was obtained from skimmed milk, which is the same raw material used for the elaboration of FbM.

125 Fb and FbM were subjected to pasteurization at 95 ºC for 45 seconds. The
126 beverages were packed hot and then cooled in a water bath. The pH was 3.94 ± 0.02.
127 Figure 1 shows the study design.
128
129 2.5. Analysis of CPPs by on-line RP-HPLC-ESI-MS/MS.
130 The analysis of CPPs (samples A, B, C and D) was carried out according to the
131 method of Miquel et al. [37], but sample concentration, and solvent gradient were
132 optimized. Solvent A was a mixture of water/TFA (1000:0.37, v/v) for the all samples,
133 and solvent B contained water/acetonitrile/TFA (200:800:0.27, v/v/v) for samples A, B
134 and C, and acetonitrile/TFA (1000:0.27, v/v) for sample D. The gradient was 60% of
135 solvent B in 90 min, after which the percentage of solvent B increased to 90% in 5 min
136 and remained constant at 90% for 5 min for samples A, B and C. For sample D the
137 gradient was modified due to the change of solvent B, and the gradient used was that
138 yielding improved separation. This gradient was 46% of solvent B in 90 min, after
139 which the percentage increased to 60% in 5 min, and increased again to 90% in 5 min.
140 The flow rate was 0.8 mL/min in the outlet of the UV detector was split 1:40 and 20
141 μL/min was directed to the mass spectrometer nebulizer. The injection volume was 100
142 μL.
143 ESI conditions were as follows: mode positive, capillary potential 4kV; dry gas
144 (N_2) temperature: 300 ºC. The other conditions (nebulization pressure and flow rate of
145 drying gas) were changed between samples to improve the sensibility. Mass spectra
146 were recorded over the m/z range 100-2500. About 25 spectra were averaged in the MS
147 analyses and about 5 spectra in the MS(n) analyses. The signal threshold to perform
148 auto MS(n) analyses (ICC Target) was 30000 and the precursor ions were isolated
149 within a range of 4 m/z and an fragmentation amplitude of 1.3 V. The m/z spectral data

were processed and transformed to spectra representing mass values by Data Analysis version 3 (Bruker Daltonik). BioTools version 2.1 (Bruker Daltonik) was used to process the MS(n) spectra and to perform peptide sequencing.

3. Results

The chromatographic separations by RP-HPLC of the samples are shown in Figure 2. In turn, Figure 3A shows the ESI-MS spectra of α_{s1}-CN(1-19)4P, ion 2455.8 m/z. Losses of 49 Da (H_3PO_4) can be seen, and Figure 3B shows the corresponding ESI-MS/MS spectra.

3.1. Evaluation of processing and simulated gastrointestinal digestion in CPP formation from milk based fruit beverage (FbM)

The identified CPPs in sample B (influence of processing) and C (influence of simulated gastrointestinal digestion) are shown in Table 1. In sample B, a total of four CPPs were identified, of which one corresponded to α_{s1}-CN, two to α_{s2}-CN, and one to β-CN. The presence of these peptides in sample B can only be justified by their presence in raw milk or due to processing. None of the identified CPPs presented the cluster (SpSpSpEE). In sample C, FbM subjected to simulated gastrointestinal digestion, a total of 10 CPPs were identified, of which five corresponded to α_{s1}-CN, three to α_{s2}-CN, and two to β-CN (Table 1). It should be noted that only one CPP contains the cluster (SpSpSpEE), specifically α_{s2}-CN(1-19)4P. The presence of α_{s1}-CN(43-52)2P and α_{s2}-CN(138-146)1P, which have been identified in sample C, could be due to the new generation of CPPs as a consequence of the simulated gastrointestinal digestion, or to the resistance of digestion, since they were found in sample B. The

174 CPPs α_{s1}-CN(40-52)2P and α_{s1}-CN(41-52)2P of sample C share part of the sequence
175 with α_{s1}-CN(43-52)2P of sample B.
176
177 3.2. Evaluation of the resistance of CPPs to simulated gastrointestinal digestion
178 Table 2 shows the CPPs identified in sample A (pool of CPPs) and sample D
179 (pool of CPPs added to Fb and subjected to simulated gastrointestinal digestion). In
180 total, 15 CPPs were identified in sample A: seven derived from α_{s1}-CN, four from α_{s2}-
181 CN, and four from β-CN. In sample D, 16 CPPs were identified: eight derived from α_{s1}-
182 CN, four from α_{s2}-CN, and four from β-CN.
183 After duplicate analysis of three different preparations of sample A, good
184 repeatability of the procedure could be confirmed. All peptides shown in Table 2 were
185 included in these three different preparations. RSD values obtained for the peak areas of
186 the extracted ions ranged from 11-25%.
187
188 **4. Discussion**
189 The CPPs identified in samples A, C and D in our study contained R, T, S, V, P,
190 K, L, M, E, Q, D, N, and A at the C-terminus (see Tables 2 and 3) that matched with
191 cleavages by the action of pancreatin and pepsin. The presence of CPPs in sample B can
192 be explained by the activity of several endogenous enzymes -being plasmin, the
193 principal representative. Plasmin in milk is associated with the CN micelles and is more
194 active for α_{s2}- and β-CN than for α_{s1}-CN, and not active for κ-CN. Plasmin is highly
195 specific for peptide bonds containing K or R at the N-terminal side, and is quite heat
196 stable and partially survives ultra high-temperature (UHT) processing [15]. The activity
197 of plasmin could explain at least one of the cleavages that justify the presence in sample
198 B of three of the four CPPs identified (α_{s1}-CN(43-52)2P, α_{s2}-CN(133-152)1P and α_{s2}-

199 CN(138-146)1P). There are cleavage sites that cannot be explained on the basis of the
200 known specificities of plasmin but they could result from proteolysis by other
201 proteinases such as cathepsin G and B or elastase. The specificity of these enzymes [9,
202 10] could explain the cleavage between residues Q-A in α_{s1}-CN, and E-L and L-S in β-
203 CN in the formation of α_{s1}-CN(43-52)P and β-CN(6-16)1P. The CPPs found in this
204 study, α_{s2}-CN(133-152)1P and α_{s2}-CN(138-146)1P, presented cleavages, which
205 incorporate these amino acids. Cleavage found between residues L16-S17 for β-CN
206 agrees with the cleavage found by Considine et al. [9] due to the action of cathepsin G.
207 Although they showed such cleavage between residues L163-S164, it is feasible that
208 cathepsin G could also cleave this bond. Cleavage between residues L16-S17 has also
209 been found as a consequence of proteolysis due to thermal treatment [17, 18]. In a
210 recent study, Gaucher et al. [16] have attributed the presence of some peptides in UHT
211 milk and UHT milk stored for 6 months at 4, 20 and 40 ºC to the activity of plasmin,
212 cathepsin D, B, G, elastase, and proteases from psychrotrophic bacteria, or to heat
213 treatment - demonstrating that they can operate in milk and may thus have importance
214 in dairy products.

215 In the sample C (influence of simulated gastrointestinal digestion in CPP
216 formation from FbM), we have not found any fragment that presented the cluster α_{s1}-
217 CN(66-70)4P. However, some studies conducted in vivo in rats [22] and minipigs [31,
218 32] fed CN have identified CPPs totally or partially containing the α_{s1}-CN sequence
219 presented by this cluster. On the other hand, the sequence of amino acids of the CPPs
220 identified corresponding to the β-CN fraction (β-CN(33-39)1P and β-CN(33-51)1P) of
221 sample C include or partially coincide with the CPPs identified by Chabance et al. [8]
222 after the ingestion of milk in humans. Besides, it must be noted that all identified CPPs
223 in FbM subjected to simulated gastrointestinal digestion (sample C) totally or partially

224 coincide with CPPs identified in previous studies of our group in infant formulas
225 (toddler and follow-up) subjected to similar simulated gastrointestinal digestion [33,
226 39].

227 The CPPs identified in sample D (pool of CPPs added to Fb and subjected to
228 simulated gastrointestinal digestion) show the resistance of CPPs to simulated
229 gastrointestinal digestion, because all peptides in sample D maintain the phosphorylated
230 residues found in the precursor peptides (CPPs of sample A). Most CPPs found in
231 sample D correspond to those found in sample A but loosing one or two residues at the
232 C- or N-terminal end. Therefore, we found a greater variety of CPPs in sample D than
233 in sample A with the cluster (SpSpSpEE). The resistance of CPPs to gastrointestinal
234 digestion has been demonstrated by in vivo and in vitro studies, as commented above in
235 the Introduction Section. The formation of the peptides β-CN(15-24)4P and β-CN(12-
236 24)4P in sample D could be explained by cleavage sites between residues E14-Sp15 and
237 T24-R25. These cleavage sites agree with the observations of Bouhallab et al. [5], who
238 evidenced the sensitivity of β-CN(1-25)4P to both proteases / peptidases and
239 phosphatases, and showed that the cleavage sites of β-CN(1-25)4P are between these
240 residues. In this sense, Hirayama et al. [20] identified in vivo the peptide β-CN(7-24)4P,
241 which presents the same cleavage. In the same way, the sequence of the peptide α_{s1}-
242 CN(64-74)4P includes the peptide identified in rat intestinal digest after the ingestion of
243 CPPs [20], and partially coincides with the CPPs identified by Brommage et al. [6] in
244 rat ileum extract after the administration of CPPs by stomach gavage.

245 Interestingly, one of the peptides identified in sample D, α_{s2}-CN(53-65)4P, did
246 not correspond to any peptide derived from those present in the added pool of CPPs
247 (sample A). This peptide could be generated from longer fragments present in sample A
248 but not identified in the HPLC-ESI-MS/MS analysis due to low ionization efficiency of

249 highly phosphorylated peptides. It is known that electronegativity of phosphate groups
250 usually reduces ionization efficiency during the positive mode of mass spectrometry
251 analysis [52].

252 Although a larger number of CPPs have been identified in CPPs added to Fb
253 (sample D) compared with FbM (sample C), the CPPs α_{s1}-CN(41-52)2P, α_{s1}-CN(43-
254 52)2P, α_{s1}-CN(73-79)1P, α_{s2}-CN(1-19)4P and α_{s2}-CN(138-146)1P were found in both
255 samples. From the results obtained, it can be seen that simulated gastrointestinal
256 digestion does not affect the generation of CPPs in a sample in which a pool of CPPs
257 precipitated with $CaCl_2$ has been added to a Fb in the same way as in a FbM. To the
258 best of our knowledge, the influence of matrix upon CPP formation had not been
259 previously evaluated. In a previous study of our group, when the same gastrointestinal
260 digestion process was used in different types of milk-based infant formulas, it was
261 evidenced a different CPPs profile according to the type of sample involved [39]. In the
262 same way as has been seen in the fruit beverages, some common CPPs or cleavages are
263 obtained in the different types of formulas. It can be seen that some cleavage sites
264 implicated in the release of CPPs were present in both samples, L40-S41, K42-D43,
265 Q52-A53, V72-P73, and K79-H80 for α_{s1}-CN; T19-Y20, K137-T138, and V146-F147
266 for α_{s2}-CN; and Q39-Q40 for β-CN. The presence of various amino acids in the
267 cleavages, such as E, K, Q, N, Sp in α_{s1}-CN, and P, G, E, I, L, Sp, T, R in β-CN,
268 implicated in the generation of CPPs (samples C and D), agrees with the generation of
269 CPPs reported by other authors in rats after the ingestion of CPP preparations [6, 20], or
270 perfused with β-CN(1-25)4P [5]. Of special interest is the greater presence of CPPs in
271 the digests of the Fb with added CPPs (sample D) versus FbM (sample C), with a
272 greater presence of the cluster (SpSpSpEE).

A protective effect of the milk versus CPPs administered with a standard milk-protein-free breakfast has been shown by Meisel et al. [29]. According to these authors, the food matrix protects CPPs from degradation in the gut, when CPPs are ingested as milk constituents and not as isolated CPP ingredient. This could explain potential differences in proteolytic susceptibility during digesta gastrointestinal transit. We have not observed the protective effect of milk, though it must be pointed out that comparison with the above study is difficult, since the food matrix was different, an in vivo model was used, and the CPPs generated were not identified.

5. Conclusion

The results obtained suggest that CPPs added to Fb and obtained by simulated gastrointestinal digestion are able to resist further degradation if digested. Thus, the use of CPPs as functional ingredients added to fruit beverages could offer a good alternative to FbMs. Future studies are needed to confirm these observations. It is important to note that this is an in vitro study. Further in vivo studies would be needed to improve our knowledge of the resistance of CPPs to digestion including their identification and the assessment of the influence of CPP formation upon mineral bioavailability, in order to apply them as functional components in foods.

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ABBREVIATIONS

CN, casein; CPPs, caseinophosphopeptides; RP-HPLC, reverse phase-high performance liquid chromatography; ESI-MS/MS, electrospray ionization tandem mass spectrometry; Fb, fruit beverage; FbM, milk based fruit beverage; RSD, relative standard deviation; SpSpSpEE, three phosphoseryl group followed by two glutamic acid residues; TFA, trifluoroacetic acid; UHT, ultrahigh-temperature.

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445 Table 1. CPPs identified in FbM: evaluation of processing (sample B), and simulated gastrointestinal digestion (sample C).

Sample B					Sample C				
Protein Fragment	Peak[a]	Amino acid sequence	Observed m/z	Ion (m/z) selected for MS/MS (charge)	Protein fragment	Peak[b]	Amino acid sequence	Observed m/z	Ion (m/z) selected for MS/MS (charge)
α_{s1}-CN(43-52)2P	1	DIG**Sp**E**Sp**TEDQ	1240.4	1240.4 (1)	α_{s1}-CN(40-52)2P	5	LSKDIG**Sp**E**Sp**TEDQ	1567.4	1568.4 (1)
					α_{s1}-CN(41-52)2P	3	SKDIG**Sp**E**Sp**TEDQ	1454.5	1455.3 (1)
					α_{s1}-CN(43-52)2P	2	DIG**Sp**E**Sp**TEDQ	1239.3	1240.3 (1)
					α_{s1}-CN(73-79)1P	8	PN**Sp**VEQK	880.3	881.3 (1)
					α_{s1}-CN(110-119)1P	6	EIVPN**Sp**AEER	1222.4	1223.4 (1)
					α_{s2}-CN(1-19)4P	9	KNTMEHV**SpSpSp**EESII **Sp**QET	2454.6	1228.3 (2)
α_{s2}-CN(133-152)1P	3	ENSKKTVDME**Sp**T EVFTKKTK	2410.1	1205.5 (1)	α_{s2}-CN(124-137)2P	4	NREQL**Sp**TSEEN**Sp**KK	1808.6	905.3 (2)
α_{s2}-CN(138-146)1P	2	TVDME**Sp**TEV	1090.4	1090.4 (1)	α_{s2}-CN(138-146)1P	7	TVDME**Sp**TEV	1089.3	1090.3 (1)
β-CN(6-16)1P	4	LNVPGEIVE**Sp**L	1249.7	1249.7 (1)					
					β-CN(33-39)1P	1	FQ**Sp**EEQQ	974.3	975.3 (1)
					β-CN(33-51)1P	10	FQ**Sp**EEQQQTEDELQD KIHP	2408.0	1205.0 (2)

446 **S** phosphoserine. **SSS**EE cluster sequence. [a,b]Chromatographic peaks were reported in Figure 2B and C.

Table 2. CPPs identified in the pool of CPPs (sample A) and CPPs resistant to digestion when added to Fb (sample D).

Sample A					Sample D				
Protein Fragment	Peak[a]	Amino acid sequence	Observed mass	Ion (m/z) selected for MS/MS (charge)	Protein Fragment	Peak[a]	Amino acid sequence	Observed mass	Ion (m/z) selected for MS/MS (charge)
α_{s1}-CN(40-52)2P	5	LSKDIGSpESpTEDQ	1567.7	1568.7 (1)	α_{s1}-CN(41-52)2P	5	SKDIGSpESpTEDQ	1454.3	1455.3 (1)
α_{s1}-CN(41-52)2P	2	SKDIGSpESpTEDQ	1454.4	1455.4 (1)	α_{s1}-CN(42-50)2P	4	KDIGSpESpTE	1124.3	1125.3 (1)
α_{s1}-CN(43-52)2P	1	DIGSpESpTEDQ	1239.5	1240.5 (1)	α_{s1}-CN(43-50)2P	2	DIGSpESpTE	996.2	997.2 (1)
					α_{s1}-CN(43-51)2P	13	DIGSpESpTED	1111.3	1112.3 (1)
α_{s1}-CN(40-60)2P	13	LSKDIGSpESpTEDQ AMEDIKQM	2515.4	1258.7 (2)	αs_1-CN(43-52)2P	3	DIGSpESpTEDQ	1239.3	1240.3 (1)
α_{s1}-CN(61-74)4P	3	EAESpISpSpSpEEIV PN	1809.0	1810.0 (1)	α_{s1}-CN(64-74)4P	10	SpISpSpSpEEIVPN	1480.2	1481.2 (1)
α_{s1}-CN(73-90)1P	10	PNSpVEQKHIQKED VPSER	2199.4	1100.7 (2)	α_{s1}-CN(73-78)1P	11	PNSpVEQ	752.3	753.3 (1)
α_{s1}-CN(73-89)1P	11	PNSpVEQKHIQKED VPSE	2043.4	1022.7 (2)	α_{s1}-CN(73-79)1P	15	PNSpVEQK	880.3	881.3 (1)
α_{s2}-CN(1-19)4P	9	KNTMEHVSpSpSpE ESIISpQET	2455.6	1228.8 (2)	α_{s2}-CN(1-19)4P	16	KNTMEHVSpSpSpEES IISpQET	2454.6	1228.3 (2)
					α_{s2}-CN(6-13)3P	1	HVSpSpSpEES	1100.1	1101.1 (1)
					α_{s2}-CN(53-65)4P	7	SIGSpSpSpEESpAEVA	1571.3	1572.3 (1)
α_{s2}-CN(124-146)3P	8	NREQLSpTSpEENS KKTVDMESpTEV	2881.0	1441.5 (2)	α_{s2}-CN(138-146)1P	14	TVDMESpTEV	1089.3	1090.3 (1)
α_{s2}-CN(138-146)1P	7	TVDMESpTEV	1089.4	1090.4 (1)					
α_{s2}-CN(133-152)1P	12	ENSKKTVDMESpTE VFTKKTK	2409.2	1205.6 (2)					
β-CN(1-24)4P	15	RELEELNVPGEIVE SpLSpSpSpEESIT	2966.6	1484.3 (2)	β-CN(11-16)1P	6	EIVESpL	768.4	769.4 (1)
β-CN(6-16)1P	14	LNVPGEIVESpL	1248.7	1249.7 (1)	β-CN(12-24)4P	12	IVESpLSpSpSpEESIT	1699.4	1700.4 (1)
					β-CN(15-24)4P	8	SpLSpSpSpEESIT	1358.2	1359.2 (1)
β-CN(30-48)1P	6	IEKFQSpEEQQQTE DELQDK	2430.4	1216.2 (2)	β-CN(30-39)1P	9	IEKFQSpEEQQ	1344.5	1345.5 (1)

β-CN(33-44)1P	4	FQSpEEQQQTEDE	1576.7	1577.7 (1)

Sp, phosphoserine, SpSpSpEE, cluster sequence. [a] Chromatographic peaks reported in Figure 2A and 2D.

Figure 1. Diagram of the study design. CN, casein; Fb, fruit beverage; FbM, milk-based fruit beverage; SGD, simulated gastrointestinal digestion; SP, selective precipitation.

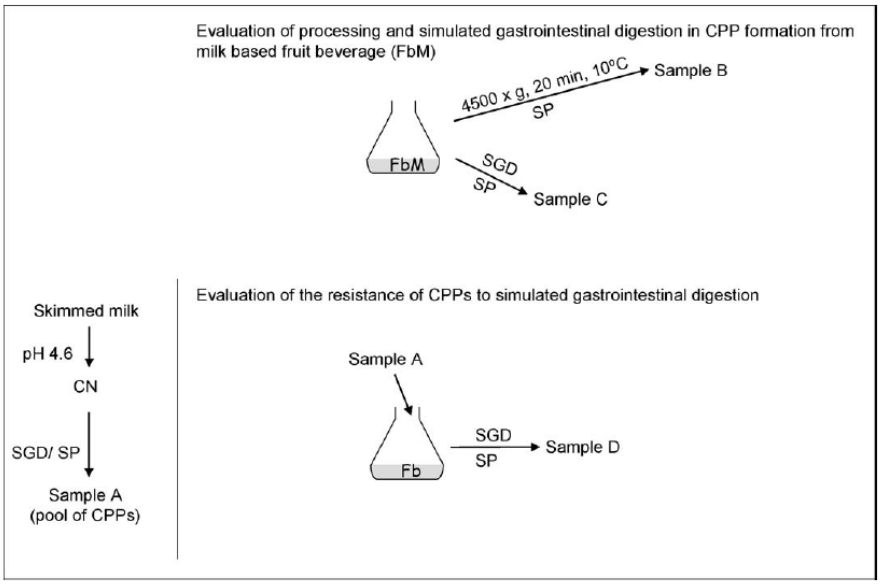


Figure 2. RP-HPLC chromatogram of samples: (A) sample A, pool of CPPs; (B) sample B, influence of processing in milk-based fruit beverage (FbM); (C) sample C, influence of simulated gastrointestinal digestion in FbM; (D) sample D, CPPs resistant to digestion when added to fruit beverage (Fb). For peaks identification see Tables 1 and 2.

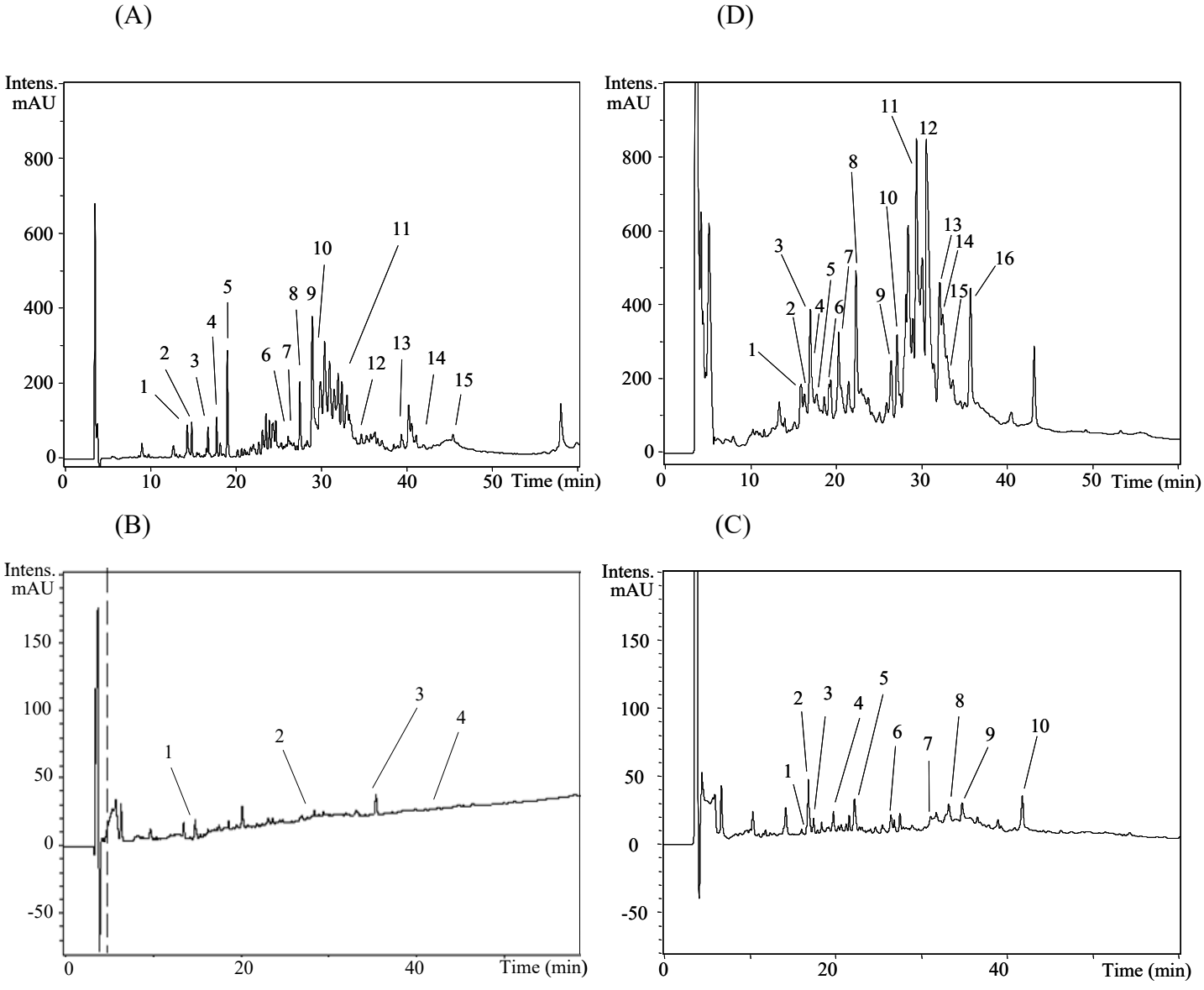
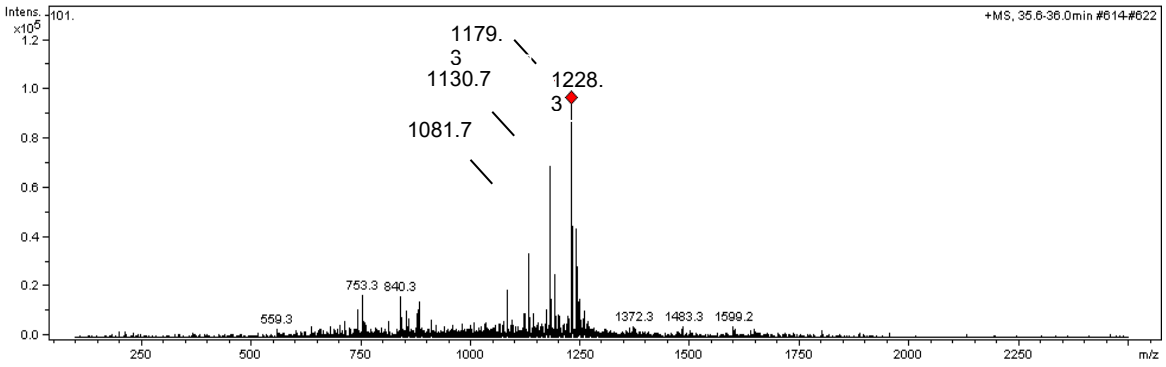
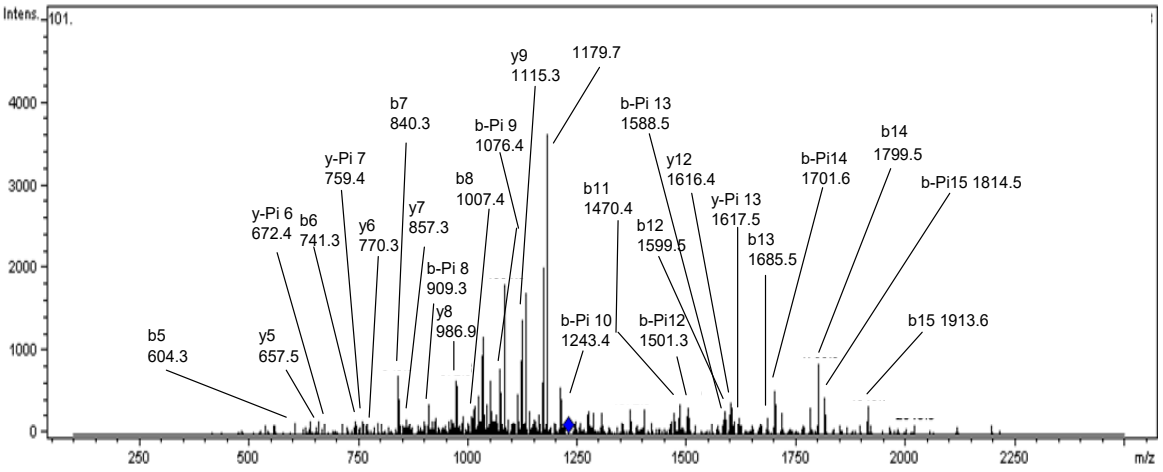


Figure 3. A) HPLC–ESI-MS spectrum of a selected peak (35.5–36.0 min) of the chromatographic separation of sample D, where losses of 49 Da from ionm/z 1228.3 are observed. (B) MS/MS spectrum of the doubly charged ion m/z 1228.3. Following sequence interpretation and data base searching, the peptide was identified as as2-CN(1–19)4P. The sequence of this peptide is displayed with the fragment ions observed in the spectrum. Fragment ions are labeled according to the nomenclature proposed by Roepstorff and Fohlman [41].

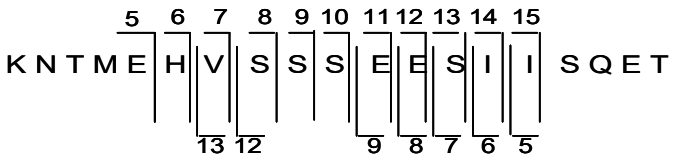
(A)



(B)



b ions



y ions