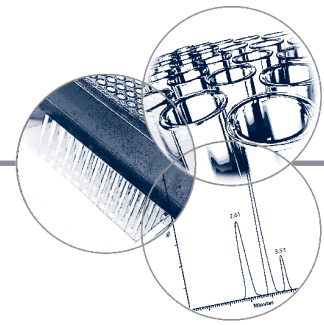
Review



Current status and bioanalytical challenges in the detection of unknown anabolic androgenic steroids in doping control analysis

Androgenic anabolic steroids (AAS) are prohibited in sports due to their anabolic effects. Doping control laboratories usually face the screening of AAS misuse by target methods based on MS detection. Although these methods allow for the sensitive and specific detection of targeted compounds and metabolites, the rest remain undetectable. This fact opens a door for cheaters, since different AAS can be synthesized in order to evade doping control tests. This situation was evidenced in 2003 with the discovery of the designer steroid tetrahydrogestrinone. One decade after this discovery, the detection of unknown AAS still remains one of the main analytical challenges in the doping control field. In this manuscript, the current situation in the detection of unknown AAS is reviewed. Although important steps have been made in order to minimize this analytical problem and different analytical strategies have been proposed, there are still some drawbacks related to each approach.

Background

■ Androgenic anabolic steroids & sport doping

Androgenic anabolic steroids (AAS) cover a wide group of substances that can be considered as synthetic derivatives of the male hormone testosterone (Figure 1). From a structural point of view, AAS are based on the cyclopentanoperhydrophenantrene four-ring structure that is three six-member rings (A, B and C) and one five-member ring (Figure 1). Similarly to testosterone, several AAS have 19 carbon atoms and two oxygen atoms located in C3 and C17, although common synthetic alterations imply the modification of the number and the location of these atoms (Figure 1).

From a biological point of view, AAS regulate a wide variety of developmental and physiological processes. Based on its denomination, AAS biological activities can be divided into anabolic and androgenic effects [1–3]. However, the detailed description of these processes is out of the scope of this review. Briefly, androgenic effects of anabolic steroids can be considered as those associated with masculinization. Hence, in male fetuses, androgens stimulate the development of the male external genitalia, and during puberty they stimulate growth of the testes. Additionally, they are also responsible for secondary sexual characteristics such as the enlargement of the larynx (cause of a deepening of the voice), the growth of terminal hair (in the pubic, axillary and facial regions), an increase in sebaceous gland activity (leading to acne) and CNS effects (libido and increased aggression) [3]. Anabolic effects can be associated with protein building in skeletal muscle and bone, resulting in an increase in body dimensions, lean body mass and muscle mass and strength. A detailed review about the effects of anabolic steroids in athletes was performed by Hartgens and Kuipers [4].

AAS are prohibited in sports because of their anabolic action. Anabolic steroids were not included in the first list of prohibited substances published by the international Olympic committee in 1967. Indeed, only in 1976 at the Montreal Olympic Games were they prohibited for the first time [5], and they have remained prohibited ever since. Nowadays, they are included as part of the anabolic agents group in the list of prohibited substances annually published by the world antidoping agency (WADA) [101]. Anabolic agents are the most frequently detected group of substances by accredited laboratories [102], demonstrating the importance of AAS in the doping control field.

Traditionally, the misuse of AAS is screened by the target detection of the parent drug and/or its metabolites in urine. MS detection plays an essential role in this, since it provides the required sensitivity and specificity. Consequently, MS instruments have been extensively used in doping control analysis for decades [6–11].

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Key Terms

Designer steroids: Steroids synthesized with the main purpose of evading doping controls. The discovery of tetrahydrogestrinone in 2003 revealed the existence of a black market producing such steroids.

Prohormones: Steroids that are biological precursors of hormones, for example, testosterone, normally commercialized as nutritional supplements.

Nutritional supplements: Preparations with the goal of being a complement of the diet. They normally contain allowed substances such as amino acids, minerals or vitamins although their contamination with steroids (either previously marketed or unmarketed) was found to be widespread in several investigations.

Precursor ion scan methods: Analytical methods based on the detection of all compounds sharing a common product ion. Using these approaches, similar compounds with a common structure are detected irrespective on their molecularweight.

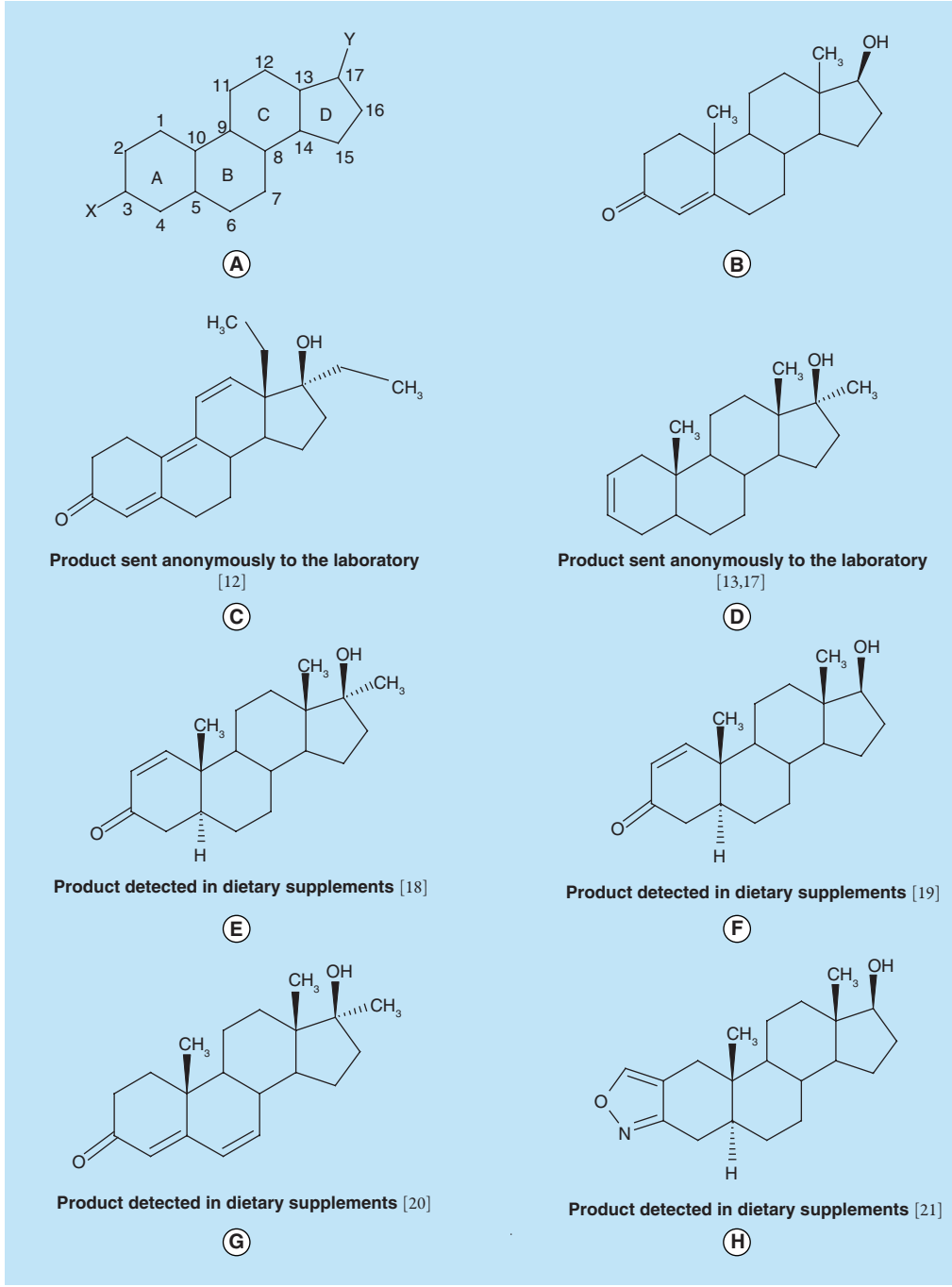

Figure 1. Androgenic anabolic steroids. (A) General structure for androgenic anabolic steroids indicating the nomenclature for carbon atoms and rings and structures of **(B)** testosterone and the designer steroids; **(C)** tetrahydrogestri-none [12]; **(D)** desoxymethyltestosterone [13,17]; **(E)** 1-methyltestosterone [18]; **(F)** 1-androsterone [19]; **(G)** 6-methyltestosterone [20]; and **(H)** isoxazole [21]. The information about how these steroids were detected is included. For more examples of designer steroids, see [14].

Common strategies for the sensitive detection of AAS normally imply the use of selected ion monitoring or SRM methods in single or triple quadrupole instruments, respectively, or the extracted ion chromatogram for specific product ions in ion trap analyzers. These methods are intrinsically designed to detect preselected compounds/metabolites and to ignore others. Therefore, they open a window for cheaters in sport competitions.

■ Anabolic steroids, designer steroids, prohormones & nutritional supplements

The ideal AAS should have the anabolic effect of testosterone (or higher) without the androgenic effects, which are unwanted, especially for women and children [12,13]. Much effort has been made in order to separate the androgenic from the anabolic effects. The most common strategy for this separation is to perform structural modifications of testosterone in order to enhance its anabolic effects and to minimize as much as possible its androgenic effect. Hence, the search for AAS with maximal anabolic effect has led to a wide variety of synthetic AAS. These modifications were reviewed by Kicman [3].

Therefore, doping control laboratories have to cope with the detection of a large number of AAS only differing in a small part of their structure. The prohibited list published in 2013 specifically mentioned 46 exogenous AAS with small structural differences [101]. However, in the prohibited list it is stated that *"other substances with a similar chemical structure or similar biological effect(s)"* are also considered as doping agents. Additionally, AAS are extensively metabolized and the urinary detection of these metabolites is normally used for the screening of AAS misuse. Therefore, doping control laboratories are faced with the detection of numerous anabolic steroids.

Modifications made in the AAS structure also change the MW of the substance, its metabolism and its MS behavior. Therefore, AAS structures can also be modified to deliberately evade either doping control or legal coverage [14]. A so-called 'designer anabolic steroid' is created. This fact was evidenced with the discovery of tetrahydrogestrinone (THG) during the Bay Area laboratory co-operative scandal in 2003 [8]. Since then, other **designer steroids** such as madol have been discovered [15,16]. All of them have a common factor: they were detected after confession of athletes who finally provided a portion of the administered drug for identification purposes. The discovery of designer steroids confirmed that classical methods are lacking for the detection of some AAS [17] and therefore analytical strategies should be reconsidered.

An additional cause for concern is the production of **prohormones**, which have been legally available on the sports nutrition market since 1996 in the USA. In 2004, the Anabolic Steroid Act was created in order to minimize this problem [103]. However, this list has several drawbacks as some important steroids (e.g., dehydroepiandrosterone) are not present. Additionally, since it is a nominative list, steroids with structure modifications (such as those described in the section titled 'Androgenic anabolic steroids & sport doping') are legally marketed because they are not included as such. In fact, several never-marketed steroids have been found in the **nutritional supplements** market [16, 18–31]. These substances are advertised as being able to increase muscle growth and strength [32]. Due to the loopholes in the regulation for these compounds, they do not pass all the controls required for drugs. In addition to the common health effects of AAS [3,4], the intake of these modified AAS can produce unpredictable health effects.

Hence, doping control laboratories also have to cope with the detection of previously nonmarketed AAS coming from designer steroids, prohormones and/or nutritional supplements. The urinary detection of such unknown steroids is currently among the main bioanalytical challenges in the doping control field. This review deals with the description and discussion of the important analytical advances performed in this direction as well as their limitations. The review focuses on the bioanalytical approaches proposed for this purpose and their analytical advantages and limitations for the detection of unknown AAS. Other important aspects, such as cost of the analysis and reporting time, are not discussed in depth.

Current analytical approaches for the detection of unknown AAS

■ Precursor ion/neutral loss scan methods using MS/MS

AAS exhibit a common MS behavior depending on their structure. Several papers and reviews have been published related to this behavior both after electron ionization (EI) using GC coupled to MS analysis and after ESI followed by collision-induced dissociation in LC–MS or MS/MS analysis [10,11, 33–41]. In summary, common fragments/product ions and neutral losses are obtained for those AAS sharing a particular structure. Therefore, using the appropriate MS instrument, it is possible to develop neutral loss and **precursor ion scan methods** for the open detection of AAS with a specific structure.

Several LC–MS/MS methods have been published using this approach, in order to detect AAS in a untargeted way, including AAS containing 4-ene-3-keto structures [42], 1-ene-3-keto structures [42], 13-methyl-4,9,11-trien-3-keto

Key Term

Neutral loss scan methods: Analytical methods based on the detection of all compounds sharing a common neutral loss. Similarly to precursor ion scan methods, compounds losing a common part of their structure are detected irrespective of their molecular weight.

structures [42], 13-ethyl-4,9,11-trien-3-keto structures [42], imidazol and hydroxyimidazol rings [43] and a fluorine atom (Table 1) [44]. Although these methods allow for the open detection of AAS, they are only able to detect those AAS sharing the preselected structure. These preselected structures are usually coming from AAS exhibiting a suitable ionization. Since some AAS metabolites, for example, fully reduced androsandiols metabolites, lack the atmospheric pressure interface (API) ionization, it is difficult to develop precursor ion/**neutral loss scan methods** for these metabolites. Therefore, the usefulness of these methods for the open detection of unknown AAS is limited.

A more general alternative is based upon the common MS behavior observed for most AAS at high collision energy in LC–MS/MS experiments. Under these circumstances, the AAS skeleton is fragmented to generate three common ions at *m/z* 77, 91 and 105, corresponding to phenyl, tropylium and methyltropylium ions. Although each ion individually has low specificty, the occurrence together at a certain retention time allows to preselect suspicious peaks with a potential steroid-like structure to be pre-selected. This was illustrated by an experiment where most of the protonable AAS could be detected using the precursor ion scan method based on the simultaneous acquisition of these ions [39]. An additional gain in specificity is related to the fact that the first quadrupole is only scanned starting from *m/z* 250. This avoids the detection of substances with *m/z* <250 Da, that are also capable of forming the three ions but that do not have a steroid structure.

Although the sensitivity of triple quadrupole instruments is limited using neutral loss/precursor ion scan, the use of this approach allowed for obtaining LODs in the range of few tens of ng/ml (Table 1). In a technical document published in 2013 [104], WADA established minimum required performance levels in the low ng/ml range for the target detection of AAS. However, it is expected that athletes would take less care in the intake of presumed undetectable AAS, such as designer steroids. Therefore LODs in the range of tens of ng/ml should theoretically be enough for the detection of unknown AAS. Precusor ion and neutral loss scanning have also been successfully applied in metabolic studies being able to detect some minor metabolites. This confirms that the sensitivity is suitable for the urinary detection of unknown AAS.

The suitability of these methods for the open detection of AAS with unknown structure allowed for the discovery of several (minor) previously unreported metabolites [43–48]. Currently, the suitability of a precursor ion scan method [39] for the routine screening of unknown AAS is being tested in our laboratories. More than 1000 routine samples, mainly coming from sports in which the misuse of AAS may greatly improve performance (weight lifting, body-building and power lifting) have been analyzed using this approach. The method was able to detect suspicious peaks in those samples declared as adverse analytical findings by other common GC–MS/LC–MS screening approaches. Additionally, some suspicious peaks were also detected. However, this application also highlighted several of the main analytical challenges that still remain in the detection of unknown AAS.

■ Full scan acquisition in high-resolution instruments

An additional alternative for the untargeted detection of AAS is the application of full acquisition methods. This can be achieved with robust high-resolution (HR) instruments (TOF and Orbitrap analyzers) due to their accurate

Table 1. Published methods based on precursor ion scan/neutral loss for the open detection of androgenic anabolic steroids.

Extraction method	Preconcentration factor	Matrix	Chromatographic separation (time)	MS method	Untargeted suitability tested	Ref.
LLE	×50	Urine	LC (10 min)	Precursor ion scan of *m/z* 109,187, 199, 227 and 241	Spiked urine	[40]
LLE	×25	Urine	LC (34 min)	Precursor ion scan of *m/z* 81, 97 and 145	Metabolic studies	[41]
LLE	×25	Urine	LC (34 min)	Neutral loss of 20 Da	Metabolic studies	[42]
LLE	×25	Urine	LC (34 min)	Precursor ion scan of *m/z* 77, 91 and 105	Spiked urine, prohormones and metabolic studies	[37, 43–46]

mass capabilities and high sensitivities obtained in scan mode [49]. Hence, the development of full acquisition methods based on HR instruments is a promising alternative, not only for the open detection of AAS but also for the comprehensive target screening in doping control analysis (TABLE 2) [50–66]. Their *modus operandi* is the acquisition of the full-scan raw data and, therefore, the detection of all compounds ionized by the source. Afterwards, extracted ion chromatograms with narrow mass window (<10 ppm) are evaluated for the desired doping agents. The fact that all the ionized compounds are detected opens the possibility to retrospective analysis once a new doping agent or metabolite is revealed.

Several different methods based on full-scan acquisition in HR instruments have been published (TABLE 2). The sensitivity obtained by full scan acquisition in HR instruments is at least similar to those obtained in precursor ion and/or neutral loss scan, that is, LODs of tens of ng/ml are reached. Using Orbitrap instruments, this sensitivity can be increased and LODs around sub ng/ml levels have been reported (TABLE 2). Therefore, they are suitable for the open detection of unknown AAS.

The benefits of HR instruments for the retrospective detection of new doping agents have been theoretically discussed in several of the published methods (TABLE 2). In many cases, this theoretical benefit has been tested by analyzing real samples (TABLE 2). Although most of these cases are not dealing with AAS, the suitability of full acquisition methods in HR instruments for retrospective analysis of AAS can be extrapolated from this behavior. At the moment, the main application of these methods in doping control analysis is focused on the target detection of known doping agents. However, their usefulness for the open detection of AAS has been successfully applied in illegal cocktails [65] and nutritional supplements [66]. Additionally, full acquisition methods using HR instruments have also been successfully applied for metabolic studies [67,68] and for the evaluation of the composition of prohormones/nutritional supplements [69].

■ Bioassays based on androgen-receptor activities

MS methods for the detection of unknown AAS are based on their structural characteristics (common product ions or neutral losses for AAS or accurate mass measurements of the ions generated). One of the main challenges for this detection is that every AAS has a different structure, and it is impossible to predict all the structures potentially used in the design of new AAS. However, despite their different molecular structure, all the AAS share the androgenic/anabolic activities (see section titled 'Androgenic anabolic steroids & sport doping'). This common androgenic activity can provide a new basis for the open detection of AAS.

AAS mostly exert their effects by binding and activating a specific receptor, the androgen receptor (AR), which is expressed in most cells. Several bioassays based on the expression of AR (mainly mammalian cell-based bioassays and yeast cell-based bioassays) in the presence of AAS have been developed and recently reviewed [70]. The sensitivity of bioassays based on AR activities mainly depends on the androgenic activity of the AAS. Hence, the response for testosterone is around five-times lower than that obtained for dihydrotestosterone [71]. The use of these bioassays commonly allows for the detection of AAS at concentrations similar to precursor ion/neutral loss scan methods (tens of ng/ml), which is enough for the detection of unknowns AAS. In some cases an improvement in retrospectivity has been reported when using assays based on AR activities [72].

A suitable alternative for routine purposes is the measurement of androgenic activity of fractions collected after LC separation, using the so-called biogram [73]. By this approach, every fraction is collected and its activity measured. Endogenous AAS are collected in known fractions and almost no activity is exhibited in the rest of the fractions. Therefore, unknown AAS that are not eluting in the same fraction as endogenous AAS will be easily detected. Despite the potential usefulness of this alternative, there are critical drawbacks for its application in routine analysis. The most important ones are the low throughput and the high global cost of this approach. For this reason, the use of bioassays with AR activities for doping control purposes has been limited to the study of the content of prohormones/nutritional supplements [69,74,75].

The suitability of this approach for the detection of unknown AAS has been demonstrated by their application in the analysis for contaminants of nutritional supplements. The detection of AR activities of nutritional supplements has been used as a preliminary step to select supplements that potentially contain AAS [69,74,75].

Table 2. Published methods based on full scan with high-resolution instruments for the open detection of doping agents.

Extraction method	Preconcentration factor	Matrix	Chromatographic separation	MS method	Target doping agents (n)	Targeted AAS (n)	LOD for targeted AAS (ng/ml)	Untargeted suitability tested	Ref.
LLE	×50	Urine	LC + GC	TOF	138	37	<2–40	Metabolic study	[48]
SPE	×7	Urine	LC	TOF	124	17	ND	Sample after methylphenidate administration	[49]
SPE	×15	Urine	LC	TOF	56	5	LOQ 2–15	Theoretically application	[50]
Dilute and shoot	/0.5	Urine	LC	TOF	103	ND	ND	Positive samples	[51]
LLE	×40	Urine	GC × GC	TOF	5	5	ND	Theoretically discussed	[52]
LLE	×25	Urine	LC	TOF and QTOF	10	10	1–20	Positive samples	[53]
LLE	×50	Urine	LC	TOF	ND	ND	ND	Spiked urine	[54]
SPE	×7	Urine	LC	TOF	207	17	ND	ND	[55]
LLE	×50	Urine	LC	TOF	22	4	ND	ND	[56]
LLE	×6	Urine	GC	TOF	7	7	ND	Blank urine samples	[57]
SPE	×100	Urine	GC	TOF	8	8	ND	ND	[58]
LLE	×30	Urine	LC	Orbitrap	29	23	0.05–00.1	Positive samples	[59]
Dilute and shoot	/10	Urine	LC	Orbitrap	122	ND	ND	ND	[60]
SPE	×10	Urine	LC	Orbitrap	182	11	0.5–5	ND	[61]
Protein precipitation	ND	Blood	LC	Orbitrap	32	4	ND	ND	[62]
Dilute and shoot	/100	Illegal cocktail	LC	TOF	Desconeguts	Desconeguts	ND	Illegal cocktails	[63]
SPE	×5	Blank matrix without steroids	GC × GC	TOF	25	25	ND	Nutritional supplements	[64]

AAS: Androgenic anabolic steroids.

Analytical challenges for the detection of unknown AAS

Sample treatment

Sample treatment is one of the first steps of the analytical method, and together with sample collection one of the most critical. Its obvious importance lies in the fact that it is responsible for preconcentration of analytes, removal of interferents and, if required, release of Phase II metabolites. Due to the low concentration level at which AAS can be present in urine (their concentration decreases to the low ng/ml range quickly after administration) and their high hydrophobicity, a liquid–liquid extraction (LLE) with organic solvents or a SPE with hydrophobic cartridges are usually performed as extraction method for the target detection of AAS reaching preconcentration factors usually between ten- and 50-fold. Extraction strategies based on LLE and SPE are also common in the detection of unknown AAS (Tables 1 & 2).

The most obvious characteristic of LLE with nonpolar solvents is that polar substances are not co-extracted. However, LLE can fail in the extraction of polar substances.

In order to avoid undesirable losses for any doping agent present in urine, the most logical sample treatment is the **dilute and shoot** approach. Using this strategy, every analyte present in the urine and exhibiting sufficient ionization behavior can be detected. Additionally, sample treatment is fast and cheap making it ideal for doping control analysis where a large amount of samples have to be analyzed with short reporting times during major events, including championships or Olympic Games. However, dilute and shoot approaches have strong limitations for the detection of unknown AAS. The most important one is the sensitivity of the method. As stated above, a preconcentration factor between ten- and 50-fold is normally required to reach the low ng/ml range in the target detection of AAS. In dilute and shoot methods applied to urine, a dilution factor between two- and ten-fold is applied (Table 2). That, together with the absence of a clean-up step, would imply around 100-times less sensitivity and, therefore, it can be extrapolated that LODs for most of the AAS are between hundreds of ng/ml and few µg/ml. Additionally, the poor ionization of some AAS metabolites makes this loss of sensitivity even more critical for fully reduced metabolites. It is probably for this reason that no target AAS were included in the hundreds of doping agents tested in the validation of these urinary methods (Table 2).

An additional potential limitation of dilute and shoot methods in the detection of unknown AAS is the absence of a hydrolysis step. Although Phase II metabolites can be directly detected by LC–MS strategies, in the hypothetic case of a new designer steroid discovered, it will be less straightforward to retrospectively look for a theoretical Phase II metabolite than directly for the parent compound or for an expected Phase I metabolite (released in urine after enzymatic hydrolysis in traditional sample treatments).

Enzymatic hydrolysis prior to LLE is a required step for the detection of AAS conjugated with glucuronides by neutral loss/precursor ion scan methods. This hydrolysis also releases endogenous steroids and other endogenous interferents. Therefore, noisier chromatograms are obtained after hydrolysis and the sensitivity for neutral loss/precursor ion scan methods is substantially increased when this step is omitted [39]. However, skipping hydrolysis will exclude the detection of glucuronide metabolites, and only those present in urine as unconjugated will be detected. The analyst should, therefore, decide between sensitivity and the number of potential AAS detected. Performing two analyses per sample (one with hydrolysis and the other without) could be an option, although it duplicates the total analysis time and cost.

Since glucuronidation normally implies deactivation of the metabolite, glucuronidated AAS would not exhibit AR activities. Therefore, enzymatic hydrolysis is compulsory before applying bioassays with AR activities.

An alternative to avoid undesired losses of polar analytes and to maintain both the preconcentration factor and the hydrolysis step, is SPE. Unfortunately, SPE usually involves higher ion suppression than LLE, with suppression values that can reach almost 100% [76–78]. Despite this limitation, it has mainly been used in analytical approaches based on full acquisition with HR instruments (Table 2). Nonpolar phases, such as C18, or phases involving mixed modes, such as cation exchange reverse-phase mode, have normally been selected for the combined extraction and preconcentration of a wide variety of doping agents, including AAS.

Therefore, the selection of sample treatment (preconcentration and extraction versus dilute and shoot; use of hydrolyzed versus nonhydrolyzed urine) is a key step of the method and it can limit the suitability of the method for the detection of unknown AAS. Ideally, a combination of them will provide the most comprehensive

Key Term

Dilute and shoot: Analytical approach based on dilution of the sample with mobile phase and injecting it into the LC–MS system. Due to the absence of sample treatment, no analytes are lost during this process.

Key Term

Double bond equivalent: Value indicating the number of insaturations (double bonds or rings) that contain a molecular formula. Double bond equivalent (DBE) is calculated as: DBE = C - H/2 - X/2 + N/2 + 1, where X are halogens. DBE provides valuable information for structural elucidation. In the case of androgenic anabolic steroids, a DBE of at least 4 is present because they have four rings in their structure (Figure 1).

set of data, maximizing the chance of detecting unknown AAS. However, that would increase the cost, the amount of urine needed and it would make data management difficult. Preconcentration and extraction of hydrolyzed urine seems to be the best individual option for this purpose. The extensive knowledge about this step means it can be considered as one of the easiest steps in the process (Figure 2).

Detection & selection of suspicious peaks

Despite the similarities in structure and activity of AAS and all the advances made in their detection, there is currently no analytical approach that allows for the simultaneous detection of all AAS and metabolites.

Inactivated AAS metabolites exhibit almost negligible AR activities. As an example, testosterone has two main urinary metabolites – androsterone and etiocholanolone. Andtrosterone demonstrates 40-times less AR activity than testosterone, whereas etiocholanolone exhibited almost no activity [71]. The complexity of the matrix is one of the biggest obstacles in the detection of the unknown AAS with AR activities. The presence of endogenous steroids with AR activities results in positive responses in every urine sample and it is not straightforward to differentiate between negative and suspicious samples. Therefore, although spiking with 50 ng/ml THG significantly increased ($p < 0.05$) the androgenic activity of the urine, the values obtained were similar to other male blank urines [71]. This fact demonstrates the limitations of fixing a threshold AR activity value to use the androgenic activity of the whole urine as a direct screening method.

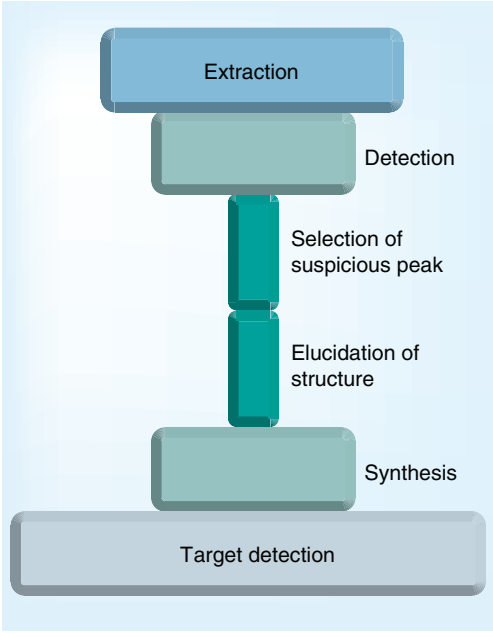

Figure 2. Analytical steps in the detection of unknown androgenic anabolic steroids. The width of the box illustrates the difficulty of the step.

Routine application of neutral loss/precursor ion scan methods is limited because they are developed only for the specific detection of AAS. Therefore, contrary to full acquisition methods using HR instruments, its implementation in routine strategies cannot be combined with the target detection of other classes of doping agents. For this reason, the application of neutral loss/precursor ion scan methods in doping control analysis has normally focused on metabolic studies (Table 1).

Additionally, despite the universality of MS detectors, those AAS without a carbonyl group, such as fully reduced metabolites, cannot be easily ionized by common API sources used in LC–MS(/MS) methods [35]. Those AAS with high conjugation in the keto function, such as THG, present serious difficulties in the common derivatization step previous to GC–MS analysis [10,11].

Therefore, the use of a unique analytical technique can fail in the detection of specific AAS. However, since AAS are largely metabolized in the liver [79], it is expected that at least one of the urinary metabolites of a hypothetical designer steroid could be detected, even by the selection of only one of the approaches. Therefore, despite the difficulties of detecting unknown AAS, this step cannot be considered as the most difficult one in the whole process (Figure 2).

More difficult is the selection of suspicious peaks when using MS-based approaches. Most of the published methods were tested by spiking known AAS into urine at an adequate concentration and evaluating if this small variation introduced in the sample can be revealed by the method. However, the fact that an analytical approach is able to detect small amounts of spiked AAS does not imply that every suspicious peak detected by the method will correspond to an exogenous AAS. In fact, every ionizable compound present in the extract will be detected by full acquisition methods in HR instruments. Several thousands of peaks are usually detected in blank urine samples analyzed by this approach [80]. The administration of an unknown AAS will only add a few peaks to this complex matrix (those coming from detectable

metabolites). The detection, *ab initio,* of those peaks is a very tedious task and almost unaffordable from a practical point of view. For this reason, full acquisition methods in HR instruments are normally seen as a tool for retrospective analysis once a new doping substance appears.

Although neutral loss/precursor ion scan methods are more specific for the detection of AAS, other compounds can be also detected by this strategy, together with potential exogenous AAS and with always-occurring endogenous steroids [39]. Therefore, precursor ion scan chromatograms for blank urine samples show several peaks arising from endogenous compounds. Several of them can be identified as metabolites of endogenous steroid hormones (corticosteroids, androgens and progestagens) as shown in Figure 3. The presence and concentration of these endogenous compounds can vary depending on several uncontrolled factors, such as administration of allowed medications (e.g., contraceptives), ethnicity, degradation of the sample or circadian and menstrual status of the subject. Therefore, chromatograms for blank urine samples can dramatically differ between them. At that point, the experience of the operator is critical to discern between endogenous compounds and potential exogenous AAS.

Even in the case that an experienced operator picks up a suspicious peak among the large amount of endogenous compounds, the peak is not necessarily an AAS. Some substances without steroidal structure can be detected by precursor ion scan methods, which can originate from different sources including diet, allowed drugs, nutritional supplements or traditional medicine [39]. All of them can be considered as potential sources of false-positive peaks. Therefore, additional experiments are needed in order to demonstrate the steroidal nature of a peak before selecting it as suspicious.

One of the most attractive approaches for this purpose is the use of bioassays with AR activities. As mentioned above, in the section titled, 'Bioassays based on androgen receptor activities', the main drawback of using this approach as a direct screening tool can be minimized when collecting fractions from an LC separation [73]. This alternative seems to be suitable in order to discern between steroidal and nonsteroidal suspicious peaks. Hence, the suspicious peak coming from the precursor ion scan can be collected in a single fraction. The analysis of this fraction by bioassays will reveal its AR activity, and indirectly its steroidal structure.

A suitable experiment to determine the steroidal structure of a suspicious peak is to perform a complete MS characterization and compare the results with the common structural features and MS behavior exhibited by AAS (see section titled 'Structural elucidation & synthesis'). First, a full-scan experiment with HR instrument will provide the molecular formula of the substance. With this formula, the **double bond equivalent** can be established. Due to the cyclopentanoperhydrophenantrene four-ring structure (Figure 1), AAS have a minimum of four insaturations and this can be used as a tool to discard those peaks without AAS structure. In a study performed by Nielen *et al.* [73], three out of five peaks found in a single fraction presenting AR activities could be discarded based on the double bond equivalent values obtained facilitating the assignment of the suspicious peak.

The product ion scan of the detected precursor ion provides structural information complementary to that obtained by the study of the full-scan spectrum. Both fragmentation after EI and collision-induced dissociation behavior after API of AAS have been extensively studied, and a general MS behavior for all AAS can be established (see section titled 'Structural elucidation & synthesis'). Therefore, the absence of losses of methyl (15 Da) and trimethylsilyl (90 Da) groups in GC–EI-MS experiments can be associated with a nonsteroidal structure. In the same way, a large number of odd electron ions and the absence of the typical molecular explosion (with several peaks differing 12–14 Da between them) can also be associated with a nonsteroidal compound in LC–API-MS/MS experiments [36].

In order to increase the chance of detecting a potential new designer steroid, it is advisable to analyze the largest number of samples. For this purpose, the simultaneous application of currently available strategies in several accredited doping control laboratories seems to be the option of choice. However, this simultaneous application also has several drawbacks regarding inter-laboratory harmonization, mainly in those strategies using LC–MS.

Doping control analysis using GC–MS is clearly standardized and similar approaches are used in most of the WADA-accredited laboratories. This fact means that data obtained in different places can be compared with regard to retention time (by using relative retention times) and MS spectrum. However, LC–MS approaches are more difficult to standardize, since different chromatographic systems

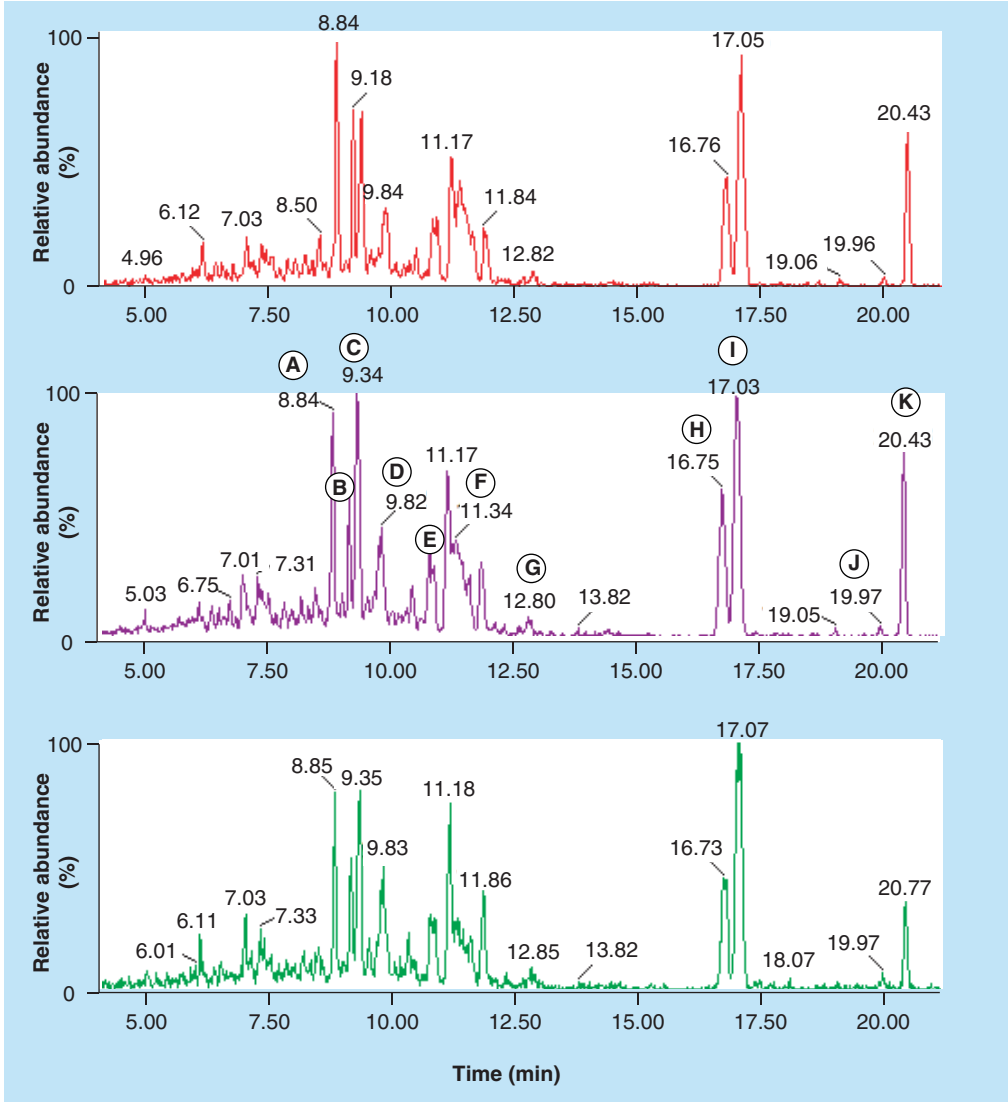

Figure 3. Precursor ion scan chromatogram (bottom: *m/z* 77; middle: *m/z* 91; and top: *m/z* 105) of a blank urine showing the peaks coming from endogenous steroids.
(A) 5a-pregnane-3b,11b,17a,21-tetrol-20-one coming from the [M-2H_2O]+ at *m/z* 331; **(B)** 5b -pregnane-3b,11b,17a,21-tetrol-20-one, coming from the [M-2H_2O]+ at *m/z* 331; **(C)** 5a -androstane-3a,11b -diol-17-one/ 5b -androstane-3a,11b -diol-17-one coming from the [M-2H_2O]+; **(D)** 5b-pregnan-3a, 11b, 20b, 21-tetrol; **(E)** 4-androsten-17b -ol-3-one coming from the [M+H]+ at *m/z* 289; **(F)** 17a -methyl-4-androsten-17b -ol-3-one (IS) coming from the [M+H]+ at *m/z* 303; **(G)** 4-androsten-17a -ol-3-one, coming from the [M+H]+ at *m/z* 289; **(H)** 5b -androstan-3a -ol-17-one, coming from [M-2H_2O]+ at *m/z* 273; **(I)** 5a -androstan-3a -ol-17-one, coming from [M-2H_2O]+ at *m/z* 273; **(J)** 5b -pregnane-3a,17b -diol-20-one coming from [M+H-2H_2O]+ at *m/z* 299; and **(K)** 5b -pregnane-3a,17a,20a -triol coming from [M+H-2H_2O]+ at *m/z* 301.

(basically UHPLC and common HPLC), columns, mobile phases, column temperatures, modifiers and gradients are used. In addition, different instruments can differ in ionization properties. This plays against inter-laboratory harmonization. As an example, Figure 4 shows the chromatograms and spectra obtained in two different instruments for the precursor ion scan analysis of the same positive sample (for experimental details see Figure 4 caption). Despite the use of the same gradient and similar columns (both C18 based columns), the relative retention times of the suspicious peak differ by approximately 20%. Additionally, different relative abundances were found for the ions detected, highlighting the potential difficulties of transferring data from one laboratory to the other.

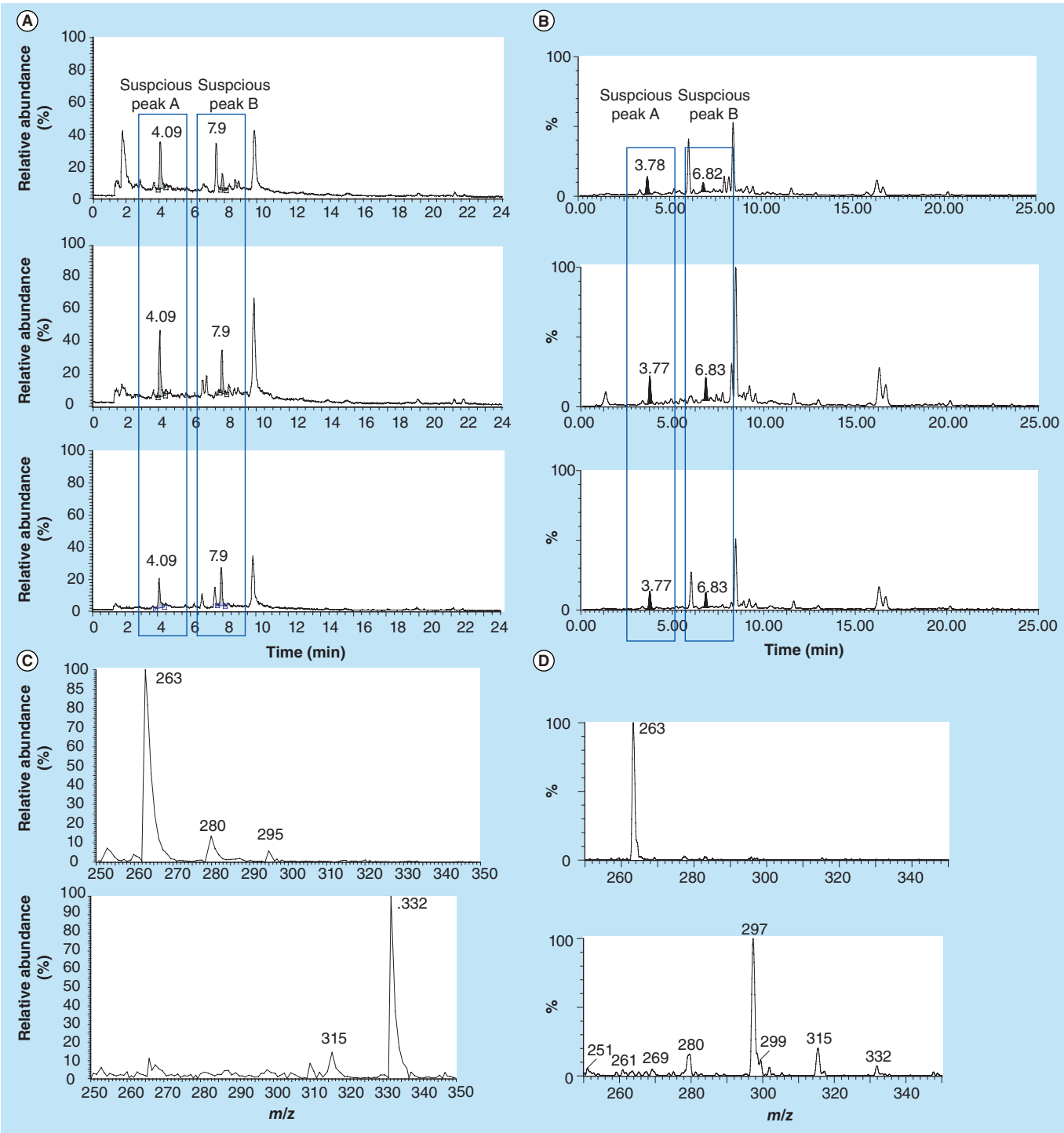

Figure 4. (A&B) Precursor ion scan chromatogram (bottom: *m/z* 77; middle: *m/z* 91; and top: *m/z* 105) of a positive urine sample containing two suspicious peaks analyzed in two different laboratories. (C&D) Ions observed for suspicious peak A (top) and suspicious peak B (bottom) in the two laboratories. Experimental conditions: laboratory A (right part of the figure) Surveyor MS-pump and Surveyor autosampler interfaced to a Quantum Discovery™ mass spectrometer (Thermo, CA, USA). Separation was performed on a SunFire™ C18-column (100 x 2 mm, 3 µm) from Waters (Zellik, Belgium). The column was maintained at 35°C. The mobile phase consisted of: **(A)** water and **(B)** MeOH, both containing 1 mM NH_4OAc and 0.1% HOAc. Gradient elution at a flow rate of 0.250 ml/min. Laboratory B (left part of the figure) UPLC system, Acquity interfaced to a Quattro Premier XE™ (Waters Associates, MA, USA). Separation was performed using an Acquity BEH C18 column (100 mm ´ 2.1 mm i.d., 1.7 µm, Waters), at a flow rate of 300 µl/min. Water and methanol both with formic acid (0.01%) and ammonium formate (1 mM) were selected as mobile phase solvents. The same gradient as in [37] was used in both experiments.

Despite these obvious analytical advances and the increasing amount of knowledge on this topic, the selection of peaks suspected to have steroidal structure remains one of the bottlenecks of the detection of unknown AAS (Figure 2).

■ Structural elucidation & synthesis.

Once a detected peak has been identified as suspicious, another difficult key step in the detection of unknown AAS starts: the structural elucidation of the compound. Due to the low concentration of the AAS and metabolites in urine, and the complexity of the matrix, it is almost impossible to reach this goal with NMR. Therefore, elucidation of the structure should be based on MS strategies.

The wide knowledge reached in the last decades on MS behavior of AAS facilitates this purpose. Therefore, the fragmentation after EI of AAS both underivatized and derivatized with several reagents has been comprehensively studied [10,11]. In these studies, several common losses and ions have been reported, depending on the AAS structure. Similarly, the collision-induced dissociation behavior after API of AAS also revealed common ionization and dissociation patterns for AAS depending on their structure [45]. Additionally, the effect of some derivatization agents in the ionization and fragmentation of AAS by LC–API-MS techniques has been reviewed recently [81].

The wide knowledge on MS behavior of AAS has been successfully applied for the structural elucidation of AAS metabolites in metabolic studies. Hence, using GC–MS approaches, the occurrence of the ions at *m/z* 218 and *m/z* 231 allowed for the proposal of 16-hydroxy metabolites of methylstenbolone [82] and the loss of 103 Da was essential in the proposal of several 18-nor-17-methyl-17-hydroxymethyl metabolites in the study of 4-chloromethandienone metabolism [83]. Similar results were obtained by LC–MS methods where the structure of several AAS metabolites were proposed based on the presence of neutral losses; for example, 26 Da for 6-ene-metabolites [46] and 30 Da for 18-nor-17-methyl-17-hydroxymethyl metabolites [84], or ions, for example, *m/z* 121 for 1,4-diene-3-keto metabolites [47,48] and *m/z* 149 or 151 for 4,6-diene-3-keto metabolites [47]. Despite this success, application of this knowledge is drastically more difficult when dealing with unknown AAS. In metabolic studies, the parent compound is known and, therefore, critical information is directly extracted from the mass of the metabolite, for example, a mass increase of 2 Da is related with a reduction process whereas a mass increase of 16 Da corresponds to a hydroxylation. Analytical efforts can then be focused on the position of the metabolic variation, for example, reduction or hydroxylation. This information is obviously missed in the detection of unknown AAS and the analyst should construct *ab initio* the structure of the substance. For this purpose, the acquisition of as much structural information as possible is compulsory.

The most straightforward structural information to be obtained is the molecular formula of the substance. The use of full-acquisition methods in HR instruments easily allows for this goal. As stated above (section titled 'Detection & selection of suspicious peaks'), the molecular formula can be used for discarding substances without AAS structure. Additionally, it provides valuable structural information on the substance, such as the number of insaturations (using the double bond equivalent), the number of oxygen atoms and the presence of uncommon atoms for AAS such as halogens or nitrogen.

Although the molecular formula indicates the number of oxygen atoms of the substance, it does not provide information regarding whether they are present as hydroxyl, ether or carbonyl. Performing different derivatization reactions can be of help for this purpose. Thus, the presence of hydroxyl and carbonyl groups can be simultaneously revealed by derivatization with *N*-methyl-*N*-trimethylsilyl trifluoroacetamide (MSTFA) [85]. Other widely used derivatives reagents specifically derivatize hydroxyl or keto functions [86]. Therefore, the combination of several derivatization processes can provide valuable structural information about the functional groups of the unknown AAS.

A promising and elegant alternative is the use of mathematical models to predict the expected instrumental behavior of an unknown AAS based on the information collected from known AAS. This strategy has been studied in depth by Fragkaki *et al.* [87–90]. Several mathematical models have been proposed for predicting relative retention times [87,88], fragmentation after EI [90] and metabolism [90] of unknown AAS. The use of this approach can help to provide additional information about the structure of an unknown AAS.

Despite the unquestionable advances made in the structural elucidation of AAS based exclusively on MS strategies, this step probably

remains the bigger bottleneck in the detection of unknown AAS (Figure 2).

Since the AAS structure arising from the elucidation performed by MS can be considered only as a proposal, the synthesis of the unknown AAS/metabolite is compulsory for the ultimate confirmation of the AAS structure [91]. Once this reference material has been synthesized the new AAS in common target procedures can be considered as the easiest step in the whole process (Figure 2).

Conclusions & future perspective

Despite the advances made in the last decade in the detection and characterization of unknown AAS, it remains one of the main analytical challenges in the field of doping control. Taking into account the last analytical advances in this field, a general approach can be used (Figure 5). First, the urine sample is extracted and the extract analyzed twice. A first analysis performed in full scan acquisition in HR instruments would be used for both the target detection of known doping agents and the full data acquisition, to make the retrospective analysis possible. A second analysis based on precursor ion scan methods would be performed in parallel in order to actively look for suspicious peaks. Once a suspicious peak is detected, two complementary experiments can be performed in order to confirm/discard the steroidal nature of the peak. On one hand, a bioassay based on AR activity can be carried out in the LC fraction containing the suspicious substance. On the other hand, an in-depth study of the MS behavior of this substance can also help for this purpose. If there is evidence about the steroidal structure of the unknown, its MS characterization is, at the moment, the most powerful tool to propose a structure. Other additional experiments (see section titled 'Structural elucidation and synthesis') can also help for structural elucidation. The proposed structure should be confirmed by synthesis. Finally, in order to close the circle, once the suspicious substance is characterized, or even better once it has been synthesized, it can be retrospectively searched in every sample routinely screened by full acquisition methods using HR instruments. Although this approach still has some limitations, it is currently the most comprehensive strategy for the detection of unknown AAS.

The evaluation of the suitability of precursor ion scan methods for routine purposes has started. The analysis of both unconjugated and conjugated fractions for more than 1000 routine samples revealed the occurrence of suspicious peaks in less than 1% of the samples. At the moment, the steroidal nature of all suspicious

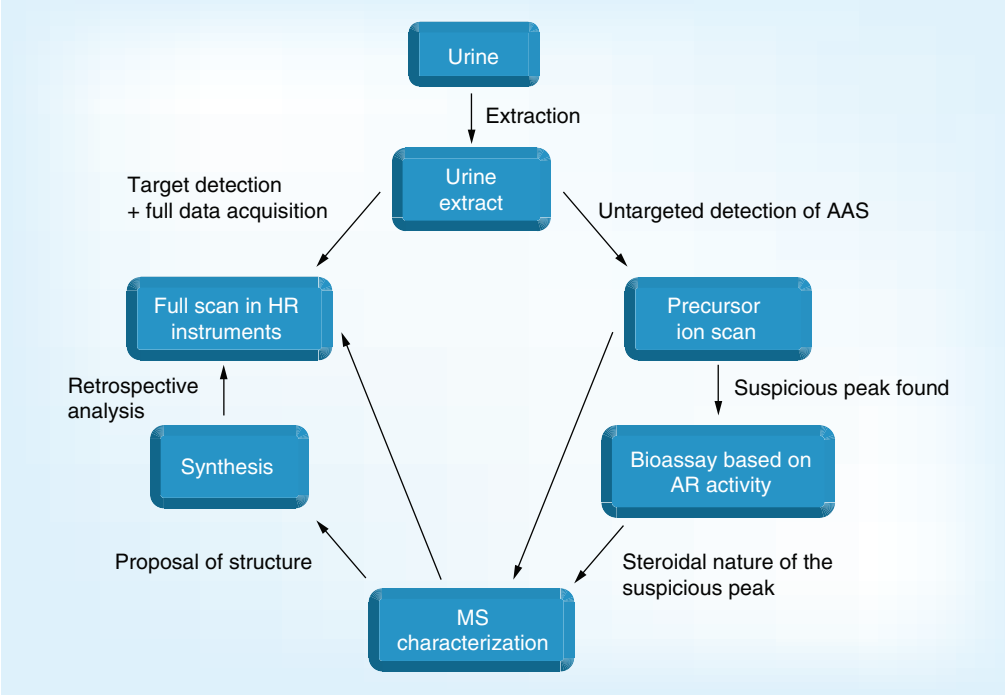

Figure 5. Comprehensive analytical strategy for the urinary detection of unknown androgenic anabolic steroids.
AAS: Androgenic anabolic steroid; AR: Androgen receptor; HR: High-resolution.

peaks could be discarded based on their MS characterization.

New instrumentation is currently available due to recent advances in analytical chemistry. It is expected that these instruments will also start to play a role in the detection of unknown AAS. Hence, triple quadrupole instruments coupled with GC are starting to be widely used in the doping control field. At the moment, their main application is the target detection of known doping agents but neutral loss/precursor ion scan methods can be also developed with these instruments. These GC–MS/MS methods can complement the existing LC–MS/MS strategies.

The recent development of metabolomic approaches can also be valuable in the detection of unknown AAS. Hence, metabolomic studies can reveal the presence of biomarkers able to show the biological effect of AAS. They would indicate the administration of AAS irrespective of the structure. These biomarkers could be ideally included in the so-called Athlete Biological Passport and would be useful as screening methods in order to select the suspicious samples, which afterwards would be studied in-depth by comprehensive strategies such as the one shown in Figure 5.

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No writing assistance was utilized in the production of this manuscript.

Executive summary

Androgenic anabolic steroids & sport doping

- Doping control laboratories are faced with the detection of unknown androgenic anabolic steroids (AAS), and common target methods based on MS are not suitable for this purpose.

Anabolic steroids, designer steroids, prohormones & nutritional supplements

- Unknown AAS can be synthesized to deliberately evade either doping control or legal coverage.

Current analytical approaches for the detection of unknown AAS

- Precursor ion/neutral loss scan methods using MS/MS: several neutral loss/precursor ion scan LC–MS/MS methods have been developed for the open detection of AAS.

Full scan acquisition in high-resolution instruments

- The use of full acquisition methods in high-resolution instruments allows for the open detection of doping substances together with the target detection of preselected ones.

Bioassays based on androgen-receptor activities

- Bioassays based on androgen-receptor activities have been developed for the detection of AAS based on their androgenic activities. Every AAS can be potentially detected by this approach without preselecting any structure.

Analytical challenges for the detection of unknown AAS

- Sample treatment: preconcentration steps are normally needed for the detection of AAS at the required sensitivity. Some doping agents can be lost during sample treatment and therefore the sample treatment should be carefully selected before developing strategies for the detection of unknown AAS.

Detection & selection of suspicious peaks

- The application of current approaches for routine purposes is rather limited. The most widely used approaches are the full acquisition methods with high-resolution instruments, but these methods are normally focused on the target detection of known doping agents.
- One of the main drawbacks of current methods is the selection of a suspicious peak, such as an exogenous peak with steroidal structure.
- LC–MS methods lack the reproducibility of GC–MS methods. This can be the cause of difficulties in the inter-laboratory harmonization.

Structural elucidation & synthesis

- MS is necessary for the structural elucidation of steroids although it cannot confirm the structure. Synthesis of the proposed structure has to be considered as the ultimate confirmation.

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