RSC Advances

REVIEW

View Article Online
View Journal | View Issue

Cite this: *RSC Adv.*, 2025, **15**, 17548

Advances in steroid purification for novel techniques in carbon isotope ratio mass spectrometry of doping control

Zhongquan Li,†[a] Jiahui Cheng, †[a] Chaomin Zhao, [b] Qing Chen, *[c] Bing Liu *[a] and Peijie Chen*[a]

Doping detection is a critical tool for maintaining fairness in competitive sports. Steroids are widely abused due to their ability to promote protein synthesis, enhance muscle growth, and improve athletic performance, leading to significant unfair competition. Carbon isotope ratios (CIRs) are effective in distinguishing between endogenous and exogenous steroid sources, making steroids an ideal target for isotope ratio analysis in anti-doping analysis. High-precision isotope ratio measurements using Gas Chromatography-Isotope Ratio Mass Spectrometry (GC-IRMS) require high separation between the target steroid and adjacent compounds to avoid interference. However, steroid concentrations in biological matrices like urine and blood are often trace and accompanied by a large number of coexisting interferents, making purification challenging. These limitations have partially restricted the use of compound-specific isotope analysis in doping detection. This review highlights the latest advancements over the past decade in sample preparation, liquid-phase purification, and both gas-phase and liquid-phase isotope ratio mass spectrometry (IRMS) techniques. By summarizing the application of these methods in anti-doping efforts and exploring future research directions, this review aims to enhance the precision and reliability of steroid doping detection technologies, providing scientific support for anti-doping efforts and identifying the abuse of steroids.

Received 1st February 2025
Accepted 10th May 2025

DOI: 10.1039/d5ra00751h

rsc.li/rsc-advances

1. Introduction

Anabolic steroids (AS) are compounds that enhance athletic performance by mimicking or amplifying the effects of endogenous hormones in the human body. The most common and widely studied category of these substances is anabolic-androgenic steroids (AAS).[1,2] AAS are synthetic compounds with structures and functions similar to endogenous male hormones. AAS have two main effects: anabolic effects, which promote protein synthesis and increase muscle mass, strength, and bone density; and androgenic effects, which drive the development and maintenance of male secondary sexual characteristics.[3] In addition to AAS, other substances with similar effects such as selective androgen receptor modulators (SARMs) are also considered steroid stimulants. While SARMs are not structurally steroids, they selectively bind to androgen receptors, producing anabolic effects similar to those of AAS.[1,2] The abuse of stimulants can be traced back to the mid-20th century, when reports first emerged of athletes using testosterone and its derivatives to enhance their physical performance.[4] In modern competitive sports, the misuse of steroid stimulants is most prevalent in strength-based and explosive events, such as weightlifting and track and field throws,[5] at the same time, there are also reports of abuse in endurance sports.[6] These substances can significantly improve an athlete's muscle mass, strength, explosiveness, and potentially aid recovery, providing an unfair advantage in competition. Athletes typically use synthetic anabolic steroids through injection or oral administration, often in long-term or cyclical regimens. Despite ongoing updates to the World Anti-Doping Agency (WADA) prohibited list of substance and enhanced detection methods for anabolic agents, athletes continue to find new ways to evade detection.[7] For instance, some athletes may use novel steroids not yet listed on the banned list or modify their drug usage cycles, employ masking agents, or use other techniques to reduce the concentration of steroids in their bodies and avoid detection.[8] The abuse of AAS not only violates the principles of fair competition but also poses severe health risks to athletes. Long-term or high-dose use of AAS can result in liver damage, increased risk of cardiovascular diseases, reproductive system dysfunction and other serious adverse effects. It may also lead

[a]*Research Institute for Doping Control, Shanghai University of Sport, Shanghai 200438, China. E-mail: liubing2019@sus.edu.cn; chenpeijie@sus.edu.cn*

[b]*Technical Center for Animal, Plant and Food Inspection and Quarantine, Shanghai Customs, Shanghai 201210, China*

[c]*Department of Pharmacy, Shenyang Medical College, Shenyang 110034, China. E-mail: chenqing.0906@163.com*

† These authors contributed equally to this work.

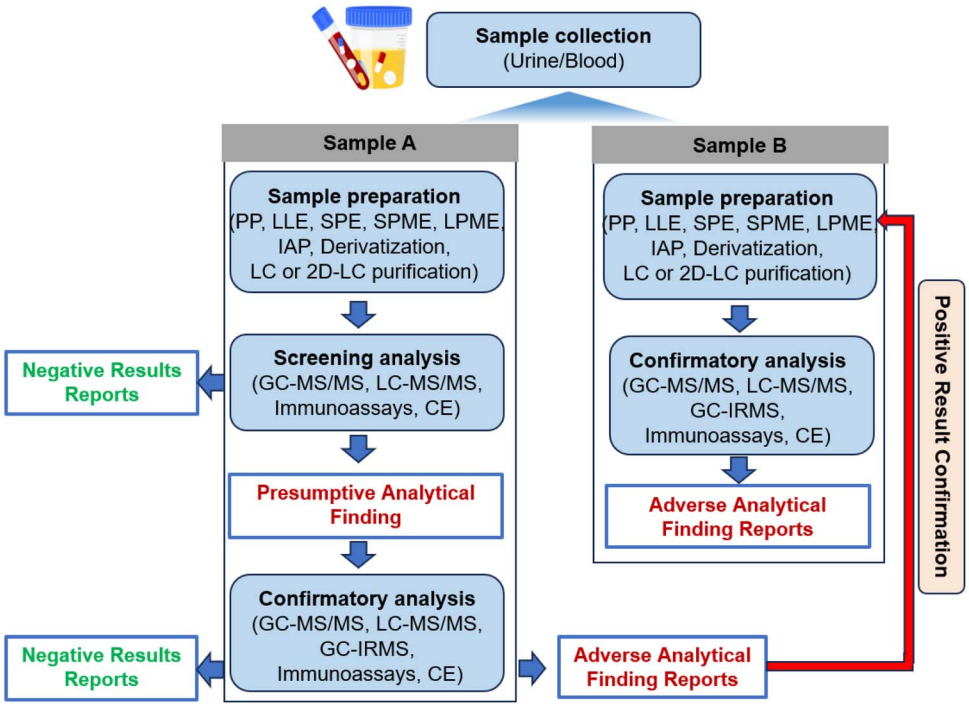

Fig. 1 Typical workflow of doping control analysis.

to psychological and physiological dependence, increasing the risk of sudden death.[1,3,5]

Doping detection is an important strategy for ensuring fairness in sports competitions and protecting athletes' health. Through rigorous testing, the use of prohibited substances by athletes can be identified and prevented in a timely. However, current doping detection still faces several technical and practical challenges,[9] including the detection of low-concentration drug residues, interference from complex biological matrices, and the identification of metabolites.[10,11] Firstly, detecting low concentrations of drug residues remains a significant challenge in the field of doping detection. Many banned substances and their metabolites are present in body fluids at extremely low concentrations, typically at the ng mL^{-1} or even at pg mL^{-1} level. This imposes strict requirements on the sensitivity and accuracy of detection techniques.[12] Secondly, the interference from endogenous substances in biological matrices of human body is another critical issue in doping detection. Biological samples such as blood and urine contain a wide range of endogenous substances that are chemically or physically similar to exogenous steroids, making it difficult to accurately distinguish between them.[13] Moreover, steroids undergo complex metabolic processes in the body, generating various metabolites that closely resemble the parent compounds in structure. This further complicates detection and introduces additional uncertainties.[14] Therefore, sample preparation and purification steps are crucial in the detection process, as they directly impact the accuracy, sensitivity, and reliability of the results. To address these challenges, optimizing existing technologies and developing novel detection methods will be the focus field for future doping analysis.

To address the various challenges in steroid doping detection, researchers have developed and applied a range of advanced techniques to enhance the sensitivity, specificity, and accuracy of detection methods[15] (Fig. 1). For example, protein precipitation (PP), solid-phase extraction (SPE) and liquid–liquid extraction (LLE), which are commonly used sample preparation techniques, effectively remove interfering substances from biological matrices and concentrate the targets, thereby significantly improving the accuracy of subsequent analyses.[16] These techniques in simplifying sample matrices, reducing potential interferences and improving analyte recovery, making them indispensable in the processing of complex biological samples such as urine and blood. In addition, IRMS has gained widespread application in steroid detection in recent years.[17–19] By precisely measuring the CIRs ($^{13}C/^{12}C$) in steroid compounds, IRMS can effectively distinguish between endogenous and exogenous steroids, overcoming the limitations of traditional mass spectrometry techniques in this regard and greatly enhancing the specificity of detection. Meanwhile, the Athlete Biological Passport (ABP) has become an innovative tool for detecting doping abuse in the anti-doping field.[20] The ABP monitors long-term changes in an athlete's biomarkers, such as blood parameters and hormone levels, to establish an individual baseline.[21] Unlike traditional one-time testing methods, the ABP can identify abnormal fluctuations that deviate from an athlete's normal physiological variations, thus indirectly indicating the use of performance-enhancing substances. This approach not only improves detection sensitivity but also compensates for the limitations of single-sample testing, providing robust support for steroid doping detection.

Table 1 List of abbreviations

Abbreviation	Full term	Abbreviation	Full term
AS	Anabolic steroids	LPME	Liquid-phase microextraction
AAS	Anabolic-androgenic steroids	MIPs	Molecularly imprinted polymers
ABP	Athlete biological passport	MD-LC	Multidimensional liquid chromatography
AAFs	Adverse analytical findings	MSPE	Magnetic solid phase extraction
CIRs	Carbon isotope ratios	MSTFA	*N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide
DES	Deep eutectic solvents	MTBE	Methyl *tert*-butyl ether
DHEA	Dehydroepiandrosterone	PP	Protein precipitation
EpiA	Epiandrosterone	PSL	Prednisolone
EpiT	Epitestosterone	PS	Prednisone
ERC	Endogenous reference compound	SARMs	Selective androgen receptor modulators
GC-IRMS	Gas chromatography-isotope ratio mass spectrometry	SFE	Supercritical fluid extraction
GC-MS/MS	Gas chromatography-tandem mass spectrometry	SLE	Supported liquid extraction
HRMS	High-resolution mass spectrometry	SPE	Solid phase extraction
HTC	High-temperature conversion	SPME	Solid phase microextraction
ILs	Ionic liquids	T	Testosterone
IRMS	Isotope ratio mass spectrometry	TC	Target compound
LLE	Liquid–liquid extraction	VPDB	Vienna Pee dee belemnite
LC-IRMS	Liquid chromatography-isotope ratio mass spectrometry	WADA	World Anti-Doping Agency
LC-MS/MS	Liquid chromatography-tandem mass spectrometry	WCOC	Wet chemical oxidation conversion
LOD	Limit of detection		

This review aims to summarize the latest advancements in sample purification, analytical techniques, and IRMS in steroid doping detection. It will also explore the technical challenges and solutions encountered in steroid detection over the past decade. Furthermore, the review will look ahead to potential future developments to further enhance the scientificalness and fairness of steroid detection. All the abbreviations used throughout the manuscript are listed in Table 1.

2. Advances in sample preparation techniques

Purification and analysis methods for steroid stimulants are crucial for their detection and identification. Since steroids are often present at low concentrations in biological samples with complex matrices, using highly sensitive and specific analytical techniques is key to achieving accurate results. In recent years, advancements in sample preparation methods have not only improved sensitivity and specificity, but also simplified workflows, reduced processing time and lowered operational complexity (in Table 2). Common sample preparation techniques include protein PP, LLE, SPE, supercritical fluid extraction (SFE), and liquid-phase microextraction (LPME).[22,23] PP and LLE remain classical sample pretreatment methods. PP involves the addition of organic solvents or salts to disrupt the hydration layer of proteins, leading to protein denaturation and precipitation, thereby releasing the analytes into the supernatant. On the other hand, LLE achieves separation and purification based on differences in partition coefficients of the target analytes between two immiscible solvents. In steroid detection, urine and plasma are often used as the aqueous phase, with appropriate organic solvents selected for protein precipitation and extraction. The solvents including acetonitrile, methanol and inorganic salts are the commonly reagents of PP methods,[24–26] while the frequently organic solvents for LLE are mainly including methyl *tert*-butyl ether (MTBE), ethyl acetate and dichloromethane and *etc.*[26–28] Urine or blood samples often require enzymatic hydrolysis to convert conjugated steroids into their free forms, thereby improving extraction efficiency. For instance, Mazzarino *et al.* used β-glucuronidase to hydrolyze urine samples before employing LLE to extract endogenous corticosteroids, achieving a limit of detection (LOD) below 1 ng mL^{-1} and demonstrating excellent sensitivity for certain steroids.[29] Similarly, Makvandi *et al.* utilized methanol–acetonitrile (1 : 5) for PP, followed by methanol-based LLE, to detect steroids in serum samples. Compared to urine testing, their method identified anabolic-androgenic steroids (AAS) in 80% of serum samples, their method identified anabolic-androgenic steroids (AAS) in 80% of serum samples, highlighting the potential of serum as a complementary matrix for AAS detection.[25] Furthermore, Yuan *et al.* developed an MTBE-based LC-MS/MS method for detecting 12 steroid hormones in serum, achieving limits of quantification ranging from 0.005 ng mL^{-1} to 1 ng mL^{-1} and recovery rates between 86.4% and 115.0%, demonstrating high sensitivity and specificity.[30]

However, traditional liquid–liquid extraction (LLE) has several limitations, including high solvent consumption, low selectivity and a tendency to form emulsions. To address these issues, researchers have begun exploring environmentally friendly solvent alternatives, such as deep eutectic solvents (DES),[31] ionic liquids (ILs)[32] and bio-based solvents.[33] These innovative solutions offer potential advantages in terms of sustainability and efficiency. Whereas, their application in doping detection remains limited, primarily due to the need for further validation of protocols and assessment of their compatibility with current analytical workflows.

SPE is another widely used sample preparation technique that separates and enriches target compounds based on their selective interactions with solid-phase adsorbents. It can effectively remove matrix interferences, concentrates target analytes

Table 2 Summary of sample pretreatment techniques

Sample	Sample extraction	Solvent	Derivatization reagent	Analytical technique	Substances	Stimulants	Ref.	Cite
Serum	LLE	Dichloromethane	Dansyl chloride	LC-MS/MS	15 Estrogens metabolites	No	2014	56
Saliva	SPME (PAN-HLB and PDMS-HLB)	—	—	LC-MS/MS	6 Steroids	Yes	2014	48
Water	SPME (PDMS-DVB)	—	—	GC-MS/MS	8 Steroids	Yes	2014	47
Urine	Hydrolysis, automated-SPE	Isopropyl alcohol–methanol (1 : 1)	—	LC-MS/MS	18 Exogenous anabolic steroids	Yes	2014	54
Serum	LLE	Chloroform	MSTFA	GC-MS/MS	4 Steroids	Yes	2015	27
Urine/plasma	(Hollow fiber)-LPME	Ethylene glycol, ((Me Ph)3PBr), ((Me Ph)3PI)	—	HPLC-UV	2 Steroids	Yes	2017	50
Urine	SPE-C18, hydrolysis, derivat.	Methanol	MSTFA	GC-MS/MS	22 Endogenous steroids	Yes	2017	55
Urine	Hydrolysis, LLE, derivat.	MTBE (LLE)	MSTFA	GC-Orbitrap	30 Exogenous steroids	Yes	2018	57
Serum	SLE	Dichloromethane	MSTFA	GC-MS/MS	37 Steroids	Yes	2018	44
Foods	(Multiple monolithic fiber)-SPME	Methanol	—	HPLC-UV	5 Steroids	Yes	2019	41
Urine	LLE, hydrolysis, LLE	MTBE (LLE)	—	LC-MS/MS	8 Gglucocorticoids	Yes	2019	29
Urine	SPE-MIPs	Methanol	—	HPLC-UV	2 Estrogens	Yes	2019	43
Serum	PP, LLE	Acetonitrile (PP), MTBE (LLE)	Isonicotinoyl chloride (INC)	LC-MS/MS	12 Steroids	Yes	2020	30
Urine	Hydrolysis, SPE-HLB	Acetonitrile	—	LC-MS/MS	23 Adrenal steroids	Yes	2020	36
Urine	SPE-C18, hydrolysis, LLE	Methanol (SPE), ether (LLE)	—	UHPLC-MS/MS	9 Testosterone metabolites	Yes	2020	35
Serum	PP, LLE, derivat., SPE-C18	Acetonitrile(PP), hexane/ethyl acetate (LLE)	Girard T (GT) and Girard P (GP)	LC-MS/MS	14 Testosterone esters	Yes	2021	61
Hair	LLE, automated-SLE	Methanol (LLE), ethyl acetate (SLE)	—	LC-MS/MS	5 Steroid hormones	Yes	2021	45
Serum	Magnetic-SPE	Methanol	Hydroxylamine hydrochloride (HAHC)	LC-MS/MS	2 Steroids	Yes	2022	42
Urine	Hydrolysis SLE, derivat.	MTBE (SLE)	MSTFA	GC-MS/MS	14 Steroids	Yes	2022	46
Serum	PP, LLE	Methanol-acetonitrile (1 : 5), methanol (LLE)	—	LC-MS/MS	8 Anabolic steroids	Yes	2023	25
Serum	PP, derivat., LLE	Methanol–acetonitrile (1 : 1), MTBE (LLE)	Hydroxylamine hydrochloride (HAHC)	LC-MS/MS	2 Steroids	Yes	2023	26
Urine/Serum	SPE-MIPs	Methanol–acetic acid (9 : 1)	—	HPLC-UV	Megestrol acetate	No	2023	39
Serum	Hydrolysis, LLE, SPE-C18	Chloroform (LLE)	MSTFA	GC-MS/MS, UHPLC-MS/MS	34 Ssteroids	Yes	2024	58
Plasma	Hydrolysis, (thin film)-SPME	—	—	LC-MS/MS	4 Androgens	Yes	2024	49
Blood	PP, SPE-(C18 and PSA)	Water–methanol (95 : 5) (PP)	—	UHPSFCMS/MS	9 Steroids	Yes	2024	52
Urine	Hydrolysis, SLE, derivat.	Dichloromethane (SLE)	Pyridine and acetic anhydride	UHPSFCMS/MS	10 Steroids	Yes	2024	53
Serum	SPE-MIPs	Acetone–acetonitrile (4 : 1)	—	HPLC-UV	4 Estrogens	No	2024	40

and then enhance the sensitivity of detection. The choice of sorbent material is critical for SPE performance, with commonly used materials including reversed-phase C18 and amino-functionalized columns.[34]

For example, Derly *et al.* employed C18 SPE cartridge to analyze testosterone (T) and hydroxylated metabolites in human urine, revealing age-dependent changes, with most metabolite levels decreasing in older men.[35] Similarly, Pussard *et al.* utilized Oasis HLB as an SPE adsorbent to extract and enrich endogenous anabolic steroids (EAAS) in human urine, including both free and conjugated forms of glucocorticoids and mineralocorticoids. This method demonstrated good linearity and accuracy, making it suitable for single-injection quantification of multiple steroids.[36]

In recent years, researchers have focused on developing novel SPE materials to further improve the separation and purification efficiency of steroids.[37] One significant advancement is the application of molecularly imprinted polymers (MIPs) in SPE.[38] MIPs are functional polymers prepared through polymerization in the presence of template molecules (*i.e.*, target analytes), enabling highly specific recognition similar to antibodies. Using MIPs as SPE adsorbents allows for the selective adsorption of target steroid molecules, significantly improving purification efficiency and sensitivity. For instance, Pournamdari *et al.* synthesized MIPs for medroxyprogesterone using methacrylic acid and applied them to selectively adsorb the drug in human serum and urine, demonstrating the potential of MIPs-SPE for trace analysis in complex matrices.[39] Guo *et al.* utilized an MIPs-SPE method to efficiently enrich and quantify four estrogens in serum, enhancing the sensitivity of HPLC and reducing matrix interferences.[40] In addition to MIPs, researchers are actively exploring other innovative SPE materials. New adsorbents, such as graphene-based materials, nanomaterials, and magnetic media, show promise in steroid separation and purification due to their unique pore structures and surface chemistry.[40–43] Magnetic solid-phase extraction (MSPE) has also garnered significant attention for its use of magnetic nanomaterials as sorbents. By applying an external magnetic field, MSPE enables to simplify the workflow and shorten sample preparation time.[42] Additionally, supported-liquid extraction (SLE) has been applied as an alternative method. In SLE, sample solutions form a thin film on the surface of inert porous sorbents, and target analytes are extracted using a solvent immiscible with the film. This technique is particularly effective for extracting nonpolar and moderately polar compounds from biological fluids.[44–46] These above growing array of advanced materials and techniques highlights the ongoing innovation in sample preparation for steroid analysis, offering solutions to improve efficiency, sensitivity and environmental sustainability in doping detection.

3. Advances in semi-preparative liquid chromatography purification techniques

Samples are typically derived from complex biological matrices, such as urine and serum, which contain numerous potential interferences that pose significant challenges to subsequent analysis.[51,59,60,62–64] Table 3 summarized the research progress of steroid purification and preparation. Semi-preparative HPLC is a powerful purification tool that effectively isolates and purifies target steroids and their metabolites from complex matrices, providing high-quality samples for structural identification, standard preparation and IRMS analysis. The principles of semi-preparative HPLC are similar to HPLC, relying on differences in partition coefficients of target compounds between the stationary and mobile phases. The key difference lies in the use of larger diameter columns and higher flow rates in semi-preparative HPLC, allowing for greater sample loading and improved separation efficiency to yield milligram-scale purified substances.[65]

The choice of stationary phase is critical for the separation efficiency and purity achieved with semi-preparative HPLC. Common stationary phases include reversed-phase C18, C8, phenyl columns, normal-phase silica columns, amino columns, and other specialized functionalized materials.[66] For steroid separation, C18 columns are the most widely used, particularly for their hydrophobicity, which makes them suitable for separating most steroid compounds. For example, in the separation of hydrophobic steroids such as T and dehydroepiandrosterone (DHEA), C18 stationary phases effectively distinguish these compounds, ensuring high-purity separation in complex biological matrices such as urine and serum.[67–71] Due to their superior resolution, C18 columns are commonly used for precise quantification and identification of steroids. C8 columns, with weaker hydrophobicity than C18 columns, are better suited for separating smaller or more polar steroid compounds.[72] Normal-phase silica relies on the interaction of silanol groups (Si–OH) with the polar functional groups in steroid molecules, such as hydroxyl or keto groups, through hydrogen bonding,.[73] However, C8 and normal-phase silica columns are less commonly used for steroid purification. Phenyl columns, which utilize hydrophobic interactions and π–π interactions are particularly effective for nonpolar and weakly polar compounds, especially those with aromatic rings.[74–77] Amino columns, featuring medium polarity through aminopropyl-functionalized silica, are mainly used for separating steroids with hydroxyl or keto groups. The amino groups on the stationary phase form hydrogen bonds with these functional groups, enabling effective retention and high-efficiency separation. For example, amino columns have been used to separate glucocorticoids such as prednisone (PS) and prednisolone (PSL) and finally achieved efficient separation of these polar compounds.[78,79] For steroids with chiral centers, chiral stationary phases are ideal for enantiomeric separation. For instance, in separating chiral molecules such as dexamethasone and betamethasone in urine an plasma, the Lux i-Cellulose-5 chiral column has been shown to achieve complete separation.[81]

Traditional one-dimensional liquid chromatography (1D-LC) often struggles to achieve sufficient resolution when separating structurally similar steroids and their metabolites with close polarity, which limit its ability to meet the demands of highly sensitive and selective analysis.[75,79,80,82,83] Semi-preparative two-

Table 3 Summary of purification and IRMS techniques

Sample	Sample extraction and purification	Methods	LC column	IRMS column	Ref.	Cite
Urine (^{13}C-T)	Hydrolysis → LLE →derivatization → 1D-HPLC	GC-IRMS	Zorbas C18 (250 mm × 4.6 mm × 5 μm)	HP-5MS	2014	67
Urine (steroids in musk)	Hydrolysis → LLE → 1D-HPLC	GC-IRMS	Zorbas C18 (250 mm × 4.6 mm × 5 μm)	HP-5MS	2017	98
Urine (T and metabolites)	SPE → hydrolysis → LLE → SPE → derivatization → LLE → Offline-2D-HPLC	GC-IRMS	1D: Shield RP18 (250 mm × 4.6 mm × 5 μm) 2D: Shield RP18 (250 mm × 4.6 mm × 5 μm)	HP ultra 1	2017	83
Urine (5bAdiol, 5aAdiol)	LLE → hydrolysis → LLE → 1D-HPLC	GC-IRMS	ACE C18 (250 mm × 4.6 mm × 5 μm)	HP-5MS	2017	70
Urine (8 steroids)	SPE → LLE → hydrolysis → LLE → IAC → derivatization → SPE	GC-IRMS	—	DB-17MS	2019	90
Urine (9 steroids)	SPE → hydrolysis → LLE → online-2D-HPLC	GC-IRMS	1D: Eclipse XDB phenyl (150 mm × 0.46 mm × 3.5 μm) 2D: Eclipse XDB phenyl (150 mm × 0.46 mm × 3.5 μm)	DB-5MS	2020	77
Urine (11 steroids including T and metabolites, B, BM, 19NA)	SPE → hydrolysis → LLE → derivatization → online-3D-HPLC	GC-IRMS	1D: Poroshell 120 EC-C18 (100 mm × 4.6 mm × 2.7 μm) 2D: Poroshell 120 EC-CN (50 mm × 4.6 mm × 2.7 μm) 3D: Poroshell 120 EC-C18 (50 mm × 4.6 mm × 2.7 μm)	DB-17 MS	2021	84
Urine (T and metabolites)	LLE → hydrolysis → LLE → 1D-HPLC	GC-IRMS	ACE C18 (5 μ m, 4.6 × 250 mm)	HP-5MS	2022	68
Urine (19NA)	SPE → hydrolysis → LLE → online-2D-HPLC	GC-IRMS	1D: Eclipse XDB-phenyl (250 mm × 4.6 mm × 5 μm) 2D: Zorbas C18 (250 mm × 4.6 mm × 5 μm)	DB-5MS	2020	88
Urine (19NA, 19NE)	Hydrolysis → LLE → offline-2D-HPLC	GC-IRMS	1D: Ascentis phenyl column (150 mm × 4.6 mm × 5 μm) 2D: ACE C18 (250 mm × 4.6 mm × 5 μm)	HP-5MS	2021	82
Urine (19NA, 19NE)	SPE → hydrolysis →LLE → online-2D-HPLC	GC-IRMS	1D: Eclipse XDB-phenyl (250 mm × 4.6 mm × 5 μm) 2D: Zorbas C18 (250 mm × 4.6 mm × 5 μm)	DB-5MS	2023	76
Urine (19NA, 19NE)	SPE → hydrolysis → LLE → derivatization → online-3D-HPLC	GC-IRMS	1D: Poroshell 120 EC-C18 (100 mm × 4.6 mm × 2.7 μm) 2D: Poroshell 120 EC-CN (50 mm × 4.6 mm × 2.7 μm) 3D: Poroshell 120 EC-C18 (50 mm × 4.6 mm × 2.7 μm)	DB-17 MS	2024	85
Urine (glucocorticoids)	LLE → hydrolysis → LLE → 1D-HPLC	GC-IRMS	ACE C18 (250 mm × 4.6 mm × 5 μm)	HP-5MS	2015	86
Urine (PS, PSL)	Hydrolysis → LLE → offline-2D-HPLC	GC-IRMS	1D: ACE C18 (250 mm × 4.6 mm × 5 μm) 2D: ACE C18 amide (250 mm × 4.6 mm × 5 μm)	HP-5MS	2019 2020	79 80
Urine (PS, PSL)	SPE → hydrolysis → LLE → online-2D-HPLC	GC-IRMS	1D: ACE C18 (250 mm × 4.6 mm × 5 μm) 2D: Eclipse XDB-phenyl (250 mm × 4.6 mm × 5 μm)	DB-5MS	2024	74

Table 3 (*Contd.*)

Sample	Sample extraction and purification	Methods	LC column	IRMS column	Ref.	Cite
Urine (6α-OH-Adion)	SPE → hydrolysis → LLE → online-2D-HPLC	GC-IRMS	1D: Zorbax Extend-C18 (250 mm × 4.6 mm × 5 μm) 2D: Gemini C6-Phenyl (150 mm × 4.6 mm × 5 μm)	DB-17 MS	2014	75
Urine (EpiA-S)	LLE → 1D-HPLC	GC-IRMS	Hypersil Gold C18 (150 mm × 4.6 mm × 5 μm)	DB-17 MS	2020	71
Urine (EpiA-S)	LLE → hydrolysis → LLE → derivatization → LLE	2D-GC-IRMS	— —	Optima 1 DB-17 MS	2020	101
Urine (10 steroids)	SPE → LLE → hydrolysis → derivatization	2D-GC-IRMS	— —	Optima 1 DB-17 MS	2018	100
Serum (A, EpiA)	PP → LLE → derivatization → 1D-HPLC	GC-IRMS	Eclipse XDB-C18 (150 mm × 4.6 mm × 5 μm)	DB-17 MS	2024	69
Urine (Bo, BoM)	Hydrolysis → LLE → 1D-HPLC → derivatization	Online-LC-GC-IRMS	ACE C18 (250 mm × 4.6 mm × 5 μm)	Silica capillary tube	2014	107
Standards (5 steroids)	—	LC-IRMS	—	Xbridge BEH300C4	2014	104
Standards (steroids)	—	LC-IRMS	—	Sachtopore-RP	2024	105
Urine (4 steroids)	SPE → hydrolysis → LLE → derivat. → online-2D-HPLC → hydrolysis → SPE	LC-IRMS	1D: Poroshell 120 EC-C18 (100 mm × 4.6 mm × 2.7 μm) 2D: Poroshell 120 EC-CN (50 mm × 4.6 mm × 2.7 μm)	Sachtopore-RP	2024	87

dimensional liquid chromatography (2D-LC) and multi-dimensional liquid chromatography (MD-LC) have emerged as powerful tools for steroid purification, significantly enhancing peak capacity and resolution by integrating orthogonal separation mechanisms.[74,76–78,84–88] The core principle of 2D-LC is the use of two chromatographic columns with different separation mechanisms, such as a combination of reversed-phase chromatography with hydrophilic interaction chromatography (HILIC) or ion exchange chromatography (IEC). Fractions separated in the first dimension are automatically collected and transferred to the second dimension for further separation. This orthogonal approach effectively removes matrix interferences and enables the separation of structurally similar steroid stimulants.[89] For example, Lalonde *et al.* developed an automated 2D-HPLC method for the purification of steroids in urine samples. Using an XDB Phenyl column in the first dimension and a reversed-phase C18 column in the second dimension, the method achieved efficient separation anabolic steroids and their metabolites, including 9 urinary steroids such as T and DHEA.[77] MD-LC builds upon the foundation of 2D-LC by incorporating more than two dimensions and combining multiple separation mechanisms. This approach allows for highly efficient purification of trace steroid stimulants from complex samples. For instance, Honesova *et al.* and Polet *et al.* established a three-dimensional semi-preparative LC purification method for the analysis of T and its metabolites in urine samples as well as the purification of nandrolone metabolites.[84,85] This method used two six-port switching valves to enable two independent, non-interfering loops for multidimensional chromatographic collection and rapid sample cleanup. While multidimensional LC systems offer higher peak capacity and resolution, these systems are inherently more complex and require more professional operating skills and method development experience. To address these challenges, immunoaffinity chromatography (IAC) has been developed as an alternative sample preparation technique for gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) analysis.[90] However, HPLC methods has its advantages in stability and purification quality, so it is still the preferred method for precise analytical workflows.

4. Advances in IRMS techniques

IRMS can distinguish endogenous steroids from exogenous synthetic steroids by measuring the CIRs, providing robust evidence for doping detection.[91] To enhance the interpretability of isotope ratio data, CIRs are typically reported using the delta notation ($\delta^{13}C$), expressed in per mil (‰) relative to the international standard Vienna Pee Dee Belemnite (VPDB).[92] The delta value is calculated using the formula: $\delta^{13}C = [(R_{sample}/R_{standard}) - 1] \times 1000$, where R is the ratio of $^{13}C/^{12}C$ in the sample or standard.[93] Recent updates in reference materials for $\delta^{13}C$ calibration should be noted. The certified reference material VPDB has undergone re-evaluation, which slightly affects the absolute isotope ratio scales and reinforces the importance of precise two-point calibration using well-characterized reference gases.[94] In anti-doping analysis, the

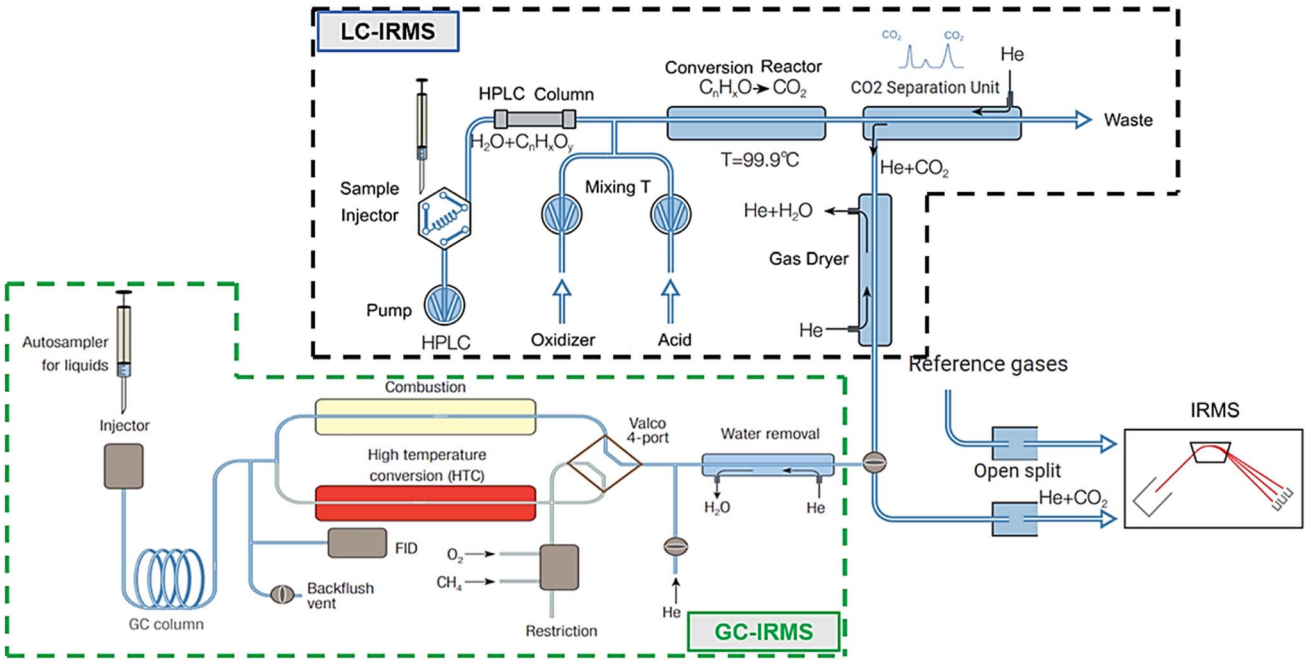

Fig. 2 Principles of the GC-IRMS and LC-IRMS interfaces.[96,102]

differentiation between endogenous and exogenous steroids relies not on absolute $\delta^{13}C$ values, but on Δ-values, defined as the isotopic difference between a target compound (TC) and an endogenous reference compound (ERC): $\Delta = \delta^{13}C_{ERC} - \delta^{13}C_{TC}$. WADA recommends using a Δ-value greater than 3‰ to indicate exogenous origin, due to the small but consistent differences in carbon isotope ratios between synthetic steroids (typically <−27‰) and endogenous compounds (usually between −16‰ and −26‰).[95] According to the sample introduction methods, IRMS is mainly classified into GC-IRMS and liquid-phase IRMS (LC-IRMS)[96] (Fig. 2). GC-IRMS combines gas chromatography (GC) with IRMS and is suitable for analyzing volatile or derivatized compounds. In doping control, target compounds often require derivatization to enhance volatility for GC separation. The separated compounds are then introduced into a combustion or high-temperature pyrolysis furnace, where they are converted into simple gases (*e.g.*, CO_2, H_2, O_2) for isotopic ratio measurements by IRMS.[97] For example, Wang *et al.* utilized GC-C-IRMS to investigate the effects of musk intake on anti-doping controls. They found that varying doses of musk altered steroid profiles and CIRs, potentially leading to abnormal analytical findings (AAFs). This study was the first to confirm that musk intake could result in positive doping test results.[98] In terms of other element determination, such as H and O isotopes, environmental factors during sample pretreatment and separation can lead to significant isotope fractionation. Therefore, it requires very delicate operations during sample pretreatment to ensure the accuracy and reproducibility of H and O isotopes.[99]

Two-dimensional gas chromatography (2D-GC) combined with IRMS enables higher-resolution analysis of steroids in complex samples. By employing two chromatographic columns with different separation mechanisms (*e.g.*, polarity and non-polarity), 2D-GC significantly enhances separation efficiency and analytical capability.[100,101] Putz *et al.* have reported the application of 2D-GC-IRMS for steroid analysis.[100] The first GC dimension used a low-polarity column (Optima 1, 30 m length, 0.25 mm ID) for preliminary purification of urinary steroids, while the second dimension employed a moderately polar column (DB-17 MS, 30 m length, 0.25 mm ID) for compound separation. This method was applied to 74 routine doping control samples to establish population-based thresholds, reducing manual workload and time on sample preparation. Epiandrosterone (EpiA), which is sulfated in the body, requires enzymatic hydrolysis followed by acidic solvolysis for analysis, making its sample preparation highly complex. In order to simplify the sample preparation of sulfonated compounds, Piper *et al.* tested the enzymatic cleavage of arylsulfatase from *Pseudomonas aeruginosa* and further simplified the pretreatment process by using MD-gas chromatography to ensure the peak purity required for CIRs.[101] While GC-IRMS is a mature technique with high sensitivity and excellent reproducibility, derivatization steps can introduce isotope fractionation effects, potentially affecting measurement accuracy. Additionally, thermally unstable steroid compounds may degrade during derivatization or GC separation, limiting the applicability of this method.

LC-IRMS overcomes the limitations of GC-IRMS, as it eliminates the need for derivatization, allowing direct analysis of non-volatile and thermally unstable compounds.[102] The LC-IRMS workflow begins with LC separation, where different steroid compounds in complex samples are separated based on their physicochemical properties. The eluate is then introduced into an online interface that converts the target analytes into gas forms detectable by IRMS. Common interface technologies

include wet chemical oxidation (WCOC) and high-temperature conversion (HTC).

WCOC method employs strong oxidizing agents (*e.g.*, sodium persulfate) to oxidize organic compounds into CO_2, while HTC pyrolyzes compounds at high temperatures into CO_2 and other small gas molecules. The generated CO_2 is subsequently introduced into the IRMS for measurement of the $^{13}C/^{12}C$ isotope ratio.[103] Zhang *et al.* developed a novel high-temperature liquid chromatography coupled with photodiode array detection and isotope ratio mass spectrometry (HT-LC/PDA/IRMS) for the CIRs analysis of non-derivatized steroid hormones. Using a C4 column at high temperatures with ultrapure water as the sole mobile phase, the method achieved complete separation of a mixture of five steroids, including T and epitestosterone (EpiT). The applicability of the method was validated using testosterone-containing ointments.[104] Recently, Honesova *et al.* evaluated six analytical columns for their suitability in separating steroids using similar conditions. They identified ZirChrom-PBD and Sachtopore-RP as the only two columns suitable for steroids separation.[105] Subsequently, the team employed a 2D-LC purification method, combining Sachtopore-RP columns with conditions up to 200 °C and pure water as the mobile phase. This approach successfully measured the CIRs of steroids in urine, enabling differentiation between endogenous and synthetic steroids.[87] Since LC-IRMS converts analytes into CO_2 *via* wet oxidation and accurately determines CIRs using water as the mobile phase, the current design of LC interfaces imposes strict limitations on flow rates and particularly the composition of the mobile phase. Addressing these limitations will require the development of novel, high-efficiency interface technologies combined with advanced multi-dimensional chromatography methods to achieve precise analysis of trace steroid stimulants in complex matrices.[106,107] Additionally, coupling IRMS with high-resolution mass spectrometry (HRMS) has emerged as a promising approach. This combination provides both structural and isotopic ratio information, offering more comprehensive evidence for doping detection and enhancing the reliability of analytical results.[108]

5. Conclusion

This review provides a summary of the latest research advancements in the purification, analysis, and application of IRMS techniques for steroid doping detection. In the field of purification and analysis, the combination of separation techniques such as HPLC and GC with mass spectrometry has significantly improved the sensitivity and specificity. These techniques enable rapid separation of targets from complex biological samples and differentiate between endogenous and exogenous steroids. With the optimization of sample preparation, particularly the combined use of LLE and SPE, detection efficiency has been further enhanced. Meanwhile, the application of IRMS techniques (*e.g.*, GC-C-IRMS and LC-IRMS) has provided a scientific basis for distinguishing between endogenous and exogenous steroids through the measurement of CIRs, thus further strengthening the ability to prevent detection evasion. However, given the complex structures of novel steroid stimulants and increasingly abuse strategies, existing detection methods still require further optimization, particularly in the areas of rapid screening, sensitivity enhancement, and the identification of unknown stimulants. Future advancements include the development of innovative sample preparation materials, such as molecularly imprinted polymers, for detecting new synthetic steroids. Additionally, integrating AI-assisted mass spectrometry for enhanced screening capabilities, optimizing mass spectrometry parameters and exploring new biomarkers will help improve the sensitivity for detecting micro-dose stimulants. Moreover, enhancing international collaboration and establishing standardized testing protocols and databases are essential for advancing the global standardization of doping detection technologies.

Data availability

Data will be made available on request.

Author contributions

Zhongquan Li: research concept, literature review, data collection, data analysis and interpretation, statistical analysis, writing of the manuscript; Jiahui Cheng: literature review, data analysis and interpretation, visualization; Chaomin Zhao: supervision, writing–review, project administration; Qing Chen: writing–review, project administration (corresponding author). Bing Liu: research concept, supervision, writing–review, project administration (corresponding author); Peijie Chen: supervision, writing–review, project administration (correspongding author).

Conflicts of interest

The authors declare that they have no known competing financial interests.

Acknowledgements

This research was funded by Research Project of the National Key Research and Development Program of China (No. 2023YFE0106900), National Natural Science Foundation of China (21804093), Liaoning Science and Technology General Projects by Central Government (2023-MS-324) and Shenyang Young and Middle-aged Science and Technology Innovation Talent Project (RC230168) and Ministry of Science and Technology of the People's Republic of China (2020YFF0304500).

References

1 B. Ribeiro, Anabolic-androgenic steroid use in sports, health, and society, *Med. Sci. Sports Exercise*, 2021, **53**(8), 1778–1794.

2 M. Dutka, R. Bernacki, P. Bernacki, W. Zardzewiały, K. Skrzyńska, O. Zaremskyi and K. Mendyk, Anabolic-

androgenic steroids in sport, *Quality in Sport*, 2024, **21**, 54158.
3 C. Maravelias, A. Dona, M. Stefanidou and C. Spiliopoulou, Adverse effects of anabolic steroids in athletes. A constant threat, *Toxicol. Lett.*, 2005, **158**(3), 167–175.
4 C. E. Yesalis and M. S. Bahrke, History of doping in sport, *Int. Sports Stud*, 2002, **24**(1), 42–76.
5 F. Hartgens and H. Kuipers, Effects of androgenic-anabolic steroids in athletes, *Sports Med.*, 2004, **34**, 513–554.
6 NIDA (2018), *Steroids and Other Appearance and Performance Enhancing Drugs (APEDs)*, National Institute on Drug Abuse, 2018, available to: **https://nida.nih.gov/sites/default/files/815-steroids-and-other-appearance-and-performance-enhancing-drugs-apeds.pdf**.
7 Y. Lu, J. Yan, G. Ou and L. Fu, A review of recent progress in drug doping and gene doping Control analysis, *Molecules*, 2023, **28**(14), 5483.
8 H. Alquraini and R. J. Auchus, Strategies that athletes use to avoid detection of androgenic-anabolic steroid doping and sanctions, *Mol. Cell. Endocrinol.*, 2018, **464**, 28–33.
9 J. Dvorak, M. Saugy and Y. P. Pitsiladis, Challenges and threats to implementing the fight against doping in sport, *Br. J. Sports Med.*, 2014, **48**(10), 807–809.
10 F. Badoud, D. Guillarme, J. Boccard, E. Grata, M. Saugy, S. Rudaz and J. L. Veuthey, Analytical aspects in doping control: challenges and perspectives, *Forensic Sci. Int.*, 2011, **213**(1–3), 49–61.
11 C. R. Harrison, The analytical challenges of anti-doping analysis, *Anal. Bioanal. Chem.*, 2013, **405**(30), 9615–9617.
12 M. Thevis, T. Kuuranne and H. Geyer, Annual banned-substance review: Analytical approaches in human sports drug testing 2019/2020, *Drug Test. Anal.*, 2021, **13**(1), 8–35.
13 T. Kuuranne, M. Saugy and N. Baume, Confounding factors and genetic polymorphism in the evaluation of individual steroid profiling, *Br. J. Sports Med.*, 2014, **48**(10), 848–855.
14 Z. Q. Li, B. Liu, Y. R. Wang, C. Yu, X. Xu and P. J. Chen, Metabolic Characterization of Vamorolone in Human Liver Microsomes: Implications for Anti-Doping, *Drug Test. Anal.*, 2024, DOI: **10.1002/dta.3819**.
15 R. Nicoli, D. Guillarme, N. Leuenberger, N. Baume, N. Robinson, M. Saugy and J. L. Veuthey, Analytical strategies for doping control purposes: needs, challenges, and perspectives, *Anal. Chem.*, 2016, **88**(1), 508–523.
16 Y. Song and X. S. Feng, Sample preparation and analytical methods for steroid hormones in environmental and food samples: An update since 2012, *Crit. Rev. Anal. Chem.*, 2023, **53**(1), 69–87.
17 S. Kelly, C. Brodie and A. Hilkert, Isotopic-spectroscopic technique: Stable isotope-ratio mass spectrometry IRMS, in *Modern Techniques for Food Authentication*, 2018, pp. 349–413.
18 M. P. Matos and G. P. Jackson, Isotope ratio mass spectrometry in forensic science applications, *Forensic Chem.*, 2019, **13**, 100154.
19 Z. Giannioti, N. Ogrinc, M. Suman, F. Camin and L. Bontempo, Isotope ratio mass spectrometry (IRMS) methods for distinguishing organic from conventional food products: A review, *TrAC, Trends Anal. Chem.*, 2024, **170**, 117476.
20 A. R. Vernec, The Athlete Biological Passport: an integral element of innovative strategies in antidoping, *Br. J. Sports Med.*, 2014, **48**(10), 817–819.
21 D. Dragčević, V. P. Jakšić and O. Jakšić, Athlete biological passport: longitudinal biomarkers and statistics in the fight against doping, *Arch. Ind. Hyg. Toxicol.*, 2024, **75**(1), 24–31.
22 Y. Song and X. S. Feng, Sample preparation and analytical methods for steroid hormones in environmental and food samples: An update since 2012, *Crit. Rev. Anal. Chem.*, 2023, **53**(1), 69–87.
23 F. Merlo, A. Profumo and A. Speltini, Sample treatment for the determination of steroid hormone contaminants in water samples: Analytical greenness assessment, *TrAC, Trends Anal. Chem.*, 2023, **169**, 117393.
24 P. Novák and V. Havlíček, Protein extraction and precipitation, in *Proteomic Profiling and Analytical Chemistry*, 2016, pp. 51–62.
25 B. Makvandi, A. Pohanka, M. Bergström, A. Börjesson, M. Lehtihet, L. Ekström and Y. Zheng, Detection of anabolic androgenic steroids in serum samples, *Drug Test. Anal.*, 2023, **15**(6), 678–688.
26 R. Huang, Y. Hong, Y. Wu, W. Li and W. Liu, Simultaneous quantification of total and free testosterone in human serum by LC–MS/MS, *Anal. Bioanal. Chem.*, 2023, **415**(27), 6851–6861.
27 S. Matysik and G. Schmitz, Determination of steroid hormones in human plasma by GC–triple quadrupole MS, *Steroids*, 2015, **99**, 151–154.
28 B. Hashemi, P. Zohrabi, K. H. Kim, M. Shamsipur, A. Deep and J. Hong, Recent advances in liquid-phase microextraction techniques for the analysis of environmental pollutants, *TrAC, Trends Anal. Chem.*, 2017, **97**, 83–95.
29 M. Mazzarino, C. Piantadosi, F. Comunità, X. de la Torre and F. Botrè, Urinary excretion profile of prednisone and prednisolone after different administration routes, *Drug Test. Anal.*, 2019, **11**(11–12), 1601–1614.
30 T. F. Yuan, J. Le, S. T. Wang and Y. Li, An LC/MS/MS method for analyzing the steroid metabolome with high accuracy and from small serum samples [S], *J. Lipid Res.*, 2020, **61**(4), 580–586.
31 A. Shishov, A. Bulatov, M. Locatelli, S. Carradori and V. Andruch, Application of deep eutectic solvents in analytical chemistry. A review, *Microchem. J.*, 2017, **135**, 33–38.
32 S. K. Singh and A. W. Savoy, Ionic liquids synthesis and applications: An overview, *J. Mol. Liq.*, 2020, **297**, 112038.
33 F. G. Calvo-Flores, M. J. Monteagudo-Arrebola, J. A. Dobado and J. Isac-García, Green and bio-based solvents, *Top. Curr. Chem.*, 2018, **376**, 1–40.
34 S. Ötles and C. Kartal, Solid-Phase Extraction (SPE): Principles and applications in food samples, *Acta Sci. Pol., Technol. Aliment.*, 2016, **15**(1), 5–15.

35 D. C. Escobar-Wilches, A. Ventura-Bahena, M. de Lourdes López-González, L. Torres-Sánchez, M. Figueroa and A. Sierra-Santoyo, Analysis of testosterone-hydroxylated metabolites in human urine by ultra high performance liquid chromatography-mass spectrometry, *Anal. Biochem.*, 2020, **597**, 113670.

36 E. Pussard, S. Travers, C. Bouvattier, Q. Y. Xue, C. Cosson, S. Viengchareun and M. Lombès, Urinary steroidomic profiles by LC-MS/MS to monitor classic 21-hydroxylase deficiency, *J. Steroid Biochem. Mol. Biol.*, 2020, **198**, 105553.

37 B. H. Fumes, M. R. Silva, F. N. Andrade, C. E. D. Nazario and F. M. Lanças, Recent advances and future trends in new materials for sample preparation, *TrAC, Trends Anal. Chem.*, 2015, **71**, 9–25.

38 A. Mpupa, S. K. Selahle, B. Mizaikoff and P. N. Nomngongo, Recent advances in solid-phase extraction (SPE) based on molecularly imprinted polymers (MIPs) for analysis of hormones, *Chemosensors*, 2021, **9**(7), 151.

39 E. Pournamdari and A. Geramizadegan, Assessment and selective extraction megestrol drug by molecularly imprinted polymers method in human fluid samples using liquid chromatography, *Anal. Bioanal. Chem. Res.*, 2023, **10**(2), 169–182.

40 P. Guo, M. Xu, F. Zhong, C. Liu, X. Cui, J. Zhang and D. Fan, Molecularly imprinted solid-phase extraction combined with non-ionic hydrophobic deep eutectic solvents dispersed liquid-liquid microextraction for efficient enrichment and determination of the estrogens in serum samples, *Talanta*, 2024, **269**, 125480.

41 R. Mirzajani, F. Kardani and Z. Ramezani, A nanocomposite consisting of graphene oxide, zeolite imidazolate framework 8, and a molecularly imprinted polymer for (multiple) fiber solid phase microextraction of sterol and steroid hormones prior to their quantitation by HPLC, *Microchim. Acta*, 2019, **186**, 1–14.

42 R. Huang, *et al.*, Simultaneous quantification of total and free testosterone in human serum by LC–MS/MS, *Anal. Bioanal. Chem.*, 2023, **415**(27), 6851–6861.

43 H. L. de Oliveira, L. S. Teixeira, L. A. F. Dinali, B. C. Pires, N. S. Simões and K. B. Borges, Microextraction by packed sorbent using a new restricted molecularly imprinted polymer for the determination of estrogens from human urine samples, *Microchem. J.*, 2019, **150**, 104162.

44 J. Y. Moon, H. S. Lee, J. H. Kim, J. H. Lee and M. H. Choi, Supported liquid extraction coupled to gas chromatography-selective mass spectrometric scan modes for serum steroid profiling, *Anal. Chim. Acta*, 2018, **1037**, 281–292.

45 C. D. Voegel, M. R. Baumgartner, T. Kraemer, S. Wüst and T. M. Binz, Simultaneous quantification of steroid hormones and endocannabinoids (ECs) in human hair using an automated supported liquid extraction (SLE) and LC-MS/MS–Insights into EC baseline values and correlation to steroid concentrations, *Talanta*, 2021, **222**, 121499.

46 M. Liu, Y. Ge, X. Xu and L. Liao, Quantification of urinary steroids by supported liquid extraction with GC-MS/MS: Unravelling cyclic fluctuations of steroid profiling in regular menstrual cycle, *J. Pharm. Biomed. Anal.*, 2022, **216**, 114789.

47 S. Chopra, L. Gomes, R. Dhandapani and N. H. Snow, Analysis of steroids using solid phase microextraction-gas chromatography-mass spectrometry-mass spectrometry (SPME-GC-MS-MS), *Sci. Chromatogr.*, 2014, **6**(2), 105–116.

48 V. Bessonneau, E. Boyaci, M. Maciazek-Jurczyk and J. Pawliszyn, In vivo solid phase microextraction sampling of human saliva for non-invasive and on-site monitoring, *Anal. Chim. Acta*, 2015, **856**, 35–45.

49 A. Rajska, J. Raczak-Gutknecht, W. Struck-Lewicka, M. Buszewska-Forajta, P. Wityk, P. Verding, *et al.*, Determination of urinary androgens in women with polycystic ovary syndrome using LC-QqQ/MS and the application of thin film solid-phase microextraction (TF-SPME), *J. Chromatogr. A*, 2024, **1718**, 464735.

50 M. M. Khataei, Y. Yamini, A. Nazaripour and M. Karimi, Novel generation of deep eutectic solvent as an acceptor phase in three-phase hollow fiber liquid phase microextraction for extraction and preconcentration of steroidal hormones from biological fluids, *Talanta*, 2018, **178**, 473–480.

51 M. S. Uddin, M. Z. I. Sarker, S. Ferdosh, M. J. H. Akanda, M. S. Easmin, S. H. Bt Shamsudin and K. B. Yunus, Phytosterols and their extraction from various plant matrices using supercritical carbon dioxide: a review, *J. Sci. Food Agric.*, 2015, **95**(7), 1385–1394.

52 P. Devo, V. Cretu, H. Radhakrishnan, D. Hamilton-Pink, S. Boussios and S. V. Ovsepian, An orthogonal approach for analysis of underivatized steroid hormones using ultrahigh performance supercritical fluid chromatography-mass spectrometry (UHPSFC-MS), *J. Neural Transm.*, 2024, 1–9, DOI: **10.1007/s00702-024-02862-3**.

53 T. Langer, R. Nicoli, D. Guillarme, C. Schweizer-Grundisch, S. Rudaz, S. Grabherr, *et al.*, A novel ultra-high-performance supercritical fluid chromatography hyphenated to tandem mass spectrometry method for the analysis of urinary endogenous steroids in the anti-doping context, *J. Chromatogr. A*, 2024, **1734**, 465224.

54 D. W. Andersen and K. Linnet, Screening for anabolic steroids in urine of forensic cases using fully automated solid phase extraction and LC–MS-MS, *J. Anal. Toxicol.*, 2014, **38**(9), 637–644.

55 J. Robles, J. Marcos, N. Renau, L. Garrostas, J. Segura, R. Ventura, *et al.*, Quantifying endogenous androgens, estrogens, pregnenolone and progesterone metabolites in human urine by gas chromatography tandem mass spectrometry, *Talanta*, 2017, **169**, 20–29.

56 B. J. Fuhrman, X. Xu, R. T. Falk, C. M. Dallal, T. D. Veenstra, L. K. Keefer, *et al.*, Assay reproducibility and interindividual variation for 15 serum estrogens and estrogen metabolites measured by liquid chromatography–tandem mass spectrometry, *Cancer Epidemiol., Biomarkers Prev.*, 2014, **23**(12), 2649–2657.

57 G. de Albuquerque Cavalcanti, L. M. Rodrigues, L. Dos Santos, X. Zheng, A. Gujar, J. Cole, *et al.*, Non-targeted acquisition strategy for screening doping compounds based on GC-EI-hybrid quadrupole-Orbitrap mass spectrometry: A focus on exogenous anabolic steroids, *Drug Test. Anal.*, 2018, **10**(3), 507–517.

58 M. Raps, C. Kleider and L. Lehmann, Quantitative analysis of 34 sex (Pro) hormones, conjugates and bioactive oxidation products thereof in human plasma by GC-and LC-MS/MS and systematic investigation of overestimations of analyte concentrations not accounted for by method validation, *Steroids*, 2024, 109441.

59 J. Marcos and O. J. Pozo, Derivatization of steroids in biological samples for GC–MS and LC–MS analyses, *Bioanalysis*, 2015, **7**(19), 2515–2536.

60 I. Athanasiadou, P. Kiousi, N. Kioukia-Fougia, E. Lyris and Y. S. Angelis, Current status and recent advantages in derivatization procedures in human doping control, *Bioanalysis*, 2015, **7**(19), 2537–2556.

61 X. de la Torre, M. Iannone and F. Botrè, Improving the detection of anabolic steroid esters in human serum by LC–MS, *J. Pharm. Biomed. Anal.*, 2021, **194**, 113807.

62 Q. Wang, L. Bottalico, C. Mesaros and I. A. Blair, Analysis of estrogens and androgens in postmenopausal serum and plasma by liquid chromatography–mass spectrometry, *Steroids*, 2015, **99**, 76–83.

63 Y. Shibata, S. Arai and S. Honma, Methodological approach to the intracrine study and estimation of DHEA and DHEA-S using liquid chromatography-tandem mass spectrometry (LC-MS/MS), *J. Steroid Biochem. Mol. Biol.*, 2015, **145**, 193–199.

64 Y. Ke, R. Gonthier and F. Labrie, A sensitive and accurate LC-MS/MS assay with the derivatization of 1-amino-4-methylpiperazine applied to serum allopregnanolone, pregnenolone and androsterone in pre-and postmenopausal women, *Steroids*, 2017, **118**, 25–31.

65 H. Schulenberg-Schell and A. Tei, *Principles and Practical Aspects of Preparative Liquid Chromatography*, Agilent Technologies, 2015, pp. 9–45.

66 I. Dioumaeva, S. B. Choi, B. Yong, D. Jones and R. Arora, *Understanding Orthogonality in Reversed-Phase Liquid Chromatography for Easier Column Selection and Method Development*, Application Note, Agilent Technologies, 2010, available from: **https://www.agilent.com/cs/library/applications/SI-02425.pdf**.

67 J. Wang, R. Yang, W. Yang, X. Liu, Y. Xing and Y. Xu, IRMS detection of testosterone manipulated with 13C labeled standards in human urine by removing the labeled 13C, *Anal. Chim. Acta*, 2014, **852**, 146–152.

68 X. de la Torre, D. Jardines and F. Botrè, Evaluation of longitudinal 13C isotope ratio mass spectrometric data in antidoping analysis, *Drug Test. Anal.*, 2022, **14**(11–12), 1877–1890.

69 T. Piper and M. Thevis, Improving the Determination of Carbon Isotope Ratios of Endogenous Steroids Found in Human Serum, *Drug Test. Anal.*, 2024, DOI: **10.1002/dta.3793**.

70 X. de la Torre, C. Colamonici, D. Curcio and F. Botrè, Fast IRMS screening of pseudoendogenous steroids in doping analyses, *Drug Test. Anal.*, 2017, **9**(11–12), 1804–1812.

71 L. De Wilde, P. Van Renterghem, P. Van Eenoo, *et al.*, Development and validation of a fast gas chromatography combustion isotope ratio mass spectrometry method for the detection of epiandrosterone sulfate in urine, *Drug Test. Anal.*, 2020, **12**(8), 1006–1018.

72 H. A. Claessens, M. A. Van Straten, C. A. Cramers, M. Jezierska and B. Buszewski, Comparative study of test methods for reversed-phase columns for high-performance liquid chromatography, *J. Chromatogr. A*, 1998, **826**(2), 135–156.

73 S. Jain and A. Jain, A Brief Review on Different Chromatography Techniques, *J. Pharma Insight Res.*, 2024, **2**(1), 072–077.

74 Z. Li and B. Liu, Two-dimensional high performance liquid chromatography purification of underivatized urinary prednisone and prednisolone for compound-specific stable carbon isotope analysis, *Analyst*, 2024, **149**(19), 4899–4907.

75 M. Polet, P. Van Renterghem, W. Van Gansbeke and P. Van Eenoo, Studies on the minor metabolite 6a-hydroxy-androstenedione for doping control purposes and its contribution to the steroid profile, *Drug Test. Anal.*, 2014, **6**(10), 978–984.

76 C. Wen, T. Zhu, X. Liu and S. Wang, Isolation and enrichment of Boldenone and its main metabolite in urinary samples to further determine the 13C/12C ratios by gas chromatography/combustion/isotope ratio mass spectrometry, *J. Chromatogr. A*, 2023, **1707**, 464297.

77 K. Lalonde, A. Barber and 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.*, 2021, **13**(3), 558–570.

78 Y. Wang, J. Fan, X. Fang, Y. Zhuo, S. Xu, L. Liao, *et al.*, Two-Dimensional Liquid Chromatography Purified GC/C/IRMS Doping Control Method: Analysis of Endogenous and Exogenous Sources in Urine Samples from Asian Subjects Administered a Low Dose of AICAR, *J. Chromatogr. A*, 2024, **1735**, 465312.

79 L. Iannella, F. Botrè, C. Colamonici, D. Curcio and X. de la Torre, Development and validation of a method to confirm the exogenous origin of prednisone and prednisolone by GC-C-IRMS, *Drug Test. Anal.*, 2019, **11**(11–12), 1615–1628.

80 L. Iannella, F. Botrè, C. Colamonici, *et al.*, Carbon isotopic characterization of prednisolone and prednisone pharmaceutical formulations: implications in antidoping analysis, *Drug Test. Anal.*, 2020, **12**(11–12), 1587–1598.

81 T. K. Karatt, R. Sayed, J. Nalakath, Z. Perwad, P. H. Albert and K. A. Khader, Separation and identification of the epimeric doping agents–Dexamethasone and betamethasone in equine urine and plasma: A reversed phase chiral chromatographic approach, *Steroids*, 2018, **140**, 77–82.

82 L. Iannella, C. Colamonici, D. Curcio, F. Botrè and 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.*, 2021, **13**(4), 770–784.

83 T. Piper, M. Putz, P. Delahaut and M. Thevis, Carbon isotope ratios of endogenous steroids in Belgian Blue and Holstein cattle: Method development, reference population studies and application to steroid misuse control, *Rapid Commun. Mass Spectrom.*, 2017, **31**(21), 1793–1802.

84 L. Honesova, P. Van Eenoo and M. Polet, A uniform sample preparation procedure for gas chromatography combustion isotope ratio mass spectrometry for all human doping control relevant anabolic steroids using online 2/3-dimensional liquid chromatography fraction collection, *Anal. Chim. Acta*, 2021, **1168**, 338610.

85 M. Polet, P. Van Eenoo, L. Brooker, C. Edey, V. Agon, J. Grainger, *et al.*, Prevalence of nandrolone preparations with endogenous carbon isotope ratios in Australia, *Drug Test. Anal.*, 2024, **16**(11), 1345–1349.

86 X. de la Torre, D. Curcio, C. Colamonici, F. Molaioni, M. Cilia and F. Botrè, Development and validation of a GC-C-IRMS method for the confirmation analysis of pseudo-endogenous glucocorticoids in doping control, *Drug Test. Anal.*, 2015, **7**(11–12), 1071–1078.

87 L. Honesova, W. Viaene, P. Van Eenoo and M. Polet, High-temperature liquid chromatography-isotope ratio mass spectrometry methodology for carbon isotope ratio determination of anabolic steroids in urine, *Anal. Chim. Acta*, 2024, **1324**, 343092.

88 C. Wen, T. Zhu, J. Wang, X. Liu, S. Wang and 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.*, 2021, **13**(2), 338–347.

89 R. Agrawal, S. Belemkar and C. Bonde, Orthogonal separations in reversed-phase chromatography, *Chromatographia*, 2018, **81**(4), 565–573.

90 M. Putz, T. Piper and M. Dubois, Analysis of endogenous steroids in urine by means of multi-immunoaffinity chromatography and isotope ratio mass spectrometry for sports drug testing, *Anal. Bioanal. Chem.*, 2019, **411**, 7563–7571.

91 S. Kelly, C. Brodie and A. Hilkert, Isotopic-spectroscopic technique: Stable isotope-ratio mass spectrometry (IRMS), in *Modern Techniques for Food Authentication*, 2018, pp. 349–413.

92 Y. Zhang, J. T. Herbert and J. T. Brenna, Steroid isotopic standards for gas chromatography-combustion isotope ratio mass spectrometry (GCC-IRMS), *Steroids*, 2009, **74**(3), 369–378.

93 Z. Giannioti, N. Ogrinc, M. Suman, F. Camin and L. Bontempo, Isotope ratio mass spectrometry (IRMS) methods for distinguishing organic from conventional food products: A review, *TrAC, Trends Anal. Chem.*, 2024, **170**, 117476.

94 P. J. H. Dunn, D. Malinovsky, N. Ogrinc, D. Potočnik, L. Flierl, O. Rienitz, *et al.*, Re-determination of R (13C/12C) for Vienna Peedee belemnite (VPDB), *Rapid Commun. Mass Spectrom.*, 2024, **38**(16), e9773.

95 T. Piper, C. Emery and M. Saugy, Recent developments in the use of isotope ratio mass spectrometry in sports drug testing, *Anal. Bioanal. Chem.*, 2011, **401**, 433–447.

96 T. C. Moerdijk-Poortvliet, H. Schierbeek, M. Houtekamer, T. Van Engeland, D. Derrien, L. J. Stal, *et al.*, Comparison of gas chromatography/isotope ratio mass spectrometry and liquid chromatography/isotope ratio mass spectrometry for carbon stable-isotope analysis of carbohydrates, *Rapid Commun. Mass Spectrom.*, 2015, **29**(13), 1205–1214.

97 W. Meier-Augenstein, Applied gas chromatography coupled to isotope ratio mass spectrometry, *J. Chromatogr. A*, 1999, **842**(1–2), 351–371.

98 J. Wang, Y. He, X. Liu, Z. Yang and W. Yang, Steroid profile and IRMS analysis of musk administration for doping control, *Drug Test. Anal.*, 2017, **9**(11–12), 1779–1787.

99 S. Zhao, Y. Zhao, K. M. Rogers, G. Chen, A. Chen and S. Yang, Application of multi-element (C, N, H, O) stable isotope ratio analysis for the traceability of milk samples from China, *Food Chem.*, 2020, **310**, 125826.

100 M. Putz, T. Piper, A. Casilli, F. R. de Aquino Neto, F. Pigozzo and M. Thevis, Development and validation of a multidimensional gas chromatography/combustion/isotope ratio mass spectrometry-based test method for analyzing urinary steroids in doping controls, *Anal. Chim. Acta*, 2018, **1030**, 105–114.

101 T. Piper, M. Putz, W. Schänzer, *et al.*, Epiandrosterone sulfate prolongs the detectability of testosterone, 4-androstenedione, and dihydrotestosterone misuse by means of carbon isotope ratio mass spectrometry, *Drug Test. Anal.*, 2017, **9**(11–12), 1695–1703.

102 T. C. Moerdijk-Poortvliet, L. J. Stal and H. T. Boschker, LC/IRMS analysis: A powerful technique to trace carbon flow in microphytobenthic communities in intertidal sediments, *J. Sea Res.*, 2014, **92**, 19–25.

103 M. Perini and L. Bontempo, Liquid chromatography coupled to isotope ratio mass spectrometry (LC-IRMS): a review, *TrAC, Trends Anal. Chem.*, 2022, **147**, 116515.

104 L. Zhang, M. Thevis, T. Piper, M. Jochmann, J. Wolbert, D. Kujawinski, *et al.*, Carbon isotope ratio analysis of steroids by high-temperature liquid chromatography-isotope ratio mass spectrometry, *Anal. Chem.*, 2014, **86**(5), 2297–2302.

105 L. Honesova, P. Van Eenoo and M. Polet, Evaluation of analytical columns suitable for high-temperature liquid-chromatography-isotope-ratio-mass-spectrometry analysis of anabolic steroids, *J. Chromatogr. A*, 2024, **1731**, 465191.

106 J. P. Godin and J. S. McCullagh, Current applications and challenges for liquid chromatography coupled to isotope ratio mass spectrometry (LC/IRMS), *Rapid Commun. Mass Spectrom.*, 2011, **25**(20), 3019–3028.

107 R. M. Toledano, E. M. Díaz-Plaza, J. M. Cortes, *et al.*, Development of an analytical method for the determination of the misuse in sports of boldenone through the analysis of urine by on-line coupling liquid chromatography–gas chromatography–combustion–isotope ratio mass spectrometry, *J. Chromatogr. A*, 2014, **1370**, 171–178.
108 K. Kantnerová, N. Kuhlbusch, D. Juchelka, *et al.*, A guide to precise measurements of isotope abundance by ESI-Orbitrap MS, *Nat. Protoc.*, 2024, **19**(8), 2435–2466.