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Recent developments in peptidomics for the quali-quantitative analysis of food-derived peptides in human body fluids and tissues

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ABSTRACT

Background: Various bioactive peptides are present in foods and food protein hydrolysates, or are generated in the stomach/intestine of organisms after digestion of dietary proteins. Those resisting gastrointestinal degradation can exert local effects in the gut or systemic effects in the organism body as result of their transport across the intestinal epithelium in the bloodstream, and subsequent adsorption in various organs. For most of these molecules, no concentration data regarding body fluids/tissues are available; this information is essential to rationalize their bioavailability and putative bioactivity.

Scope and approach: The main purpose of this study is to provide an exhaustive overview of the bioactive food-derived peptides identified in the gastrointestinal tract, blood, body tissues, urine, breastmilk and feces of animal models or humans fed specific diets, as well as a description of the adsorption mechanisms and metabolic processes eventually affecting their fate. Untargeted and targeted peptidomic methods used for their quali-quantitative description are also reported, together with recent technological advances that have partially solved various analytical challenges in this research field and have disclosed future promising scenarios in nutrition and physiology.

Key findings and conclusions: Available information emphasizes that organism tissues/body fluids are pervaded of food-derived species resulting from the digestion of dietary proteins, including some already proved having a specific biological activity. For some for which blood concentration was measured, ascertained data highlight levels in the nanomolar range, which are lower than those generally used for *in vitro* functional assays. Conversely, few peptides have shown concentration values compatible with a substantial molecular bioavailability and a putative bioactivity. Thus, it remains uncertain if the presence of bioactive food-derived peptides in the body fluids/tissues can be associated with a significant functional effect. Accordingly, the actual study of these exogenous peptides in the human body is more relevant than ever, with the ultimate aim of tangling the complex relationship between diet and health.

1. Introduction

Various raw food materials contain some bioactive peptides that exert a specific physiological function in tissues and fluids of living organisms (Arena et al., 2020; Arena & Scaloni, 2018; Capriotti et al., 2016; Cerrato et al., 2021; Halim et al., 2016; Lafarga & Hayes, 2014; Nongonierma & FitzGerald, 2015; Yu et al., 2014) (Fig. 1). In parallel, gastrointestinal digestion of dietary proteins generates a huge number of peptides. Some of them are inactive when encrypted in the parent molecules but become bioactive once released by proteolysis in the human gut (Chakrabarti et al., 2018; Mora et al., 2019). The above-mentioned bioactive peptides that escape further gastrointestinal

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degradation can exert local effects within the intestine, through the interaction with specific receptors/cells, or systemic effects in the body, as result of their transport across the intestinal epithelium into the blood stream, and subsequent adsorption in target tissues/organs (Miner-Williams et al., 2014; Matsui, 2018; Xu et *al.*, 2019; Amigo & Hernández-Ledesma, 2020). In these cases, they can affect endocrine, immune, digestion and cardiovascular systems as result of their antihypertensive, anti-inflammatory, antimicrobial, antioxidant, opioid, immunomodulatory, anti-adhesive, antidiabetic, antithrombotic and osteoanabolic activities, contributing to the modulation exerted by the diet on some physiological functions (Shimizu & Hachimura, 2011; Hernández-Ledesma & Hsieh, 2017; Sultan et al., 2018; Fernández-Tomé & Hernández-Ledesma B. 2020). These functional components have attracted increasing interest as nutraceutical and therapeutic agents. They have the potential to be used as food additives or nutraceutical ingredients for preventing, delaying or modulating various human chronic diseases. Thus, various natural peptides are currently under trials or have already reached the market as dietary supplements or health foods (Patil et al., 2022).

Recognition of the health-promoting characteristics for food-derived peptides as well as the increased knowledge on functional properties of traditional fermented products promoted the development of technological processes for the generation of bioactive food protein hydrolysates through the use of proteolytic enzymes or microbial fermentation with starter cultures (Aaslyng et al., 1998; Nongonierma & FitzGerald, 2018; Toldrà et al., 2020; Manzoor et al., 2022) (Fig. 1). These functional protein hydrolysates were investigated at molecular level, and various peptides were isolated and characterized for their composition and bioactive properties (Lafarga & Hayes, 2014; Yu et al., 2014; Nongonierma et *al.*, 2015; Capriotti et al., 2016; Halim et al., 2016). Most of these molecules as well as natural bioactive peptides present in raw food materials have been listed in dedicated databases, among which: i) FeptideDB (Panyayai et al., 2019), which collects data from other bioactive peptide databases; ii) MBPDB (Nielsen et al., 2017); iii) AHTPDB (Kumar et al., 2015); iv) AntiCP (Tyagi et al., 2013) v) AHT_{PIN} (Kumar et al., 2015) vi) Antinflam (Gupta et al., 2017) vii) $CAMP_{R3}$ (Waghu et al., 2016) viii) dPABBs (Sharma et al., 2016); ix) AVPpred (Thakur et al., 2012). Some of these databases also include protein cleavage tools and allow *in silico* prediction of potential functional molecules, taking advantage of support-vector machine supervised learning models working on amino acid composition, sequence and binary profile features. A prediction of peptide bioactivity based on structural features was also obtained with PeptideRanker (Mooney et al., 2012) and PeptideLocator (Mooney et al., 2013) software.

To confirm the causal nexus between food-derived bioactive peptides and human health, dedicated investigations on the absorption of these compounds are needed, which might systematically demonstrate the occurrence of (pro)active molecular form(s) within the intestine, as well as the passage of these species across the intestinal epithelial membrane, and their occurrence in the human blood and body tissues/fluids. In analogy with other omics fields, peptidomics has two major goals: i) the identification of all peptides present within a biological fluid/tissue at a certain time; ii) the quantification (relative or absolute) of all these molecules in the above-mentioned context (Schrader & Schulz-Knappe, 2001). In addition to generate information regarding molecules involved in important pathophysiological processes (Dallas et al., 2015; Maes et al., 2019; Foreman et al., 2021), peptidomic studies were also proved essential for elucidating degradation of ingested food proteins and distribution of resulting fragments within various anatomical body districts. In the last two decades, several studies have contributed to address some challenging issues on dietary protein processing in the body of human or related animal models, and the absorption of resulting peptides. Untargeted investigations were aimed to identify all peptides present in different biological fluids/tissues, to relate them to ingested foods as well as to specific physiological functions. Once untargeted studies were performed and marker candidates were identified, targeted peptidomic investigations provided an accurate quantitation of the compound(s) of interest in various body districts. This review article

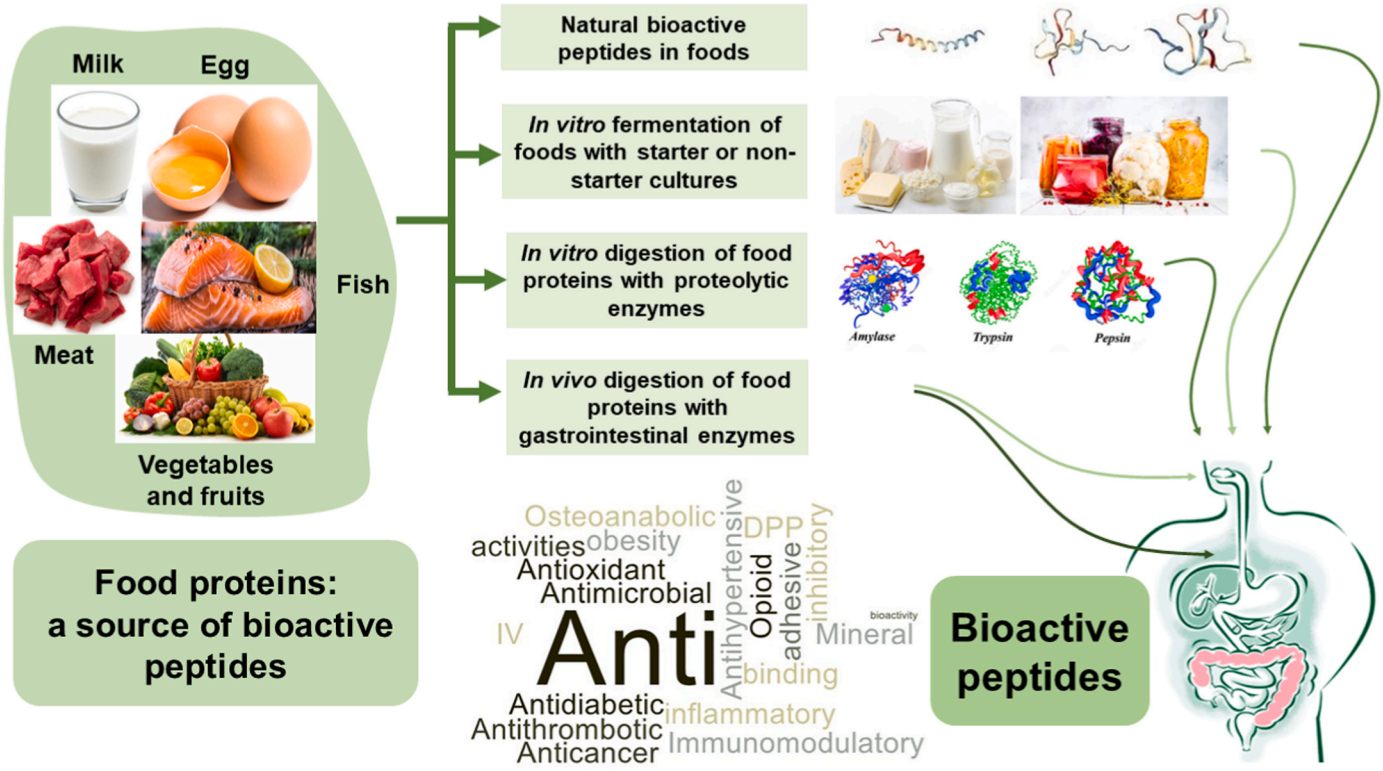

Fig. 1. Schematic overview of the different sources of food-derived bioactive peptides. Information is provided on bioactive peptides deriving from raw food materials, fermented food products, food protein hydrolysates and/or deriving from gastrointestinal digestion of food proteins.

aims to provide a comprehensive picture of relevant findings in this research field, summarizing bioactive peptides already detected in the body of humans or related model organisms following the ingestion of specific foods, as well as recent technological advances in peptidomics that may assist future research in nutritional science.

2. Peptidomics of gastric and intestinal aspirates

Ingested peptides and proteins are partially released from the starchy matrix in saliva and then are digested within the stomach as result of the action of pepsin. The products of pepsinolysis pass into various intestine segments, where they are subjected to the activity of trypsin, chymotrypsin, elastase and various epithelial-associated peptidases (Sultan et al., 2018), which generate a huge number of peptides of various size, among which some that may be bioactive. The surface of the intestinal striated membrane is covered of microvilli contributing with their large surface to host various metabolic activities essential for adsorption of nutrients, including enzyme secretion and presentation to transporter proteins (Woodley, 1994). Dedicated studies have demonstrated that less than 10–15% of total nitrogen present in the human small intestine after protein ingestion occurs in the form of urea, ammonia, or free amino acids, about 20–30% in the form of peptides, and the remaining 50% in the form of proteins (Chacko & Cummings, 1988).

Information on bioactive peptides present within the stomach and the intestine mostly derived from untargeted peptidomic studies, which were performed *in vivo* on animal models or human generally fed with milk or other dairy products. In animal-focused investigations, initial data derived from pilot experiments accomplished on piglets fed milk infant formula, whose content of stomach, proximal and median jejunum, and ileum was collected after the last meal and characterized by nano-liquid chromatography coupled with electrospray tandem mass spectrometry (nanoLC-ESI-MS/MS) (Bouzerzour et al., 2012). In the stomach, caseins (CNs) were proved being hydrolyzed more rapidly than β-lactoglobulin (β-Lg) and α-lactalbumin (α-La). Most of the peptides identified in the animal jejunum and ileum originated from β-CN, among which the antihypertensive TPVVVPPFLQP and opioid/immunomodulatory YPFPGPI components (Supplementary Table 1). Later, Barbè and coworkers investigated milk peptides released in the gastrointestinal tract of piglets during the digestion of 6 dairy matrices (Barbé et al., 2014). Duodenal effluents were collected over a 5 h-period and analyzed by nanoLC-ESI-MS/MS. In the whole, about 16,000 peptides were identified, among which a number having emulsifying, antihypertensive, anti-stress, antimicrobial, transport, antioxidative, opioid agonist, immunomodulating, anti-thrombotic, protease/peptidase-inhibitory and mineral-binding properties (Supplementary Table 1). This study originally suggested that the structure of dairy products has a little influence on the location of cleavage sites in the protein sequences, but strongly impacts on the kinetics of milk protein digestion *in vivo* and the number of identified peptides. Very recently, Miralles and colleagues monitored by peptidomics the digestion of bovine CNs in the porcine upper intestinal tract and matched the outcome with the gastric *in vitro* digestion following the INFOGEST harmonized protocol (Miralles et al., 2021). The peptide profile generated after *in vitro* and *in vivo* digestion showed clear similarities. Various identified α_{S1}, α_{S2}- and β-CN-derived phosphorylated peptides coincided with some already associated with a mineral-binding activity (Supplementary Table 1). Finally, a peptidomic analysis was conducted by Wada and colleagues that identifed in a suckling rat pup model peptides released from human milk proteins and milk infant formula (Wada et al., 2017). Several hundred peptides were detected in the upper and the lower part of the animal intestine, mostly covering specific protein regions resistant to gastrointestinal digestion. Some of the identified peptides coincided with molecules already known having opioid (YPFPGPI), immunomodulatory (SPTIPFFDPQIPK and FPGPIPN), antibacterial (SDISLLDAQSAPLR, VLVLDTDYKK and LLYQEPVLGPVRGPF), antioxidant (VPYPQ and NNPYVPR), prolyl-endopeptidase inhibitory (PLIYPFVEPIP and VYPFPGPI), dipeptidyl-peptidase (DPP) IV inhibitory (ELKPTPEGDLE and SLPQNIPPLTQ), antihypertensive (YPFPGPIPN) and antithrombotic (MAIPPK) activity (Supplementary Table 1). Above-mentioned studies definitively demonstrated the occurrence of various bioactive peptides in the stomach and intestine of animal models after the ingestion of dairy products.

Analogous studies were accomplished on human stomach and duodenum liquid aspirates. Initial investigations were performed by Chabance and colleagues on volunteers fed milk or yogurt (Chabance et al., 1998). Some peptides deriving from α_{S1}-, β- or κ-CN, including the inhibitor of platelet aggregation κ-caseinoglycopeptide, were detected in the stomach using combined LC and Edman degradation approaches (Supplementary Table 2). Smaller peptides derived from various CNs and lactoferrin were similarly identified in the duodenum, among which antioxidant YFYPEL from α_{S1}-CN and antithrombotic MAIPPKKNQDK from κ-CN. Few years later, mass spectrometry-based peptidomics was originally applied to investigate the peptides present in jejunum liquid aspirates from adult subjects fed with ^{15}N-labeled casein or whey proteins (Boutrou et al., 2013), identifying about 356 medium-size peptides and 146 larger peptides, respectively. Detected molecules included opioid β-casomorphins (YPFPGP and YPFPGPI), angiotensin converting enzyme 1 (ACE1)-inhibitory peptides (LNVPGEIVE, NVPGEIVE, DKIHPF, YPFPGPIPN, FVAPFPEVF and YKVPQL), antihypertensive compounds (EMPFPK, LHLPLP and SKVLPVPQ), antithrombotic species (VPNSA and VPQLEIVPNSA), and the immunomodulatory peptide PGPIPN (Supplementary Table 2). The use of ^{15}N-labeled proteins allowed original peptide quantification in jejunal aspirates. According to author's view, the amounts of β-casomorphins and ACE1-inhibitory peptides released in the postprandial window were sufficient to elicit their opioid and antihypertensive action in the jejunum. On the other hand, Dallas and colleagues analyzed by nanoLC-ESI-MS/MS the gastric aspirates of infants fed breast milk (Dallas et al., 2014). Authors demonstrated that about 603 peptides differed significantly in abundance with respect to compounds present in parent milk, among which β-CN-derived SPTIPFFDPQIPK with immunomodulatory activity, casomorphin 8 (YPFVEPIP) with opioid action, QELLLNPTHQIYPVTQPLAPVHNPISV with antibacterial action, DKIHP, VPKAKD, VPKAKDT, KVLPVPQ and VVPYPQR having antihypertensive properties, and YPYY with opioid antagonist activity (Supplementary Table 2). Later, Sanchon and colleagues analyzed with similar technologies human jejunal aspirates after oral ingestion of CN and whey protein powders (Sanchón et al., 2018). Hundreds of peptides were assigned in the different digests based on time-course experiments, also identifying polypeptide domains resistant to digestion. Among assigned peptides, noteworthy were again some compounds having opioid, antihypertensive, mineral-binding, hypocholesterolemic and DPP inhibitory activities (Supplementary Table 2).

More recently, Dallas and coworkers took advantage of the development of high-resolution tribrid mass spectrometers to investigate the peptides released overtime in preterm infant stomach during gastric digestion of proteins from mother's milk alone or added with bovine milk fortifier (Beverly et al., 2019; Nielsen et al., 2018). Authors assigned various thousand peptides in infant gastric samples, among which 92 were identical to known bioactive compounds, and 678 showed a sequence homology $\geq$80% with functional molecules. The number of identified antimicrobial, ACE1-inhibitory, antioxidant and immunomodulatory peptides was greater for bovine milk proteins than for human counterparts (Supplementary Table 2); this finding was tentatively associated with the poor knowledge on functional compounds in the latter. The hypothetical, preferential release of bioactive bovine peptides was suggested to impact on overall health outcomes in premature infants (Nielsen et *al.*, 2018). When the release of human milk peptides in the stomach of preterm and term infants was quantitatively compared, significant protein digestion differences were observed (Beverly et al., 2021). While preterm infants were proved to release more total peptides during gastric digestion, term infants were shown to

release specific bioactive peptides at higher abundance (Supplementary Table 2). This is the case of the antimicrobial peptide QELLLNPTHQIYPVTQPLAPVHNPISV, whose abundance was significantly higher in the gastric samples from term newborns than in preterm counterparts. The same authors used untargeted peptidomics to investigate molecules present in the intestinal tract of breast milk-fed infants (Beverly et al., 2021), focusing on peptides having antimicrobial and bifidogenic activities. They analyzed 29 intestinal samples and assayed the antibacterial activities of the bulk peptide extracts against *Escherichia coli*, *Staphylococcus aureus*, and *Bifidobacterium longum* spp. *infantis*. Peptidomic analysis identified 6645 milk peptides from 223 proteins, which occurred with a calculated mean concentration of 4 ± 2 mg/mL; among that, some were able inhibiting *E. coli* and *S. aureus* growth at concentrations of 2.5 and 3.0 mg/mL, respectively. Identified antimicrobial peptides were: HLPLPLLQPLMQQVPQPI, LLNPTHQIYPVTQPLAPVHNPIS, HQIYPVTQPL, LAPVHNPI, EPIPLESREE, YANPAVVRPHAQIPQR, RPNLHPS, EKFGKDKSPKFQ, DMLVVDPK, MTSALPIIQK and FKDLGEENFK (Supplementary Table 2).

Supplementary Table 2 summarize many peptides with bioactive properties identified in the last decade in the human stomach and different intestine districts after intake of dairy products. Notwithstanding the difference related to the specific organism under investigation, and the variation in both food composition and intake, the intestine sites where sample aspirates were taken as well as the technology used for peptidomic experiments, various bioactive molecules were commonly identified in the gastrointestinal apparatus (Wada & Lönnerdal, 2020). For example, the opioid agonist casomorphin 8 YPFVEPIP, the immunomodulatory compound SPTIPFFDPQIPK, ACE1-inhibitory peptides LENLHLPLP and YANPAVVRP, the antioxidant species WSVPQPK, the antimicrobial molecule QELLLNPTHQIYPVTQPLAPVHNPISV and the opioid antagonist YPYY were commonly detected (Dallas et al., 2014; Wada & Lönnerdal, 2014; Nielsen et al., 2018; Beverly et al., 2019; Beverly, Huston, et al., 2021; Beverly, Woonnimani, et al., 2021).

Studies on milk-derived bioactive peptides were not paralleled with analogous investigations on fish- and meat-derived molecules. Indeed, only Bauchart and colleagues reported the identification of various protein-derived compounds in pig duodenal and jejunal aspirates after animal ingestion of trout flesh or beef meat (Bauchart et al., 2007). Among all the identified peptides, some of them derived from major structural/sarcoplasmic muscle proteins and contained peculiar sequence motifs already associated with antihypertensive activity.

Overall, above-reported studies demonstrate that food-derived bioactive peptides occur in the stomach, jejunum, and ileum of various organisms, and suggest that these molecules can play a functional role within the human gut, such as the regulation of some intestinal barrier functions and epithelial cell activities. In this context, CN-derived, egg yolk-derived and soybean-derived peptides have already been described increasing mineral absorption and exerting an antioxidant/anti-inflammatory action in human gut (Bao & Wu, 2021; Fernández-Tomé & Hernández-Ledesma, 2020). Similarly, different milk-derived peptides and CN hydrolysates were reported regulating intestinal chemical barrier function by improving brush border membrane functionality and increasing local secretion of mucins (Bao & Wu, 2021). On the other hand, various milk-derived (casein macropeptide, casoxin and β-casomorphins), soy β-conglycin-derived (soymorphins) and gluten-derived (exorphins) peptides were shown to exhibit an opioid agonist/antagonist functions, affecting gut motility, mucus production and gastrointestinal transit (Bao & Wu, 2021; Fernández-Tomé & Hernández-Ledesma, 2020; Moughan et al., 2007). Finally, some CN-derived compounds, fish protein hydrolysates, and wheat gluten- or soybean-derived peptides were already described regulating the intestinal physical barrier function by modulating adherent junction formation and intestinal permeability, as well as the gut biological barrier function by modifying microbiota composition (Bao & Wu, 2021). The above-mentioned information complements previous data on the effect of egg white, meat, wheat gluten and CN hydrolysates, as well as of peptides from these foods and from soybean β-conglycinin, bean and potato in stimulating gut secretion of insulinotropic polypeptide, cholecystokinin, glucagon-like peptide 1 and peptide YY (Calbet & Holst, 2004; Chen et al., 2018; Hira et al., 2009; Nakajima et al., 2010, 2012; Pizarroso et al., 2021; Sufian et al., 2006). Thus, dedicated *in-vivo* studies and clinical trials are needed to prove the actual physiological role(s) of the above-mentioned food-derived bioactive peptides in different gastrointestinal districts, disclosing future applications for these molecules in the possible treatment of digestive system diseases (Bao & Wu, 2021; Fernández-Tomé & Hernández-Ledesma, 2020).

3. Transport of food-derived peptides across the intestinal epithelium

Various food-derived peptides have been identified in the blood of model organisms and humans (see subsequent chapter). To reach the blood stream, peptides generated by proteases present in the stomach or in the brush-border membrane of the small intestine must move across the intestinal epithelial cell barrier. The process generally develops along one or more pathways as follows: i) carrier-mediated permeation; ii) paracellular transport via tight junctions (TJs); iii) transcytosis via vesicles; iv) passive transcellular diffusion (Fig. 2). Dedicated review articles have summarized the characteristics of the peptide transport pathways reported so far as well as the optimal substrates for each process (Wu et *al.*, 2019).

In the case of carrier-mediated permeation, the proton-dependent transmembrane protein PepT1 plays a pivotal role in the transport of peptides using a H^+ electrochemical gradient (Newstead, 2017) (Fig. 2). Dedicated experiments with molecules having a different size indicated that the transport capacity of this carrier protein is significant for neutral and hydrophobic di- and tripeptides, but limited for free amino acids and other peptides (Pedretti et al., 2008; Xu et al., 2019). PepT1 was proved effective in transporting various antihypertensive and antioxidant molecules. On the other hand, the paracellular transport pathway is mediated by TJs mostly made of zonula occludens-1, occludin and claudin proteins, which form a tight biological barrier having a pore size of 50–60 Å (in crypt cells) and <6 Å (in villus cells) allowing the selective passage of water and electrolytes through energy-independent passive diffusion (Brunner et al., 2021; Xu et al., 2019) (Fig. 2). This transport route was proved effective in transferring hydrophilic peptides made of 2–9 amino acids, mostly negatively charged, including some food-derived antihypertensive, antioxidant, opioid, anti-inflammatory and connective tissue-regenerating molecules. The transcytotic pathway involves an energy-dependent transcellular transport based on the apical endocytic uptake of external peptides, their intracellular transport through inner lipid vesicles, and final molecular basolateral effusion (Komin et al., 2017) (Fig. 2). This process is considered the main transport route of large-size peptides across the intestinal epithelium (Shen et al., 1992; Regazzo et al., 2010); optimal substrates are hydrophobic 4-amino acids long molecules (Shimizu et al., 1997). Some antihypertensive, antioxidant, immunomodulatory and antimicrobial compounds made of hydrophobic amino acids were described to cross the intestinal brush-border membrane via transcytosis (Xu et al., 2019). Finally, different peptides pass across the intestinal epithelial membrane via passive transcellular diffusion, which generally occurs for most compounds and includes passive molecular uptake into cells, intracellular transport, and basolateral secretion (Pappenheimer & Michel, 2003) (Fig. 2). Molecular size, charged state and hydrophobicity are the physicochemical properties determining effective transport (Liu et al., 2009); in general, only hydrophobic peptides are transported through this pathway. Since no mediators modulate passive transcellular diffusion, quantification of peptide transport via this pathway is difficult to be accomplished.

Quali-quantitative information on the transport of food-derived bioactive peptides across the human intestinal epithelium was

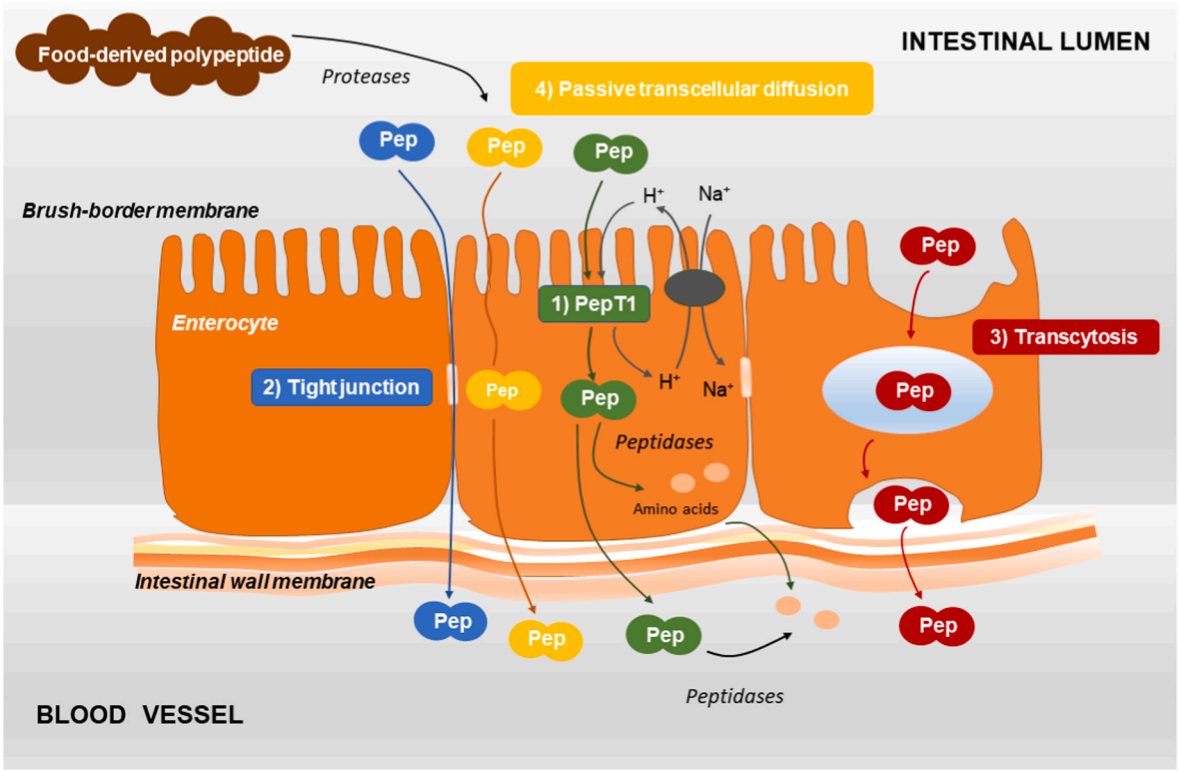

Fig. 2. Alternative and concomitant pathways allowing the transport of food-derived peptides across intestinal epithelial cell monolayers. Food-derived polypeptides resulting from gastrointestinal digestion of ingested proteins are eventually hydrolyzed to smaller components by proteases present at the intestinal brush-border membrane. Resulting peptides can be transported across intestinal epithelial cell monolayers through independent and/or concomitant routes (shown in different color), such as the PepT1 carrier-mediated pathway (green), the paracellular pathway via tight junctions (blue), the vesicle-mediated transcytosis (red), and the passive transcellular diffusion (orange). Adapted from previous reports (Wada & Lönnerdal, 2014; Xu et *al.*, 2019). PepT1 utilizes the gradient of H^+ between the intestinal lumen and epithelial cells. The gradient is maintained through the H^+/Na^+ exchanger that is balanced by the basolateral Na^+/K^+ ATPase (both in grey). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

generally obtained by using Caco-2 cell monolayers grown on permeable supports, which were used as a suitable experimental model due to their dynamic polar surface (Hubatsch et al., 2007; Xu et al., 2019). Notwithstanding the intrinsic limitations associated with the reduced peptide permeability of this model, with respect to that of intestinal epithelium, the measurement of kinetic parameters at the apical and the basolateral side of the cell monolayer allowed discriminating between the different transport routes reported above, and determining the molecular apparent permeability coefficient (Sugano et al., 2010; Xu et al., 2019). In alternative, selective inhibitors were used to exclude some specific absorption routes (Regazzo et al., 2010). Many peptides were demonstrated being transported across Caco-2 cell monolayers via dual routes, with the combined PepT1-paracellular ones or the paracellular-transcytotic ones being the most common. In general, the carrier-mediated transport was the main transport pathway at low peptide concentrations, and the contribution of passive transport became significant when compound concentrations increased. Notwithstanding the successful application of Caco-2 cell monolayers in the study of peptide absorption, these systems do not consider the impact of the mucus on the diffusion of proteins and peptides. To overcome this drawback, some researchers have evaluated the peptide uptake with co-cultures of Caco-2 with mucus-producing cells (Ding et al., 2021) or Ussing chambers with *ex vivo* animal or human intestinal tissues (Ozorio et al., 2020).

4. Peptidomics of food-derived peptides in the blood and other body fluids/tissues

To exert a functional activity in body districts other than the gastrointestinal system, food-derived bioactive peptides must reach the circulation system. Since the poor absorption, distribution, metabolism and excretion properties of peptides result in low peptide bioavailability, the possibility that these molecules can exert systemic activities has been often questioned (Foltz et al., 2010; Miner-Williams et al., 2014). With the aim to investigate the absorption and metabolic fate of these components, various studies have been carried out on animal models or humans; individuals under investigation were subjected to a specific diet and then analyzed overtime for the occurrence of food-derived peptides in the blood and other biological fluids/tissues (Matsui, 2018; Sato, 2018, 2022). Depending on the goal of the study and the peptides under investigation, untargeted and targeted peptidomic approaches were used. In the latter case, peptide maximum observed concentration (C_{max}), elimination half-life ($t_{1/2}$) and bioavailability values were often measured, as similarly done in pharmacological studies on oral administered peptide-based immunomodulating agents (Mika & Stepnowski, 2016), growth hormone precursors (Thevis & Schaenzer, 2014; van den Broek et al., 2015), and mushroom-related toxins (Sun et al., 2018). These parameters allowed evaluating how long a peptide resists to plasmatic degradation and whether this time range allows it permeating eventual target organs.

To evaluate the possible transport in the blood of specific food-derived peptides already known having specific functional properties, preliminary studies were performed in animal models subjected to acute nutritional interventions including the bioactive synthetic compound. This is the case of the antihypertensive peptide VY deriving from various foods, whose distribution in the blood and tissues of spontaneously hypertensive rats (SHR) was investigated by Matsui and colleagues (Matsui et al., 2004). Peptide administration resulted in a prolonged

reduction of systolic blood pressure and a 10-fold increment of peptide levels at 1 h, followed by a rapid concentration decline (Supplementary Table 3). Long-lasting accumulation of VY was observed in kidney, lung, heart, mesenteric artery and abdominal aorta. A significant reduction of tissue ACE activity was measured in the abdominal aorta as well as in the kidney, suggesting these organs as target sites of the antihypertensive action of VY. Blood concentration data on this peptide (C_{max} = 4 nM) were confirmed with a more sophisticated targeted LC-ESI-MS/MS method based on a dedicated multiple reaction monitoring (MRM) procedure and inclusion of the ^{13}C-labeled dipeptide in food (Nakashima et al., 2011) (Supplementary Table 3). Close concentration values of labeled MY and LY peptides in blood were also ascertained after the inclusion of these compounds in the rat diets.

Plasma concentration and kinetics of the β-CN-derived antihypertensive peptide HLPLP were similarly evaluated in rat after oral administration of this compound (Sanchez-Rivera et al., 2014) (Supplementary Table 3). Plasma concentration of HLPLP (C_{max} = 35 nM), and of the two derived fragments LPLP and HLPL were determined by LC-ESI-MS/MS as well. An initial untargeted procedure for peptide identification was followed by a targeted one involving selected reaction monitoring (SRM) experiments. Results suggested a considerable and rapid blood distribution throughout the body of HLPLP and its derivatives, and a significant corresponding peptide uptake into tissues. The oral bioavailability of HLPLP was found to be about 5%. Finally, two independent studies from Deutz's group were performed on piglets to evaluate the absorption, distribution, and elimination the food-derived antihypertensive peptides IPP, LPP and VPP. Quantitative levels of these molecules in blood were measured by using a targeted LC-ESI-MS/MS approach and ^{13}C-labeled standards. In the first case, synthetic peptides were administered with intragastric dosing (van der Pijl et al., 2008); they all reached the blood circulation as intact compounds and showed comparable C_{max} and $t_{1/2}$ values, being in the ranges 9–12 nM and 9–15 min, respectively (Supplementary Table 3). Corresponding peptide bioavailability was about 0.1%. In the second study, a 3-fold higher dose of peptides was orally administered to piglets using different meal types (Ten Have et al., 2015) (Supplementary Table 3). Although no significant differences were observed for C_{max}, $t_{1/2}$ and bioavailability values when peptides were administered in saline buffer, with respect to experiments based on intragastric dosing system, the values of parameters substantially changed when a meal including protein and fiber was orally co-administered. Authors concluded that the co-presence of the latter ingredients increases the systemic absorption of IPP, LPP and VPP peptides. In the whole, the above-reported studies demonstrate that food-derived antihypertensive peptides administered to animals can enter quickly the bloodstream, where they are present for short times at very low (nanomolar range) concentrations, to reach the target organs as intact compounds.

Similar studies based on the administration of synthetic peptides in the diet were performed to evaluate the absorption in the blood of rats of the anti-atherosclerotic molecule WH (Hanh et al., 2017), the hepatoprotective wheat gluten-derived component pyroglutamyl-leucine (<EL) (Sato et al., 2013), the antioxidant compound glutathione (Yamada et al., 2018) and the flaxseed-derived phosphodieterase-inhibitory species AGA and RWIQ (Nwachukwu et al., 2019). Depending on molecules tested in the assays, the optional use of ^{13}C-labeled standard compounds and the inclusion in the analytical procedure of precolumn derivatization of sample extracts paralleled in all cases LC-ESI-MS/MS determinations in MRM mode. Specific peptide oral doses, and corresponding measured C_{max} and $t_{1/2}$ values are reported in Supplementary Table 3; the highest C_{max} value (0.4 μM) was observed for glutathione. In general, all peptides quickly reached the animal bloodstream, where they persisted at nanomolar concentrations for less (WH) or more (<EL, glutathione, AGA and RWIQ) than 1 h after ingestion. In the case of <EL, AGA and RWIQ, corresponding functional effects were also detected in various body districts (Sato et al., 2013; Nwachukwu et al., 2019), while induction of PepT1 expression was measured for WH (Hanh et al., 2017).

Additional studies on food-derived peptides in the rat blood were performed on animals fed proteins or protein hydrolysates. In particular, an untargeted peptidomic approach was used to identify β-conglycinin (βCGC)-derived peptides in blood samples of rat fed this soybean protein (Sheng et al., 2021). Plasma samples taken from the tail vein overtime were analyzed by nanoLC-ESI-MS/MS. Among detected newly formed species, SEL, KGPL, SILGA, DSEL, GDANI, SYFV, CLQSC, GEQPRPF, and LVINEGDA were successfully identified as βCGC-derived peptides present in plasma at 0.75–756 nM concentration values (Supplementary Table 3).

Targeted studies were performed on rats fed food protein hydrolysates. Collagen hydrolysates and peptides have been used as supplementary health foods based on their action as enhancers of fibroblast cell proliferation and hyaluronic acid production, and adjutants in managing wound healing and joint pain disfunctions in knees (Moskowitz, 2000). In this context, Sontakke and coworkers evaluated the absorption of collagen-derived hydroxyproline (O)-containing peptides GPO and PO in the rat blood, after the intake by animals of a collagen hydrolysate (Sontakke et al., 2016). It was demonstrated that food-derived peptides were stable in gastrointestinal fluids, efficiently transported across the intestinal cell monolayers and quickly reached micromolar concentrations in plasma. As far as we know, concentrations measured in the micromolar range were the highest ever reported in animal models (Supplementary Table 3).

The positive results regarding the application of collagen hydrolysates as skin fibroblast promoters as well as the detection of O-containing peptides in the blood of animal models exposed to a collagen-based diet prompted researchers performing similar and more detailed experiments on humans. In this context, significant results were obtained by Sato and coworkers, which deeply investigated the adsorption of food-derived peptides in the blood of volunteers fed different diets based on collagen hydrolysates. These studies at first identified PO, POG, IO, LO, PG, OG, EO and AO dipeptides in blood samples from adult subjects fed with single or multiple doses of collagen hydrolysates, and then determined corresponding quantitative levels (Shigemura et al., 2014; Shigemura et al., 2018; Shigemura et al., 2018). For peptide determinations, an *ad hoc* analytical procedure was developed, which involved compound precolumn derivatization with phenyl isothiocyanate and subsequent LC-ESI-MS/MS quantitative determination in MRM mode, using standard molecules for external calibration (Shigemura et al., 2011). The effect of variable amounts of ingested collagen hydrolysates was also evaluated in terms of measured C_{max}, $t_{1/2}$ and bioavailability values, confirming for these compounds micromolar concentrations in the human blood, an appreciable stability to plasmatic proteases and a persistence for 3–4 h after ingestion (Asai et al., 2019) (Supplementary Table 4). These authors also evaluated the amount of collagen in Japanese daily dishes, determining that the meat in one serving of most plates contains 0.2–2.5 g of this protein (Asai et al., 2019). After ingestion of a single dose of cooked shark meat by human volunteers, 9 collagen-derived peptides were detected in their plasma. The amount found for most peptides, except for OG and POG, was almost 30% of that after ingestion of a collagen hydrolysate containing an equivalent dose of the biopolymer. Likewise, only about 30% of the total collagen in the meat was liberated into solution by pepsin and pancreatin digestion. Thus, the authors concluded that the levels of collagen-derived peptides in the blood depend not only on the protein content of the ingested food but also on its susceptibility to digestive enzymes.

The above-reported peptidomic approach was also applied for investigating peptide absorption after ingestion of gelatin (heat-denatured collagen) and gelatin hydrolysates (Ohara et al., 2007; Ichikawa et *al.*, 2010). In particular, Sato and coworkers identified and quantified PO, AOG, SOG, AO, FO, LO, IO, GPO and POG in the blood specimens of adult human individuals fed with a single dose of collagen hydrolysates from fish scale, fish skin, chicken cartilage and porcine skin. Different

amounts of ingested gelatin hydrolysates were tested to this purpose. Peptide C_{max}, $t_{1/2}$ and bioavailability values were determined (Supplementary Table 4), confirming that analytes occurred at micromolar concentrations and their persistence in the blood was similar to that ascertained for orally ingested intact collagen. In all cases, PO dipeptide was identified as the most abundant food-derived peptide, while AOG and SOG tripeptides were detected only with fish scale gelatin hydrolysates. Authors verified that the total area under the concentration-time curve of the fish scale gelatin hydrolysate was significantly higher than that of the porcine/chicken counterparts. Thus, they claimed that the quantity and structure of O-containing peptides in human blood after oral administration of gelatin hydrolysates depends on the gelatin source. A similar peptidomic approach was also used to evaluate PG dipeptide in human blood of healthy volunteers fed with an elastin hydrolysate (Shigemura et al., 2012). This compound was the major food elastin-derived peptide detected in this biological fluid, which reached a C_{max} value of 18 μM at 30 min after ingestion and decreased to approximately 20% 4 h after food administration (Supplementary Table 4).

More recently, Hattori and colleagues developed a more accurate and sensitive method for the quantification of plasmatic hydroxyproline-containing peptides after oral administration of gelatin (Taga et al., 2014). To this purpose, they prepared a stable isotope-labeled collagen obtained from HEL cells cultured in Dulbecco's modified eagle medium supplemented with $^{13}C_6$-Lys, $^{13}C_6$ $^{15}N_4$-Arg, $^{13}C_5$ ^{15}N-Pro; resulting collagen was subjected to digestion with trypsin/chymotrypsin and plasma proteases to mimicking biopolymer degradation pathways in the body. This labeled collagen digest was then used as an internal standard mixture for accurate, simultaneous quantification of 13 O-containing peptides in the blood of volunteers subjected to a single dose oral administration of fish gelatin hydrolysate (Taga et al., 2014). The measured area under the plasma concentration−time curve of O-containing peptides ranged from 0.663 ± 0.022 μM h for POG to 163 ± 1 μM h for PO. Corresponding C_{max}, and $t_{1/2}$ values are shown in Supplementary Table 4. This method was used to test a novel gelatin hydrolysate developed by using a cysteine-type ginger protease, which contained increased levels of XOG and GPX tripeptides (Taga et al., 2016). When *in vivo* absorption of these hydrolysates was tested in mice, the authors verified that plasmatic XOG peptide concentrations were significantly higher than those of conventional counterparts. Novel O-containing peptides, namely LOG, FOG, TOG and GPOG, were also detected in human blood. By using this approach, absorption of cyclic collagen-specific O-containing dipeptides having a heat-dependent 2, 5-diketopiperazine moiety was also verified (Taga et al., 2017).

The promising results obtained on the adsorption and bioavailability of the antihypertensive peptide VY and the hepatoprotective wheat gluten-derived < EL in animals (see above) prompted scientists to shift research focus on humans. In the first case, the correlation between varying concentrations of orally administered VY to healthy and mild hypertensive subjects, and time-course plasma absorption and concentration of peptide were investigated (Matsui et al., 2002; Matsui et al., 2006). Maximal plasma VY concentrations peaked at 2 h postprandially, with a dose-dependent increase in concentrations (Supplementary Table 4). However, no marked changes of blood pressure paralleled the observed increase of the plasma levels of VY, suggesting that peptide treatment did not exert an acute hypotensive effect on human subjects. In the second case, a targeted and untargeted peptidomic analysis was carried out on the blood of healthy volunteers fed corn and wheat gluten hydrolysates (Ejima et al., 2018). Authors verified that 6 peptides, namely PA, PG, PQ, <EP, <ELP and <EQP, showed increased plasmatic concentrations up to 10–100 nM, compared to the baseline, which persisted for several hours after cessation of treatment. Corresponding C_{max}, and $t_{1/2}$ values were deduced (Supplementary Table 4). Authors claimed a significant increase of plasmatic levels of peptides bearing a C-terminal proline, making corn and wheat gluten hydrolysate-based meal a good source of putative bioactive peptides.

The broad research activity assessing the occurrence of O-containing peptides in the human blood after oral administration of gelatin hydrolysates was paralleled by similar efforts aimed at identifying and quantifying milk protein-derived species in human plasma/serum following the ingestion of dairy products. Initial studies explored newly generated CN-derived (poly)peptides in the blood of healthy newborns and adult individuals fed milk and milk/yogurt, respectively (Chabance et al., 1995; Chabance et al., 1998). By taking advantage of the combined use of LC and Edman degradation, authors demonstrated in both sample groups the occurrence of the large antithrombotic κ-caseinoglycopeptide (106–169) and, only in adult volunteers, of the antibacterial peptides RPKHPIKHQGLPQEVLNENLLRF and RPKHPIKHQGLPQEVLNENLL (Supplementary Table 4). κ-Caseinoglycopeptide (106–169) was also detected in the stomach and duodenum of adult individuals (Supplementary Table 2), suggesting its direct passage across the intestinal epithelium into the bloodstream. The hypothesis of a direct route of milk-derived peptides in the human blood was confirmed few years later by Foltz and coworkers, who investigated the formation of the antihypertensive dipeptides IPP, LPP, AW, IW, LW, FY and IY in adult volunteers fed yogurt, or similar IPP- and LPP-enriched products (Foltz et al., 2007). By using a targeted LC-ESI-MS/MS approach based on a dedicated MRM procedure and ^{13}C-labeled standard molecules, authors measured quantitative levels of these molecules in the blood. They demonstrated a selective rise of the peptide concentrations after meal administration with respect to basal levels. Corresponding C_{max}, $t_{1/2}$ and bioavailability values were also deduced, ascertaining for these compounds plasmatic nanomolar concentration values, and a common persistence for at least 1 h (Supplementary Table 4). This study showed that IPP and other milk-derived antihypertensive dipeptides escape from intestinal degradation and reach the bloodstream, a finding also tested and validated for IPP and LPP in animal models (van der Pijl et al., 2008). This result was not surprising and may derive from the likelihood for these molecules to be translocated across the intestinal barrier by peptide transporters.

β-Casomorphins are important peptides during the first year of life, when postnatal formation is predominant and milk is the main source of both nutritive and bioactive molecules. In addition to exert a possible local μ-opioid agonist activity in the gastrointestinal tract, as reported above according to their evident detection by peptidomics (Supplementary Tables 1 and 2), these molecules have also been associated overtime with specific actions in other body districts, as recently reviewed (Daniloski et al., 2021). To verify the latter hypotheses as related to food ingestion, the levels of human and bovine β-casomorphins 7 (YPFVEPI and YPFPGPI) were initially evaluated in the blood of infants fed breast milk and bovine milk formula, respectively (Kost et al., 2009). To this purpose, authors used a dedicated approach based on a combined use of LC, radioimmunoassay and enzyme-linked immunosorbent assays. In both cases, increased levels of both β-casomorphins-7 forms were detected in the blood during first 3 h after feeding. This phenomenon was significant in the first 3 months of life of infants. These preliminary studies were in good agreement with an investigation on the intestinal transit and plasma levels of the β-casomorphin analog casokefamide, which was performed on human volunteers (Schulte–Frohlinde et al., 2000). Very recently, β-casomorphins 7 was also evaluated in the plasma of children fed with pasteurized bovine milk to confirm a possible relationship between peptide and DPP-IV contents, and autism spectrum disorders (Jarmołowska et al., 2019). Authors used enzyme-linked immunosorbent assays that demonstrated concentration values of about 33 nM in the control children group; concentration increased by a 1.6-fold in the disease group. Despite this evidence and multiple dedicated attempts, no data generated by nanoLC-ESI-MS/MS procedures have proved the occurrence of β-casomorphins in human blood of individuals fed with dairy products.

To bridge this gap and to evaluate the possible passage of peptides deriving from ingested milk proteins across the intestinal epithelium into the human bloodstream, we recently used an untargeted peptidomic

approach. In a pilot study, we investigated at first the absorption of CN-derived peptides in the human blood of healthy subjects fed cheese for one-week after an identical period of a dairy products-free diet (Caira et al., 2016). The use of a dedicated extraction and enrichment treatment with hydroxyapatite allowed trapping phosphopeptides for subsequent nanoLC-ESI-MS/MS analysis. This approach originally identified phosphopeptides HVsssEE, VsssEE, VSssEE, sssEE, SssEE, DIGsEsTEDQ and DIGsEsTE (where phosphoserine is reported with a lowercase letter) as well as non-modified peptides YQEPVLGPVRGFPIIV, QEPVLGPVRGFPIIV and PVRGPFPIIV in blood samples (Supplementary Table 4). Some of these molecules can derive from phosphopeptide precursors already identified in the ingested cheese (Ferranti et al., 1997). Data indicated that CN-derived oligopeptides are adsorbed and bioavailable in the human blood after a continued intake of cheese. As expected, most of the assigned peptides contained the phosphorylated motif sssEE, which is common to α_{S1}-, α_{S2}- and β-CN, and it is responsible for the protein ability to maintain calcium and phosphate ions in a soluble form (Gravaghi et al., 2007). Very recently, we investigated the food-derived peptide profile of blood samples from 3 volunteers subjected to a single dose of milk after a 10-day wash out of dairy products (Caira et al., 2022). Untargeted peptidomics over an 8 h-period after milk ingestion allowed identifying 758, 273 and 212 unique peptides in the human blood, which originated from 15, 15 and 18 bovine milk proteins, respectively. Among identified bioactive peptides, worth mentioning are: i) ACE-inhibitory FFVAPFPEVFGK, PFPEVFGK, FVAPFPEVFG, FALPQYLK, YQKFPQYLQY, VYPFPGPIPN, YPFPGPIPN, VRGPFPIIV, VLGPVRGPFP, LVYPFPGPIPNSLPQ, QEPVLGPVRGPFPIIV, LLYQEPVLGPVRGPFPIIV, LLYQEPVLGPVR, RDMPIQAF, SQSKVLPVPQ, LHLPLPL, NLHLPLPLL, YQEPVLGPVRGPFPIIV, YQEPVLGPVR, AVPYPQR, VLPVPQK, AIPPKKNQD and DAQSAPLRVY; ii) antimicrobial VLNENLLR, SDIPNPIGSENSEK, RPKHPIKHQGLPQEVLNENLLRF, RPKHPIKHQGLPQEVLNENLLRFF, TKVIPYVRYL, KAMKPWIQPKTKVIPYVRYL, TVYQHQKAMKPWIQPKTKVIPYVRYL, YQEPVLGPVRGPFPIIV, AVPYPQR, VLPVPQK and TPEVDDEALEK; iii) antioxidant APSFSDIPNPIGSENSE, AYFYPEL, FALPQYLK, VYPFPGPIPN, YPFPGPIPN, IHPFAQTQ, FPKYPVEPF, HKEMPFPKYPVEPFTESQ, SQSKVLPVPQKAVPYPQ, AVPYPQR, VLPVPQK, ARHPHPHLSFM and INNQFLPYPYYAKPA; iv) antihypertensive AYFYPEL; v) immunomodulatory RPKHPIKHQGLPQEVLNENLLRF, LYQEPVLGPVRGPFPIIV, YQEPVLGPVRGPFPIIV and YQEPVLGPVR; vi) PEP-inhibitory IHPFAQTQ; vii) anxiolytic YLGYLEQLLR; viii) antithrombotic YQEPVLGPVRGPFPIIV and YQEPVLGPVR; ix) anti-inflammatory YQEPVLGPVR; x) antiapoptotic VLPVPQK; xi) osteoanabolic VLPVPQK; xii) cytotoxic LIVTQTMK; xiii) DPP-IV inhibitory TPEVDDEALEK; xiv) opioid YIPIQYVLSR; xv) C3a receptor agonist YIPIQYVLSR (Supplementary Table 4). No β-casomorphins 7 and 8 were detected in blood samples probably as result of peptide susceptibility to the action of plasmatic proteases and/or their low amounts in human plasma. The size of the peptides detected in plasma clearly demonstrated the passage across the intestinal epithelium of large compounds. Peptide representation data suggested protein degradation phenomena deriving from the concomitant action of endoproteases and exopeptidases. It remains to be established whether half-life and concentration values of circulating milk-derived peptides may have any impacts on human health. Future studies are needed to confirm these findings on a wider population and provide quantitative information to clarify the mechanisms behind molecular absorption and bioaccessibility of milk-derived peptides.

In the whole, most of the studies in this research field highlighted concentration values for most peptides detected in the human blood that were generally in the nanomolar range, which is lower than that (about 1–100 μM) associated with positive results during *in vitro* functional assays (Supplementary Tables 3 and 4) (Matsui, 2018; Sato, 2022). On the other hand, some peptides exhibited a certain resistance to exopeptidases and showed molecular levels compatible with a certain bioavailability. On this basis, it remains questionable if peptide detection in the human blood can be associated with a significant functional action on humans; future quantitative studies are necessary to address definitively this question.

5. Peptidomics of food-derived peptides in biological fluids/materials ejected from the organism body

Exogenous peptides circulating in the bloodstream of investigated animal models or humans can reach specific organs and possibly elicit bioactive activities at these sites. Depending on their structure, they can be eventually processed by specific proteases/peptidases in the blood and/or other body districts, and then be excreted in the urines. At the same time, some (poly)peptides and/or residual amounts of non-absorbed molecules present in the intestine do not cross the brush-border epithelial membrane and then move from duodenum to colon, where they can be extensively degraded by resident microflora (Amaretti et al., 2019). Products of corresponding metabolism are finally excreted with feces, together with eventual undigested proteins and fibers. Different targeted methods were developed and used to measure the occurrence of oral administered peptide-based drugs/toxins in animal/human urines and/or feces, including immunomodulating agents (Fang et al., 2010; Mika & Stepnowski, 2016), growth hormone precursors (Thevis & Schaenzer, 2014; van den Broek et al., 2015), antidiuretic hormones (Thomas et al., 2011), mushroom-related toxins (Li et al., 2018; Sun et al., 2018; Park et al., 2021), and muscle performance adjuvants (Gardner et al., 1991). Conversely, a very limited number of peptidomic studies was accomplished on food-derived peptides. Probably, this is the result of the general technical challenge of detecting unknown molecules present at a low abundance in complex matrices such as urine and feces (Foreman et al., 2021). On the other hand, the analytical determination of a bioactive peptide during its permanence in the body, starting from its generation within the gastrointestinal tract and ending up with its excretion in urine/feces, is essential to understand or even imagine its possible functional effect(s).

To address some of these knowledge gaps, recently Dallas and co-workers investigated peptides released inside the gastrointestinal tract and/or present in the stool of infants fed milk (Beverly et al., 2020; Beverly, Huston, et al., 2021). By analyzing stool samples with an untargeted nanoLC-ESI-MS/MS approach, authors identified 8132 peptides, among which 118 exclusively deriving from milk CNs and α-La, including molecules with known or potential antimicrobial properties eventually active within the final tract of the intestine (Supplementary Table 5). When stool samples were compared with corresponding gastric counterparts according to peptide abundance and count values, it was evident that molecular levels decreased from stomach to stool. To test the hypotheses that the milk peptide content of stools is different between preterm and term infants at different days of life, authors also performed a comparative peptidomic analysis between the different groups based on corresponding peptide counts and abundances (Beverly et al., 2020). Although many individual milk peptides were significantly different between preterm infants at 8/9 and 21/22 days of life, and between preterm and term infants, total peptide abundance and count values were similar for all groups. This study confirmed the survival of milk peptides in the stool of infants and suggested that some compounds have potential bioactivities that might influence infant gut development.

Recent studies on food-derived peptides present in stool samples of infants also allowed obtaining information on the influence of food thermal processing, and the consequent complex network of reactions between food proteins and sugars (also known as the Maillard reaction) yielding non-enzymatic glycated products, on microbiota populations present in gut and the corresponding fermentation processes. In fact, investigations on glycated dipeptides present in feces of formula-fed or breastfed infants demonstrated that these modified compounds occur only in the intestine of the former group (Sillner et al., 2019), which also become enriched in specific bacteria able metabolizing dietary glycated

components through the activation of specific catabolic pathways (Bui et al., 2015). Metabolomic analysis of the same samples demonstrated that unsaturated fatty acids and human milk oligosaccharides are increased in breastfed infants, whereas glycated peptides along with secondary bile acids are accumulated in formula-fed childrens (Sillner et al., 2021).

Although not identified according to a real peptidomic perspective but through ELISA assay, the occurrence of food-derived peptides was also ascertained in the stool samples of volunteers fed with a diet containing gluten (Silvester et al., 2020). Gluten-derived peptides were also detected in the urine of test participants, demonstrating for the first time the occurrence of food-derived protein fragments in this body fluid. In both cases, excretion kinetics were highly variable between individuals, and molecules were not assigned by mass spectrometry procedures. Very recently, Elias and coworkers used an optimized workflow for the analysis of the urine peptidome of healthy volunteers fed wheat gluten to identify peptides derived from dietary proteins (Palanski et al., 2022). When peptidomic experiments were performed on individuals experiencing for 2 days a food gluten-free diet followed by ingestion of wheat-based food, urine samples yielded an average of 24 unique food-derived peptides per participant, all of which were identified only in the post-challenge samples. The number of unique wheat peptides varied between tested individuals. Among identified molecules, the authors assigned various peptides with known celiac disease (CeD) relevance, together with some compounds never implicated in this disease or already identified as being resistant to gastrointestinal digestion. When a similar experiment was done on volunteers experiencing an overnight fasting followed by a significant gluten assumption, about 123 food-derived peptides were identified, among which GQQQPFPPQQPYPQPQPFPS and GQQQPFPPQQPYPQPQPFP (Supplementary Table 5) from α-gliadins that were already recognized to stimulate an innate immune response. Additional assigned peptides harbored known CeD-relevant T-cell epitopes, such as QQPQQPFPQ, QQPFPQQPQ, PQQSFPQQQ, QQPQQPYPQ and PFSQQQQPV. Different molecules showed a large size, demonstrating the passage across the intestinal and the kidney glomerulus epithelium not only of compounds having low-medium mass value. Similar peptidomic experiments were performed after ingestion of rye- or barley-derived gluten; they provided comparable results, highlighting also for these cereals the occurrence of food-derived peptides in the urine of the challenged volunteers. When the same approach was used to investigate eventual differences in the urinary peptidome between wheat-consuming healthy individuals and patients suffering CeD, gluten peptides in the latter case differed qualitatively and quantitatively from controls, being higher in number either for the assigned sequences or molecules including T-cell epitopes. This phenomenon was explained as result of an increased concentration of gluten-derived molecules in the systemic circulation and in urine of CeD patients, thereby enhancing investigator's capability to detect them, or depending on a different metabolism of wheat between healthy and diseased individuals. In this case, peptidomic analysis identified peptides containing 17 distinct immunotoxic epitopes, which are known to be presented on MHC class II molecules and recognized by T cells residing in the lamina propria of patients with CeD. Many of these epitopes occurred more frequently in patients with CeD than in healthy subjects (Supplementary Table 5).

Since exclusively breast-fed infants can exhibit clear signs of IgE or non-IgE-mediated cow's milk allergy, a complete characterization of mother's milk was also accomplished to investigate whether dietary cow's proteins can survive the maternal digestive tract to be absorbed in the bloodstream and secreted into the breast fluid (Picariello et al., 2016). To this purpose, we used an untargeted nanoLC-ESI-MS/MS approach that allowed identifying in breast milk samples from mothers receiving a cup of bovine milk daily two bovine β-Lg-derived and one α_{S1}-CN-derived peptides, which were absent in counterparts from mothers subjected to a dairy product-free diet (Supplementary Table 5). These preliminary results about the possible sensitization and elicitation of allergic responses to bovine milk peptides in the neonate through mother's milk was confirmed later, when we analyzed by the same peptidomic approach the peptide fraction of breast milk samples collected from a single non-atopic donor on different days after receiving an oral load of cow's milk (Picariello et al., 2019). Control milk samples from the same individual were taken after a prolonged milk- and dairy product-free diet. Overall, 21 peptides exclusively originating from bovine CNs and whey proteins were identified in the breast milk samples of the mother fed bovine material, which were otherwise absent in control (Supplementary Table 5). Time course analysis of selected cow's milk-derived peptides in breast milk, as determined from the MS ion intensity of the molecular signals, demonstrated that bovine compounds were secreted up to 4 h after food ingestion. β-Lg, which is not expressed by humans, was monitored as a marker of cow's milk in breast counterpart. While no intact β-Lg was detected by dedicated Western blotting experiments, dot-blot analysis confirmed the occurrence of protein fragments in the enriched peptide fraction of breast milk. Similar findings were obtained by Heck and coworkers, which also analyzed human milk taken from mothers not subjected to a special diet to detect the occurrence of generic food-derived peptides (Zhu et al., 2018). By using shotgun proteomics, authors identified 109 non-human peptides, of which 71 were grouped as related to 36 exogenous proteins (Supplementary Table 5). In analogy to what detected in the above-mentioned studies from our group, peptides from major bovine proteins (α_{S1}-, α_{S2}-, β- and κ-CNs as well as β-Lg) present in dairy products were the dominant non-human components. Results from these articles suggested a shifting the analytical perspective for the detection of dietary food allergens in breast milk from intact proteins to digested peptide fragments. The potential sensitization and elicitation or the tolerogenic properties of such low amounts of dietary peptides for the breastfed newborns remain to be explored.

6. Methodological and technological improvements for the detection of food-derived peptides in the organism body

All the studies surveyed above well reflect the technological improvements that were developed in the last two decades to improve chromatography- and mass spectrometry-based untargeted peptidomic studies centered at identifying novel molecules resulting from dietary proteins within various anatomical districts as well as at quantifying them with targeted approaches (Fig. 3). Both results represent essential information for the final assignment of newly generated, exogenous functional peptides into specific body fluids/tissues, and provide preliminary data for further studies related to the nutrition-gut microbiome-physiology axis and allergic diseases.

Notwithstanding the misleading idea that proteomics and peptidomics deal with the same molecules (polypeptides) and share several analytical workflows, a dedicated search in PubMed for the corresponding published articles revealed a significant lower number of publications for the latter case (data not shown). This finding well reflects the challenging nature of peptidomic analysis and data interpretation, which are highly dependent on peptide characteristics (Fig. 4). In fact, peptides can differ their representation within complex biological samples for: i) the order of magnitude values for concentration; ii) the size range, from 2 to about 50–60 amino acids, often overlapping isobaric metabolites and lipids; iii) variable physicochemical properties. Depending on the latter properties, in particular the molecular charge state and hydrophobicity, optimization of peptide chromatographic separation by liquid chromatography can be challenging, often requesting trial and error exercise (Fig. 4). Because of the charge state of peptides, mass spectrometric characterization of molecules can also be a complex process since each compound can in principle occur as singly to multiply charged species, making MS/MS-based sequence assignment not trivial as result of complications in instrument acquisition methods and data analysis. This condition does not occur during proteomic experiments, in which doubly to triply charge tryptic peptides are

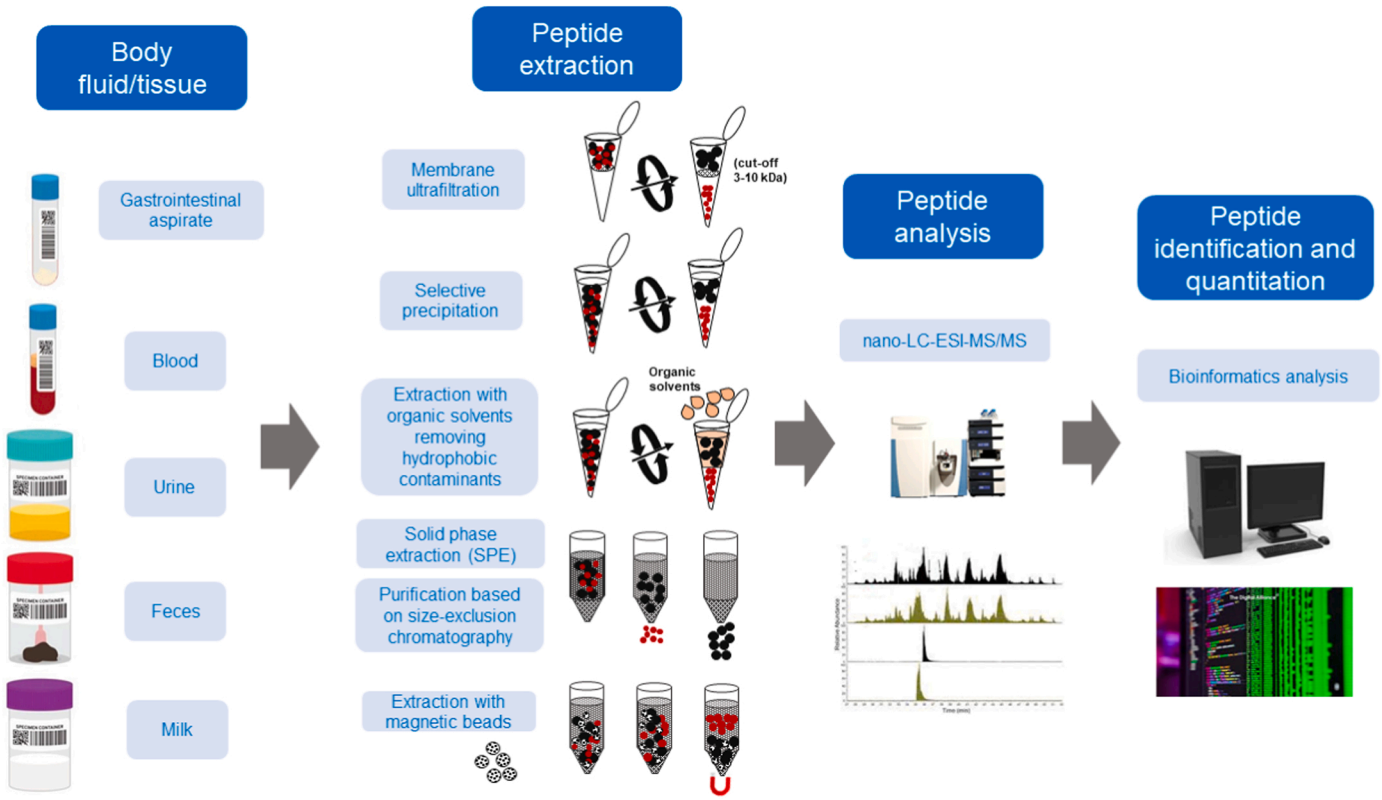

Fig. 3. Typical workflow of peptidomic analysis used for identification and quantitation of food protein-derived peptides present in various human body fluids/tissues. Selective peptide enrichment procedures include: ultrafiltration onto polymeric membranes; extraction with immiscible organic solvents; selective precipitation of contaminants; extraction with stationary solid phases packed in dedicated cartridges; purification based on size exclusion chromatography; extraction with magnetic beads functionalized for affinity purification.

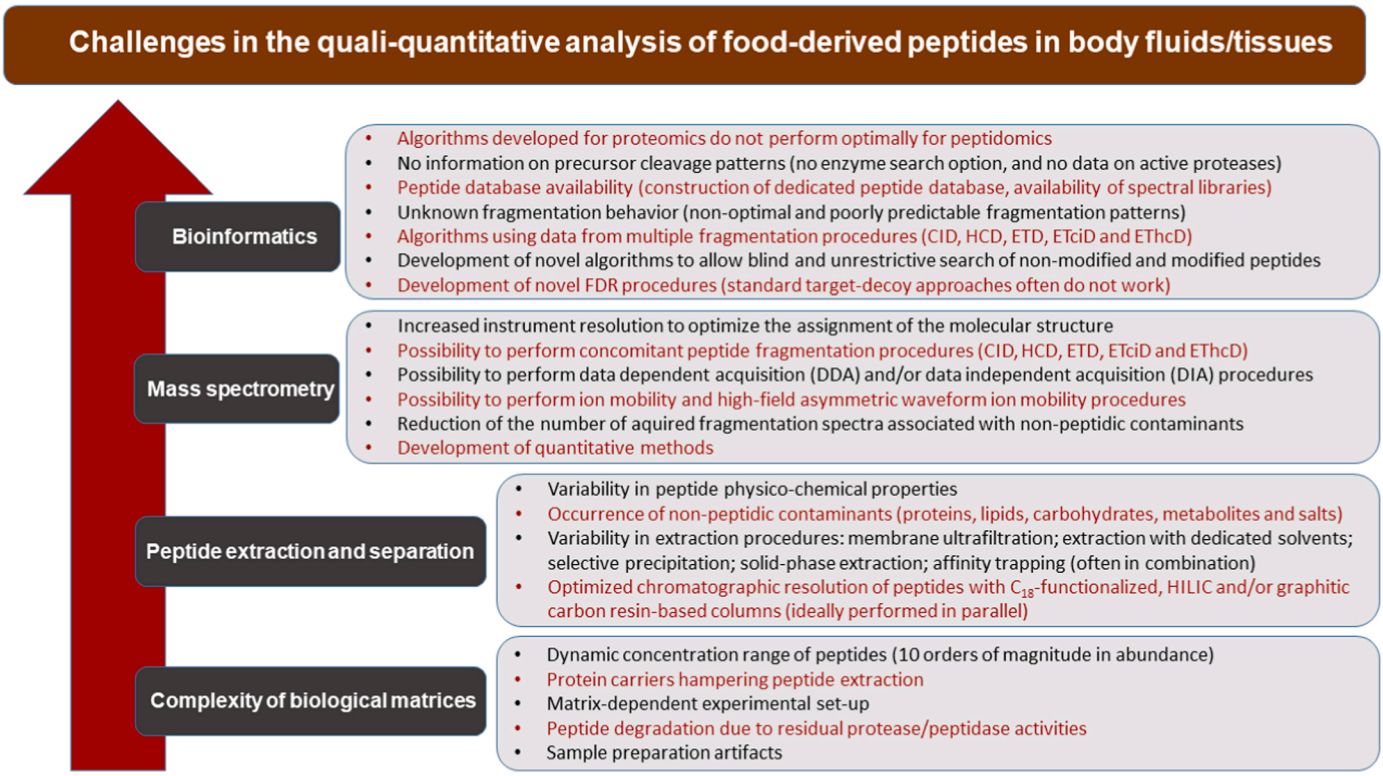

Fig. 4. Overview of the major technological challenges during quali-quantitative analysis of food-derived peptides from various human body fluids/tissues.

generally observed. The latter point is extremely important since molecular assignment in peptidomics generally depends on 1 to 2 mass spectrometric measurements, whereas protein identification in proteomics is generally the result of multiple peptide fragmentation determinations (Fig. 4). To this purpose, the chance to detect an individual peptide in a complex mixture is much lower, if compared to that of a protein that in a bottom-up proteomic approach generally produces a multitude of peptides, each of which allows to infer the presence of the parent molecule.

To overcome the specific drawbacks of peptidomic analysis, various technological improvements have been developed overtime, considering that methods need to be tuned depending on the biological matrix under investigation. The general features of the peptidomic approaches are reviewed below in the following sections.

6.1. Peptide extraction methods

Sample preparation procedures and related variables have a dramatic impact on final peptidomic results (Vitorino et al., 2012; Leichtle et al., 2013); this is particularly important when minor components must be detected, as in the case food-derived peptides. At first, timing of sample collection, eventual addition of chemicals (anticoagulants and protease inhibitors) to biological samples, and freeze-thaw cycles have to be carefully considered (Dallas et al., 2015; Maes et al., 2019; Foreman et al., 2021). Abrogation of residual protease/peptidase activity using dedicated inhibitors is crucial in peptidomic investigations to avoid degradation artifacts (Fig. 4). However, inhibitor concentrations must be carefully evaluated, since these molecules are often peptide derivatives that can affect the original peptidomic profile.

In general, biological samples for peptidomics must be highly enriched in peptide components and ideally should be removed of protein, lipid, carbohydrate and salt contaminants, whose presence can deleteriously impact on subsequent liquid chromatographic and mass spectrometric analysis, due to molecular separation-interfering phenomena and ionization suppression effects, respectively (Fig. 3). Since the target peptides can differ for their molecular size, charge and hydrophobicity, different molecular preparation methods must be comparatively tested at the beginning of a peptidomic study and optimized for the biological matrix under investigation. Differently from proteomics, the concept that one sample preparation method generally is widely applicable to most experimental setups is not valid in peptidomics. The most common peptide extraction procedures developed overtime (often used in combination) (Fig. 3) derived from clinical studies (Maes et al., 2019; Foreman et al., 2021), and generally include: 1) ultrafiltration with polymeric membranes differing for their pore size (with molecular cut-off values of 3 and 10 kDa), which resolve filtrated and retained compounds according to their mass, removing high-molecular weight contaminants. This purification step is fast and easy to be applied, but it suffers from partial peptide losses and/or significant molecular contamination (Li et al., 2020), 2) Extraction with immiscible organic solvents (ether or chloroform) to remove hydrophobic contaminants (generally lipids and other organic compounds) (Li et al., 2020). 3) Selective precipitation of contaminant proteins with organic acids, *e.g.*, trichloroacetic acid (TCA) and trifluoroacetic acid (TFA), or solvents, namely methanol, acetonitrile (ACN) and/or acetone (Vitorino et al., 2012; Dallas et al., 2015). This fractionation step is also fast and easy to be accomplished, but it can suffer from an incomplete protein removal and an eventual precipitation of peptide aggregates (Dallas et al., 2015). To limit the latter inconveniences, the addition of aqueous solutions of organic solvents has been suggested (Kay et al., 2008). 4) Extraction with stationary solid phases packed in dedicated cartridges or pipet tips, which selectively retain peptides based on their hydrophilic/hydrophobic characteristics. These procedures allow the wash out of contaminants and concomitant concentration of peptide solutions. The most used solid phase extraction devices are based on porous C_{18}-functionalized silica or hydrophilic-lipophilic balance (HLB) resins, but other materials are also available (Dias & Poole, 2002; Sigdel et al., 2018). 5) Purification based on size exclusion chromatography (SEC) or SDS-PAGE, which allow the recovery of peptides directly and indirectly (after gel extraction), respectively, and optimal resolution with respect to high-molecular mass molecules (Foreman et al., 2021). 6) Extraction with magnetic beads functionalized for affinity purification of target peptides (Hoofnagle & Wener, 2009).

Comparative studies to optimize existing peptidomic protocols have been performed for adapting the extraction procedure to the biological matrix under investigation. A prototype example of these investigations is represented by the recent procedure developed by Heck and colleagues for the untargeted study of the human milk peptidome (Dingess et al., 2019), in which 7 peptide extraction procedures based on fluid ultrafiltration with polymeric membranes, liquid extraction and acid precipitation were comparatively evaluated according to corresponding final mass spectrometric results. Qualitative and quantitative reproducibility of extraction methods in both technical and biological replicates was also assessed. An optimized procedure based on protein precipitation with 20% TCA was proposed; aside from slight modifications, this protocol integrates experimental procedures already set up for milk peptidomics (Dallas et al., 2013; Dingess et al., 2017) and allows detection of minor components. The latter feature is very important in the case of low abundant food-derived peptides. Very recently, an extraction procedure dedicated to the analysis of di-to tetra-peptides present in milk was also developed (Montone et al., 2021). It involves the precipitation of proteins and lipids by addition of TFA to samples, followed by peptide trapping on SPE devices packed with graphitized carbon black, and final molecular elution.

Since blood reflects peptide secretion of several tissues/organs and contains various proteases/peptidases, it represents a very complex molecular matrix in which analytes occur in a wide and dynamic representation range. Evident peptide differences were observed by comparing the peptidomic profile of plasma and serum, which well reflected the activities of proteolytic enzymes involved in clotting (Tammen et al., 2005). In general, plasma is the preferred biological matrix for blood peptidomics, and sampling is accomplished in tubes containing additives (EDTA, citrate, or heparin) that minimize coagulation phenomena, degradation of (poly)peptides and non-specific coprecipitation of molecules within clots. This choice prolongs peptide stability and reduces sample complexity and/or variability (Maes et al., 2019). Different methods for preparation of plasma samples were compared and minor differences in pre-extraction conditions were proved to significantly affect final peptidomic results (Tammen et al., 2005). Further implementations included the use of sampling tubes containing protease/peptidase inhibitor cocktails, the addition of acids or sample snap freezing (Maes et al., 2019). Recently, a sequential protein precipitation and delipidation procedure based on optimized use of methyl-*tert*-buthyl ether/methanol/water solvent mixtures enabled efficient enrichment of polypeptides in human plasma samples (Lin et al., 2020). When compared with TCA-, ACN- and membrane ultrafiltration-based methods, it allowed optimal recovery of large peptides. On the other hand, a recent clean-up strategy was developed to purify short peptides present in blood for further mass spectrometric characterization (Piovesana et al., 2020). It involves the sequential treatment of diluted plasma samples with phospholipid cartridges allowing simultaneously removal of proteins and phospholipids, followed by application of corresponding eluates on graphitized carbon black-packed SPE devices; final elution from the latter cartridges allowed optimal recovery of dipeptides and tripeptides. In both above-mentioned cases, performance results were obtained after dedicated mass spectrometric analysis. Very recently, a dedicated method was proposed for extraction of exogenous medium to large-size peptides from blood samples (Caira et al., 2022); it is based on the addition of protein denaturants to samples followed by SEC-based peptide enrichment procedure. This experimental set up avoided the binding of peptides to carrier proteins and allowed original detection of milk-derived peptides in the presence of largely dominant

blood-related ones. Notwithsanding above-reported technological advances, the major challenge in peptidomic studies on blood samples resides on the evaluation of the general origin of peptides from protein degradation processes. In fact, it remains difficult to distinguish peptides that originate from physiological phenomena with respect to those that derive from plasma collection and/or sample handling, which must be considered as sampling artifacts (Aristoteli et al., 2007).

Molecular complexity also characterizes the urinary peptidome, and complicates corresponding analysis (Martelli et al., 2014; Trindade et al., 2021). Urine contains large amounts of small peptides present in plasma, which are excreted by filtration in the kidney, originate from the urogenital tract or are generated in the bladder as result of the proteolytic action of endogenous proteases on proteins (Barbé et al., 2014). Additional variations in the urine peptidome were associated with daily food intake and related metabolic processes as well as between first and second void or between first and mid-stream (Martelli et al., 2014; Delanghe & Speeckaert, 2014; Trindade et al., 2021). All these elements generate a significant sample variability that is highly related to sampling times. Notwithstanding above-mentioned variability, the urine peptide pattern seems more stable than that of blood at room temperature, likely due to the reduced presence of peptidases (Fiedler et al., 2007). With the aim to optimize urine peptidomics, Sarwal and colleagues adapted a standard solid-phase extraction (SPE) method to a modified approach in which peptides isolated from SPE were subjected to a second purification step using a processed silicon carbide resin in a pH dependent manner (Sigdel et al., 2018). This sample preparation method avoided clogging of chromatographic columns due to metabolite/salt contaminants and ensured a high recovery of peptides, thus increasing yield and sensitivity of the nanoLC-ESI-MS/MS analysis, and the number of identified peptides. Similar improvements in the number of identified peptides (about 6500 in number) were achieved with the sequential use of membrane ultrafiltration (10 kDa cutoff)-based enrichment of reduced and carboxyamidomethylated peptides, analyte trapping on HLB cartridges, extraction of urinary pigments with ethyl acetate, and strong cation exchange chromatography resolution into fractions for final nanoLC-ESI-MS/MS analysis (Van et al., 2020). Overall, preparation of peptide fractions took about 48–72 h. Very recently, Elias and coworkers have developed an optimized extraction technique to remove efficiently interfering urinary compounds in about 6 h, with evident improvements in performance measurement capabilities of urinary peptides originating from physiological or dietary sources (Palanski et al., 2022). After reduction and alkylation of thiols, proteins were depleted through sample ultrafiltration with a membrane having a molecular cutoff of 10 kDa under acid denaturing conditions, and resulting permeates was processed using a mixed cation exchange SPE column to remove contaminant metabolites/salts before mass spectrometric analysis. This optimized peptidomic workflow allowed for the identification of approximately 10 times more endogenous human peptides from typical (1–10 mL) urine samples than previous SPE-based cleanup procedures, determining the largest collection of urinary peptides assigned to date (about 30,000 compounds). More importantly, it allowed the original demonstration of food-derived peptides in urine samples from volunteers fed wheat, rye, and barley gluten.

In addition to blood, urine and milk, other biofluids and tissues have the potential to provide important physiological information related to food-derived peptides. In this context, peptidomic studies reporting analysis of human stool samples from individuals fed specific diets have been recently reported (Sillner et al., 2019; Beverly et al., 2020; Beverly, Huston, et al., 2021). Generally, stool samples for peptidomic analysis were prepared by carefully dissolving material in water and removing solid, cell debris and lipid contaminants by centrifugation. Peptide fractions were then isolated through a protein precipitation step in acid conditions.

Notwithstanding the participation of saliva in early phases of food digestion, very few peptidomic studies have been performed so far on food-derived peptides in this biological fluid; the limited examples in this context are associated with recent studies on the generation of bitter- and salty-taste associated peptides in this fluid (Stolle et al., 2018; Sebald et al., 2020). Such a lack is probably the result of the complexity and variability of the salivary peptidome as related to gender, age, and circadian/seasonal rhythms (Bauça et al., 2014). Similarly, only very few peptidomic studies were performed on food-derived peptides in animal tissues/biopsies, namely antioxidant glutathione in liver (Yamada et al., 2018), antihypertensive VY in liver, kidney, heart and lung (Matsui et al., 2004; Tanaka et al., 2015), and adiponectin-receptor agonist YP in brain tissues (Tanaka et al., 2019; Lee et al., 2021). This may be due to the anatomical distance of target organs with respect to the gastrointestinal districts, which *a priori* should affect the degradation of peptides as result of the activity of various proteases/peptidases in brush border membrane, blood, and different tissues, highly impacting on the final nature and concentration of peptides in the specimens. Accordingly, studies were generally performed in targeted mode for evaluating the levels of ingested peptides in different organs. In general, problems related to tissue subdissection, homogenization and peptide extraction were solved by mechanical fragmentation of specimens with ultraturrax and/or lysing beads in the presence of chaotropic salts. Other methods involved snap freezing of tissue samples in liquid N_2, and then their grinding into fine powder for final peptide extraction.

6.2. Analytical methods and instruments for identification and quantitation of food-derived peptides

A careful analysis of the available scientific literature shows that peptidomic studies have been widely performed overtime through the combination of highly-resolutive liquid chromatography with electrospray ionization-based tandem mass spectrometry, which is one of the most sensitive and powerful technique for the detection and identification of peptides, also providing reliable quantitation. This combination allowed optimal analysis of thousands of peptides present in a complex mixture and having a wide dynamic range of concentration, as already verified in proteomics (Dallas et al., 2015; Maes et al., 2019; Foreman et al., 2021). Accordingly, LC-ESI-MS/MS-based approaches have found the widest use in peptidomic studies; based on recent technological improvements (see below), it is reasonable to predict that they will cover most of the future applications in this research field.

Regarding peptide separation, molecules have been generally resolved on reversed-phase high performance chromatographic columns that ensure a reproducible separation of analytes with a high resolution. These columns are available as nanoflow devices (~50–100 nL/min), which allow obtaining an increased compound concentration in the ionization source of the mass spectrometer thus maximizing sensitivity, or as microflow counterparts (~100–200 μL/min) that enable suitable flow rates for excellent spray stability in the source to ensure reliable quantification of the target analytes. Nanoflows were generally used in untargeted analysis of peptide mixtures to obtain the greatest molecular identification numbers, while microflows historically were preferably used for targeted peptide determinations. A good compromise has recently been achieved in modern chromatographic instruments allowing to maximize sensitivity and specificity of analyte detection and quantitation at nanoflows of about 300 nL/min.

Focusing on chromatography columns, optimal peptide separation always requires efficient retention and gradual elution of analytes. Although medium to large-size peptides are generally retained on C_{18}-functionalized silica-based columns used for proteomics, short and very hydrophilic peptides elute in the chromatographic void volume, hampering their reliable mass spectrometric characterization. Small peptides generated by multiple proteolytic processing events on food proteins are important to trace the relatively short life of parental molecules in physiological fluids. To face this inconvenience, various groups modified peptides with reagents introducing a lipophilic moiety in the analyte structure, thus increasing their hydrophobicity before final chromatographic separation. To this end, derivatization with

naphthalene 2,3-dialdehyde (Matsui et al., 2002), phenyl isothiocyanate (Ohara et al., 2007), 3,4,6-trinitrobenzensulfonate (Hanh et al., 2017), fluorenylmethoxycarbonyl-chloride (Ten Have et al., 2015) or 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (Ejima et al., 2018) was accomplished. The latter reagent allowed increasing the intensity of a_1 fragment ions during subsequent tandem mass spectrometric analysis of derivatized peptides aiding their full-length sequencing.

To improve separation of hydrophilic peptides without pre-column derivatization, dedicated C_{18}-functionalized silica-based resins bearing hydrophobic side chains and TMS end-capping as well as porous graphitic carbon resins (PGC) have recently been introduced, combined with conventional reversed-phase solvents (Alley et al., 2009; Cavaliere et al., 2021; Cerrato et al., 2021; Montone et al., 2021) (Fig. 4). Similarly, zwitterionic, diol, amide, amino and silica hydrophilic interaction liquid chromatography (HILIC) procedures have been recently optimized for peptidomic analysis of short peptides (Spicer & Krokhin, 2018); they use hydrophilic stationary phases interacting with charged residues in peptides, and acetonitrile and gradients of water as typical eluents including volatile salts (Piovesana et al., 2020). Very recently, chromatographic runs through dedicated chromatographic systems connecting simultaneously in parallel conventional C_{18}- and PGC-based columns have allowed to obtain deeper peptidomic profiles and concomitant, comprehensive characterizations of medium to large-size and small peptides, respectively (Jennings et al., 2021). These combined systems have been set up at μL/min flow rates so far but promise future, interesting applications in the nanoLC scale applied to the detection of food-derived peptides in body fluids/tissues. They will balance the need for high sensitivity with ease of implementation as compared to other two-dimensional LC systems that use nanocolumns with multiple trapping columns and multiport valves.

Optimal peptide separation was also obtained with capillary electrophoresis (CE), whose connection with ESI-based mass spectrometers however did not find its way into a widespread use, since the method does not ensure reproducibility in sample processing during high-throughput analysis. Thus, CE has been generally used in combination with salt-compatible matrix-assisted laser desorption ionization (MALDI)-based mass spectrometers. The latter molecular ionization technique found the widest application in peptidomic studies generally centered at rapidly screening large cohorts of samples containing complex peptide mixtures to identify discriminant singly charged ions, with the final goal of highlighting eventual biomarkers on a high-throughput basis (Hortin, 2006). This was due to the high rate of spectral acquisition in MALDI-based instruments. The latter characteristics also allowed peptidomic imaging applications, which measured the distribution of various peptides in a bidimensional space, generally a tissue section (Seeley & Caprioli, 2008). MALDI-based peptide imaging was widely applied in clinical studies, while it found a unique application for food-derived peptides, limited to detection of the distribution of the anti-atherosclerotic peptide WH in the rat intestinal membrane (Tanaka et al., 2015).

An additional level of analyte separation, useful to limit the ion suppression effect of singly charged interferences related to lipid and metabolite contaminations, is represented by recent ion mobility (IM) (Harvey et al., 2011) and high-field asymmetric waveform ion mobility (FAIM) (Swearingen & Moritz, 2012) interfaces, which have been introduced within the ionization source of modern mass spectrometers (Fig. 4). These electrophoretic gas-phase separation technologies allow to resolve molecules based on their charge, mass and mobility. The application of these techniques is currently enabling the separation of coeluting contaminant species and discrimination between differentially modified forms of the same peptide (Roberto et al., 2020). Thus, they have the potential to ensure interesting future applications also for the analysis of food-derived peptides in complex body fluids/tissues.

Further recent improvements in mass spectrometry-centered peptidomics derived from the availability of high-resolution (240,000–480,000)/high-accuracy (<1 ppm) mass spectrometers (Fig. 4). Resolution and accuracy are essential parameters for reducing the number of concomitant molecular possibilities during peptide assignment in untargeted determinations, but also are fundamental for improved acquisition selectivity during targeted peptidomic measurements. Further advances in the peptide assignment have also derived from the recent introduction of different peptide fragmentation methods, including collision induced dissociation (CID), electron-transfer dissociation (ETD), high-energy collision dissociation (HCD), ETD with supplemental CID (ETciD), ETD with supplemental HCD (EThcD) (Shliaha et al., 2018). Execution of experiments on the same peptide ion according to diverse (often sequential) fragmentation modes allowed expanding molecular information on the same analyte, assigning molecular identity with a higher probability degree (Shen et al., 2011) (Fig. 5). This point is extremely important for peptidomics, in which molecular identification is limited to a very poor number of mass spectrometric measurements. In the above-mentioned contexts, high-resolution mass spectrometers have recently found a widespread use also for the analysis of food-derived peptides in complex body fluids/tissues, as proved by various studies reviewed in this study. Conversely, very limited are the examples in which CID, ETD, HCD, ETD,

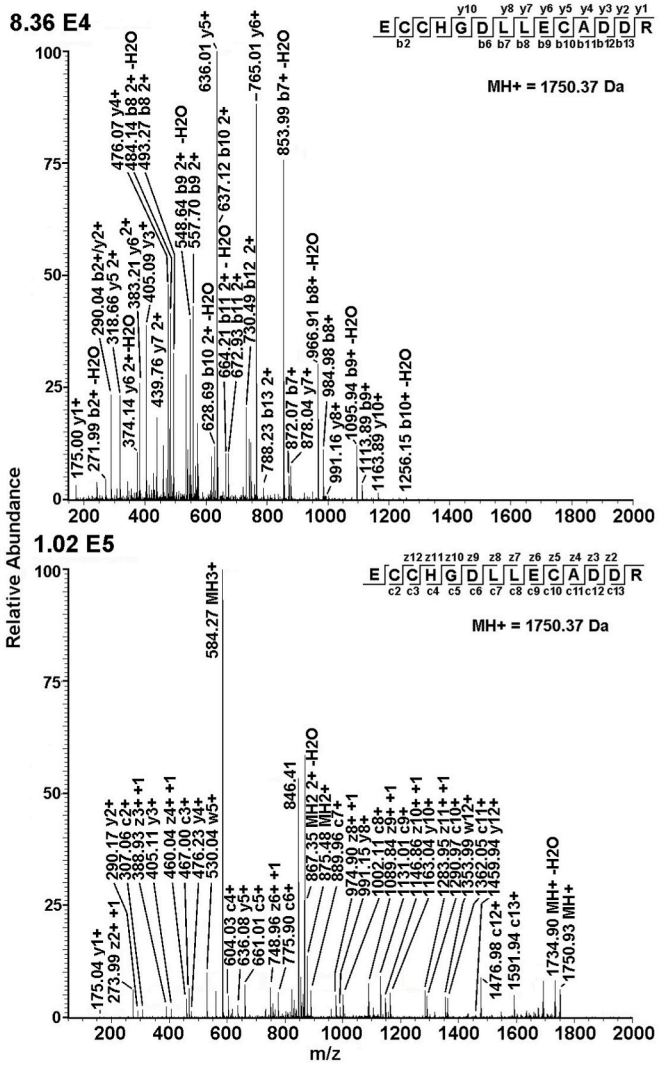

Fig. 5. CID (top) and ETD (bottom) fragmentation spectra of the milk-derived peptide ECCHGDLLECADDR from serum albumin. Tandem mass spectra derived from the fragmentation of the $[M\text{-}3H]^{+3}$ ion at *m/z* 584.27; identified fragment ions are labeled above and below the peptide sequence in both panels. The peptide extract was reduced and alkylated with iodoacetamide before nanoLC-ESI-MS/MS.

ETciD and EThcD procedures have been simultaneously applied to the analysis of food-derived peptides (Dingess et al., 2019). Combination of different peptide fragmentation methods can also be crucial for the proper assignment of peptides bearing post-translational modifications (PTMs).

Recent mass spectrometers also have allowed performing different mass spectral acquisition routines based either on data dependent acquisition (DDA) or data independent acquisition (DIA) scan functions (Bilbao et al., 2015) (Fig. 4). In DDA approaches, precursor MS survey scans are continuously performed, relative to retention time ranges, and peptides are detected. Then, MS/MS spectra of most intense *m/z* features are discontinuously but rapidly collected, also as related to their repetition over time intervals, ensuring the recording of a huge number of combined MS and MS/MS information for effective peptidome characterization. This acquisition mode often suffers from inconsistency across sample measurements, as result of the stochastic way by which the instrument selects the ions for further fragmentation. This inconvenience has been partially compensated with the development of fast scanning instruments and implemented software. However, DDA approaches are still affected by quantification, reproducibility, and accuracy limitations. These drawbacks have recently been overcome with DIA scan functions (Bilbao et al., 2015), in which precursor selection does not follow stochasticity but deterministic duty cycles according to an unbiased manner (Fig. 4). A large precursor isolation window is chosen to select and fragment all parent ions in a multiplexed manner, yielding an intricate combination of fragmentation spectra from several precursors. Spectral signals must be deconvoluted; this is achieved based on a retention time-based fitting of linked parent and product ions. When compared with DDA, DIA allowed improving both sensitivity and reproducibility of peptide measurements. While finding multiple applications in proteomics, DIA-based approaches have found a partial, promising use in peptidomics, mostly in clinical applications (Ritz et al., 2017; Lin et al., 2020). Their sensitivity promises interesting future applications also for the analysis of food-derived peptides in complex body fluids/tissues, as revealed by recent original studies (Stolle et al., 2018; Sebald et al., 2020).

Once untargeted analysis of food-derived peptides in different biological fluids/tissues has been accomplished and potential marker molecules have been identified, quantitative peptidomics can provide important information on eventual compound lifetime in the human body (Fig. 4). This is a very challenging task because in most cases exogenous peptides are present at a much lower concentration with respect to endogenous ones. Although peptide quantification based on molecular labeling (isobaric and isotopic) have been recently developed (Boonen et al., 2018; Wang et al., 2017), most of the applications used so far included label-free procedures (Romanova et al., 2013; Wang et al., 2017). In previous sections, we have reported a number of studies that used targeted SRM or MRM approaches to provide accurate quantification of food-derived peptides of interest in animal and human blood (Foltz et al., 2007; van der Pijl et al., 2008; Ichikawa et al., 2010; Nakashima et al., 2011; Sato et al., 2013; Sanchez-Rivera et al., 2014; Taga et al., 2014; Tanaka et al., 2015; Ten Have et al., 2015; Sontakke et al., 2016; Hanh et al., 2017; Ejima et al., 2018; Yamada et al., 2018; Asai et al., 2019; Nwachukwu et al., 2019). In general, molecular standards (even isotopically labeled) were used to optimize MS/MS conditions, which allowed achieving optimal sensitivity and suitable calibration curves for accurate peptide quantification. Experimental parameters were generally adjusted in a trial-and-error fashion to take into account peptide and biological matrix diversities. Due to mass spectrometers technical characteristics, most of these studies were performed with triple quadrupole or Q-Trap instruments to obtain high-sensitivity determinations, as already done in clinical peptidomics (Romanova et al., 2013; Wada et al., 2017). Recently, Heck and coworkers estimated the concentration of food-derived peptides in human milk also by combining data-dependent shotgun proteomics and parallel reaction monitoring (PRM) (Zhu et al., 2018). Targeted quantitative analysis of 9 exogenous molecules was obtained with PRM applying stable isotope heavy peptide standards and retention times of a peptide calibration mixture.

6.3. Bioinformatic analysis of peptidomic mass spectrometric data

Although always referring to polypeptide chain analysis, most popular mass spectrometric data search engines used for proteomics, *e.g.* MASCOT, SEQUEST, X!Tandem and MaxQuant, have shown partial peptide recognition capabilities and additional limits when used for peptidomics, probably as result of their protein-centered focus (Menschaert et al., 2010) (Fig. 4). They generate candidate peptides from an *in silico* digestion of the sequence entries present in a protein database, which are then used for subsequent experimental to theoretical parent (initial) and fragment (subsequent) mass matching. Putatively identified peptides are ranked according to their probability scores, and parent proteins are then assigned by inference as result of multiple peptide identifications (Eng et al., 2011). The latter process is done according to the number of peptide spectrum matches (PSMs) and unique recognized peptides, and tolerates a certain error in single peptide assignment. Conversely, the peptide assignment itself is the final target of the search process in peptidomics, which generally is done by matching parent and fragment mass values from very few experimental spectra with theoretical counterparts. Errors in this process fully compromise the final molecular assignment; accordingly, quality and accuracy of fragmentation spectra are critical factors for proper peptide identification. Further limitations to the application of the above-mentioned proteomic search engines are associated with the unpredictable nature of the amino acid at N- and C-terminus in natural peptides, due to the lack of information on proteolytic enzymes involved in physiological processing of molecules (Fig. 4). Thus, a search of the protein sequence database with the option "no-enzyme" should be performed to consider all possible cleavage sites in the parent molecule. This unconstrained parametrization significantly complicates the whole identification process and generates an enormous search space, which generally requests augmented computing time and memory. Preformed peptide databases have been used to reduce this search space, such in the case of clinically relevant compounds present in SATPdb (Singh et al., 2016) and SwePep (Fülth et al., 2006); no similar databases are available for medium-size to large food-derived peptides (Fig. 4). A peptide database building process should be facilitated by explorative experiments in which a portion of the sample under investigation is digested with trypsin and analysis of resulting peptides is performed according to a classical proteomic bottom-up experiment. As expected, most of the resulting peptides should originate from trypsinolysis, allowing the final recognition of the parent proteins and facilitating the construction of a preformed, size-reduced database. Semi-tryptic peptides caused by N-terminus and C-terminus processing should also be considered, and explored in a subsequent searching process limited to already characterized parent species.

Only recently, a novel customized procedure based on implementation of Compound Discoverer 3.0 software dedicated to the identification of metabolites was proposed for the recognition of short peptides (Cerrato et al., 2020). This software tool was set up with the purpose of leading to a comprehensive identification of all combinations of di-, tri- and tetra-peptides in various biological matrices, as deriving both from natural and modified amino acids. To this purpose, using MatLab authors compiled a database with all short peptide molecular formulas and mass values deriving from the combination of the 20 natural amino acids and 14 residues presenting the most common PTMs, and used it for data processing on Compound Discoverer 3.0 software. The developed data processing workflow was successfully applied to short peptide profiling in the human urine, comprehensive of various PTMs on amino acids. Later, it was also used for the analysis of short peptides in dairy products (Montone et al., 2021) and antioxidant peptides in soy flour-simulated gastrointestinal hydrolysates (Cavaliere et al., 2021).

Additional search engines used for proteomics, like MS InsPecT, SpectraST and BiblioSpec, are based on strategies focused on matching observed fragment spectra to a library of previously detected and high-quality annotated spectra based on the precursor mass (Falkner et al., 2008) (Fig. 4). To be effective, this kind of software requests the availability of comprehensive peptidomic spectral libraries (Wang & Bandeira, 2013). Some libraries exist for clinically relevant molecules but not yet for food-derived peptides. This factor can be considered as a limiting issue to the general applicability of the above-mentioned search engines to nutrition themes.

Peptide identification can also be obtained by using *de novo* sequencing algorithms, such as PepNovo (Frank, 2009) and Novor (Ma, 2015), which are based on the recognition of tandem spectrum peak patterns (Fig. 4). These search methods do not require *a priori* knowledge on protein database or reference libraries, and mathematical interpretation of fragmentation spectra allows establishing putative relationships between the mass values of the fragment ions and series of amino acids. However, these algorithms suffer from limitations due to the length of the peptide to be assigned and are highly affected by the eventual occurrence of PTMs. To bridge these gaps, novel search algorithms dedicated to peptidomics have recently been proposed (Budamgunta et al., 2018), which combine concepts of the above-mentioned strategies, such as sequence-tag assisted database searching and its variations using "spectral dictionaries" and gapped peptides, with classical procedures (Menschaert et al., 2010). In particular, Peaks and Peaks DB software have obtained a broad application in peptidomic studies, due to their initial pass *de novo* search that significantly reduces the search space for the subsequent database matching process. This user-friendly software can match peptides and their PTMs in complex data sets, with lengths up to 65 residues (Zhang et al., 2012). Similar routines are performed by the commercial Byonic software, which initially performs a small amount of *de novo* analysis to identify likely b- and y-ion peaks that are then used to extract candidate peptides from the database, with the number of candidates tunable to fit a computing budget. We successfully used the latter software to identify a high number of natural bioactive peptides in hen egg white and yolk (Arena & Scaloni, 2018; Arena et al., 2020). Above-mentioned data interpretation methods mixing *de novo* algorithms with classical database searching are nowadays reaching optimal results, especially when used to analyze data from mass spectrometers having very high mass accuracy and multiple peptide fragmentation outputs.

In proteomics, the generation of thousands of experimental and theoretical spectra have determined the need of controlling the confidence of peptide-to-spectrum-matches and filtering the search results. This is done by calculating the false discovery rate (FDR), which can be done specifically, by assigning error probabilities to each peptide-spectrum match *a posteriori* (Choi & Nesvizhskii, 2008), or globally, by calculating the proportion of decoy count-based identifications from the target database of interest and a related decoy (null) counterpart through a target-decoy approach (TDA) (Elias & Gygi, 2007). Very frequently, used decoy database includes the reverted protein sequences of the target database of interest. This computing effort provides a probabilistic score of the number of false positives obtained in the real experiment by using the target database. In general, the sampling statistics are enough to assess confidently the FDR value in proteomics; this is not the case of peptidomics, in which the FDR strategy might fail and/or suffer of evident limitations (Fig. 4). This result depends on the fact that the number of chance findings from the decoy database is often close to the number of PSMs from the target database. Accordingly, various authors suggested that the TDA method is designed not properly for evaluating the reliability of individual peptide identifications (Colaert et al., 2011; Gupta et al., 2011). Thus, most scientists have considered the standard FDR value of 1% adopted in proteomics not applicable to peptidomic studies, and have used higher values for peptide data search filtering and validation (Eng et al., 2011). Where forced to use TDA, they proposed that a randomized decoy strategy is the most appropriate, in which random sequences with the same distribution of amino acids, cleavage sites and peptide lengths as the target database are generated. To limit above-mentioned problems, similar decoy strategies have been used during spectral library searching with decoy library methods (Zhang et al., 2018). Nevertheless, this research field shows evident needs of dedicated solutions (Fig. 4).

As already stated above, additional difficulties may derive from the occurrence of PTMs or digestion-induced modifications of exogenous or endogenous origin that may hinder peptide assignment. In this context, *N*-terminal pyroglutamatylation (−17 Da), *C*-terminal amidation (−0.98 Da), deamidation of asparagine/glutamine residues (+1 Da), and isomerization of aspartic acid-containing motifs (+0 Da, but affecting fragmentation) have been described to occur in food-derived peptides after ingestion (Sato, 2018, 2022), whereas a number of PTMs (phosphorylation, acetylation, enzymatic/non-enzymatic glycation, oxidation, and others) are already present in food proteins before their administration. In addition to affect detrimentally peptide ionization properties, resulting in lower ion intensities, PTMs can determine production of less informative tandem mass spectra, especially when fragmentation spectra of multiply charged ions (as in the case of natural non-tryptic peptides) are considered. This unpredicted behavior can eventually complicate the peptide identification process whenever common bioinformatic tools for proteomics are used (Menschaert et al., 2010). To face these inconveniences, the concomitant use of multiple fragmentation methods has been proposed, although the acquisition rate of spectra from different peptides is affected. In this case, searching algorithms able using data from CID, ETD, HCD, ETD, ETciD and EThcD spectra are requested (Fig. 4). Notwithstanding above-mentioned issues, the inclusion of possible PTMs in the database search process results in a combinatorial growth of the search space. This increase is dependent on the number of considered PTMs, and yields is a significant rise in search time and false positive hits. Whereas the introduction of PTMs or process-induced modifications do not hinder final protein identification in proteomics, because it is very improbable that all tryptic peptides would carry a modification, the same condition has a significant impact in peptidomics. Possible peptide PTMs must be considered and the use of advanced algorithms allowing for blind and unrestrictive searches is suggested (Kong et al., 2017; Menschaert et al., 2009).

When putative peptides present in studied sample have been assigned, further verification and validation of experimental data can be accomplished. For example, recognition of the concomitant occurrence of related longer or shorter molecular homologues clearly increases the confidence for a certain peptide assignment, even if this condition cannot be considered a fully independent confirmation. Nevertheless, PSMs from similar peptides can increase the confidence of PSMs that are based on low quality fragmentation spectra. Additional peptide confirmation can be acquired by evaluating fragmentation patterns and fragment ion intensities (Budamgunta et al., 2018). To this purpose, a predicted fragmentation spectrum can be calculated, including fragment relative intensities, and compared to the real one. Various accurate software tools have been developed overtime for proteomic applications, such as MS2PIP (Degroeve et al., 2015), which perform well also for natural peptides. Final confirmation can be obtained by synthesizing a peptide standard, which will present identical chromatographic and mass spectrometric fragmentation characteristics with respect to the natural counterpart, when assayed under the same experimental setup.

It has been widely recognized that different search engines can yield variable results in peptidomices. Based on what reported above and similarly to what described also for proteomics (Kwon et al., 2011), the combined use of different search engines and strategies in peptidomics can highly augment the confidence in peptide identification (Menschaert et al., 2010). However, particular attention is need when combining information from multiple search methods. This can be especially true for FDR and evaluation of result's threshold confidence. In fact, some authors verified that the same set of peptide mass spectra studied with multiple search strategies have sometime resulted in

different assigned molecules with the same low FDR level (Shen et al., 2011). Accordingly, additional studies are necessary for the development of statistical error rate estimation methods (Fig. 4). Moreover, further algorithm implementations for the treatment of data from peptides bearing modifications are requested (Fig. 4). In this context, the recognition of modified peptides has been greatly improved by using spectral matching/clustering strategies (Menschaert et al., 2009). Finally, the development of additional bioinformatics tools for the prediction of proteases/peptidases involved in the generation of specific, experimentally observed degradomes might be highly valuable (Song et al., 2012). Regarding contaminants, software improvements allowed the recognition of non-peptide molecules prior to data interpretation (Dittwald et al., 2014).

7. Future scenarios and conclusions

Information described in this review emphasizes the occurrence of food-derived peptides in human and animal model tissues/body fluids, which can resist to gastrointestinal digestion, pass the epithelial membrane reaching the bloodstream and some organs, and are even excreted in breast milk and/or urine. These findings suggest that animal and human tissues/body fluids are pervaded of food-derived species, among which various peptides already proved having a specific biological activity. For most of these compounds, no information is available on their levels in the blood. For those few ones that were investigated for corresponding plasmatic concentration values after ingestion, results highlighted levels in the nanomolar range, which are lower than that (about 1–100 μM) generally used for *in vitro* assays (Matsui, 2018; Sato, 2022). Conversely, few peptides exhibited a certain resistance to proteases/peptidases and showed concentrations compatible with a certain molecular bioactivity. Thus, it remains uncertain if the presence of food-derived peptides in the blood can be associated with a significant biological effect. Accordingly, the actual study of the exogenous peptide content in tissues/body fluids is more relevant than ever. A variety of hurdles related to peptide extraction, complexity of biological samples, mass spectrometric and bioinformatic analysis had made in the past peptidomic research less straightforward, compared to proteomics. Recently, several of these problems have been partially circumvented with some technological improvements, which include dedicated sample preparation procedures, more sensitive and high-performing mass spectrometers, and advanced bioinformatic solutions. These recent advances in peptidomics, together with those that probably will be developed in the next future, hold the promise to disclose the possible functional role of ingested food proteins and corresponding digestion-derived peptides with the ultimate aim of tangling the complex relationship between diet and human health.

Contribution

S.C.: Data curation; Investigation; Writing - review & editing. G.Pic.: Data curation; Investigation; Writing - review & editing. G.R.: Data curation; Software; Validation; Visualization. S.A.; Data curation; Funding acquisition; Software; Validation; Visualization. A.D.T.: Data curation; Funding acquisition; Writing - review & editing. S.D.P.: Data curation; Resources; Software; Validation; Visualization. V.C.: Data curation; Resources; Software; Validation; Visualization. G.Pin.: Conceptualization; Data curation; Investigation; Writing - review & editing. F.A.: Investigation; Writing - review & editing. A.S.: Conceptualization; Data curation; Funding acquisition; Investigation; Supervision; Roles/Writing - original draft; Writing - review & editing.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tifs.2022.06.014.

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