Journal of Chromatography B 1229 (2023) 123904

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/jchromb

Tutorial review for peptide assays: An ounce of pre-analytics is worth a pound of cure

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ARTICLE INFO

Keywords:
Peptides
Stability
Adsorption
Guidelines
Surfactants
Proteases

ABSTRACT

The recent increase in peptidomimetic-based medications and the growing interest in peptide hormones has brought new attention to the quantification of peptides for diagnostic purposes. Indeed, the circulating concentrations of peptide hormones in the blood provide a snapshot of the state of the body and could eventually lead to detecting a particular health condition. Although extremely useful, the quantification of such molecules, preferably by liquid chromatography coupled to mass spectrometry, might be quite tricky. First, peptides are subjected to hydrolysis, oxidation, and other post-translational modifications, and, most importantly, they are substrates of specific and nonspecific proteases in biological matrixes. All these events might continue after sampling, changing the peptide hormone concentrations. Second, because they include positively and negatively charged groups and hydrophilic and hydrophobic residues, they interact with their environment; these interactions might lead to a local change in the measured concentrations. A phenomenon such as nonspecific adsorption to lab glassware or materials has often a tremendous effect on the concentration and needs to be controlled with particular care. Finally, the circulating levels of peptides might be low (pico- or femtomolar range), increasing the impact of the aforementioned effects and inducing the need for highly sensitive instruments and well-optimized methods. Thus, despite the extreme diversity of these peptides and their matrixes, there is a common challenge for all the assays: the need to keep concentrations unchanged from sampling to analysis. While significant efforts are often placed on optimizing the analysis, few studies consider in depth the impact of pre-analytical steps on the results. By working through practical examples, this solution-oriented tutorial review addresses typical pre-analytical challenges encountered during the development of a peptide assay from the standpoint of a clinical laboratory. We provide tips and tricks to avoid pitfalls as well as strategies to guide all new developments. Our ultimate goal is to increase pre-analytical awareness to ensure that newly developed peptide assays produce robust and accurate results.

Abbreviations: ACE, Angiotensin-converting enzyme; ACN, Acetonitrile; AcOH, Acetic acid; ADS, Analytical data sheet; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride; ANG, Angiotensin; ANP, Atrial natriuretic peptide; AVP, Arginine-vasopressine; BK, Bradykinin; BSA, Bovine serum albumin; BNP, B-type natriuretic peptide; CCK-85, Cholecystokinin-85; CHAPS, 3-[(3-cholamido-propyl)dimethylammonio]-1-propanesulfonat; CLSI, Clinical and Laboratory Standards Institute; CoA, Certificate of analysis; CRF, Corticotropin-releasing factor; CSF, Cerebrospinal fluid; DBS, Dried blood spot; DMF, Dimethylformamide; DMSO, Dimethyl sulfoxide; DoE, Design of experiment; DPP-4, Dipeptidyl peptidase 4; EC, Enzyme Commission; EDTA, Ethylenediaminetetraacetic acid; EMA, European Medicines Agency; EtOH, Ethanol; FA, Formic acid; FANG, FA 0.1%–NG 0.1%; FDA, Food and Drug Administration; GIP, Glucose-dependent insulinotropic polypeptide; GLP-1, Glucagon-like peptide-1; GnRH, Gonadotropin-releasing hormone; HCl, Hydrochloric acid; hProNPY, human pro-neuropeptide Y; IS, Internal standard; IUBMB, International Union of Biochemistry and Molecular Biology; IVS, Invitrosol; LC, Liquid chromatography; LLOQ, Lower limit of quantification; MaSDeS, MS-compatible degradable surfactant; MeOH, Methanol; MS, Mass-spectrometry; MS/MS, Tandem mass-spectrometry; NaOH, Sodium hydroxide; NG, *n*-nonyl-β-d-glucopyranoside; NP 40, Nonidet P-40; NPY, Neuropeptide Y; QC, Quality control; OG, Octylglycoside; OT, Oxytocin; PE, Polyethylene; PEG, Polyethylene glycol; PMSF, Phenylmethylsulfonyl fluoride; PP, Polypropylene; POMC, Pro-opio melanocortin peptide; PPS, PPS Silent Surfactant; PRA, Plasma renin activity; PSTI, Pancreatic secretory trypsin inhibitor; PTH, Parathyroid hormone; PTMs, Post-translational modifications; PYY, Peptide YY; RAAS, Renin–angiotensin–aldosterone system; RPG, RapiGest SF, and MaSDeS; ROS, Reactive oxygen species; SBTI, Soybean trypsin inhibitor; SDS, Sodium dodecyl sulfate; SIL, Stable isotope-labeled; SOP, Standard operating procedure; SPE, Solid phase extraction; TFA, Trifluoroacetic acid; WCH, White coat hypertension.

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https://doi.org/10.1016/j.jchromb.2023.123904
Received 7 September 2023; Received in revised form 4 October 2023; Accepted 5 October 2023
Available online 6 October 2023

1. Introduction

Peptide hormones are at the center of many biological processes, such as energy metabolism (insulin and glucagon), social bonding (oxytocin [OT]), bone remodeling (parathormone), reproductive function (GnRH), and blood pressure regulation (angiotensin [ANG]). They are composed of a chain of amino acids, making them a class of small proteins. If they are secreted by the body into the blood circulation and have a signaling effect, they are called peptide hormones. These hormones are usually secreted from a biologically inactive pre-propeptide precursor composed of an N-terminal signal peptide followed by the pro-peptide. The signal peptide is used to target the peptide to the endoplasmic reticulum. The inactive pro-peptide will then undergo several post-translational modifications (PTMs) along the *trans*-Golgi network to be activated and secreted from neuroendocrine cells. Through series of cleaving enzymes known as proteases, biologically active peptides are usually metabolized into fragments, a change that may abolish their function or switch their bioactivity into agonists for specific receptors or even antagonists for other receptors. Therefore, one pro-peptide may produce several peptide hormones with different activities and functions (Fig. 1), with the pro-opio melanocortin peptide (POMC) as the most emblematic example in endocrinology.

Recently, interest in quantifying peptide hormones (and their analogs) has increased dramatically because their biological function has attracted pharmaceutical companies to market peptidomimetics for treating diseases. However, quantifying fragments derived from the native peptide has not been possible until recently, given that immunoassays are not able to distinguish the different species present in biological fluids or tissues, and physical methods were not sensitive enough for their quantification. Technological advances with mass spectrometry have made it possible to quantify peptides down to the picomolar range, such as parathyroid hormone (PTH) and incretins [1–3]. In addition, there is a need to perform accurate pharmacokinetic studies on peptidomimetics during their preclinical and clinical development prior to their commercialization as new treatments based on peptide hormones or analogs, such as teriparatide (PTH1–34) [4], octreotide (somatostatin analog) [5], and semaglutide (a glucagon-like peptide-1 [GLP-1] analog) [6]. Hence, their levels in biological fluids must be quantified. A PubMed search with keywords "peptides AND quantification" provided 19,131 results between 1965 and September 2023, of which 54 % were from the last 10 years (10,382). The use of peptides as biomarkers or treatment is out of the scope of this review.

Immunoassays have been used for decades to quantify peptide hormones, but their lack of specificity [7–9] due to cross-reactivity with precursors and fragments of peptides often results in an biased quantification of the target peptide and explains the recent popularity of liquid chromatography coupled to mass spectrometry (LC-MS) [10,11]. This technology provides excellent specificity, especially when used in the tandem mode (LC-MS/MS). Moreover, it allows multiplex analysis, that is, measuring several compounds simultaneously—for example, all peptides of a particular metabolic pathway including those with PTMs. Because each fragment has different bioactivity, being able to quantify the fragments separately is of great interest. LC-MS/MS has been used successfully to diagnose tuberculosis using *Mycobacterium tuberculosis* antigen peptide [12], neurological diseases using amyloid β peptide [13], Wilson disease using ATP7B peptide [14], asthma, heart disease, and depression using cytokines [15].

Quantifying peptide hormones is a difficult and tedious task. The obvious reason is their very low circulating concentrations, mostly in the 0.1–100 pM range. Besides, peptide quantification is challenging for three other reasons. First, peptides are composed of amino acids linked together by peptide bonds prone to chemical modifications or degradation by hydrolysis, oxidation, glycation, and other post-translational events. Second, amino acids that comprise the peptide chain interact with their environment because of their positively and negatively charged groups and hydrophobic and hydrophilic residues. Third, the proteases involved in their cleavage are present in many biological tissues and, if soluble, may be also secreted and found in the samples. This is a concern because peptide degradation occurring *ex vivo* alters the peptide composition that was initially present at the sampling time.

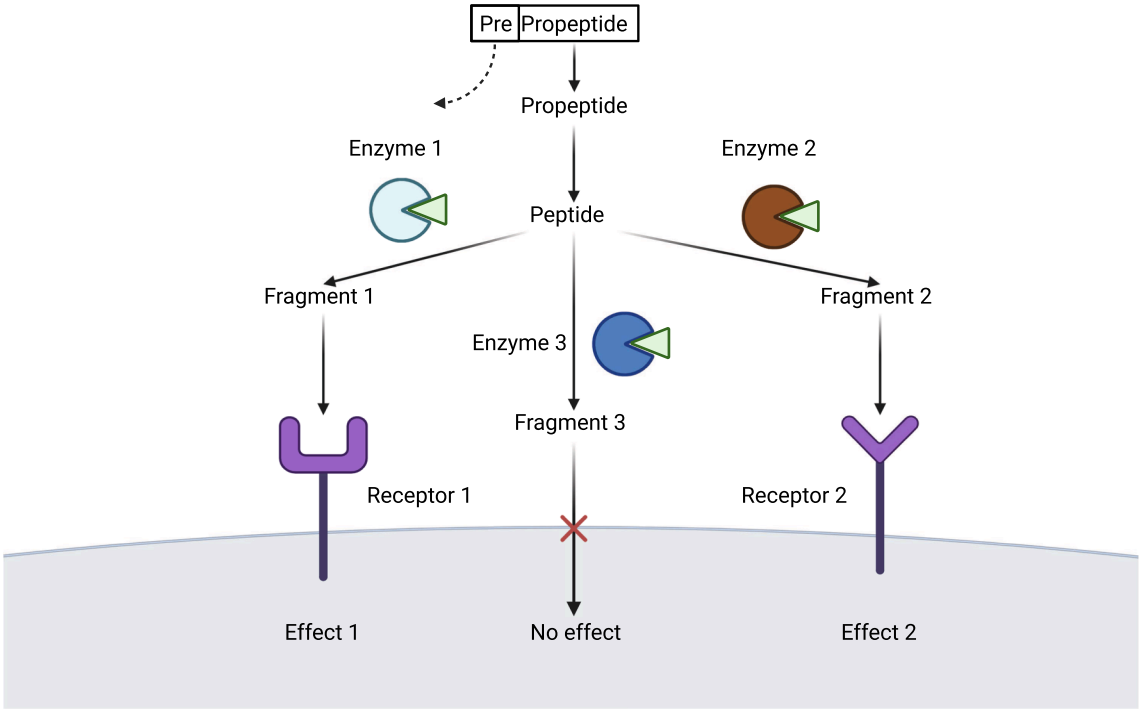

Fig. 1. Schematic representation of the switching bioactivity of peptides. It starts with an inactive pre-propeptide composed of a signaling peptide and a propeptide. The signaling peptide is used to target the peptide to the endoplasmic reticulum. The propeptide then undergoes post-translational modifications, which transform it into the active peptide. This peptide is further metabolized with enzyme 1, 2, or 3, leading to the production of peptide fragments. These fragments can have a different effect or no effect at all on receptors. Created with BioRender.com.

Thus, particular attention must be paid to preserve the concentrations of the native peptide and its fragments throughout the entire process, from the sampling to the quantification, so that what is measured reflects the actual peptide concentration. While great efforts are often placed on developing the sample preparation and of the mass spectrometry protocol, the impact of pre-analytical steps on the results is often underestimated and sometimes not even studied by laboratories unaware of these concerns during method development. The sampling time, the patient's diet and posture, sample handling, storage conditions, the container material, and protease activity are all factors that can lead to significant loss or increase the peptide concentrations. These pre-analytical steps may be difficult to control because sample collection and handling are often performed outside the laboratory. As highlighted by several independent groups, there is an urgent need for a standardized procedure as well as traceability to allow comparing between studies and to produce data that reflect circulating concentrations of peptides at a given time [8,16–30]. Therefore, pre-analytical preparation is still a challenge for laboratories developing peptide assays.

Guidelines for bioanalytical method validation tend to increasingly consider the pre-analytical steps as part of the validation process. While this increasing importance given to the pre-analytical steps in the assay validation has improved considerably, these guidelines still do not cover crucial aspects specific to peptides assays. For example, the 2018 Food and Drug Administration (FDA) guideline for bioanalytical method validation does not address the question of adsorption or stability during sampling, and the 2014 Clinical and Laboratory Standards Institute (CLSI) C62-A guideline only briefly discusses adsorption and stability [31,32]. The most recent 2022 European Medicines Agency (EMA) guideline on bioanalytical method validation and study sample analysis provides more details on how stability should be evaluated for the assay validation, but the adsorption aspects are lacking [33]. Finally, the 2021 CLSI C64 guideline "Quantitative Measurement of Proteins and Peptides by Mass Spectrometry" is the only guideline that addresses peptide stability and adsorption in detail, and discusses the issues related to PTMs [34]. Because of the lack of harmonization from one guideline to another, relying on them to validate a new assay might have tremendous effects on the standardization of the results obtained and does not guarantee their reproducibility.

By going through practical examples, the scope of this solution-oriented tutorial review is to report usual pre-analytical challenges encountered during the development of an assay from the standpoint of a clinical laboratory, and to propose strategies to facilitate and guide assay developments. We focus on MS-based assays because their specificity allows targeting peptides and highlights stability issues, whereas the pre-analytical considerations are also applicable to immunoassays on a case-by-case basis. This article is divided into two main chapters. The first focuses on handling standard peptides, from ordering them to method development, including assessing and controlling nonspecific adsorption to containers and evaluating the stability of neat peptides. The second main chapter focuses on biological samples, including peptide stability, sampling variations, and matrix effects. We do not consider the important aspect of calibrator standardization and their commutability because certified reference materials are not yet available. We believe that this tutorial will increase the awareness of analysts regarding the pre-analytical steps and pitfalls that may occur during peptide assay development and thus improve the reliability of their measurements.

2. Handling of standards

With a few rare exceptions, there are no commercial kit for peptide MS-based assays. Therefore, every assay is a laboratory-developed test, requiring in-house preparation of calibration curves and quality control samples from peptide standards.

To that end, laboratories have to purchase neat peptides with a certificate of analysis (CoA). Upon receipt, these peptides are deconditioned to be weighed, solubilized, transferred, dried, or diluted several times throughout the development of the analytical process. Because of their specific physicochemical properties, peptide losses may occur during these steps. The amino acid composition of the peptide defines its structure in such a way that it may induce nonspecific adsorption to containers, resulting in a significant loss of the peptide. The conditions in which peptides are stored for periods ranging from minutes to months also have tremendous effects on their stability. Residues such as methionine, tryptophan, and histidine are sensitive to thermal and photo-degradation, oxidation, and glycation depending on the matrix and pH of the storage solution. While some of these nonenzymatic modifications are most often not detected with immunoassays, the slightest change in peptide mass is enough to exclude them in MS-based assays, leading to underestimation of the results. Therefore, it is critical to assess the stability of the peptides to deal with every deviation or loss. Because no guidelines have previously been established to ensure the integrity of peptides in a matrix prior to their measurement, we propose an empirical methodological approach to stabilize endogenous peptides present in a biological fluid during their storage and extraction from their matrix before LC-MS/MS injection.

We discuss in detail the main problems encountered when handling standard peptides. Each subchapter is dedicated to commonly known physical or chemical degradation that has a strong impact on concentrations. Starting with methods to measure peptide stability, we provide in-house examples, followed by practical information to avoid pitfalls and to limit peptide degradation.

2.1. Peptide content and purity

Commercial peptide standards are provided as freeze-dried lyophilizates. This ensures the best stability (see subchapter 2.3) because peptides are sensitive to moisture and lyophilizates are the most convenient form to store and work with. Most providers offer an exact weight option, sometimes with aliquoting (e.g., 10 × 1.00 mg), performed on the solutions before freeze-drying. This approach is highly recommended because determining the amount of peptide is more accurate when measuring a volume than when weighing a lyophilizate. Alternatively, we suggest a method to perform this operation (see subchapter 4.3).

Along with the freeze-dried peptide, an analytical data sheet (ADS) or a CoA is provided. This document must include two important characteristics of the lyophilizate: the purity and the peptide content. Purity describes the amount of the target peptide compared with the total amount of peptide in the lyophilizate. Indeed, the lyophilizate contains not only the desired peptide, but also numerous by-products from peptide synthesis such as single amino acids or truncated peptides. Although very efficient, peptide purification after synthesis cannot completely remove these by-products. Purity is expressed as a percentage of the total amount of peptides in the lyophilizate (e.g., 80 %) and is usually determined by high-performance LC coupled to UV detection at the peptide bond wavelength (220 nm) by adding the area under the curves of chromatographic peaks on the chromatogram. The peptide content is the amount of the lyophilizate that is effectively peptides or amino acids because other compounds such as salts and synthesis intermediates (e.g., trifluoroacetic acid) are difficult to eliminate during peptide synthesis and purification. Finally, the peptide content is also expressed as a percentage (e.g., 70 %) and is determined by an amino acid analysis.

As a result, the net weight of the desired peptide in a preparation can be dramatically lower than the weight of the lyophilizate, termed the gross weight. This net weight of peptide is calculated as:

$$Netweight = Grossweight*Peptidepurity*Peptidecontent$$

This net weight must be used for the preparation of stock. With the previous example, the net peptide weight is:

$Netweight = 1.00mg*80\%*70\% = 0.56mg$

This means that only 56 % of the total mass is actually composed of the target peptide. Therefore, to obtain a 1 mg/mL solution, a 1.79 mg aliquot should be reconstituted in 1 mL of the adapted solvent.

2.2. Nonspecific adsorption

Adsorption is a very common phenomenon when working with peptides, but it is not often considered during assay development by analysts. Nonspecific adsorption of peptides to surfaces is multifactorial, may considerably affect results, and is one of the reasons for discrepancies observed between studies measuring peptides. It can happen in single use containers, pipette tips, other consumables, or in the LC system, and result in a complete loss of the peptide. For this reason, it is crucial to be aware of this matter and to establish strategies to control this concern.

The following sections describe the mechanisms of this physicochemical process using practical examples and provide ideas to prevent nonspecific peptide adsorption. Of note, because this tutorial focuses on pre-analytical steps, we do not cover adsorption in the analytical apparatus. However, this issue can usually be avoided in LC if several highly concentrated samples and/or samples originating from plasma are injected prior to peptide analysis. Fig. 2 presents representative chromatograms after injecting a standard peptide (angiotensin 1–9) into a new LC-MS/MS column. The chromatograms were obtained for that peptide before (top) and after (bottom) two injections of an extract from a plasma sample. The injection of such samples on a new column acts as a coating step (also called passivation) of the tubing and other parts of the LC system, and thus limits the subsequent adsorption of the target analyte. Readers interested in this LC issue are referred to any of the existing valuable contributions [35–37].

2.2.1. Definition and mechanism

Nonspecific adsorption is a physical and chemical surface phenomenon by which molecules or ions (adsorbates) from a liquid or a gas (fluids) adhere to a solid surface (adsorbent). This results in the formation of an adsorbate film on the adsorbent, with a concomitant local change in the adsorbate concentration in the fluid. This makes nonspecific adsorption a concern for every quantitative assay involving contact between surfaces and molecules with a propensity for adsorption.

Nonspecific adsorption occurs due to the noncovalent interactions (electrostatic and hydrophobic) between adsorbates and adsorbent [38,39], and is related to the equilibrium constant between surface and fluid. Therefore, it is not only influenced by the nature of the adsorbates (peptides containing basic amino acids, such as arginine or lysine, being more prone to adsorption) and adsorbent, but also by temperature, sample volume, and pH. Moreover, adsorption is time dependent, as a longer interaction period induces more binding events until a plateau is reached. For example, Willemse et al. [40] observed adsorption of amyloid β (1–42) after only 30 s, Law et al. [41] observed a plateau of calcitonin adsorption after 2 h, and Van Wanseele et al. [42] recovered only few percent of neurotensin after 3 h in water. Finally, this is a concentration-dependent phenomenon, with a less pronounced effect at high concentrations, when the analyte is in excess compared with the number of binding sites on a particular surface. This means that the lower the adsorbate concentration, the higher the impact of adsorption on the residual analyte in the fluid [43].

Any molecule can undergo adsorption, but proteins and peptides are more prone to it because of their ratios of charged to uncharged and hydrophobic to hydrophilic residues [44–47]. Although the impact of peptide adsorption is well known [46,48], only a few studies have discussed in detail its effect on quantitative results [42,49–52]. However, this tendency to bind to nonspecific binding sites is ubiquitous in every peptide assay: This phenomenon occurs in tips and vials during sample preparation, or directly in the analytical system, which results in carryover.

2.2.2. Evaluation of adsorption in containers

Because of the multifactorial nature of adsorption, each solution, material, and matrix used during an assay should be tested to measure its propensity to induce peptide adsorption. Moreover, because peptide hormones circulate at low concentrations, many of them in the range of 0.1–100 pM, it is crucial to test their adsorption at expected circulating concentrations, and especially at the lower limit of quantification (LLOQ). Indeed, the lower the concentration, the higher the impact of adsorption on the final concentration. Finally, it is important to study adsorption in every step of the assay. Because adsorption is a time-dependent phenomenon, strategies to reduce adsorption during

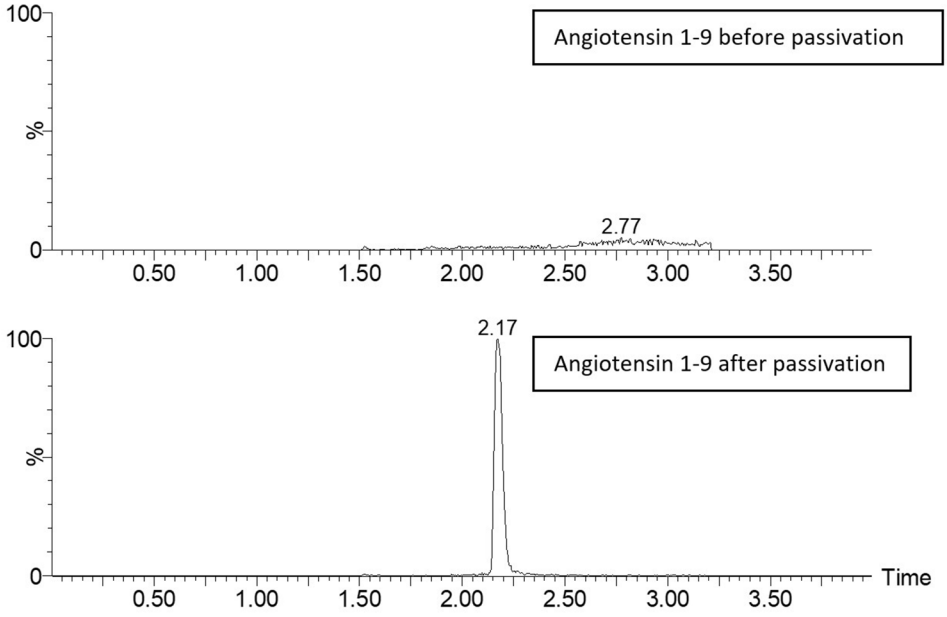

Fig. 2. Two LC-MS/MS chromatograms of a 1000 pM solution of angiotensin 1–9. They were obtained before (top) and after (bottom) two injections of an extract from a plasma sample. The plasma samples act as a coating agent, reducing the adsorption of angiotensin I in the LC system and increasing its detection.

storage (weeks to months), extraction (a few hours), or analysis (hours to days) will not be the same. For example, Murphy et al. [53] demonstrated that acetonitrile (ACN) 20 % prevent the adsorption of Vn96 and apomyoglobin, but only for samples stored for less than 2 h.

One simple and fast way to test the adsorption of a particular peptide in a container is to perform a multiple transfer test [34,54,55]. For this endeavor, a solution of the peptide, ideally at a concentration close to the LLOQ, is serially transferred in native vials. The adsorption is then determined by comparing the peptide concentrations in the transferred and untransferred solutions (see Fig. 3).

The classical protocol to study the adsorption in a container is as follows:

- Prepare a typical working solution of the peptide at the expected LLOQ concentration.
- Pipette up and down a few times and keep this tip for the next steps. This will ensure proper coating of the tip and that any adsorption observed is related to the container and not to the tip.
- Transfer 60 µL of solution to the container (vial, plate) to be tested; wait 10 min.
- Transfer this solution to another a container; wait 10 min.
- Repeat for a total of five times.
- Transfer this solution to a vial for injection in the LC-MS system → solution (a).
- Transfer 60 µL of the initial (untransferred) solution to another vial for injection → solution (b).
- Quantify the peptide in both solutions.
- Adsorption is calculated with the following equation (Eq. (1) and Fig. 3).
- Adsorptive loss should not exceed the imprecision of the considered method [34].

$$\%ofadsorption = \frac{a}{b} * 100 \quad (1)$$

Adsorption might also occur in the pipette tip, so it is important to test that by using a similar protocol: The peptide is transferred in several tips, with an unchanged vial. A possible protocol for tip adsorption is as follows:

- Prepare a solution of the peptide at the expected LLOQ concentration and split the solution into two vials → solutions (a) and (b)
- Take the solution of one of the two vial in a tip for 10 s → solution (a).
- Put back the solution in the original vial and discard the tip.
- Repeat 10 times in a row.
- Quantify the peptide in solutions (a) and (b).
- Adsorption is calculated with the previously presented equation (Eq. (1) and Fig. 3).
- Adsorptive loss should not exceed the imprecision of the considered method [34].

2.2.3. How to prevent adsorption

Once evaluated, nonspecific adsorption must be suppressed before the sampling procedure. Indeed, if nonspecific adsorption is not mastered, a modification of the number of transfers, the material type, or the matrix between two samples will alter the real measured concentration, precluding comparison between samples and overestimating the LLOQ of the assay.

Strategies to cope with nonspecific adsorption are based on modifying the equilibrium constant between surface and solution. Therefore, solvents, pH, adsorption competitors, vial types, pipette types, and surfactants are all factors that can be used to reduce adsorption. Here we describe the main strategies to cope with adsorption. For more details, we strongly recommend the review article by Maes et al. [35].

2.2.3.1. Influence of solvents and pH. The easiest and least expensive way to decrease adsorption is to improve the solubility of the peptides. Indeed, the higher the solubility, the lower the adsorption [35]. Most peptides are composed of ionizable and hydrophobic groups, which means that their solubility and adsorption change according to the proportion of organic solvent and pH. On the one hand, ACN or methanol (MeOH) are often used at concentrations between 5 % and 60 % by volume to increase solubility and decrease adsorption of peptides [9,56–61]. Solvents with the ability to solubilize both polar and nonpolar compounds, such as dimethyl sulfoxide (DMSO) and dimethylformamide (DMF), might also be good alternative, but they are not suitable for every peptide because they may induce uncontrolled oxidation of the peptides (see Section 2.3.1.1) [62,63]. It is noteworthy that the addition of a high percentage of organic solvent might make the samples unsuitable for the extraction and/or the LC-MS analysis.

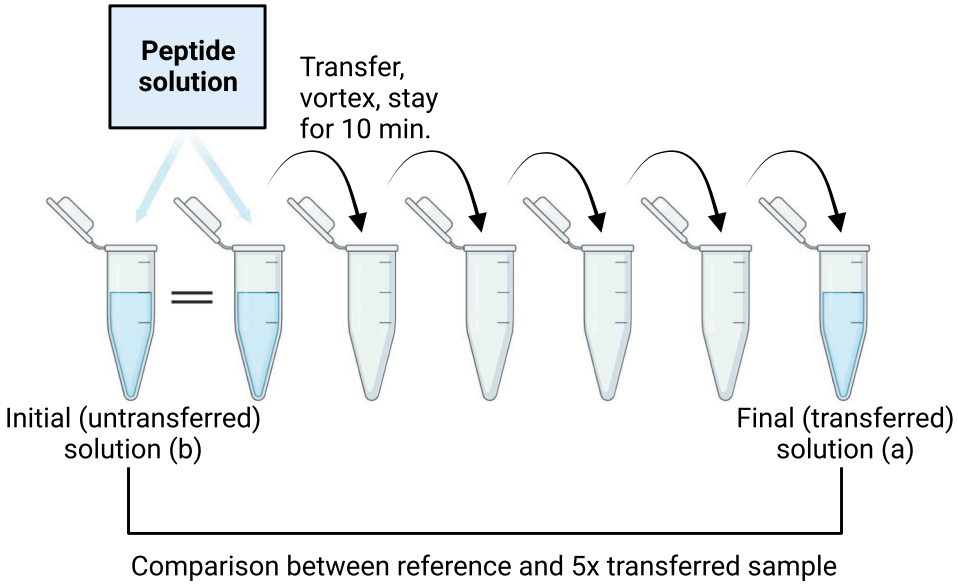

Fig. 3. Schematic representation of the transfer test to evaluate adsorption loss. A mother solution is added to two vials. In one, the solution is kept inside without transfer. In the other, the solution is transferred to a new vial, kept for 10 min, and then transferred to another new vial. This is done serially five times. To evaluate the loss caused by adsorption, the peptide remaining in the last vial is compared to the initial untransferred one. Created with BioRender.com.

Antibodies used in immunoassays requiring a sample treatment prior to their analyses do not tolerate organic solvents. On the other hand, ionic and dipole–dipole interactions might be reduced when the pH is increased or decreased, leading to a change in the adsorption. For example, Law and Shih [41] showed that the adsorption of calcitonin to glass increased when pH raises. This effect was interpreted as a change in the adsorption mechanism on glass that, above an acidic pH, goes from a monolayer to a multilayer formation. Ammonium bicarbonate is often used to alkalinize the solutions [37,64], while acetic acid (AcOH), formic acid (FA), or trifluoroacetic acid (TFA) (0.1 %–5% v/v) are used to acidify diluents [62,65–67].

To find the optimal conditions that limit nonspecific adsorption, designs of experiment (DOE) might be of great help. DOE provide a better understanding of the influence of several factors using as few experiments as possible. By plotting a three-dimensional response surface, it becomes easy to find the optimal conditions. For readers interested in the way DOE can be applied to adsorption issues, we recommend an article by Maes et al. [68].

As a general practice rule, we recommend testing various compositions of solvent and pH to see if it changes solubility and adsorption of peptides, before going deeper into the troubleshooting. This will show, without any optimization, whether a change in solvent composition can help. As seen in Fig. 4 with the reconstitution solution used for endogenous human proNPY extracted from plasma sample, an increase in the FA concentration is associated with higher areas after three transfers, while ACN 20 % does not change the adsorption. However, in this example, the solvent composition has a 50-times lower effect than the use of a surfactant (for surfactants, see Section 2.2.3.3).

2.2.3.2. Influence of the materials. Because adsorption is the result of an interaction between an adsorbate in a fluid and an adsorbent, it is also influenced by the adsorbents properties—that is, the properties of the inner surface of containers and tips that are used to transfer and store the standard peptide. Usually, tubes and vials are either plastic or glass based, while tips are plastic based. Plastic (polypropylene [PP], polyethylene [PE], and polymethylpentene) induces hydrophobic interactions with the peptide hydrophobic residues, while glass (borosilicate glass, etc.) induces electrostatic interactions between the negatively charged silanol groups and the positively charged peptide [35,43,69]. Overall, peptides tend to adsorb more onto hydrophobic surfaces, making the choice of plastics a real challenge [46].

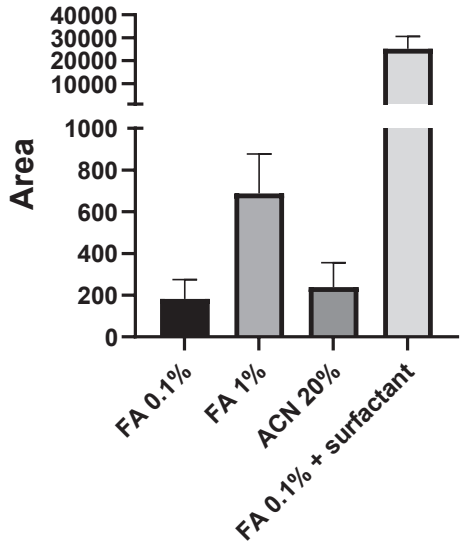

Fig. 4. hProNPY adsorption test. Endogenous level of human proneuropepetide Y (hProNPY) recovered from plasma, expressed as LC-MS/MS peak areas. Each bar represents a solubilization solution used before injection in the LC-MS/MS system. FA. formic acid; ACN. acetonitrile.

Companies are aware of the adsorption issues and have developed vials and tips of various nature that may reduce the adsorption. For polymer-based materials, either the tips and vials are coated with resin to create a barrier between the surface and the peptide, or their surface and/or composition are modified by using different combination of polymers and thus take advantage of their various properties to reduce the surface hydrophobicity. For glass-based materials, the aim is to prevent adsorption caused by the negatively charged silanol groups on the surface. To do so, glass might be coated with alkoxysilane molecules that will form covalent bond with the surface (a process called silanization), or the manufacturing process is changed to limit the formation of silanol groups. These vials and tips are summarized in the non-exhaustive lists presented in Tables 1 and 2.

The adsorption on a given material should always be considered in combination with a solvent composition. Indeed, the state of the ionizable groups of a peptide, which is dependent on the solvent composition, changes its propensity to stick to a particular surface [70]. Thus, in a given material, the adsorption of a peptide may be low when in a solvent A but high in a solvent B. For instance, silanol groups in lab glasswares have a mean pka of 4. The addition of acids will neutralize those groups, reducing the ionic interactions with the positively charged groups of

Table 1
A nonexhaustive list of the main vials available, with their composition and the specificity given by the supplier. The data were collected in May 2023.

Product name	Material	Specificity	Supplier
QuanRecovery with MaxPeak HPS	PP	High-performance surface	Waters
Eppendorf LoBind Tube	Polymer	Two compound polymers with a hydrophilic surface	Eppendorf
Low Protein Binding Microcentrifuge Tubes	PP		Thermo Scientific
Posi-Lock™ Low-Binding Microcentrifuge Tubes		Low-binding resin	LabForce
MiniSorp™	PE		Thermo Scientific
Ultra High Recovery Microcentrifuge Tube	PP		Starlab
Corning® Costar® low binding microcentrifuge tubes	PP	Bonded polymer technology	Merck
Low Binding MCT	PP		BioScience Inc.
Kinesis Snap Top Vial	Polymethylpentene	Silanized fused insert	Antylia Scientific
Total Recovery	Glass		Waters
Low Adsorption vial	Glass	Silanization	Shimadzu
TORAST-H glass vial	Glass	Torast-H special processing	Shimadzu
RSA™ Glass	Glass	Reduced surface activity glass	MicroSolv Technology Corporation
RSA-Pro X™ Glass	Glass	Reduced surface activity glass coupled with a surface treatment	MicroSolv Technology Corporation
KimShield Deactivated Glass	Glass	Surface deactivated for samples when silanization is not effective	Capital Analytical
National Silanized sample vial	Glass	Silanization	Thermo Scientific

PP. polypropylene; PE. polyethylene.

Table 2
A nonexhaustive list of the main pipette tips available, with their composition and their supplier. The data were collected in May 2023.

Product name	Material	Supplier
Qualitix low binding tips	Silicone-free resin	Socorex
Corning® DeckWorks™ low binding tips	PP	Merck
Low Retention Pipette Tips	PP	Sartorius
Low Retention Pipette Tips	PP	Thomas Scientific
Low Binding Pipette Tips	PP	BioScience Inc.
TipOne® tips	PP	Starlab

PP. polypropylene.

peptides.

In the following example (Fig. 5), we performed an adsorption test with a 1.2 nM solution of angiotensin I (ANG I) in FA 0.1 %. After being serially transferred five times in Eppendorf tubes, Minisorp tubes, or a Waters 700 µL collection plate, we transferred triplicates of 10 µL of the remaining solution to a collection plate. We added an internal standard solubilized in a solution of 0.1 % FA and 0.1 % *n*-nonyl-beta-d-glucopyranoside (NG), FA 0.1 %–NG 0.1 %, to the sample before we evaporated it to dryness. Another sample remained untransferred and we added it directly to the collection plate before drying. We solubilized samples in a solution of FA 0.1 %–NG 0.1 %, injected them into a LC-MS/MS, and compared the ratio between ANG I and its internal standard. The results showed that 20 % of the peptide in FA 0.1 % was lost after five transfers in a Waters 700 µL collection plate or an Eppendorf tube. On the other hand, adsorption was prevented when using the Minisorp tube.

In conclusion, we recommend having a few samples of different vials and tips of various materials to rapidly test their effect on peptide adsorption. It is also important to keep in mind that adsorption depends on the surface and volume. Therefore, the containers should be as small as possible to contain the peptide, and the volume should be kept identical between samples. Finally, because the pipette tips suffer from the same adsorption issues than vials [40], a general good practice rule is to prerinse the tips by pipetting up-and-down with the sample several times before transferring the desired volume. This will act as a coating step, reducing the adsorption.

2.2.3.3. Adding surfactants. Changing solvents, vials, and/or pH is sometimes not sufficient to limit adsorption in containers. Therefore, a second strategy to reduce nonspecific adsorption in containers is the addition of surfactants in every solution containing the sample. For example, OT is completely lost when water is used as solvent, but fully recovered when a surfactant is added (Fig. 6). This effect is independent on the number of transfers.

Surfactants, or surface-active agents, are amphiphilic compounds that contains both hydrophilic and hydrophobic groups (see Fig. 7). By adsorbing the inner surface of vials or tips, surfactants create a barrier between the solid and the liquid phase, changing the surface hydrophilic or hydrophobic properties, decreasing the surface tension, and thus modifying peptide adsorption [38,46]. Moreover, their amphiphilic nature increases unfolding and dissolution of peptides [46,47,71], and might also help de-adsorbing an already adsorbed peptide [72].

Although surfactants may lower the peptide adsorption, they are rarely used in MS-based assays. Indeed, surfactants such as Tween-20, Tween-80, Triton X-100, octylglycoside (OG), sodium dodecyl sulfate (SDS), 3-[(3-cholamido-propyl)dimethylammonio]-1-propanesulfonat (CHAPS), Nonidet P-40 (NP 40), and NG (also known as C9-Glu or NNGP) strongly decrease the LC-MS signal due to their effect on ionization, or affecting the extraction procedure [35,71,73–75]. However, Barco et al. [76] studied the ion suppression effect of various surfactants and show no ion suppression with cationic and nonionic surfactants. They observed low ion suppression due to ion pairing when different types of surfactants were combined.

These nuanced findings regarding the suitability of surfactants in MS-based assays explains why more and more surfactants are used by various groups. As shown in Table 3, low amounts (0.005 %–1%) of surfactants (mostly Tweens, polyethylene glycol, and NG) added to the

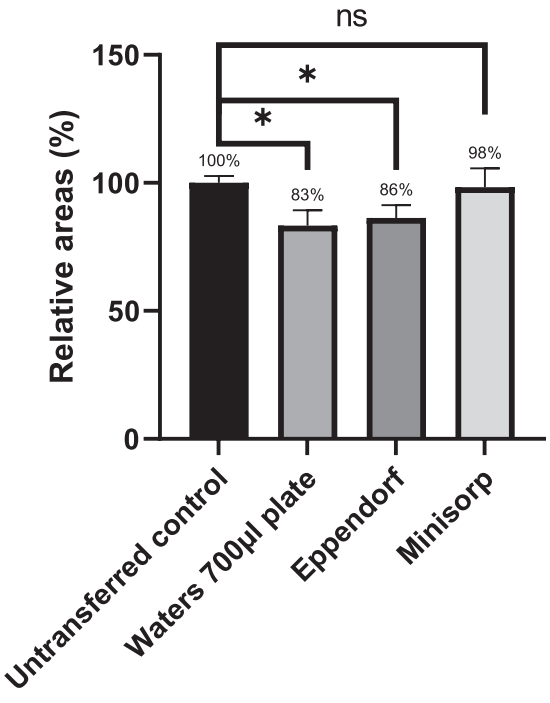

Fig. 5. ANG I ratio area/IS in 0.1 % FA. Relative areas recovered from 60 µL of a 1.2 nM angiotensin I (ANG I) solution in 0.1 % formic acid, following an adsorption test in four different vials. The untransferred control condition is in a Waters 700 µL collection plate. Each condition was done in triplicate. There was a 17 % and 14 % loss when ANG I was serially transferred to a Waters 700 µL collection plate and Eppendorf tubes, respectively. The loss was negligible in Minisorp tubes.

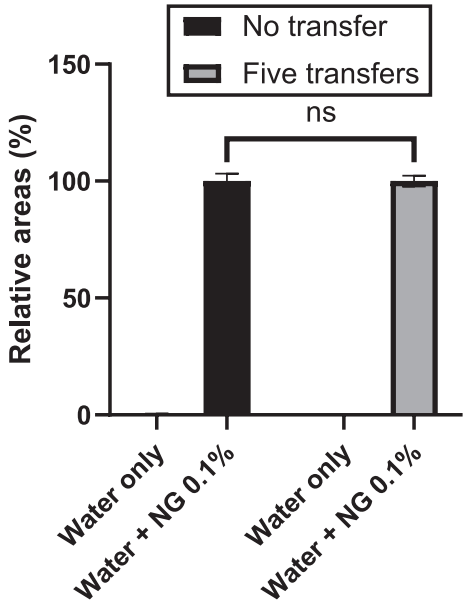

Fig. 6. Oxytocin adsorption test. Relative areas recovered from 1 ng/mL oxytocin (OT) solubilized in water, with or without the addition of n-nonyl-beta-d-glucopyranoside (NG) 0.1 % (v/v). The black columns indicate no oxytocin transfer while the gray columns indicate OT was transferred five times. Adsorption of OT occurs even when no transfer is done. NG 0.1 % efficiently prevents OT adsorption.

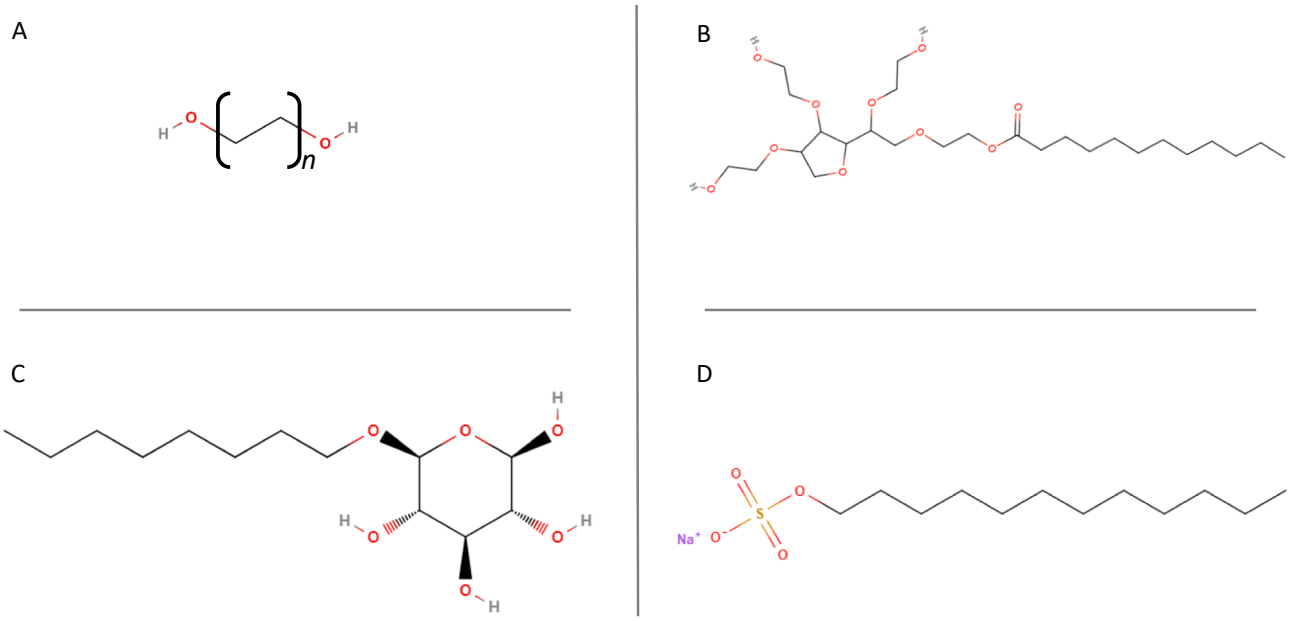

Fig. 7. Chemical structure of the main surfactants used in peptide assays. A. Polyethylene glycol; B. Tween 20; C. Glucoside; D. Sodium dodecyl sulfate. Structure obtained with molview.org.

solutions in contact with the peptide (but not the mobile phases) are usually enough to lower the adsorption or de-adsorb a peptide from a surface without affecting the sensitivity [72]. To our knowledge, some surfactants are retained in the LC in reverse phase mode, and they elute with a high organic composition. Therefore, if the target peptides are eluted sooner, it might be quite easy to use the divert valve to send the surfactants to the waste and thus avoid any troubles with the mass spectrometer. Moreover, MS-compatible detergents such as RapiGest SF (RPG, Waters Corporation), Invitrosol (IVS, Invitrogen), PPS Silent Surfactant (PPS, Protein Discovery, Inc.), and MaSDeS are available on the market and might be good options to avoid ionization issue [71,77–79]. Finally, sample preparation might integrate a step in which surfactants are removed from the sample. Yeung et al. [80] presented an efficient way to remove OG, SDS, NP-40, or Triton X-100 by using ethyl acetate extraction which reduces the interferences; Xu et al. [81] optimized solid phase extraction (SPE) to get rid of Tween-20; Quirino [82] removed SDS by adding cyclodextrins to the sample; and Antharavally et al. [83] used Thermo Scientific Pierce Detergent Removal Resin to remove efficiently SDS, Triton X-100, NP-40, and CHAPS prior to MS detection. However, these extra detergent removal steps increase the cost and time needed for the assays as well as peptide loss. Because the effect of surfactant on peptide recovery is dependent on the peptide quantified, the type of surfactant, and its concentration, an empirical approach for each compound is appropriate.

A protocol used routinely in our lab consists of adding NG to the sample and every solution used for elution and solubilization. For peptides with strong adsorption behavior, such as NPY [66], we add 5 µL of NG 10 % to 500 µL plasma samples prior to the extraction. The elution solution for SPE and the reconstitution solution always contain NG 0.1 %. This completely prevents NPY adsorption without affecting the analysis. For peptide with lower adsorption behavior, NG 0.1 % is sufficient to prevent adsorption in the elution and solubilization solution.

Fig. 8 shows a transfer test in Eppendorf tubes with 1000 pM vasopressin (AVP) solubilized in MeOH 10 %–acetic acid 1 %, with or without the addition of NG 0.1 % (v/v). The results showed that 50 % of the AVP adsorbed to the Eppendorf tubes when no NG was added, while loss was abolished with 0.1 % NG added to the solution (unpublished data).

The combination of several approaches often gives the best results. In the following example (Fig. 9), 3.75 ng of ANG 1–10 lyophilized in Minisorp tubes were solubilized in 250 µL of FA 0.1 %, FA 0.1 %–NG 0.1 % (FANG), or MeOH 20 %–FA 0.1 %–NG 0.1 %. After vortexing the sample, 10 µL of the solution was added to a 700 µL Waters collection plate, dried, resolubilized in 30 µL of FANG, and injected. The worst solubilization of ANG I occurred when FA 0.1 % was used. The addition of NG 0.1 % as a surfactant greatly helped the solubilization. Finally, the combined use of NG 0.1 % and MeOH 20 % provided the highest recovery.

2.2.3.4. Surface coating with competitors. During the development of a PTH 1–34 LC-MS/MS assay, we found that PTH 1–34 immunoextracted and purified by SPE from its plasma matrix adsorbed strongly to the vial's surfaces within minutes, precluding proper analysis of more than five samples. The addition of organic solvents and modification of the pH did not solve the problem. The use of surfactant was also not satisfactory. Finally, we prevented the adsorption by filling the vials with a solution of bovine serum albumin (BSA) 0.5 % (m/v) overnight at room temperature. We then washed the vials three times with water and dried under a nitrogen flow and used then for the analysis. Fig. 10 shows that BSA coating efficiently prevented the adsorption of PTH 1–34 from a 100 pM solution [99].

This third strategy to cope with nonspecific adsorption in containers is based on the competition for the binding sites between analytes and the so-called adsorption competitors, such as BSA or Prionex, a protein stabilizer. These molecules have a high tendency to adsorb onto surfaces. Therefore, by saturating the nonspecific binding sites of the containers, the competitors limit the peptide adsorption.

The general procedure for a coating step of a container is as follows:

- Incubate the selected containers with highly concentrated solutions of competitors for hours. The incubation time is important and must be optimized, as the binding process is time dependent.
- The containers are then emptied, rinsed (e.g. with water) to get rid of the unbound competitor molecules, and dried.
- With most binding sites occupied, the containers are then ready to be filled with the solution containing the analyte.

The same effect occurs when pipetting the peptide up-and-down to prevent changes in concentration due to adsorption on pipette tips: The peptide itself acts like a competitor, saturating the tip's binding sites and

Table 3
Summary of the main surfactants used in mass spectrometry–based assays to reduce adsorption. Surfactants are sorted in five categories that represent their structure. tween, polyethylene glycol, glucoside, sodium dodecyl sulfate, and others.

Surfactant category	Analytes (concentration)	Matrix	Surfactants	References
Tween	Thyroglobulin (6 pM)	Human serum	Tween-20 0.005 %	[84]
	3-[5-(4-Methanesulfonyl-piperazin-1-ylmethyl)-1H-indol-2-yl]-1H-quinolin-2-one (1.5 ng/mL)	Urine	Tween-20 0.2 % (v/v)	[81]
	Lactate dehydrogenase (0.02 mg/mL)		Tween-80 0.01 % (w/v)	[85]
	BAF312 (0.02 ng/mL)	Urine	Tween-80 0.5 % (v/v)	[72]
PEG	MMP-12		DDM, PEG400-C8, and PEG600-C8 at 0.0033 %	[86]
	Tryptic digest of six bovine proteins (100 pM)	0.1 % FA	PEG 2000 0.001 % (w/v)	[69]
Glucoside	Model peptide MP1 (20 µg/mL)	0.1 % TFA	NG 0.03 %–0.05 % (w/v)	[55]
	NPY and fragments (0.03–0.16 pM)	Human plasma	NG 0.1 % (w/v)	[66]
	NPY and precursors (0.16–8.72 pM)	Human plasma	NG 0.1 % (w/v)	[87]
	Leucine-enkephalin Goserelin Bovine insulin Buserelin Mouse obestatin (2 µg/mL)	Water	NG 0.05 % (w/v)	[43]
	AP102 (somatostatin analog) (pg/mL level)	Plasma	NG 1 % (v/v)	[88]
	PYY, PP, NPY (0.5–3 pM)	Human plasma	NG 0.1 % (w/v)	[67]
	Protease digest		OG 0.4 % (v/v)	[73]
	Protease digest		OG 0.1 % (v/v)	[89]
	Hela cell lysate protein (0.05 ng/µL)	0.1 % FA	DDM 0.015 %	[90]
	Protease digest		Laurylmaltoside 0.1 %	[91]
SDS	Protein in *Escherichia coli*		SDS 1 % (w/v)	[92]
	Trypsin digestion		SDS 0.1 % (v/v)	[74]
	Mouse liver proteins	Mouse liver	SDS 0.1 % (v/v)	[93]
Other	Tropomyosin	Black tiger prawn	RapiGest SF	[94]
	Kn2-7/dKn2-7	Human serum	RapiGest SF surfactant 10 % (v/v)	[95]
	Ubiquitin		Prionex 0.1 % and Triton X-100 0.01 %	[96]
	Insulin and C-peptide (0.09 and 0.06 ng/mL)	Plasma	Zwittergent 3–16 0.001 % (v/v)	[97]
	Tryptic BSA	BSA and albumin/IgG-depleted serum	IVS used to resolubilize the lyophilized peptides	[79]
	Protein digest		Sodium 3-((1-(furan-2-yl) undecyloxy) carbonylamino) propane-1-sulfonate	[98]

BSA. bovine serum albumin; DDM. n-dodecyl-β-D-maltoside; FA. formic acid; MMP-12. Matrix metallopeptidase 12; NG. n-nonyl-beta-d-glucopyranoside; NPY. Neuropeptide Y; OG. octylglycoside; PEG. polyethylene glycol; PYY. peptide YY; PP. pancreatic polypeptide; SDS. sodium dodecyl sulfate; TFA. trifluoroacetic acid.

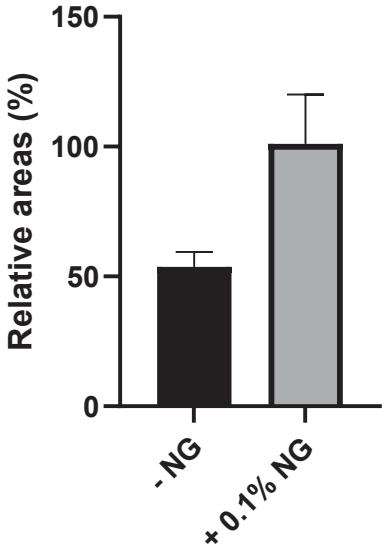

Fig. 8. AVP adsorption test. Relative areas recovered from 1000 pM vasopressin (AVP) solubilized in methanol 10 %–acetic acid 1 %, with or without the addition of n-nonyl-beta-d-glucopyranoside (NG) 0.1 % (v/v). This surfactant efficiently prevented the loss of AVP during an adsorption test.

limiting adsorption. It is noteworthy that this peptide adsorption might be reversible, as demonstrated by Penna et al. [100]. Therefore, to avoid any contamination issue related to the release of the bound peptide, we strongly recommend saturating the binding sites of the tip with a different peptide than the one targeted by the method. For instance, it is possible to use a stable isotope-labeled peptide with a molecular weight different from both the internal standard and the target peptide as coating agent. This method can also be employed to coat other containers.

Verbeke et al. [101] reported a 2-hour incubation of their vials with a highly concentrated hydrolyzed and precipitated BSA solution acidified with FA 0.1 %. This method showed promising results on a set of 36 representative peptides. Other groups have used Prionex 0.1 %–0.01 % [96,102] or PEG 20000 1 % (w/v) [103] to limit adsorption. Another strategy proposed by Goebel-Stengel et al. [104] relies on the addition of BSA directly to the tubes containing the sample. The recovery was improved for each tested peptide (nesfatin-1, peptide YY [PYY], leptin, insulin, GLP-1, ghrelin, corticotropin-releasing factor [CRF], and Cholecystokinin-85 [CCK-85]) when BSA 1 % was added to the solutions, regardless of the used vial type. Finally, Suzuki et al. [105] reported that the adsorption of ANG peptides was efficiently prevented when aqueous ACN 5 % containing 50 mM arginine was used during sample preparation and MS analysis.

This coating procedure presents some limitations. First, it is time consuming because every container has to be prepared prior to use.

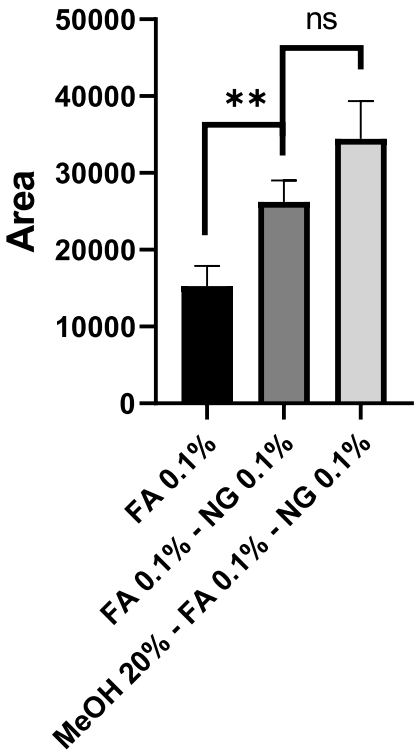

Fig. 9. ANG I solubilization comparison. Areas recovered from 1500 pg/mL angiotensin I (ANG I) after a solubilization test in formic acid (FA) 0.1 %, FA 0.1 %– n-nonyl-beta-d-glucopyranoside (NG) 0.1 %, or methanol (MeOH) 20 %–FA 0.1 %–NG 0.1 %. The experiment was performed in triplicate.

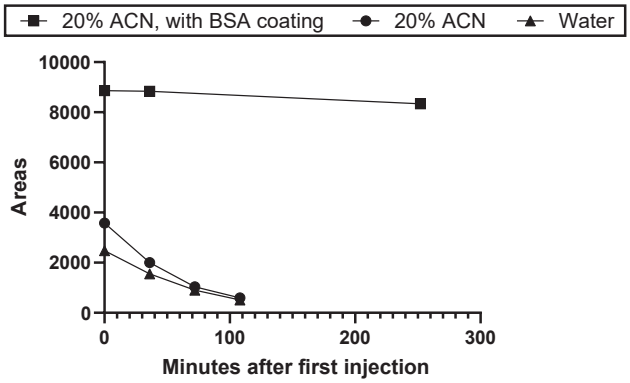

Fig. 10. PTH 1–34 adsorption. Areas obtained after injecting a 100 pM solution of parathyroid hormone (PTH) 1–34 solubilized in water, acetonitrile (ACN) 20 %, or 20 % ACN in a bovine serum albumin (BSA)-coated vial. The same solution was kept in the autosampler and injected at T0 and after 35, 70, and 110 min. The solution in the coated vial was also injected after 250 min. The graph highlights the adsorption over time. It was slightly reduced by ACN, but greatly prevented by the BSA coating step.

Second, the conditions of the sample preparation might be extreme and affect the competitor stability. If the competitor undergoes desorption from the surface during the sample preparation, it loses its effect, and the analyte will adsorb onto the surface. Finally, the type of material used can affect extent to which the competitors will stay onto the surface, and thus the duration of their effect on adsorption. This is why materials should be selected cautiously. For example, Murphy et al. [53] combined a wise choice of vials and the addition of carbonic anhydrase and ubiquitin to limit adsorption of the synthetic peptide Vn96 and apomyoglobin. We found that after 72 h, adsorption of PTH 1–34 was still efficiently prevented by BSA 0.5 % (m/v) in PP vials (Brown Chromatography Supplies) but not in Total Recovery Vials (Waters Corporation) or in clear glass vials (Interchim) (Fig. 11).

2.2.3.5. Alternative strategies. When adsorption cannot be reduced, one can estimate its effect to correct the analytical results. For example, when working with cerebrospinal fluid (CSF) to evaluate amyloid β (1–42), a strategy proposed by several authors relies on the use of amyloid β (1–40), which has similar adsorption behavior as the targeted one. By calculating the ratio of the two molecules, they were able to estimate the adsorption and reduce its impact on the results [40,106]. Another group working with these CSF biomarkers considered the surface-to-volume ratio of the storage tubes [107]. While these strategies help to obtain robust results, they do not cope with the analyte loss, eventually resulting in lower sensitivity of the assay.

2.2.4. Conclusion

Peptide nonspecific adsorption might occur on every surface, from pipette tips to vials, and at any time from sampling to extraction to analysis, resulting in irreversible loss of the peptide and eventually inducing lower sensitivity and precision and biased results [108,109].

To study the behavior of the peptide regarding adsorption, we propose a technique based on serial transfer of the solution containing the peptide into vials and pipettes used for the extraction. Once adsorption has been determined and quantified, several strategies might be involved to limit nonspecific adsorption. Most of the time, a combination of strategies is the best choice (see Fig. 12).

First, the easiest way to limit the adsorption is to change the solvent composition. By increasing the organic proportion (MeOH/ANC 5 %–60 %) or changing the pH (0.1 %–5% acid or base), peptide solubility may increase and adsorption decrease. Second, because adsorption is strongly related to the properties of the surface, changing, for example, from glass to PP might strongly reduce adsorption. Therefore, we recommend any lab working with peptide to have a large collection of different vials ready for adsorption studies, including glass, PP, and Minisorp, and to test them with various solvents.

If changing the solvents composition or the materials does not help to decrease adsorption, the addition of surfactants (Tween, NG, and PEG) at low concentrations often dramatically reduces adsorption by creating a barrier between the surface and the peptide. This is a technically easy and cost-effective trick, but the effects of the surfactant on sample

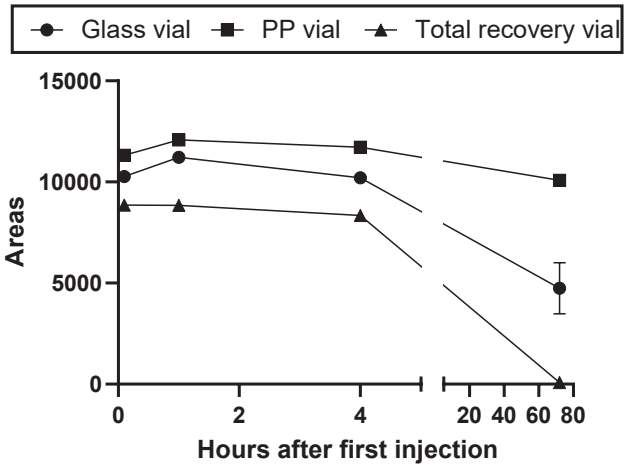

Fig. 11. PTH 1–34 adsorption after BSA coating. Areas obtained after the injection of a 100 pM solution of parathyroid hormone (PTH) 1–34 solubilized in acetonitrile (ACN) 20 % in three different bovine serum albumin (BSA)-coated vials (glass, polypropylene [PP], and Total recovery vials). The same solution was kept in the autosampler and injected at T0 and after 1, 4, and 70 h. The efficiency of the coating step to prevent adsorption loss was greatly affected by the type of vial used.

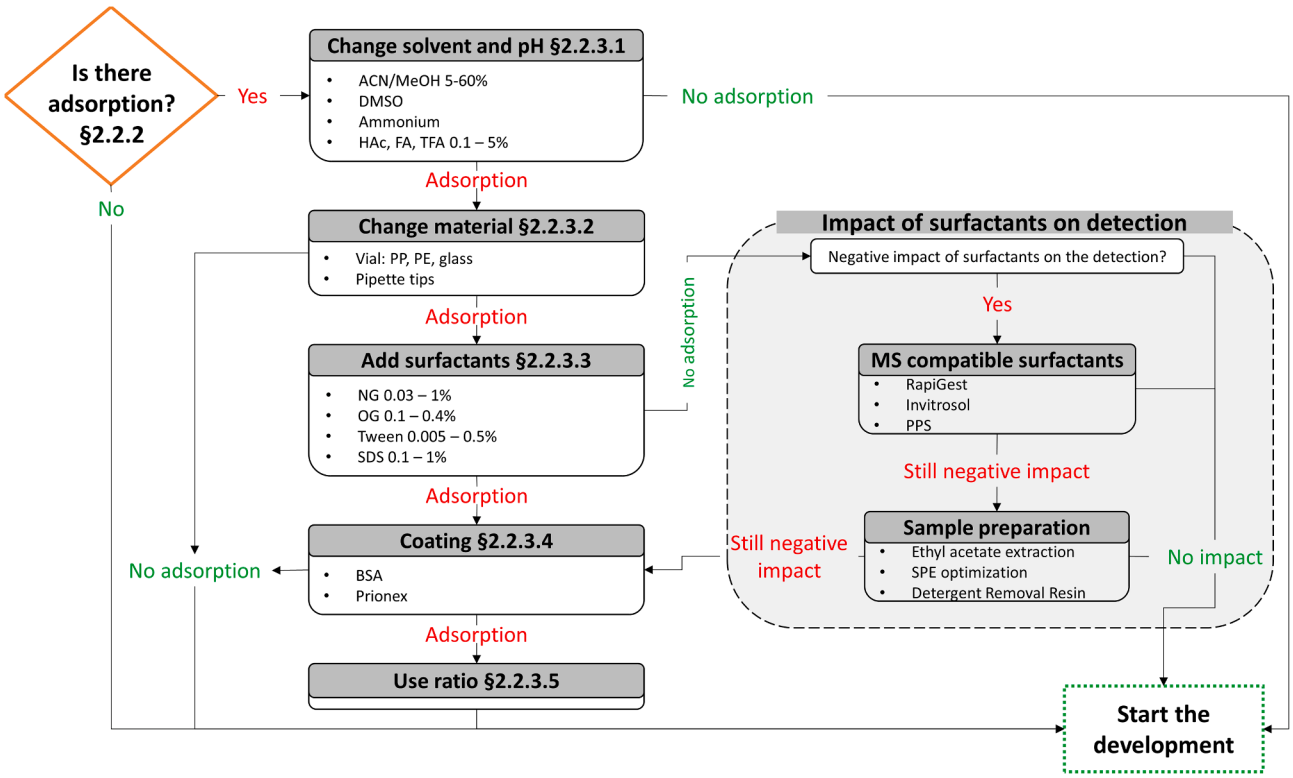

Fig. 12. Suggested workflow for the control of peptide nonspecific adsorption. The square box represents the starting point, and grey-colored boxes indicate the strategies adopted with their corresponding Sections.

extraction and ionization must be carefully studied. We strongly recommend having NG in any lab working with peptides because this surfactant shows excellent anti-adsorptive properties for almost every peptide we have tested in our laboratory, with almost no effect on the LC-MS/MS assay performance.

Finally, coating the containers with competitors is another way to prevent adsorption of peptides. By allowing molecules with known adsorption behavior (BSA and Prionex) to remain in the containers, one can saturate the binding sites prior to the addition of the peptides, reducing its adsorption. From our perspective, this should be a method of last resort. Indeed, the process is long, fastidious, and requires perfect optimization to ensure that the competitors stay in place during storage and extraction.

2.3. *Neat peptide stability*

Whether in a hydro-organic solution or as a freeze-dried standard, peptides are affected by their environment, such as the energy provided by heat or by oxidizing agents such as oxygen. This enables many reactions that ultimately lead to changes in the structure of the peptides. Therefore, it is extremely important to test and assess the stability of the peptides of interest and to fully understand and prevent these modifications.

As a preamble, we would like to draw the reader's attention to the fact that the notion of stability depends on each application. For example, a 10 % loss of a peptide prevents its use for the preparation of a calibration curve, while it still allows the use for qualitative bioactivity studies. It is crucial to define the stability requirements and the error tolerance before initiating any study.

The following sections address theoretical considerations on peptide stability, present typical tests to assess their stability, and provide suggestions on how to store and handle the peptides during the various stages of assay development and peptide extraction.

2.3.1. *Peptide degradation*

Peptide degradation mainly depends on the amino acids of which they are composed. A few typical degradation reactions are described below. Knowledge of these reactions enables predicting and preventing peptide degradation but requires a stability evaluation (see subchapter 4.1).

2.3.1.1. Oxidation. Oxidation results from a covalent modification of an amino acid by a reactive oxygen. For example, the so-called reactive oxygen species (ROS) are extensively studied *in vivo* and known to play an important role in protein and peptide metabolism, but also in diseases and aging. The ratios of the oxidized to unoxidized forms of proteins and peptides have been proposed as a means to evaluate oxidative stress involved in inflammation, psoriasis, and rheumatoid arthritis, among other conditions [110–113].

Peptide oxidation also occurs *in vitro* and in the absence of a biological matrix. It can occur at cysteine, tryptophan, and histidine residues, but the most sensitive amino acid to oxidation is methionine [114–116]. Oxidizing agents are mainly the oxygen from air and chemicals present in solutions. For example, DMSO—used to increase the solubilization of peptides [117]—has been reported to oxidize amino acids [118]. Because peptides are in contact with oxygen during storage and extraction, the proportions of oxidized forms might fluctuate over time, resulting in lower precision of the LC-MS/MS assay [66] and even immunoassays given that antibody recognition is affected by subtle changes of the oxidized epitope. The analysis itself, such as electrospray ionization in a mass spectrometer, might induce oxidation and create artefacts [119].

Oxidation of the peptides used in a laboratory-developed test may have various consequences. For immunoassays, it depends on whether the oxidized residues are part of the epitope. A robust immunoassay should rely on the recognition of epitopes without residues that may be oxidized. For MS-based assays, the situation is more dramatic because the detection is based on the *m/z* ratio, which increases for every oxygen

captured. This results in underestimation of the peptide concentration because the oxidized forms are no longer quantified by MS. Similarly, if the standards used to prepare a calibration curve are partially oxidized, then the concentrations measured in an unknown specimen will be overestimated.

When the peptides of interest are prone to oxidation and the assay is sensitive to it, it is more convenient to prevent or control oxidation of the standard than to modify the assay. Oxidation of dried peptides is efficiently prevented by adding an inert gas such as argon in the sealed container. In aqueous solutions, an efficient strategy is the addition of an antioxidant agent or a reducing agent, which prevents oxidation by competition. Miyachi et al. [9] reported 1.4 % oxidized glucagon, a peptide with methionine in position 27, in aqueous samples after extraction, that is, without a biological matrix. To circumvent this phenomenon, they added methionine in the extracted samples to a final concentration of 5 mM. The free methionine acted as an oxidation competitor by reacting with the oxygen and reduced the level of oxidized glucagon to 0.2 %.

Another strategy is to favor one oxidized state over the others by a controlled oxidation step, as proposed for the quantification of NPY by Vocat et al. [66]. NPY is a peptide of 36 amino acids with a methionine at position 17 [120]. Because of its functional group, this methionine can be unoxidized (sulfide), singly oxidized (sulfoxide), or doubly oxidized (sulfone). We found that NPY in its circulating form is approximately 15 % unoxidized and 85 % singly oxidized, but with a high variability that results in low precision. We forced the oxidation *in vitro* with an incubation of the sample with hydrogen peroxide (H_2O_2) for 20 min, resulting in 95 % singly oxidized form. This led to a net increase in the sensitivity (Fig. 13) and robustness of our assay. Of note, the addition of H_2O_2 to a sample containing FA should be avoided because it results in the formation of performic acid, a strong oxidizing agent that doubly oxidizes NPY and ultimately degrades the peptide.

In other situations, only a particular oxidized form may be of interest. For example, PTH is an 84 amino acid peptide with two methionine residues at position 8 and 18. Oxidation diminishes its bioactivity and therefore might be related to health conditions such as renal disease [121]. On the other hand, it seems that only the active form is useful to adapt the treatment strategy for hyperparathyroidism in patients receiving dialysis [122]. Therefore, immunoassays and spectrometric methods have been used to quantify the various oxidized forms of PTH for different purposes [8,122–125]. For example, Hocher et al. [122] proposed using an antibody against oxidized PTH to remove the oxidized form prior to the analysis of unoxidized PTH. However, poor oxidation control, low sensitivity of PTH immunoassays, or a general absence of standardized procedure resulted in a heterogenous concentration of oxidized forms with high discrepancies, ranging from 0 % to > 90 % [8,20]. Therefore, before the assay is developed, it is crucial to know its purpose to limit the time wasted on pre-analytical considerations that are irrelevant for a particular application. This is true for oxidation as well as other neat peptide degradation mechanisms.

In summary, we recommend identifying the amino acids subjected to oxidation in the peptide prior to developing the method as this may strongly affect the quantification. Special attention should be paid to methionine residues that are highly sensitive to oxidation. Finally, either the assay can be designed to measure the different oxidation states of the peptides, or oxidation can be blocked or strictly controlled.

2.3.1.2. Deamidation. Deamidation is the second most prevalent degradation reaction of peptides and occurs *in vitro* and *in vivo*. The two amide-containing residues, Asn and Gln, are converted to Asp and Glu residues, respectively [126–128], resulting in a + 1 Da mass increase. This mechanism has raised major concerns about peptide and protein stability [129].

To limit deamidation, one can work on the physical state of the peptide. Lyophilizates of standards are less prone to deamidation because of a reduced water content and reduced peptide mobility that leads to fewer interactions. The temperature is also positively correlated with the reactivity of Asn residues, especially if the peptides are not in a solid state [130]. For storage purpose, the temperature should be as low as possible. Finally, deamidation is affected by the hydroxide ion concentration, and thus by the pH of the solution [131]. For instance, Asn in PTH is frequently deamidated at pH > 5 [125,132]. However, because cleavage of other residues (such as Asp) occurs at a lower pH, a pH close to 5 seems to be the optimum for peptides prone to Asn deamidation, without increasing the risk of aggregation and hydrolysis [129]. Of note, lyophilization does not change the pH of the lyophilizate that maintains the pH of the solution from which it was lyophilized [133]. For more details on deamidation, we strongly recommend the reviews by Wakankar et al. [129] and Lindner et al. [134].

2.3.1.3. Other degradation mechanisms. Other less common modifications of a peptide's amino acids might happen *in vitro*. We briefly mention them in this review. For those interested in more details on the main stability concerns occurring with solid-state peptides, we recommend the review by Lai and Topp [135].

Peptides, as well as proteins, are prone to aggregation or fibrillization, which means that they tend to associate into larger polypeptide chains. This is well known to happen *in vivo* and has important health consequences regarding neurodegenerative diseases [136,137]. However, this phenomenon is also known to occur *in vitro* and is a frequent problem encountered during drug development. From an analytical point of view, aggregation might change the epitope availability, leading to a poor recovery of immunoassays, and change the mass of the peptide, which precludes its detection by a mass spectrometer. For more

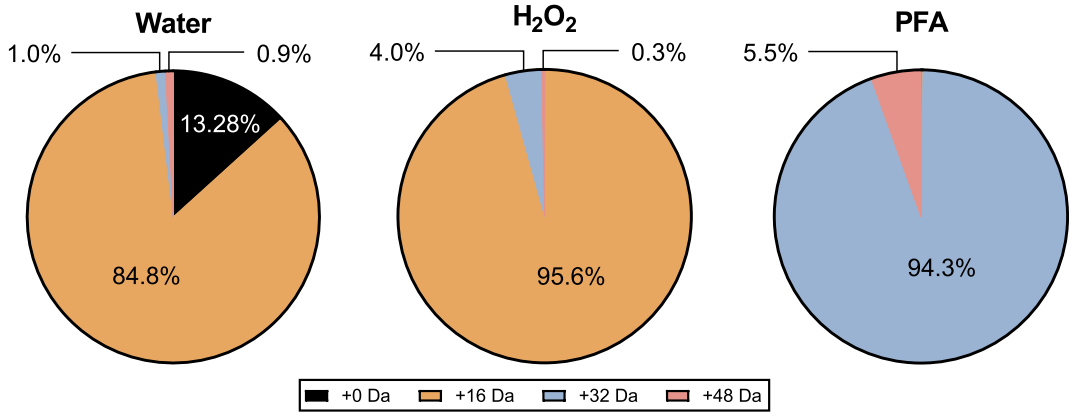

Fig. 13. Oxidation forms of NPY. Human Neuropeptide Y (hNPY) areas obtained for its unoxidized (+0 Da), singly oxidized (+16 Da), doubly oxidized (+32 Da), and triply oxidized (+48 Da) forms, after incubation for 20 min in water, hydrogen peroxide (H_2O_2), or performic acid (PFA = H_2O_2 + FA).

information on aggregation, we recommend the valuable published contributions [138,139].

Finally, other environmental aggression might induce peptide degradation. Extreme temperature or pH induce nonenzymatic hydrolysis of peptide bond, which is not common and a relative slow process in usual lab conditions [140–142]. Light can also greatly reduce the stability of peptides, because peptide bonds and side chains of tryptophan, phenylalanine, and tyrosine absorb ultraviolet (UV) light [143]. Mozziconacci and Schoneich [144] reported this denaturation: They showed a degradation of OT in contact with UV light when pH was 4.0–5.0 or 7.0–8.0.

2.3.2. Peptide stability in solutions

As neat solutions at room temperature, peptides present highly variable stability. Long-term storage should be avoided in such conditions. For example, AVP in aqueous solutions is stable for only 24 h at room temperature [145], and ANG peptides stored in MeOH are stable for only 30 h at 20 °C [146].

In general, degradation of peptides in solutions is efficiently reduced when stored at 4 °C, that is, in a fridge or on ice during handling. This temperature usually allows much longer stability of peptides than room temperature. For example, ANG peptide stability in MeOH is increased from 30 h at room temperature to 4 days at 4 °C, and it is likely that if they had tested a longer storage period, they would have found a longer stability at 4 °C [146].

Some processes involve temperatures above room temperature, such as drying with heat and a nitrogen flow, which may result in significant degradation. We observed that 50 % AVP was lost during such drying at 50 °C, while this degradation was reduced down to 30 % at 25 °C. We ultimately used a freeze-dryer to fully prevent degradation.

Peptides are usually not exposed to extreme temperatures (>100 °C) in lab conditions, unlike in the food industry, where this is extensively studied. In these conditions, peptide bonds are rapidly cleaved and amino acids are modified [147,148]. We do not discuss this issue in this review.

For long-term storage, solutions should be frozen. At very low temperatures such as −80 °C, chemical and biological degradation is very limited. Storage at −80 °C might extend the stability of peptides to years in most cases. For example, 1 mg/mL solutions of PYY 1–36, PYY 3–36, oxyntomodulin, GLP-1 7–36 amide, and GLP-1 9–36 amide in 20 µg/mL BSA in MeOH 10 %–acetic acid 20 % were stable for 20 months at −80 °C [149]. Temperatures closer to 0 °C slightly increase the chemical reactions, but as long as the solutions remain frozen, the chemical reaction rates are limited [150]. However, long-term storage of peptides in solutions, even at −80 °C, is not recommended; most authors report storage in dried form (see Section 2.3.3).

2.3.3. Peptide stability in dried forms

As lyophilizates, typically freeze-dried, peptides are considered by most authors to be stable for years, and always more stable than in solutions. Indeed, the absence of water greatly reduces the mobility and thus chemical reaction rates, microbial degradation, and hydrolysis, and limits the overall effect of temperature on stability [151,152]. Freeze-dried NPY is stable for more than 1 year at −20 °C with desiccant [66], and freeze-dried PTH is stable for 2 years at room temperature [153]. Moreover, repeated freeze-drying usually does not reduce the stability of peptides. For example, 10 nM solutions of Neuropeptide Y, peptide YY and pancreatic polypeptide were freeze-dried and reconstituted three times with no significant degradation [67]. However, as oxidation, deamidation, and other chemical reactions might continue even on solid-state peptides, it is still important to evaluate peptide stability in dried forms [143].

Freeze-dried peptide stability depends on the sequence of the peptide, as reported in Section 2.3.1, and on the storage conditions. Storing peptide standards as freeze-dried lyophilizates requires specific precautions. Peptides containing Ser, Gln, or Asp (among others) are hygroscopic, which means that they tend to absorb water from the environment in a process called deliquescence, resulting in an increased risk of hydrolysis and a change in their mass [154]. Deliquescence is efficiently prevented by storing peptides with a desiccant in a tightly sealed container. It is also important to let the frozen lyophilizates reach room temperature before opening the vial, to avoid water condensation.

Evaluating peptide stability should be one of the very first steps of any method development. In subchapter 4.1 we present a general protocol to determine the stability of peptides in any condition. Importantly, and as recommended by all major bioanalytical method validation guidelines, these studies should cover all conditions encountered during the entire analysis—for example, stability at −20 °C in the dried/lyophilized form for 2 years, stability of stock and working solutions in FA 1 % on ice for 4 h, and stability during a lyophilization-reconstitution cycle.

2.3.4. Freeze-thaw stability

The development and use of laboratory-developed tests require the frequent use of stock solutions for different purposes, including preparing internal standards and calibration curves. As a result, stock solutions are frequently thawed and frozen. Because these changes may greatly affect the matrix by inducing crystallization, cold denaturation, salt precipitation, and pH modification, the freeze–thaw cycle must be studied carefully [155,156]. Studies are especially needed to assess the extent to which a particular peptide suffer from repeated freeze–thaw cycles. Typically, these studies compare samples subjected to multiple versus no or a single freeze–thaw cycle. See subchapter 4.2 for an example of such a protocol.

2.3.5. Conclusion

In summary, predicting the stability of a peptide in solution is not possible, and stability studies should be among the first tasks of any method development to be assessed. As a rule of thumb, one can consider “the colder, the better.” A second rule is to freeze-dry peptides and/or to prepare single-use aliquots for long-term storage and to avoid freeze–thaw cycles. Finally, for long-term storage of peptides, we recommend storing them in dried form, at −20 °C, away from bright light and with a desiccant. For peptides containing Cys, Met, or Trp, storage should be under oxygen-free conditions to prevent oxidation. For more details, see the dedicated protocol (see subchapters 4.1, 4.2, and 4.3).

3. How to work with biological samples

Once the neat peptide stability has been established and any unwanted degradation has been prevented, the development of the quantification method and the work on real samples may start. In a clinical environment, the samples are usually plasma, serum, or urine, which are composed of a complex biological matrix. This matrix has important effects on the stability of peptides. Moreover, the sampling and the additives used change the stability of peptides and their recovery during sample preparation. Going through the main parameters that can affect biological samples, the following subchapters address the problems related to the matrix, the proteases, the chemical stability of peptides, and the patient.

3.1. Effect of the matrix

3.1.1. Blood, plasma, and serum

When starting the development of an ANG I assay, we initially observed nice LC peaks for spiked ANG I in both water and plasma. A few days later, the peak areas dropped completely for spiked plasma. We rapidly dismissed any issue with MS because we observed nice peaks when ANG I was extracted from water. We then realized that the first experiments were carried out using plasma collected in ethylenediaminetetraacetic acid (EDTA) tubes, and the subsequent

experiments used heparin tubes. We tested our hypothesis and found the following results (Fig. 14), later confirmed by other authors [157].

The loss of ANG I is explained here by a change in the matrix properties that leads to a lower retention during the extraction procedure, and a change in the ionization properties of the analytes that leads to a lower MS signal.

The impact of heparin on ANG I recovery is impressive and highlights the importance of considering the matrix as part of the method development. Anticoagulants are one of the first exogenous substances added to the sample, and thus it is important to understand the way they will modify the matrix behavior and how they can interact with the peptide, to choose wisely which anticoagulant to use in which situation. For more information on the blood sample handling, we strongly recommend the valuable contributions available for metabolomic and proteomic studies [158–160].

Most peptide assays are based on peptide extraction from serum or plasma. Serum is the supernatant obtained after complete coagulation of blood, while plasma is obtained by separating the cells through centrifugation of the blood in the presence of anticoagulants such as EDTA, heparin, or citrate [161]. Therefore, serum and plasma differ in many ways that will affect a peptide assay. After coagulation, the serum is free from fibrinogen and other clotting factors that may interfere with measuring peptides. However, the clotting step increases the amount of peptides in serum, because many abundant proteins are cleaved during coagulation, resulting in a high number of fragments [162]. The clotting step is also responsible for important enzymatic activity that eventually affects the peptides concentration if no protease inhibitors are added to the samples. On the other hand, plasma samples have not been subjected to coagulation, therefore avoiding the release of intracellular content that might affect the recovery of peptide and impact MS-based assays [163,164]. However, anticoagulants might interfere with peptide extraction and detection [165]. In a study related to progastrin-releasing peptide, the authors showed that this peptide stability over 3 days was greatly affected by the type of anticoagulant used [166]. Because plasma is the most used matrix for peptide quantification, we address the question related to the use of anticoagulants in the following paragraphs. For readers interested in the differences between serum and plasma, we recommend the valuable contribution of Hernandes et al. [167].

The three most frequently used anticoagulants are potassium EDTA, sodium citrate, and lithium heparin [168]. While the first two block the coagulation by chelating the calcium ions necessary for the coagulation cascade [169], heparin inhibits coagulation by binding to and activating antithrombin. This protease inhibitor inactivates thrombin, factor Xa, and other proteases, stopping the formation of fibrin; this inactivation is increased by 2000-fold when heparin is bound to antithrombin [170,171].

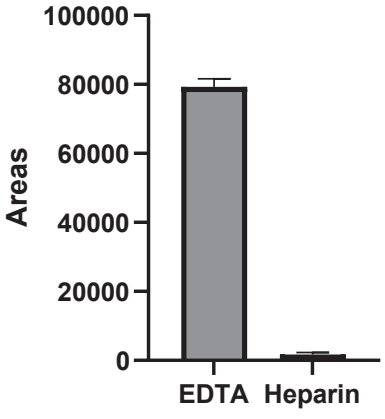

Fig. 14. Anticoagulants influence on ANG I recovery. Recovered areas from 1000 pg/mL angiotensin I (ANG I) spiked in ethylenediaminetetraacetic acid (EDTA) or heparin plasma sample. Heparin used as anticoagulant preclude a proper detection of ANG I. The experiment was performed in triplicate.

Because the mechanisms of action of these anticoagulants are different, their effects on peptide stability, extraction, and detection may differ [172]. On the one hand, EDTA and citrate have an enzyme inhibitor-like effect because their chelating properties preclude the use of calcium ions by other enzymes. Therefore, they enhance the stability of some proteins or peptides [173]. On the other hand, heparin might bind peptides other than antithrombin because of its negatively charged sulfo- and carboxyl groups [174]. Moreover, heparin is known to nonspecifically bind peptides, which influences MS detection, and to modify peptide elution in the LC system due to its polyanionic nature [175,176]. Finally, heparin inhibits carboxypeptidases, increasing the stability of a few peptides [177].

For all these reasons, selecting the most appropriate anticoagulant for an assay is not straightforward, and authors are quite divided on the topic. For some of them, EDTA is a better choice than citrate because of its high chelating power, its low proteolytic activity, and its independence on the subject's hematocrit [176,178]. EDTA is often recommended for metabolomic studies, for hormones assays, amyloid β peptide, procalcitonin, and ANG assays [179–182]. For others, heparin seems to be an overall better choice for metabolomics studies [183]. Moreover, heparin nicely correlates with EDTA for the measurement of brain-derived neurotrophic factor [184].

In this chapter, we would like to bring attention to another method of blood sampling known as Dried Blood Spot (DBS). This biosampling technique involves allowing drops of blood to air-dry on a filter paper substrate. The key advantage of this approach is its ability to simplify sample handling significantly. While DBS has proven effective for efficiently collecting endogenous biomarkers or exogenous peptides that circulate at high concentrations, such as insulin analogues, it is generally not well-suited for the analysis of endogenous peptides [185–187]. This limitation arises from the typically low concentrations of endogenous peptides, the potential for the drying process to induce matrix effects due to cellular disruption, and the continued occurrence of oxidation and other degradation processes. However, it is worth noting that DBS could potentially evolve into a gold-standard sampling method for endogenous peptides in the years ahead. This transformation may occur as analytical instrument sensitivity improves, allowing for the quantification of peptides in low volumes, and as stability issues are addressed.

In summary, there is no perfect anticoagulant that fits all situations. Hence, we will not recommend one anticoagulant. Instead, we recommend determining the most suitable anticoagulant for a given assay based on experimental data. In other words, we recommend studying their impact on the different characteristics of the assay by comparing the peptide recovery in different plasma samples, and to think about other sampling technique, such as DBS, to overcome some sampling issues.

3.1.2. Cerebrospinal fluid

CSF is a fluid mainly produced by the choroid plexus and is highly related to neuronal function. It acts as a shock absorber and regulates the intracranial pressure and the chemical environment of the central nervous system [188–191]. Because its circulation is separated from the lymph and the blood, CSF is known as the third circulation [192]. Because the blood–brain and blood–CSF barriers are quite impermeable to peptides in the bloodstream, peptide biomarkers from the CSF are exclusively used to diagnose neurodegenerative diseases such as Alzheimer's and Parkinson's disease [193].

CSF and plasma share common issues, although a major difference is that CSF has a low protein concentration. Moreover, CSF might be contaminated by blood during the sampling, leading to an important change in the matrix and the addition of more enzymes to the sample. Blood-contaminated samples should be discarded as a safety recommendation [23].

For readers specifically interested in CSF handling for peptide

quantification, we strongly recommend the valuable contributions available [23,194–196].

3.1.3. Urine

Peptide hormones are rarely quantified in urine. Measuring peptides in urine presents many advantages compared with measuring them in blood: Urine collection is easier, peptide stability is better in urine because all the proteolytic events already occurred within the bladder, and there is a large dataset available for comparative studies [197–199]. On the other hand, urine composition is greatly influenced by renal glomerular filtration and the bladder, where it is stored for several hours. The drawback of that storage is that enzymatic activity continues in the bladder, resulting in important proteolysis that eventually leads to degradation of proteins and peptides. This results in very stable peptides with strong resistance to endogenous enzymes, which eases the pre-analytical steps that can be limited to control microbial contamination, filtration of large proteins, along with usual stability and adsorption tests [197,200]. What might seem to be an advantage of urine over blood is in fact a strong limitation. Indeed, it leads to a peptidome composed almost exclusively of random degradation products, which is difficult to associate with any biological condition, apart from those directly related to the kidneys or bladder [197,201–204]. Moreover, peptide concentration should be normalized with creatininuria to take into account urine concentration.

3.1.4. Tissue

As a material with high peptide contents, tissues samples are of great interest to understand biological processes. Tissue analysis is challenging because the extraction procedure involves cell lysis at elevated temperatures, tissue structure disruption with a homogenizer (such as a potter or turrax), followed by solvent precipitation and peptide recovery in supernatants, or with the assistance of sonication. Moreover, the procedure might require a cutting step prior to the extraction to reduce the size of the sample and to allow proper solubilization. These steps are responsible for protein and peptide breakdown either enzymatically or nonenzymatically, leading to modifications in the measured concentrations [205]. The extraction itself is tricky because cells are difficult to lyse completely, which leads to poor peptide recovery. This issue cannot be properly evaluated because it is not possible to calculate the yield of the extraction by artificially adding the peptide into the cells.

For the cutting step, we strongly recommend working on dry ice to keep the tissue frozen for as long as possible. Then, the tissue should be warmed up by taking the sample out of dry ice for a few seconds, just enough to cut through it with a razor blade, and rapidly put back on dry ice. Lysing the tissue with either by potterization or by using a fast and cold mechanical lysing tool such as the TissueLyser from Qiagen will help break the tissue into small pieces without keeping them at room temperature for a prolonged period. To disrupt the neuroendocrine vesicles in cells, we suggest sonicating the samples in an ice-cold buffer and recovering the supernatant. As highlighted by the following example of NPY extraction from prostate tissue, repeating the above-mentioned procedure will drastically increase the recovery of the peptide (Fig. 15). A minimum of two consecutive extractions and a pooled of the obtained supernatants are strongly recommended. These different steps will limit peptide degradation and ensure greater recovery.

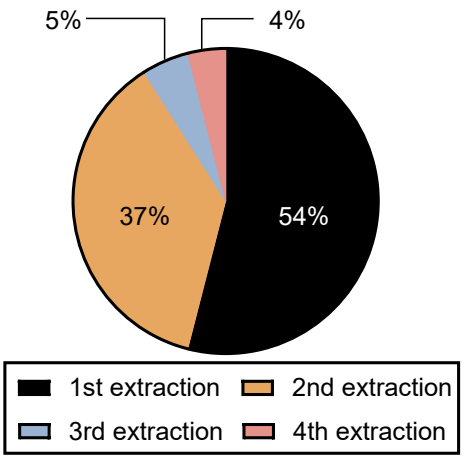

Fig. 15. NPY recovery in tissue. Human Neuropeptide Y (hNPY) recovered after extraction from prostate tissue. The pie chart represents the relative recoveries from four extractions of the same tissue sample, with the hypothesis that after four extractions, all the hNPY was extracted.

3.2. Enzymes

3.2.1. Function and mechanism

Enzymes are a class of proteins able to accelerate a chemical reaction to convert one or a few substrate(s) into one or a few product(s). This catalytic activity is at the center of almost all metabolic processes that enable the organism to work, either as highly selective enzymes controlling metabolic pathways or nonspecific catalytic activity involved in the degradation of numerous proteins [206]. Enzymes that hydrolyze peptides bonds are called peptidases, proteinases, or proteases. To simplify, we will use only the term protease in this review. Proteases cleave peptide hormones to generate smaller fragments with different activity regarding their specific receptors and are responsible for the generation of the peptidome (see chapter 1). Proteases cleave the signaling peptide from a pre-pro-peptide to form the pro-peptide. The latter is cleaved again by other proteases to form the peptide hormone with biological activity. This peptide hormone might then be metabolized again whether to change or stop its activity. We focus on proteases, their localization, their activity, and the way to prevent unwanted peptide hormone degradation that may occur during sample preparation.

Proteases can be separated in two major classes. Endopeptidases cleave internal bonds in a peptide chain, and exopeptidases cleave one or a few residues at one of the two peptide termini. Exopeptidases can be further classified into aminopeptidases, dipeptidyl peptidases, or tripeptidyl peptidases if one, two, or three amino acids are liberated from the N-terminus, respectively. If one or two amino acids are liberated from the C-terminus, they are called carboxypeptidases or peptidylpeptidases, respectively [207]. More than 200 proteases have been identified in plasma [208]. Exopeptidases are responsible for the majority of peptide degradation because they are more active and stable in plasma than endopeptidases [209]. Similarly, a classification has been proposed to identify the cleavage sites and the related amino acids [210]. Fig. 16 shows an illustration of a peptide from the N- to the C-terminus. Amino acids on each side of the cleavage site are named P1 to P4 when near the N-terminus, and P1′ to P4′ when near the C-terminus. This nomenclature allows highlighting the specificity of an enzyme for the cleavage of a particular peptide bond. Indeed, the amino acids close

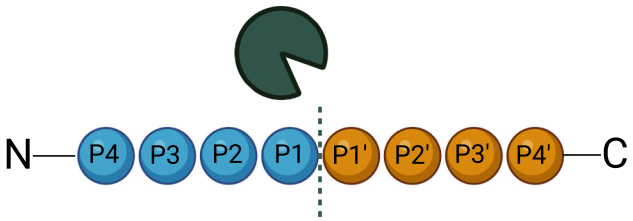

Fig. 16. Graphical representation of a cleavage site in an amino acid chain. N represents the chain side with an amine group, and C represents the chain side with a carboxyl group. Amino acids on each side of the cleavage site are named P1 to P4 when near the N-terminus, and P1′ to P4′ when near the C-terminus. The green partial circle represents a protease. Created with BioRender.com. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to the cleavage site greatly affect the availability of the bond and thus change the possible enzymes that are able to reach the site.

Enzymatic activity activates and regulates biological processes by changing a peptide's structure and thus its ability to bind to receptors. As presented in Fig. 1 a peptide might be cleaved by many enzymes, resulting in multiple fragments with different activities, or no activity at all. Knowing these metabolic pathways and their functions is the key to understand many diseases and to adapt their treatment. For example, the renin–angiotensin–aldosterone system (RAAS) is at the center of blood pressure regulation (see Fig. 17). More specifically, angiotensin-converting enzyme (ACE) metabolizes inactive ANG I to ANG II, which has vasoconstrictive activity. Consequently, ACE inhibitors are one of the most common antihypertensive treatments prescribed because their ability to inhibit conversion of ANG I to ANG II leads to a blood pressure reduction. Because of their functions, enzymes are ubiquitous in the body: They are found in tissues, organs, and blood. They might be in the cell cytoplasm, on the cell surface, or secreted and thus solubilized in biological fluids [207,211].

There are two analytical consequences of peptide metabolism. First, several peptides with very similar structures might coexist in a biological sample. In such a case, immunoassays often suffer from lack of specificity because of cross-reactivity, when the epitopes of the peptides are identical, precluding a proper quantification. In contrast, MS-based assays do not suffer from the same problem, as even the slightest change in the structure results in a different mass-to-charge ratio. Second, in the absence of adequate measures, the activity of proteases continues after sample collection, and may significantly bias the results of the assay. We discuss this issue in Section 3.2.3.

3.2.2. Classification

With several thousands of identified enzymes, a classification was needed. The first one, based on the enzyme's activity, was proposed by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB) and is used in several databases (https://enzyme.expasy.org, https://www.uniprot.org, and https://www.brenda-enzymes.org) [212]. It is based on an Enzyme Commission (EC) tag, composed of four numbers separated by a dot. Each number characterizes a level of classification related to a particular chemical reaction. Seven main categories are defined: oxidoreductase (EC 1), transferase (EC 2), hydrolase (EC 3), lyase (EC 4), isomerase (EC 5), ligase (EC 6), and translocase (EC 7).

Another classification, suggested by Rawlings et al. [208], is available at https://www.ebi.ac.uk/merops/index.shtml. It classifies proteases into families with homologous proteolytic activity, and attributes a letter corresponding to the catalytic type of the proteolytic enzyme combined with a unique number to separate the families. Nine different families have been identified: aspartic (A), cysteine (C), glutamic (G), metallo (M), asparagine (N), mixed (P), serine (S), threonine (T), and unknown (U). Families sharing an evolutionary relationship can be grouped into clans [213]. Clans are identified with two letters, the first being the catalytic type of the families included in the clan.

Let's consider the well-known dipeptidyl peptidase 4 (DPP-4) enzyme. It is a serine protease with hydrolase activity that acts on peptide bonds and specifically cleaves dipeptides from the N-terminus. Hence, it can be classified as EC 3.4.14.5, or in the SC clan, family S9 (S09.003). In this review, for clarity we only use the EC number. A summary is presented in Table 4.

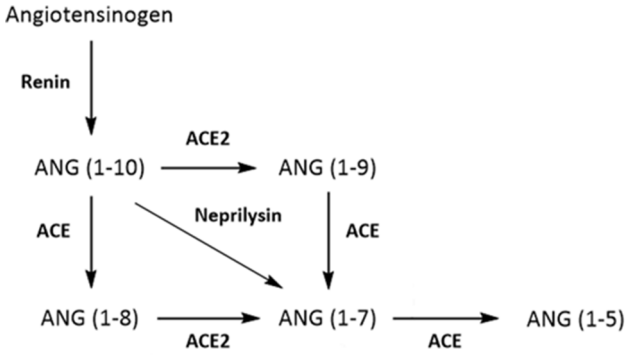

Fig. 17. Simplified representation of the metabolism of angiotensins. Renin forms ANG 1–10 from Angiotensinogen. ACE metabolizes ANG 1–10 in ANG 1–8, ANG 1–9 in ANG 1–7, and ANG 1–7 in ANG 1–5. ACE2 metabolizes ANG 1–10 in ANG 1–9, and ANG 1–8 in ANG 1–7. NEP metabolizes ANG 1–10 in ANG 1–7. ACE. angiotensin-converting enzyme; ACE2. angiotensin-converting enzyme 2; NEP. neprilysin.

3.2.3. Impact on analytical results

A diagnosis based on quantifying circulating peptides relies on the hypothesis that a pathological state will result in a local or systemic change of a peptide secretion, or vice versa. Blood samples represent a perfect snapshot of these circulating concentrations. However, peptide metabolism still occurs *ex vivo* through circulating proteases present in the sample. This leads to a change in peptide concentrations that will no longer represent the true circulating concentrations, precluding any accurate diagnosis.

Fig. 18 shows the relative areas of a peptide (ANG I) in EDTA plasma, in three conditions. One sample was stored frozen (used as a control) with a mix of inhibitors, an identical sample was stored at room temperature for 1 h, and a third sample without inhibitors was stored at room temperature for 1 h. We observed significant peptide degradation after 1 h at room temperature, which was efficiently prevented by inhibitors.

To provide the true concentration of a peptide in a biological fluid—that is, the *in vivo* concentration—it is critical to stop any enzymatic activity at the time of collection to prevent any *ex vivo* modification of the peptides of interest. For this endeavor, the first step is to study the stability of the peptides *ex vivo* (subchapter 4.4) and then, if necessary, take actions to stop any degradation (subchapter 4.5).

Table 4
Main types of proteases with an explanation of their enzymatic activity, and the corresponding IUBMB EC number. X, Y, and Z. cleaved amino acids; -¦-. representation of the cleavage site; Peptide. remaining peptide after the cleavage.

Type of protease	Enzymatic activity	IUBMB nomenclature
Aminopeptidase	Cleaves one amino acid from the N-terminus (X -¦- Peptide)	EC 3.4.11
Carboxypeptidase	Cleaves one amino acid from the C-terminus (Peptide -¦- X)	EC 3.4.16–18
Dipeptidase	Hydrolyses a dipeptide	EC 3.4.13
Dipeptidyl peptidase	Cleaves two amino acids from the N-terminus (XY-¦-Peptide)	EC 3.4.14
Endopeptidase	Hydrolyses peptide bonds of nonterminal amino acids (Peptide-¦-Peptide)	EC 3.4.21–25
Exopeptidase	General name for all proteases that hydrolyses a peptide bond not more than three residues away from the terminus	–
Metallopeptidase	Protease with a metallic ion in its active site	EC 3.4.17/24
Peptidyl dipeptidase	Cleaves two amino acids from the C-terminus (Peptide-¦-XY)	EC 3.4.15
Tripeptidyl peptidase	Cleaves three amino acids from the N-terminus (XYZ-¦-Peptide)	EC 3.4.14

IUBMB. International Union of Biochemistry and Molecular Biology.

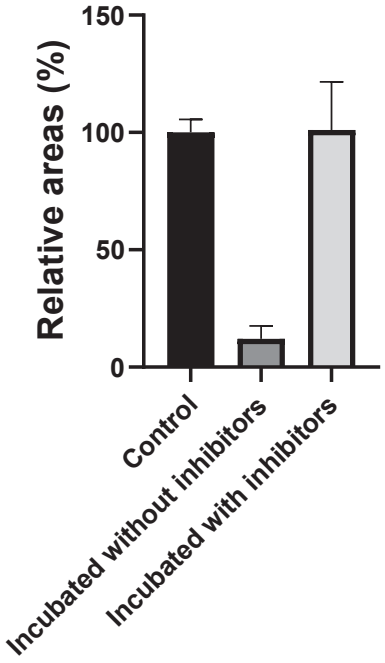

Fig. 18. Stability of ANG I in plasma. Relative areas of angiotensin I obtained after an incubation period, to show its stability. The reference sample was frozen, and the conditions evaluated were incubation for 1 h at room temperature in the presence or absence of a dedicated mix of inhibitors. The experiment was performed in triplicate.

3.2.4. How to study enzymatic activity

As discussed in the previous subchapters, enzymatic activity undoubtedly continues after sample collection, potentially leading to an important modification of the peptide concentration over time. Because of this, every development of an assay for a peptide in a biological fluid should include an evaluation of the peptide stability during the pre-analytical and analytical steps of the assay, such as sampling, centrifugation, transport, storage, and sample preparation. In the following paragraphs, we cover the methodology used for plasma samples because plasma is the most frequently used matrix, but the general approach might be applied to any type of matrix.

Fig. 19 shows the general approach to test peptide stability in a biological matrix. To be as efficient as possible, we recommend starting with a pilot study aimed at testing the stability of the peptide during an incubation of 30 min at 37 °C. These conditions mimic sample collection where blood at body temperature is sampled and slowly cooled to 4 °C. Ensuring stability during the first 30 min is the most crucial milestone to obtain reliable results. The general idea of the pilot study is to spike two samples with the same amount of the peptide. One sample is kept frozen and the other is incubated for 30 min at 37 °C. Then the two samples are extracted, and peptide concentrations are compared. For more details, see the dedicated protocol (Section 4.4.1).

Once the stability during sample collection has been confirmed, a comprehensive stability study is mandatory to assess whether the peptide is stable during transport and sample preparation; this endeavor involves reproducing the conditions (temperature, pH, and duration) of these steps. Therefore, the samples should be incubated at times and temperatures representing the above-mentioned steps adapted to the need of the assay (4 °C for 24 h to mimic sample transportation on ice, room temperature for 1 h to mimic the sample preparation on a bench, etc.). Stability is evaluated by comparing the recovered peptides in those conditions and in a sample kept frozen at −80 °C. A detailed protocol to evaluate the peptide stability is available in the Section 4.4.2. Then,

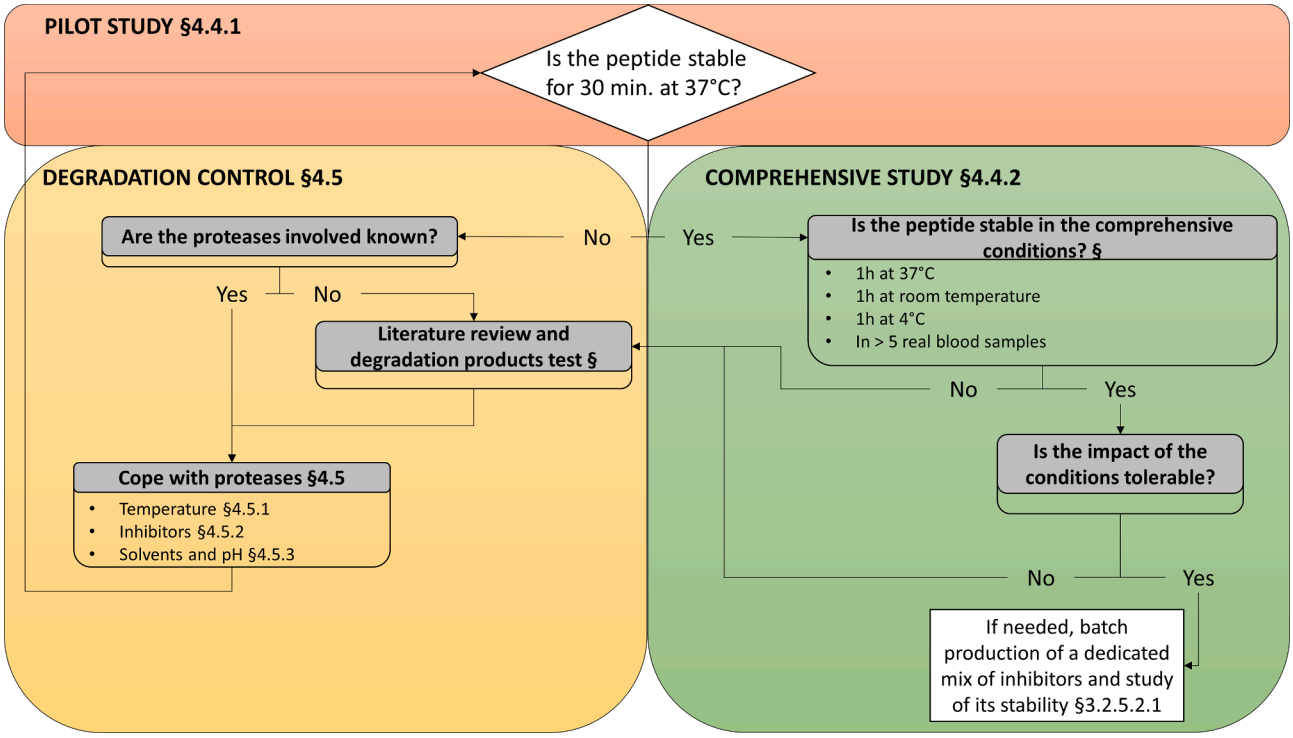

Fig. 19. General procedure to evaluate peptide stability. It starts with a pilot study aimed at evaluating the stability when sampling blood (at 37 °C) and cooling it to 4 °C. Then, if the stability is confirmed in the pilot study, the comprehensive study aims to evaluate the stability in the other steps of the assay, such as transport and sample preparation, by mimicking their temperature and duration. At any point in the study, degradation control strategies are applied if the required stability is not reached. Moreover, the impact of these strategies must be evaluated to ensure they do not lower the recovery of the analytes. Finally, if inhibitors are used, then their stability and batch production must be studied.

depending on the results of this study, strategies to stabilize the peptides must be applied (see subchapter 4.5).

3.2.5. How to work around enzymatic activity

If the target peptide is unstable in the expected handling conditions because of enzymatic activity, one must find strategies to limit its impact on results. This is the most difficult and time-consuming step of the assay development for several reasons. First, one peptide may be cleaved by several proteases with different optimal working conditions. Second, the degradation must be stopped immediately after collecting the sample, using a robust and technically easy methodology for a clinical environment. Third, the strategy deployed to prevent degradation should not interfere with the extraction or induce a matrix effect, to avoid losing LC-MS/MS signal.

An initial interesting test is to study the degradation products obtained. We recommend spiking samples with a stable isotopically labeled (SIL, typically with ^{13}C) analog of the precursor peptide. By allowing this precursor to stay in contact with the biological matrix, and by measuring the SIL peptides at several time points, it is possible to evaluate degradation of the peptide and the metabolites obtained, and it might provide insights into the enzymes responsible for that cleavage. Of note, it is necessary to have every analog labeled at the same position so that enzymatic degradation does not cleave the labeled amino acid. For example, if a method targets ANG I, ANG II, and ANG 1–5, then the five first amino acids are shared by the three peptides and should be labeled (see Fig. 17 for metabolism of angiotensins).

As highlighted previously, the development of such strategies is an iterative process that should be repeated until a satisfying solution is found (see Fig. 19). We describe different strategies to ensure peptide stability by blocking protease activity. These strategies must be tested in this iterative process.

3.2.5.1. Temperature. The easiest way to limit the action of proteases is by lowering the temperature at which the samples are stored and handled. Indeed, the optimal working conditions for most proteases are close to what is found in the human body. By storing samples at a temperature below the human body temperature (around 36 °C), the activity of some proteases is completely abolished [214–216].

As a rule of thumb, the colder the better. This is easily applied for the analytical steps: The samples should be stored at −80 °C or −20 °C, the samples should be centrifuged at 4 °C, and the sample preparation should be performed on ice. In contrast, this is most difficult to apply and control during the pre-analytical steps, especially during sample collection that often takes place in a clinical environment. Nurses typically collect blood while performing several other tasks and handle the samples when all bedside tasks have been completed. Moreover, adequate instruments and material such as ice machines and refrigerated centrifuges are often not available on site. Typically, in a clinical environment, blood samples are placed on a tray for a few minutes at room temperature after collection. The tube is then placed in a centrifuge, perhaps after transfer to the central laboratory. The sample temperature remains higher than 20 °C during this time. Then, the sample is centrifuged and frozen. In summary, samples originating from the real world often remain in ideal conditions for enzymatic activity for 15–30 min, resulting in unreliable laboratory results for peptides that are not stable in such conditions.

Systematic storage of samples on ice immediately after collection, and the availability of refrigerated centrifuges and freezers in units close to the patients, may reduce the risks of degradation. Nonetheless, unforeseen clinical circumstances often prevent samples from being properly processed, without the laboratory being informed. Therefore, to circumvent the matter with temperature we recommend the addition of protease inhibitors to ensure the stability of the peptides during the pre-analytical steps.

3.2.5.2. Proteases inhibitors. Protease inhibitors are molecules that reduce or block enzymatic activity. They produce this effect by binding to an enzyme, either directly on its binding site or to another site, thus preventing metabolism of the substrate. In the case of metalloproteases, the inhibitors might also chelate the metal ions needed for the catalytic activity. It is noteworthy that inhibitors can affect multiple proteases. For example, EDTA is a chelating agent that binds to iron and calcium ions. Therefore, it can be used to inhibit the activity of many different metalloproteases. Of note, inhibitors might also be replaced by competitors that will saturate the enzymes, reducing their effects on the target peptide. This strategy has been applied by using the tripeptides Tyr-Pro-Ala-NH_2 and Tyr-Pro-Ala-OH as enzymatic competitors for DPP-4, to increase the stability of endomorphins [217].

The prerequisite to the use of an enzyme inhibitor is to have a comprehensive understanding of the involved metabolic pathways. The first step is an extensive literature review to identify the enzymes that could degrade the peptide. These proteases might be nonspecific and cleave many substrates or be specific to a limited number of substrates. After that, one can use various databases such as MEROPS (https://www.ebi.ac.uk/merops/index.shtml) and BRENDA (https://www.brenda-enzymes.org) that provide lists of proteases and their related inhibitors. To help find enzymes that might cleave one particular peptide and the possible inhibitors to block it, Table 5 summarizes the most common plasma proteases, the cleavage sites, examples of substrates, and suggestions of inhibitors that could be used in bioanalytical assays.

Once one or a few inhibitors have been selected, it is important to optimize their use and storage. In the following examples, we focus on inhibitors used for plasma samples, but the strategies deployed are the same for other matrixes such as serum and tissue. For urine, peptides are in contact with proteases for a long time in the bladder, which means that the peptides have already been cleaved. Therefore, the use of inhibitors with urine is generally not necessary, and the peptidome is stable for several hours even at room temperature [198].

During the iterative process and the many tests needed to find the optimal inhibitor mix, inhibitors should be weighed and prepared in low volumes that can be added directly to the plasma. This approach will ensure fresh and nondegraded inhibitors and is the fastest option to run several tests in a row. However, in real clinical conditions, inhibitors are added directly to the blood during the collection. This greatly impacts preparation of the inhibitor mix. First, because blood is about 50 % plasma, the optimal concentrations found during the testing phase should be divided by two when working with blood: Elimination of the cells will increase the concentrations of inhibitors in the remaining plasma by a factor of two. Second, the addition of the inhibitor mix to the sample will dilute the concentration of the target peptide, increasing the LLOQ. To limit this dilution, we recommend using a lyophilized inhibitor mix that is resolubilized in the lowest possible volume when added to the collection tube. However, in this concentrated solution, the inhibitor concentrations should not be too high, or there is a risk that they will precipitate and not properly solubilize in the blood. Finally, the inhibitor mix must be added directly in the collection tube so it can rapidly exert its effect. We do not recommend vacuumed vials, such as Vacutainer, because inhibitors cannot be added easily. Monovette-type vials are more appropriate, but it is important to tighten the cap while pushing the plunger to ensure that the maximum volume of blood is collected.

Once a good compromise between stability and recovery has been found, we strongly recommend evaluating several different plasma samples (each in triplicate). Indeed, metabolic variations or unknown drug therapy may increase the interindividual variability toward a particular metabolic pathway. In Fig. 20, we spiked ANG I in the plasma of six donors, with no known medication, along with a mix of protease inhibitors (aliskiren, captopril, EDTA, and 2,2′-bipyridyl). Plasma samples were then frozen or kept at 37 °C for up to 1 h. In this experiment, enzymatic activity was sufficiently prevented by the inhibitors in only half of the subjects (1, 2, and 6). Therefore, the protease inhibitor mix

Table 5
Summarizing table of the most common proteases sorted by the amino acids they cleave, in the following order. N-terminal proteases; C-terminal proteases; endopeptidases. If available, their cleavage site, known substrates, localization, and specific or nonspecific inhibitors are given.⊕. amino acids cleaved by the protease; -|-. cleavage site; O. nonspecific amino acids; …. other amino acids that form the peptide; PSTI. pancreatic secretory trypsin inhibitor; SBTI. soybean trypsin inhibitor.

Amino acids concerned (⊕)	Cleavage site	Proteases	Known substrates	Localization	Optimal pH for proteolysis	Inhibitors
Ala and Arg	⊕-|-O…	Aminopeptidase N (previously M)(EC 3.4.11.2)	Angiotenin-3 and somatostatin [211,223]			Amastatin, probestin, and actinonin [224]
Arg, Asp, and Glu	⊕-|-O…	Aminopeptidase A (EC 3.4.11.7)	Angiotensin I, angiotensin II, cholecystokinin-8, and neurokinin B [211,225]		7.4	Amastatin and probestin [224]
Pro	O⊕-|-O…	Aminopeptidase P (EC 3.4.11.9)	Bradykinin, substance P, and neuropeptide Y [226–228]	Anchored in the plasma membrane [229]		Apstatin [226–228]
Not specific	OO–|-O…	Dipeptidyl peptidase 1 or cathepsin C (EC 3.4.14.1) [230]		Bone narrow, spleen, kidney, and serum [231]	Acidic pH [232]	Brensocatib and vildagliptin [233,234]
Ala-Pro and Lys-Pro [235] Substrate preference for tripeptides	O⊕-|-O…	Dipeptidyl peptidase 2 (EC 3.4.14.2)	Substance P, bradykinin, and casomorphin [236,237]	Intracellular (lysosomal) [236]	5.5–6 [236]	Talobostat, sitagliptin, diprotin A, AX8819, and Diisopropyl fluorophosphate [236–239]
Pro or Ala at the penultimate position [217,240]	O⊕-|-O…	Dipeptidyl peptidase 4 (EC 3.4.14.5)	Bradykinin, neuropeptide Y, substance P, glucagon-like peptide 1, glucose-dependent insulinotropic polypeptide, and endormorphins [217,226,241]	Cell membrane [242], and as a soluble form in plasma and urine [238,243].		Vildagliptin, linagliptin, and diprotin A [238,241]
Pro at the penultimate position	O⊕-|-O…	Dipeptidyl peptidase 8 and 9 (EC 3.4.14)	Neuropeptide Y			Talobostat and vildagliptin [238]
Large hydrophobic side chains (Phe) Does not cleave Lys, Arg, Pro	…O-|-⊕	Carboxypeptidase A (EC 3.4.17.1)			7–8	Chelating agent [244,245]
Basic amino acids (Arg > Lys > His)	…O-|-⊕	Carboxypeptidase E (EC 3.4.17.10)	Insulin, vasopressin, oxytocin	Mainly in the brain and pituitary	5.6	Chelating agent [245]
Basic amino acids (Arg > Lys > Gly)	…O-|-⊕	Carboxypeptidase B (EC 3.4.17.2)			7–8	Chelating agent [245]
Arg and Lys	…O-|-⊕	Carboxypeptidase U (EC 3.4.17.20)			7.5–7.8	Chelating agent [177,246]
Ile, Leu, Phe, and Val	…⊕-|–OO (O)	Neprilysin (EC 3.4.24.11)	Bradykinin, neuropeptide Y, and angiotensin [211,247]	Found in epithelial cells, fibroblasts, neutrophils, and in soluble form in the circulation, urine, and cerebrospinal fluid		Sacubitril, valsartan, and omapatrilat/ ethylenediaminetetraacetic acid [211,247]
Tyr	…O-|-⊕	Kallikrein (EC 3.4.21.34)	Neuropeptide Y and bradykinin [226,248]	Blood, urine [249,250] and in many organs to different extents [251].		DX-88 [252], aprotinin, and SBTI [253]
Not specific	…O-|–OO	Angiotensin-converting enzyme (3.4.15.1)	Angiotensin, substance P, enkephalins, bradykinin [254], and amyloid β [255]	Lungs and vascular tissues [256] and plasma [257,258]		Captopril, enalapril, quinapril, ethylenediaminetetraacetic acid [211]
Not specific	…O-|-O	Angiotensin-converting enzyme 2 (EC 3.4.17.23)	Angiotensin	Vascular endothelial cells of the heart and kidneys [259–261] and plasma [257]	6.5	DX600 [262], MLN4760 [263,264] and ethylenediaminetetraacetic acid [265]
Pro (but not Pro-Pro) [266]	…O-|-⊕	Prolidase or peptidase P (EC 3.4.13.9)		Human kidney		*p*-Hydroxymercuribenzoate [266]
Not specific	…O-|-O	Cathepsin A (EC 3.4.16.5)	Endothelin-1 and angiotensin I [267]			Pepstatin [268]
Endopeptidase. two residues with large hydrophobic side chains (Phe) or Arg [269]	…⊕⊕-|-O…	Cathepsin B (EC 3.4.22.1)		Intracellular [269]	4–8.5 [269]	Pepstatin [268]
Endopeptidase. bonds involving residues with hydrophobic, preferably aromatic, side chains	…⊕⊕-|-O…	Cathepsin D (EC 3.4.23.5)	Endothelin, tachykinin, enkephalin, insulin, prolactin	Intracellular [270]		Pepstatin [268]
Endopeptidase. aromatic and strongly positively charged residues (Phe, Lys, Arg, or Leu) [271]	… O-|-⊕…	Cathepsin G (EC 3.4.21.20)	Angiotensin I		7–8 [271]	Pepstatin [268]

(*continued on next page*)

Table 5 (*continued*)

Amino acids concerned (⊕)	Cleavage site	Proteases	Known substrates	Localization	Optimal pH for proteolysis	Inhibitors
Endopeptidase. cleave after a basic amino acid (Lys or Arg)	… ⊕-⊣-O…	Trypsin (EC 3.4.21.4)			7.5–8.5	PSTI, SBTI, and serpins [272].

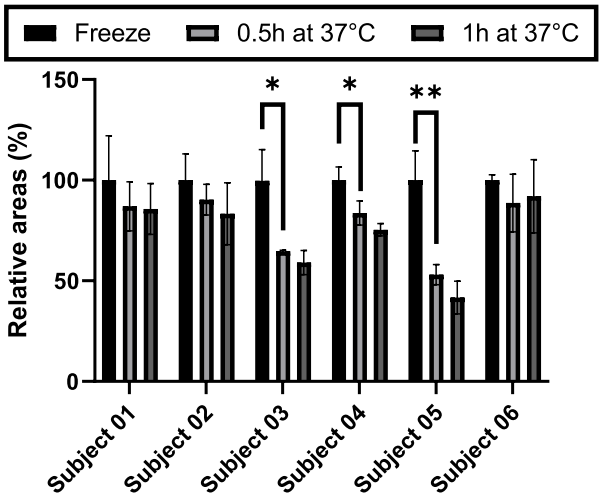

Fig. 20. Angiotensin I. Relative areas recovered from 1000 pg/mL angiotensin I spiked in the plasma of six subjects, with or without an incubation step. All the samples contained a mix of protease inhibitors (aliskiren, captopril, EDTA, and 2,2′-bipyridyl). The reference samples are the frozen one. Three out of six subjects showed an enzymatic activity not controlled by the inhibitors. The experiment was performed in triplicate.

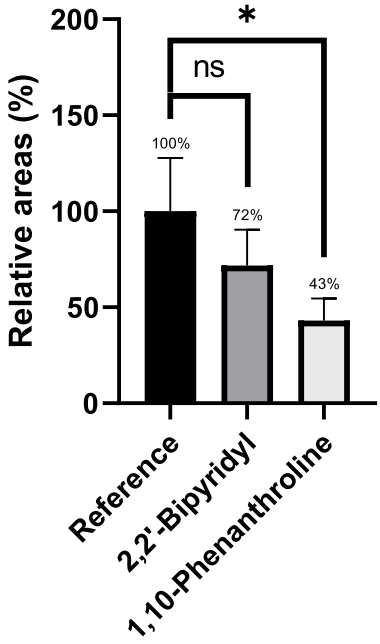

Fig. 21. Inhibitors effect on ANG I recovery. Relative areas recovered from 1000 pg/mL angiotensin I spiked in plasma samples when 2,2′-bipyridyl or 1,10-phenanthroline was added. The 1,10-phenanthroline induces a higher loss during the extraction than 2,2′-bipyridyl. The experiment was performed in triplicate.

was re-evaluated using blood samples, and the conditions of preparing the mix were studied to ensure that there was no degradation of the inhibitors.

The addition of protease inhibitors to the matrix is by far the most efficient method to prevent the degradation or the production of the peptides of interest. However, this use is not without risk, as the inhibitors change the composition of the matrix, and they can have a tremendous effect on the sample preparation efficiency or on the detection of the sample. They may change the retention in SPE, induce stronger precipitation of proteins, or change the ionization of the electrospray source. With its great impact on peptide recovery, the effect of the inhibitors must be extensively studied. We propose a protocol dedicated to this evaluation in Section 4.5.2.3.

In the following example, we added 1,10-phenanthroline to plasma samples to limit the degradation of ANG (Fig. 21). This chelating agent blocks the activity of metalloproteases such as neprilysin and aminopeptidase A, ensuring that peptides are stable for a much longer time. However, after SPE, ANG I recovery was 57 % lower when *o*-phenanthroline was added compared with the control samples. In cases like this, it might be very rewarding to identify an alternative inhibitor with similar inhibitory properties as the problematic inhibitor. Here, 2,2′-bipyridyl stabilized ANG to the same extent as *o*-phenanthroline but did not decrease the efficiency of the extraction.

The effect on recovery is also related to the concentration and may specifically affect one peptide. This phenomenon occurs with 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), a serine protease inhibitor that reacts with the hydroxy group of the active site of serine and forming a sulfonyl group. AEBSF has an inhibitory effect on ACE, among other enzymes. At a low concentration (0.05 mg/mL), it did not change the recovery of ANG 1–9 extracted from plasma using SPE. However, a 10-fold increase of AEBSF to 0.5 mg/mL resulted in a total loss of the peptide (Fig. 22). In this case, we replaced AEBSF with phenylmethylsulfonyl fluoride (PMSF), another inhibitor of the same family with similar inhibitory power that did not affect ANG 1–9 recovery during sample preparation. Interestingly, the recovery of other ANG peptides was not affected by the addition of AEBSF to the matrix. In conclusion, it is important to determine the optimal concentrations of the inhibitors needed to reduce the enzymatic activity. It should be as low as possible to limit their effect on recovery. Generally, using a lower concentration of an inhibitor with a greater inhibitory power might be a better option than a higher concentration of an inhibitor with a lower inhibitory power.

The second important limitation of inhibitors is that they need time to exert their effect because they must interact with the enzymes or metal ions. To speed up the process, once collected, blood samples should be mixed gently to allow a proper distribution of the inhibitors in the sample, but not shaken at the risk of causing hemolysis. We recommend gently turning the tubes up and down five times. Because of this slight delay between the collection and the actual effect of inhibitors on enzymes, inhibitors might not be suitable for highly and quickly metabolized peptides such as bradykinin (BK), whose half-life is 17 s [218]. In such a case, changing the matrix for a one that will instantly block enzymatic activity by inducing a fast precipitation, such as solvents or extreme pH, might be the only way to reach the desired stability. These strategies are covered in Section 4.5.3.

3.2.5.2.1. Inhibitor cocktail preparation. Enzyme inhibitors are usually provided in a solid state to increase their stability. It is better to store them in that form at −20 °C until further use. A very convenient method

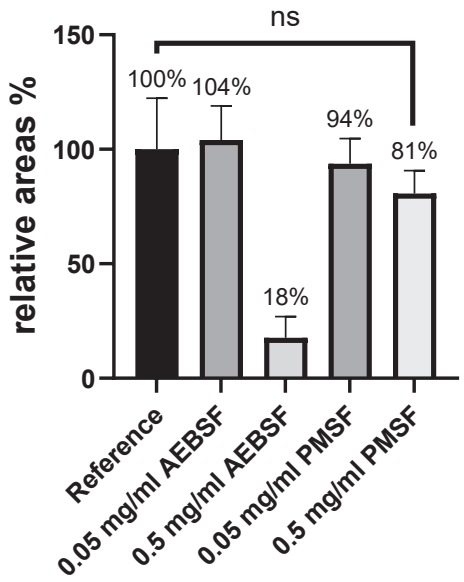

Fig. 22. AEBSF and PMSF effects on ANG 1–9 recovery. Relative areas recovered from 1000 pg/mL angiotensin 1–9 spiked in plasma samples with or without the addition of 4-benzenesulfonyl fluoride hydrochloride (AEBSF) and phenylmethylsulfonyl fluoride (PMSF). The concentration of inhibitors has a great impact on the recovery, with a higher loss when 0.5 mg/mL AEBSF is used. The experiment was performed in triplicate.

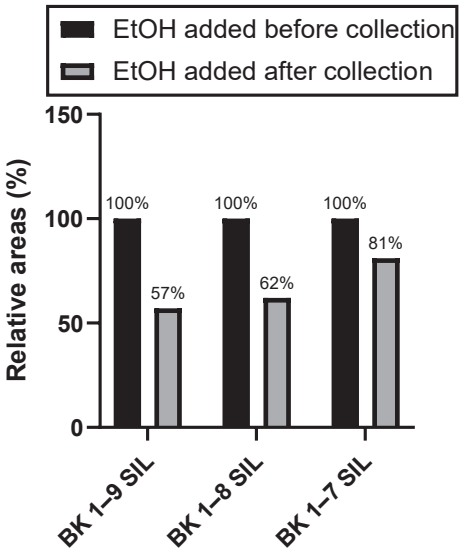

Fig. 23. BKs SIL stability during sampling collection. Relative areas recovered from 1000 pg/mL SIL Bradykinin 1–9 (BK1–9), BK1–8, and BK1–7 spiked in blood collection tubes. For the black and grey conditions, precipitation was induced by spiking the tubes with ethanol (EtOH) before or after the blood collection, respectively.

is to prepare freeze-dried aliquots of the inhibitors in a ready-to-use quantity to be thawed and reconstituted when needed. Moreover, as several inhibitors often have to be added to the samples because several different proteases degrade the peptide(s) of interest, we recommend preparing aliquots that contain all the required inhibitors. The quantity of each tube depends on the type of inhibitors and the application; the inhibitory power is dependent on the inhibitors themselves. It is also important to remember that blood is approximately 50 % plasma, and because the inhibitors are soluble in the plasma, their concentration is approximately two times the concentration spiked in blood. A protocol dedicated to the use of protease inhibitors is available in Section 4.5.2.

To integrate the inhibitors in the blood samples, we suggest solubilizing the lyophilized inhibitors in the lowest volume of water and adding them directly in the tubes used for blood collection. Thus, the inhibitors are mixed instantly with the blood, and they will rapidly exert their action on enzymes without significantly diluting the sample.

The stability of lyophilized aliquots of inhibitors must be controlled after long-term storage and during freeze-drying. In the absence of a convenient direct method to measure the concentration of the inhibitors, we suggest using an indirect method by comparing the inhibitory power of inhibitors from aliquots stored at −20 °C for some time versus control aliquots (e.g., freshly prepared). The stability of the mix must also be evaluated when reconstituted and added to the blood collection tube. For example, a protease inhibitor cocktail mix developed to ensure the stability of NPY was stable for 1 year at −20 °C in its solid form, but for only 1 week once solubilized [67].

3.2.5.3. Change the matrix composition. In some very specific cases, the addition of protease inhibitors is not adapted to prevent peptide degradation, such as BK with its 17-second half-life [218]. In such a case, inhibitors will not be mixed quickly enough in the plasma to inhibit the enzymes. Although this might not be an issue when BK is spiked in plasma containing well solubilized inhibitors, rapid degradation of BK will undoubtedly occur during sample collection [57]. This will eventually result in a change in concentration, precluding a proper comparison between sample concentrations that will be affected by the availability and quantity of proteases. Therefore, a faster option is to add a precipitating agent such as ethanol directly to the collection tube so that blood precipitates instantly, stopping the enzymatic activity. In this example (Fig. 23), we spiked SIL metabolites of BK (BK 1–9, BK 1–8, and BK 1–7) in two blood collection tubes. We added ethanol in the tubes before or after the blood collection to induce precipitation. To mimic normal sampling conditions, we left the tubes at room temperature for 15 min after the collection. We compared the conditions by measuring the areas obtained. Instantaneous precipitation during collection produced higher relative areas, suggesting that BK is not stable during the blood sampling unless ethanol is added beforehand.

Another option is to change the pH to reduce the enzymatic activity. Indeed, most enzymes have an ideal working pH around 7.4. Increasing or decreasing the pH will possibly stop this activity. In the following example, we incubated ANG I in plasma spiked with a combination of inhibitors (Fig. 24). The plasma contained aliskiren, a renin inhibitor, so we could monitor the degradation of ANG I and not its production by renin. After the incubation, we compared the recovery with a frozen plasma sample used as a reference. Almost all ANG I was lost after incubation for 3 h (-80 %). Moreover, the addition of EDTA and captopril, to block metalloproteases and ACE, respectively, only slightly improved the stability (-64 %). However, increasing the pH to 12 by adding a solution of sodium hydroxide 40 % almost completely stopped the degradation (-15 %). While this method works theoretically, in practice, a pH that is too high or too low might not be suitable to collect blood because it could lead to massive hemolysis.

Finally, Murphey et al. [65] proposed another method to cope with the rapid degradation of peptides such as BK. They found that BK 1–5 is a stable *in vivo* metabolite obtained from the degradation of BK. They postulated that the BK 1–5 concentration may be used as a marker for BK production. Although this approach provides an approximation of BK secretion, it cannot be used to obtain reliable quantitative data and may result in poor reproducibility [58].

3.2.6. The importance of setting a goal for the method

The previous section is dedicated to finding the best inhibitor mix and conditions to completely stop the enzymatic degradation. Nevertheless, this strategy requires additional considerations.

In a recent review concerning GLP-1 quantification, the author stated that protease inhibitors are crucial when measuring plasma levels of its

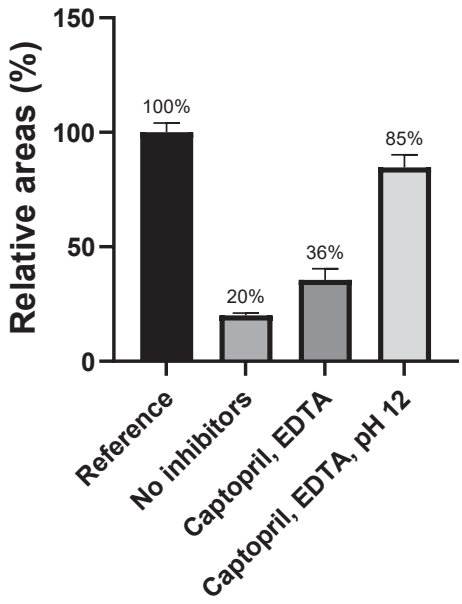

Fig. 24. ANG I stability in aliskiren plasma. Relative areas recovered from 1000 pg/mL angiotensin I (ANG I) in plasma containing aliskiren. The reference was a frozen plasma sample containing aliskiren. The no inhibitors condition contained aliskiren to prevent the production of ANG I by renin. The two other conditions contained captopril and EDTA as inhibitors, with the lighter gray condition set at a pH of 12. Except the reference, all the samples were incubated at 37 °C for 3 h. The experiment was performed in triplicate.

active forms, but they can be omitted when evaluating secretion using a C-terminal-specific assay [219]. Indeed, as DPP-4 is responsible for almost all GLP-1 degradation, and as it only cleaves the N-terminus of GLP-1, the epitope situated at the C-terminus of the peptide will still be recognized by the antibody. If immunoassays detect the two forms indistinctly, MS-based assays should quantify the two forms and only their addition would represent GLP-1 secretion. Of note, DPP-4-cleaved GLP1 is biologically inactive.

Another example is from Cegla et al. [220], who highlighted that glucagon is stable without protease inhibitors in plasma when it is quantified via LC-MS. This finding is contradictory to other studies using immunoassays. The discrepancies regarding glucagon stability from previous studies may be related to variation in antibody specificity that results in cross-reactivity with other peptides that are more sensitive to enzymes.

These examples show the importance of understanding the impact that degradation could have on the analytical method used and the results. Defining acceptable stability specific to the applications and goals of the method will save time and increase confidence in the results.

3.2.7. *Summary table*

Table 5 summarizes the most common proteases, their cleavage sites, and the inhibitors that block their activity. You may want to use Table 5 as follows:

1) In your peptide, identify the first three amino acids on the N-terminus.
2) Use the first column to see what type of exopeptidases might cleave the first three amino acids.
3) Once you have found the proteases, use the inhibitor column to get an idea of potential candidates that could block their activity and thus increase the stability of the peptide.
4) Do the same for the three amino acids on the C-terminus to find possible cleaving exopeptidases.
5) Finally, search for endopeptidases that may reduce the stability of the peptide.

Once you have found the list of inhibitors, we recommend testing their effect on stability by using the dedicated protocol (see Section 4.5.2.3) and evaluating their impact on the detection by comparing the recovery with and without inhibitors. Of note, Pro-Pro bonds are extremely resistant to degradation, even for proteases specialized for Pro cleavage, because it induces a conformation change that has a protective effect [221,222]. For more details, Page et al. [206] summarized the most common eukaryote proteases and Mentlein [207] reviewed the cell-surface proteases.

3.3. *Nonenzymatic modifications*

Peptides are prone to the same stability issues in biological matrixes and as a neat solution. Oxidation, deamidation, and other degradation mechanisms still occur in patient samples and therefore must be carefully controlled. However, biological matrixes add more complexity as peptides can react with the matrix components. This subchapter discusses peptide stability in biological matrixes and the effect of freeze–thaw cycles and storage temperature.

Nonenzymatic modifications are reactions between the peptide and its environment that induce PTMs of the amino acid chains, but they are not linked to enzymatic activity [273]. We cover enzymes in subchapter 3.2. PTMs can occur *in vivo* and *in vitro*. As long as these PTMs do not change the amino acids targeted by the antibody or their availability, they are not an issue for immunoassays. However, the slightest change in mass will be detected by an MS-based assay, resulting in inaccurate measurements. Whether the ratio of post-translationally modified to unmodified peptides or the total amount of peptide are of interest, PTMs must be evaluated and controlled [113]. In the first case, PTMs must be stopped, while in the second case, PTMs can also be forced to favor one peptide form over others. This will lead to a unique transition for a peptide, and therefore greater sensitivity. The following sections address the issues of glycation, oxidative crosslinking, and phosphorylation in peptide assays. For a deeper understanding of the reactions and mechanisms presented here, we recommend the review by Lai and Topp [135].

First, glycation may occur and modify the peptide structure by the Maillard reaction, a reduction of carbohydrates with amino compounds [274]. This reaction occurs in circulating human pancreatic peptide [275]. There has been a recent interest in these nonenzymatic modifications for the diagnosis of type 2 diabetes mellitus [276,277]. The direct consequence of this modification is that the clinical diabetic state of the patient might be a confounder when measuring peptides. For more detail, we recommend the valuable review by Thornalley and Rabbani [113].

Second, oxidative crosslinking, a reaction in which residues are crosslinked with others, might occur between Lys residues and C-terminal Asp residues [278]. Crosslinks in peptides might be revealed by using labeled ^{18}O that will be incorporated two times in the crosslinked peptides because of their double C-terminal ends, but only one time in the non-crosslinked peptide [279,280]. For more information about oxidative crosslinks, we direct readers to a valuable published review [281].

Third, phosphorylation is the addition of a phosphoryl group to the lateral chains of amino acids with an alcohol group (Ser and Thr). For example, up to 20 % of the Ser residues at the N-terminus of PTH can be phosphorylated [8,282].

3.3.1. *From cold to hot: What is the influence of storage temperature?*

The general rule for peptide stability in biological matrixes is the colder, the better, similarly to the storage temperature for neat peptides. At a very low temperature (-80 °C), chemical and biological degradation is very limited, although examples of metabolome alterations even at

−80 °C have been reported, meaning that even in this condition, peptide stability needs to be checked to fit the purpose of the assay [283,284]. In most cases though, storage at −80 °C might extend the stability of both biological samples and standard lyophilizates to years.

Increasing the temperature close to 0 °C will slightly increase biological and chemical reactions, but because the samples will still be frozen, it will limit the degradation of peptides by limiting the rates of biological and chemical reactions. There is a drastic change above 0 °C because the samples are no longer frozen. Usually, 4 °C is the temperature tested because it represents the temperature of a refrigerator as well as samples kept on ice. This temperature usually allows much longer stability of peptides such as procalcitonin, glucagon, and AVP, among others, than room temperature [180,182,285]. Room temperature might be suitable for some peptides even in a biological matrix, for short periods, as shown for B-type natriuretic peptide (BNP) [286].

A temperature of 37 °C (body temperature) is close to the optimal working condition for enzymes (see Section 3.2.1). This temperature completely changes the stability of peptides in biological samples and might result in the unwanted formation or complete degradation of peptides. Protocol in subchapter 4.4 might be used to test the stability in various temperature settings.

3.3.2. Freeze-thaw

In usual lab conditions, samples are frozen after collection and then thawed for analysis. Sometimes, one sample undergoes repeated freezing and thawing steps because it needs to be re-analyzed, which might change the stability of plasma constituents. In addition, more time at a temperature above 0 °C will reactivate the enzymes, and the matrix itself might change by protein precipitation and recrystallization [287]. Zhang et al. [85] highlighted that proteins tend to aggregate irreversibly during freeze/thaw cycles. Several groups have reported that more than two freeze–thaw cycles induce changes in the metabolic or protein profiles [24,288,289]. Other studies have reported no effect on the peptides measured [290]. In our experiments, multiple freeze–thaw cycles of plasma samples induce protein aggregation that eventually increases the probability of clogging issues during the extraction. In addition, extracted samples that have been frozen and thawed several times before injection seem to have a higher chance of clogging the instrument.

An example of the freeze–thaw issue is measuring plasma renin activity (PRA) [291]. In this case, when the sample temperature reaches −5°C to + 4 °C, prorenin is cryoactivated, inducing the production of renin and eventually ANG 1–10 [292–294]. This phenomenon is attributed to the cleavage by plasma proteases of the unfolded prosegment of prorenin, leading to its active form. Thus, activation of enzymes in thawed samples might be an important issue. We address this issue in the subchapter 3.2 dedicated to enzymes.

Therefore, a freeze–thaw stability study is necessary to assess the extent to which a particular peptide will suffer from repeated freeze–thaw cycles and to allow comparison between samples subjected to a single and multiple freeze–thaw cycles. It is also of great importance to monitor cautiously the number of freeze/thaw cycles that each sample undergoes to estimate its influence on the results. The general idea of evaluating the freeze–thaw effect relies on comparing two identical samples, one of which is serially thawed and frozen. For more details, see the dedicated protocol (see subchapter 4.2).

To cope with freeze–thaw effects as much as possible, the samples must be stored in multiple aliquots of the smallest possible volume for the extraction, so one sample is never frozen and thawed more than once. Plasma samples should also be centrifuged for 10 min at 2500 *g* prior to their use so that all the precipitated proteins remain at the bottom of the vial. This approach will lead to fewer problems by avoiding clogging of the filters used during the extraction and limit the matrix effect. Finally, a crucial point is to keep a record of the number of freeze–thaw undergone by a sample, to evaluate the validity of the results.

3.3.3. Conclusion

All the reactions that might occur to a peptide that change its mass, charge, or sequence must be kept in mind when developing an MS-based assay. The most impactful parameters are certainly temperature, the state of the peptide (solid or in a media), and pH. Because each peptide is different, there is no one-size-fits-all approach. However, the usual optimal conditions to store a peptide is to lyophilize the peptide, to keep it in a well-sealed container, and to store it at a low temperature (<-20 °C), protected from light. These conditions will limit nonenzymatic modifications and increase the stability of standards. It is not always possible to achieve these optimal conditions, and it is crucial to keep a record of the life of a peptide to highlight the modifications it might have experienced. This is especially the case when working with peptides composed of fragile amino acids such as Met for oxidation, Asn for deamidation, and Ser/Thr for phosphorylation.

3.4. The patient

Recently, our lab was developing a method to measure ANG I in plasma. One of the challenges was optimizing a mixture of protease inhibitors to improve ANG I stability in plasma. Indeed, ANG I is produced from angiotensinogen by renin, a very efficient enzyme, leading to a massive increase in ANG I over time. This is well studied because PRA is used to screen and diagnose secondary forms of hypertension [295]. To evaluate the capacity of the mixture of inhibitors to stop ANG I production, we incubated a random plasma sample with and without inhibitors in different conditions and then measured ANG I. We expected a significant increase in ANG I in the absence of inhibitors (control samples), and hopefully constant concentrations of ANG I with inhibitors. However, the outcomes were unexpected (Fig. 25). The inhibitors efficiently prevented ANG I degradation (black bars). However, surprisingly and against all odds, ANG I decreased in the control group without inhibitors.

After intense investigation, we found that the plasma was from a patient taking aliskiren to treat his hypertension. Aliskiren is a powerful renin inhibitor. This information was missing from the medical record and affected all the stability experiments. This example shows how impactful the patient's history, health conditions, and behavior are on the results. Moreover, it is not always possible to detect the presence of such bias in clinical practice, potentially leading to dramatic diagnostic failures.

Below we address the questions and challenges related to medication, stress, and other sampling conditions related to the patient that affect peptide concentrations.

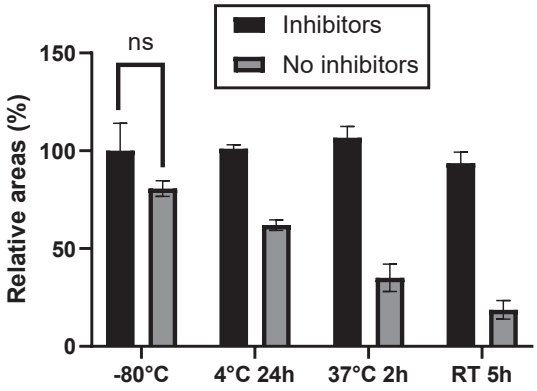

Fig. 25. ANG I incubation. Recovery of angiotensin I (ANG I) after different incubation times and temperatures, in the presence or absence of a dedicated inhibitor mix composed of aliskiren, captopril, ethylenediaminetetraacetic acid (EDTA), and 2,2′-bipyridyl. The ANG I decrease in the condition without inhibitors was unexpected because renin should produce ANG I from angiotensinogen. The experiment was performed in triplicate. RT. room temperature.

3.4.1. Treatment

As illustrated by the example with PRA, medications may strongly impact the concentration and stability of peptides. Indeed, many of the widely used treatments are protease inhibitors. For example, gliptins (sitagliptin, vildagliptin, saxagliptin, alogliptin, linagliptin, etc.) are used to treat type 2 diabetes. They are highly selective inhibitors of DPP-4, a protease that cleaves Pro or Ala at penultimate position and thus that rapidly degrades two major gastrointestinal hormones (GLP-1 and glucose-dependent insulinotropic polypeptide [GIP]) into inactive products. By stopping their degradation, gliptins reduce the blood glucose concentration and improve glycemic control [296,297]. However, DPP-4 degrades many other peptides, such as BK, NPY, and substance P. Thus, treatment with a gliptin will change the production and degradation of these peptides and lead to a change in their circulating concentration and stability.

Depending on peptide and drug half-lives and protease renewal, it might take minutes to days to return to baseline levels after a treatment. Understanding the mechanism of action of the medication and the metabolism of the peptides, and keeping a record of the drugs that alter the production or metabolism of the peptide is crucial to ensure reliable and comparable results. Medical treatments should always be checked for possible bias prior to measuring any peptide.

Other parameters may impact the quantification of peptides. For example, glucagon concentrations are reduced after hemodialysis in patients with type 2 diabetes mellitus [298]. The intense oxidative stress induced by dialysis increases oxidation of peptides such as PTH [20]. Acute ACE inhibitor–associated angioedema reduces DPP-4 activity and thus might influence the concentrations of many cleaved peptides [299]. Anesthesia might also increase or decrease the concentration of several peptides, such as natriuretic peptides that decrease as a consequence of anesthesia [300,301].

3.4.2. Stress

Whether physical or emotional, stress significantly modifies metabolism and, as a result, biomarkers. This is the basis for clinical chemistry, where the measurement of biomarkers modified by a stress such a bacterial infection participates in the diagnosis. On the contrary, stress induced by sample collection or the clinical environment is not desirable.

3.4.2.1. Emotional stress. The typical example is white coat hypertension (WCH), a well-studied condition in which patients have high blood pressure at their health care provider's office, but normal blood pressure at home [302,303]. The stress induced by simply being in a health care environment is sufficient to increase blood pressure. Such emotional stress modifies blood pressure as well as the concentrations of many biomarkers, including peptides. Conen et al. [304] showed that BNP is increased in patients with WCH in the same range as for patients with sustained hypertension. Karter et al. [305] showed that endothelin-1 concentrations were higher in patients with WCH compared with normotensive patients. Nystrom et al. [306] showed that NPY correlates with systolic tension in patients with WCH, while Pedrazzini et al. [307] highlighted its relation to stress.

3.4.2.2. Posture and physical exercise. Should I wait a few minutes to collect blood if the patient has rush to come to the hospital? Should I collect blood in the supine or upright position? Answers to these questions are not always straightforward, and it might be quite a challenge to integrate that into standard operating procedures (SOPs). We review the main consequences of the position and the physical efforts on blood sampling.

Exercise induces a change in peptide concentrations [308,309]. Barletta et al. [310] showed that atrial natriuretic peptide (ANP) and BNP were increased during bicycle and hand-grip exercises. While ANP seems to drop rapidly after exercise, other peptides might take minutes to hours to reach baseline levels after exercise [311]. Moreover, the metabolites of a peptide might increase after exercise because of a delayed production from their precursors. For example, while NPY increases with effort, its metabolite NPY 3–36 increases during the recovery period [312,313]. Grossman et al. [314] showed that many hormones (such as growth factor, cortisol, prolactin, and follicle-stimulating hormone) increase during effort.

Similarly, the posture, supine or upright, as well as postural changes might significantly impact circulating peptide concentrations. Several groups have shown that ANP and BNP increase in the supine posture and decrease in the upright posture [315–317]. NPY is also affected by posture, as highlighted by Vocat et al. [66] who showed increased concentrations when patients standing upright. The leg position plays a significant role in the distribution of blood in the body. Upright posture means more blood is pooled to the lower parts of the body, resulting in a reduction in renal blood, PRA, plasma flows, and urinary salt excretion. The supine position, however, increases the central blood volume [315].

3.4.2.3. Other stresses. To collect blood, should I wait after introducing the needle? Physical efforts and postural changes are not the only physical stress that may affect peptide concentrations. Almost any parameter may result in such a change. The introduction of a needle into the body to collect blood or to deliver an injection results in a dramatic increase in BK, which is produced from kininogen by kallikrein, an enzyme generated through activation of the contact activation system [58]. A delay between needle introduction and sampling is needed in such cases. Concentration changes may also occur when the patient is not conscious. Katan et al. [318] showed that copeptin correlates with stress in patients 30 min after extubation. Finally, extreme temperature might also change the peptide concentrations. For example, Damianaki et al. [319] showed that NPY increased during a cold pressure test.

To simplify sample collection, we recommend placing the patient in the supine position, immediately placing a venous catheter, and then allowing the patient to rest for 15 min in a temperature-controlled room. This approach will standardize the procedure and limit the impact of the patient's behavior before the collection.

3.4.3. Time and food

Should I collect the sample during daytime or nighttime? Should the patient abstain from food, caffeine, and alcohol? What is more impactful: smoking, or the stress provided by its abstinence?

When it comes to circadian fluctuations, it is easy to think about melatonin and its night/day concentration variations [320]. However, many other peptides are affected to a greater or a lesser extent by the circadian rhythm, including PTH [321–323], CCK, gastrin [324], and ANP [325]. Amyloid β has also been reported to have diurnal variations [181], but these results are controversial [194]. Being able to quantify biomarkers and to understand the effect of the time of day has been proposed as a way to improve drug administration in the so-called chronotherapy [326].

Food intake can also greatly affect the concentration of peptides. Obviously, the pharmacokinetics depends on the presence of food in the digestive track. In addition, many peptides are secreted during or after food intake, including ghrelin, CCK, GLP-1, PYY, and insulin, among others [327]. Moreover, the increase will depend on the type of food taken (lipid, carbohydrate, protein, or fiber) [328]. Therefore, a short period of time is needed to return to the baseline concentration, supporting the introduction of a fasting period before sample collection.

3.4.4. Conclusion

We have highlighted that the patient's treatments, level of stress, posture, food intake, and sampling time impact peptide concentrations. Of course, this is not an exhaustive list. Moreover, the impact of these pre-analytical considerations is difficult to predict, so they should be evaluated on a case-by-case basis. Clinical studies considering

pathophysiological regulation of the secretion of a given peptide are required to determine the critical parameters to control. Moreover, strict standardization of the sampling conditions is crucial point. As general advice, we recommend collecting blood samples in the morning after overnight fasting. The patient should be in a supine position, a catheter should be placed immediately, and then the patient should rest for a period of time corresponding to at least 5 half-lives in a temperature-controlled room before sample collection. Moreover, a record of any element that might have influenced the results (smoking, medications, etc.) should always be kept when a sample is collected.

4. Protocols

The following homemade protocols, developed over the years at our lab, have been refined to address the unique challenges of peptide analysis. These protocols adhere to the guidelines of CLSI, FDA, and EMA, offering comprehensive and detailed step-by-step instructions. They are based on our findings and insights from prior research in the field [34,35,54,55]. They should be applied on a case-by-case basis to fit the need of the assay developed.

4.1. Evaluation of the stability of standards

The strategy involves comparing the concentrations of samples prepared at the same time and subjected to different conditions. This approach allows evaluating the stability of a peptide in plasma at room temperature compared with reference samples stored at −80 °C.

1. Determine a storage temperature (e.g., +4°C) and the nature of the sample to be tested (e.g., 50 µg freeze-dried sample, 10 nM solution in ACN 50 %, or 100 pM in heparin plasma). These conditions should match the conditions encountered during analysis.
2. Determine the number of timepoints to be tested (e.g., control + days 1, 5, and 10).
3. Prepare n samples, with n = 3x the number of time-points (e.g., 12). We usually prepare an additional triplicate as a backup (e.g., 15).
4. Pool the samples and re-aliquot them. This will ensure they are all identical.
5. Store the control samples in the reference condition (e.g., −80 °C), and all other in the studied condition (e.g., +4°C).
6. Store in the reference condition (e.g., −80 °C) each triplicate after the duration of the test (e.g., 1, 5, and 10 days).
7. Thaw all samples and then quantify the peptide.
8. Calculate the ratio of concentrations in all triplicates versus control samples, as well as the p-value.

4.2. Evaluation of the freeze–thaw stability

1. Determine the nature of the sample to be tested (e.g., 10 nM solution in ACN 50 %, or 100 pM in heparin plasma). These conditions should match with conditions encountered during analysis.
2. Prepare six samples: label three samples “FT” and three samples “control.”
3. Pool the samples together and re-aliquot them. This will ensure they are all identical.
4. Freeze the six samples.
5. After > 12 h, thaw the three FT samples at room temperature.
6. After > 15 min, refreeze the three FT samples.
7. Repeat steps 5 and 6 at least three times. The number of freeze–thaw cycles should be equal to or exceed the number of cycles expected for the samples in lab conditions.
8. Thaw all six samples and add an internal standard.
9. Quantify the peptide.
10. Calculate the ratio of concentrations in FT versus control samples, as well as the p-value.

4.3. Procedure to aliquot standards

Here we present our procedure for handling of standards to ensure the best stability:

- Order an exact amount of peptide (e.g., 2.00 mg). Ask for a CoA with the purity and peptide content, which is not always included by default.
- Calculate the net amount of peptide as follows: gross weight × purity × peptide content (e.g., 2.00 mg × 95 % × 75 % = 1.425 mg).
- Solubilize the peptide by adding an exact volume of solution to get 1 mg/mL (e.g., 1425 µL). Keep on ice. The solution used to solubilize the lyophilizate should be ice cold, and its composition should respect the recommendations from the manufacturer or literature (e.g., ACN 20 % in water).
- Prepare 50 µL (=50 µg net peptide) aliquots in tightly sealable tubes.
- Freeze-dry.
- If oxidation may occur, then add an inert gas in the tube, such as argon or N_2.
- Store at −20 °C.
- The tube contains exactly 50 µg of net peptide and is meant for a single use.
- When necessary, place an aliquot at room temperature for 15 min.
- Add 500 µL of an adapted solution to get a 0.1 mg/mL stock solution, which is ready to use.
- When work with the stock solution is completed:
  - o If the stability of the peptide in this stock solution has been assessed, then prepare aliquots as necessary for further use and store at −80 °C.
  - o If the stability of the peptide has not been assessed, then do not keep it.

We recommend that long-term storage be in dried forms, frozen, and with a desiccant. If long-term storage of a stock solution is required, then consider storing small aliquots at −80 °C to prevent the need to thaw and refreeze the stock solution. Keep working solutions on ice. Finally, stability studies should be performed in the early steps of any development if robust stability data are not available.

4.4. Evaluation of stability in a biological matrix

Stability should be tested in different conditions—variable temperature and several durations—to reflect the relevant situations the samples are subjected to during the pre-analytical and analytical steps. For the sake of efficiency, when testing the effect of a new protease inhibitor, it is more efficient to test one pilot condition. We recommend testing the stability of the peptide for 30 min at 37 °C because it mimics sample collection and represents highly aggressive conditions. If the peptide is stable in these conditions, it will likely also be stable in other conditions, such as 4 °C for 2 h. If the stability during the pilot study is confirmed, then perform a comprehensive evaluation of other conditions to ensure the stability of the peptide during the entire assay. In addition, used other plasma samples to determine inter-sample variability. If the peptide is not stable during the pilot study, then review the literature and determine which proteases degrade it. Adjust the conditions to ensure peptide stability (see protocols 5.4.1 and 5.4.2).

4.4.1. Pilot study

1) If the target peptide circulates at very low concentrations, then spike plasma samples with the target peptide at a detectable concentration. If it is detectable, simply use native plasma samples. Create six identical samples: Label three as “Control” and three as “Incubation.”
2) Freeze all the samples at −80 °C for more than 2 h.
3) Thaw the three “Incubation” samples in front of a fan for 10 min and incubate them for 30 min at 37 °C.

4) Ten minutes before the end of the incubation, thaw the remaining three "Control" samples in front of a fan for 10 min so that they will be thawed when incubation is over.
5) Spike all samples with a SIL analog right before the extraction so that it will correct for extraction and analytical variations. Thus, only the incubation will induce a change in the determined concentrations.
6) Compare the conditions using the analyte/SIL ratio to evaluate the stability. If the peptide is stable, then the ratio in the "Incubation" tubes should not be different from the one in the "Control" tubes.
7) If the peptide is not stable, in addition to an extensive literature review, we suggest a degradation product study to find the proteases of the metabolic pathway:
   - o Spike samples with a SIL (typically with ^{13}C) analog of the precursor peptide. Of note, it is necessary to have every analog labeled at the same position so that enzymatic degradation does not cleave and eliminate the labeled amino acid. For example, if a method targets ANG I, ANG II, and ANG 1–5, the first five amino acids common to the three peptides should be labeled.
   - o Incubate the samples for 1 h at 37 °C and measure the peptides every 15 min.
   - o Identify the proteases that could produce the results obtained in term of peptide production and degradation.

4.4.2. Comprehensive study

At a second time, the conditions in which the sample will be handled from collection to preparation should be tested. It is important to clearly specify the conditions that will be specific to an assay. For a peptide assay in a clinical context, we recommend:

- 1 h at 4 °C or room temperature to mimic sample preparation on ice or on a bench;
- 12 h at 4 °C to mimic sample transport.

Ideally, a few more tests should be done to study the behavior of the analyzed peptide in unusual conditions; this approach allows assessing the limits in which the samples are still considered stable. It must fit the specificity of the analysis developed and the handling conditions particular to it. We suggest the following incubation times:

- 24 h at 4 °C for a leftover sample in a fridge;
- 24 h at room temperature for a leftover sample on a bench;
- 1 h at 37 °C to increase the confidence in the stability during the first hour after sampling, which is critical because the laboratory does not handle the samples at that time.

Once chosen, each condition should be tested using the following protocol:

1) If the endogenous target peptide is undetectable, then spike plasma samples with the target peptide at a detectable concentration. If it is detectable, simply use native plasma samples. Run every condition in triplicate; one triplicate used as a reference should be kept frozen at −80 °C until extraction.
2) Freeze all the samples at −80 °C for more than 2 h.
3) For each condition to test, thaw samples as they are processed for incubation in front of a fan for 10 min and incubate them for the corresponding time so that they all finish the incubation at the same time.
4) Spike all samples with a SIL analog right before the extraction so that it will correct for extraction and analytical variations. Thus, only the incubation will induce a change in the concentrations.

Finally, the conditions that ensure peptide stability should be tested on at least five different plasma samples. This will confirm that the inter-sample variability in terms of enzymatic activity does not change the peptide stability.

4.5. How to cope with enzymatic degradation

If the stability study (subchapter 4.4) shows that the peptide is unstable during one of the assay steps, then several parameters might be changed to improve the peptide's stability. As soon as a parameter is changed, the stability should be retested by following the dedicated testing protocols (Sections 4.4.1 and 4.4.2). The following protocol covers the way parameters, such as temperature and the addition of protease inhibitors or solvents, might be changed and how to evaluate the impact of these changes on the results.

4.5.1. Temperature and duration

Enzymatic activity is closely linked to the time a sample spends at a specific temperature. The closer to the optimal working condition of the protease (usually 37 °C), the higher the enzymatic activity. If the peptide is not stable under the expected handling conditions, then it might be rewarding to reduce the temperature or the duration spent at that temperature.

1) After blood collection, place the tubes directly on crushed ice.
2) Make sure that centrifugation is performed within 30 min after blood collection in a centrifuge cooled to 4 °C.
3) If the peptide is not stable for 1 h at room temperature, then the sample preparation might lead to great degradation. Keep the samples as well as the solvent used for sample preparation on crushed ice. Everything should be at around 4 °C.
4) If these conditions are not sufficient to stabilize the peptides, then use protease inhibitors.

4.5.2. Protease inhibitors

Protease inhibitors are used when the temperature and duration of the various steps of the assay are not sufficient to ensure stability of the peptide. Tests are first done directly on plasma using solubilized inhibitors. Then, the inhibitor mix is tested in real conditions, directly on blood, and from a lyophilized batch. Finally, the impact on results of the addition of inhibitors in the matrix is evaluated.

4.5.2.1. Utilization of protease inhibitors during the pilot study.

1) Following the literature review and the degradation test, calculate the required amount of each inhibitor based on its inhibitory power.

2) Weigh out the necessary amounts of the selected inhibitors in the solid form.

3) Combine and solubilize inhibitors in the lowest possible volume of water. If they do not solubilize, then shake or sonicate the solution for a few minutes.

4) Inhibitors in solution are added directly to the plasma to reach the desired concentration.

5) Perform stability tests using the dedicated protocol (Sections 4.4.1 and 4.4.2).

4.5.2.2. Utilization of protease inhibitors during the comprehensive study.

In real conditions, to avoid diluting the sample and to ensure proper solubilization of the inhibitors in the blood, we first recommend freeze-drying the optimal mix in ready-to-use vials. Then, resolubilize the mix and add it directly to the collection tubes.

1) Following the literature review and the degradation test, calculate the required amount of each inhibitor based on its inhibitory power.

2) Split the inhibitors into two categories: highly polar (soluble in water) and apolar.
3) Weigh the previously calculated amount of each polar inhibitor together.
4) Weigh the previously calculated amount of each apolar inhibitor together.
5) Solubilize the polar and apolar inhibitors in the lowest possible volume of water and *tert*-butyl alcohol, respectively, with the *tert*-butyl alcohol volume equal to 25 % of the water volume. *Tert*-Butyl alcohol is miscible with water and has a high melting point (25 °C). It will help solubilize the inhibitors and ensure that they stay frozen during the freeze-drying process, thus avoiding boiling of the liquid. A few tests might be needed to adapt the proportion of this solvent to the batch volume and composition. If the inhibitors are not properly solubilized in their respective solvent, then shake and sonicate the solution for few minutes.
6) Prepare aliquots of the solution and freeze-dry the aliquots. Of note, the aliquot volume must be adapted to the volume of the tubes used for blood collection. Indeed, an 8 mL collection tube will need two times the inhibitors used for a 4 mL collection tube.
7) Once freeze-dried, store inhibitors at −20 °C until further use.
8) For the tests, resolubilize the freeze-dried inhibitors in the lowest possible volume of water, to avoid dilution of the sample (ideally, the volume of the added inhibitors should not be more than 5 % of the collection tube volume).
9) Add the solubilized inhibitors directly to the blood collection tube. Tighten the cap while pushing the plunger to ensure that the maximum volume of blood can be collected.
10) Collect the blood using the prepared collection tubes, ideally from different people (at least five) to highlight possible inter-sample variability in term of enzymatic activity.
11) Once the blood is collected in the prepared collection tubes, they are mixed gently to distribute the inhibitors and then quickly centrifuged.
12) The plasma is collected and ready to be used as described in the stability test protocol (Sections 4.4.1 and 4.4.2).

4.5.2.3. Evaluation of the impact of inhibitors on recovery. The inhibitors might affect the recovery of peptide by reducing the retention during the extraction or inducing matrix effects. Therefore, it is important to ensure that the target peptide is not affected by the mix.

1) If the target peptide circulates at very low concentrations, spike plasma samples with the target peptide at a detectable concentration. If it is detectable, simply use native plasma samples.
2) Spike the plasma samples with the selected inhibitors solubilized in water. The concentration needed will depend on the inhibitory power of the inhibitors. Create two conditions:
   o Without inhibitors
   o With inhibitors
3) Spike all samples with a SIL analog right before the extraction so that it will correct for extraction and analytical variations. Thus, the comparison between SIL analog recovery in samples with and without inhibitors will provide insight into the effects of the inhibitors on the recovery.
4) Compare the conditions using the analyte-to-SIL analog ratio to evaluate the matrix effect.

4.5.3. Solvent and pH

If no inhibitors can ensure the stability of the target peptide, then it might be rewarding to stop the protease activity by drastically changing the pH or the solvent used. This is the usually the case for quickly metabolized peptides, and these strategies should therefore be used directly in the blood collection tubes.

1) Add acid or base (typically hydrochloric acid (HCl) or sodium hydroxide (NaOH), respectively) directly to the blood collection tubes, just enough to change the pH and to stop the enzymatic activity. The volume added will depend on the collection tube volume. You could also add a solvent such as ACN, ethanol (EtOH), or MeOH to precipitate the blood directly during the collection.
2) Follow the usual protocol to test the stability (Sections 4.4.1 and 4.4.2).
3) Watch out: Such additives might induce strong hemolysis, leading to changes in the measured concentrations or difficulties during sample preparation because of the changed matrix.

5. Conclusion

In recent decades, the quantification of peptide hormones has gained significant interest for diagnostic and drug development applications. Guidelines for peptide handling have evolved to emphasize pre-analytical steps. However, notable gaps exist in current main guidelines (FDA, EMA, CLSI), particularly regarding neat peptide handling and stability in biological matrixes. This tutorial-review addresses peptide work challenges from a clinical laboratory stance. It covers handling neat peptides, focusing on stability and adsorption, and delves into analyzing complex biological samples, addressing matrix effects, protease-mediated degradation, and patient-specific impacts. The review offers precise protocols to assess and mitigate pre-analytical concerns. By amalgamating these insights with existing peptide analysis guidelines, our aim is to enhance assay reliability and improve the accuracy of clinical results.

CRediT authorship contribution statement

Jonathan Maurer: Writing – original draft, Visualization. **Eric Grouzmann:** Writing – review & editing. **Philippe J. Eugster:** Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

We gratefully acknowledge Léon Martin (Geneva University), Anke de Groot, Delphine Cappelle, and Charlotte Van Gyseghem (Antwerpen University) for their work on angiotensins, PTH, and AVP assays, respectively, and Dr Gemma Reverter-Branchat, Marielle Dunand, Adeline Herren and Céline Vocat (Lausanne University Hospital) for their work on NPY. Prof. Jean-Luc Veuthey (Geneva University) is warmly thanked for his critical review of the manuscript.

Funding

We acknowledge the Bryn Turner-Samuels Foundation (Lausanne, Switzerland) for funding Jonathan Maurer's doctoral thesis.

References

[1] S. Gandhi, R.B.H. Myers, Can parathyroid hormone be used as a biomarker for heart failure? Heart Fail. Rev. 18 (2013) 465–473.
[2] F.M. Khattab, M. Fawzy, Incretin serum level as a biomarker of insulin resistance in acne vulgaris patients, J. Cosmet. Dermatol. 21 (2022) 5181–5186.

[3] A. Latosinska, M. Frantzi, J. Siwy, Peptides as "better biomarkers"? Value, challenges, and potential solutions to facilitate implementation, Mass Spectrom. Rev., DOI 10.1002/mas.21854(2023).
[4] E.E. Chambers, M.E. Lame, J. Bardsley, S. Hannam, C. Legido-Quigley, N. Smith, K.J. Fountain, E. Collins, E. Thomas, High sensitivity LC-MS/MS method for direct quantification of human parathyroid 1–34 (teriparatide) in human plasma, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 938 (2013) 96–104.
[5] A. Capron, J. Destree, D. Maiter, P. Wallemacq, Validation of a rapid liquid chromatography-tandem mass spectrometric assay for the determination of octreotide plasma concentrations, Clin. Biochem. 47 (2014) 139–141.
[6] A. Abramson, F. Halperin, J. Kim, G. Traverso, Quantifying the Value of Orally Delivered Biologic Therapies: A Cost-Effectiveness Analysis of Oral Semaglutide, J. Pharm. Sci. 108 (2019) 3138–3145.
[7] G. Antonelli, M. Marinova, C. Artusi, M. Plebani, Mass spectrometry or immunoassay: est modus in rebus, Clin. Chem. Lab. Med. 55 (2017) 1243–1245.
[8] L. Couchman, D.R. Taylor, B. Krastins, M.F. Lopez, C.F. Moniz, LC-MS candidate reference methods for the harmonisation of parathyroid hormone (PTH) measurement: a review of recent developments and future considerations, Clin. Chem. Lab. Med. 52 (2014) 1251–1263.
[9] A. Miyachi, M. Kobayashi, E. Mieno, M. Goto, K. Furusawa, T. Inagaki, T. Kitamura, Accurate analytical method for human plasma glucagon levels using liquid chromatography-high resolution mass spectrometry: comparison with commercially available immunoassays, Anal. Bioanal. Chem. 409 (2017) 5911–5918.
[10] C. Seger, L. Salzmann, After another decade: LC-MS/MS became routine in clinical diagnostics, Clin. Biochem. 82 (2020) 2–11.
[11] S.K.G. Grebe, R.J. Singh, Clinical peptide and protein quantification by mass spectrometry (MS), TrAC, Trends Anal. Chem. 84 (2016) 131–143.
[12] C. Liu, Z. Zhao, J. Fan, C.J. Lyon, H.-J. Wu, D. Nedelkov, A.M. Zelazny, K. N. Olivier, L.H. Cazares, S.M. Holland, E.A. Graviss, Y. Hu, Quantification of circulating Mycobacterium tuberculosis antigen peptides allows rapid diagnosis of active disease and treatment monitoring, PNAS 114 (2017) 3969–3974.
[13] R. Farah, H. Haraty, Z. Salame, Y. Fares, D.M. Ojcius, N. Said Sadier, Salivary biomarkers for the diagnosis and monitoring of neurological diseases, Biomed. J. 41 (2018) 63–87.
[14] C.J. Collins, F. Yi, R. Dayuha, P. Duong, S. Horslen, M. Camarata, A.K. Coskun, R. H.J. Houwen, T.L. Pop, H. Zoller, H.W. Yoo, S.W. Jung, K.H. Weiss, M.L. Schilsky, P. Ferenci, S.H. Hahn, Direct Measurement of ATP7B Peptides Is Highly Effective in the Diagnosis of Wilson Disease, Gastroenterology 160 (2021) 2367–2382 e2361.
[15] C. Liu, D. Chu, K. Kalantar-Zadeh, J. George, H.A. Young, G. Liu, Cytokines: From Clinical Significance to Quantification, Adv. Sci. 8 (2021) 2004433.
[16] M.C. Chappell, Reply to "Letter to the editor: Angiotensin quantification by mass spectrometry", Am. J. Phys. Heart Circ. Phys. 310 (2016) H454.
[17] E. Grouzmann, P.J. Eugster, LC-MS/MS Peptide Assay Validation: A Plea for Robust Stability Studies, Clin. Chem. 68 (2022) 727–728.
[18] G. Lippi, J.J. Chance, S. Church, P. Dazzi, R. Fontana, D. Giavarina, K. Grankvist, W. Huisman, T. Kouri, V. Palicka, M. Plebani, V. Puro, G.L. Salvagno, S. Sandberg, K. Sikaris, I. Watson, A.K. Stankovic, A.M. Simundic, Preanalytical quality improvement: from dream to reality, Clin. Chem. Lab. Med. 49 (2011) 1113–1126.
[19] G.L. Salvagno, E. Danese, G. Lippi, Preanalytical variables for liquid chromatography-mass spectrometry (LC-MS) analysis of human blood specimens, Clin. Biochem. 50 (2017) 582–586.
[20] C.Z. Ulmer, K. Kritmetapak, R.J. Singh, H.W. Vesper, R. Kumar, High-Resolution Mass Spectrometry for the Measurement of PTH and PTH Fragments: Insights into PTH Physiology and Bioactivity, J. Am. Soc. Nephrol. 33 (2022) 1448–1458.
[21] B. Sabbagh, S. Mindt, M. Neumaier, P. Findeisen, Clinical applications of MS-based protein quantification, Proteomics - Clin. Appl. 10 (2016) 323–345.
[22] A.J. Rai, F. Vitzthum, Effects of preanalytical variables on peptide and protein measurements in human serum and plasma: implications for clinical proteomics, Expert Rev. Proteomics 3 (2006) 409–426.
[23] O. Hansson, A. Mikulskis, A.M. Fagan, C. Teunissen, H. Zetterberg, H. Vanderstichele, J.L. Molinuevo, L.M. Shaw, M. Vandijck, M.M. Verbeek, M. Savage, N. Mattsson, P. Lewczuk, R. Batrla, S. Rutz, R.A. Dean, K. Blennow, The impact of preanalytical variables on measuring cerebrospinal fluid biomarkers for Alzheimer's disease diagnosis: A review, Alzheimers Dement. 14 (2018) 1313–1333.
[24] A.N. Hoofnagle, J.R. Whiteaker, S.A. Carr, E. Kuhn, T. Liu, S.A. Massoni, S. N. Thomas, R.R. Townsend, L.J. Zimmerman, E. Boja, J. Chen, D.L. Crimmins, S. R. Davies, Y. Gao, T.R. Hiltke, K.A. Ketchum, C.R. Kinsinger, M. Mesri, M. R. Meyer, W.J. Qian, R.M. Schoenherr, M.G. Scott, T. Shi, G.R. Whiteley, J. A. Wrobel, C. Wu, B.L. Ackermann, R. Aebersold, D.R. Barnidge, D.M. Bunk, N. Clarke, J.B. Fishman, R.P. Grant, U. Kusebauch, M.M. Kushnir, M. S. Lowenthal, R.L. Moritz, H. Neubert, S.D. Patterson, A.L. Rockwood, J. Rogers, R.J. Singh, J.E. Van Eyk, S.H. Wong, S. Zhang, D.W. Chan, X. Chen, M.J. Ellis, D. C. Liebler, K.D. Rodland, H. Rodriguez, R.D. Smith, Z. Zhang, H. Zhang, A. G. Paulovich, Recommendations for the Generation, Quantification, Storage, and Handling of Peptides Used for Mass Spectrometry-Based Assays, Clin. Chem. 62 (2016) 48–69.
[25] A. Padoan, The Impact of Pre-Analytical Conditions on Human Serum Peptidome Profiling, Proteomics - Clin. Appl. 12 (2018) 1700183.
[26] E.S. Nakayasu, M. Gritsenko, P.D. Piehowski, Y. Gao, D.J. Orton, A.A. Schepmoes, T.L. Fillmore, B.I. Frohnert, M. Rewers, J.P. Krischer, C. Ansong, A.M. Suchy-Dicey, C. Evans-Molina, W.-J. Qian, B.-J.-M. Webb-Robertson, T.O. Metz, Tutorial: best practices and considerations for mass-spectrometry-based protein biomarker discovery and validation, Nat. Protoc. 16 (2021) 3737–3760.
[27] J. Maurer, E. Grouzmann, P.J. Eugster, The road to reliable peptide assays is paved with good guidelines, Clin. Endocrinol. (Oxf.), DOI 10.1111/cen.14863 (2022).
[28] L.M. McShane, D.G. Altman, W. Sauerbrei, S.E. Taube, M. Gion, G.M. Clark, REporting recommendations for tumour MARKer prognostic studies (REMARK), Br. J. Cancer 93 (2005) 387–391.
[29] S.E. Abbatiello, B. Schilling, D.R. Mani, L.J. Zimmerman, S.C. Hall, B. Maclean, M. Albertolle, S. Allen, M. Burgess, M.P. Cusack, M. Gosh, V. Hedrick, J.M. Held, H.D. Inerowicz, A. Jackson, H. Keshishian, C.R. Kinsinger, J. Lyssand, L. Makowski, M. Mesri, H. Rodriguez, P. Rudnick, P. Sadowski, N. Sedransk, K. Shaddox, S.J. Skates, E. Kuhn, D. Smith, J.R. Whiteaker, C. Whitwell, S. Zhang, C.H. Borchers, S.J. Fisher, B.W. Gibson, D.C. Liebler, M.J. Maccoss, T.A. Neubert, A.G. Paulovich, F.E. Regnier, P. Tempst, S.A. Carr, Large-Scale Interlaboratory Study to Develop, Analytically Validate and Apply Highly Multiplexed, Quantitative Peptide Assays to Measure Cancer-Relevant Proteins in Plasma, Mol. Cell. Proteomics 14 (2015) 2357–2374.
[30] R. González-Domínguez, Á. González-Domínguez, A. Sayago, Á. Fernández-Recamales, Recommendations and Best Practices for Standardizing the Pre-Analytical Processing of Blood and Urine Samples in Metabolomics, Metabolites 10 (2020) 229.
[31] FDA, Guidance for Industry - Bioanalytical Method Validation, in: U.S.F.a.D. Administration, (Ed.) 2018.
[32] CLSI, C62-A Liquid Chromatography-Mass Spectrometry Methods, (Ed.) 2014.
[33] EMA, ICH guideline M10 on bioanalytical method validation and study sample analysis, (Ed.) 2022.
[34] CLSI, C64 Quantitative Measurement of Proteins and Peptides by Mass Spectrometry, (Ed.) 2021.
[35] K. Maes, I. Smolders, Y. Michotte, A. Van Eeckhaut, Strategies to reduce aspecific adsorption of peptides and proteins in liquid chromatography–mass spectrometry based bioanalyses: An overview, J. Chromatogr. A 1358 (2014) 1–13.
[36] W. Bittremieux, D.L. Tabb, F. Impens, A. Staes, E. Timmerman, L. Martens, K. Laukens, Quality control in mass spectrometry-based proteomics, Mass Spectrom. Rev. 37 (2018) 697–711.
[37] N.C. Hughes, E.Y.K. Wong, J. Fan, N. Bajaj, Determination of carryover and contamination for mass spectrometry-based chromatographic assays, AAPS J. 9 (2007). E353–E360.
[38] M. Duncan, Influence of surfactants upon protein/peptide adsorption to glass and polypropylene, Int. J. Pharm. 120 (1995) 179–188.
[39] S.V. Patwardhan, F.S. Emami, R.J. Berry, S.E. Jones, R.R. Naik, O. Deschaume, H. Heinz, C.C. Perry, Chemistry of aqueous silica nanoparticle surfaces and the mechanism of selective peptide adsorption, J. Am. Chem. Soc. 134 (2012) 6244–6256.
[40] E. Willemse, K. Uffelen, B. Brix, S. Engelborghs, H. Vanderstichele, C. Teunissen, How to handle adsorption of cerebrospinal fluid amyloid β (1–42) in laboratory practice? Identifying problematic handlings and resolving the issue by use of the Aβ42 / Aβ40 ratio, Alzheimers Dement. 13 (2017) 885–892.
[41] S.L. Law, C.L. Shih, Adsorption of calcitonin to glass, Drug Dev. Ind. Pharm. 25 (1999) 253–256.
[42] Y. Van Wanseele, K. Maes, K. Lanckmans, J. Van Schoors, I. Smolders, A. Van Eeckhaut, Surface and Solvent Dependent Adsorption of Three Neuromedin-Like Peptides in Glass and Plastic Syringes, Chromatographia 81 (2018) 65–72.
[43] A. Pezeshki, V. Vergote, S. Van Dorpe, B. Baert, C. Burvenich, A. Popkov, B. De Spiegeleer, Adsorption of peptides at the sample drying step: influence of solvent evaporation technique, vial material and solution additive, J. Pharm. Biomed. Anal. 49 (2009) 607–612.
[44] K.L. Prime, G.M. Whitesides, Self-Assembled Organic Monolayers: Model Systems for Studying Adsorption of Proteins at Surfaces, Science (1991).
[45] G.B. Sigal, M. Mrksich, G.M. Whitesides, Effect of Surface Wettability on the Adsorption of Proteins and Detergents, J. Am. Chem. Soc. (1998).
[46] F. Staniscia, H.V. Guzman, M. Kanduč, Tuning Contact Angles of Aqueous Droplets on Hydrophilic and Hydrophobic Surfaces by Surfactants, J. Phys. Chem. B 126 (2022) 3374–3384.
[47] G. Lefebvre, A. Maze, R. Alvarez-Palencia Jimenez, F. Bruckert, V. Filipe, S. Huille, M. Weidenhaupt, Surfactant Protection Efficacy at Surfaces Varies with the Nature of Hydrophobic Materials, Pharm. Res., DOI (2021).
[48] Y. Van Wanseele, A. De Prins, I.S. B., A. Van Eeckhaut, Challenges for the in vivo quantification of brain neuropeptides using microdialysis sampling and LC–MS, Bioanalysis, DOI (2016).
[49] P. Hyenstrand, J.S. Metcalf, K.A. Beattie, G.A. Codd, Effects of adsorption to plastics and solvent conditions in the analysis of the cyanobacterial toxin microcystin-lr by high performance liquid chromatography, Water Res, DOI, 2001.
[50] H.R. Cho, J.S. Park, T.D. Wood, Y.S. Choi, Longitudinal Assessment of Peptide Recoveries from a Sample Solution in an Autosampler Vial for Proteomics, Bull. Kor. Chem. Soc. 36 (2015) 312–321.
[51] J. Bongaerts, D. De Bundel, D. Mangelings, I. Smolders, Y. Vander Heyden, A. Van Eeckhaut, Sensitive targeted methods for brain metabolomic studies in microdialysis samples, J. Pharm. Biomed. Anal. 161 (2018) 192–205.
[52] M. Feickert, B.B. Burckhardt, A design of experiments concept for the minimization of nonspecific peptide adsorption in the mass spectrometric determination of substance P and related hemokinin-1, J. Sep. Sci. 43 (2020) 818–828.

[53] E.L. Murphy, A.P. Joy, R.J. Ouellette, D.A. Barnett, Improved intact peptide and protein quantitation by LC-MS: Battling the deleterious effects of analyte adsorption, Anal. Sci. Adv. 2 (2021) 299–307.

[54] J. Toombs, R.W. Paterson, J.M. Schott, H. Zetterberg, Amyloid-beta 42 adsorption following serial tube transfer, Alzheimers Res. Ther. 6 (2014) 5.

[55] J. Harald, M. Walden, S. Schaefer, G. Sandra, F. Wolf-Georg, Analytical procedures for quantification of peptides in pharmaceutical research by liquid chromatography-mass spectrometry, Anal. Bioanal. Chem. 378 (2004) 883–897.

[56] A. Thomas, M. Thevis, Analysis of insulin and insulin analogs from dried blood spots by means of liquid chromatography–high resolution mass spectrometry, Drug Test. Anal. 10 (2018) 1761–1768.

[57] M. Lindström, M. Valkonen, N. Tohmola, R. Renkonen, T. Strandin, A. Vaheri, O. Itkonen, Plasma bradykinin concentrations during septic shock determined by a novel LC-MS/MS assay, Clin. Chim. Acta 493 (2019) 20–24.

[58] K.F. Seip, K.C. Bjerknes, H.T. Johansen, E.W. Nielsen, L. Landro, L. Reubsaet, Bradykinin analysis revived–a validated method for determination of its stable metabolite in whole blood by LC-MS/MS, J. Chromatogr. B Analyt. Technol. Biomed. Life. Sci., 947–948 (2014) 139–144.

[59] D. Liu, X. Han, X. Liu, M. Cheng, M. He, G. Rainer, H. Gao, X. Zhang, Measurement of ultra-trace level of intact oxytocin in plasma using SALLE combined with nano-LC–MS, J. Pharm. Biomed. Anal. 173 (2019) 62–67.

[60] G. Zhang, Y. Zhang, D.M. Fast, Z. Lin, R. Steenwyk, Ultra sensitive quantitation of endogenous oxytocin in rat and human plasma using a two-dimensional liquid chromatography-tandem mass spectrometry assay, Anal. Biochem. 416 (2011) 45–52.

[61] C. Wai-Shan Lo, T. Kam-Chi Tsui, R. Ching-Wan Ma, M. Ho-Ming Chan, C.S. Ho, Quantitation of plasma angiotensin II in healthy Chinese subjects by a validated liquid chromatography tandem mass spectrometry method, Biomed. Chromatogr., DOI 10.1002/bmc.5318(2022).

[62] P.M. Van Midwoud, L. Rieux, R. Bischoff, E. Verpoorte, H.A.G. Niederlander, Improvement of Recovery and Repeatability in Liquid Chromatography-Mass Spectrometry Analysis of Peptides, J. Proteome Res. (2007).

[63] H.J. Issaq, K.C. Chan, J. Blonder, X. Ye, T.D. Veenstra, Separation, detection and quantitation of peptides by liquid chromatography and capillary electrochromatography, J. Chromatogr. A (2009).

[64] K. Delaney, D. Jia, L. Iyer, Z. Yu, S.B. Choi, P.J. Marvar, P. Nemes, Microanalysis of Brain Angiotensin Peptides Using Ultrasensitive Capillary Electrophoresis Trapped Ion Mobility Mass Spectrometry, Anal. Chem. 94 (2022) 9018–9025.

[65] L.J. Murphey, D.L. Hachey, D.E. Vaughan, N.J. Brown, J.D. Morrow, Quantification of BK1-5, the stable bradykinin plasma metabolite in humans, by a highly accurate liquid-chromatographic tandem mass spectrometric assay, Anal. Biochem. 292 (2001) 87–93.

[66] C. Vocat, M. Dunand, S.A. Hubers, N. Bourdillon, G.P. Millet, N.J. Brown, G. Wuerzner, E. Grouzmann, P.J. Eugster, Quantification of Neuropeptide Y and Four of Its Metabolites in Human Plasma by Micro-UHPLC-MS/MS, Anal. Chem. 92 (2020) 859–866.

[67] G. Reverter-Branchat, P.J. Eugster, C. Kuenzli, B. Rindlisbacher, T. Stauffer, C. T. Nakas, D. Herzig, E. Grouzmann, L. Bally, Multiplexed Assay to Quantify the PP-Fold Family of Peptides in Human Plasma Using Microflow Liquid Chromatography-Tandem Mass Spectrometry, Clin. Chem. 68 (2022) 584–594.

[68] K. Maes, J. Van Liefferinge, J. Viaene, J. Van Schoors, Y. Van Wanseele, G. Bechade, E.E. Chambers, H. Morren, Y. Michotte, Y. Vander Heyden, J. Claereboudt, I. Smolders, A. Van Eeckhaut, Improved sensitivity of the nano ultra-high performance liquid chromatography-tandem mass spectrometric analysis of low-concentrated neuropeptides by reducing aspecific adsorption and optimizing the injection solvent, J. Chromatogr. A (2014, 1360,) 217–228.

[69] K. Stejskal, D. Potesil, Z. Zdrahal, Suppression of peptide sample losses in autosampler vials, J. Proteome Res. 12 (2013) 3057–3062.

[70] X. Wang, X. Yang, H. Chen, X. Yang, Z. Xu, Entropy-Enthalpy Compensation in Peptide Adsorption on Solid Surfaces: Dependence on Surface Hydration, Langmuir 36 (2020) 10822–10829.

[71] E.I. Chen, D. Cociorva, J.L. Norris, J.R. Yates, Optimization of Mass Spectrometry-Compatible Surfactants for Shotgun Proteomics, J. Proteome Res. (2007).

[72] W. Li, S. Luo, H.T. Smith, F.L. Tse, Quantitative determination of BAF312, a S1P-R modulator, in human urine by LC-MS/MS: prevention and recovery of lost analyte due to container surface adsorption, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 878 (2010) 583–589.

[73] Y.-G. Yeung, E. Nieves, R.H. Angeletti, E.R. Stanley, Removal of detergents from protein digests for mass spectrometry analysis, Anal. Biochem. 382 (2008) 135–137.

[74] N. Zhang, L. Li, Effects of common surfactants on protein digestion and matrix-assisted laser desorption/ionization mass spectrometric analysis of the digested peptides using two-layer sample preparation, Rapid Commun. Mass Spectrom. 18 (2004) 889–896.

[75] B. Bobaly, E. Sipko, J. Fekete, Challenges in liquid chromatographic characterization of proteins, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 1032 (2016) 3–22.

[76] M. Barco, C. Planas, O. Palacios, F. Ventura, J. Rivera, J. Caixach, Simultaneous Quantitative Analysis of Anionic, Cationic, and Nonionic Surfactants in Water by Electrospray Ionization Mass Spectrometry with Flow Injection Analysis, Anal. Chem. 75 (2003) 5129–5136.

[77] Y.-H. Chang, Z.R. Gregorich, A.J. Chen, L. Hwang, H. Guner, D. Yu, J. Zhang, Y. Ge, New Mass-Spectrometry-Compatible Degradable Surfactant for Tissue Proteomics, J. Proteome Res. 14 (2015) 1587–1599.

[78] M. Waas, S. Bhattacharya, S. Chuppa, X. Wu, D.R. Jensen, U. Omasits, B. Wollscheid, B.F. Volkman, K.R. Noon, R.L. Gundry, Combine and Conquer: Surfactants, Solvents, and Chaotropes for Robust Mass Spectrometry Based Analyses of Membrane Proteins, Anal. Chem. 86 (2014) 1551–1559.

[79] Y. Kawashima, N. Takahashi, M. Satoh, T. Saito, S. Kado, F. Nomura, H. Matsumoto, Y. Kodera, Enhanced recovery of lyophilized peptides in shotgun proteomics by using an LC-ESI-MS compatible surfactant, Proteomics 13 (2013) 751–755.

[80] Y.G. Yeung, E.R. Stanley, Rapid Detergent Removal from Peptide Samples with Ethyl Acetate for Mass Spectrometry Analysis, Curr. Protoc. Protein Sci. 59 (2010).

[81] Y. Xu, L. Du, M.J. Rose, I. Fu, E.J. Woolf, D.G. Musson, Concerns in the development of an assay for determination of a highly conjugated adsorption-prone compound in human urine, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 818 (2005) 241–248.

[82] J.P. Quirino, Sodium dodecyl sulfate removal during electrospray ionization using cyclodextrins as simple sample solution additive for improved mass spectrometric detection of peptides, Anal. Chim. Acta 1005 (2018) 54–60.

[83] B. Antharavally, R. Bomgarden, J. Rogers, A Versatile High-Recovery Method For Removing Detergents From Low-Concentration Protein or Peptide Samples For Mass Spectrometry Sample Preparation And Analysis, Insulin (2012).

[84] A.N. Hoofnagle, J.O. Becker, M.H. Wener, J.W. Heinecke, Quantification of thyroglobulin, a low-abundance serum protein, by immunoaffinity peptide enrichment and tandem mass spectrometry, Clin. Chem. 54 (2008) 1796–1804.

[85] A. Zhang, W. Qi, S.K. Singh, E.J. Fernandez, A New Approach to Explore the Impact of Freeze-Thaw Cycling on Protein Structure: Hydrogen/Deuterium Exchange Mass Spectrometry (HX-MS), Pharm. Res. 28 (2011) 1179–1193.

[86] R. Freije, T. Klein, B. Ooms, H.F. Kauffman, R. Bischoff, An integrated high-performance liquid chromatography-mass spectrometry system for the activity-dependent analysis of matrix metalloproteases, J. Chromatogr. A 1189 (2008) 417–425.

[87] P.J. Eugster, J. Maurer, C. Vocat, K. Abid, M. Matter, G. Wuerzner, R. Trepp, S. Fischli, C. Henzen, W. Kolb, S. Bilz, S. Sigrist, F. Beuschlein, S. Nölting, A. Reul, I. Schütze, S.A. Hubers, N.J. Brown, E. Grouzmann, Proneuropeptide Y and neuropeptide Y metabolites in healthy volunteers and patients with a pheochromocytoma or paraganglioma, Clin. Chim. Acta 534 (2022) 146–155.

[88] P.J. Eugster, C.N. Boyle, S. Prod'Hom, E. Tarasco, T. Buclin, T.A. Lutz, A. G. Harris, E. Grouzmann, Sensitive quantification of the somatostatin analog AP102 in plasma by ultra-high pressure liquid chromatography-tandem mass spectrometry and application to a pharmacokinetic study in rats, Drug Test. Anal. 10 (2018) 1448–1457.

[89] H. Katayama, T. Nagasu, Y. Oda, Improvement of in-gel digestion protocol for peptide mass fingerprinting by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, Rapid Commun. Mass Spectrom. 15 (2001) 1416–1421.

[90] S. Nie, R. O'Brien Johnson, Y. Livson, T. Greer, X. Zheng, N. Li, Maximizing hydrophobic peptide recovery in proteomics and antibody development using a mass spectrometry compatible surfactant, Anal. Biochem. 658 (2022), 114924.

[91] M. Rühl, S. Schönborn, M. Karas, Detergent-assisted sample preparation for MALDI-MS: Investigation of octylglucoside and docecylmaltoside for matrix crystallization, on-plate digestion, and trypsin activity, J. Mass Spectrom. 53 (2018) 675–679.

[92] N. Zhang, A. Doucette, L. Li, Two-Layer Sample Preparation Method for MALDI Mass Spectrometric Analysis of Protein and Peptide Samples Containing Sodium Dodecyl Sulfate, Anal. Chem. (2001).

[93] Y. Shimazaki, T. Manabe, Detection of activity and mass spectrometric identification of mouse liver carboxylesterase and aldehyde dehydrogenase separated by non-denaturing two-dimensional electrophoresis after extraction with detergents, Biochim. Biophys. Acta 1749 (2005) 95–101.

[94] A.M.A. Rahman, S. Kamath, A.L. Lopata, R.J. Helleur, Analysis of the allergenic proteins in black tiger prawn (Penaeus monodon) and characterization of the major allergen tropomyosin using mass spectrometry, Rapid Commun. Mass Spectrom. 24 (2010) 2462–2470.

[95] W. Chen, D. Kirui, N.J. Millenbaugh, Optimized peptide extraction method for analysis of antimicrobial peptide Kn2-7/dKn2-7 stability in human serum by LC–MS, Future Sci. OA 8 (2022).

[96] L. Kategaya, P. Di Lello, L. Rougé, R. Pastor, K.R. Clark, J. Drummond, T. Kleinheinz, E. Lin, J.-P. Upton, S. Prakash, J. Heideker, M. McCleland, M. S. Ritorto, D.R. Alessi, M. Trost, T.W. Bainbridge, M.C.M. Kwok, T.P. Ma, Z. Stiffler, B. Brasher, Y. Tang, P. Jaishankar, B.R. Hearn, A.R. Renslo, M.R. Arkin, F. Cohen, K. Yu, F. Peale, F. Gnad, M.T. Chang, C. Klijn, E. Blackwood, S. E. Martin, W.F. Forrest, J.A. Ernst, C. Ndubaku, X. Wang, M.H. Beresini, V. Tsui, C. Schwerdtfeger, R.A. Blake, J. Murray, T. Maurer, I.E. Wertz, USP7 small-molecule inhibitors interfere with ubiquitin binding, Nature 550 (2017) 534–538.

[97] N. Foulon, E. Goonatilleke, M.J. MacCoss, M.A. Emrick, A.N. Hoofnagle, Multiplexed quantification of insulin and C-peptide by LC-MS/MS without the use of antibodies, J. Mass. Spectrom. Adv. Clin. Lab. 25 (2022) 19–26.

[98] S.V. Saveliev, C.C. Woodroofe, G. Sabat, C.M. Adams, D. Klaubert, K. Wood, M. Urh, Mass Spectrometry Compatible Surfactant for Optimized In-Gel Protein Digestion, Anal. Chem. 85 (2013) 907–914.

[99] P.J. Eugster, H. Chtioui, A. Herren, M. Dunand, D. Cappelle, J. Bourquin, T. Buclin, E. Grouzmann, Sub-picomolar quantification of PTH 1–34 in plasma by UHPLC-MS/MS after subcutaneous injection of teriparatide and identification of PTH 1–33, its degradation product, J. Pharm. Biomed. Anal. 166 (2019) 205–212.

[100] M.J. Penna, M. Mijajlovic, M.J. Biggs, Molecular-level understanding of protein adsorption at the interface between water and a strongly interacting uncharged solid surface, J. Am. Chem. Soc. 136 (2014) 5323–5331.

[101] F. Verbeke, N. Bracke, N. Debunne, E. Wynendaele, B. De Spiegeleer, LC-MS Compatible Antiadsorption Diluent for Peptide Analysis, Anal. Chem. 92 (2020) 1712–1719.
[102] D. Martínez, V. Morera, M. DallaSerra, Y. García, C. Potrich, S. Schreier, F. Casallanovo, E. Lissi, M. Raida, C. Álvarez, G. Menestrina, M.E. Lanio, N-terminal peptide (1–92) of Sticholysin II generated by the cleavage with cyanogen bromide interacts with lipid bilayer but does not promote pore formation, Biotecnol. Apl. (2006).
[103] K.J. Kramer, P.E. Dunn, R.C. Peterson, H.L. Seballos, L.L. Sanburg, J.H. Law, Purification and characterization of the carrier protein for juvenile hormone from the hemolymph of the tobacco hornworm Manduca sexta Johannson (Lepidoptera: Sphingidae), J. Biol. Chem. 251 (1976) 4979–4985.
[104] M. Goebel-Stengel, A. Stengel, Y. Taché, J.R. Reeve, The importance of using the optimal plasticware and glassware in studies involving peptides, Anal. Biochem. 414 (2011) 38–46.
[105] S. Suzuki, T. Goto, S.H. Lee, T. Oe, Robust analysis of angiotensin peptides in human plasma: Column switching-parallel LC/ESI-SRM/MS without adsorption or enzymatic decomposition, Anal. Biochem. 630 (2021), 114327.
[106] J. Toombs, M.S. Foiani, H. Wellington, R.W. Paterson, C. Arber, A. Heslegrave, M. P. Lunn, J.M. Schott, S. Wray, H. Zetterberg, Amyloid β peptides are differentially vulnerable to preanalytical surface exposure, an effect incompletely mitigated by the use of ratios, Alzheimers Dement.: Diagn, Assess. Dis. 10 (2018) 311–321.
[107] C. Delaby, L. Munoz, S. Torres, A. Nadal, N. Le Bastard, S. Lehmann, A. Lleo, D. Alcolea, Impact of CSF storage volume on the analysis of Alzheimer's disease biomarkers on an automated platform, Clin. Chim. Acta 490 (2019) 98–101.
[108] S. Warwood, A. Byron, M.J. Humphries, D. Knight, The effect of peptide adsorption on signal linearity and a simple approach to improve reliability of quantification, J. Proteomics 85 (2013) 160–164.
[109] M. Bjerke, E. Portelius, L. Minthon, A. Wallin, H. Anckarsäter, R. Anckarsäter, N. Andreasen, H. Zetterberg, U. Andreasson, K. Blennow, Confounding Factors Influencing Amyloid Beta Concentration in Cerebrospinal Fluid, Int. J. Alzheimers Dis. 2010 (2010) 1–11.
[110] C. Yazici, K. Köse, S. Utaş, E. Tanrikulu, N. Taşlidere, A novel approach in psoriasis: first usage of known protein oxidation markers to prove oxidative stress, Arch. Dermatol. Res. 308 (2016) 207–212.
[111] G. Baskol, H. Demir, M. Baskol, E. Kilic, F. Ates, C. Karakukcu, M. Ustdal, Investigation of protein oxidation and lipid peroxidation in patients with rheumatoid arthritis, Cell Biochem. Funct. 24 (2006) 307–311.
[112] I. Verrastro, S. Pasha, K. Jensen, A. Pitt, C. Spickett, Mass Spectrometry-Based Methods for Identifying Oxidized Proteins in Disease: Advances and Challenges, Biomolecules 5 (2015) 378–411.
[113] P.J. Thornalley, N. Rabbani, Detection of oxidized and glycated proteins in clinical samples using mass spectrometry–a user's perspective, Biochim. Biophys. Acta 2014 (1840) 818–829.
[114] R. Matthiesen, G. Bauw, K.G. Welinder, Use of Performic Acid Oxidation To Expand the Mass Distribution of Tryptic Peptides, Anal. Chem. 76 (2004) 6848–6852.
[115] K.L. Schey, E.L. Finley, Identification of Peptide Oxidation by Tandem Mass Spectrometry, Acc. Chem. Res. (2000).
[116] D. Houde, P. Kauppinen, R. Mhatre, Y. Lyubarskaya, Determination of protein oxidation by mass spectrometry and method transfer to quality control, J. Chromatogr. A 1123 (2006) 189–198.
[117] H. Hahne, F. Pachl, B. Ruprecht, S.K. Maier, S. Klaeger, D. Helm, G. Médard, M. Wilm, S. Lemeer, B. Kuster, DMSO enhances electrospray response, boosting sensitivity of proteomic experiments, Nat. Methods 10 (2013) 989–991.
[118] Y. Shechter, Selective oxidation and reduction of methionine residues in peptides and proteins by oxygen exchange between sulfoxide and sulfide, J. Biol. Chem. 261 (1986) 66–70.
[119] M. Chen, K.D. Cook, Oxidation Artifacts in the Electrospray Mass Spectrometry of AB Peptide, Anal. Chem. (2007).
[120] C.D. Minth, S.R. Bloom, J.M. Polak, J.E. Dixon, Cloning, characterization, and DNA sequence of a human cDNA encoding neuropeptide tyrosine, PNAS 81 (1984) 4577–4581.
[121] K.A. Hruska, A. Korkor, K. Martin, E. Slatopolsky, Peripheral Metabolism of Intact Parathyroid Hormone, J. Clin. Invest. 67 (1981) 885–892.
[122] B. Hocher, F.P. Armbruster, S. Stoeva, C. Reichetzeder, H.J. Grön, I. Lieker, D. Khadzhynov, T. Slowinski, H.J. Roth, Measuring Parathyroid Hormone (PTH) in Patients with Oxidative Stress – Do We Need a Fourth Generation Parathyroid Hormone Assay? PLoS One 7 (2012) e40242.
[123] J.W. Chu, J. Yin, B.R. Brooks, D.I. Wang, M.S. Ricci, D.N. Brems, B.L. Trout, A comprehensive picture of non-site specific oxidation of methionine residues by peroxides in protein pharmaceuticals, J. Pharm. Sci. 93 (2004) 3096–3102.
[124] K. Kritmetapak, L.A. Losbanos, J.M. Hines, K.L. O'Grady, C.Z. Ulmer, H. W. Vesper, F.T. Enders, R.J. Singh, R. Kumar, Chemical Characterization and Quantification of Circulating Intact PTH and PTH Fragments by High-Resolution Mass Spectrometry in Chronic Renal Failure, Clin. Chem. 67 (2021) 843–853.
[125] C. Pan, J.J. Valente, R. LoBrutto, J.S. Pickett, M. Motto, Combined application of high resolution and tandem mass spectrometers to characterize methionine oxidation in a parathyroid hormone formulation, J. Pharm. Sci. 99 (2010) 1169–1179.
[126] N.E. Robinson, A.B. Robinson, Deamidation of human proteins, PNAS 98 (2001) 12409–12413.
[127] L. Huang, J. Lu, V.J. Wroblewski, J.M. Beals, R.M. Riggin, In Vivo Deamidation Characterization of Monoclonal Antibody by LC/MS/MS, Anal. Chem. 77 (2005) 1432–1439.
[128] R. Bischoff, H.V. Kolbe, Deamidation of asparagine and glutamine residues in proteins and peptides: structural determinants and analytical methodology, J. Chromatogr. B (1994).
[129] A.A. Wakankar, R.T. Borchardt, Formulation considerations for proteins susceptible to asparagine deamidation and aspartate isomerization, J. Pharm. Sci. 95 (2006) 2321–2336.
[130] A.L. Pace, R.L. Wong, Y.T. Zhang, Y.H. Kao, Y.J. Wang, Asparagine deamidation dependence on buffer type, pH, and temperature, J. Pharm. Sci. 102 (2013) 1712–1723.
[131] K. Patel, R.T. Borchardt, Chemical Pathways of Peptide Degradation. III. Effect of Primary Sequence on the Pathways of Deamidation of Asparaginyl Residues in Hexapeptides, Pharm. Res. 07 (1990) 787–793.
[132] Y. Nabuchi, E. Fujiwara, H. Kuboniwa, Y. Asoh, H. Ushio, The stability and degradation pathway of recombinant human parathyroid hormone: deamidation of apsaraginyl residue and peptide bond cleavage at aspartyl and apsparaginyl residues, Pharm. Res. 14 (1997) 1685–1690.
[133] H.R. Costantino, K. Griebenow, R. Langer, A.M. Klibanov, On the pH Memory of Lyophilized Compounds Containing Protein Functional Groups, Biotechnol. Bioeng. 53 (1996).
[134] H. Lindner, W. Helliger, Age-dependent deamidation of asparagine residues in proteins, Exp. Gerontol. (2001).
[135] M.C. Lai, E.M. Topp, Solid-state chemical stability of proteins and peptides, J. Pharm. Sci. 88 (1999) 489–500.
[136] D. Thirumalai, D.K. Klimov, R.I. Dima, Emerging ideas on the molecular basis of protein and peptide aggregation, Curr. Opin. Struct. Biol. 13 (2003) 146–159.
[137] R.M. Murphy, Peptide Aggregation in Neurodegenerative Disease, Annu. Rev. Biomed. Eng. 4 (2002) 155–174.
[138] K.L. Zapadka, F.J. Becher, A.L. Gomes Dos Santos, S.E. Jackson, Factors affecting the physical stability (aggregation) of peptide therapeutics, Interface Focus 7 (2017) 20170030.
[139] I.W. Hamley, Peptide Fibrillization, Angew. Chem. Int. Ed. 46 (2007) 8128–8147.
[140] R.M. Smith, D.E. Hansen, The pH-Rate Profile for the Hydrolysis of a Peptide Bond, J. Am. Chem. Soc. 120 (1998).
[141] D. Kahne, W.C. Still, Hydrolysis of a Peptide Bond in Neutral Water, J. Am. Chem. Soc. 110 (1988).
[142] Y. Qian, M.H. Engel, S.A. Macko, S. Carpenter, J.W. Deming, Kinetics of peptide hydrolysis and amino acid decomposition at high temperature, Geochim. Cosmochim. Acta 57 (1993).
[143] A. Shirwaikar, K. Srinivasan, J. Alex, S. Prabu, R. Mahalaxmi, R. Kumar, S. Jacob, Stability of proteins in aqueous solution and solid state, Indian J. Pharm. Sci. 68 (2006) 154.
[144] O. Mozziconacci, C. Schoneich, Photodegradation of oxytocin and thermal stability of photoproducts, J. Pharm. Sci. 101 (2012) 3331–3346.
[145] D. Zhang, D.R. Rios, V.H. Tam, D.S. Chow, Development and validation of a highly sensitive LC-MS/MS assay for the quantification of arginine vasopressin in human plasma and urine: Application in preterm neonates and child, J. Pharm. Biomed. Anal. 99 (2014) 67–73.
[146] Y. Shen, M. Liu, M. Xu, Z. Xu, Y. Na, N. Zhang, F. Geng, Simultaneous determination of nine trace concentration angiotensin peptides in human serum using ultra high performance liquid chromatography with tandem mass spectrometry with sephadex LH-20 gel solid-phase extraction, J. Sep. Sci. 42 (2019) 2247–2254.
[147] M. Bikaki, R. Shah, A. Muller, N. Kuhnert, Heat induced hydrolytic cleavage of the peptide bond in dietary peptides and proteins in food processing, Food Chem. 357 (2021), 129621.
[148] X. Lan, P. Liu, S. Xia, C. Jia, D. Mukunzi, X. Zhang, W. Xia, H. Tian, Z. Xiao, Temperature effect on the non-volatile compounds of Maillard reaction products derived from xylose–soybean peptide system: Further insights into thermal degradation and cross-linking, Food Chem. 120 (2010) 967–972.
[149] R.E.J. Clarke, The development and application of liquid chromatography/tandem mass spectrometry (LC-MS/MS) methods for the quantification of the gut hormones peptide YY, glucagon like peptide-1 and oxyntomodulin, Division of Diabetes, Endocrinology and Metabolism, Faculty of Medicine, Imperial College London, 2018.
[150] I.W. Smith, The temperature-dependence of elementary reaction rates: beyond Arrhenius, Chem. Soc. Rev. 37 (2008) 812–826.
[151] J.F. Carpenter, M.J. Pikal, B.S. Chang, T.W. Randolph, Rational design of stable Lyophilized protein formulations: some practical advice, Pharm. Res. 14 (1997).
[152] M.J. Pikal, K.M. Dellerman, A.M.L. Roy, R.M. Riggin, The effects of formulation variables on the stability of freeze-dried human growth hormone, Pharm. Res. 08 (1991) 427–436.
[153] M. Ameri, P.E. Daddona, Y.-F. Maa, Demonstrated Solid-State Stability of Parathyroid Hormone PTH(1–34) Coated on a Novel Transdermal Microprojection Delivery System, Pharm. Res. 26 (2009) 2454–2463.
[154] S. Yang, X. Liu, M. Zhang, S. Lin, F. Chen, Moisture-Absorption and Water Dynamics in the Powder of Egg Albumen Peptide, Met-Pro-Asp-Ala-His-Leu, J. Food Sci. 82 (2017) 53–60.
[155] N. Murase, F. Franks, Salt precipitation during the freeze-concentration of phosphate buffer solutions, Biophys. Chem. 34 (1989).
[156] M.J. Akers, N. Milton, S.R. Byrn, S.L. Nail, Glycine crystallization during freezing: the effects of salts form, pH, and ionic strength, Pharm. Res. 12 (1995) 1457–1461.
[157] S. Khajeh Pour, C. Scoville, S.S. Tavernier, A. Aghazadeh-Habashi, Plasma angiotensin peptides as biomarkers of rheumatoid arthritis are correlated with anti-ACE2 auto-antibodies level and disease intensity, Inflammopharmacology, DOI 10.1007/s10787-022-01008-9(2022).

[158] H. Gika, G. Theodoridis, Sample preparation prior to the LC–MS-based metabolomics/metabonomics of blood-derived samples, Bioanalysis (2011).
[159] T. Barri, L.O. Dragsted, UPLC-ESI-QTOF/MS and multivariate data analysis for blood plasma and serum metabolomics: effect of experimental artefacts and anticoagulant, Anal. Chim. Acta 768 (2013) 118–128.
[160] M. Ilies, C.A. Iuga, F. Loghin, V.M. Dhople, T. Thiele, U. Volker, E. Hammer, Impact of blood sample collection methods on blood protein profiling studies, Clin. Chim. Acta 471 (2017) 128–134.
[161] R. Böttger, R. Hoffmann, D. Knappe, Differential stability of therapeutic peptides with different proteolytic cleavage sites in blood, plasma and serum, PLoS One 12 (2017) e0178943.
[162] H. Tammen, I. Schulte, R. Hess, C. Menzel, M. Kellmann, T. Mohring, P. Schulz-Knappe, Peptidomic analysis of human blood specimens: Comparison between plasma specimens and serum by differential peptide display, Proteomics 5 (2005) 3414–3422.
[163] Z.-Q. Wu, J. Lu, H.-G. Xu, Hemolysis Affects C-Peptide Immunoassay, J. Clin. Lab. Anal. 30 (2016) 1232–1235.
[164] V.L. Stevens, E. Hoover, Y. Wang, K.A. Zanetti, Pre-Analytical Factors that Affect Metabolite Stability in Human Urine, Plasma, and Serum: A Review, Metabolites 9 (2019) 156.
[165] R.A.R. Bowen, A.T. Remaley, Interferences from blood collection tube components on clinical chemistry assays, Biochem. Med., DOI 10.11613/bm.2014.006(2014) 31–44.
[166] M.S. Nordlund, J. Bjerner, D.J. Warren, K. Nustad, E. Paus, Progastrin-Releasing Peptide: Stability in Plasma/Serum and Upper Reference Limit, Tumor Biol. 29 (2008) 204–210.
[167] V.V. Hernandes, C. Barbas, D. Dudzik, A review of blood sample handling and pre-processing for metabolomics studies, Electrophoresis 38 (2017) 2232–2241.
[168] A.J. Rai, C.A. Gelfand, B.C. Haywood, D.J. Warunek, J. Yi, M.D. Schuchard, R. J. Mehigh, S.L. Cockrill, G.B.I. Scott, H. Tammen, P. Schulz-Knappe, D. W. Speicher, F. Vitzthum, B.B. Haab, G. Siest, D.W. Chan, HUPO Plasma Proteome Project specimen collection and handling: Towards the standardization of parameters for plasma proteome samples, Proteomics 5 (2005) 3262–3277.
[169] G. Banfi, G.L. Salvagno, G. Lippi, The role of ethylenediamine tetraacetic acid (EDTA) as in vitro anticoagulant for diagnostic purposes, Clin. Chem. Lab. Med. 45 (2007) 565–576.
[170] I. Björk, U. Lindahl, Mechanism of the anticoagulant action of heparin, Mol. Cell. Biochem. 48 (1982) 161–182.
[171] J.A. Oates, A.J.J. Wood, J. Hirsh, Heparin, New Engl. J. Med. 324 (1991) 1565–1574.
[172] R.E. Banks, A.J. Stanley, D.A. Cairns, J.H. Barrett, P. Clarke, D. Thompson, P. J. Selby, Influences of blood sample processing on low-molecular-weight proteome identified by surface-enhanced laser desorption/ionization mass spectrometry, Clin. Chem. 51 (2005) 1637–1649.
[173] B.B. Haab, B.H. Geierstanger, G. Michailidis, F. Vitzthum, S. Forrester, R. Okon, P. Saviranta, A. Brinker, M. Sorette, L. Perlee, S. Suresh, G. Drwal, J.N. Adkins, G. S. Omenn, Immunoassay and antibody microarray analysis of the HUPO Plasma Proteome Project reference specimens: Systematic variation between sample types and calibration of mass spectrometry data, Proteomics 5 (2005) 3278–3291.
[174] I. Capila, R.J. Linhardt, Heparin-Protein Interactions, Angew. Chem. Int. Ed. 41 (2002) 390–412.
[175] H. Tammen, R. Hess, Collection and handling of blood specimens for peptidomics, Methods Mol. Biol. 1023 (2013) 161–168.
[176] G.S. Omenn, D.J. States, M. Adamski, T.W. Blackwell, R. Menon, H. Hermjakob, R. Apweiler, B.B. Haab, R.J. Simpson, J.S. Eddes, E.A. Kapp, R.L. Moritz, D. W. Chan, A.J. Rai, A. Admon, R. Aebersold, J. Eng, W.S. Hancock, S.A. Hefta, H. Meyer, Y.-K. Paik, J.-S. Yoo, P. Ping, J. Pounds, J. Adkins, X. Qian, R. Wang, V. Wasinger, C.Y. Wu, X. Zhao, R. Zeng, A. Archakov, A. Tsugita, I. Beer, A. Pandey, M. Pisano, P. Andrews, H. Tammen, D.W. Speicher, S.M. Hanash, Overview of the HUPO Plasma Proteome Project: Results from the pilot phase with 35 collaborating laboratories and multiple analytical groups, generating a core dataset of 3020 proteins and a publicly-available database, Proteomics 5 (2005) 3226–3245.
[177] D. Hendriks, S. Sharpé, M. van Sande, M.P. Lommaert, Characterisation of a Carboxypeptidase in human serum distinct from Carboxypeptidase N, J. Clin. Chem. Clin. Biochem. 27 (1989).
[178] K. Jambunathan, A.K. Galande, Sample collection in clinical proteomics-Proteolytic activity profile of serum and plasma, Proteomics - Clin. Appl. 8 (2014) 299–307.
[179] M. Khadka, A. Todor, K.M. Maner-Smith, J.K. Colucci, V. Tran, D.A. Gaul, E. J. Anderson, M.S. Natrajan, N. Rouphael, M.J. Mulligan, C.E. McDonald, M. Suthar, S. Li, E.A. Ortlund, The Effect of Anticoagulants, Temperature, and Time on the Human Plasma Metabolome and Lipidome from Healthy Donors as Determined by Liquid Chromatography-Mass Spectrometry, Biomolecules 9 (2019) 200.
[180] M.J. Evans, J.H. Livesey, J.M. Ellis, T.G. Yandle, Effect of anticoagulants and storage temperatures on stability of plasma and serum hormones, Clin. Biochem. 34 (2001).
[181] M. Rózga, T. Bittner, R. Batrla, J. Karl, Preanalytical sample handling recommendations for Alzheimer's disease plasma biomarkers, Alzheimers Dement.: Diagn. Assess. Dis. 11 (2019) 291–300.
[182] M. Meisner, K. Tschaikowsky, S. Schnabel, J. Schmidt, A. Katalinic, J. Schattier, Procalcitonin — Influence of Temperature, Storage, Anticoagulation and Arterial or Venous Asservation of Blood Samples on Procalcitonin Concentrations, Eur. J. Clin. Chem. Clin. Biochem. (1997).
[183] Z. Zhou, Y. Chen, J. He, J. Xu, R. Zhang, Y. Mao, Z. Abliz, Systematic evaluation of serum and plasma collection on the endogenous metabolome, Bioanalysis (2017).
[184] S. Tsuchimine, N. Sugawara, M. Ishioka, N. Yasui-Furukori, Preanalysis Storage Conditions Influence the Measurement of Brain-Derived Neurotrophic Factor Levels in Peripheral Blood, Neuropsychobiology 69 (2014) 83–88.
[185] A.G. Chambers, A.J. Percy, J. Yang, A.G. Camenzind, C.H. Borchers, Multiplexed quantitation of endogenous proteins in dried blood spots by multiple reaction monitoring-mass spectrometry, Mol. Cell. Proteomics 12 (2013) 781–791.
[186] M.F. Ewles, P.E. Turpin, L. Goodwin, D.M. Bakes, Validation of a bioanalytical method for the quantification of a therapeutic peptide, ramoplanin, in human dried blood spots using LC-MS/MS, Biomed. Chromatogr. 25 (2011) 995–1002.
[187] T. Lange, A. Thomas, K. Walpurgis, M. Thevis, Fully automated dried blood spot sample preparation enables the detection of lower molecular mass peptide and non-peptide doping agents by means of LC-HRMS, Anal. Bioanal. Chem. 412 (2020) 3765–3777.
[188] R. Di Terlizzi, S. Platt, The function, composition and analysis of cerebrospinal fluid in companion animals: part I - function and composition, Vet. J. 172 (2006) 422–431.
[189] C.E. Johanson, J.A. Duncan, P.M. Klinge, T. Brinker, E.G. Stopa, G.D. Silverberg, Multiplicity of cerebrospinal fluid functions: New challenges in health and disease, Cerebrospinal Fluid Res. 5 (2008) 10.
[190] M. Pollay, The function and structure of the cerebrospinal fluid outflow system, Cerebrospinal Fluid Res. 7 (2010) 9.
[191] R. Spector, S. Robert Snodgrass, C.E. Johanson, A balanced view of the cerebrospinal fluid composition and functions: Focus on adult humans, Exp. Neurol. 273 (2015) 57–68.
[192] T. Brinker, E. Stopa, J. Morrison, P. Klinge, A new look at cerebrospinal fluid circulation, Fluids Barriers CNS 11 (2014) 10.
[193] K. Blennow, B. Dubois, A.M. Fagan, P. Lewczuk, M.J. Leon, H. Hampel, Clinical utility of cerebrospinal fluid biomarkers in the diagnosis of early Alzheimer's disease, Alzheimers and Dement. 11 (2015) 58–69.
[194] H. Vanderstichele, M. Bibl, S. Engelborghs, N. Le Bastard, P. Lewczuk, J. L. Molinuevo, L. Parnetti, A. Perret-Liaudet, L.M. Shaw, C. Teunissen, D. Wouters, K. Blennow, Standardization of preanalytical aspects of cerebrospinal fluid biomarker testing for Alzheimer's disease diagnosis: A consensus paper from the Alzheimer's Biomarkers Standardization Initiative, Alzheimers Dement. 8 (2012) 65–73.
[195] A. Fourier, E. Portelius, H. Zetterberg, K. Blennow, I. Quadrio, A. Perret-Liaudet, Pre-analytical and analytical factors influencing Alzheimer's disease cerebrospinal fluid biomarker variability, Clin. Chim. Acta 449 (2015) 9–15.
[196] A.H. Simonsen, J.M. Bahl, P.B. Danborg, V. Lindstrom, S.O. Larsen, A. Grubb, N. H. Heegaard, G. Waldemar, Pre-analytical factors influencing the stability of cerebrospinal fluid proteins, J. Neurosci. Methods 215 (2013) 234–240.
[197] A. Latosinska, J. Siwy, S. Faguer, J. Beige, H. Mischak, J.P. Schanstra, Value of Urine Peptides in Assessing Kidney and Cardiovascular Disease, Proteomics - Clin. Appl. 15 (2021) 2000027.
[198] D.M. Good, P. Zürbig, À. Argilés, H.W. Bauer, G. Behrens, J.J. Coon, M. Dakna, S. Decramer, C. Delles, A.F. Dominiczak, J.H.H. Ehrich, F. Eitner, D. Fliser, M. Frommberger, A. Ganser, M.A. Girolami, I. Golovko, W. Gwinner, M. Haubitz, S. Herget-Rosenthal, J. Jankowski, H. Jahn, G. Jerums, B.A. Julian, M. Kellmann, V. Kliem, W. Kolch, A.S. Krolewski, M. Luppi, Z. Massy, M. Melter, C. Neusüss, J. Novak, K. Peter, K. Rossing, H. Rupprecht, J.P. Schanstra, E. Schiffer, J.-U. Stolzenburg, L. Tarnow, D. Theodorescu, V. Thongboonkerd, R. Vanholder, E. M. Weissinger, H. Mischak, P. Schmitt-Kopplin, Naturally Occurring Human Urinary Peptides for Use in Diagnosis of Chronic Kidney Disease, Mol. Cell. Proteomics 9 (2010) 2424–2437.
[199] F. Trindade, A.S. Barros, J. Silva, A. Vlahou, I. Falcão-Pires, S. Guedes, C. Vitorino, R. Ferreira, A. Leite-Moreira, F. Amado, R. Vitorino, Mining the Biomarker Potential of the Urine Peptidome: From Amino Acids Properties to Proteases, Int. J. Mol. Sci. 22 (2021) 5940.
[200] H. Mischak, A. Vlahou, J.P. Ioannidis, Technical aspects and inter-laboratory variability in native peptide profiling: the CE-MS experience, Clin. Biochem. 46 (2013) 432–443.
[201] V. Sirolli, L. Pieroni, L. Di Liberato, A. Urbani, M. Bonomini, Urinary Peptidomic Biomarkers in Kidney Diseases, Int. J. Mol. Sci. 21 (2019) 96.
[202] J. Klein, J.-L. Bascands, H. Mischak, J.P. Schanstra, The role of urinary peptidomics in kidney disease research, Kidney Int. 89 (2016) 539–545.
[203] P. Magalhães, J.P. Schanstra, E. Carrick, H. Mischak, P. Zürbig, Urinary biomarkers for renal tract malformations, Expert Rev. Proteomics 13 (2016) 1121–1129.
[204] Z.Y. Zhang, E. Nkuipou-Kenfack, J.A. Staessen, Urinary Peptidomic Biomarker for Personalized Prevention and Treatment of Diastolic Left Ventricular Dysfunction, Proteomics - Clin. Appl. 13 (2019) 1800174.
[205] L.D. Fricker, J. Lim, H. Pan, F.-Y. Che, Peptidomics: Identification and quantification of endogenous peptides in neuroendocrine tissues, Mass Spectrom. Rev. 25 (2006) 327–344.
[206] M.J. Page, E. Di Cera, Evolution of Peptidase Diversity, J. Biol. Chem. 283 (2008) 30010–30014.
[207] R. Mentlein, Cell-Surface Peptidases, Int. Rev. Cytol., http://doi.org/10.1016/s0074-7696(04)35004-7(2004) 165–213.
[208] N.D. Rawlings, A.J. Barrett, P.D. Thomas, X. Huang, A. Bateman, R.D. Finn, The MEROPS database of proteolytic enzymes, their substrates and inhibitors in 2017 and a comparison with peptidases in the PANTHER database, Nucleic Acids Res. 46 (2018).
[209] E. Maffioli, Z. Jiang, S. Nonnis, A. Negri, V. Romeo, C.B. Lietz, V. Hook, G. Ristagno, G. Baselli, E.B. Kistler, F. Aletti, A.J. O'Donoghue, G. Tedeschi, High-

Resolution Mass Spectrometry-Based Approaches for the Detection and Quantification of Peptidase Activity in Plasma, Molecules 25 (2020) 4071.
[210] I. Schechter, A. Berger, On the size of the active site in proteases. I Papain, Biochem. Biophys. Res. Commun. (1967).
[211] V.H.J. Van Der Velden, A.R. Hulsmann, Peptidases: structure, function and modulation of peptide-mediated effects in the human lung, Clin Exp Allergy 29 (1999) 445–456.
[212] A. Bairoch, The ENZYME database in 2000, Nucleic Acids Res. 28 (2000).
[213] N.D. Rawlings, A.J. Barrett, Evolutionary families of peptidases, Biochem. J 290 (1993) 205–218.
[214] R.M. Daniel, M.J. Danson, Temperature and the catalytic activity of enzymes: A fresh understanding, FEBS Lett. 587 (2013) 2738–2743.
[215] R.M. Daniel, M.J. Danson, A new understanding of how temperature affects the catalytic activity of enzymes, Trends Biochem. Sci 35 (2010) 584–591.
[216] N.A. Turner, E.N. Vulfson, At what temperature can enzymes maintain their catalytic activity? Enzyme Microb. Technol. 27 (2000).
[217] A. Janecka, R. Staniszewska, K. Gach, J. Fichna, Enzymatic degradation of endomorphins, Peptides 29 (2008) 2066–2073.
[218] S.H. Ferreira, J.R. Vane, The disappearance of bradykinin and eledoisin in the circulation and vascular beds of the cat, Br. J. Pharmacol. Chemother. 30 (1967) 417–424.
[219] N.J.W. Albrechtsen, Measurement of Gastrointestinal Hormones, Dan. Med. J. (2017).
[220] J. Cegla, B.J. Jones, J. Howard, R. Kay, C.S. Creaser, S.R. Bloom, T.M. Tan, The preanalytical stability of glucagon as measured by liquid chromatography tandem mass spectrometry and two commercially available immunoassays, Ann. Clin. Biochem. 54 (2017) 293–296.
[221] G. Vanhoof, F. Goossens, I. De Meester, D. Hendriks, S. Scharpé, Proline motifs in peptides and their biological processing, FASEB J. (1995).
[222] K. Jambunathan, A.K. Galande, Design of a Serum Stability Tag for Bioactive Peptides, Protein Pept. Lett. (2014).
[223] P. Ward, I.F. Benter, L. Dick, S. Wilk, Metabolism of vasoactive peptides by plasma and purified renal aminopeptidase M, Biochem. Pharmacol. 40 (1990).
[224] S. Tieku, N.M. Hooper, Inhibition of aminopeptidases N, A and W A re-evaluation of the actions of bestatin and inhibitors of angiotensin converting enzyme, Biochem. Pharmacol. 44 (1992).
[225] Y. Goto, A. Hattori, S. Mizutani, M. Tsujimoto, Asparatic Acid 221 Is Critical in the Calcium-induced Modulation of the Enzymatic Activity of Human Aminopeptidase A, J. Biol. Chem. 282 (2007) 37074–37081.
[226] K. Abid, B. Rochat, P.-G. Lassahn, R. Stöcklin, S. Michalet, N. Brakch, J.-F. Aubert, B. Vatansever, P. Tella, I. De Meester, E. Grouzmann, Kinetic Study of Neuropeptide Y (NPY) Proteolysis in Blood and Identification of NPY3–35, J. Biol. Chem. 284 (2009) 24715–24724.
[227] A. Yaron, D. Mlynar, Aminopeptidase-P, Biochem. Biophys. Res. Commun. 32 (1968).
[228] W.H. Simmons, A.T. Orawski, Membrane-bound Aminopeptidase P from bovine lung, J. Biol. Chem. 267 (1992).
[229] N.M. Hooper, J. Hryszko, A.J. Turner, Purification and characterization of pig kidney aminopeptidase P. A glycosyl-phosphatidylinositol-anchored ectoenzyme, Biochem. J 267 (1990) 509–515.
[230] X.B. Shen, X. Chen, Z.Y. Zhang, F.F. Wu, X.H. Liu, Cathepsin C inhibitors as anti-inflammatory drug discovery: Challenges and opportunities, Eur. J. Med. Chem. 225 (2021), 113818.
[231] I. Dolenc, B. Turk, G. Pungercic, A. Ritonja, V. Turk, Oligomeric Structure and Substrate Induced Inhibition of Human Cathepsin C, J. Biol. Chem. 270 (1995) 21626–21631.
[232] N.V. Rao, G.V. Rao, J.R. Hoidal, Human Dipeptidyl-peptidase I, J. Biol. Chem. 272 (1997) 10260–10265.
[233] J.D. Chalmers, C.S. Haworth, M.L. Metersky, M.R. Loebinger, F. Blasi, O. Sibila, A. E. O'Donnell, E.J. Sullivan, K.C. Mange, C. Fernandez, J. Zou, C.L. Daley, Phase 2 Trial of the DPP-1 Inhibitor Brensocatib in Bronchiectasis, New Engl. J. Med. 383 (2020) 2127–2137.
[234] D.I. Laine, J. Busch-Petersen, Inhibitors of cathepsin C (dipeptidyl peptidase I), Expert Opin. Ther. Pat. 20 (2010) 497–506.
[235] R. Gossrau, Z. Lojda, Study on dipeptidylpeptidase II (DPP II), Histochemistry 70 (1980) 53–76.
[236] B. Leiting, K.D. Pryor, J.K. Wu, F. Marsilio, R.A. Patel, C.S. Craik, J.A. Ellman, R. T. Cummings, N.A. Thornberry, Catalytic properties and inhibition of proline-specific dipeptidyl peptidases II, IV and VII, Biochem. J 371 (2003) 525–532.
[237] M.B. Maes, S. Scharpe, I. De Meester, Dipeptidyl peptidase II (DPPII), a review, Clin. Chim. Acta 380 (2007) 31–49.
[238] Y. Waumans, L. Baerts, K. Kehoe, A.M. Lambeir, I. De Meester, The Dipeptidyl Peptidase Family, Prolyl Oligopeptidase, and Prolyl Carboxypeptidase in the Immune System and Inflammatory Disease, Including Atherosclerosis, Front. Immunol. 6 (2015) 387.
[239] O. Danilova, B. Li, A.K. Szardenings, B.T. Huber, J.S. Rosenblum, Synthesis and activity of a potent, specific azabicyclo [3.3.0] octane-based DPP II inhibitor, Bioorg. Med. Chem. Lett. 17 (2007) 507–510.
[240] G. Kreil, M. Umbach, V. Brantl, H. Teschemacher, Studies on the enzymatic degradation of B-casomorphins, Life Sci. 33 (1983).
[241] A.-M. Lambeir, C. Durinx, S. Scharpé, I. De Meester, Dipeptidyl-Peptidase IV from Bench to Bedside: An Update on Structural Properties, Functions, and Clinical Aspects of the Enzyme DPP IV, Crit. Rev. Clin. Lab. Sci. 40 (2008) 209–294.
[242] R. Thoma, B. Löffler, M. Stihle, W. Huber, A. Ruf, M. Hennig, Structural Basis of Proline-Specific Exopeptidase Activity as Observed in Human Dipeptidyl Peptidase-IV, Structure 11 (2003) 947–959.
[243] S. Iwaki-Egawa, Y. Watanabe, Y. Kikuya, Y. Fujimoto, Dipeptidyl peptidase IV from human serum: Purification, characterization, and N-terminal amino acid sequence, J. Biochem. 124 (1998).
[244] W.N. Lipscomb, Carboxypeptidase A, Acc. Chem. Res. 22 (1989).
[245] L.D. Fricker, Carboxypeptidase E, Ann. Rev. Physiol. 50 (1988).
[246] W. Wang, D. Hendriks, S. Sharpé, Carboxypeptidase U, a plasma Carboxypeptidase with high affinity for plasminogen, J. Biol. Chem. 269 (1994).
[247] A. Bayes-Genis, J. Barallat, M. Richards, A Test in Context: Neprilysin Function, Inhibition, and Biomarker, J. Am. Coll. Cardiol. 68 (2016).
[248] M.E. Moreau, N. Garbacki, G. Molinaro, N.J. Brown, F. Marceau, A. Adam, The Kallikrein-Kinin System: Current and Future Pharmacological Targets, J. Pharmacol. Sci. 99 (2005) 6–38.
[249] H. Kato, N. Adachi, S. Iwanaga, K. Abe, K. Takada, T. Kimura, S. Sakakibara, A new fluorogenic substance method for the estimation of kallikrein in urine, J. Biochem. 87 (1980).
[250] G. Sotiropoulou, G. Pampalakis, E.P. Diamandis, Functional Roles of Human Kallikrein-related Peptidases, J. Biol. Chem. 284 (2009) 32989–32994.
[251] Å. Lundwall, M. Brattsand, Kallikrein-related peptidases, Cell. Mol. Life Sci. 65 (2008) 2019–2038.
[252] J.H. Levy, P.S. O'Donnell, The therapeutic potential of a kallikrein inhibitor for treating hereditary angioedema, Expert Opin. Invest. Drugs 15 (2006) 1077–1090.
[253] Z. Xie, Z. Li, Y. Shao, C. Liao, Discovery and development of plasma kallikrein inhibitors for multiple diseases, Eur. J. Med. Chem. 190 (2020), 112137.
[254] A. Kuoppala, K.A. Lindstedt, J. Saarinen, P.T. Kovanen, J.O. Kokkonen, Inactivation of bradykinin by angiotensin-converting enzyme and by carboxypeptidase N in human plasma, Am. J. Physiol. Heart Circ. Physiol. (2000).
[255] X. Sun, M. Becker, K. Pankow, E. Krause, M. Ringling, M. Beyermann, B. Maul, T. Walther, W.E. Siems, Catabolic attacks of membrane-bound angiotensin-converting enzyme on the N-terminal part of species-specific amyloid-beta peptides, Eur. J. Pharmacol. 588 (2008) 18–25.
[256] J.C. Velez, The importance of the intrarenal renin-angiotensin system, Nat. Clin. Pract. Nephrol. 5 (2009) 89–100.
[257] R.A. Lew, F.J. Warner, I. Hanchapola, M.A. Yarski, J. Manohar, L.M. Burrell, A. I. Smith, Angiotensin-converting enzyme 2 catalytic activity in human plasma is masked by an endogenous inhibitor, Exp. Physiol. 93 (2008) 685–693.
[258] K.E. Bernstein, Z. Khan, J.F. Giani, D.-Y. Cao, E.A. Bernstein, X.Z. Shen, Angiotensin-converting enzyme in innate and adaptive immunity, Nat. Rev. Nephrol. 14 (2018) 325–336.
[259] N.E. Clarke, A.J. Turner, Angiotensin-converting enzyme 2: the first decade, Int. J. Hypertens. 2012 (2012), 307315.
[260] F. Salamanna, M. Maglio, M.P. Landini, M. Fini, Body Localization of ACE-2: On the Trail of the Keyhole of SARS-CoV-2, Front. Med. 7 (2020), 594495.
[261] F.C. Luft, ACE in the hole, J. Mol. Med. 92 (2014) 793–795.
[262] L. Huang, D.J. Sexton, K. Skogerson, M. Devlin, R. Smith, I. Sanyal, T. Parry, R. Kent, J. Enright, Q.-L. Wu, G. Conley, D. Deoliveira, L. Morganelli, M. Ducar, C. R. Wescott, R.C. Ladner, Novel Peptide Inhibitors of Angiotensin-converting Enzyme 2, J. Biol. Chem. 278 (2003) 15532–15540.
[263] K.M. Elased, T.S. Cunha, S.B. Gurley, T.M. Coffman, M. Morris, New Mass Spectrometric Assay for Angiotensin-Converting Enzyme 2 Activity, Hypertension 47 (2006) 1010–1017.
[264] N. Grobe, K.M. Elased, D.R. Cool, M. Morris, Mass spectrometry for the molecular imaging of angiotensin metabolism in kidney, Am. J. Phys. Endocrinol. Metab. 302 (2012). E1016–E1024.
[265] C. Vickers, P. Hales, V. Kaushik, L. Dick, J. Gavin, J. Tang, K. Godbout, T. Parsons, E. Baronas, F. Hsieh, S. Acton, M. Patane, A. Nichols, P. Tummino, Hydrolysis of Biological Peptides by Human Angiotensin-converting Enzyme-related Carboxypeptidase, J. Biol. Chem. 277 (2002) 14838–14843.
[266] D.A. Priestman, J. Butterworth, Prolinase and non-specific dipeptidase of human kidney, Biochem. J 231 (1985) 689–694.
[267] N. Kolli, S.C. Garman, Proteolytic Activation of Human Cathepsin A, J. Biol. Chem. 289 (2014) 11592–11600.
[268] C.E. Chwieralski, T. Welte, F. Bühling, Cathepsin-regulated apoptosis, Apoptosis 11 (2006) 143–149.
[269] J.S. Mort, D.J. Buttle, Cathepsin B, Int. J. Biochem. Cell Biol. (1997).
[270] N. Zaidi, A. Maurer, S. Nieke, H. Kalbacher, Cathepsin D: a cellular roadmap, Biochem. Biophys. Res. Commun. 376 (2008) 5–9.
[271] T. Burster, H. Macmillan, T. Hou, B.O. Boehm, E.D. Mellins, Cathepsin G: Roles in antigen presentation and beyond, Mol. Immunol. 47 (2010) 658–665.
[272] M. Hirota, M. Ohmuraya, H. Baba, The role of trypsin, trypsin inhibitor, and trypsin receptor in the onset and aggravation of pancreatitis, J. Gastroenterol. 41 (2006) 832–836.
[273] R. Bischoff, H. Schluter, Amino acids: chemistry, functionality and selected non-enzymatic post-translational modifications, J. Proteomics 75 (2012) 2275–2296.
[274] M. Hellwig, T. Henle, Baking, Ageing, Diabetes: A Short History of the Maillard Reaction, Angew. Chem. Int. Ed. 53 (2014) 10316–10329.
[275] A.F. Maus, E.M., R. Taylor, B.J. Larson, A. Algeciras-Schimnich, R.J. Singh, S.K. Grebe, Identification, Measurement, and Assessment of the Clinical Utility of Human Pancreatic Polypeptide by Liquid Chromatography–Tandem Mass Spectrometry, J. Proteome Res., DOI (2023).
[276] J. Hong, S. Xin, R. Min, Y. Zhang, Y. Deng, The tryptic peptides of hemoglobin for diagnosis of type 2 diabetes mellitus using label-free and standard-free LC-ESI-DMRM, Redox Biol. 43 (2021), 101985.
[277] A.M. Korwar, Q. Zhang, Comprehensive Quantification of Carboxymethyllysine-Modified Peptides in Human Plasma, J. Am. Soc. Mass Spectrom. 32 (2021) 744–752.

[278] Z. Wang, M.G. Friedrich, R.J.W. Truscott, K.L. Schey, Cleavage C-terminal to Asp leads to covalent crosslinking of long-lived human proteins, Biochim. Biophys. Acta. Proteins. Proteom. 2019 (1867) 831–839.
[279] M. Liu, Z. Zhang, T. Zang, C. Spahr, J. Cheetham, D. Ren, Z.S. Zhou, Discovery of Undefined Protein Cross-Linking Chemistry: A Comprehensive Methodology Utilizing 18O-Labeling and Mass Spectrometry, Anal. Chem. 85 (2013) 5900–5908.
[280] M. Mariotti, F. Leinisch, D.J. Leeming, B. Svensson, M.J. Davies, P. Hagglund, Mass-Spectrometry-Based Identification of Cross-Links in Proteins Exposed to Photo-Oxidation and Peroxyl Radicals Using (18)O Labeling and Optimized Tandem Mass Spectrometry Fragmentation, J. Proteome Res. 17 (2018) 2017–2027.
[281] E. Fuentes-Lemus, P. Hägglund, C. López-Alarcón, M.J. Davies, Oxidative Crosslinking of Peptides and Proteins: Mechanisms of Formation, Detection, Characterization and Quantification, Molecules 27 (2021) 15.
[282] S.A. Rabbani, R. Kremer, H.P. Bennett, D. Goltzman, Phosphorylation of parathyroid hormone by human and bovine parathyroid glands, J. Biol. Chem. 259 (1984) 2949–2955.
[283] D. Rouy, I. Ernens, C. Jeanty, D.R. Wagner, Plasma storage at -80 degrees C does not protect matrix metalloproteinase-9 from degradation, Anal. Biochem. 338 (2005) 294–298.
[284] M. Haid, C. Muschet, S. Wahl, W. Romisch-Margl, C. Prehn, G. Moller, J. Adamski, Long-Term Stability of Human Plasma Metabolites during Storage at -80 degrees C, J. Proteome Res. 17 (2018) 203–211.
[285] J.C. Bradley, J. Simoni, R.H. Bradley, D.L. McCartney, S.M. Brown, Time- and Temperature-Dependent Stability of Growth Factor Peptides in Human Autologous Serum Eye Drops, Cornea (2009).
[286] M.G. Buckley, N.J. Marcus, M.H. Yacoub, D.R.J. Singer, Prolonged stability of brain natriuretic peptide: importance for non-invasive assessment of cardiac function in clinical practice, Clin. Sci. (1998).
[287] E. Cao, Y. Chen, Z. Cui, P.R. Foster, Effect of freezing and thawing rates on denaturation of proteins in aqueous solutions, Biotechnol. Bioeng. 82 (2003) 684–690.
[288] O. Fliniaux, G. Gaillard, A. Lion, D. Cailleu, F. Mesnard, F. Betsou, Influence of common preanalytical variations on the metabolic profile of serum samples in biobanks, J. Biomol. NMR 51 (2011) 457–465.
[289] B.L. Mitchell, Y. Yasui, C.I. Li, A.L. Fitzpatrick, P.D. Lampe, Impact of Freeze-thaw Cycles and Storage Time on Plasma Samples Used in Mass Spectrometry Based Biomarker Discovery Projects, Cancer Inform., 1 (2005) 117693510500100.
[290] L.J. Zimmerman, M. Li, W.G. Yarbrough, R.J.C. Slebos, D.C. Liebler, Global Stability of Plasma Proteomes for Mass Spectrometry-Based Analyses, Mol. Cell. Proteomics, 11 (2012) M111.014340.
[291] J. Hillebrand, A. Heijboer, E. Endert, Effects of repeated freeze–thaw cycles on endocrine parameters in plasma and serum, Ann. Clin. Biochem. 54 (2017) 289–292.
[292] L. Bernstone, J.E. Adaway, B.G. Keevil, An LC-MS/MS assay for analysis of equilibrium angiotensin II in human serum, Ann. Clin. Biochem. 58 (2021) 422–433.
[293] D.J. Campbell, J. Nussberger, M. Stowasser, A.H.J. Danser, A. Morganti, E. Frandsen, J.L. MéNard, Activity Assays and Immunoassays for Plasma Renin and Prorenin: Information Provided and Precautions Necessary for Accurate Measurement, Clin. Chem. 55 (2009) 867–877.
[294] S. Hepburn, C. Munday, K. Taylor, D.J. Halsall, Stability of direct renin concentration and plasma renin activity in EDTA whole blood and plasma at ambient and refrigerated temperatures from 0 to 72 hours, Clin. Chem. Lab. Med. 60 (2022) 1384–1392.
[295] A.G. Camenzind, J.G. Van Der Gugten, R. Popp, D.T. Holmes, C.H. Borchers, Development and evaluation of an immuno-MALDI (iMALDI) assay for angiotensin I and the diagnosis of secondary hypertension, Clin. Proteom. 10 (2013) 20.
[296] A.J. Scheen, A review of gliptins in,, Expert Opin, Pharmacother. 13 (2012) (2011) 81–99.
[297] L.J. Scott, Sitagliptin: A Review in Type 2 Diabetes, Drugs 77 (2017) 209–224.
[298] Y. Yoshizawa, M. Hosojima, H. Kabasawa, N. Tanabe, A. Miyachi, H. Hamajima, E. Mieno, M. Kobayashi, T. Kitamura, I. Narita, A. Saito, Measurement of Plasma Glucagon Levels Using Mass Spectrometry in Patients with Type 2 Diabetes on Maintenance Hemodialysis, Kidney Blood Press. Res. 46 (2021) 652–656.
[299] J. Lefebvre, L.J. Murphey, T.V. Hartert, R. Jiao Shan, W.H. Simmons, N.J. Brown, Dipeptidyl Peptidase IV Activity in Patients With ACE-Inhibitor-Associated Angioedema, Hypertension 39 (2002) 460–464.
[300] L.A. Krichevskiy, I.A. Kozlov, Natriuretic Peptides in Cardiac Anesthesia and Intensive Care, J. Cardiothorac. Vasc. Anesth. 33 (2019) 1407–1419.
[301] Y. Heishima, Y. Hori, S. Chikazawa, K. Kanai, F. Hoshi, N. Itoh, Influence of storage conditions on in vitro stability of atrial natriuretic peptide and of anesthesia on plasma atrial natriuretic peptide concentration in cats, Am. J. Vet. Res. 77 (2016) 854–859.
[302] C.A. Martin, B.P. McGrath, White-coat hypertension, Clin. Exp. Pharmacol. Physiol. 41 (2014) 22–29.
[303] K. Kario, L. Thijs, J.A. Staessen, Blood Pressure Measurement and Treatment Decisions, Circ. Res. 124 (2019) 990–1008.
[304] D. Conen, T. Dieterle, K. Utech, M. Rimner, B. Martina, C-Reactive Protein and B-Type Natriuretic Peptides in Never-Treated White Coat Hypertensives, Hypertens. Res. 29 (2006) 411–415.
[305] Y. Karter, S. Aydın, A. Curgunlu, H. Uzun, N. Ertürk, S. Vehid, A. Kutlu, G. Simsek, R. Yücel, A. Arat, E. Ozturk, S. Erdine, Endothelium and angiogenesis in white coat hypertension, J. Hum. Hypertens. 18 (2004) 809–814.
[306] F. Nyström, E. Aardal, K.P. Öhman, A population-based study of the white-coat blood pressure effect: Positive correlation with plasma cortisol, Clin. Exp. Hypertens. 20 (1998) 95–104.
[307] T. Pedrazzini, F. Pralong, E. Grouzmann, Neuropeptide Y: the universal soldier, Cell. Mol. Life Sci. (2002).
[308] J. Autio, V. Stenbäck, D.D. Gagnon, J. Leppäluoto, K.-H. Herzig, (Neuro) Peptides, Physical Activity, and Cognition, J. Clin. Med. 9 (2020) 2592.
[309] G.D. Lewis, L. Farrell, M.J. Wood, M. Martinovic, Z. Arany, G.C. Rowe, A. Souza, S. Cheng, E.L. McCabe, E. Yang, X. Shi, R. Deo, F.P. Roth, A. Asnani, E.P. Rhee, D. M. Systrom, M.J. Semigran, R.S. Vasan, S.A. Carr, T.J. Wang, M.S. Sabatine, C. B. Clish, R.E. Gerszten, Metabolic Signatures of Exercise in Human Plasma, Sci. Transl. Med. 2 (2010).
[310] G. Barletta, L. Stefani, R. Del Bene, C. Fronzaroli, S. Vecchiarino, C. Lazzeri, F. Fantini, G. La Villa, Effects of exercise on natriuretic peptides and cardiac function in man, Int. J. Cardiol. 65 (1998).
[311] M. Follenius, G. Brandenberger, Increase in atrial natriuretic peptide in response to physical exercise, Eur. J. Appl. Physiol. 57 (1988) 159–162.
[312] J.M. Lundbergl, A. Martinsson, A. Hemsenl, E. Theodorsson-Norheim, J. Svedenhag, B. Ekblom, P. Hjemdahl, Co-release of neuroppeptide Y and catecholamines during physical exercise in man, Biochem. Biophys. Res. Commun. 133 (1985).
[313] P.J. Eugster, N. Bourdillon, C. Vocat, G. Wuerzner, T. Nguyen, G.P. Millet, E. Grouzmann, Kinetics of neuropeptide Y, catecholamines, and physiological responses during moderate and heavy intensity exercises, Neuropeptides 92 (2022), 102232.
[314] A. Grossman, P. Bouloux, P. Price, P.L. Drury, K.S.L. Lam, T. Turner, J. Thomas, G. M. Besser, J. Sutton, The role of opioid peptides in the hormonal responses to acute exercise in man Clin, Sci. 67 (1984).
[315] L.R. Solomon, J.C. Atherton, H. Bobinski, R. Green, Effect of posture on plasma immunoreactive atrial natriuretic peptide concentrations in man, Clin. Sci. 71 (1986).
[316] G. La Villa, S. Vena, A. Conti, C. Fronzaroli, A. Brat, C. Lazzeri, C. Tosti Guerra, S. Madiai, N. Mama, F. Franchi, Plasma levels of brain natriuretic peptide in healthy subjects and patients with essential hypertension: response to posture, Clin. Sci. 85 (1993).
[317] H. Perrault, M. Cantin, G. Thibault, G.R. Brisson, G. Brisson, M. Beland, Plasma atrial natriuretic peptide during brief upright and supine exercise in humans J, Appl. Physiol. (1989).
[318] M. Katan, N. Morgenthaler, I. Widmer, J.J. Puder, C. König, B. Müller, M. Christ-Crain, Copeptin, a stable peptide derived from the vasopressin precursor, correlates with the individual stress level, Neuroendocrinol. Lett. 29 (2008).
[319] A. Damianaki, M. Hendriks-Balk, W. Brito, E. Polychronopoulou, K. Theiler, M. Maillard, J. Maurer, P. Eugster, M. Pruijm, G. Wuerzner, Contrast enhanced ultrasonography reveals a lower cortical perfusion and a decreased renal flow reserve in hypertensive patients, Nephrol. Dial. Transplant., DOI 10.1093/ndt/gfad158(2023).
[320] G. Magliocco, F. Le Bloc'h, A. Thomas, J. Desmeules, Y. Daali, Simultaneous determination of melatonin and 6-hydroxymelatonin in human overnight urine by LC-MS/MS, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 1181 (2021), 122938.
[321] S.S. Diemar, S.S. Dahl, A.S. West, S.A. Simonsen, H.K. Iversen, N.R. Jørgensen, A Systematic Review of the Circadian Rhythm of Bone Markers in Blood, Calcif. Tissue Int. 112 (2022) 126–147.
[322] W. Jubiz, J.M. Canterbury, E. Reiss, F.H. Tyler, Circadian rhythm in serum parathyroid hormone concentration in human subjects: correlation with serum calcium, phosphate, albumin, and growth hormone levels, J. Clin. Invest. 51 (1972) 2040–2046.
[323] G. El-Haji Fuleihan, E.B. Klerman, E.N. Brown, Y. Choe, E.M. Brown, C. A. Czeisler, The Parathyroid Hormone Circadian Rhythm Is Truly Endogenous—A General Clinical Research Center Study, J. Clin. Endocrinol. Metab. 82 (1997).
[324] J.F. Rehfeld, H.P. Sennels, H.L. Jørgensen, J. Fahrenkrug, Circadian variations in plasma concentrations of cholecystokinin and gastrin in man, Scand. J. Clin. Lab. Invest. 80 (2020) 546–551.
[325] F. Portaluppi, L. Montanari, B. Bagnib, E.D. Uberiia, G. Trasforinia, A. Margull, Circadian Rhythms of Atrial Natriuretic Peptide, Blood Pressure and Heart Rate in Normal Subjects, Cardiology 76 (1989).
[326] Y. Minamia, T. Kasukawab, Y. Kakazuc, M. Ligod, M. Sugimotoc, S. Ikedac, A. Yasuif, G.T.J. van der Horstg, T. Sogac, H.R. Ueda, Measurement of internal body time by blood metabolomics, Proc. Natl. Acad. Sci. USA (2009).
[327] J.J. Lim, S.D. Poppitt, How Satiating Are the 'Satiety' Peptides: A Problem of Pharmacology versus Physiology in the Development of Novel Foods for Regulation of Food Intake, Nutrients 11 (2019) 1517.
[328] L.J. Karhunen, K.R. Juvonen, A. Huotari, A.K. Purhonen, K.H. Herzig, Effect of protein, fat, carbohydrate and fibre on gastrointestinal peptide release in humans, Regul. Pept. 149 (2008) 70–78.