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Review

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

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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.
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1. Introduction

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

In a global context of increased health-related problems which are notably linked with the metabolic syndrome, adherence to a healthier diet has been acknowledged as one of the key components for maintenance of a healthy status in humans (O'Neill & O'Driscoll, 2015). Consequently, the requirement for high quality dietary proteins has increased significantly due to consumer's demand. Several food processing protein-rich by-products have conventionally been used for animal nutrition either in their intact or hydrolysed format (Martínez-Alvarez, Chamorro, & Brenes, 2015). However, there is an increasing interest in valorising these underutilised protein sources in human nutrition (Lemes et al.,

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2016). Therefore, a wide range of dietary proteins originating from animal, plant and algal sources are currently being investigated for their potential to act as BAP precursors (Udenigwe & Aluko, 2012). Conventionally, food-grade BAPs have been released from dietary proteins using enzymatic hydrolysis, microbial fermentation and/or physical processes (e.g., high pressure, sonication, etc.). However, currently enzymatic hydrolysis and fermentation are the most common means to produce dietary BAPs (Udenigwe & Aluko, 2012).

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

Due to uncertainties surrounding the demonstrated efficacy and the mode of action of BAPs/hydrolysates, their regulatory approval in many countries is extremely difficult. Health claims are granted to food components by the European Food Safety Authority (EFSA) based on scientific substantiation while the Food Drug Administration (FDA, USA) and the Ministry of Health, Labour and Welfare (MHLW, Japan) may grant health claims based on suggested scientific evidence (Lalor & Wall, 2011). To date, in Europe and the USA it appears that no protein-derived BAP-based ingredient/food has been granted health claims by EFSA or the FDA. However, in Japan, the situation is quite different, where a number of BAP-based foods have been recognised as so-called "food for specified health uses" (FOSHU) by the MHLW (Arai, Yasuoka, & Abe, 2008; Shimizu & Hettiarachchy, 2012). The negative opinions provided by EFSA to regulatory application dossiers for peptide-based ingredients with health promoting activity have either been due to a lack of full characterisation of the active ingredients or an inconsistent link between BAP ingestion and the proposed health benefit in the specific target population (De Noni et al., 2009). Guidelines in relation to health claim dossiers have been made available by EFSA to assist applicants in the regulatory dossier preparation process (EFSA, 2011). These guidelines have recently been updated (EFSA, 2016).

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

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

2. Conventional approach to study dietary BAPs and its limitations

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

2.1. Generation of food protein hydrolysates

The first step of the conventional approach consists in the selection of the protein substrate and the proteolytic/peptidolytic enzyme preparation for hydrolysate generation. The selection of protein and enzyme combinations are mainly aided with information from the scientific literature, knowledge of protein sequences and enzyme specificity (Udenigwe & Aluko, 2012). In the conventional approach, selection of the protein substrate may be quite empirical (linked to substrate availability, potential source of BAPs, underutilised protein, cost, etc.). Several commercially available food-grade enzyme preparations have been described in the development of protein hydrolysates. Lemes et al. (2016) recently reviewed the main enzyme preparations which have been employed for the release of BAPs from food by-products. Generally, the enzyme preparations used for the development of food protein hydrolysates originate from animal, microbial or plant sources (Nongonierma & FitzGerald, 2011). Currently, selection of the enzyme preparation arises from (1) knowledge of the specificity of the main activity therein, if available, or (2) indications in the literature of its potential to release certain BAPs.

Following selection of the substrate(s) and enzyme preparation(s), the hydrolysis conditions (i.e., pH, temperature, time, enzyme to substrate (E:S) ratio, total solids, etc.) need to be chosen. These conditions may influence the release of BAPs as they can modify both substrate conformation and enzyme activity and therefore the accessibility of specific peptide bonds in proteins. For instance, it was demonstrated that the protein concentration (0.1–10.0% (w/v)) at which whey protein isolate (WPI) was hydrolysed by a *Bacillus licheniformis* protease, modified the cleavage specificity of certain peptide bonds within β-lactoglobulin (β-Lg) (Butré, Sforza, Gruppen, & Wierenga, 2014). Similarly, β-Lg peptide bond selectivity for cleavage by a *B. licheniformis* protease depended on the pH (7.0–9.0) at which the hydrolysis reaction was conducted (Butré, Sforza, Wierenga, & Gruppen, 2015). To date, optimisation of the *in vitro* generation of food protein hydrolysates appears to have been conducted following modifications of the hydrolysis parameters one at a time. This approach has some limitations as it can be relatively time consuming, generating a large number of samples. In addition, it does not allow an understanding

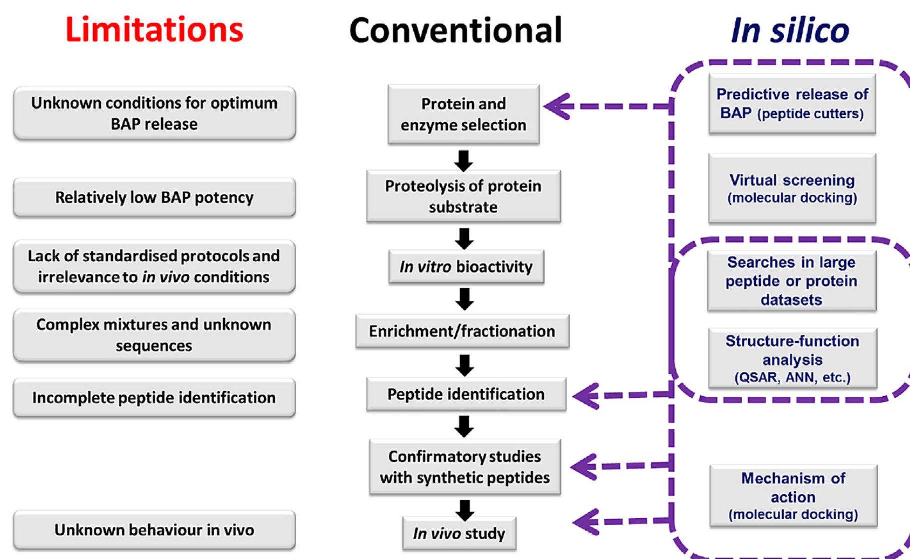

Fig. 1. Components employed for the identification and validation of food protein-derived bioactive peptides (BAPs). ANN: artificial neural network; QSAR: quantitative structure activity relationship.

of the contribution of each parameter, nor the interactive effects between them. Furthermore, the optimum hydrolysis parameters for BAP release are ultimately not likely to be determined with this empirical approach. For this reason a more comprehensive approach to BAP generation has been described which involve the use of multifactorial design of experiments (DOE) and response surface methodologies (RSM) (van der Ven, Gruppen, de Bont, & Voragen, 2002). Specific examples of RSM used as an optimisation tool for BAP release will be described in section 4.

2.2. *In vitro bioactivity assessment of hydrolysates*

Dietary BAPs have been assessed for their biological potential using a wide range of *in vitro* bioassays involving cells grown in culture along with specific chemical or enzymatic reactions. These bioassays are essentially based on the same protocols as those routinely used in drug discovery. The different bioassays used for BAP analysis evaluate the extent of chemical reactions, enzyme inhibition, receptor binding as well as metabolite profiling (for reviews, see: de Castro & Sato, 2015; Nongonierma & FitzGerald, 2016b). However, several parameters may influence the bioactivity results. These include the reactant concentration (hydrolysates/peptides and other test reagents), purity and origin as well as temperature, pH, number of passages of cells in culture, etc. For example, in enzyme inhibition assays, a large variability in the half maximal inhibitory concentration (IC_{50}) of BAPs/hydrolysates has been demonstrated when tested at different E:S ratios in the angiotensin converting enzyme (ACE, EC 3.4.15.1) inhibitory assay (Murray, Walsh, & FitzGerald, 2004). Furthermore, in the presence of specific peptide substrates or inhibitors, variability in enzyme activity has been reported which depended on the organism from which the enzyme originated. This was the case for different bacterial sources of prolyl endopeptidases (EC 3.4.21.26) (Shan, Marti, Sollid, & Khosla, 2004) and for porcine and human recombinant dipeptidyl peptidase IV (DPP-IV, EC 3.4.14.5) (Lacroix & Li-Chan, 2015).

Nevertheless, communalities between different research groups performing the same assay are found. Generally, specific bioassays are conducted with similar positive/negative controls to allow for the comparison of different test samples. These controls may consist of reference chemical compounds which may be a pharmaceutical drug (e.g., Captopril for ACE inhibition, Sitagliptin for DPP-IV inhibition, Allopurinol for xanthine oxidase (EC 1.2.3.2) inhibition), a synthetic compound (e.g., Trolox™, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) for antioxidant assays) or peptides known to positively impact on bioactivity (e.g., Ile-Pro-Pro for ACE inhibition, Ile-Pro-Ile (Diprotin A) for DPP-IV inhibition, Glu-Cys-Gly (glutathione) or Ala-His (carnosine) for antioxidant properties). In antioxidant assays for example, it is common practice to express the potency of the BAPs as an equivalent of the positive control (Trolox). Nevertheless, for most bioassays, this is not the case, making it challenging to compare data across different studies, due to the large variability in the setup of the *in vitro* bioassays.

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

There is currently a relatively low level of confidence regarding the appropriateness of different *in vitro* bioassays for assessment of relevant markers to specific disease/health conditions. In most cases, the aetiology of specific diseases is quite complex and therefore involves several mechanisms of action. Therefore, using a single bioassay to identify a disease reducing agent is questionable as most *in vitro* bioassays are quite specific. For this reason, recent publications are beginning to combine different bioassays to study food protein hydrolysates/BAPs in order to target a wider range of

biological markers linked with a specific disease/health condition (Girgih et al., 2014; Lacroix, Meng, Cheung, & Li-Chan, 2016; Majumder et al., 2015; Nongonierma & FitzGerald, 2013a; Yousr & Howell, 2015).

2.3. Enrichment and fractionation of BAPs

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

A bioactivity-driven fractionation approach is often used in order to determine that enrichment/purification of the BAPs has occurred (Carrasco-Castilla et al., 2012). Fractionation of BAPs is a time consuming process and often requires combination of several techniques to bring about separation of BAPs from other inactive peptides within complex hydrolysates. Generally, peptide fractionation is accompanied by an increased activity in selected fractions compared to the starting hydrolysate. In addition, modifications of peptide-peptide physicochemical (i.e., hydrophobic and electrostatic) interactions may prevail in complex peptide mixtures which may in turn hinder overall bioactivity (Groleau, Morin, Gauthier, & Pouliot, 2003; Mercier, Gauthier, & Fliss, 2004). In certain instances, a reduction in peptide compositional complexity following fractionation may be accompanied with a loss/reduction in bioactive properties. In fact, certain hydrolysates contain peptide sequences which concomitantly contribute to the overall bioactivity as they are able to interact in various ways, e.g., in an additive, synergistic or antagonistic manner (Nongonierma & FitzGerald, 2015b; Schanbacher, Talhouk, Murray, Gherman, & Willett, 1998).

2.4. Peptide identification

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

To date, most MS systems are able to accurately detect peptides $\geq$5 amino acid residues in length. In contrast, shorter peptides (2–4 amino acids) have been underreported, to date, in the literature. This is possibly due to analytical limitations. The under or over fragmentation of short peptides in tandem MS has made their detection complicated and challenging (Dallas et al., 2015; Lahrichi, Affolter, Zolezzi, & Panchaud, 2013). However, the bioavailability and stability of short peptides is thought to be higher compared to larger peptides which are more likely to be further processed during gastrointestinal digestion, intestinal permeation and in the circulation (Foltz, Van Buren, Klaffke, & Duchateau, 2009; Webb, Matthews, & DiRienzo, 1992). The identification of short peptides is therefore crucial for research involving dietary BAPs as these sequences are likely to be relevant to human health.

2.5. In vivo studies

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

Based on the outcomes of all the above experiments, *in vivo* studies may initially be performed in animal models (Fernández-Musoles et al., 2014; Gaudel et al., 2013; Mukhopadhya et al., 2015; Yamada et al., 2015; Zhang, Chen, Jiang, Yin, & Zhang, 2016a). Different animal models of disease are available for testing biological compounds (for reviews, see: Aydin et al., 2014; Nelson & Reusch, 2014). Animal studies are often used before human studies for mechanistic purposes and to evaluate possible toxic effects *in vivo*. Several hydrolysates have also been directly evaluated in humans notably for their antihypertensive, antidiabetic and mineral binding properties (for reviews, see: Boutrou, Henry, & Sanchez-Rivera, 2015; Fekete, Givens, & Lovegrove, 2013; Nongonierma & FitzGerald, 2012a, 2015b). *In vivo* trials are generally performed at the last stage of the study, due to their high cost, time requirement and for ethical reasons (Foltz, van der Pijl, & Duchateau, 2010). *In vivo* studies ultimately serve to confirm the bioactive potential of hydrolysates. While positive outcomes have been reported in several human studies, conflicting results have been found, sometimes making it difficult to understand the beneficial role of BAPs in human health. This conflicting information may arise from the poor bioavailability/stability of the BAPs as well as human interindividual variability (genetic, phenotypic, non-responders, gut microbiota, health status, etc.) (Nongonierma & FitzGerald, 2015b).

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

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

3. Targeted approaches for the discovery and identification of BAPs

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

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

A rapid means to interrogate the dietary proteome is to systematically search for known BAP sequences within a dataset of protein sequences (Dziuba & Darewicz, 2007; Dziuba, Iwaniak, & Minkiewicz, 2003). *In silico* analyses have been conducted on dietary proteins originating from plant (Chang & Alli, 2012; Cheung, Nakayama, Hsu, Samaranayaka, & Li-Chan, 2009; Udenigwe, 2016; Vecchi & Añón, 2009), milk (Dziuba & Dziuba, 2014; Tulipano, Faggi, Nardone, Cocchi, & Caroli, 2015), fish (Darewicz, Borawska, & Pliszka, 2016; Huang, Lin, & Chang, 2015), meat (Keska & Stadnik, 2016; Lafarga, O'Connor, & Hayes, 2015) and insects (Vercruysse et al., 2009) to study their potential to act as substrates for the generation of BAPs. This approach has also been specifically described for the identification of staple food proteins which may be useful for the generation of ACE (Gu, Majumder, & Wu, 2011b; Vermeirssen, van der Bent, Van Camp, van Amerongen, & Verstraete, 2004) and DPP-IV (Lacroix & Li-Chan, 2012; Nongonierma & FitzGerald, 2014) inhibitory peptides. Different methodologies have been employed to rank proteins for their bioactive potential (Table 1). Protein ranking based on the frequency of occurrence of BAPs (A) within their sequence (Table 1) has been described by Dziuba et al. (2003).

The main limitations of ranking proteins based on this approach are that the frequency of BAP occurrence may incorporate overlapping peptide sequences and that bioactive potency is not taken into account. To address these issues, different indexes incorporating peptide potency have been proposed in the literature. The potential bioactivity index of food proteins was first introduced by Dziuba et al. (2003), which incorporated parameters such as the half maximal effective concentration (EC_{50}) of the peptides identified within protein sequences (Table 1). Vermeirssen et al. (2004) employed a scoring approach taking into account the ACE IC_{50} value and molecular mass of the protein (pea and milk proteins) in their *in silico* study (Table 1). They developed two scoring systems to take into account the presence of overlapping peptide sequences within protein sequences, by prioritising the most potent peptides (score 1) and subsequently prioritising the shortest and most potent peptides (score 2). An *in silico* model was developed by Nongonierma and FitzGerald (2014) to take account of the fact that each amino acid residue can only contribute to one peptide sequence at a time within a given region of the protein containing overlapping BAP sequences. In this model, only the most potent DPP-IV inhibitory peptide was considered (regardless of its size). Furthermore, a potency index was developed incorporating peptide potency (i.e., IC_{50}) and molecular mass of the protein (Table 1). In another study on milk proteins, Tulipano et al. (2015) also incorporated a peptide DPP-IV IC_{50} value in their *in silico* analysis. Peptide candidates identified within α-lactalbumin (α-La) and β-Lg were classified into four groups (i.e., DPP-IV IC_{50} < 100, 100–200, 200–500 and 500–1600 μM) based on the structural similarity of their N-terminal dipeptide sequence to previously identified DPP-IV inhibitory peptides. In both models developed by Vermeirssen et al. (2004) and Nongonierma and FitzGerald (2014), discrepancies were found between BAP occurrence and potential predicted bioactivity of proteins. For this reason, it was concluded that the number of BAPs was not a good predictor of the bioactivity potential of proteins (Vermeirssen et al., 2004), suggesting that it may be more accurate to routinely incorporate BAP potency in *in silico* predictions.

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

Parameter estimated	Model	Unit	Reference
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_E)	$A_E = \frac{d}{N}$	none	(Minkiewicz et al., 2011)
Potency score (Score)	$Score = \frac{\sum_i \left(\frac{1}{IC_{50,i}}\right)}{MW}$	mL mg^{-1}	(Vermeirssen et al., 2004)
Half maximal inhibitory potential (IC_{50}) 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^{-1}	(Pripp, 2005)
Potency index (PI)	$\mathrm{PI} = \frac{\sum_i \left(\frac{1}{IC_{50,i}} \times n_{i,corrected}\right)}{MW}$	μM^{-1} g^{-1}	(Nongonierma & FitzGerald, 2014)

a: number of bioactive peptide (BAP) sequences found within the protein; A: frequency of BAP occurrence in a protein; A_E: frequency of BAP occurrence as predicted to be released *in silico* following digestion of a protein by a specific enzyme activity; a_i: 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; $IC_{50,i}$: half maximal inhibitory concentration of peptide i (expressed in M (Vermeirssen et al., 2004) or μM (Nongonierma & FitzGerald, 2014; Pripp, 2005)); $IC_{50,protein}$: half maximal inhibitory concentration of the *in silico* protein digest; MW: molecular mass of the protein (g mol^{-1}); N: number of amino acid residues in the protein; $n_{i,corrected}$: corrected occurrence of peptide i per mol of protein.

One of the most common *in silico* tools consists in the use of peptide cutter programmes. These software are used to predict amino acid/peptide release from specific protein substrates based on knowledge of cleavage specificity of specific enzyme activities. Peptide cutters have been employed to predict starting protein substrate(s) or enzyme(s) which should release previously reported BAPs (Chang & Alli, 2012; Cheung et al., 2009; Fu, Wu, Zhu, & Xiao, 2015; Majumder & Wu, 2010; Minkiewicz, Dziuba, & Michalska, 2011; Nongonierma, Le Maux, Hamayon, & FitzGerald, 2016a; Tulipano et al., 2015; Udenigwe, Gong, & Wu, 2013; Vermeirssen et al., 2004). Peptide cutter outputs may be employed to determine the frequency of BAP release from proteins by a given enzyme activity (A_E, Table 1) (Minkiewicz et al., 2011). Specific features of BAPs may be incorporated in the model when the bioactivity of the peptides is not available in the literature. For instance, peptides predicted to be released from bovine caseins, having an N-terminal Xaa-Pro/Ala (with Xaa an amino acid) were specifically taken into account to calculate a frequency of occurrence for DPP-IV inhibitory peptides (Hsieh et al., 2016). Another strategy has been described to overcome the variability seen in the biological data as well as the availability of specific peptide sequences within BAP databases. For instance, Pripp (2005) combined C-terminal cleavage post each of the 20 conventional amino acids in milk protein sequences, followed by *in silico* gastrointestinal digestion of the resulting peptides predicted to be released and estimation of their ACE IC_{50} values using quantitative structure activity relationship (QSAR) modelling. They developed a model to predict the ACE IC_{50} of protein hydrolysates which was based on *in silico* prediction of peptide release and their predicted ACE IC_{50} value (Table 1).

In many instances, the *in silico* enzymatic digestion of proteins was confirmed by the *in vitro* generation of hydrolysates. This was the case for pea, egg and bovine whey proteins (Majumder & Wu, 2010; Nongonierma, Le Maux et al., 2016a; Tulipano et al., 2015; Vermeirssen et al., 2004). In several studies, validation of the *in silico* prediction was demonstrated at a macromolecular level with the *in vitro* generation of bioactive protein hydrolysates. For example, the ability of thermolysin to produce more potent ACE inhibitory oat protein hydrolysates than subtilisin (EC 3.4.21.62) or pepsin (EC 3.4.4.1) was validated by the *in vitro* evaluation of the corresponding hydrolysates (Cheung et al., 2009). Similarly, confirmation of a higher ACE inhibitory potency following gastrointestinal digestion of whey proteins compared to pea proteins has been demonstrated (Vermeirssen et al., 2004). *In vitro* gastrointestinal digests of β-Lg yielded higher DPP-IV inhibition than those of α-La as predicted *in silico* (Tulipano et al., 2015). A higher DPP-IV inhibition of tryptic (EC 3.4.21.4) caprine compared to tryptic bovine casein hydrolysates was found after 3 h enzymatic hydrolysis as per *in silico* prediction (Zhang et al., 2016b).

Studies based on *in silico* predictions for the release of potent BAPs have proved very useful as a decision tool for the selection of both protein substrates and enzyme activities. However, at a molecular level, a direct translation between predicted and *in vitro* peptide release has not been systematically verified, as demonstrated in a number of studies. For example, Nongonierma, Le Maux et al. (2016a) showed that 60% of the peptides which were predicted to be released following digestion of α-La with pancreatic elastase (EC 3.4.21.36) were actually identifiable by LC-MS/MS. Similar results have been reported with peptic digests of ovotransferrin, where only the precursors of the target ACE inhibitory peptides predicted to be released *in silico* could be identified by LC-MS/MS within the hydrolysate (Majumder & Wu, 2010). Recently, differences in ACE inhibitory peptides predicted to be released *in silico* and those detected by LC-MS/MS in a papain digest of bovine collagen were also reported (Fu et al., 2016). Similarly, potato proteins were cleaved differently by pepsin *in vitro* compared to the *in silico* prediction, generally resulting in less peptide bonds cleaved *in vitro* (Rajendran, Mason, & Udenigwe, 2016). Variable results between *in vitro* and *in silico* predictions have been explained by the inability of peptide cutters to accurately predict peptide release from globular proteins (e.g., α-La and ovotransferrin). In addition, peptide cutters assume that all cleavable peptide bonds would be accessible to the enzyme and be readily hydrolysed. The purity of the enzyme activity employed to generate hydrolysates may also affect peptide release *in vitro*. Furthermore, as already outlined, cleavage of peptide bonds during protein hydrolysis is dependent of their accessibility as well as the enzyme activity which is dictated by the experimental hydrolysis conditions (Butré et al., 2014, 2015; Kalyankar, Zhu, O'Cuinn, & FitzGerald, 2013). Following protein denaturation, which may be caused by aggregation during heat-treatment (Dupont et al., 2010; Rahaman, Vasiljevic, & Ramchandran, 2017; Rinaldi, Gauthier, Britten, & Turgeon, 2014; Wada & Lönnerdal, 2014), enzymatic cross-linking (Havenaar et al., 2013; Monogioudi et al., 2011), glycation (Bouhallab, Morgan, Henry, Mollé, & Léonil, 1999; Cattaneo, Stuknytė, Masotti, & De Noni, 2017; Pinto et al., 2014) or interfacial interactions (Maldonado-Valderrama, Wilde, Mulholland, & Morris, 2012), peptide bond accessibility and cleavage during hydrolytic reactions may be modified, which may subsequently impact on bioactivity. Finally, the role of post-translational modifications (PTMs) on peptide release and bioactivity is still not fully understood. PTMs may occur during processing and storage. As they are not taken into account in *in silico* digestion of proteins, there is a need to determine PTMs to fully characterise the peptides within protein hydrolysates (Rajendran et al., 2016).

4. Optimisation of BAP release during enzymatic hydrolysis

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

Selection of the starting substrate and the enzyme may be aided by the use of peptide cutters, as already outlined. The optimisation of hydrolysis parameters has been mainly based on the utilisation of multifactorial DOE combined with RSM. A number of studies using these approaches have allowed the systematic evaluation of the contribution of various hydrolysis parameters (i.e., temperature, time, E:S ratio, pH, etc.) to the release of BAPs during enzymatic hydrolysis of dietary proteins (Abedin et al., 2015; Cheung et al., 2009; del Mar Contreras, Hernández-Ledesma, Amigo, Martín-Álvarez, & Recio, 2011; Naik, Mann, Bajaj, Sangwan, & Sharma, 2013; Nikolaev et al., 2016; Nongonierma, Le Maux, Esteveny, & FitzGerald, 2017; Quirós, Hernández-Ledesma, Ramos, Martín-Álvarez, & Recio, 2012; van der Ven et al., 2002). Other advantages of hydrolysing food proteins using DOE approaches include a significant reduction in the number of experiments required while providing the ability to study interactive effects

between hydrolytic parameters. Predictive models linking hydrolysis parameters to bioactivity can be developed using DOEs. These models can subsequently be used to build RSM profiles and allow determination of the optimum parameters for the generation of protein hydrolysates having enhanced bioactive potency (Abedin et al., 2015; Naik et al., 2013). In general, a good agreement between the predicted and the experimentally determined bioactivity has been reported.

To date, most DOE-based studies have evaluated the bioactivity of the whole hydrolysate. Therefore, the contribution of its individual constituents is unknown. An interesting study has described the application of DOE (using as variables the enzyme preparation (Corolase PP and Peptidase 433P) and hydrolysis duration) and RSM for optimisation of the release of a bovine casein-derived ACE inhibitory peptide (His-Leu-Pro-Leu-Pro) during enzymatic hydrolysis (Quirós et al., 2012). In this study, it was reported that within the experimental design, a higher amount of peptide would be released from caseinate using Corolase PP for an hydrolysis duration of 24 h.

5. Developing strategies for the identification of peptides in complex mixtures with a focus on short peptides

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

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

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

The selective isolation of short peptides has been employed on several occasions to separate them from other materials which are present in the sample and which may hinder their detection (i.e., larger peptides, proteins and non proteinaceous materials). Membrane separation such as ultrafiltration (Eisele, Stressler, Kranz, & Fischer, 2012; Pampanin et al., 2012; Schlichtherle-Cerny, Affolter, & Cerny, 2003) and nanofiltration (Le Maux, Nongonierma, & FitzGerald, 2015a) or adsorbent-based fractionation (Gu, Li, Liu, Yi, & Cai, 2011a; Le Maux et al., 2015a; Sánchez-Rivera et al., 2014a) may be used to specifically separate short peptides from complex samples prior to analysis.

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

Other improvements which may be employed during LC separations consist in the combination of several separation modes, e.g., reverse-phase (RP), hydrophilic interaction liquid chromatography (HILIC) and CE (Harscoat-Schiavo et al., 2012; Le Maux, Nongonierma, Murray, Kelly, & FitzGerald, 2015b; van Platerink, Janssen, & Haverkamp, 2008). As separation of peptides using these different analytical tools is based on their physicochemical properties (i.e., hydrophobicity, hydrophilicity and molecular mass), the information gained during these different analytical methods can be combined to increase the confidence in peptide identification. This may be particularly relevant with isobaric peptides or peptides with similar hydrophobicity or hydrophilicity. It may therefore be important to optimise the LC separative method in order to avoid the potential for co-eluting peptides and facilitate their subsequent MS detection. Modifications (i.e., HILIC, RP, CE separation modes) of front end separation methodologies have been described in several instances to improve the analytical separation of short peptides, which generally involve the utilisation of relatively long duration LC-gradients (Lahrichi et al., 2013; Le Maux et al., 2015a).

During MS detection, different optimisation steps have been described to analyse short peptides. These may be classified in two groups including alterations which are performed pre or post MS analysis. Before MS analysis, different analyser settings may be altered. One common means is the selective monitoring of ions from known peptide sequences. Using LC-MS/MS, Morifuji, Koga, Kawanaka, and Higuchi (2009) reported the identification of seven branched-chain amino acid (BCAA)-containing dipeptides (Ile-Val, Leu-Val, Val-Leu, Ile-Ile, Leu-Ile, Ile-Leu and Leu-Leu) within a whey protein hydrolysate. This was achieved employing a multiple reaction monitoring (MRM) approach based on the detection of specific fragment (i.e., diagnostic) ions arising from these dipeptides. This methodology also allowed quantification of the seven peptides (with amounts ranging between 0.03 and 3.69 mg for Ile-Ile and Ile-Leu, respectively) within a whey protein hydrolysate. Similarly, several hydroxyproline (Hyp) containing di- and tripeptides were quantified in plasma of humans following the consumption of fish scale gelatin hydrolysates (Ichikawa et al.,

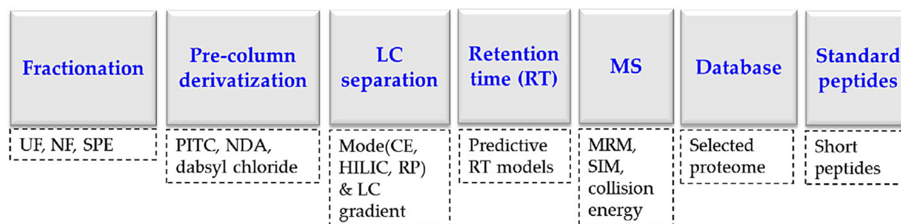

Fig. 2. Summary of the strategies employed to improve the detection and identification of short peptides within complex mixtures. CE: capillary electrophoresis; HILIC: hydrophilic interaction liquid chromatography; LC: liquid chromatography; MRM: multiple reaction monitoring; MS: mass spectrometry; NDA: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

2010; Shigemura et al., 2011; Taga, Kusubata, Ogawa-Goto, & Hattori, 2014) or porcine skin gelatin hydrolysates (Sugihara et al., 2012) using an MRM approach. In another study, the use of peptide retention time during LC separation, MRM and variation in the collision energy has been described for the analysis of the 117 di- and tripeptides representing all possible combinations of Val, Leu and Ile (Lahrichi et al., 2013). Based on the fragmentation pattern of short peptides, this approach allowed the ability to distinguish isobaric peptides containing BCAAs (Leu and Ile), which are generally very difficult to identify due to their identical mass and similar structure (Kaiser et al., 2016). Other studies based on the monitoring of specific fragments using selected ion monitoring (SIM) and pseudoselected reaction monitoring (SRM) (Eisele et al., 2012; Stressler et al., 2013; Sánchez-Rivera et al., 2014a) have proved successful in allowing short peptide identification in complex mixtures (Table 2). Ion monitoring approaches have yielded quite promising results. However, they necessitate previous knowledge of the target peptides in order to determine the specific fragments of interest to be monitored. Alterations of the collision energy and transfer time have also proved beneficial for the fragmentation and subsequent detection of short peptides within milk protein hydrolysates (Chen et al., 2015; Kaiser et al., 2016; Lahrichi et al., 2013; Morifuji et al., 2009; O'Keeffe & FitzGerald, 2015).

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

Metabolites present the same challenges as short peptides in terms of the difficulties associated with their MS identification. Therefore, MS analysers developed for metabolites detection may be employed for the study of short peptides. Studies describing the successful identification of short peptides using ion mobility spectrometry are found in the literature. Ion mobility spectrometry allows separation of peptides based on both their m/z and mobility (drift time) in the MS analyser. Therefore, ion mobility spectrometry is suited for the detection of isobaric peptides or peptides presenting similar masses. The use of ion mobility spectrometry in the discrimination of isobaric dipeptides (Gly-Ala/Ala-Gly and Gly-Ser/Ser-Gly (Blagojevic, Chramow, Schneider, Covey, & Bohme, 2011)), tripeptides (Tyr-Gly-Trp, Trp-Gly-Tyr and Tyr-Trp-Gly (Pollard et al., 2011)) or isotopomer dipeptides (Kaszycki, Bowman, & Shvartsburg, 2016) has been reported. While it may facilitate the detection of short peptides within complex mixtures, applications of ion mobility spectrometry to the study of BAPs appear to be rare.

The identification of peptides within complex mixtures may be facilitated by various *in silico* approaches. Peptidomic methodologies which are currently used for studying BAPs have been recently reviewed (Dallas et al., 2015; Iwaniak et al., 2015). *In silico* methods are very useful for the study of short peptide sequences. As the quality of peptide fragments may not always be sufficient to identify the amino acid sequence, different *in silico* tools have been employed. MS data analysis is based on different algorithms, software and databases which allow processing of large amounts of data in an automated and comprehensive manner. These software may be provided with the MS instrument or by independent suppliers. The use of BAP databases is also very helpful in the identification of peptides with previously reported bioactivities, truncated/precursor peptide sequences or sequences presenting features of BAPs (Iwaniak et al., 2015; Nongonierma & FitzGerald, 2016c). In addition to automated searches, peptide retention time models during LC separation have allowed the identification of short peptide sequences within complex mixtures (Harscoat-Schiavo et al., 2012; Le Maux et al., 2015a; Le Maux et al., 2015b). A limited number of studies have also described how computational methods, such as QSAR, could be used to predict the bioactivity of peptides identified in complex samples and achieve a more targeted identification of BAP sequences identified by LC-MS (Majumder & Wu, 2009; Sagardia, Iloro, Elortza, & Bald, 2013).

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

6. Understanding interactive effects between BAPs as well as matrix effects

As already mentioned, a large number of peptide sequences may be found within food protein hydrolysates. These peptides may interact to decrease or increase the bioactive properties of the hydrolysate. For this reason it is important to consider the potential for interactive effects between peptides. While scientists have often highlighted the possibility that peptides in a mixture might

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

Sample	Peptides identified[a]	Workflow[b]	Bioactivity of selected sequences	Reference
Human plasma collected after oral intake of a sardine muscle hydrolysate	Val-Tyr	• RP-HPLC fractionation • NDA-derivatisation • fluorescence detection (excitation and emission wavelengths 420 and 490 nm, respectively)	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)	• unbound fraction on a C18 column (gluten hydrolysate) and 3 kDa permeate (cheese water soluble extract) • separation on an HILIC column • analysis of MS/MS fragments	n.d.	(Schlichtherle-Cerny et al., 2003)
Milk protein hydrolysate	71 compounds (amino acids and di- to tetra peptides)	• 2D LC separation (C18 and HILIC columns) • accurate mass • retention time • MS/MS fragmentation • synthetic peptides	antihypertensive	(van Platerink et al., 2008)
Whey protein hydrolysate	Ile-Val, Leu-Val, Val-Leu, Ile-Ile, Leu-Ile, Ile-Leu and Leu-Leu	• MRM of 7 target peptides • synthetic peptides	glucose uptake in myotubes and isolated skeletal muscles	(Morifuji et al., 2009)
Commercial fish scale gelatin hydrolysate	Hyp containing di- and tripeptides	• MRM of 9 target peptides • synthetic peptides		
Atlantic salmon skin collagen hydrolysate	11 di- to pentapeptides	• C18 fractionation • LC-MS/MS identification	antihypertensive	(Gu et al., 2011a)
Commercial fish scale gelatin hydrolysate	Pro-Hyp and Hyp-Gly	• SEC fractionation • PITC derivatisation • MRM • Synthetic peptides	fibroblast growth	(Shigemura et al., 2011)
Commercial porcine skin gelatin hydrolysate	Pro-Hyp and Hyp-Gly	• SEC fractionation • PITC derivatisation • MRM • Synthetic peptides	fibroblast growth	(Sugihara et al., 2012)
Atlantic herring by-products (skin and left over material after filleting)	66 di- to hexapeptides	• centrifugation and ultrafiltration (10 kDa) • nanoflow LC-MS Orbitrap • in-house database	numerous	(Pampanin et al., 2012)
Commercial and experimental *L. helveticus* ATCC fermented milks	Phe-Pro, Val-Pro-Pro, Ile-Pro-Pro and Leu-Pro-Pro	• ultrafiltration (10 kDa) • pre-column derivatisation with dabsyl chloride • SIM	antihypertensive	(Eisele et al., 2012)
Synthetic peptides in a mixture and rapeseed protein hydrolysate	numerous	• retention time prediction (HILIC, C18 and CE columns) • all possible amino acid combinations corresponding to the detected mass. • peptide occurrence in rapeseed proteome	n.d.	(Harscoat-Schiavo et al., 2012)
β-casein incubated with a cell free extract of *L. helveticus*	12 di- (Xaa-Pro) and tripeptides (Xaa-Pro-Pro)	• ultrafiltration (10 kDa) • pre-column derivatisation with dabsyl chloride • SIM during LC-MS • external standards	antihypertensive	(Stressler et al., 2013)
Synthetic peptides in a mixture and spiked in a whey protein hydrolysate	117 di- and tripeptides (all possible combinations of Val, Leu and Ile)	• MRM of 117 target peptides • retention time on C18 column • modification of the collision energy	n.d.	(Lahrichi et al., 2013)
Gly-Sar and Val-Tyr in rat intestinal sections	Gly-Sar and Val-Tyr	• MALDI-IMS • phytic acid matrix additive	antihypertensive (Val-Tyr)	(Hong et al., 2013)
His-Leu-Pro-Leu-Pro (β-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	• SPE concentration • SRM • retention time • fragmentation profile	antihypertensive	(Sánchez-Rivera et al., 2014a)
Enzymatic hydrolysates of *Laminaria japonica*	8 Tyr containing di- to pentapeptides	• MRM • modification of collision energy • synthetic peptides	antihypertensive	(Chen et al., 2015)
Synthetic peptides in a mixture and whey protein hydrolysate	numerous	• SPE fractionation • accurate mass • all possible amino acid combinations corresponding to the detected mass. • peptide occurrence in milk proteome and *de novo* searches • retention time prediction (HILIC column) • analysis of MS/MS fragments	n.d.	(Le Maux et al., 2015a)
Whey protein hydrolysate	numerous	• nanofiltration (300 Da) fractionation • accurate mass • all possible amino acid combinations corresponding to the detected mass.	DPP-IV inhibition	(Le Maux et al., 2015a)

(*continued on next page*)

Table 2 (*continued*)

Sample	Peptides identified[a]	Workflow[b]	Bioactivity of selected sequences	Reference
		• peptide occurrence in milk proteome and *de novo* searches • retention time prediction (HILIC and C18 columns) • analysis of MS/MS fragments		
Whey and casein hydrolysates	numerous	• modification of collision energy • modification of transfer time • analysis of MS/MS fragments	n.d.	(O'Keeffe & FitzGerald, 2015)
Human plasma (fed with Ile-Trp and Trp-Leu)	Ile-Trp and Trp-Leu	• isotopic labelling • MRM • modification of collision energy • synthetic peptides	antihypertensive	(Kaiser et al., 2016)

[a] Peptides identified by their three letter code.
[b] CE: capillary electrophoresis; C18: octodecyl 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.

interact, modifying the bioactive properties, very few studies have actually demonstrated which mechanisms of action were involved.

An isobole approach has been described, showing *in vitro* additive or antagonistic effects between binary mixtures of DPP-IV inhibitory peptides (Nongonierma & FitzGerald, 2015c). The identification of peptide sequences which are able to interact in a synergistic manner to increase the bioactive properties of hydrolysates is of interest. Peptide mixtures with synergistic effects may allow the formulation of more potent samples. With this in mind, mixtures of other components (foods, lipid-based carriers or drugs) with dietary hydrolysates have shown promising results in increasing the overall bioactive properties both *in vitro* and *in vivo*. The concomitant administration of the ACE inhibitory lactotripeptides (LTPs - Ile-Pro-Pro, Leu-Pro-Pro and Val-Pro-Pro) with foods (different meals having various protein, carbohydrate, fat and fiber contents) have resulted in increased bioavailability in pigs. This has been attributed to the potential modification of the kinetics of digestion and gut metabolism in the presence of foods (Ten Have, van der Pijl, Kies, & Deutz, 2015).

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

7. Determining the mechanism of action of BAPs

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

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

A number of limitations of QSAR when applied to the study of BAPs have been reported in the literature. These comprise incorporation of biological activity data obtained using different experimental conditions as well as the lack of discrimination of peptides which may act through different modes of action. In this context, it has been suggested that the mode of inhibition of ACE should be taken into account in structure activity relationship studies in order to develop more meaningful models (Jao, Huang, & Hsu, 2012). Recently, it was shown that statistically significant structure activity relationship models could only be obtained by the inclusion of competitive DPP-IV inhibitors and IC_{50} values obtained under the same experimental conditions (Nongonierma & FitzGerald, 2016c).

Molecular docking has been used to virtually screen peptide sequences with ACE (Norris, Casey, FitzGerald, Shields, & Mooney, 2012; Pan, Cao, Guo, & Zhao, 2012; Pripp, 2007), DPP-IV (Nongonierma, Mooney, Shields, & FitzGerald, 2013; Nongonierma, Mooney, Shields, & FitzGerald, 2014) and xanthine oxidase (XO) (Nongonierma et al., 2013) inhibitory properties. Molecular docking has also been employed to better understand specific binding of peptides to enzymes such as ACE (Asoodeh et al., 2014; He, Aluko, & Ju, 2014; Li et al., 2014; Norris et al., 2012; Pan

et al., 2012; Pina & Roque, 2009; Zhang et al., 2015), renin (He et al., 2014) and DPP-IV (Velarde-Salcedo et al., 2013). While providing interesting results and allowing, in certain cases, the identification of enzyme cofactors (e.g., Zn^{2+} for ACE inhibition) (Pina & Roque, 2009) or novel BAPs (Norris et al., 2012), some limitations of molecular docking have been highlighted. Several molecular docking studies assume peptide binding to the active site of metabolic enzymes (competitive inhibition). However, this has not always been confirmed following their *in vitro* evaluation (Nongonierma et al., 2014). Therefore, it has been suggested that the mode of inhibition of the peptides should be taken into account in conjunction with molecular docking analyses. The main difficulty of this approach is that the mode of interaction of peptides with metabolic enzymes is generally unknown. However, there are a growing number of studies which have evaluated the mode of inhibition of metabolic enzymes by peptides using *in vitro* protocols based on the Lineweaver and Burke approach. The different modes of ACE inhibition (competitive, non-competitive and uncompetitive) with dietary peptides have been reviewed by Jao et al. (2012). Determination of the mode of inhibition of other metabolic enzymes appears to have been studied to a lesser extent. However, a few examples of peptide mode of inhibition have been reported for DPP-IV (for review, see: Lacroix & Li-Chan, 2016), XO (Nongonierma & FitzGerald, 2012b; Nongonierma et al., 2013) and cathepsin B (EC 3.4.22.1) (Lee & Lee, 2000). In general, there is a requirement for more data to be generated to understand how these BAPs may interact with the enzymes and subsequently inhibit or activate them. Understanding the mode of interaction between enzymes and peptides is of central importance in order to determine if these BAPs may act through the same mechanism of action in comparison to specific pharmaceutical drugs (Pina & Roque, 2009) or whether they may interact with drugs to further inhibit enzyme activity (Nongonierma & FitzGerald, 2015c).

8. Demonstrating effects of BAPs in humans

Current knowledge on the *in vivo* bioavailability of BAPs is limited (for reviews, see: Boutrou et al., 2015; Nongonierma & FitzGerald, 2015b, 2016b). However, the number of nutritional intervention studies reporting on the occurrence of peptides in human fluids or tissues is increasing (Nongonierma & FitzGerald, 2015a). Examples of studies which have detected peptides in humans following the ingestion of peptides, food protein hydrolysates or intact proteins are listed in Table 3. Several peptides with previously reported *in vitro* ACE inhibitory, opioid (Boutrou et al., 2013), mineral binding (Meisel et al., 2003) and antithrombic (Chabance et al., 1995, 1998) activities have been identified in the gastrointestinal contents of humans. Peptides previously identified in the gastrointestinal tract of humans have been analysed for their potential to act as inhibitors of DPP-IV *in vitro*. This has allowed the discovery of two relatively potent DPP-IV inhibitors, Leu-Pro-Val-Pro-Gln and Ile-Pro-Met having IC_{50} values of 43.8 and 69.5 μM, respectively (Nongonierma & FitzGerald, 2016c).

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

To date, it appears that only a limited number of studies have quantified peptide levels in humans and in certain instances determined their bioavailability (Table 3). The quantification of BAPs in human samples have been described using calibrated ELISA methods (Chabance et al., 1995, 1998; Meisel et al., 2003) or synthetic peptides in the format of internal or external (generally isotopically labelled) standards (Foltz et al., 2007; Kaiser et al., 2016; Taga et al., 2014). Caseinomacropetide (CMP) was quantified (16 and 21 μg mL^{-1}) in the plasma of newborns after ingestion of human milk and bovine milk-based infant formula (Chabance et al., 1995). Lower levels (0.5–2 and 1.05–10 μg mL^{-1}) were reported in adults following milk and yogurt ingestion (Chabance et al., 1998). Differences between adults and infants were attributed to the maturity and permeability of their gut and to the feeding pattern (Chabance et al., 1998). Concentrations of CPPs between 0.2 and 7.10 nmol mL^{-1} were reported in the ileostomy fluid of humans following ingestion of CPPs or milk, respectively (Meisel et al., 2003). Concentrations of 17 and 900 μM for the opioid peptide β-casomorphin-7 (β-CN (f60-66)) and the ACE inhibitory peptide β-CN (f108-113) were reported in the jejunum of humans who had ingested bovine milk (Boutrou et al., 2013).

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

Lunasin, an anticancer peptide, was detected in the plasma of humans following ingestion of soy proteins administered in the format of a soy chilli meal (Dia, Torres, De Lumen, Erdman, & De Mejia, 2009). Concentrations of 50.2–110.6 and 33.5–122.7 ng mL^{-1} were detected in plasma 30 and 60 min following feeding, respectively. It was estimated that these levels would correspond to an absorption of 4.5% of the amount of lunasin reaching the small intestine. In another study, it was hypothesised that Bowman-Birk inhibitor (BBI), a serine protease (8 kDa) with anticancer properties, may be bioavailable as some of its metabolites (23.6 and 21.6 μg detected 6 h post ingestion at 36 h apart, respectively) were detected by ELISA in the urine of humans following soy milk intake (Wan, Lu, Anderson, Ware, & Kennedy, 2000).

Cell proliferative collagen/gelatin-derived peptide bioavailability in humans has been investigated. Various levels of Hyp containing di- and tripeptides (Table 3) were reported in the plasma of humans following the ingestion of fish scale or porcine skin gelatin hydrolysates (Ichikawa et al., 2010; Shigemura et al., 2011; Sugihara et al., 2012). While many of these studies have allowed the quantification of a limited number of Hyp-containing peptides, a method employing the utilisation of stable isotope Hyp-containing peptides has recently been described (Taga et al., 2014). This method has allowed the simultaneous accurate quantification of free Hyp and 13 Hyp-containing peptides in plasma of humans who had ingested a fish scale gelatin hydrolysate. It was

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

Feed	Protein source	Peptide(s)	Location	BAP maximal concentration (C_{max})	Bioavailability (%)	Bioactivity	Reference
Human milk	κ-casein	CMP (f106-169)	plasma of newborns	16 μg mL^{-1}	n.d.	antithrombic	(Chabance et al., 1995)
Bovine milk-based infant formula	κ-casein	CMP (f106-169)	plasma of newborns	22 μg mL^{-1}	n.d.	antithrombic	
Bovine milk	κ-casein	CMP (f106-169)	plasma of adults	0.5–2 μg mL^{-1}	n.d.	antithrombic	(Chabance et al., 1998)
Bovine yogurt	κ-casein	CMP (f106-169)	plasma of adults	1.05–10 μg mL^{-1}	n.d.	antithrombic	
CPP preparations or bovine milk	milk	n.d.	ileostomy fluid of adults	0.02 and 7.10 nmol mg^{-1}	1.8% (120 mg CPP/250 g milk)	mineral binding	(Meisel et al., 2003)
Lactotripeptide 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^{-1} (Ile-Pro-Pro) 152 ± 85 pmol L^{-1} (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^{-1} (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^{-1}	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^{-1}	n.d.	fibroblast growth	(Shigemura et al., 2011)
Porcine skin gelatin hydrolysate	porcine gelatin	Hyp-Gly	plasma of adults	4.2 nmol mL^{-1}	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^{-1}	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^{-1} (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	

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

also shown in this study that the level of free and total Hyp detected were significantly enhanced (>1.6 times more) when using the stable isotope internal standard method.

Overall, the bioavailability of BAPs in humans is generally very low (Table 3). Low bioavailability has been explained by the fact that peptides may be prone to degradation by peptidases/proteinases in the gastrointestinal tract and in the serum while low peptide permeation at the intestinal level has also been described (for reviews, see: Picariello, Mamone, Nitride, Addeo, & Ferranti, 2013b; Sánchez-Rivera et al., 2014b). Different strategies may be employed to increase bioavailability of peptides. More particularly, encapsulation of peptides (for review, see: Mohan, Rajendran, He, Bazinet, & Udenigwe, 2015) may increase their stability to digestive enzymes (Giroux, Robitaille, & Britten, 2016; Li, Paulson, & Gill, 2015). In addition, permeation enhancers may help to increase peptide absorption in the ileum (for reviews, see: Gleeson, Ryan, & Brayden, 2016; Niu, Conejos-Sánchez, Griffin, O'Driscoll, & Alonso, 2016). Lipid-based intestinal permeation enhancers (e.g., sodium caprate and the sodium salt of 10-undecylenic acid) were shown to increase the permeation of ACE inhibitory peptides (Ile-Pro-Pro and Leu-Lys-Pro) in Ussing chambers containing rat jejunum and colon (Gleeson, Heade, Ryan, & Brayden, 2015).

The low bioavailability of peptides raises the question of how much of the peptide(s) should be ingested to cause an *in vivo* effect. Utilisation of dose-response approaches to evaluate BAPs in humans may allow assessment of their *in vivo* potency. A limited number of human studies have employed a dose-response approach to understand the level of hydrolysates/BAPs required to observe a bioactive effect *in vivo*. Several human intervention studies conducted with the ACE inhibitory LTPs (Ile-Pro-Pro and Val-Pro-Pro) have demonstrated a dose-response relationship between their ingestion and SBP reduction. This was the case with a casein hydrolysate, obtained on casein hydrolysis with an *Aspergillus oryzae* protease, administered to subjects with mild and high hypertension at dosages of 1.8, 2.5 and 3.6 mg LTPs for 6 weeks. A reduction in SBP of 5.8, 6.2 and 9.3 mmHg, respectively, was obtained with the three treatments (Mizuno et al., 2005). Similarly, de Leeuw, Van der Zander, Kroon, Rennenberg, and Koning (2009) have demonstrated a significant reduction ($p < 0.05$) in SBP in a dose-dependent manner following LTPs intake (2.3, 4.56 and 9.30 mg/200 g yogurt drink formulated with a casein hydrolysate containing the LTPs) by mildly hypertensive subjects. A decrease in arterial stiffness was reported in hypertensive subjects who consumed a *Lactobacillus helveticus* fermented milk drink containing 5 or 50 mg/day LTPs for 12 weeks (Jauhiainen et al., 2010). Different doses of a proprietary casein-derived hydrolysate (InsuVida™) with antidiabetic properties have been evaluated in humans (for review, see: Nongonierma & FitzGerald, 2015b). In the

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

While several studies have identified peptides with *in vitro* bioactive effects, understanding of the relevance of these results to *in vivo* behaviour of peptides is not straightforward. This is mainly due to major differences in terms of their *in vivo* bioavailability, stability and transfer kinetics (Foltz et al., 2010). In order to develop more meaningful *in vitro* evaluation of peptides, it has been proposed to reverse the strategy of BAP discovery starting from *in vivo* knowledge to inform the release of peptides during *in vitro* hydrolysis. This may, in turn, improve the correlation between *in vitro* bioassays and *in vivo* results (Foltz et al., 2010; Nongonierma & FitzGerald, 2016b).

9. Conclusions

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

Conflicts of interest

The authors declare that they have no conflict of interest.

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