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Fast analysis of doping agents in urine by ultra-high-pressure liquid chromatography–quadrupole time-of-flight mass spectrometry. II: Confirmatory analysis

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ABSTRACT

For doping control, analyses of samples are generally achieved in two steps: a rapid screening and, in the case of a positive result, a confirmatory analysis. A two-step methodology based on ultra-high-pressure liquid chromatography coupled to a quadrupole time-of-flight mass spectrometry (UHPLC–QTOF-MS) was developed to screen and confirm 103 doping agents from various classes (e.g., β-blockers, stimulants, diuretics, and narcotics). The screening method was presented in a previous article as part I (i.e., Fast analysis of doping agents in urine by ultra-high-pressure liquid chromatography–quadrupole time-of-flight mass spectrometry. Part I: screening analysis). For the confirmatory method, basic, neutral and acidic compounds were extracted by a dedicated solid-phase extraction (SPE) in a 96-well plate format and detected by MS in the tandem mode to obtain precursor and characteristic product ions. The mass accuracy and the elemental composition of precursor and product ions were used for compound identification. After validation including matrix effect determination, the method was considered reliable to confirm suspect results without ambiguity according to the positivity criteria established by the World Anti-Doping Agency (WADA). Moreover, an isocratic method was developed to separate ephedrine from its isomer pseudoephedrine and cathine from phenylpropanolamine in a single run, what allowed their direct quantification in urine.

1. Introduction

The procedure generally used to detect and identify doping agents in an athlete's urine is performed in two steps. First, a rapid screening is conducted and, when a sample is presumed positive for a prohibited substance, a confirmatory test is carried out. In a previous paper [1], a fast screening method using ultra-high-pressure liquid chromatography coupled to a quadrupole time-of-flight mass spectrometry (UHPLC–QTOF-MS) was developed to detect 103 doping agents from different pharmaceutical classes (e.g., stimulants, diuretics, anti-estrogens, β-blockers, and narcotics) from the World Anti-Doping Agency (WADA) prohibited list [2]. The method has allowed a reduction of analysis time up to 5-fold compared to accredited methods (STS 288), meeting the minimal required performance limit (MRPL) concentration of the WADA [3].

Generally, the confirmatory analysis is conducted for one specific analyte found positive during the screening step. In certain cases, the determination of the major metabolite or of a concomitant drug intake is simultaneously achieved. Commonly, qualitative results are required, as trace of drugs of abuse detected in a urine sample is considered as the final result. However, an estimation of the concentration found in urine was required for threshold compounds (e.g., cathine, ephedrine and methylephedrine), which were considered doping agents only above a given cut-off value. Criteria must be established at the confirmatory level for the complete identification of a prohibited substance by high-pressure liquid chromatography (HPLC) coupled to MS [4,5]. First, all materials should be submitted to the entire analytical process with a strict sample injection order. The first sample to be analysed is a negative blank urine, followed by the suspect sample, a second negative blank urine, a quality control (QC) and finally a reference collection sample (administration study sample) or a reference material [6]. The retention time (t_R) tolerance window must be within the range of ±2% between the suspect analyte

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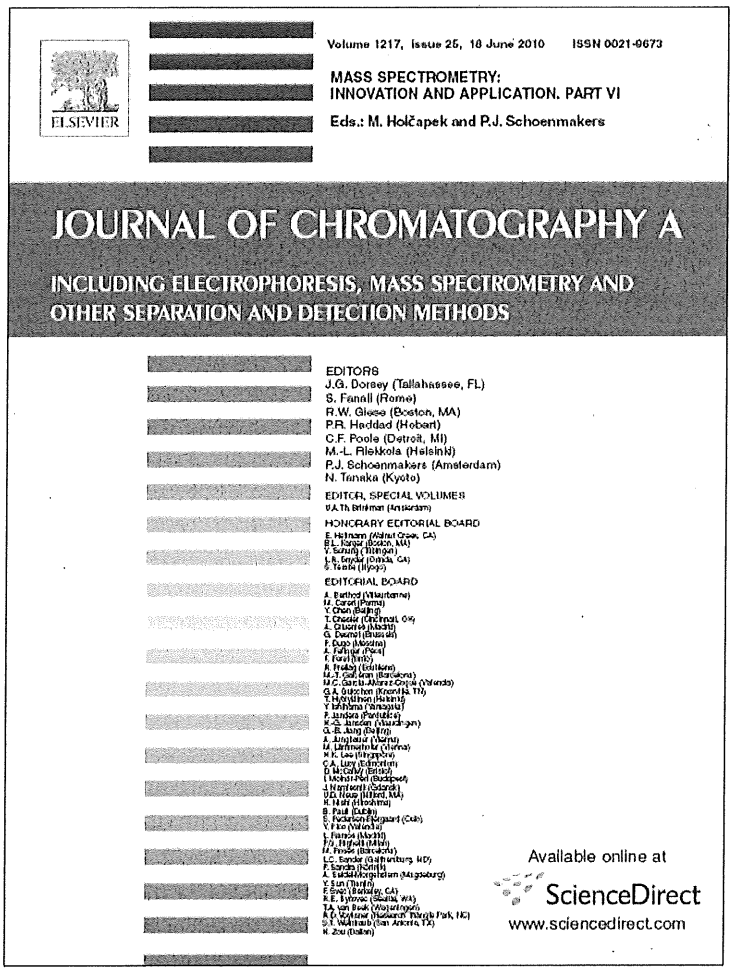

and the QC of the same batch. Finally, for MS/MS experiments there must be three diagnostic ions that may include the precursor ion, which must have intensity equal to or greater than 5% of that of the most intense diagnostic ion of the MS/MS spectrum. These must be considered with a S/N ratio >3 and the relative intensity of any of the ions shall not differ by more than 10% (absolute) or 25% (relative) from that of the positive control urine [7].

Today, different separative techniques, such as gas chromatography (GC), capillary electrophoresis (CE) and HPLC, are used to confirm or quantify doping agents in urine matrix. GC is the most frequently employed for the confirmatory step (e.g., cannabis [8], ephedrine and related substances [9], and anabolic steroids [10]). This technique has been known for years and the coupling of GC with MS detectors is reliable with electron ionisation (EI) sources. Indeed, it allows the construction of worldwide spectral reference libraries and, with the development of fast-GC technologies, analysis time could be drastically shortened. However, the major drawback of GC is its incompatibility with thermolabile substances, the necessity of hydrolysing conjugate molecules and derivatising polar analytes.

Methods by CE coupled to laser-induced fluorescence (LIF) detector or to MS were also used to quantify or detect some stimulants [11,12] and furosemide [13] and for separating chiral isomers (e.g., ephedrine and related compounds) [14]. Finally, HPLC–MS/MS currently constitutes the method of choice for anti-doping analysis. Indeed, it allows the straightforward determination of polar analytes excreted in urine. Therefore, HPLC–MS/MS methods were successfully developed in the anti-doping field to confirm or quantify amphetamine and derivatives [15], diuretics [16], ephedrines [17], or corticosteroids and anabolic agents [18].

Fast analyses are emerging for anti-doping purposes, since the number of samples to be screened is continuously increasing. Moreover, the time delivery response to give results is required to be 24 h or less after sample reception during major sporting events.

The use of fast HPLC techniques, such as UHPLC, is of particular interest for screening and confirmatory analysis. UHPLC is a recognized approach to reduce analysis time and improve or maintain chromatographic performance by using columns packed with small particles (i.e., sub-2 μm diameters). This technique is especially recommended because of its high resolution and excellent retention time repeatability [19]. Benefits of the UHPLC approach have been experimentally highlighted using fast duty cycle mass analysers such as triple quadrupole or time-of-flight (TOF) mass spectrometers in the anti-doping field [20–22].

The hyphenation of the QTOF mass spectrometer with UHPLC is a very attractive tool for performing confirmatory analysis. Indeed, the QTOF mass spectrometer can acquire MS/MS spectra with high reproducibility and give accurate mass measurements, allowing the determination of the analyte elemental composition. Moreover, it ensures high selectivity in complex biological matrices and is also proven to be a satisfactory tool for quantitative analysis [23–25].

This study is therefore the second part of a complete dedicated procedure to screen and confirm 103 doping agents. The hyphenation of UHPLC with QTOF-MS was evaluated in the anti-doping field as it combines speed, high repeatability and accurate mass measurement. All of these parameters are required for the fast unambiguous identification of a prohibited compound. Moreover, the technique allowed to baseline separate threshold compounds ephedrine and its isomer pseudoephedrine, together with cathine and phenylpropanolamine. Each isomer can be directly quantified, what was really relevant since the reintroduction of pseudoephedrine in the WADA's prohibited list in January 2010 at a threshold of 150 μg/mL.

2. Experimental

2.1. Chemicals

The origin of all compounds is listed in the experimental part of Ref. [1]. Ultra-pure water was provided by a Milli-Q system from Millipore (Bedford, MA, USA) or obtained from Biosolve (Chemie Brunschwig, Basel, Switzerland) for ULC/MS quality. ACN of ULC/MS quality was purchased from Biosolve (Chemie Brunschwig, Basel, Switzerland). Formic acid, hydrochloric acid and ammonium hydroxide were supplied by Fluka (Buchs, Switzerland). Methanol was obtained from Merck (Darmstadt, Germany).

The three I.S.s, methyltestosterone, nalorphine, and mefruside, were obtained from Biosolve (Chemie Brunschwig, Basel, Switzerland), SERB (Paris, France) and Bayer (Zürich, Switzerland), respectively.

2.2. Solutions

Stock standard solutions of the 103 substances were prepared at a concentration of 1 mg/mL in methanol and kept at −20 °C in glass tubes fitted with PTFE caps. The stock standard solutions were diluted with ultra-pure water to obtain diluted standard solutions at the following concentrations: 25 μg/mL for stimulants and β-blockers, 12.5 μg/mL for diuretics and oxygen transfer enhancers, 10 μg/mL or 0.5 μg/mL for narcotics and some stimulants, 5 μg/mL for β-agonists, and 2.5 μg/mL for aromatase inhibitors and anti-estrogens.

I.S. solution at 10 μg/mL containing two I.S.s for the positive mode (methyltestosterone and nalorphine) and one for the negative mode (mefruside) was prepared from 10 μL of 3 stock I.S. solutions in methanol at a concentration of 1 mg/mL to 1000 μL with ultra-pure water.

2.3. Quality controls

Quality controls (QCs) solutions (103) were prepared by spiking 10 μL of the diluted standard solutions in an aliquot of 500 μL of urine to obtain a final concentration at the MRPL level for each analyte, following the sample preparation procedure described in Section 2.4.

2.4. Sample preparation

2.4.1. Basic and neutral analytes

Oasis® MCX cartridges of 30 mg (30 μm particle size) were selected in the 96-well plate format to extract basic and neutral analytes (Table 1). The method was adapted from the generic Waters® protocol for solid-phase extraction (SPE), as illustrated in Table 2. Urine samples were centrifuged at 2500 rpm for 5 min. The loading solution was prepared by adding 500 μL of 0.5 M HCl to an aliquot of urine (500 μL). The I.S. solution (10 μL), at a concentration of 10 μg/mL, was spiked in the acidified sample. For cartridge conditioning and equilibrium, 500 μL of CH_3OH and 500 μL of 120 mM HCl were successively used. A quantity of 1 mL of the acidified urine solution was loaded on the wells and was eluted at a flow rate of approximately 400 μL/min. A washing step with 1 mL of 120 mM HCl was performed, followed by the elution of neutral compounds with 250 μL of CH_3OH. The bases were eluted with 250 μL of 5% NH_4OH in CH_3OH. The elution phases were collected into injection plates and directly injected into the UHPLC–QTOF-MS.

2.4.2. Acidic analytes

A 96-well plate format Oasis® sorbent MAX 30 mg (30-μm particle size) was used to extract acidic compounds (Table 1). After urine sample centrifugation (2500 rpm, 5 min), 500 μL of urine was

Table 1
Detailed list of the investigated compounds.

No.	Compound	Class	Ionization mode	Chemical formula $[M+H]^+$ or $[M-H]^-$	Experimental mass (*m/z*)	In-source fragmentation	In-source fragment ion (*m/z*)	Precursor ion (*m/z*)	Product ions (*m/z*)				Cone voltage [V]	Collision energy [eV]	PE%	Extraction sorbent
1	Acebutolol	β-Blocker	Positive	$C_{18}H_{29}N_2O_4$	337.2120	No		337.2120	319.2376	260.1550	116.1205		40	20	101	MCX
2	Acetazolamide	Diuretic	Negative	$C_4H_5N_4O_3S_2$	220.9795	No		220.9795	178.0940	139.0935			40	15	65	MAX
3	Adrafinil	Stimulant	Negative	$C_{15}H_{14}NO_3S$	288.0698	No		288.0698	121.0423	104.0159	74.0675		40	15	150	MAX
4	Amfepramone	Stimulant	Positive	$C_{13}H_{20}NO$	206.1543	No		206.1543	133.0856	105.0988	100.1411		40	15	87	MCX
5	Amfetaminil	Stimulant	Positive	$C_{17}H_{19}N_2$	251.1547	Yes	106.0650	251.1547	106.0650				40	20	47	MCX
6	Amiloride	Diuretic	Positive	$C_6H_9ClN_7O$	230.0557	No		230.0557	171.0356	143.0434	116.0249		40	20	62	MCX
7	Aminogluthetimide	Aromatase inhibitor	Positive	$C_{13}H_{17}N_2O_2$	233.1290	No		233.1290	205.1624	188.1354	160.1290	146.1126	40	15	103	MCX
8	Amiphenazole	Stimulant	Positive	$C_9H_{10}N_3S$	192.0598	No		192.0598	159.1014	150.0494	133.1057	105.0900	40	15	71	MCX
9	Amphetamine	Stimulant	Positive	$C_9H_{14}N$	136.1134	No		136.1134	119.0954	91.0717			40	5	66	MCX
10	Anastrozole	Aromatase inhibitor	Positive	$C_{17}H_{20}N_5$	294.1709	No		294.1709	225.1734				40	15	82	MCX
11	Atenolol	β-Blocker	Positive	$C_{14}H_{23}N_2O_3$	267.1711	No		267.1711	225.1162	190.0869	145.0678	116.1081	40	15	62	MCX
12	Bendroflumethiazide	Diuretic	Negative	$C_{15}H_{13}F_3N_3O_4S_2$	420.0292	No		420.0292	327.9927	289.0658	239.0682		40	25	99	MAX
13	Benzoylecgonine	Stimulant	Positive	$C_{16}H_{20}NO_4$	290.1404	No		290.1404	168.1093	105.0459			40	20	111	MCX
14	Benzylpiperazine	Stimulant	Positive	$C_{11}H_{17}N_2$	177.1402	No		177.1402	91.0758	85.0906			40	15	115	MCX
15	Bumetanide	Diuretic	Positive	$C_{17}H_{21}N_2O_5S$	365.1180	No		365.1180	284.1505	240.1584	184.0943		40	15	115	MAX
16	Buprenorphine	Narcotic	Positive	$C_{29}H_{42}NO_4$	468.3135	No		468.3135	396.2640	187.1040	101.1078		40	40	105	MCX
17	Bupropion	Stimulant	Positive	$C_{13}H_{19}ClNO$	240.1159	Yes	184.0529	184.0529	166.0706	139.0858	131.1174		40	10	120	MCX
18	Caffeine	Stimulant	Positive	$C_8H_{11}N_4O_2$	195.0882	No		195.0882	138.0870	110.0919			40	25	77	MCX
19	Canrenone	Diuretic	Positive	$C_{23}H_{28}O_3$	341.2130	No		341.2130	283.1700	205.1221	187.1150	107.0921	40	20	104	MCX
20	Carphedon	Stimulant	Positive	$C_{12}H_{15}N_2O_2$	219.1130	Yes	174.0924	174.0924	145.0942	129.0675	117.0856		40	15	61	MCX
21	Carteolol	β-Blocker	Positive	$C_{16}H_{25}N_2O_3$	293.1877	No		293.1877	237.1521	202.1222	164.0979		40	15	96	MCX
22	Cathine	Stimulant	Positive	$C_9H_{14}NO$	152.1084	Yes	134.0968	134.0968	115.1207	91.0755			40	10	89	MCX
23	Celiprolol	β-Blocker	Positive	$C_{20}H_{34}N_{304}$	380.2552	No		380.2552	324.1966	307.1895	251.1274	233.1212	40	20	119	MCX
24	Chlorothiazide	Diuretic	Negative	$C_7H_5ClN_3O_4S_2$	293.9414	No		293.9414	214.0091	179.0299			40	30	46	MAX
25	Chlorphentermine	Stimulant	Positive	$C_{10}H_{15}ClN$	184.0896	No		184.0896	167.0761	125.0395			40	7	91	MCX
26	Chlorthalidone	Diuretic	Negative	$C_{14}H_{10}ClN_2O_4S$	337.0045	No		337.0045	319.0278	283.0569	190.0070	146.0625	40	15	43	MAX
27	Clobenzorex	Stimulant	Positive	$C_{16}H_{19}ClN$	260.1205	No		260.1205	119.1153	64.9811			40	15	145	MCX
28	Clomiphen	Anti-estrogenic	Positive	$C_{26}H_{29}ClNO$	406.1958	No		406.1958	297.1285	100.1287			40	20	73	MCX
29	Clopamide	Diuretic	Positive	$C_{14}H_{20}ClN_3O_3S$	346.0992	No		346.0992	250.0279	169.0385	112.1222		40	25	81	MCX
30	Cocaine	Stimulant	Positive	$C_{17}H_{22}NO_4$	304.1541	No		304.1541	182.1222	150.0978	105.0421		20	20	132	MCX
31	Cropropamide	Stimulant	Positive	$C_{13}H_{25}N_2O_2$	242.1994	Yes	100.1130	242.1994	196.1635	168.1669	100.1189		40	10	69	MCX
32	Crotetamide	Stimulant	Positive	$C_{12}H_{23}N_2O_2$	227.1759	No		227.1759	196.1069	86.1170			40	22	47	MCX
33	Dextromoramide	Narcotic	Positive	$C_{25}H_{33}N_2O_2$	393.2552	No		393.2552	306.2014	236.1577			40	30	97	MCX
34	Dichlorphenamide	Diuretic	Negative	$C_6H_5Cl_2N_2O_4S_2$	302.9066	No		302.9066	266.9588	238.9710	224.0156		40	15	57	MAX
35	Dimethamphetamine	Stimulant	Positive	$C_{11}H_{18}N$	164.1442	No		164.1442	119.1212	91.0963			40	10	94	MCX
36	Ephedrine	Stimulant	Positive	$C_{10}H_{16}NO$	166.1235	Yes	148.1126	148.1126	133.0898	117.0712	115.0616	91.0638	40	20	78	MCX
37	Esmolol	β-Blocker	Positive	$C_{16}H_{26}NO_4$	296.1868	No		296.1868	219.1124	145.0773	116.1174		40	20	100	MCX
38	Etafedrine	Stimulant	Positive	$C_{12}H_{20}NO$	194.1544	No		194.1544	176.1719	135.0902	117.1225		40	15	63	MCX
39	Ethacrynic acid	Diuretic	Negative	$C_{13}H_{13}Cl_2O_4$	304.0269	Yes	242.9979	242.9979	207.0553	192.0400	171.0961		40	20	68	MAX
40	Etamivan	Stimulant	Positive	$C_{12}H_{18}NO_3$	224.1291	No		224.1291	151.0597	100.0679			40	15	108	MCX
41	Ethylamphetamine	Stimulant	Positive	$C_{11}H_{18}N$	164.1438	No		164.1438	119.1320	91.1187			40	10	81	MCX
42	Etilefrine	Stimulant	Positive	$C_{10}H_{16}NO_2$	182.1165	Yes		164.1110	135.0715	91.0565			40	10	88	MCX
43	Exemestane	Aromatase inhibitor	Positive	$C_{20}H_{25}O_2$	297.1862	No		297.1862	279.1803	149.1026	121.1016		40	15	177	MCX
44	Fenbutrazate	Stimulant	Positive	$C_{23}H_{30}NO_3$	368.2244	No		368.2244	204.1555	191.1252	119.0928		40	20	102	MCX
45	Fencamfamine	Stimulant	Positive	$C_{15}H_{22}N$	216.1752	No		216.1752	171.1769	129.1103	91.1041		40	15	102	MCX
46	Fenetylline	Stimulant	Positive	$C_{18}H_{24}N_5O_2$	342.1925	No		342.1925	224.1447	207.1116	119.1362		40	20	131	MCX
47	Fenfluramine	Stimulant	Positive	$C_{12}H_{17}F_3N$	232.1316	No		232.1316	187.0912	159.0647			40	10	13	MCX
48	Fenproporex	Stimulant	Positive	$C_{12}H_{17}N_2$	189.1388	No		189.1388	149.9821	119.1153	91.0786		40	5	65	MCX
49	Fentanyl	Narcotic	Positive	$C_{22}H_{29}N_2O$	337.2281	No		337.2281	188.1756	105.0947			40	20	105	MCX
50	Finasteride	A-reductase inhibitor	Positive	$C_{23}H_{37}N_2O_2$	373.2860	No		373.2860	317.2310	305.2685			40	20	81	MCX
51	Furfenorex	Stimulant	Positive	$C_{15}H_{20}NO$	230.1546	No		230.1546	148.1291	81.0470			40	10	113	MCX
52	Furosemide	Diuretic	Negative	$C_{12}H_{10}ClN_2O_5S$	328.9997	No		328.9997	285.0210	204.9929			40	15	70	MAX

Table 1 (*Continued*)

No.	Compound	Class	Ionization mode	Chemical formula $[M+H]^+$ or $[M-H]^-$	Experimental mass (*m/z*)	In-source fragmentation	In-source fragment ion (*m/z*)	Precursor ion (*m/z*)	Product ions (*m/z*)				Cone voltage [V]	Collision energy [eV]	PE%	Extraction sorbent
53	Heptaminol	Stimulant	Positive	$C_8H_{20}NO$	146.1551	Yes	128.1449	146.1687	128.1489	69.0834			40	10	56	MCX
54	Hydrochlorothiazide	Diuretic	Negative	$C_7H_7ClN_3O_4S_2$	295.9567	No		295.9567	268.9785	205.0170	126.0572		40	25	20	MAX
55	Hydroxybromantan	Stimulant	Positive	$C_{16}H_{21}BrNO$	322.0816	No		322.0816	151.1174	133.1067	91.0634		40	15	100	MCX
56	Indapamide	Diuretic	Negative	$C_{16}H_{15}ClN_3O_3S$	364.0528	No		364.0528	215.9776	188.9951	132.0846		40	20	77	MAX
57	Isometheptene	Stimulant	Positive	$C_9H_{20}N$	142.1597	No		142.1597	69.1065				40	15	72	MCX
58	MDA[a]	Stimulant	Positive	$C_{10}H_{14}NO_2$	180.1033	Yes	163.0758	163.0758	135.0839	105.1077	79.0613		40	15	56	MCX
59	MDMA[b]	Stimulant	Positive	$C_{11}H_{16}NO_2$	194.1181	Yes	163.0758	163.0758	135.0758	105.0965	79.1261		40	15	97	MCX
60	Mefenorex	Stimulant	Positive	$C_{12}H_{19}ClN$	212.1214	No		212.1214	119.1169	91.0789			40	10	104	MCX
61	Mesocarb	Stimulant	Positive	$C_{18}H_{19}N_4O_2$	323.1513	No		323.1513	177.0928	119.0983	91.0682		40	7	138	MCX
62	Metamphetamine	Stimulant	Positive	$C_{10}H_{16}N$	150.1283	No		150.1283	119.1116	91.1073			40	10	71	MCX
63	Methadone	Narcotic	Positive	$C_{21}H_{28}NO$	310.2171	Yes	265.1589	265.1589	223.1140	105.0540			40	15	61	MCX
64	Methoxyphenamine	Stimulant	Positive	$C_{11}H_{18}NO$	180.1388	Yes	121.0665	121.0665	91.1001				40	15	102	MCX
65	*para*-Methylamphetamine	Stimulant	Positive	$C_{10}H_{16}N$	150.1280	No		150.1280	105.0971	79.0566			40	10	81	MCX
66	Methylecgonine	Stimulant	Positive	$C_{10}H_{18}NO_3$	200.1294	No		200.1294	182.1241	154.1934	82.0752		40	15	68	MCX
67	Methylephedrine	Stimulant	Positive	$C_{11}H_{18}NO$	180.1389	Yes	162.1284	162.1284	146.1307	132.4878	115.0937	91.0700	40	20	103	MCX
68	Methylphenidate	Stimulant	Positive	$C_{14}H_{20}NO_2$	234.1500	No		234.1500	196.0378	84.0970			40	15	94	MCX
69	Metipranolol	β-Blocker	Positive	$C_{17}H_{28}NO_4$	310.2025	No		310.2025	233.1318	191.1126	116.2663		40	20	100	MCX
70	Metolazone	Diuretic	Negative	$C_{16}H_{15}ClN_3O_3S$	364.0516	No		364.0516	257.0068	231.0366	152.0719		40	25	46	MAX
71	Metoprolol	β-Blocker	Positive	$C_{15}H_{26}NO_3$	268.1924	No		268.1924	191.1543	159.1085	116.1318		40	15	101	MCX
72	Modafinil	Stimulant	Positive	$C_{15}H_{16}NO_2S$	274.0918	Yes	167.0868	274.0918	296.0834	128.9958	167.0889		15	5	112	MCX
73	Nadolol	β-Blocker	Positive	$C_{17}H_{28}NO_4$	310.2033	No		310.2033	254.1536	236.1418	201.1043		40	15	100	MCX
74	Nikethamide	Stimulant	Positive	$C_{10}H_{15}N_2O$	179.1185	No		179.1185	108.0619	80.0641			40	20	62	MCX
75	Norbuprenorphine	Narcotic	Positive	$C_{25}H_{36}NO_4$	414.2647	No		414.2647	265.1083	183.0763	101.1086		40	30	37	MCX
76	Norfenfluramine	Stimulant	Positive	$C_{10}H_{13}F_3N$	204.1003	Yes	159.0421	159.0421	139.0984	119.0720	109.0715		40	15	90	MCX
77	Norfentanyl	Narcotic	Positive	$C_{14}H_{21}N_2O$	233.1651	No		233.1651	177.1814	150.1149	84.1007		40	15	105	MCX
78	Oxilofrine	Stimulant	Positive	$C_{10}H_{16}NO_2$	182.1181	Yes	164.1081	164.1081	149.1322	133.0709	105.1221		40	15	81	MCX
79	Pemoline	Stimulant	Positive	$C_9H_9N_2O_2$	177.0670	No		177.0670	106.0795	79.0753			40	20	81	MCX
80	Pentazocine	Narcotic	Positive	$C_{19}H_{28}NO$	286.2161	No		286.2161	218.1735	173.1091	159.0939		40	20	270	MCX
81	Pentetrazole	Stimulant	Positive	$C_6H_{11}N_4$	139.0984	No		139.0984	96.1246	79.1634			40	15	31	MCX
82	Pethidine	Narcotic	Positive	$C_{15}H_{22}NO_2$	248.1646	No		248.1646	220.1664	174.1567			40	20	110	MCX
83	Phendimetrazine	Stimulant	Positive	$C_{12}H_{18}NO$	192.1387	No		192.1387	146.1300	133.0906	115.0863		40	20	81	MCX
84	Phenpromethamine	Stimulant	Positive	$C_{10}H_{16}N$	150.1285	No		150.1285	119.1016	91.0866			40	10	93	MCX
85	Phentermine	Stimulant	Positive	$C_{10}H_{16}N$	150.1283	Yes	133.1016	133.1016	105.1635	91.0856			40	5	88	MCX
86	Phenylpropanolamine	Stimulant	Positive	$C_9H_{14}NO$	152.1082	Yes	134.0968	134.0968	115.0821	91.0739			40	20	85	MCX
87	Pholedrine	Stimulant	Positive	$C_{10}H_{16}NO$	166.1241	Yes	135.0820	135.0820	107.0949				40	10	78	MCX
88	Pipradol	Stimulant	Positive	$C_{18}H_{22}NO$	268.1702	Yes	250.1591	250.1591	167.0952	144.1609	130.1046		40	25	104	MCX
89	Piretanide	Diuretic	Positive	$C_{17}H_{19}N_2O_5S$	363.1016	No		363.1016	282.1341	236.1276	210.1451		40	20	140	MCX
90	Probenecide	Uricosuric	Negative	$C_{13}H_{18}NO_4S$	284.0954	No		284.0954	240.1344	140.0432			40	15	77	MAX
91	Prolintane	Stimulant	Positive	$C_{15}H_{24}N$	218.1907	No		218.1907	105.0933	91.0741			40	15	105	MCX
92	Propylhexedrine	Stimulant	Positive	$C_{10}H_{22}N$	156.1757	No		156.1757	83.1960	69.1107			40	15	77	MCX
93	Pseudoephedrine	Stimulant	Positive	$C_{10}H_{16}NO$	166.1240	Yes		148.1126	148.1126	132.1365	115.0750	91.1242	40	20	80	MCX
94	Ritalinicacid	Stimulant	Positive	$C_{13}H_{18}NO_2$	220.1342	No		220.1342	174.1308	84.0919			40	15	48	MCX
95	RSR 13[c]	Oxygen transfer enhancer	Positive	$C_{20}H_{24}NO_4$	342.1696	No		342.1696	296.1863	256.1518	122.1208		40	15	85	MAX
96	Salmeterol	β-Agonist	Positive	$C_{25}H_{38}NO_4$	416.2806	No		416.2806	398.3032	380.2928	232.1902		40	15	94	MCX
97	Sibutramine	Stimulant	Positive	$C_{17}H_{27}ClN$	280.1835	No		280.1835	153.0761	139.0645	125.0381		40	10	109	MCX
98	Sotalol	β-Blocker	Positive	$C_{12}H_{21}N_2O_3S$	273.1277	Yes	255.1178	255.1178	213.0942	133.1158			40	15	81	MCX
99	Spironolactone	Diuretic	Positive	$C_{24}H_{33}O_4S$	418.2178	Yes	341.2130	341.2130	303.2697	187.1370	107.1021		40	20	60	MCX
100	Strychnine	Stimulant	Positive	$C_{21}H_{23}N_2O_2$	335.1759	No		335.1759	184.0756	156.0834	129.0756		40	35	55	MCX
101	Torasemide	Diuretic	Positive	$C_{16}H_{21}N4O_3S$	349.1325	Yes	264.0810	264.0810	219.0852	183.1154	168.0804		40	20	86	MCX
102	Triamterene	Diuretic	Positive	$C_{12}H_{12}N_7$	254.1153	No		254.1153	237.1317	195.0877	104.0698		40	25	58	MCX
103	Xipamide	Diuretic	Negative	$C_{15}H_{14}ClN_2O_4S$	353.0363	No		353.0363	273.0858	170.0284	127.0376		40	25	94	MAX

[a] Methylenedioxyamphetamine.

[b] Methylenedioxymetamphetamine.

[c] Efaproxiral.

Table 2
Optimised solid-phase extraction protocol to extract selectively basic, neutral and acidic compounds.

Protocol	MAX (mixed mode anion exchange) acids (pK_a 2–8)	MCX (mixed mode cation exchange) bases (pK_a 2–10)	MCX neutrals
Conditioning	500 μL CH_3OH		
Equilibrium	500 μL NH_4OH 4%	500 μL HCl 120 mM	
Load	500 μL urine – 500 μL NH_4OH 4% – (pH > 9.5) + 10 μL IS (mefruside)	500 μL urine – 500 μL HCl 0.5 M – (pH < 2) + 10 μL I.S. (nalorphine/methyltestosterone)	
Wash 1	1 mL NH_4OH 4%	1 mL HCl 120 mM	
Wash 2/elute 1	250 μL CH_3OH		250 μL CH_3OH
Elute 2	250 μL 2% formic acid in CH_3OH	250 μL 5% NH_4OH in CH_3OH	

diluted with 500 μL of 4% NH_4OH. The I.S. solution (10 μL) at a concentration of 10 μg/mL was spiked in the loading solution. The cartridge was conditioned with 500 μL of CH_3OH and equilibrated with a solution of NH_4OH at 4%. The basified urine samples (1 mL) were loaded. The sorbent was washed first with 1 mL of NH_4OH at 4% and then with 250 μL of CH_3OH at a flow rate of 400 μL/min. Acidic compounds were finally eluted with 250 μL of 2% FA in CH_3OH and injected into the UHPLC–QTOF-MS.

2.4.3. Threshold analytes

A concentration threshold is set for physiological substances (e.g., epitestosterone) or for substances presenting a doping effect above a certain concentration (e.g., ephedrines). Among the set of the investigated analytes, cathine, ephedrine and methylephedrine are considered doping agents when their urinary concentration exceeds 5, 10 and 10 μg/mL, respectively [3]. A quantitative analysis is thus required for these compounds.

The three threshold analytes were extracted by SPE on Oasis® MCX cartridges of 30 mg (30 μm particle size). Urine samples were centrifuged at 2500 rpm for 5 min. To avoid saturation of the detector response, a urine aliquot was diluted 60-fold (10–600 μL of ultra-pure water). The loading solution was prepared by adding 500 μL of 0.5 M HCl to 500 μL of a 60-fold diluted urine. Finally, 10 μL of the I.S. solution at a concentration of 10 μg/mL was spiked in the acidified sample. The extraction method described in Section 2.4.1 was then followed.

2.5. UHPLC

Separations were carried out on an Acquity UPLC System (Waters, Milford, MA, USA) with an Acquity column (BEH C_{18} 50 mm × 2.1 mm, 1.7 μm). The mobile phase flow rate was set at 400 μL/min and the column temperature was maintained at 30 °C. The mobile phase was (A) 0.1% FA in water, and (B) 0.1% FA in ACN, linearly programmed from 5% to 95% B in 3 min, with 1.5 min of equilibration time. A Van Guard precolumn (BEH C_{18} 5 mm × 2.1 mm, 1.7 μm) was used and the injection volume was fixed at 5 μL in the partial loop with needle overfill mode. Samples were maintained at 4 °C in the autosampler.

Selective conditions were required for the separation of two pairs of isomers (ephedrine/pseudoephedrine and cathine/phenylpropanolamine). The separation was performed in isocratic mode at 30 °C with 95% of water containing 0.1% FA and 5% of ACN with 0.1% FA (v/v) using a flow rate of 300 μL/min and an injection volume of 2 μL on a Van Guard precolumn (BEH C_{18} 5 mm × 2.1 mm, 1.7 μm) followed by an Acquity column (BEH C_{18} 100 mm × 2.1 mm, 1.7 μm).

2.6. QTOF-MS and MS/MS

Analyte detection was performed with a Micromass-Q-Tof Premier mass spectrometer (Waters) equipped with an electrospray ionisation (ESI) source.

The QTOF was operated as follows. The desolvation gas flow was set at 800 L/h and 300 °C, and the capillary voltages at 3.0 kV in positive mode and 2.4 kV in negative mode. The micro-channel plates (MCPs) were operated at 1800 V in positive mode and 1750 V in negative mode. The source temperature was adjusted at 100 °C, the cone gas flow at 10 L/h, and the collision gas flow at 0.32 mL/min in positive mode and 0.25 mL/min in negative mode.

Data were collected in V-optics centroid mode over an *m/z* range of 50–1000 with a scan time of 0.25 s and an interscan delay of 0.02 s. For the dynamic range enhancement (DRE) lockmass, a solution of leucine-enkephalin at 2 ng/mL (Sigma–Aldrich, Buchs, Switzerland) was infused through the Lock Spray probe at a flow rate of 5 μL/min, and acquired every 20 scans (5 scans were averaged).

Two separate channels were acquired in the same analytical run. In the first function, the instrument was working in wide pass quadrupole mode (MS mode). While in the second function, a specific MS/MS method for a selective precursor ion was achieved. In the tandem mode, collision energies and cone voltages were set individually for each analyte.

2.7. Software

Data acquisition, data handling and instrument control were performed by MassLynx Software (Waters).

2.8. Matrix effect

The investigation of the matrix effect (ME) was performed based on the approach proposed by Matuszewski et al. [26]. ME could be assigned to a specific part of the analytical process. Indeed, it could occur during either the sample preparation or the ionisation step. The authors recommended analysing three sets of samples to determine the ME and the extraction process. A recent study suggests to add a supplementary solution to estimate the extraction yield contributions [27]. Four sets of solutions were prepared for each analyte at the MRPL concentration. The first set consisted of a neat solution with standards spiked in ultra-pure water (a). The three other sets were standards spiked in extracted urine (b), standards spiked in urine before the extraction step (c), and a set of standards spiked in a neat solution before extraction (d). Four phenomena can be highlighted by comparison between the absolute peak areas of two sets of solutions, as reported below (Eqs. (1)–(4)).

$$\text{Process Efficiency (PE)} = \frac{c}{a} \quad (1)$$

$$\text{Matrix Effect (ME)} = \frac{b}{a} \quad (2)$$

$$\text{Extraction Recovery (RE)} = \frac{c}{b} \quad (3)$$

$$\text{Extraction Yield (EY)} = \frac{d}{a} \quad (4)$$

All experiments were performed in triplicate at the MRPL concentration on 3 batches of urine to take the inter-individual variability into account. Finally, for the three threshold analytes, the experiments were carried out at their respective cut-off value diluted 60-fold.

2.9. Quantitative analysis

Experiments were conducted in compliance with the Internal Standards for Laboratories (ISL) of the World Anti-Doping Code [6]. Detailed procedures for validating were found in the International Conference for Harmonisation (ICH) guideline [28], the US Food and Drug Administration (FDA) guidelines and the 3rd American Association of Pharmaceutical Scientists (AAPS)/FDA Bioanalytical Workshop in 2006 [29].

Quantitative analysis was performed for cathine, methylephedrine and ephedrine. Two pools of three negative urines were used for the calibration procedure and the validation assays, respectively. The validation was performed on 3 consecutive series. For each series, calibration standard at three concentration levels ($k=3$) and validator standard at four concentration levels ($k=4$) were prepared in duplicate ($n=2$) and triplicate ($n=3$), respectively. To avoid the detector response saturation at the cut-off concentration (5–10 μg/mL), a dilution factor of 60 was included in the validation process to detect the analytes in the detector response dynamic range. A calibration curve was generated over the range from 0.1 to 2 μg/mL for ephedrine and methylephedrine, and over the range from 0.05 to 2 μg/mL for cathine.

Calibration curves were built from the peak area ratio of each analyte to the I.S. (nalorphine). Trueness, repeatability and intermediate precision were determined at each concentration level. Trueness was expressed in percent as the ratio between the theoretical and the average measured concentration. Repeatability was defined as the relative standard deviation (RSD) of the ratio of the intra-day standard deviation and the exact value at each concentration level as indicated in Rozet et al. [30]. Intermediate precision was expressed as the RSD of the ratio of the inter-day standard deviation on the theoretical value at each concentration level.

3. Results and discussion

3.1. Method development

3.1.1. Strategy

An approach for the screening and the pre-confirmatory analysis of 103 prohibited substances was previously developed and published as part I of this study [1]. For the screening, the conditions were optimised to detect the highest number of analytes in the shortest time. In the confirmatory analysis, the method should be sensitive and selective enough to meet the WADA's criteria. The method is dedicated and developed to confirm one doping agent, including eventually its major metabolites and/or concomitant drug intake. As the confirmatory analysis is the second step of a procedure to identify doping agents, the analyte identity is already strongly presumed. Therefore, a selective sample preparation and MS/MS method with generic UHPLC conditions must be employed and the general strategy is presented in Fig. 1.

The entire analytical process (sample preparation, chromatographic separation and detection) was optimised to confirm the analytes with the highest selectivities and sensitivities within a short time.

3.1.2. SPE

For performing the selective extraction of the 103 investigated compounds, a sample preparation based on SPE was used. The method was developed on mixed-mode extraction supports and was performed in the 96-well plate format. Mixed-mode cation exchange (MCX) cartridges were used for basic and neutral compounds. The latter were extracted after an elution step with 100% MeOH, as illustrated in Table 2, and a second elution performed with 5% NH_4OH in MeOH to collect basic compounds. A synergic procedure, detailed in Table 2, was performed for acidic compounds on mixed-mode anion exchange (MAX) sorbents. It can be noted that the detection mode (ESI-MS) was a function of the extraction procedure. Indeed, basic and neutral compounds extracted on MCX cartridges were ionised in positive mode, whereas acidic compounds were extracted on MAX sorbent and detected in negative mode.

Ten representative analytes of the investigated compounds were selected to optimise the SPE method in terms of elution volume, solvent composition and pH of washing steps. The analytes were chosen for their different chromatographic behaviour (t_R), ionisation mode (ESI positive or negative) and physico-chemical properties (MW, pK_a). Seven of these compounds were ionised in positive mode (e.g., amiphenazole, exemestane, heptaminol, methylecgonine, modafinil, spironolactone and sotalol) and three

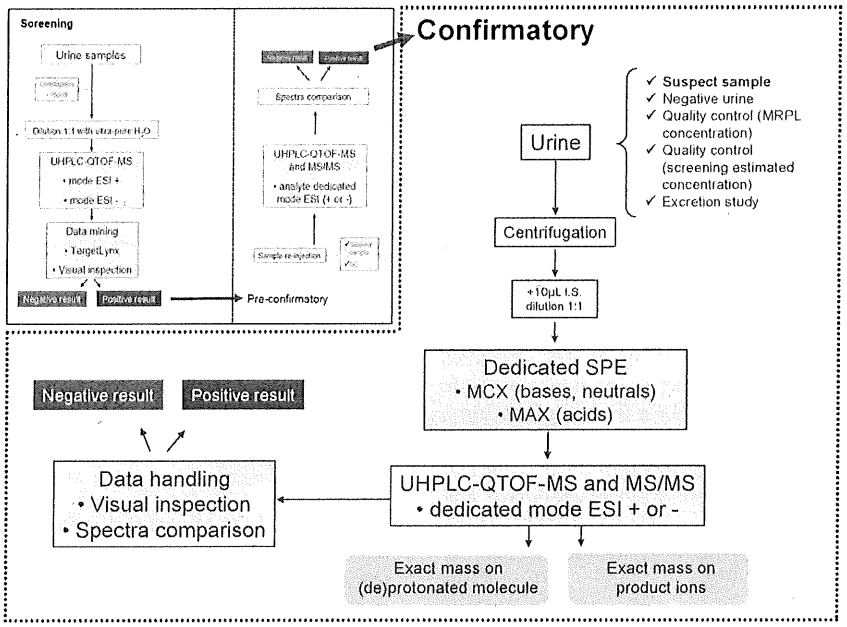

Fig. 1. Detailed confirmatory analysis procedure.

in negative mode (e.g., bendroflumethiazide, hydrochlorothiazide and dichlorphenamide).

The final method, described in Table 2, was successfully applied to the set of 103 analytes. Extraction recoveries calculated from peak area ratios between extracted urine samples spiked at the MRPL concentration before and after SPE (Eq. (3), data not shown) ranged between 50% and 120%. However 10 compounds were extracted with recoveries lower than 50% (i.e., hydroxybromantan, isometheptene, phenpromethamine, phentermine, norfentanyl, sibutramine, heptaminol and metolazone). Methylecgonine was obtained with a recovery of only 8% because of its extreme polarity ($\log D_{3.0}$ −3.33 [31]). However, even with this low recovery, the observed signal-to-noise ratio (S/N) was higher than 3 at the MRPL concentration. Moreover, methylecgonine is one of the two major metabolites of cocaine found in urine, with benzoylecgonine. Cocaine and benzoylecgonine were detected with acceptable SPE recovery (86% and 111%, respectively). An intake of cocaine was thus confirmed by following the cocaine itself and one of its two major metabolites excreted in urine.

The developed strategy has also the advantage of being selective and generic. Indeed, as an example, in the case of a parent compound with basic properties (e.g., methylphenidate) metabolized in a molecule with acidic properties (e.g., ritalinic acid), the analyte is extracted on MCX cartridges and the elution phase 1 (CH_3OH containing acidic and neutral analytes) and 2 (basified CH_3OH containing basic compounds) are pooled together and directly injected. This allowed easy implementation of new compounds in the procedure, and a consideration of drugs metabolites. Moreover, the method used small quantity of urine sample (500 μL) and allowed a pre-concentration step by a factor of 2. The reduced urine quantity in comparison with classical SPE cartridges is of utmost importance, as anti-doping analyses often require different tests to screen and confirm several kinds of doping agents. The high throughput and possibility of sample scalability offered by the 96-well plate format are additional benefits when several samples must be extracted simultaneously [32]. Indeed, with the developed method, the time for extraction *per* sample can be drastically reduced by the use of 96-well plate format. For example, 50 samples can be extracted within 30 min and the time for UHPLC–QTOF-MS and MS/MS analysis required is then 300 min. The time *per* sample can thus be estimated at around 7 min.

3.1.3. UHPLC

UHPLC allows an increase in resolution, throughput and sensitivity using sub-2 μm particles. Therefore, a fast gradient of 3 min with 1.5 min of equilibration time was generated on a short column (50 mm). It is worth mentioning that the gradient time cannot be further reduced because of the complexity of the mixture and the limitation of the QTOF instrument in terms of the acquisition rate, which can be critical with very narrow peaks (*ca.* 3 s).

3.1.4. QTOF-MS and MS/MS

3.1.4.1. Acquisition mode. A selective QTOF-MS and MS/MS detection was performed for each analyte to meet the WADA's identification criteria. With the QTOF mass analyser, it was possible to obtain a QTOF-MS full scan acquisition in a first channel and a QTOF–MS/MS spectrum in a second channel in the same analytical run. The acquisition of simultaneous MS and MS/MS methods at two collision energies allows the determination of precursor and product ions with high mass accuracy. A dedicated MS/MS method was developed for each analyte by setting the cone voltage and the collision energy at the analyte expected t_R to obtain at least three diagnostic ions, including the protonated molecule. The MS and MS/MS settings determined for each doping agent are reported in Table 1.

3.1.4.2. MS and MS/MS sensitivity. It was important to reach the highest sensitivity on the precursor peak to obtain suitable and reproducible tandem mass spectra. The QTOF mass spectrometer is able to work either in the V-optics mode with a resolution of about 8000–10,000 full-width at half maximum (FWHM) or in the W-optics mode with 15,000–17,500 FWHM obtained by approximately doubling the path length [33]. The highest mass resolution gained in W-optics mode was not mandatory for the confirmatory purpose because the analyte identity was already suspected (screening analysis). Furthermore, when working in tandem MS, a gain in sensitivity in terms of S/N ratio was obtained in V-optics mode for most of the analytes compared to the W-optics mode, despite an acceptable loss in resolution and in mass accuracy (5–10 ppm instead of 2–5 ppm of mass accuracy). The precursor ions were chosen according to their intensities and are reported in Table 1. As already observed in the screening part, some molecules were prone to in-source fragmentation. Indeed, the selected precursor ion for MS/MS experiments was sometimes already a fragment ion of the (de)protonated molecule.

3.1.4.3. MS/MS fragmentation. The concomitant MS and MS/MS mode is illustrated in Fig. 2 for atenolol (A) and dextromoramide (B). The WADA required 3 diagnostic ions, including the precursor ion, which must match the reference material. As presented in Fig. 2(A), the protonated molecule of atenolol (*m/z* 267.17) was observed at low collision energy (5 eV) in the first channel, while 3 product ions were obtained in the second channel by applying a collision energy of 15 eV. A sufficient number of diagnostic ions (*m/z* 267.17, *m/z* 225.12, *m/z* 190.08 and *m/z* 145.06) with intensities higher than 5% of the base peak were obtained in the second channel, satisfying WADA's recommendations. The simultaneous MS and MS/MS method was especially useful for obtaining the 3 diagnostic ions for dextromoramide (*m/z* 393.25, *m/z* 306.19 and *m/z* 236.15). Indeed, the protonated molecule (*m/z* 393.25) and 1 product ion (*m/z* 306.19), obtained by in-source fragmentation, were observed in the first channel using the MS acquisition at low collision energy (5 eV). Higher collision energy (30 eV) was applied for the MS/MS mode acquired in the second channel. This led to the entire fragmentation of the protonated molecule, used as precursor ion, into 2 product ions (*m/z* 306.19 and *m/z* 236.15). The 3 diagnostic ions were thus obtained for dextromoramide by combining the information obtained in both channels. However, four compounds fragmented in only one moiety, even at high collision energy. These last, namely methylphenidate, isometheptene, *para*-methylamphetamine and anastrozole, were characterised by the precursor ion and one product ion. Nevertheless, an intake of methylphenidate was confirmed by the presence of its major metabolite (ritalinic acid), which is predominantly excreted in urine and met WADA requirements in terms of identification criteria. The three other compounds must be treated by an orthogonal method to obtain at least 3 diagnostic ions for each analyte. GC–MS with EI sources will be a helpful method as the fragmentation leads to additional cleavage reactions. However, with the QTOF mass analyser, it was possible to measure the exact mass of the analyte and to predict its elemental composition with its isotopic pattern. This was considered sufficient to ensure the identity of the presumed positive compounds, but, up to now, is not accepted by the WADA as an official criterion.

3.2. Matrix effect

The ME was measured according to the original work of Matuszewski et al. [26]. An exhaustive classification of the presumed ME could be established in the case of an SPE prior to HPLC or UHPLC–ESI-MS analysis as presented elsewhere [34]. Indeed, large signal suppression can be expected by using an ESI source [35]. The 103 analytes were classified as a function of the type of

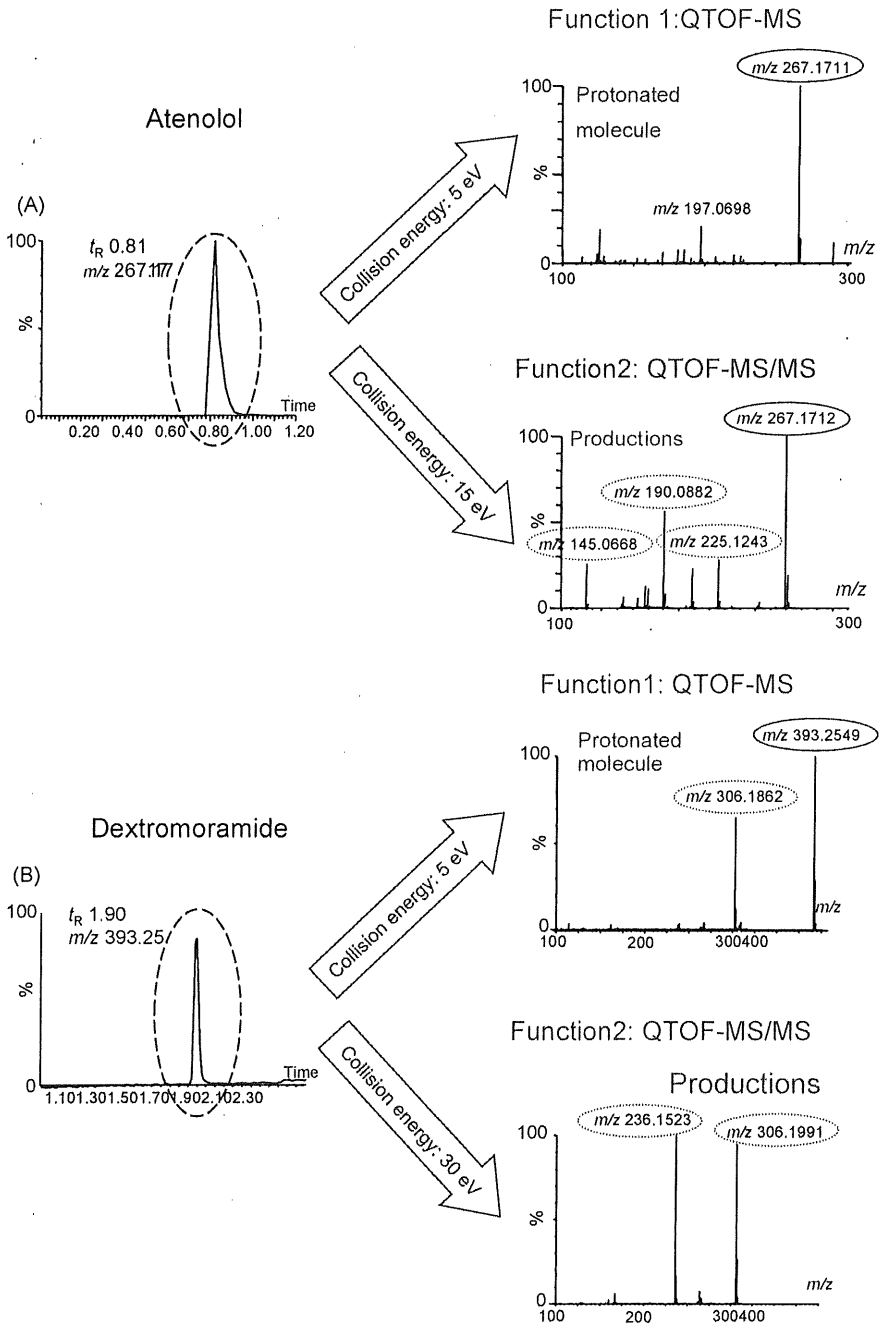

Fig. 2. QTOF-MS and MS/MS spectra for atenolol (A) and dextromoramide (B). (A) Chromatogram obtained at low collision energy of 5 eV to obtain the protonated molecule of atenolol (*m/z* 267.17). A higher collision energy of 15 eV was applied to obtain product ions from the precursor ion (*m/z* 225.12, *m/z* 190.08 and *m/z* 145.06). (B) A low collision energy of 5 eV allowed to keep the protonated molecule of dextromoramide (*m/z* 393.25) in the function 1, whereas, in the function 2, product ions (*m/z* 306.19 and *m/z* 239.15) were obtained with a higher collision energy of 30 eV from the precursor ion.

matrix interferences, as illustrated in Fig. 3. The process efficiency is defined as PE_{RE}^{ME}, with the exponent expresses ME while the suffix expresses RE. PE between 90% and 110% corresponded to a negligible effect of the matrix on the analyses for a particular compound (PE_0^0). The major ME was observed in the UHPLC–MS process (43%), namely ion suppression or enhancement (PE− or PE+) compared to the effect on the SPE method (PE−) or on both process (PE_-^- or PE_-^+). As shown in Fig. 3, for many compounds, the signal was influenced by the matrix (66%) and only 19% of the analytes were not altered by co-eluting compounds from the matrix in the entire process. Two phenomena could be highlighted in the extraction step. First, the urine matrix interfered with some analytes (e.g., clomifen and mefenorex) and second, the small and highly polar analytes were partly eluted during the washing step of the extraction method (e.g., heptaminol and methylecgonine). For some compounds, the ion suppression observed during the UHPLC–MS step was compensated by a high SPE recovery, attributable to a salting-out effect which could occur and increase the extraction recovery in urine samples compared to water sample. This demonstrates the importance of evaluating the matrix effect for each analyte even after a selective sample preparation, especially for quantitative analyses.

3.3. Application to a real case (qualitative analysis)

The applicability of the method was demonstrated with an authentic doping control sample found positive for the prohib-

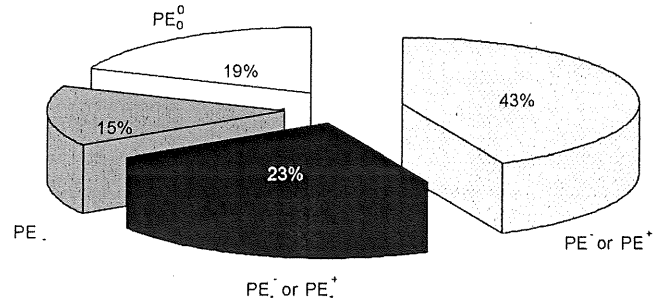

Fig. 3. Relative distribution in % of ME and RE on the whole set of data. PE_0^0 indicates cases with no ME and good RE, PE+ or PE− is related to cases with high or low ME, PE− refers to cases with low RE and $PE^{\pm}$ or PE^- is related to cases with high or low ME and low RE.

ited β-blocker atenolol at the screening and pre-confirmatory level. This basic compound was extracted on MCX cartridges in triplicate and identified by its t_R (0.80 min), its protonated molecule (*m/z* 267.17) and 3 product ions (*m/z* 225.12, *m/z* 190.08 and *m/z* 145.06) as illustrated in Fig. 4. The extracted ion chromatogram at *m/z* 267.17 ± 0.05 Da was highlighted at the same t_R on the suspect sample as well as on the quality control but, as expected, was absent on the negative urine spectrum in the t_R tolerance window (±2%). By comparing MS/MS spectra from the suspect and the QC sample, the relative intensity of the three diagnostic ions was also similar. The cluster of ions from the suspect sample matched that of the QC, whereas none of the diagnostic ions were observed in the negative urine. The abundance ratios were found equivalent as expressed by their differences in relative and absolute response reported in Table 3. Indeed, the relative intensity of any of the ions did not differ by more than 10% (absolute) and 25% (relative) as required by the WADA [7]. The identification criteria were reached and the analyte confirmed without ambiguity.

3.4. Quantitative validation

A quantitative validation was conducted for three of the investigated analytes (cathine, ephedrine and methylephedrine) following ISL, ICH and FDA recommendations [6,28,29]. It is noteworthy that cathine and ephedrine were co-eluting with their respective isomers (phenylpropanolamine and pseudoephedrine) in the selected gradient conditions. An isocratic method was thus developed to obtain the baseline separation of the isomers in 5 min, as presented in Fig. 5. Thanks to the high resolution of the UHPLC, it was possible to quantify the threshold analytes without the contribution of their respective isomer. Quantitative analyses were performed by diluting the samples in the dynamic range of the detector. Indeed, the signal of the analytes at their cut-off concentrations (i.e., 5 μg/mL for cathine and 10 μg/mL for ephedrine

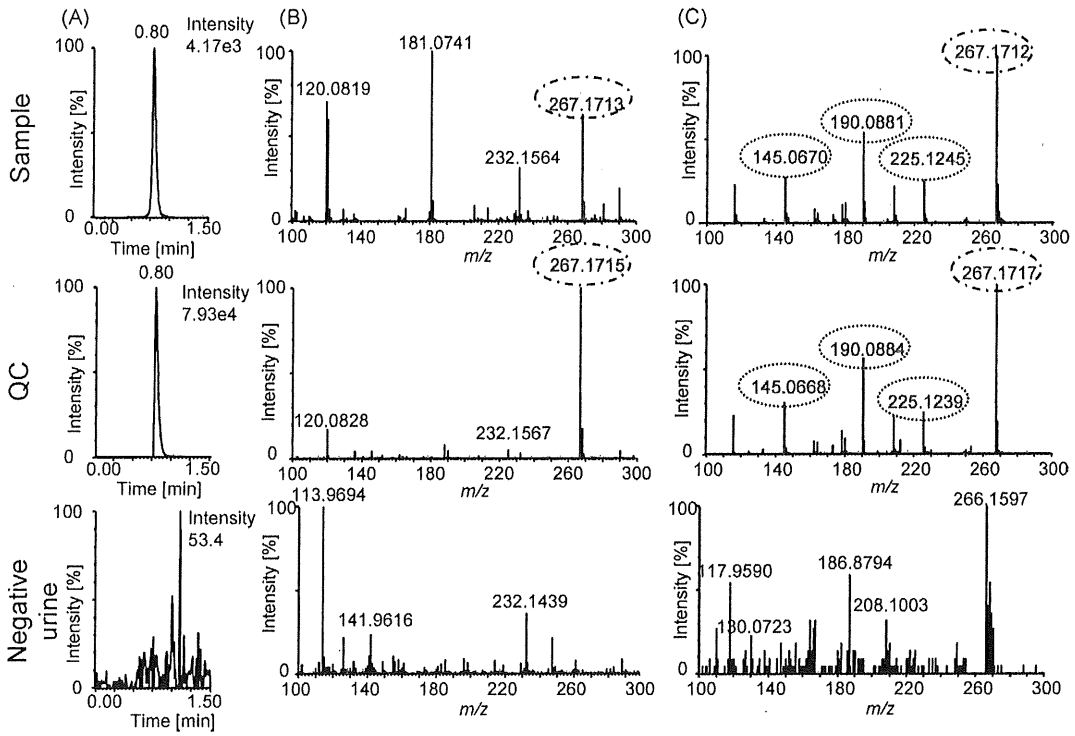

Fig. 4. Confirmatory analysis of atenolol. Comparison of spectra from the suspect sample, the QC and a negative blank urine. (A) The peak of atenolol is highlighted in the suspect and the QC samples at t_R = 0.80 min, whereas no peak was observed in the negative blank urine. (B) The protonated molecule of atenolol (*m/z* 267.17) was found in the mass spectra of the two first samples. (C) The four diagnostic ions of atenolol, namely the precursor ion (*m/z* 267.17) and the product ions (*m/z* 225.12, *m/z* 190.08 and *m/z* 145.06) obtained in the QTOF–MS/MS spectra with a collision energy set at 15 eV. None of them was found in the negative urine sample.

Table 3
Ion intensity ratios for identification criteria.

m/z	Suspect sample		QC sample		Difference (absolute) [%]	Difference (relative) [%]
	Absolute intensity	Relative intensity [%]	Absolute intensity	Relative intensity [%]		
145.06	6,990	27.6	2,520	23.8	3.9	14.0
190.08	13,900	54.9	5,230	49.3	5.6	10.2
225.12	6,410	25.3	2,660	25.1	0.2	1.0
267.17	25,300	100.0	10,600	100.0	0.0	0.0

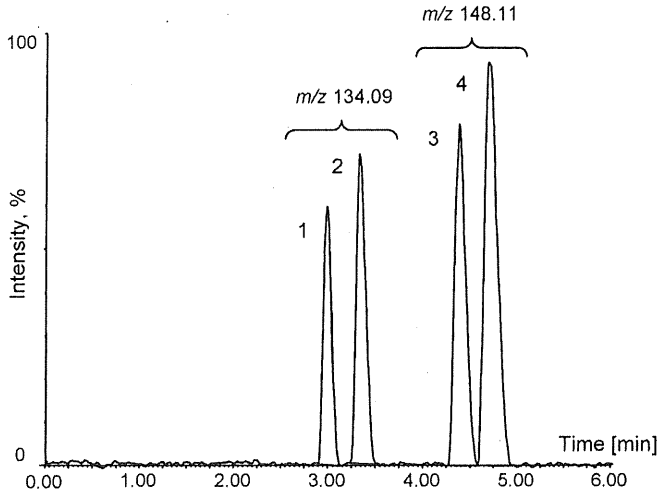

Fig. 5. Isocratic separation of 2 pairs of isomers: phenylpropanolamine (1) (t_R = 3.24 min)/cathine (2) (t_R = 3.57 min) and ephedrine (3) (t_R = 4.62 min)/pseudoephedrine (4) (t_R = 4.93 min) on an Acquity BEH C_{18} (2.1 mm × 50 mm, 1.7 μm) at 300 μL/min with an injection volume of 2 μL. The mobile phase consists of 0.1% formic acid (v/v) in 5:95 ACN/water.

and methylephedrine) saturated the detector response. A dilution factor of 60 was selected to obtain a concentration domain ranging from 0.05 to 2 μg/mL. Calibration curves were generated with 3 levels including the lower and upper limit of quantification and the middle concentration of the investigated range. A linear regression after logarithmic transformation was obtained with coefficients of determination (R^2) above 0.991 as reported in Table 4. Trueness was found acceptable at each concentration level except for cathine at the lowest concentration, where the threshold of ±15% was exceeded. The precision of the method was evaluated by calculating the repeatability and the intermediate precision as recommended in the validation guidelines [30]. The RSD values presented in Table 4 were acceptable at each concentration level and were in the range of 4.1–11.7% for repeatability and of 5.0–16.8% for intermediate precision, demonstrating acceptable performance of the method. The linearity, expressed as the calculated concentration as a function of the introduced concentration, was finally estimated by the slope, intercept and R^2. Since the slope values were between 0.95 and 1.08, the intercepts were between −0.0144 and 0.0021 and the R^2 values were all above 0.991, the method was considered linear and valid over the selected range.

Table 4
Validation parameters for ephedrine, methylephedrine and cathine.

Validation criterion	Ephedrine	Methylephedrine	Cathine
Trueness			
Relative bias (%)			
0.08 μg/mL	–	–	18.3
0.16 μg/mL	3.0	−11.3	−12.2
0.33 μg/mL	3.8	−1.1	–
0.50 μg/mL	–	–	2.6
1.0 μg/mL	0.4	−1.7	–
1.7 μg/mL	9.0	5.1	−7.4
Precision			
Repeatability/intermediate precision (RSD, %)			
0.08 μg/mL	–	–	11.7/9.9
0.16 μg/mL	11.1/12.0	4.1/7.4	8.0/9.8
0.33 μg/mL	8.9/16.8	4.6/7.6	–
0.50 μg/mL	–	–	4.6/7.2
1.0 μg/mL	6.0/5.0	4.7/9.2	–
1.7 μg/mL	6.7/16.7	8.3/10.7	6.3/6.4
Linearity			
Range (μg/mL)	0.1–2.0	0.1–2.0	0.05–2.0
Slope	1.0028	1.0852	0.9541
Intercept	0.0021	−0.0007	−0.0144
R^2	0.9940	0.9972	0.9914

3.5. Application to a real case (quantitative analysis)

A real case example of doping with ephedrine was presented. The suspect sample, the QC, an excretion study and the negative urine were extracted on MCX cartridges in triplicate analysis. The compound was identified according to the WADA's identification criteria. The t_R of the protonated molecule in the suspect sample as well as in the QC and the excretion study samples were identical and no peak was found in the t_R tolerance window (±2%) of the negative urine sample. The QTOF-MS/MS spectra were acquired with a collision energy set at 20 eV and 4 diagnostic ions of more than 5% intensity of the base peak were obtained (*m/z* 148.11, *m/z* 133.08, *m/z* 115.05 and *m/z* 91.05). The concentration of ephedrine in the suspect sample was estimated from the MS trace at 29.6 ± 1.8 μg/mL. This value was not statistically different (Student's *t* test, $\alpha = 0.05$) to the one obtained with an accredited method (30.9 ± 2.8 μg/mL determined by GC–MS, STS 288). The method is currently applied to a higher number of real case samples thanks to its reliability and suitability for quantitative analysis. Moreover, this developed method allowed a real gain in time compared to the GC–MS accredited method, as it was not necessary to derive the analytes to obtain their baseline separation.

4. Conclusion

The method proposed for the confirmatory analysis of 103 doping agents in urine allowed us to obtain a time *per* sample of about 7 min. Indeed, the time to extract 50 samples in the 96-well plate format is evaluated to around 30 min, while the UHPLC–QTOF-MS and MS/MS analysis requires 300 min and the spectra comparison can be performed within 20 min. The dedicated SPE procedure allowed a pre-concentration factor and use of low sample capacity (500 μL). Moreover, a gain in the overall throughput was observed by collecting the sample directly in the injection plates. For an overall confirmatory process, including the 6 samples (e.g., a negative blank urine, the suspect sample, a negative blank urine, two QCs and an excretion study or reference material) in triplicate, the required time is around 2 h. The method also proved to be a satisfactory tool for quantitative analysis and demonstrates reliability and time saving compared to classical confirmatory analysis. The use of simultaneous acquisition of MS and MS/MS spectra in the same run allowed us to reach the criteria for confirmatory analysis. Moreover, mass accuracy and elemental composition were certified as innovative identification criteria for the unambiguous confirmation of the intake of drugs of abuse in the anti-doping field. Furthermore, the baseline separation of ephedrine and pseudoephedrine together with cathine and phenylpropanolamine was of utmost importance, as it allowed their direct determination in urine within a short time, and will be very useful since the reintroduction of pseudoephedrine in the WADA's prohibited list from January 2010.

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