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Advances and perspectives in the analytical technology for small peptide hormones analysis: A glimpse to gonadorelin

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ABSTRACT

In the last twenty years, we have witnessed an important evolution of bioanalytical approaches moving from conventional lab bench instrumentation to simpler, easy-to-use techniques to deliver analytical responses on-site, with reduced analysis times and costs. In this frame, affinity reagents production has also jointly advanced from natural receptors to biomimetic, abiotic receptors, animal-free produced. Among biomimetic ones, aptamers, and molecular imprinted polymers (MIPs) play a leading role. Herein, our motivation is to provide insights into the evolution of conventional and innovative analytical approaches based on chromatography, immunochemistry, and affinity sensing referred to as peptide hormones. Indeed, the analysis of peptide hormones represents a current challenge for biomedical, pharmaceutical, and anti-doping analysis. Specifically, as a paradigmatic example, we report the case of gonadorelin, a neuropeptide that in recent years has drawn a lot of attention as a therapeutic drug misused in doping practices during sports competitions.

1. Introduction

The detection of peptide hormones represents a current challenge for biomedical, pharmaceutical, and anti-doping analysis. Since the discovery of the first peptide hormone insulin occurred in 1992, this class of peptides (composed of 10–100 amino acids), has been recognized as one of the major classes of chemical signaling with multi-spectra physiological activities [1]. They are physiologically spawned by specialized glands [2,3] as preprohormones, which were then proteolytically cleaved by removing the signal peptide sequence, the N-terminal flag, to obtain the mature peptide hormones [3]. Once the peptide hormones are secreted and released into the bloodstream, they are directed to bind cell-specific surface receptors. This event triggers multiple physiological functions and signaling pathways. In this frame, the development of peptide drug analogs, eventually with improved features (e.g., biological activity and resistance to biodegradation), as therapeutic agents for clinical applications, is associated with the advances in biochemistry and molecular biology [4]. Mostly, the detection of peptide hormones is challenging, and it should be addressed with quick and straightforward methods in relation to the very short peptides' half-lives in biological fluids that may require a multi-sampling over time. Some studies dealing with this issue have been reported during the last years focusing mainly on angiotensin, insulin, oxytocin, and vasopressin [5–9]. In this direction, the goal of this review is to provide a broad overview of the current methodologies for hypothalamic-releasing hormones' detection, with particular attention to gonadorelin, a key member of the aforementioned class. Specifically, a journey from reference benchtop-based approaches looking towards recent promising strategies to implement the analytical workflow aiming at in-situ peptide hormones analysis is conducted, identifying gaps that potentially can be filled by directing future research. A comprehensive summary outline of the main points addressed in this review is depicted in Fig. 1.

1.1. Gonadorelin overview: structure and pharmaceutical role

Gonadotropin-releasing hormone (GnRH) over decades has assumed multiple aliases name in the medical literature, some of these are: gonadotropin-releasing hormone I (GnRH I), luteinizing hormone-releasing hormone (LHRH), gonadotropin-releasing factor (GnRF), gonadoliberin, luliberin, and gonadorelin [10], which are widely used referring to the bioactive principle of a pharmaceutical drug [11]. Herein we use the following terminology, i.e., GnRH indicates the tropic neuropeptide endogenously produced, whereas gonadorelin refers to the analogue synthetic peptide. Gonadorelin (G) is a bioactive synthetic

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decapeptide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 MW = 1182.33 Da, Uniprot accession number #P01148) with an isoelectric point (pI) in the alkaline pH range (pI around 9) [12] and a chemical structure that coincides with the endogenous one (GnRH) with a pyroglutamic acid at the N-terminus and an amidated C-terminal. The UV-Vis optical characterization of gonadorelin displays a peak of absorbance at 280 nm associated with the presence of a tyrosine and a tryptophan in its sequence [13]. Besides, the amino acid residues -ProGly- at the C-terminus can undergo a beta-turn motif [14], through hydrogen bonds, which can be relevant for the three-dimensional arrangement. Physiologically, GnRH is a highly conserved decapeptide encoded by the GnRH1 gene located on chromosome 8.p11.2-p21 [15]. It is the pivotal central regulator of the hypothalamic-pituitary-gonadal (HPG) axis and plays a significant role in the processes of mammalian reproduction [16, 17]. The pulsatile secretion of GnRH, by a small number of neurons located in the mediobasal hypothalamus, stimulates the synthesis and the release of pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), both in adult males and females, by binding to the GnRH receptors (GnRH-R) which are mainly expressed in the anterior pituitary [16,18] (Fig. 2). Detailing, the latter receptors belong to the G-protein coupled receptors family (GPCRs, 328 amino acids (aa) [19]), which are integral membrane proteins (IMPs) that are used by cells to transduce extracellular signals into intracellular signaling pathways for the regulation of the reproductive axis functions [20]. Besides, it is now well established that GnRH-R overexpression takes place in gonadal steroid-dependent (responsive) tumors, such as prostate, breast, endometrial tumors, and ovarian adenocarcinomas [19–21]. The molecular pathway, triggered by pituitary GnRH-R, proceeds with the production of both steroid hormones (testosterone in males and progesterone in females) and gametes, triggered by the two gonadotropins which in turn act on the gonads, regulating the endocrine functions [22]. From a structural point of view, GnRH is first synthesized and released by the hypothalamus as pre-prohormone, a large precursor protein named progonadoliberin-1 precursor (MW = 10,380 Da, length 92 aa), that undergoes post-translational modifications to obtain the mature form [23,24]. Firstly, the N-terminal signaling sequence of 23 amino acids (1–23 aa position) is removed and then the residual peptide (24–92 aa), the prohormone or pro-neuropeptide, is proteolytically cleaved by specific endopeptidases in two fragments, which are separated by a small polymer chain as dibasic cleavage site (Gly-Lys-Arg) [1, 25,26]: GnRH bioactive peptide (24–33 aa position, 10 aa length, MW = 1182.33 Da), with amidation of the carboxy-terminal portion, and GnRH-associated peptide (GAP 37–92 aa position, 45 aa, MW = 1492 Da) [22] (Fig. 2).

To date, in addition to the hypothalamic GnRH form, other isoforms (chicken GnRH II and sea lamprey GnRH III) exist among vertebrates that have conserved the 10 amino acids length during their evolution [25]. GnRH is released from the hypothalamus in an episodic mode in which the frequency and the amplitude of the single pulse play an important role in controlling the loop-feedback mechanism of the hypothalamic-pituitary-gonadal (HPG) axis to avoid the downregulation of the GnRH-R in the pituitary [22]. Alterations in the normal pulsatile GnRH secretion can cause several reproductive disorders [27]. Since 1971, when the GnRH isolation and characterization occurred from the hypothalamus of pigs and sheep [25,28], structural analogues of the endogenous GnRH have been developed. In some cases, L-peptides (e.g., native GnRH) are vulnerable to enzymatic degradation in vivo and can be replaced by their analogues D-enantiomers which are more resistant towards proteolysis [29]. The GnRH analogues synthesized in the recent years, e.g., gonadorelin, buserelin, goserelin, leuprorelin, nafarelin, and triptorelin [30], are used as pharmaceutical drugs to treat different pathophysiological states, e.g., hormonal dysfunctions, such as endometriosis, uterine fibroids, precocious puberty, infertility, and hormone-sensitive tumors whose cells overexpress the GnRH-R [31–34]. The reverse side is that these peptides with a wide spectrum of physiological activity, including the capacity to trigger testosterone secretion to unnatural high levels [28], have been misused by male athletes to improve athletic performance (e.g., promoting muscle growth). Continuous administration of gonadorelin and analogues drugs, available as intranasal “pulse-dose” spray (e.g., kryptocur), leads to the desensitization and down-regulation of GnRH-R pituitary gonadotrophs, the so called “flare effect”, with impact on health. Accordingly, to protect the athletes’ health and ensure equal competitive opportunities, all these small peptides have been listed, since 2014, among the substances banned in sports, specifically in the S2 class of the World-Anti-Doping (WADA) code, which is annually reviewed and updated [30,35,36]. Peptide-derived drugs represent an emerging class of prohibited substances in modern doping controls as reported by Thevis et. al. [37]. In this scenario, WADA labs currently lack unambiguous criteria regarding gonadorelin (the so-called “pseudo-endogenous” peptide because it is

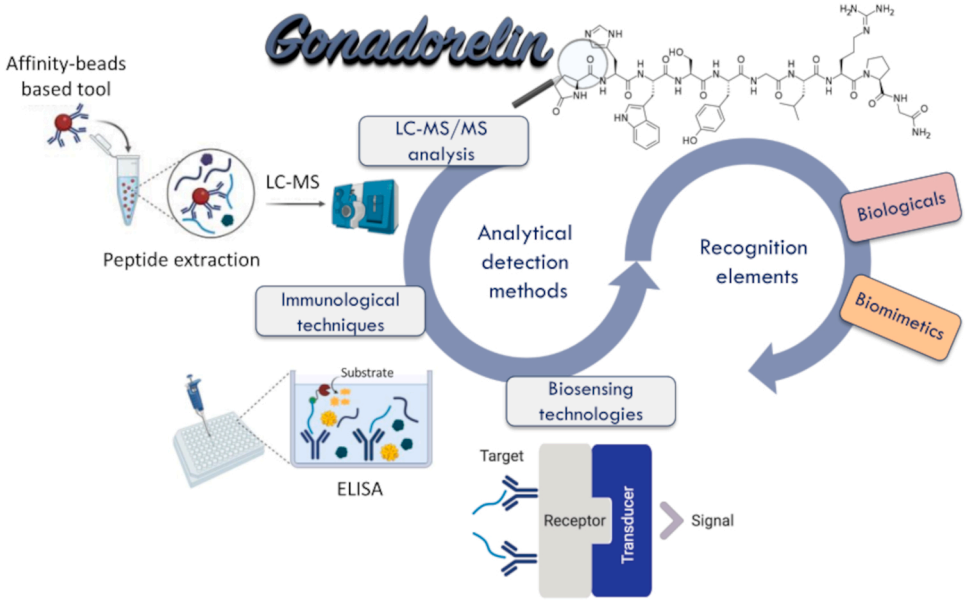

Fig. 1. Schematic diagram of the contents addressed in this review: analytical methods for gonadorelin detection with a particular focus on the affinity recognition tools integrable into different diagnostic platforms.

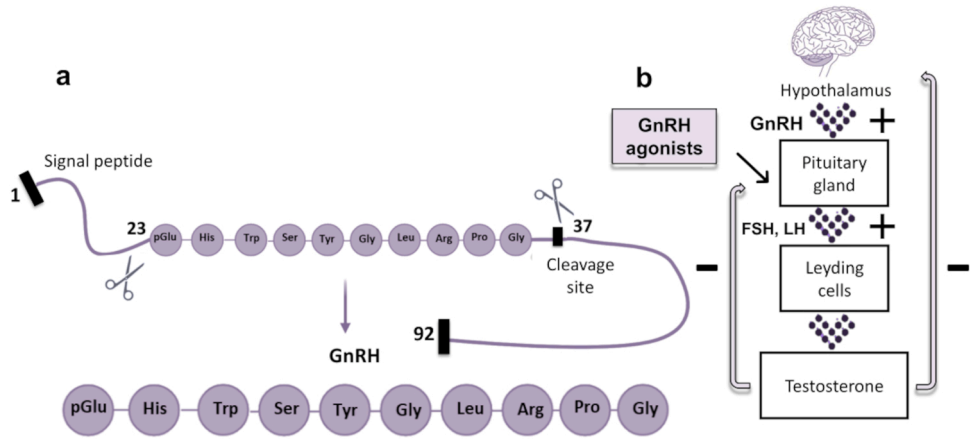

Fig. 2. (a) Schematic depiction of progonadoliberin-1 precursor processing to generate the mature bioactive peptide GnRH by removing the signaling sequence (1–23aa), the GnRH-associated peptide and the tripeptide cleavage site. (b) Mechanism involved in the hypothalamic-pituitary-gonadal axis.

endogenously produced and is prohibited if it is administered exogenously) to report adverse analytical findings. The ongoing introduction of an endocrine module for the athlete's biological passport (ABC) should further help to tackle doping and thus to detect peptide hormones. For this reason, gonadorelin is currently classified among the non-threshold substances and the minimum required performance levels (MRPL) = 2 ng/mL (1.69 nmol/L), a mandatory analytical parameter of technical performance, was established to guarantee that all WADA-accredited laboratories can report the presence of gonadorelin, in their routine daily analysis, in a uniform way [38,39]. It is important to note that gonadorelin is also included in veterinary medicine to treat animals with infertility secondary disorders, cystic ovarian disease, and to induce medical castration [40]. Different pharmaceutical drugs, containing gonadorelin as active principle, are available on the market: Cystorelin® (gonadorelin diacetate tetrahydrate), Factrel® (gonadorelin hydrochloride), Fertagyl® (gonadorelin diacetate tetrahydrate), Gonabreed® (gonadorelin acetate). All of them have been approved by the Food and Drug Administration since 2013 (except Cystorelin approved in 2018) for dairy and beef cows [41,42]. In this context, performance-enhancing drugs used for animals' race, especially used in horses and greyhounds, are not regulated yet by a Worldwide legislation, but they are only prohibited at the state level (e.g., Australia under the Rules of Racing) [43].

2. Analytical detection methods: state-of-the-art

The main goal of this review is to provide an overview of the state-of-art of analytical methods for the detection of peptide hormones starting from the reference methods for small peptides' analysis and looking towards novel promising bioanalytical strategies. Here affinity-based approaches, dealing with natural and synthetic receptors, namely DNA/RNA- and polymeric- based biomimetic receptors, will be described. Specifically, the use of affinity receptors coupled to biosensing-based approaches, as well as the use of simple, quick and low-cost methods with application to conventional clinical laboratory instrumentation, will be reported and discussed.

2.1. Liquid chromatography-mass spectrometry analysis

The scientific literature dedicated to the synthetic peptide hormones detection is very limited in terms of type of techniques and most of the studies reported in addressed the quantitative determination of these short peptides through high-performance liquid chromatography (HPLC) [44,45], tandem mass spectrometry (MS/MS) [46–49], or capillary zone electrophoresis (CZE) [50,51]. Notwithstanding, a dominant role in the current studies, concerning the analytical field, belongs to liquid chromatography, eventually coupled to mass spectrometry (Fig. 3). Only a few studies involve conventional immunoassays and sensing platforms for the detection of peptides. Currently, in clinical diagnostic and anti-doping settings, mass spectrometry is considered the gold standard analytical technique for the unambiguous detection of small molecules thanks to its high sensitivity, which ensures a high sample throughput. In addition, from a technical and logistical standpoint, this technique allows timely analysis of several drugs in biological fluids processed every day. The chromatographic assets can be coupled to various detection approaches, i.e., optical, electrochemical, spectrometric, etc. Among optical detection, fluorescence is suitable for sensitive peptides' detection [52] and the chemical derivatization is usually the pretreatment requested. Conventional fluorogenic reagents used for the peptides' derivatization are o-phthalaldehyde, naphthalene-2,3-dicarboxaldehyde, fluorescamine, ninhydrin, and phenanthrenequinone, which react with primary or secondary amine groups [53–56]. Benzoin or ninhydrin, newer derivatization reagents, can react with arginine present in the peptide, as in gonadorelin, under strongly basic conditions (pH 12) to generate a fluorescent product which is measured inline [53–56]. Despite the wide variety of detectors available, the mass spectrometer has emerged as selective, sensitive, and universal.

In this context, different chromatographic approaches, available in the scientific literature, are compared mainly in terms of detection limit (LOD) in real biological matrices and recovery, when specified. Appropriate sample preparation represents a critical and long step for LC-MS techniques with a relevant impact on the high-throughput screening as well as analysis times. Solid-phase extraction (SPE) is a single-use low-resolution chromatographic process, and it is widely involved upstream of LC-MS allowing the extraction/concentration of the target from standard solutions or biological matrix. Various methods are used to purify/enrich peptides from complex biological samples and among them, affinity chromatography (or affinity purification) represents a powerful tool. Affinity purification process employs affinity ligands, natural or biomimetic receptors, directed towards a specific target or an entire class, which are immobilized on the stationary phase. Here, we refer to antibodies as natural receptors whilst aptamers and molecular imprinted polymers (MIPs) are included in the biomimetic class, which are further described in the following 2.4 paragraph with application to gonadorelin analysis. Specifically, streptavidin-functionalized magnetic beads (MBs) are involved as stationary phase for gonadorelin clean-up/concentration from human and equine urine samples respectively, using specific biotinylated antibodies [54] or aptamers (e.g., spiegelmer NOX-1255) [58]. The enrichment of peptide drugs by biomimetic receptors currently is an effective/outstanding alternative to the natural counterparts overcoming some of their limiting features (extremely high

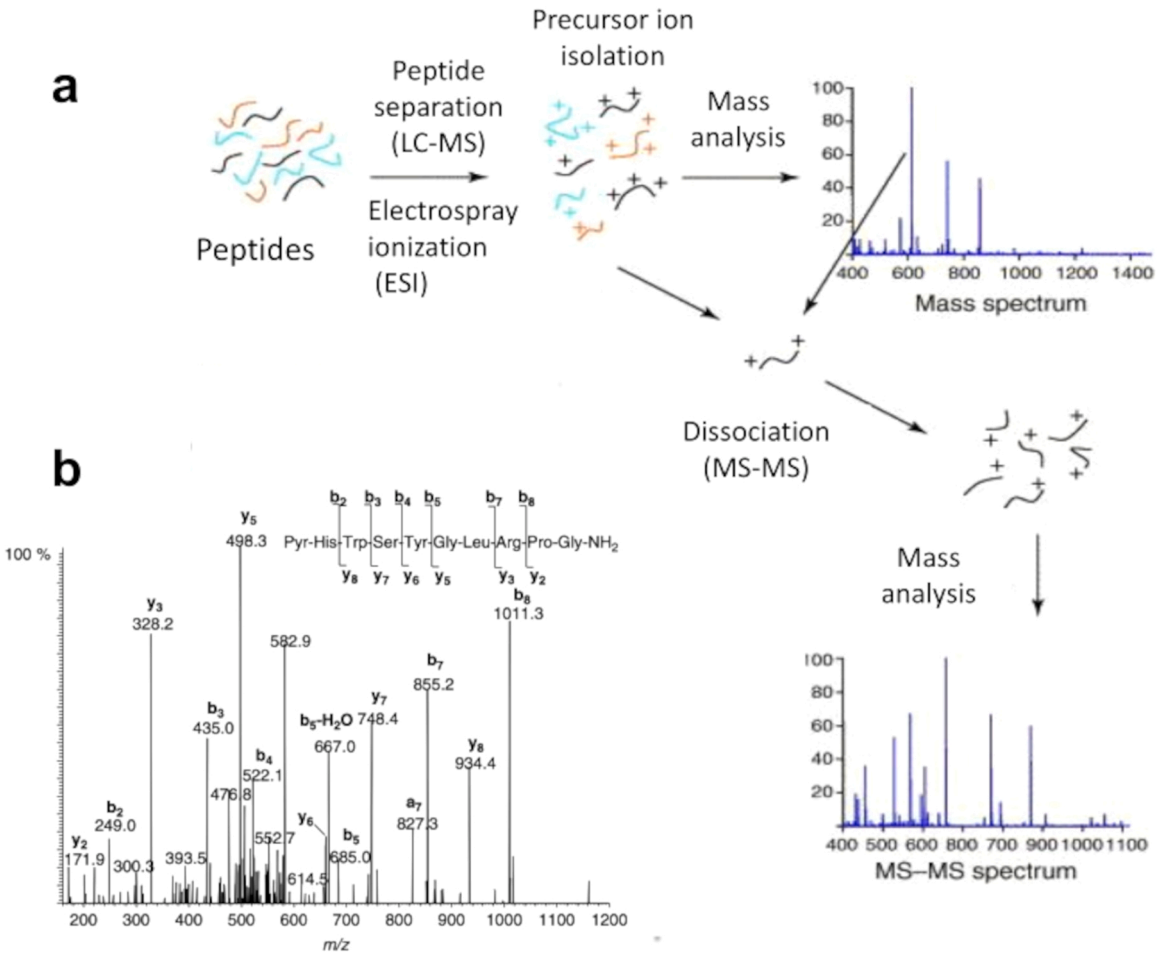

Fig. 3. (a) Schematic LC-MS/MS workflow for peptides' analysis. (b) Representative MS GnRH spectra (LTQ Orbitrap) of a urine sample acquired as product ion scan experiment of the precursor (doubly charged precursor at *m/z* 592) at the characteristic retention time (27 min). Figures reproduced and modified with permission of [55] and [57], respectively.

costs for disposable use, sensitive to environmental conditions, etc.). In particular, the growing availability of biomimetic affinity-based receptors against peptide hormones will positively impact not only the samples' pre-treatment step, before HPLC analysis, but also the peptides' detection strategies itself, proving to be a chance in developing biosensors, disposable low-cost and/or lab-on-chip platforms. This topic will be discussed deeper in the following sections, firstly introducing biological ligands and then biomimetic ones simultaneously going to reveal affinity-binders' properties in bioanalysis (Table 1).

2.2. Bio-based methods: immunological techniques

Immunoassays denote a very powerful analytical techniques field. These are widely involved in clinical laboratories, as diagnostic tests for the analysis of multiple analytes, or as immunoaffinity tools for pre-analytical samples' preparation due to its straightforwardness protocol and small samples' amounts required. Immunological techniques conventionally rely on antibody-antigen affinity reaction able to form a stable complex (Ab-Ag) that can be described according to the law of mass action: Ag+Ab ⇌Ag-Ab; where Ag/Ab/Ag-Ab is the antigen/antibody/complex concentration, respectively; k_a and k_d correspond to the kinetic constant of association and dissociation, respectively [69]. The ratio of the two kinetic constants expresses the equilibrium constant (K_D). Besides, this technique allows the multiplexed screening of different biological samples (e.g., blood, urine, saliva, etc.) or different analytes on disposable plastic microplates, offering many practical advantages. These microplates are usually constituted by several wells used singly as micro test tubes (till a maximum of 384 micro-test tubes for a single plate). In this scenario, enzyme-linked immunosorbent assay (ELISA) is the most established technology which integrates the specificity of antibodies and the sensitivity of a simple enzymatic signal amplification reporter. The ELISA assay has undergone numerous welcomed changes from the first one described by Engvall and Perlmann in 1971 [70]. As a result, the term ELISA now refers to a wide range of micro-welled plate assays, some of which do not involve enzymatic reactions [71–73] and immune complex formation [74,75]. The sensitivity and specificity of immunoassay have allowed the detection of analytes at very low concentrations not easily measured by other analytical techniques [69]. Nowadays, the smartphone technology, coupled with microplates-based assays enables rapid point-of-care (POC) diagnosis (or mHealth, mobile health) for in situ analysis, namely mobile phone-based ELISA (MELISA) [76]. In this context, the bottleneck of immunoassays for the detection of small molecules (MW < 2 kDa), such as gonadorelin, is related to the production of bioreceptors. Small molecules do not represent good immunogens mainly due to their size (e.g., haptens) and elicit antibody titers only when combined with a larger carrier molecule such as bovine serum albumin (BSA), ovalbumin (OVA), keyhole limpet hemocyanin (KLH), or linked to other peptide fragments. Moreover, small molecules lack multiple epitopes for response enhancement by sandwich format and therefore the assays can be designed only in a competitive mode. In the competitive assay, the antigen competes with a chemically identical molecule (competitor molecule) tethered to a signal reporter for the antibody's binding sites immobilized onto a solid microtiter plate. Up to this point, there are only

Table 1
Comparison of different chromatographic methods for quantitative detection of gonadorelin in biological matrices.

Target company	Detection method	LOD (ng/mL)	Matrix	Ref.
LHRH ICN pharmaceuticals (Costa Mesa, CA)	HPLC system UV detector (210 nm)	1	Not tested	[59]
LHRH Bachem (Bubendorf, Switzerland)	Sample clean-up: SPE methodology UPLC-MS/MS analysis Ion source: heated electrospray (HESI) Targeted method positive ion mode	0.1	Urine samples spiked Recovery: 87 ± 10%	[40]
Gonadorelin Bachem Americas Inc. (Torrance, CA, USA)	Sample clean-up: SPE methodology HILIC-HRMS Ion source: electrospray positive ion mode Untargeted method MS full-scan range *m/z* 200–2000	0.02 in plasma 0.04 in urine	Equine plasma and urine spiked with 44 peptides Extraction efficiency % = 78% (plasma) and 85% (urine)	[60]
LHRH Sigma-Aldrich (Saint Louis, MO, USA)	Online sample clean-up using a Dionex UltiMate 3000 separation LC system LC-HRMS/MS Ion source: ESI MS full-scan range *m/z* 300–1500	< 0.1	Human urine samples	[38]
GnRH Abbiotec, LLC (San Diego, CA, USA)	Sample clean-up: SPE methodology HPLC-MS (Q-TOF) Ion source: ESI MS full-scan range *m/z* 50–1350	0.1	Human urine samples spiked. Matrix effect: 59%	[61]
LH-RH Australian Sports Drug Testing Laboratory (Sydney, Australia)	Sample clean-up: SPE methodology LC-MS Ion source: ESI MS full-scan range *m/z* 50–1350	0.25	Human urine samples spiked	[62]
Kryptocur Sanofi-Aventis (Frankfurt, Germany)	LC-MS/MS (Orbitrap) Ion source: ESI MS full-scan range *m/z* 300–2000	0.005	Human urine samples	[52]
LHRH Bachem (Bubendorf, Switzerland)	Sample clean-up: SPE methodology UPLC-MS/MS (TSQ) Ion source: HESI MS full-scan range *m/z* 300–2000	0.05	Human urine samples Recovery: 86%	[63]
GnRH AusPep (Melbourne, Australia)	Nano LC-MS/MS (TSQ) Ion source: ESI	0.001	Equine urine spiked Matrix effect: 170 ± 8%	[64]
LHRH Sigma-Aldrich (Saint Louis, MO, USA)	ECD-Tandem FTICR MS Ion source: microESI	X	Not tested	[65]
LH-RH Sigma-Aldrich (Milan, Italy)	Sample clean-up: SPE methodology LC-MS/MS (TSQ) Ion source: ESI	0.10	Artificial urine samples spiked Matrix effect: 35%	[55]
		50	Not tested	[66]
Gonadorelin European Pharmacopoeia reference standards	Screening method: LC-MS/MS			
Kryptocur Sanofi-Aventis (Frankfurt, Germany)	Sample clean-up: SPE methodology nano-UHPLC-MS (Orbitrap) Ion source: nano-ESI	0.002	Urine samples Recovery: 49%	[36]
LHRH	LC-MS (LTQ linear ion trap mass spectrometer)	59	Plasma samples spiked	[67]
GnRH	Sample clean-up: SPE methodology HPLC-HRMS	0.008	Ewe plasma samples (Healthy and prenatal androgen exposed ewe) Recovery: 65.4%	[68]

a few competitive inhibition ELISA assays for gonadorelin dosage, available on the market as a research tool. In this regard, a lack of clearness transpires, from our viewpoint, about the target employed in the kit and the immunogen used to produce antibodies. Some handbook kits report the name of different peptides portions (gonadorelin, progonadoliberin-1, GAP, etc.) belonging to the prohormone but their sequences do not coincide with that of gonadorelin, for this reason understanding the kits' effectiveness in detecting gonadorelin is difficult. Moreover, the unknown antibody-binding epitope and the scarce information about the immunogen used to trigger the immune response limit the applicability of the present gonadorelin kits. In this scenario, despite the significant role that GnRH physiologically plays, its detection does not take place directly from blood specimens since the hypophyseal portal blood sampling, a small and relatively inaccessible blood system, is extremely difficult and invasive [77]. For this reason, examining the research literature concerning immunoassays to dose GnRH, a lot of tests have been conducted in vivo animal models. The pioneered immunoassay developed for GnRH quantification exploited a radioactive signal label [78]. As a first example, Rosenblum et al. [79] propose a radioimmunoassay (RIA) for the measurement of GnRH in human plasma, Morris et al. [78] measured hypothalamic GnRH in rat, Inaba et al. [16] measured it in canine plasma while Mosby et al. [80] quantify GnRH in human plasma and urine (Table 2). Nonetheless, regarding the safe handling of radioactive reagents, regulatory disposal, and waste of working with radioactive materials have led to the development of alternative non-isotopic labeling strategies, such as enzymatic tracers [69]. Moreover, studies focused on the quantification of GnRH, through a competitive ELISA, were conducted in vivo by sampling hypothalamic-hypophyseal portal blood in dogs and seabreams. Inaba et al. [16] proposed a newly competitive ELISA for GnRH detection in the canine hypothalamus and plasma, whilst Holland et al. [81] developed three competitive ELISA assays to measure various GnRH isoform (GnRH I, GnRH II, and GnRH III) in male and female seabreams' pituitary (sbGnRH) [16,81,82] (Table 2). Rodríguez et al. [83] reported the measurement of sbGnRH levels by applying the same assay methodology of Inaba et al. [16]. Immunoassays represent an established technique in the clinical field and arise as promising portable and high performing bioanalytical tools also in the forensic field. However, since current research interest is dictated by the requirement of greater throughput and sensitivity alternatives, as well as cheaper, conventional immunoassays could gradually be replaced by abiotic pseudo-ELISA tests. In this context, several efforts have already been directed towards discovering

Table 2
A comprehensive survey on affinity-based methods available in the scientific literature addressing gonadorelin detection is provided in the following table.

Strategy	Sample investigated	Ref.
Radioimmunoassay (RIA)	Human plasma	[79]
RIA	Medial basal hypothalamus and posterior pituitary tissues	[78]
RIA	Human plasma and urine	[80]
RIA	Canine plasma	[16]
Enzyme-linked immunosorbent assay (ELISA) Competitive format	Hypothalamic-hypophyseal portal blood in dogs and seabreams	
ELISA	Seabreams' pituitary	[82, 83]
Stripping-based electrochemical sensor	Standard solutions	[91]
Amperometric sensor	Blood serum samples	[92]
DNA aptamer-based approach for clean-up before LC-MS/MS analysis	Equine urine	[64]
Aptamer-NASBA-based lab-on-chip (LOC)	Standard solution	[114]
Molecularly imprinted polymer coupled to surface plasmon resonance (SPR) sensor	Artificial Urine	[119]
Biomimetic-ELISA	Human urine	[74]

novel signal reporters to increase the sensitivity of the assay [71] and to pinpoint abiotic capturing agents, i.e., mimetics [84,85], able to flank and/or supplant bioreagents. Moreover, it has been estimated that almost 1 million animals are used annually for antibody production in the EU alone. In accordance with the EU directive 2010/63/EU on protecting animals used for scientific purposes, the EU Reference Laboratory for alternatives to animal testing (EURL ECVAM) published new recommendations on non-animal-derived antibodies in 2020. The recommendation clearly states that "animals should no longer be used for the development and production of antibodies for research, regulatory, diagnostic and therapeutic applications". This statement clearly opens and/or strengthens the way in weighing alternative strategies to bioreagents in diagnostic and bioanalysis. In the following paragraph, biosensor technology, another important tool in the analytical field, was purposely addressed to the state-of-art of gonadorelin detection.

2.3. Biosensing technologies

Biosensing represents a well-established and extremely modulable analytical technology. Since its introduction occurred in 1956 [86], biosensors [87] have witnessed a fast growth becoming a field thriving worldwide for advancing human healthcare, for pharmaceutical companies to screen different drugs or vaccines' libraries, food, and environmental analysis, but also for anti-doping control analysis. Sensing technology gained attention over conventional analytical techniques because they enable quick and in real-time detection of a plethora of analytes (spanning from biomarkers, environmental, food and drug targets) by monitoring the interactions (qualitative ranking, calculation of kinetic and affinity constants), without the need of labeling and with a minimal (or no) sample pretreatment [88]. Specifically, a biosensor is defined as an analytical device constituted by a biological or biomimetic element integrated within a transducer system, which may be optical, electrochemical, thermometric, piezoelectric, magnetic, or micromechanical [89]. When the optimal features of sensing devices are achieved, they offer remarkable advantages over conventional analytical techniques principally in terms of cost, time-consuming, and clean-up of samples. Biosensors can be classified on the basis of biorecognition elements (e.g., tissues, microorganisms, organelles, enzymes, cell receptors, antibodies and biomimetic receptors) and signal transducers. In addition, they can be divided into two classes: the first class enclosed affinity-based biosensors established on the specific binding of an analyte to the receptor; on the other hand, the second class comprises enzyme-based biosensors that rely on the enzymatic turnover, i.e., the enzyme converts the substrate into a product [90]. In a general analysis, the receptor element binds the analyte, and this recognition event is converted by the transducer into a measurable signal (signalization). The latter, in turn, is processed to a readable analytical signal by electronic devices. Narrowing the scenario to the case of gonadorelin detection, a stripping-based electrochemical sensor was described by Cai et al. [91]. They reported a potentiometric stripping analysis (PSA) which was successfully applied to determine gonadorelin in buffer solution (0.2 M sodium phosphate, pH 7.0) [91]. The tryptophan and tyrosine residues in the gonadorelin sequence produce two-well resolved potentiometric peaks at the corresponding potentials, tryptophan shows a peak around 0.7 V (against Ag/AgCl reference electrode) tyrosine around 0.55 V (Table 2). This sensing platform provides high sensitivity, down to picomole peptide level. In another example, Zhou et al. [92] based their approach on an electrochemical amperometric sensor in which the glassy carbon electrode (GCE) surface was modified with Ag-graphene oxide-poly(L-serine) nanocomposite for GnRH agonists detection (Table 2). The porous surface and the larger surface area facilitates a high electron transfer and electro-catalytic performance, which enabled the achievement of a linear range 1–15 $\times$ 10^7 μg/mL and a detection limit of 20 pg/mL for GnRH in buffer (0.1 M PBS, pH 7.4) at − 0.15 V [92]. In addition, the sensor was successfully applied to GnRH detection directly from blood serum samples (average recovery around 85%) obtained from patients that were administered for the treatment of uterine fibroids. The same samples were analyzed in parallel with an ELISA kit showing a good agreement between the two methods. Likewise, optical (e.g., fluorescence spectroscopy, Surface Plasmon Resonance (SPR) including imaging and localized, surface-enhanced Raman scattering (SERS), interferometric spectroscopy and guided mode resonance (GMR)) [90] and acoustic biosensors (e.g., quartz crystal microbalance (QCM) and surface acoustic wave (SAW) devices) [93–95] are an effective, affordable, and non-invasive alternative to electrochemical ones. Comprehensively, affinity-based biosensors, which traditionally require a selective receptor (biologically or biomimetic derived) to capture the target antigen, may offer a valid approach to expand the cohort of techniques applied to the detection of small peptides for biomedical and forensic diagnostic purposes [48,96–99]. In this sense, it is important to spotlight that the same assay molecular architecture can be combined with different transducer technologies or signal output measurements (e.g., colorimetric analysis ELISA-like formats).

2.4. Abiotic-based analytical strategies

This section primarily focuses on biomimicry (from the Greek *bios* = life and *mimesis* = to imitate [100]), a field that seeks to mimic natural mechanisms, structures, and functions to exploit them into several scientific applications. Over the last two decades, extensive research on alternative "abiotic" affinity receptors, namely biomimetics, such as aptamers and molecularly imprinted polymers (MIP), has been carried out highlighting interesting and promising solutions intended to comply with EU directive. Looking at these results, various formats/analytical approaches employing biomimetic receptors have been developed. From a more comprehensive standpoint, the next section will provide an overview of the existing biomimetic receptors against gonadorelin addressing the broad need for simple, cost-effective, and accurate bioanalytical approaches.

2.4.1. Biomimetic receptors' features

The trend and challenge of the last decade in bioanalytical chemistry is the growing need for rapid, robust, and inexpensive platforms. The use of synthetic receptors in this circumstance offers several advantages. Novel biomimetic receptor elements can be successfully synthesized and integrated for compound analysis (e.g., ELISA assays and biosensing platforms) but also for sample purification intended for clinical and forensic investigations. In the case study of gonadorelin, there are only few existing examples in literature of biomimetic receptors, such as

aptamers (and/or spiegelmers) and molecularly imprinted polymers (MIPs) (Fig. 4), specific for the latter peptide. An overlook to the current available strategies is presented, from an analytical point of view, in the following paragraphs.

2.4.2. Aptamers and spiegelmers

Aptamers are short synthetic single-stranded (ss) nucleic acid sequences (i.e., DNA or RNA in the range of 15–60 nucleotides in length) able to fold into a well-defined three-dimensional structure capable of binding a plethora of target molecules non-covalently, with high affinity and selectivity [101,102]. Aptamers can also be classified as peptide aptamers constituted by few amino acidic residues (5–20 aa) embedded into a stable protein scaffold [103–105]. The difference between these two classes of aptamers is simply related to the random libraries (nucleic acids or peptides) used to generate them during the synthesis process. The structural interactions between the aptamers and the targets (via van der Waals forces, hydrogen bonds and electrostatic interactions, etc.) are similar to the way in which antibodies bind antigens. Unlike their natural counterparts' receptors, aptamers are synthesized in vitro with no animal sacrifice and in a shorter time, by reducing the batch-to-batch variability associated to distinct immune responses, and also, they are cost-effective and amenable to modifications [74]. Dealing mainly with oligonucleotides-based aptamers, they display an improved stability compared to antibodies, on account of the presence of robust phosphodiester backbone. Besides, they have a flexible structure (able to fold and unfold an unlimited number of times) over an extensive range of physical-chemical conditions (pH, ionic strength, temperature, denaturants, chemicals, etc.), whereas antibodies are subjected to irreversible denaturation [106] in the same conditions. Chemical modifications of aptamers can impart resistance to nuclease cleavage in biological matrices. Therefore, aptamers are a versatile multifunctional tool that find increasing application in various fields of research such as targeted therapeutic agents, as affinity ligands and as a tool for cell tracking. Aptamers can be potentially generated for a multitude of macromolecules (proteins, whole cells, bacteria, and viruses) as well as for small molecules (peptides, metabolites, small drugs, metal ions, toxins etc.) that don't trigger an immune response [101,105] but compressively, aptamers for small molecules represent a small percentage of all existing. Traditionally, aptamers are produced via an in vitro screening process, namely, systematic evolution of ligands by exponential enrichment (SELEX), involving several rounds of alternating steps of partitioning of candidate oligonucleotides and their PCR amplification (Fig. 5). In particular, the SELEX process is characterized

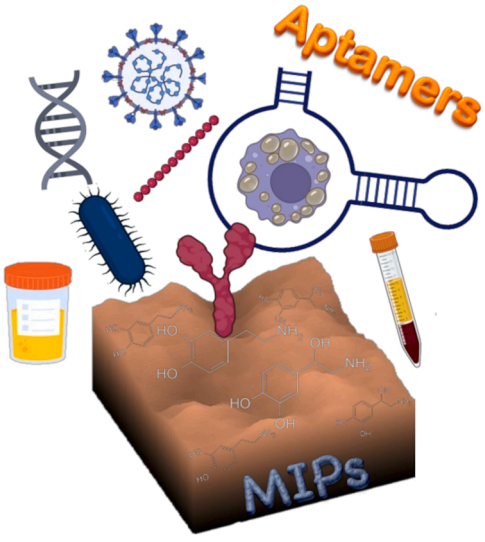

Fig. 4. Sketched overview of biomimetic receptors (MIPs and Aptamers) targeting very different biomolecules.

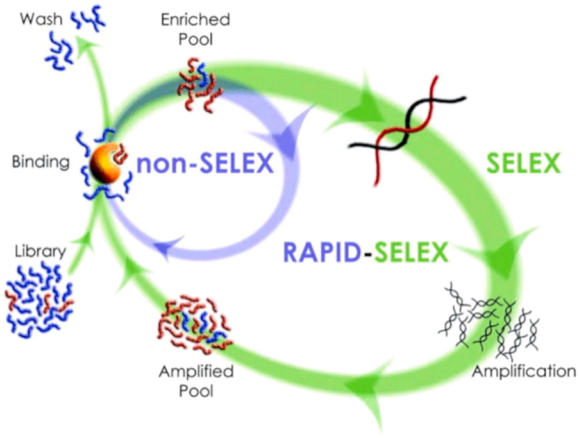

Fig. 5. Schematic illustration of SELEX and non-SELEX protocols for DNA-based aptamers generation. Figure reproduced with permission of [108].

by iterative cycles consisting in the repetition of sequential steps: selection, amplification, and conditioning (purification of the relevant ssDNA) to produce selective aptamers fit for a certain non-nucleic acid target. The SELEX procedure begins with a starting "pool", a randomized library of oligonucleotides (DNA and RNA) consisting of about 10^{13} – 10^{15} different sequence motifs [107,108]. Each DNA sequence is composed of a central region flanked by two constant primer binding sites, which are used for polymerase chain reaction (PCR) amplification. In a general process, at the beginning, the pool and the target of interest are incubated in solution. Conversely, the same reaction for small molecules involves their immobilization onto a solid-support matrix to enable DNA (or RNA)-binding. Several washing steps are further employed to remove unbound sequences, to yield an enriched library, which then are PCR-amplified to form the starting pool for the next selection round (4–20 rounds).

Recent advances were performed in improving also the non-SELEX selection process of aptamers (Fig. 5), which includes the same multiple rounds of partitioning with no amplification between them [108,109].

The equilibrium dissociation constants of aptamers against macromolecules typically fall in the pico- or nano-molar range whilst Ruscito et al. [101] report, concerning small molecules binding aptamers, that averagely recognize the low-MW targets with K_D in the low-mid micromolar range.

Notwithstanding, small molecules binding aptamers are still an active research field of great relevance and among these, Leva et al. [110] reported for the first time two spiegelmers, based respectively on L-RNA and L-DNA, for gonadorelin. Spiegelmers are L-enantiomeric synthetic oligonucleotides, the mirror image of D-oligonucleotide ligands, built of L-2'-deoxyribose unit [111] and their synthesis is more complicated than the conventional one because it requires an appropriate non-natural mirror-image of the target [104]. These spiegelmers are able to bind GnRH with high affinity, as stated by K_D in the nmol/L range, and with high specificity [110–113], as attested by the ability to discriminate a single amino acid exchange in the chicken GnRH sequence. Specifically, spiegelmers' affinity has been tested through isothermal calorimetry, while the ligands' selectivity *via* Surface Plasmon Resonance (SPR) analysis [110]. The selectivity study of the selected receptors was carried out using 5'-biotinylated RNA and DNA spiegelmers, which were immobilized on a streptavidin-coated SPR sensor chip and tested against different peptides (concentration range 0.1–100 μmol/L), such as decapeptide chicken GnRH and nonapeptide buserelin (both differing for one amino acids residue and also for the C-terminal moiety in the case of buserelin). DNA-based spiegelmer did not bind buserelin while RNA-spiegelmer recognized buserelin, albeit exhibited a reduced affinity for it compared to the target. These receptor

elements for specific recognition of targets are extremely versatile for applications in various areas. In a first study, the potential application of anti-GnRH spiegelmers was devoted to the therapeutic field, as reported by Leva et al. [110]. They explored the potential use of spiegelmers as bioactive tools for cancer treatment in order to block the GnRH binding to its cell surface receptor, overexpressed in hormone sensitive cancers, reducing in this way the release of the sexual hormones. A more recent study by Richards et al. [64] proposed a DNA aptamer (NOX-1255)--based approach for extraction/enrichment of gonadorelin directly from equine urine coupled to LC-MS/MS analysis [110]. In this case, a low analytical recovery (29 ± 15%) and significant matrix interference was obtained with the aptamer based SPE-cleanup. Behind these applications, some attempts to develop lab on chip (LOC) devices are reported by Zhao et al. [114]. A prototype device, named aptamer-NASBA-based lab-on-chip (LOC), was designed and preliminarily tested for gonadorelin detection in standard solutions [106]. The chip, made of PDMS/silicon and coated with a gold layer, is divided into four parallel functional areas for multiple analytes detection and modeled on a 384-well microplate fitting with common microplate readers. The chip, belonging to one of the functional areas, is further functionalized by aptamers for the specific recognition of GnRH (gonadotropin-releasing hormone). Except for the aforementioned preliminary study, no diagnostics devices (e.g., aptasensors) have been developed yet for analyzing gonadorelin, which instead could really emphasize and broaden the biochemical properties of the aptamers presented by Leva et al. [110].

Comprehensively, the aptamers technology has continuously progressed in the past 20 years and still today is an intriguing tool for biomimetic receptor source. However, the research in overcoming immunoassay limitation continues, eventually exploring new biomimetic approaches, based on biopolymers.

2.4.3. *Molecular imprinting technology*

Molecular imprinting is a versatile technology to synthesize affinity reagents, named "artificial antibodies", with tailor-made binding sites mimicking the ability of natural receptors to recognize target molecules. Imprinted mimetic receptors can be developed in multiple formats (e.g., film layers or nanostructures) gaining a lot of interest in different research fields. These receptors are easy to prepare and lack biological components, which leads to a far superior shelf life [115]. The possibility of tailoring the MIP to ensure the coverage of a broad range of analytes/applications, makes this technology a powerful and interesting tool in pharmaceutical, biomedical, and forensic sciences. MIPs gained a significant interest in the bioanalysis field as recognition elements directly integrated in bioassays to quantify drugs or as solid phase imprinted surface (MIP-SPE) to clean-up several biological samples, which then are massively processed with standard analytical techniques [111–114]. In addition, MIPs flexibility has allowed the development of several analytical tools for the detection/extraction of very different bioactive drugs, for example belonging to different classes of prohibited substances by WADA [30,116–118]. Bioanalytical portable assays also represent an important tool for upstream in-situ screening. In this context, nowadays, the challenges rely on identifying newer rapid, specific, and sensitive strategies to quantify gonadorelin in urine samples. Hence, in response to this emerging issue, our research group developed the first imprinted biopolymeric receptor based on neurotransmitters-derived biopolymers (e.g., polynorepinephrine), following a very simple reliable procedure, for gonadorelin detection.

Firstly, the mimetic receptor against gonadorelin was integrated on an optical sensing platform (SPR) for the development of a label-free quantitative assay [119] (Fig. 6). Undoubtedly, the core of SPR affinity biosensing is based on the study of biomolecular interactions in which recognition elements can be immobilized onto the metal surface, through various chemistries, and capture analytes present in a sample solution, or vice versa. This carries to a measurable increase of the refractive index at the metal surface which is directly proportional to the concentration of the interacting molecules with the immobilized counterparts. We believe that SPR technology represents a powerful technique to characterize novel "household" receptors, in terms of analytical performances, and to design original abiotic approaches, taking

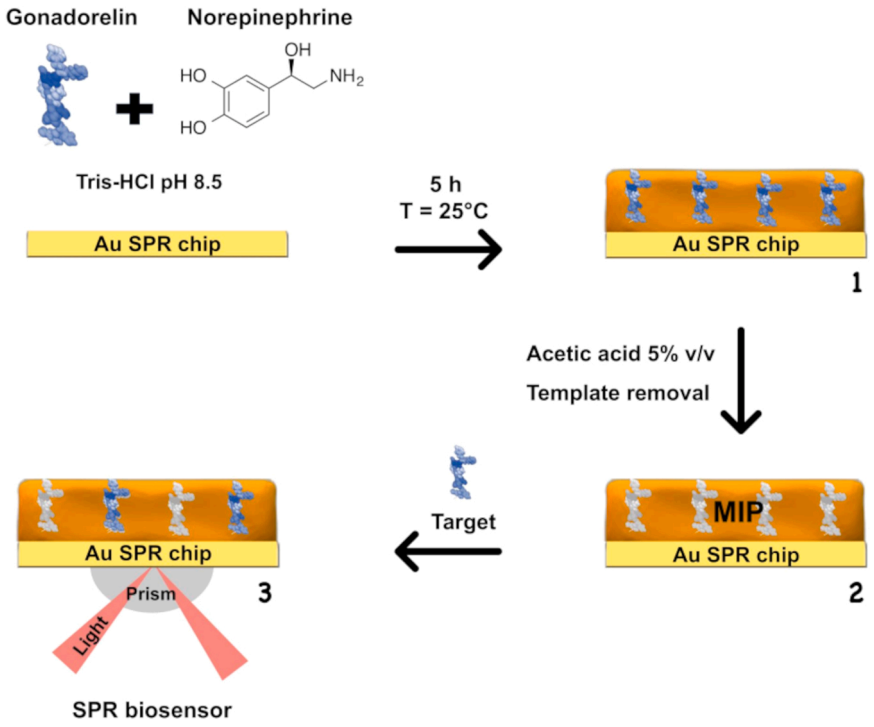

Fig. 6. Schematic depiction of the imprinting process on the SPR sensing platform. (1) Norepinephrine (functional monomer) was polymerized around the template/target, gonadorelin. (2) The template was removed from the polymeric matrix by leaving binding sites able to recognize (3) the target (gonadorelin) itself in real-time *via* SPR sensing platform. Figure reproduced with permission of [119].

inspiration from convention ELISA assays' format. The aforementioned mimetic receptors represent an effective alternative to bioreagents and, in the specific case of gonadorelin, is a simple and cost-effective solution to the lack of natural receptors. In this study, the novel biomimetic receptor was integrated in establishing a two-steps competitive assay for real-time and label-free gonadorelin detection achieving a high sensitivity (LOD = 52.0 ± 6.0 pmol/L), in line with the WADA analytical requirements (MRPL = 1.69 nmol/L), in artificial urine samples [119]. A direct comparison with a commercial antibody highlights the excellent features of this polynorepinephrine (PNE)-based MIP in terms of selectivity, reusability, and cost of the overall synthesis procedure. As with any technology, many hurdles must be overcome to "translate" a benchtop-based biosensor into a portable device. SPR reference instrumentations commercially available hold large size (benchtop equipment), but with advances in modern biosensing, few portable SPR devices have been developed (e.g., Plasmetrix) [120]. So gradually SPR biosensing technology is moving from off-site laboratory to on-site diagnosis, for point-of-care testing (POCT). In our case, motivated by the promising results of PNE-based MIP and the well-known versatility of the material (catecholamine-based polymers), we transferred the mimetic receptor to a disposable micro welled platform to set up a biomimetic enzyme linked assay (BELISA) (Fig. 6).

The growth of the PNE-based mimetic receptor was directly performed on each microwell and the gonadorelin quantification has been achieved via setting up a competitive binding assay between biotinylated gonadorelin linked to the colorimetric reporter enzyme (HRP-conjugated streptavidin) and the unlabeled analyte. Finally, this novel assay (LOD = 277 pmol/L) was successfully validated by mass-spectrometry, a reference platform for peptides detection, analyzing in parallel the same gonadorelin spiked real urine samples [74]. In the last decade, MIPs have beheld a great advance and few articles have already been published for analysis/pre-cleaning of peptides in biological fluids via MIP-based on hard materials (e.g., polyethylene terephthalate, polybutylene terephthalate, polycarbonate, etc.). Notwithstanding, to the best of our knowledge, no MIP-based tools (e.g., sensing devices or ELISA-like assays) were developed to detect gonadorelin. In this context, it is also important to emphasize the building block material, neurotrasmitters, used to fabricate the aforementioned mimetic receptor against gonadorelin, which is in line with the perspective of MIPs "*greenification*" of the synthesis process. The neurotrasmitters-derived biopolymers are soft and biocompatible materials (e.g., polydopamine (PDA), polynorepinephrine (PNE) etc.), which are considered a very attractive and promising tool in bioanalytical settings thanks to key features such as the green simple one-pot synthesis, low-cost and reusability. Comprehensively, the MIP-based approach could be also implemented to simultaneously detect other bioactive peptides or could be used to develop tailorable stationary phases for SPE to clean-up/concentrate the samples in the analytical upstream step. (Fig. 7).

3. Pre-analytical challenges: towards miniaturization

Currently, we are witnessing to the progressive streamlining and decentralization (out-of-the-lab) of the analytical workflow in various research fields. The advances in modern bio-analytical methods, described in the previous paragraphs, are striving to push toward straightforward and miniature implementation of the detection phase. In this context, also the processes involving samples collection, handling, and storage, key issues belonging to the pre-analytical phase, are increasingly advanced in the same direction combining a miniaturization level with minimally invasive sampling procedure e.g., dried blood spots (DBS) micro-sampling technique. The use of filter papers to collect blood samples, in clinical chemistry, dates back to the early 1960 [121,122] when DBS was introduced as part of the neonatal screening programs [123]. Since then, DBS is considered a valuable tool to collect several analytes (proteins, peptides, metabolites, lipids, and nucleic acids for different purposes [122]. Undoubtedly, the main advantageous features of DBS sampling are associated with the speed and ease of specimen collection *via* finger sticks (few microliter samples are required), which is less invasive for the patient, combined with the overall reduction of analysis costs as it not requires a phlebotomy station, trained personnel, and compliance with the cold chain protocol [124]. Moreover, the small size of the filters (reduced storage space), the reduced bio-hazard risk during handling operations (e.g., transmission of bloodborne pathogens [125]) as well as the dry samples stability during the storage and shipping process, make DBS a safe, easy, reliable,

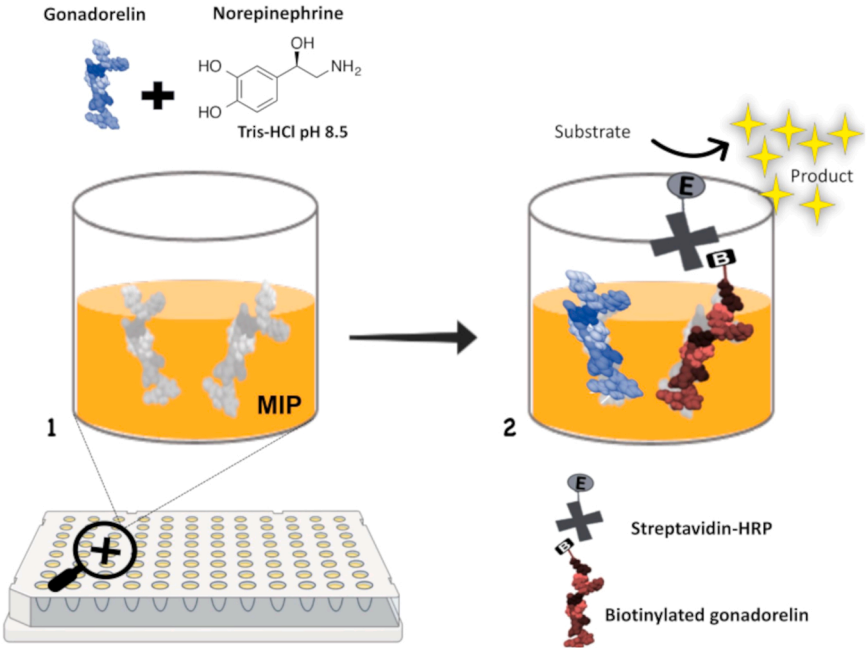

Fig. 7. Schematic depiction of the imprinting process on the ELISA plate (1) and colorimetric competitive two-steps assay (2). Figure reproduced and modified with permission of [74].

inexpensive and logistics savings tool [122,124,126] that can be exploited for decentralized (out-of-the-lab) large-scale sampling and also for retrospective analysis. DBS really supports the quantification of circulating drugs and enhances the stability of labile markers in blood passport analysis [127]. Specifically, after sampling, the filter cards are dried in proper conditions and then the small spots are punched out from the filter cards directly into 96-microtiter plates or tubes before being soaked with buffers to eluate the investigated analytes. Presently, DBS technology is not limited to the whole blood, but other biological matrices are collected (dried matrix spots – DMS [128]), such as saliva (dried saliva spots – DSS) [129], urine (dried urine spots - DUS) [130], milk (dried milk spots) [131], feces (dried fecal spots - DFS) [131,132], tears, synovial fluid, and cerebrospinal fluid [133]. Further, it is not surprising that this technology has gained several potentials and emerging applications in various research areas spanning from clinical diagnostics, therapeutic drug monitoring (TDM), pharmacokinetic and toxicokinetic studies, clinical pharmacology, forensic toxicology, and doping controls [132]. Referring to the latter field, the number of scientific research concerning DMS, mainly coupled to mass spectrometry analysis, began to grow in the last few years discussing its increasing potential to support result management processes in routine testing. Additionally, this collecting/testing method has been fully introduced in anti-doping setting to screen athletes for illegal non-threshold substances compounds on large scale at the 2022 winter Olympics in Beijing, following the mandatory guidelines established *for DBS sample collection, transport, analytical testing, and storage* reported in the WADA technical document – TD2021DBS [134]. Herein, we summarize recent advancements in DMS methods combined with LC-MS/MS analysis for doping-relevant drugs, since no studies were reported for gonadorelin. Besides, we outline the challenges and prospects of such technology in practical applications related to gonadorelin. Protti et al. [134] compared the stability of peptide hormones and growth factors in urine sampled by two miniaturized and matrix dried-based techniques, namely DUS and volumetric absorptive micro-sampling (VAMS). The latter approach is based on the absorption of a liquid sample into a porous polymeric tip connected to a handler, by wicking [134]. They highlighted the enhanced analytes' stability (20 days at RT) in DUS and urine VAMS, which are prone, especially peptides, to proteolytic degradation under common storage conditions (−20 °C and −80 °C), broadening their detection window. Among all the promising features of these miniaturized sampling tools, Protti et al. [134] pointed out a few drawbacks, in order to further advance and improve these technologies, such as the low sensitivity, related to the impossibility of analytes pre-concentration, and the need for multiple microsamples for the analysis of several classes of prohibited substances [134]. Gerace et al. [135] successfully tested ipamorelin, a decapeptide ghrelin mimetic, in DBS at low concentration (ng/mL) by a UHPLC-HRMS detection. Also in this study, DBS was spotlighted as a doable micro-sampling strategy in remote world areas, avoiding the cold chain, which at the same time offers the opportunity to carry out a retrospective analysis. GnRH analogues (e.g., goserelin, leuprolide, triptorelin, buserelin, and nafarelin) along with other lower molecular mass analytes (MW $<$ 2 kDa) were mainly detected, after being extracted from an automated DBS sample preparation, below the WADA's MRPL in the study of Lange et al. [136]. DBS sampling was also involved for the sensitive (LOD = 0.5 ng/L) and reproducible detection of insulin and its synthetic analogs by mass spectrometry [137,138]. On the road of dried matrix spot samplers for anti-doping peptides analysis, all the recent studies discussed can be extended to gonadorelin testing in different biological matrices. Additionally, looking ahead to the next generation of DMS samplers and beyond, they could be potentially coupled (e.g., elution of analytes on ELISA plate modified with abiotic receptors) and eventually in-line integrated to portable and easy-to-use measuring devices to accomplish all the analytical process in situ on-demand. Concurrently, the same analysis can be performed in a retrospective modality to validate the analytical data, eventually obtained outside the lab, with benchtop reference instrumentation.

4. Conclusions and perspectives: from laboratories to in situ-analysis

We have presented and discussed the analytical conventional and innovative approaches presently in the scientific literature for the detection of gonadorelin, taken as a paradigmatic example of small peptide hormone. The excursus moved from laboratory-based instrumentation as chromatographic analysis coupled to mass spectrometry to bioanalytical approaches. Among others, electrochemical and optical sensing have been employed and specifically the label free and real-time surface plasmon resonance (SPR) technology was applied in combination with new abiotic receptors, which could be considered as an alternative to bioreagents. Following this path, we have to face the new EU 2010/63/EU Directive on the protection of animals used for scientific purposes, limiting their use only "where a non-animal alternative exists'' and that is why the research is pushing toward bio-synthetic binders, which can be integrated in affinity-based assays, including conventional plate assays, biosensors and lab-on-a-chip devices. These synthetic solutions have to be developed in a green environment, possibly selecting a water environment, as in the case of catecholamine-based MIPs, showing interesting affinity properties even for the small peptide hormone gonadorelin. In this context, aptamers can also deliver interesting solutions as biomimetics for gonadorelin, as in the case of specific selected spiegelmers. In conclusion, the combination of simple analytical devices with cheap, green and thermo-stable affinity receptors, within micro-sampling techniques, is a current research line that is pushing to try opening pathways for in-situ/remote analysis, where the cold chain is an issue. We may finally imagine point-of-care (POC) devices based on biomimetic receptors, designed with various substrate materials coupled to very different output signal technologies, for in-situ clinical and/or antidoping analysis as a close goal to screen the populations on large scale.

CRediT authorship contribution statement

Francesca Torrini Conceptualization, Investigation, Writing – original draft, Writing – review & editing. **Simona Scarano** Conceptualization, Investigation, Writing – review & editing, Project administration. **Pasquale Palladino** Conceptualization, Investigation, Writing – review & editing. **Maria Minunni** Conceptualization, Investigation, Writing – original draft, Writing – review & editing, Supervision, Project administration.

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

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

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