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Screening and confirmation capabilities of liquid chromatography-time-of-flight mass spectrometry for the determination of 200 multiclass sport drugs in urine

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ABSTRACT

In this article, a screening method for the determination of 200 sport drugs in human urine has been developed using liquid-chromatography electrospray time-of-flight mass spectrometry (LC-TOFMS). The chromatographic separation of the targeted doping agents was carried out by fast liquid chromatography using a C_{18} column (4.6 × 50 mm) with 1.8 μm particle size. Accurate mass measurements of the selected ion (typically $[M+H]^+$ and $[M-H]^-$) along with retention time matching was used for the screening and detection of the targeted species. The proposed methodology comprised also a simple sample treatment stage based on solid-phase extraction (SPE) with polymeric cartridges. The SPE method displayed satisfactory recoveries rates (between 70 and 120%) for the majority of the compounds at both concentration levels tested (2.5 and 25 μg L^{-1}). The overall performance of the method was satisfactory with all 200 compounds fulfilling WADA minimum required performance levels (MRPLs), with limits of quantitation lower than 1 μg L^{-1} for 80% of the compounds, and showing an appropriate linearity ($r^2 > 0.99$) in most cases. Additionally, the ability of "in-source" collision induced dissociation (CID) for confirmatory purposes was examined using as criterion the presence of two high-resolution ions with relevant abundances for unambiguous confirmation. This stringent criterion was fulfilled for 75% of the species using in-source CID fragmentation. The use of an improved approach based on CID performed on a dedicated collision cell without precursor ion selection (using a Q-TOF) provided at least two ions in all cases with the exception of 2-aminoheptane. Finally, based on the use of diagnostic fragment ions, a workflow for the comprehensive screening and identification of non-targeted compounds (*viz.* compounds with no primary standards or retention time information available, such as metabolites) has been also examined using rat urine samples. The proposed screening method has proved to be effective for the analysis of targeted compounds, and also for the identification of metabolites, expanding easily the search for doping agents not only limited to specific banned parent compounds but also to derivate compounds with similar structure as well as metabolites.

1. Introduction

The World Anti-Doping code defines doping as the occurrence of one or more of the anti-doping rule violations set forth in Article 2.1 through Article 2.8 of *The Code*, including, but not limited to the presence of a Prohibited Substance or its Metabolites or Markers in an Athlete's Sample [1]. The World Anti-Doping Agency (WADA) is responsible of publication and revision of the *Prohibited List* [2], which is an international standard in which all the substances and methods prohibited are listed. *The List* is divided into three sections: substances and methods prohibited at all times (in- and out-of-competition); substances and methods prohibited in-competition; and substances prohibited in particular sports.

Liquid chromatography/mass spectrometry (LC–MS) has nowadays a compelling role in sport drug testing, given the features of most of the targeted analytes [3–6]. LC–MS ability of fast and sensitive targeted analysis has dramatically expanded the tools available for comprehensive sport drug testing. In addition, liquid chromatography provides some advantages in ease of sample treatment compared to gas chromatography (GC), although GC methods are still in use particularly for class-specific applications [7–9]. Amongst the assays described for sport drug testing, the initial approach followed by official laboratories was the validation of class-specific methods, due to the relatively large number of

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compounds to trace and their different physicochemical properties. Most of these methods were based on the use of either GC–MS or LC–MS to determine class-specific groups of doping agents such as anabolic steroids and glucocorticosteroids [10–17], stimulants and narcotics [18–22], diuretics and masking agents [23–27], β_2-agonists and β-blockers [28–34], or emerging sport drugs such as selective androgen receptor modulators (SARMs) [35–42].

In the last decade, advances in instrumentation (sensitivity, speed and resolution) have led to the development of generic wide-scope multiclass screening methods [3–6], covering several dozens of sport drugs from different families within the same run [43–61]. For this purpose, there are different LC–MS(MS) technologies, based on: (i) predefined list of analytes under optimized conditions (approach limited to targeted species) or (ii) non-predefined masses or conditions (approach covering targeted and eventually non-targeted analysis). Amongst the first option, the use of triple quadrupole operated in multiple-reaction monitoring mode is the standard approach for either class-specific methods or multiclass methods [3–5,47,58]. As an alternative, the use of high resolution mass spectrometry (LC-HRMS) as screening tool for sport drug testing purposes has been investigated in recent years [3–6]. The main advantage of this approach in comparison with targeted measurements based on the acquisition of fixed parent/fragment ion transitions is the comprehensive acquisition of the entire data, which provides the possibility to retrospectively scrutinize the analytical data for formerly unknown compounds or new species that eventually become relevant from the point of view of sport drug testing. This feature maps well against the requirements of doping control laboratories. Actually *The List* specifies that substances with similar structure or biological effects of any prohibited substances are also prohibited [2]. This fact has prompted that these instruments have become highly used for screening of doping agents [3–6].

Amongst LC-HRMS methods using full-scan high-resolution mass spectrometry, liquid chromatography time-of-flight mass spectrometry (LC-TOFMS) was first applied for the screening of 64 drugs using liquid–liquid extraction with diethyl ether [44]. An update of this methodology was reported by the same authors, but extended to 173 compounds validated at WADA MRPLs [52]. Kolmonen et al. proposed a multiclass screening method based on the combined use of SPE and LC-TOFMS [45], validated at WADA MRPL standards for 97 compounds. The same authors proposed an improved screening method validated at MRPLs for up to 197 compounds using LC-TOFMS after a dedicated 2-step SPE procedure using two mixed mode (cation and anion) exchange cartridges [59]. Similarly, Badoud et al. described a screening method for 103 compounds using Q-TOF instrumentation, but without the inclusion of steroids [48,49]. As an alternative to TOFs, orbital ion trap (Orbitrap) is a high resolution analyzer also used for the screening of sport drugs in urine [46,54,60,61]. Musenga et al. developed a method for the screening of 182 sport drugs using a mixed-mode cation exchange SPE procedure and Orbitrap [60]. Jimenez-Girón et al. skipped the sample treatment by simply using 1:10 dilution for the screening of 120 sport drugs [61]. Moulard et al. developed a method for equine urine covering 235 compounds using SPE with C18 cartridges using an Exactive Orbitrap [54]. The method is so far the more comprehensive in terms of number of analytes included, although not detailed extraction recoveries were provided, and given the nature of the cartridge used, the sample preparation can be considered somewhat biased towards less polar species, being the more polar compounds/metabolites not eventually recovered with this approach.

In this article, a screening method for the determination of 200 sport drugs in urine using LC-TOFMS has been developed and fully validated at WADA MRPLs. The proposed methodology comprises a generic single-stage sample preparation with commercial polymeric SPE cartridges followed by LC-TOFMS analysis. A dedicated study and evaluation of the fragmentation displayed using in-source CID fragmentation was performed and contrasted with the fragmentation exhibited using a dedicated collision cell with a Q-TOF instrument. Additionally, based on the use of diagnostic ions (obtained by in-source CID fragmentation or CID without precursor selection) and a database of predefined biotransformations, an automated workflow for the comprehensive screening and identification of non-targeted compounds (*viz.* with no standards or retention time information available, such as metabolites) has been examined using rat urine samples.

2. Experimental

2.1. Chemicals and reagents

HPLC grade acetonitrile (MeCN) and methanol (MeOH) were obtained from Sigma-Aldrich (Madrid, Spain). Drug analytical standards were purchased from Cerilliant (Round Rock, TX), Dr. Ehrenstorfer (Madrid, Spain), European Pharmacopeia, National Measurement Institute (Australia) and Sigma-Aldrich (Madrid, Spain). Individual stock solutions were prepared in MeOH or MeCN and were stored at −18 °C. Formic acid was purchased from Fluka (Madrid, Spain). A Milli-*Q*-Plus ultra-pure water system from Millipore (Milford, MA) was used throughout the study to obtain HPLC water used during the analyses and to prepare all the solutions. Bond Elut PLEXA SPE cartridges (200 mg, 6 mL) were purchased from Agilent Technologies (Santa Clara, CA) and a Supelco (Bellefonte, PA) Visiprep™ SPE vacuum system was used for SPE experiments.

2.2. Sample preparation

Untreated human urine sample aliquots were subjected to an SPE procedure using Bond Elut PLEXA cartridges (200 mg, 6 mL). The cartridges were preconditioned with 4 mL of MeOH/MeCN (1:1) and 4 mL of HPLC grade Milli-*Q* water. After the conditioning step, 2 mL of urine buffered with 2 mL of formic acid/formate (50 mM) at pH 2.6, were passed through the SPE cartridge. 4 mL of 5% MeOH in Milli-*Q* water was then added to rinse the cartridge prior to elution. The cartridges were dried under vacuum in order to remove the excess water and the analytes were finally eluted with 4 mL of MeOH/MeCN (1:1, v/v). The extracts were evaporated until near dryness using a Turbo Vap LV from Zymark (Hopkinton, MA), with a water bath temperature of 37 °C and a N_2 pressure of 15 psi. The samples were then taken up with 0.5 mL of MeOH/water (10:90 v/v) to achieve a preconcentration of 4:1. The reconstituted extracts were filtered through a 0.45 μm syringe filter and then transferred to a 2-mL analysis vial.

2.3. LC-TOFMS and LC-QTOFMS

The separation of the analytes from the urine extract was carried out using a high-performance liquid chromatography (HPLC) system (consisting of vacuum degasser, auto sampler and a binary pump) (Agilent 1200, Agilent Technologies, Santa Clara, CA) equipped with a reversed-phase XDB-C18 analytical column of 4.6 mm × 50 mm and 1.8 μm particle size (Agilent Technologies, Santa Clara, CA). 20 μL of the extract were injected in each run. Mobile phases A and B were water with 0.1% formic acid and MeCN. The chromatographic method held the initial mobile phase composition (10% B) constant for 3 min, followed by a linear gradient to 100% B up to 15 min and kept for 3 min at 100% B. The flow rate used was 0.5 mL min^{-1}. The HPLC system was connected to a time-of-flight mass spectrometer Agilent 6220 accurate mass TOF

(Agilent Technologies, Santa Clara, CA) equipped with an electrospray interface operating in either positive or negative ion mode, using the following operation parameters: capillary voltage, 4000 V; nebulizer pressure, 40 psig; drying gas flow rate, 9.0 L min^{-1}; gas temperature, 325 °C; skimmer voltage, 65 V; octapole 1 rf, 250 V; fragmentor voltage: 190 V (screening method) and 220 V (for in-source CID fragmentation and confirmatory purposes evaluation). LC–MS accurate mass spectra were recorded across the *m/z* range of 50–1000 in positive ion mode and 50–1100 in negative ion mode. The instrument performed the internal mass calibration automatically, using a dual-nebulizer electrospray source, which introduced a low flow (approximately 5 μL min^{-1}) of calibrating internal reference masses solution ($TFANH_4$ (ammonium trifluoroacetate, $C_2O_2F_3NH_4$, at *m/z* 112.9856 in negative ion mode), purine ($C_5H_4N_4$, at *m/z* 121.0509, in ESI(+)) and HP-0921 [hexakis-(1*H*,1*H*,3*H*-tetrafluoropropoxy)phosphazine], $C_{18}H_{18}O_6N_3P_3F_{24}$, at *m/z* 922.0098 in positive ion mode and 1033.9881 in negative mode). To perform CID experiments with a dedicated collision cell, an Agilent 1260 Infinity HPLC system was connected to a hybrid quadrupole time-of-flight (Q-TOF) mass spectrometer Agilent 6530 (Agilent Technologies, Santa Clara, CA), equipped with the same dual spray interface, applying the same chromatographic method and MS parameters described for the TOF instrument except fragmentor voltage, set at 90 V. *"All-ion mode"* full-scan acquisition was used at different collision energy conditions (0, 10, 20 and 30 V). All data was recorded with Agilent Mass Hunter Data Acquisition software (version B.04.00) and processed with Agilent Mass Hunter Qualitative Analysis software (version B.04.00) which included both *"Molecular Feature Extractor"* and *"Find by Formula"* applications used.

2.4. Method validation

A pool of human urine samples (specific gravity 1.015, pH 7.0) were checked for the presence of any of the 200 sport drugs tested. Seven replicates of spiked urine samples were prepared to measure extraction recovery and method precision. Extraction recovery was determined by analyzing samples spiked before and after SPE extraction at two concentration levels (2.5 and 25 μg L^{-1}). Spiking after the extraction process (10 and 100 μg L^{-1} respectively considering the preconcentration factor) corresponded to 100% recovery. The results were obtained by comparing mean values of peak areas of analytes. Repeatability of the extraction method was expressed as relative standard deviations (RSD (%)) of peak areas. In the case of endogenous substances, the background value was considered and corrected for method performance evaluation.

The limits of detection (LODs) and quantitation (LOQs) were calculated as the minimum concentration of analyte whose extracted ion chromatogram (with a narrow mass window extraction of ± 20 ppm without smooth filters) showed a signal-to-noise ratio at (*S*/*N*)=3:1 and 10:1, respectively. LODs were empirically calculated using the data acquired from recovery experiments (2.5 and 25 μg L^{-1}), or by subsequent dilutions of these extracts with non-spiked matrix. LOD and LOQ were not calculated for endogenous steroids testosterone and 19-norandrosterone. In the case of other endogenous substances, they were not detected in the pooled urine tested. Matrix effects (ME) were also estimated in order to assess the impact of the matrix on the ionization suppression/enhancement on the analytes (compared to neat standards). For this purpose, the slopes obtained in the calibration with urine extracts were compared with those obtained with solvent-based standards, calculating slope ratios matrix/solvent (ME) for each of the targeted compounds. ME significantly > 1 means signal enhancement while ME significantly < 1 means signal suppression (the more common phenomenon).

2.5. Development of the automated targeted screening method

An automated screening approach based on accurate masses of selected ions and retention time matching was used based on "Find by Formula" application of the software used. The selected 200 drugs were divided in 10 mixtures containing *ca.* 20 drugs each at a concentration of *ca.* 200 μg L^{-1}. These solutions were analyzed by LC-TOFMS to collect the retention time data and relevant mass spectra information. For the automatic screening method, an excel spreadsheet was constructed containing the elemental composition and exact mass data for each drug and their retention times. This file was put into *csv* (*comma separated values*) file format for use by the Agilent TOF automated data analysis (*"Find by Formula"*) software. The data included are summarized in Table 1, where the retention time, molecular formula and accurate masses of the selected ions are shown for each compound tested.

2.6. Non-targeted analysis of rat urinary metabolites

Besides the standard screening method for targeted detection of sport drugs, the performance of LC-TOFMS for the comprehensive screening and identification of non-targeted compounds (*viz.* preliminary identification of components with neither primary standards nor retention time information available (e.g. such as derivate species and/or metabolites)) has been examined using rat urine samples. The non-targeted approach is based on the use of diagnostic ions (obtained by in-source CID fragmentation or CID without precursor selection) and a list of predefined biotransformations that parent compounds usually undergo.

2.6.1. Sample collection

The study was performed on adult male Wistar rats (250–300 g) (Charles River Laboratories, Barcelona, Spain). The animals (n=5) were weighed and placed in individual metabolic cages 48 h prior to treatment to acclimatize them to this environment, maintained under standard conditions of light and temperature and allowed *ad libitum* access to food and water to the end of the experiment. All the procedures followed the Spanish guidelines on the use of animals for research (RD 1201/2005) and were approved by the institutional Committee for Ethics. The rats were treated with bumetadine at 8 mg kg^{-1} body weight (intraperitoneal). After drug administration, urine was daily collected in graduate cylinders for 2 days (24, 48 and 72 h). The urine collected 24 h prior to treatment was used as control. The SPE procedure was applied omitting intentionally the enzymatic hydrolysis/deconjugation step in order to keep the metabolites in their conjugated forms.

2.6.2. Compilation of diagnostic ions and data analysis

For untargeted detection, the full-scan high-resolution mass spectrometry data was processed by two main approaches:

(a) *"Molecular Feature Extractor"* software tool. Raw LC–MS data is scrutinized thoroughly by an algorithm, which treats the entire sample mass spectral data from the experiment as a large, three-dimensional array of retention time, *m/z* and abundance values). Background and other incoherent signals are disregarded and only chromatographic peaks (*viz.* transient signals with coherent chromatographic peak profile behavior) are finally included in the list of sample features. The results provided for each compound detected are: a mass spectrum containing the ions with the same elution time and explainable relationships, and an extracted compound chromatogram that is effectively an extracted ion chromatogram

Table 1
LC-TOFMS accurate mass database of the studied sport drugs, including retention times, elemental composition of the detected ions, theoretical and experimental accurate mass and their error (fortification level: 25 μg L^{-1}).

Compound	R_t	Ioniz. mode	Ion	Ion formula	Theoretical (*m*/*z*)	Experimental (*m*/*z*)	Error (ppm)
11-Nor-11-carboxy-Δ^9-THC	*15.07*	ESI +	$[M+H]^+$	$C_{21}H_{29}O_4$	345.2060	345.2062	0.58
16-β-Hydroxyfurazabol	*13.23*	ESI +	Fragment 1	$C_{20}H_{29}N_2O_2$	329.2224	329.2225	0.30
17,17-Dimethyl-18-androsta-1,4,13(14)-trien-3-one	*17.59*	ESI +	$[M+H]^+$	$C_{20}H_{27}O$	283.2056	283.2056	0.00
17-α-Ethynil estradiol	*12.33*	ESI +	Fragment 1	$C_{20}H_{23}O$	279.1743	279.1742	−0.36
17-α-Methyltestosterone	*12.62*	ESI +	$[M+H]^+$	$C_{20}H_{31}O_2$	303.2319	303.2318	−0.33
17-α-Hydroxyprogesterone	*12.80*	ESI +	$[M+H]^+$	$C_{21}H_{31}O_3$	331.2268	331.2271	0.91
17-Epioxandrolone	*12.89*	ESI +	Fragment 1	$C_{19}H_{29}O_2$	289.2162	289.2166	1.38
19-Norandrosterone	*13.43*	ESI +	Fragment 1	$C_{18}H_{27}O$	259.2056	259.2054	−0.77
19-Norethiocholanone	*13.00*	ESI +	Fragment 1	$C_{18}H_{27}NO$	259.2056	259.2057	0.39
2-Aminoheptane	*6.93*	ESI +	$[M+H]^+$	$C_7H_{18}N$	116.1434	116.1436	1.72
2-Hydroxymethyl-17α-methylandrosta-1, 4-diene-11α,17β-diol-3-one	*9.35*	ESI +	$[M+H]^+$	$C_{21}H_{31}O_4$	347.2217	347.2213	−1.15
2α-Hydroxymethylethisterone	*11.48*	ESI +	$[M+H]^+$	$C_{22}H_{31}O_3$	343.2268	343.2269	0.29
2α-Methyl-5α-Androstan-3α-ol-17-one	*15.04*	ESI +	Fragment 1	$C_{20}H_{31}O$	287.2369	287.2372	1.04
4-Androstene-3,17-dione	*12.78*	ESI +	$[M+H]^+$	$C_{19}H_{27}O_2$	287.2006	287.2003	−1.04
4-Hydroxyandrostendione (Formestane)	*13.06*	ESI +	$[M+H]^+$	$C_{19}H_{27}O_3$	303.1958	303.1962	1.32
4-Hydroxytestosterone	*12.33*	ESI +	$[M+H]^+$	$C_{19}H_{23}O_3$	305.2111	305.2114	0.98
5-α-Dihydroxytestosterone	*13.22*	ESI +	$[M+H]^+$	$C_{19}H_{31}O_2$	291.2319	291.2315	−1.37
6α-Methylprednisolone	*10.48*	ESI +	$[M+H]^+$	$C_{22}H_{31}O_5$	375.2166	375.2165	−0.27
Acebutolol	*7.97*	ESI +	$[M+H]^+$	$C_{18}H_{29}N_2O_4$	337.2122	337.2120	−0.59
Acepromazine	*9.95*	ESI +	$[M+H]^+$	$C_{19}H_{23}N_2OS$	327.1526	327.1530	1.22
Acetazolamide	*3.58*	ESI +	Fragment 1	$C_2H_5N_4O_2S_2$	180.9848	180.9848	0.00
Acethylmorphine	*6.00*	ESI +	$[M+H]^+$	$C_{19}H_{22}NO_4$	328.1543	328.1542	−0.30
Adrafinil	*9.68*	ESI +	Fragment 1	$C_{13}H_{11}$	167.0855	167.0856	0.60
α-Zearalanol	*11.79*	ESI +	$[M+H]^+$	$C_{18}H_{27}O_5$	323.1853	323.1856	0.93
Alprenolol	*9.43*	ESI +	$[M+H]^+$	$C_{15}H_{24}NO_2$	250.1802	250.1800	−0.80
Althiazide	*10.91*	ESI +	$[M+H]^+$	$C_{11}H_{15}ClN_3O_4S_3$	383.9908	383.9906	−0.52
Altrenogest	*13.11*	ESI +	$[M+H]^+$	$C_{19}H_{27}O_2$	311.2006	311.2005	−0.32
Amcinonide	*14.03*	ESI +	$[M+H]^+$	$C_{28}H_{36}FO_7$	503.2440	503.2446	1.19
Aminoglutethimide	*3.95*	ESI +	$[M+H]^+$	$C_{13}H_{17}N_2O_2$	233.1285	233.1281	−1.70
Amphetamine	*3.75*	ESI +	Fragment 1	C_9H_{11}	119.0858	119.0862	2.20
Anastrazole	*11.03*	ESI +	$[M+H]^+$	$C_{17}H_{20}N_5$	294.1713	294.1714	0.34
Androsterone	*13.84*	ESI +	Fragment 1	$C_{19}H_{29}O$	273.2213	273.2211	−0.73
Atenolol	*1.78*	ESI +	$[M+H]^+$	$C_{14}H_{23}N_2O_3$	267.1703	267.1701	−0.75
Bambuterol	*8.62*	ESI +	$[M+H]^+$	$C_{18}H_{30}N_3O_5$	368.2180	368.2183	0.81
Beclomethasone	*10.93*	ESI +	$[M+H]^+$	$C_{22}H_{30}ClO_5$	409.1776	409.1774	−0.49
Bendroflumethiazide	*11.84*	ESI +	$[M+H]^+$	$C_{15}H_{15}F_3N_3O_4S_2$	422.0451	422.0453	0.47
Benthiazide	*11.36*	ESI −	$[M-H]^-$	$C_{15}H_{13}ClN_3O_4S_3$	429.9771	429.9775	0.93
Benzoylecgonine	*7.69*	ESI +	$[M+H]^+$	$C_{16}H_{20}NO_4$	290.1387	290.1390	1.03
β-Estradiol	*11.85*	ESI +	Fragment 1	$C_{18}H_{23}O$	255.1743	255.1746	1.18
Betaxolol	*9.47*	ESI +	$[M+H]^+$	$C_{18}H_{20}NO_3$	308.2220	308.2219	−0.32
Bis(4-cyanophenyl) methanol	*11.46*	ESI +	$[M+H]^+$	$C_{15}H_{11}N_2O$	238.0866	238.0864	−0.84
Bisoprolol	*8.94*	ESI +	$M+H]^+$	$C_{18}H_{32}NO_4$	326.2326	326.2324	−0.61
Boldenone	*11.48*	ESI +	$[M+H]^+$	$C_{19}H_{27}O_2$	287.2006	287.2006	0.00
Boldine	*7.22*	ESI +	$[M+H]^+$	$C_{19}H_{22}NO_4$	238.1543	238.1542	−0.42
Brucine	*7.47*	ESI +	$[M+H]^+$	$C_{23}H_{27}N_2O_4$	295.1966	295.1969	1.02
Bumetanide	*12.23*	ESI +	$[M+H]^+$	$C_{17}H_{21}N_2O_5S$	365.1166	365.1168	0.55
Buprenorphine	*9.82*	ESI +	$[M+H]^+$	$C_{29}H_{42}NO_4$	468.3108	468.3107	−0.21
Bupropion	*8.90*	ESI +	Fragment 1	$C_9H_{11}ClNO$	184.0524	184.0526	1.09
Butabarbital	*9.84*	ESI +	Fragment 1	$C_6H_9N_2O_3$	157.0608	157.0609	0.64
Cannabidiol	*16.11*	ESI +	$[M+H]^+$	$C_{21}H_{32}O_2$	315.2319	315.2314	−1.59
Canrenone	*13.02*	ESI +	$[M+H]^+$	$C_{22}H_{29}O_3$	341.2111	341.2111	0.00
Capsaicin	*12.92*	ESI +	Fragment 2	$C_8H_9O_2$	137.0597	137.0599	1.46
Carphedone	*8.49*	ESI +	Fragment 1	$C_{11}H_{12}NO$	174.0913	174.0910	−1.72
Carvedilol	*10.20*	ESI +	$[M+H]^+$	$C_{24}H_{27}N_2O_4$	407.1965	407.1968	0.74
Celecoxib	*13.85*	ESI +	$[M+H]^+$	$C_{17}H_{15}F_3N_3SO_2$	382.0832	382.0830	−0.52
Celiprolol	*8.55*	ESI +	$[M+H]^+$	$C_{20}H_{34}N_3O_4$	380.2544	380.2547	0.79
Chlorothiazide	*4.75*	ESI −	$[M-H]^-$	$C_7H_5ClN_3O_4S_2$	293.9421	293.9426	1.70
Clenbuterol	*8.14*	ESI +	$[M+H]^+$	$C_{12}H_{19}Cl_2N_2O$	277.0869	277.0874	1.80
Clopamide	*9.37*	ESI +	$[M+H]^+$	$C_{14}H_{21}ClN_3O_3S$	346.0987	346.0988	0.29
Clostebol acetate	*16.06*	ESI +	$[M+H]^+$	$C_{21}H_{30}ClO_3$	365.1878	365.1880	0.55
Cocaethylene	*9.19*	ESI +	$[M+H]^+$	$C_{18}H_{24}NO_4$	318.1700	318.1706	1.89
Cocaine	*8.59*	ESI +	$[M+H]^+$	$C_{17}H_{22}NO_4$	304.1543	304.1542	−0.33
Codeine	*3.13*	ESI +	$[M+H]^+$	$C_{18}H_{22}NO_3$	300.1594	300.1596	0.67
Cotinine	*1.37*	ESI +	$[M+H]^+$	$C_{10}H_{12}N_2O$	177.1022	177.1023	0.56
Cyclofenil	*16.33*	ESI +	$[M+H]^+$	$C_{23}H_{28}NO_4$	382.2013	382.2013	0.00
Cyclothiazide	*11.50*	ESI +	Fragment 1	$C_9H_{11}ClN_3O_4S_2$	323.9874	323.9877	0.93
Danazol	*14.35*	ESI +	$[M+H]^+$	$C_{22}H_{29}NO_2$	338.2115	338.2116	0.30
Dehydroandrosterone	*12.66*	ESI +	Fragment 1	$C_{19}H_{27}O$	271.2056	271.2052	−1.47
Δ9-THC	*17.47*	ESI +	$[M+H]^+$	$C_{21}H_{31}O_2$	315.2319	315.2322	0.95
Dexamethasone	*10.72*	ESI +	$[M+H]^+$	$C_{22}H_{30}FO_5$	393.2072	393.2076	1.02
Diazepam	*12.40*	ESI +	$[M+H]^+$	$C_{16}H_{14}ClN_2O$	285.0789	285.0791	0.70
Diethylnicotinamide(Niketamide)	*4.23*	ESI +	$[M+H]^+$	$C_{10}H_{15}N_2O$	179.1179	179.1175	−2.23
Dimethylphenetylamine	*5.22*	ESI +	Fragment 1	$C_{10}H_{13}$	133.1013	133.1016	2.25

Table 1 (*continued*)

Compound	R_t	Ioniz. mode	Ion	Ion formula	Theoretical (m/z)	Experimental (m/z)	Error (ppm)
Doxapram	8.93	ESI +	$[M+H]^+$	$C_{24}H_{31}N_2O_2$	379.2380	379.2382	0.53
EDDP	10.16	ESI +	$[M]^+$	$C_{20}H_{24}N$	278.1909	278.1906	−1.08
Ephedrine	2.79	ESI +	Fragment 1	$C_{10}H_{14}N$	148.1121	148.1120	−0.68
Epitestosterone	12.76	ESI +	$[M+H]^+$	$C_{19}H_{29}O_2$	289.2162	289.2163	0.35
Eplerenone	10.87	ESI +	$[M+H]^+$	$C_{24}H_{31}O_6$	415.2115	415.2117	0.48
Esmolol	8.57	ESI +	$[M+H]^+$	$C_{16}H_{26}NO_4$	296.1856	296.1854	−0.68
Ethacrinic acid	12.77	ESI +	$[M+H]^+$	$C_{13}H_{12}Cl_2O_4$	303.1850	303.1852	0.66
Ethiocholanone	13.56	ESI +	Fragment 1	$C_{19}H_{29}O$	273.2213	273.2214	0.37
Ethisterone	12.70	ESI +	$[M+H]^+$	$C_{21}H_{29}O_2$	313.2162	313.2162	0.00
Ethylamphetamine	6.99	ESI +	$[M+H]^+$	$C_{11}H_{17}N$	164.1434	164.1430	−2.44
Ethylmorphine	6.95	ESI +	$[M+H]^+$	$C_{19}H_{23}NO_3$	314.1751	314.1753	0.64
Famprofazone	10.21	ESI +	$[M+H]^+$	$C_{24}H_{32}N_3O$	378.2540	378.2538	−0.53
Fenbutrazate	11.01	ESI +	$[M+H]^+$	$C_{23}H_{30}NO_3$	368.2220	368.2221	0.27
Fenfluramine	9.21	ESI +	$[M+H]^+$	$C_{12}H_{16}F_3N$	232.1308	232.1305	−1.29
Fenspiride	4.83	ESI +	$[M+H]^+$	$C_{15}H_{21}N_2O_2$	261.1598	261.1594	−1.53
Fentanyl	9.60	ESI +	$[M+H]^+$	$C_{22}H_{29}N_2O$	337.2274	337.2273	−0.30
Finasteride	12.28	ESI +	$[M+H]^+$	$C_{23}H_{37}N_2O_2$	373.2850	373.2851	0.27
Flumethasone	10.79	ESI +	$[M+H]^+$	$C_{22}H_{29}F_2O_5$	411.1978	411.1978	0.00
Flunisolide	11.19	ESI +	$[M+H]^+$	$C_{24}H_{32}FO_6$	435.2177	435.2172	−1.15
Fluocinolone acetonide	11.30	ESI +	$[M+H]^+$	$C_{24}H_{31}F_2O_6$	453.2083	453.2082	−0.22
Fluorometholone	11.54	ESI +	$[M+H]^+$	$C_{22}H_{30}FO_4$	377.2123	377.2122	−0.27
Fluoxymesterone	10.90	ESI +	$[M+H]^+$	$C_{20}H_{30}FO_3$	337.2173	337.2174	0.30
Flurandrenolide	11.32	ESI +	$[M+H]^+$	$C_{24}H_{34}FO_6$	437.2334	437.2335	0.23
Fluticasone propionate	14.07	ESI +	$[M+H]^+$	$C_{25}H_{32}F_3O_5S$	501.1917	501.1919	0.40
Formoterol	8.28	ESI +	$[M+H]^+$	$C_{19}H_{25}N_2O_4$	345.1809	345.1810	0.29
Furosemide	10.74	ESI −	$[M-H]^-$	$C_{12}H_{10}ClN_2O_5S$	329.0004	329.0004	0.00
Gestrinone	12.54	ESI +	$[M+H]^+$	$C_{21}H_{25}O_2$	309.1849	309.1848	−0.32
Glibenclamide	13.35	ESI +	$[M+H]^+$	$C_{23}H_{29}ClN_3O_5S$	494.1511	494.1513	0.40
Glipizide	11.46	ESI +	$[M+H]^+$	$C_{21}H_{28}N_5O_4S$	446.1857	446.1859	0.45
Heptaminol	1.53	ESI +	Fragment 1	$C_8H_{18}N$	128.1434	128.1435	0.78
Heroin	8.39	ESI +	$[M+H]^+$	$C_{21}H_{24}NO_5$	370.1649	370.1645	−1.08
Hexobarbital	10.91	ESI +	Fragment 1	$C_6H_9N_2O_3$	157.0608	157.0605	−1.91
Hydrochlorothiazide	5.73	ESI −	$[M-H]^-$	$C_7H_7ClN_3O_4S_2$	295.9572	295.9570	−0.68
Hydrocortison	9.98	ESI +	$[M+H]^+$	$C_{21}H_{31}O_5$	363.2166	363.2160	−1.65
Hydroflumethiazide	8.49	ESI −	$[M-H]^-$	$C_8H_7F_3N_3O_4S_2$	329.9836	329.9840	1.21
Hydromorphone	1.94	ESI +	$[M+H]^+$	$C_{17}H_{20}NO_3$	286.1438	286.1439	0.35
Ibuprofen	13.69	ESI +	Fragment 1	$C_{12}H_{17}$	161.1325	161.1326	0.62
Indapamide	11.20	ESI +	$[M+H]^+$	$C_{16}H_{17}ClN_3O_3S$	366.0674	366.0677	0.82
Isoetharine	1.85	ESI +	$[M+H]^+$	$C_{13}H_{22}NO_3$	240.1594	240.1596	0.83
Ketamine	7.48	ESI +	$[M+H]^+$	$C_{13}H_{17}ClNO$	238.0993	238.0994	0.42
Labetalol	9.02	ESI +	$[M+H]^+$	$C_{19}H_{25}N_2O_3$	329.186	329.1861	0.30
Letrozole	11.13	ESI +	Fragment 1	$C_{15}H_9N_2$	217.076	217.0758	−0.92
Lidocaine	7.10	ESI +	$[M+H]^+$	$C_{14}H_{23}N_2O$	235.1805	235.1801	−1.70
LSD	8.86	ESI +	$[M+H]^+$	$C_{20}H_{26}N_3O$	324.2070	324.2073	0.93
MDA	5.85	ESI +	Fragment 1	$C_{10}H_{11}O_2$	163.0754	163.0753	−0.61
MDEA	7.43	ESI +	Fragment 1	$C_{10}H_{11}O_2$	163.0754	163.0755	0.61
MDMA	6.26	ESI +	Fragment 1	$C_{10}H_{11}O_2$	163.0754	163.0754	0.00
Medroxyprogesterone	13.55	ESI +	$[M+H]^+$	$C_{22}H_{33}O_3$	345.2424	345.2423	−0.29
Mefenorex	8.70	ESI +	$[M+H]^+$	$C_{12}H_{18}ClN$	212.1201	212.1203	1.10
Meloxicam	12.39	ESI +	$[M+H]^+$	$C_{14}H_{14}N_3O_4S_2$	352.0420	352.0421	0.28
Meperidine	8.67	ESI +	$[M+H]^+$	$C_{15}H_{22}NO_2$	248.1645	248.1644	−0.40
Mesterolone	13.57	ESI +	$[M+H]^+$	$C_{20}H_{33}O_2$	305.2475	305.2477	0.66
Metaproterenol (Orciprenaline)	1.37	ESI +	Fragment 1	$C_{11}H_{16}NO_2$	194.1176	194.1177	0.52
Methadone	10.58	ESI +	$[M+H]^+$	$C_{21}H_{28}NO$	310.2165	310.2163	−0.64
Methamphetamine	5.89	ESI +	Fragment 1	C_9H_{11}	119.0858	119.0854	−3.36
Methandienone	11.90	ESI +	$[M+H]^+$	$C_{20}H_{29}O_2$	301.2162	301.2165	1.00
Methoxyphenamine	7.53	ESI +	Fragment 1	$C_{10}H_{13}O$	149.0961	149.0962	0.67
Methylephedrine	3.28	ESI +	$[M+H]^+$	$C_{11}H_{18}NO$	180.1383	180.1382	−0.56
Methylphenidate	8.18	ESI +	$[M+H]^+$	$C_{14}H_{20}NO_2$	234.1489	234.1487	−0.85
Meticrane	8.36	ESI +	$[M+H]^+$	$C_{10}H_{13}NO_4S_2$	276.0359	276.0359	0.00
Metolazone	10.68	ESI +	$[M+H]^+$	$C_{16}H_{17}ClN_3O_3S_2$	366.0674	366.0671	−0.82
Metoprolol	8.11	ESI +	$[M+H]^+$	$C_{15}H_{26}NO_3$	268.1907	268.1904	−1.12
Morphine	1.50	ESI +	$[M+H]^+$	$C_{17}H_{20}NO_3$	286.1438	286.1435	−1.05
Nadolol	6.39	ESI +	$[M+H]^+$	$C_{17}H_{28}NO_4$	310.2013	310.2015	0.64
Nandrolone(19-Nortestosterone)	11.71	ESI +	$[M+H]^+$	$C_{18}H_{27}O_2$	275.2006	275.2001	−1.82
Nateglinide	13.45	ESI +	$[M+H]^+$	$C_{19}H_{28}NO_3$	318.2064	318.2066	0.63
N-Desmethylselegiline	7.29	ESI +	Fragment 1	C_9H_{11}	119.0858	119.0861	2.52
Nicotine	1.36	ESI +	$[M+H]^+$	$C_{10}H_{15}N_2$	163.1230	163.1232	1.23
Norbolethone	14.39	ESI +	$[M+H]^+$	$C_{21}H_{33}O_2$	317.2475	317.2477	0.63
Norcocaine	8.73	ESI +	$[M+H]^+$	$C_{16}H_{20}NO_4$	290.1387	290.1386	−0.34
Norcodeine	2.76	ESI +	$[M+H]^+$	$C_{17}H_{20}NO_3$	286.1438	286.1439	0.35
Norethandrolone	13.38	ESI +	$[M+H]^+$	$C_{20}H_{31}O_2$	303.2319	303.2320	0.33
Norethindrone	12.28	ESI +	$[M+H]^+$	$C_{20}H_{27}O_2$	299.2006	299.2004	−0.67
Norfenfluramine	8.73	ESI +	Fragment 2	$C_{10}H_9N$	187.0729	187.0732	1.60
Norfentanyl	7.58	ESI +	$[M+H]^+$	$C_{14}H_{20}N_2O$	233.1648	233.1647	−0.43
Norgestrel	13.20	ESI +	$[M+H]^+$	$C_{21}H_{29}O_2$	313.2162	313.2162	0.00

Table 1 (*continued*)

Compound	R_t	Ioniz. mode	Ion	Ion formula	Theoretical (*m*/*z*)	Experimental (*m*/*z*)	Error (ppm)
Normorphine	1.38	ESI +	$[M+H]^+$	$C_{16}H_{18}NO_3$	272.1281	272.1282	0.37
Noroxycodone	4.10	ESI +	$[M+H]^+$	$C_{17}H_{20}NO_4$	302.1387	302.1386	−0.33
Noroxymorphone	1.52	ESI +	$[M+H]^+$	$C_{16}H_{28}NO_4$	288.1230	288.1234	1.39
Norpseudoephedrine(Cathine)	2.24	ESI +	Fragment 1	$C_9H_{12}N$	134.0964	134.0962	−1.49
Octopamine	1.02	ESI +	Fragment 1	$C_8H_{10}NO$	136.0757	136.0758	0.73
Oxilofrine	1.37	ESI +	Fragment 1	$C_{10}H_{14}NO$	164.1070	164.1072	1.22
Oxycodone	4.46	ESI +	$[M+H]^+$	$C_{18}H_{22}NO_4$	316.1545	316.1543	−0.63
Oxymorphone	1.65	ESI +	$[M+H]^+$	$C_{17}H_{20}NO_4$	302.1387	302.1389	0.66
Penbutolol	10.58	ESI +	$[M+H]^+$	$C_{18}H_{30}NO_2$	292.2271	292.2273	0.68
Pentobarbital	10.77	ESI +	Fragment 1	$C_6H_9N_2O_3$	157.0608	157.0607	−0.64
Pentoxyfilline	8.59	ESI +	$[M+H]^+$	$C_{13}H_{19}N_4O_3$	279.1452	279.1450	−0.72
Pentylenetetrazole	5.44	ESI +	$[M+H]^+$	$C_6H_{11}N_4$	139.0978	139.0978	0.00
Phenobarbital	9.67	ESI +	$[M+H]^+$	$C_{12}H_{13}N_2O_3$	233.0921	233.0922	0.43
Phenylephrine	1.37	ESI +	Fragment 1	$C_9H_{12}NO$	150.0913	150.0914	0.67
Picrotin	9.02	ESI +	$[M+H]^+$	$C_{15}H_{19}O_7$	311.1125	311.1129	1.29
Picrotoxinin	10.18	ESI +	$[M+H]^+$	$C_{15}H_{17}O_6$	293.1020	293.1023	1.02
Pindolol	6.25	ESI +	$[M+H]^+$	$C_{14}H_{21}N_2O_2$	249.1598	249.1597	−0.40
Piretanide	11.79	ESI +	$[M+H]^+$	$C_{17}H_{19}N_2O_5S$	363.1009	363.1007	−0.55
Prednisolone	9.89	ESI +	$[M+H]^+$	$C_{21}H_{29}O_5$	361.2010	361.2014	1.11
Prednisone	9.99	ESI +	$[M+H]^+$	$C_{21}H_{27}O_5$	359.1853	359.1855	0.56
Probenecid	12.51	ESI +	$[M+H]^+$	$C_{13}H_{20}NO_4S$	286.1108	286.1107	−0.35
Propafenone	10.29	ESI +	$[M+H]^+$	$C_{21}H_{28}NO_3$	342.2064	342.2066	0.58
Propoxyphene	10.50	ESI +	Fragment 1	$C_{19}H_{24}N$	266.1903	266.1903	0.00
Propranolol	9.36	ESI +	$[M+H]^+$	$C_{16}H_{22}NO_2$	260.1645	260.1646	0.38
Propylhexedrine	8.61	ESI +	$[M+H]^+$	$C_{10}H_{22}N$	156.1747	156.1747	0.00
Repaglinide	11.74	ESI +	$[M+H]^+$	$C_{27}H_{37}N_2O_4$	453.2748	453.2746	−0.44
Ritodrine	4.71	ESI +	$[M+H]^+$	$C_{17}H_{22}NO_3$	288.1594	288.1596	0.69
Salbutamol	1.65	ESI +	$[M+H]^+$	$C_{13}H_{22}NO_3$	240.1594	240.1598	1.67
Salicylamide	7.96	ESI +	$[M+H]^+$	$C_7H_8NO_2$	138.0550	138.0553	2.17
Salmeterol	10.58	ESI +	$[M+H]^+$	$C_{25}H_{38}NO_4$	416.2795	416.2796	0.24
Selegiline (Deprenil)	7.67	ESI +	$M+H]^+$	$C_{13}H_{18}N$	188.1434	188.1430	−2.12
Sotalol	2.03	ESI +	Fragment 1	$C_{12}H_{19}N_2O_2S$	255.1162	255.1160	−0.78
Spironolactone	12.86	ESI +	Fragment 1	$C_{22}H_{29}O_3$	341.2111	341.2113	0.59
Stanozol	11.89	ESI +	$[M+H]^+$	$C_{21}H_{33}N_2O$	329.2587	329.2590	0.91
Strychnine	7.28	ESI +	$[M+H]^+$	$C_{21}H_{23}N_2O_2$	335.1754	335.1755	0.30
Tamoxifen	12.39	ESI +	$[M+H]^+$	$C_{26}H_{30}NO$	372.2332	372.2330	−0.54
Terbutaline	1.72	ESI +	$[M+H]^+$	$C_{12}H_{20}NO_3$	226.1438	226.1440	0.88
Testosterone	12.16	ESI +	$[M+H]^+$	$C_{19}H_{29}O_2$	289.2162	289.2163	0.35
Tibolone	13.66	ESI +	$[M+H]^+$	$C_{21}H_{29}O_2$	313.2162	313.2164	0.64
Timolol	7.92	ESI +	$[M+H]^+$	$C_{13}H_{25}N_4O_3S$	317.1642	317.1644	0.63
Tolazamide	12.09	ESI +	$[M+H]^+$	$C_{14}H_{21}N_3O_3S$	312.1376	312.1375	−0.32
Tolbutamide	11.80	ESI +	$[M+H]^+$	$C_{12}H_{19}N_2O_3S$	271.1111	271.1110	−0.37
Torasemide	9.52	ESI +	$[M+H]^+$	$C_{16}H_{20}N_4O_3S$	349.1329	349.1330	0.29
Toremifene	12.15	ESI +	$[M+H]^+$	$C_{26}H_{29}ClNO$	406.1932	406.1930	−0.49
Triamcinolone	9.17	ESI +	$[M+H]^+$	$C_{21}H_{28}FO_6$	395.1864	395.1865	0.25
Triamterene	7.20	ESI +	$[M+H]^+$	$C_{12}H_{12}N_7$	254.1149	254.1149	0.00
Trichlormethiazide	10.19	ESI −	Fragment 1	$C_8H_5ClN_3O_4S_2$	305.9415	305.9416	0.33
Turinabol	13.04	ESI +	$[M+H]^+$	$C_{20}H_{28}ClO_2$	335.1772	335.1771	−0.30
Tyramine	1.40	ESI +	Fragment 1	C_8H_9	105.0699	105.0700	0.95
Vanillic acid diethylamide	9.32	ESI +	$[M+H]^+$	$C_{12}H_{18}NO_3$	224.1281	224.1280	−0.45
Warfarin	12.76	ESI +	$[M+H]^+$	$C_{19}H_{17}O_4$	309.1121	309.1125	1.29

which computes all of the masses in the compound spectrum (and only those masses). Once the compounds were automatically extracted, its molecular formula was generated and may be confronted against any user-created database. These databases should contain specific information such as diagnostic ions or the use of accurate-mass shifts from parent species due to typical biotransformations.

(b) *"Find by formula"*. This software tool can be also used for non-targeted detection, similarly to the targeted approach, but without retention time information included in the spreadsheet. A list of elemental compositions/ions to be searched (without retention time restriction), including diagnostic fragment ions and eventually a list of potential metabolites from parent species based on an *ab-initio* list prepared considering formula modifications/mass shifts due to biotransformations were prepared.

To obtain the specific information (diagnostic ions) required for this approach, spectral features of each compound were also studied. In-source CID fragmentation was investigated at different fragmentor voltages (160, 190 and 220 V), obtaining diagnostic fragment ions (Table S-1, Suppl. data), which can be used to identify compounds with similar structure, degradation products or metabolites [62–64].

3. Results and discussion

3.1. Identification of targeted sport drugs by LC-TOFMS accurate mass measurements

For screening purposes, the identification of the selected sport drugs was accomplished by combining retention time matching and accurate mass measurements of the selected ion, criteria in compliance with current WADA guidelines [65]. Table 1 summarizes the data of the 200 compounds tested listed in alphabetical order including retention time, elemental composition of the detected ions, theoretical and experimental accurate mass and their relative mass error at 25 μg L^{-1}

concentration level. Most of the compounds were detected in positive ion mode except 6 detected in the negative ion mode (benthiazide, chlorothiazide, furosemide, hydrochlorothiazide, hidroflumethiazide and trichlormethiazide). A single high-resolution ion (either a protonated ($[M+H]^+$) or deprotonated molecule ($[M-H]^-$)) were used for screening (detection) and quantitation purposes, except in some cases including EDDP (already charged ($[M]^+$)) and other compounds for those a fragment ion was found more appropriate due to higher signal than the respective molecule, avoiding the use of non-specific ions such as tropyllium cation (*m/z* 91) and selecting the higher mass value as possible in order to preserve selectivity. On the other hand, only a few compounds exhibited sodium adducts (e.g. 4-androstene-3,17-dione, norethandrolone, 16-β-hydroxyfurazabol) although these ions were not selected (Table S1, Suppl. data).

For the automated targeted screening, "Find by formula" software application was used. This tool is implemented to search the selected targeted list of retention time/accurate mass (elemental composition) pairs in the LC–MS raw datafile. Two main parameters affecting search criteria were optimized: accurate mass tolerance and retention time tolerance. A ± 10-mDa mass window was selected for screening purposes, although final confirmation within 5 ppm relative mass error tolerance is required for positive identification. Different retention time tolerance windows were also considered, and finally it was fixed at ± 0.2 min for screening/identification purposes. The RSD deviation of retention time were typically well below this tolerance (e.g. < 0.1%).

To each individual positive finding, the retention time and accurate mass bias is provided along with the isotope pattern matching score. This is an additional tool to further confirm the identity of a positive, based on the comparison of the experimental data with the theoretical values of the assigned elemental composition of the tentative positive. A score coefficient (scale up to 100), which considers the relative abundance of the different isotope signals of the detected species, the space (*m/z* gap) between these signals and the relative mass error is generated per positive finding.

Finally, with respect to the identification criteria, it is worthwhile mention the high mass accuracy attained with the LC-TOFMS instrument used. As shown in Table 1, the relative mass errors were below 2 ppm in most cases, being the average mass error as low as 0.72 ppm in the studied urine matrix-matched standard spiked at 25 μg L^{-1}.

Another key part of the LC-TOFMS screening method is the chromatography which is of paramount importance for appropriate identification of each individual sport drug. Several isobaric (same nominal mass) or isomeric species (same elemental composition) can be easily distinguished with an optimized LC separation. A 18-min run gradient using a short C_{18} column (50 mm) with small particle size (1.8 μm particle size) was finally selected. The use of shorter methods may yield some isobaric coeluting species and also stronger matrix effects. With the selected method, only 8 compounds eluted near the void volume (e.g. retention time ≤ 1.5 min). The targeted compounds are distributed mainly (> 80%) in the retention time range from 5 to 15 min, with the following distribution: 15% compounds between 0 and 5 min; 35% compounds between 5 and 10 min; 46% compounds between 10 and 15 min and 4% compounds from 15 to 18 min. With the selected method, no coeluting isobaric species were found amongst the selected compounds. A summary of tentative coeluting isobaric species resolved is shown in Table S2 (Suppl. data). All the species could be distinguished between themselves by retention time, mass resolving power and/or characteristic fragmentation.

3.2. Evaluation of LC-TOFMS approach for confirmatory purposes

The only document available so far related to criteria and guidelines for identification of substances by chromatographic/mass spectrometric assays [6,65] states that for molecules < 800 Da, prohibited substances with a concentration greater than approximately 100 ng mL^{-1} should have a full or partial scan acquired or shall have accurate mass determined such that the elemental composition can be determined. Whenever possible, a full-scan is the preferred option [6]. The WADA guidelines are thus very non-specific in terms of identification criteria by HRMS [6,65].

Table 1 includes the selected ion for screening purposes. Unlike other applications such as food or feed testing [66,67], yet there are no detailed guidelines in sport drug testing requiring for instance two high resolution ions for confirmation purposes) or even ion ratios. Most of the literature published so far (preventive screening method with HRMS) only included one single ion [6,44,48,52,54,59–61], with the exception of Virus et al. [46] (20 compounds using LTQ-Orbitrap) and Badoud et al. [49] (103 compounds using Q-TOF), both using dedicated CID MS/MS experiments providing at least two HR ions. Even, it is often easy to find LC–MS/MS methods based on a single screening MRM MS/MS transition [55,58]. As an alternative, Jimenez-Girón et al. [61] used an HRMS Orbitrap without collision cell and considered Na adducts and chlorine isotope signals instead of actual fragment ions. One of the drawbacks reported was the extent of matrix effects which was remarkable for these secondary ions used for confirmatory purposes.

With the aim to evaluate LC-TOFMS capabilities for confirmatory purposes, considering the measurement of 2 HR ions (within 5 ppm error) as criterion for unambiguous confirmation, two approaches were tested:

(a) In-source CID fragmentation using two different fragmentor voltages (190 and 220 V), and;

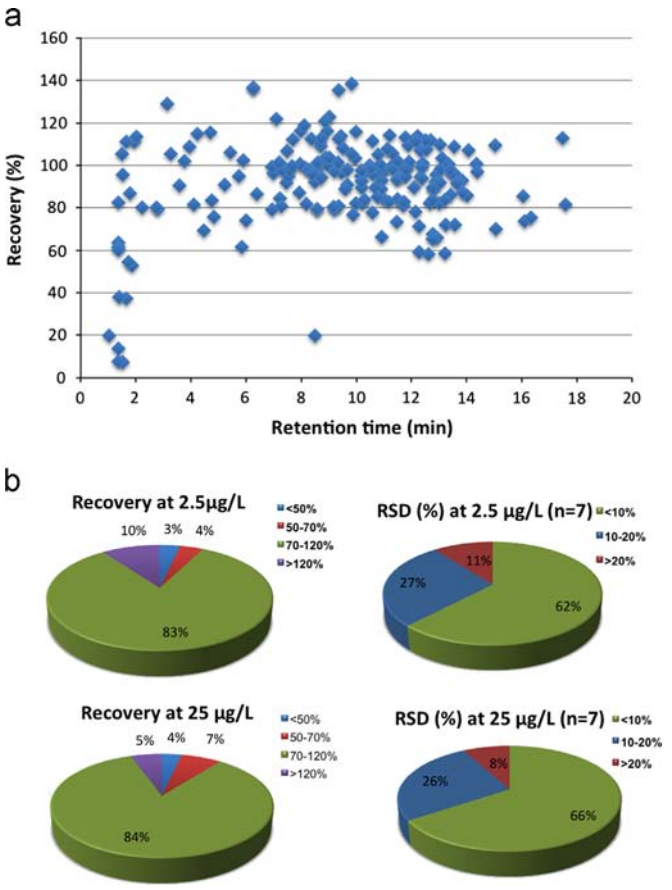

Fig. 1. (a) 2D plot representing recovery percentages for all targeted compounds over the entire LC run. (b) Cake diagrams summarizing recovery rates of the SPE at both concentration levels tested and the corresponding RSD values.

Table 2
Analytical parameters of the LC-TOFMS method for screening and quantitation of 200 sport drugs in urine.

Compound	*R_t*	*Molecular formula*	*Class*	*MRPL*[a] *(ng mL^{-1})*	*LOD (ng mL^{-1})*	*LOQ (ng mL^{-1})*	*R^2*	*Matrix effect*[b]
11-Nor-11-carboxy-Δ9-THC	15.07	$C_{21}H_{28}O_4$	Cannabinoid	15	0.12	0.41	0.9907	0.991
16-β-Hydroxyfurazabol	13.23	$C_{20}H_{30}N_2O_3$	Steroid	10	0.38	1.3	0.9935	1.021
17,17-Dimethyl-18-androsta-1,4,13(14)-trien-3-one	17.59	$C_{20}H_{26}O$	Steroid	10	0.16	0.53	0.9971	0.914
17-α-Ethynil estradiol	12.33	$C_{20}H_{24}O_2$	Strogen agonist	50	7.26	24.0	0.9970	0.607
17-α-Methyltestosterone	12.62	$C_{20}H_{30}O_2$	Steroid	10	0.061	0.20	0.9987	0.777
17-α-Hydroxyprogesterone	12.80	$C_{21}H_{30}O_3$	Steroid	10	0.030	0.10	0.9993	0.964
17-Epioxandrolone	12.89	$C_{19}H_{30}O_3$	Oxandrolone Met. (Steroid)	10	0.13	0.42	0.9983	1.000
19-Norandrosterone	13.43	$C_{18}H_{28}O_2$	Steroid	10	–[c]	–[c]	0.9991	0.823
19-Norethiocholanone	13.00	$C_{18}H_{28}O_2$	Steroid	10	0.30	1.0	0.9981	0.953
2-Aminoheptane	6.93	$C_7H_{17}N$	Stimulant	500	0.091	0.30	0.9888	1.274
2-Hydroxymethyl-17α-methylandrosta-1,4-diene-11α,17β-diol-3-one	9.35	$C_{21}H_{30}O_4$	Formebolone Met. (Steroid)	10	0.032	0.11	0.9979	0.808
2α-Hydroxymethylethisterone	11.48	$C_{22}H_{30}O_3$	Steroid	10	0.030	0.10	0.9992	0.983
2α-Methyl-5α-Androstan-3α-ol-17-one	15.04	$C_{20}H_{32}O_2$	Drostanolone Met. (Steroid)	10	0.28	0.93	0.9988	0.981
4-Androstene-3,17-dione	12.78	$C_{19}H_{26}O_2$	Steroid	10	0.024	0.081	0.9979	0.741
4-Hydroxyandrostendione (Formestane)	13.06	$C_{19}H_{26}O_3$	Steroid	10	0.18	0.60	0.9995	0.934
4-Hydroxytestosterone	12.33	$C_{19}H_{28}O_3$	Steroid	10	0.13	0.43	0.9991	0.912
5-α-Dihydroxytestosterone	13.22	$C_{19}H_{30}O_2$	Steroid	10	0.21	0.69	0.9998	0.833
6α-Methylprednisolone	10.48	$C_{22}H_{30}O_5$	Glucocorticosteroid	30	0.032	0.11	0.9993	1.004
Acebutolol	7.97	$C_{18}H_{28}N_2O_4$	β-Blocker	500	0.0060	0.021	0.9949	1.263
Acepromazine	9.95	$C_{19}H_{22}N_2OS$	Narcotic	200	0.012	0.041	0.9983	0.948
Acetazolamide	3.58	$C_4H_6N_4O_3S_2$	Diuretic	250	1.2	4.1	0.9942	0.789
Acethylmorphine	6.00	$C_{19}H_{21}NO_4$	Narcotic	200	0.12	0.40	0.9930	0.854
Adrafinil	9.68	$C_{15}H_{15}NO_3S$	Stimulant	500	0.32	1.1	0.9892	0.972
α-Zearalanol	11.79	$C_{18}H_{26}O_5$	Strogen agonist	50	0.21	0.71	0.9927	0.937
Alprenolol	9.43	$C_{15}H_{23}NO_2$	β-Blocker	500	0.016	0.052	0.9984	0.857
Althiazide	10.91	$C_{11}H_{14}ClN_3O_4S_3$	Diuretic	250	0.45	1.5	0.9960	1.736
Altrenogest	13.11	$C_{21}H_{26}O_2$	Steroid	10	0.026	0.088	0.9920	0.954
Amcinonide	14.03	$C_{28}H_{35}FO_7$	Glucocorticosteroid	30	0.015	0.048	0.9984	0.862
Aminoglutethimide	3.95	$C_{13}H_{16}N_2O_2$	Aromatase Inhibitor	50	0.9	3.0	0.9990	0.808
Amphetamine	3.75	$C_9H_{13}N$	Stimulant	500	7.6	25.0	0.9870	1.176
Anastrazole	11.03	$C_{17}H_{19}N_5$	Aromatase Inhibitor	50	0.013	0.045	0.9996	0.948
Androsterone	13.84	$C_{19}H_{30}O_2$	Steroid	10	0.23	0.78	0.9996	0.963
Atenolol	1.78	$C_{14}H_{22}N_2O_3$	β-Blocker	500	0.053	0.18	0.9997	0.891
Bambuterol	8.62	$C_{18}H_{29}N_3O_5$	β2-Agonist	100	0.012	0.040	0.9992	1.064
Beclomethasone	10.93	$C_{22}H_{29}ClO_5$	Glucocorticosteroid	30	0.024	0.078	0.9995	1.336
Bendroflumethiazide	11.84	$C_{15}H_{14}F_3N_3O_4S_2$	Diuretic	250	0.28	0.94	0.9981	0.858
Benthiazide	11.36	$C_{15}H_{14}ClN_3O_4S_3$	Diuretic	250	0.030	0.099	0.9994	0.779
Benzoylecgonine	7.69	$C_{16}H_{19}NO_4$	Cocaine Met. (Stimulant)	500	0.011	0.035	0.9989	0.575
β-Estradiol	11.85	$C_{18}H_{24}O_2$	Strogen agonist	50	0.26	0.86	0.9936	0.785
Betaxolol	9.47	$C_{18}H_{29}NO_3$	β-Blocker	500	0.006	0.019	0.9955	1.050
Bis(4-cyanophenyl) methanol	11.46	$C_{15}H_{10}N_2O$	Letrozole Met. (Aromatase Inhibitor)	50	0.71	2.4	0.9969	0.706
Bisoprolol	8.94	$C_{18}H_{31}NO_4$	β-Blocker	500	0.005	0.016	0.9994	0.964
Boldenone	11.48	$C_{19}H_{26}O_2$	Steroid	10	0.084	0.28	0.9990	0.830
Boldine	7.22	$C_{19}H_{21}NO_4$	Broncodilatador	–	0.027	0.092	0.9990	1.140
Brucine	7.47	$C_{23}H_{26}N_2O_4$	Stimulant	500	0.035	0.12	0.9569	0.711
Bumetanide	12.23	$C_{17}H_{20}N_2O_5S$	Diuretic	250	0.035	0.12	0.9987	0.955
Buprenorphine	9.82	$C_{29}H_{41}NO_4$	Narcotic	10	0.005	0.017	0.9971	0.828
Bupropion	8.90	$C_{13}H_{18}ClNO$	Stimulant	500	0.062	0.21	0.9981	0.712
Butabarbital	9.84	$C_{10}H_{16}N_2O_3$	Narcotic	200	2.1	7.1	0.9893	0.363
Cannabidiol	16.11	$C_{21}H_{30}O_2$	Cannabinoid	15	0.45	1.5	0.9938	1.152
Canrenone	13.02	$C_{22}H_{28}O_3$	Masking Agent	250	0.029	0.096	0.9976	0.939
Capsaicin	12.92	$C_{18}H_{27}NO_3$	Analgesic	–	0.033	0.11	0.9992	1.005
Carphedone	8.49	$C_{12}H_{14}N_2O_2$	Stimulant	500	0.12	0.42	0.9941	0.696
Carvedilol	10.20	$C_{24}H_{26}N_2O_4$	β-Blocker	500	0.012	0.040	0.9991	1.028
Celecoxib	13.85	$C_{17}H_{14}F_3N_3SO_2$	Anti-inflamatory	–	0.032	0.11	0.9915	0.990
Celiprolol	8.55	$C_{20}H_{33}N_3O_4$	β-Blocker	500	0.005	0.016	0.9959	1.098
Chlorothiazide	4.75	$C_7H_6ClN_3O_4S_2$	Diuretic	250	0.32	1.1	0.9981	0.679
Clenbuterol	8.14	$C_{12}H_{18}Cl_2N_2O$	Anabolic agent	2	0.044	0.15	0.9992	0.968
Clopamide	9.37	$C_{14}H_{20}ClN_3O_3S$	Diuretic	250	0.010	0.035	0.9991	0.940
Clostebol acetate	16.06	$C_{21}H_{29}ClO_3$	Steroid	10	0.14	0.47	0.9951	1.052
Cocaethylene	9.19	$C_{18}H_{23}NO_4$	Cocaine Met. (Stimulant)	500	0.006	0.019	0.9990	0.927
Cocaine	8.59	$C_{17}H_{21}NO_4$	Stimulant	500	0.16	0.53	0.9948	0.822
Codeine	3.13	$C_{18}H_{21}NO_3$	Narcotic	200	0.047	0.16	0.9994	0.716
Cotinine	1.37	$C_{10}H_{12}N_2O$	Stimulant	500	0.048	0.16	0.9977	0.828
Cyclofenil	16.33	$C_{23}H_{24}O_4$	SERM	50	0.34	1.1	0.9983	1.130
Cyclothiazide	11.50	$C_{14}H_{16}ClN_3O_4S_2$	Diuretic	250	3.7	12.0	0.9989	0.781
Danazol	14.35	$C_{22}H_{27}NO_2$	Steroid	10	0.020	0.066	0.9967	0.961
Dehydroandrosterone	12.66	$C_{19}H_{28}O_2$	Steroid	10	1.4	4.7	0.9949	0.733
Δ9-THC	17.47	$C_{21}H_{30}O_2$	Cannabinoid	15	4.1	13.0	0.9962	1.124
Dexamethasone	10.72	$C_{22}H_{29}FO_5$	Glucocorticosteroid	30	0.050	0.17	0.9965	0.426
Diazepam	12.40	$C_{16}H_{13}ClN_2O$	Narcotic	200	0.016	0.054	0.9996	0.936
Diethylnicotinamide (Niketamide)	4.23	$C_{10}H_{14}N_2O$	Stimulant	500	0.15	0.51	0.9985	1.183

Table 2 (*continued*)

Compound	R_t	*Molecular formula*	*Class*	*MRPL*[a] (*ng mL*$^{-1}$)	*LOD* (*ng mL*$^{-1}$)	*LOQ* (*ng mL*$^{-1}$)	R^2	*Matrix effect*[b]
Dimethylphenetylamine	5.22	$C_{10}H_{15}N$	Stimulant	500	3.0	10.0	0.9975	1.233
Doxapram	8.93	$C_{24}H_{30}N_2O_2$	Stimulant	500	0.021	0.071	0.9988	0.851
EDDP	10.16	$C_{20}H_{24}N$	Methadone Met. (Narcotic)	200	0.007	0.024	0.9988	1.115
Ephedrine	2.79	$C_{10}H_{15}NO$	Stimulant	500	0.082	0.27	0.9991	0.936
Epitestosterone	12.76	$C_{19}H_{28}O_2$	Steroid	10	0.071	0.24	0.9983	1.000
Eplerenone	10.87	$C_{24}H_{30}O_6$	Diuretic	250	0.065	0.22	0.9933	0.774
Esmolol	8.57	$C_{16}H_{25}NO_4$	β-Blocker	500	0.009	0.028	0.9967	1.005
Ethacrinic acid	12.77	$C_{13}H_{12}Cl_2O_4$	Diuretic	250	0.78	2.6	0.9941	0.788
Ethiocholanone	13.56	$C_{19}H_{30}O_2$	Testosterone Met.(Steroid)	10	1.3	4.2	0.9993	1.080
Ethisterone	12.70	$C_{21}H_{28}O_2$	Steroid	10	0.048	0.16	0.9994	0.987
Ethylamphetamine	6.99	$C_{11}H_{17}N$	Stimulant	500	2.4	8.0	0.9935	0.884
Ethylmorphine	6.95	$C_{19}H_{23}NO_3$	Narcotic	200	0.020	0.066	0.9989	0.879
Famprofazone	10.21	$C_{24}H_{31}N_3O$	Stimulant	500	0.009	0.031	0.9988	0.916
Fenbutrazate	11.01	$C_{23}H_{29}NO_3$	Stimulant	500	0.008	0.025	0.9968	1.154
Fenfluramine	9.21	$C_{12}H_{16}F_3N$	Stimulant	500	0.054	0.18	0.9967	0.780
Fenspiride	4.83	$C_{15}H_{20}N_2O_2$	α-Adrenergic blocker	50	0.083	0.28	0.9987	0.733
Fentanyl	9.60	$C_{22}H_{28}N_2O$	Narcotic	10	0.013	0.044	0.9997	0.910
Finasteride	12.28	$C_{23}H_{36}N_2O_2$	Steroid	10	0.006	0.021	0.9988	0.968
Flumethasone	10.79	$C_{22}H_{28}F_2O_5$	Glucocorticosteroid	30	0.050	0.17	0.9974	0.852
Flunisolide	11.19	$C_{24}H_{31}FO_6$	Glucocorticosteroid	30	0.038	0.13	0.9968	0.980
Fluocinolone acetonide	11.30	$C_{24}H_{30}F_2O_6$	Glucocorticosteroid	30	0.033	0.11	0.9958	0.915
Fluorometholone	11.54	$C_{22}H_{29}FO_4$	Glucocorticosteroid	30	0.025	0.084	0.9996	0.982
Fluoxymesteorone	10.90	$C_{20}H_{29}FO_3$	Steroid	10	0.065	0.22	0.9968	0.889
Flurandrenolide	11.32	$C_{24}H_{33}FO_6$	Glucocorticosteroid	30	0.069	0.23	0.9968	0.914
Fluticasone propionate	14.07	$C_{25}H_{31}F_3O_5S$	Glucocorticosteroid	30	0.017	0.058	0.9997	0.937
Formoterol	8.28	$C_{19}H_{24}N_2O_4$	β2-Agonist	100	0.026	0.086	0.9998	1.082
Furosemide	10.74	$C_{12}H_{11}ClN_2O_5S$	Diuretic	250	0.85	2.8	0.9934	0.133
Gestrinone	12.54	$C_{21}H_{24}O_2$	Steroid	10	0.038	0.13	0.9996	0.998
Glibenclamide	13.35	$C_{23}H_{28}ClN_3O_5S$	Antidiabetic	–	0.020	0.066	0.9923	0.991
Glipizide	11.46	$C_{21}H_{27}N_5O_4S$	Antidiabetic	–	0.016	0.054	0.9967	0.956
Heptaminol	1.53	$C_8H_{19}NO$	Stimulant	500	0.063	0.21	0.9987	1.110
Heroin	8.39	$C_{21}H_{23}NO_5$	Narcotic	200	0.039	0.13	0.9907	0.633
Hexobarbital	10.91	$C_{12}H_{16}N_2O_3$	Narcotic	200	2.0	6.6	0.9997	0.554
Hydrochlorothiazide	5.73	$C_7H_8ClN_3O_4S_2$	Diuretic	250	0.24	0.80	0.9953	0.545
Hydrocortison	9.98	$C_{21}H_{30}O_5$	Cortisol Met. (Glucocorticosteroid)	30	0.032	0.11	0.9957	0.762
Hydroflumethiazide	8.49	$C_8H_8F_3N_3O_4S_2$	Diuretic	250	0.26	0.86	0.9925	0.151
Hydromorphone	1.94	$C_{17}H_{19}NO_3$	Narcotic	200	0.044	0.15	0.9984	0.809
Ibuprofen	13.69	$C_{13}H_{18}O_2$	Anti-inflamatory	–	0.64	2.1	0.9989	0.774
Indapamide	11.20	$C_{16}H_{16}ClN_3O_3S$	Diuretic	250	0.028	0.092	0.9990	0.684
Isoetharine	1.85	$C_{13}H_{21}NO_3$	β2-Agonist	100	0.056	0.18	0.9971	3.810
Ketamine	7.48	$C_{13}H_{16}ClNO$	Stimulant	500	0.049	0.16	0.9974	0.838
Labetalol	9.02	$C_{19}H_{24}N_2O_3$	β-Blocker	500	0.038	0.13	0.9985	1.029
Letrozole	11.13	$C_{17}H_{11}N_5$	Aromatase Inhibitor	50	0.14	0.48	0.9953	0.924
Lidocaine	7.10	$C_{14}H_{22}N_2O$	Anesthetic	–	0.045	0.15	0.9954	0.969
LSD	8.86	$C_{20}H_{25}N_3O$	Narcotic	200	0.011	0.038	0.9990	0.831
MDA	5.85	$C_{10}H_{13}NO_2$	Stimulant	500	0.31	1.0	0.9961	0.469
MDEA	7.43	$C_{12}H_{17}NO_2$	Stimulant	500	0.11	0.36	0.9987	0.545
MDMA	6.26	$C_{11}H_{15}NO_2$	Stimulant	500	0.096	0.32	0.9957	1.599
Medroxyprogesterone	13.55	$C_{22}H_{32}O_3$	Steroid	10	0.026	0.088	0.9988	1.001
Mefenorex	8.70	$C_{12}H_{18}ClN$	Stimulant	500	0.08	0.30	0.9991	1.423
Meloxicam	12.39	$C_{14}H_{13}N_3O_4S_2$	Anti-inflamatory	–	0.003	0.009	0.9989	0.973
Meperidine	8.67	$C_{15}H_{21}NO_2$	Narcotic	200	0.017	0.056	0.9982	0.930
Mesterolone	13.57	$C_{20}H_{32}O_2$	Steroid	10	0.15	0.50	0.9998	1.007
Metaproterenol (Orciprenaline)	1.37	$C_{11}H_{17}NO_3$	β2-Agonist	100	0.10	0.34	0.9855	0.949
Methadone	10.58	$C_{21}H_{27}NO$	Narcotic	200	0.009	0.030	0.9967	0.939
Methamphetamine	5.89	$C_{10}H_{15}N$	Stimulant	500	3.0	10.0	0.9934	0.955
Methandienone	11.90	$C_{20}H_{28}O_2$	Steroid	2	0.027	0.089	0.9989	0.945
Methoxyphenamine	7.53	$C_{11}H_{17}NO$	Stimulant	500	0.10	0.34	0.9987	0.712
Methylephedrine	3.28	$C_{11}H_{17}NO$	Stimulant	500	0.15	0.49	0.9997	1.096
Methylphenidate	8.18	$C_{14}H_{19}NO_2$	Stimulant	500	0.027	0.091	0.9983	1.161
Meticrane	8.36	$C_{10}H_{13}NO_4S_2$	Diuretic	250	0.55	1.8	0.9921	0.485
Metolazone	10.68	$C_{16}H_{16}ClN_3O_3S$	Diuretic	250	0.028	0.092	0.9980	0.936
Metoprolol	8.11	$C_{15}H_{25}NO_3$	β-Blocker	500	0.034	0.11	0.9994	1.008
Morphine	1.50	$C_{17}H_{19}NO_3$	Narcotic	200	0.029	0.096	0.9975	0.519
Nadolol	6.39	$C_{17}H_{27}NO_4$	β-Blocker	500	0.033	0.11	0.9994	1.192
Nandrolone (19-Nortestosterone)	11.71	$C_{18}H_{26}O_2$	Steroid	10	0.043	0.14	0.9989	0.968
Nateglinide	13.45	$C_{19}H_{27}NO_3$	Antidiabetic	–	0.020	0.068	0.9977	1.025
N-Desmethylselegiline	7.29	$C_{12}H_{15}N$	Selegiline Met. (Stimulant)	500	1.2	4.0	0.9938	0.882
Nicotine	1.36	$C_{10}H_{14}N_2$	Stimulant	500	0.66	2.2	0.9956	0.192
Norbolethone	14.39	$C_{21}H_{32}O_2$	Steroid	10	0.006	0.021	0.9977	1.054
Norcocaine	8.73	$C_{16}H_{19}NO_4$	Cocaine Met. (Stimulant)	500	0.43	1.4	0.9977	0.653
Norcodeine	2.76	$C_{17}H_{19}NO_3$	Codeine Met. (Narcotic)	200	0.054	0.18	0.9970	0.727
Norethandrolone	13.38	$C_{20}H_{30}O_2$	Steroid	10	0.063	0.21	0.9992	1.014
Norethindrone	12.28	$C_{20}H_{26}O_2$	Steroid	10	0.032	0.11	0.9997	1.003

Table 2 (*continued*)

Compound	R_t	*Molecular formula*	*Class*	*MRPL*[a] (*ng mL*$^{-1}$)	*LOD* (*ng mL*$^{-1}$)	*LOQ* (*ng mL*$^{-1}$)	R^2	*Matrix effect*[b]
Norfenfluramine	8.73	$C_{10}H_{12}F_3N$	Fenfluramine Met. (Stimulant)	500	0.067	0.22	0.9989	1.000
Norfentanyl	7.58	$C_{14}H_{20}N_2O$	Fentanyl Met.(Narcotic)	10	0.033	0.11	0.9979	0.747
Norgestrel	13.20	$C_{21}H_{28}O_2$	Steroid	10	0.052	0.18	0.9992	1.001
Normorphine	1.38	$C_{16}H_{17}NO_3$	Morphine Met. (Narcotic)	200	0.029	0.096	0.9946	0.648
Noroxycodone	4.10	$C_{17}H_{19}NO_4$	Narcotic	200	0.24	0.80	0.9932	0.838
Noroxymorphone	1.52	$C_{16}H_{17}NO_4$	Narcotic	200	0.062	0.21	0.9988	0.578
Norpseudoephedrine(Cathine)	2.24	$C_9H_{13}NO$	Stimulant	500	0.058	0.20	0.9966	0.918
Octopamine	1.02	$C_8H_{11}NO_2$	Stimulant	500	1.7	5.6	0.9909	0.921
Oxilofrine	1.37	$C_{10}H_{15}NO_2$	Stimulant	500	0.065	0.22	0.9966	0.624
Oxycodone	4.46	$C_{18}H_{21}NO_4$	Narcotic	200	0.096	0.32	0.9868	0.737
Oxymorphone	1.65	$C_{17}H_{19}NO_4$	Narcotic	200	0.081	0.27	0.9966	0.927
Penbutolol	10.58	$C_{18}H_{29}NO_2$	β-Blocker	500	0.012	0.039	0.9968	0.945
Pentobarbital	10.77	$C_{11}H_{18}N_2O_3$	Narcotic	200	1.8	6.1	0.9997	0.567
Pentoxyfilline	8.59	$C_{13}H_{18}N_4O_3$	Anticoagulant	–	0.16	0.52	0.9995	0.790
Pentylenetetrazole	5.44	$C_6H_{10}N_4$	Stimulant	500	0.49	1.6	0.9988	0.584
Phenobarbital	9.67	$C_{12}H_{12}N_2O_3$	Narcotic	200	2.0	6.5	0.9907	0.596
Phenylephrine	1.37	$C_9H_{13}NO_2$	Stimulant	500	0.051	0.17	0.9968	0.733
Picrotin	9.02	$C_{15}H_{18}O_7$	Stimulant	500	1.5	4.9	0.9988	0.253
Picrotoxinin	10.18	$C_{15}H_{16}O_6$	Stimulant	500	15	50	0.9935	0.287
Pindolol	6.25	$C_{14}H_{20}N_2O_2$	β-Blocker	500	0.072	0.24	0.9952	0.686
Piretanide	11.79	$C_{17}H_{18}N_2O_5S$	Diuretic	250	0.013	0.044	0.9964	0.994
Prednisolone	9.89	$C_{21}H_{28}O_5$	Glucocorticosteroid	30	0.043	0.14	0.9950	0.809
Prednisone	9.99	$C_{21}H_{26}O_5$	Glucocorticosteroid	30	0.061	0.20	0.9939	0.791
Probenecid	12.51	$C_{13}H_{19}NO_4S$	Diuretic	250	0.058	0.19	0.9997	0.933
Ppropafenone	10.29	$C_{21}H_{27}NO_3$	Antiarrhythmic Agent	–	0.010	0.035	0.9982	0.921
Propoxyphene	10.50	$C_{22}H_{29}NO_2$	Analgesic	–	0.050	0.17	0.9967	1.017
Propranolol	9.36	$C_{16}H_{21}NO_2$	β-Blocker	500	0.015	0.051	0.9992	0.805
Propylhexedrine	8.61	$C_{10}H_{21}N$	Stimulant	500	0.043	0.14	0.9958	0.990
Repaglinide	11.74	$C_{27}H_{36}N_2O_4$	Antidiabetic	–	0.002	0.006	0.9991	1.114
Ritodrine	4.71	$C_{17}H_{21}NO_3$	β2-Agonist	100	0.046	0.15	0.9983	0.810
Salbutamol	1.65	$C_{13}H_{21}NO_3$	β2-Agonist	100	0.020	0.067	0.9971	1.007
Salicylamide	7.96	$C_7H_7NO_2$	Analgesic	–	0.18	0.60	0.9971	0.874
Salmeterol	10.58	$C_{25}H_{37}NO_4$	β2-Agonist	100	0.010	0.032	0.9967	1.096
Selegiline (Deprenil)	7.67	$C_{13}H_{17}N$	Stimulant	500	0.3	1.0	0.9931	0.755
Sotalol	2.03	$C_{12}H_{20}N_2O_3S$	β-Blocker	500	0.081	0.27	0.9945	6.540
Spironolactone	12.86	$C_{24}H_{32}O_4S$	Diuretic	250	0.058	0.19	0.9976	0.939
Stanozol	11.89	$C_{21}H_{32}N_2O$	Steroid	2	0.006	0.019	0.9940	0.911
Strychnine	7.28	$C_{21}H_{22}N_2O_2$	Stimulant	200	0.035	0.12	0.9959	0.896
Tamoxifen	12.39	$C_{26}H_{29}NO$	SERM	50	0.016	0.055	0.9963	1.421
Terbutaline	1.72	$C_{12}H_{19}NO_3$	β2-Agonist	100	0.11	0.35	0.9979	1.055
Testosterone	12.16	$C_{19}H_{28}O_2$	Steroid	10	–[c]	–[c]	0.9993	1.012
Tibolone	13.66	$C_{21}H_{28}O_2$	Steroid	10	0.52	1.8	0.9928	0.962
Timolol	7.92	$C_{13}H_{24}N_4O_3S$	β-Blocker	500	0.026	0.087	0.9978	0.867
Tolazamide	12.09	$C_{14}H_{21}N_3O_3S$	Antidiabetic	–	0.014	0.045	0.9972	1.046
Tolbutamide	11.80	$C_{12}H_{18}N_2O_3S$	Antidiabetic	–	0.12	0.39	0.9989	0.957
Torasemide	9.52	$C_{16}H_{20}N_4O_3S$	Diuretic	250	0.011	0.037	0.9956	0.843
Toremifene	12.15	$C_{26}H_{28}ClNO$	SERM	50	0.012	0.039	0.9926	1.296
Triamcinolone	9.17	$C_{21}H_{27}FO_6$	Glucocorticosteroid	30	0.20	0.68	0.9989	0.821
Triamterene	7.20	$C_{12}H_{11}N_7$	Diuretic	250	0.017	0.057	0.9990	0.873
Trichlormethiazide	10.19	$C_8H_8Cl_3N_3O_4S_2$	Diuretic	250	0.30	1.0	0.9965	0.216
Turinabol	13.04	$C_{20}H_{27}ClO_2$	Diuretic	250	0.055	0.18	0.9991	1.001
Tyramine	1.40	$C_8H_{11}NO$	Stimulant	500	5.2	17	0.9940	0.968
Vanillic acid diethylamide	9.32	$C_{12}H_{17}NO_3$	Stimulant	500	0.22	0.72	0.9987	0.879
Warfarin	12.76	$C_{19}H_{16}O_4$	Anticoagulant	–	0.036	0.12	0.9987	0.885

[a] MRPLs: WADA minimum required performance levels.
[c] LOD/LOQ not calculated (endogenous species).
[b] Calculated using the ratio of the calibration curve slopes obtained with urine extracts and with solvent-based standards (slope matrix/slope solvent (ME)). ME > 1 means signal enhancement while ME < 1 means signal suppression (the more common phenomenon).

(b) CID MS/MS fragmentation without precursor ion isolation (commercially called "all ion mode" or "MS^E"), an approach analogous to in-source CID carried out in a dedicated collision cell (instead of the ion transportation region), fully compatible with full-scan acquisition of LC-TOFMS instruments,

In-source CID fragmentation was studied for each individual compound and is summarized in Table S1 (Suppl. data), including the assigned elemental composition for each fragment ion. Data was acquired at two fragmentor voltages (190 and 220 V), using either a single injection for each voltage, or both experiments in the same run, which involved a slight decrease of sensitivity (*ca.* 20%) due to reduced acquisition time (500 ms in each experiment). Nearly 70% of the studied compounds exhibited at least one fragment ion with relevant relative abundance (e.g. > 10%) at the tested fragmentor voltages. In contrast, about 60 compounds only displayed the protonated molecule at the optimized fragmentor voltages. In a few cases (benthiazide, chlorothiazide, clopamide, diazepam, ethacrinic acid, indapamide and toremifene) these compounds displayed additional information due to A+2 signals

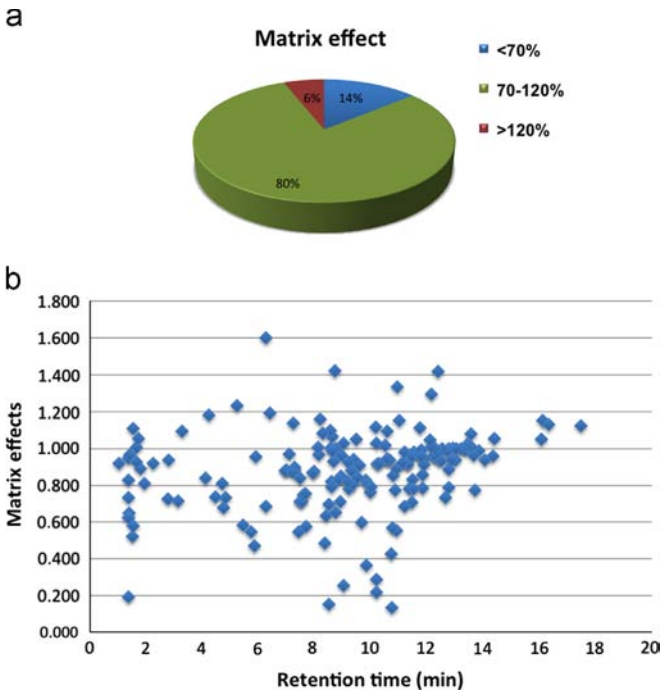

Fig. 2. (a) Cake diagrams summarizing matrix effects. (b) 2D plot representing matrix effects for all targeted compounds over the entire LC run. Data from isoethanine (3.81) and selegiline (6.54) not represented.

from chlorine atoms, that can also be used for confirmation purposes. The list of compounds difficult to be fragmented with in-source CID is detailed in Table S3 (Suppl. data).

At this point it is important to mention that the use of "all ion mode" preserves full-scan acquisition flexibility and benefits of acquiring all the information all the time without time window boundaries (scheduled precursor ion lists), but also adding the ability to cleave molecules which requires high energy to provide fragmentation due to the use of a dedicated collision cell. Given the number of potentially coeluting analyes, and considering the fact that 80% of the targeted species are concentrated on the middle section of the chromatographic run, the use of all ion mode seems to be better suited than dedicated CID MS/MS with precursor isolation, since several MS/MS features can be collected from coeluting species without loss of sensitivity. A dedicated MS/MS method development would be required instead, and eventually may produce a significant loss of sensitivity and also of information of the sample. Without precursor selection, this is no longer a problem, although at the expense of an inherent loss of specificity compared to precursor ion isolation MS/MS spectra. The fragmentation of the species which were difficult to cleave with in-source CID were satisfactorily accomplished, providing at least an additional fragment ion in all cases with the exception of 2-aminoheptane, a low-molecular weight compound which could not be fragmented whatsoever.

Therefore, LC-TOFMS using in-source CID provided information for 70%, a value reasonably high, but not as comprehensive as results obtained with "all ion mode" CID MS/MS fragmentation without precursor isolation. The use of two experiments at for

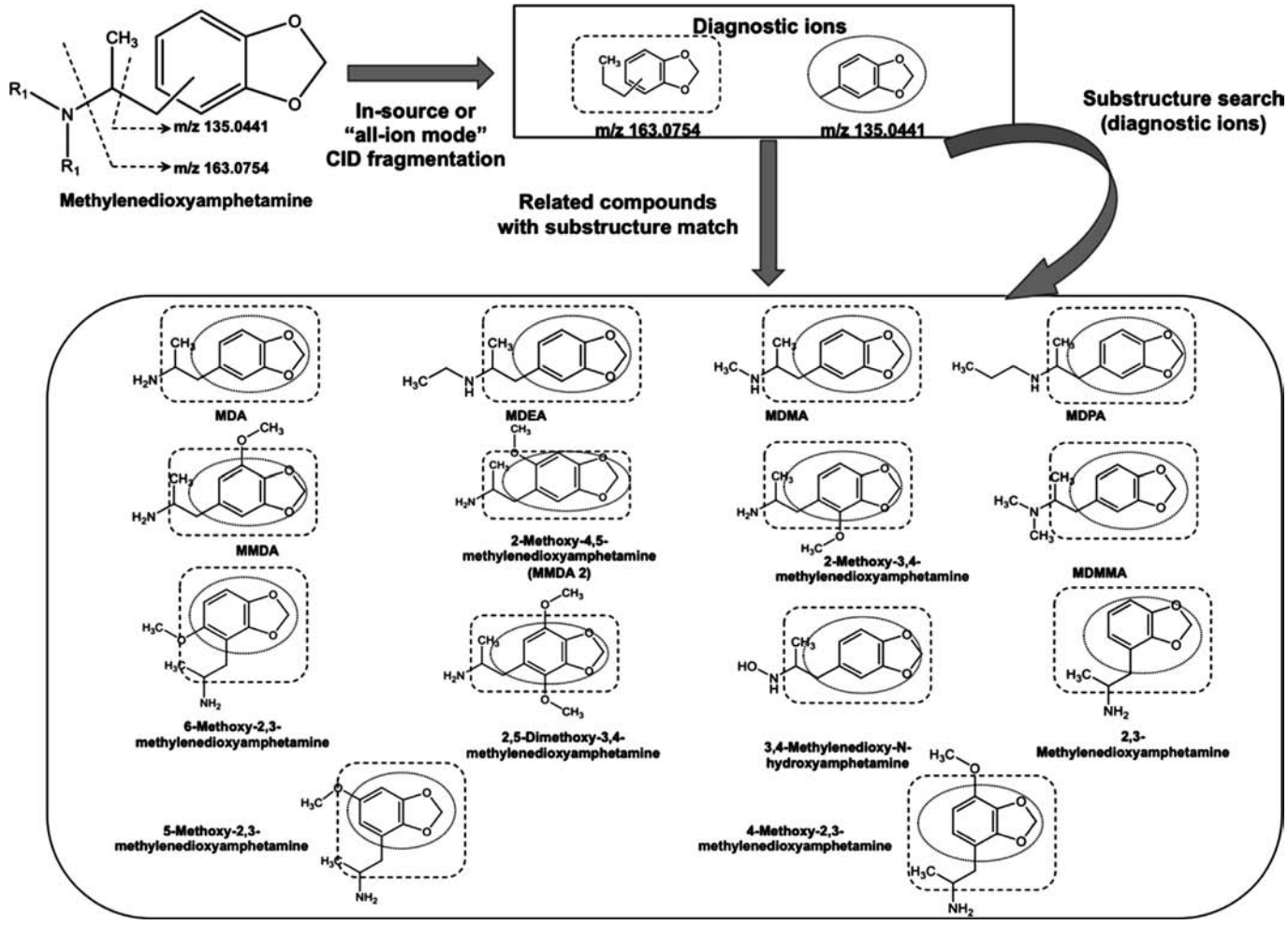

Fig. 3. Fragmentation of a generic methylenedioxyamphetamine (MDMA) generating two diagnostic ions and set of related compounds with the same moiety/substructure.

instance 5 and 20 V (collision energy) provided detailed information for confirmation purposes in a single run.

3.3. Method performance for quantitation purposes

The performance of the extraction method was studied at two concentration levels (2.5 and 25 μg L^{-1}) ($n=7$). The results obtained are summarized in Fig. 1, with all the detailed recovery data included in Table S4 (Suppl. data) The SPE method exhibited high recoveries for the majority of the sport drugs at both concentration levels tested (2.5 and 25 μg L^{-1}), ranging between 70 and 120% for over 80% of the compounds tested, displaying also appropriate precision with relative standard deviation below 20% for approximately 90% of the compounds at both concentration levels tested.

The overall results are positive given the variety of compounds included in the method This can be partly attributed to the SPE cartridge used, which displays different retention mechanisms, both ionic-exchange type and nonpolar adsorption mechanisms. Only lower recoveries were obtained for some compounds eluting near the void (Fig. 1(b)), perhaps due to poor retention in the cartridge because of their relatively high hydrophobicity. Anyway, the recoveries were enough to fulfill WADA minimum required performance levels. It should be remarked that no deconjugation step was included in the present method. In order to recover highly metabolized (e.g. yielding glucuronide derivatives) compounds such as norandrosterone, ethiocholanone, epitestosterone or gestrinone, an additional enzymatic hydrolysis step should be included prior to SPE step.

In order to evaluate the linearity of the proposed method, calibration curves of the 200 targeted drugs were constructed at 9 different concentrations in the range 0.25–125 μg L^{-1} using blank urine extracts. The obtained results are shown in Table 2 where the limits of detection and quantitation are summarized together with the matrix effects and the linearity for each compound. LOQs were empirically calculated using the data acquired from recovery experiments (2.5 and 25 μg L^{-1}), or by subsequent dilutions of these extracts with non-spiked matrix. The results obtained were satisfactory with all analytes tested in compliance with the minimum performance required by WADA

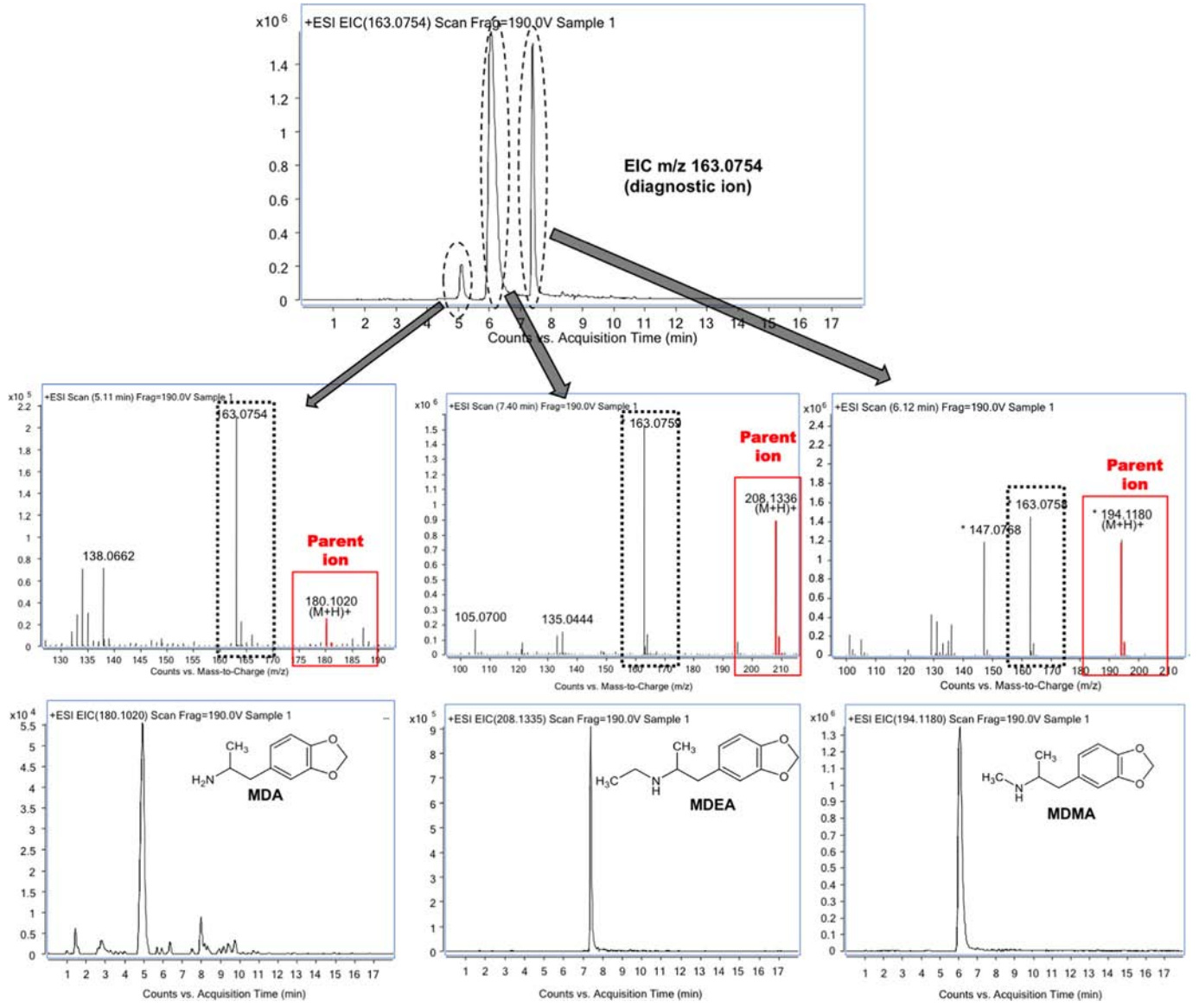

Fig. 4. Example of a urine sample spiked with three methylenedioxyamphetamine derivatives. EIC of the diagnostic ion at *m/z* 163.0754 and the compounds detected with this diagnostic ion.

standards (Table 2). The proposed method showed good quantitation limits, lower than 0.5 μg L^{-1} for 72% of the compounds and lower than 1 μg L^{-1} for 81% of the included compounds.

No isotopically labelled internal standard (ILIS) were used in the present method, as it is difficult to address and compensate matrix effects (ME) for 200 species using a reduced number of non-analogue ILIS for all the species included. We considered that the use of 3–4 ILIS distributed throughout the LC run would not improve and compensate ME, which vary a significantly during the run as shown in Fig. 2(a), even amongst compounds with similar retention time. Given the complexity and variability of the sample, it is difficult to compensate ME even using the specific analogue ILIS of an analyte, due to the variability of urine matrix (e.g. specific gravity values strongly altered ME compensation by ILIS) [68] or even due to significant isotope effects [69]. For this reason, the correction of ME was accomplished via matrix-matched calibration. ME were evaluated by comparing the slopes of the calibration with matrix-matched standards (urine extracts) with standards prepared in pure solvent.

Detailed ME are represented as a 2D-plot in Fig. 2(b). Two compounds with significant signal enhancement (isoethanine (3.81 min) and selegiline (6.54 min) not represented) were observed. Their behaviour can be attributed to their early elution near the void. This section is probably the more subjected to ME as noticed in the 2D-plot. Besides this exception, not clear tendencies could be observed when mapping the occurrence of ME throughout the LC run. Only a section from 8 to 10 min suffered particularly relevant signal suppression that may be attributed to coeluting species from the matrix. In overall terms, the results were satisfactory nearly 80% of the compounds exhibiting soft ME in the range of 25% signal suppression or lower (Fig. 2).

The main issue of matrix-matched standards for ME correction is the representativeness of the matrix used, particularly difficult to predict given the different features urine samples may pose (specific gravity, pH, composition, etc). The best approach to minimize matrix effects and the impact of matrix variability on ME correction is dilution. The lower the relative amount of urine in the injected extracts the lower these effects so that the representativeness of the

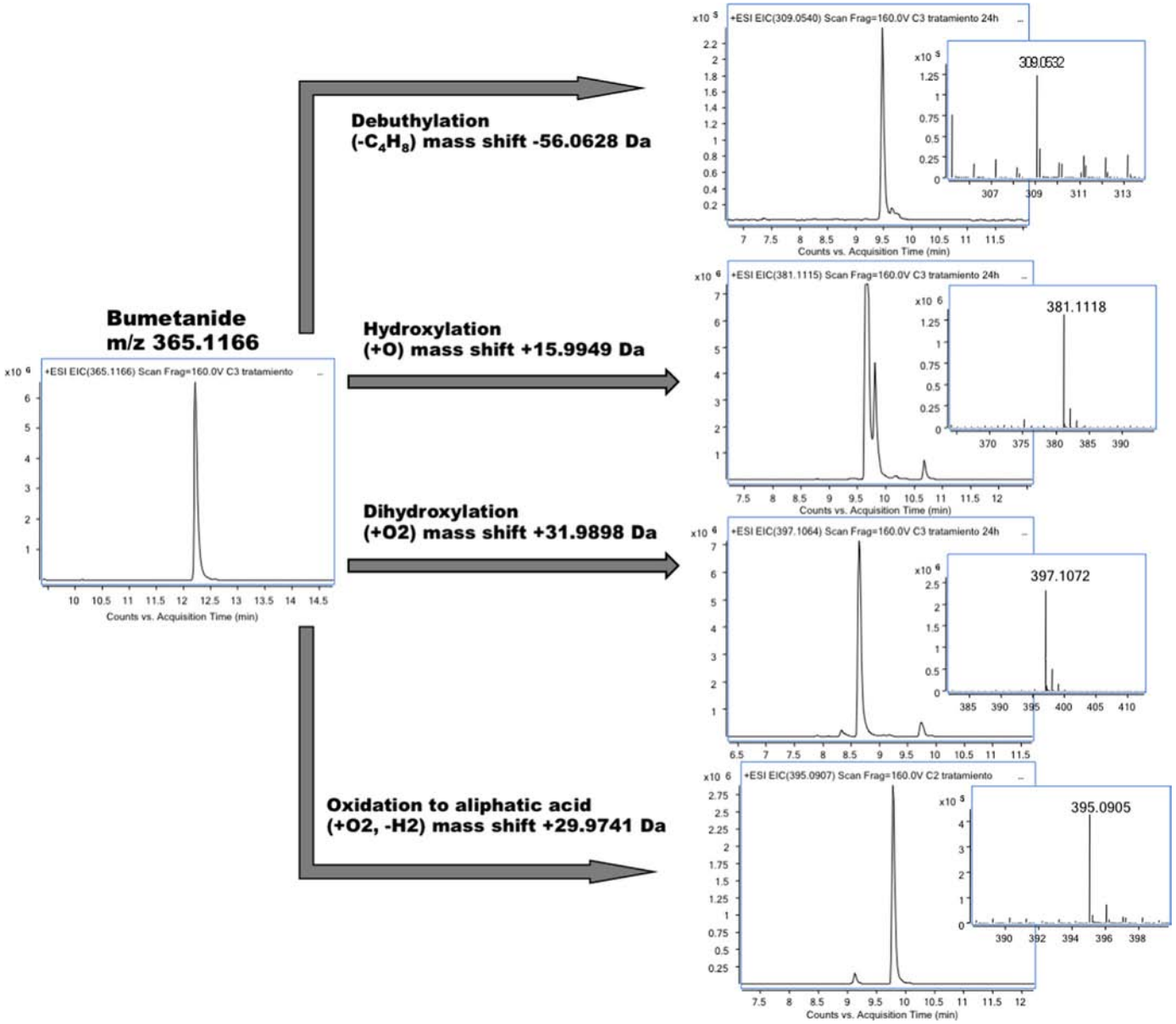

Fig. 5. Example of typical biotransformations search in a real urine sample after the treatment with the diuretic bumetanide where six metabolites were identified starting from the original molecule and applying different biotransformations.

sample is less problematic. For instance a 1:10 or 1:20 dilution may minimize ME in most cases so that external calibration with solvent standards could be feasible. The implementation of these dilutions depends strongly on instrument sensitivity and performance. Preliminary experiments accomplished with a state-of-the-art instrument (Agilent 6550 Q-TOF) provided both increase in sensitivity and minimized ME when using 1:10 dilution of the SPE extracts. Anyway, it should be noticed that these quantitative issues are more critical only for threshold substances, which require a dedicated assessment of ME and the uncertainty associated. For these particular cases, the use of ILIS may be considered and tested in first place [68].

3.4. Systematic data analysis for detection of metabolites and other derivate species of sport drugs

After a detailed study of fragmentation detection of the 200 sport drugs, the search of metabolites and other derivate species of sport drugs was addressed through the use of diagnostic fragment ions, which are defined as molecular ion or fragment ions whose presence and abundance are characteristic of the substance and thereby may assist in its identification [62–64]. Hence, related compounds or metabolites with similar structure, preserving some of these diagnostic fragments, may be easily tracked using narrow-mass window extraction of diagnostic ions or even by means of an automated search of diagnostic ions. For this *substructure search*, a database with the masses of the diagnostic ions for each family of class of compounds can be created, grouping them because similar compounds may have the same diagnostic ions. In this case, only accurate mass is used as search criterion. In most cases, the compounds displayed characteristic information for the search of derivative compounds or metabolites which are also targeted compounds of WADA methods since the list of prohibited species includes not only the species listed but also any other substances with similar structure or biological effects of any prohibited substances.

An example of this searching strategy is depicted in Fig. 3, where the structure and tentative fragmentation of a generic methylenedioxyamphetamine (MDMA) is illustrated yielding two diagnostic ions at *m/z* 163.0754 and 135.0441. These *diagnostic ions* or substructures are common in many compounds derived from methylenedioxyamphetamine as shown in Fig. 3, where up to 14 compounds described share these diagnostic ions. Data from a urine sample spiked with three derivatives of methylenedioxyamphetamine is shown in Fig. 4. The extracted ion chromatogram (EIC) of diagnostic ion *m/z* 163.0754 is represented, with three chromatographic peaks, and extracting their mass spectra is possible to find in them the mass of the diagnostic ion and the mass of the parent compound. Extracting their EICs confirms that the compounds present in the sample are MDA, MDEA and MDMA.

In addition, for a thorough metabolism study of an individual sport drug, the use of accurate mass shifts due to typical biotransformations is also a convenient tool [64]. Potential metabolites starting from the original drug were also studied by applying typical biotransformations with calculated mass shifts and the corresponding chemical formula modification [64]. Only the transformations plausible for a molecule were taken into account, being a csv format excel file created for each compound with all feasible biotransformations, and their accurate masses. This file is then used with "Find by formula" search tool of the software (MassHunter Qualitative Analysis). For instance, a set of typical biotransformations is summarized in Table S5 (Supplementary Data). The defined criteria search were the same applied to targeted species but without using retention time data, since no pure standards are available in first place. An example of this approach is shown in Fig. 5, with data obtained from a rat urine sample after intraperitoneal dose of diuretic bumetanide. Up to six metabolites were identified based on their possible biotransformations, including three hydroxylated metabolites, a dihydroxylated species, one oxidized to acid and the metabolite formed by the dealkylation of the alkyl chain.

4. Concluding remarks

In this work, a method for the screening and quantitation of 200 sport drugs using LC-TOFMS has been developed. The proposed SPE method provided adequate recoveries and RSD while providing a clean extract that not causes important matrix effects. The screening method based on accurate-mass/retention time pairs enabled the detection of all the species tested at WADA MRPLs levels using a single high resolution ion.

For confirmatory analysis, the use of in-source CID fragment provided appropriate results for the unambiguous confirmation of *ca.* 70% of the compounds if the 2-high resolution ion criterion is used. In contrast, the use of CID fragmentation on a dedicated collision cell without precursor ion selection provided comprehensive fragmentation information for all the compounds. This approach is definitely the best suited for this type of application as the full-scan acquisition and their advantages are not altered, while the fragmentation obtained is thorough. Finally, the use of this characteristic fragmentation along with the *ab initio* calculation of typical biotransformations allowed the straightforward identification of relevant non-targeted compounds such as drugs with similar structure to the WADA targeted compounds or metabolites. Furthermore, the validated method can be expanded towards new targeted species with high flexibility and also permits retrospectively inspect former samples for new or unknown doping agents added to the WADA list without the need of re-processing the sample, just the data acquired.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.10.050.

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