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Review Article

Analytical Detection Methods of Performance Enhancing Drugs in Athletes, Review Study

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Abstract: The detection and deterrence of the abuse of performance-enhancing drugs in sport are important to maintaining a level playing field among athletes and to decreasing the risk to athletes' health. The World Anti-Doping Program consists of six documents, three of which play a role in analytical development: The World Anti-Doping Code, The List of Prohibited Substances and Methods, and The International Standard for Laboratories. Among the classes of prohibited substances, three have given rise to the most recent analytical developments in the field: anabolic agents; peptide and protein hormones; and methods to increase oxygen delivery to the tissues, including recombinant erythropoietin. Methods for anabolic agents, including designer steroids, have been enhanced through the use of liquid chromatography/tandem mass spectrometry and gas chromatography/combustion/isotope-ratio mass spectrometry. Protein and peptide identification and quantification have benefited from advances in liquid chromatography/tandem mass spectrometry. Incorporation of techniques such as flow cytometry and isoelectric focusing have supported the detection of blood doping.

Keywords: Detection methods, drugs, Athletes

List of abbreviations

AAS	Anabolic androgenic steroids
APCI	Atmospheric pressure chemical ionization
APPI	Atmospheric pressure photo ionization
B	Blood
DBS	Dried blood spots
CI	Chemical ionization
DHEA	Dehydroepiandrosterone
DL	Decision limits
EI	Electron ionization
EPO	Erythropoietin
EQAS	External quality assessment scheme
ESI	Electro spry ionization
GC-EI-MS	Gas chromatography electron ionization mass spectrometry
GC-MS	Gas chromatography mass spectrometry
GC-MS/MS	Gas chromatography mass spectrometry/ mass spectrometry
GLC	Gas liquid chromatography
LC-UV	Liquid chromatography Ultraviolet
HBOC	Hemoglobin based oxygen carriers
hCG	Human Chorionic Gonadotropin
HGH	Human growth hormone
HFB	Heptafluorobutyrated
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectrometry
IDCR	Identification criteria
IOC	International Olympic Committee

IR	Isotope ratio
ISL	International standard for laboratories
LC-MS	Liquid chromatography mass spectrometry
LC-MS/MS	Liquid chromatography mass spectrometry/mass spectrometry
LH	Luteinizing Hormone
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantitation
MDMA	Methylene dioxymetamphetamine
mg/L	milligram per litter
MRM	Multiple reaction monitoring
m/z	Mass to charge ratio
NESP	Novel erythropoiesis stimulating protein
ng/mL	nanogram per milliliter
NPD	Nitrogen phosphorus detector
No	Number
PEDs	Performance enhancing drugs
PVE	Plasma volume expanders
Qs	Quadrapoles
QTOFMS	Quadra poles time of flight mass spectrometry
rEPO	recombinant erythropoietin
RT	Retention time
SIM	Selected ion monitoring
SPE	Solid phase extraction
SRM	Selected reaction monitoring
TD	Technical documents
TFA	Trifluoro acetylated
TMS	Trimethylsilylation
TQs	triple Quadrapoles
UHPLC	Ultra high performance liquid chromatography
USADA	United States Anti-Doping Agency
WADA	World Anti-Doping Agency

1. INTRODUCTION

Performance enhancing drugs are referred to as “body enhancement drugs”. When an athlete takes these products to acquire supernormal abilities, it is called “doping.” There are many reasons that athletes use drugs to aid performance. The main factor is pressure, which comes in several forms. All athletes put pressure on themselves because they have a basic desire to be successful. Additionally, coaches, family, and friends with high expectations can add more pressure. Beyond this, pressure can come from other athletes, spectators, and the media (Santella, 2005). Because the rewards (both status elevation and financial) are so great, athletes are often willing to build mass and strength of muscles and/or bones, increase delivery of oxygen to exercising tissues, mask pain, stimulate his or her body (increase alertness, reduce fatigue, increase aggressiveness), relax, reduce weight and hide their use of other drugs (Haley, 2003). Doping has many short-term and long-term risks. A few of the many serious consequences (harmful effects) an athlete may experience include: liver disease, cardiovascular disease, high blood pressure, sexual side effects, weakening of the immune system, nausea, tremors, increased risk of stroke, heart attack (USADA, 2016).

The World Anti-Doping Agency (WADA) was established in 1999 to promote, coordinate, and monitor the fight against performance enhancing drugs like anabolic androgenic steroids (AAS), human growth hormone (HGH), diuretics, erythropoietin (EPO), stimulants and many others. The analytical chemistry associated with deterrence and detection of performance enhancing substances is also advancing at a rapid pace. Based on the mass spectrometric characterization of target analytes, numerous assays have been established allowing the comprehensive identification of performance enhancing drugs in doping control specimens and the majority of anti-doping tests rely on the combination of gas chromatography combined with mass spectrometry (GC-MS), high performance liquid chromatography linked with mass spectrometry (HPLC-MS) and many procedures employ GC-EI-MS, GC-MS/MS, HPLC-MS/MS, LC-MS and ever since LC-MS/MS techniques have proven sensitivity and robustness, the determination of several classes of compounds has been accomplished using this comparably new strategy (Bowers, 2009).

2. World Anti-Doping Agency (WADA)

WADA is an independent, international agency which promotes, coordinates and monitors the fight against doping in sport in all its forms. There were four main reasons that led to the foundation of WADA. First was the lack of harmonization of anti-doping rules (Catlin *et al.,* 2008). Second, some doping substances had spread among amateurs, producing an alarming public health problem. The third reason was to promote research to keep abreast of developments in the pharmaceutical industry. Fourth was the desire to centralize collaboration between national and international anti-doping activities. In 2002, WADA’s World Anti-Doping Program was approved at an international congress in Helsinki. This consisted of six documents of which three are relevant for accredited laboratories: the World Anti-Doping Code (1), the list of Prohibited substances and methods

(2), international standard for laboratories (ISL) (3), the international standard for the protection of privacy and personal information (4), the international standard for testing (5) and the international standard for therapeutic use exemptions (6) (WADA Anti-Doping Program, 2010). The main activities of WADA include scientific research, education, development of anti-doping capacities and monitoring the compliance with the World Anti-Doping Code. WADA, a Swiss law foundation, sits in Lausanne, Switzerland and has its headquarters in Montreal, Canada. The Anti-Doping Community consists of several stakeholder groups including athletes, National Anti-Doping Organizations, major event organizations, governments, and anti-doping laboratories. As the custodian of the World Anti-Doping Code, WADA has the duty to oversee and monitor stakeholders' activities in relation to the Code and to ensure the integrity of the Code (Kolmonen, 2011).

3. Analytical challenges and advances

As it has occurred in many fields of scientific endeavor, the development of reliable analytical tools for anti-doping has preceded advances in detection, measurement, and interpretation. The first testing at an Olympic games occurred in Mexico City in 1968. At the 1972 Munich Olympics, GC/MS was used for confirmation of stimulants. The introduction of the fused silica capillary GC column and robust bench top mass analyzers in the early 1980s facilitated extensive study of steroid profiles in urine and allowed faster analysis of the increasing numbers of anabolic steroids. The application of this sensitive technique, which can identify trace amounts of anabolic steroids, resulted in a large number of last minute withdrawals from competition and in sanctions for anabolic steroid use at the 1983 Caracas Pan American Games.

The metabolism of synthetic anabolic steroids became better understood, primarily as a result of research in the anti-doping laboratories. Similarly, the development of high performance liquid chromatography (HPLC) coupled to a tandem mass spectrometer (MS/MS) has allowed the detection of polar small molecules and peptides and proteins. Thus, the analytical techniques available for testing in anti-doping laboratories have broadened dramatically in the past decade. Another analytical challenge arises from the fact that in anti-doping testing, some individuals being tested actively attempt to avoid detection. For example, after the development of urine test for recombinant erythropoietin (rEPO), it was apparent from the isoelectric focusing patterns observed over time that some athletes changed from standard dosing regimens to "micro dosing" to beat the test.

Advisors with scientific or medical expertise have counseled athletes to provide minimum volumes of urine in the hopes that the volume is insufficient to allow confirmation. They have also advised athletes on drug dosages and kinetics to avoid detection. Athletes have also received advice about the newest undetectable drugs, with varied success. The fact that there are active attempts to mask drug use means that the selection of analytical approaches to testing schemes must take so called masking into account. The majority of anti-doping tests rely on the combination of GC or HPLC and MS (Bowers, 2009).

3.1. Principles of doping control sample analysis

Unlike many other analytical areas, the analysis of a doping control sample has certain very specific features. Hundreds of compounds and their metabolites have to be detected and identified from a limited aliquot of sample in a short period of time. During major sporting events the results have to be ready in just 24 hours. Trout and Kazlauskas presented a scheme of several issues that have to be considered before establishing an analysis method for a doping agent (Trout and Kazlauskas, 2004). These involve drug properties, metabolism, applicability to an existing method, and the cost and availability of standards. The characteristics of the method performance are also dependent on whether non-threshold or threshold substances have to be determined (Peters *et al.,* 2010). For non-threshold compounds, the laboratory has to identify, not quantify, the compound's presence in urine. For threshold compounds, the concentration in urine has to be measured following identification. An adverse analytical finding is reported if the result obtained exceeds the decision limit, which includes maximum combined standard uncertainty as defined by WADA.

The analysis is predominantly performed on a urine sample, although blood is collected at present to test for the use of novel erythropoiesis stimulating protein (NESP) or autologous blood transfusions. Serum samples are used to detect the prohibited use of human growth hormone (HGH) and hemoglobin based oxygen carriers (HBOC). Other specimens such as hair and saliva have been proposed (Kintz and Samyn, 2002). However, urine is still the specimen of choice since the collection is non-invasive, the volume available is quite large, the concentrations of drugs are higher than in blood, and since hydrophilic metabolites are also excreted in urine, thus enlarging the detection time window (Trout and Kazlauskas, 2004).

The doping control sample is split, sealed and labeled as A and B samples. In the laboratory testing begins with the A sample, while the B sample

is stored. Both A and B samples are stored for a minimum of 3 months up to a maximum of 8 years depending on the request of the testing authority. In long term storage the samples are kept frozen. The B sample is used to confirm the results of the A sample. The urine samples are first tested for possible adulteration or manipulation by observing color, odor, turbidity or foam and by measuring pH and specific gravity. The primary analysis of the A samples takes place in two phases: screening and confirmation analysis (Kolmonen, 2011).

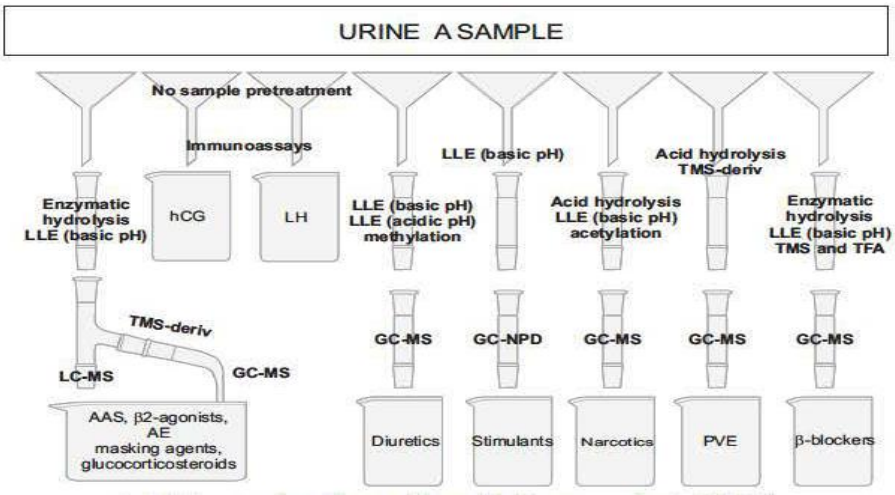
3.2. Screening

Screening analysis, known as initial testing in WADA's documentation, is used to find samples containing prohibited substances, the presence of which is then confirmed with more specific methods. The critical aspects of a good screening method include high throughput, sensitivity, selectivity, specificity, coverage and suitability for automation. In addition, the sample consumption in the screening phase should be reasonable, and the results should be simple to interpret.

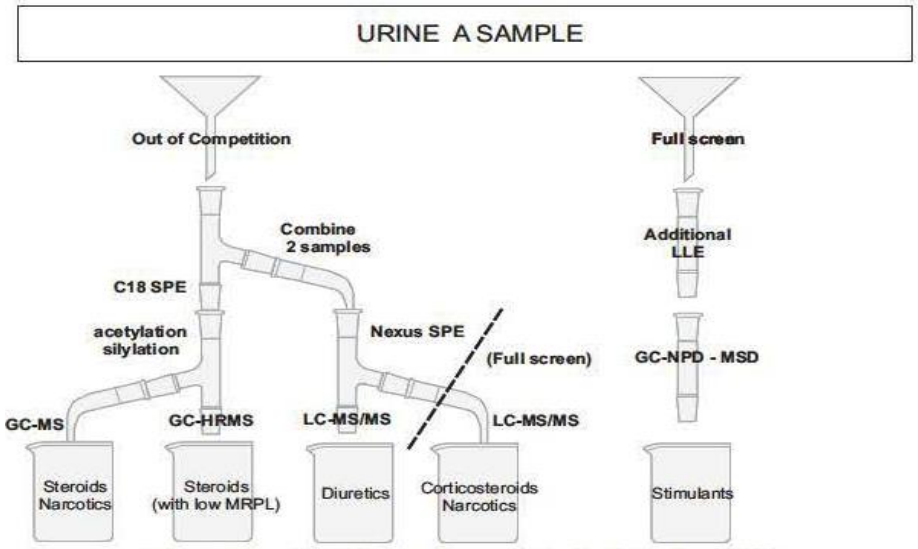
Most often screening is performed with chromatographic mass spectrometric methods. For this reason the samples are normally cleaned prior to the analysis to concentrate and to remove interfering matrix compounds. Sample preparation usually starts with an enzymatic or acidic hydrolysis of the samples to release conjugated metabolites in their free forms. The most used sample preparations techniques are liquid-liquid extraction (LLE) and solid phase extraction (SPE). LLE at alkaline pH has commonly been used for steroids (Borges *et al*., 2007) and stimulants (Trout and Kazlauskas, 2004. However, while LLE provides a robust performance, hydrophilic compounds like diuretics and metabolites have poor recoveries, and expansion of the analyte selection therefore requires an additional extraction at acidic pH or salting out, e.g. with sodium sulfate (Georgakopoulos *et al*., 2007). SPE is well suited for urine analysis, since cells and proteins are not usually present in this matrix. The use of SPE has increased because of its greater suitability for hydrophilic compounds and automation compared with LLE. An automatic SPE for steroids was presented over ten years ago. Several different types of sorbent materials are commercially available (polar, non-polar, ion exchange, mixed mode), enabling more selective extractions. However, there are several parameters affecting the recoveries that make optimization a complex process. Non-polar (C8 and C18) and ion exchange sorbent materials have been used to extract steroids and chemically heterogeneous diuretics (Goebel *et al*., 2004). Since many of the target analytes are either thermo labile or nonvolatile compounds, they have to be derivatized into a more volatile form prior to gas chromatographic (GC) analysis. Unfortunately, this step is usually laborious and time consuming and does not always suit for the target compound. Recently, LC-MS methods without sample preparation have been published for comprehensive screening of diuretics masking agents, narcotics, oxygen transfer enhancers, and stimulants (Guddat *et al*., 2011). These approaches require instruments with high sensitivity and resolution, careful evaluation of matrix effects, and more frequent instrument clean up.

Since the number and nature of target analytes in screening is huge, several different methods have to be applied. The common strategy is to screen chemically similar compounds within one method. GC based methods have been important in doping control for decades. Traditionally, GC combined with a nitrogen phosphorus detector (NPD) has been used to detect nitrogen containing stimulants and narcotics, the first prohibited compound classes. However, the complexity of the matrix and the ever increasing number of target analytes has required more specific detectors, leading to the use of MS. GC-MS based methods have been used to detect anabolic androgenic steroids (AAS) (Marcos *et al*., 2002), and stimulants (Thuyne *et al*., 2007). Electron ionization (EI) is traditionally routinely used for ionization in GC-MS methods producing characteristic spectral information on the analytes. Chemical ionization (CI) is a softer technique and results in reduced fragmentation in contrast to EI. However, it is used merely for specific issues (Choi *et al*., 1998). The use of GC is limited to small, volatile and thermostable compounds. Nevertheless, many doping agents, such as diuretics and higher molecular weight analytes such as polysaccharide based plasma volume expanders (PVE) have polar functionalities and need to be derivatized prior to GC-MS analysis. Due to these limitations of GC, LC-MS methods have become a fundamental part of sports drug testing, providing fast, robust, sensitive and specific performance to complement GC-MS and immunological methods. In addition, LC analysis can be more suitable than GC for some target analytes. For a single class screening, LC-MS has been used to analyze some AAS (Pozo *et al*., 2007), and stimulants (Thomas *et al*., 2008). Reversed phase C18 columns are the most frequently used, although hydrophilic compounds pose problems because of their poor retention. Electrospray ionization (ESI) is a soft ionization technique which is widely used in LC-MS methods in doping controls. It allows the detection of small as well as large polar molecules, although its suitability for neutral and nonpolar compounds is limited. Atmospheric pressure chemical ionization (APCI) is better suited for stable and nonpolar compounds

and is used for specific applications such as analysis of some AAS and PVE (Deventer *et al*., 2006). Because the number of prohibited substances is constantly increasing, high throughput methods are needed to rationalize and simplify the work in laboratories to make screening schemes more effective. Lately, comprehensive screening procedures have been published based on both GC-MS (van Eenoo *et al*., 2011) and LC-MS/MS (Thevis *et al*., 2011). LC-MS/MS measurements have been made using triple quadrupoles (TQs) (van Eenoo *et al*., 2011), and hybrid MS techniques such as TQ ion trap analyzers (Guddat *et al*., 2011). However, quadrupoles (Qs) and TQs are scanning instruments and can measure one m/z ratio at a time. In multi target analysis, the number of target analytes is therefore limited because of the need for an adequate number of data points across a chromatographic peak, which also affects the sensitivity of the method (Thurman and Ferrer, 2009). In these targeted multiple reaction monitoring (MRM) analyses, the number of analytes has often been between 50 and 150 (Mazzarino *et al*., 2010). Moreover, the complete collection of raw data opens up the possibility for retrospective evaluation of the analytical data and allows reprocessing and reanalysis of a doping sample for formerly unknown compounds in a fast and cost effective manner. The methods applied for doping agents in urine have been based on the use of ultra-high performance liquid chromatography (UHPLC) column designs and hybrid MS techniques such as linear TQ ion trap and QTOFMS or a single stage TOFMS. LC run times vary between 5 and 16 min, and dual polarity is employed in a few approaches (Badoud *et al*., 2010). Laboratories have the freedom to choose the techniques and methods that are fit for purpose and consequently there are several different screening schemes; these are illustrated in Scheme 1 and 2.



Scheme 1: Urine A sample screening (Leinonen *et al*., 2005)



Scheme 2: Urine A sample screening (Geobel *et al*., 2006).

The technical improvements in MS have allowed the development of more sensitive analysis methods for doping control (Thevis, 2010). The first monosector instrument employed had been achieved a scan rate of 12 s and µg/mL concentration level for stimulants. A faster scan speed was achieved with low resolution Q analyzers, which became state of the art analytical tools in combination with GC. The use of selected ion monitoring (SIM) increased sensitivity by decreasing the biological background. Higher resolution (5,000-20,000) was obtained with double focusing sector instruments with different geometries (Hemmers Bach *et al*., 2006). High resolution permitted the discrimination of background signals, since narrow mass windows could be used. Using a double focusing magnet sector analyzer it was possible to measure accurate mass and resolution over 20,000 was achieved. However, rapid exact mass analysis over a narrow GC peak was not possible due to the low scan rate. A magnet sector analyzer was used to identify metabolites of clostebol in urine and used accurate mass measuring as part of the process. Tandem MS measurements were introduced in the late 1990's, and GC and LC instruments were combined with ion trap and TQ analyzers, allowing isolation and characterization of the specific fragments of the original molecular structures (Hemmers Bach, 2008). Isotope ratio (IR) MS has been used since the mid 1990s to reveal the abuse of endogenous steroids. Lately, high resolution/high mass accuracy instruments such as TOFMS and orbitrap with resolution from 10,000 to 100,000 and mass accuracies below 5 mg/L , have been used in doping control mainly for screening (Peters *et al*., 2010). One drawback of orbitrap analyzers is their poor suitability for multi target screening due to their longer duty cycles and equilibration times (Kolmonen, 2011).

3.3. Confirmation

If the screening of an A sample results in a presumptive analytical finding, the result has to be confirmed using an additional aliquot of the A sample. The ISL states that in most cases confirmation analysis must be based on a chromatographic (GC or LC/MS) method that can also be used for screening (ISL, 2009). However, the confirmation method is often more specifically optimized for the analyte in question. The results are compared with reference material and are considered an adverse finding if the identification criteria are fulfilled (TD2010IDCR, 2010).

The identification criteria for chromatography include tolerance windows for retention time (RT) and chromatographic separation efficiency (retention factors, selectivity). If the concentrations of prohibited substances detected in urine are approximately over 100 ng/mL, their MS detection must have a full or partial scan acquired or an accurate mass measured so that elemental composition can be determined. Whenever possible a full scan is preferred, SIM can be used when low concentrations of prohibited substances need to be detected in urine. Tandem MS can be used to increase specificity in either full scan or selected reaction monitoring (SRM) mode. In general, two precursor product ion transitions should be monitored. The minimum criteria for single MS measurements are the need for three diagnostic ions with signal to noise ratios (S/N) > 3 and relative ion abundances within the given tolerance windows. For accurate mass measurements, relative mass accuracies (mg/L) should be used, and information about the analyzer employed, lock masses, mass range and resolution should be provided. Optional parameters, such as isotope pattern, can be used to decrease the number of possible compositions. For threshold substances, quantification is needed in addition to qualitative identification. The results of quantification are expressed as the mean of three replicates. If the results exceed WADA's decision limits, an adverse analytical finding is reported (ISL, 2009). For this purpose, WADA has published a technical document including threshold levels, decision limits and directions for evaluating measurement uncertainty (TD2010DL, 2010).

4. Mass spectrometry of target analytes

4.1. Anabolic androgenic steroids (AAS)

Anabolic androgenic steroids are similar in structure to the male sex hormone, testosterone. So they enhance male reproductive and secondary sex characteristics (testicle development, hair growth, thickening of the vocal cords). Athletes use anabolic steroids because they increase muscle strength by encouraging new muscle growth and allow them to train harder and longer at any given period. Examples of anabolic steroids involve testosterone, dihydrotestosterone, and rostenedione (Andro), dehydroepiandrosterone (DHEA), clostebol, nandrolne. These substances can be injected or taken as pills (Haley, 2003).

4.1.1. Some potential side effects of anabolic steroid abuse

Physiological and psychological side effects of anabolic steroid abuse have the potential to impact any user, while other side effects are gender specific. Few of the physiological harmful effects are male pattern baldness**,** liver damage**,** premature closure of the growth, centers of long bones (in adolescents) which may result in**,** stunted (short) growth and some of the Psychologicaleffects are increased aggressiveness and sexual appetite,

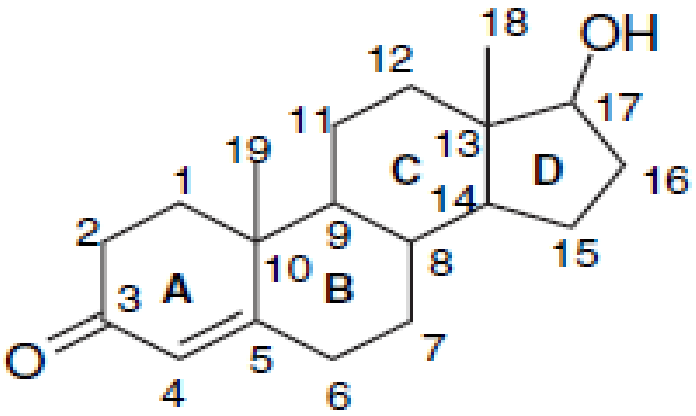
sometimes resulting in abnormal sexual and criminal behavior, often referred to as "Raid Rage", Withdrawal from anabolic steroid use can be associated with depression and suicide (USADA, 2016).

4.2. Electron ionization (EI)

Traditionally, EI is one of the most commonly employed ionization techniques in doping controls (Thevis and Schänzer, 2005). Ever since sports drug testing was introduced on a regular basis in 1967, GC-MS has been used extensively to identify banned drugs or their metabolites in athletes' urine specimens based on the significance of mass spectral results. Hence, studies on fragmentation pathways of relevant drugs have been of particular interest for doping control laboratories as they have provided the necessary data to unambiguously determine prohibited compounds in collected samples. In the following, typical dissociation pathways after EI of underivatized target analytes as well as some of their common derivatives are presented.

4.3. α,β-unsaturated 3-keto-steroids

Extensive work on the determination of dissociation pathways of derivatized and underivatized steroids upon EI was conducted. Detailed studies for the elucidation of fragmentation pathways were performed providing fundamental information on steroid dissociation behaviors, a selection of which is compiled with more recent studies using trimethylsilylation derivatization. The basis of most steroidal agents relevant for doping controls is the testosterone nucleus (Scheme 3) that has allowed numerous modifications leading to a huge variety of anabolic androgenic steroids and respective metabolites.



Scheme 3: Typical steroid relevant for doping control analysis: testosterone (mol wt = 288),

The EI mass spectrum of testosterone is depicted in Figure 1a, and characteristic ions are observed at m/z 288 ($M^{+\cdot}$), 273 (M^{+}-15), 270 (M^{+}-18), 246 ($M^{+\cdot}$-42), and 124. The major dissociation route leading to the base peak at m/z 124 was suggested to start with ionization at the carbonyl oxygen followed by homologous fission of the bond between C-9 and C-10. Deuterium labeling at C-8 proved the migration of the respective hydrogen to carbon C-10 followed by a McLafferty rearrangement and formation of the fragment ion at m/z 124 as illustrated in Scheme 4a (Shapiro and Djerassi, 1964).

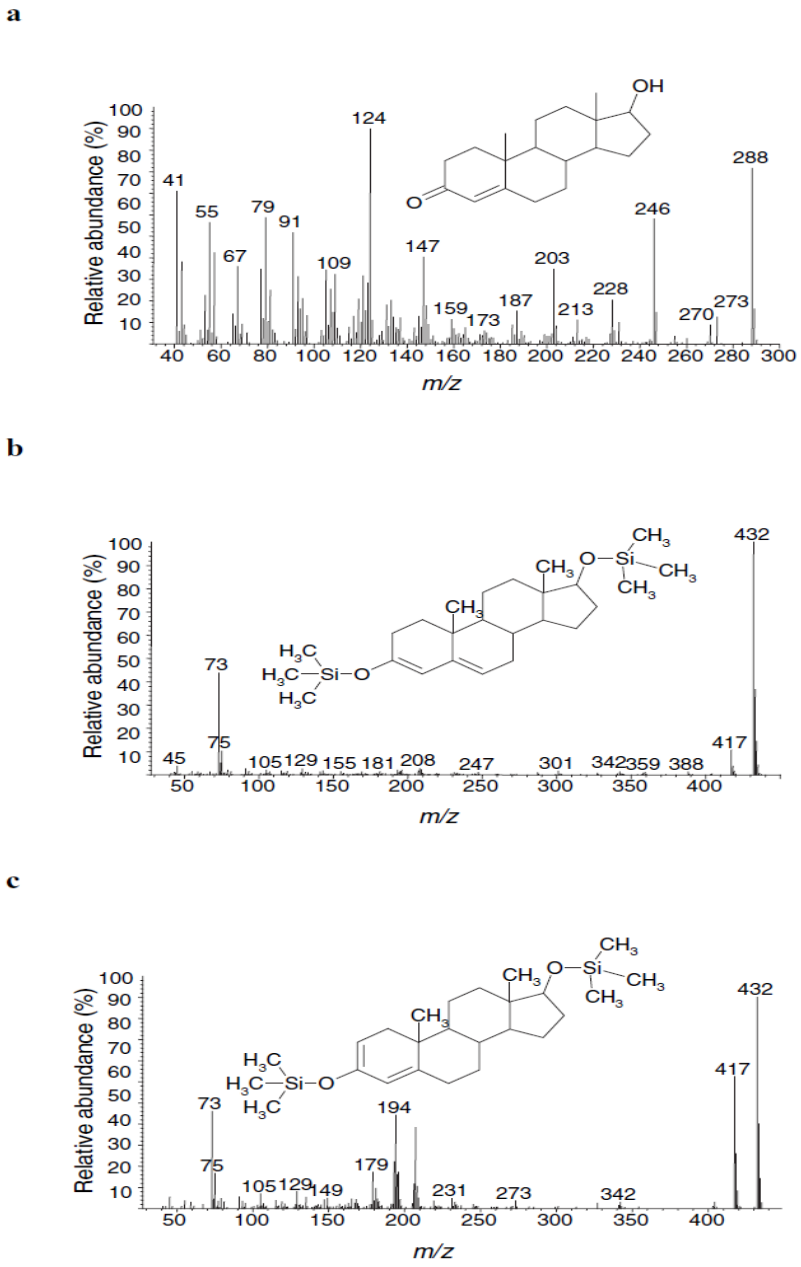
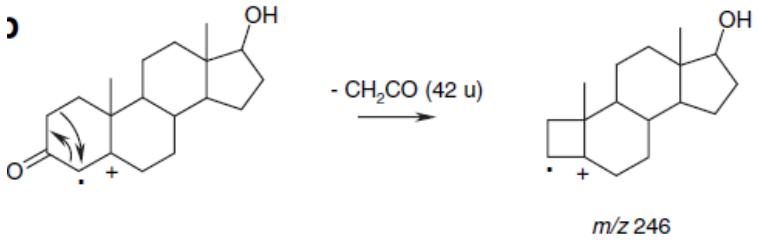


Figure 1: EI mass spectra of (a) testosterone (mol wt = 288), (b) testosterone-bis-*O*-TMS (androsta-3,5-diene-3,17β-diol-bis-*O*-TMS isomer, mol wt = 432), and (c) testosterone-bis-*O*-TMS (androsta-2,4-diene-3,17β-diol-bis-*O*-TMS isomer, mol wt = 432)



Scheme 4: Proposed dissociation pathway of underivatized testosterone after electron ionization yielding characteristic fragment ions

The fragmentation to *m/z* 246 was postulated to be initiated by the removal of a π-electron from the α,β-unsaturated system followed by the elimination of ketene (−42 *m/z*) forming a 4-

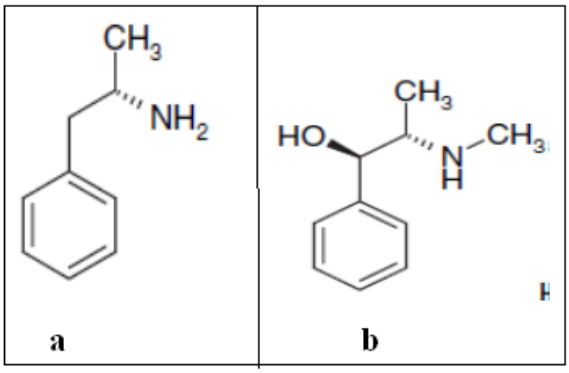
member ring structure (Scheme 4), and the common losses of a water molecule or a methyl radical give rise to m/z 270 and 273, respectively. While the eliminations of 15, 18, or 42 have also been observed with other α,β-unsaturated nuclei (e.g., 3-keto-1-ene steroids), the fragment ion at m/z 124 has demonstrated considerable specificity for a testosterone related steroid structure (Horning, 1968). The location of a double bond between C-1 and C-2 instead of C-4 and C-5 causes the formation of a fragment ion at m/z 122 (Table 1, 6), the formation of which is in accordance to the mechanism described for 3-keto-4-ene structures but includes the migration of the C-5-positioned hydrogen. These mass spectrometric peculiarities of ring isomers of testosterone provide one important tool for the determination of double bond locations in steroids related to testosterone (Budzikiewicz *et al.*, 1964).

Table 1: Characteristic fragment ions of selected underivatized steroids using electron ionization

Steroid nucleus	Representative compound	No	Mol. wt (Da)	Fragment ions (m/z) M^+ M^+-15 M^+-18						
3-Keto	5α-Androstane-3,17-dione	**1**	288	288	273	270	255	244	224	217
	5α-Dihydrotestosterone	**2**	290	290	275	272	231	199	163	123
3-Keto-4-ene	Testosterone	**3**	288	288	273	270	246	203	124	109
	Methyltestosterone	**4**	302	302	287	284	269	245	229	124
	Nandrolone	**5**	274	274	-	256	231	215	160	110
3-Keto-1-ene	1-Testosterone	**6**	288	288	273	270	246	204	122	109
3-Keto-1,4-diene	1-Dehydrotestosterone	**7**	286	286	-	-	253	227	147	122
	Metandienone	**8**	300	300	-	282	267	242	161	122
3-Keto-4,6-diene	6-Dehydrotestosterone	**9**	286	286	271	268	253	227	151	136
3-Keto-4,9-diene	17α-Methyl-androsta-4,9(11)-Diene-17β-ol-3-one	**10**	300	300	285	282	267	242	227	215
17-Keto	Androsterone	**11**	290	290	275	272	257	246	139	215
	Etiocholanolone	**12**	290	290	-	272	257	246	244	215

4.4. Stimulants

The class of stimulants consists of a heterogeneous group of compounds, the majority of which is structurally related to amphetamine (Scheme 5). Amines, in general, have been one of the early subjects of mass spectrometric investigations (McLafferty and Turecek, 1993), and due to their relevance for forensic and toxicological investigations, numerous studies have been performed regarding their determination in biological matrices.



Scheme 5: Typical representatives of compounds categorized as stimulants and prohibited in sports: amphetamine (a, mol wt = 135) and ephedrine (b, mol wt = 165)

Amines are likely to be ionized by EI at the nitrogen atom, which induces a typical α-cleavage (also referred to as β-bond cleavage) yielding dominant fragment ions upon electron bombardment (Gohlke and McLafferty, 1962). In Figure 2, the EI mass spectra of amphetamine (a) and ephedrine (b) are depicted, both of which contain base peaks resulting from respective eliminations due to fissions of carbon-carbon bonds adjacent to the nitrogen (i.e., m/z 44 and 58, respectively). As a rule of thumb, the largest possible group is lost preferentially, and because of the

comprehensiveness of the class of stimulants several studies were undertaken to elucidate the principle fragmentation pathway of phenyl alkyl amines (Valentine and Middleton, 2000). A postulated dissociation route is shown in Scheme 6 for ephedrine, the general aspects of which shall provide information on unknown or new "designer drugs" or their metabolites comprising a phenyl alkyl amine structure that may be detected in specimens analyzed for doping control or forensic purposes. The molecular ion ($M^{\cdot+}$) is not visible in the mass spectrum of ephedrine generated by electron ionization, but its intermediate existence has been postulated allowing the elimination of a hydrogen atom to m/z 164 as described by (Barry and Pet zinger, 1977). The subsequent loss of water (−18 m/z) is responsible for the formation of m/z 146 (Scheme 6). Here, a migration of the proton located at the nitrogen atom is suggested as substantiated by H/D exchange experiments. H/D exchange conditions allowed the substitution of two mobile hydrogens by deuterium atoms located at the benzylic hydroxyl function and the secondary amine, and corresponding mass spectra demonstrated the lack of both deuterium atoms in the fragment ion at m/z 146. The fragment ions at m/z 117 and 115 have been proposed to result from further dissociation of m/z 146 eliminating HCN (−27 m/z) and one or two hydrogen molecules, respectively (Scheme 6), and accurate mass measurements provided evidence for the loss of nitrogen. A complementary fragmentation route was suggested to yield the fragment ion at m/z 132 via consecutive losses of a methyl group (−15 m/z) and water (−18 m/z), and suggested structures for m/z 107, 105, 91, and 77 as hydroxyl benzyl, benzoyl, tropylium, and phenyl cations, respectively (Scheme 6), were substantiated by stable isotope labeling of the phenyl residue (Baba and Kawai, 1974). As indicated above, the base peak of the spectrum at m/z 58 results from characteristic α-cleavage yielding the ethylidene methyl ammonium ion (Scheme 6). The modification of particular sites of amphetamine or ephedrine causes characteristic mass shifts of diagnostic fragment ions, hence providing important information on possible alterations of known and prohibited compounds and enabling their detection and identification (Table 2). The addition of an ethyl function to amphetamine in addition to a trifluoromethyl substitution of the phenyl residue yields fenfluoramine (Scheme 6), which generates a base peak at m/z 72 and a minor fragment at m/z 159 that correspond to m/z 44 and 91, respectively (Brownsill *et al.*, 1991).

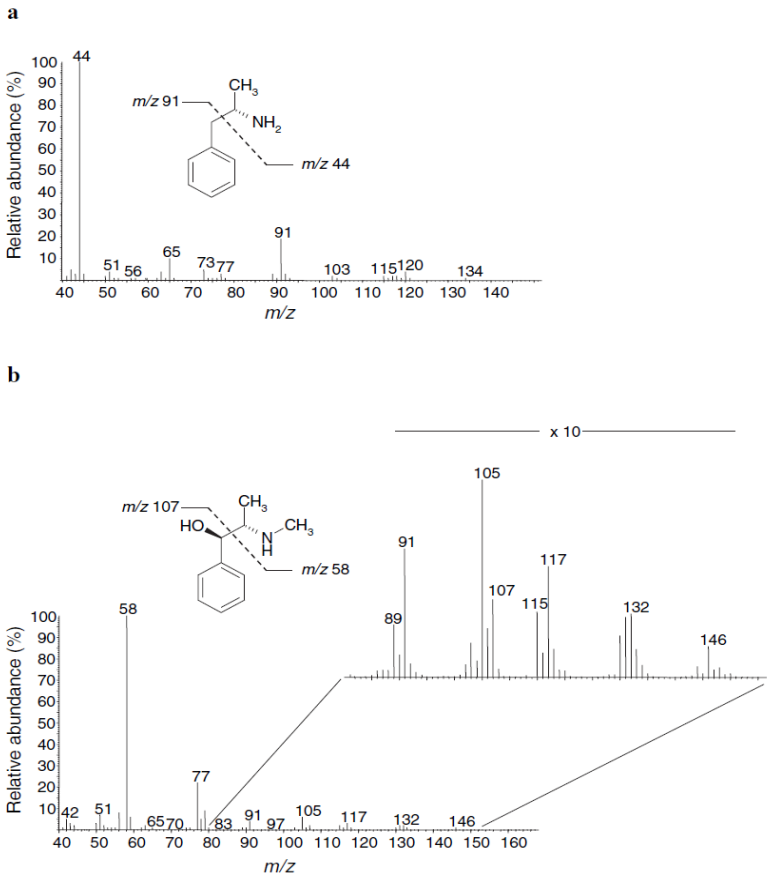
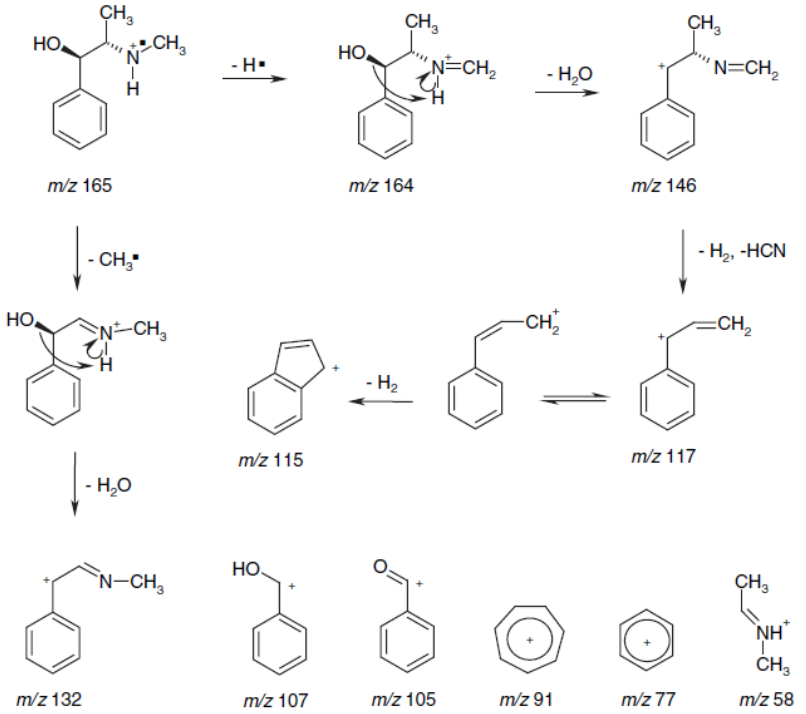


Figure 3: EI mass spectra of (a) amphetamine (mol wt = 135), and (b) ephedrine (mol wt = 165) with an inset showing the tenfold enlarged region m/z 80–150



Scheme 6: Proposed fragmentation pathway of ephedrine after electron ionization yielding characteristic fragment ions.

Table 2: Characteristic fragment ions of selected stimulants with and without derivatization using electron ionization

Compound	Underivatized compound				N-TFA,O-TMS or mixed N-TFA/O-TMS derivative			
	Mol. wt (Da)	Fragment ions (*m/z*)			Mol. wt (Da)	Fragment ions (*m/z*)		
Amphenepramone	205	190	100	105	-	-	-	-
Amphetamine	135	91	65	44	231	140	118	91
Benzphetamine	239	224	148	91	-	-	-	-
Cathine	151	107	105	44	319	179	163	140
Chlorphentermine	183	125	107	58	279	166	154	114
Dimethylamphetamine	163	91	72	65	-	-	-	-
Ephedrine	165	105	77	58	333	179	154	110
Ethylamphetamine	163	148	91	72	259	168	140	91
Etilefrine	181	121	77	58	421	406	267	179
Fencamphamine	215	186	115	98	311	242	170	142
Fenfluramine	231	216	159	72	327	308	168	140
Fenproporex	188	97	92	57	284	193	140	118
Furfenorex	229	138	91	81	-	-	-	-
Hydroxyephedrine	181	107	71	58	421	267	193	154
MDA	179	136	77	44	275	162	140	135
MDMA	193	193	135	58	289	162	154	135
Methylamphetamine	149	134	91	58	245	154	118	110
Methylephedrine	179	105	77	72	251	236	149	72
Methylphenidate	233	91	84	56	329	180	126	67
Phendimetrazine	191	176	85	57	-	-	-	-
Phenmetrazine	177	177	77	71	273	167	98	70
Phentermine	149	134	91	58	245	230	154	91

Owing to the moderately low yield of abundant and diagnostic fragment ions of underivatized stimulants related to amphetamine or ephedrine, their limited gas chromatographic properties, and the need to meet identification requirements based on mass spectrometric results, various derivatization strategies have been developed during the last decades. Acylating properties of bisacyl amides, preferably fluorinated acyl derivatives have been extensively used to modify amphetamine and related drugs for chromatographic and mass spectrometric analyses. In particular trifluoroacetylated (TFA) and heptafluorobutyrated (HFB) compounds have commonly been prepared andfor improved separation of enantiomers, hydroxylated amphetamine or ephedrine like substances, also mixed derivatives obtained from the reaction with α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (Mosher's acid) and *O*-trimethylsilylation (TMS) and/or *N*-acylation have been employed (Donike, 1996). The mass spectrometric behavior of the derivatizedanalytes differs considerably from underivatized compounds (Table 3), although the principle dissociation pathway via α-cleavage remains in most spectra. In Figure 3, the EI mass spectra of amphetamine-*N*-TFA (a) and ephedrine-*N*-TFA-*O*-TMS (b) are illustrated demonstrating the influence of chemically modified functional groups on the general fragmentation route. Ionization of amphetamine-*N*-TFA at the nitrogen triggers the loss of the benzyl radical (−91 *m/z*) yielding the base peak at *m/z* 140, which corresponds to *m/z* 44 in Figure 3a. The tropylium ion at *m/z* 91 is present in both spectra of amphetamine with and without derivatization of the amine function, and in contrast to Figure 3a, the *N*-TFA derivative of amphetamine (Figure 3a) gives rise to a fragment ion at *m/z* 118 that represents the propyl benzene cation after elimination of 2,2,2-trifluoroacetamide (−113 *m/z*).

The modification of ephedrine to its mixed *N*-TFA-*O*-TMS derivative yields more significant changes in the fragmentation behavior as the base peak in Figure 3b is not generated by an α-cleavage initiated by an ionization of the nitrogen atom. The fragment ion at *m/z* 179 is suggested to result from ionization and subsequent α-cleavage starting at the ether oxygen. Nevertheless, an ion at *m/z* 154 is found that corresponds to *m/z* 58 in Figure 3b representing the classical nitrogen induced α-cleavage of underivatized ephedrine analogs.

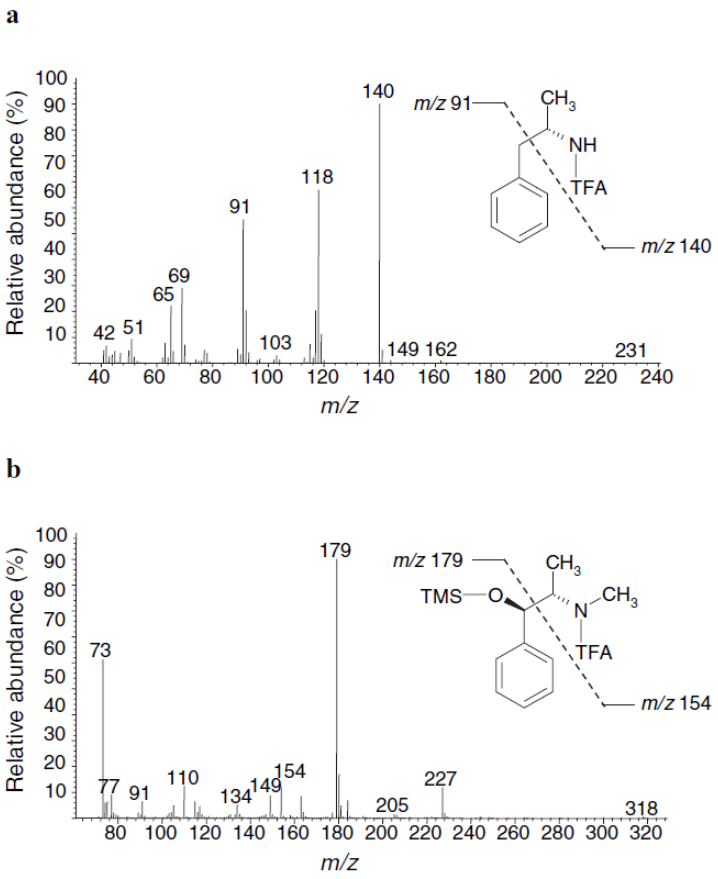


Figure 3: EI mass spectra of (a) amphetamine-*N*-TFA (mol wt = 231), and (b) ephedrine-*N*-TFA-*O*-TMS (mol wt = 333)

Accordingly, fragmentation routes have been described for HFB derivatives (Ariniemi, 2005). Due to the heptafluorobutyration of amine functions, the above described common base peaks, for example, at m/z 44 or 58 resulting from α-cleavages of amphetamine or metamphetamine are shifted by 196 m/z to m/z 240 or 254, respectively, while principal dissociation routes correspond to those described for TFA derivatives.

Some of the analytical results in the blood (B) and dried blood spot (DBS) samples tested by different methods and different authors are given in Table 3.

Table 3: Some analytical results tested by different detection methods in blood and dried blood spot samples

No	Compound	Method	Sample	LOD (ng/mL)	LOQ (ng/mL)	Linear range (ng/mL)	Reference
1	Amphetamine	GC-MS	B	11	22	14-2700	Moeller *et al.*, 1998
2	Clenbuterol	GC-MS	DBS	0.05	0.25	0-20	Thomas *et al.*, 2012
3	Coca ethylene	GC-NPD	B	20	50	50-10,000	Moeller *et al.*, 1998
4	Tetra hydrocannabinol	GC-MS	DBS	0.25	1	0-20	Thomas *et al.*, 2012
5	Cocaine	GC-MS	B	1	5	1-100	Moeller *et al.*, 1998
6	Salbutamol	GC-MS	DBS	0.5	2	0-20	Thomas *et al.*, 2012
7	Methamphetamine	GC-MS	B	13	34	15-3000	Moeller *et al.*, 1998
8	Cocaine	LC-UV	B	24	70	0-2000	Moeller *et al.*, 1998

5. CONCLUSION

In sport drug testing, mass spectrometry with electron ionization in combination with chromatographic techniques has become an indispensable tool for unambiguous determinations of performance enhancing drugs. Robustness but also sensitivity and selectivity of mass spectrometric analyses employed in comprehensive screening and specific confirmation procedures has been the basis of doping control analysis. Characteristic fragmentation pathways of drugs provide identification items of utmost importance and significance, which have been studied and elucidated for numerous classes of drugs in the past. The concerted use of MS with chromatography has been the method of choice for screening procedures covering more than 200 target analytes plus new and unknown derivatives or designer drugs. A considerable bias to transfer traditional GC-MS based approaches to LC-MS/MS has been observed owing to significantly reduced sample preparation times and simplicity of detection strategies. However, LC-MS/MS requires a sufficient proton affinity of target analytes, which is not provided by some important therapeutics and their metabolic products, in particular several AAS. Hence, the complementary use of GC-MS and LC-MS/MS will be necessary to allow the comprehensive determination of drugs possibly misused in amateur and professional sport.

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