Monitoring drugs in sport testing: an insight of current trends and recent findings from the Drug Control Centre, the UK's Anti-Doping Laboratory

by Erika Castrignanò, Scarlett Devey, Alan Brailsford and Kim Wolff*

Drug Control Centre, King's Forensics, Department of Analytical, Environmental & Forensic Sciences, School of Population Health & Environmental Sciences, King's College London, London SE1 9NH

** Corresponding author: Erika Castrignanò, e-mail: erika.castrignano@kcl.ac.uk*

The requirement to monitor banned substances and maintain analytical competency in sport means Anti-Doping is a continuous challenge for scientists, due to the diversity of doping agents used and the challenges faced for proving the presence of prohibited substances, their metabolites and doping methodologies (i.e. blood doping). The chemistry of doping agents includes a wide range of substances, from low to high molecular weight molecules, that requires an Anti-Doping Laboratory to be at the top of its analytical performance, but also be a research centre with interests in (i) understanding excretion profiles of new banned drugs, (ii) exploring several "-omics" areas and (iii) developing methods to detect new forms of doping. This article will show recent findings from the Drug Control Centre, the UK's only WADA accredited Anti-Doping Laboratory, and compare them against those reported by International Laboratories within the context of advanced analytical methodologies to provide an insight into new form of doping.

1. Introduction

Tackling doping in sport is a dynamic challenge that is continuously evolving over time. The input to harmonise strategies and policies in tackling doping worldwide originated when the World Anti-Doping Agency (WADA) was established in 1999. Along with educational and social activities aimed at increasing the awareness of the danger of doping for the health of those participating in sport at any level, the scientific aspect needs to cope with various analytical challenges when a prohibited substance is present in an athlete's sample or when a banned method (such as blood doping) has been attempted or used by the athlete. These analytical challenges involve the detection of an ever greater number of prohibited substances particularly synthetic analogues of anabolic steroids and new biomarkers, and the need to improve assay detection limits. For this purpose, advanced analytical techniques need to keep up with the "multifaceted" nature of doping as doping agents vary from low molecular weight molecules to large proteins. This article will provide an insight of (i) the most recent findings from the UK's WADA accredited Anti-Doping Laboratory with the relative state-of-the-art of analytical methodologies used and (ii) consider the impact of new forms of doping.

2. A perspective from an Anti-Doping Laboratory

Along with an increased percentage of 7.1% in the number of samples analysed from 2016 and 2017 by WADA-accredited laboratories, a decrease in the number of Adverse Analytical Findings (AAFs) has been observed in 2017 [1]. An Adverse Analytical Finding (AAF) is a report defining the presence of a prohibited substance or its metabolites or biomarkers in an athlete's sample or the use of a prohibited method of doping by the athlete [1]. In the report "2017 Anti-Doping Testing Figures" [1], an AAF does not imply a sanctioned Anti-Doping Rule Violation (ADRV) as Therapeutic Use Exemption (TUE) approval processes might be included. It is important to note that the decrease in AAFs from 2016 (1.60%) to 2017 (1.43%) was ascribed in a large part to a decrease in reported cases of meldonium (prescribed to treat coronary artery disease), prohibited in 2016 because of its metabolic modulator activity and known use. Immediately following its ban there were many AAFs for meldonium as athletes had not ceased to take the drug following its change in status, however by 2017 increased awareness meant this was no longer a problem (i.e. 6.5 times less cases reported in 2017 since it was first banned in 2016).

In 2017, 78 AAFs were reported in the WADA-accredited Drug Control Centre based in London (United Kingdom, UK) that accounted for 1.9% of the total findings (Figure 1) [1]. In agreement with the majority of AAFs found in other WADA-accredited laboratories in the world with exception for two laboratories based in Los Angeles (United States of America, USA) and in Stockholm (Sweden), anabolic agents were the substances with most frequently detected prohibited substances (n = 28). The stimulants were the second most commonly reported drug class with 25 AAFs, followed by narcotics with 10. Those figures show a different profile with respect to the overall percentage of reported findings as diuretics and other masking agents are slightly more prevalent in other laboratories than stimulants, whilst narcotics are less commonly reported (9th most common class).

3. Analytical advances in drug monitoring in sport

Anabolic Agents. Stanozolol was the highest reported compound among the anabolic agents in 2017 [1]. Before 2014, the trend for this drug class showed a higher occurrence for "testosterone/epitestosterone –T/E-ratio > 4". While numbers relating to T/E findings have probably decreased due to the adaptation of the athlete biological passport (ABP), the increased frequency in stanozolol findings is a product of the use of improved liquid chromatography mass spectrometry (LC-MS) protocols, which facilitate the detection of more polar steroids, together with the implementation of data interpretation on their metabolism through the usage of high-resolution mass spectrometry (HRMS) [2]. Progress has also been made recently on the increasing effective use of gas chromatography combustion isotope ratio mass spectrometry (GC/C/IRMS), a technique that is fundamental in distinguishing between the exogenous and endogenous origin of steroids such as testosterone [3]. In this regard, GC/C/IRMS is the gold standard technique particularly when used in combination with the ABP, as IRMS can effectively distinguish between atypical steroid profiles that result from doping, and those that result from other factors such as alcohol consumption. Despite a few limitations in the evaluation of ABP, such as in some female athlete cases where steroid concentrations may be very low, transdermal testosterone application and DHEA [4], its adoption has been successful. As the ABP requires numerous data points to be collected, the number of steroidal ABP tests increased by 13% in 2017 [1] and will increase further. Moreover, steroidal ABP would benefit from the inclusion of other steroid markers to make it more effective.

Stimulants. Methylphenidate (19 %), amphetamine (18%) and cocaine (12 %) are compounds that have seen the highest occurrence in AAFs [1]. The WADA prohibited list has been modified several times in order to comply with new emerging trends in misused stimulants, as in the case of the introduction of synthetic cathinones and the re-classification of 3,4-Methylenedioxymethamphetamine (MDMA) and 3,4-Methylenedioxyamphetamine (MDA) as doping agents. This list has also been updated to re-classify substances for which metabolism studies have clarified their involvement in the production of the banned amphetamine and methamphetamine [1]. *Ad-hoc* analytical methods are often used for their screening (e.g. those using HRMS) and confirmation is performed by LC-MS/MS (i.e. triple quadrupole) or GC-MS.

Peptide hormones, Growth Factors and Related Substances. In 2017, within this drug class, the detection of erythropoietin (EPO) was high in samples analysed (48%), followed by the analogues and human chorionic gonadotropin (hCG) at 12% [1]. The latter is a heterodimeric glycoprotein, used to stimulate natural production of steroids after the intake of synthetic ones, and is analysed mainly by immunoassays. However, recently one of the first confirmation methods by LC-MS/MS for proteins was applied to hCG in urine. An increased number of tests for Erythropoiesis Stimulating Agents (ESAs), human Growth Hormone (hGH) and GH Releasing Factors (GHRF) has been performed in recent years despite the relative low number in AAFs for monitoring purposes. Major research on this drug class has been undertaken in London to investigate more sensitive analytical methods suitable to screen and confirm with low detection limits, and to explore the excretion profile of these substances. After hCG, ibutamoren, a GH secretagogue that mimics the endogenous GH ghrelin, is the next most prevalent substance and has been reported with an occurrence of 8% [1].

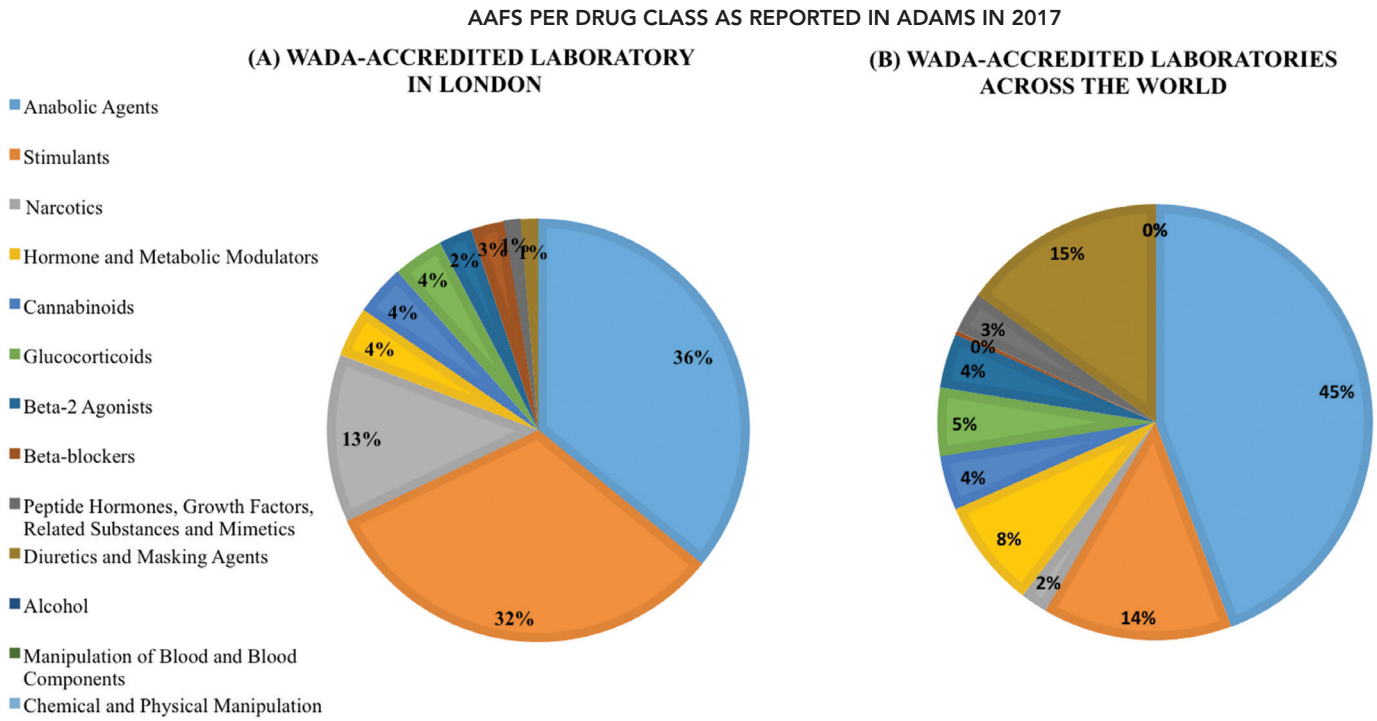

Figure 1: AAFs per drug class as reported in ADAMS in 2017 by (a) the WADA-accredited Laboratory in London (UK) (n=78) (b) compared to total AAFs reported by all WADA-accredited laboratories (n=4076). Data from the pie chart have been extrapolated by Table reported in '2017 Anti-Doping Testing Figures' report and re-arranged.

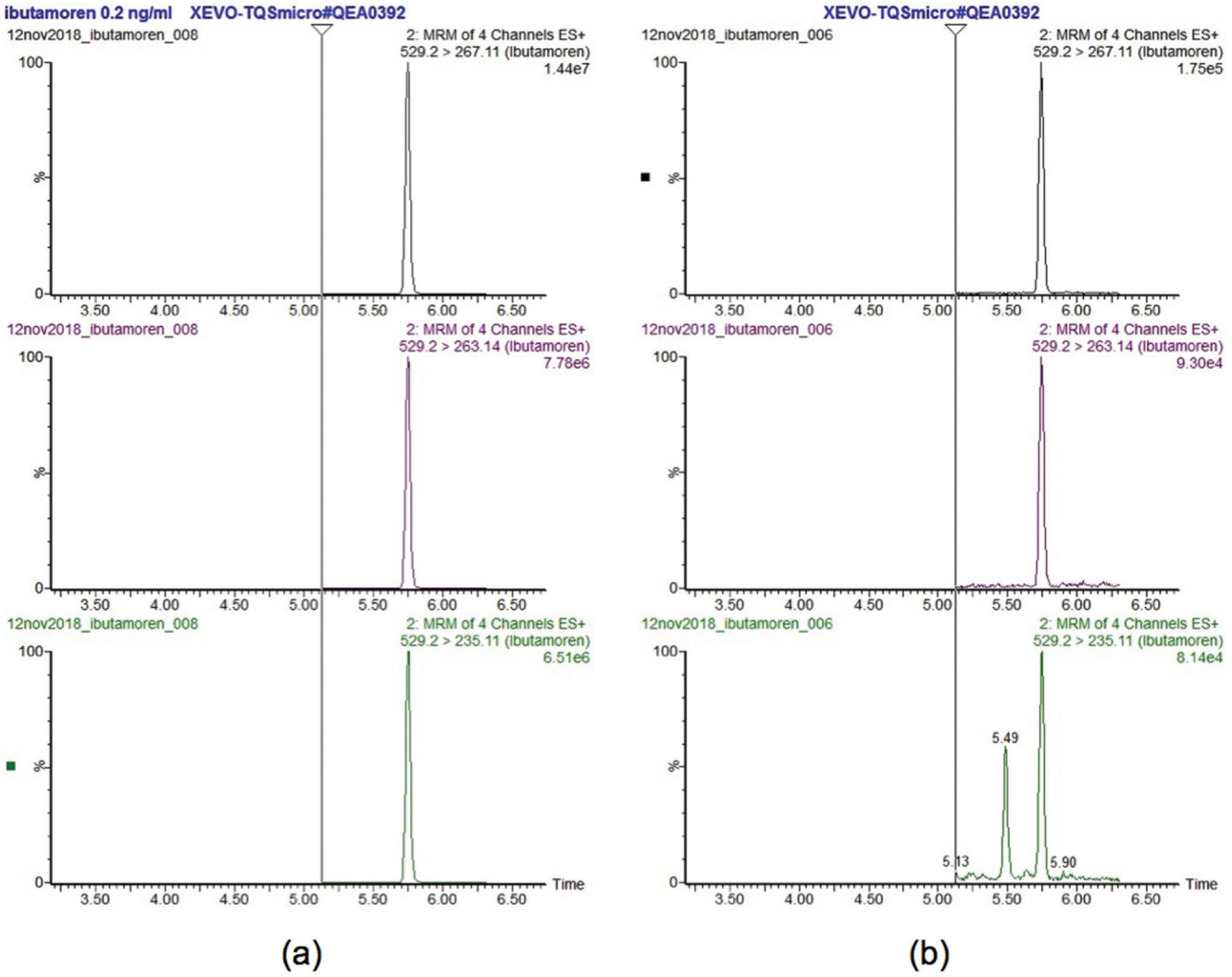

Figure 2: Chromatograms showing the confirmation of ibutamoren in a real urinary sample (b) compared to a standard at 0.2 ng mL^{-1}(a).

Figure 2 shows a confirmation of this non-peptide agonist by the Drug Control Centre in a urine sample by LC-MS/MS against a standard prepared at 0.2 ng mL^{-1}. Recently, the "GH-omics" approach has been developed to propose alternative forms of screening for co-administration of EPO and hGH [5]. This methodology is based on HRMS and evaluates metabolic profiles and relative changes characteristic of doping.

Hormone and Metabolic Modulators. Meldonium is most commonly detected in this drug class, although as mentioned in the previous Section there has been a decrease in AAFs with respect to 2016 down to 25% of class, followed by clomiphene and tamoxifen at 20% (Figure 3). Large molecules, such as insulin and Insulin-like Growth Factor (IGF), belong to this group with the detection of these large molecules adding more challenges from an analytical perspective. Indeed, insulin tests need to be performed in urine and blood with complex sample preparation procedures including immunopurification, prior to analysis by high end LC-MS systems to achieve the necessary sensitivity for both screening and confirmation analyses.

4. Future directions

Biological matrices, such as oral fluid, dried blood spots and exhaled breath (EB), are currently being investigated to evaluate their suitability as alternative matrices in Anti-Doping Testing. The drive to assess the suitability of these matrices in the Anti-Doping context is that compared to urine and venous blood collection they offer less invasive sample collection and reduced costs. It is therefore considered their use may facilitate an increase in testing through the more effective use of existing resource.

However, as new alarming frontiers in doping are rising, the scientific community is also looking at unconventional doping approaches such as "brain doping". As with other doping methods, it is based on the principle of an enhancement in performance. The novelty relies in the application that uses electrical brain stimulations to modulate the responses on targeted brain areas, thus "interfering" with a number of physiological activities. Such technique, named transcranial direct current stimulation (tDCS), has been historically used in neuroscience and in psychiatry [6] since it allows understanding of the role of specific brain areas affecting certain activities. In particular, a weak constant direct electric current is applied by two (or more) electrodes on the scalp for longer than nine minutes and the polarity-specific effects on the cortical excitability, caused by the change in resting membrane potential, might take place [7]. Usually, tDCS that are responsible for cortical excitability are anodal, whilst those that produce cortical inhibition are catodal [8]. Several advantages are also acknowledged, such as being painless, non-invasive and a reversible technique [9], that may appeal a wider range of users.

The potential effects produced by tDCS seem to be comparable to those produced

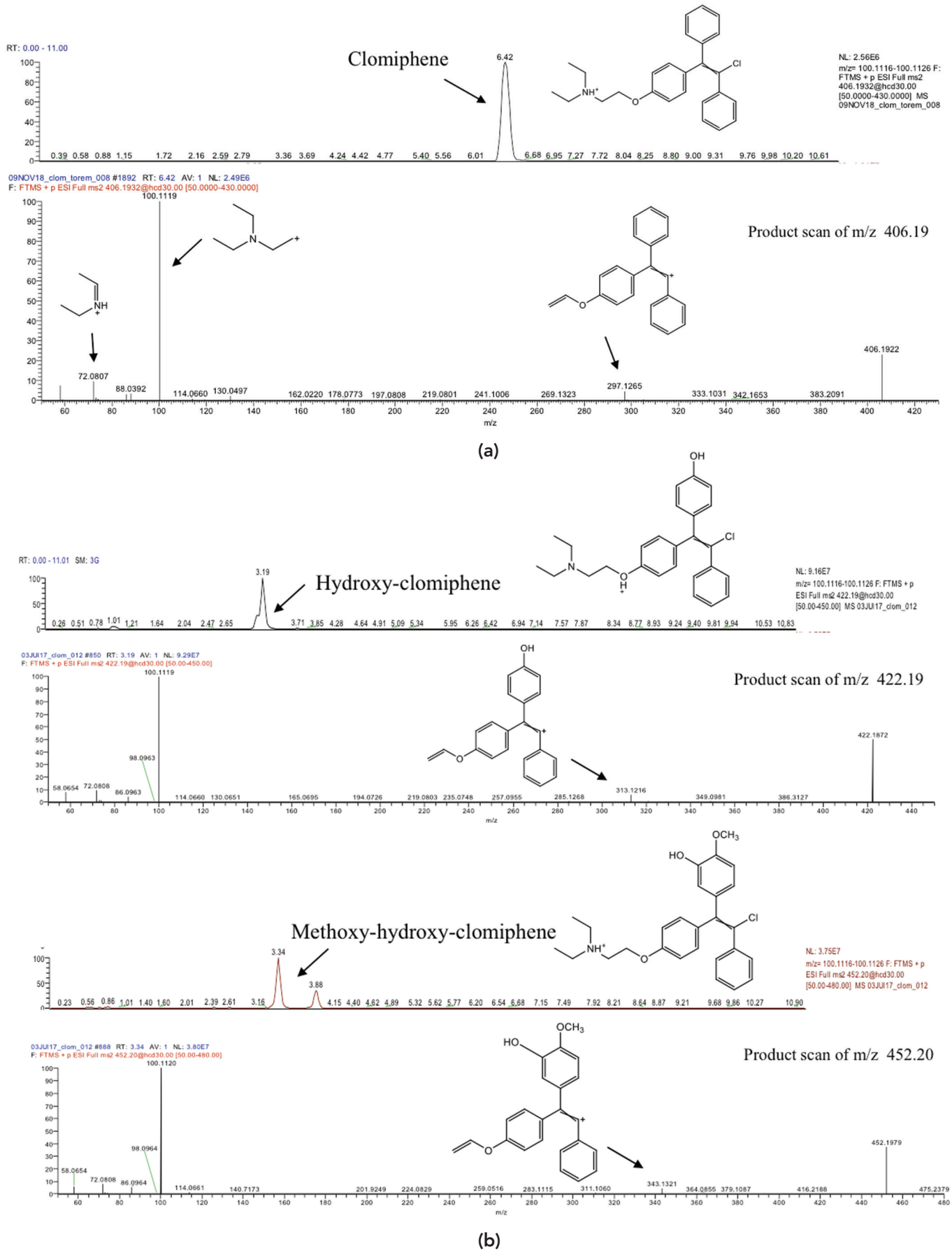

Figure 3: Chromatograms showing the confirmation of clomiphene in a real sample (a) and the presence of its metabolites from a different real sample (b).

by many substances currently listed on the WADA Prohibited List. Indeed, the decreased perception of the athlete's fatigue is being considered one of the major effects. The modulation of neuromuscular fatigue with tDCS has been investigated by Cogiamanian *et al.* and showed an overall decrease of muscle fatigue, an improvement of the muscle endurance and an increase of motivation [10]. Other studies revealed that exertion and exercise performance can be modulated by brain stimulation [11], such as the temporarily increase of isometric strength of shoulder rotators muscles [12] and quadriceps [13] after anodal tDCS.

Despite these findings, the effectiveness of tDCS has been questioned by a number of scientists who expressed their concern on the limited population size involved in the research [14] and whether the enhancement on endurance exercise performance [15], exercise tolerance or perception [16] is significant. However, as highlighted by Alix-Fages *et al.*, a non-uniform approach has been used to directly compare such studies based on (i) tDCS protocols applied, (ii) stimulated brain area and (iii) evaluated skill, thus results appeared even more controversial.

5. Conclusions

The recent findings from the UK's WADA accredited Anti-Doping Laboratory pointed out that anabolic agents, stimulants and narcotics were the most frequently detected prohibited substances. This showed a different profile with respect to the overall percentage of AAFs reported by all WADA-accredited laboratories, highlighting the diversity of doping profiling across the world. Future directions in Anti-Doping Testing will look at enhancing the development of new analytical methodologies for keeping up with the evolving nature of doping and at evaluating the suitability of alternative biological matrices.

Nevertheless the potential use of tDCS remains a concern and the Anti-Doping community must consider methods that would facilitate its detection. One area which may be adapted to address this problem is the ABP. A key difference between an ABP adverse finding compared to a "traditional" AAF is that it is no longer a requirement to prove a specific substance has been taken. Instead it is established that the athletes biological markers are outside their individual specific limits (as defined through the long term monitoring of their own markers), thus resulting from the use of a banned substance or method. It may be therefore that the detection of tDCS could be performed by monitoring markers which would be known to increase through.

6. References

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