Challenges in the quantitation of naturally generated bioactive peptides in dry-cured meats

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Abstract

Background

The final characteristics of dry-cured meats depend on many factors but one of the most important is the intense proteolysis occurred in muscle proteins due to the action of endogenous enzymes in ham, and also microbial peptidases in the case of dry-fermented meats, that not only affects taste and flavour but also the generation of bioactive peptides.

Scope and approach

In this review main difficulties in the identification of bioactive peptides in dry-cured meats have been described. This study highlights the novel strategies used during the last years to identify naturally generated peptides, and emphasises the need of robust quantitative methodologies for the adequate characterisation of their bioavailability. In fact, the most common and well established quantitation approaches using proteomics are not adapted for peptidomics analysis, so alternative strategies need to be considered.

Key findings and conclusions

The progress in the identification and characterisation of the activity of natural bioactive peptides is highly dependent on modern instruments and bioinformatics tools as well as updated protein databases. In fact, the use of *in silico* approaches and proteomics can be complementary tools in the identification of peptides from meat protein sources as the empirical experimental design can be simplified by using bioinformatics for computer simulation in most of the steps. Finally, Multiple Reaction Monitoring mass spectrometry methodology previously used in the quantitation of therapeutic peptides and biomarkers arises as a powerful tool for absolute quantitation or semi-quantitation of bioactive peptides.

Keywords: peptidomics, quantitation, small peptides, naturally generated peptides, bioactive peptides, dry-cured meat, dry-cured ham, dry-fermented sausages

Highlights

- Dry-cured meat described as a source of naturally-generated bioactive peptides.
- Antioxidant, antihypertensive and antimicrobial peptides identified in dry-cured ham.
- Quantitation is still a challenge due to complex matrix, small size and low abundance of peptides.
- Modifications in multiple reaction monitoring approaches as an alternative to peptides quantitation.

1. Introduction

Bioactive peptides have been of great interest in last decades from the point of view of different disciplines such as pharmaceutical, clinical, functional foods or nutriomics. In fact, synthetic bioactive peptides are being used in therapeutic applications contributing to the treatment of cardiovascular, gastrointestinal, immunosuppression, diabetic, osteoporotic, obesity, antibacterial or oncologic diseases (Fosgerau & Hoffmann, 2015). However, despite the convenience of synthesising/modifying peptides to convert them in more therapeutical and cost-effective compounds, synthetic peptides have resulted to cause numerous and severe side-effects. In this sense, new tendencies in the discovery of drug candidates followed during the last years involve the study of natural bioactive peptides as their accumulation in organs such like kidney and liver is very low, resulting in a minimal development of the most severe toxic side-effects.

Pork raw meat has been previously identified as a source of ACE inhibitory peptides when digested under controlled conditions of hydrolysis using eight different proteases (Arihara, Nakashima, Mukai, Ishikawa, & Itoh, 2001; Katayama et al., 2003) as well as after simulated *in vitro* gastrointestinal digestion (Escudero, Sentandreu, Arihara, & Toldrá, 2010; Escudero, Toldrá, Sentandreu, Nishimura, & Arihara, 2012). The generation of certain bioactive peptides from dry-cured meat products depends on the activity of endogenous muscular enzymes, which is very affected by differences in the type of muscle and genetics of raw material as well as processing conditions including added ingredients and time of curing (Mora, Calvo, Escudero & Toldrá, 2016). Recently, peptide extracts of Spanish Teruel, Italian Parma and Belgian dry-cured ham have been evaluated for their ACE-inhibitory and antioxidant activity, showing differential peptide sequences according to the type of ham (Mora, Escudero, & Toldrá, 2016). Despite the observed differences in the peptides profile, the bioactive potential of the tested fractions was very similar in the three types of ham although a significant increase in ACE-inhibitory activity of Spanish Teruel dry-cured ham was observed, probably due to the longer time of curing of its process. **Table 1** shows the sequences of bioactive peptides identified in dry-cured meat products ham and their IC_{50} values.

However, the study of these peptides with the aim to be included as a part of a functional food also involves the challenge of reaching the blood stream and/or organ of interest to exert its biological activity, especially considering the difficulties due to the low availability of the peptides when orally administered. This fact occurs as a result of its inactivation by saliva, gastric acid, and proteases participating in the gastrointestinal (GI) digestion as well as the difficulty of crossing intact the intestinal barrier which very frequently ends in the degradation of the bioactive peptides by the action of transepithelial transport cells or blood peptidases (Gallego, Grootaert, Mora, Aristoy, Van Camp, & Toldrá, 2016).

Several databases compiling the sequences of bioactive peptides described in the literature are frequently used and result very useful in the identification by comparison of similarities of most active peptides in bottom-up approaches (Carrasco-Castilla, Hernández-Álvarez, Jiménez-Martínez, Gutiérrez-López & Dávila-Ortiz, 2012). However, there is a lack of information about the concentration of these bioactive peptides in the generated food as well as the amount of peptides bioavailable in the blood stream after GI digestion. In fact, the quantitation of bioactive peptides faces up to several difficulties due to their small molecular size, that is between metabolomics and proteomics disciplines, and the complexity of food matrices. Thus, the use of advanced mass spectrometry techniques is critical to identify the sequence of these small peptides and to develop fast, precise and sensitive methodologies for their quantitation from difficult matrices such as dry-cured meat products.

2. Dry-cured meat as a source of naturally generated peptides

The food-derived peptidome comprises an immense source of peptides showing an unlimited combination of residues with high bioactive potential. Most of these peptides form part of a parent protein, being inactive in that form and needing to be released in order to be transported into the blood stream and exert their activity.

Food peptides can be naturally generated and be present in food products that are consumed fresh or raw. In this sense, specific food processes including curing or fermentation like cheese, wine, dry-cured meats, etc., have been widely described as a source of bioactive peptides (Corrêa et al., 2014; Mohanty, Mohapatra, Misra, & Sahu, 2016). Other key mechanisms to obtain bioactive peptides are GI digestion due to the action of salivary, stomachal, intestinal and pancreatic enzymes (Capriotti et al., 2015; Pepe et al., 2016) and the most widely extended *in vitro* digestion using controlled and commercial peptidases or microorganisms mainly used for revalorisation of food by-products (Ryder, Bekhit, McConnell, & Carne, 2016), as described in **Figure 1**.

Dry-cured meats are elaborated through a salting process followed by ripening/drying, which give to these products their characteristic organoleptic properties as well as good stability at room temperature. There are 2 major groups of dry-cured meats: Those based on whole muscles (i.e. dry-cured ham), or those using grinded meat that is cased (i.e. salami-type sausages, chorizo, etc).

Dry-curing of ham is a long process that can take from several months up to a few years. It occurs in chambers and following the stages of salting, post-salting, and ripening/curing. The water activity of the product decreases during its processing and different biochemical phenomena take place. Proteolysis is considered the most important mechanism responsible for the degradation of muscle proteins and for main changes observed in texture and flavour of the final product (Toldrá, 2012). In this sense, endopeptidases, essentially cathepsins and calpains, are able to cleave myofibrillar proteins, affecting the texture and also giving rise to large polypeptides which are later degraded by exopeptidases, such as aminopeptidases or carboxipeptidases among others, into small peptides and free amino acids (Toldrá & Flores, 1998; Toldrá, 2006).

On the other hand, dry-fermented sausages are elaborated using shorter processes with microorganisms such as lactic acid bacteria (LAB), responsible of fermentation followed by ripening/drying. The proteolytic system of LAB comprises a cell wall-bound proteinase, peptide transporters, and various intracellular peptidases, including endopeptidases, aminopeptidases, tripeptidases and dipeptidases (Christensen, Dudley, Pederson, & Steele, 1999), which has been proved to mainly influence the last period of curing and also contributing to the generation of small peptides and free amino acids (Toldrá & Flores, 2011).

Previous studies have described the role of peptidases in the generation of peptides in dry-cured meat products (Toldrá, 2012) and the intense protein degradation that occurrs (Di Luccia, et al., 2005). Moreover, proteolysis in dry-fermented products has been studied and the degradation of main myofibrillar and sarcoplasmic proteins has been described using SDS-PAGE analysis (Hughes et al., 2002; López, Bru, Vignolo, & Fadda, 2015; Chen, Kong, Han, Liu, & Xu, 2016). The generated peptides have been less studied in dry-fermented meat products. In this sense, dry-fermented sausages have been recently characterised using a peptidomic approach that reported the intense proteolysis occurred during its processing due to the action of peptidases from muscle and LAB added in the starter (Mora, Escudero, Aristoy & Toldrá, 2015; Mora et al., 2015). In this study, the influence of added sodium caseinate on the amount of generated peptides at the end of the curing has been described.

However, the specific generated sequences from myofibrillar and sarcoplasmic proteins have only been described more recently in dry-cured ham (Mora, Gallego, Aristoy, Fraser, & Toldrá, 2015) due to the need of modern mass spectrometry (MS) techniques in the analysis of naturally generated peptides. Thus, peptides from the myofibrillar proteins titin (Gallego, Mora, Aristoy & Toldrá, 2015), myosin light chain (Mora, Sentandreu, & Toldrá, 2011), troponin T (Mora, Sentandreu, & Toldrá, 2010), LIM domain-binding protein 3 (Gallego, Mora, Fraser, Aristoy, & Toldrá, 2014), and actin (Sentandreu et al., 2007), as well as sarcoplasmic proteins creatine kinase (Mora, Sentandreu, Fraser, Toldrá, & Bramley, 2009), glycolytic enzymes (Mora, Valero, Del Pino, Sentandreu, & Toldrá, 2011), and myoglobin (Mora & Toldrá, 2012) have been described using peptidomic strategies based on MS/MS analysis.

Thus, thousands of peptides with sizes ranging from 3 to 30 amino acids length have been identified in dry-cured meats, proving the capacity of these food products as a source of naturally generated peptides that could potentially exert bioactive capacity due to their properties.

3. Difficulties in the analysis of naturally generated bioactive peptides

First approaches in proteomics were developed and optimised for the identification of protein biomarkers in pharmacology and medicine applications. The most frequently used strategy includes a first step of SDS-PAGE separation, where proteins were isolated according to their isoelectric point and molecular mass (Bantscheff, Schirle, Sweetman, Rick & Kuster, 2007).

In proteomics there are two main strategies depending on the use of single MS or MS in tandem. Thus, after separation, proteins of interest are selected and in-gel digested with specific proteases like trypsin, which specifically cleaves the protein on the C-terminal side of the basic amino acids Arg and Lys. The peptides obtained were analysed by MS, achieving a list of peaks named peptide mass fingerprint. This experimental mass profile is matched against the theoretical masses obtained from the *in silico* digestion of all protein sequences in the database. The search engine ranks the identified proteins according to a score number that is calculated from the number of fragment masses matching the theoretical peptide masses contained in the protein databases. On the other hand, the use of modern MS techniques with analysers in tandem allows a faster and more reliable identification of a protein using very small number of peptide fragments (Aebersold & Mann, 2003). The most used search engines are Mascot (http://www.matrixscience.com/search_form_select.html), SEQUEST (http://fields.scripps.edu/sequest/), Phenyx (http://www.ionsource.com/functional_reviews/Phenyx/phenyx-web.htm), MassWiz (Yadav, Kumar, & Dash, 2011), Hydra (Lewis et al., 2012), and the free softwares OMSSA (https://www.ncbi.nlm.nih.gov/Web/Newsltr/V14N2/) and X!Tandem (http://www.thegpm.org/tandem/). A scheme of main analytical strategies used in mass spectrometry is shown in **Figure 2**.

Bioactive peptides are small sequences usually between 2 and 20 amino acids that can belong, especially the shortest, to hundreds of protein sequences. The identification of bioactive peptides relies on the detection of a very small molecule showing low abundance and hidden behind the complex matrix of food. This fact is a challenge as the most common bottom-up proteomics strategies followed in the identification of protein biomarkers from trypsinated peptides cannot be used. In fact, differences in the hydrolysis of naturally generated peptides during dry-curing process in comparison with controlled enzyme proteolysis impacts on the identification of peptides (Panchaud, Affolter &

Kussmann, 2012). It is mainly due to the wide combination of sizes of the generated peptides and complex hydrolysis that results in a mixture of peptides with unspecific cleavage sites and broader physical and chemical properties showing a wide variety of charges (from 1+ to 6+) when ionising using the very common electrospray ionisation (ESI) in mass spectrometric approaches. Then, there is a need of specific strategies and procedures as well as adapted methodologies and bioinformatics tools for the management of peptidomics data as compared to proteomics.

4. Novel strategies in the identification of naturally generated bioactive peptides

Bioactive peptides present in food matrices are currently identified following strategies very different in cost, time-consuming and effectivity that very frequently require the use of modern bioinformatics tools and processes to analyse data. Traditionally, bioactive peptides have been empirically identified starting with the selection of a protein and its subsequent hydrolysis with proteases. The obtained pull of peptides is fractionated and purified for the identification of the generated sequences by MS in tandem. Then, peptides were synthesised and tested *in vitro* for their bioactivities and later deposited in open access databases such as BIOPEP (Minkiewicz, Dziuba, Iwaniak, Dziuba, & Darewicz, 2008), PEPBANK (Shtatland, Guettler, Kossodo, Pivovarov, & Weissleder, 2007), or ERP-Moscow (Zamyatnin, Borchikov, Vladimirov ,& Voronina, 2006; http://erop.inbi.ras.ru/). The information contained in these databases together with the sometimes limited bibliographic description of the characteristics of peptides exerting certain activities provide data about which of the identified sequences by MS/MS could be good potential candidates to exert the studied bioactivity (Pihlanto-Leppälä, 2001). In this way, ACE-inhibitory peptides have been described to contain Pro, Lys or aromatic residues preferably in any of the three positions closest to the C-terminal site, whereas the antioxidant activity of peptides has been described to be closely related to their molecular mass, amino acid composition, sequence length and hydrophobicity (Rajapakse, Mendis, Jung, Je, & Kim, 2005; Escudero, Mora, Fraser, Aristoy, & Toldrá, 2013). However, the screening of bioactive peptides from novel substrates using the empirical approach is expensive and time-consuming because it involves using previous reported studies to select proteases that demonstrate the highest potential to liberate the bioactive peptides, and later assay the *in vitro* activity of the generated peptides.

The experimental design can be simplified by using bioinformatics for computer simulation in most of the steps. Thus, the selection of the protein could be done by determining the occurrence frequency of bioactive sequences in the protein which results very useful as bioactive peptide sequences comply with certain requisites according to the type of activity. Certain tools such as PATTINPROT from PBIL server (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_pattinprot.html) scan a protein sequences or database searching for one or several patterns previously established (Combet, Blanchet, Geourjon, & Deléage, 2000). Then, different tools were used to obtain the theoretical potential prediction of several known protein sequences to generate bioactive peptides, using enzymes with known cleavage specificities. The hydrolysis of the selected protein is simulated to generate *in silico* peptide profiles using bioinformatics tools such as BIOPEP, where it is possible to simulate the digestion with up to three different enzymes simultaneously; PeptideMass from ExPASy (Wilkins et al., 1997; Gasteiger et al., 2005), very intuitive and extended; or PoPS (Boyd, Garcia de la Banda, Pike, Whisstock, & Rudy, 2005), a tool that allows the prediction of substrate cleavages using protease specificity models that have to be uploaded by the user. Finally, the sequences generated by simulation are matched with the peptides of bioactive databases looking for coincidences. Some authors have reported the optimisation of Quantitative

Structure-Activity Relationship (QSAR) models to predict the most interesting food proteins for the generation of ACE-inhibitory peptides (Wu, Aluko, & Nakai, 2006a,b) from the *in silico* digested profiles obtained using PeptideMass from ExPASy (Gu, Majumder, & Wu, 2011).

But when considering the exclusive use of predictive approaches, many potentially bioactive sequences could be ignored as they are not included in the bioactive peptide databases. To avoid this situation, peptides can be analysed *in silico* to identify desirable amino acids at certain position or interesting characteristics that make them potent candidates to exert bioactivity using the software PeptideRanker, which identify among a set of peptides those that may be more likely to be bioactive by giving a list of scores. This predictive tool is based on such general shared features of bioactive peptides across different functional classes and aid in the improved design of existing bioactive peptides (Mooney, Haslam, Pollastri, & Shields, 2012). On the other hand, the online tool EnzymePredictor (http://bioware.ucd.ie/~enzpred/Enzpred.php) evaluates the evidence for which enzymes are most likely to have cleaved a sample containing peptides from hydrolysed proteins, which would result very useful to drive hydrolysis processes towards the generation of certain specific bioactive peptides (Vijayakumar et al., 2012).

Finally, the prediction of the three-dimensional structure of peptides from their amino acid sequence and post-traductional modifications is very important in peptidomics because the structure of the peptide can affect its functionality. Thus, on-line tools such as PEPstrMOD (http://osddlinux.osdd.net/raghava/pepstrmod/) or PEP-FOLD (http://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD/) for peptides with length range between 7-25 (Kaur, Garg, & Raghava, 2007) and 9-36 residues (Thévenet et al., 2012), respectively, allow the prediction of multiple *de novo* peptide structures for linear and cyclic peptides.

One of the most studied bioactivity in foods is the ACE-inhibitory activity due to the relation of this enzyme with the regulation of blood pressure and its importance as a controllable risk factor for cardiovascular diseases. In this sense, BIOPEP database (http://www.uwm.edu.pl/biochemia/) contains a classification of main bioactivities described in the literature, including a total of 3285 sequences of bioactive peptides mainly showing ACE-inhibitory, antioxidant and antibacterial activities in a percentage distribution of 21.6, 16.1 and 14.1%, respectively (Minkiewicz et al., 2008), as indicated in **Figure 3**.

Several studies of Spanish dry-cured ham have described ACE-inhibitory and antioxidant activity in certain of the naturally generated peptides determined empirically using consecutive fractionation steps to isolate the most active fractions and, thus, identify the peptides by MS in tandem. Some of the identified peptides were selected as potential bioactive based on their length, amino acid composition, and amino acid location in the sequence (Pihlanto-Leppälä, 2001). **Figure 4** shows the peptides profile obtained after MALDI-ToF mass spectrometry analysis of an aqueous extract of dry-cured ham. Regarding the bioactive potential of meat products, a peptide extract from Iberian dry-cured ham showed a significant decrease in systolic blood pressure (SBP) of 12 mmHg after 8h of ingestion in spontaneously hypertensive rats (SHR). The analysis resulted in the identification of 2632 sequences of peptides containing the previously described ACE-inhibitory fragments PPK, PAP, and AAP repeated a total of 322, 302, and 119 times, respectively (Mora, Escudero, Arihara, & Toldrá, 2015).

Additionally, the most active fractions and peptides identified in Spanish dry-cured ham have also been tested *in vivo* for their antihypertensive activity using the SHR model. Fractions from size-exclusion chromatography corresponding to molecular masses lower than 1700 Da showed the most intense antihypertensive activity with a decrease in systolic blood pressure (SBP) of 38.38 mmHg in spontaneous hypertensive rats after 8 hours of ingestion (Escudero, Aristoy, Nishimura, Arihara & Toldrá, 2012), whereas peptide AAATP with an *in vitro* IC_{50} value of 100 μM showed a decrease in the SBP of 25.62 mmHg after 8h administration in SHR (Escudero, Mora, Fraser, Aristoy, Arihara & Toldrá, 2013). Also fractions obtained after GI digestion of Parma dry-cured ham of 18 and 24 months of curing were analysed to identify ACE-inhibitory peptides using a combination of an *in silico* model and the traditional *in vitro* approach, resulting in the identification of several small peptides such as LGL and SFVTT with IC50 values of 145 and 395 μM, respectively (Dellafiora, Paolella, Dall'Asta, Dossena, Cozzini, & Galaverna, 2015). On the other hand, the influence of starter culture and protease addition on the bioactive capacity of dry-fermented sausages has also been recently evaluated (Fernández et al.,2016). In this study, dry-fermented sausages were prepared using *Pediococcus acidilactici* MS200 and *Staphylococcus vitulus* RS34 as starter culture and were also inoculated with the protease Erg222. Results showed an increase in the ACE-inhibitory and antioxidant activities in those batches with the protease after 63 days of ripening, showing a very good stability after simulated *in vitro* GI digestion.

Dry-cured meats have also been studied as a natural source of antioxidant peptides. In fact, apart from antioxidant peptides generated during processing, dry-cured hams have been described to contain antioxidants that are naturally present in meat as, for example, some free amino acids, dipeptides carnosine and anserine, ubiquinone, or alpha-tocopherol among others (Marušić, Aristoy, & Toldrá, 2013). In this sense, Jinhua ham and Xuanwei ham from China have been described to show antioxidant activity resulting in the identification of some natural peptide sequences. The sequence GKFNV was presented as the main peptide playing a key role as a scavenger of free radicals in a Jinhua ham extract (Zhu et al., 2013), whereas the tetrapeptide DLEE was identified in Xuanwei ham as one of the key peptides responsible for the observed antioxidant activity, showing a DPPH radical scavenging activity of 74.45% at a concentration of 0.5 mg/mL (Xing, et al., 2016). In a recent study, different antioxidant peptides from Jinhua ham were isolated and identified such as the sequences DLEE, GKFNV, and LPGGGHGDL which were tested using DPPH radical scavenging and hydroxyl radical scavenging assays (Zhu, Zhang, Zhou, & Xu, 2016). In this sense, Escudero, Mora, Fraser, Aristoy and Toldrá (2013) reported that the water soluble extract of Spanish dry-cured ham contained a large amount of potentially antioxidant peptides. This fact was confirmed with the identification of the peptide SNAAC with an IC_{50} value of 75.2 μM in DPPH radical-scavenging assay and 205 μM in ferric-reducing antioxidant power assays (Mora, Escudero, Fraser, Aristoy, & Toldrá, 2014).

On the other hand, the presence of antimicrobial peptides in dry-meats increases the value of these products that offer the advantage of improving their safety when additional treatments such as slicing or packaging are used. In fact, the development of pathogen microorganisms such as *Listeria monocytogenes* is an important concern due to its resistance to drying and high salt content. Recently, a novel antilisterial peptide with sequence RHGYM was identified in Spanish dry-cured ham of 10 months of curing showing a MIC value of 6.25 mM (Castellano et al., 2016).

6. Current interest, requirements and main challenges in quantitative peptidomics

A better knowledge about the concentration of certain bioactive peptides identified in food products is crucial in future studies of bioavailability as well as to reach more realistic conclusions about the effect that could be expected for an active peptide in the organism. In fact, the effect and impact of a bioactive peptide in an organic system depends on the amount that is ingested and its ability to reach reach the blood stream or the organ of interest. Thus, the quantitation data of identified bioactive peptides is a necessary aim for a better understanding of the effects and mechanisms that involve the action of these compounds.

There are numerous methodologies for the study of protein levels, which can be summarised in two main approaches: the use of labelling techniques and the use of label-free techniques (Bantscheff, Schirle, Sweetman, Rick & Kuster, 2007). In peptidomics, label-free methodologies involve studies based on the comparison of peptide amounts between two or more samples. It can be performed by spectral counting or by extracting peptide intensities. Spectral counting is based on counting the total number of times a peptide is selected for fragmentation and spectra is identified in an MS/MS experiment. The peak intensity-based approach correlates the extracted ion chromatogram area with the concentration of that particular peptide by comparison between samples. However, the abundance estimation in a comparative analysis is subjected to variations because the ionisation of peptides that depends on their sequence, the presence of predominant peptides can suppress bioactive peptides signals, and the search engine algorithm and settings could limit the detection/identification in the data analysis step (Zapata & Wick, 2012). Despite this fact, there are a number of advantages as it does not require extra sample preparation, can be performed with very low amounts of sample, is less time-consuming and has a lower cost. This methodology has been used in food for the relative quantitation of protein abundances between two or more sets of samples. Regarding bioactive peptides, a spectral counting label-free approach has been recently described to characterise differences in the peptide profile of raw and pasteurised ovine milk cheese and to relate it with the bioactive potential (Pisanu et al., 2015). Moreover, label-free quantitation has been used to determine differences in the peptide profile of Spanish Teruel, Italian Parma and Belgian dry-cured hams and its potential bioactivity (Mora et al., 2016).

On the other hand, a semiquantitative methodology has been also described with oligopeptides from Grana Padano cheese, using the extracted ion chromatograms (XICs) obtained from the total ion chromatogram (TIC) and by comparison with the XIC of the internal standard Phe-Phe, showing the result as a ratio value (Sforza, Ferroni, Galaverna, Dossena, & Marchelli, 2003). A similar approach has also been described to study the cocoa oligopeptide fraction after fermentation (Marseglia et al., 2014).

Labelling techniques are considered the most accurate method available for quantitation. However, these approaches require expensive labelling reagents, high amounts of sample and a long and tedious preparation. Most common stable isotope labelling techniques comprise the modification of the peptides with isobaric tags (iTRAQ or Tandem Mass Tags) and the labelling of proteins with isotope tags (ICAT or SILAC) (Rauh, 2012). However, one of the challenges using labelling techniques in naturally generated peptides is the lack of Cys residues in most of endogenous peptides as thiol groups of Cys are necessary to react with the reagents that form isotopic labels. Complementarily, nearly all endogenous peptides have either a free N-terminus or a Lys residue to label free amine groups so result optimal for this type of labelling. However, if free amine is converted into a neutral or negative residue and the peptide does not contain Arg or His residue, it cannot be positively ionised and detected in the mass spectrometer. The Multiple Reaction Monitoring (MRM) is currently the most used methodology in the quantitation of bioactive peptides. It has been used during decades for the quantitation of therapeutic peptides and biomarker candidates in plasma, serum and urine (Rauh, 2012) and nowadays has started to be introduced in the area of food science. The MRM is usually performed on an ion trap or triple quadrupole mass spectrometer where the ion of interest is selected with the first mass analyser Q1 and induced to fragment by collision-activated dissociation in the collision cell Q2. The resulting ions are uniquely derived from the peptide of interest and are analysed using the third quadrupole Q3 (Picotti & Aebersold, 2015). When multiple target fragment ions resulting from multiple precursor ions are monitored, the overall process is termed multiple reaction monitoring. The development of the assay for the quantitation of bioactive peptides is based on the selection of several transitions per peptide through the optimisation of the MS parameters, the calculation of its concentration range and linear response, and the synthesis of heavy isotope labeled analogues to be used as internal standards. The absolute quantitation using MRM has been applied to bioactive ACE-inhibitory tripeptides extracted from rye malt sourdoughs, showing the highest concentrations those gluten sourdoughs fermented with *Lactobacillus reuteri* TMW 1.106 and added protease (Hu et al., 2011), as well as during the bread-making process (Zhao, Hu, Schieber, & Gänzle, 2013). This methodology has also been used to determine the intact absorption of the ACE-inhibitory dipeptide Val-Tyr into the blood of SHR after administration of 30 mg/kg rat, and detecting a maximal absorption amount of 1.1 ng/mL plasma (Nakashima et al., 2011). Longer peptides such as the bioactive peptides beta-casomorphin 5 and beta-casomorphin 7 were also quantified using a MRM approach in yoghurt by labelling the peptides with deuterium (Nguyen, Solah, Johnson, Charrois, & Busetti, 2014). Despite some studies reflect the increase in data of bioactive peptides quantitation, there is still an important challenge to overcome when analysing complex matrices where the peptides have been generated with no control on the enzymatic action. In this sense, main difficulty is in the optimisation of MRM parameters to perform a sensitive and accurate quantitation without the interferences of other peptides or signal suppression.

7. Key prospects in the quantitation of dry-cured meat bioactive peptides

One of main challenges in current status of peptidomics is to improve the understanding of naturally generated bioactive peptides generation and their interactions in order to reach more accurate conclusions about their bioavailability and effect in the organism. Despite the discovery and identification of bioactive peptides in food matrices has experienced an important development together with the advance of analytical techniques, data analysis software, and computational prediction, there is still a challenge in the quantitation of bioactive peptides from complex matrices such as dry-cured meat. Conversely, most bioinformatics tools are focused on the analysis of proteins that have been hydrolysed under controlled enzymatic conditions and there is a need for the development of more adequate tools for data processing in peptidomics. In this respect, bioactive peptides databases such as the previously described BIOPEP, PEPBANK, or eBASIS count on a wide report of bioactive peptides identified in plant or animal-based food, very well categorised according to their activity, origin, and IC_{50} value, but only a few of them have been quantified. Current mass spectrometry analysers such as triple quadrupoles and ion trap (QQQ and Q/Trap) together with new analytical strategies in peptidomics are contributing to advance in the quantitation of bioactive peptides generated during food processing such as fermentation, being the MRM, based on the labelling of the corresponding heavy ions, the current method of choice for their quantitation.

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LEGENDS FOR THE FIGURES

Figure 1. Scheme of main sources of food-derived peptides that could exert biological activity when liberated from the protein of origin.

Figure 2. Proteomics vs peptidomics. Main differences are in the generation and identification of peptides. Different approaches are needed when objectives are the identification of protein biomarkers and the identification of protein-derived bioactive peptides.

Figure 3. Activity profile of main bioactive peptides included in the free access BIOPEP database.

Figure 4. MALDI-ToF spectra of the peptide extract of Spanish dry-fermented sausages. A) Values from 200 to 900 *m/z* [M-H+] and B) values from 850 to 3500 *m/z* [M-H+]. Reproduced from *Mora, Escudero, Aristoy and Toldrá (2015). A peptidomic approach to*

study the contribution of added casein proteins to the peptides profile in Spanish dry-fermented sausages. International Journal of Food Microbiology, 212, 41-48 with permission from Elsevier.

Figure 5. MALDI ToF analysis of water soluble extract of Iberian dry-cured ham. A) from 150 to 800 *m/z* [M-H+] and B) from 800 to 1800 *m/z* [M-H+]. Reproduced from *Mora, Escudero, Arihara, and Toldrá (2015). Antihypertensive effect of peptides naturally generated during Iberian dry-cured ham processing. Food Research International, 78, 71–78* with permission from Elsevier.

Table 1. Sequences of bioactive peptides identified from dry-cured meat products.

Sequence	Activity	IC_{50} (µM)	MIC (mM)	Protein of origin	Dry-cured meat product	References
PAPPK	ACE-inhibitory	199.58	-	Myosin light chain	Spanish dry-cured ham	Escudero et al. (2014)
KAAAAP	"	19.79	-	Myosin light chain	"	"
AMNPP	"	304.50	-	Myosin	"	"
IKLPP	"	193.90	-	Myosin	"	"
AAPLAP	"	14.38	-	Myosin	"	"
KPVAAP	"	12.37	-	Myosin	"	"
VPPAK	"	>1000	-	Titin	"	"
KPGRP	"	67.08	-	Titin	"	"
PSNPP	"	192.27	-	Titin	"	"
IAGRP	"	25.94	-	Titin	"	"
EAPPK	"	>1000	-	Titin	"	"
PAAPPK	"	>1000	-	Titin	"	"
KVLPG	"	265.44	-	Phosphoglicerate kinase	"	"
TGLKP	"	51.57	-	Aspartate aminotransferase	"	"
KAAAATP	"	25.64	-	PR domain zinc finger protein	"	"
GNGGA	"	>1000	-	Carbamoyl-phosphate synthase	"	Escudero et al. (2013)
DVITGA	"	900	-	Myosin light chain	"	"
KDQGSYEDF	"	>1000	-	Ca2+ binding protein	"	"
GVDNPGHPF	"	>1000	-	Creatine kinase	"	"
LNSLT	"	>1000	-	Creatine kinase	"	"
KAEEEYPDL	"	>1000	-	Creatine kinase	"	"
EEYPDL	"	>1000	-	Creatine kinase	"	"
ASGPINFT	"	975	-	Myosin regulatory light chain	"	"
LGL	"	145	-	-	Parma dry-cured ham	Dellafiora et al. (2015)
ALM	"	>1100	-	-	"	"
SFVTT	"	395	-	-	"	"
GVVPL	"	956	-	-	"	"
NSIM	"	>1100	-	-	"	"
AAATP	Antihypertensive	100	-	Allantoicase	Spanish dry-cured ham	Escudero et al. (2013)
SNAAC	Antioxidant	75.2	-	Myosin heavy chain	"	Mora et al. (2013)
AEEEYPDL	"	2 mg/mL	-	Creatine kinase	"	"
GKFNV	"	n.d.	-	*De novo* sequence	Jinhua dry-cured ham	Zhu et al. (2013)
LPGGGHGDL	"	n.d.	-	*De novo* sequence	"	Zhu et al. (2016)
LPGGGT	"	~1 mg/mL	-	*De novo* sequence	"	"
HA	"	~1 mg/mL	-	*De novo* sequence	"	"
KEER	"	n.d.	-	*De novo* sequence	"	"
SAGNPN	"	1.5 mg/mL	-	Zinc finger-X protein	Spanish dry-cured ham	Escudero et al. (2013)
GLAGA	"	1 mg/mL	-	Collagen	"	"
DLEE	"	n.d.	-	*De novo* sequence	Xuanwei ham	Xing et al. (2016)
RHGYM	Antilisterial	-	6.25	Dynein heavy chain	Spanish dry-cured ham	Castellano et al. (2016)

Figure 1

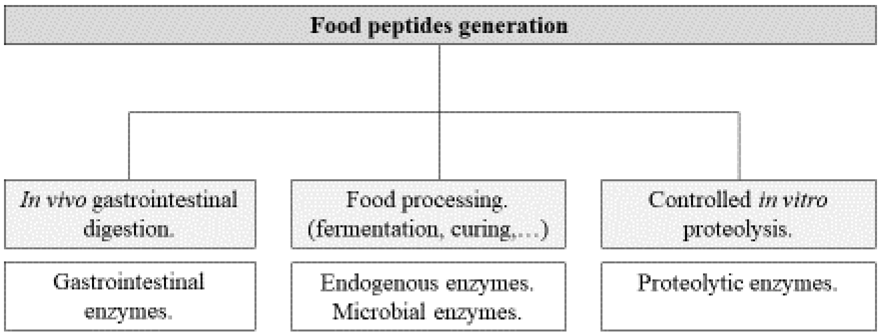
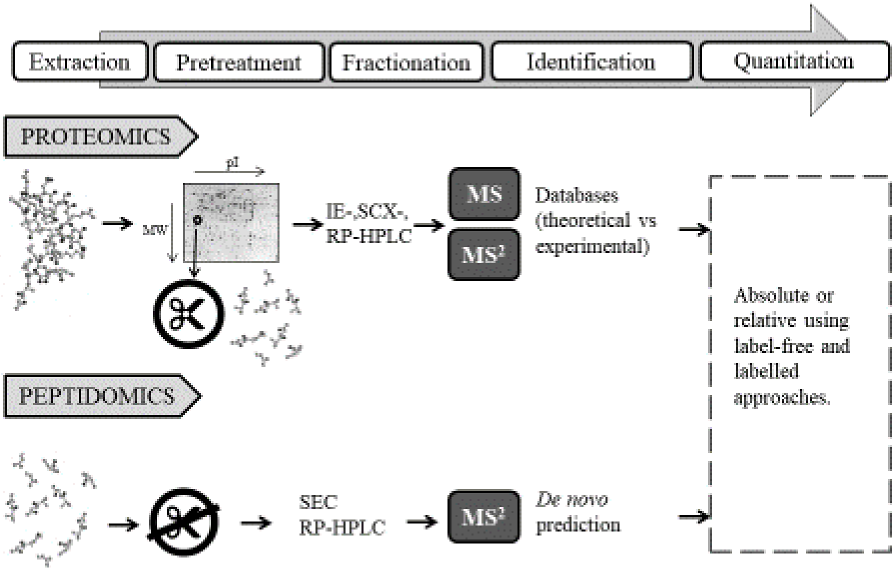

Food peptides generation
In vivo gastrointestinal digestion.
Food processing. (fermentation, curing,…)
Controlled *in vitro* proteolysis.
Gastrointestinal enzymes.
Endogenous enzymes. Microbial enzymes.
Proteolytic enzymes.

Figure 2



Extraction
Pretreatment
Fractionation
Identification
Quantitation
PROTEOMICS
pI
MW
IE-,SCX-,
RP-HPLC
MS
MS²
Databases
(theoretical vs
experimental)
Absolute or
relative using
label-free and
labelled
approaches.
PEPTIDOMICS
SEC
RP-HPLC
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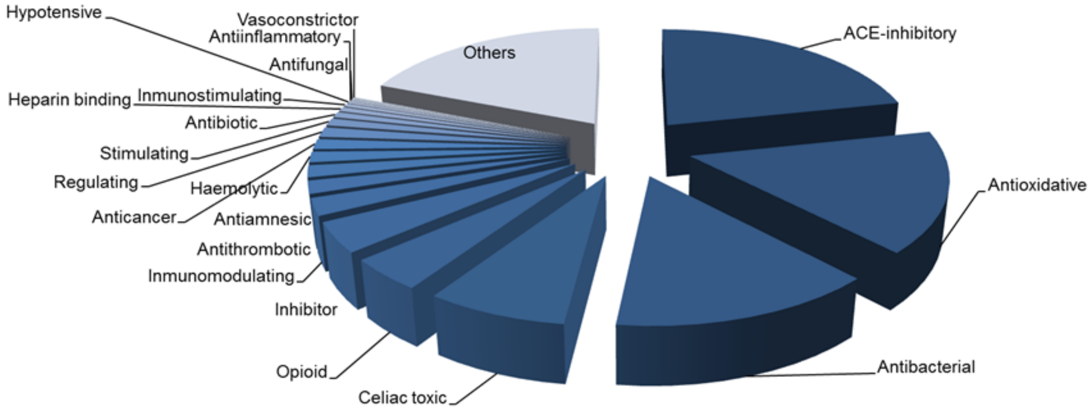
De novo
prediction

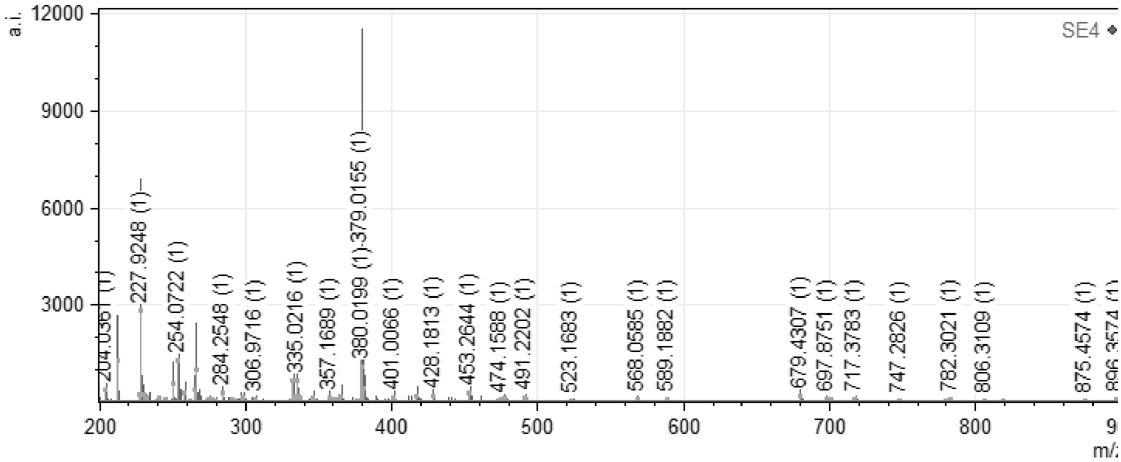
Figure 3



Hypotensive
Vasoconstrictor
Antiinflammatory
Antifungal
Others
ACE-inhibitory
Heparin binding
Inmunostimulating
Antibiotic
Stimulating
Regulating
Haemolytic
Anticancer
Antiamnesic
Antithrombotic
Inmunomodulating
Inhibitor
Opioid
Celiac toxic
Antioxidative
Antibacterial

Figure 4

A)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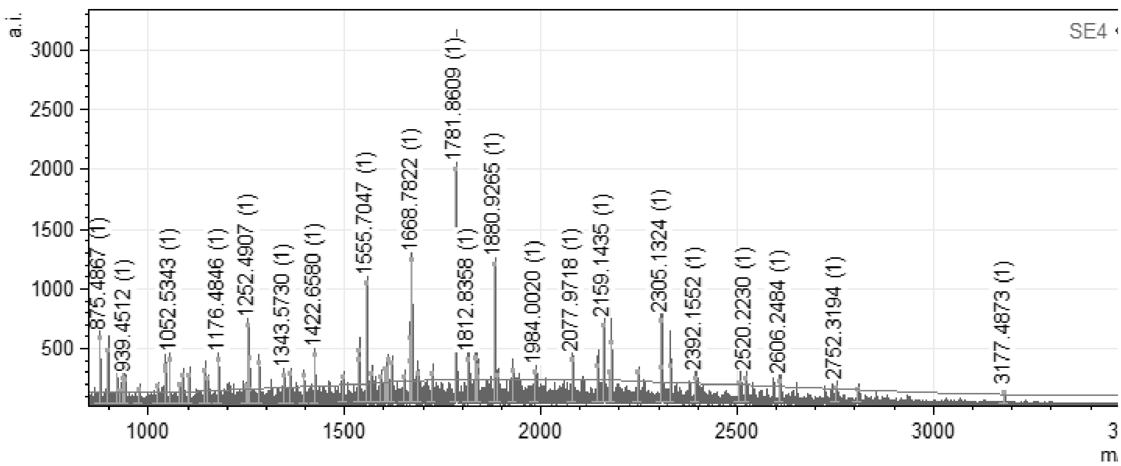
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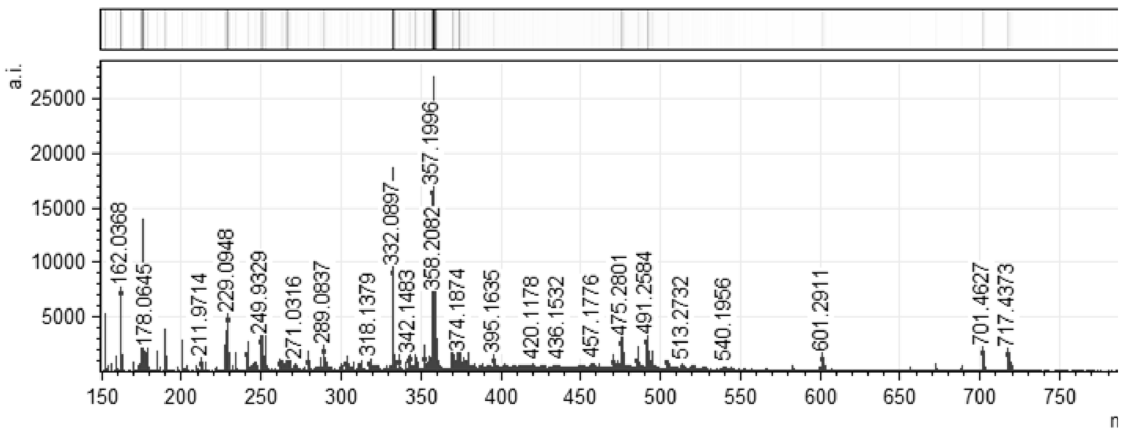
Figure 5

A)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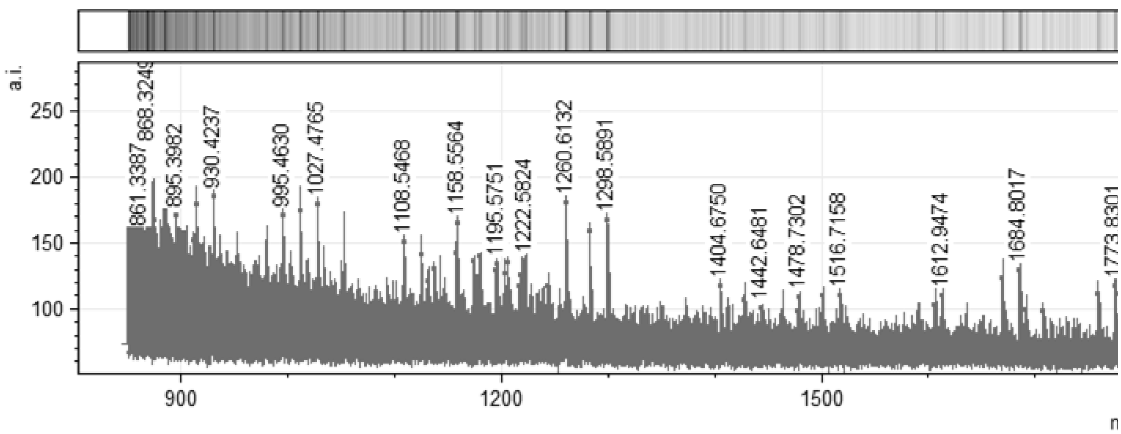
B)


a.i.
250
200
150
100
861.3387
868.3249
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995.4630
1027.4765
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1158.5564
1195.5751
1222.5824
1260.6132
1298.5891
1404.6750
1442.6481
1478.7302
1516.7158
1612.9474
1684.8017
1773.8301
900
1200
1500