



## Review Article

# A comprehensive review on current analytical approaches used for the control of drug abuse in sports

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## ABSTRACT

Doping in sports is defined as violating any anti-doping guidelines issued by World anti-doping agency (WADA), which might include ingesting illegal or illicit performance-enhancing medications or using other unlawful practices. Doping has been scientifically shown to have major adverse health impacts, such as hypertension, an increased prevalence of cardio-vascular ailments, liver damage, as well as psychological dependence. It also plainly tarnishes the spirit, prestige, and worth of sports. Moreover, it is also linked to a number of untimely deaths in mainstream sports. As a result, there is a significant priority imposed on the present globalised issue of doping in sports and thus, this problem is becoming more and more apparent to health authorities, leading to the issuance of numerous novel techniques and guidelines to control doping in many literature reviews and editorials that have been publicly released till present day, but the large percentage of them are strictly limited to the anti-doping analytical advances only within a particular class of drugs. Advances in analytical chemistry are a requisite for the majority of significant anti-doping research fields, hence numerous such analytical based experimental approaches or techniques, like mass spectrometry, gas chromatography, etc., are routinely employed to detect doping in athletes. Herein, this review aims to provide a thorough and critical assessment of the academic research on the substantial advancements in anti-doping analytical approaches over the last decade and also, provides a broader visionary for various classes and their respective subclasses of prohibited drugs/substances along with their chemical, structural, biological and analytical profile along with their utilization as per the official guidelines of World anti-doping agency (WADA).

**Abbreviations:** A, Androsterone; AAF, Adverse Analytical Finding; ABP, Athlete Biological Passport; CIR, Carbon Isotope Ratio; CLEIA, Chemiluminescence Assay; CPDA, Citrate Phosphate Dextrose Adenine solution; DBS, Dried Blood Spot; DHCMT, Dehydrochloromethyltestosterone; DHEA, Dehydroepiandrosterone; DHT, Dihydrotestosterone; DUS, Dried Urine Spots; EG, Epitestosterone Glucuronidated; ELISA, Enzyme-linked Immunosorbent Assay; EpiT, Epitestosterone; ES, Epitestosterone sulfated; FSM, Full Scan Mode; GC/C/IRMS, Gas Chromatography/Combustion/ Isotope Ratio Mass Spectrometry; GC, Gas Chromatography; GC-MS/MS, Gas Chromatography- Mass Spectrometry/ Mass Spectrometry; GC-NPD/MS, Gas Chromatography- Nitrogen Phosphate Detector/ Mass Spectrometry; GH, Growth Hormone; hCG, Human Chorionic Goadotropin; HRMS, High Resolution mass Spectrometry; IGF-1, Insulin-like Growth Factor-1; IRMA, Immunoradiometric Assay; IRMS, Isotope-Ratio Mass Spectrometry; LC, Liquid Chromatography; LC-MS/MS, Liquid Chromatography- Mass Spectrometry/ Mass Spectrometry; LLE, Liquid Liquid Extraction; LOD, Limit of Detection; LOI, Limit of identification; LOQ, Limits of Quantification; MDGC, Multi-dimensional Gas Chromatography; MGF, Mechano Growth Factor; GHS, Growth Hormone Secretagogues; MRL, Minimum Reporting Level; MRM, Multiple Reaction Monitoring; MRPL, Minimum Required Performance Level; PCA, Principal Component Analysis; PCR, Polymerase Chain Reaction; PEDs, Performance Enhancing Drugs; AAS, Anabolic Androgen Steroid; QTOF, Quadrupole Time of flight; SALDI/MS, Surface-Assisted Laser Desorption Ionisation/ Mass Spectrometry; SARM, Selective Androgen Receptor Modulators; SAR-PAGE, Sarcosyl Polyacrylamide Gel Electrophoresis; SERM, Selective Oestrogen Receptor Modulators; SPE, Solid Phase Extraction; SRM, Selected Reaction Monitoring; T, Testosterone; TG, Testosterone Glucuronidated; THC,  $\Delta^9$ -Tetrahydrocannabinol; TS, Testosterone sulfated; UHPLC, Ultra High-Performance Liquid Chromatography; VAMS, Volumetric Absorptive Micro-Sampling; WADA, World Anti-Doping Agency.

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## 1. Introduction

Doping is essentially the unethical consumption of substances that seems to enhance one's performance, once ingested in copious quantities [1]. The usage of performance-enhancing drugs (PEDs) to enhance efficiency in sport traces back to the 3rd century BCE in Greek Olympics, where sportsmen used a wide assortment of medicinal herbs like wine, halogenic mushrooms, and sometimes even animal testicles in the optimism to become physically stronger [2]. In the late 1800 s and early 1900 s, numerous athletes utilized special dopants to achieve a competitive edge. These specialized dopants contained a customizable unique blend of flavourings including such brandy, caffeine, cocaine, and heroin aimed to minimize fatigue, enhance ability to focus, restrict hunger, and ease the pain of physical extortion. The first sports body to forbid the use of performance-enhancing substances was the International Association of Athletes Federation and was established in 1928. Doping with performance-enhancing stimulants has become a progressively significant matter in both amateur and professional sporting activities [3,4]. Assessing the pervasiveness of doping in sport is pertinent for all units associated with sports organizations; but it is important to evaluate the incidence as well as the frequency of doping for anti-doping officials to assess the efficacy of their anti-doping policy initiatives and also the relevance of the socioeconomic capital invested to avoid favourable perceptions toward doping [5,6]. The assumption that doped athletes have negative effects on elite athletes has been supported and substantiated by a number of comprehensive research [7]. A range of scientific methodologies have been carried out to assess and improve the detection and treatment of the present doping situation. As the detection method for performance enhancing drugs in sports athletes has enhanced, a growing percentage of sportsmen are now being zapped as the detection limits become more substantial [8]. The World Anti-Doping Agency (WADA) was established in 1999, ever since, there

seems to be a significant anti-doping campaign among sports organisations and government authorities, with the main objective of minimizing the incidence and frequency of doping in sports. The goal of WADA is to spearhead a cooperative global movement for doping-free sport worldwide. A Prohibited List is a list of illicit substances and practices for the sportspersons is maintained on a regular basis by the WADA [9]. The prohibited list of 2022 delineates performance-enhancing substances into multiple categories, such as "Prohibited at all times", "Prohibited in-competition", and "Prohibited in particular sports" (see Table 1). The aforementioned categories further contain drugs of distinct classes, such as anabolic androgenic steroids, cannabinoids, and beta-blockers respectively [9]. Table 2.

Sports physicians must be aware of the procedure and legal requirements governing drugesting while treating an athlete [10]. Significant advancements have been documented in every aspect of anti-doping analysis. The achieved advancements were made possible by the coordinated efforts of many research projects, which improved our understanding of drug metabolism, provided new insights into emerging drug categories, provided new/complementary sample collection and preparation techniques, and significantly improved analytical instrumentation that opened up new possibilities for data quality, (re)processing, and use [11]. Drug testing procedures includes series of processes like selection process in which Athletes may be recruited at any point to undergo testing followed by sample [urine/ blood/ dried blood spots (DBS)] collection which is further subjected to analysis by anti-doping laboratories (Fig. 1) [12]. The merits of collecting urine samples over the blood samples, which remains as invasive and also has a restricted capacity, comprise their non-invasive collection and also, their ease of access to large volumes of matrices [13]. Additionally, certain specifically listed requirements are briefed in many scientific documents and also the regulatory Guidelines that highlight a number of different subjects, including assays and documentation of endogenous

**Table 1**  
Prohibited Substances according to WADA Prohibited List 2022.

| Code | Class                                                                              | Sub-class                                                | Examples                                                                                        | Specified/Non-Specified Substances | Prohibited (At all times/ in competition only) |
|------|------------------------------------------------------------------------------------|----------------------------------------------------------|-------------------------------------------------------------------------------------------------|------------------------------------|------------------------------------------------|
| S0   | Non-approved substances                                                            | –                                                        | AdipoRon, BPC-157                                                                               | Specified                          | At all times                                   |
| S1   | Anabolic androgenic steroids                                                       | –                                                        | Testosterone, Stanozolol, Methandriol, Nandrolone, Epitestosterone                              | Non-specified                      | At all times                                   |
| S2   | Other anabolic substances<br>Peptide hormones, growth hormones, related substances | –                                                        | Clenbuterol, Osilodrostat, Zeranor                                                              | Non-specified                      | At all times                                   |
|      |                                                                                    | Erythropoietin (EPO) and agents affecting erythropoiesis | Darbepoetins, CNT-530, Peginesatide, Cobalt, Molidustat, K-11706, Sotatercept, carbamylated EPO | Non-specified                      | At all times                                   |
|      |                                                                                    | Peptide hormones and their releasing factors             | Gonadorelin, Corticorelin hGH-176–191, Alexamorelin, Lenomorelin                                | Non-specified                      | At all times                                   |
| S3   | Beta-2 agonists                                                                    | Growth factors and growth factor modulators              | FGFs, HGF, IGF-1, MGFs, VEGF, TB-500                                                            | Non-specified                      | At all times                                   |
|      |                                                                                    | –                                                        | Arformoterol, Higenamine, Salbutamol, Terbutaline                                               | Specified                          | At all times                                   |
| S4.1 | Hormone and metabolic modulators                                                   | Aromatase inhibitors                                     | 2-Androstenoil, Anastrozole, Letrozole, Testolactone                                            | Specified                          | At all times                                   |
| S4.2 | Hormone and metabolic modulators                                                   | Anti-estrogenic substances                               | Cyclofenil, Fulvestrant, Tamoxifen                                                              | Specified                          | At all times                                   |
| S4.3 | Hormone and metabolic modulators                                                   | Agents preventing activin receptor IIB activation        | Activin A-neutralizing antibodies, Anti-activin receptor IIB antibodies (Bimagrumab)            | Specified                          | At all times                                   |
| S4.4 | Hormone and metabolic modulators                                                   | Metabolic modulators                                     | Insulin and mimetics, Trimetazidine                                                             | Specified                          | At all times                                   |
| S5   | Diuretics and Masking agents                                                       | –                                                        | Acetazolamide, spironolactone, furosemide                                                       | Specified                          | At all times                                   |
| S6.A | Stimulants                                                                         | –                                                        | Amphetamine, Fencamine, Cocaine, Fenfluramine                                                   | Non-Specified                      | In competition only                            |
| S6.B | Stimulants                                                                         | –                                                        | Ethylphenidate, Pentetrazol, Selegiline, Strychnine                                             | Specified                          | In competition only                            |
| S7   | Narcotics                                                                          | –                                                        | Buprenorphine, Hydromorphone, Oxycodone, Methadone                                              | Specified                          | In competition only                            |
| S8   | Cannabinoids                                                                       | –                                                        | Hashish, Marijuana, Natural and synthetic tetrahydrocannabinols                                 | Specified                          | In competition only                            |
| S9   | Glucocorticoids                                                                    | –                                                        | Dexamethasone, Hydrocortisone, Prednisolone, Flunisolide                                        | Specified                          | In competition only                            |
| P1   | Beta Blockers                                                                      | –                                                        | Alprenolol, Propranolol, Esmolol, Pindolol                                                      | Specified                          | In Particular sports                           |

androgenic anabolic agents (EAAS), analytical parameters for the athlete biological passport (ABP), and recognition standards for gas chromatography (GC) that has been coupled with mass spectrometry (MS) techniques [14–17]. Furthermore, various immunoassays and electrophoresis methods have also been explored for controlling doping [18,19]. Various review articles, editorials, tutorials have been published till now but most of the are limited to specific category of drugs [20–24]. Herein, this review aims to offer an in-depth and critical evaluation of the academic research on the significant improvements in anti-doping analytical techniques over the previous ten years. The explanation of recent developments, which mostly rely on chromatographic methods coupled with mass spectroscopy like GC–MS(/MS) and LC–MS(/MS) techniques and sample preparation in certain examples, are given special consideration. Also this narrative review summarises and discusses developments in the detection and utilisation of those metabolites newly identified categorised by the molecular mass and/or their relevant biochemical substance class.

## 2. Performance enhancing drugs (PEDs)

### 2.1. Anabolic-androgenic steroids (AASs)

AASs are chemically synthesized testosterone derived analogues that have been altered to enhance their anabolic properties. These testosterone-based compounds have a number of effects like: They encourage a high quantity of nitrogen in a muscle, which in turn, encourages an anabolic state; the agents prevent the muscle catabolic glucocorticoid binding, protecting and preserving muscular mass and preventing muscle deterioration. Furthermore, they influence aggression while supporting athletes to work out more and use more effort [25–27]. Some the commonly used AASs as PEDs includes methyl-testosterone, fluoxymesterone, methandriol, testosterone, stanozolol etc. (Fig. 2) [9,27]. In accordance with the prohibited list 2022 issued by World Anti-Doping Agency, there are more than 50 AASs which are prohibited for use in sports personals [9]. According to a recent meta-analysis by Sagoe et al., 6.4 percent of males and 1.6 percent of females worldwide reported using AASs in their lifetime [28]. The likelihood of

using AAS rose by 91% with participation in at least one activity, and the consumption of AASs has been most common amongst both the recreational as well as the competitive sportspersons. The survey also discovered that AASs utilization was marginally more common in the 2000 s than it was in the 1990 s. Furthermore, according to Al-Harbi et. al. that among male gym members in Riyadh, Iran with modifiable risk factors, the lifetime prevalence of AASs usage is substantial [29]. In a survey of high school football players in the USA, 6.3% said they were currently using or had previously used AASs, with 14 years being the age range for the first consumption [30]. Thus, it can be concluded that young boys, adult men, and women are the main groups who are interested in AASs [31].

Adverse consequences associated with the AASs consumption include potential behavioural changes, cardiotoxicity, arrhythmias, abnormalities associated with reproductive system like decrease in gonadotropic hormones and sex hormone binding globulin resulting in atrophy of testicles, increased aggression and many more [32–35]. The usage of such AASs can have negative health effects on the consumer whether the AASs are purposely added or accidentally contaminated during production of dietary supplements (DSs). Accidental use of AASs not only interferes with the healthy competition among sportspeople, but it also runs the risk of ruining their professional careers if they are found guilty of prohibited doping. The regulatory treatment of DSs, that do not have to be evaluated for the inclusion of hazardous ingredients prior to getting sold in the market, leaves unanswered the subject of dangerous DSs that are still unknown in the market.

### 2.2. Analytical approaches for detection of AASs:

Identification of the consumption of AASs and perception of the structures of pertinent target analytes begin with initial testing procedures which includes screening and metabolism studies followed by steroid profiling in urine and serum and confirmatory testing procedures involving isotope ratio mass spectrometry [36]. However, because there are so many potential structural alterations for the steroidal pharmacophore, their detection and identification are difficult. Liquid chromatography-electrospray ionization-(tandem)mass spectrometry

**Table 2**  
Summarised characteristics of doping control procedures.

| S. No. | Matrix            | Sample preparation and analytical Method adopted                    | Detection mode/ Detector | LOD/LOQ                                                       | Compounds detected                                                                                                        | Reference |
|--------|-------------------|---------------------------------------------------------------------|--------------------------|---------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------|-----------|
| 1.     | Urine             | SPE by LC to a quadrupole/orbitrap mass analyser                    | ESI+                     | 100 pg/ml                                                     | Stanozolol (Anabolic steroid)                                                                                             | [35]      |
| 2.     | Urine             | SPE and HPLC- MS/MS                                                 | MRM±                     | 10 pg/ml<br>50 pg/ml<br>100 pg/ml<br>20 pg/ml<br>20–250 pg/ml | Stanozolol<br>3'-hydroxy-stanozolol<br>4-hydroxy-stanozolol<br>16-hydroxy-stanozolol<br>T, EpiT, androstenedione, and DHT | [38]      |
| 3.     | Dried blood spots | Extracted by sonication and analysed by LC-MS/MS                    | ESI and MRM              |                                                               |                                                                                                                           | [46]      |
| 4.     | Urine             | SPE followed by GC-C-IRMS                                           | –                        | 2 ng/ml                                                       | 19-Norandrosterone and 19-Noreticholanolone (AAS)                                                                         | [51]      |
| 5.     | Urine and blood   | SPE, LC-MS/MS analysis, and enzymatic hydrolysis                    | MRM±                     | 0.025 ng/ml<br>0.05 ng/ml                                     | Salmeterol (β- agonist)<br>Hydroxysalmeterol                                                                              | [63]      |
| 6.     | Beef sausages     | UHPLC-MS/MS                                                         | ESI+                     | 5 pg/g                                                        | Clenbuterol                                                                                                               | [67]      |
| 7.     | Human serum       | LC-HRMS                                                             | –                        | 20 ng/ml                                                      | IGF-1                                                                                                                     | [84]      |
| 8.     | Urine             | SPE-LC-MS/MS                                                        | SRM                      | 0.1–4 ng/ml                                                   | HIF activating compounds                                                                                                  | [92]      |
| 9.     | Dried blood spots | Ultra-sonication followed by LC-MS                                  | ESI±                     | 0.05 and<br>0.125 ng/mL                                       | Hormone and metabolic modulators (Example: clomiphene, exemestane, and anastrozole)                                       | [97]      |
| 10.    | Urine             | SPE followed by LC-HRMS                                             | EI+ & FSM                | 10–25 pg/ml                                                   | Synthetic insulin analogues                                                                                               | [102]     |
| 11.    | Urine             | LLE at pH = 9.5 followed by UHPLC-MS/MS                             | MRM±                     | 50–200 ng/ml<br>50–100 ng/ml                                  | Diuretics<br>Stimulants                                                                                                   | [112]     |
| 12.    | Urine             | SPE-LC-MS/MS                                                        | SRM                      | 10–100 ng/ml                                                  | Stimulants                                                                                                                | [121]     |
| 13.    | Urine             | Sample preparation by dilute and shoot method followed by GC-NPD/MS | FSM                      | 25–100 ng/ml                                                  | Stimulants and narcotics                                                                                                  | [127]     |
| 14.    | Urine             | LLE followed by GC-MS                                               | MRM±                     | 500 pg/ml                                                     | Anabolic steroids                                                                                                         | [138]     |
| 15.    | Urine             | Microwave assisted LLE followed by GC-MS and LC-MS                  | MRM±                     | 40–50 ng/ml<br>50–100 ng/ml<br>3–15 ng/ml                     | Beta-blockers<br>Diuretics<br>Corticosteroids                                                                             | [139]     |

(LC-ESI-MS/MS) and gas chromatography-electron ionization-(tandem) mass spectrometry (GC-EI-MS(/MS)) are the two main methods employed by anti-doping laboratories at the moment for the study of AASs and their metabolites [37,38]. These methods are effective at identifying endogenous steroids and have good sensitivity and specificity. Furthermore, these techniques offer one-of-a-kind instruments for convicting athletes of unlawful doping and play a significant role in the examination of impounded suspicious items [39]. Endogenous unconjugated and glucuronidated AASs steroids as derivatives of trimethylsilyl (TMS) can be evaluated by GC-MS(/MS) employed by the WADA-accredited Cologne anti-doping laboratory [40]. By leveraging the idea of androgen receptor activation, androgen bioassays also seem to offer special qualities in determining the presence and quantity of anabolic substances in biological specimens. There have recently been publications on both cell-based and cell-free experiments [41–43].

According to the study done by Leogrande et. al., a system was developed to categorise structural characteristics of “unknown” steroids

found in investigative sources [44]. A total of 136 anabolic substances were divided into five categories of steroidal moiety containing compounds including A-ring saturated 3-oxo-4-ene-, and A-ring substituted, 5-ene-, 3-hydroxylated or 3-oxygenated androgens. The molecular spectrum were acquired with a GC/quadrupole-time-of-flight (QTOF) high resolution mass spectrometry (HRMS) system using conventional energy of 70 and 15 eV for ionisation. 115 of these 136 analytes were utilised to produce statistical sets centred on common mass spectrometric traits (including such as fragmentations/fragment ions common to specific subclasses), that were then implemented to the 21 steroidal substances to illustrate the approach's broad applicability [44]. However, further research is necessary to take use of chemometrics' continually expanding capability in the interpretation of results in anti-doping, as it is currently just a technique for comparably pure drugs. The characterization of AASs' metabolites also forms an important platform in detection of AAS in athletes. For example, to investigate the well-known phases I and II metabolism of stanozolol and

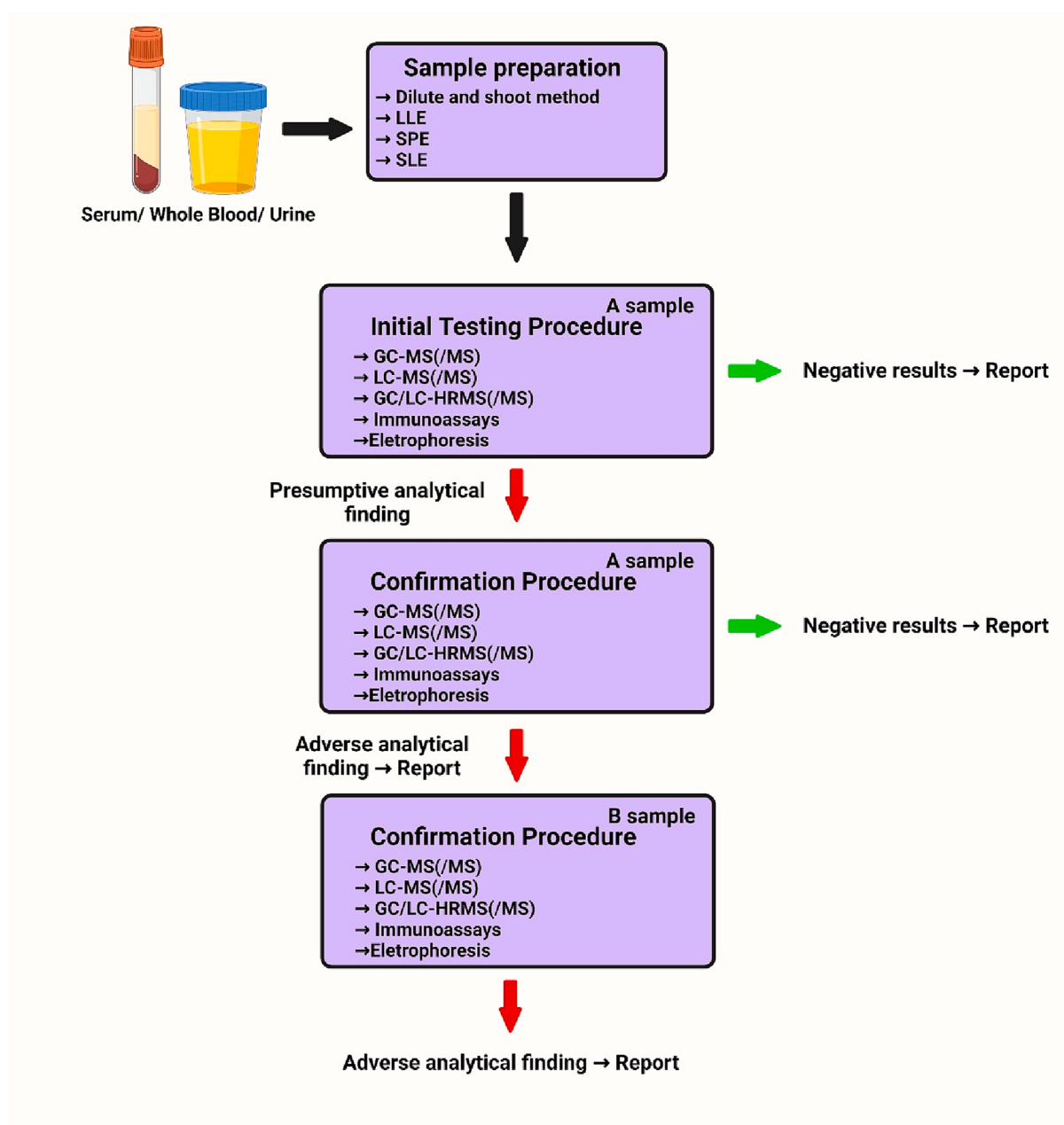


Fig. 1. A schematic illustration of the doping analysis workflow.

### Anabolic Androgenic Steroids

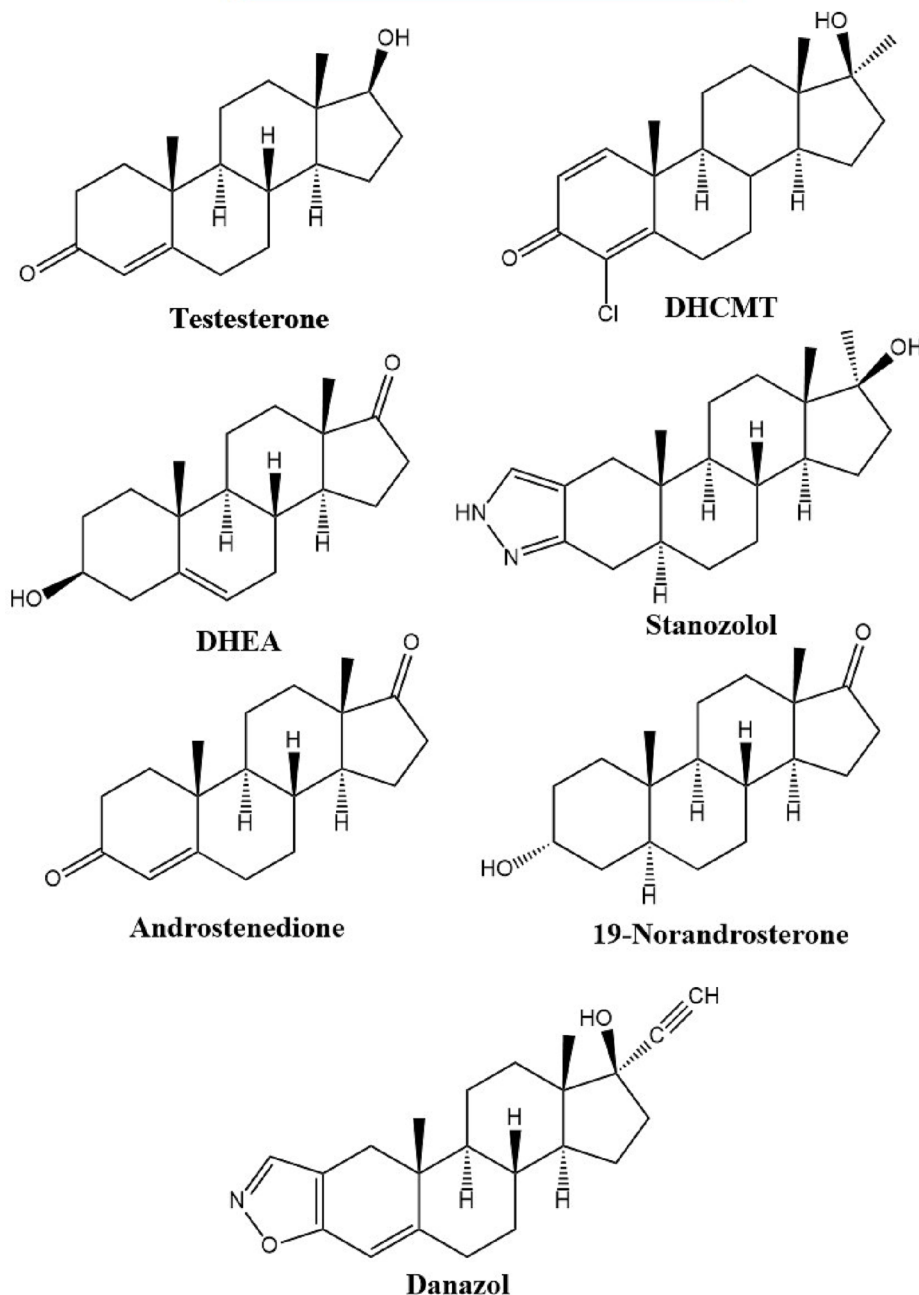


Fig. 2. Chemical structures of various anabolic androgenic steroids.

dehydrochloromethyltestosterone (DHCMT), Gorgens et al. used human HepaRG and primary human hepatic stellate cells [45]. Here, the physiology of higher order systems is mimicked using a multichannel microfluidic cell culture chip with connected 3-dimensional tissue spheroids (or “organoids”). The identified metabolite pattern for stanozolol, which includes stanozolol-N-glucuronides and 3-OH-stanozolol-glucuronide, was shown to closely mirror the drug’s urinary metabolic profile. Although multi-hydroxylated metabolites and a long-term marker were absent, the findings for DHCMT were discovered to be in acceptable accordance with metabolite information gathered from the evaluation of post-administration samples from humans. The “organ-on-a-chip” approach is a very intriguing approach for future metabolic investigations because it can overcome certain constraints of the

conventional in vitro as well as in vivo methodologies due to the observed significant level of convergence of findings from human in vivo studies. Loke et. al. studied the elimination profile of DHCMT in by performing various instrumental analysis like GC/MS triple quadrupole (QQQ) mass spectrophotometric analysis, GC/QTOF, HPLC etc. and monitored common metabolite, of known structure, of DHCMT, which was confirmed to be an appropriate urine long-term marker for consumption of the prohibited AAS., exhibiting significant inter-individual variability in the metabolism of drug and clearance, validating the usefulness of previously found long-term metabolites [46]. The authors were also able to identify, characterize and elucidate structures of two additional metabolites of DHCMT [46]. Hence, for conclusion, based on administration studies, all the metabolites (4-chloro-17β-

hydroxymethyl-17 $\alpha$ -methyl-18-nor-androstane-1,13-dien-3 $\alpha$ -ol, 3 $\alpha$ ,6 $\beta$ ,17 $\beta$ -trihydroxy-17 $\alpha$ -methyl-4 $\xi$ -chloro-5 $\beta$ -androst-1-en-16-one, and 4-chloro-17 $\beta$ -hydroxymethyl-17 $\alpha$ -methyl-18-nor-androstane-4,13-dien-3 $\alpha$ -ol as well as its epimer 4-chloro-17 $\alpha$ -hydroxymethyl-17 $\beta$ -methyl-18-norandrostane-4,13-dien-3 $\beta$ -ol) of DHCMT can be used as long term marker for detection of DHCMT. An innovative method based on High performance liquid chromatography coupled with triple quadrupole mass spectrometer (HPLC–MS/MS) and Multiple reaction monitoring (MRM) was used by Wang et al. to examine stanozolol post-administration samples and identified 48 metabolites, including 9 long term markers detectable for more than 15 days as well as 13 novel phase I and 14 novel phase II derivatives [47]. In conclusion this study shows that the HPLC- MS/MS approach used by the research can be of great significance in establishing metabolic profiles of the athletes.

In individual, longitudinal monitoring, blood and urine variables were no longer just used as direct screening measures but rather as trustworthy markers of either erythropoietic stimulation or steroid intake, thanks to the haematological and steroidal modules of an athlete biological passport (ABP) [48]. Because indirect biological markers may show abnormal fluctuations in blood and urine that may be caused by doping, this indirect screening method is important [49,50]. Research on enhancing the ABP's robustness and sensitivity is still ongoing, and sulfo-conjugated (pseudo)endogenous steroids have drawn a lot of attention in this context [36]. Sakellariou et al. examined post-administration samples using LC-MS/MS, GC–MS/MS and LC/Q/TOF-MS in negative ionization to study the metabolism of methyl-nortestosterone [51]. In particular, 17 $\alpha$ -methyl-5 $\beta$ -estrane-3 $\alpha$ , 17 $\beta$ -diol 3 $\alpha$  sulfate (S2), which was detectable until the completion of the excretion study (192 h), was shown to be a promising supplementary marker that might be used to support the use of methyl-nortestosterone. They also discovered a number of unique glucuronidated and sulfated metabolites. Salamin et al., who combined the established analysis of the urine steroid profile with the longitudinal monitoring of blood levels of testosterone (T), dihydrotestosterone (DHT), and androstenedione [52]. In order to evaluate the efficacy of the proposed approach, a study was carried out, wherein 14 healthy women who weren't taking hormonal contraceptives engaged in three study phases spaced across 3 menstrual cycles. The first cycle served as the control phase. The second cycle comprised the transdermal administration of 10 mg of T, once daily for 28 days whereas the third cycle was developed to evaluate the outcomes of the administration. The routine collection of blood and urine samples for analysis using LC-MS/MS (for serum steroid profiling) or GC–MS/MS was followed by standard test procedures (for urine steroid profiling) [52]. The long-term monitoring of serum levels of testosterone, DHT, and T/androstenedione was found to be a promising complement, offering steady and menses-independent indicators that enable the flagging of possible doping control serum samples in support of more investigation. Invasive venous blood extraction might well be replaced by the utilization of dried blood spots (DBSs). This was further supported by a study done by Salamin et. al. [53]. In their study, DBSs of volume of 20  $\mu$ l were isolated, extracted by sonication into a 95% v/v methanol and water solution, dried, and reconstituted for analysis using an LC-MS/MS. Using a C-18 analytical column (150  $\times$  2.1 mm, 1.7  $\mu$ m particle size) and 5 mM ammonium formate in water (solvent A) and methanol, chromatographic separation was carried out (solvent B). Target analytes were detected after ESI in polarity switching mode using diagnostic precursor/product ion pairs in MRM, allowing for lower LOQs for T, EpiT, androstenedione, and DHT between 20 and 250 pg/mL. Furthermore, a study performed by Yuan et. al. developed and validated a novel UHPLC-Q-Orbitrap-MS employing Girard's Reagent P (GRP) derivatization method for the detection of 20 endogenous anabolic steroids esters in DBS [54]. This study also supports the fact that DBS can be used as an effective complementary matrix for the detection of endogenous steroids in athletes. Hence, from the studies discussed above comprehensive screening technologies are needed to cover the entire spectrum of these steroids. In the context, the use of

mass spectrometric methods like GC–MS provides the opportunity to detect unidentified steroid by keeping track of common fragment ions or losses that reveal the fundamental structure and functional groups. A helpful technique for the discovery of unknowns is the use of triple-quadrupole mass analyzers, specifically when the precursor ion scanning option is used. Although it has a high degree of selectivity and sensitivity, GC–MS equipment is still expensive and the technique demands a high level of skill.

IRMS is being used in doping control laboratories to establish the carbon isotope ratio (CIR) of urine steroid hormones and demonstrate that they have an exogenous source [55,56]. Furthermore, in certain circumstances including 19-norandrosterone (19-NA) doping, anomalous passport findings, and suspicious steroid profile data, confirmatory studies using GC/C/IRMS are used to evaluate if there are substantial changes exist between the target chemicals' carbon isotope fingerprints as well as endogenous reference substances (ERCs). In order to boost automation and decrease the time commitment required for sample preparation, Casilli et. al. examined the use of multidimensional gas chromatography (MDGC) in IRMS analysis [57]. A pressure-controlled heart-cutting tool (Deans switch) and two separate GC ovens were employed to enable a highly effective and selective 2D separation with a non-polar column as the first and a mid-polar column as the second dimension. Before using MDGC-C-IRMS, samples were prepared using SPE, LLE followed by enzymatic hydrolysis, and acetylation instead of the more usual HPLC purification processes. As a proof-of-concept, a urine sample acquired after the injection of T undecanoate was examined, and the outcomes were compared to those attained by utilising the procedure regularly used by anti-doping laboratories. The matching of the obtained  $\delta$  and  $\Delta\delta$  values amply illustrated the potential of MDGC to accelerate IRMS analysis and provide a higher sample throughput [57]. Lalonde et al. developed a method and procedure that allowed the purification of six TCs (T, Dehydroepiandrosterone (DHEA), A, E, 5 $\alpha$ Adiol, and 5 $\beta$ Adiol) and two ERCs (PD and 16-ene) in an integrated two-dimensional LC fractionation setup, accelerating and automating the central processes [58]. Prior to fractionation, urine samples passed SPE, the extracts followed  $\beta$ -glucuronidase's enzymatic hydrolysis, and the unconjugated steroids underwent an extraction into hexane and concentration. Only one of the four first-dimension fractions, fraction 1, needed to be subjected to the second-dimension fractionation, which produced another four fractions for GC/C/IRMS analysis. This has been facilitated by merging multiple hyphenated C-18 HPLC columns with a stationary phase constructed of phenyl-silane (first dimension, 150  $\times$  4.6 mm, 3.5  $\mu$ m particle size). A DB5-MS capillary column (25 m  $\times$  0.2 mm, 0.33  $\mu$ m film thickness) was inserted in the GC in order to produce baseline segregated as well as intrusion of signals but without necessity for steroid derivatization. This, in turn, allowed for the creation of a test method that was incredibly quick and required comparatively little manual sample handling work. The use of the assay on more than 10,000 regular doping control samples further supported the robustness of the methodology [58]. The major metabolite of nandrolone and/or its prohormones is 19-NA, and urine naturally contains trace levels of this compound. In order to determine the source of the drug, urine samples with 19-NA values between 2.5 and 15 ng/mL must be evaluated by GC-C-IRMS, as this compound can also be detected at elevated levels during pregnancy. The endogenous reference compounds (ERCs) pregnanediol (in this particular study) A, as well as the 19-NA compound and its isomer 19-noretiocholanolone (19-NE), were the subject of a special sample preparation protocol described by Iannella et al., target analytes were extracted into n-pentane after enzymatic hydrolysis, concentrated, and put through two HPLC purification stages [59]. Four fractions were generated and concentrated following the initial fractionation on a phenyl-silane-based analytical column (150  $\times$  4.6 mm, 5.0  $\mu$ m particle size), and three of these fractions underwent a second HPLC purification. In this case, a C-18 amide analytical column (250  $\times$  4.6 mm, 5.0  $\mu$ m particle size) was used. It was run isocratically with 100% acetonitrile and gave purified fractions for GC/C/IRMS analysis after 30 min of

HPLC purification. The TCs and ERCs were separated for GC/C/IRMS on a 5% phenylmethylpolysiloxane capillary column (30 m  $\times$  0.25 mm, 0.25 m film thickness), and the assay's LOQ of 2 ng/ml as well as the analysis of post-administration urine samples obtained from volunteers who received a single oral dose of norandrostenedione served to demonstrate the presented approach's suitability for routine analysis [59]. The methylstenbolone, a nutritional supplement, is also a widely used AAS in athletes. It was found that this steroid undergoes metabolism in the host to give three specific metabolites i.e. methasterone, 2,17-dimethylandrosterone-16,17-diol-3-one and dihydroxylated and reduced methylstenbolone which can be detected over a long period of time in plasma and urine samples [60]. Piper et. al. also conducted an investigational study on detection of long term metabolites methylstenbolone and reported 40 different metabolites of it with the help of hydrogen IRMS. It was found that out of the 40 metabolites, two metabolites [ $2\alpha,17\alpha$ -dimethyl-5 $\alpha$ -androst-1-ene-3 $\alpha,17\beta$ -diol (NEW\_LTM) and  $2\alpha,17\alpha$ -dimethyl-5 $\alpha$ -androst-1-ene-3 $\beta,17\beta$ -diol (Epi-NEW\_LTM)] seems to be a potential candidate which can be used for the detection of methylstenbolone in doping subjects. Furthermore, these metabolites were also characterized by NMR (Fig. 2)[61]. Another study done by Torre et. al. showed that the IRMS screening approach developed during the analysis of different steroids in the urine samples suggested has appropriate selectivity, yields results that overlap with the currently used validated method, and allows for analysis of a significantly higher volume of samples even during a major event without affecting the detection capability [62]. A sample pre-treatment approach employing an online two-dimensional (2D)-HPLC 19-NA confirmation method was developed using GC-C-IRMS by Wen et. al. Since in the developed method no chemical derivatization stage is involved, thereby the detection can be done of 19-NA in less time. The method can be a potential detection technique for 19-NA as it can detect the minute level (LOD – 2 ng/ml) of drug in very less amount of urine samples [63].

This can be concluded from studies discussed above involving IRMS that isotope tagging of pharmacological compounds has proven to be effective and established methods that aid in structure elucidation when used for metabolite identification utilising IRMS. Despite being quite useful for sports drug testing in general, IRMS analyses are nevertheless distinguished by laborious sample preparation, a constrained batch size, and lengthy instrument run durations that are necessary to guarantee proper peak purities. However, the requirements for sample purity and GC resolution (in case of GC-C-IRMS) in terms of instrument maintenance need to be considered. Similarly, stable isotope-based inference turned out to be very complex. Although it was anticipated that endogenous and synthetic steroids in urine might be distinguished by their differential  $^{13}\text{C}$  values, it has since become clear that different baseline values call for rigorous research and evaluation of reference populations [64,65].

A new class of AR ligands known as selective androgen receptor modulators (SARMs) have favourable oral bioavailability, few androgenic side effects, high receptor affinity, and tissue-specificity [66]. SARM abuse in sports is forbidden both in- and out-of-competition as of 2008. Different SARMs are widely available on the black market even without clinical authorisation, and some dietary supplements have also been demonstrated to have been tainted or illegally enhanced with these compounds [67]. GC-MS can be used to detect SARMs and/or their metabolites in doping control urine samples, depending on the molecular structure, but since most SARMs' physico-chemical properties allow for a decent LC separation and ESI in either a positive or negative mode, most tests employ the latter technique [68,69]. Furthermore, LC-HRMS/MS and various immunoassays like ELISA, CLEIAs have also been utilized for the analysis of SARMs and AAS [70–72].

### 3. $\beta_2$ -agonists

Athletes with asthma and exercise-induced bronchoconstriction (EIB) are treated with beta-2 agonists [73]. However, skeletal muscle

tremor has been recognized for years as one of the most frequent side effects of beta-2 agonists, but beta-2 agonists as a medication class are also well-known to have considerable effects on muscle mass [74]. Thus, because of the incidence of asthma-related ailments both within the general community and among elite athletes, as well as the need to maintain respiratory health while preserving the integrity of sport, anti-doping considerations for asthma treatment, notably the usage of  $\beta_2$ -agonists, have been particularly important [75]. As per the prohibited list 2022 issued by WADA, there are 15 beta-2 agonists which are prohibited for use in sports personnel with certain conditions [9]. Clenbuterol, A beta-2 agonist categorized as an anabolic agent, remains restricted both in and out of competition, with a minimum level of performance at 0.2 ng/mL. The inhalation of salbutamol is authorised under specific restrictions and up to an urine threshold concentration of 1  $\mu\text{g/mL}$  [76]. The properties for the inhalative usage are further defined in the Prohibited List: inhaled salmeterol: maximal 200 g in 24 h; inhaled salbutamol: maximal 1600 g in 24 h in divided doses; not to exceed 600 g over 8 h commencing from every dose, Inhaled formoterol: maximal administered dose of 54  $\mu\text{g}$  in 24 h; inhaled vilanterol: maximal 25 g in 24 h.

#### 3.1. Analytical approaches for detection of $\beta_2$ -agonists

Various research studies have been done on earlier detection of beta-2 agonists in doping subjects (Fig. 3). For example, Jessen et al. carried out a focused administration trial using single inhalative doses of 200  $\mu\text{g}$  and 400  $\mu\text{g}$  as well as seven consecutive days of daily inhalative doses of 200  $\mu\text{g}$  administered to 11 trained healthy subjects. Most drug administration possibilities were supported by properly structured workout programmes, that occasionally go unappreciated as a component of elimination study procedures [77]. Urine (and blood) specimens were collected beforehand and for up to 24 h just after drugs were administered. SPE, LC-MS/MS analysis, and enzymatic hydrolysis were used to assess the levels of salmeterol and its primary hydroxylated metabolite in urine samples. The sample extracts were placed onto an analytical C-18 column with 1.2  $\times$  150 mm, 3 m particle size, and gradient-eluted with acetonitrile and an ESI source utilising 1 mM ammonium formate in a solution of 0.1% aqueous formic acid (solvent A) and formic acid (solvent B). Salmeterol had a LOQ of 0.025 ng/ml and  $\alpha$ -hydroxysalmeterol had a LOQ of 0.05 ng/ml when the target analytes were found and quantified in MRM mode. The anti-doping rule-compliant scenario (200 g, single dosage, and every 24 h) showed mean maximum salmeterol urine concentrations of 2.1–2.2 ng/ml, while the supra-therapeutic dose (400 g) produced a mean urinary concentration of 4.0 ng/ml. In light of the fact that  $\alpha$ -hydroxysalmeterol was detected at these concentrations 5.7–6.5 and 11.6 ng/ml, respectively a combination of two thresholds—each set at 3.3 ng/ml—applicable to salmeterol and its metabolite was proposed. Adverse analytical finding (AAF) test results would only be disclosed if the doping control urine sample contained both analytes at quantities greater than 3.3 ng/ml, thereby excluding rule-compliant drug usage [77]. Furthermore, enantioselective liquid chromatography and fluorimetric detection were already used in early investigations on doping controls to separate (R)-/(S)-salbutamol and make it possible to distinguish between oral (prohibited) and inhalation (permitted) administration [78–80]. Bejarano et. al. developed and validated UHPLC-MS/MS method for detection of unintentional doping in sport fields by clenbuterol in beef sausages [81]. The developed UHPLC-MS/MS technology combines quick chromatographic run times with great selectivity and sensitivity. Additionally, capillary electrophoresis techniques were used to permit the chiral separation of several illegal  $\beta_2$  agonists, but unlike analytical methods, including GC-MS and/or LC-MS, weren't always accessible across all doping control institutions [76,82–84]. Fig. 4. Fig. 5. Fig. 6. Fig. 7. Fig. 8..

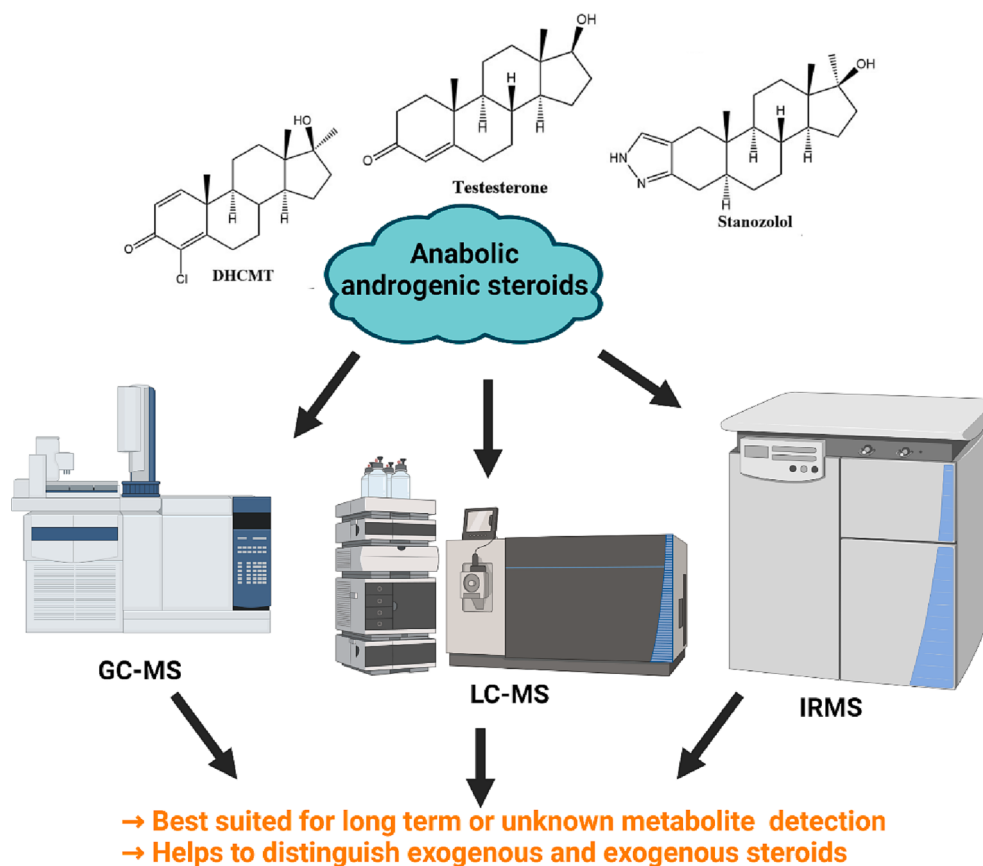


Fig. 3. Schematic illustration of various analytical techniques for detection of AASs.

#### 4. Peptide hormones, growth hormones, related substances and mimetics

This category of PEDs is prohibited throughout all times, both in as well as out competition. Non-specified substances are also included among all of the substances in this class [9]. This class is further classified into following categories:

##### 4.1. Growth hormone, its fragments and releasing factors, gonadotropins and lutenizing hormone

GH (growth hormone) along with its primary transmitter, insulin-like growth factor-I (IGF-I), are required for various physiological functions [85]. Growth hormone (GH) treatment has gained interest as a possible performance-enhancing medicine since it reduces body fat, increases lean body mass, and improves strength as well as fitness in individuals with GH insufficiency [86]. It is discovered that Growth hormone (GH) is a prominent performance-enhancing substance in athletics [87,88]. Additionally, growth factors can be released by blood platelets in response to signals of damage. These are believed to be beneficial for athletes in good health but may also be used to treat sports injuries [89,90]. However, the majority of growth factors have no impact on athletes' performance. One exception is IGF-1, as mentioned previously, which has been studied as an ergogenic aid and is expected to have ergogenic benefits primarily through the anabolic pathway that GH and IGF-1 share. Athletes might use luteinizing hormone (LH) or human chorionic gonadotropin (hCG) by parenteral injection to increase muscle strength since these gonadotropins boost the synthesis of testosterone in males [91]. Furthermore, the WADA forbids its usage in athletic competition, and competitors are compelled to submit to testing for GH exposure.

##### 4.1.1. Analytical approaches

The identification of orally active growth hormone secretagogues (GHS) based on a variety of distinct pharmacophores, such as benzolactame, capromorelin, macimorelin, 4-spiropiperidine, and oxindole derivatives like CP-424391, MK-0677 [92]. These GHS and growth hormone releasing peptides (GHRPs) are reported to be available in numerous black-market which emphasises the significance of doping control assays permitting the sensitive and thorough examination of GHS [93]. According to a thorough summary by Judak et. al., mass spectroscopy methods have been significantly improved over the past decade, and in some situations, metabolites (and/or catabolites) have been demonstrated to reflect the better target analytes in both blood and urine for the doping control analysis of peptides [94]. Pinyot et al. offered a general strategy for dealing with this problem in and used a competitive receptor-binding assay [95,96]. A detection window of roughly 4.5 h was suggested for this specific medicine based on the measurement of the growth hormone-releasing peptide GHRP-2 in post-administration study urine samples. It was advised to use specific LC-MS/MS assays to confirm the presence of a restricted compound from the GHS class. Okano et. al. developed mass spectrometry-based screening technology focuses specifically on the identification of GHRP-2 and its primary metabolite (d-Ala-d(-naphthyl)-Ala-Ala-OH) in human urine [97]. Prior to the specimen being exposed to SPE and subsequent LC-MS/MS analysis, the appropriate sample preparation and analytical conditions were ensured using an isotope-labelled GHRP-2 internal standard. The excretion research, which had ten volunteers who received an intravenous bolus of 100 g of GHRP-2 dihydrochloride, was conducted using the test, which allowed LODs of 20–50 pg/ml. The intact GHRP-2 was discovered for up to 13 h, although the established method allowed for the detection of the aforementioned metabolite for up to 24 h [97]. A UHPLC-HRMS approach was developed and verified by to screen for ipamorelin in DBS [98]. Ipamorelin, a small peptide, was

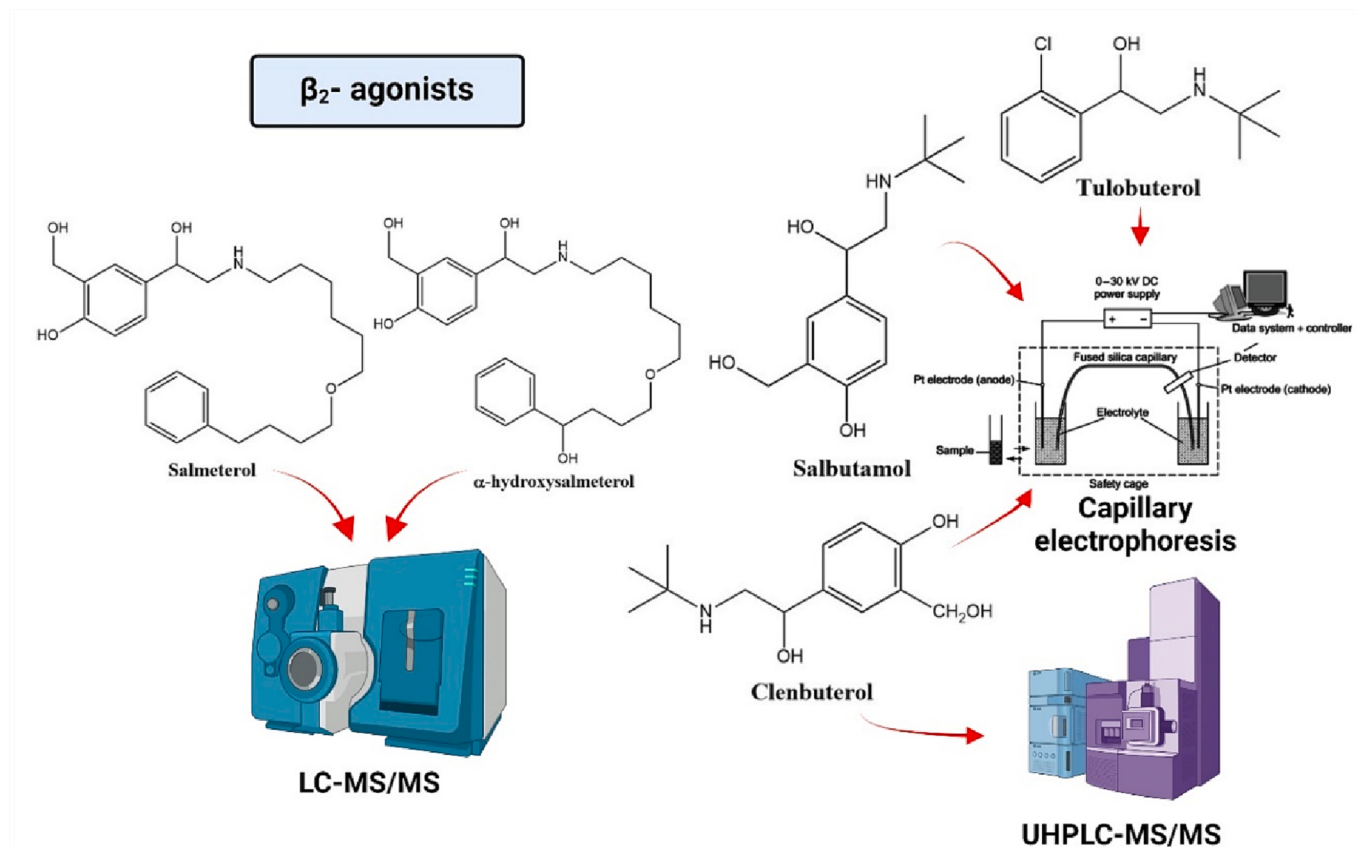


Fig. 4. Schematic illustration of various analytical techniques for detection of  $\beta_2$ - agonists.

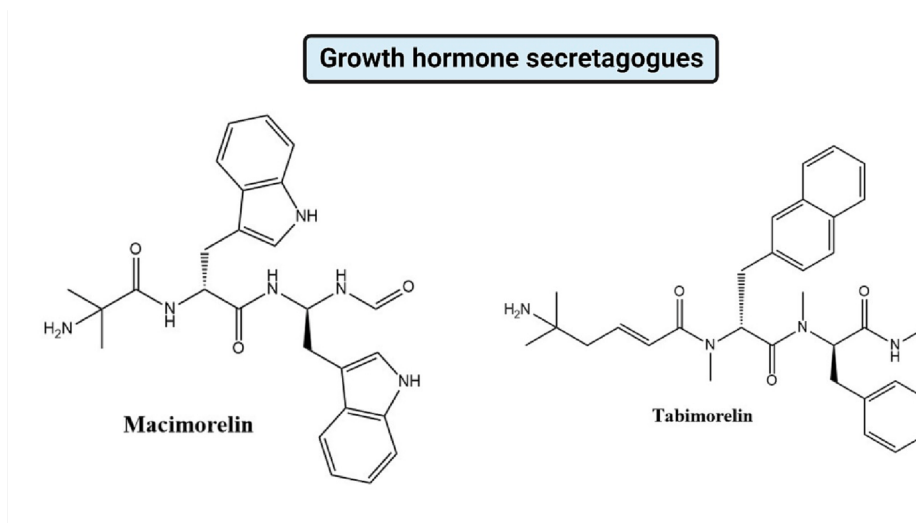
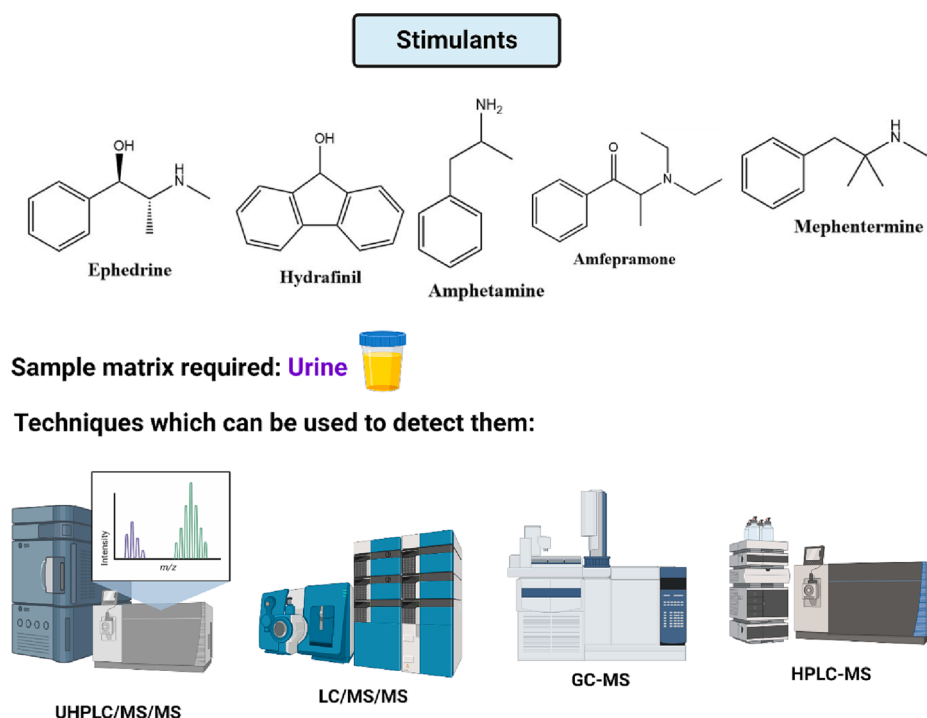


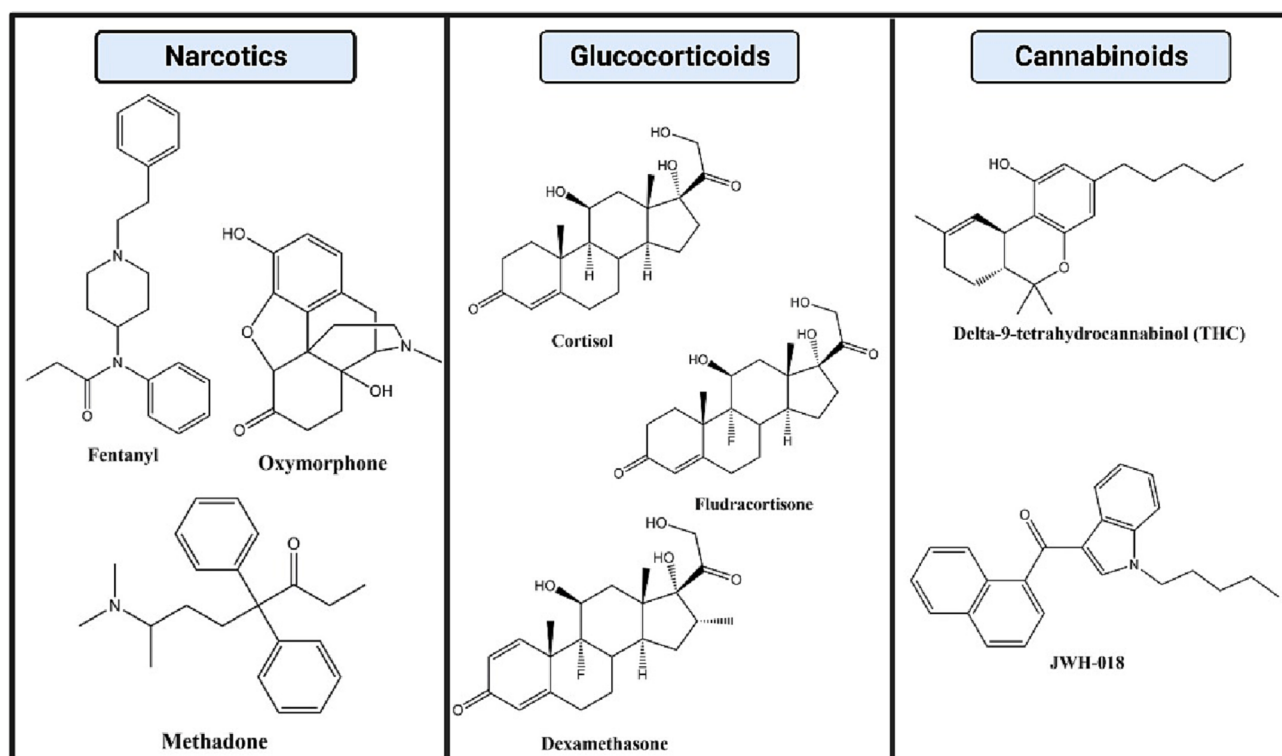
Fig. 5. Chemical structures of growth hormone secretagogues.

isolated from DBS in this study via liquid extraction and ultrasonic treatment, and it was subsequently discovered by acquiring high-resolution fragmentation spectra. In terms of sensitivity, specificity, linearity, recovery, accuracy, and ion suppression/enhancement effects, the method performed satisfactorily. The precursor and fragment ions' mass errors were less than 5 ppm and ipamorelin was detected with LOD of 2.5 g/nl. Hence, it could be concluded that in order to detect small peptides in blood at low levels, the combination of DBS with high-resolution mass spectrometry has proven to be a viable and efficient technology. Especially when unidentified synthetic peptides are the

target analytes, this method appears potential for targeted and untargeted analysis in clinical monitoring and sports drug testing [98]. Moreover, a LC-HRMS-based technique that allows the measurement of IGF-I from 25 l of human serum was developed by using 0.1% aqueous formic acid (solvent A) and 0.1% formic acid in methanol as the gradient eluents, serum was subjected in order to accomplish protein precipitation, followed by ultracentrifugation (10 kDa) [99]. The retentate was then loaded onto a C-18 analytical column that was  $2.1 \times 100$  mm in diameter and 2.6 m in particle size (solvent B). The seven-fold protonated molecule was detected at  $m/z$  1093.5 and the stable isotope-labeled



**Fig. 6.** Chemical structures of various narcotic PEDs which are prohibited in sports and various analytical techniques which can be adopted for their detection in athletes.



**Fig. 7.** Chemical structures of various narcotics, glucocorticoids and cannabinoids.

internal standard at  $m/z$  1,106.9 when the used Q/orbitrap MS was run with a dedicated narrow mass range configuration, scanning from  $m/z$  1,090 to  $m/z$  1,100 exclusively, with a resolution of 70,000 (@  $m/z$  200). A single run of the assay took place in less than 10 min, and it was validated with a LOQ of 20 ng/ml. The approach was tested on a set of

209 blood samples and proven to be reliable. It also showed good correlation ( $r = 0.85$ ) when compared to an immune-radiometric assay (IRMA), especially when a single-point calibrator was used [99]. Thevis et. al. used modern analytical techniques, such as mass spectrometric (top-down and bottom-up) sequencing and gel electrophoresis to

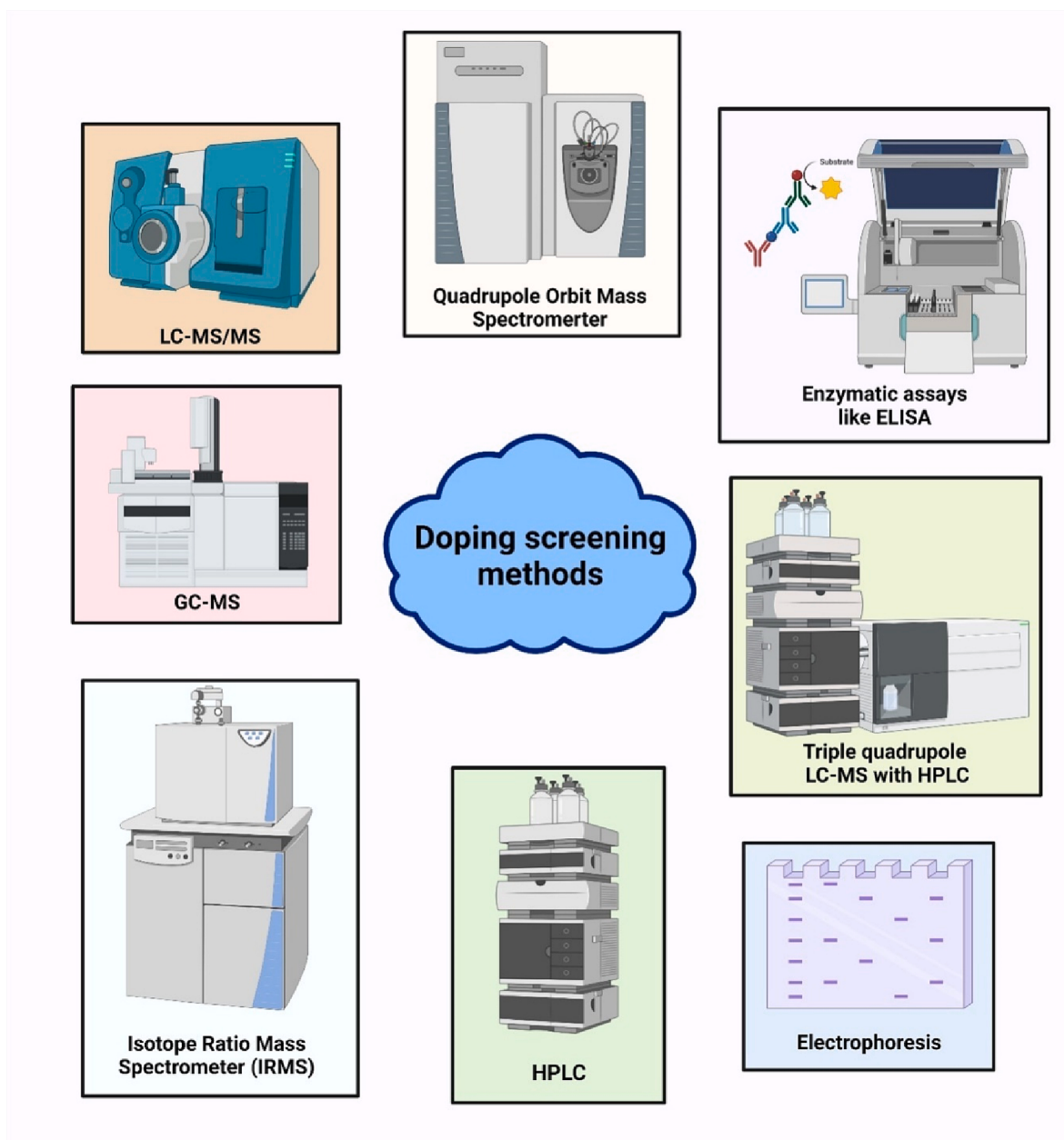


Fig. 8. Summary of detection approaches available for doping control.

characterise the content of an aliquot of the product. After thorough analysis, the detection of mechano growth factor (MGF) was developed employing modified routine doping controls using ultrafiltration, immunoaffinity-based separation, and nano-liquid chromatography-high resolution/high accuracy mass spectrometry [100]. For the detection of gonadotropins, immunoassays and spectroscopic methods have been widely used for doping control [91].

#### 4.2. Erythropoietin-receptor agonists and hypoxia-inducible factor (HIF) activating agents

Erythropoietin receptor agonists are intended to increase red blood cell volume by stimulating erythropoiesis, potentially improving performance [101]. Recombinant human erythropoietins (rHuEPOs), an erythropoietin-receptor agonist, induce erythropoiesis by raising haemoglobin levels. This could boost haemoglobin's capacity to carry oxygen, which could improve endurance performance [102]. In addition to this, a relatively similar explanation for possible performance-

enhancing impacts in correlation with direct rHuEPO delivery applies to HIF activating drugs since they directly affect erythropoietin production by boosting erythropoietin gene expression [103].

##### 4.2.1. Analytical approaches

Even though it was demonstrated that a single intravenous dose of 60,000 IU of recombinant EPO (rEPO) had no impact on short-term aerobic exercise efficiency (in neither submaximal nor maximal cycling test circumstances), refining and streamlining existing testing strategies were deemed particularly significant and was followed utilising direct as well as indirect methodological approaches [104]. Also, EPO can be detected in blood, however interferences, such as excessively abundant proteins, make this difficult (e.g., ion suppression). Combining nanoLC-MS with selective sample preparation, such as immunoaffinity extraction, is one potential solution to this issue. A study done by Reichel et. al. discovered that utilization of modified sarcosyl polyacrylamide gel electrophoretic approach (SAR-PAGE) facilitates nearly doubled window detection period, compared to earlier developed methods

[105]. Additionally, various attempts have been made for the better detection of the physiological effects of micro-dosed rEPO have been thoroughly investigated through the investigation of numerous biomarkers. For example, 5'-aminolevulinic synthase 2 (ALAS2) mRNA levels have been shown by Loria et. al. using the ABP's haematological module, they developed sensitive and unique transcriptome indicators for detecting rhEPO microdosing. This methodology is appropriate with the utilization of DBSs for anti-doping studies. This method is also compatible with the use of DBSs for anti-doping tests. ELISA technique has also been utilized for quantification of EPO followed by electrophoresis as confirmatory analysis [106]. Furthermore, De Wilde et al. demonstrated a potential method for monitoring HIF activating compounds in an automated and multiplexed test using online SPE-LC-MS/MS. Following this, the isolated analytes were transported to a C-8 analytical column ( $2 \times 150$  mm, 5  $\mu$ m particle size) during gradient elution utilizing both methanol and water that served as solvents A and B and contained, respectively, formic acid (0.001%) and ammonium formate (1 mM). Eight specific constituents were identified in MRM mode having LODs that ranged from 0.1 to 4 ng/ml in as short as 6.5 min of total run time using an online SPE column with such a hydrophilic-lipophilic balanced copolymer stationary phase ( $2.1 \times 20$  mm, 25  $\mu$ m particle size) [107]. A recent study conducted by Heiland et. al. reported that DBS can be used as a potential sample matrix for detection erythropoietin receptor agonists. They reported the optimal conditions required for accurate detection of illegal erythropoietin receptor agonists prohibited by WADA from DBSs and contrasted detection limits to the MRPLs set by WADA for ERAs in serum/plasma samples using SAR-PAGE electrophoresis and western blotting. The results obtained from DBS samples showed less variations in comparison to urine and venous blood sample [108].

## 5. Hormone and metabolic modulators

A subcategory of the term "hormone and metabolic modulators" includes selective oestrogen receptor modulators (SERMs), aromatase inhibitors (arimistane, formestane), other anti-estrogenic medicines, substances that alter, metabolic modulators as well as myostatin activities (insulins) [9]. These pharmaceuticals, which come in a variety of forms, are taken by men and women in distinct ways. SERMs block oestrogen receptors, and aromatase inhibitors lower oestrogen levels in the bloodstream. while toremifene and tamoxifen have anti-oestrogenic actions [109]. Increasing male testosterone levels is also a benefit as well. Tamoxifen appears to have a masculinizing impact on females; however, it has also been demonstrated to be hazardous and raises the risk of thromboembolism.

### 5.1. Analytical approaches

In sports drug testing, comprehensive surveillance has been provided by combining urine and blood methodological approaches. Exemestane, formestane, tamoxifen, and toremifene are just a few examples of anti-estrogens that can be found using traditional analytical chromatographic techniques like GC-MS for anti-doping purposes (e.g. aminoglutethimide, trimetazidine) [110,111]. Arimistane could be detected in urine as evidence of drug use, and LC-MS/MS has been proven to be more sensitive than GC-MS/MS in detecting arimistane and its primary metabolite androst-3,5-dien-7 $\beta$ -ol-17-one [112]. Furthermore, DBS have proven to be quite useful in anti-doping analytical methods including anti-estrogenic substances. Thomas et al. devised the standardized multi-analyte testing protocol for doping checks of clomiphene, exemestane, and anastrozole were detected at LODs between 0.05 and 0.125 ng/mL utilising the aforementioned LC-ESI-quadrupole-orbitrap-based equipment [113]. Moving on, commercially accessible radioimmunoassays or enzyme-linked immunosorbent tests were used to detect insulin in urine [114–117]. Furthermore, for analysis of C-peptide and synthetic analogues of insulin in human urine, mixed

cation-exchange SPE, protein precipitation, as well as LC-MS/MS analysis were all performed on 3 ml of urine by Thomas et al. in 2021 [118]. Here, C-18 analytical column ( $3 \times 50$  mm, 2.7  $\mu$ m particle size) and phenyl-hexyl-based trapping column ( $3 \times 10$  mm, 2.7  $\mu$ m particle size) were used for chromatography with gradient elution taking place over a 15-minute period using 0.1% aq. HCOOH (containing 1% DMSO, solvent A) and acetonitrile (containing 1% DMSO + 0.1% HCOOH, solvent B). A Q/orbitrap MS operating in full scan (resolution 60,000 @  $m/z$  200) and targeted MS/MS (resolution 45,000 @  $m/z$  200) was given the effluent through positive ESI. With the exception of bovine insulin, which had a LOI of 50 pg/ml, other targeting analytes were shown to permit for LOIs of 25 pg/ml. LODs ranged from 10 to 25 pg/ml for all analytes. Diagnostic observations from urine samples taken from type I and type II diabetics employing, insulin degludec, insulin aspart, insulin glargine as well as combinations thereof were provided as proof-of-concept data [118]. The method developed does not require antibody based sample purification step, thereby reduces the overall time required for detection of Insulin. Furthermore, all the parameters including LOI, LOD reported showed that method developed complies with the requirements of WADA. Therefore, this technique can be a potential doping test for the detection of insulin in athletes.

## 6. Diuretics and masking agents

Diuretics and masking chemicals can be misused to cause diuresis to shed weight (most frequently in weight-classified sports like judo) or to hide the ingestion of illicit substances [119]. As a result, they are prohibited by the WADA [9].

### 6.1. Analytical approaches

Extraction and dilute-and-shoot conjunction employing high resolution mass spectrometry (HRMS), liquid chromatography (LC), or LC-triple quadrupole mass spectrometry (LC-TQMS) are the most utilised techniques for the identification of diuretics [119–121]. While the advantages of dilute-and-shoot include time and labour savings, this method also has drawbacks such the matrix effect and retention time shift [119]. For authorised laboratories, for the detection of diuretics in urine, WADA has created a single absolute minimum level of performance i.e (MRPL) of 200 ng/mL [122]. In 2018, De Wilde et al. designed and tested a turbulent SPE approach linked with 2D-LC-MS/MS for assessing 50 diuretics [123]. Furthermore, techniques like capillary electrophoresis as well as micellar electro-kinetic chromatography (EKC) tests for diuretics have also been researched [124–127]. From the studies cited, it was discovered that capillary zone electrophoresis based techniques were used to determine non-chiral APIs and compounds related to them, whereas EKC-based techniques were used for enantio-selective separations and optical purity testing. Ventura et. al. showed that direct injection following urine dilution allowed for the competitive detection of 34 target chemicals at the requisite detection ranges (10–250 ng/mL) [128].

## 7. Stimulants

According to some theories, stimulants may enhance performance by altering the brain's levels of the neurotransmitters dopamine and norepinephrine [129,130]. Therefore, stimulants are only permitted during competition in sports [9].

The ability to distinguish between a drug consumption that pharmacologically impacts the timeframe during in-competition as well as causes irrelevant drug residues is made possible in this situation by analytical retrospective accuracy [131]. Given the massive array of stimulants that are tracked by anti-doping laboratories, it can be challenging to build a single screening strategy to detect all of the relevant compounds, notably at the minimal performance standards prescribed by WADA [122]. Following the LLE or SPE of urine samples, GC-MS is

frequently used to identify a variety of stimulants [21,132]. Because some underivatized stimulants only create a small number of diagnostic ions, which are frequently in low abundance, urine extract is typically made [133]. For thermolabile, volatile, and polar chemicals can be found using LC-MS/MS, that has an excess of diagnostic ions and doesn't need sample derivatization for successful chromatographic separation. Regular use of LC-MS/MS for the detection of stimulants has the significant benefit of enabling the development of high-throughput screening techniques without the requirement for a sample concentration or clean-up phase (dilute and shoot methods)[121,134,135]. For example, De Wilde et al. used an established instrument configuration of online-SPE-LC-MS/MS to further expand their framework already in place to facilitate the identification of 80 stimulants, allowing LODs of 10 to 100 ng/ml [136]. Anti-doping research facilities must be capable of responding fast to new information and legislation, hence adaptable and extensible techniques (based on both LC-MS/MS as well as GC systems) have been found to be particularly important [136,137]. In 2021, Knoop et. al. conducted a pilot eliminated study on three healthy male volunteers who received a single oral dose of 50 mg of hydralinil. Prior to delivery and for up to 72 h after, urine samples were obtained, and analysed using GC-MS and LC-MS enabled for the detection of the intact drug in addition with Phase I as well as Phase II metabolites [138]. A recent study by Zhu et. al. devised an ultra-performance liquid chromatography-tandem mass spectrometry (UHPLC/MS/MS) technique to find five ephedrine analogues using a tandem mass spectrometer with an electrospray ionisation source in multiple reaction detection mode after one-step dilution. The method developed was found to be effective and more accurate as LOD was found to be below 0.5 ng/ml [139].

## 8. Narcotics, Glucocorticoids, and cannabinoids

Similar to the group of stimulants, intake of narcotic agents is prohibited during competition [9]. Significant emphasis has been paid to measuring drug exhalation and drug metabolites, particularly with regard to narcotics in the contexts of clinical monitoring and drug-abuse testing [140]. Furthermore, due to the significant proton affinity of stimulants and narcotics, it was possible to identify and quantify these substances in doping controls using robust and sensitive apparatus made up of tandem mass spectrometers (LC-MS/MS) as well as liquid chromatography (LC) [21,22]. Additionally, LC-MS/MS dilute and shoot techniques can be used to detect narcotics since they provide the evaluatory analytical sensitivity required to distinguish fentanyl and their derivatives at the threshold detectable level of 2 ng/mL [121,141]. For the identification of 80 stimulants, narcotics, and a few other prohibited PEDs discharged in free form in human urine, Dubey et. al. offered a thorough, specific and sensitive gas chromatography- nitrogen phosphorus detector/ mass spectroscopy (GC-NPD/MS) technique. For all drugs, LOD was found to be ranged from 25 to 100 ng/ml [142]. Minhas et. al. utilized nanostructured silicon (nano-Si) based surface-assisted laser desorption/ionisation mass spectrometry (SALDI-MS) for the detection of narcotics, AAS and peptide hormones at very low concentration from the biological samples [143].

An increasing body of research suggests that glucocorticoids do indeed have the ability to improve athletic performance, depending on sport-specific factors and dosages of glucocorticoids employed. However, glucocorticoids can also be harmful to an athlete's health [144]. In particular, salivary cortisol (and testosterone) levels in relation to sportsperson performance and diagnosis have been examined, needing quick and sensitive detection techniques [145,146]. Additionally, quantitative as well as qualitative methodologies for assessing corticoids, such as dexamethasone or budesonide, for DBS in a systematic doping control setting have been documented [113]. More research is still thought to be necessary to investigate the possibility of measuring additional glucocorticoids at pertinent blood quantities, ideally using multi-analyte test methods [147].

Natural as well as synthetic cannabinoids like  $\Delta^9$ -tetrahydrocannabinol (THC) are also prohibited in competition [9]. Only when the primary urinary metabolite of the THC, known as 11-nor-9-carboxy-THC (THCCOOH), surpasses a limit of 150 ng/mL are AAFs for 9-tetrahydrocannabinol (THC) issued. In addition to THC, the possibility of detecting substances from the vast array of synthetically derived cannabinoids in oral fluid has been specifically observed for toxicological as well as forensic research studies. All of these findings have demonstrated and validated the efficacy of this matrix as a substitute for conventional urine and blood testing, often permitting LODs under 1 ng/mL while using LC-MS/MS-based test methodologies using QqQ analyzers [148,149]. Despite the fact that the only published data so far only include JWH-018 and THC and its metabolites, DBS have been proved to be effective as doping controls [150]. In 2017, Protti et. al. utilized volumetric absorptive micro-sampling (VAMS) followed by the concept and authorization of the LC-MS/MS technique for the identification of synthetically derived cannabinoids [151]. For the purpose of detecting JWH-018 and its metabolites, Möller et. al. developed and validated a detection method based on the analysis involving electrospray tandem mass spectrometry- liquid chromatography, liquid-liquid extraction, as well as the enzymatic hydrolysis. The method was tested on around 7500 urine doping control samples, and it led to the discovery of JWH-018. It demonstrated the method's ability to detect JWH-018 and also its derivatives in human urine with sensitivity and specificity [152–154]. However, it will take more research that uses synthetic cannabis to examine the proper specificities and sensitivities.

## 9. Prohibited methods in sports

### 9.1. Blood doping

The World Anti-Doping Agency (WADA) defines blood doping as “the practice of utilizing specific methods and/or substances to increase one's red blood cell count, thereby permitting the body to carry more oxygen throughout the body”. Numerous athletes employ underhand tactics as well as medications with the purpose of enhancing their haemoglobin mass and thereby increase their physical stamina as well as their performance [156]. Moreover, the utilization of artificial O<sub>2</sub> carriers, red blood cell transfusion (RBCs), infusion of haemoglobin (Hb), as well as synthetic activation of erythropoiesis are all explicitly banned procedures in Blood doping [157]. In the International Standard Prohibited List, issued by WADA numerous blood doping formulations and their related methodologies are included in method “M1-MANIPULATION OF BLOOD AND BLOOD COMPONENTS” [9,158]. Many novel advancements like total RNA sequencing (RNA-Seq) was employed in a recent preclinical study to uncover new RNA markers for detecting blood doping within red blood cells (RBCs) as a research design before human trials. Blood samples from wistar rats were blended with a citrate-phosphate-dextrose-adenine (CPDA) mixture and were stored at 4 °C for 0 (control), 10, or 20 days in this study. Following RNA extraction and RBC purification, total RNA-Seq as well as bioinformatics was executed after the prolonged storage of the samples. According to these findings, there were clear patterns of drastically decreased expression of RNA markers like *Lyz2*, *Ifit3*, and *Il1b* genes. As a result, it is anticipated that the methods utilized in this study and the detected RNA markers will be valuable for subsequent research for creating testing procedures for blood doping [159–161].

To evaluate the athlete's haematological blood profile score, ABP (Athlete Biological Passport) was developed with the idea of longitudinal blood parameter monitoring in tandem with direct detection techniques to discover biological markers that are suggestive of doping [162]. This direct detection technique involves the detection of a second population of erythrocytes in a blood sample and serves as the foundational analytical approach for the blood doping procedure [163]. Many techniques like metabolomic, proteomic, or transcriptome investigations emerges for the evaluation of ABP and act as a new source of

optimism in the fight against blood doping. This novel approach of blood profile score evaluation, enables the tracking of individual modifications over time and also distinguishes the anomalies that are both naturally caused by physiological changes within a specific range and even perhaps owing to any outside factors, such as doping [164]. The recent studies have found Hepcidin, a peptide released by the liver, that can serve as an characteristic indirect marker in blood doping detection [165,166]. Hence, many recent advances have been made in strengthening the protocols to avert the practice of blood doping. These recent advances act as the detection algorithms or systems that will be beneficial for future research studies in designing efficient and comprehensive testing procedures against blood doping.

## 10. Gene doping

Sports medicine is deeply concerned about the possibility of gene doping, which is described as the swapping of sequences of nucleic acid along with the utilization of genetically engineered or unmodified cells to increase athletic performance [167]. Several proteins including EPO, IGF, endorphin, vascular endothelial growth factor, human growth hormone, fibroblast growth factor, enkephalin, and myostatin, have been identified as possible targets of gene doping [167]. For such a process, a number of delivery techniques have been suggested and investigated. In general, the athlete's cells are removed from the body, altered in a lab, and then directly transplanted back into the athlete [168]. Cells from the athlete can be modified in vitro using one of two techniques [169,170]. Considering how new gene doping is, many of the potential negative effects are merely hypothetical. Immune response is one potential negative impact. An immunological reaction that could potentially result in the endogenous protein being destroyed could be brought on by the virus or the protein itself. Another issue is that the virus may integrate in such a way that it increases proto-oncogene synthesis, raising the risk of cancer. Additionally, gene doping may influence germ cells and have both planned and unexpected consequences that may be passed on to progeny. Finally, it is challenging to regulate the gene's expression, and overexpression may result in an excess of protein that may accumulate to dangerous amounts [167].

As in 2021 in the month of January, WADA announced laboratories recommendations for genome doping identification approaches utilising polymerase chain reaction (PCR), including various reports, summarising the existing systems as well as methodological approaches for recognizing transgenic gene for sports doping control objectives, and their advantages and disadvantages have also been reported [171–174]. Since gene editing methods based on the CRISPR/Cas system have been demonstrated to be extremely adaptable in modifying genomic information and also, modulating activity of gene using catalytically inactive Cas, evaluation as a doping strategy is also required here to prevent possible subsequent efforts to subvert the approach for performance-enhancing objectives. For example, the appropriateness of the test technique for the analysis of real doping control samples was confirmed by a proof-of-concept administration investigation done by Paßreiter et. al. using an in vivo mouse model that exhibited a SpCas9 detection window for up to 8 h after injection [155]. Moreover, an HPLC-HRMS/MS technique for detecting SpCas9 was devised and tested.

## 11. Conclusion

This review highlights important developments in different analytical approaches, particularly chromatography-and mass spectrometry-based sports drug doping testing. Furthermore, research into bio-transformations has continued to unveil a succession of new metabolites in support of international anti-doping efforts, and mass spectrometry has been the most often used technique to define and detect these metabolites in routine doping controls. Utilizing new knowledge on human drug metabolism and disposition id continuously updated and expanded. Additionally, novel strategies focusing on marker-based test

methods have been evaluated, developed, and put into practise. Moreover, doping control also makes use of immunoassays or electrophoretic techniques as discussed in literature. These direct screening and verification methodologies should be updated frequently, which would include tracking of additional substances with promising performance enhancing assets and constituents of already discovered compounds with expansive sensing windows, this is done to recognize the most notable, extremely developed doping strategies. Yet, the increasing number of novel substances and drug candidates with a wide range of physicochemical properties that have the potential to be abused in sports will continue to be a challenge for sports drug testing labs. Furthermore, anti-doping laboratories should adopt a variety of sample preparation procedures that allow speedier and automated workflow like LLE, SPE, dilute and shoot methods, given the short reporting periods, because there is a requirement to examine a significant quantity of urine and blood samples. Furthermore, DBS was discovered to be a suitable substitute for invasive venous blood sample collection. However, the key analytical combination must be used, but this frequently necessitates costly instrument and personnel with specialised training.

## Declaration of Competing Interest

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

## Data availability

Data will be made available on request.

## References

- [1] A. Momaya, M. Fawal, R. Estes, Performance-Enhancing Substances in Sports: A Review of the Literature, *Sport. Med.* 45 (2015) 517–531, <https://doi.org/10.1007/s40279-015-0308-9>.
- [2] R. Holt, I. Erotokritou-Mulligan, P. Sonksen, The history of doping and growth hormone abuse in sport, *Growth Horm. IGF Res.* 19 (2009) 320–326, <https://doi.org/10.1016/j.ghir.2009.04.009>.
- [3] J. Dvorak, D. Kirkendall, M. Vouillamoz, Therapeutic use exemption, *Br. J. Sports Med.* 40 (Suppl 1) (2006) i40–i42, <https://doi.org/10.1136/bjsm.2006.027490>.
- [4] J. Savulescu, B. Foddy, M. Clayton, Why we should allow performance enhancing drugs in sport, *Br. J. Sports Med.* 38 (2004), <https://doi.org/10.1136/bjsm.2003.005249>.
- [5] O. de Hon, H. Kuipers, M. van Bottenburg, Prevalence of doping use in elite sports: a review of numbers and methods, *Sports Med.* 45 (2015) 57–69, <https://doi.org/10.1007/s40279-014-0247-x>.
- [6] M. Aguilar-Navarro, J. Muñoz-Guerra, M. del Mar Plara, J. Del Coso, Analysis of doping control test results in individual and team sports from 2003 to 2015, *J. Sport Heal. Sci.* 9 (2020) 160–169, <https://doi.org/https://doi.org/10.1016/j.jshs.2019.07.005>.
- [7] J. Shelley, S.N. Thrower, A. Petróczi, Racing Clean in a Tainted World: A Qualitative Exploration of the Experiences and Views of Clean British Elite Distance Runners on Doping and Anti-Doping, *Front. Psychol.* 12 (2021). <https://www.frontiersin.org/articles/10.3389/fpsyg.2021.673087>.
- [8] H. Mortimer, J. Whitehead, M. Kavussanu, B. Gürpınar, C. Ring, Values and clean sport, *J. Sports Sci.* 39 (2021) 533–541, <https://doi.org/10.1080/02640414.2020.1835221>.
- [9] WADA, Prohibited methods (prohibited at all times), *World Anti-Doping Code Prohibited List 2022*. (2022) 13. [https://www.wada-ama.org/sites/default/files/2022-01/2022list\\_final\\_en\\_0.pdf](https://www.wada-ama.org/sites/default/files/2022-01/2022list_final_en_0.pdf).
- [10] G.A. Green, Doping control for the team physician: a review of drug testing procedures in sport, *Am. J. Sports Med.* 34 (2006) 1690–1698, <https://doi.org/10.1177/0363546506293022>.
- [11] M. Thevis, K. Walpurgis, A. Thomas, Analytical Approaches in Human Sports Drug Testing: Recent Advances, Challenges, and Solutions, *Anal. Chem.* 92 (2020) 506–523, <https://doi.org/10.1021/acs.analchem.9b04639>.
- [12] M. Bundy, A. Leaver, Chapter 4 - Drugs and sport, in: M. Bundy, A.B.T.-A.G. to S. and I.M. Leaver (Eds.), *Churchill Livingstone, Edinburgh*, 2010: pp. 61–76. <https://doi.org/https://doi.org/10.1016/B978-0-443-06813-3.00007-7>.
- [13] R. Nicoli, D. Guillaume, N. Leuenberger, N. Baume, N. Robinson, M. Saugy, J. L. Veuthey, Analytical Strategies for Doping Control Purposes: Needs, Challenges, and Perspectives, *Anal. Chem.* 88 (2016) 508–523, <https://doi.org/10.1021/acs.analchem.5b03994>.
- [14] World Anti-Doping Agency, *World Anti-Doping Code International Standard Laboratories (ISL) 2021* (2021) 1–160.

- [15] World Anti-Doping Agency (WADA), World Anti-Doping Code International Standard Testing and Investigations, 2021.
- [16] S. Verma, H. Gupta, O. Alam, P. Mullick, N. Siddiqui, S.A. Khan, Spectrophotometric methods for the estimation of mycophenolate mofetil, *J. Appl. Spectrosc.* 76 (2009) 876–882, <https://doi.org/10.1007/s10812-010-9272-1>.
- [17] M.K. Parr, F. Botrè, Supercritical fluid chromatography mass spectrometry as an emerging technique in doping control analysis, *TrAC Trends Anal. Chem.* 147 (2022) 116517.
- [18] K.M. Welker, B. Lassetter, C.M. Brandes, S. Prasad, D.R. Koop, P.H. Mehta, A comparison of salivary testosterone measurement using immunoassays and tandem mass spectrometry, *Psychoneuroendocrinology*. 71 (2016) 180–188, <https://doi.org/10.1016/j.psyneuen.2016.05.022>.
- [19] A. Breidbach, D.H. Catlin, G.A. Green, I. Tregub, H. Truong, J. Gorzek, Detection of recombinant human erythropoietin in urine by isoelectric focusing, *Clin. Chem.* 49 (2003) 901–907, <https://doi.org/10.1373/49.6.901>.
- [20] M. Thevis, T. Piper, A. Thomas, Recent advances in identifying and utilizing metabolites of selected doping agents in human sports drug testing, *J. Pharm. Biomed. Anal.* 205 (2021) 114312.
- [21] P. Hemmersbach, R. de la Torre, Stimulants, narcotics and beta-blockers: 25 years of development in analytical techniques for doping control, *J. Chromatogr. B. Biomed. Appl.* 687 (1996) 221–238, [https://doi.org/10.1016/s0378-4347\(96\)00276-9](https://doi.org/10.1016/s0378-4347(96)00276-9).
- [22] M. Thevis, W. Schänzer, Examples of doping control analysis by liquid chromatography-tandem mass spectrometry: ephedrine, beta-receptor blocking agents, diuretics, sympathomimetics, and cross-linked hemoglobins, *J. Chromatogr. Sci.* 43 (2005) 22–31, <https://doi.org/10.1093/chromsci/43.1.22>.
- [23] L. Rivier, M. Saugy, Peptide hormones abuse in sport: state of the art in the detection of growth hormone and erythropoietin, *J. Toxicol. Toxin Rev.* 18 (1999) 145–176.
- [24] O. Barroso, D.J. Handelsman, C. Strasburger, M. Thevis, Analytical challenges in the detection of peptide hormones for anti-doping purposes, *Bioanalysis*. 4 (13) (2012) 1577–1590.
- [25] D.E. Greydanus, C. Feucht, Chapter 7. Performance-Enhancing Drugs and Supplements, in: D.R. Patel, D.E. Greydanus, R.J. Baker (Eds.), *Pediatr. Pract. Sport. Med.*, The McGraw-Hill Companies, New York, NY, 2009. <http://accesspediatrics.mhmedical.com/content.aspx?aid=6973163>.
- [26] S. Bhasin, T.W. Storer, N. Berman, C. Callegari, B. Clevenger, J. Phillips, T. J. Bunnell, R. Tricker, A. Shirazi, R. Casaburi, The effects of supraphysiologic doses of testosterone on muscle size and strength in normal men, *N. Engl. J. Med.* 335 (1996) 1–7, <https://doi.org/10.1056/NEJM199607043350101>.
- [27] J.B. Ding, M.Z. Ng, S.S. Huang, M. Ding, K. Hu, N.J. Rehrer, Anabolic-Androgenic Steroid Misuse: Mechanisms, Patterns of Misuse, User Typology, and Adverse Effects, *J. Sports Med.* 2021 (2021) 1–9.
- [28] D. Sague, H. Molde, C.S. Andreassen, T. Torsheim, S. Pallesen, The global epidemiology of anabolic-androgenic steroid use: a meta-analysis and meta-regression analysis, *Ann. Epidemiol.* 24 (2014) 383–398, <https://doi.org/10.1016/j.annepidem.2014.01.009>.
- [29] F.F. Al-Harbi, I. Gamaleddin, E.G. Alsabaie, K.M. Al-Surimi, Prevalence and Risk Factors Associated with Anabolic-androgenic Steroid Use: A Cross-sectional Study among Gym Users in Riyadh, Saudi Arabia., *Oman, Med. J.* 35 (2) (2020) e110.
- [30] A.J.M. Gregory, R.W. Fitch, Sports Medicine: Performance-Enhancing Drugs, *Pediatric Clinics of North America* 54 (4) (2007) 797–806.
- [31] J.M.X. Amaral, M.C. Padilha, S.V. Chagas, J.S. Baker, C. Mullen, L. Vieira Neto, F. R. Aquino Neto, M.S. Cruz, Effective treatment and prevention of attempted suicide, anxiety, and aggressiveness with fluoxetine, despite proven use of androgenic anabolic steroids, *Drug Test. Anal.* 13 (1) (2021) 197–202.
- [32] C.D. Rahneima, L.I. Lipshultz, L.E. Crosnoe, J.R. Kovac, E.D. Kim, Anabolic steroid-induced hypogonadism: diagnosis and treatment, *Fertil. Steril.* 101 (5) (2014) 1271–1279.
- [33] E. Turillazzi, G. Perilli, M. Di Paolo, M. Neri, I. Riezzo, V. Fineschi, Side effects of AAS abuse: an overview, *Mini Rev. Med. Chem.* 11 (2011) 374–389.
- [34] J.G. Oberlander, L.P. Henderson, The Sturm und Drang of anabolic steroid use: angst, anxiety, and aggression, *Trends Neurosci.* 35 (2012) 382–392.
- [35] M. Montisci, R. El Mazloum, G. Cecchetto, C. Terranova, S.D. Ferrara, G. Thiene, C. Basso, Anabolic androgenic steroids abuse and cardiac death in athletes: morphological and toxicological findings in four fatal cases, *Forensic Sci. Int.* 217 (2012) e13–e18.
- [36] M. Thevis, T. Kuuranne, H. Geyer, Annual banned-substance review: Analytical approaches in human sports drug testing 2020/2021, *Drug Test. Anal.* 14 (2022) 7–30.
- [37] M. Polet, W. Van Gansbeke, A.D. Albertsdóttir, G. Coppieters, K. Deventer, P. Van Eenoo, Gas chromatography-mass spectrometry analysis of non-hydrolyzed sulfated steroids by degradation product formation, *Drug Test. Anal.* 11 (2019) 1656–1665, <https://doi.org/10.1002/dta.2606>.
- [38] Y. Zhang, X. Wu, W. Wang, J. Huo, J. Luo, Y. Xu, J. Lu, Simultaneous detection of 93 anabolic androgenic steroids in dietary supplements using gas chromatography tandem mass spectrometry, *J. Pharm. Biomed. Anal.* 211 (2022) 114619.
- [39] D. Thiene, P. Hemmersbach, Doping in sports, Springer Science & Business Media, 2009.
- [40] U. Marek, H. Geyer, G. Opfermann, M. Thevis, W. Schänzer, Factors influencing the steroid profile in doping control analysis, *J. Mass Spectrom.* 43 (2008) 877–891, <https://doi.org/10.1002/jms.1457>.
- [41] E.R. Cooper, G. Hughes, A. Kauff, E. Sutherland, Z. Ashley, A.K. Heather, A cell-free bioassay for the detection of androgens, *Drug Test. Anal.* 13 (2021) 903–915.
- [42] A. Zschiesche, C. Chundela, D. Thiene, A.M. Keiler, HepG2 as promising cell-based model for biosynthesis of long-term metabolites: Exemplified for metandienone, *Drug Test. Anal.* 14 (2022) 298–306.
- [43] M. Patt, K.R. Beck, T. Di Marco, M.-C. Jäger, V. González-Ruiz, J. Boccard, S. Rudaz, R.W. Hartmann, M. Salah, C.J. van Koppen, M. Grill, A. Odermatt, Profiling of anabolic androgenic steroids and selective androgen receptor modulators for interference with adrenal steroidogenesis, *Biochem. Pharmacol.* 172 (2020) 113781.
- [44] P. Leogrande, F. Botrè, X. de la Torre, D. Jardines, M.K. Parr, F. Marini, Coupling high-resolution mass spectrometry and chemometrics for the structural characterization of anabolic-androgenic steroids and the early detection of unknown designer structures, *Talanta*. 227 (2021) 122173, <https://doi.org/10.1016/j.talanta.2021.122173>.
- [45] C. Görgens, A.P. Ramme, S. Guddat, Y. Schrader, A. Winter, E.-M. Dehne, R. Horland, M. Thevis, Organ-on-a-chip: Determine feasibility of a human liver microphysiological model to assess long-term steroid metabolites in sports drug testing, *Drug Test. Anal.* 13 (2021) 1921–1928, <https://doi.org/10.1002/dta.3161>.
- [46] S. Loke, X. de la Torre, M. Iannone, G. La Piana, N. Schlöf, F. Botrè, M. Bureik, M.K. Parr, Controlled administration of dehydrochloromethyltestosterone in humans: Urinary excretion and long-term detection of metabolites for anti-doping purpose, *J. Steroid Biochem. Mol. Biol.* 214 (2021) 105978.
- [47] Z. Wang, X. Zhou, X. Liu, Y. Dong, J. Zhang, A novel HPLC-MRM strategy to discover unknown and long-term metabolites of stanozolol for expanding analytical possibilities in doping-control, *J. Chromatogr. B.* 1040 (2017) 250–259.
- [48] M. Saugy, N. Leuenberger, Antidoping: From health tests to the athlete biological passport, *Drug Test. Anal.* 12 (2020) 621–628, <https://doi.org/10.1002/dta.2773>.
- [49] P.-E. Sottas, N. Robinson, M. Saugy, The athlete's biological passport and indirect markers of blood doping, *Doping Sport, Biochem. Princ. Eff. Anal.* (2010) 305–326.
- [50] T. Piper, H. Geyer, N. Haenelt, F. Huelsemann, W. Schaezner, M. Thevis, Current Insights into the Steroidal Module of the Athlete Biological Passport, *Int. J. Sports Med.* 42 (2021) 863–878, <https://doi.org/10.1055/a-1481-8683>.
- [51] P. Sakellariou, P. Kiousi, A.G. Fragkaki, E. Lyris, M. Petrou, C. Georgakopoulos, Y. S. Angelis, Alternative markers for Methylornestosterone misuse in human urine, *Drug Test. Anal.* 12 (2020) 1544–1553, <https://doi.org/10.1002/dta.2887>.
- [52] O. Salamin, R. Nicoli, T. Langer, J. Boccard, C.S. Grundisch, C. Xu, S. Rudaz, T. Kuuranne, N. Pitteloud, M. Saugy, Longitudinal evaluation of multiple biomarkers for the detection of testosterone gel administration in women with normal menstrual cycle, *Drug Test. Anal.* 14 (2022) 833–850, <https://doi.org/10.1002/dta.3040>.
- [53] O. Salamin, R. Nicoli, C. Xu, J. Boccard, S. Rudaz, N. Pitteloud, M. Saugy, T. Kuuranne, Steroid profiling by UHPLC-MS/MS in dried blood spots collected from healthy women with and without testosterone gel administration, *J. Pharm. Biomed. Anal.* 204 (2021) 114280.
- [54] Y. Yuan, Y. Xu, J. Lu, Detection of 20 endogenous anabolic steroid esters with Girard's Reagent P derivatization in dried blood spots using UPLC-Q-Orbitrap-MS, *J. Chromatogr. B.* 1213 (2022) 123535.
- [55] W.S./L.W. Group, WADA Technical Document – TD2021IRMS - Detection of Synthetic Forms of Prohibited Substances by GC/C/IRMS, (2021) 1–15. [https://www.wada-ama.org/sites/default/files/resources/files/td2021irms\\_final\\_eng\\_v\\_2.0.pdf](https://www.wada-ama.org/sites/default/files/resources/files/td2021irms_final_eng_v_2.0.pdf) (accessed September 24, 2022).
- [56] M. Polet, P. Van Eenoo, GC-C-IRMS in routine doping control practice: 3 years of drug testing data, quality control and evolution of the method, *Anal. Bioanal. Chem.* 407 (2015) 4397–4409, <https://doi.org/10.1007/s00216-014-8374-7>.
- [57] A. Casilli, T. Piper, F.A. de Oliveira, M.C. Padilha, H.M. Pereira, M. Thevis, F.R. de Aquino Neto, Optimization of an online heart-cutting multidimensional gas chromatography clean-up step for isotopic ratio mass spectrometry and simultaneous quadrupole mass spectrometry measurements of endogenous anabolic steroid in urine, *Drug Test. Anal.* 8 (2016) 1204–1211.
- [58] K. Lalonde, A. Barber, C. Ayotte, Two-dimensional high performance liquid chromatography purification of underivatized urinary testosterone and metabolites for compound-specific stable carbon isotope analysis, *Drug Test. Anal.* 13 (2021) 558–570.
- [59] L. Iannella, C. Colamonici, D. Curcio, F. Botrè, X. de la Torre, Detecting the abuse of 19-norsteroids in doping controls: A new gas chromatography coupled to isotope ratio mass spectrometry method for the analysis of 19-norandrosterone and 19-noretiocholanolone, *Drug Test. Anal.* 13 (2021) 770–784.
- [60] T.L.S. Choi, J.K.Y. Wong, W.H. Kwok, P. Curl, S. Mechie, T.S.M. Wan, Metabolic study of methylstenbolone in horses using liquid chromatography-high resolution mass spectrometry and gas chromatography-mass spectrometry, *J. Chromatogr. A.* 1546 (2018) 106–118.
- [61] T. Piper, G. Fusshöller, W. Schänzer, A. Lagojda, D. Kuehne, M. Thevis, Studies on the in vivo metabolism of methylstenbolone and detection of novel long term metabolites for doping control analysis, *Drug Test. Anal.* 11 (2019) 1644–1655.
- [62] X. de la Torre, C. Colamonici, D. Curcio, F. Botrè, Fast IRMS screening of pseudoendogenous steroids in doping analyses, *Drug Test. Anal.* 9 (2017) 1804–1812.
- [63] C. Wen, T. Zhu, J. Wang, X. Liu, S. Wang, Y. Zhang, Application of online two-dimensional high-performance liquid chromatography as purification procedure to determine the origin of 19-norandrosterone in urine by gas

- chromatography–combustion–isotope ratio mass spectrometry, *Drug Test. Anal.* 13 (2021) 338–347.
- [64] A.T. Cawley, U. Flenker, The application of carbon isotope ratio mass spectrometry to doping control, *J. Mass Spectrom.* 43 (2008) 854–864.
- [65] K.J. Goodman, Hardware Modifications to an Isotope Ratio Mass Spectrometer Continuous-Flow Interface Yielding Improved Signal, Resolution, and Maintenance, *Anal. Chem.* 70 (1998) 833–837, <https://doi.org/10.1021/ac970887q>.
- [66] M. Thevis, W. Schänzer, Mass spectrometry of selective androgen receptor modulators, *J. Mass Spectrom.* 43 (2008) 865–876, <https://doi.org/10.1002/jms.1438>.
- [67] K. Walpurgis, A. Thomas, H. Geyer, U. Mareck, M. Thevis, Dietary Supplement and Food Contaminations and Their Implications for Doping Controls., *Foods* 9 (2020), <https://doi.org/10.3390/foods9081012>.
- [68] M. Thevis, W. Schänzer, Detection of SARMS in doping control analysis, *Mol. Cell. Endocrinol.* 464 (2018) 34–45.
- [69] P. Kintz, The forensic response after an adverse analytical finding (doping) involving a selective androgen receptor modulator (SARM) in human athlete, *J. Pharm. Biomed. Anal.* 207 (2022) 114433.
- [70] B. Holubová, P. Kubešová, L. Huml, M. Vlach, O. Lapčík, M. Jurašek, L. Fukal, Tailor-Made Immunochromatographic Test for the Detection of Multiple 17 $\alpha$ -Methylated Anabolics in Dietary Supplements, *Foods* 10 (2021) 741.
- [71] J.-P. Salvador, F. Sánchez-Baeza, M.-P. Marco, A high-throughput screening (HTS) immunochemical method for the analysis of stanozolol metabolites in cattle urine samples., *J. Chromatogr. B, Anal. Technol. Biomed. Life Sci.* 878 (2010) 243–252, <https://doi.org/10.1016/j.jchromb.2009.08.027>.
- [72] K. Kowalczyk, J.C. Torres-Elguera, A. Jarek, A. Konopka, D. Kwiatkowska, E. Bulska, In vitro metabolic studies of novel selective androgen receptor modulators and their use for doping control analysis, *Drug Test. Anal.* 14 (2022) 122–136, <https://doi.org/10.1002/dta.3151>.
- [73] M.L. Decramer, N.A. Hanania, J.O. Lötvall, B.P. Yawn, The safety of long-acting  $\beta_2$ -agonists in the treatment of stable chronic obstructive pulmonary disease, *Int. J. Chron. Obstruct. Pulmon. Dis.* 8 (2013) 53–64, <https://doi.org/10.2147/COPD.S39018>.
- [74] J.N. Marchant-Forde, D.C.J. Lay, R.M. Marchant-Forde, K.A. McMunn, B. T. Richert, The effects of R-salbutamol on growth, carcass measures, and health of finishing pigs, *J. Anim. Sci.* 90 (2012) 4081–4089, <https://doi.org/10.2527/jas.2011-4423>.
- [75] H. Allen, O.J. Price, J.H. Hull, S.H. Backhouse, Asthma medication in athletes: a qualitative investigation of adherence, avoidance and misuse in competitive sport, *J. Asthma Off. J. Assoc. Care Asthma.* 59 (2022) 811–822, <https://doi.org/10.1080/02770903.2021.1881968>.
- [76] A. Thomas, M. Thevis, Stereoisomers in sports drug testing: Analytical strategies and applications, *J. Chromatogr. A.* 1674 (2022) 463154, <https://doi.org/10.1016/j.chroma.2022.463154>.
- [77] S. Jessen, V. Becker, S. Rzeppa, V. Backer, K.H. Bengtsen, I. Hullstein, Y. Dehnes, M. Hostrup, Pharmacokinetics of salmeterol and its main metabolite  $\alpha$ -hydroxysalmeterol after acute and chronic dry powder inhalation in exercising endurance-trained men: Implications for doping control, *Drug Test. Anal.* 13 (2021) 747–761, <https://doi.org/10.1002/dta.2978>.
- [78] R. Bergés, J. Segura, X. de la Torre, R. Ventura, Analytical methodology for enantiomers of salbutamol in human urine for application in doping control Presented at the 27th International Meeting of the Spanish Group of Chromatography and Related Techniques, *Lugo, July 8–10, 1998, J. Chromatogr. B Biomed. Sci. Appl.* 723 (1999) 173–184.
- [79] R. Bergés, J. Segura, R. Ventura, K.D. Fitch, A.R. Morton, M. Farré, M. Mas, X. de la Torre, Discrimination of prohibited oral use of salbutamol from authorized inhaled asthma treatment, *Clin. Chem.* 46 (2000) 1365–1375.
- [80] R. Ventura, J. Segura, R. Bergés, K.D. Fitch, A.R. Morton, S. Berrueto, C. Jiménez, Distinction of inhaled and oral salbutamol by urine analysis using conventional screening procedures for doping control, *Ther. Drug Monit.* 22 (2000) 277–282, <https://doi.org/10.1097/00007691-200006000-00008>.
- [81] B. Velasco-Bejarano, R. Velasco-Carrillo, E. Camacho-Frias, J. Bautista, R. López-Arellano, L. Rodríguez, Detection of clenbuterol residues in beef sausages and its enantiomeric analysis using UHPLC-MS/MS: A risk of unintentional doping in sport field, *Drug Test. Anal.* 14 (2022) 1130–1139, <https://doi.org/10.1002/dta.3235>.
- [82] V.L. Campo, L.S.C. Bernardes, I. Carvalho, Stereoselectivity in drug metabolism: molecular mechanisms and analytical methods, *Curr. Drug Metab.* 10 (2009) 188–205, <https://doi.org/10.2174/138920009787522188>.
- [83] J. Vela, E.G. Yanes, A.M. Stalcup, Quantitative determination of clenbuterol, salbutamol and tulobuterol enantiomers by capillary electrophoresis, *Fresenius, J. Anal. Chem.* 369 (2001) 212–219, <https://doi.org/10.1007/s002160000584>.
- [84] J. Caslavská, W. Thormann, Stereoselective determination of drugs and metabolites in body fluids, tissues and microosomal preparations by capillary electrophoresis (2000–2010), *J. Chromatogr. A.* 1218 (2011) 588–601.
- [85] J. Bailes, M. Soloviev, Insulin-Like Growth Factor-1 (IGF-1) and Its Monitoring in Medical Diagnostic and in Sports, *Biomolecules.* 11 (2) (2021), <https://doi.org/10.3390/biom11020217>.
- [86] L.J. Woodhouse, A. Mukherjee, S.M. Shalet, S. Ezzat, The influence of growth hormone status on physical impairments, functional limitations, and health-related quality of life in adults, *Endocr. Rev.* 27 (2006) 287–317, <https://doi.org/10.1210/er.2004-0022>.
- [87] V.I. Rickert, C. Pawlak-Morello, V. Sheppard, M.S. Jay, Human growth hormone: a new substance of abuse among adolescents? *Clin. Pediatr. (Phila)* 31 (1992) 723–726, <https://doi.org/10.1177/000992289203101206>.
- [88] C. Ehrnborg, B.A. Bengtsson, T. Rosén, Growth hormone abuse., *Baillieres, Best Pract. Res. Clin. Endocrinol. Metab.* 14 (2000) 71–77, <https://doi.org/10.1053/beem.2000.0054>.
- [89] R. Morishita, H. Makino, M. Aoki, N. Hashiya, K. Yamasaki, J. Azuma, Y. Taniyama, Y. Sawa, Y. Kaneda, T. Ogiwara, Phase I/IIa clinical trial of therapeutic angiogenesis using hepatocyte growth factor gene transfer to treat critical limb ischemia, *Arterioscler. Thromb. Vasc. Biol.* 31 (2011) 713–720, <https://doi.org/10.1161/ATVBAHA.110.219550>.
- [90] D.L. Steed, Clinical evaluation of recombinant human platelet-derived growth factor for the treatment of lower extremity ulcers, *Plast. Reconstr. Surg.* 117 (SUPPLEMENT) (2006) 143S–149S.
- [91] U.H. Stenman, K. Hotakainen, H. Alfthan, Gonadotropins in doping: Pharmacological basis and detection of illicit use, *Br. J. Pharmacol.* 154 (2008) 569–583, <https://doi.org/10.1038/bjp.2008.102>.
- [92] C.Y. Bowers, F.A. Momany, G.A. Reynolds, A. Hong, On the in vitro and in vivo activity of a new synthetic hexapeptide that acts on the pituitary to specifically release growth hormone, *Endocrinology.* 114 (1984) 1537–1545, <https://doi.org/10.1210/endo-114-5-1537>.
- [93] A. Thomas, M. Kohler, J. Mester, H. Geyer, W. Schänzer, M. Petrou, M. Thevis, Identification of the growth-hormone-releasing peptide-2 (GHRP-2) in a nutritional supplement, *Drug Test. Anal.* 2 (2010) 144–148, <https://doi.org/10.1002/dta.120>.
- [94] P. Judák, S. Esposito, G. Coppieters, P. Van Eenoo, K. Deventer, Doping control analysis of small peptides: A decade of progress., *J. Chromatogr. B, Anal. Technol. Biomed. Life Sci.* 1173 (2021) 122551, <https://doi.org/10.1016/j.jchromb.2021.122551>.
- [95] A. Pinyot, Z. Nikolovski, J. Bosch, G. Such-Sanmartín, S. Kageyama, J. Segura, R. Gutiérrez-Gallego, Growth hormone secretagogues: out of competition, *Anal. Bioanal. Chem.* 402 (2012) 1101–1108, <https://doi.org/10.1007/s00216-011-5544-8>.
- [96] A. Pinyot, Z. Nikolovski, J. Bosch, J. Segura, R. Gutiérrez-Gallego, On the use of cells or membranes for receptor binding: growth hormone secretagogues, *Anal. Biochem.* 399 (2010) 174–181, <https://doi.org/10.1016/j.jab.2010.01.003>.
- [97] M. Okano, Y. Nishitani, M. Sato, A. Ikekita, S. Kageyama, Influence of intravenous administration of growth hormone releasing peptide-2 (GHRP-2) on detection of growth hormone doping: growth hormone isoform profiles in Japanese male subjects, *Drug Test. Anal.* 2 (2010) 548–556, <https://doi.org/10.1002/dta.166>.
- [98] E. Gerace, J. Modaffari, P. Negri, D. Di Corcia, E. Amante, A. Salomone, M. Vincenti, Detection of the synthetic peptide ipamorelin in dried blood spots by means of UHPLC-HRMS, *Int. J. Mass Spectrom.* 462 (2021) 116531.
- [99] Y. Seo, J. Park, M. Kim, C. Sung, O.-S. Kwon, H.J. Lee, H. Min, 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.* 13 (2021) 451–459, <https://doi.org/10.1002/dta.2944>.
- [100] M. Thevis, A. Thomas, H. Geyer, W. Schänzer, Mass spectrometric characterization of a biotechnologically produced full-length mechano growth factor (MGF) relevant for doping controls, *Growth Horm. IGF Res.* 24 (2014) 276–280, <https://doi.org/10.1016/j.ghir.2014.10.004>.
- [101] C. Lundby, G.P. Millet, J.A. Calbet, P. Bärtsch, A.W. Subudhi, Does “altitude training” increase exercise performance in elite athletes? *Br. J. Sports Med.* 46 (2012) 792–795, <https://doi.org/10.1136/bjsports-2012-091231>.
- [102] J.A.A.C. Heuberger, A.F. Cohen, Review of WADA Prohibited Substances: Limited Evidence for Performance-Enhancing Effects, *Sport. Med.* 49 (2019) 525–539, <https://doi.org/10.1007/s40279-018-1014-1>.
- [103] J.A.A.C. Heuberger, J.I. Rotmans, P. Gal, F.E. Stuutman, J. van 't Westende, T. E. Post, J.M.A. Daniels, M. Moerland, P.L.J. van Veldhoven, M.L. de Kam, H. Ram, O. de Hon, J.A. Posthuma, J. Burggraaf, A.F. Cohen, Effects of erythropoietin on cycling performance of well trained cyclists: a double-blind, randomised, placebo-controlled trial, *Lancet. Haematol.* 4 (8) (2017) e374–e386.
- [104] T. Haider, V. Diaz, J. Albert, M. Alvarez-Sanchez, M. Thiersch, M. Maggiorini, M. P. Hilty, C.M. Spengler, M. Gassmann, A Single 60,000 IU Dose of Erythropoietin Does Not Improve Short-Term Aerobic Exercise Performance in Healthy Subjects: A Randomized, Double-Blind, Placebo-Controlled Crossover Trial, *Front. Physiol.* 11 (2020) 537389, <https://doi.org/10.3389/fphys.2020.537389>.
- [105] B. Chapuy, M.R. McKeown, C.Y. Lin, S. Monti, M.G.M. Roemer, J. Qi, P.B. Rahl, H. H. Sun, K.T. Yeda, J.G. Doench, E. Reichert, A.L. Kung, S.J. Rodig, R.A. Young, M. A. Shipp, J.E. Bradner, Discovery and characterization of super-enhancer-associated dependencies in diffuse large B cell lymphoma, *Cancer Cell.* 24 (2013) 777–790, <https://doi.org/10.1016/j.ccr.2013.11.003>.
- [106] A. Rocca, L. Martin, T. Kuuranne, M. Ericsson, A. Marchand, N. Leuenberger, A fast screening method for the detection of CERA in dried blood spots, *Drug Test. Anal.* 14 (2022) 820–825, <https://doi.org/10.1002/dta.3142>.
- [107] L. De Wilde, K. Roels, K. Deventer, P. Van Eenoo, Automated sample preparation for the detection and confirmation of hypoxia-inducible factor stabilizers in urine, *Biomed. Chromatogr.* 35 (2021) e4970.
- [108] C.E. Heiland, M. Ericsson, A. Pohanka, L. Ekström, A. Marchand, Optimizing detection of erythropoietin receptor agonists from dried blood spots for anti-doping application, *Drug Test. Anal.* 14 (2022) 1377–1386.
- [109] D.A. Seehusen, J.E. Glorioso, Tamoxifen as an ergogenic agent in women body builders, *Clin. J. Sport Med. Off. J. Can. Acad. Sport Med.* 12 (2002) 313–314, <https://doi.org/10.1097/00042752-200209000-00010>.
- [110] U. Mareck, G. Sigmund, G. Opfermann, H. Geyer, W. Schänzer, Identification of the aromatase inhibitor aminoglutethimide in urine by gas chromatography/mass spectrometry, *Rapid Commun. Mass Spectrom.* 16 (2002) 2209–2214, <https://doi.org/10.1002/rcm.838>.

- [111] M. Okano, A. Miyamoto, M. Ota, S. Kageyama, M. Sato, Doping control analysis of trimetazidine in dried blood spot, *Drug Test. Anal.* 2022.
- [112] D. Martínez Brito, P. Leogrande, F. Botré, X. de la Torre, Detection of urinary arimistane metabolites in humans using liquid chromatography-mass spectrometry: Complementary results to gas chromatography mass spectrometric data and its application to antidoping analyses, *Rapid Commun. Mass Spectrom.* 35 (2021) e9080.
- [113] A. Thomas, H. Geyer, W. Schänzer, C. Crone, M. Kellmann, T. Moehring, M. Thevis, 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.* 403 (2012) 1279–1289, <https://doi.org/10.1007/s00216-011-5655-2>.
- [114] D. Nedelkov, E.E. Niederkofler, P.E. Oran, S. Peterman, R.W. Nelson, Top-down mass spectrometric immunoassay for human insulin and its therapeutic analogs, *J. Proteomics.* 175 (2018) 27–33, <https://doi.org/10.1016/j.jprot.2017.08.001>.
- [115] N. Rosli, H.-J. Kwon, J. Lim, Y.A. Yoon, J.-S. Jeong, Measurement comparability of insulin assays using conventional immunoassay kits, *J. Clin. Lab. Anal.* 36 (2022) e24521.
- [116] R. Abellan, R. Ventura, S. Pichini, J.A. Pascual, R. Pacifici, S. Di Carlo, A. Bacosi, J. Segura, P. Zuccaro, Evaluation of immunoassays for the measurement of insulin-like growth factor-I and procollagen type III peptide, indirect biomarkers of recombinant human growth hormone misuse in sport, *Clin. Chem. Lab. Med.* 43 (2005) 75–85, <https://doi.org/10.1515/CCLM.2005.012>.
- [117] L. Andersen, P.N. Jørgensen, L.B. Jensen, D. Walsh, A New Insulin Immunoassay Specific for the Rapid-Acting Insulin Analog, Insulin Aspart, Suitable for Bioavailability, Bioequivalence, and Pharmacokinetic Studies, *Clin. Biochem.* 33 (2000) 627–633.
- [118] A. Thomas, L. Benzenberg, L. Bally, M. Thevis, Facilitated Qualitative Determination of Insulin, Its Synthetic Analogs, and C-Peptide in Human Urine by Means of LC-HRMS, *Metabolites* 11 (5) (2021), <https://doi.org/10.3390/metabo11050309>.
- [119] K. Deventer, O.J. Pozo, P. Van Eenoo, F.T. Delbeke, Qualitative detection of diuretics and acidic metabolites of other doping agents in human urine by high-performance liquid chromatography-tandem mass spectrometry: comparison between liquid-liquid extraction and direct injection, *J. Chromatogr. A* 1216 (2009) 5819–5827, <https://doi.org/10.1016/j.chroma.2009.06.003>.
- [120] A.J. Girón, K. Deventer, K. Roels, P. Van Eenoo, Development and validation of an open screening method for diuretics, stimulants and selected compounds in human urine by UHPLC-HRMS for doping control, *Anal. Chim. Acta* 721 (2012) 137–146, <https://doi.org/10.1016/j.aca.2012.02.002>.
- [121] S. Guddat, E. Solymos, A. Orlovius, A. Thomas, G. Sigmund, H. Geyer, M. Thevis, W. Schänzer, High-throughput screening for various classes of doping agents using a new “dilute-and-shoot” liquid chromatography-tandem mass spectrometry multi-target approach, *Drug Test. Anal.* 3 (2011) 836–850, <https://doi.org/10.1002/dta.372>.
- [122] World Anti-Doping Agency (WADA), WADA Technical Document – TD2022MRPL Minimum Required Performance Levels and applicable Minimum Reporting Levels for non-threshold substances analyzed by Chromatographic - Mass Spectrometric analytical methods. 1.0, (2022) 1–12.
- [123] L. De Wilde, K. Roels, M. Polet, P. Van Eenoo, K. Deventer, Identification and confirmation of diuretics and masking agents in urine by turbulent flow online solid-phase extraction coupled with liquid chromatography-triple quadrupole mass spectrometry for doping control, *J. Chromatogr. A* 1579 (2018) 31–40, <https://doi.org/10.1016/j.chroma.2018.10.032>.
- [124] R. Remínek, F. Foret, Capillary electrophoretic methods for quality control analyses of pharmaceuticals: A review, *Electrophoresis* 42 (2021) 19–37.
- [125] L. Zheng, L. Zhang, P. Tong, X. Zheng, Y. Chi, G. Chen, Highly sensitive transient isotachopheresis sample stacking coupling with capillary electrophoresis-ampereometric detection for analysis of doping substances, *Talanta* 81 (2010) 1288–1294.
- [126] C.R. Harrison, Role of Capillary Electrophoresis in the Fight Against Doping in Sports, *Anal. Chem.* 85 (2013) 6982–6987, <https://doi.org/10.1021/ac302821x>.
- [127] P. Nan, Detection of Diuretic Doping by Capillary Electrophoresis and Electrochemical Technology: A Mini-Review, *Curr. Pharm. Anal.* 18 (1) (2022) 34–42.
- [128] R. Ventura, M. Roig, N. Monfort, P. Sáez, R. Bergés, J. Segura, High-throughput and sensitive screening by ultra-performance liquid chromatography tandem mass spectrometry of diuretics and other doping agents, *Eur. J. Mass Spectrom.* 14 (3) (2008) 191–200.
- [129] J. Swart, R.P. Lamberts, M.I. Lambert, A. St Clair Gibson, E.V. Lambert, J. Skowno, T.D. Noakes, Exercising with reserve: evidence that the central nervous system regulates prolonged exercise performance, *Br. J. Sports Med.* 43 (10) (2009) 782–788.
- [130] J.V. Chandler, S.N. Blair, The effect of amphetamines on selected physiological components related to athletic success, *Med. Sci. Sports Exerc.* 12 (1980) 65–69.
- [131] M. Thevis, H. Geyer, L. Tretzel, W. Schänzer, Sports drug testing using complementary matrices: Advantages and limitations, *J. Pharm. Biomed. Anal.* 130 (2016) 220–230, <https://doi.org/10.1016/j.jpba.2016.03.055>.
- [132] A. Solans, M. Carnicero, R. de la Torre, J. Segura, Comprehensive screening procedure for detection of stimulants, narcotics, adrenergic drugs, and their metabolites in human urine, *J. Anal. Toxicol.* 19 (1995) 104–114, <https://doi.org/10.1093/jat/19.2.104>.
- [133] J. Segura, R. Ventura, C. Jurado, Derivatization procedures for gas chromatographic-mass spectrometric determination of xenobiotics in biological samples, with special attention to drugs of abuse and doping agents, *J. Chromatogr. B Biomed. Sci. Appl.* 713 (1998) 61–90.
- [134] J.J. Pitt, Principles and applications of liquid chromatography-mass spectrometry in clinical biochemistry, *Clin. Biochem. Rev.* 30 (2009) 19–34, <https://pubmed.ncbi.nlm.nih.gov/19224008>.
- [135] C. Mantzourani, M.G. Kokotou, Liquid Chromatography-Mass Spectrometry (LC-MS) Derivatization-Based Methods for the Determination of Fatty Acids in Biological Samples, *Molecules* 27 (17) (2022), <https://doi.org/10.3390/molecules27175717>.
- [136] L. De Wilde, K. Roels, P. Van Eenoo, K. Deventer, Online Turbulent Flow Extraction and Column Switching for the Confirmatory Analysis of Stimulants in Urine by Liquid Chromatography-Mass Spectrometry, *J. Anal. Toxicol.* 45 (2021) 666–678, <https://doi.org/10.1093/jat/bkaa136>.
- [137] P.D. de O. Sardela, V.F. Sardela, A.M. dos S. da Silva, H.M.G. Pereira, F.R. de Aquino Neto, A pilot study of non-targeted screening for stimulant misuse using high-resolution mass spectrometry, *Forensic Toxicol.* 37 (2019) 465–473, <https://doi.org/10.1007/s11419-019-00482-1>.
- [138] A. Knoop, G. Fußhöller, N. Haenelt, C. Goergens, S. Guddat, H. Geyer, M. Thevis, Mass spectrometric characterization of urinary hydralin metabolites for routine doping control purposes, *Drug Test. Anal.* 13 (2021) 1915–1920, <https://doi.org/10.1002/dta.3137>.
- [139] T. Zhu, Y. Jin, J. Zhao, J. Jing, T. Tian, Y. Shan, X. Xu, Y. Wang, Qualitative and quantitative analysis of ephedrine stimulants in urine by ultra-performance liquid chromatography-tandem mass spectrometry, *Rapid Commun. Mass Spectrom.* 36 (2022) e9229.
- [140] P. Kintz, F. Mathiaux, P. Villéger, J.-M. Gaulier, Testing for Drugs in Exhaled Breath Collected With ExaBreath in a Drug Dependence Population: Comparison With Data Obtained in Urine After Liquid Chromatographic-Tandem Mass Spectrometric Analyses, *Ther. Drug Monit.* 38 (2016) 135–139, <https://doi.org/10.1097/FTD.0000000000000228>.
- [141] B.D. Ahrens, Y. Kucherova, A.W. Butch, Detection of Stimulants and Narcotics by Liquid Chromatography-Tandem Mass Spectrometry and Gas Chromatography-Mass Spectrometry for Sports Doping Control BT - Clinical Applications of Mass Spectrometry in Drug Analysis: Methods and Protocols, in: U. Garg (Ed.), Springer New York, New York, NY, 2016: pp. 247–263, [https://doi.org/10.1007/978-1-4939-3252-8\\_26](https://doi.org/10.1007/978-1-4939-3252-8_26).
- [142] S. Dubey, S. C.P., K. Tejinder, S. S.P., C. M., A. Beotra, S. Jain, Development and validation of a comprehensive method for detection of eighty stimulants, narcotics & other drugs by gas chromatography nitrogen phosphorous detector/mass spectrometry in sports doping analysis, (2016).
- [143] R.S. Minhas, D.A. Rudd, H.Z. Al Hmoud, T.M. Guinan, K.P. Kirkbride, N. H. Voelcker, Rapid Detection of Anabolic and Narcotic Doping Agents in Saliva and Urine by Means of Nanostructured Silicon SALDI Mass Spectrometry, *ACS Appl. Mater. Interfaces* 12 (2020) 31195–31204, <https://doi.org/10.1021/acsaami.0c07849>.
- [144] K.V. Trinh, K.J.Q. Chen, D. Diep, Effect of Glucocorticoids on Athletic Performance: A Systematic Review and Meta-Analysis, *Clin. J. Sport Med. Off. J. Can. Acad. Sport Med.* 32 (2022) e151–e159, <https://doi.org/10.1097/JSM.0000000000000911>.
- [145] L.D. Hayes, F.M. Grace, J.S. Baker, N. Sculthorpe, Exercise-induced responses in salivary testosterone, cortisol, and their ratios in men: a meta-analysis, *Sports Med.* 45 (2015) 713–726, <https://doi.org/10.1007/s40279-015-0306-y>.
- [146] T. Cevada, P.E. Vasques, H. Moraes, A. Deslandes, Salivary cortisol levels in athletes and nonathletes: a systematic review, *Horm. Metab. Res. = Horm. Und Stoffwechselforsch. = Horm. Metab.* 46 (2014) 905–910, <https://doi.org/10.1055/s-0034-1387797>.
- [147] K. Tou, A. Cawley, C. Bowen, K. Sornalingam, S. Fu, Measurements of hydrocortisone and cortisone for longitudinal profiling of equine plasma by liquid chromatography-tandem mass spectrometry, *Drug Test. Anal.* 14 (2022) 943–952.
- [148] S. Kneisel, V. Auwärter, J. Kempf, Analysis of 30 synthetic cannabinoids in oral fluid using liquid chromatography-electrospray ionization tandem mass spectrometry, *Drug Test. Anal.* 5 (2013) 657–669, <https://doi.org/10.1002/dta.1429>.
- [149] S. Kneisel, M. Speck, B. Moosmann, T.M. Corneillie, N.G. Butlin, V. Auwärter, LC/ESI-MS/MS method for quantification of 28 synthetic cannabinoids in neat oral fluid and its application to preliminary studies on their detection windows, *Anal. Bioanal. Chem.* 405 (2013) 4691–4706, <https://doi.org/10.1007/s00216-013-6887-0>.
- [150] L. Mercolini, R. Mandrioli, V. Sorella, L. Somaini, D. Giocondi, G. Serpelloni, M. A. Raggi, Dried blood spots: liquid chromatography-mass spectrometry analysis of  $\Delta^9$ -tetrahydrocannabinol and its main metabolites, *J. Chromatogr. A* 1271 (2013) 33–40, <https://doi.org/10.1016/j.chroma.2012.11.030>.
- [151] M. Protti, J. Rudge, A.E. Sberna, G. Gerra, L. Mercolini, Dried haematic microsamples and LC-MS/MS for the analysis of natural and synthetic cannabinoids, *J. Chromatogr. B* 1044–1045 (2017) 77–86.
- [152] I. Möller, A. Wintermeyer, K. Bender, M. Jübner, A. Thomas, O. Krug, W. Schänzer, M. Thevis, Screening for the synthetic cannabinoid JWH-018 and its major metabolites in human doping controls, *Drug Test. Anal.* 3 (2011) 609–620.
- [153] L. Göschl, G. Gmeiner, P. Gärtner, G. Stadler, V. Enev, M. Thevis, W. Schänzer, S. Guddat, G. Forsdahl, Stanazolol-N-glucuronide metabolites in human urine samples as suitable targets in terms of routine anti-doping analysis, *Drug Test. Anal.* 13 (2021) 1668–1677.
- [154] N. Homer, S. Kothiyi, A. Rutter, B.R. Walker, R. Andrew, Gas chromatography tandem mass spectrometry offers advantages for urinary steroids analysis, *Anal. Biochem.* 538 (2017) 34–37, <https://doi.org/10.1016/j.ab.2017.09.002>.
- [155] M. Mazzarino, S. Riggi, X. de la Torre, F. Botré, Speeding up the process urine sample pre-treatment: some perspectives on the use of microwave assisted

- extraction in the anti-doping field, *Talanta*. 81 (2010) 1264–1272, <https://doi.org/10.1016/j.talanta.2010.02.019>.
- [156] W. Jelkmann, C. Lundby, Blood doping and its detection, *Blood*. 118 (2011) 2395–2404, <https://doi.org/10.1182/blood-2011-02-303271>.
- [157] World Anti-Doping Agency (WADA), Athlete Biological Passport Operating Guidelines, 2021. [https://www.wada-ama.org/sites/default/files/resources/files/guidelines\\_abp\\_v8\\_final.pdf](https://www.wada-ama.org/sites/default/files/resources/files/guidelines_abp_v8_final.pdf).
- [158] K. Tibe, A. Dabholkar, D. Bhandari, BLOOD DOPING AND GENE DOPING : A REVIEW ON RECENT TRENDS IN DOPING, *Int. J. Curr. Res.* (2017).
- [159] R. Staff, Olympics-Bahrain runner Alsadik Mikhoul suspended for suspected blood doping, (2021). <https://www.reuters.com/article/olympics-2020-doping-idUSL8N2PF04N> (accessed October 15, 2022).
- [160] T. Pottgiesser, Y.O. Schumacher, H. Funke, K. Rennert, M.W. Baumstark, K. Neunuebel, S. Mosig, Gene expression in the detection of autologous blood transfusion in sports—a pilot study, *Vox Sang.* 96 (2009) 333–336, <https://doi.org/10.1111/j.1423-0410.2009.01169.x>.
- [161] A. D'Alessandro, J.A. Reisz, Y. Zhang, S. Gehrke, K. Alexander, T. Kanas, D. J. Triulzi, C. Donadee, S. Barge, J. Badlam, S. Jain, M.G. Risbano, M.T. Gladwin, Effects of aged stored autologous red blood cells on human plasma metabolome, *Blood Adv.* 3 (2019) 884–896, <https://doi.org/10.1182/bloodadvances.2018029629>.
- [162] O. Salamin, T. Kuuranne, M. Saugy, N. Leuenberger, Erythropoietin as a performance-enhancing drug: Its mechanistic basis, detection, and potential adverse effects, *Mol. Cell. Endocrinol.* 464 (2018) 75–87, <https://doi.org/10.1016/j.mce.2017.01.033>.
- [163] J. Bejder, G. Gürdeniz, C. Cuparencu, F. Hall, M. Gybel-Brask, A. Breenfeldt Andersen, L.O. Dragsted, N.H. Secher, P.I. Johansson, N.B. Nordsborg, An Untargeted Urine Metabolomics Approach for Autologous Blood Transfusion Detection, *Med. Sci. Sports Exerc.* 53 (2021) 236–243, <https://doi.org/10.1249/MSS.0000000000002442>.
- [164] R. Faiss, J. Saugy, A. Zollinger, N. Robinson, F. Schuetz, M. Saugy, P.-Y. Garnier, Prevalence Estimate of Blood Doping in Elite Track and Field Athletes During Two Major International Events, *Front. Physiol.* 11 (2020). <https://www.frontiersin.org/articles/10.3389/fphys.2020.00160>.
- [165] N. Leuenberger, E. Bulla, O. Salamin, R. Nicoli, N. Robinson, N. Baume, M. Saugy, Hepcidin as a potential biomarker for blood doping, *Drug Test. Anal.* 9 (2017) 1093–1097, <https://doi.org/10.1002/dta.2122>.
- [166] C. Tsitsimpikou, D. Kouretas, K. Tsarouhas, K. Fitch, D.A. Spandidos, A. Tsatsakis, Applications and biomonitoring issues of recombinant erythropoietins for doping control, *Ther. Drug Monit.* 33 (2011) 3–13, <https://doi.org/10.1097/FTD.0b013e31820032c4>.
- [167] T. van der Gronde, O. de Hon, H.J. Haisma, T. Pieters, Gene doping: an overview and current implications for athletes, *Br. J. Sports Med.* 47 (11) (2013) 670–678.
- [168] E. Brill-Almon, B. Stern, D. Afik, J. Kaye, N. Langer, S. Bellomo, M. Shavit, A. Pearlman, Y. Lippin, A. Panet, N. Shani, Ex vivo transduction of human dermal tissue structures for autologous implantation production and delivery of therapeutic proteins, *Mol. Ther.* 12 (2005) 274–282.
- [169] W. Wang, W. Li, N. Ma, G. Steinhoff, Non-viral gene delivery methods, *Curr. Pharm. Biotechnol.* 14 (2013) 46–60.
- [170] P.L. Sinn, S.L. Sauter, P.B. McCray, Gene therapy progress and prospects: development of improved lentiviral and retroviral vectors—design, biosafety, and production, *Gene Ther.* 12 (2005) 1089–1098.
- [171] T. Tozaki, N.A. Hamilton, Control of gene doping in human and horse sports, *Gene Ther.* 29 (2022) 107–112, <https://doi.org/10.1038/s41434-021-00267-5>.
- [172] World Anti-Doping Agency (WADA), Laboratory Guidelines Gene Doping Detection based on Polymerase Chain Reaction (PCR), 2021. [https://www.wada-ama.org/sites/default/files/resources/files/wada\\_guidelines\\_for\\_gene\\_doping\\_pcr\\_test\\_v1\\_jan\\_2021\\_eng.pdf](https://www.wada-ama.org/sites/default/files/resources/files/wada_guidelines_for_gene_doping_pcr_test_v1_jan_2021_eng.pdf).
- [173] T. Sugawara, T. Nakano, S.-I. Fujita, Y. Matsumoto, G. Ishihara, K. Aoki, K. Yanazawa, S. Ono, S. Tamai, L. Manevich, H. Ueda, N. Ishibashi, K. Tamai, Y. Kanki, Y. Yoshida, K. Watanabe, T. Takemasa, Y. Kawakami, K. Takekoshi, Proof of Gene Doping in a Mouse Model with a Human Erythropoietin Gene Transferred Using an Adenoviral Vector., *Genes (Basel)*. 12 (2021). <https://doi.org/10.3390/genes12081249>.
- [174] J. Maniego, B. Pesko, J. Habershon-Butcher, J. Huggett, P. Taylor, J. Scarth, E. Ryder, Screening for gene doping transgenes in horses via the use of massively parallel sequencing, *Gene Ther.* 29 (5) (2022) 236–246.