

# Current role of LC–MS(/MS) in doping control

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**Abstract** Liquid chromatography–(tandem) mass spectrometry (LC–MS/MS) has revolutionized the detection assays used in doping control analysis over the last decade. New methods have enabled the determination of drugs that were formerly difficult to detect or undetectable at preceding sample concentrations, and complex and/or time-consuming procedures based on alternative chromatographic–mass spectrometric or immunochemical principles have been replaced by faster, more comprehensive and robust assays. A critical overview of the contributions of LC–MS(/MS) to sports drug testing is provided, including recent developments regarding low and high molecular weight drugs.

**Keywords** Doping · Sport · Mass spectrometry · Criteria · Peptide · Protein

## Introduction

The technique of liquid chromatography used in concert with (tandem) mass spectrometry (LC–MS/MS) has complemented sports drug testing strategies ever since soft ionization interfaces such as electrospray or atmospheric pressure chemical ionization (ESI or APCI, respectively) became commercially available. Numerous applications have been developed that allow the determination of

prohibited therapeutics that are barely detectable or undetectable with conventional gas chromatographic–mass spectrometric techniques (GC–MS), and comprehensive summaries of the methods commonly employed in doping controls have been published in the past [1–6]. Due to the progressive nature of doping controls, the continuously changing demands originating from the dynamic pharmaceutical market, new illegal approaches that presumably increase athletic performance, and modifications to the lists of prohibited compounds of regulative authorities such as the World Anti-Doping Agency (WADA) [7], numerous new applications and drug-testing strategies based on LC–MS(/MS) are frequently developed in order to improve the portfolios of drug-testing laboratories.

In the current review, analytical assays for anabolic agents, stimulants and peptide hormones that utilize LC–MS/MS and were established within the last two years are presented and discussed. Special attention is focused on recent developments regarding peptide and protein analysis that require particular consideration in terms of target analyte identification.

## Anabolic agents

Over the past two decades, among all of the drugs that are misused in sports, anabolic agents (as classified by WADA) have been the ones most frequently determined. Numerous findings of the misuse of anabolic androgenic steroids, natural steroid hormones (e.g., testosterone) or designer steroids, as well as other anabolic agents such as the  $\beta_2$ -agonist clenbuterol have been reported. Hence, there has been a strong push to enhance screening and confirmation assays for this particular class of compounds, which has

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yielded several new applications utilizing the advantages of LC–MS/MS.

Steroids with large conjugated or cross-conjugated electron systems such as trenbolone and gestrinone or boldenone, respectively, have demonstrated marginal gas chromatographic properties under commonly employed derivatization and analytical conditions. These problems have resulted in relatively high detection limits using GC–MS approaches. However, their particular structures provide them with considerable proton affinities [8, 9] and so they are well suited to LC–MS/MS approaches. Consequently, anabolic steroids as well as glucocorticosteroids that are difficult to assay using GC have been analyzed using LC–MS/MS methods [3, 10–12] that yield detection limits matching the minimum required performance limits (MRPL) as defined by WADA. Also, the designer steroid tetrahydrogestrinone (THG), which bears the same steroidal nucleus as gestrinone, has been determined using LC–MS/MS ever since it was discovered by Catlin et al. [13]. The fact that THG was not detected for several years illustrates a drawback of the doping control screening protocols usually used, which are based on target analysis. Known drugs and/or metabolic products are determined via precursor/product ion pair measurements, which provide the utmost sensitivity but reduce the analytical result to a limited number of compounds. Drugs that have unknown molecular weights and dissociation pathways under conventional collision-induced dissociation (CID) conditions are provided with a *cloak of invisibility* and remain undetected. Hence, complementary analyses have been suggested that are based on precursor ion scanning of the product ions that characterize particular steroid structures [14] or that utilize androgen bioassays in concert with high-resolution MS (HRMS) [15], allowing broader views of urinary steroids. These proposals provide a deeper insight into potentially misused anabolic androgenic steroids, but they can still not ensure the determination of surreptitiously altered steroids prepared solely for doping purposes, as primarily metabolic reactions may reduce or even impede the effectiveness of these assays.

New anabolic agents termed *selective androgen receptor modulators* (SARMs) have recently entered phase-II and -III clinical trials and possess a considerable potential for misuse in sports [16–18]. Their structural diversity (they encompass at least six chemical categories) has necessitated the extension of doping control assays through the introduction of new target analytes as well as precursor ion scanning in order to make sure that this emerging class of drugs is comprehensively screened for [19]. Detection assays for arylpropionamide-derived SARMs were reported for between 1 and 50 ng per mL of urine.

Screening assays are of particular importance in doping controls, as they provide the information necessary to

consider a sample suspicious for drug abuse. They have been updated and extended frequently based on new requirements as well as new findings, such as additional long-term metabolites for improved retrospectives, as recently reported for methandienone [20]. However, the requirements of obligatory confirmation analyses are strict and have also led to the development of alternative sample preparation procedures for LC–MS/MS measurements that are specifically optimized for just a few analytes. Compounds are efficiently purified and this leads to unambiguous analytical results, as for instance demonstrated for clenbuterol [21] or stanozolol and its metabolic products recently [22].

### Stimulants

Stimulants have traditionally been measured by conventional GC–MS and GC–nitrogen/phosphorus detection (NPD) techniques in doping control analysis [23–26]. Straightforward liquid–liquid extractions followed by full-scan and nitrogen/phosphorus-specific analysis have provided fast and comprehensive screening tools. Limitations were observed primarily when a distinction between isomeric compounds was required, for instance in the case of ephedrine and pseudoephedrine. These drugs are considered differently during sports drug testing and require quantitative measurements. Here, derivatization has been necessary in order to obtain baseline separation of target analytes under GC conditions [27]. Due to the ease of use of modern LC–MS/MS instruments, the reduction in effort needed for sample preparation, and the ability to simultaneously determine an enormous number of analytes, numerous screening and qualitative as well as quantitative confirmation assays have recently been established. The detection of 27 drugs related to amphetamine has been reported using an ion trap analyzer with a sensitivity, specificity and speed of analysis comparable to conventional GC–MS procedures [28]. Novel approaches employing ultrahigh-performance liquid chromatography (UPLC) yielded a screening assay that required only very short run times of less than two minutes to assay for 11 amphetamine-like compounds [29]. In addition, qualitative and quantitative determinations of selected compounds such as mesocarb [30], sibutramine metabolites [31] or ephedrines [32] have been reported, the latter of which employed stable isotope-labeled internal standards [33]. Despite the apparent utility of LC–MS/MS for measuring stimulating agents, no screening procedure has been presented that includes hydroxylated phenolalkylamines such as 4-hydroxyamphetamine, 4-hydroxyephedrine or 4-hydroxyphenylethylamine. The enhanced polarities of these compounds are assumed to complicate the chromatographic separation of target analytes from potentially interfering matrix substances, as their retentions

on commonly employed C-8 or C-18 reverse-phase HPLC columns are rather low.

### Peptide hormones and proteins

Throughout the history of doping control, peptide hormones and proteins have primarily been determined using immunological approaches. However, due to enormous improvements in sample pretreatment as well as instrumental sensitivity and specificity, several assays based on LC–MS/MS have been reported recently [6]. These procedures include blood or urine analyses for bovine hemoglobin-based oxygen carriers (so-called HBOCs) [34–37], rapid and slow-acting insulins [38, 39], human chorionic gonadotropin [40], as well as the corticotrophin synacthen [41], and they provide unambiguous results for the respective target analytes.

#### HBOCs

HBOCs such as Hemopure and Oxyglobin (approximate average mol weight=250 kDa) consist of inter- and intramolecularly covalently crosslinked bovine hemoglobin, and they have been prohibited from use in sports due to the potential increase in blood oxygen transport capacity that they afford [42, 43]. Owing to their xenobiotic nature, several different LC–MS/MS approaches have been established to determine their presence in human serum. Chemical stabilization of the hemoglobin subunits using crosslinking agents resulted in an N-terminal derivatization of methionines of beta chains, which have been identified as potential targets in analytical approaches employing proteasomal degradation of serum with endoproteinase Glu-C [35]. The presence of product ions at  $m/z$  170 and 172 has been attributed to the characteristically altered peptide MLTAE, which yields precursor ions at  $m/z$  759 and 761. These ion pairs have been utilized in a screening assay for HBOCs, yielding detection limits of 2 mg/mL for compounds such as Oxyglobin and Hemopure. Alternative procedures have exploited the differences in the primary structures of bovine and human hemoglobins, as well as the presence of crosslinks in hemoglobin molecules. Due to a sequence homology of only 85%, several proteotypical peptides uniquely derived from bovine hemoglobin upon trypsin digestion are detectable using LC–MS/MS approaches and provide evidence for the presence of HBOCs at concentration levels of 2 mg/mL [32, 37]. Additionally, crosslinks introduced at lysines of hemoglobins impede the activity of trypsin at the respective residues and considerably change the ratios of tryptic peptides obtained from bovine hemoglobin and Hemopure [36]. The latter information in particular will be useful for

differentiating between human hemoglobin and its cross-linked analogs (e.g., PolyHeme) if these are launched by pharmaceutical companies.

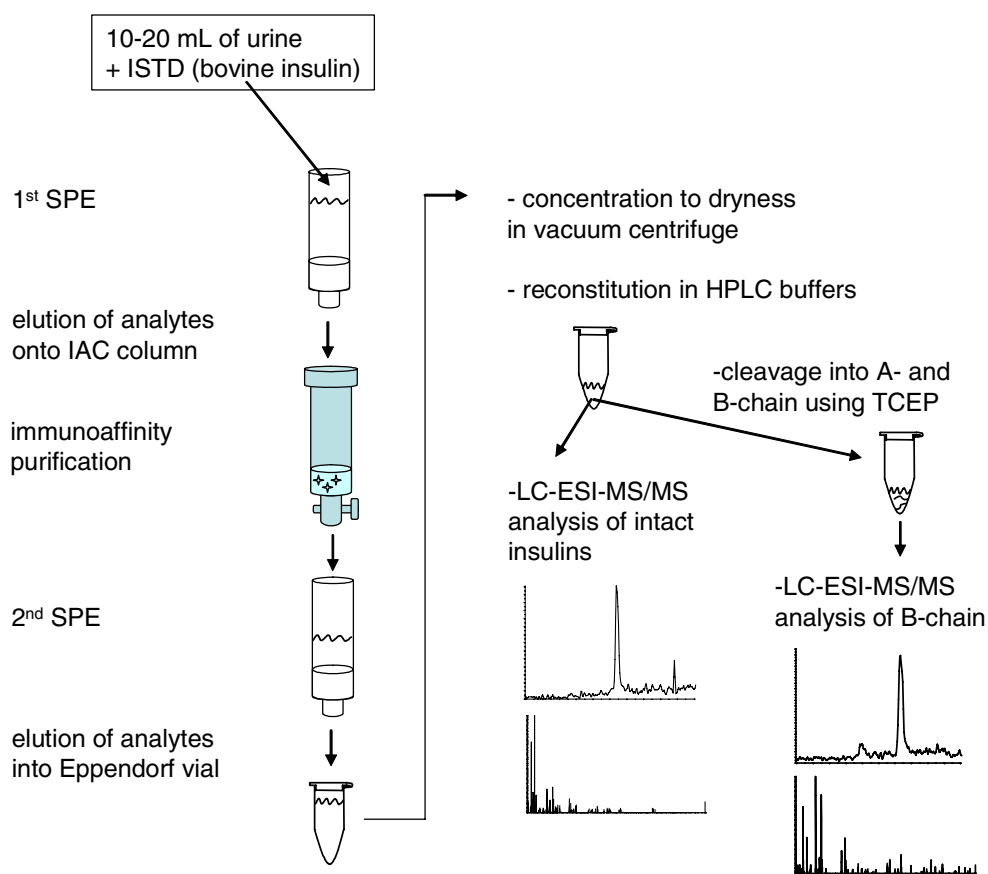
#### Insulins

Human insulin has been considered relevant for sports drug testing since 1999 due to its assumed positive effects on muscle glycogen formation, anticatabolic (so-called *chalone*) actions on muscle protein, and improvements in protein biosynthesis [44–46]. Besides conventional formulations consisting of recombinantly produced human insulin, numerous synthetic analogs, termed rapid- and long-acting insulins, have been introduced that possess improved injection-to-onset and pharmacokinetic profiles. In particular, the rapid-acting insulins such as Humalog, Novolog and Apidra have been targeted by new analytical methods, and top-down sequencing-based approaches have enabled their determination in human plasma and urine using immunoaffinity chromatography (IAC), solid-phase extraction (SPE) and microbore LC–MS/MS procedures [38, 39], as illustrated in Fig. 1. While Humalog differs from human insulin only by the switched positions of proline B28 and lysine B29, Novolog and Apidra comprise substituted amino acid residues, resulting in them having different molecular weights compared to that of human insulin (5807 Da). Product ion scan experiments yielded diagnostic ions that unequivocally identify the synthetic nature of these insulin analogs, and detection limits of 0.5 and 0.05 ng/mL, respectively, were obtained, enabling the determination of normal insulin levels in both matrices. Several athletes selected for doping control sample collection suffer from *diabetes mellitus*, and numerous authentic specimens from these tested “positive,” providing the proof-of-principle of these new procedures.

#### Human chorionic gonadotropin

Human chorionic gonadotropin (hCG) is a 37-kDa glycoprotein predominantly produced by the placenta during pregnancy, and its use has been prohibited for male athletes since 1987. Its ability to induce the secretion of endogenously produced testosterone from testes has necessitated its detection in doping controls, which has primarily been accomplished using immunological methods. A new approach was recently presented by Gam et al. [40], based on immunoaffinity purification followed by trypsin digestion and subsequent LC–MS/MS analysis of marker peptide  $\beta T5$ . Thus, qualitative and

**Fig. 1** Schematic illustration of the preparation of a urine sample for the determination of synthetic insulins in relation to sports drug testing



quantitative evidence for hCG was obtained, allowing detection limits of 5 mIU/mL of urine to be obtained. This approach provided the discriminative power required, which common immunological test methods lack due to possible cross-reactivity.

### Synacthen

A peptide hormone termed synacthen, which belongs to the class of corticotrophins, has also recently been determined in human plasma using immunoaffinity purification and LC-MS/MS analysis [41]. The peptide consists of 24 amino acids and mimics the adrenocorticotrophic hormone ACTH, which triggers the secretion of endogenously produced cortisone. Due to its relatively small size, no further enzymatic hydrolysis was required and unambiguous information on its presence was obtained by multiple reaction monitoring experiments, including those monitoring the quadruply charged precursor ion and the diagnostic product ion  $a_2$  at  $m/z$  223. Using administration study specimens as well as spiked blank plasma samples, detection limits of 0.3 ng/mL have been obtained, while sampling and storage have been identified as key factors regarding the stability of the target analyte. Only specimens stored at  $-20$  °C were stable for up to three months, which demonstrates that blood sampling

requires different conditions than the collection of urine specimens in doping controls.

### Mass spectrometric identification criteria for peptides and proteins

The importance of mass spectrometry to doping control analysis has been evident for decades, and most assays enabling the screening and confirmation of hundreds of drugs and/or their metabolites have been established based on chromatographic separation techniques combined with mass spectrometric detection. Depending on the methodology applied [i.e., gas chromatography / (tandem) mass spectrometry or liquid chromatography / (tandem) mass spectrometry], examples of acceptance criteria for the unambiguous determination of prohibited compounds have been proposed by respective authorities such as the WADA [47] or the Commission of European Communities [48], and comparable guidelines are available for forensic toxicology or veterinary medicine (US FDA Guidance for Industry) [49], as recently summarized by Van Eenoo and Delbeke [50] and challenged by Stein and Heller [51]. Chromatographic retention times (RTs) as well as relative abundances of diagnostic fragment ions resulting from electron or chemical ionization or product ions generated

by collision-induced dissociation are important items that can be used to identify the target analyte by comparing to reference material or characterized metabolites obtained from authentic administration study specimens.

The present criteria—including distinct guidelines and so-called identification points (IPs)—are defined for low molecular weight substances. However, with the advent of the application of LC–MS/MS to doping control analysis for peptide hormone and protein identification, there is a need for new rules regarding this class of compounds, because new/complementary facts and parameters must be considered.

Numerous proteomics applications have utilized various approaches and criteria for the identification of peptides and proteins, primarily using MS techniques [52–56]. Procedures have been developed to identify unknown proteins from complex mixtures by strategies including protein fractionation and multidimensional protein identification technology (MudPIT), which incorporates multidimensional high-performance liquid chromatography (LC/LC), tandem mass spectrometry (MS/MS) and database-searching algorithms [57–63]. However, sports drug testing is focused on targeted analysis, i.e., known compounds are sought and determined with the utmost sensitivity and confidence, yielding unambiguous analytical results. Hence, it is important that criteria for the unequivocal identification of compounds are provided that demonstrate the *fitness for purpose* of the applied technology.

The protocol applied to mass spectrometric identifications of target analytes performed in the doping control laboratory in Cologne (Germany) [64] follows a modified version of the WADA technical document TD2003IDCR. Firstly, the importance of molecular mass determination is stressed due to the considerable difference between the singly charged precursor ions usually observed with low molecular weight compounds and peptide or protein-derived molecular ions (e.g., those from electrospray or matrix-assisted laser desorption ionization). Molecular masses of target analytes as determined by deconvolution in confirmatory measurements should not deviate by more than  $\pm 0.5$  Da between doping control urine samples and reference specimens. Secondly, a minimum of three structure-specific product ions is recommended when applying MS/MS technologies, and  $m/z$  values should also not deviate by more than  $\pm 0.5$  Da to ensure that the uncertainties obtained are acceptable. In addition to the mere presence of these product ions in the spectra obtained from a suspicious doping control sample and a reference specimen, the relative abundances of the product ions are considered, as proposed in the WADA technical document TD2003IDCR for LC–MS<sup>n</sup> experiments. Maximum deviations of 15% (absolute), 25% (relative) and 10% (absolute) are allowed for product ions with relative abundances of >50%, between 25 and 50%, and <25%, respectively. Structure-specific product ions

derived from precursor ions are needed to provide confidence in the analytical result with respect to the above-mentioned criteria for relative abundances. However, the preparation of additional derivatives of target peptides or proteins providing different precursor ions and/or product ions could provide substantiating information in order to unequivocally identify an analyte. In all cases, the signal-to-noise ratio of the product ions should be greater than 3:1, and assignments of origin should be provided.

### Identification points (IPs)

A complementary approach based on IPs was recently proposed [48, 65]. The identification of low molecular weight target compounds was based on the different identification powers of low-resolution and high-resolution/high-accuracy MS and MS<sup>n</sup> techniques, and it was suggested that a minimum of three IPs was needed for analyte identification. This can also be accomplished via different derivatives and analyses. In Table 1, the values of the mass spectrometric IPs corresponding to each of the analytical strategies applied are listed.

Considering the complexity of peptide and protein identification, a minimum of five IPs is recommended for sports drug testing applications, should they be extended to peptide/protein characterization in the future. Additionally, the general requirements for each ion or product ion (as outlined above), such as for the S/N ratio or relative abundance, must also be fulfilled.

### Bottom-up sequencing for protein identification

In addition to defining which MS and MS/MS criteria to fulfil, one must also specify the number of peptides derived from a particular protein (mol weight > 8 kDa) that characterize the target protein. Different requirements should be fulfilled, depending on the type of analysis, i.e., peptide mass fingerprinting using single MS vs. amino acid

**Table 1** IP values for each mass spectrometric technique applied (modified from [48])

Mass spectrometric technique	IP value
Low-resolution MS <sup>n</sup> precursor ion/ molecular weight determination	1.0
Low-resolution MS <sup>n</sup> product ion	1.5
High-resolution/high-accuracy MS <sup>n</sup> precursor ion/ molecular weight determination	2.0
High-resolution/high-accuracy MS <sup>n</sup> product ion	2.5

**Table 2** Summary of the characteristics of current LC–MS(/MS) procedures used in doping control

Drug class / Compound	Sample	Work-up	Stationary phase	Mobile phase	Detection mode	LOD	Refs.
<b>Anabolic agents</b>							
Anabolic steroids	urine	LLE	C-18 RP	formic acid / MeOH	SRM +	1–10 ng/mL	[10]
Anabolic steroids (and corticosteroids)	urine	LLE	C-18 RP	acetic acid / ACN	MRM +	1–30 ng/mL	[12]
Anabolic steroids	urine	LLE	C-18 RP	acetic acid, ammonium acetate / ACN	MRM +	0.1–2.0 ng/mL	[11]
Designer steroids	urine	LLE	C-8 RP	acetic acid, ammonium acetate / ACN	Precursor +	~ 50 ng/mL	[14]
Designer steroids	urine	SPE (2x)	C-18 RP	formic acid / ACN	TOFMS +	n.d.	[15]
THG	urine	LLE	C-18 RP	acetic acid / CAN	MRM +	5 ng/mL	[13]
Methandienone metabolite	urine	LLE	C-8 RP	acetic acid, ammonium acetate / ACN	MRM +	n.d.	[20]
Stanozolol and metabolites	urine	LLE	C-8 RP	acetic acid, ammonium acetate / ACN	MRM +	0.1–0.2 ng/mL	[22]
Clenbuterol	urine / blood	LLE (2x)	C-18 RP	acetic acid, ammonium acetate / ACN	MRM +	0.1 ng/mL	[21]
SARMs	urine	SPE	C-18 RP	acetic acid, ammonium acetate / ACN	MRM - / precursor -	1–50 ng/mL	[19]
<b>Stimulants</b>							
Stimulants	urine	LLE	C-18 RP	formic acid / MeOH	SRM + / full MS	25–250 ng/mL	[28]
Stimulants	blood	SPE	C-18 RP	acetic acid, pyrrolidine / MeOH	SIR	n.d.	[29]
Mesocarb and metabolites	urine	LLE	C-18 RP	ammonium acetate / MeOH	SRM +	0.1 pg/mL	[30]
Sibutramine metabolites	urine	LLE	C-18 RP	acetic acid, ammonium acetate / ACN	MRM +	6–40 ng/mL	[31]
Ephedrine	urine	direct injection	C-18 RP	acetic acid, ammonium acetate / ACN	MRM +	~ 500 ng/mL	[32]
<b>Peptides and proteins</b>							
HBOCs	serum	Glu-C digest	C-18 RP	formic acid / ACN	precursor +	2 mg/mL	[35]
	serum	HCl hydrolysis	C-18 RP	formic acid / ACN	SRM +	4 mg/mL	[34]
	plasma	trypsin digest	C-18 RP	formic acid / ACN	SIR +	1–2 mg/mL	[36]
	plasma	trypsin digest	C-18 RP	acetic acid, trifluoroacetic acid / ACN	MRM +	2 mg/mL	[37]
Insulins	plasma	IAC / SPE	C-18 RP	acetic acid, trifluoroacetic acid / ACN	EPI +	0.5 ng/mL	[38]
	urine	SPE / IAC / SPE	C-18 RP	acetic acid, trifluoroacetic acid / ACN	EPI +	0.05 ng/mL	[39]
hCG	urine	IAC / trypsin digest	C-18 RP	trifluoroacetic acid / ACN	SRM+	5 mIU/mL	[40]
Synacthen	plasma	IAC / SPE	C-18 RP	acetic acid, trifluoroacetic acid / ACN	MRM +	0.3 ng/mL	[41]

Abbreviations: “+” = positive ionization, “-” = negative ionization, ACN = acetonitrile, EPI = enhanced product ion, HBOCs = hemoglobin-based oxygen carriers, hCG = human chorionic gonadotropin, IAC = immunoaffinity chromatography, LLE = liquid–liquid extraction, LOD = limit of detection, MeOH = methanol, MRM = multiple reaction monitoring, n.d. = not determined, SARMs = selective androgen receptor modulators, SIR = selected ion reaction, SPE = solid-phase extraction, SRM = selected reaction monitoring, THG = tetrahydrogestrinone

sequencing using MS/MS techniques. Owing to the importance of the unambiguous determination of compounds, the identification of just four peptides characterized by their molecular mass should be provided (accounting for four IPs). In the case of MS/MS analysis, the identification of two peptides according to the suggestions described above is considered sufficient due to the significantly increased sequence information.

## Conclusion

LC–MS(/MS) has considerably influenced doping control analysis by providing powerful tools for low and high molecular weight analysis. Numerous drugs that have not been detectable or have barely been detectable using alternative approaches based on, say, GC–MS or immunological techniques have been determined with high sensi-

tivities and specificities, as summarized in Table 2. Sample preparation efforts have been significantly reduced and detection limits have improved enough to allow prolonged retrospectives for the determination of drug abuse. Moreover, peptide hormones and proteins were unambiguously analyzed using top-down or bottom-up sequencing approaches that provide evidence for the presence or absence of synthetic therapeutics. The discriminative power of mass spectrometry in particular is a great benefit and highly advantageous compared to conventional immunological assays, although the sample throughput is currently much smaller than for such assays due to long preparation and analysis times. Hence, only established peptide and protein detection procedures have been considered for confirmatory analyses so far.

Analytical assay sensitivity and throughput are key factors for the LC-MS(/MS) approaches used in sports drug testing. Considerable improvements in liquid chromatography (such as using monolithic or UPLC columns) have resulted in shorter analysis times and significantly narrower peaks, which in turn lead to enhanced signal-to-noise ratios, better detection limits, and increased productivity. However, these fast analytical runs require very short mass spectrometric cycle times in order to obtain sufficient data points per compound and corresponding peak.

In order to incorporate newly developed methods into sports drug testing systems, identification criteria are required, which have only been established for low molecular weight drugs. Several aspects are applicable and transferable to peptide and protein analysis, but specific issues—such as molecular weight determination from multiply charged species or bottom-up sequencing for protein identification—are still to be addressed.

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