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## **Strategies for the discovery and identification of food protein-derived biologically active peptides**

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## **Abstract**

*Background:* The widespread application of protein-derived bioactive peptides (BAPs) with health promoting properties in human nutrition is currently limited. This may be due to the fact that several challenges exist in the discovery and identification of BAPs both *in vitro* and *in vivo*.

*Scope and approach:* To date, most BAP studies have been conducted following a so-called “conventional” approach. This is based on the non-targeted release of BAPs *in vitro*, followed, in certain instances, with their subsequent evaluation *in vivo*. However, more targeted approaches have recently been described for the release of specific BAPs in a more predictable and efficient manner. These targeted approaches are mostly based on *in silico* protocols (e.g., peptide cutters, molecular docking, quantitative structure activity relationship (QSAR) models) aimed at predicting the release and/or the bioactivity of specific peptides.

*Key findings:* Targeted approaches have, in certain instances, resulted in the development of particularly potent BAPs/hydrolysates and the discovery of novel BAP sequences. In addition, significant progress has been made in the identification of short peptides, involving the utilisation of multi-stage processes combining various physicochemical, analytical and *in silico* tools. This has allowed identification of novel sequences which are more relevant to human health from a bioavailability and stability perspective. BAPs have successfully been detected and quantified in human samples (e.g., serum, intestinal contents and urine) using different liquid chromatography-mass spectrometric (LC-MS) methodologies. In addition, human dose-response studies have allowed determination of their *in vivo* potency and efficacy, which in turn contributes to the development of scientific dossiers for regulatory approval.

*Key words:* dietary proteins; bioactive peptides; *in silico*; bioavailability; short peptides; human studies.

## 1 **1 Introduction**

2 Food proteins have been studied widely for their positive contribution to human health.  
3 Subsequent to protein digestion, amino acids are incorporated into proteins within the human  
4 body. In addition, dietary proteins also contain specific peptide sequences, called bioactive  
5 peptides (BAPs). A large number of *in vitro* studies has demonstrated that these BAPs may  
6 beneficially modulate markers of health (for reviews, see: Bhat, Kumar, & Bhat, 2015; Halim,  
7 Yusof, & Sarbon, 2016; Hernández-Ledesma, García-Nebot, Fernández-Tomé, Amigo, & Recio,  
8 2014; Li-Chan, 2015; Maestri, Marmiroli, & Marmiroli, 2016; Suleria, Gobe, Masci, & Osborne,  
9 2016). In addition, some of these peptides have been evaluated in humans, and positive health  
10 outcomes have been reported in certain instances. To date, most human studies evaluating the  
11 health benefits of dietary BAPs appear to have been carried out with milk and soy protein  
12 hydrolysates or fermentates (for reviews, see: Hsieh et al., 2015; Nongonierma & FitzGerald,  
13 2012a; Nongonierma & FitzGerald, 2015a, 2015b; Udenigwe & Aluko, 2012).

14 In a global context of increased health-related problems which are notably linked with the  
15 metabolic syndrome, adherence to a healthier diet has been acknowledged as one of the key  
16 components for maintenance of a healthy status in humans (O'Neill & O'Driscoll, 2015).  
17 Consequently, the requirement for high quality dietary proteins has increased significantly due to  
18 consumer demand. Several food processing protein-rich by-products have conventionally been  
19 used for animal nutrition either in their intact or hydrolysed format (Martínez-Alvarez,  
20 Chamorro, & Brenes, 2015). However, there is an increasing interest in valorising these  
21 underutilized protein sources in human nutrition (Lemes et al., 2016). Therefore, a wide range of  
22 dietary proteins originating from animal, plant and algal sources are currently being investigated  
23 for their potential to act as BAP precursors (Udenigwe & Aluko, 2012). Conventionally, food-

24 grade BAPs have been released from dietary proteins using enzymatic hydrolysis, microbial  
25 fermentation and/or physical processes (e.g., high pressure, sonication, etc.). However, currently  
26 enzymatic hydrolysis and fermentation are the most common means to produce dietary BAPs  
27 (Udenigwe & Aluko, 2012).

28 BAPs may be included in foods in order to provide additional health benefits to the general  
29 population. Nevertheless, several limitations/challenges have been reported for the development  
30 of BAPs targeted at human nutrition (for reviews, see: Li-Chan, 2015; Nongonierma &  
31 FitzGerald, 2016b). These include limited knowledge of BAP sequences when they are released  
32 within protein hydrolysates, which is particularly related to the high level of compositional  
33 complexity of protein hydrolysates. In addition, poor knowledge of the mode of action of BAPs  
34 currently exists. Finally, there appears to be a limited number of human studies which have  
35 shown a direct relationship between the ingestion of food protein hydrolysates and enhanced  
36 health-related properties (Nongonierma & FitzGerald, 2015b).

37 Due to uncertainties surrounding the demonstrated efficacy and the mode of action of  
38 BAPs/hydrolysates, their regulatory approval in many countries is extremely difficult. Health  
39 claims are granted to food components by the European Food Safety Authority (EFSA) based on  
40 scientific substantiation while the Food Drug Administration (FDA, USA) and the Ministry of  
41 Health, Labour and Welfare (MHLW, Japan) may grant health claims based on suggested  
42 scientific evidence (Lalor & Wall, 2011). To date, in Europe and the USA it appears that no  
43 protein-derived BAP-based ingredient/food has been granted health claims by EFSA or the FDA.  
44 However, in Japan, the situation is quite different, where a number of BAP-based foods have  
45 been recognised as so-called “food for specified health uses” (FOSHU) by the MHLW (Arai,  
46 Yasuoka, & Abe, 2008; Shimizu & Hettiarachchy, 2012). The negative opinions provided by

47 EFSA to regulatory application dossiers for peptide-based ingredients with health promoting  
48 activity have either been due to a lack of full characterisation of the active ingredients or an  
49 inconsistent link between BAP ingestion and the proposed health benefit in the specific target  
50 population (De Noni et al., 2009). Guidelines in relation to health claim dossiers have been made  
51 available by EFSA to assist applicants in the regulatory dossier preparation process (EFSA,  
52 2011). These guidelines have recently been updated (EFSA, 2016).

53 Over the past number of years, the scientific community and the food ingredient sector have  
54 attempted to close the gap between ongoing BAP research and their application in functional  
55 foods. Significant efforts have been made in the development of more potent dietary protein  
56 hydrolysates, the characterisation of BAP sequences within these hydrolysates, along with  
57 mechanistic and human intervention studies to assess their bioactive properties. To date, the  
58 study of BAPs has been carried out in a rather conventional manner. The workflow describing  
59 the conventional production of dietary protein hydrolysates starts with *in vitro* generation to  
60 peptide identification followed by *in vivo* testing of selected hydrolysates (Figure 1). This  
61 conventional approach may be time consuming and inefficient in the discovery and development  
62 of potent bioactive food protein hydrolysates. However, the development of more powerful  
63 computers and software has allowed the use of computational methodologies in the study of  
64 BAPs. More targeted alternative approaches have been developed which are mainly based on the  
65 utilisation of *in silico* methodologies and/or knowledge of BAPs which have been identified in  
66 humans (for reviews, see: Agyei, Ongkudon, Wei, Chan, & Danquah, 2016; Capriotti, Cavaliere,  
67 Piovesana, Samperi, & Laganà, 2016; Carrasco-Castilla, Hernández-Álvarez, Jiménez-Martínez,  
68 Gutiérrez-López, & Dávila-Ortiz, 2012; Iwaniak, Minkiewicz, Darewicz, Protasiewicz, &  
69 Mogut, 2015; Li-Chan, 2015; Nongonierma & FitzGerald, 2016b; Udenigwe, 2014; Udenigwe &

70 Aluko, 2012). In many instances, the alternative approaches have shown promise in the  
71 generation and identification of BAPs with enhanced potency, higher bioavailability and/or of  
72 more relevance to humans.

73 The aim of this review was to assess the main limitations in the conventional approach to the  
74 discovery and identification of dietary BAPs and to outline alternative strategies to enhance this  
75 process.

## 76 **2 Conventional approach to study dietary BAPs and its limitations**

77 Conventional approaches for BAP studies have been described in the scientific literature (Agyei  
78 et al., 2016; Capriotti et al., 2016; Carrasco-Castilla et al., 2012; Li-Chan, 2015; Nongonierma &  
79 FitzGerald, 2016b). These approaches involve a stepwise process (Figure 1) which has yielded  
80 the successful identification and evaluation of dietary BAPs. However, several limitations exist  
81 with the conventional approach which are summarised in Figure 1. These will be discussed in  
82 further detail in the following subsections.

### 83 **2.1 Generation of food protein hydrolysates**

84 The first step of the conventional approach consists in the selection of the protein substrate and  
85 the proteolytic/peptidolytic enzyme preparation for hydrolysate generation. The selection of  
86 protein and enzyme combinations are mainly aided with information from the scientific  
87 literature, knowledge of protein sequences and enzyme specificity (Udenigwe & Aluko, 2012).  
88 In the conventional approach, selection of the protein substrate may be quite empirical (linked to  
89 substrate availability, potential source of BAPs, underutilised protein, cost, etc.). Several  
90 commercially available food-grade enzyme preparations have been described in the development  
91 of protein hydrolysates. Lemes et al. (2016) recently reviewed the main enzyme preparations

92 which have been employed for the release of BAPs from food by-products. Generally, the  
93 enzyme preparations used for the development of food protein hydrolysates originate from  
94 animal, microbial or plant sources (Nongonierma & FitzGerald, 2011). Currently, selection of  
95 the enzyme preparation arises from (1) knowledge of the specificity of the main activity therein,  
96 if available, or (2) indications in the literature of its potential to release certain BAPs.

97 Following selection of the substrate(s) and enzyme preparation(s), the hydrolysis conditions (i.e.,  
98 pH, temperature, time, enzyme to substrate (E:S) ratio, total solids, etc.) need to be chosen.  
99 These conditions may influence the release of BAPs as they can modify both substrate  
100 conformation and enzyme activity and therefore the accessibility of specific peptide bonds in  
101 proteins. For instance, it was demonstrated that the protein concentration (0.1-10.0% (w/v)) at  
102 which whey protein isolate (WPI) was hydrolysed by a *Bacillus licheniformis* protease, modified  
103 the cleavage specificity of certain peptide bonds within  $\beta$ -lactoglobulin ( $\beta$ -Lg) (Butré, Sforza,  
104 Gruppen, & Wierenga, 2014). Similarly,  $\beta$ -Lg peptide bond selectivity for cleavage by a *B.*  
105 *licheniformis* protease depended on the pH (7.0-9.0) at which the hydrolysis reaction was  
106 conducted (Butré, Sforza, Wierenga, & Gruppen, 2015). To date, optimisation of the *in vitro*  
107 generation of food protein hydrolysates appears to have been conducted following modifications  
108 of the hydrolysis parameters one at a time. This approach has some limitations as it can be  
109 relatively time consuming, generating a large number of samples. In addition, it does not allow  
110 an understanding of the contribution of each parameter, nor the interactive effects between them.  
111 Furthermore, the optimum hydrolysis parameters for BAP release are ultimately not likely to be  
112 determined with this empirical approach. For this reason a more comprehensive approach to  
113 BAP generation has been described which involve the use of multifactorial design of  
114 experiments (DOE) and response surface methodologies (RSM) (van der Ven, Gruppen, de Bont,

115 & Voragen, 2002). Specific examples of RSM used as an optimisation tool for BAP release will  
116 be described in section 4.

## 117 **2.2 *In vitro* bioactivity assessment of hydrolysates**

118 Dietary BAPs have been assessed for their biological potential using a wide range of *in vitro*  
119 bioassays involving cells grown in culture along with specific chemical or enzymatic reactions.  
120 These bioassays are essentially based on the same protocols as those routinely used in drug  
121 discovery. The different bioassays used for BAP analysis evaluate the extent of chemical  
122 reactions, enzyme inhibition, receptor binding as well as metabolite profiling (for reviews, see:  
123 de Castro & Sato, 2015; Nongonierma & FitzGerald, 2016b). However, several parameters may  
124 influence the bioactivity results. These include the reactant concentration (hydrolysates/peptides  
125 and other test reagents), purity and origin as well as temperature, pH, number of passages of cells  
126 in culture, etc. For example, in enzyme inhibition assays, a large variability in the half maximal  
127 inhibitory concentration (IC<sub>50</sub>) of BAPs/hydrolysates has been demonstrated when tested at  
128 different E:S ratios in the angiotensin converting enzyme (ACE, EC 3.4.15.1) inhibitory assay  
129 (Murray, Walsh, & FitzGerald, 2004). Furthermore, in the presence of specific peptide substrates  
130 or inhibitors, variability in enzyme activity has been reported which depended on the organism  
131 from which the enzyme originated. This was the case for different bacterial sources of prolyl  
132 endopeptidases (EC 3.4.21.26) (Shan, Marti, Sollid, & Khosla, 2004) and for porcine and human  
133 recombinant dipeptidyl peptidase IV (DPP-IV, EC 3.4.14.5) (Lacroix & Li-Chan, 2015).  
134 Nevertheless, communalities between different research groups performing the same assay are  
135 found. Generally, specific bioassays are conducted with similar positive/negative controls to  
136 allow for the comparison of different test samples. These controls may consist of reference  
137 chemical compounds which may be a pharmaceutical drug (e.g., Captopril for ACE inhibition,

138 Sitagliptin for DPP-IV inhibition, Allopurinol for xanthine oxidase (EC 1.2.3.2) inhibition), a  
139 synthetic compound (e.g., Trolox<sup>TM</sup>, butylated hydroxyanisole (BHA), butylated hydroxytoluene  
140 (BHT) for antioxidant assays) or peptides known to positively impact on bioactivity (e.g., Ile-  
141 Pro-Pro for ACE inhibition, Ile-Pro-Ile (Diprotin A) for DPP-IV inhibition, Glu-Cys-Gly  
142 (glutathione) or Ala-His (carnosine) for antioxidant properties). In antioxidant assays for  
143 example, it is common practice to express the potency of the BAPs as an equivalent of the  
144 positive control (Trolox). Nevertheless, for most bioassays, this is not the case, making it  
145 challenging to compare data across different studies, due to the large variability in the setup of  
146 the *in vitro* bioassays.

147 In many research areas, standard methodologies have been established to provide a greater level  
148 of confidence and cross validation in the characterisation and evaluation of test compounds. This  
149 is the case for instance with methods related to analysis of food composition which have been  
150 certified by international scientific associations such as the International Organisation for  
151 Standardisation (ISO), the International Dairy Federation (IDF) or the Association of Official  
152 Analytical Chemists (AOAC). To date, such standardised methods do not seem to be available  
153 for the bioassays used in the evaluation of BAPs. Standardisation of bioassay protocols is  
154 ultimately required to facilitate the study of BAPs and increase the level of understanding of the  
155 compounds which are likely to be highly potent.

156 There is currently a relatively low level of confidence regarding the appropriateness of different  
157 *in vitro* bioassays for assessment of relevant markers to specific disease/health conditions. In  
158 most cases, the aetiology of specific diseases is quite complex and therefore involves several  
159 mechanisms of action. Therefore, using a single bioassay to identify a disease reducing agent is  
160 questionable as most *in vitro* bioassays are quite specific. For this reason, recent publications are

161 beginning to combine different bioassays to study food protein hydrolysates/BAPs in order to  
162 target a wider range of biological markers linked with a specific disease/health condition (Girgih  
163 et al., 2014; Lacroix, Meng, Cheung, & Li-Chan, 2016; Majumder et al., 2015; Nongonierma &  
164 FitzGerald, 2013a; Yousr & Howell, 2015).

### 165 **2.3 Enrichment and fractionation of BAPs**

166 Bioactivity determination is used as a means to rank different hydrolysates in terms of their  
167 biological potency. The peptide composition of many hydrolysates may be highly complex  
168 (Capriotti et al., 2016). Therefore, this presents a difficulty in understanding which peptides  
169 within complex mixtures are exerting a specific bioactivity. For this reason, fractionation and/or  
170 peptide enrichment techniques may be employed to reduce the compositional complexity of  
171 hydrolysates and subsequently allow identification of those peptides contributing to the bioactive  
172 properties. Peptides may be further fractionated with techniques based on different  
173 physicochemical properties such as molecular mass, hydrophobicity or charge (for reviews, see:  
174 Dallas et al., 2015; Lemes et al., 2016; Nongonierma, O’Keeffe, & FitzGerald, 2016b; Panchaud,  
175 Affolter, & Kussmann, 2012).

176 A bioactivity-driven fractionation approach is often used in order to determine that  
177 enrichment/purification of the BAPs has occurred (Carrasco-Castilla et al., 2012). Fractionation  
178 of BAPs is a time consuming process and often requires combination of several techniques to  
179 bring about separation of BAPs from other inactive peptides within complex hydrolysates.  
180 Generally, peptide fractionation is accompanied by an increased activity in selected fractions  
181 compared to the starting hydrolysate. In addition, modifications of peptide-peptide  
182 physicochemical (i.e., hydrophobic and electrostatic) interactions may prevail in complex  
183 peptide mixtures which may in turn hinder overall bioactivity (Groleau, Morin, Gauthier, &

184 Pouliot, 2003; Mercier, Gauthier, & Fliss, 2004). In certain instances, a reduction in peptide  
185 compositional complexity following fractionation may be accompanied with a loss/reduction in  
186 bioactive properties. In fact, certain hydrolysates contain peptide sequences which concomitantly  
187 contribute to the overall bioactivity as they are able to interact in various ways, e.g., in an  
188 additive, synergistic or antagonistic manner (Nongonierma & FitzGerald, 2015c; Schanbacher,  
189 Talhouk, Murray, Gherman, & Willett, 1998).

## 190 **2.4 Peptide identification**

191 When a highly bioactive fraction has been obtained, it can be further characterised by  
192 identification of the peptide sequences therein. This generally involves the utilisation of front end  
193 separative techniques (e.g., liquid chromatography (LC) or capillary electrophoresis (CE))  
194 coupled with mass spectrometry (MS) (Dallas et al., 2015; Panchaud et al., 2012). As fractions  
195 are less complex than the starting hydrolysate from a peptide composition point of view,  
196 conducting the MS characterisation on the fractions is a means to narrow down the peptide  
197 candidates responsible for specific bioactive properties (Panchaud et al., 2012). Confirmatory  
198 studies may then be carried out with selected sequences using synthetic peptides which are  
199 assessed *in vitro* for their bioactive properties.

200 To date, most MS systems are able to accurately detect peptides  $\geq 5$  amino acid residues in  
201 length. In contrast, shorter peptides (2-4 amino acids) have been underreported, to date, in the  
202 literature. This is possibly due to analytical limitations. The under or over fragmentation of short  
203 peptides in tandem MS has made their detection complicated and challenging (Dallas et al.,  
204 2015; Lahrichi, Affolter, Zolezzi, & Panchaud, 2013). However, the bioavailability and stability  
205 of short peptides is thought to be higher compared to larger peptides which are more likely to be  
206 further processed during gastrointestinal digestion, intestinal permeation and in the circulation

207 (Foltz, Van Buren, Klaffke, & Duchateau, 2009; Webb, Matthews, & DiRienzo, 1992). The  
208 identification of short peptides is therefore crucial for research involving dietary BAPs as these  
209 sequences are likely to be relevant to human health.

## 210 **2.5 *In vivo* studies**

211 To date, there does not appear, in many instances, to be a direct link between the occurrence of  
212 dietary peptides in humans and their presupposed health benefits (for reviews: see Miner-  
213 Williams, Stevens, & Moughan, 2014; Nongonierma & FitzGerald, 2015b). Additional *in vitro*  
214 studies may need to be performed in order to enhance the relevance of *in vitro* findings to human  
215 health. These include *in vitro* simulated gastro-intestinal digestion of the hydrolysates,  
216 assessment of their cytotoxicity, or peptide permeation through cell culture-based intestinal layer  
217 models (De Noni, Stuknyte, & Cattaneo, 2015; Egger et al., 2016; Mat, Le Feunteun, Michon, &  
218 Souchon, 2016; Minekus et al., 2014; Picariello, Ferranti, & Addeo, 2016; Picariello et al., 2010;  
219 Picariello et al., 2013a; Stuknyte, Cattaneo, Masotti, & De Noni, 2015; Walsh et al., 2004).

220 Based on the outcomes of all the above experiments, *in vivo* studies may initially be performed  
221 in animal models (Fernández-Musoles et al., 2014; Gaudel et al., 2013; Mukhopadhyaya et al.,  
222 2015; Yamada et al., 2015; Zhang, Chen, Jiang, Yin, & Zhang, 2016a). Different animal models  
223 of disease are available for testing biological compounds (for reviews, see: Aydin et al., 2014;  
224 Nelson & Reusch, 2014). Animal studies are often used before human studies for mechanistic  
225 purposes and to evaluate possible toxic effects *in vivo*. Several hydrolysates have also been  
226 directly evaluated in humans notably for their antihypertensive, antidiabetic and mineral binding  
227 properties (for reviews, see: Boutrou, Henry, & Sanchez-Rivera, 2015; Fekete, Givens, &  
228 Lovegrove, 2013; Nongonierma & FitzGerald, 2012a, 2015b). *In vivo* trials are generally  
229 performed at the last stage of the study, due to their high cost, time requirement and for ethical

230 reasons (Foltz, van der Pijl, & Duchateau, 2010). *In vivo* studies ultimately serve to confirm the  
231 bioactive potential of hydrolysates. While positive outcomes have been reported in several  
232 human studies, conflicting results have been found, sometimes making it difficult to understand  
233 the beneficial role of BAPs in human health. This conflicting information may arise from the  
234 poor bioavailability/stability of the BAPs as well as human interindividual variability (genetic,  
235 phenotypic, non-responders, gut microbiota, health status, etc.) (Nongonierma & FitzGerald,  
236 2015b).

237 Promising studies based on LC-MS characterisation of human fluids, both in the gastrointestinal  
238 tract (Boutrou et al., 2013) and in the circulation (Foltz et al., 2007; Kaiser et al., 2016; Morifuji  
239 et al., 2010), have demonstrated that certain BAPs were present within the human body  
240 following intake of intact or hydrolysed proteins. However, further quantitative studies are  
241 needed to verify that these BAPs are present in sufficient quantities to induce their biological  
242 effects.

243 Overall, the conventional approach currently in use for dietary protein hydrolysate evaluation is a  
244 time-consuming and complicated process which may not guarantee the identification of specific  
245 BAPs (Capriotti et al., 2016). For these reasons, many research groups have attempted to  
246 improve the workflow used both during BAP generation and identification. The following  
247 sections outline examples of methodological approaches alternative to the conventional strategy  
248 which have been described in the literature to improve the release, identification and *in vivo*  
249 validation of BAPs.

### 250 **3 Targeted approaches for the discovery and identification of BAPs**

251 The components of an alternative more targeted approach are outlined in Figure 1. These  
252 approaches may help to improve the generation, discovery and validation of BAPs. They  
253 comprise the utilisation of different *in silico* tools at the peptide generation, identification and  
254 validation stages. As already outlined, *in silico* tools are computational tools which are based on  
255 the utilisation of computers and various software (Iwaniak et al., 2015). The broad application of  
256 *in silico* methodologies to study peptides has been facilitated by the large number of freely  
257 available software and peptide databases. One commonly used peptide repository for *in silico*  
258 analysis of BAPs is the BIOPEP database (Minkiewicz, Dziuba, Iwaniak, Dziuba, & Darewicz,  
259 2008). Other BAP databases and software have also been reported in the literature, which may  
260 aid in the design of novel and more potent peptides (for review, see: Iwaniak et al., 2015;  
261 Nongonierma & FitzGerald, 2016b).

262 Numerous enzyme preparations and dietary proteins have been described for the generation of  
263 BAPs. Therefore, a large number of enzyme×substrate combinations may be employed to  
264 generate protein hydrolysates. The predicted release of BAPs from food proteins has been  
265 employed in order to develop more targeted approaches (Capriotti et al., 2016). *In silico*  
266 approaches may be used to guide researchers/industry in the selection of the most promising  
267 protein substrates.

268 A rapid means to interrogate the dietary proteome is to systematically search for known BAP  
269 sequences within a dataset of protein sequences (Dziuba, Iwaniak, & Minkiewicz, 2003; Dziuba  
270 & Darewicz, 2007). *In silico* analyses have been conducted on dietary proteins originating from  
271 plant (Chang & Alli, 2012; Cheung, Nakayama, Hsu, Samaranayaka, & Li-Chan, 2009;  
272 Udenigwe, 2016; Vecchi & Añón, 2009), milk (Dziuba & Dziuba, 2014; Tulipano, Faggi,

273 Nardone, Cocchi, & Caroli, 2015), fish (Darewicz, Borawska, & Pliszka, 2016; Huang, Lin, &  
274 Chang, 2015), meat (Keska & Stadnik, 2016; Lafarga, O'Connor, & Hayes, 2015) and insects  
275 (Vercruyssen et al., 2009) to study their potential to act as substrates for the generation of BAPs.  
276 This approach has also been specifically described for the identification of staple food proteins  
277 which may be useful for the generation of ACE (Gu, Majumder, & Wu, 2011b; Vermeirssen, van  
278 der Bent, Van Camp, van Amerongen, & Verstraete, 2004) and DPP-IV (Lacroix & Li-Chan,  
279 2012; Nongonierma & FitzGerald, 2014) inhibitory peptides. Different methodologies have been  
280 employed to rank proteins for their bioactive potential (Table 1). Protein ranking based on the  
281 frequency of occurrence of BAPs (A) within their sequence (Table 1) has been described by  
282 Dziuba et al. (2003).

283 The main limitations of ranking proteins based on this approach are that the frequency of BAP  
284 occurrence may incorporate overlapping peptide sequences and that bioactive potency is not  
285 taken into account. To address these issues, different indexes incorporating peptide potency have  
286 been proposed in the literature. The potential bioactivity index of food proteins was first  
287 introduced by Dziuba et al. (2003), which incorporated parameters such as the half maximal  
288 effective concentration ( $EC_{50}$ ) of the peptides identified within protein sequences (Table 1).  
289 Vermeirssen et al. (2004) employed a scoring approach taking into account the ACE  $IC_{50}$  value  
290 and molecular mass of the protein (pea and milk proteins) in their *in silico* study (Table 1). They  
291 developed two scoring systems to take into account the presence of overlapping peptide  
292 sequences within protein sequences, by prioritising the most potent peptides (score 1) and  
293 subsequently prioritising the shortest and most potent peptides (score 2). An *in silico* model was  
294 developed by Nongonierma and FitzGerald (2014) to take account of the fact that each amino  
295 acid residue can only contribute to one peptide sequence at a time within a given region of the

296 protein containing overlapping BAP sequences. In this model, only the most potent DPP-IV  
297 inhibitory peptide was considered (regardless of its size). Furthermore, a potency index was  
298 developed incorporating peptide potency (i.e.,  $IC_{50}$ ) and molecular mass of the protein (Table 1).  
299 In another study on milk proteins, Tulipano et al. (2015) also incorporated a peptide DPP-IV  $IC_{50}$   
300 value in their *in silico* analysis. Peptide candidates identified within  $\alpha$ -lactalbumin ( $\alpha$ -La) and  $\beta$ -  
301 Lg were classified into four groups (i.e., DPP-IV  $IC_{50} < 100$ , 100-200, 200-500 and 500-1600  
302  $\mu$ M) based on the structural similarity of their N-terminal dipeptide sequence to previously  
303 identified DPP-IV inhibitory peptides. In both models developed by Vermeirssen et al. (2004)  
304 and Nongonierma and FitzGerald (2014), discrepancies were found between BAP occurrence  
305 and potential predicted bioactivity of proteins. For this reason, it was concluded that the number  
306 of BAPs was not a good predictor of the bioactivity potential of proteins (Vermeirssen et al.,  
307 2004), suggesting that it may be more accurate to routinely incorporate BAP potency in *in silico*  
308 predictions.

309 One of the most common *in silico* tools consists in the use of peptide cutter programmes. These  
310 software are used to predict amino acid/peptide release from specific protein substrates based on  
311 knowledge of cleavage specificity of specific enzyme activities. Peptide cutters have been  
312 employed to predict starting protein substrate(s) or enzyme(s) which should release previously  
313 reported BAPs (Chang & Alli, 2012; Cheung et al., 2009; Fu, Wu, Zhu, & Xiao, 2015;  
314 Majumder & Wu, 2010; Minkiewicz, Dziuba, & Michalska, 2011; Nongonierma, Le Maux,  
315 Hamayon, & FitzGerald, 2016a; Tulipano et al., 2015; Udenigwe, Gong, & Wu, 2013;  
316 Vermeirssen et al., 2004). Peptide cutter outputs may be employed to determine the frequency of  
317 BAP release from proteins by a given enzyme activity ( $A_E$ , Table 1) (Minkiewicz et al., 2011).  
318 Specific features of BAPs may be incorporated in the model when the bioactivity of the peptides

319 is not available in the literature. For instance, peptides predicted to be released from bovine  
320 caseins, having an N-terminal Xaa-Pro/Ala (with Xaa an amino acid) were specifically taken into  
321 account to calculate a frequency of occurrence for DPP-IV inhibitory peptides (Hsieh et al.,  
322 2016). Another strategy has been described to overcome the variability seen in the biological  
323 data as well as the availability of specific peptide sequences within BAP databases. For instance,  
324 Pripp (2005) combined C-terminal cleavage post each of the 20 conventional amino acids in milk  
325 protein sequences, followed by *in silico* gastrointestinal digestion of the resulting peptides  
326 predicted to be released and estimation of their ACE IC<sub>50</sub> values using quantitative structure  
327 activity relationship (QSAR) modelling. They developed a model to predict the ACE IC<sub>50</sub> of  
328 protein hydrolysates which was based on *in silico* prediction of peptide release and their  
329 predicted ACE IC<sub>50</sub> value (Table 1).

330 In many instances, the *in silico* enzymatic digestion of proteins was confirmed by the *in vitro*  
331 generation of hydrolysates. This was the case for pea, egg and bovine whey proteins (Majumder  
332 & Wu, 2010; Nongonierma et al., 2016a; Tulipano et al., 2015; Vermeirssen et al., 2004). In  
333 several studies, validation of the *in silico* prediction was demonstrated at a macromolecular level  
334 with the *in vitro* generation of bioactive protein hydrolysates. For example, the ability of  
335 thermolysin to produce more potent ACE inhibitory oat protein hydrolysates than subtilisin (EC  
336 3.4.21.62) or pepsin (EC 3.4.4.1) was validated by the *in vitro* evaluation of the corresponding  
337 hydrolysates (Cheung et al., 2009). Similarly, confirmation of a higher ACE inhibitory potency  
338 following gastrointestinal digestion of whey proteins compared to pea proteins has been  
339 demonstrated (Vermeirssen et al., 2004). *In vitro* gastrointestinal digests of  $\beta$ -Lg yielded higher  
340 DPP-IV inhibition than those of  $\alpha$ -La as predicted *in silico* (Tulipano et al., 2015). A higher  
341 DPP-IV inhibition of tryptic (EC 3.4.21.4) caprine compared to tryptic bovine casein

342 hydrolysates was found after 3 h enzymatic hydrolysis as per *in silico* prediction (Zhang et al.,  
343 2016b).

344 Studies based on *in silico* predictions for the release of potent BAPs have proved very useful as a  
345 decision tool for the selection of both protein substrates and enzyme activities. However, at a  
346 molecular level, a direct translation between predicted and *in vitro* peptide release has not been  
347 systematically verified, as demonstrated in a number of studies. For example, Nongonierma et al.  
348 (2016a) showed that 60% of the peptides which were predicted to be released following  
349 digestion of  $\alpha$ -La with pancreatic elastase (EC 3.4.21.36) were actually identifiable by LC-  
350 MS/MS. Similar results have been reported with peptic digests of ovotransferrin, where only the  
351 precursors of the target ACE inhibitory peptides predicted to be released *in silico* could be  
352 identified by LC-MS/MS within the hydrolysate (Majumder & Wu, 2010). Recently, differences  
353 in ACE inhibitory peptides predicted to be released *in silico* and those detected by LC-MS/MS in  
354 a papain digest of bovine collagen were also reported (Fu et al., 2016). Similarly, potato proteins  
355 were cleaved differently by pepsin *in vitro* compared to the *in silico* prediction, generally  
356 resulting in less peptide bonds cleaved *in vitro* (Rajendran, Mason, & Udenigwe, 2016). Variable  
357 results between *in vitro* and *in silico* predictions have been explained by the inability of peptide  
358 cutters to accurately predict peptide release from globular proteins (e.g.,  $\alpha$ -La and  
359 ovotransferrin). In addition, peptide cutters assume that all cleavable peptide bonds would be  
360 accessible to the enzyme and be readily hydrolysed. The purity of the enzyme activity employed  
361 to generate hydrolysates may also affect peptide release *in vitro*. Furthermore, as already  
362 outlined, cleavage of peptide bonds during protein hydrolysis is dependant of their accessibility  
363 as well as the enzyme activity which is dictated by the experimental hydrolysis conditions (Butré  
364 et al., 2014; Butré et al., 2015; Kalyankar, Zhu, O’Cuinn, & FitzGerald, 2013). Following

365 protein denaturation, which may be caused by aggregation during heat-treatment (Dupont et al.,  
366 2010; Rahaman, Vasiljevic, & Ramchandran, 2017; Rinaldi, Gauthier, Britten, & Turgeon, 2014;  
367 Wada & Lönnerdal, 2014), enzymatic cross-linking (Havenaar et al., 2013; Monogioudi et al.,  
368 2011), glycation (Bouhallab, Morgan, Henry, Mollé, & Léonil, 1999; Cattaneo, Stuknytè,  
369 Masotti, & De Noni, 2017; Pinto et al., 2014) or interfacial interactions (Maldonado-Valderrama,  
370 Wilde, Mulholland, & Morris, 2012), peptide bond accessibility and cleavage during hydrolytic  
371 reactions may be modified, which may subsequently impact on bioactivity. Finally, the role of  
372 post-translational modifications (PTMs) on peptide release and bioactivity is still not fully  
373 understood. PTMs may occur during processing and storage. As they are not taken into account  
374 in *in silico* digestion of proteins, there is a need to determine PTMs to fully characterise the  
375 peptides within protein hydrolysates (Rajendran et al., 2016).

#### 376 **4 Optimisation of BAP release during enzymatic hydrolysis**

377 Many commercially available food-grade enzyme preparations are crude extracts and contain  
378 several activities which have not been fully characterised. Therefore, the hydrolytic specificity of  
379 several of these enzyme preparations is essentially unknown (Li-Chan, 2015). Conventionally,  
380 optimisation of BAP release during protein hydrolysis reactions generally appears to be  
381 conducted by modification of hydrolysis conditions one at a time. During hydrolysis, various  
382 parameters may affect enzyme activity and therefore the release of BAPs. Because BAP potency  
383 has been generally reported to be relatively low in comparison to drug compounds, the  
384 development of potent hydrolysates is of significant interest. More potent hydrolysates may be  
385 formulated at a lower dosage in food products while still inducing a significant biological effect.  
386 Therefore, several studies have attempted to improve the bioactive potency of hydrolysates by

387 carefully selecting the substrate or enzyme preparation or by optimising the hydrolysis  
388 conditions.

389 Selection of the starting substrate and the enzyme may be aided by the use of peptide cutters, as  
390 already outlined. The optimisation of hydrolysis parameters has been mainly based on the  
391 utilisation of multifactorial DOE combined with RSM. A number of studies using these  
392 approaches have allowed the systematic evaluation of the contribution of various hydrolysis  
393 parameters (i.e., temperature, time, E:S ratio, pH, etc.) to the release of BAPs during enzymatic  
394 hydrolysis of dietary proteins (Abedin et al., 2015; Cheung et al., 2009; del Mar Contreras,  
395 Hernández-Ledesma, Amigo, Martín-Álvarez, & Recio, 2011; Naik, Mann, Bajaj, Sangwan, &  
396 Sharma, 2013; Nikolaev et al., 2016; Nongonierma, Le Maux, Esteveny, & FitzGerald, 2017;  
397 Quirós, Hernández-Ledesma, Ramos, Martín-Álvarez, & Recio, 2012; van der Ven et al., 2002).  
398 Other advantages of hydrolysing food proteins using DOE approaches include a significant  
399 reduction in the number of experiments required while providing the ability to study interactive  
400 effects between hydrolytic parameters. Predictive models linking hydrolysis parameters to  
401 bioactivity can be developed using DOEs. These models can subsequently be used to build RSM  
402 profiles and allow determination of the optimum parameters for the generation of protein  
403 hydrolysates having enhanced bioactive potency (Abedin et al., 2015; Naik et al., 2013). In  
404 general, a good agreement between the predicted and the experimentally determined bioactivity  
405 has been reported.

406 To date, most DOE-based studies have evaluated the bioactivity of the whole hydrolysate.  
407 Therefore, the contribution of its individual constituents is unknown. An interesting study has  
408 described the application of DOE (using as variables the enzyme preparation (Corolase PP and  
409 Peptidase 433P) and hydrolysis duration) and RSM for optimisation of the release of a bovine

410 casein-derived ACE inhibitory peptide (His-Leu-Pro-Leu-Pro) during enzymatic hydrolysis  
411 (Quirós et al., 2012). In this study, it was reported that within the experimental design, a higher  
412 amount of peptide would be released from caseinate using Corolase PP for an hydrolysis  
413 duration of 24 h.

## 414 **5 Developing strategies for the identification of peptides in complex mixtures** 415 **with a focus on short peptides**

416 Peptide sequence identification has conventionally been carried out by Edman degradation or  
417 enzyme-linked immunoassay (ELISA) and these techniques are still being reported for the  
418 identification of specific peptides (Chabance et al., 1995; Chabance et al., 1998; Ledoux et  
419 al., 1999; Meisel et al., 2003). These methodologies, are however quite complex, as they  
420 generally necessitate significant fractionation work prior to peptide identification. The difficulty  
421 of isolating specific peptides from hydrolysates arises from the fact that hundreds of peptides  
422 may be present in any given hydrolysate. Because some peptides may have very similar  
423 physicochemical properties (mass, hydrophobicity, charge, solubility, etc.), they may be very  
424 difficult to separate from each other. More recently, with increased accuracy of MS analysers  
425 and the development of various bioinformatic tools, the detection of peptides has become less  
426 challenging and numerous peptide sequences may be detected simultaneously (Capriotti et al.,  
427 2016; Dallas et al., 2015; Panchaud et al., 2012; Sánchez-Rivera, Martínez-Maqueda,  
428 Cruz-Huerta, Miralles, & Recio, 2014b). Nowadays, unfractionated hydrolysates are routinely  
429 characterised by LC-MS/MS, allowing assessment of gross peptide composition. Fractionation  
430 prior to MS characterisation has been employed to help in the determination of the BAPs present  
431 within dietary hydrolysates (Capriotti et al., 2016). The application of fractionation methods may

432 result in enrichment in specific peptide sequences, allowing, in certain instances, easier detection  
433 of the BAPs.

434 The detection of short peptides ( $\leq 4$  amino acids) within food protein hydrolysates appears to  
435 have been underreported in the area of dietary BAPs (Lahrichi et al., 2013). This may be because  
436 analytical issues are encountered in the detection of short peptides. These issues are linked with  
437 poor UV absorption and the difficulty to obtain meaningful fragmentation during MS analysis. In  
438 addition, short peptides display a low structural diversity which increases the probability of  
439 detecting isobaric fragments within the same sample or samples generated from different protein  
440 substrates, making searches against protein databases and the subsequent peptide identification  
441 quite complicated.

442 To date, there appears to be a low level of confidence regarding the identification of these short  
443 sequences within dietary hydrolysates. This may be the reason why only a few studies have  
444 attempted to analyse short peptides in complex matrices. However, the combination of different  
445 fractionation approaches, derivatisation methods, front end analytical separation, *in silico* tools  
446 and altered MS settings has proved beneficial in the identification of short peptide sequences  
447 within complex mixtures (Figure 2). Studies which have successfully identified short peptides  
448 within complex mixtures (model solutions, hydrolysates/fermentates and biological samples) are  
449 summarised in Table 2. It is evident from the literature that short peptide identification requires a  
450 number approaches which are based on their (1) selective fractionation/isolation, (2)  
451 derivatisation, (3) LC separation, (4) retention time prediction and (5) enhanced MS detection  
452 (Figure 2)

453 The selective isolation of short peptides has been employed on several occasions to separate  
454 them from other materials which are present in the sample and which may hinder their detection

455 (i.e., larger peptides, proteins and non proteinaceous materials). Membrane separation such as  
456 ultrafiltration (Eisele, Stressler, Kranz, & Fischer, 2012; Pampanin et al., 2012; Schlichtherle-  
457 Cerny, Affolter, & Cerny, 2003) and nanofiltration (Le Maux, Nongonierma, & FitzGerald,  
458 2015a) or adsorbent-based fractionation (Gu, Li, Liu, Yi, & Cai, 2011a; Le Maux et al., 2015a;  
459 Sánchez-Rivera et al., 2014a) may be used to specifically separate short peptides from complex  
460 samples prior to analysis.

461 Following sample preparation, precolumn derivatisation has been employed to improve the  
462 detection and identification of amino acids and short peptides. Different tagging reagents such as  
463 phenyl isothiocyanate (PITC), naphthalene-2,3-dialdehyde (NDA) or dabsyl chloride have been  
464 reported in this instance (Eisele et al., 2012; Matsui et al., 2002; Shigemura et al., 2011;  
465 Stressler, Eisele, & Fischer, 2013; Sugihara, Inoue, Kuwamori, & Taniguchi, 2012).

466 Other improvements which may be employed during LC separations consist in the combination  
467 of several separation modes, e.g., reverse-phase (RP), hydrophilic interaction liquid  
468 chromatography (HILIC) and CE (Harscoat-Schiavo et al., 2012; Le Maux, Nongonierma,  
469 Murray, Kelly, & FitzGerald, 2015b; van Platerink, Janssen, & Haverkamp, 2008). As separation  
470 of peptides using these different analytical tools is based on their physicochemical properties  
471 (i.e., hydrophobicity, hydrophilicity and molecular mass), the information gained during these  
472 different analytical methods can be combined to increase the confidence in peptide identification.  
473 This may be particularly relevant with isobaric peptides or peptides with similar hydrophobicity  
474 or hydrophilicity. It may therefore be important to optimise the LC separative method in order to  
475 avoid the potential for co-eluting peptides and facilitate their subsequent MS detection.  
476 Modifications (i.e., HILIC, RP, CE separation modes) of front end separation methodologies  
477 have been described in several instances to improve the analytical separation of short peptides,

478 which generally involve the utilisation of relatively long duration LC-gradients (Lahrichi et al.,  
479 2013; Le Maux et al., 2015a).

480 During MS detection, different optimisation steps have been described to analyse short peptides.  
481 These may be classified in two groups including alterations which are performed pre or post MS  
482 analysis. Before MS analysis, different analyser settings may be altered. One common means is  
483 the selective monitoring of ions from known peptide sequences. Using LC-MS/MS, Morifuji,  
484 Koga, Kawanaka, and Higuchi (2009) reported the identification of seven branched-chain amino  
485 acid (BCAA)-containing dipeptides (Ile-Val, Leu-Val, Val-Leu, Ile-Ile, Leu-Ile, Ile-Leu and  
486 Leu-Leu) within a whey protein hydrolysate. This was achieved employing a multiple reaction  
487 monitoring (MRM) approach based on the detection of specific fragment (i.e., diagnostic) ions  
488 arising from these dipeptides. This methodology also allowed quantification of the seven  
489 peptides (with amounts ranging between 0.03 and 3.69 mg for Ile-Ile and Ile-Leu, respectively)  
490 within a whey protein hydrolysate. Similarly, several hydroxyproline (Hyp) containing di- and  
491 tripeptides were quantified in plasma of humans following the consumption of fish scale gelatin  
492 hydrolysates (Ichikawa et al., 2010; Shigemura et al., 2011; Taga, Kusubata, Ogawa-Goto, &  
493 Hattori, 2014) or porcine skin gelatin hydrolysates (Sugihara et al., 2012) using an MRM  
494 approach. In another study, the use of peptide retention time during LC separation, MRM and  
495 variation in the collision energy has been described for the analysis of the 117 di- and tripeptides  
496 representing all possible combinations of Val, Leu and Ile (Lahrichi et al., 2013). Based on the  
497 fragmentation pattern of short peptides, this approach allowed the ability to distinguish isobaric  
498 peptides containing BCAAs (Leu and Ile), which are generally very difficult to identify due to  
499 their identical mass and similar structure (Kaiser et al., 2016). Other studies based on the  
500 monitoring of specific fragments using selected ion monitoring (SIM) and pseudoselected

501 reaction monitoring (SRM) (Eisele et al., 2012; Sánchez-Rivera et al., 2014a; Stressler et al.,  
502 2013) have proved successful in allowing short peptide identification in complex mixtures (Table  
503 2). Ion monitoring approaches have yielded quite promising results. However, they necessitate  
504 previous knowledge of the target peptides in order to determine the specific fragments of interest  
505 to be monitored. Alterations of the collision energy and transfer time have also proved beneficial  
506 for the fragmentation and subsequent detection of short peptides within milk protein hydrolysates  
507 (Chen et al., 2015; Kaiser et al., 2016; Lahrichi et al., 2013; Morifuji et al., 2009; O’Keeffe &  
508 FitzGerald, 2015).

509 To date, very few studies have used matrix-assisted laser desorption ionisation (MALDI)  
510 analysers to study short peptides. This has been mainly linked with the relatively high signal to  
511 noise ratio (S/N) seen with short peptides in MALDI. Hong, Tanaka, Yoshii, Mine, and Matsui  
512 (2013) have described a modified MALDI-based method for the identification of short peptides.  
513 Visualisation of dipeptides (Gly-Sar and Val-Tyr) absorbed on rat intestinal membrane has been  
514 reported with a methodology involving phytic acid-aided MALDI-imaging mass spectrometry  
515 (IMS). The role of phytic acid was to interact with salts (e.g., Na<sup>+</sup> and K<sup>+</sup>), thereby reducing the  
516 formation of Na- or K-peptide adducts, which are known to increase the S/N ratio and interfere  
517 with short peptide detection. This novel approach has allowed the determination of dipeptide  
518 permeation through intestinal epithelium as well as their stability (Hong et al., 2013).

519 Metabolites present the same challenges as short peptides in terms of the difficulties associated  
520 with their MS identification. Therefore, MS analysers developed for metabolites detection may  
521 be employed for the study of short peptides. Studies describing the successful identification of  
522 short peptides using ion mobility spectrometry are found in the literature. Ion mobility  
523 spectrometry allows separation of peptides based on both their m/z and mobility (drift time) in

524 the MS analyser. Therefore, ion mobility spectrometry is suited for the detection of isobaric  
525 peptides or peptides presenting similar masses. The use of ion mobility spectrometry in the  
526 discrimination of isobaric dipeptides (Gly-Ala/Ala-Gly and Gly-Ser/Ser-Gly (Blagojevic,  
527 Chramow, Schneider, Covey, & Bohme, 2011)), tripeptides (Tyr-Gly-Trp, Trp-Gly-Tyr and Tyr-  
528 Trp-Gly (Pollard et al., 2011)) or isotopomer dipeptides (Kaszycki, Bowman, & Shvartsburg,  
529 2016) has been reported. While it may facilitate the detection of short peptides within complex  
530 mixtures, applications of ion mobility spectrometry to the study of BAPs appear to be rare.

531 The identification of peptides within complex mixtures may be facilitated by various *in silico*  
532 approaches. Peptidomic methodologies which are currently used for studying BAPs have been  
533 recently reviewed (Dallas et al., 2015; Iwaniak et al., 2015). *In silico* methods are very useful for  
534 the study of short peptide sequences. As the quality of peptide fragments may not always be  
535 sufficient to identify the amino acid sequence, different *in silico* tools have been employed. MS  
536 data analysis is based on different algorithms, software and databases which allow processing of  
537 large amounts of data in an automated and comprehensive manner. These software may be  
538 provided with the MS instrument or by independent suppliers. The use of BAP databases is also  
539 very helpful in the identification of peptides with previously reported bioactivities,  
540 truncated/precursor peptide sequences or sequences presenting features of BAPs (Iwaniak et al.,  
541 2015; Nongonierma & FitzGerald, 2016c). In addition to automated searches, peptide retention  
542 time models during LC separation have allowed the identification of short peptide sequences  
543 within complex mixtures (Harscoat-Schiavo et al., 2012; Le Maux et al., 2015a; Le Maux et al.,  
544 2015b). A limited number of studies have also described how computational methods, such as  
545 QSAR, could be used to predict the bioactivity of peptides identified in complex samples and

546 achieve a more targeted identification of BAP sequences identified by LC-MS (Majumder &  
547 Wu, 2009; Sagardia, Iloro, Elortza, & Bald, 2013).

548 Overall, a majority of the studies which have reported on the identification of short peptides in  
549 complex mixtures appear to have combined different approaches to validate their analysis. Multi-  
550 stage processes have been employed combining the utilisation of (1) accurate mass  
551 determination, (2) possible peptide sequences matching this mass, (3) occurrence of these  
552 peptides in selected protein sequences combined with *de novo* searches, (4) retention time  
553 validation, (5) good quality MS/MS spectra and (6) confirmatory studies with synthetic peptides  
554 (Figure 2). Utilisation of these sequential approaches have allowed a narrowing down of peptide  
555 candidates within complex samples which has, in several instances, led to the identification of  
556 short peptides.

## 557 **6 Understanding interactive effects between BAPs as well as matrix effects**

558 As already mentioned, a large number of peptide sequences may be found within food protein  
559 hydrolysates. These peptides may interact to decrease or increase the bioactive properties of the  
560 hydrolysate. For this reason it is important to consider the potential for interactive effects  
561 between peptides. While scientists have often highlighted the possibility that peptides in a  
562 mixture might interact, modifying the bioactive properties, very few studies have actually  
563 demonstrated which mechanisms of action were involved.

564 An isobole approach has been described, showing *in vitro* additive or antagonistic effects  
565 between binary mixtures of DPP-IV inhibitory peptides (Nongonierma & FitzGerald, 2015c).  
566 The identification of peptide sequences which are able to interact in a synergistic manner to  
567 increase the bioactive properties of hydrolysates is of interest. Peptide mixtures with synergistic

568 effects may allow the formulation of more potent samples. With this in mind, mixtures of other  
569 components (foods, lipid-based carriers or drugs) with dietary hydrolysates have shown  
570 promising results in increasing the overall bioactive properties both *in vitro* and *in vivo*. The  
571 concomitant administration of the ACE inhibitory lactotripeptides (LTPs - Ile-Pro-Pro, Leu-Pro-  
572 Pro and Val-Pro-Pro) with foods (different meals having various protein, carbohydrate, fat and  
573 fiber contents) have resulted in increased bioavailability in pigs. This has been attributed to the  
574 potential modification of the kinetics of digestion and gut metabolism in the presence of foods  
575 (Ten Have, van der Pijl, Kies, & Deutz, 2015).

576 Food-drug interactions have been studied *in vitro* in the case of DPP-IV inhibition (Nongonierma  
577 & FitzGerald, 2013b). It was shown *in vitro* that Sitagliptin, an antidiabetic drug, and DPP-IV  
578 inhibitory peptides from milk proteins could interact in an additive or synergistic manner to  
579 inhibit DPP-IV (Nongonierma & FitzGerald, 2015c). *In vivo* studies have also been conducted  
580 where pharmaceutical drugs have been administered together with food protein hydrolysates.  
581 One study has recently evaluated the effect of the concomitant administration of Enaprilat (an  
582 antihypertensive drug) combined with the LTPs (Ile-Pro-Pro and Val-Pro-Pro) or a fish protein  
583 hydrolysate to spontaneous hypertensive rats (SHRs) (Watanabe et al., 2015). A single  
584 administration of the LTPs or the fish protein hydrolysate reduced the antihypertensive effects of  
585 Enaprilat, suggesting that competition during intestinal absorption may occur. However, a  
586 modest additional antihypertensive effect was seen, which was not significant ( $p > 0.05$ ), when  
587 the LTPs were administered during a long-term (6 weeks) study at day 29 to the Enaprilat group.

## 588 **7 Determining the mechanism of action of BAPs**

589 *In silico* methodologies have been utilised to study structure-activity relationships between BAPs  
590 and various biological activities or organoleptic properties (e.g., bitterness). QSAR approaches  
591 applied to the study of ACE, renin (EC 3.4.23.15) and DPP-IV inhibitory as well as antioxidant,  
592 antimicrobial and bitter peptides have been described in the literature. Peptide structural features  
593 have been determined by QSAR for certain bioactive properties such as ACE, renin and DPP-IV  
594 inhibition (for reviews, see: Iwaniak et al., 2015; Nongonierma & FitzGerald, 2016a). However,  
595 for other biological properties (e.g., antimicrobial, antioxidant and bitterness), while the overall  
596 physicochemical properties of the peptides have been suggested as being important, no clear  
597 favourable peptide sequence has been identified. Interestingly, the importance of hydrophobic  
598 amino acids (Ala, Ile, Leu, Pro, Trp and Val) in BAPs able to inhibit metabolic enzymes has  
599 often been reported in QSAR outcomes (Nongonierma & FitzGerald, 2016a).

600 To date, it appears that most QSAR analyses have not subsequently employed confirmatory  
601 studies with synthetic peptides to validate the models developed. Not only can QSAR help to  
602 identify novel and potent BAPs, it may also be combined to predict their sensory attributes such  
603 as bitterness to select sequences with enhanced bioactivity and acceptable organoleptic  
604 properties (Pripp & Ardö, 2007; Zhou, Yang, Ren, Wang, & Tian, 2013). In addition, QSAR has  
605 been described as a decision tool to help select potential BAP sequences within large datasets  
606 (Carrasco-Castilla et al., 2012). This may be the case for peptides identified within humans  
607 (Nongonierma & FitzGerald, 2016c) or in peptide sets identified by LC-MS/MS (Majumder &  
608 Wu, 2010; Sagardia et al., 2013).

609 A number of limitations of QSAR when applied to the study of BAPs have been reported in the  
610 literature. These comprise incorporation of biological activity data obtained using different

611 experimental conditions as well as the lack of discrimination of peptides which may act through  
612 different modes of action. In this context, it has been suggested that the mode of inhibition of  
613 ACE should be taken into account in structure activity relationship studies in order to develop  
614 more meaningful models (Jao, Huang, & Hsu, 2012). Recently, it was shown that statistically  
615 significant structure activity relationship models could only be obtained by the inclusion of  
616 competitive DPP-IV inhibitors and IC<sub>50</sub> values obtained under the same experimental conditions  
617 (Nongonierma & FitzGerald, 2016c).

618 Molecular docking has been used to virtually screen peptide sequences with ACE (Norris, Casey,  
619 FitzGerald, Shields, & Mooney, 2012; Pan, Cao, Guo, & Zhao, 2012; Pripp, 2007), DPP-IV  
620 (Nongonierma, Mooney, Shields, & FitzGerald, 2013; Nongonierma, Mooney, Shields, &  
621 FitzGerald, 2014) and xanthine oxidase (XO) (Nongonierma et al., 2013) inhibitory properties.

622 Molecular docking has also been employed to better understand specific binding of peptides to  
623 enzymes such as ACE (Asoodeh et al., 2014; He, Aluko, & Ju, 2014; Li et al., 2014; Norris et al.,  
624 2012; Pan et al., 2012; Pina & Roque, 2009; Zhang et al., 2015), renin (He et al., 2014) and DPP-  
625 IV (Velarde-Salcedo et al., 2013). While providing interesting results and allowing, in certain  
626 cases, the identification of enzyme cofactors (e.g., Zn<sup>2+</sup> for ACE inhibition) (Pina & Roque,  
627 2009) or novel BAPs (Norris et al., 2012), some limitations of molecular docking have been  
628 highlighted. Several molecular docking studies assume peptide binding to the active site of  
629 metabolic enzymes (competitive inhibition). However, this has not always been confirmed  
630 following their *in vitro* evaluation (Nongonierma et al., 2014). Therefore, it has been suggested  
631 that the mode of inhibition of the peptides should be taken into account in conjunction with  
632 molecular docking analyses. The main difficulty of this approach is that the mode of interaction  
633 of peptides with metabolic enzymes is generally unknown. However, there are a growing number

634 of studies which have evaluated the mode of inhibition of metabolic enzymes by peptides using  
635 *in vitro* protocols based on the Lineweaver and Burke approach. The different modes of ACE  
636 inhibition (competitive, non-competitive and uncompetitive) with dietary peptides have been  
637 reviewed by Jao et al. (2012). Determination of the mode of inhibition of other metabolic  
638 enzymes appears to have been studied to a lesser extent. However, a few examples of peptide  
639 mode of inhibition have been reported for DPP-IV (for review, see: Lacroix & Li-Chan, 2016),  
640 XO (Nongonierma & FitzGerald, 2012b; Nongonierma et al., 2013) and cathepsin B (EC  
641 3.4.22.1) (Lee & Lee, 2000). In general, there is a requirement for more data to be generated to  
642 understand how these BAPs may interact with the enzymes and subsequently inhibit or activate  
643 them. Understanding the mode of interaction between enzymes and peptides is of central  
644 importance in order to determine if these BAPs may act through the same mechanism of action  
645 in comparison to specific pharmaceutical drugs (Pina & Roque, 2009) or whether they may  
646 interact with drugs to further inhibit enzyme activity (Nongonierma & FitzGerald, 2015c).

## 647 **8 Demonstrating effects of BAPs in humans**

648 Current knowledge on the *in vivo* bioavailability of BAPs is limited (for reviews, see: Boutrou et  
649 al., 2015; Nongonierma & FitzGerald, 2015b; Nongonierma & FitzGerald, 2016b). However, the  
650 number of nutritional intervention studies reporting on the occurrence of peptides in human  
651 fluids or tissues is increasing (Nongonierma & FitzGerald, 2015a). Examples of studies which  
652 have detected peptides in humans following the ingestion of peptides, food protein hydrolysates  
653 or intact proteins are listed in Table 3. Several peptides with previously reported *in vitro* ACE  
654 inhibitory, opioid (Boutrou et al., 2013), mineral binding (Meisel et al., 2003) and antithrombic  
655 (Chabance et al., 1995; Chabance et al., 1998) activities have been identified in the

656 gastrointestinal contents of humans. Peptides previously identified in the gastrointestinal tract of  
657 humans have been analysed for their potential to act as inhibitors of DPP-IV *in vitro*. This has  
658 allowed the discovery of two relatively potent DPP-IV inhibitors, Leu-Pro-Val-Pro-Gln and Ile-  
659 Pro-Met having IC<sub>50</sub> values of 43.8 and 69.5 μM, respectively (Nongonierma & FitzGerald,  
660 2016c).

661 Identification of peptides, with previously reported *in vitro* ACE inhibition, anticancer and cell  
662 proliferative effects, in the plasma has also been achieved in a number of human intervention  
663 studies (Table 3). Recently, a very promising study reported for the first time on the detection of  
664 caseinophosphopeptides (CPPs, mineral binding peptides) in the plasma of humans following  
665 ingestion of Parmigiano Reggiano cheese (100 g/day for 7 days) (Caira et al., 2016). Several  
666 CPPs [ $\alpha_{s1}$ -CN (f43-52)2P and  $\alpha_{s1}$ -CN (f43-50)2P,  $\alpha_{s2}$ -CN (f8-12)2P/3P,  $\alpha_{s2}$ -CN (f7-12)2P/3P and  
667  $\alpha_{s2}$ -CN (f6-12)3P] as well as non-phosphorylated casein-derived peptides [ $\beta$ -CN (f193-209),  $\beta$ -  
668 CN (f194-209) and  $\beta$ -CN (f200-209)] were identified in plasma by LC-MS/MS. The presence of  
669 BAPs *in vivo* is, however, not sufficient to guarantee that they can exert a positive effect on  
670 human health. In fact, there is still a level of uncertainty as to what concentration of a BAP is  
671 required for biological effects to be seen in humans.

672 To date, it appears that only a limited number of studies have quantified peptide levels in humans  
673 and in certain instances determined their bioavailability (Table 3). The quantification of BAPs in  
674 human samples have been described using calibrated ELISA methods (Chabance et al., 1995;  
675 Chabance et al., 1998; Meisel et al., 2003) or synthetic peptides in the format of internal or  
676 external (generally isotopically labelled) standards (Foltz et al., 2007; Kaiser et al., 2016; Taga et  
677 al., 2014). Caseinomacropetide (CMP) was quantified (16 and 21 μg mL<sup>-1</sup>) in the plasma of  
678 newborns after ingestion of human milk and bovine milk-based infant formula (Chabance et al.,

679 1995). Lower levels (0.5-2 and 1.05-10  $\mu\text{g mL}^{-1}$ ) were reported in adults following milk and  
680 yogurt ingestion (Chabance et al., 1998). Differences between adults and infants were attributed  
681 to the maturity and permeability of their gut and to the feeding pattern (Chabance et al., 1998).  
682 Concentrations of CPPs between 0.2 and 7.10  $\text{nmol mL}^{-1}$  were reported in the ileostomy fluid of  
683 humans following ingestion of CPPs or milk, respectively (Meisel et al., 2003). Concentrations  
684 of 17 and 900  $\mu\text{M}$  for the opioid peptide  $\beta$ -casomorphin-7 ( $\beta$ -CN (f60-66)) and the ACE  
685 inhibitory peptide  $\beta$ -CN (f108-113) were reported in the jejunum of humans who had ingested  
686 bovine milk (Boutrou et al., 2013).

687 Different ACE inhibitory peptides (Table 3) were quantified by LC-MS/MS using the method  
688 developed by van Platerink, Janssen, Horsten, and Haverkamp (2006) in the plasma of adults  
689 following the consumption of an LTP-enriched yoghurt drink (Foltz et al., 2007). The ACE  
690 inhibitory peptides Ile-Trp and Trp-Leu were fed to humans and detected in their plasma at  
691 levels of 2.4 and 29-36 nM, respectively, 30 min following ingestion (Kaiser et al., 2016). In this  
692 study, it was demonstrated for the first time that following ingestion of 50 mg, Ile-Trp could  
693 reduce human ACE activity in plasma by  $32 \pm 8\%$ . In another study, Val-Tyr, an ACE inhibitory  
694 peptide, was identified in plasma following ingestion of a sardine muscle hydrolysate fraction  
695 (containing 12 mg Val-Tyr), reaching a maximal concentration of 1934  $\text{fmol mL}^{-1}$  after 2 h  
696 (Matsui et al., 2002).

697 Lunasin, an anticancer peptide, was detected in the plasma of humans following ingestion of soy  
698 proteins administered in the format of a soy chilli meal (Dia, Torres, De Lumen, Erdman Jr, &  
699 De Mejjia, 2009). Concentrations of 50.2-110.6 and 33.5-122.7  $\text{ng mL}^{-1}$  were detected in plasma  
700 30 and 60 min following feeding, respectively. It was estimated that these levels would  
701 correspond to an absorption of 4.5% of the amount of lunasin reaching the small intestine. In

702 another study, it was hypothesised that Bowman-Birk inhibitor (BBI), a serine protease (8 kDa)  
703 with anticancer properties, may be bioavailable as some of its metabolites (23.6 and 21.6 µg  
704 detected 6 h post ingestion at 36 h apart, respectively) were detected by ELISA in the urine of  
705 humans following soy milk intake (Wan, Lu, Anderson, Ware, & Kennedy, 2000).

706 Cell proliferative collagen/gelatin-derived peptide bioavailability in humans has been  
707 investigated. Various levels of Hyp containing di- and tripeptides (Table 3) were reported in the  
708 plasma of humans following the ingestion of fish scale or porcine skin gelatin hydrolysates  
709 (Ichikawa et al., 2010; Shigemura et al., 2011; Sugihara et al., 2012). While many of these  
710 studies have allowed the quantification of a limited number of Hyp-containing peptides, a  
711 method employing the utilisation of stable isotope Hyp-containing peptides has recently been  
712 described (Taga et al., 2014). This method has allowed the simultaneous accurate quantification  
713 of free Hyp and 13 Hyp-containing peptides in plasma of humans who had ingested a fish scale  
714 gelatin hydrolysate. It was also shown in this study that the level of free and total Hyp detected  
715 were significantly enhanced (> 1.6 times more) when using the stable isotope internal standard  
716 method.

717 Overall, the bioavailability of BAPs in humans is generally very low (Table 3). Low  
718 bioavailability has been explained by the fact that peptides may be prone to degradation by  
719 peptidases/proteinases in the gastrointestinal tract and in the serum while low peptide permeation  
720 at the intestinal level has also been described (for reviews, see: Picariello, Mamone, Nitride,  
721 Addeo, & Ferranti, 2013b; Sánchez-Rivera et al., 2014b). Different strategies may be employed  
722 to increase bioavailability of peptides. More particularly, encapsulation of peptides (for review,  
723 see: Mohan, Rajendran, He, Bazinet, & Udenigwe, 2015) may increase their stability to digestive  
724 enzymes (Giroux, Robitaille, & Britten, 2016; Li, Paulson, & Gill, 2015). In addition,

725 permeation enhancers may help to increase peptide absorption in the ileum (for reviews, see:  
726 Gleeson, Ryan, & Brayden, 2016; Niu, Conejos-Sánchez, Griffin, O’Driscoll, & Alonso, 2016).  
727 Lipid-based intestinal permeation enhancers (e.g., sodium caprate and the sodium salt of 10-  
728 undecylenic acid) were shown to increase the permeation of ACE inhibitory peptides (Ile-Pro-  
729 Pro and Leu-Lys-Pro) in Ussing chambers containing rat jejunum and colon (Gleeson, Heade,  
730 Ryan, & Brayden, 2015).

731 The low bioavailability of peptides raises the question of how much of the peptide(s) should be  
732 ingested to cause an *in vivo* effect. Utilisation of dose-response approaches to evaluate BAPs in  
733 humans may allow assessment of their *in vivo* potency. A limited number of human studies have  
734 employed a dose-response approach to understand the level of hydrolysates/BAPs required to  
735 observe a bioactive effect *in vivo*. Several human intervention studies conducted with the ACE  
736 inhibitory LTPs (Ile-Pro-Pro and Val-Pro-Pro) have demonstrated a dose-response relationship  
737 between their ingestion and SBP reduction. This was the case with a casein hydrolysate, obtained  
738 on casein hydrolysis with an *Aspergillus oryzae* protease, administered to subjects with mild and  
739 high hypertension at dosages of 1.8, 2.5 and 3.6 mg LTPs for 6 weeks. A reduction in SBP of  
740 5.8, 6.2 and 9.3 mmHg, respectively, was obtained with the three treatments (Mizuno et al.,  
741 2005). Similarly, de Leeuw, Van der Zander, Kroon, Rennenberg, and Koning (2009) have  
742 demonstrated a significant reduction ( $p < 0.05$ ) in SBP in a dose-dependent manner following  
743 LTPs intake (2.3, 4.56 and 9.30 mg/200 g yogurt drink formulated with a casein hydrolysate  
744 containing the LTPs) by mildly hypertensive subjects. A decrease in arterial stiffness was  
745 reported in hypertensive subjects who consumed a *Lactobacillus helveticus* fermented milk drink  
746 containing 5 or 50 mg/day LTPs for 12 weeks (Jauhainen et al., 2010). Different doses of a  
747 proprietary casein-derived hydrolysate (InsuVida™) with antidiabetic properties have been

748 evaluated in humans (for review, see: Nongonierma & FitzGerald, 2015b). In the study of Jonker  
749 et al. (2011) lower doses of 6 and 12 g hydrolysate were ingested compared to 25-35 g reported  
750 in earlier studies (Manders et al., 2006a; Manders et al., 2005; Manders et al., 2006b; Manders,  
751 Praet, Vikstrom, Saris, & van Loon, 2007; van Loon et al., 2003). Higher insulin secretion and  
752 reduced blood glucose levels were observed in type 2 diabetic subjects only after 12 but not 6 g  
753 hydrolysate intake, showing that certain BAP levels may be required to observe the antidiabetic  
754 properties (Jonker et al., 2011). Matsui et al. (2002) fed humans with three doses (3, 6 and 12  
755 mg) of Val-Tyr (present within a sardine muscle hydrolysate fraction). Val-Tyr could be  
756 absorbed in the blood in a dose-dependent manner. A commercial cod skin gelatin hydrolysate  
757 was fed to humans at three different doses, i.e., 30.8, 153.8 and 384.6 mg per kg body weight  
758 (Shigemura, Kubomura, Sato, & Sato, 2014). Hydrolysate doses > 30.8 mg per kg body weight  
759 were required in order to observe a change in concentration in free Hyp and Hyp-bound peptides  
760 in plasma. In addition, a dose-response pattern was seen between the amount of hydrolysate  
761 ingested and the level of Hyp and Hyp-containing peptides in plasma. It was also shown that at  
762 the highest dose tested (384.6 mg per kg body weight), no limit of absorption of Hyp and Hyp-  
763 containing peptides was reached, suggesting that higher doses would yield an increased level in  
764 plasma.

765 While several studies have identified peptides with *in vitro* bioactive effects, understanding of  
766 the relevance of these results to *in vivo* behaviour of peptides is not straightforward. This is  
767 mainly due to major differences in terms of their *in vivo* bioavailability, stability and transfer  
768 kinetics (Foltz et al., 2010). In order to develop more meaningful *in vitro* evaluation of peptides,  
769 it has been proposed to reverse the strategy of BAP discovery starting from *in vivo* knowledge to  
770 inform the release of peptides during *in vitro* hydrolysis. This may, in turn, improve the

771 correlation between *in vitro* bioassays and *in vivo* results (Foltz et al., 2010; Nongonierma &  
772 FitzGerald, 2016b).

## 773 **9 Conclusions**

774 Different targeted approaches have attempted to develop alternative strategies to overcome the  
775 limitations of the conventional approach used in BAP studies. There is a need to develop more  
776 robust scientific hypotheses leading to targeted release of peptides with specific features,  
777 physicochemical properties or predicted bioactivity. To better understand the role of dietary  
778 BAPs in human health, a number of issues still needs to be dealt with, i.e., insufficiently  
779 characterised enzyme preparations, non-standardised protocols (for bioassay assessment, peptide  
780 identification and *in vivo* evaluation of BAPs), inadequate study of short peptides and lack of  
781 mechanistic studies. A focus on these challenges may allow a “closing of the gap” between *in*  
782 *vitro* bioassays, bioavailability and efficacy of BAPs in humans. In addition, knowledge of the  
783 sequence and level of BAPs identified *in vivo* may constitute the basis for a reversed discovery  
784 approach starting from *in vivo* bioavailability to inform the generation of BAPs during enzymatic  
785 hydrolysis. A focus on peptides identified within humans is of significant interest as such studies  
786 provide a better understanding of the mechanism of action of BAPs in humans, which is a  
787 requirement from a regulatory point of view.

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792 **Conflicts of interests**

793 The authors declare that they have no conflict of interest.

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## Table and figure captions

**Table 1.** *In silico* predictive models used to rank dietary proteins based on their potential to act as a source of bioactive peptides (BAPs).

**Table 2.** Examples of studies reporting on short peptide sequence identification within model solutions, food protein hydrolysates or plasma samples.

**Table 3.** Summary of human intervention studies describing the identification and quantification of peptides *in vivo*.

**Figure 1.** Components employed for the identification and validation of food protein-derived bioactive peptides (BAPs). ANN: artificial neural network; DOE: design of experiments; QSAR: quantitative structure activity relationship; RSM: response surface methodology.

**Figure 2.** Summary of the strategies employed to improve the detection and identification of short peptides within complex mixtures. CE: capillary electrophoresis; HILIC: hydrophilic interaction liquid chromatography; LC: liquid chromatography; MRM: multiple reaction monitoring; MS: mass spectrometry; NDA: naphthalene-2,3-dialdehyde; PITC: phenyl isothiocyanate; RP: reverse-phase; SIM: selected ion monitoring; SPE: solid phase extraction;; UF: ultrafiltration.

**Table 1**

<b>Parameter estimated</b>	<b>Model</b>	<b>Unit</b>	<b>Reference</b>
Frequency of occurrence of BAP (A)	$A = \frac{a}{N}$	none	(Dziuba et al., 2003)
Potential biological activity (B)	$B = \frac{\sum_i \left( \frac{a_i}{EC_{50,i}} \right)}{N}$	none	(Dziuba et al., 2003)
Frequency of occurrence of BAP release (A <sub>E</sub> )	$A_E = \frac{d}{N}$	none	(Minkiewicz et al., 2011)
Potency score (Score)	$Score = \frac{\sum_i \left( \frac{1}{IC_{50,i}} \right)}{MW}$	mL mg <sup>-1</sup>	(Vermeirssen et al., 2004)
Half maximal inhibitory potential (IC <sub>50</sub> ) of a protein hydrolysate	$IC_{50,hydrolysate} = \frac{10^{-6} \times MW}{\frac{1}{a} \times \sum_{i=1}^p \left( \frac{1}{IC_{50,i}} \right)}$	mg mL <sup>-1</sup>	(Pripp, 2005)
Potency index (PI)	$PI = \frac{\sum_i \left( \frac{1}{IC_{50,i}} \times n_{i,corrected} \right)}{MW}$	μM <sup>-1</sup> g <sup>-1</sup>	(Nongonierma & FitzGerald, 2014)

a: number of bioactive peptide (BAP) sequences found within the protein; A: frequency of BAP occurrence in a protein; A<sub>E</sub>: frequency of BAP occurrence as predicted to be released *in silico* following digestion of a protein by a specific enzyme activity; a<sub>i</sub>: number of repetitions of peptide i within the protein sequence; B: potential biological activity of the protein; d: number of BAP sequences released *in silico* following digestion of a protein by a specific enzyme activity; IC<sub>50,i</sub>: half maximal inhibitory concentration of peptide i (expressed in M (Vermeirssen et al., 2004) or μM (Nongonierma & FitzGerald, 2014; Pripp, 2005)); IC<sub>50,protein</sub>: half maximal inhibitory concentration of the *in silico* protein digest; MW: molecular mass of the protein (g mol<sup>-1</sup>); N: number of amino acid residues in the protein; n<sub>i,corrected</sub>: corrected occurrence of peptide i per mol of protein.

**Table 2**

<b>Sample</b>	<b>Peptides identified*</b>	<b>Workflow**</b>	<b>Bioactivity of selected sequences</b>	<b>Reference</b>
Human plasma collected after oral intake of a sardine muscle hydrolysate	Val-Tyr	<ul style="list-style-type: none"> <li>• RP-HPLC fractionation</li> <li>• NDA-derivatisation</li> <li>• fluorescence detection (excitation and emission wavelengths 420 and 490 nm, respectively)</li> </ul>	antihypertensive	(Matsui et al., 2002)
Model solution, gluten protein hydrolysate and Parmesan cheese	10 amino acids, 9 di- and 1 tripeptide (gluten hydrolysate) 9 dipeptides (parmesan cheese)	<ul style="list-style-type: none"> <li>• unbound fraction on a C18 column (gluten hydrolysate) and 3 kDa permeate (cheese water soluble extract)</li> <li>• separation on an HILIC column</li> <li>• analysis of MS/MS fragments</li> </ul>	n.d.	(Schlichtherle-Cerny et al., 2003)
Milk protein hydrolysate	71 compounds (amino acids and di- to tetra peptides)	<ul style="list-style-type: none"> <li>• 2D LC separation (C18 and HILIC columns)</li> <li>• accurate mass</li> <li>• retention time</li> <li>• MS/MS fragmentation</li> <li>• synthetic peptides</li> </ul>	antihypertensive	(van Platerink et al., 2008)
Whey protein hydrolysate	Ile-Val, Leu-Val, Val-Leu, Ile-Ile, Leu-Ile, Ile-Leu and Leu-Leu	<ul style="list-style-type: none"> <li>• MRM of 7 target peptides</li> <li>• synthetic peptides</li> </ul>	glucose uptake in myotubes and isolated skeletal muscles	(Morifuji et al., 2009)
Commercial fish scale gelatin hydrolysate	Hyp containing di- and tripeptides	<ul style="list-style-type: none"> <li>• MRM of 9 target peptides</li> <li>• synthetic peptides</li> </ul>	fibroblast growth	(Ichikawa et al., 2010)
Atlantic salmon skin collagen hydrolysate	11 di- to pentapeptides	<ul style="list-style-type: none"> <li>• C18 fractionation</li> <li>• LC-MS/MS identification</li> </ul>	antihypertensive	(Gu et al., 2011a)

Commercial fish scale gelatin hydrolysate	Pro-Hyp and Hyp-Gly	<ul style="list-style-type: none"> <li>• SEC fractionation</li> <li>• PITC derivatisation</li> <li>• MRM</li> <li>• Synthetic peptides</li> </ul>	fibroblast growth	(Shigemura et al., 2011)
Commercial porcine skin gelatin hydrolysate	Pro-Hyp and Hyp-Gly	<ul style="list-style-type: none"> <li>• SEC fractionation</li> <li>• PITC derivatisation</li> <li>• MRM</li> <li>• Synthetic peptides</li> </ul>	fibroblast growth	(Sugihara et al., 2012)
Atlantic herring by-products (skin and left over material after filleting)	66 di- to hexapeptides	<ul style="list-style-type: none"> <li>• centrifugation and ultrafiltration (10 kDa)</li> <li>• nanoflow LC-MS Orbitrap</li> <li>• in-house database</li> </ul>	numerous	(Pampanin et al., 2012)
Commercial and experimental <i>L. helveticus</i> ATCC fermented milks	Phe-Pro, Val-Pro-Pro, Ile-Pro-Pro and Leu-Pro-Pro	<ul style="list-style-type: none"> <li>• ultrafiltration (10 kDa)</li> <li>• pre-column derivatisation with dabsyl chloride</li> <li>• SIM</li> </ul>	antihypertensive	(Eisele et al., 2012)
Synthetic peptides in a mixture and rapeseed protein hydrolysate	numerous	<ul style="list-style-type: none"> <li>• retention time prediction (HILIC, C18 and CE columns)</li> <li>• all possible amino acid combinations corresponding to the detected mass.</li> <li>• peptide occurrence in rapeseed proteome</li> </ul>	n.d.	(Harscoat-Schiavo et al., 2012)
$\beta$ -casein incubated with a cell free extract of <i>L. helveticus</i>	12 di- (Xaa-Pro) and tripeptides (Xaa-Pro-Pro)	<ul style="list-style-type: none"> <li>• ultrafiltration (10 kDa)</li> <li>• pre-column derivatisation with dabsyl chloride</li> <li>• SIM during LC-MS</li> <li>• external standards</li> </ul>	antihypertensive	(Stressler et al., 2013)
Synthetic peptides in a mixture and spiked in a whey protein	117 di- and tripeptides (all possible combinations of Val, Leu and Ile)	<ul style="list-style-type: none"> <li>• MRM of 117 target peptides</li> <li>• retention time on C18 column</li> </ul>	n.d.	(Lahrichi et al., 2013)

hydrolysate		<ul style="list-style-type: none"> <li>• modification of the collision energy</li> </ul>		
Gly-Sar and Val-Tyr in rat intestinal sections	Gly-Sar and Val-Tyr	<ul style="list-style-type: none"> <li>• MALDI-IMS</li> <li>• phytic acid matrix additive</li> </ul>	antihypertensive (Val-Tyr)	(Hong et al., 2013)
His-Leu-Pro-Leu-Pro ( $\beta$ -casein (f134-138)) after oral and intravenous administration and breakdown products detected in rat plasma	His-Leu-Pro-Leu-Pro, Leu-Pro-Leu-Pro and His-Leu-Pro-Leu	<ul style="list-style-type: none"> <li>• SPE concentration</li> <li>• SRM</li> <li>• retention time</li> <li>• fragmentation profile</li> </ul>	antihypertensive	(Sánchez-Rivera et al., 2014a)
Enzymatic hydrolysates of <i>Laminaria japonica</i>	8 Tyr containing di- to pentapeptides	<ul style="list-style-type: none"> <li>• MRM</li> <li>• modification of collision energy</li> <li>• synthetic peptides</li> </ul>	antihypertensive	(Chen et al., 2015)
Synthetic peptides in a mixture and whey protein hydrolysate	numerous	<ul style="list-style-type: none"> <li>• SPE fractionation</li> <li>• accurate mass</li> <li>• all possible amino acid combinations corresponding to the detected mass.</li> <li>• peptide occurrence in milk proteome and <i>de novo</i> searches</li> <li>• retention time prediction (HILIC column)</li> <li>• analysis of MS/MS fragments</li> </ul>	n.d.	(Le Maux et al., 2015a)
Whey protein hydrolysate	numerous	<ul style="list-style-type: none"> <li>• nanofiltration (300 Da) fractionation</li> <li>• accurate mass</li> <li>• all possible amino acid combinations corresponding to the detected mass.</li> <li>• peptide occurrence in milk proteome and <i>de novo</i> searches</li> <li>• retention time prediction (HILIC and C18 columns)</li> </ul>	DPP-IV inhibition	(Le Maux et al., 2015b)

Whey and casein hydrolysates	numerous	<ul style="list-style-type: none"> <li>• analysis of MS/MS fragments</li> <li>• modification of collision energy</li> <li>• modification of transfer time</li> <li>• analysis of MS/MS fragments</li> </ul>	n.d.	(O’Keeffe & FitzGerald, 2015)
Human plasma (fed with Ile-Trp and Trp-Leu)	Ile-Trp and Trp-Leu	<ul style="list-style-type: none"> <li>• isotopic labelling</li> <li>• MRM</li> <li>• modification of collision energy</li> <li>• synthetic peptides</li> </ul>	antihypertensive	(Kaiser et al., 2016)

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\*Peptides identified by their three letter code

\*\*CE: capillary electrophoresis; C18: octadecyl silane; HILIC: hydrophilic interaction liquid chromatography; LC-MS: liquid chromatography- mass spectrometry; MALDI-IMS: matrix-assisted laser desorption ionisation-imaging mass spectrometry; MRM: multiple reaction monitoring; n.d.: not disclosed; NDA: naphthalene-2,3-dialdehyde; PITC: phenyl isothiocyanate; RP-HPLC: reverse-phase high pressure liquid chromatography; SEC: size exclusion chromatography; SIM: selected monitoring; SPE: solid phase extraction; SRM: pseudoselected reaction monitoring; UF: ultrafiltration; 2D: two dimension.

**Table 3**

<b>Feed</b>	<b>Protein source</b>	<b>Peptide(s)</b>	<b>Location</b>	<b>BAP maximal concentration (C<sub>max</sub>)</b>	<b>Bioavailability (%)</b>	<b>Bioactivity</b>	<b>Reference</b>
Human milk	κ-casein	CMP (f106-169)	plasma of newborns	16 µg mL <sup>-1</sup>	n.d.	antithrombic	(Chabance et al., 1995)
Bovine milk-based infant formula	κ-casein	CMP (f106-169)	plasma of newborns	22 µg mL <sup>-1</sup>	n.d.	antithrombic	
Bovine milk	κ-casein	CMP (f106-169)	plasma of adults	0.5-2 µg mL <sup>-1</sup>	n.d.	antithrombic	(Chabance et al., 1998)
Bovine yogurt	κ-casein	CMP (f106-169)	plasma of adults	1.05-10 µg mL <sup>-1</sup>	n.d.	antithrombic	
CPP preparations or bovine milk	milk	n.d.	ileostomy fluid of adults	0.02 and 7.10 nmol mg <sup>-1</sup>	1.8 % (120 mg CPP/250 g milk)	mineral binding	(Meisel et al., 2003)
Lactotriptide enriched yoghurt drink	caseins	Ala-Trp, Ile-Trp, Val-Tyr, Ile-Tyr, Phe-Tyr, Leu-Trp Ile-Pro-Pro and Leu-Pro-Pro	plasma of adults	897-973 pmol L <sup>-1</sup> (Ile-Pro-Pro) 152 ± 85 pmol L <sup>-1</sup> (Leu-Pro-Pro)	n.d.	antihypertensive	(Foltz et al., 2007)
Bovine milk	β-casein	β-casomorphin-7 (f60-66)	jejunal effluent	17 µM	n.d.	opioid	(Boutrou et al., 2013)
	β-casein	(f108-113)	jejunal effluent	900 µM	n.d.	ACE inhibition	

Sardine muscle protein hydrolysate	n.d.	Val-Tyr	plasma of adults	933 ± 201 to 1934 ± 145 fmol mL <sup>-1</sup> (for 3 and 12 mg Val-Tyr intake, respectively)	0.006-0.014%	ACE inhibition	(Matsui et al., 2002)
Commercial fish scale gelatin hydrolysate	fish gelatin	Hyp containing di- and tripeptides	plasma of adults	60.65 ± 5.74 nmol mL <sup>-1</sup>	n.d.	diverse	(Ichikawa et al., 2010)
Commercial fish scale gelatin hydrolysate	fish gelatin	Pro-Hyp and Hyp-Gly	plasma of adults	120 nmol mL <sup>-1</sup>	n.d.	fibroblast growth	(Shigemura et al., 2011)
Porcine skin gelatin hydrolysate	porcine gelatin	Hyp-Gly	plasma of adults	4.2 nmol mL <sup>-1</sup>	n.d.	cell proliferation	(Sugihara et al., 2012)
Commercial fish scale gelatin hydrolysate	fish gelatin	Hyp and 13 Hyp containing di- and tripeptides	plasma of adults	315 ± 15 (Hyp) and 219 ± 4 (total Hyp) nmol mL <sup>-1</sup>	n.d.	fibroblast growth	(Taga et al., 2014)
Soy protein (50 g for 5 days)	soy	Lunasin	plasma of adults	50.2-110.6 and 33.5-122.7 ng mL <sup>-1</sup> (after 30 and 60 min ingestion, respectively)	2.2-7.8%	anticancer	(Dia et al., 2009)
Soy milk (105 and 175 mg BBI)	soy	BBI metabolites	urine	23.6 and 21.6 µg (measured 6 h after two feeds 36 h apart)	< 0.02% of the ingested BBI	anticancer	(Wan et al., 2000)
Trp-containing dipeptides	n/a (synthetic peptides)	Ile-Trp	plasma of adults	2.4 nM	0.0027 ± 0.001%	ACE inhibition	(Kaiser et al., 2016)

Trp-Leu

29-36 nM

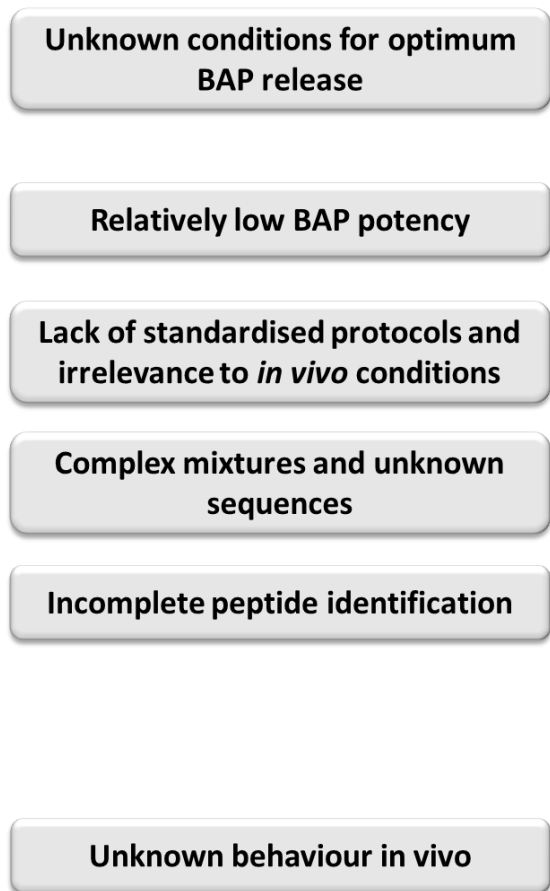
0.021-0.022%

ACE inhibition

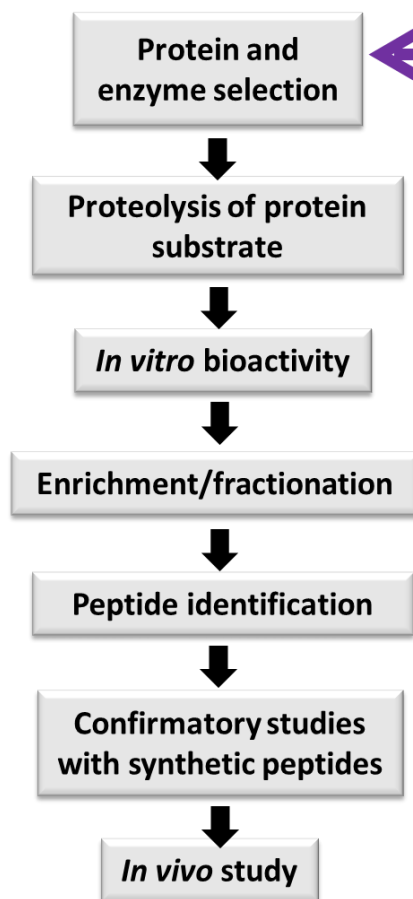
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ACE: angiotensin converting enzyme; BBI: Bowman-Birk inhibitor; CPP: caseinophosphopeptide; CMP: caseinomacropetide

## Limitations



## Conventional



## *In silico*

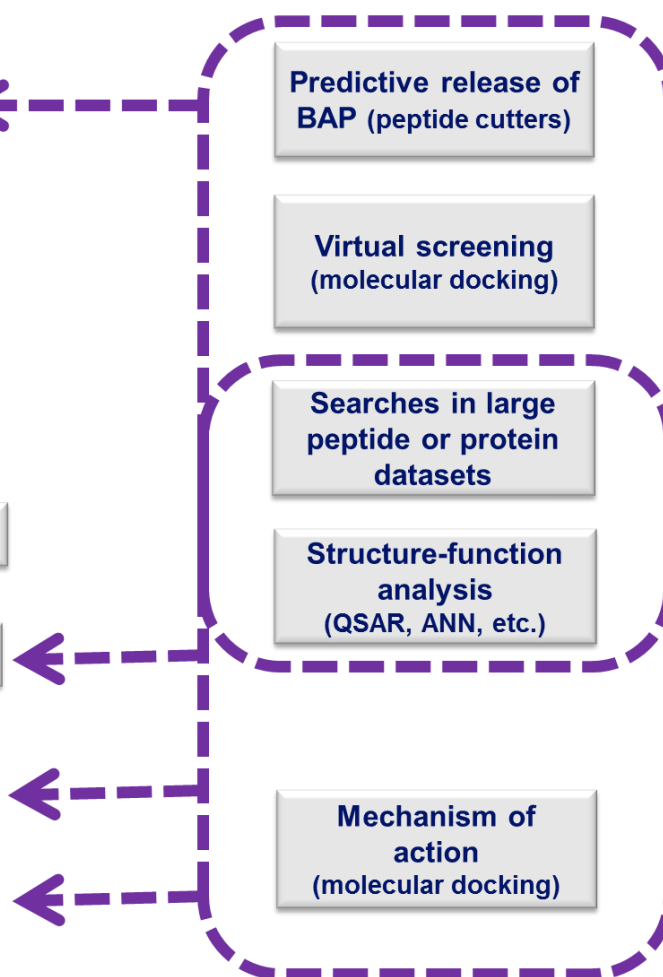
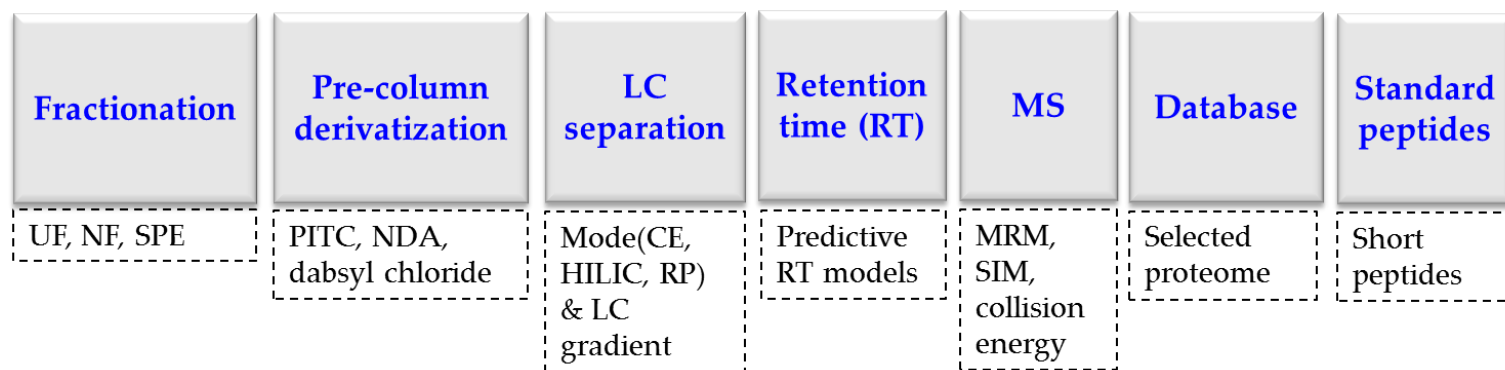


Fig. 1



**Fig. 2**