Screening and quantitation of multiclass drugs of abuse and pharmaceuticals in hair by fast liquid chromatography electrospray time-of-flight mass spectrometry

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ABSTRACT

In this work, an automated screening method for the simultaneous identification and quantitation of 30 representative multiclass drugs (including opiates, cocaine and its main metabolite, cannabinoids, amphetamines and other stimulants in hair samples) has been developed using fast liquid-chromatography time-of-flight mass spectrometry (LC-TOFMS). The identification and quantitation of the drugs were carried out by liquid chromatography using a C_{18} column (4.6×50 mm) with $1.8\,\mu m$ particle size. Accurate mass measurements of ions of interest (typically $[M+H]^+$) by electrospray time-of-flight mass spectrometry in the positive ionization mode were used for unambiguous confirmation of the targeted species. Three sample preparation methodologies were evaluated: (a) direct methanolic extraction by sonication, (b) acidic extraction, and (c) alkaline digestion. Direct methanolic extraction showed better recoveries and cleaner extracts. The limits of detection obtained in hair matrix were as low as $5\,\mathrm{pg\,mg^{-1}}$ for cocaine and cannabidiol, ranging from 5 to $75\,\mathrm{pg\,mg^{-1}}$ for the studied species while the LOQ ranged from 15 to $250\,\mathrm{pg\,mg^{-1}}$. The method has been applied to six hair samples from drug consumer volunteers, where the presence of at least one drug was confirmed by accurate mass measurements within 2 ppm (mass error) in most cases. The present study demonstrates the usefulness of LC-TOFMS for both screening and quantitation purposes in drug testing in hair. In addition, the possibility of non-target or *a posteriori* data analysis of samples or the extension of the procedure for testing for additional compounds offers interesting features for forensic analysis.

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1. Introduction

Drug testing in hair samples has become of increasing importance in recent years, since it has potential applications in forensic and clinical toxicology [1,2]. Hair analysis is a complementary approach for drug of abuse and pharmaceutical testing in biofluids, that offers some advantages over urine or blood assays [3,4], as time frame (sample collection period) in blood and urine samples is in the hour-range after drug consumption, while hair samples can be sampled much later, since drugs and metabolites remain in the hair an indefinite time [5,6]. Proof of this are the studies of Báez et al. [5], which tested positive main cocaine metabolite (benzoylecgonine) in hair samples from ancient mummies of more than 2000 years. Another advantaging feature is that collection of hair samples is less intrusive and embarrassing than blood or urine samples, and the stability of hair samples do not need special storage conditions as biofluids, and can be maintained in a simple paper envelope. Hair samples also allow to recognize the recent history of drug consumption, this is due to the hair growing speed is about 1 cm per month, so it is possible to make a segmental analysis of hair and to know the distribution of drug consumption in last months [6,7]. For all these reason, this sampling and testing method has gain wide acceptance in forensics.

In order to increase the performance of drug testing, development of a rapid and simultaneous method for the analysis of multiple drugs is required. The generally proposed mechanism for drug incorporation into hair is based in three different ways: during the hair growing, via the bloodstream; after the formation, via secretions of the sebaceous gland; and an external contamination due to the exposition to the environment [8]. The more dedicated part of drug testing methods in hair is sample treatment which requires the drug extraction from the hair matrix (inside). There are different extraction methods and comparisons between the different extraction protocols have been made [9–13]. The extraction step must be studied with special interest because it is not possible to make a recovery study, although some authors have published methods were they perform a recovery study only soaking the surface of the hair with a drug solution, thus always obtained good recoveries [12,14,15], but the Society of Hair Testing does not recommend this methodology. Its proposed methodology is to

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expose drug-free hair to aqueous solutions of drugs at high concentrations, for several days and then thoroughly wash the hair before drying and analyzing [16], additionally, these spiked samples can be used for precision studies, routine quality controls, and as internal degradation controls, but not for recovery studies.

Different methods have been proposed for the testing of class-specific groups of compounds as amphetamines and other stimulants [15,17–21], sedatives [22,23], cocaine and opiates [6,15,21,24,25], or cannabis and derivatives [26,27], but there are only a few papers describing extensive screening of multiclass drugs [14,28]. The testing of hair extracts has been made by gas chromatography/mass spectrometry [7,12,19,26,29,30], which has the disadvantage inherent to the gas chromatography, due to the need of derivatization of polar analytes such as most of these drugs. This is solved when using liquid chromatography with traditional detectors [6,18,22,29], or coupled to different mass spectrometry detectors as triple quadrupole [23,24], ion trap [15], time-of-flight mass spectrometry [14] or hybrid linear ion trap–orbitrap mass spectrometry [31,32]. The use of direct mass spectrometric techniques such as ambient ionization mass spectrometry [33] and Matrix-Assisted Laser Desorption Ionization Mass Spectrometry (MALDI-MS) [34] have been also described for drug testing in hair extracts.

In this article, an automated screening method for the simultaneous identification and quantitation of 30 representative multiclass drugs (including opiates, cocaine and metabolite, cannabinoids, amphetamines and other stimulants in hair samples) has been developed using fast liquid-chromatography time-of-flight mass spectrometry (LC-TOFMS). The identification and quantitation of the drugs were carried out by rapid resolution liquid chromatography using a C_{18} column (4.6 mm × 50 mm) with 1.8 μm particle size and mass spectrometry detection using accurate mass measurements of ions of interest by electrospray time-of-flight mass spectrometry in the positive ionization mode. The use of high resolving power mass spectrometry provides high sensitivity in full scan mode, allowing the analysis of both targets and non-targets, so that untargeted retrospective analysis is possible in order to extend the test for new (unexpected) compounds. Three generic multiclass sample preparation methodologies were evaluated: (a) direct methanolic extraction by sonication, (b) acidic extraction, and (c) alkaline digestion. Direct methanolic extraction showed better recoveries and cleaner extracts. The method has been applied to six hair samples from drug consumer volunteers in which the presence of at least one drug has been confirmed by accurate mass measurements within 2 ppm (mass error). The present study demonstrates the usefulness of LC-TOFMS for both screening and quantitation purposes in drug testing in hair.

2. Experimental

2.1. Chemicals and reagents

Standards were purchased from Cerilliant (Round Rock, TX). Individual drug stock solutions (*ca.* 500 μg mL^{-1}) were prepared in pure methanol and stored at −18 °C. HPLC grade acetonitrile was obtained from J-T. Baker (Constantí, Tarragona, Spain). HPLC grade methanol was acquired from Sigma–Aldrich (Madrid, Spain). Formic acid and ammonium formate were purchased from Fluka (Madrid, Spain). A Milli-Q-Plus ultra-pure water system from Millipore (Milford, MA) was used throughout the study to obtain the HPLC water used during the analyses. Oasis HLB^{TM} SPE cartridges (200 mg, 6 mL) were purchased from Waters (Milford, MA) and a Supelco (Bellefonte, PA) Visiprep^{TM} SPE vacuum system was used for SPE experiments.

2.2. Hair sample collection

Hair samples were collected from six drug consumer volunteers (men and women, older than 18 years). Strands of hair were cut as close as possible to the skin from the posterior vertex region of the head, as close as possible to the scalp, according to the recommendations for hair testing in forensic cases by the Society of Hair Testing [16,35]. Hair samples were preserved at room temperature until analysis in closed paper envelopes. Blank hair samples were obtained from a healthy man with no drug of abuse or pharmaceutical history.

2.3. Sample preparation

Human hair segments were washed with shampoo and rinsed with deionized water, then rinsed with acetone and air dried overnight. Dried samples were cut into 1 mm pieces and about 20 mg of segments were weighted in glass tubes. Three different extraction protocols were used in order to evaluate the most appropriate extraction method.

2.3.1. Direct methanol extraction
Hair samples (20 mg) were incubated with 4 mL of methanol. The mixture was ultrasonicated for 8 h at 50 °C and then incubated at room temperature overnight, the extract was centrifuged and the supernatant was evaporated under N_2 stream until almost dry. The residue was reconstituted with 1 mL of methanol/water (1:1) and filtered through a 0.45 μm PTFE syringe filter and then transferred to an analysis vial. 20 μL of the extract was analyzed with the LC-TOFMS system.

2.3.2. Acidic extraction with hydrochloric acid
Two mL of 0.1 N HCl was added to the hair segments (20 mg) and incubated for 18 h at 50 °C. The extract was diluted with water and neutralized with NaOH. An additional SPE clean-up step was performed with Oasis HLB^{TM} cartridges. The cartridges were previously conditioned with 4 mL of methanol and 4 mL of MilliQ water, then the neutralized extract was passed through the cartridge, the cartridges were washed with 5% MeOH in water and then dried with a N_2 stream, finally the analytes were eluted from the cartridge with 4 mL of methanol. Clean extracts were evaporated under N_2 stream until almost dryness and the residue was reconstituted with 1 mL of methanol/water (1:1) and filtered through a 0.45 μm PTFE syringe filter and then transferred to an analysis vial. 20 μL of the extract was analyzed with the LC-TOFMS system.

2.3.3. Alkaline digestion
Two mL of 0.5 N NaOH was added to the hair samples (20 mg) and incubated at 100 °C for 30 min. This produces the complete dissolution of the hair matrix, yielding a very dirty extract. For this reason, the same SPE clean up step used in the acidic extraction, using Oasis HLB^{TM} cartridges was performed. 20 μL of the extract was analyzed with the LC-TOFMS system.

2.4. Liquid chromatography time-of-flight mass spectrometry

The separation of the drugs from the hair extract was carried out using a high-performance liquid chromatography (HPLC) system (consisting of vacuum degasser, auto sampler and a binary pump) (Agilent series 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 hair extract was injected in each run. Mobile phases A and B were water with 0.1% formic acid and acetonitrile. The chromatographic method held the initial mobile

Table 1
Accurate mass analysis of the selected drugs of abuse and pharmaceuticals in a blank hair extract, spiked at 100 μg L^{-1} (190 V fragmentor voltage).

Name	RT	Ion	Elemental composition	Theoretical m/z	Experimental m/z	Error	
						mDa	ppm
Acetaminophen	2.26	[M+H]$^+$	$C_8H_{10}NO_2$	152.0706	152.0707	−0.11	−0.70
Acetazolamide	3.82	[M+H]$^+$	$C_4H_7N_4O_3S_2$	222.9954	222.9952	−0.21	−0.96
Amiloride	2.17	[M+H]$^+$	$C_6H_9ClN_7O$	230.0552	230.0555	0.3	1.33
Amphetamine	4.02	Fragment 2	C_7H_7	91.0542	91.0545	0.31	3.48
Atenolol	1.79	[M+H]$^+$	$C_{14}H_{23}N_2O_3$	267.1703	267.1706	0.29	1.05
Benzoylecgonine	7.72	[M+H]$^+$	$C_{16}H_{20}NO_4$	290.1387	290.1391	0.43	1.48
β-Estradiol	11.77	Fragment 1	$C_{18}H_{23}O$	255.1743	255.1744	0.09	0.35
Boldenone	11.38	[M+Na]$^+$	$C_{19}H_{26}O_2Na$	309.1825	309.1824	−0.07	−0.24
Cannabidiol	16.13	[M+H]$^+$	$C_{21}H_{30}O_2$	315.2319	315.2318	−0.07	−0.22
Cocaine	8.70	[M+H]$^+$	$C_{17}H_{21}NO_4$	304.1543	304.1543	0.05	0.17
Codeine	3.26	[M+H]$^+$	$C_{18}H_{21}NO_3$	300.1594	300.1597	0.3	1.01
Δ^9-THC	17.71	[M+H]$^+$	$C_{21}H_{31}O_2$	315.2319	315.2317	−0.15	0.49
Diazepam	12.33	[M+H]$^+$	$C_{16}H_{14}N_2OCl$	285.0789	285.0792	0.3	1.06
Dobutamine	7.77	[M+H]$^+$	$C_{18}H_{24}NO_3$	302.1751	302.1570	−0.1	−0.35
Ephedrine	2.86	Fragment 5	$C_{10}H_{14}N$	148.1121	148.1122	0.15	1.02
Fluoxymesteorone	10.81	[M+Na]$^+$	$C_{20}H_{29}FO_3Na$	359.1993	359.1895	0.2	0.59
Heroin	8.48	[M+H]$^+$	$C_{21}H_{24}NO_5$	370.1649	370.1650	0.04	0.12
Ketamine	7.58	[M+H]$^+$	$C_{13}H_{17}NOCl$	238.0993	238.0996	0.23	0.98
Methadone	10.69	[M+H]$^+$	$C_{21}H_{28}NO$	310.2165	310.2170	0.44	1.41
Methamphetamine	5.17	Fragment 2	C_7H_7	91.0542	91.0545	0.3	3.33
Morphine	1.51	[M+H]$^+$	$C_{17}H_{20}NO_3$	286.1438	286.1441	0.29	1
Nandrolone	11.61	[M+H]$^+$	$C_{18}H_{27}O_2$	275.2006	275.2009	0.32	1.18
Propranolol	9.41	[M+H]$^+$	$C_{16}H_{22}NO_2$	260.1645	260.1648	0.26	1.01
Salbutamol	1.66	[M+H]$^+$	$C_{13}H_{22}NO_3$	240.1594	240.1590	−0.39	−1.61
Stanozol	11.82	[M+H]$^+$	$C_{21}H_{33}N_2O$	329.2587	329.2592	0.43	1.31
Terbutaline	1.74	[M+H]$^+$	$C_{12}H_{20}NO_3$	226.1438	226.1432	−0.58	−2.6
Testosterone	12.06	[M+Na]$^+$	$C_{19}H_{28}O_2Na$	311.1982	311.1986	0.43	1.48
Triamcinolone	9.12	[M+Na]$^+$	$C_{21}H_{27}FO_6Na$	417.1679	417.1673	−0.59	−1.49
Triamterene	7.37	[M+H]$^+$	$C_{12}H_{12}N_7$	254.1149	254.1152	0.34	1.35
Warfarin	12.73	[M+Na]$^+$	$C_{19}H_{16}O_4Na$	331.0941	331.0945	0.39	1.25

phase composition (10% B) constant for 3 min, followed by a linear gradient to 100% B up to 15 min and kept for 5 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 positive 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 (in-source CID fragmentation), 160, 190, 220 and 250 V. LC-MS accurate mass spectra were recorded across the m/z range of 50–1000. The instrument performed the internal mass calibration automatically, using a dual-nebulizer electrospray source with an automated calibrant delivery system, which introduces the flow from the outlet of the chromatograph together with a low flow (approximately 40 μL min^{-1}) of a calibrating solution which contains the internal reference masses purine ($C_5H_4N_4$, at m/z 121.050873, in positive ion mode) and HP-0921 (Hexakis-(1H,1H,3H-tetrafluoropropoxy)phosphazine, $C_{18}H_{18}O_6N_3P_3F_{24}$, at m/z 922.009798 in positive ion mode). The instrument provided a typical resolution better than 10,000 at m/z 118 and better than 18,000 at m/z 1522. The full scan data recorded with Agilent MassHunter Data Acquisition software (version B.02.00, Patch 3) and processed with Agilent MassHunter Qualitative Analysis software (version B.02.00, Patch 3) and Agilent MassHunter Quantitative Analysis software (version B.01.04, Patch 2).

2.5. Development of an automated screening method based on accurate mass of selected ions, retention time and characteristic fragmentation of the targeted species

A mixture standard with the 30 selected multiclass drugs at an individual concentration of *ca.* 200 μg L^{-1} was prepared. This solution was analyzed using the LC-TOFMS to collect the retention time using the proposed gradient method. For the automatic screening

method, an Excel spreadsheet was constructed containing the exact mass data for each drug and their retention times (Table 1). This file was put into CSV format for use by the Agilent TOF automated data analysis software (MassHunter Qualitative Analysis, version B.02.00, Patch 3) (Fig. 1).

The spectral features of each compound were studied, in-source CID fragmentation was investigated at four different fragmentor voltages (160, 190, 220 and 250 V), obtaining diagnostic fragment ions for the analytes, and the database was built including also these diagnostic ions for confirmation purposes. The fragmentation pattern can be used to identify compounds with similar structure (e.g. family of derivates of ephedrine or amphetamines), degradation products or metabolites, thus can be used for the identification of unknown metabolites or degradation products without the need of primary standards.

To find and identify the presence of target compounds in the sample the searching database tool of the software (MassHunter Qualitative Analysis) was used. This tool is based on the search on the raw data (LC-TOFMS file) of the selected retention time/accurate mass pairs included on the database. When a positive is found, its extracted ion chromatogram and mass spectrum were extracted automatically. The defined search criteria were accurate mass and retention time tolerance, two mass tolerances were selected, 10 mDa for screening purposes and 5 ppm or 1 mDa for confirmation. The retention time tolerance was fixed at ±0.2 min in both screening and confirmation analysis.

2.6. Application of the automated screening method for the identification and confirmation of multiclass drugs of abuse and pharmaceuticals in hair samples

Six hair samples from volunteer drug consumers and a blank hair sample from volunteers were extracted following described procedures in Section 2.3 and analyzed by the LC-TOFMS screening method based on the created accurate mass database.
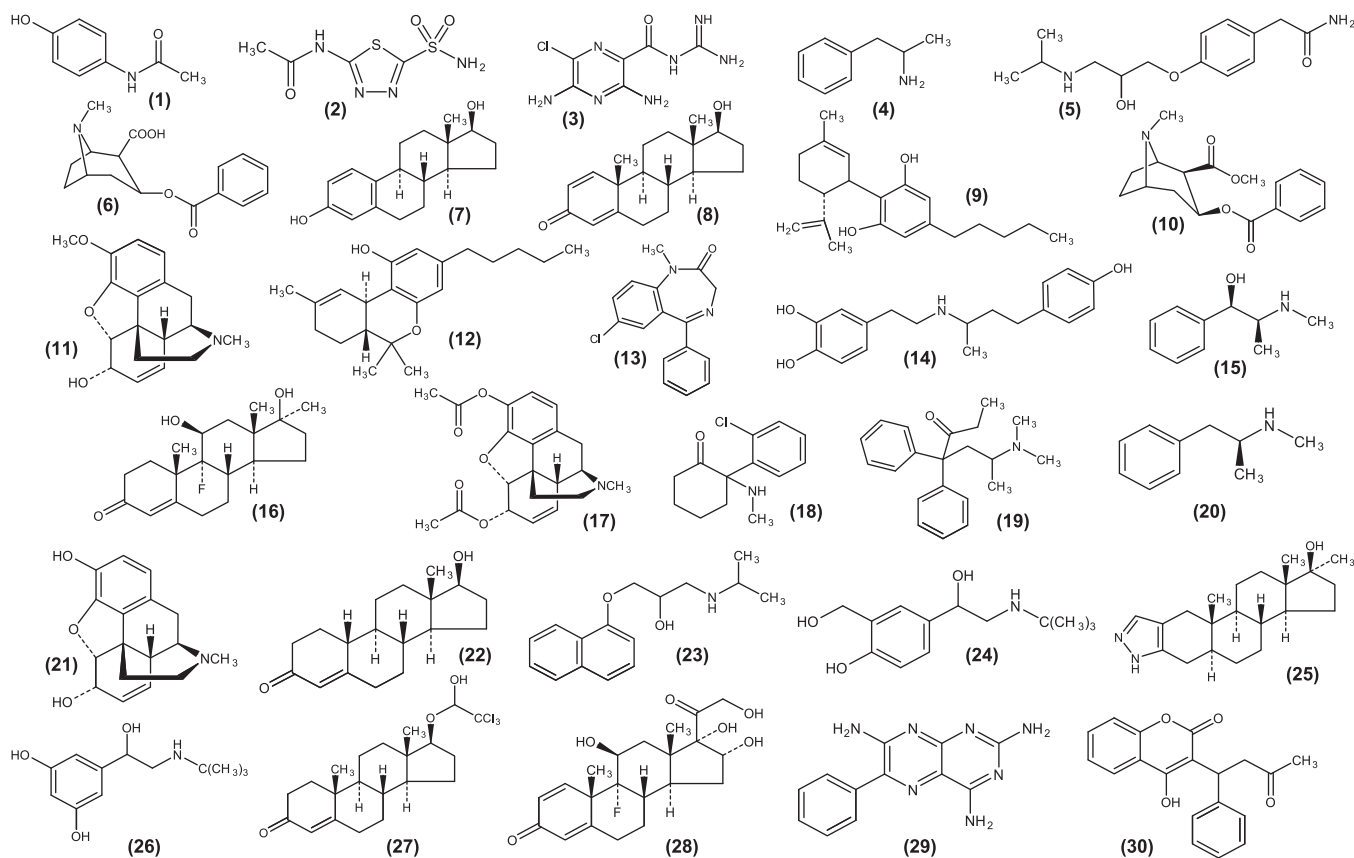
Fig. 1. Structures of compounds included in the method. (1) Acetaminophen, (2) acetazolamide, (3) amiloride, (4) amphetamine, (5) atenolol, (6) benzoylecgonine, (7) β-estradiol, (8) boldenone, (9) cannabidiol, (10) cocaine, (11) codeine, (12) Δ⁹-THC, (13) diazepam, (14) dobutamine, (15) ephedrine, (16) fluoxymesterone, (17) heroin, (18) ketamine, (19) methadone, (20) methamphetamine, (21) morphine, (22) nandrolone, (23) propranolol, (24) salbutamol, (25) stanozol, (26) terbutaline, (27) testosterone, (28) triamcinolone, (29) triamterene, and (30) warfarin.

3. Results and discussion

3.1. Identification and confirmation of the targeted drugs by LC-TOFMS: in-source CID fragmentation and accurate mass measurements

Standard electrospray ionization conditions were selected to obtain the best possible sensitivity and selectivity for the selected compounds. Standard values were set for drying and nitrogen flow rates, vaporizer and drying temperatures, and capillary voltage. Besides the typical electrospray parameters, the parameter associated with in-source CID fragmentation which had a strong influence on the sensitivity and relative abundance of protonated molecules were carefully studied.

Table 2 shows the fragmentation of the studied drugs and the relative abundances of the different species formed. The fragmentor voltage is the parameter that establishes the extent in which in-source CID fragmentation is carried out. These values are usually tested with the instrument used: 160 V (mild conditions), 190 V (medium fragmentation), 230 V (high fragmentation) and 250 V (extensive fragmentation). The extent of the fragmentation is primarily compound-dependent. For instance compounds with a related structure as amphetamine, methamphetamine and ephedrine yield several fragment ions even under mild conditions, while other compounds such as codeine, heroin and morphine, that also have a similar structure, are difficult to fragment despite a high fragmentor voltage is applied.

The highest fragmentor voltage value (250 V) gave extensive fragmentation of the protonated molecules in most cases. Only 11 out of 30 compounds still presented the protonated molecule as

base peak under these conditions. On the contrary, 160 V produced little or no fragmentation, so no additional structure information could be achieved for unambiguous confirmation of the target species. For this reason, the fragmentor voltage was set at 190 V, as a compromise value between sensitivity for quantitation and additional mass spectrum information for confirmation purposes.

In-source CID fragmentation is an interesting feature to add specific analyte information for unambiguous confirmation of the positive findings. Using the selected conditions, 14 out of 30 compounds (47%) gave useful fragmentation. It should be noted that the primary identification of the targeted species is performed by retention time matching and accurate mass measurements of the main characteristic ion with accuracy typically better than 2 ppm in almost all cases. In-source CID was characterized for complementary tool for confirmatory purposes. By using high resolution mass spectrometry data with high mass accuracies, unambiguous identification of the targeted species can be accomplished despite some of them might not have additional fragments ions.

Table 1 shows the results obtained for the accurate mass analysis of the selected drugs and pharmaceuticals in a blank hair extract, spiked with 100 μg L⁻¹ (final concentration). From the data obtained, it can be concluded that the method offers a high confirmation degree because of its very high mass accuracy, enabling accurate mass measurements of target ions within 2 ppm (mass error) in most cases. For identification and quantitation purposes, we used extracted ion chromatograms (XICs) using a mass-window width of 20 mDa ([M+H]⁺ ±10 mDa). The protonated molecule ([M+H]⁺) was used for both confirmation and quantitation purposes in most of the species except when the relative intensity of a sodium adduct ([M+Na]⁺) (e.g. acetaminophen) or characteristic

Table 2
Fragmentation study on the selected 30 drugs of abuse and pharmaceuticals: effect of the fragmentor voltage on CID fragmentation.

Compound	Ion	Theoretical m/z	Elemental composition	Relative abundance (%)			
				160 V	190 V	220 V	250 V
Acetaminophen	[M+H]$^+$	152.0706	$C_8H_{10}NO_2$	100	46	38	36
	Fragment 1	110.0600	C_6H_8NO	–	80	100	100
Acetazolamide	[M+Na]$^+$	244.9774	$C_4H_6N_4O_3S_2Na$	84	39	–	–
	[M+H]$^+$	222.9954	$C_4H_7N_4O_3S_2$	100	44		
	Fragment 1	180.9848	$C_2H_5N_2O_2S_2$	31	100		
Amiloride	[M+H]$^+$	230.0552	$C_6H_9ClN_7O$	100	100	18	–
	Fragment 1	171.0068	$C_5H_4ClN_4O$	–	62	100	50
	Fragment 2	143.0119	$C_4H_4ClN_4$	–	7	27	46
	Fragment 3	116.0010	$C_3H_3ClN_3$	–	5	27	100
Amphetamine	[M+H]$^+$	136.1121	$C_9H_{14}N$	47	–	–	–
	Fragment 1	119.0855	C_9H_{11}	92	12	–	–
	Fragment 2	91.0542	C_7H_7	100	100	100	100
Atenolol	[M+H]$^+$	267.1703	$C_{14}H_{23}N_2O_3$	100	100	100	100
	Fragment 1	225.1234	$C_{11}H_{17}N_2O_3$	–	–	10	28
	Fragment 2	190.0863	$C_{11}H_{12}NO_2$	–	–	9	65
Benzoylecgonine	[M+Na]$^+$	312.1206	$C_{16}H_{19}NO_4Na$	19	22	26	14
	[M+H]$^+$	290.1387	$C_{12}H_{20}NO_4$	100	100	100	18
	Fragment 1	168.1019	$C_9H_{14}NO_2$	–	9	75	100
β-Estradiol	[M+H]$^+$	273.1849	$C_{18}H_{25}O_2$	16	13	–	–
	Fragment 1	255.1743	$C_{18}H_{23}O$	100	100	49	9
	Fragment 2	159.0804	$C_{11}H_{11}O$	–	31	100	100
	Fragment 3	133.0648	C_9H_9O	–	7	33	41
	Fragment 4	107.0491	C_7H_7O	31	40	64	41
Boldenone	[M+Na]$^+$	309.1825	$C_{19}H_{26}O_2Na$	100	100	100	100
	[M+H]$^+$	287.2006	$C_{19}H_{27}O_2$	76	29	7	–
Cannabidiol	[M+H]$^+$	315.2319	$C_{21}H_{31}O_2$	100	100	100	100
	Fragment 1	193.1223	$C_{12}H_{17}O_2$	–	–	–	78
Cocaine	[M+H]$^+$	304.1543	$C_{17}H_{22}NO_4$	100	100	100	20
	Fragment 1	182.1176	$C_{10}H_{16}NO_2$	–	8	64	100
Codeine	[M+H]$^+$	300.1594	$C_{18}H_{22}NO_3$	100	100	100	100
Δ^9-THC	[M+H]$^+$	315.2319	$C_{21}H_{31}O_2$	100	100	100	100
Diazepam	[M+H]$^+$	285.0789	$C_{16}H_{14}ClN_2O$	100	100	100	100
Dobutamine	[M+H]$^+$	302.1751	$C_{18}H_{24}NO_3$	100	100	100	25
	Fragment 1	137.0597	$C_8H_9O_2$	–	–	40	65
	Fragment 2	107.0491	C_7H_7O	–	–	47	100
	Fragment 3	91.0542	C_7H_7	–	–	5	17
Ephedrine	[M+H]$^+$	166.1226	$C_{10}H_{16}NO$	92	5	–	–
	Fragment 1	148.1121	$C_{10}H_{14}N$	100	100	100	39
	Fragment 2	133.0886	$C_9H_{11}N$	–	7	46	90
	Fragment 3	117.0699	C_9H_9	–	6	25	37
	Fragment 4	115.0542	C_9H_7	–	–	13	70
	Fragment 5	91.0542	C_7H_7	–	–	17	100
Fluoxymesterone	[M+Na]$^+$	359.1993	$C_{20}H_{29}FO_3Na$	100	100	100	100
	[M+H]$^+$	337.2173	$C_{20}H_{30}FO_3$	55	24	20	6
Heroin	[M+H]$^+$	370.1649	$C_{21}H_{24}NO_5$	100	100	100	100
Ketamine	[M+H]$^+$	238.0993	$C_{13}H_{17}ClNO$	100	100	8	–
	Fragment 1	220.0888	$C_{13}H_{15}ClN$	–	7	10	–
	Fragment 2	207.0571	$C_{12}H_{12}ClO$	–	33	17	–
	Fragment 3	125.0153	C_7H_6Cl	–	45	100	100
Methadone	[M+H]$^+$	310.2165	$C_{21}H_{28}NO$	100	100	36	–
	Fragment 1	265.1587	$C_{19}H_{21}O$	–	9	100	100
	Fragment 2	223.1117	$C_{16}H_{15}O$	–	–	9	39
Methamphetamine	[M+H]$^+$	150.1277	$C_{10}H_{16}N$	100	7	–	–
	Fragment 1	119.0855	C_9H_{11}	33	18	–	–
	Fragment 2	91.0542	C_7H_7	48	100	100	100
Morphine	[M+H]$^+$	286.1438	$C_{17}H_{20}NO_3$	100	100	100	100
Nandrolone	[M+H]$^+$	275.2006	$C_{18}H_{27}O_2$	100	100	100	100
	Fragment 1	257.1900	$C_{18}H_{25}O$	–	–	5	18
Propranolol	[M+H]$^+$	260.1645	$C_{16}H_{22}NO_2$	100	100	100	69
	Fragment 1	218.1176	$C_{13}H_{16}NO_2$	–	–	6	12
	Fragment 2	183.0804	$C_{13}H_{11}O$	–	–	12	75
	Fragment 3	157.0648	$C_{11}H_9O$	–	–	9	100
	Fragment 4	145.0648	$C_{10}H_9O$	–	–	–	33
	Fragment 5	116.107	$C_6H_{14}NO$	–	–	11	47
Salbutamol	[M+H]$^+$	240.1594	$C_{13}H_{22}NO_3$	100	100	–	–
	Fragment 1	222.1489	$C_{13}H_{20}NO_2$	8	51	7	–
	Fragment 2	166.0863	$C_9H_{12}NO_2$	8	88	51	10
	Fragment 3	148.0757	$C_9H_{10}NO$	6	65	100	100
Stanozol	[M+H]$^+$	329.2587	$C_{21}H_{33}N_2O$	100	100	100	100
Terbutaline	[M+H]$^+$	226.1438	$C_{12}H_{20}NO_3$	100	76	–	–
	Fragment 1	152.0706	$C_8H_{10}NO_2$	13	100	100	100
	Fragment 2	107.0491	C_7H_7O	–	5	10	79

Table 2 (*Continued*)

Compound	Ion	Theoretical m/z	Elemental composition	Relative abundance (%)			
				160 V	190 V	220 V	250 V
Testosterone	$[2M+Na]^+$	599.4071	$C_{38}H_{56}O_4Na$	100	67	58	43
	$[M+Na]^+$	311.1982	$C_{19}H_{28}O_2Na$	75	100	100	100
	$[M+H]^+$	289.2162	$C_{19}H_{29}O_2$	87	47	37	20
Triamcinolone	$[M+Na]^+$	417.1684	$C_{21}H_{27}FO_6Na$	57	100	100	100
	$[M+H]^+$	395.1864	$C_{21}H_{28}FO_6$	100	56	10	–
Triamterene	$[M+H]^+$	254.1149	$C_{12}H_{12}N_7$	100	100	100	100
	Fragment 1	237.0883	$C_{12}H_9N_6$	–	–	–	21
Warfarin	$[2M+Na]^+$	639.1989	$C_{38}H_{32}O_8Na$	72	49	15	–
	$[M+Na]^+$	331.0941	$C_{19}H_{16}O_4Na$	100	100	100	100
	$[M+H]^+$	309.1121	$C_{19}H_{17}O_4$	13	7	–	–

common fragment ion (e.g. ephedrine) was higher than that of the protonated molecule in the selected conditions.

3.2. Hair extraction experiments

Unlike previous studies involving spiking of hair just by soaking the surface of the hair, we did not consider to perform a recovery study due to is not possible to spike correctly the hair samples because the standard solution applied to hair is not going to penetrate into the hair matrix as it would happen under real conditions. In these studies, the hair surface is wetted with the solution being the analyte deposited on the surface, so that recoveries obtained usually approached 100% and the complete extraction is achieved within a very short extraction time. This scenario is completely different from real hair samples because drugs are not going to be in the surface, but in the hair matrix. For this reason, in order to evaluate different multiclass generic extraction methods of drugs in hair, a sample with positive findings on multiclass analytes was used to perform a relative comparison of peak areas of the extracted drugs from real samples, using the different extraction method under comparable conditions (in terms of extract volume/weight hair ratios).

The results obtained from this study are shown in Fig. 2. Direct methanolic sonication was selected as the extraction method because the extraction yields of the detected drugs from the
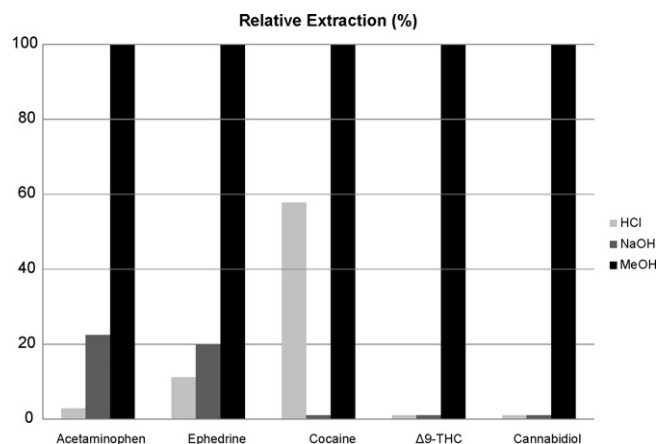

Fig. 2. Comparison of the relative recovery of different extractions protocols.

hair matrix was distinctly higher with methanolic extraction. Fig. 2 shows the relative extraction of the drugs present on a real sample (sample 4, not spiked) is shown, taking as 100% the recovery of the best extraction protocol. The best recoveries were obtained when direct methanolic extraction is used. The sample contained acetaminophen, cocaine, ephedrine, Δ^9-THC and
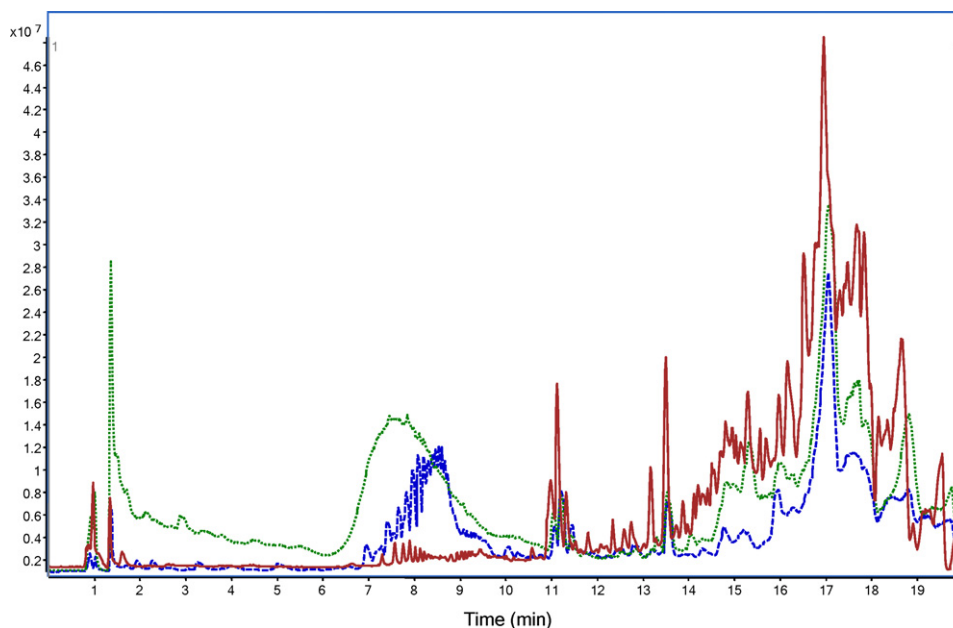

Fig. 3. Overlapped Total Ion Chromatograms (TICs) of methanolic sonication (red continuous line), alkaline digestion (green dotted line) and acidic extraction (blue discontinuous line) extracts of the same sample. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)
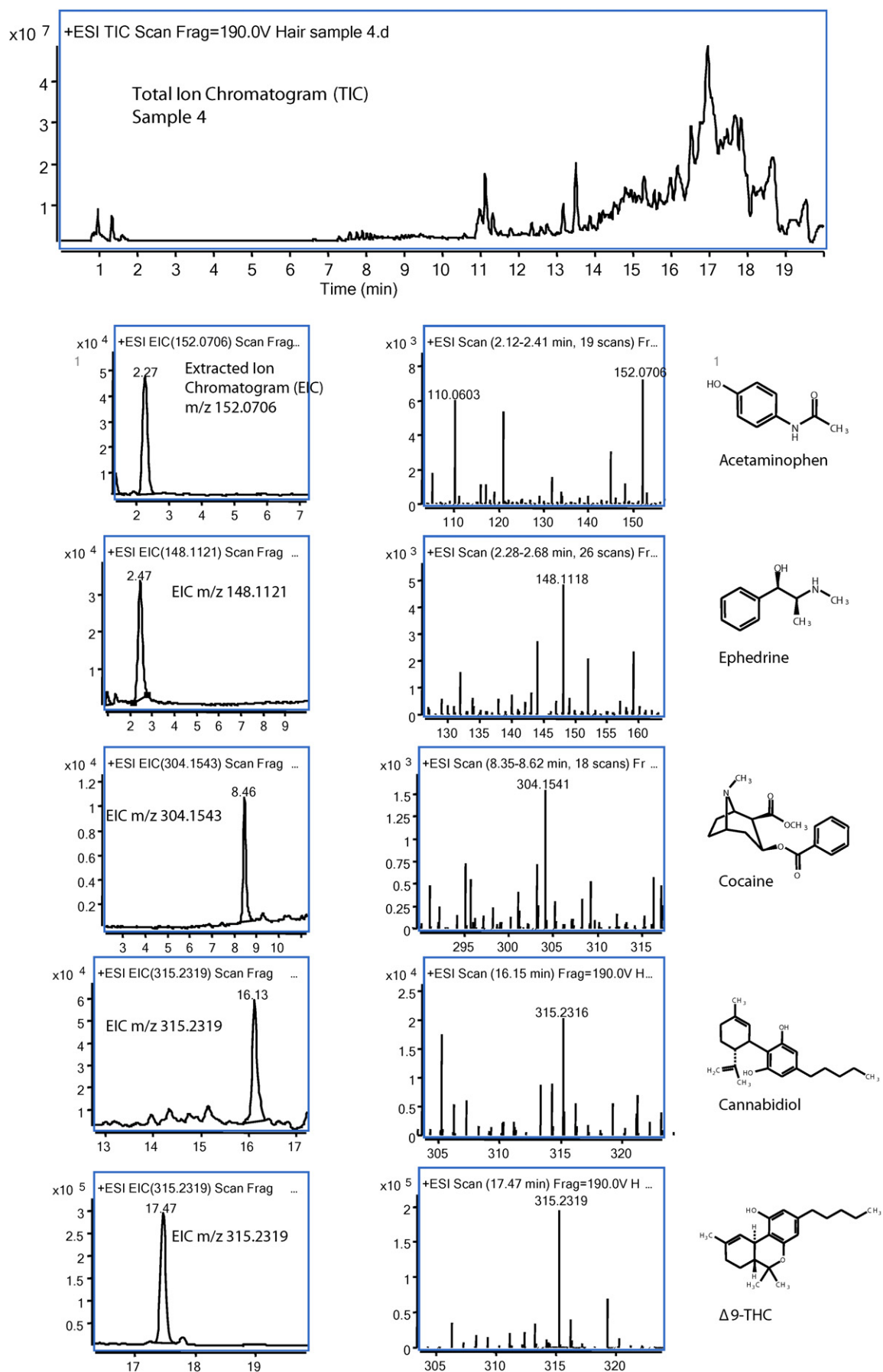
Fig. 4. Example of a hair sample containing acetaminophen (1081 pg mg^{-1}), ephedrine (321 pg mg^{-1}), cocaine (23 pg mg^{-1}), cannabidiol (2456 pg mg^{-1}) and Δ^9-THC (2979 pg mg^{-1}).

Table 3
Analytical parameters for the analysis of selected 30 drugs of abuse and pharmaceuticals in hair samples by LC-TOFMS.

Compound	LOD (pg mg^{-1})	LOQ (pg mg^{-1})	Calibration curve			RSD (%) ($n=6$)			Matrix effect
			R^2	Slope	Intercept	Intra day ($C=$ LOQ)	Intra day ($C=100 \times$ LOQ)	Inter day ($C=100 \times$ LOQ)	
Acetaminophen	50	150	0.9996	5425.8	13150.2	1.70	0.93	21.73	0.87
Acetazolamide	50	150	0.9992	2637.8	14693.2	1.52	2.61	28.73	0.85
Amiloride	12.5	40	0.9996	9681.3	42865.8	2.03	1.45	16.04	0.82
Amphetamine	50	150	0.9999	32,483	−275,940	4.15	2.61	10.92	1.08
Atenolol	5	15	0.9998	38422.9	8187.6	3.27	2.40	15.32	0.87
Benzoylecgonine	12.5	40	0.9996	49507.7	24096.8	1.50	0.86	18.44	0.67
β-Estradiol	20	60	0.9996	2658.0	3231.1	2.69	3.30	6.53	0.94
Boldenone	25	80	0.9997	2004.1	1353.1	3.42	0.97	20.98	0.94
Cannabidiol	5	15	0.9987	2329.6	6222.9	2.65	3.27	6.26	0.96
Cocaine	5	15	0.9999	9113.6	−15350.5	2.31	0.79	13.93	1.17
Codeine	25	75	0.9991	37517.0	−203085.5	1.84	1.59	7.92	1
Δ^9-THC	12.5	40	0.9994	9565.1	15708.1	4.69	5.14	7.83	0.95
Diazepam	12.5	40	0.9994	82975.1	−108887.1	5.12	1.14	10.75	0.99
Dobutamine	50	150	0.9926	4022.3	−15710.9	7.23	6.33	12.23	0.49
Ephedrine	50	150	0.9972	26671.7	−51061.8	3.25	1.31	16.83	0.96
Fluoxymesterone	50	150	0.9997	7779.1	36.0	1.56	1.14	22.06	0.98
Heroin	12.5	40	0.9987	50188.8	−55500.9	0.94	1.45	14.51	1.16
Ketamine	12.5	40	0.9993	25104.5	518.1	1.78	1.45	17.56	1
Methadone	50	150	0.9973	29383.7	22732.9	3.41	4.00	6.64	0.28
Methamphetamine	50	150	0.9994	5051.3	15029.4	1.85	2.29	13.67	0.97
Morphine	12.5	40	0.9991	26690.0	5481.1	3.79	1.23	15.66	0.57
Nandrolone	12.5	40	0.9988	20757.6	13594.0	2.45	1.38	13.87	0.98
Propranolol	5	15	0.9996	144128.9	10998.3	1.50	0.44	6.03	0.94
Salbutamol	25	80	0.9997	22357.2	17380.8	3.29	1.56	22.07	0.88
Stanozol	50	150	0.9976	37057.9	49966.6	2.17	1.05	21.97	0.77
Terbutaline	5	15	0.9990	20331.0	50803.9	0.84	1.11	11.90	0.84
Testosterone	75	250	0.9994	24687.5	14411.5	1.14	1.53	11.95	0.99
Triamcinolone	5	15	0.9993	1039.4	1829.9	3.54	2.11	23.85	1.10
Triamterene	50	150	0.9982	60344.2	−50099.3	2.43	1.50	17.82	1.85
Warfarin	5	15	0.9999	2958.3	15002.4	0.99	1.20	11.61	1

cannabidiol. In addition, the extracts obtained by this methanolic method were cleaner than alkaline or acidic digestions as shown in Fig. 3, so that the method would be less affected by matrix effects.

3.3. Validation of the method

In order to evaluate the analytical performances of the proposed method, calibration curves of the targeted 30 drugs were constructed at different concentrations in the range 50–2500 pg mg^{-1}

using blank hair extract to prepare matrix-matched standards at 8 concentration levels.

The LOD was defined as the lower concentration whose extracted ion chromatogram with a window of 20 mDa without smoothing showing a signal-to-noise ratio at least 3 and was empirically determined by fortifying hair extracts at decreasing analyte concentrations. The LOQ was the lowest concentration that could be quantified with acceptable precision and accuracy with a signal-to-noise ratio at least 10. This was experimentally calculated from the injection of spiked blank matrix extracts at low concentration levels. The LOQs obtained were as low as

Table 4
Concentration in pg mg^{-1} of positive results for all the tested hair samples.

Sample	Detected compounds	Retention time	Measured m/z	Theoretical m/z	Error		Calculated concentration (pg mg^{-1})
					mDa	ppm	
1	Cannabidiol	17.52	315.2314	315.2319	−0.49	−1.57	267
	Cocaine	8.60	304.1548	304.1543	0.49	1.63	16
	Δ^9-THC	16.15	315.2329	315.2319	0.99	3.16	104
	Ephedrine	2.62	148.1122	148.1121	0.09	0.62	162
2	Cannabidiol	17.36	315.2330	315.2319	1.13	3.59	50
3	Acetaminophen	2.23	152.0703	152.0706	−0.33	−2.19	317
	Cocaine	8.69	304.1550	304.1543	0.68	2.25	<LOQ
	Ephedrine	2.42	148.1121	148.1121	0.04	0.29	322
4	Acetaminophen	2.21	152.0707	152.0706	0.11	0.72	1081
	Cannabidiol	17.47	315.2315	315.2319	−0.42	−1.13	2456
	Cocaine	8.65	304.1545	304.1543	0.14	0.46	23
	Δ^9-THC	16.13	315.2317	315.2319	−0.21	−0.067	2979
	Ephedrine	2.47	148.1118	148.1121	−0.31	−2.11	321
5	Cocaine	8.72	304.1535	304.1543	−0.79	−2.74	15
	Ephedrine	2.74	148.1114	148.1121	−0.69	−4.69	<LOQ
6	Acetaminophen	2.32	152.0709	152.0706	0.37	2.1	12,953
	Cocaine	8.82	304.1547	304.1543	0.41	1.35	<LOQ

15 pg mg^{-1} for atenolol, cannabidiol, cocaine, propranolol and triamcinolone and were lower than 250 pg mg^{-1} for all the studied analytes.

The coeluting species in the matrix can cause an effect of enhancing or decreasing the signal of the analytes, this effect was evaluated by comparing the slope of the calibration with matrix-matched standards with those obtained in solvent. 25 of the 30 (83%) analytes showed a matrix effect lower than 25%. Intra-day relative standard deviation (RSD) was evaluated by analyzing six replicates at two concentration levels (LOQ and 100 × LOQ) in the same run, inter-day RSD was assessed by analyzing for six different days the same concentration level (100 × LOQ). Intra-day RSD percentages were better than 4% in most cases, while inter-day RSD (%) values were below 20% in most cases. The analytical parameters obtained are summarized in Table 3.

3.4. Application to the analysis of real hair samples

In order to demonstrate the applicability of the method, six real samples were analyzed according to the previously described method. The samples were provided by volunteer drug abusers. Fig. 4 shows the analysis of a hair sample which contained acetaminophen (1081 pg mg^{-1}), cannabidiol (2456 pg mg^{-1}), cocaine (23 pg mg^{-1}), Δ^9-THC (2979 pg mg^{-1}) and ephedrine (321 pg mg^{-1}).

The positive findings of the detected drugs were confirmed by LC-TOFMS accurate mass analysis (obtaining mass accuracy <2 ppm error in most cases). This data provides an evidence of the reliability of the present approach for unambiguous identification and confirmation of the studied drugs of abuse and pharmaceutical in hair samples The results obtained for the six tested hair samples are shown in Table 4, all the samples contained at least one drug, the analytes detected were acetaminophen, cannabidiol, cocaine, Δ^9-THC and ephedrine. These results show the applicability of the method for drug testing in hair.

4. Conclusions

A fast LC-TOFMS screening method has been developed for the simultaneous identification and quantitation of 30 representative multiclass drugs of abuse and pharmaceuticals. The applicability of the method has been demonstrated by analyzing different hair samples from drug consumers. Direct methanolic extraction was found to be the more efficient method tested for the extraction of drugs from hair, considering both relative recovery rates and cleanliness of the extracts. The developed method was applied to six hair samples from drug consumer volunteers, in which the presence of at least one drug has been confirmed by accurate mass measurements within 2 ppm (mass error) in most cases. The method allowed detecting the target compounds in the picogram range (per milligram of hair). The present study demonstrates the usefulness of LC-TOFMS for qualitative and quantitative drug testing in hair.

The use of high resolving power mass spectrometry provides high sensitivity in full scan mode, allowing untargeted retrospective analysis in order to extend the test for new (unexpected) compounds.

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