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Interest of HRMS systems in analytical toxicology: focus on doping products

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Abstract

Performance and image enhancing drugs are misused by top athletes, young people and amateurs like bodybuilders. Their misuses lead to adverse analytical findings in sport but also physical and mental health risks.

Anti-doping laboratories must set up analytical strategies and develop precise, robust and sensitive methods to detect all analytes prohibited by the World Anti-Doping Agency (WADA). In recent years, high resolution mass spectrometry (HRMS) systems have emerged and are gradually replacing more traditional systems for screening and quantification. The targeted compounds in this review are the drugs prohibited by the WADA in-and out-of competition and glucocorticoids. Advanced technologies were applied for anti-doping, forensic and clinical purposes.

The contribution of HRMS in the analysis of doping products allows a retrospective analysis to detect new metabolites in order to increase the detection windows and to identify new synthetic products.

These systems are also used for new applications like study of metabolomics, characterisation of metabolites and applications in alternative matrices (hair, dried blood spots). Moreover, the misuses of these substances lead the consumers to purchase on the black market.

Keywords: high resolution mass spectrometry, doping, clinical, forensic, toxicology

1. Introduction

In human sport, lots of products are prohibited because of their misuse to enhance physical and/or psychological performance. After the scandal that took place in the world of cycling with the Festina case, the World Anti-Doping Agency (WADA) was created in 1999. WADA is an independent international foundation. It is responsible for promoting, coordinating, and monitoring the fight against doping in sport, the management of scientific research, education, anti-doping development and the supervision of compliance with the World Anti-Doping Code.

The Code is a document that harmonizes doping-related practices and prohibited substances across countries and sports. The prohibited substances are classified by ban period and by family of substances. The first category includes six families that are permanently banned and classified in section S0 up to S5 (non-approved substances, anabolic agents, peptide hormones, beta-2-agonists, hormones, and diuretics). The second includes four families, S6 to S9, which are only prohibited in competition (stimulants, narcotics, cannabinoids, and glucocorticoids). And finally, there is a third category in which beta-blockers are banned in-and out-of-competition or only depending on the sport [1].

Concerning animal sports, all products are prohibited in-and out-of-competition. Moreover, some products are prohibited by the European Union (EU) as growth promoters [2].

Anti-doping laboratories work on urine for screening drugs and blood for testing peptide hormone. In urine, the main analytes present are the transformation products or metabolites. Metabolites are not always commercially available and/or can be very expensive. Moreover, false negative tests are not allowed by WADA. In general, all substances were detected and quantified by liquid or gas chromatography coupled with tandem mass spectrometry (LC-MS/MS or GC-MS/MS). In recent years, high resolution mass spectrometry (HRMS) systems have emerged and are gradually replacing more traditional systems for screening [3]. Today, there are two types of high-resolution technologies widely used: the time-of-flight (TOF) and the orbitrap, launched by ThermoFisher Scientific.

These systems allow precise mass measurements with an accuracy of less than 5 ppm. In addition, they allow full scan acquisitions and avoid the optimization of multiple reaction monitoring transitions required in LC-MS/MS and GC-MS/MS. Finally, it is easy to add new substances in the libraries and it is possible to make retrospective analyses. Furthermore, for some products, there is no information in literature especially for new compounds such as designer steroids. Moreover, in terms of doping, samples are kept for up to 10 years [4].

The misuse of performance drugs can lead to severe health problems. For example, this is the case for anabolic steroids for which their misuse can lead to severe health effects like cardiac diseases. The issues and the interest of HRMS are also stackable to clinical and forensic laboratories issues for which it is necessary to know the cause of a disease or death.

All these products are therapeutic indications but no clinical approval for all, therefore the authors focused on applications in anti-doping, forensic and clinical toxicology in humans and animals.

Method

The authors review the state of the art in the use of HRMS systems for doping products analysis. In this study, products that are prohibited only in-competition (i.e., stimulants), are not included because they will be the subject of another bibliographic review excluding glucocorticoids. Therefore, the authors are interested in the analysis of doping substances by HRMS systems and more particularly in those permanently prohibited by the WADA and glucocorticoids banned only in competition. For each family, we sought to know what existed for their analysis in the doping, clinical and forensic fields, and what place HRMS occupied. Therefore, we used open access articles on Pubmed, Web of Science or Science Direct.

2. Actual applications in anti-doping, forensic and clinical toxicology

2.1. Screening for anabolic steroids

Anabolic androgenic steroids (AAS) are a class of steroidal hormones developed from a natural and endogenous human hormone: testosterone. Testosterone is secreted by the gonads, the testes in males and the ovaries in females, in smaller quantities. Initially, they were developed for therapeutic purposes to stimulate bone growth, increase muscle mass, and induce male puberty in cases of hypogonadism. Because of their anabolic properties, AAS are used for doping purposes. Indeed, WADA has prohibited these substances and classified them as S1 class. The number of adverse analytical findings (AAFs) is more important for this class than the other class. In 2019, anabolic agents represented 44 % (1825 on 4180) of all AAFs in all sports [5]. Moreover, their easy access through the internet has attracted a larger public. Indeed, amateurs also use these products for aesthetic purposes. The misuse of these substances can lead to health problems such as cardiac arrest but also mental problems such as the increasing of violence. Therefore, the interest to identify them is multiple. S1 class includes endogenous and exogenous AAS but also other anabolic agents. These substances are cited in the non-exhaustive list of the Code [1].

The authors have reviewed the literature related to high resolution for these three groups of substances in the fields of human and animal doping, clinical and forensic. The applications described in this paragraph are only applications in biological fluids (blood, urine), and meat.

2.1.1. Exogenous AAS

In sport, top athletes misuse AAS to increase muscle mass, decrease recuperation time or to increase their aggressiveness in fight sport, i.e., boxing. To fight these misuse, anti-doping controls in urine are set up in-an out-of-competition period. Anti-doping laboratories must follow two important rules. The first is to identify all exogenous AAS (including metandienone, trenbolone, testosterone esters, steroid esters...). The prohibited list of WADA is updated every year. The second is to be able to identify them at low concentrations in the urine. These concentrations, called minimum required performance level (MRPL) are described in the WADA Technical Document - TD2019MRPL [6]. For exogenous AAS, MRPL is fixed at 5 ng/mL in urine for all AAS excluding dehydrochlormethyltestosterone, metandienone, 17<-methyltestosterone and stanozolol for which MRPL is fixed at 2 ng/mL.

Mass spectrometry is the technique of choice for the analysis of these small molecules. The triple quadrupole systems were chosen and used by several laboratories for decades. Nevertheless, these techniques have a big disadvantage. Indeed, it is a targeted approach that can only include a limited number of compounds to safeguard a good sensitivity. Therefore, laboratories are turning to HRMS to be able to add new substances in libraries and do retrospective analysis. Retrospective analysis showed its strength after the 2008 Beijing Olympics. Moreover, HRMS enables continuous scan and to detect non-target compounds compared with tandem MS methods.

Many studies in scientific literature describe the screening of doping products namely exogenous AAS in urine. Indeed, there are several articles that mention the development of screening methods for performance substances by liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) [7- 8, 10] and gas chromatography coupled to high resolution mass spectrometry (GC-HRMS) [8, 9, 11]. Concerning sample preparation, methods such as solid phase extraction (SPE) [9], dilute-and-shoot [7] and liquid-liquid extraction (LLE) [8-9, 11] are widely used.

Novel approaches are also developed to enhance the specificity and sensitivity. For example, He *et al.* analysed 13 steroids, including trenbolone, by liquid chromatography coupled to quadrupole-orbitrap mass spectrometry (LC-Q-Orbitrap) coupled with parallel reaction monitoring (PRM). This method allowed to reach limits of detection (LOD) lower than 25 times the MRPL required by the WADA and showed that the use of this coupling allowed increasing the specificity of the method [12]. Raro *et al.* compared two analytical methods: liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (LC-Q-TOF) and LC-Q-Orbitrap to study metabolomics related to the misuse of testosterone cypionate (T cypionate). Two methods gave different mass characteristics but the same conclusion and the same marker of T cypionate: 1-cyclopentenoylglycine [13].

Concerning GC-methods, the derivatization step is needed. Chemical modification with TMS is the most used derivatization and is known to improve sensitivity and selectivity. In another study performed by Cha *et al.* a

new method was presented to analyse TMS derivatives of AAS by gas chromatography coupled to linear trap quadrupole- Orbitrap (GC-LTQ-Orbitrap) . This study was applied to spiked urine and showed good sensitivity and selectivity. It could be applied to the simultaneous detection of AAS [14].

Otherwise, due to the short half-life of most AAS, a great interest has been shown by anti-doping laboratories to the research of long-term metabolites in urine. For that, several laboratories developed different strategies.

The first study is about stanozolol. Stanozolol remains the most widely used AAS by athletes. It is therefore important for laboratories to increase the specificity and the detection window. In this study, Göschl *et al.* present an online SPE method coupled with LC-Q-Orbitrap. This method allows the identification of glucuronide metabolites of stanozolol until 28 days without derivatization step [15].

In another study, it has been shown that longer detection times can be achieved by focusing on the sulfated metabolites of AAS rather than the gluco-conjugates. The authors presented an analytical method capable of identifying unhydrolyzed sulfated metabolites and precursor AAS with GC-Q-TOF [16]. Another strategy consists of the use of the hydrogen isotope ratio mass spectrometry and LC or GC - high resolution/high accuracy mass spectrometry. This method was applied for the identification of metandienone and trenbolone metabolites [17-18].

Another issue is the identification of the new synthetic steroids. Some authors have developed strategies to identify designer steroids without reference material. Nielen *et al.* described an androgenic bioactivity/LC-Q-TOF mixed method to test for synthetic steroids in urine [19].

The use of AAS is also prohibited in animal sports and is closely monitored, especially in horse racing. Furthermore, the use of growth-promoting compounds (such as AAS that increase muscle mass) has been strictly prohibited in the EU since 1986 [2]. Several authors described the identification of AAS by LC-HRMS. Methods were applied in several matrices such as equine testis, bovine bile, urine and equine plasma [20-23].

On the other hand, long-term abuse of anabolic agents can lead to adverse health effects. Physical health problems have been noted in the literature: necrosis due to injections of anabolic esters [24], cardiovascular problems and even death [25]. Because of the hazardous nature of these products, forensic and clinical laboratories must test for these drugs. Screening for the analysis of AAS in forensic context was described. For example, Fabresse *et al.* developed a method in whole blood by LC-Q-Orbitrap to test for 11 AAS with limit of quantification (LOQ) at 5 ng/mL. Method was applied to two forensic cases in blood and urine [26].

2.1.2. Endogenous AAS

Endogenous AAS are derived from testosterone and are physiologically present in the human body. In view of the difficulties associated with the determination of these compounds, WADA has advised anti-doping laboratories to use the ratio testosterone/epitestosterone (T/E). T/E ratio is evaluated to assess testosterone misuse ($T/E > 4$) as dosage of testosterone is not sufficient. However, there are factors that influence these values such as inter-individual variation, so anti-doping laboratories have introduced the steroid profile assay [27]. The analysis of these substances (testosterone, epitestosterone, androsterone, etiocholanolone, dehydroepiandrosterone (DHEA)...) allows to establish the athlete's biological passport (APB) and to follow the fluctuation of their value throughout their careers. Identification and quantification of endogenous AAS is also an indirect approach to detect exogenous AAS use. Indeed, it has been shown that the administration of exogenous AAS can suppress the excretion of endogenous AAS [28]. Moreover, these substances are also consumed exogenously. The analysis of the steroid profile is therefore important for the follow-up of the APB, to detect new products such as designer steroids and endogenous AAS. The screening and quantification of endogenous substances in urine has been described in GC-HRMS [29-30] and LC-Q-Orbitrap [31].

Abushareeda *et al.* presented in 2017 a comparison between GC-Q-TOF and GC-MS/MS. They showed that methods of analysis were similar in terms of LOD [29]. In a second study of the same group two types of GC-HRMS are evaluated and compared: Q-Orbitrap and Q-TOF systems [30]. The Q-Orbitrap generally produces higher resolution spectra at a lower scan rate and higher dynamic range. This study showed very good results for both systems (low LOQ, below MRPL). Nevertheless, the Q-Orbitrap presented better mass accuracies due to its higher resolution and less matrix effect [29]. The use of full scan HRMS allows to decrease the background noise coming from the urine matrix, to increase the resolution power and the mass accuracy.

While anti-doping laboratories perform routine analyses on urine, serum could be an alternative because it is less prone to manipulation or contamination. The use of serum has been described by Elmongy *et al.* using LC-Q-Orbitrap for which LOQ ranged from 0.006 and 7.904 ng/mL for non-conjugated, and gluco- and sulfato-conjugated endogenous AAS [32]. Ponzetto *et al.* also described a quantitative method in serum for 5 endogenous AAS using LC-LTQ-Orbitrap with targeted selected ion monitoring (t-SIM) mode and presented lower LOQ ranging from 50 to 1000 pg/mL [27].

The analysis of these substances also has clinical relevance. Indeed, the measurement of steroid hormones including endogenous AAS can help in the diagnosis of endocrine disorders (hirsutism, infertility...) [33]. For example, Kaabia *et al.* have developed a comprehensive steroid profile in urine using SPE and LLE steps followed by analysis with LC-Q-Orbitrap with LOQ at 0.01 ng/mL [31].

In addition, an alteration in endogenous AAS (DHEA, testosterone, androstenedione) levels may be caused by exposure to environmental pollutants such as dioxin, which appears to cause endocrine disruption. In this respect endogenous AAS could be analysed to indirectly test exposure to dioxin.

Two studies have described analysis in human serum and breast milk for testing testosterone and DHEA by GC-HRMS [34-35]. In the first study described by Dong *et al.* in 2020 [34], the authors showed that the perinatal exposure to dioxins results in a change in steroidogenesis. The results of their study suggested that an increase of dioxins in breast milk was associated with a reduction in testosterone levels in boys' serum samples and progesterone levels in girls.

2.1.3. Screening for other anabolic steroids

Class S1 also includes other anabolic agents classified as S1.2 by the WADA [1]. The following molecules are included in this class: clenbuterol, tibolone, zeranol, zilpaterol and selective androgen modulator receptors (SARMs). MRPL is fixed at 2 ng/mL for all compounds of this class excluding clenbuterol for which MRPL is at 5 ng/mL [6]. Tibolone and zilpaterol were added to the prohibited list in 2005 and SARMs in 2008. Clenbuterol is a β -2 agonist and will be discussed in section 2.3.

SARMs are the new anabolic steroids and are considered as designer steroids. They are derivatives of AAS and of bicalutamide, an anti-androgen. Due to their anabolic properties, they are misused by top athletes and amateurs to enhance physical performance (fat burning, increase muscle mass). Anti-doping laboratories have developed analytical methods for testing SARMs in urine. Stacchini *et al.* presented a method to test for SARMs in urine. The big challenge was to analyse all SARMs simultaneously because there are many different structures [36]. In another study, Thevis *et al.* presented the search of mass characteristics for YK-11, a steroidal SARM; HRMS is used as a tool of elucidation of mass characterisation [37]. Other publications related to SARMs and HRMS concern the research and characterisation of metabolites *in vivo* and *in vitro*. This part will be the subject of the following paragraph (section 3.1.).

Only one article was published for testing zilpaterol in a screening for β -agonists (including clenbuterol). Method was applied in feed and animal body fluid and tissues with LC-Q-Orbitrap but no zilpaterol was found in biological or feed samples [38].

Tibolone is a synthetic steroid which mimics the activity of estrogen and progesterone. Only Son *et al.* have presented a study about the pharmacokinetic of tibolone and have shown that the use of GC-HRMS enabled to detect 3α -hydroxytibolone and 3β -hydroxytibolone, collected at 15 and 24h after oral administration which was not detected by GC-MS [39]. In the literature, there is no application in HRMS for zilpaterol.

2.2. Screening for peptides hormones, growth factors, related substances, and mimetics

In recent years, peptide hormones have gained an important role in pharmaceutical research. Although these drugs have been developed for specific therapeutic uses, some of them can be used to improve performance in both human and animal sport. Hence, the list of substances banned by the WADA nowadays also includes substances of higher molecular weight described under the section S2: "Peptides hormones, growth factors and related substances" [1].

Advances in molecular biology, particularly DNA recombination techniques, have led to a misuse of peptide hormones and related substances. This has challenged doping control laboratories, which have found themselves developing methods to identify and quantify peptides that may be structurally identical to their endogenous counterparts or differ slightly in their peptide sequence or glycosylation pattern.

These substances may be either of natural origin, when extracted and purified from human tissues or fluids, or produced by DNA recombination techniques.

Additional difficulties are related to the identification of peptide hormones whose release is stimulated by other substances, and which often have a very short half-life that greatly reduces the time to detect them in body fluids [40-41].

Another issue is the misuse of peptides purchased on the black market. As these peptides were originally synthesized for therapeutic purposes, their use by healthy individuals may lead to adverse effects. Some of these preparations are not clinically approved and others are simply of poor quality [42].

To enforce the ban on these substances in the athlete's world and given the health risk they pose, it was necessary to implement identification and quantification procedures using sensitive methods which can identify proteins in picomolar concentrations with unequivocal specificity.

While the analysis of low molecular weight compounds has been successfully carried out using methods based on mass spectrometry, progress is starting to be made for peptides [41].

However, the identification and quantification of peptides is in most cases still carried out by means of immunological tests developed primarily for clinical purposes [43-44]. These tests differ in the instrumentation used, the specificity of the antibodies and the quality of the standards.

These variations lead to a high variability in the results of the tests, which triggers the need to develop uniform analytical procedures.

Several methods for the analysis of various matrices such as plasma, serum and urine have been developed to identify and quantify numerous drugs of peptide nature. These substances represent a considerable challenge for doping control laboratories with regard to the ability to distinguish the synthetic substance from the natural analogue as well as the ability to provide the analytical sensitivity required to reveal pharmacologically relevant concentrations in control samples.

HRMS allows peptides and proteins with high peptide sequence homology to be distinguished by its ability to measure very small mass differences.

This section of the paper reviews the HRMS methodologies used for the identification of peptide hormones and their releasing factors described in section S2 of the WADA Prohibited Substances List (Table 1).

2.2.1 Erythropoietins and agents affecting erythropoiesis

The first category in section S2 concerns erythropoietins and agents affecting erythropoiesis.

Erythropoietin (EPO) is a glycoprotein hormone that regulates the production of red blood cells, whose release is stimulated by a reduction in oxygen in the blood. Since the 1980s, the development of synthetic recombinant EPO (rHuEPO) for therapeutic purposes has allowed the treatment of various types of anaemia as well as renal and tumour diseases. Because of their ability to increase oxygen transport in the blood and thus increase muscle oxygenation, they have been misused by athletes, especially to boost their physical endurance.

The high similarity between human and equine EPO (84 % identical at the protein level) has also led to the use of these substances in horse doping [45].

Detection methods for these substances are mainly based on immunological methods such as enzyme-linked immunosorbent assay (ELISA), electrophoresis and western blotting [46-49].

As the use of these substances implies an increase in certain blood parameters, such as hematocrit, erythrocyte count and hemoglobin, the dosage of the latter is used to indirectly trace the administration of an erythropoiesis-stimulating agent.

Nevertheless, the methods need high sensitivity and specificity. For this purpose, several methods using HRMS have been described and applied to both humans and animals [45, 50-54].

In 2017 Joré *et al.* [45] described a metabolomic approach to prove CERA (Continuous erythropoietin receptor activator) intake in horses 74 days after administration.

The authors' hypothesis was that most ESAs (Erythropoiesis-stimulating agents) have an effect on the metabolome, and the study of the variations in the metabolome following the intake of ESAs could prove their use. After administration of CERA on horses, the latter was analysed by a LC-Q-TOF, following protein precipitation of the plasma and filtration and dilution of urine. This technique enabled the detection of CERA up to 31 days after administration, and using urine and plasma metabolomic fingerprints, it was possible to discriminate between pre- and post-CERA administration samples up to 74 days (43 days longer than with an ELISA method). A new generation of ESAs is represented by EPO-mimetic peptides, of which peginesatide is the major representative. This protein has a completely different structure compared to EPO but a high affinity for the EPO receptor.

Since it is structurally different from human EPO, its detection would not be compatible with classical EPO detection tests using anti-EPO antibodies that would not recognize peginesatide [40].

Knowing that conventional doping control assays for EPO will fail in detecting peginesatide, a sensitive assay was developed by Möller *et al.* [54]. A pegylated EPO-mimetic peptide was synthesised as a model compound and based on the latter, a detection method employing an enzymatic digestion with subtilisin and a mass spectrometric detection of a pentapeptide fragment was developed.

A LOD of 1 ng/mL was accomplished using a LC-Q-Orbitrap instrument with a small volume of plasma (100 µL).

2.2.2 Peptide hormones and their releasing factors

2.2.2.1 Chorionic gonadotropin (CG) and luteinizing hormone (LH) and their releasing factors in males

In the second category of section S2 of the substances prohibited in sport (for males only), one of the representatives is the human chorionic gonadotropin (hCG).

hCG is a peptide hormone produced during pregnancy by the placenta. Consequently, it is not normally present in healthy subjects, except in certain tumour diseases. Its use for indirect androgenic doping is well known, since like other hormones such as luteinising hormone (LH), it acts on the pituitary gland by inducing the release of testosterone by testicular Leydig cells [41]. hCG is composed of two subunits: alpha and beta. The alpha subunit is common to many hormones such as the thyroid stimulating hormone (TSH) and LH, while the beta subunit is characteristic only of hCG. Having highly homologous primary structures, hCG and LH bind to the same receptor [55].

The recombinant version of the LH hormone (rLH) has recently come available on the market, but it is not investigated in the anti-doping world because injections of high doses of rLH results in negligible levels of LH. Unlike the latter, hCG has a longer half-life and a single dose of its recombinant form causes a high stimulation of testosterone levels in blood and urine [41].

hCG is commonly determined in urine by immunoassays, but recently, methods involving tandem mass spectrometry analysis following an immunopurification step have also been used [56-57].

The post-translational glycosylations which exist in the recombinant version of the LH hormone (rhCG) results in the presence of residues which are not present in endogenous hCG, making it useful to test an exogenous administration of hCG. [41].

WADA requires a specific test in which intact hCG is detected. Recently, a method based on the analysis of hCG glycoforms at the intact level by nanoLC- Q-TOF was presented [55].

An improvement of this method has recently been described by Al Matari *et al.* [58] which allowed for the first time the identification of 33 glycoforms of hCG beta at the intact level and 42 glycoforms of the alpha subunit. This method involves a pre-concentration step on the trapping column to increase the injected volume and an acidification of the mobile phase to promote ionisation.

The developed method also allows its use for the semi-quantification of these isoforms. The LOQ, determined as a function of the overall concentration of rhCG, has been defined at 12.5 µg/mL for the alpha subunit and 25 µg/mL for the beta subunit and is proposed by the authors for rapid quality control to detect and compare the glycoforms present in recombinant synthetic hCG.

The secretion of gonadotropins such as LH and the follicle stimulating hormone (FSH) is regulated by GnRH (Gonadotropin-Releasing Hormone), a decapeptide secreted by the hypothalamus which controls the secretion of gonadotropins by the pituitary gland. Synthetic GnRH agonists are now used in the form of long-acting depot injections to treat prostate cancer.

Although the long-acting form would be deleterious to athletic performance, administration for short periods triggers a temporary increase in serum of LH and testosterone levels that persists for 5-10 days [41]. The dosage of GnRH analogues is performed by means of immunological tests, but these are not applicable for anti-doping detection due to their lack of specificity [41]. Given the low concentrations to be found in complex biological matrices, sensitive and specific methods are required.

Several mass spectrometry-based methods have been reported for the identification of GnRH and its agonists [59-60].

In 2020, a method is reported [61] that allows the detection of 40 bioactive peptides including GnRH analogues at low pg/mL level in horse urine using LC-Q-Orbitrap. Analytes are extracted from urine by mixed-mode cation exchange SPE cartridges. In fact, most methods use SPE technology as sample clean-up, as the polar nature of these peptides does not allow for LLE. The assay allowed for LODs between 5 and 10 pg/mL.

A second approach is reported by Judák *et al.* in 2017 [62], which involves a method based on simple dilute-and-shoot of urine prior to analysis by a LC-Q-Orbitrap system. As there is no pre-clean-up, nor initial sample concentration, the ability to identify small concentrations depends on the efficiency of ionization. The addition of DMSO reagent to the mobile phase enhances peptide ionization and allows applying a dilute-and-shoot strategy, making this method advantageous in time and cost-saving by the omission of the sample preparation step.

The applicability of the method was demonstrated by the analysis of urine from patients treated with desmopressin and leuprolide, the presence of which was demonstrated in all the urines analysed.

2.2.2.2 Corticotropins and their releasing factors

In elite sport “Corticotropins and their releasing factors” are also banned by the WADA.

Corticotropin releasing hormone (CRH) is a peptide hormone with a molecular weight of approximately 4.7 kDa composed of 41 amino acids. This hormone is released by the hypothalamus as an adaptive response to stress. CRH in turn promotes gene transcription and the synthesis of the precursor of the adrenocorticotrophic hormone (ACTH), which in turn stimulates the adrenal gland to produce glucocorticoids such as cortisone and cortisol [63].

CRH and analogues are commonly analysed by immunological analysis, in particular radio-immunological analysis (RIA).

In a 2017 study [63], a method for identifying CRH in plasma and serum using a nanoLC-Q-Orbitrap was presented. Sample preparation involved immunopurification followed by analysis in HRMS.

The accomplished LOD was 200 pg/mL and allowed for detection in plasma of 3 patients after intravenous administration of 100 µg of CRH finding concentrations between 7.6 and 18.9 ng/mL.

2.2.2.3 Growth Hormone (GH), its fragments and releasing factors

One of the first-generation peptide hormones that has received the most attention in terms of efforts by anti-doping authorities is the human growth hormone (hGH).

hGH is a polypeptide hormone consisting of 190 amino acids and secreted by the pituitary gland. It is present in two isoforms: a 20 kDa isoform and a 22 kDa isoform.

The 22 kDa isoform is the most abundant and is responsible for most of hGH biological activity. The development of molecular biology technologies has led to large-scale production of recombinant growth hormone (rhGH) to treat mainly hGH deficiencies in humans. Because of its properties as a promoter of muscle and bone growth and its ability to accelerate healing processes, it is used not only as a doping substance in human and animal sports, but also to increase meat production in farms.

Consequently, various analytical methods have been developed in order to demonstrate short- and long-term rhGH abuse. The identification of rhGH is carried out by means of two methods. The first is an immunological method which measures the two main isoforms of hGH. As the synthetic version presents only the 22 kDa isoform, exogenous administration of hGH would lead to an increase in the ratio of the two isoforms. The second approach is based on the dosage of two biomarkers: insulin-like growth factor I (IGF-I) and procollagen III amino-terminal propeptide (P-III-NP).

However, the increase in these markers proves manipulation of the hypothalamic-pituitary axis regardless of the substance used [41, 64].

Over the last decade, various methods have been developed in MS and HRMS [64-66].

In 2016, a method has been described [65] in which three recombinant GHs of different species (human, porcine and equine) are simultaneously identified using a SPE method following precipitation with ammonium sulphate and proteolysis by trypsin. The proteotypic peptides obtained were then analysed by a nanoLC-Q-Orbitrap. The fully validated method resulted in LODs ranging from 0.5 to 1.25 ng/mL.

Some authors consider that in order to highlight the long-term effect of a hormone treatment, it is necessary to evaluate the metabolic changes associated with the administration of a given hormone. In some studies, a metabolomic approach is used to measure the global biological effect following GH treatment. Traditionally, nuclear magnetic resonance (NMR) has been used for this purpose, but recent advances in mass spectrometry have contributed to the expansion of its use. HRMS allows detection of low concentrations and accurate mass measurement for efficient subsequent structural elucidation.

HRMS methods have been developed and applied to animal matrices mainly to highlight metabolome changes after hGH administration [64, 66].

The release of GH is regulated by the growth hormone-releasing hormone (GHRH). This hypothalamic hormone of 44 amino acids has an enhancing effect on the physical performance of athletes and is therefore included in the S2 section of substances prohibited by WADA along with its analogues.

The problems associated with its analysis relate to its instability in matrices such as blood and urine and the very low concentrations at which it is present (around pg/mL).

LC-MS is the preferred technique for the analysis of GHRH analogues in doping control but requires concentration and immunopurification steps of the sample [67-72].

In a recently published study [67], GHRH analogues are analysed after immunopurification of human urine using magnetic beads and subsequent analysis with a LC-Q-Orbitrap system. The method provides a LOD of 200 pg/mL.

In 2015, Knoop *et al.* [68] developed a method involving immunoaffinity purification using multichannel pipettes equipped with protein A/G mass spectrometric immunoassay (MSIA)-Tips and conditioned with polyclonal anti-GHRH antibodies and subsequent LC-Q-Orbitrap analysis for the identification of 4 GHRH analogues (sermorelin, CJC-1293, CJC-1295 and tesamorelin).

The method not only provided a better LOD than the previous method (< 50 pg/mL), but also allowed the identification of intact GHRH analogues up to 4 hours after intravenous administration.

Other substances included in the same section are the ghrelin receptor agonists to which the growth hormone secretagogues (GHS) and growth hormone releasing peptides (GHRPs) belong. These substances were mainly developed for the treatment of growth hormone deficiency [73]. Because they stimulate GH secretion, they are considerably abused in sport, as evidenced by the 26 AAFs reported in 2019 for GHRPs and GHS by the WADA [5] and for this reason there is a need to develop and improve analytical methods for these substances.

The identification and quantification of these substances is carried out by anti-doping laboratories using both matrices such as blood and urine and alternative matrices such as dried blood spots (DBS) as a preliminary analysis. Subsequently, either 'dilute-and-shoot' approaches or purification and concentration using SPE prior to mass spectrometry analysis are used.

In order to improve the detection of this type of substances by offering a wider detection window, particular attention has been given to the detection of metabolites. This part is discussed in section 3.1.

2.2.3. Growth factors and growth factor modulators

Due to the short half-life of hGH, its measurement after exogenous administration results in a narrow detection window. For this reason, WADA has implemented tests for hGH biomarkers to increase the detection window.

IGF-I is the primary mediator of the effects of GH. An increase in IGF-I corresponds to an increase in GH levels. Its detection in serum is carried out in anti-doping laboratories, due to its direct use either as an anabolic or as a marker of GH use, since serum concentrations remain elevated for several days after hGH intake, and in the clinical field for the follow-up of patients with hypothalamic-pituitary growth hormone axis disorders. The measurement of IGF-I is based both on immunological approaches and more often on mass spectrometry [41]. However, immunological methods require rather demanding standardisation between different anti-doping laboratories, thus mass spectrometric methods are preferred. The latter can use two types of approach, the bottom-up, approved by WADA, and the top-down. The bottom-up approach involves enzymatic digestion using trypsin and quantification of the peptides obtained. However, this approach is very laborious, time-consuming and induces high analytical variability between laboratories [74]. Due to the relatively low molecular weight (7.6 kDa) and the improved performance of the recent mass spectrometers, tryptic digestion has become an unnecessary step. For this reason, most laboratories prefer the top-down approach where the protein is found intact. Several top-down approaches have been developed in different matrices [74-76]. Recently, Coppieters *et al.* [74] developed a method for the quantification of intact IGF-I in serum prior to analysis by LC-Q-Orbitrap.

Detection of intact protein in serum prior to mass spectrometry analysis requires sample preparation, in this case by precipitation combined with SPE. This type of preparation minimises the workload and provides a method that can be used for anti-doping or clinical purposes to handle the large sample numbers.

Considering that IGF-I is found in the bloodstream at fairly high concentrations (> 100 ng/mL), the method developed provides an interesting LOQ of 50 ng/mL.

Since peptide drugs reach low concentrations in blood and urine, information about their metabolism may increase their window of detection.

However, there are some peptide hormones such as TB-500 that are not approved and for which there are no excretion studies in humans. TB-500 is a peptide isolated in 1981 from the thymus with an anabolic effect, as it regulates actin synthesis in muscle. It has often been used in racehorses where it has been shown to increase physical performance, but it has not been tested on humans [40]. As WADA prohibits any type of substance that affects muscle growth, this substance is nevertheless included in the S2 section of prohibited substances. For this type of product, blood tests are the only proof of intake. Several methods have been developed for this purpose in low resolution mass spectrometry and some also in HRMS. Notably, Esposito *et al.* [77] developed a HRMS method involving extraction by precipitation followed by SPE for blood and SPE only for urine. HRMS was used only for the identification of the intact molecule, and later also for the identification of 5 metabolites produced by TB-500 following incubation with HLM [78].

2.3. Screening for β -2 agonist

β ₂-adrenergic agonists are among the oldest therapeutic agents most prescribed for the treatment of asthma but also for numerous respiratory diseases such as the chronic obstructive pulmonary disease due to their bronchodilator activity. Their action is mediated by binding β ₂-adrenergic receptors which are present in airway smooth muscle. β ₂-adrenergic agonists are derivatives of the phenyl- β -ethanol amines with different substituents in the aromatic ring or in the terminal amine group.

They exist in two pharmaceutical classes: short-acting- β ₂-agonists and long-acting- β ₂-agonists. β ₂-agonists belong to a class of drugs that is widely abused both as a therapeutic agent and for their possible anabolic effects for doping purposes [79].

WADA has prohibited the use of this class of drugs and set MRPL for β ₂-agonists at 20 ng/mL in urine, except for formoterol and salbutamol for which a finding of their presence is considered an AAF if their concentration exceeds 1000 ng/mL for salbutamol and 40 ng/mL for formoterol [1, 80].

In 2019, WADA reported 153 cases of AAFs for β ₂-agonists, with terbutaline accounting for 52 % of its group and being the most abused [5].

Possible anabolic effects in humans are linked to their ability to cause fatty mass loss through acceleration of the fatty acid degradation process and inhibition of protein degradation, which would contribute to muscle mass gain.

Due to their ability to redistribute nutrients, these drugs are used illicitly in the livestock industry to reduce fat mass and increase muscle mass in farm animals such as pigs and ruminants [81].

Recent studies have also shown an increased risk of unintentional doping following the consumption of meat contaminated mainly with clenbuterol [82].

In addition, β ₂-agonists have several adverse effects when consumed orally, in particular irritation of the throat, cardiovascular effects measurable in terms of increased blood pressure and pulse.

In order to control the illicit use of these drugs, several methods have been developed for the identification and quantification.

Concerning sample preparation, methods such as SPE and purification using immunoaffinity cartridges (IAC) are quite efficient and widely used.

Regarding analytical methods, RIA and enzymatic tests have been developed and are still used today. Different types of biosensors have also been developed as an alternative approach in livestock meat.

These sensors usually contain an antibody that interacts with the analyte. The binding is then measured optically and converted into an electronic signal. This method allows multiple residues to be identified in several samples simultaneously.

However, the sensitivity of these tests is lacking and is often not compatible with the very low levels required in urine and tissue.

For this purpose, various methods have been developed in GC or LC/MS often with high-resolution configurations that have been found to be the most powerful approaches to measure β_2 -agonists [79].

Recently, HRMS has received more attention for their sensitivity and ng/mL quantification capability.

This type of technology has proven to be very powerful for screening and quantifying trace amounts of β_2 -agonists in some livestock meats and fluids to ensure food safety and public health [81, 83-85].

Using one of the latest HRMS technologies (Q-Orbitrap), Li *et al.* [81] achieved a LOD of 0.02-1.2 ng/mL and 0.07-12.85 $\mu\text{g}/\text{kg}$ for solids.

Although the use of β_2 -agonists is banned in some countries such as China and Europe, they are still abused by some breeders, illegally.

Guo *et al.* [83] in 2015 developed a method using the same technology to detect β_2 -agonist residues in some farm meat samples which tested positive for salbutamol, clenbuterol, ractopamine and chlorprenaline. The method exhibited a LOD in the range of 0.0033-0.01 g/ kg.

Given the short half-life of these drugs, some studies have exploited HRMS technology for the determination of metabolites and degradation products [86-87].

Georgakopoulos *et al.* [86] performed a study of reprotoleol excretion in equine urine. Following administration of 20 mg of reprotoleol in a horse, only the main metabolite is detected in the urine but not the parent drug. Using gas chromatography coupled to a reverse geometry double focusing mass spectrometer (GC-HRMS), the presence of the metabolite was confirmed after accurate measurement of diagnostic ions.

2.4. Screening for hormone and metabolic modulators

According to the WADA list of the prohibited substances in sports, the hormone section is composed of aromatase inhibitors, anti-estrogens and selective estrogen receptor modulators (SERMs) and agents

preventing activin receptor IIB activation. With metabolic modulators (adenosine monophosphate-activated protein kinase (AMPK), peroxisome proliferator-activated receptors (PPAR- δ), insulins, and insulin-mimetics, meldonium and trimetazidine) they constitute the S4 class of prohibited substances [1].

2.4.1. Hormone

Hormones count numerous compounds with various masses, properties, and metabolism. HRMS has been used in various fields for different goals. Kollmeier *et al.* reported LC-Q-TOF use for structural confirmation of fragments ions of formestane and its fragment ion pathway elucidation [88].

In biological matrices, HRMS had demonstrated its interest to formally identify compounds, as in case of agents preventing activin receptor IIB activation, for which differentiation from endogenous antibodies consists of a detection challenge. In a first analytical step, this kind of compounds have to be reduced to be observed as their molecular weight is too high. In case of follistatin, bimagrumab and domagrozumab, tryptic digestion was required before analysis [89-91]. Then, using LC-HRMS or nanoLC-HRMS, two or three peptides related to the hormone were monitored to formally identify the compounds. In case of bimagrumab, addition of ion mobility as orthogonal separation technique has facilitated unambiguous detection of the hormone. For a more sensitive detection and to facilitate isolation of compound from serum, combination of immunoaffinity purification using antigen coated magnetic beads, tryptic digestion and detection by HRMS was performed.

With the accurate mass data, HRMS was also used in metabolism studies. Mainly in urine, as it is the major matrix used in doping control, metabolism studies were conducted for several compounds with HRMS such as tamoxifen [92-93], toremifene [92-93], cyclofenil [94], clomiphene [93, 95], arimistane [96]. Most of these studies were performed in human matrices and some in animals such as horses [94]. Dahmane *et al.* reported identification of tamoxifen metabolites in serum in 20 clinical samples [97]. Instrumentations used were Q-Orbitrap [92] or Q-TOF [93, 95-96] coupled to liquid [92-93, 95] or gas [96] chromatography. In all cases, spectrometric accurate information was obtained, offering the possibility to identify new metabolites, which could be potential biomarkers for monitoring compound administration in matrices.

To highlight the presence of hormones in real biological samples, HRMS were already largely used for its high sensitivity. First, in a qualitative way as reported by Ameline *et al.* who used LC-Q-TOF as confirmation technique to highlight presence of letrozole in hair samples (LOD 1 pg/mg) [98]. Thus, in a quantitative way, as Favretto *et al.* who demonstrated that LC- LTQ-Orbitrap was recommended to detect weak concentrations of the same compound in hair samples (LOD 5 pg/mg) and to discriminate a chronic use to an occasional administration [99]. In both cases, samples were analysed in the context of doping. Fabresse *et al.* reported a validated LC-Q-Orbitrap method for numerous compounds applied to whole blood and hair

samples [26]. Tamoxifen LOD of 1 ng/mL and 10 pg/mg respectively in blood and hair allowed to highlight it in two forensic *postmortem* cases. Deaths were not related to tamoxifen, but doping products were expected and confirmed thanks to HRMS.

2.4.2. Metabolic modulators

AMPK activators and PPAR- δ agonists activate the transcriptional receptors which are involved in processes enhancing aerobic capacity and increasing mitochondrial genesis. Administration of this kind of substances could improve physical performances and endurance. Thus, these compounds are monitored by doping labs. The endogenous substance 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside (AICAR) is an AMPK activator. This polar compound was studied by HRMS after dilute-and-shoot injection whatever the biological matrix plasma [100] or urine [100-101]. As AICAR is an endogenous substance, a reliable quantification is required to distinguish between endogenous concentrations and illicit application. Görgens *et al.* validated LOD inferior to 10 ng/mL in urine using LC-Q-Orbitrap and a HILIC column [101], whereas Kwok *et al.* reported LOD of 2.1 ng/mL and 13.7 ng/mL in plasma and urine, respectively, using LC-Q-Orbitrap [100].

Further applications of this family of substances concern the study of *in vitro* and *in vivo* metabolism which will be described in section 3.1.

2.4.3. Insulins

Human insulin is an endogenous anabolic hormone essential to regulate carbohydrate metabolism, leading to regulation of blood glucose level. Insulin therapy is required for type 1 and many type 2 diabetic patients. Two types of insulin analogues were synthesised according to onset and half-life: the rapid-acting analogues such as aspart, glulisine and lispro; and the long-acting analogues such as glargine, detemir and degludec. In addition to use in a medical context, insulins were misused in years following their commercialization as in doping to provide energy reserves as glycogen and to decelerate catabolism [102-103]. Therefore, use of insulins in sports has been forbidden since 1999 [103]. The MRPL is 50 pg/mL in human urine [6]. Moreover, insulins have also been used for suicidal or criminal purposes. Identification and quantification of insulins are complex in biological matrices. In doping the most analysed matrix is urine. However, as insulins are reabsorbed at 99% by proximal tubular cells, a small amount of protein is urinary excreted. In case of blood analysis (doping/clinical/forensic context), insulins undergo a rapid degradation in presence of hemolysis which requires a specific sample management and leads to low concentrations.

To formally identify insulins, the use of mass spectrometry is required as only few amino acids modifications exist between human insulin and their analogues. Last years, development of insulin analysis by mass spectrometry has extended, particularly in doping. Recently, reviews of literature dedicated to insulins

presented the used technologies to analyse these compounds: quadrupole, orbitrap or TOF [102, 104]. The limitation of TOF systems is their limited resolution generally around 40,000 FWHM, whereas Orbitrap systems could rise resolution until > 400,000 FWHM. This point is interesting as to distinguish glulisine from aspart a minimum resolution of 400,000 FWHM is required [102]. Without this resolution, distinction could be done by comparison of product ions after fragmentation. In the same way, as the difference between lispro and human insulin is only a switch between two aminoacids, they share the same molecular mass. Thus, no distinction in full-scan or SIM mode is possible and fragmentation must be performed to distinguish the first from the second. The product ion m/z 217.118, corresponding to fragment B (y_2), is specific to lispro.

In doping, the most recent methods reach very low concentrations of insulins. Thomas *et al.* proposed insulins quantification in plasma using an LC-Q-Orbitrap method with LODs between 0.2 to 0.5 ng/mL [105] or using an LC-Q-TOF method presenting LODs between 0.1 to 0.8 ng/mL [106]. As long-acting insulins detemir and degludec have a different structure with a fatty acid group acylated to their peptic chain, LODs are generally more important for these two insulins. In urine samples, LODs of 10 pg/mL were validated for rapid-acting insulins by coupling nanoLC with a Q-Orbitrap [107] or an LTQ-Orbitrap [108]. To reach these concentrations several milliliters of samples were extracted. Concerning forensic context, a LOD was validated at 1.0 ng/mL for rapid-acting insulins (2.5 ng/mL and 10 ng/mL for degludec and detemir, respectively) in *postmortem* blood (250 μ L) by LC-Q-Orbitrap [109]. Using the same system, a qualitative method was validated in *postmortem* tissues with a LOD between 25 to 80 ng/g according to insulins and matrices (kidney, liver, muscle, injection site) [110]. Insulins were also quantified in vitreous humor (500 μ L) by an LC-Q-Orbitrap method with LOD of 0.2 ng/mL [111].

Using HRMS coupled with sample pretreatment such as immunoaffinity or immunopurification, LOD were considerably diminished. Thus, insulins are ever better detected in all contexts. Finally, HRMS offers the possibility to some authors to study insulin metabolism particularly in urine samples in doping [112].

2.4.4. Insulin mimetics

Among metabolic modulators, some compounds are considered as insulin mimetics as they mimic the action of insulins by activation of tyrosine kinase domain of the insulin-receptor or by inhibition of the protein tyrosine phosphatases [113]. Some of them were synthesised from plants or fungal extract such as the benzoquinone derivative L-783,281 [113] or generated from screening of phage display peptide libraries such as s597 [114]. These compounds are not authorized in sports because they have properties enhancing athlete's performances. Globally, HRMS have been used to identify structure of these compounds and to monitor their presence in samples in the laboratory as they are potential doping agents [114].

2.4.5. Meldonium

Meldonium was synthesised in the 1970s as a growth-promoting agent for animals. Later, the drug was approved for its medical indications such as its anti-ischemic properties [115]. It has been then misused in sports for its positive effect on the endurance performance of athletes, its enhancement of rehabilitation after exercise, its protection against stress and its activation of central nervous system functions [115]. Meldonium has been included in the WADA list of prohibited substances since 2016. MRPL is 200 ng/mL in human urine [6]. As meldonium is a highly polar compound its extraction from biological samples simultaneously with other major doping agents presenting different properties is a real challenge. However, its excretion in urine under unchanged form makes it an ideal compound for “dilute-and-shoot” analytical process. Thus, recently, a screening method of meldonium and over 300 doping agents was published. To detect meldonium at a satisfactory concentration, 10 µL of fresh urine was added to an SPE (C18) extract before injection on a LC-Q-Orbitrap, resulting in a LOD of 100 ng/mL [116]. In 2015, Görgens *et al.* reported a simple dilution of urine sample before injection on LC-MS/MS (initial testing assay) and on LC-Q-Orbitrap (confirmatory analysis). The use of HRMS allowed validation of a LOD of 10 ng/mL from 270 µL of urine sample, compared to 200 ng/mL with LC-MS/MS [115]. The disadvantage of the simple dilution is the presence of endogenous interferences which could interfere with analysis and lead to false positives as experienced by Kim *et al.* with acetylcholine [117]. However, product ions of meldonium can be separated in HRMS. Indeed, positive mode is used to analyse meldonium and the monitored ions are the precursor ion m/z 147.1128 [115-116] and the most abundant product ion m/z 58.0654 [115, 117]. A LC-Q-Orbitrap method was also used to determine urinary detection windows of meldonium for doping control purposes after single or multiple oral administration [118].

2.4.6. Trimetazidine

Trimetazidine is a coronary vasodilator and was prescribed from 1963 for the treatment of angina pectoris [125]. It has been then misused in sports for its mechanism of action, which imply a decrease on demand for oxygen when glucose is used instead of free fatty acids [119]. Thus, trimetazidine has been included in the WADA list of prohibited substances since 2014. To perform its analysis several types of urine sample pre-treatment were reported: a simple dilution [120] or a direct injection after addition of internal standard and centrifugation [119] or a SPE extraction from 5 mL of sample [121]. Sigmund *et al.* used an LTQ-Orbitrap method to measure accurate mass of trimetazidine and its urinary metabolites. The method was applied to four real samples from athletes and the authors obtained elemental composition and structure information on main trimetazidine metabolites in urine [119]. They observed that the most abundant signal was generated from the unchanged drug rather than its metabolites. Apart following trimetazidine intake, the

compound could be detected after lomerizine administration. This substance is permitted in sports. Thus, in doping laboratories it is essential to distinguish lomerizine from trimetazidine intake.

2.5. Screening for diuretics and masking agents

Masking agents modify urine excretion or induce hemodilution and thus, could be used in sports to falsify the results of doping controls. The substance class of masking agents comprises primarily diuretics but also includes probenecid and plasma expanders, as well as substances with a similar chemical structure or biological effect.

Diuretics family is composed of substances which increase urine flow and volume and decrease body mass [122]. These characteristics have for consequence to dilute residues of potential sport doping agents; thus, diuretics are included in the WADA list of prohibited substances in sports (S5 class) [1]. The MRPL is 250 ng/mL for this class of substances [6]. Giancotti *et al.* tried to elucidate the fragmentation pattern of diuretics by using Q-Orbitrap. In this class of substances, all compounds have not the same chemical properties and acid-base behavior, as amiloride which contains no acid function conversely to ethacrynic acid [123]. These differences required specific method adjustments. Indeed, intensity of amiloride was better with a positive ionization whereas intensity of ethacrynic acid, furosemide and bendroflumethiazide was better with negative ionization. This particularity was also described by Peters *et al.* [122]. Diuretics analyses have been developed by using HRMS in plasma [124], DBS [125], and urine [126], as well as in human [123, 125] and animal [124, 126] matrices. Wasfi *et al.* studied pharmacokinetic and metabolism of furosemide in camels after intravenous administration, as this substance is prohibited in camel racing. None of the expected metabolites were detected in plasma suggesting a possible urine excretion as unchanged or metabolized with a small extent [124]. Metabolism of bumetanide was also studied by using LC-Q-TOF in urine of rat and only 6 metabolites were identified [126].

Excepted plasma expanders and diuretics, desmopressin is to our best knowledge the masking agent the most studied by HRMS. Desmopressin induces haemodilution by decreasing haematocrit and haemoglobin concentration [127]. During numerous years, desmopressin was detected by immunoassay. Even if the risk of cross-reaction with its endogenous analog, vasopressin, was weak, it became mandatory to formally identify compounds in doping control. Thus, several methods were developed in HRMS to analyse specifically desmopressin in urine [125, 128-130] and in plasma [131]. LODs ranged from 50 pg/mL [128] to 500 pg/mL [130]. In order to formally identify desmopressin and to discriminate its detection from those of vasopressin, it was required to perform analysis on the reduced peptide which allowed to target specific ions [128]. With desmopressin analysis by using Q-TOF, it was demonstrated that HRMS was a reliable technique for large molecules such as peptides quantification [132].

Plasma expanders significantly increase plasmatic volume. They prevent dehydration and decrease hematocrit values to conceal erythropoietin abuse [133]. Hydroxyethyl starch (HES) and dextran are the major representative compounds of this class of substances and have been the most studied by using HRMS to this day. HRMS methods have been developed for this type of substances because with other detection techniques it was impossible in matrix to discriminate oligosaccharides and glucose from endogenous compounds. For example, in diabetic patients, concentration of glucose is often abnormally high [134]. HRMS allowed to formally distinguish the different polysaccharides. These large substances were easily fragmented and produced protonated charged fragments [135]. Nevertheless, due to their high molecular weight, these compounds produce numerous precursor ions in the source. To overcome this issue, a strategy based on in-source collision-induced dissociation (CID) has been developed [133, 135]. This technique leads to specific fragments in source that could be used to formally identify substances. More recently, Wang *et al.* observed that using the maximum energy of 100 eV in ionization source, HES and dextran induced large fragments ($> m/z 1000$) implying a weak intensity. The authors developed a technique coupling all ion fragmentation (AIF) and in quadrupole fragmentation to achieve high fragmentation efficiency (HCD). Obtained fragments were lighter ($< m/z 1100$) and peak intensity was 100 times higher. By coupling in-source CID at 100 eV and HCD fragmentation (NCE 40%), Wang *et al.* improved intensity of plasma expanders detection to reach a LOD of 3 $\mu\text{g/mL}$ for dextran in rat plasma [136].

2.6. Screening for glucocorticoids (prohibited only in competition)

Glucocorticoids are a group of low molecular weight lipophilic drugs belonging to the steroid hormone class, a category of hormones produced by the adrenal cortex under stress conditions.

These substances have an analgesic and anti-inflammatory effect and are therefore used to treat inflammatory diseases such as arthritis, asthma or rhinitis. However, in some areas they may be misused in livestock feed as growth promoters or as doping substances in the world of sport to improve athletic performance.

Thus, glucocorticoids promote gluconeogenesis by stimulating the breakdown of fatty acids and proteins and suppress the feeling of tiredness and pain [137].

These drugs have a wide range of adverse effects on the cardiovascular system, bone tissue and central nervous system, and therefore uncontrolled use of these drugs could be harmful to human health. In addition, some hormone-regulated tissues, such as the breast, ovaries and prostate, may respond with abnormal cell proliferation leading to hyperplasia [138].

Glucocorticoids are tested by a wide variety of analytical methods including immunological methods such as RIA, but also methods such as GC and LC - MS or UV.

Although immunoassays are sensitive and characterised by ease and speed of use, their disadvantages are their lack of specificity and the risk of cross-reactions with precursors or metabolites.

As confirmatory methods, GC and LC - MS are preferred. However, GC is not optimal for measuring glucocorticoids in complex biological matrices due to the lack of suitable derivative agents [139].

Due to their sensitivity and selectivity, LC-MS is considered the gold standard for the analysis of glucocorticoids in complex biological matrices.

However, glucocorticoids have to be researched in trace amounts in biological matrices due to their short half-life. For this reason, in recent years HRMS is gaining interest in research laboratories and laboratories performing routine analyses.

Herrero *et al.* in 2014 [140] compared triple quadrupole mass spectrometry and orbitrap for the determination of 9 glucocorticoids in complex matrices such as wastewater and obtained a good correlation between the two methods. However, HRMS prevented false results due to full-scan acquisition, so that interferences from the matrix can be identified and evaluated. Parameters such as LOD and LOQ proved to be similar. For some analytes, LOD and LOQ were even better using the HRMS. The methods developed provided a LOD of 5 pg/mL and a LOQ of 20 ng/mL for most of the substances researched.

Because of their abuse in the field of sport, WADA has published a list of substances belonging to this class that are prohibited [1].

Due to their short half-life, finding these substances in blood and urine samples is very difficult and if detected they are found in very low concentrations.

For this purpose, various methods have been developed for the identification and dosage of these substances in serum, plasma and urine samples using high-resolution analysers [141-145].

The preferred extraction method for serum samples is generally LLE using MTBE as extraction solvent. Some authors using Q-TOF as detection systems have obtained LOQs of 5 ng/mL for molecules such as prednisone and prednisolone [142], while others using Q-Orbitrap have achieved LOQs of less than 1 ng/mL for substances such as cortisone, corticosterone and cortisol [141].

The misuse of these substances in farmed meat has raised several fears about the presence of steroid hormones in foods of animal origin.

To ensure the safety and health of consumers, it has been necessary to develop methods capable of identifying both these substances and their degradation products in the meat of various animals consumed by humans.

For the qualitative and quantitative determination of these substances, GC and LC-MS are the most widely used. However, due to the difficulties associated with the derivatization reaction, LC has become more popular for the analysis of these compounds.

The need to search for traces has led in recent years to the expanded use of HRMS, which not only allows retrospective data analysis but also the identification of residues of these substances at very low concentrations.

Lopez *et al.* [138] developed a method based on a LC-Q-Orbitrap for the determination of steroid hormones and their metabolites in three types of animal meat. Using the QuEChERS extraction method, good results and recovery were obtained. The authors achieved LOQs ranging from 1.0 to 2.0 µg/kg. To prove the suitability of the method, it was applied to samples of farm animal meat to see if there was any misuse of these substances. Two steroids were found in two different meat samples. The development of sensitive methods provides information on the misuse of these substances and since most glucocorticoids have many fragments in common, this can be used to identify new compounds not present in the databases.

In fact, the ban on the use of glucocorticoids for non-medical purposes has led to the search for counterfeit products on the black market that pose a risk to public health [146-147].

Another important analytical challenge in the anti-doping field is the efficient separation of isomers which are often the cause of legal arguments

In this respect, HRMS allows the discrimination of each isomer at very low concentrations from complex biological matrices [148-150].

Karatt *et al.* [148] have discussed the issue of three glucocorticoids: dexamethasone, betamethasone and paramethasone. The first two are mainly used to treat metabolic and inflammatory diseases, while the last one is used to treat asthmatic diseases and allergies. The similarity of these molecules also results in similar behaviour at chromatographic level, which is the reason for the difficulty in separating the isomers using a classical chromatographic system.

In the study, they were able to distinguish these isomers on the fragmentation pattern of each isomer, which represents the fingerprint of a molecule, and the ratios between the various ions formed, using a LC-Q-Orbitrap.

The fragmentation pattern they proposed was then applied to complex matrices such as blood and horse urine, treated with LLE for the first one and SPE for the second.

The authors have demonstrated that they can distinguish these co-eluting isomers even in samples in which all three are present at very low concentrations.

3. Innovative approaches

3.1. *In vitro* and *in vivo* drug metabolism studies

The increasing variety of HRMS techniques based on soft ionization and TOF single/triple-stage or hybrid MS-instruments, gives precious information to propose or assign structures to supposedly generated metabolites. In recent years, an increasing number of research projects have been carried out focusing on the structural elucidation of presumed metabolites of doping substances using HRMS. Considering the huge amount of data produced by these contributions, only an overview of these studies is presented below. The analytical workflows used in the reviewed studies were summarized in Table 2.

In a research focused on the detection and characterization of stanozolol metabolites in human urine, Pozo *et al.* reported the application of a LC-Q-TOF method to mice and human excretion studies. A total of 19 stanozolol metabolites were detected and a possible structure for each metabolite has been proposed on the basis of the product-ion spectra with accurate TOF mass measurements [151]. Another excretion study based on the urine analysis from four volunteers receiving the aromatase inhibitor exemestane was carried out to detect new exemestane metabolites in human urine by GC-HRMS, after derivatization with methoxyamine and MSTFA/TMS-imidazole [152]. The metabolism of the stimulant benfluorex was investigated in 2010 by Thevis *et al.* LC-Q-Orbitrap experiments were carried out for the characterisation of four metabolites detected in urine samples coming from a patient treated with a single oral dose of 300 mg benfluorex [153].

In vitro models simulating human, or animal metabolic pathways were used to obtain information on drug metabolism in the early stages of clinical studies. These methodologies have been applied to the so-called new designer drugs under clinical approval.

For example, the metabolism of the drug candidate S107 (a substance tested for the treatment of cardiac arrhythmia that improves cardiac and skeletal muscle functions) was investigated using *in vitro* models, and numerous generated phase I and II metabolites were characterised by LC-LTQ-Orbitrap experiments [154]. Another class of non-approved drugs with an increasing appeal in the sports doping community is SARMs. Arylpropionamide-derived SARMs were investigated using human liver microsomal (HLM) preparations and the structures of the metabolites were identified by HRMS [155-156]. *In vitro* generated metabolites were confirmed by *in vivo* experiments, and several metabolites were selected as target analytes for sports drug testing [157]. Similarly, the metabolism of LGD-4033 (Ligandrol), a SARM with a pyrrolidiny-benzonitrile core, was extensively studied by *in vitro* metabolism experiments employing HLM and *Cunninghamella elegans* (*C.elegans*) preparations, as well as electrochemical metabolism simulations [158]. Five mono and bis-hydroxylated metabolites were identified and structurally confirmed by LC-HRMS experiments. In following studies, some of these metabolites were detected and characterised by LC-HRMS in human and

equine urine samples, after the controlled administration of LGD-4033 [159-160]. The *in vivo* metabolism of the steroidal SARM YK-11 and the anabolic steroids methylstenbolone and trenbolone was investigated by Piper *et al.* by analysing post-administration urine specimens. Several metabolites, relevant to sports drug testing, were characterised using GC-HRMS (as trimethylsilyl derivatives) and/or LC-HRMS instruments [18, 161-162]. Moreover, GC-HRMS and GC-MS/MS techniques were recently employed for detecting novel A-Ring reduced metabolites of the anabolic steroids methyltestosterone and metandienone [163].

Regarding the development of analytical methods able to detect small peptides for anti-doping purposes, the *in vitro* metabolism of GnRH was studied by HRMS [61,164]. Ten synthetic analogs of GnRH were investigated using human kidney microsomes (HKM), and the structures of the metabolites were identified by LC-Q-Orbitrap [164]. In the same study, the *in vitro* obtained metabolites were added into UPLC-MS/MS method for GnRH analogs determination, and the presence of one of these was confirmed in the real urine sample.

Several *in vitro* studies have been carried out on HLM to investigate *in vitro* metabolism of GHS and GHRPs, and various methods have been developed for this purpose, particularly using HRMS [62, 73, 165-167].

Guan *et al.* [166] describe an approach using liquid chromatography coupled to a quadrupole-orbitrap mass spectrometer, in which they highlight the interest of using a HILIC column instead of classical reversed-phase chromatography columns due to the high hydrophilicity of metabolites that are poorly retained in this type of column. For the same purpose Lange *et al.* [167] performed an *in vitro* study for metabolites of 3 ghrelin mimetics and identified 76 metabolites.

SR-9009 is a synthetic AMPK activator. HRMS was used to study its metabolism in urine [168-169]. Cutler *et al.* identified *in vitro* SR-9009 metabolites in horse plasma and urine. The metabolites were confirmed by *in vivo* analysis of the same matrices in real samples after SR-9009 administration. The authors described HRMS as an important tool to metabolism investigation [169].

PPAR- δ agonists potentially used in sports are synthetic compounds such as adiporon and 112254 for which *in vitro* and *in vivo* metabolism was studied in rat urine. Characterisation of metabolites was possible thanks to HRMS use [170]. The most studied PPAR- δ agonist by HRMS is GW1516 or GW501516. Its detection by MS is possible with a positive [171-172] and a negative mode [173]. However, Ishii *et al.* reported that addition of 10 mmol/L of glycerin in acetonitrile to the final extract could facilitate yield of [M-H]⁻. Then, using the negative ionization mode with this modification, MS intensities were 10-fold higher than those obtained in the positive mode [173]. The two major identified metabolites of GW1516 are the sulfoxide and the sulfone. In addition, metabolism study by HRMS allowed to identify seven other metabolites in horse plasma and urine [174].

Okano *et al.* developed an LC-Q-TOF method to determine lomerizine metabolites structures in urine. They concluded that trimetazidine was only detected in *in vivo* samples [121]. More recently, using a LC-Q-

Orbitrap the same team developed a very sensitive method to detect lomerizine, trimetazidine and another specific metabolite of lomerizine (M6) in human urine. From 0.5 mL, LOD and LOQ of trimetazidine were assessed at 0.02 ng/mL and 0.05 ng/mL, respectively. Their previous method without high resolution could not reach these limits. The proof of concept was performed on ten real doping cases, concluding that after lomerizine intake trimetazidine is systematically detected in association with the M6 [120].

3.2. Alternative matrices: hair and dried blood spots (DBS)

The study of alternative specimens (e.g., oral fluid, hair, exhaled breath, DBS, nails) has gained significant relevance in forensic and clinical chemistry, including sports drug testing. In the last years, among the alternative matrices, hair and DBS have received particular attention in doping analyses, showing advantages but also exhibiting limitations if compared to the currently employed routine doping control samples consisting principally of urine and blood.

Supporting urine and blood analysis, hair testing demonstrated to provide complementary information in selected cases of AAFs with banned substances, differentiating the misuse of the drug from a single inadvertent and/or accidental intake [175]. On the other hand, the speed and ease of sampling of DBS, together with its low invasiveness, intrusiveness, risk of adulteration, and low transportation and storage costs, make the DBS attractive for the application in sports drug testing [176]. Therefore, several analytical methods were recently developed for the detection of doping agents in hair and DBS, including methods based on HRMS detection. Today, HRMS able to scan all masses in a predefined m/z mass range for the duration of the analysis at high resolution without loss of sensitivity are present on the market. This makes it possible to search a very large number of compounds simultaneously and allows for retrospective analysis of compounds that were not originally included in the method.

3.2.1 Hair

One of the first applications of HRMS for the analysis of anabolic steroids in hair samples was presented by Strano-Rossi *et al.* in 2013 [177]. The procedure for the detection of 25 among anabolic steroids and their esters consisted of an overnight methanolic extraction of the hair sample (30 mg) by sonication, followed by a LC-HRMS screening using a Q-Orbitrap mass spectrometer operating in full scan mode (m/z 110–800) in APCI under positive ionization mode, with nominal resolving power of 100,000 FWHM. In case of screening positive results, a confirmation method based on in-source CID was developed, acquiring ions from 70 to 500 m/z with a resolving power of 50,000 FWHM, and obtaining the accurate masses of both precursor and fragment ions. The characteristic fragmentation of the analytes, the exact masses of the precursor and fragment ions (mass error within ± 1 ppm) together with the characteristic isotopic cluster allowed the

correct identification of the analytes. Moreover, the method was validated for quantitative purposes with LODs values in the interval 0.01-0.05 ng/mg and a LOQ of 0.5 ng/mg for all analytes. The method was successfully applied to a real-life case, resulting in the identification of testosterone undecanoate (measured accurate mass of 457.3678; mass error < 0.5 ppm) in the hair of a suspect at a 5 ng/mg concentration.

A similar study on the detection and quantification of 11 anabolic steroids in the blood (androstenedione, dihydrotestosterone, boldenone, epitestosterone, mesterolone, methandienone, nandrolone, stanozolol, norandrostenedione, testosterone, trenbolone) and eight more in hair samples (nandrolone phenylpropionate, nandrolone decanoate, testosterone propionate, testosterone benzoate, testosterone cypionate, testosterone decanoate, testosterone phenylpropionate, testosterone undecanoate) using a Q-Orbitrap mass spectrometer was published by Fabresse *et al.* in 2017 [26]. Again, the ionization was carried out with an APCI ion source operating in positive ionization mode, and the protonated molecular ions of the steroids were identified with exact mass (mass error < 3 ppm for all analytes) and retention time. Data were acquired in full scan mode over a mass range of 100–800 m/z at the maximal resolving power of 140,000 FWHM. The method was also validated for the quantification of the steroids in different matrices. LODs values in the hair matrix were in the interval 2-20 pg/mg and LOQs were between 10 and 100 pg/mg, with the higher LOD/LOQ values obtained for the esters. The method allowed the detection and quantification of testosterone (18 pg/mg), trenbolone (10 pg/mg), and nandrolone (23 pg/mg) in two *postmortem* hair samples.

Favretto *et al.* published an HRMS method for the detection and quantification of the aromatase inhibitor letrozole in hair [99]. The hair analysis was performed on an LC-LTQ-Orbitrap system with the scope of discriminating voluntary doping vs inadvertent ingestion of letrozole in a real case. The detection was carried out with the mass spectrometer operating in positive ESI mode and the mass acquisition in the range of m/z 80–600 using a target mass resolution of 60,000 FWHM and a scan time of 0.65 sec. The detection of the analytes (letrozole and its main metabolite bis(4-cyanophenyl)methanol) was based on accurate mass measurements of the protonated ions (286.1087 and 235.086), the correspondence between the observed isotopic pattern and the calculated one, and the relative retention time. Starting from 10 mg of hair sample, values of 5 pg/mg and 15 pg/mg were calculated for LOD and LOQ, respectively, for letrozole. The sensitivity of this method made possible the detection of letrozole in head hair, after a single intake of 0.62-2.5 mg of letrozole, at a 16 to 60 pg/mg concentration. No presence of letrozole metabolite was revealed in the real samples.

An analogous study concerning the detection of letrozole in hair samples by LC-MS/MS and LC-HRMS was recently published by Ameline *et al.* [98]. LC-Q-TOF technique was employed as a confirmatory method after the LC-MS/MS analysis. Letrozole was extracted from hair (20 mg) after 1 h methanolic extraction at room temperature and solvent evaporation. The HRMS system consisted of a Q-TOF mass spectrometer operating

in ESI positive ion mode. The data were acquired in full-scan mode from m/z 50 to 1000, at 6 eV, while for MS/MS fragmentation of target ions, collision energy ranging from 10 to 40 V was applied. Letrozole was correctly identified with a mass error less than 5 ppm from its exact mass. The combination of two independent methods for the detection of a substance (LC-MS/MS and LC-HRMS) is a requested requirement by the doping authorities to consider a valid hair test, as WADA accredited laboratories do the same for the confirmation of any adverse analytical findings.

Following this approach, several doping real-case studies regarding the application of the LC-HRMS as a confirmation method after the LC/MS-MS detection in the hair of stanozolol [178], ostarine [179] and cardarine [171] were presented by Kintz *et al.* In all studies, the HRMS detection was based on a Q-TOF instrument. Conditions for the TOF spectrometer scan mode were as follows: scan range m/z 50-1000 at 6 eV (low energy) and m/z 50-1000 with a collision energy ramp from 10-40 eV (high energy). Stanozolol was detected after that 30 mg of decontaminated hair were incubated in 1 mL of 1M NaOH for 10 min at 95°C and extracted with diethyl ether. The presence of stanozolol (73 pg/mg) was confirmed in real head hair samples detecting the mass at m/z 329.2580, with a mass error of - 2.2 ppm, when compared to a reference standard (m/z 329.2588). The presence of the SARM ostarine (S22 or MK-2866) was confirmed in real hair samples after incubation of 50 mg of the matrix in a pH 9.5 buffer, followed by extraction with organic solvents. The detection was carried out with the mass spectrometer operating in negative ESI mode. The correct identification of the analyte was achieved by monitoring the exact masses of the deprotonated molecule at m/z 388.0909 together with three diagnostic ions at m/z 269.0536, 118.0294, and 185.0323. The LOD was estimated at 0.5 pg/mg. The presence of ostarine was confirmed in real hair samples at concentrations between 12 and 168 pg/mg. Moreover, in the same context, the method was successfully applied for the detection of ostarine in real nails samples at concentrations of 111 and 61 pg/mg, in fingernails and toenails, respectively.

Likewise, the PPAR- δ agonist cardarine (also known as GW501516 or GW1516) was identified by LC-MS/MS and LC-Q-TOF in two 2-cm hair segments of a frequent user of the drug at a concentration of 32 and 22 pg/mg, after methanol incubation of 20 mg of hair with ultrasound for 1 hour [171].

Due to their capabilities to increase physical and endurance performances, PPAR- δ agonists were considered to have a high potential for use also in animal doping, including horse racing doping [173-174]. In this context, a study about the detection of cardarine and its metabolites in equine hair by LC-HRMS, after a controlled administration, was recently published by Ishii *et al.* [174]. The pulverized equine hair samples (200 mg) were extracted with methanol, followed by further purification, and the extracts were analysed using a Q-Orbitrap instrument equipped with a heated ESI source operating in both positive and negative ion mode. In order to reveal also the presence of cardarine metabolites, full MS experiments were carried out in the scan range m/z 50-750 with a nominal resolving power of 60,000 FWHM. In case of positive results, a

further parallel reaction monitoring protocol based on in-source CID was developed, acquiring product ion spectra from the following precursor ions: m/z 452.0607 (cardarine), m/z 468.0557 (cardarine sulfoxide), and m/z 484.0506 (cardarine sulfone), plus other three precursor ions from minor metabolites. The method was fully validated and the estimated LODs and limits of confirmation (LOCs) for the three target analytes were 0.02 pg/mg and 0.03 pg/mg, respectively. After a single nasoesophageal dose of cardarine (150 mg) administered to a thoroughbred female horse (mare), the hair samples collected from 2 weeks post-administration turned out positive to cardarine and its metabolites, proving that an administration of a single low dose of cardarine can be detected by hair analysis.

In racehorse or greyhound doping the administration of AAS is often carried out by intramuscular injection of esterified forms, to allow a slow release resulting in extended half-life and prolonged effect. Therefore, the ability to detect intact esterified forms of endogenous steroids in hair is appealing as it can provide unequivocal proof of their exogenous origin. For this scope, Choi *et al.* developed a comprehensive LC-Q-Orbitrap method for the detection of 72 AAS and/or their esters in equine hair [180], as an update of a previous work on the detection of 48 AAS [181]. In order to allow more target analytes to be covered in a single injection, the Q-Orbitrap mass spectrometer operated in targeted MS/MS mode, making use of the idling time during parallel the MS acquisition for filling of several precursor ions into the collisional cell for the next experiment. Starting from 50 mg of equine hair sample, and employing a combined LLE/SPE extraction procedure, LODs values were estimated between 0.25 and 80 pg/mg. The method was then applied for the analysis of two real samples coming from horse racing doping controls, allowing the detection of anabolic steroids at ppb levels, namely boldione (2 pg/mg), 4-androstenedione, and testosterone propionate (3 pg/mg).

A similar LC-HRMS approach for the detection of testosterone esters in dog hair following LLE combined with SPE clean up with Bond-Elut amino cartridges was proposed by Devi *et al.* [182]. In order to enhance the sensitivity of the method, testosterone esters were added with methoxyamine hydrochloride to form the methyloxime derivatives. The method was validated for the detection and quantification of the propionate, phenylpropionate, isocaproate, decanoate, and enanthate esters of testosterone in Greyhound hair. The LODs for the three testosterone esters, phenylpropionate, isocaproate, and decanoate was 0.05 pg/mg and the LOD for the propionate was 0.025 pg/mg while for the testosterone enanthate was 0.5 pg/mg. The Q-Orbitrap mass spectrometer was operated in heated electrospray ionization positive mode with an ion spray voltage of 3000V. The acquisition was carried out in targeted MS/MS mode with a resolution of 35,000 FWHM. The method was applied to hair samples collected from male greyhounds before and after a single administration of a mixture of testosterone esters (3-4 mg/kg). Concentrations of testosterone esters in the interval 0.03-0.60 pg/mg were measured in dogs' hair from 35 to 91 days post-administration.

β_2 -agonists such as clenbuterol can be detected in blood and urine for a relatively short period after administration. In order to improve the detection window for these substances and to know the history of consumption, some studies have been carried out on the analysis of hair matrices [183-184].

Schlupp *et al.* [183] administered 0.8 μg of clenbuterol to horses for 10 days. Up to 360 days after administration, mane and tail hair were analysed in addition to blood and urine. After 30 days, clenbuterol was no longer detectable in either blood or urine, while already on the 5th day, clenbuterol was detected in the hair. A method was developed using GC-Q-Orbitrap following extraction of the analyte by ultrasounds followed by SPE, which provided an LOD of 0.2 pg/mg and resulted in concentrations ranging from a minimum of 0.5 pg/mg to a maximum of 21 pg/mg .

In a study by Machnik *et al.* [184], better results were obtained by analysing hair collected from pregnant women under Spiropent (clenbuterol-HCl) treatment. The hair samples were digested by incubation in KOH at 70°C, after LLE, purification by IAC, the sample was derivatized with MSTFA. Using a double focusing mass spectrometer with reversed geometry, a LOD of 0.8 ng/ng was obtained and concentrations in the range of 2 to 236 ng/g were found in the hair of the analysed women.

3.2.2 Dried Blood Spots (DBS)

One of the first approaches for the determination of prohibited drugs in DBS for doping controls was presented by Thomas *et al.* in 2012 [185]. In this study, the MS detection was carried out combining a quadrupole mass filter, an HCD cell and an ion trap analyser and acquiring the single product ion mass spectra for previously selected precursors of 26 model compounds (THC, THC-COOH, methylhexaneamine, methylphenidate, cocaine, nikethamide, MDMA, MDA, strychnine, mesocarb, salbutamol, formoterol, clenbuterol, methandienone, stanozolol, bisoprolol, propranolol, metoprolol, anastrozole, clomiphene, exemestane, dexamethasone, budesonide, andarine, SARM S1, and hydrochlorothiazide), at a resolution of 17,500 FWHM. The method also included a full scan data acquisition in positive and negative mode at resolving powers of 70,000 FWHM each, making possible a non-targeted analysis for retrospective data evaluation. Starting from 20 μL of blood sample spotted on Sartorius TFN DBS card and following a double LLE with methanol/tert-butyl-methyl-ether/acetone, LODs in the range 0.05-0.5 ng/mL were obtained, making the method suitable for an initial testing procedure for prohibited substances in doping control analysis.

A multi-target approach for the screening of eight AAS esters (nandrolone phenylpropionate, trenbolone enanthate, testosterone acetate, testosterone cypionate, testosterone isocaproate, testosterone phenylpropionate, testosterone decanoate, and testosterone undecanoate) plus nandrolone in DBS was developed by Tretzel *et al.* [186]. Again, the identification and determination of the target compounds was conducted by a Q-Orbitrap operating in targeted MS/MS mode. From 20 μL of blood on a Whatman DBS

sampling card, LODs in the ppb range (0.5 ng/mL for nandrolone phenylpropionate and 0.1 ng/mL for the other analytes) were accomplished by the preparation of the methyloxime derivatives of the target compounds, as the analysis of the derivatized analytes showed a 10-fold increase of the peak area ratios if compared to the not derivatized 3-oxo-steroids. The method was then applied on DBS specimens collected after a single oral dose of 80 mg of testosterone undecanoate. The positivity of authentic samples in the time period 2-8 h post-administration substantiates the suitability of the DBS sampling technique for the analysis of intact anabolic steroids, allowing an unequivocal proof of the administration of conjugates of exogenous testosterone and its derivatives.

Another study proving the suitability of the DBS sampling coupled to HRMS detection for the analysis of anabolic steroids esters was recently proposed by Solheim *et al.* [187]. For the extraction, 20 μ L of blood were spotted on the DBS sampling cards (FTA[®] DMPK-C), and 1 mL of a mixture of ACN:MeOH (50:50 v/v) was added to the punched spot. After filtration and derivatization with 2-hydrazino pyridine, the extracts were analysed by LC-HRMS/MS. The nanoflow LC system was combined with PRM using a Q-Orbitrap mass spectrometer. The resolution of the PRM experiments was set to 35,000 FWH, monitoring the protonated precursor ions in a m/z 2 mass window. The method was validated and the LODs were estimated between 50 and 200 pg/mL. The method was successfully applied on DBS samples from men receiving two intramuscular injections of a mixture of testosterone esters, some of which were detected for at least five days in all subjects receiving testosterone ester injections.

Regarding other anabolic agents, stanozolol, dehydrochloromethyltestosterone (DHCMT, oral Turinabol), and clenbuterol were detected in DBS by LC-HRMS/MS with an LOD of 0.1, 2.5, and 0.03 ng/mL, respectively [188-189]. To qualify the developed methods for doping control purposes, authentic DBS samples were analysed after a single oral dose of 5 mg stanozolol, 5 mg DHCMT, or 80 μ g clenbuterol. The analysis of stanozolol and DHCMT in DBS resulted in peak levels of approximately 10 and 11 ng/mL in blood, respectively. Measurable concentrations of the drugs occurred within 3 days after the application. Similarly, clenbuterol was detected in DBS samples up to 24 h to 72 h after administration. These results showed that DBS could be a useful supplemental sample matrix for anabolic agents also in out-of-competition testing.

In light of the considerable blood concentrations of some therapeutic proteins, several studies on the detection of peptide hormones and growth factors in DBS by LC-HRMS were published in the last decade. In 2012, Moller *et al.* demonstrated that peginesatide can be analysed from DBS samples with a LOD of 10 ng/mL [190]. The method was based on the extraction, proteolytic digestion and cation exchange purification of DBS followed by a LC-HRMS/MS analysis monitoring the precursor ion at m/z 584.31 related to the target peptide GPIT (1-nal) resulting from the enzymatic digestion. Similarly, Lange *et al.* proposed the detection of the stimulating protein sotatercept and bimagrumab (myostatine inhibitor) from DBS samples by protocols like those applied to serum, with LODs between 250 and 500 ng/mL [191]. DBS (20 μ L of blood)

were extracted into an aqueous buffer solution supported by ultrasonication, and immunoaffinity purification was carried out by magnetic nanoparticles equipped with either immobilized Protein-G (initial testing procedure) or immobilized activin A (confirmation procedure). LC-HRMS/MS analysis, conducted on a Q-Orbitrap mass spectrometer operating in both full scan and targeted MS/MS mode, enabled the detection of the target analytes in spiked as well as authentic post-administration samples.

Another example demonstrating the suitability of the LC-HRMS technique for the analysis of higher molecular mass drugs, like insulin and its synthetic or animal analogues (human, lispro, aspart, glulisine, glargine, detemir, degludec, porcine and bovine insulin) in DBS was presented by Thomas *et al.* [192]. Starting from blood spots of 20 μ L, the extraction protocol consisted of a double LLE with a mixture of acetonitrile/3% acetic acid (60/40, v/v) followed by a SPE and immunoaffinity purifications. Mass spectrometric detection was accomplished using either an ion mobility/Q-TOF or a Q-Orbitrap-equipped instrument, in full MS and MS/MS mode or targeted MS/MS mode, respectively. The assay validation demonstrated adequate sensitivity (LOD of 0.5 ng/mL for most insulins: 1.5 ng/mL for insulin detemir) allowing the detection of normal non-fasting levels of human insulin and eight synthetic analogs in DBS. Moreover, authentic DBS collected from a type-I diabetic individual using insulin Aspart were successfully analysed providing proof-of concept data.

A simple and effective procedure based on a fast methanolic extraction and a LC-HRMS detection of the growth hormone secretagogue ipamorelin in DBS was recently proposed by Gerace *et al.* [193]. MS and MS/MS data were collected by a Q-TOF mass spectrometer operating in the positive ESI mode. A double data-independent acquisition procedure for MS acquisition was carried out, namely, i) TOF-MS scan (m/z 230-800) and ii) TOF-MS/MS scan, monitoring all the fragments in a mass range 50-720 m/z . The mass spectrometer acquired a full scan and MS/MS data at a resolving power of 35,000 FWHM. The positive electrospray ionization of ipamorelin resulted in the predominant doubly protonated ion at m/z 356.7001. To increase the sensitivity of the method, CID experiments of the precursor ion were performed, giving fragment ions derived from the γ -series at m/z 146.1292 (γ_1), 490.2820 (γ_3), 627.3396 (γ_4), and the b -series at 223.1191 (b_2), 420.2041 (b_3) and 567.2720. Starting from 40 μ L of blood, LOD and LOQ values, calculated using the Hubaux-Vos algorithm, were 2.5 ng/mL and 5.0 ng/mL, respectively.

Lange *et al.* developed a comprehensive LC-HRMS method for the quantitative detection of 46 low molecular mass (<2 kDa) peptide/non-peptide drugs and drug candidates, after a fully automated DBS sample preparation [194]. For the sample extraction, a robotic multi-purpose sampler was used for the automatic sampling, purification (SPE and strong-cation exchange cartridges) and concentration of the DBS extracts. The MS was carried out on a Q-Orbitrap analysis comprising alternating full scan MS experiments (m/z 300 - 1500) and targeted MS/MS mode with an inclusion list of 53 precursor ions. The lower molecular mass

peptides < 2 kDa were predominantly observed as doubly-charged molecules; LODs were in the range 0.5-20 ng/L. This procedure proved effective by testing GHRP-2 and GHRP-6 post-administration study samples.

Meldonium (classified in the section Hormone and Metabolic Modulators) was quantitatively detected in DBS by LC-HRMS [195]. Meldonium was tested in urine, plasma, dried plasma spots (DPS), and DBS samples, in the context of a controlled administration pilot study, by a validated LC-HR-MS/MS method with a LOD of 20 ng/mL.

The study revealed that the concentrations of meldonium in DBS were 30-times higher than those detected in DPS samples, showing that meldonium may be incorporated into erythrocytes and gradually released to plasma [195].

3.3. Black market

The desire to improve physical and mental performance has given rise to an illegal traffic on the Internet of food and dietetic supplements that can be supplemented with AAS, metabolic modulators or even peptide hormones. It is therefore necessary to analyse them to know the products in circulation. Several studies describe the identification of dietary supplements by HRMS.

Four studies in the literature describe the identification of performance products in dietary supplements over a long period.

In France, 75 pharmaceutical products were seized and analysed by LC-Q-Orbitrap over a 3 year-period from 2016 to 2019, but quantified by LC-MS/MS. The following products were identified: AAS (72%), PDE5 (tadalafil, sildenafil), β 2-mimetic, peptide hormones, SERMs, aromatase inhibitors, diuretics [196].

The following analytical strategy was developed and applied in Switzerland and Germany. The identification was done by LC-Q-TOF then on GC-Q-TOF and then by nanoLC-HRMS, but the quantification was done by LC-MS/MS [42, 197].

In Switzerland, 1190 supplements were analysed following this strategy and 75% were identified as AAS, 12% as peptide hormones, 9% as hormones and metabolic modulators. Anastrozole, aromatase inhibitor product, presenting a higher product quality compared to other seizures [197].

In Germany, 337 supplements were analysed following the same strategy. 288 were identified as AAS and 56 were other performance products. It is interesting to note that in this study, 55% of the AAS were not correctly labelled [42].

In Italy, a similar study was conducted from 2017 to 2019. Here the samples were identified and quantified by LC-Q-Orbitrap [198].

For some new compounds, like designer steroids, it is necessary to use multiple analytical tools simultaneously to accurately identify the compound, namely the liquid chromatography-high resolution

accurate mass-mass spectrometry (LC-HRAM-MS). This was the case for the identification of two designer steroids, androsta-3,5-diene-7,17-dione and 2 β -chlorotestosterone, in dietary supplements [199-200]. First, LC-MS screening allowed the detection of unknown compounds with steroid characteristics. Then, thanks to the simultaneous use of HRMS and NMR methods, unknown compounds have been identified.

Thevis *et al* have identified thanks to LC-HRMS and comparison with reference material, S4 (Andarine), SARM, in oil solution purchased as tea extract [201]. More recently, Leaney *et al.* have analysed 20 SARMs marketed online by LC-HRMS. 60% of products were adulterated with other SARMs [202].

HRMS is very useful for testing large molecules as hormones and peptides. The hormone GHRP-2 has been identified by LC-HRMS offered as an over-the-counter food supplement [203]. The use of a LC-Q-Orbitrap allows the identification not-conjugated CJC-1295 in a sealed powder. Despite the absence of reference standards, the authors were able to characterise the peptide using HRMS, combining the mass spectrum with knowledge of its structure [72].

Another new rapid and versatile approach was presented by Doué *et al.* , allowing the identification of 21 anabolic steroid esters in supplements by direct analysis in real time-high resolution mass spectrometry (DART) [204].

Finally, in a recent study, Chen *et al.* have elucidated two new corticoids (betamethasone dibutyrate and betamethasone tributyrate) thanks to the determination of exact mass using LC-Q-Orbitrap mass spectrometer [205].

To conclude, one molecule per family of dopants mentioned in this review has been identified in dietary supplements by HRMS. Nevertheless, it seems that AAS remain the most identified substances and the most popular by consumers.

4. Conclusions

Within the last decade, advances in mass spectrometry have promoted the introduction of more and more sensitive detectors and particularly HRMS. This technology counts several benefits as accurate measurements ($m/z < 5$ ppm), simultaneous untargeted screening with effective targeted accurate masses acquisition and time saving assay with possibility of retrospective data analysis. In the context of doping products analysis, HRMS has demonstrated its usefulness by formally identifying compounds from endogenous analogs and by easily distinguishing peptides and proteins with high sequence homology. With the constant evolution of prohibited substances list, another positive point of HRMS is its ability to easily add new compounds in spectral libraries and to search a very large number of compounds simultaneously. Moreover, structural elucidation of peptides and fragmentation patterns of various compounds is facilitated with these new spectrometers. With recent advances, both quantitative and qualitative analysis of doping

agents and their metabolites could be performed on a single run. Methods coupling untargeted and targeted acquisition allow to enhance sensitivity without loss of spectral information. Globally, the same LODs have been observed compared to tandem mass spectrometry but the crucial point of HRMS is its higher dynamic range of detection. With their increasing sensitivity, actual high resolution mass spectrometers discriminate some isomers at very low concentrations from complex biological matrices such as urine, blood, DBS or hair samples. Moreover, the use of full scan HRMS allows to decrease the background noise coming from the matrix, reducing sample preparation steps without losing specificity. Thereby, HRMS is more and more used for metabolism studies in urine or to determine urinary detection windows of numerous compounds. Alone or in association with other techniques such as tandem mass spectrometry, HRMS is nowadays a method of choice for both research and routine analyses.

Conflict of interest: the authors declare no conflict of interest

Reference

1. WADA prohibited list. Consulted on website <https://www.wada-ama.org/en/what-we-do/the-prohibited-list> on August 31 2021
2. Council Directive 86/469/EEC of 16 September 1986 concerning the examination of animals and fresh meat for the presence of residues. Consulted on website <https://op.europa.eu/fr/publication-detail/-/publication/729312f3-d248-4bca-b8b1-1748efde6707/language-hu> on August 31 2021.
3. Ericson M. Analytical challenges and solutions in doping control: a perspective from the Swedish doping control laboratory. *Bioanalysis* 2016;8:735-39.
4. Gosetti F, Mazzucco E, Gennaro MC, Marengo E. Ultra-high performance liquid chromatography tandem mass spectrometry determination and profiling of prohibited steroids in hfn biological matrices. A review. *J Chromatogr B* 2013;927:22-36.
5. WADA testing figure list. Consulted on website <https://www.wada-ama.org/en/resources/laboratories/anti-doping-testing-figures-report> on August 31 2021.
6. MRPL technical document. Consulted on website https://www.wada-ama.org/sites/default/files/resources/files/td2019mrpl_eng.pdf on August 31 2021.
7. Tudela E, Deventer K, Geldof L, Van Eenoo P. Urinary detection of conjugated and unconjugated anabolic steroids by dilute-and-shoot liquid chromatography-high resolution mass spectrometry. *Drug Test Anal* 2015; 7(2):95-108.
8. Kioussi MK, Lyris EM, Angelis YS, Tsiyou M, Koupparis MA, Georgakopoulos CG. A generic screening methodology for horse doping control by LC-TOF-MS, GC-HRMS and GC-MS. *J Chromatogr B Analyt Technol Biomed Life Sci* 2013;941:69-80.
9. Buiarelli F, Cartoni GP, Amendola L, Botrè F. Screening and confirmation analysis of anabolic agents in human urine by gas chromatography — hybrid mass spectrometry (high resolution — time of flight). *Anal Chim Acta* 2001;447:75-88.

10. Abushareeda W, Vonaparti A, Saad KA, Almansoori M, Meloug M, Saleh A, Aguilera R, Angelis Y, Horvatovich PL, Lommen A, Alsayrafi M, Georgakopoulos C. High resolution full scan liquid chromatography mass spectrometry comprehensive screening in sports antidoping urine analysis. *J Pharm Biomed Anal* 2018; 151:10-24.
11. de Albuquerque Cavalcanti G, Rodrigues LM, Dos Santos L, Zheng X, Gujar A, Cole J, Padilha MC, de Aquino Neto FR. Non-targeted acquisition strategy for screening doping compounds based on GC-EI-hybrid quadrupole-Orbitrap mass spectrometry: A focus on exogenous anabolic steroids. *Drug Test Anal* 2018;10(3):507-17.
12. He G, Wu Y, Lu J. Doping control analysis of 13 steroids and structural-like analytes in human urine using Quadrupole-Orbitrap LC-MS/MS with parallel reaction monitoring (g) mode. *Steroids* 2018;131:1-6.
13. Raro M, Ibáñez M, Gil R, Fabregat A, Tudela E, Deventer K, Ventura R, Segura J, Marcos J, Kotronoulas A, Joglar J, Farré M, Yang S, Xing Y, Van Eenoo P, Pitarch E, Hernández F, Sancho JV, Pozo ÓJ. Untargeted metabolomics in doping control: detection of new markers of testosterone misuse by ultrahigh performance liquid chromatography coupled to high-resolution mass spectrometry. *Anal Chem* 2015;87(16):8373-80.
14. Cha E, Jeong ES, Cha S, Lee J. Coupling of gas chromatography and electrospray ionization high resolution mass spectrometry for the analysis of anabolic steroids as trimethylsilyl derivatives in human urine. *Anal Chim Acta* 2017;964:123-33.
15. Göschl L, Gmeiner G, Gärtner P, Stadler G, Enev V, Thevis M, Schänzer W, Guddat S, Forsdahl G. Stanozolol-N-glucuronide metabolites in human urine samples as suitable targets in terms of routine anti-doping analysis. *Drug Test Anal* 2021; doi: 10.1002/dta.3109.
16. Albertsdóttir AD, Gansbeke WV, Eenoo PV, Polet M. Enabling the inclusion of non-hydrolysed sulfated long term anabolic steroid metabolites in a screening for doping substances by means of gas chromatography quadrupole time-of-flight mass spectrometry. *J Chromatogr A* 2021; <https://doi.org/10.1016/j.chroma.2021.462039>.
17. Thevis M, Piper T, Horning S, Juchelka D, Schänzer W. Hydrogen isotope ratio mass spectrometry and high-resolution/high-accuracy mass spectrometry in metabolite identification studies: detecting target compounds for sports drug testing. *Rapid Commun Mass Spectrom* 2013; 27(17):1904-12.

18. Putz M, Piper T, Thevis M. Identification of Trenbolone Metabolites Using Hydrogen Isotope Ratio Mass Spectrometry and Liquid Chromatography/High Accuracy/High Resolution Mass Spectrometry for Doping Control Analysis. *Front Chem* 2020;8:435.
19. Nielen MW, Bovee TF, van Engelen MC, Rutgers P, Hamers AR, van Rhijn JH, Hoogenboom LR. Urine testing for designer steroids by liquid chromatography with androgen bioassay detection and electrospray quadrupole time-of-flight mass spectrometry identification. *Anal Chem* 2006;78(2):424-31.
20. Viljanto MJ, Kicman AT, Walker CJ, Parkin MC, Wolff K, Pearce CM, Scarth J. Elucidation of the biosynthetic pathways of boldenone in the equine testis. *Steroids* 2019;146:79-91.
21. Moretti S, Lega F, Rigoni L, Saluti G, Giusepponi D, Gioiello A, Manuali E, Rossi R, Galarini R. Multiclass screening method to detect more than fifty banned substances in bovine bile and urine. *Anal Chim Acta* 2018;1032:56-67.
22. Baglai A, Blokland MH, Mol HGJ, Gargano AFG, van der Wal S, Schoenmakers PJ. Enhancing detectability of anabolic-steroid residues in bovine urine by actively modulated online comprehensive two-dimensional liquid chromatography - high-resolution mass spectrometry. *Anal Chim Acta* 2018;1013:87-97.
23. Hansson A, Knych H, Stanley S, *et al.* Investigation of the selective androgen receptor modulators S1, S4 and S22 and their metabolites in equine plasma using high-resolution mass spectrometry. *Rapid Comm Mass Spectrom* 2016; 30(7):833-42.
24. Friedman O, Arad E, Ben Amotz O. Body Builder's Nightmare: Black Market Steroid Injection Gone Wrong: a Case Report. *Plast Reconstr Surg Glob Open* 2016; doi: 10.1097/GOX.0000000000001040.
25. Hernández-Guerra AI, Tapia J, Menéndez-Quintanal LM, Lucena JS. Sudden cardiac death in anabolic androgenic steroids abuse: case report and literature review. *Forensic Sci Res* 2019;4(3):267-73.
26. Fabresse N, Grassin-Delyle S, Etting I, Alvarez JC. Detection and quantification of 12 anabolic steroids and analogs in human whole blood and 20 in hair using LC-HRMS/MS: application to real cases. *Int J Legal Med* 2017; 131(4):989-999. doi: 10.1007/s00414-017-1552-3.

27. Ponzetto F, Boccard J, Baume N, Kuuranne T, Rudaz S, Saugy M, Nicoli R. High-resolution mass spectrometry as an alternative detection method to tandem mass spectrometry for the analysis of endogenous steroids in serum. *J Chromatogr B Analyt Technol Biomed Life Sci* 2017;1052:34-42.
28. Donike M, Geyer H, Kraft M, *et al.* Longterm influence of anabolic steroid misuse on the steroid profile, in Official Proceedings of the IInd I.A.F. Belotti P, Benzi G, Ljungqvist A.eds *World symposium on doping in sport*. Monte Carlo: International Athletic Foundation, 1989:107-16.
29. Abushareeda W, Lyris E, Kraiem S, Wahaibi AA, Alyazidi S, Dbes N, Lommen A, Nielen M, Horvatovich PL, Alsayrafi M, Georgakopoulos C. Gas chromatographic quadrupole time-of-flight full scan high resolution mass spectrometric screening of human urine in antidoping analysis. *J Chromatogr B Analyt Technol Biomed Life Sci* 2017;1063:74-83.
30. Abushareeda W, Tienstra M, Lommen A, Blokland M, Sterk S, Kraiem S, Horvatovich P, Nielen M, Al-Maadheed M, Georgakopoulos C. Comparison of gas chromatography/quadrupole time-of-flight and quadrupole Orbitrap mass spectrometry in anti-doping analysis: I. Detection of anabolic-androgenic steroids. *Rapid Commun Mass Spectrom* 2018;32(23):2055-64.
31. Kaabia Z, Laparre J, Cesbron N, Le Bizec B, Dervilly-Pinel G. Comprehensive steroid profiling by liquid chromatography coupled to high resolution mass spectrometry. *J Steroid Biochem Mol Biol* 2018; 183:106-15.
32. Elmongy H, Masquelier M, Ericsson M. Development and validation of a UHPLC-HRMS method for the simultaneous determination of the endogenous anabolic androgenic steroids in human serum. *J Chromatogr A* 2020; doi: 10.1016/j.chroma.2019.460686.
33. Nieschlag E, Vorona E. Medical consequences of doping with anabolic androgenic steroids: effects on reproductive functions. *Eur J Endocrinol* 2015;173:47-58.
34. Dong JJ, Ruan MC, Hang JG, Nakayama SF, Jung CR, Kido T, Wang Z, Ma CC, Sun XL. The relationship between perinatal exposure to dioxins and serum steroid hormone levels in preschool-aged children at an e-waste region in China. *Int J Hyg Environ Health* 2020; doi: 10.1016/j.ijheh.2020.113580.

35. Oanh NTP, Kido T, Honma S, Oyama Y, Anh LT, Phuc HD, Viet NH, Manh HD, Okamoto R, Nakagawa H, Nakayama SF, Nhu DD, Van Tung D, Van Chi V, Minh NH, Van Toan N. Androgen disruption by dioxin exposure in 5-year-old Vietnamese children: Decrease in serum testosterone level. *Sci Total Environ* 2018; doi: 10.1016/j.scitotenv.2018.05.257.
36. Stacchini C, Botrè F, Comunità F, de la Torre X, Dima AP, Ricci M, Mazzarino M. Simultaneous detection of different chemical classes of selective androgen receptor modulators in urine by liquid chromatography-mass spectrometry-based techniques. *J Pharm Biomed Anal.* 2021 Feb 20; 195:113849. doi: 10.1016/j.jpba.2020.113849.
37. Thevis M, Piper T, Dib J, Lagojda A, Kühne D, Packschies L, Geyer H, Schänzer W. Mass spectrometric characterization of the selective androgen receptor modulator (SARM) YK-11 for doping control purposes. *Rapid Commun Mass Spectrom* 2017;31(14):1175-83.
38. Li T, Cao J, Li Z, Wang X, He P. Broad screening and identification of β -agonists in feed and animal body fluid and tissues using ultra-high performance liquid chromatography-quadrupole-orbitrap high resolution mass spectrometry combined with spectra library search. *Food Chem* 2016;192:188-96.
39. Son J, Moon JY, Kim SA, Cho YD, Kim JD, Kim DH, Choi MH. Improved detectability in pharmacokinetic study of tibolone by gas chromatography-high resolution mass spectrometry with selected ion monitoring. *Talanta* 2006;70(1):37-42.
40. Thevis M, Thomas A, Schänzer W. Targeting prohibited substances in doping control blood samples by means of chromatographic-mass spectrometric methods. *Anal Bioanal Chem.* 2013; doi:10.1007/s00216-013-7224-3.
41. Barroso O, Handelsman D, Strasburger C, Thevis M. Analytical challenges in the detection of peptide hormones for anti-doping purposes. *Bioanalysis* 2012;4(13):1577-90.
42. Krug O, Thomas A, Walpurgis K, Piper T, Sigmund G, Schänzer W, Laussmann T, Thevis M. Identification of black-market products and potential doping agents in Germany 2010-2013. *Eur J Clin Pharmacol* 2014;70(11):1303-11.

43. Erotokritou-Mulligan I, Guha N, Stow M, Bassett EE, Bartlett C, Cowan DA, Sonsken PH, Holt RIG. The development of decision limits for the implementation of the GH-2000 detection methodology using current commercial insulin-like growth factor-I and amino-terminal pro-peptide of type II collagen assays. *Growth Horm. IGF Res* 2012;22(2):53-58.
44. Bidlingmaier M, Suhr J, Ernst A. High-sensitivity chemiluminescence immunoassays for detection of growth hormone doping in sports. *Clin Chem* 2009;55(3):445-53.
45. Joré C, Loup B, Garcia P, Paris AC, Popot MA, Audran M *et al.* Liquid chromatography – high resolution mass spectrometry –based metabolomic approach for the detection of Continuous Erythropoiesis Receptor Activator effects in horse doping control. *J Chromatogr A* 2017;152:90-99.
46. Lasne F, Martin L, Crepin N, de Ceaurriz J. Detection of isoelectric profiles of erythropoietin in urine: differentiation of natural and administered recombinant hormones. *Anal Biochem* 2002;311:119-26.
47. Reichel C, Abzieher F, Geisendorger T. SARCOSYL-PAGE: a new method for the detection of MIRCERA- and EPO-doping in blood. *Drug Test Anal* 2009;1:494-504.
48. Reichel C, Kulovics R, Jordan V, Watzinger M, Geisendorfer T. SDS-PAGE of recombinant and endogenous erythropoietins: benefits and limitations of the method for application in doping control. *Drug Test Anal* 2009;1:43-50.
49. Lasne F, Popot MA, Varlet-Marie E, Martin L, Martin JA, Bonnaire Y, Audran M, Ceaurriz JD. Detection of recombinant epoetin and darbepoetin alpha after subcutaneous administration in the horse. *J Anal Toxicol* 2005;29:835-37.
50. Delcourt V, Garcia P, Chabot B, Loup B, Remy P, Popot MA, Bailly-Chouriberry L. Screening and confirmatory analysis of recombinant Human EPO for racing camels doping control. *Drug Test Anal* 2020; 12(6):763-70.
51. Walpurgis K, Thomas A, Vogel M, Reichel C, Geyer H, Schänzer W, Thevis M. Testing for the erythropoiesis-stimulating agent Sotatercept/ACE-011 (ActRIIA-Fc) in serum by means of Western blotting and LC-HRMS. *Drug Test Anal* 2016; 8(11-12):1152-61.

52. Lange T, Walpurgis K, Thomas A, Geyer H, Thevis M. Development of two complementary LC-HRMS methods for analysing sotatercept in dried blood spots for doping controls. *Bioanalysis* 2019; 11(10):923-40.
53. Möller I, Thomas A, Geyer H, Schänzer W, Thevis M. Synthesis, characterisation, and mass spectrometric detection of a pegylated EPO-mimetic peptide for sports drug testing purposes. *Rapid Commun Mass Spectrom*. 2011;25(15):2115-23.
54. Möller I, Thomas A, Wingender A, Machnik M, Schänzer W, Thevis M. Detection of peginesatide in equine serum using liquid chromatography-tandem mass spectrometry for doping control purposes. *Eur J Mass Spectrom* 2012;18(4)407-12.
55. Camperi J, Combes A, Guibourdenche J, Guillaume D, Pichon V, Fournier T, Delaunay N. An attempt to characterize the human Chorionic Gonadotropin protein by reversed phase liquid chromatography coupled with high-resolution mass spectrometry at the intact level. *J Pharm Biomed Anal* 2018;161:35-44.
56. Stenman UH, Unlika-Kallio L, Korhonen J, Alfthan H. Immunoprocedures for detecting human chorionic gonadotropin: clinical aspects and doping control. *Clin Chem* 1997;43(7):1293-98.
57. Stenman UH, Hotakainen K, Alfthan H. Gonadotropins in doping: pharmacological basis and detection of illicit use. *Br J Pharmacol* 2008; 154(3):569-83.
58. Al Matari A, Goumenou A, Combès A, Fournier T, Pichon V, Delaunay N. Identification and semi-relative quantification of intact glycoforms of human chorionic gonadotropin alpha and beta subunits by nano liquid chromatography-Orbitrap mass spectrometry. *J Chromatogr A* 2021;doi:10.1016/j.chroma.2021.461945.
59. Thomas A, Geyer H, Kamber M, Schänzer W, Thevis M. Mass spectrometric determination of gonadotropin-releasing hormone (GnRH) in human urine for doping control purposes by means of LC-ESI-MS/MS. *J Mass Spectrom* 2008; 43(7):908-15.
60. Toro I, Sanz-Nebot V, Barbosa J. Analysis of a peptide hormone mixture of therapeutic interest by liquid chromatography coupled high-flow pneumatically assisted electrospray mass spectrometry. *J Chromatogr A* 2000;893(1):95-106.

61. Kwok KY, Choi TLS, Kwok WH, Lau MY, Leung EMK, Leung GNW *et al.* Detection of bioactive peptides including gonadotrophin-releasing factors (GnRHs) in horse urine using ultra-high performance liquid chromatography-high resolution mass spectrometry (UHPLC/HRMS). *Drug Test Anal* 2020;12(9):1274-86.
62. Judák P, Grainger J, Goebel C, Eenoov PV, Deventer K, DMSO Assisted Electrospray Ionization for the Detection of Small Peptide Hormones in Urine by Dilute-and-Shoot-Liquid-Chromatography-High Resolution Mass Spectrometry. *J Am Soc Mass Spectrom* 2017;28(8):1657-65.
63. Knoop A, Thomas A, Bidlingmaier M, Delahaut P, Schänzer W, Thevis M. Probing for corticotropin-releasing hormone (CRH) in human blood for doping control purposes using immunoaffinity purification and LC-HRMS/MS. *Anal Methods* 2017;9:4304-10.
64. Boyard-Kieken F, Dervilly-Pinel G, Garcia P, Paris AC, Popot MA, le Bizec B, Bonnaire Y. Comparison of different liquid chromatography stationary phases in LC-HRMS metabolomics for the detection of recombinant growth hormone doping control. *J Sep Sci* 2011;34(24):3493-3501.
65. Wong KS, Chan GHM, Ho ENM, Wan TSM. Simultaneous detection of recombinant growth hormones in equine plasma by liquid chromatography/high-resolution tandem massspectrometry for doping control. *J Chromatogr A* 2016;1478:35-42.
66. Kieken F, Pinel G, Antignac JP, Monteau F, Paris AC, Popot MA *et al.* Development of a metabonomic approach based on LC-ESI-HRMS measurements for profiling of metabolic changes induced by recombinant equine growth hormone in horse urine. *Anal Bioanal Chem* 2009;394(8):2119-28.
67. Pont L, Alechaga E, Terrero A, Monfort N, Ventura R. Comparison of magnetic bead surface functionalities for the immunopurification of growth hormone-releasing hormones prior to liquid chromatography-high resolution mass spectrometry. *J Chromatogr A* 2020; doi: 10.1016/j.chroma.2020.461548.
68. Knoop A, Thomas A, Fichant E, Delahaut P, Schänzer W, Thevis M. Qualitative identification of growth hormone-releasing hormones in human plasma by means of immunoaffinity purification and LC-HRMS/MS. *Anal Bioanal Chem* 2016;408(12):3145-53.

69. Esposito S, Deventer K, Van Eenoo P. Identification of the growth hormone-releasing hormone analogue [Pro1, Val14]-hGHRH with an incomplete C-term amidation in a confiscated product. *Drug Test Anal* 2014;6(11-12):1155-59.
70. Henninge J, Pepaj M, Hullstein I, Hemmersbach P. Identification of CJC-1295, a growth-hormone-releasing peptide, in an unknown pharmaceutical preparation. *Drug Test Anal* 2010;2(11-12):647-50.
71. Thomas A, Walpurgis K, Tretzel L, Brinkkotter P, Fichant E, Delahaut P, Schänzer W, Thevis M. Expanded test method for peptides >2 kDa employing immunoaffinity purification and LC-HRMS/MS. *Drug Test Anal* 2015;7(11-12):990-98.
72. Fabresse N, Grassin-Delyle S, Etting I, Alvarez JC. Identification d'un analogue peptidique de la GHRH, le CJC-1295, par CL-SM/SMHR Identification of a GHRH peptide analogue, the CJC-1295, using LC-HRMS/MS. *Toxicol Anal Et Clin* 2017;29(2):205-11.
73. Lange T, Thomas A, Gorgens C, Bidlingmaier M, Schilbach K, Fichant E, Delahaut P, Thevis M. Comprehensive insights into the formation of metabolites of the ghrelin mimetics capromorelin, macimorelin and tabimorelin as potential markers for doping control purposes. *Biomed Chromatogr* 2021; doi: 10.1002/bmc.5075.
74. Coppieters G, Judak P, Haecke NV, Renterghem PV, Eenoo PV, Deventer K. A high-throughput assay for the quantification of intact Insulin-like Growth Factor I in human serum using online SPE-LC-HRMS. *Clin Chim Acta* 2020;510:391-99.
75. Seo Y, Park J, Kim M, Sung C, Kwon OS, Lee HJ, Min H. Optimization, validation, and comparison of a rapid method for the quantification of insulin-like growth factor 1 in serum using liquid chromatography-high-resolution mass spectrometry. *Drug Test Anal* 2021;13(2):451-59.
76. Mongongu C, Moussa EM, Semence F, Roulland I, Ericsson M, Coudoré F *et al.* Use of capillary dried blood for quantification of intact IGF-I by LC-HRMS for antidoping analysis. *Bioanalysis* 2020;12(11):737-52.
77. Esposito S, Deventer K, Geldof L, Eenoo PV. In vitro models for metabolic studies of small peptide hormones in sport drug testing. *J Pept Sci* 2015;21(1): -9.

78. Chen Z, Alelyunas YW, Wrona MD, Rehler JR, Szapacs ME, Evans CA. Microflow UPLC and high-resolution MS as a sensitive and robust platform for quantitation of intact peptide hormones. *Bioanalysis* 2019;11(13):1275-89.
79. Fragkaki AG, Georgakopoulos C, Sterk S, Nielen MWF. Sports doping: Emerging designer and therapeutic β_2 -agonists. *Clin Chem Acta* 2013;425:242-58.
80. WADA Technical Document - TD2021DL. Consulted on website https://www.wada-ama.org/sites/default/files/resources/files/td2021dl_final_eng_0.pdf on August 20 2021.
81. Li T, Cao J, Li Z, Wang X, He P. Broad screening and identification of β -agonists in feed and animal body fluid and tissues using ultra-high-performance liquid chromatography quadrupole-orbitrap high resolution mass spectrometry combined with spectra library search. *Food Chem* 2016;192:188-96.
82. Hemmersbach P, Tomten S, Nilsson S, Offerdo H, Havrevoll O, Oen B. Illegal use of anabolic agents in animal fattening-consequences for doping analysis. *Recent advances in doping analysis*. Köln, Germany: Sport und Buch Strauß 1995;195-201.
83. Guo C, Shi F, Gong L, Tan H, Hu D, Zhang J. Ultra-trace analysis of 12 β_2 -agonists in pork, beef, mutton and chicken by ultrahigh-performance liquid-chromatography-quadrupole-orbitrap tandem mass spectrometry. *J Pharm Biomed Anal* 2015;107:526-34.
84. Montrade MP, Bizec BSe, Monteau F, Andre F. Analysis of beta-agonists in urine and tissues by capillary gas chromatography-mass spectrometry: in vivo study of salbutamol disposition in calves. *Food Addit Contam* 1995; 12(5):625-36.
85. Léon N, Roca M, Igualada C, Martins CPB, Pastor A, Yusà V. Wide-range screening of banned veterinary drugs in urine by ultra high liquid chromatography coupled to high-resolution mass spectrometry. *J Chromatogr A* 2012;1258:55-65.
86. Georgakopoulos CG, Tsitsimpikou C, Spyridaki MH. Excretion study of the β_2 -agonist reproterol in human urine. *J Chromatogr B Biomed Sci Appl* 1999; 726(1-2):141-48.

87. Yerra NV, Dadinaboyina SB, Abbaraju LSSN V, Talluri MVNK, Thota JR. Identification and characterization of degradation products of indacaterol using liquid chromatography/mass spectrometry. *Eur J Mass Spectrom* 2020;1-7.
88. Kollmeier AS, Torre X de la, Müller C, Botrè F, Parr MK. In-depth gas chromatography/tandem mass spectrometry fragmentation analysis of formestane and evaluation of mass spectral discrimination of isomeric 3-keto-4-ene hydroxy steroids. *Rapid Commun Mass Spectrom*. 2020; DOI: 10.1002/rcm.8937.
89. Walpurgis K, Thomas A, Thevis M. Detection of the myostatin-neutralizing antibody Domagrozumab in serum by means of Western blotting and LC-HRMS. *Drug Test Anal* 2019;11(11-12):1714-23.
90. Walpurgis K, Thomas A, Dellanna F, Schänzer W, Thevis M. Detection of the Human Anti-ActRII Antibody Bimagrumab in Serum by Means of Affinity Purification, Tryptic Digestion, and LC-HRMS. *PROTEOMICS - Clin Appl* 2018; doi: 10.1002/prca.201700120.
91. Walpurgis K, Weigand T, Knoop A, Thomas A, Reichel C, Dellanna F, *et al.* Detection of follistatin-based inhibitors of the TGF- β signaling pathways in serum/plasma by means of LC-HRMS/MS and Western Blotting. *Drug Test Anal* 2020;12(11-12):1636-48.
92. Kwok KY, Chan GHM, Kwok WH, Wong JKY, Wan TSM. In vitro phase I metabolism of selective estrogen receptor modulators in horse using ultra-high performance liquid chromatography-high resolution mass spectrometry: In vitro phase I metabolism of tamoxifen and toremifene in horse. *Drug Test Anal* 2017;9(9): 1349-62.
93. Mazzarino M, Biava M, de la Torre X, Fiacco I, Botrè F. Characterization of the biotransformation pathways of clomiphene, tamoxifen and toremifene as assessed by LC-MS/(MS) following in vitro and excretion studies. *Anal Bioanal Chem* 2013;405(16):5467-87.
94. Gärtner P, Hofbauer K, Reichel C, Geisendorfer T, Gmeiner G. Synthesis and identification of hydroxylated metabolites of the anti-estrogenic agent cyclofenil. *J Mass Spectrom* 2008; 43(7): 958-64.
95. Lu J, He G, Wang X, Xu Y, Wu Y, Dong Y, *et al.* Mass spectrometric identification and characterization of new clomiphene metabolites in human urine by liquid chromatography-quadrupole time-of-flight tandem mass spectrometry. *J Chromatogr A* 2012;1243:23-32.

96. Brito DM, Torre X de la, Botrè F. Detection of urinary metabolites of arimistane in humans by gas chromatography coupled to high-accuracy mass spectrometry for antidoping analyses. *Rapid Commun Mass Spectrom* 2019;33(24):1894–1905.
97. Dahmane E, Boccard J, Csajka C, Rudaz S, Décosterd L, Genin E, *et al.* Quantitative monitoring of tamoxifen in human plasma extended to 40 metabolites using liquid-chromatography high-resolution mass spectrometry: new investigation capabilities for clinical pharmacology. *Anal Bioanal Chem* 2014; 406(11):2627–40.
98. Ameline A, Gheddar L, Raul J-S, Kintz P. Characterization of letrozole in human hair using LC-MS/MS and confirmation by LC-HRMS: Application to a doping case. *J Chromatogr B* 2021; 1162:122495. doi: 10.1016/j.jchromb.2020.122495.
99. Favretto D, Snenghi R, Pertile R, Mazloum RE, Tucci M, Visentin S, *et al.* Hair analysis to discriminate voluntary doping vs inadvertent ingestion of the aromatase inhibitor letrozole. *Drug Test Anal* 2019;11(6):762–71.
100. Kwok WH, Choi TLS, Kwok KY, Chan GHM, Wong JKY, Wan TSM. Doping control analysis of 46 polar drugs in horse plasma and urine using a 'dilute-and-shoot' ultra high performance liquid chromatography-high resolution mass spectrometry approach. *J Chromatogr A* 2016;1451:41–9.
101. Görgens C, Guddat S, Orlovius A-K, Sigmund G, Thomas A, Thevis M, *et al.* "Dilute-and-inject" multi-target screening assay for highly polar doping agents using hydrophilic interaction liquid chromatography high resolution/high accuracy mass spectrometry for sports drug testing. *Anal Bioanal Chem* 2015;407(18):5365–79.
102. Thomas A, Thevis M. Recent advances in the determination of insulins from biological fluids. In: *Advances in Clinical Chemistry* 2019;93:115-67.
103. Thevis M, Thomas A, Schänzer W. Doping control analysis of selected peptide hormones using LC-MS(/MS). *Forensic Sci Int* 2011;213(1–3):35–41.

104. Bottinelli C, Cartiser N, Bévalot F, Fanton L, Guitton J. Is insulin intoxication still the perfect crime? Analysis and interpretation of postmortem insulin: review and perspectives in forensic toxicology. *Crit Rev Toxicol* 2020;50(4):324–47.
105. Thomas A, Yang R, Petring S, Bally L, Thevis M. Simplified quantification of insulin, its synthetic analogs and C-peptide in human plasma by means of LC-HRMS. *Drug Test Anal* 2020;12(3):382–90.
106. Thomas A, Schänzer W, Thevis M. Determination of human insulin and its analogues in human blood using liquid chromatography coupled to ion mobility mass spectrometry (LC-IM-MS): LC-IM-HRMS of insulins. *Drug Test Anal* 2014;6(11–12):1125–32.
107. Judák P, Coppieters G, Lapauw B, Van Eenoo P, Deventer K. Urinary detection of rapid-acting insulin analogs in healthy humans. *Drug Test Anal* 2020;12(11–12):1629–35.
108. Thomas A, Schänzer W, Delahaut P, Thevis M. Sensitive and fast identification of urinary human, synthetic and animal insulin by means of nano-UPLC coupled with high-resolution/high-accuracy mass spectrometry. *Drug Test Anal* 2009;1(5):219–27.
109. Bottinelli C, Nicoli R, Bévalot F, Cartiser N, Roger C, Chikh K, *et al.* Development and validation of a method for quantification of human insulin and its synthetic analogues in plasma and post-mortem sera by LC-MS/HRMS. *Talanta* 2021;doi: 10.1016/j.talanta.2020.122047.
110. Bottinelli C, Bévalot F, Cartiser N, Fanton L, Guitton J. Detection of insulins in postmortem tissues: an optimized workflow based on immunopurification and LC-MS/HRMS detection. *Int J Legal Med* 2021;135(5):1813–22.
111. Beckett N, Tidy R, Douglas B, Priddis C. Detection of intact insulin analogues in post-mortem vitreous humour—Application to forensic toxicology casework. *Drug Test Anal* 2021;13(3):604–13.
112. Thomas A, Brinkkötter P, Schänzer W, Thevis M. Metabolism of human insulin after subcutaneous administration: A possible means to uncover insulin misuse. *Anal Chim Acta* 2015;897:53–61.
113. Nankar RP, Doble M. Non-peptidyl insulin mimetics as a potential antidiabetic agent. *Drug Discov Today* 2013;18(15–16):748–55.

114. Mesonzhnik N, Krotov G, Appolonova S. Mass Spectrometry Characterization of a Novel Insulin Mimetic Peptide s597. *Pharm Anal Acta* 2017; doi: 10.4172/2153-2435.1000549.
115. Görgens C, Guddat S, Dib J, Geyer H, Schänzer W, Thevis M. Mildronate (Meldonium) in professional sports – monitoring doping control urine samples using hydrophilic interaction liquid chromatography – high resolution/high accuracy mass spectrometry. *Drug Test Anal* 2015;7(11–12):973–9.
116. Oliveira D, de Araújo A, Ribeiro W, Silva D, Duarte AC, de Sousa V, *et al.* Screening method of mildronate and over 300 doping agents by reversed-phase liquid chromatography-high resolution mass spectrometry. *J Pharm Biomed Anal* 2021; doi: 10.1016/j.jpba.2020.113870.
117. Kim, Yongseok, Jeong, Dawon, Min, Hophil, Sung, Changmin, Park, Ju-hyung, Son, Junghyun, *et al.* Method for Screening and Confirming Meldonium in Human Urine by High-Resolution Mass Spectrometry and Identification of Endogenous Interferences for Anti-Doping Testing. *Mass Spectrom Lett* 2017;8(2):39–43.
118. Görgens C, Guddat S, Bosse C, Geyer H, Pop V, Schänzer W, *et al.* The atypical excretion profile of meldonium: Comparison of urinary detection windows after single- and multiple-dose application in healthy volunteers. *J Pharm Biomed Anal* 2017;138:175–9.
119. Sigmund G, Koch A, Orlovius A-K, Guddat S, Thomas A, Schänzer W, *et al.* Doping control analysis of trimetazidine and characterization of major metabolites using mass spectrometric approaches: Analysis of trimetazidine in doping controls. *Drug Test Anal* 2014;6(11–12):1197–205.
120. Okano M, Sato M, Kageyama S. Lomerizine, trimetazidine and bis-(4-fluorophenyl)-methylpiperazine in human urine after oral administration of lomerizine dihydrochloride: analysis by liquid chromatography-high resolution-tandem mass spectrometry. *Drug Test Anal* 2018;10(11–12):1689–97.
121. Okano M, Thevis M, Sato M, Kageyama S. Analytical detection of trimetazidine produced by metabolic conversion of lomerizine in doping control analysis: Detection of trimetazidine produced by metabolic conversion of lomerizine. *Drug Test Anal* 2016;8(8):869–74.

122. Peters RJB, Oosterink JE, Stolker AAM, Georgakopoulos C, Nielen MWF. Generic sample preparation combined with high-resolution liquid chromatography–time-of-flight mass spectrometry for unification of urine screening in doping-control laboratories. *Anal Bioanal Chem* 2010;396(7):2583–98.
123. Giancotti V, Medana C, Aigotti R, Pazzi M, Baiocchi C. LC–high-resolution multiple stage spectrometric analysis of diuretic compounds. *J Pharm Biomed Anal* 2008;48(2):462–6.
124. Wasfi IA, Wajid SA, Agha BA, Kamel AM, Al Biriki NA, Al Neaimi KM, *et al.* Quantification of furosemide in camel plasma by high resolution mass spectrometry, application on pharmacokinetics. *Biomed Chromatogr* 2017; 31(6) doi: 10.1002/bmc.3902.
125. Thomas A, Geyer H, Schänzer W, Crone C, Kellmann M, Moehring T, *et al.* Sensitive determination of prohibited drugs in dried blood spots (DBS) for doping controls by means of a benchtop quadrupole/Orbitrap mass spectrometer. *Anal Bioanal Chem* 2012;403(5):1279–89.
126. Domínguez-Romero JC, García-Reyes JF, Martínez-Romero R, Berton P, Martínez-Lara E, Del Moral-Leal ML, *et al.* Combined data mining strategy for the systematic identification of sport drug metabolites in urine by liquid chromatography time-of-flight mass spectrometry. *Anal Chim Acta* 2013;761:1–10.
127. Esposito S, Deventer K, T'Sjoen G, Vantilborgh A, Delbeke FT, Goessaert A-S, *et al.* Qualitative detection of desmopressin in plasma by liquid chromatography–tandem mass spectrometry. *Anal Bioanal Chem* 2012;402(9):2789–96.
128. Thomas A, Solymos E, Schänzer W, Baume N, Saugy M, Dellanna F, *et al.* Determination of Vasopressin and Desmopressin in urine by means of liquid chromatography coupled to quadrupole time-of-flight mass spectrometry for doping control purposes. *Anal Chim Acta* 2011;707(1–2):107–13.
129. Görgens C, Guddat S, Thomas A, Thevis M. Recent improvements in sports drug testing concerning the initial testing for peptidic drugs (< 2 kDa) - sample preparation, mass spectrometric detection, and data review. *Drug Test Anal* 2018;10(11–12):1755–60.
130. Thomas A, Görgens C, Guddat S, Thieme D, Dellanna F, Schänzer W, *et al.* Simplifying and expanding the screening for peptides <2 kDa by direct urine injection, liquid chromatography, and ion mobility mass spectrometry: Liquid Chromatography. *J Sep Sci* 2016;39(2):333–41.

131. Kwok WH, Ho ENM, Lau MY, Leung GNW, Wong ASY, Wan TSM. Doping control analysis of seven bioactive peptides in horse plasma by liquid chromatography–mass spectrometry. *Anal Bioanal Chem* 2013;405(8):2595–606.
132. Morin L-P, Mess J-N, Garofolo F. Large-molecule quantification: sensitivity and selectivity head-to-head comparison of triple quadrupole with Q-TOF. *Bioanalysis* 2013;5(10):1181–93.
133. Esposito S, Deventer K, Giron AJ, Roels K, Herregods L, Verstraete A, *et al.* Investigation of urinary excretion of hydroxyethyl starch and dextran by uhplc-hrms in different acquisition modes. *Biol Sport* 2014;31(2):95–104.
134. Kolmonen M, Leinonen A, Kuuranne T, Pelander A, Deventer K, Ojanperä I. Specific screening method for dextran and hydroxyethyl starch in human urine by size exclusion chromatography–in-source collision-induced dissociation–time-of-flight mass spectrometry. *Anal Bioanal Chem* 2011;401(2):563–71.
135. Görgens C, Guddat S, Thomas A, Wachsmuth P, Orlovius A-K, Sigmund G, *et al.* Simplifying and expanding analytical capabilities for various classes of doping agents by means of direct urine injection high performance liquid chromatography high resolution/high accuracy mass spectrometry. *J Pharm Biomed Anal* 2016;131:482–96.
136. Wang H, Chen H, Geng J, Zheng Y, Zhang Z, Sun L, *et al.* Quantitative analysis of dextran in rat plasma using Q-Orbitrap mass spectrometry based on all ion fragmentation strategy. *J Chromatogr B*. 2018;1095:24–31.
137. Duclos M. Glucocorticoids: A Doping Agent? *Endocrinol Metab Clin N Am* 2010; 39:107–26.
138. López-García M, Romero-Gonzalez R, Frenich AG. Determination of steroid hormones and their metabolite in several types of meat samples by ultra-high performance liquid chromatography—Orbitrap high resolution mass spectrometry. *J Chromatogr A* 2018; doi:10.1016/j.chroma.2018.01.052_
139. Fidani M, Pompa G, Mungiguerra F, Casati A, Fracchiolla ML, Arioli F. Investigation of the presence of endogenous prednisolone in equine urine by high-performance liquid chromatography mass spectrometry and high-resolution mass spectrometry. *Rapid Commun Mass Spectrom* 2012;26:879–86.

140. Herrero P, Cortés-Francisco N, Borrull F, Caixach J, Pocurull, Marcé RM. Comparison of triple quadrupole mass spectrometry and Orbitrap high-resolution mass spectrometry in ultrahigh performance liquid chromatography for the determination of veterinary drugs in sewage: benefits and drawbacks. *J Mass Spectrom* 2014;49:585-96.
141. Matysik S, Liebisch G. Quantification of steroid hormones in human serum by liquid chromatography-high resolution tandem mass spectrometry. *J Chromatogr A* 2017; <https://doi.org/10.1016/j.chroma.2017.10.042>.
142. Fung EN, Xia Y, Aubry AF, Zeng J, Olah T, Jemal M. Full-scan high resolution accurate mass spectrometry (HRMS) in regulated bioanalysis: LC-HRMS for the quantitation of prednisone and prednisolone in human plasma. *J Chromatogr B* 2011;879:2919-27.
143. Wong JKY, Chan GHM, Choi TLS, Kwok KY, Lau MY, Leung GNW *et al.* A High-Throughput and Broad-Spectrum Screening Method for Analysing Over 120 Drugs in Horse Urine by Liquid Chromatography-High Resolution Mass Spectrometry. *Drug Test Anal* 2020;12(7):900-17.
144. De Clercq N, Van Meulebroek L, Bussche JV, Croubels S, Delahaut P, Vanhaecke L. The impact of stress on the prevalence of prednisolone in bovine urine: A metabolic fingerprinting approach. *J Steroid Biochem Mol Biol* 2015;154:206-16.
145. De Clercq N, Bussche VB, Croubels S, Delahaut P, Vanhaeck L. A validated analytical method to study the long-term stability of natural and synthetic glucocorticoids in livestock urine using ultra-high performance liquid chromatography coupled to Orbitrap-high resolution mass spectrometry. *J Chromatogr A* 2013;1301:111-21.
146. Chen SY, Fang M, Lin YT, Tsai CF, Cheng HF, Wang DY. Elucidation of two new corticosteroids, betamethasone dibutyrate and betamethasone tributyrate. *Steroids* 165 2021; [doi:10.1016/j.steroids.2020.108739](https://doi.org/10.1016/j.steroids.2020.108739).
147. Zhou S, Guo C, Shi F, Jiang W, Wang L. Application of an ultrahigh-performance liquid chromatography coupled to quadrupole-orbitrap high-resolution mass spectrometry for the rapid screening, identification

and quantification of illegal adulterated glucocorticoids in herbal medicines. *J Chromatogr B* 2016;1038:34-42.

148. Karatt TK, Nalakath J, Perwad Z, Albert PH, Kader KKA, Padusha MSA, Laya S. Mass spectrometric method for distinguishing isomers of dexamethasone via fragment mass ratio: a HRMS approach. *J Mass Spectrom* 2018;53(11):1046-58.

149. Karatt TK, Sathiq MA, Laya S. Is 9β -dehydrohalogenation of betamethasone and dexamethasone hindering the detection of banned co-eluting meprednisone? A reverse-phase chiral liquid chromatography high-resolution mass spectrometry approach. *Steroids* 2019;doi:10.1016/j.steroids.2019.108572.

150. Karatt TK, Sayed R, Nalakath J, Perwad Z, Albert PH, Kader KA. Separation and identification of the epimeric doping agents -Dexamethasone and betamethasone in equine urine and plasma: A reversed phase chiral chromatographic approach. *Steroids* 2018;140:77-82.

151. Pozo OJ, Van Eenoo P, Deventer K, Lootens L, Grimalt S, Sancho JV, *et al.* Detection and structural investigation of metabolites of stanozolol in human urine by liquid chromatography tandem mass spectrometry. *Steroids* 2009;74(10-11):837-52.

152. de Albuquerque Cavalcanti G, Garrido BC, Leal FD, Padilha MC, de la Torre X, de Aquino Neto FR. Detection of new urinary exemestane metabolites by gas chromatography coupled to mass spectrometry. *Steroids* 2011;76(10):1010-5.

153. Thevis M, Sigmund G, Gougoulidis V, Beuck S, Schlörner N, Thomas A, *et al.* Screening for benfluorex and its major urinary metabolites in routine doping controls. *Anal Bioanal Chem* 2011;401(2):543-51.

154. Beuck S, Schänzer W, Thevis M. Investigation of the in vitro metabolism of the emerging drug candidate S107 for doping-preventive purposes. *J Mass Spectrom* 2011;46(2):112-30.

155. Kuuranne T, Leinonen A, Schänzer W, Kamber M, Kostianen R, Thevis M. Aryl-Propionamide-Derived Selective Androgen Receptor Modulators: Liquid Chromatography-Tandem Mass Spectrometry Characterization of the in Vitro Synthesized Metabolites for Doping Control Purposes. *Drug Metab Dispos* 2008;36(3):571-81.

156. Thevis M, Gerace E, Thomas A, Beuck S, Geyer H, Schlörer N, *et al.* Characterization of in vitro generated metabolites of the selective androgen receptor modulators S-22 and S-23 and in vivo comparison to post-administration canine urine specimens. *Drug Test Anal* 2010;2(11-12):589-98.
157. Thevis M, Thomas A, Fußhöller G, Beuck S, Geyer H, Schänzer W. Mass spectrometric characterization of urinary metabolites of the selective androgen receptor modulator andarine (S-4) for routine doping control purposes. *Rapid Commun Mass Spectrom* 2010;24(15):2245-54.
158. Thevis M, Lagojda A, Kuehne D, Thomas A, Dib J, Hansson A, *et al.* Characterization of a non-approved selective androgen receptor modulator drug candidate sold via the Internet and identification of in vitro generated phase-I metabolites for human sports drug testing *Rapid Commun Mass Spectrom* 2015;29(11):991-9.
159. Fragkaki AG, Sakellariou P, Kiouisi P, Kioukia-Fougia N, Tsiyou M, Petrou M, *et al.* Human in vivo metabolism study of LGD-4033. *Drug Test Anal* 2018;10(11-12):1635-45.
160. Cutler C, Viljanto M, Hincks P, Habershon-Butcher J, Muir T, Biddle S. Investigation of the metabolism of the selective androgen receptor modulator LGD-4033 in equine urine, plasma and hair following oral administration. *Drug Test Anal* 2020;12(2):247-60.
161. Piper T, Dib J, Putz M, Fusshöller G, Pop V, Lagojda A, *et al.* Studies on the in vivo metabolism of the SARM YK11: Identification and characterization of metabolites potentially useful for doping controls. *Drug Test Anal* 2018;10(11-12):1646-56.
162. Piper T, Fusshöller G, Schänzer W, Lagojda A, Kuehne D, Thevis M. Studies on the in vivo metabolism of methylstenbolone and detection of novel long term metabolites for doping control analysis. *Drug Test Anal* 2019;11(11-12):1644-55.
163. Loke S, Liu L, Wenzel M, Scheffler H, Iannone M, de la Torre X, *et al.* New Insights into the Metabolism of Methyltestosterone and Metandienone: Detection of Novel A-Ring Reduced Metabolites. *Molecules* 2021;26(5):1354.
164. Zvereva I, Dudko G, Dikunets M. Determination of GnRH and its synthetic analogues' abuse in doping control: Small bioactive peptide UPLC-MS/MS method extension by addition of in vitro and in vivo

metabolism data; evaluation of LH and steroid profile parameter fluctuations as suitable biomarkers. *Drug Test Anal* 2018;10(4):711-22.

165. Gajda PM, Holm NB, Hoej LJ, Rasmussen BS, Dalsgaard PW, Reitzel LA *et al.* Glycine-modified growth hormone secretagogues identified in seized doping material. *Drug test anal* 2019;11(2):350-54.

166. Guan F, Fay S, Li X, You Y, Robinson MA. Identification of ex vivo catabolites of peptides with doping potential in equine plasma by HILIC-HRMS. *Drug Test Anal* 2020;12(6):771-84.

167. Lange T, Thomas A, Gorgens C, Bidlingmaier M, Schilbach H, Fichant E, Delahaut P, Thevis M. Comprehensive insights into the formation of metabolites of the ghrelin mimetics capromorelin, macimorelin and tabimorelin as potential markers for doping control purposes. *Biomed Chromatogr* 2021;35(6):e5075.

168. Geldof L, Deventer K, Roels K, Tudela E, Van Eeno P. In Vitro Metabolic Studies of REV-ERB Agonists SR9009 and SR9011. *Int J Mol Sci* 2016;17(10):1676.

169. Cutler C, White DL, Viljanto M. In vitro metabolism of the REV-ERB agonist SR-9009 and subsequent detection of metabolites in associated routine equine plasma and urine doping control samples. *Drug Test Anal* 2021; doi: 10.1002/dta.3124.

170. Dib J, Thomas A, Delahaut P, Fichant E, Schänzer W, Thevis M. Identification and characterization of in vitro and in vivo generated metabolites of the adiponectin receptor agonists AdipoRon and 112254. *J Pharm Biomed Anal* 2016;125:68-76.

171. Kintz P, Ameline A, Gheddar L, Raul J-S. Testing for GW501516 (cardarine) in human hair using LC/MS-MS and confirmation by LC/HRMS. *Drug Test Anal* 2020;12(7):980-6.

172. Thevis M, Möller I, Thomas A, Beuck S, Rodchenkov G, Bornatsch W, *et al.* Characterization of two major urinary metabolites of the PPAR δ -agonist GW1516 and implementation of the drug in routine doping controls. *Anal Bioanal Chem* 2010; 396(7):2479-91.

173. Ishii H, Shibuya M, Leung GN, Nozawa S, Yamashita S, Yamada M, *et al.* Detection and longitudinal distribution of GW1516 and its metabolites in equine hair for doping control using liquid

chromatography/high-resolution mass spectrometry. *Rapid Commun Mass Spectrom* 2021;doi: 10.1002/rcm.9050.

174. Ishii H, Shibuya M, Leung GN, Yamashita S, Yamada M, Kushiro A, *et al.* Metabolic study of GW1516 in equine urine using liquid chromatography/electrospray ionization Q-Exactive high-resolution mass spectrometry for doping control. *Rapid Commun Mass Spectrom* 2021;doi: 10.1002/rcm.9028.

175. Kintz P, Gheddar L, Ameline A, Arbouche N, Raul J-S. Hair testing for doping agents. What is known and what remains to do. *Drug Test Anal* 2020;12(3):316–22.

176. Yuan Y, Xu Y, Lu J. Dried blood spots in doping analysis. *Bioanalysis* 2021;13(7):587–604.

177. Strano-Rossi S. Screening for exogenous androgen anabolic steroids in human hair by liquid chromatography/orbitrap-high resolution mass spectrometry. *Anal Chim Acta* 2013;793:61-71.

178. Gheddar L, Raul J-S, Kintz P. Testing for Stanozolol, Using UPLC–MS-MS and Confirmation by UPLC–q-TOF-MS, in Hair Specimens Collected from Five Different Anatomical Regions. *J Anal Toxicol* 2020;44(8):834–9.

179. Kintz P, Gheddar L, Ameline A, Raul JS. Identification of S22 (ostarine) in human nails and hair using LC-HRMS. Application to two authentic cases. *Drug Test Anal* 2020; 12(10):1508-13.

180. Choi TLS, Kwok KY, Kwok WH, Tsoi YYK, Wong JKY, Wan TSM. Detection of seventy-two anabolic and androgenic steroids and/or their esters in horse hair using ultra-high performance liquid chromatography-high resolution mass spectrometry in multiplexed targeted MS2 mode and gas chromatography-tandem mass spectrometry. *J Chromatogr A* 2018;1566:51–63.

181. Kwok KY, Choi TLS, Kwok WH, Wong JKY, Wan TSM. Detection of anabolic and androgenic steroids and/or their esters in horse hair using ultra-high performance liquid chromatography–high resolution mass spectrometry. *J Chromatogr A* 2017;1493:76–86.

182. Devi JL, Zahra P, Vine JH, Whittem T. Determination of testosterone esters in the hair of male greyhound dogs using liquid chromatography–high resolution mass spectrometry. *Drug Test Anal* 2018;10(3):460–73.

183. Schlupp A, Anielski P, Thieme D, Muller RK, Meyer H, Ellendorff F. The β -agonist clenbuterol in mane and tail hair of horses. *Equine vet J* 2004;36 (2):122-118.
184. Machnik M, Geyer H, Horning S, Breidbach A, Delahaut P, Schanzer W. Long-term detection of clenbuterol in human scalp hair by gas chromatography-high-resolution mass spectrometry. *J Chromatogr B* 1999;723:147-55.
185. Thomas A, Geyer H, Schänzer W, Crone C, Kellmann M, Moehring T, *et al.* Sensitive determination of prohibited drugs in dried blood spots (DBS) for doping controls by means of a benchtop quadrupole/Orbitrap mass spectrometer. *Anal Bioanal Chem* 2012;403(5):1279-89.
186. Tretzel L, Thomas A, Geyer H, Gmeiner G, Forsdahl G, Pop V, *et al.* Use of dried blood spots in doping control analysis of anabolic steroid esters. *J Pharm Biomed Anal* 2014;96:21-30.
187. Solheim SA, Levernaes MCS, Mørkeberg J, Juul A, Uppers EN, Nordsborg NB, *et al.* Stability and detectability of testosterone esters in dried blood spots after intramuscular injections. *Drug Test Anal* 2021; doi: 10.1002/dta.3030.
188. Tretzel L, Thomas A, Geyer H, Pop V, Schänzer W, Thevis M. Dried blood spots (DBS) in doping controls: a complementary matrix for improved in- and out-of-competition sports drug testing strategies. *Anal Methods* 2015;7(18):7596-605.
189. Solheim SA, Jessen S, Mørkeberg J, Thevis M, Dehnes Y, Eibye K, *et al.* Single-dose administration of clenbuterol is detectable in dried blood spots. *Drug Test Anal* 2020;12(9):1366-72.
190. Möller I, Thomas A, Geyer H, Schänzer W, Thevis M. Development and validation of a mass spectrometric detection method of peginesatide in dried blood spots for sports drug testing. *Anal Bioanal Chem* 2012;403(9):2715-24.
191. Lange T, Katja Walpurgis, Thomas A, Geyer H, Thevis M. Development of two complementary LC-HRMS methods for analyzing sotatercept in dried blood spots for doping controls. *Bioanalysis* 2019;11(10):923-40.

192. Thomas A, Thevis M. Analysis of insulin and insulin analogs from dried blood spots by means of liquid chromatography–high resolution mass spectrometry. *Drug Test Anal* 2018;10(11–12):1761–8.
193. Gerace E, Modaffari J, Negri P, Di Corcia D, Amante E, Salomone A, *et al.* Detection of the synthetic peptide ipamorelin in dried blood spots by means of UHPLC-HRMS. *Int J Mass Spectrom* 2021; doi.org/10.1016/j.ijms.2021.116531.
194. Lange T, Thomas A, Walpurgis K, Thevis M. 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* 2020;412(15):3765–77.
195. Tretzel L, Görgens C, Geyer H, Thomas A, Dib J, Guddat S, *et al.* Analyses of Meldonium (Mildronate) from Blood, Dried Blood Spots (DBS), and Urine Suggest Drug Incorporation into Erythrocytes. *Int J Sports Med* 2016;37(6):500–2.
196. Fabresse N, Gheddar L, Kintz P, Knapp A, Larabi IA, Alvarez JC. Analysis of pharmaceutical products and dietary supplements seized from the black market among bodybuilders. *Forensic Sci Int* 2021; doi: 10.1016/j.forsciint.2021.110771.
197. Weber C, Krug O, Kamber M, Thevis M. Qualitative and Semiquantitative Analysis of Doping Products Seized at the Swiss Border. *Subst Use Misuse* 2017;52(6):742–53.
198. Odoardi S, Mestria S, Biosa G, Valentini V, Federici S, Strano Rossi S. An overview on performance and image enhancing drugs (PIEDs) confiscated in Italy in the period 2017–2019. *Clin Toxicol (Phila)* 2021; 59(1):47–52.
199. Lorenz, Lisa & Toomey, Valerie & Lanzarotta, Adam & Flurer, Rick & Falconer, Travis. Identification of the Designer Steroid Androsta-3,5-diene-7,17-dione in a Dietary Supplement. *Drug Test Anal* 2019;11(7):1109–15.
200. Falconer TM, Flurer RA, Jones MB, Lorenz LM, Voelker SE. Identification of the anabolic steroid 6 β -chlorotestosterone in a dietary supplement. *Drug Test Anal.* 2019;11(3):422–27.

201. Thevis M, Geyer H, Kamber M, Schänzer W. Detection of the arylpropionamide-derived selective androgen receptor modulator (SARM) S-4 (Andarine) in a black-market product. *Drug Test Anal* 2009;1(8):387-92.
202. Leaney AE, Beck P, Biddle S, Brown P, Grace PB, Hudson SC, Mawson DH. Analysis of supplements available to UK consumers purporting to contain selective androgen receptor modulators. *Drug Test Anal* 2021;13(1):122-27.
203. Thomas A, Kohler M, Mester J, Geyer H, Schänzer W, Petrou M, Thevis M. Identification of the growth-hormone-releasing peptide-2 (GHRP-2) in a nutritional supplement. *Drug Test Anal* 2010;2(3):144-8.
204. Doué M, Dervilly-Pinel G, Pouponneau K, Monteau F, Le Bizec B. Direct analysis in real time - high resolution mass spectrometry (DART-HRMS): a high throughput strategy for identification and quantification of anabolic steroid esters. *Drug Test Anal* 2015;7(7):603-8.
205. Chen SY, Fang M, Lin YT, Tsai CF, Cheng HF, Wang DY. Elucidation of two new corticosteroids, betamethasone dibutyrate and betamethasone tributyrate. *Steroids* 2021; doi: 10.1016/j.steroids.2020.108739.

Table 1. Summary of methods' characteristics for peptide and protein analysis by HRMS

| Peptides | Matrix | LOD/LOQ | Sample preparation | MS instrument | Reference |
|---|-------------|--|--------------------------|--------------------|-----------|
| <u>ESAs</u> | | | | | |
| rHuEPO | Plasma | 500 pg/mL | Immunopurification | MS/MS + Q-Orbitrap | [50] |
| Sotatercept | Serum | 10 ng/mL | Immunopurification | MS/MS + Q-Orbitrap | [51] |
| Peginesatide | Whole blood | 1 ng/mL | Precipitation /digestion | MS/MS + Q-Orbitrap | [53] |
| <u>Gnrh</u> | | | | | |
| Buserelin, Deslorelin Gonadorelin Goserelin Leuprorelin Nafarelin Triptorelin | Urine | 10 pg/mL 12.5 pg/mL 25 pg/mL 10 pg/mL 10 pg/mL 50 pg/mL 10 pg/mL | Precipitation/SPE WCX | MS/MS + Q-Orbitrap | [61] |
| Buserelin Deslorelin Goserelin Nafarelin Triptorelin | Urine | 0.125 pg/mL 0.125 pg/mL 0.125 pg/mL 0.25 pg/mL 0.25 pg/mL | Dilute and shoot | MS/MS + Q-Orbitrap | [62] |

| <u>Corticotrophins</u> | | | | | |
|-----------------------------------|-------------|----------------------|--|--------------------|-------|
| CRF | Plasma | 0.5 ng/mL | SPE MCX | Full MS + TOF | [78] |
| CRH | Plasma | 200 pg/mL | Immunopurification | MS/MS + Q-Orbitrap | [63] |
| <u>GH</u> | | | | | |
| rhGH | Plasma | 2.5 ng/mL | Precipitation/SPE C4 SPE C4 | MS/MS + Q-Orbitrap | [65] |
| <u>GHRH</u> | | | | | |
| Sermorelin, Tesamorelin, CJC-1295 | Urine | 0.2 ng/mL | Immunopurification | MS/MS+ Q-Orbitrap | [67] |
| Semorelin, CJC-1293, CJC-1295 | Blood/urine | < 5 pg/mL / 0.2ng/mL | Ultrafiltration/ Immunopurification | MS/MS + Q-Orbitrap | [71] |
| Tesamorelin | | | | | |
| <u>GHRPs</u> | | | | | |
| Alexamorelin | Blood- DBS | 1 ng/mL | SPE | MS/MS + Q-Orbitrap | [167] |
| GHRP-1 | | 10 ng/mL | | | |
| GHRP-2 | | 2 ng/mL | | | |
| GHRP-3 | | 5 ng/mL | | | |
| GHRP-4 | | 2 ng/mL | | | |
| GHRP-5 | | 2 ng/mL | | | |
| GHRP-6 | | 2 ng/mL | | | |
| Hexarelin | | 10 ng/mL | | | |

| <u>GHS</u> | | | | | |
|--------------------------------------|-------|---------------------------|---------------------|--------------------|------|
| Ipamorelin Anamorelin | Urine | 0.05 ng/mL 0.125 ng/mL | Dilute-and-shoot | MS/MS + Q-Orbitrap | [62] |
| <u>Growth Factors and modulators</u> | | | | | |
| IGF-1 | Blood | 50 ng/mL | SPE C18 | MS/MS + Q-orbitrap | [74] |
| IGF-1 | Serum | 15 ng/mL | Ultracentrifugation | MS/MS + Q-Orbitrap | [76] |

Table 2: Overview of metabolism studies on doping agents using HRMS

| Compounds | Prohibited class | Sample | Pretreatment | MS-instrument | Ionization mode | Reference |
|--|------------------|---------------------------|---|-----------------|-----------------|-----------|
| Stanozolol | S1 | Mice urine Human urine | Enzymatic hydrolysis LLE (pH 9.2) | LC-QTOF | ESI+/- | [151] |
| Exemestane | S4 | Human urine | Enzymatic hydrolysis LLE (pH 10) Derivatization (TMS) | GC-QTOF | EI | [152] |
| Benfluorex | S6 | Human urine | Dilute and shoot | LC-Q-Orbitrap | ESI+ | [153] |
| S-107 | S0 | HLM and S-9 | Enzymatic hydrolysis SPE (Oasis HLB) | LC-LTQ-Orbitrap | ESI+ | [154] |
| 4 Aryl-Propionamide derived SARMs | S1 | HLM and S-9 | LLE (acidic pH) | LC-LTQ-Orbitrap | ESI- | [155] |

| | | | | | | |
|--|----|--|---|---|------------|-------|
| Ostarine Andarine | S1 | HLM and S-9 Dog urine | LLE (pH 4.5-5.0) SPE (PAD-1) | LC-Q-Orbitrap | ESI- | [156] |
| Andarine | S1 | Human urine | Enzymatic hydrolysis SPE (PAD-1) | LC-LTQ-Orbitrap | ESI- | [157] |
| LGD-4033 (Ligandrol) | S1 | HLM <i>C. Elegans</i> suspension | Dilute and shoot | LC-Q-Orbitrap | ESI- | [158] |
| LGD-4033 (Ligandrol) | S1 | Human urine | Enzymatic hydrolysis Dilute and shoot | LC-QTOF | ESI- | [159] |
| LGD-4033 (Ligandrol) | S1 | ELM Equine urine | Enzymatic hydrolysis LLE (pH 6.8) | LC-LTQ-Orbitrap | ESI- | [160] |
| YK-11 | S1 | Human urine | Enzymatic hydrolysis SPE (C18) + LLE (pH 7-10) HPLC Clean-up Derivatization (TMS) | GC-QTOF GC-Q-Orbitrap | EI | [161] |
| Methylstenbolone | S1 | Human urine | Enzymatic hydrolysis SPE (C18) + LLE (pH 7-10) HPLC Clean-up Derivatization (TMS) | GC-QTOF GC-Q-Orbitrap | EI | [162] |
| Trenbolone | S1 | Human urine | Enzymatic hydrolysis SPE (C18) + LLE (pH 7-10) HPLC Clean-up Derivatization (TMS) for GC | LC-Q-Orbitrap GC-QTOF GC-Q-Orbitrap | ESI+ EI | [18] |
| Methyltestosterone Metandienone | S1 | Human urine | Enzymatic hydrolysis LLE (Basic pH) Derivatization (TMS) | GC-QTOF | EI | [163] |
| 10 GnRH | S2 | HKM Human urine | SPE (Oasis WCX) | LC-Q-Orbitrap | ESI+ | [164] |
| 27 bioactive peptides | S2 | Equine plasma | SPE (anion exchange) | HILIC-Q-Orbitrap | ESI+ | [166] |

| | | | | | | |
|--|----|---------------------------------|--|---------------|---------|-------|
| Capromorelin Macimorelin Tabimorelin | S2 | Rat urine Human serum HLM | Dilute and shoot/SPE (urine) Protein precipitation (serum, HLM) | LC-Q-Orbitrap | APCI+ | [167] |
| SR-9009 SR-9011 | S4 | HLM | Dilute and shoot | LC-Q-Orbitrap | ESI +/- | [168] |
| AdipoRon 112254 | S4 | HLM and S-9 Rat urine | <u>Protein precipitation and evaporation (HLM, S-9) Direct injection (urine)</u> | LC-Q-Orbitrap | ESI+ | [170] |
| GW1516 | S4 | HLM | <u>Protein precipitation + LLE</u> | LC-Q-Orbitrap | ESI+ | [172] |
| Lomerizine/ Trimetazidine | S4 | HLM Human urine | <u>SPE</u> | LC-QTOF | ESI+ | [121] |
| <p>HLM: Human Liver Microsomes, HKM: Human Kidney Microsomes, ELM: Equine Liver Microsomes, LLE: Liquid-Liquid Extraction, SPE: Solid-Phase Extraction, S-9: S9 Fraction, ESI: Electrospray Ionization, EI: Electron Ionization, TMS: Trimethyl-silylation, LTQ: Linear Trap Quadrupole, QTOF: Quadrupole time-of-flight</p> | | | | | | |

Table of acronyms

| AAFs | Adverse analytical findings |
|---------------|--|
| AAS | Anabolic androgenic steroids |
| ACTH | Adrenocorticotrophic hormone |
| AIF | All ion fragmentation |
| AMPK | Adenosine monophosphate activated protein kinase |
| APB | Athlete's biological passport |
| AAFs | Adverse analytical findings |
| CEBA | Continuous erythropoietin receptor activator |
| CID | Collision-induced dissociation |
| DART | Direct analysis in real time-high resolution mass spectrometry |
| DHEA | Dehydroepiandrosterone |
| ELISA | Enzyme linked immunosorbent assay |
| EPO | Erythropoietin |
| ESI | Electrospray ionization |
| EPO | Erythropoietin |
| FSH | Follicle stimulating hormone |
| GC | Gas chromatography |
| GHRH | Growth hormone releasing hormone |
| GHRPs | Growth hormone releasing peptides |
| GnRH | Gonadotropin-Releasing Hormon |
| HCD | High energy C trap dissociation |
| hCG | Human chorionic gonadotropin |
| hGH | Human growth hormone |
| HLM | Human liver microsomes |
| HKM | Human kidney microsomes |
| HRMS | High resolution mass spectrometry |
| IAC | Immunoaffinity cartridges |
| IGF- I | Insulin-like growth factor I |
| LH | Luteinising hormone |
| LLE | Liquid liquid extraction |
| LOD | Limit of detection |

| | |
|---------------------------------|--|
| LOQ | Limit of quantification |
| MRPL | Minimum required performance level |
| MS/MS | Tandem mass spectrometry |
| MSIA | Mass spectrometry immunoassay |
| NCE | Normalized collision energy |
| Pro-III-NP | Procollagen III amino-terminal propeptide |
| PPAR-α | Peroxisome proliferator activated receptors |
| PRM | Parallel reaction monitoring |
| Q-ToF | Quadrupole time-of-flight mass spectrometry |
| Q-Exactive | Quick, Easy, Cheap, Efficient, Rugged and Safe |
| rHuEPO | Recombinant Erythropoietin |
| RIA | Radioimmunoassay |
| rHh | Recombinant luteinizing hormone |
| SARMs | Selective androgen modulator receptors |
| SPE | Solid-phase extraction |
| TSU | Thyroid stimulating hormone |
| UV | Ultraviolet |
| WADA | World Anti-Doping Agency |