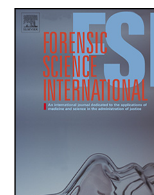
Forensic Science International 243 (2014) 117–125

Contents lists available at ScienceDirect

Forensic Science International

journal homepage: www.elsevier.com/locate/forsciint



Validation and application of an UPLC–MS/MS method for the quantification of synthetic cannabinoids in urine samples and analysis of seized materials from the Portuguese market☆

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ARTICLE INFO

Article history:
Available online 27 July 2014

Keywords:
Synthetic cannabinoids
Spice
UPLC–MS/MS
Urine

ABSTRACT

An UPLC–MS/MS method using ESI + ionization and MRM was developed and fully validated according to international guidelines for the qualitative and quantitative analysis of nine synthetic cannabinoids and/or their metabolites in urine samples (1 mL). Prior to extraction the samples were subjected to an enzymatic hydrolysis using β-glucuronidase followed by a SPE procedure using Oasis® HLB 3 cc (60 mg) columns. The chromatographic separation was performed with an Acquity UPLC® HSS T3 (50 mm × 2.1 mm i.d., 1.8 μm) reversed-phase column using a gradient with methanol–ammonium formate 2 mM (0.1% formic acid) and with a run time of 9.5 min. The method was validated in terms of selectivity, capacity of identification, limits of detection (0.01–0.5 ng/mL) and quantification (0.05–0.5 ng/mL), recovery (58–105%), carryover, matrix effect, linearity (0.05–50 ng/mL), intra-assay precision, inter-assay accuracy and precision (CV < 20%). The method was applied to 80 authentic samples, five of them (6.2%) were confirmed or suspected to be positive for the metabolites JWH-018 N-hydroxypentyl and JWH-018 N-pentanoic acid of JWH-018 and for the metabolite JWH-122 N-(5-hydroxypentyl) of JWH-122, and three of them in association with THC and/or THCCOOH (substances included in the method, together with the 11-OH-THC). Additionally, 17 spice products were analyzed, for which were confirmed the presence of the following substances: AM-2201, JWH-018, JWH-022 JWH-073, JWH-122, JWH-203, JWH-210, JWH-250, HU-210 and RCS-4, according to the comparison with authentic reference material and published data. The analytical method developed allowed the analysis of synthetic cannabinoids and the notification of the first cases in Portugal.

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1. Introduction

Over de last decade, the abuse of herbal blend incense products and other "legal high" products, such as powders and pills containing "designer drugs" available to purchase on the Internet and in various head shops, has increased. Since the introduction into the market in 2004 as "legal highs" the synthetic cannabinoids have gained popularity first in Europe but rapidly spread all over the world. The synthetic cannabinoids are synthesized in laboratories and are formulated to interact with the endogenous cannabinoid receptors (CB1 and CB2) in the brain to produce psychoactive cannabis-like effects that have been reported to include acute adverse effects, such us, agitation, anxiety, confusion, hypertension, sedation, psychosis, hallucinations, tachycardia, and seizures. Despite the fact that the exact pharmacokinetic and pharmacodynamic profiles of most synthetic cannabinoids have not been adequately described, it is widely known that most synthetic cannabinoids are potent CB1 agonists, possessing higher binding affinity for cannabinoid receptors than cannabis, which means greater potency, greater adverse effects and longer duration of action. Moreover, it seems that tolerance may develop fairly rapid, might being associated with relatively high potential to cause dependence and accidental overdosing is more likely to occur because the amount and type of compounds in the herbal mixtures vary considerably, even from batch to batch [1–3].

Meanwhile, almost all European countries, United States, Chile, South Korea, New Zealand and Australia, scheduled one or more of the most commonly abused synthetic cannabinoids or structural classes of compounds. In Portugal, only during the last year the

☆ This paper is part of the special issue entitled "The 51st Annual Meeting of the International Association of Forensic Toxicologists (TIAFT)". September 2–3, 2013, Funchal, Medeira, Portugal. Guest edited by Professor Helena Teixeira, Professor Duarte Nuno Vieira and Professor Francisco Corte Real.

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http://dx.doi.org/10.1016/j.forsciint.2014.07.022
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public opinion has been alert by the media to the growing problem of the abuse of these compounds, mainly by young people, a result of the increasing number of "smart shops" that commercialize these products. Patients admitted at Emergency Departments with symptoms of intoxication and even suspicious of drug related deaths after the abuse of these substances have been reported. In April 2013 a new law was published to regulate the commercialization of these kinds of products in Portugal. This recent law includes a list with a total number of 159 new psychoactive substances that includes: phenylethylamines, piperazines, cathinones and cocaine derivatives, plants and respective active compounds and synthetic cannabinoids ($n = 46$) [4].

Since the introduction of synthetic cannabinoids into the "designer drugs" market, laboratories worldwide have developed distinctive analytical methods to respond to the increasing demand for synthetic cannabinoids analysis in seized materials [5–19] and biological matrices, such as, whole blood or serum [20–28], oral fluid [29–33], hair [34–36], and mainly in urine that provides a longer detection window for synthetic cannabinoids metabolites than serum or oral fluid for parent drugs [20,23,37–53]. To accomplish this, a variety of mass spectrometry techniques have been used, gas chromatography–mass spectrometry (GC–MS) [5,6,9,11,13–15,17,19,23,37,48,52,53], gas chromatography–tandem mass spectrometry (GC–MS/MS) [10,51], liquid chromatography–mass spectrometry (LC–MS) [17], liquid chromatography–tandem mass spectrometry (LC–MS/MS) [8,9,16,21–34,36,38,40–52] and high resolution mass spectrometry techniques [7–12,18,35,39,47,52]. The GC–MS and GC–MS/MS have proven to be sensitive and selective enough for the detection of a wide range of compounds, however they also present some limitations when dealing with thermolabile compounds or artifacts and co-eluting chromatographic peaks. The LC–MS/MS is highly sensitive and selective. In addition, it allows the direct analysis of aqueous solutions and is better suited for non-volatile compounds. Therefore, it has been predominantly used to identify and quantify synthetic cannabinoids, especially for the metabolites. However, the LC–MS/MS technology works better with targeted methods, when the instrument is instructed to acquire specific data related with ion transitions at certain collision energies. Therefore, it is not considered the best choice as a screening tool for unknown compounds and/or their metabolites. The high resolution techniques, such as the liquid chromatography–time-of-flight–mass spectrometry (LC–TOF-MS), present high-sensitivity allied to selectivity providing accurate mass that allows identifying a wide range of substances, metabolites and other unknown compounds in a single run. On the other hand, this is a relatively new technique, very expensive and therefore not available in many laboratories.

The aim of this paper was to report the ultra performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) method that was developed and fully validated that allowed the confirmation and quantification of the first positive cases for synthetic cannabinoids in urine samples collected in the clinical and forensic context in Portugal. Additionally, we report the results obtained for the analysis of several seized products available on the Portuguese market.

2. Experimental

2.1. Standards, reagents and materials

JWH-018, JWH-018 4-hydroxyindole, JWH-018 5-hydroxyindole, JWH-018 N-(4-hydroxypentyl), JWH-018 N-(5-hydroxypentyl), JWH-073, JWH-073 N-(3-hydroxybutyl), JWH-073 N-(4-hydroxybutyl), JWH-250 and HU-210 were purchased from Cayman Chemicals (Ann Arbor, MI, USA). 11-Hydroxy-Δ^9-tetrahydrocannabinol (11-OH-THC), 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THCCOOH) and Δ^9-tetrahydrocannabinol (THC) were supplied by Cerilliant (Round Rock, TX, USA) and Lipomed (Arlesheim, Switzerland). The internal standards JWH-018-d9, JWH-018 4-hydroxyindole-d9, JWH-073-d7 and JWH-073 N-(3-hydroxybutyl)-d5 were purchased from Cayman Chemicals (Ann Arbor, MI, USA) and 11-hydroxy-Δ^9-tetrahydrocannabinol-d3 (11-OH-THC-d3), 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol-d3 (THCCOOH-d3) and Δ^9-tetrahydrocannabinol-d3 (THC-d3) were supplied by Cerilliant (Round Rock, TX, USA). Acetonitrile, methanol and water LC–MS grade and ammonium formate puriss ($\geq$99.0%) were purchased from Fluka (Buchs, Switzerland). Formic acid (98–100%), ethyl acetate (for chromatography), acetic acid (glacial) 100% anhydrous, *n*-hexane and ammonia solution 25% were obtained from Merck (Darmstadt, Germany). Sodium phosphate dibasic and sodium dihydrogen phosphate monohydrate from Sigma–Aldrich (Steinheim, Germany) and β-glucuronidase (from *E. coli K12*) was purchased from Roche (Penzberg, Germany). Solid-phase extraction columns Oasis® HLB 3 cc (60 mg) were purchased from Waters (Wexford, Ireland). From individual stock solutions, mixed working solutions in methanol at 1 μg/mL, 0.1 μg/mL, 0.01 μg/mL and 0.001 μg/mL were prepared by dilution and used for the validation, preparation of calibration curves and quality control samples. The deuterated internal standards were conveniently diluted in methanol to give a working solution of 1 μg/mL for the 11-OH-THC-d3, THCCOOH-d3 and THC-d3 and a mixed working solution of 0.1 μg/mL for the other deuterated internal standards used. The stock and working solutions were kept at −20 °C when not in use.

2.2. Specimens

Urine blank samples obtained from living healthy volunteers and from our department of forensic pathology were used for development and validation of the procedure. Real urine samples were analyzed in a total number of 80 samples, that include samples collected from an emergency department of a Central Hospital in Lisbon (Portugal) ($n = 73$) and samples collected from the clinical and forensic activities of the National Institute of Legal Medicine and Forensic Sciences ($n = 7$). The samples from the emergence department were supplied with a code number without any personal information from the subjects, prior drug history or regarding intake of "legal high" products. All urine samples were stored in polypropylene tubes at −10 °C. In addition, it was necessary to analyze some herbal blends and powders seized by the authorities in smartshops, in a total of 17 products. After receipt by the laboratory the products were stored at room temperature, in dark and dry conditions until analysis.

2.3. Sample preparation

2.3.1. Urine samples

After adding 50 μL of the internal standard working solutions, 2 mL of phosphate buffer 0.2 M (pH 6.5) and 50 μL of β-glucuronidase to 1 mL of urine, the samples were incubated at 55 °C for 1 h for enzymatic hydrolysis. The calibrators and quality control samples were prepared by spiking blank urine samples with the appropriate working solution volumes. Subsequently, the samples were centrifuged at 2122 × *g* for 10 min and were extracted using a solid-phase extraction (SPE) procedure. The SPE columns Oasis® HLB 3 cc (60 mg) were conditioned with 2 mL of methanol and 2 mL of water. After loading the prepared samples, the columns were washed with 2 mL of water/acetonitrile/ammonia solution (90:10:1, v/v/v). The columns were allowed to dry under pressure for 20 min and then 2 mL of ethyl acetate was applied to the columns and the eluent collected to conical

glass tubes. The columns were dried again under pressure for 10 min, then 2 mL of *n*-hexane/ethyl acetate/acetic acid (88:10:2, v/v/v) was added to the columns and the eluent collected to the same elution tube. The samples were evaporated to dryness at 45 °C under a stream of nitrogen. The dried extract was reconstituted with 50 μL of methanol. Afterwards, 50 μL of water was added, the samples were centrifuged at 2122 × g for 3 min, transferred into vials and analyzed.

2.3.2. "Legal high" products

The "legal high" products were prepared by weighting approximately 50 mg of each sample, transferred to a glass tube, and 5 mL of acetonitrile-methanol (1:1, v/v) were added. The mixture was sonicated in a water bath for 10 min and vortex-mixed for 2 min. After vortex mixing, 100 μL were transferred to a clean glass tube and were diluted 1:50 with methanol–water (1:1, v/v). The solution was vortex-mixed for few seconds and a portion was transferred into a vial and analyzed.

2.4. UPLC–MS/MS

A Waters Acquity UPLC separation module with a column heater (Waters, Milford, MA, USA) system was used. The chromatographic separation was performed by a Waters Acquity UPLC® HSS T3 (50 mm × 2.1 mm, 1.8 μm) column at 45 °C using gradient elution with methanol/2 mM ammonium formate (formic acid 0.1%) (95:5, v/v) (A) and 2 mM ammonium formate (formic acid 0.1%)/methanol (95:5, v/v) (B) at a flow rate of 0.4 mL/min. The gradient was programmed as follows: 45% A at 0 min, linearly increased to 60% A in 4.5 min and to 95% A in 1.5 min, isocratic for 1.5 min followed by a decrease to the initial conditions in 0.05 min and equilibration time for 1.95 min, which resulted in a total run time of 9.5 min. An injection volume of 10 μL was used. The UPLC system was combined with a TQ Detector (triple quadrupole mass spectrometer, Waters) equipped with an electrospray ionization probe. The system operation and data acquisition were controlled by the MassLynx™ V4.1 SCN 714 and data were processed by TargetLynx V4.1 SCN 714 software (Waters). The equipment was operated in electrospray positive ionization mode (ESI+) and in the multiple reaction monitoring mode (MRM). The source parameters were optimized for the analysis and the following conditions were found: capillary voltage, 1.0 kV; source block temperature, 120 °C; desolvation gas (nitrogen) heated to 450 °C and delivered at a flow rate of 600 L/h; no cone gas was used. The appropriate MRM transitions, cone voltages and collision energies for the individual analytes and internal standards were determined by direct infusion into the mass spectrometer. The cone voltage was adjusted to maximize the intensity of the protonated molecular ion and collision induced dissociation was performed. Collision gas (argon) pressure was maintained at 3.5×10^{-3} mbar, two product ions were selected and the collision energy optimized for each substance. The corresponding MRM transitions were monitored using different time windows depending on the analytes retention time. The substances for which no standards or reference retention time were initially available the data acquisition was performed using a retention time window corresponding to the total run time. The MRM transitions with the corresponding cone voltage, collision energy and retention time of the analytes and internal standards are presented in Table 1. For the "legal high" products analysis, 27 compounds were selected among the most prevalent according with the literature [12]. For the substances for which standards were not available, the analysis was based on the data concerning the precursor mass ion and respective fragment mass ions expected for each compound according to the literature and the data acquisition was performed using a retention time window corresponding to the total run time, see Table 2. Taking into consideration the values obtained during optimization for similar substances, the cone voltage was maintained at 45 V and for the collision energy two values were used (25 and 45 eV). The equipment was operated in ESI+ mode and in the MRM and product ion scan mode.

2.5. Method validation

The method was validated according to the international guidelines in terms of selectivity, capacity of identification, limits

Table 1
MRM transitions, cone voltage, collision energy (Coll) and retention time (RT) of the analytes and internal standards (IS).

Compound	Transitions (*m/z*)	Cone (V)	Coll (eV)	RT (min)	IS
JWH-073 N-(4-hydroxybutyl)	344.2 > 155.0/127.0	45	22/45	4.59	JWH-073 N-(3-hydroxybutyl)-d5
JWH-073 N-(3-hydroxybutyl)	344.2 > 155.0/127.0	45	24/45	5.13	JWH-073 N-(3-hydroxybutyl)-d5
JWH-073 N-(3-hydroxybutyl)-d5	349.2 > 155.0	45	24	5.13	_
JWH-018 N-pentanoic acid[a,b]	372.2 > 155.0/127.0	45	22/45	5.13	JWH-018-d9
JWH-018 N-(5-hydroxypentyl)	358.2 > 155.0/127.0	45	22/45	5.34	JWH-018-d9
JWH-122 N-(5-hydroxypentyl)[a,b]	372.2 > 169.0/141.0	45	25/25	5.80	JWH-073-d7
AM-2201[a,b]	360.2 > 155.0/232.0	45	25/25	6.03	JWH-073-d7
JWH-018 5-hydroxyindole	358.2 > 155.0/127.0	50	26/45	6.04	JWH-018 4-hydroxyindole-d9
JWH-073	328.2 > 155.0/127.0	45	24/45	6.26	JWH-073-d7
JWH-073-d7	335.2 > 155.0	45	25	6.26	_
11-OH-THC[a]	331.2 > 193.0/201.0	30	25/25	6.26	11-OH-THC-d3
11-OH-THC-d3	334.2 > 196.0	30	25	6.26	_
JWH-250	336.2 > 121.0/200.0	40	22/22	6.26	JWH-073-d7
JWH-022[a,b]	340.2 > 155.0/212.0	45	25/25	6.26	JWH-073-d7
THCCOOH[a]	345.2 > 193.0/299.0	35	25/20	6.34	THCCOOH-d3
THCCOOH-d3	348.2 > 302.0	35	20	6.34	_
JWH-018	342.2 > 155.0/127.0	45	26/45	6.43	JWH-018-d9
JWH-018-d9	351.0 > 155.0	45	26	6.44	_
JWH-018 4-hydroxyindole	358.2 > 155.0/127.0	50	28/45	6.44	JWH-018 4-hydroxyindole-d9
JWH-018 4-hydroxyindole-d9	367.2 > 155.0	50	28	6.44	_
JWH-122[a,b]	356.2 > 169.0/214.0	45	25/25	6.57	JWH-018-d9
JWH-210[a,b]	370.2 > 183.0/214.0	45	22/22	6.68	JWH-018-d9
HU-210	387.3 > 201.0/243.0	45	25/25	6.72	THC-d3
THC[a]	315.2 > 123.0/193.0	40	30/25	6.76	THC-d3
THC-d3	318.2 > 196.0	40	25	6.76	_

[a] Substances included in the method but no validation data presented.
[b] No standards available. The data concerning the MRM transitions and expected retention time in these cases was based on the comparison with data obtained from the literature [12,41,43,44,50] and data collected from the analysis of real samples.

Table 2
Substances monitored for the analysis of "legal high" products.

Compound	Transitions (m/z)
AM-251	555.0 > 454.0/472.0
AM-694	436.1 > 230.0/243.0
AM-1241	504.1 > 98.0/275.0
AM-2201	360.2 > 155.0/232.0
AM-2233	459.1 > 98.0/112.0/230.0/362.0
HU-210	387.3 > 201.0/243.0
JWH-007	356.2 > 155.0/228.0
JWH-011	384.2 > 155.0/286.0
JWH-015	328.2 > 155.0/200.0
JWH-018	342.2 > 155.0/127.0
JWH-019	356.2 > 155.0/228.0
JWH-022	340.2 > 155.0/212.0
JWH-030	292.2 > 155.0/168.0
JWH-073	328.2 > 155.0/127.0
JWH-081	372.2 > 185.0/198.0/214.0
JWH-098	386.2 > 185.0/198.0/228.0
JWH-122	356.2 > 169.0/214.0
JWH-182	384.2 > 197.0/214.0
JWH-200	385.2 > 114.0/155.0/168.0
JWH-201	336.2 > 121.0/135.0/214.0
JWH-203	340.1 > 125.0/166.0/214.0
JWH-210	370.2 > 183.0/214.0
JWH-250	336.2 > 121.0/200.0
JWH-251	320.2 > 105.0/144.0/214.0
MPPP	218.2 > 91.0/98.0/132.0
RCS-4	322.2 > 135.0/214.0
RCS-8	376.2 > 121.0/132.0/144.0/254.0

The MRM transitions were selected based on the article of Shanks et al. [12], except for HU-210, JWH-018, JWH-073 and JWH-250, for which standards were available.

of detection and quantification, recovery, carryover, matrix effect, linearity, intra-assay precision, inter-assay accuracy and precision prior to the application to authentic samples [54].

Selectivity and capacity of identification was evaluated by analyzing 10 pools of blank urine samples, each pool being a mixture of four different urine samples collected from living subjects and from autopsies, and comparing them with the same urine samples spiked at a final concentration of 5 ng/mL. The negative samples were analyzed to confirm the absence of potential interferences and the positive samples were evaluated according to the identification criteria (relative retention time, ion ratio and signal to noise ratio (S/N > 3). [55] Limit of detection (LOD) and quantification (LOQ) were established fortifying blank samples with decreasing concentrations. The acceptance criteria for the LOD included relative retention time, ion ratio and S/N > 3. The LOQ was set as the lowest concentration with a S/N > 10 and that can be quantified with acceptable imprecision (coefficient of variation (CV %) and accuracy (deviation of the mean from the theoretical value) (E%) < 20%). Recovery and matrix effect were determined at three different concentrations preparing three sets of samples with three replicates for each concentration: (A) urine samples spiked before the extraction procedure; (B) urine samples spiked after the extraction procedure and before the evaporation step; and (C) standards added to the same amount of elution solvents, dried and redissolved in the reconstitution solution. The recovery was obtained by comparing the analyte/internal standard peak areas of A and B samples (Rec% = B rel.area/A rel.area $\times$ 100) and the matrix effect by comparing the areas of B and C samples (ME% = [(B area/C area) − 1] $\times$ 100). Meaning that, a matrix effect with a negative result indicates ion suppression whereas a positive result indicates ion enhancement. Additionally, the following experiment was also performed: (B) nine different urine samples from healthy volunteers were collected and prepared according to the sample preparation procedure and spiked before the evaporation step at a concentration of 5 ng/mL, and (C) standards prepared in the reconstitution solution. The ME% was calculated for each different urine sample the same way as in the first experiment. Carryover was investigated by injecting extracted blank samples between the samples from the recovery experiment at the highest concentration level. Linearity was studied preparing five calibration curves (ranging from the respective LOQ and 50 ng/mL), in five different days and using a simple (unweighted) and a weighted least squares linear regression model based on the method described by Almeida et al. [56] The following weighting factors were tested: 1 (unweighted), $1/x^{0.5}$, $1/x$, $1/x^2$, $1/y^{0.5}$, $1/y$ and $1/y^2$. The weighting factor chosen was the one that presented the lowest percentage relative error (%RE) and fulfilled the criterion of a coefficient of determination (r^2) higher than 0.99. In order to evaluate the intra-assay precision five replicates at two concentration levels (2 and 20 ng/mL) were analyzed and a CV% less than 10% (for the high concentration level) and 20% (for the low level) was accepted. For the inter-accuracy and precision samples at two concentration levels (0.5 and 10 ng/mL) were analyzed in sets of three replicates on five different days. Precision was expressed as the CV% and accuracy as the bias (with acceptable values of <20% and ±20%, respectively).

3. Results and discussion

3.1. Sample preparation

According to previous published papers hydroxylation and carboxylation are the main phase I biotransformations of the synthetic cannabinoids. The hydroxyl and carboxyl metabolites are excreted in urine mainly as ether and ester glucuronides and therefore it is important to hydrolyze the urine samples prior to extraction otherwise the urinary metabolite concentrations may be under estimated [43,45,48]. The hydrolysis of glucuronides is commonly performed by chemical or enzymatic treatment. However, the enzymatic hydrolysis using β-glucuronidase from different sources is much more convenient and gentle than chemical hydrolysis and therefore is the most prevalent procedure for the deconjugation of the metabolites. There are several β-glucuronidase pretreatment protocols and therefore to better obtain the adequate sample pretreatment conditions the assessment of the hydrolysis efficiency should be done whenever it is possible. Because no glucuronide certified materials, nor authentic positive samples were initially available it was not possible to determine the efficiency of the deconjugation and therefore the enzymatic hydrolysis procedure was chosen based on the conditions suggested in the instructions for use of the β-glucuronidase (from *E. coli K12*) purchased from Roche, and the protocols used by Möller et al. [49] and Sobolevsky et al. [51], where the same β-glucuronidase source was used with complete hydrolysis efficiency results.

The most frequently used techniques for the extraction of synthetic cannabinoids and/or their metabolites from different biological specimens, including urine samples, are the liquid–liquid extraction (LLE) and solid-phase extraction (SPE). Both techniques have advantages and disadvantages. The LLE could represent a simple and faster method when compared to SPE. On the other hand, using the SPE it is possible to obtain cleaner extracts and therefore better analytical results concerning matrix effect. In this study a SPE procedure was applied based on a previously successfully established and validated sample preparation procedure for the LC–MS/MS analysis of cannabinoids in whole blood samples. Some minor modifications, allowed a rapid implementation of the new target analytes into the existing protocol and the simultaneous analysis of THC, synthetic cannabinoids and respective metabolites.

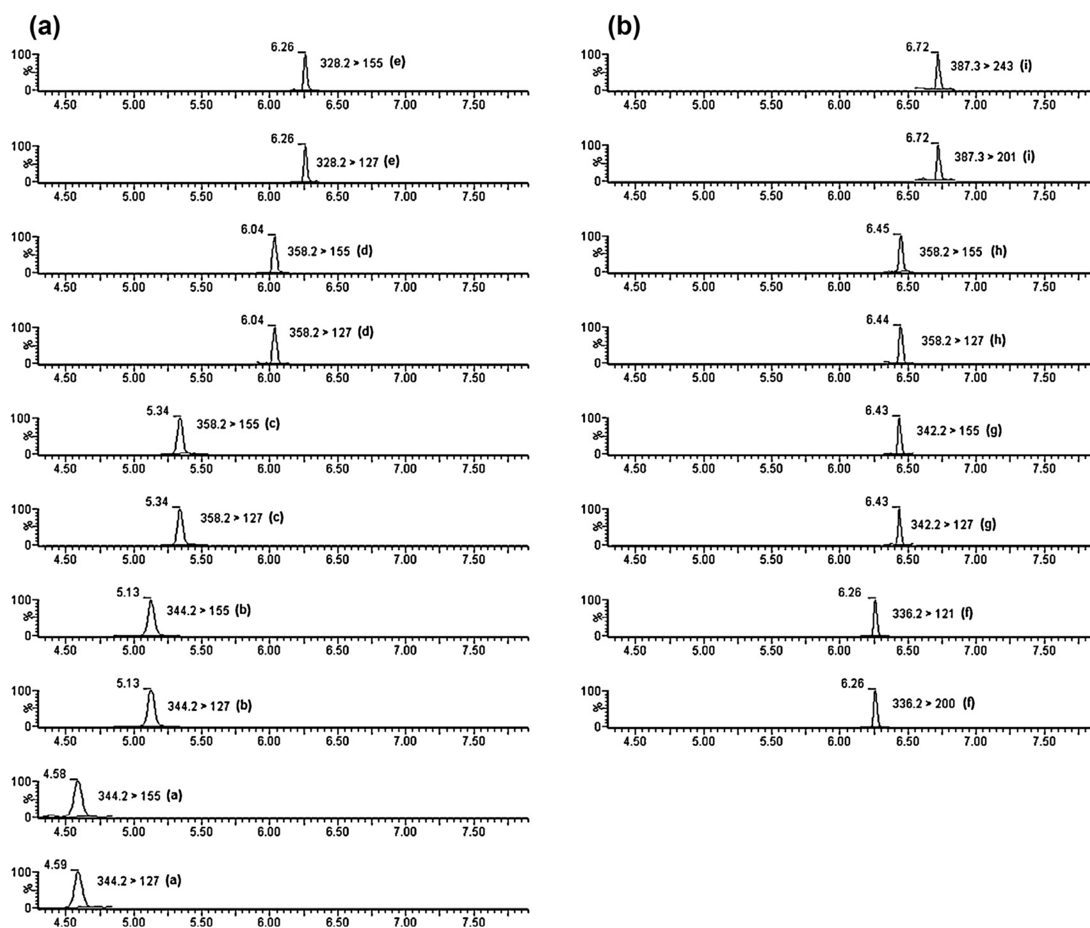

Fig. 1. (a) MRM chromatograms of a calibrator at the correspondent LOQ for the substances: (a) JWH-073 N-(4-hydroxybutyl), (b) JWH-073 N-(3-hydroxybutyl), (c) JWH-018 N-(5-hydroxypentyl), (d) JWH-018 5-hydroxyindole and (e) JWH-073. (b) MRM chromatograms of a calibrator at the correspondent LOQ for the substances: (f) JWH-250, (g) JWH-018, (h) JWH-018 4-hydroxyindole and (i) HU-210.

3.2. Method validation

Fig. 1a and b shows the MRM chromatograms of a calibrator with all the synthetic cannabinoids at the LOQ concentrations. The main results of the method validation are summarized in Tables 3 and 4. The selectivity and capacity of identification were ensured by a combination of relative retention time and ion ratio criteria that guaranteed the substance identification for the positive samples, and by the analysis of the negative samples that showed no peaks that could interfere with the detection of the compounds of interest. However, based on this method, it was not possible to distinguish between the two main hydroxyl metabolites of JWH-018, the JWH-018 N-(5-hydroxypentyl) and JWH-018 N-(4-hydroxypentyl), due to the fact that they share the same ion transitions and similar retention times. The option was to validate the method for the JWH-018 N-(5-hydroxypentyl), despite the fact that the method revealed not to be suitable for the quantification of this compound. Therefore, the results obtained in real samples for the JWH-018 N-(5-hydroxypentyl) might represent the sum of both hydroxyl metabolites. The LOD and LOQ values achieved the relative retention time, ion ratio and S/N criteria and moreover the LOQ values tested for precision and accuracy presented CV% and *E*% always below 15%. Comparing the present method with those reported by other authors using similar technology and for the same substances it is possible to establish that the LOQ values obtained in this study are of the lowest

Table 3
Method validation data: LOD (ng/mL), LOQ (ng/mL), recovery (for three levels of concentration: 0.5, 5 and 50 ng/mL) and matrix effect (for three levels of concentration: 0.5, 5 and 50 ng/mL, and for nine different urine samples).

Compound	LOD	LOQ	LOQ test		Recovery (%)			ME%			ME% (CV%)
			CV%	*E*%							Diff. urine samples
JWH-073 N-(4-hydroxybutyl)	0.01	0.05	2.3	0.8	91	97	105	4	−6	6	5 (15)
JWH-073 N-(3-hydroxybutyl)	0.05	0.05	1.9	5.2	89	96	96	−5	−10	−2	−4 (15)
JWH-018 N-(5-hydroxypentyl)	0.01	0.05	6.5	2.8	82	95	89	46	38	43	10 (19)
JWH-018 5-hydroxyindole	0.05	0.05	7.2	13.2	73	78	59	10	13	26	0 (16)
JWH-073	0.01	0.05	4.9	14.8	79	80	84	−6	−6	−2	−24 (16)
JWH-250	0.1	0.5	4.4	10.2	81	85	92	−12	−12	7	−30 (17)
JWH-018	0.01	0.05	7.8	0.4	62	72	77	−7	−7	4	−12 (14)
JWH-018 4-hydroxyindole	0.05	0.05	8.2	4.4	58	60	62	−1	−9	2	−2 (14)
HU-210	0.5	0.5	14.6	7.4	60	64	70	2	−6	4	−6 (12)

Table 4
Method validation data: linearity (coefficients of determination using a $1/x$ – weighting factor), intra-assay precision (for two levels of concentration: 2 and 20 ng/mL), inter-assay precision and accuracy (expressed as the bias) (for two levels of concentration: 0.5 and 10 ng/mL).

Compound	Linearity (r^2)	Intra-assay precision (CV%)		Inter-assay precision (CV%)		Inter-assay accuracy (bias)	
JWH-073 N-(4-hydroxybutyl)	0.996	2.6	2.4	8.7	11.6	10	9
JWH-073 N-(3-hydroxybutyl)	0.995	2.7	2.2	9.4	7.9	12	7
JWH-018 N-(5-hydroxypentyl)	0.994	19.4	8.8	14.5	14.3	15	1
JWH-018 5-hydroxyindole	0.994	18.8	8.8	19.1	16.1	1	3
JWH-073	0.996	8.0	3.6	6.0	7.5	14	6
JWH-250	0.998	16.4	4.4	9.7	8.3	-8	3
JWH-018	0.997	3.5	7.6	4.7	7.5	12	8
JWH-018 4-hydroxyindole	0.997	6.0	2.6	5.5	8.9	4	3
HU-210	0.995	10.6	6.5	14.6	19.4	−10	2

presented in the literature and fulfill the minimum expected concentration levels for the metabolites of synthetic cannabinoids in human urine, according to the published quantitative data and obtained from the real samples analysis included in this study (see Table 5) [38,40–45,47–51]. The recoveries obtained for all nine substances at three different concentration levels ranged from 58% to 105% and no significant differences between different concentration levels was observed. The blank samples included in the recovery experiment did not show any relevant peak as a result of an eventual contamination by carryover. The results obtained for the matrix effect using three levels of concentration for the same urine sample ranged from −12% to 26% showing a slight ion suppression in some cases or a slight enhancement in others, with the exception of the JWH-018 N-(5-hydroxypentyl) that ranged from 38% to 46%, showing a moderate ion enhancement for the three levels of concentration. The results obtained using different urine samples showed that the matrix effect depends on the urine sample ranging from −30% to 10% with CV% ranging from 12% to 19%. Therefore, it was possible to conclude that in general the method did not present significant matrix effects with some exceptions. In those cases the use of deuterated internal standards compensate the samples with a higher coefficient of variation and allow an accurate quantification. All calibration curves showed good linearity with coefficients of determination above 0.994 for all the synthetic cannabinoids over the range investigated using a $1/x$ – weighting factor. For the intra-assay precision the CV% values varied between 2.6% and 19.4% for the low concentration level and 2.2% and 8.8% for the high concentration level meaning that were within the acceptance criteria. The inter-assay precision was considered adequate for the tested concentrations levels with a CV% value below 20% (varying between 4.7% and 19.4%) and the accuracy of the method ranged from 90% to 115% for all the compounds.

3.3. Urine sample analysis

The validated UPLC–MS/MS method previously described is being routinely applied. In a total number of 80 samples analyzed, that include samples collected from an emergency department of a Central Hospital in Lisbon (n = 73) and samples collected from the clinical and forensic activities of the National Institute of Legal Medicine and Forensic Sciences (NILMFS) (n = 7), five were positive for synthetic cannabinoids, three of them in association with THC and/or THCCOOH (substances that were included in the method, together with the 11-OH-THC). The confirmation of the presence of THC and/or metabolites together with synthetic cannabinoids is important information confirming that the cannabis users are many times the same "Spice" drugs abusers and that could also imply a concomitant consumption representing a potential increase in the adverse side effects and in extreme cases accidental overdosing. Table 5 presents the results obtained for each individual authentic sample. Although the consumption of designer drugs is usually associated to the younger people, in spite of the small number of positive samples, we observed that users can be older. The synthetic cannabinoids detected were, mainly, the N-hydroxypentyl metabolites of JWH-018. As previously mentioned, it was not possible to differentiate between the JWH-018 N-(4-hydroxypentyl) and the JWH-018 N-(5-hydroxypentyl), since they co-elute according to the method and therefore the quantitative result is merely an indicator given that it corresponds to the sum of both metabolites that might be present. Additionally, a tentative identification of other main metabolites of the synthetic cannabinoids included in the method (such as, the JWH-018 N-pentanoic acid or the JWH-073 N-butanoic acid) and of other JWH compounds with structural similarities and thus common mass spectral fragmentation patterns was applied. The tentative identification was based on the expected

Table 5
Analytical results for the positive samples.

Sample	Gender	Age	Information	Substances
1	Male	15	Possible intoxication with mephedrone	JWH-018 N-hydroxypentyl (0.5 ng/mL) JWH-018 N-pentanoic acid
2	Female	28	Student; Sexual assault	THCCOOH (54 ng/mL) JWH-018 N-hydroxypentyl (<0.05 ng/mL)
3	Male	51	Teacher; possible intoxication with plant fertilizer "Smooth Golol" obtained in a smartshop	THCCOOH (7.7 ng/mL) THC (1.1 ng/mL) JWH-018 N-hydroxypentyl (1.1 ng/mL) JWH-018 N-Pentanoic acid
4	–	–	–	THCCOOH (22 ng/mL) JWH-018 N-hydroxypentyl (3.9 ng/mL) JWH-018 N-pentanoic acid JWH-122 N-(5-hydroxypentyl)
5	–	–	–	JWH-018 N-hydroxypentyl (0.1 ng/mL) JWH-018 N-pentanoic acid

Table 6
Analytical results for the dried plant materials and powders analysis.

Product name	Form	Substances
Gorby Mix Incenso	Dried plant material	AM-2201; JWH-018; JWH-122; JHW-210
Incenso Herbal Magic	Dried plant material	JWH-018; JWH-073; JWH-122; JWH-210; JWH-250
CM21	Dried plant material	AM-2201; JWH-018; JWH-022; JWH-210
Hulk Power	Dried plant material	JWH-122
Mascara	Dried plant material	–[a]
Texas	Dried plant material	AM-2201; HU-210; JWH-018; JWH-022; JWH-210
Reggae Love	Dried plant material	–[a]
Troll	Dried plant material	AM-2201; JWH-210
Mega Ninja	Dried plant material	AM-2201; JWH-018; JWH-022; JWH-073; JWH-122
U79	Dried plant material	AM-2201; JWH-018; JWH-203; JWH-250
Dnb	Dried plant material	AM-2201; JWH-018; JWH-022; JWH-122; JWH-203; JWH-250; RCS-4
Ninja Ultra Strong	Dried plant material	AM-2201; JWH-203
Blind Heat	Dried plant material	AM-2201; JWH-122; JWH-210
Salvia Divinorum	Dried plant material	AM-2201; JWH-018; JWH-073; JWH-250
Happy Caps–Space-E	Powder	_[a]
Happy Caps–Sex-E	Powder	_[a]
Happy Caps–Lounge-E	Powder	_[a]

[a] None of the substances included in the method were suspected to be present in the product.

MRM transitions and relative retention times expected taking as a reference the retention time of the substances for which standards were available [41,43,44,50]. For the urine samples analyzed in this study, the JWH-018 N-pentanoic acid was detected in four of the five JWH-018 N-hydroxypentyl metabolite positive samples and the JWH-122 N-(5-hydroxypentyl) metabolite was suspected to be present in one of the positive samples. The results obtained were considered mainly as preliminary results, since it was not possible to obtain a full confirmation and quantification, for the reason that the proper analytical standard was not available, no questionnaire with admittance of intake was available in any case, and therefore no positives results for these substances were notified in any circumstance. We also notice that the concentration values obtained for the hydroxyl metabolites of JWH-018 were very low when compared with the majority of the results presented by other authors, such as Jang et al. [38], Yanes et al. [44], ElSohly et al. [47] and Rigdon et al. [50] this could be explained by the dosage used, the time elapsed between substance intake and sample collection and the type of product used, including the amount of JWH-018 present in the formulation.

The five positive samples in a total number of 80 samples analyzed, corresponds to a 6.2% of positive samples. However, considering the 7 samples collected on the clinical and forensic activities of the NILMFS, due to suspicion of the legal high products intake, the positive cases corresponds to 42.8% of cases analyzed in clinical and forensic context. In the case of samples collected from the emergence department without any specific information of synthetic cannabinoids intake, the percentage of positive samples obtained corresponds to 2.5%. This result is also important as a possible indicator of the consumption of these substances, since reflects general population assisted in the emergency department for several reasons and not by suspicion of intoxication. This source of information is even more relevant because in Portugal didn't exist reliable data about the prevalence of consumption of these substances among the general population. A number of surveys in EU countries report the use of synthetic cannabinoids. Although they are not comparable because of different methods and sampling frames, these studies indicate that the percentage obtained (2.5%) is higher than in United Kingdom (England and Wales) with prevalence levels for adults (16–64 years) at 0.1% in 2011/2012, and in Spain among students aged 14–18 years, that revealed a consumption prevalence between 0.6 and 1.4%, according to a 2012 national survey. On the other hand, it is lower than in Frankfurt (Germany) with lifetime levels of use at 7% in 2012 among students aged 15–18 years. The students reporting the consumption of synthetic cannabinoids were, the majority, experienced cannabis consumers. In spite of the new law of April 2013 to regulate the commercialization of these kinds of products in Portugal, resulting in the closing of several "smartshops", the possibility of acquisition of these products through the internet should be a concern, particularly among the younger ages, revealing the importance of indicators that allow monitoring the consumption of these products.

We consider that the results presented in this paper represent important alert data, meaning that the consumption of this type of products is a reality in Portugal and that in the future it will be very important to invest in the analysis of new synthetic cannabinoids and/or metabolites, since according to the EMCDDA (European Monitoring Centre of Drugs and Drug Addiction) the number of synthetic cannabinoids detected in Europe continues to grow with 30 new substances reported in 2012, and 29 in 2013 [57].

3.4. "Legal high" products analysis

To analyze commercially available "legal high" products suspected to contain synthetic cannabinoids, several researchers have published GC–MS, LC–MS/MS and high resolution mass spectrometry methods [5–19]. These methods allowed the qualitative identification of numerous synthetic cannabinoids in different product formulations. For the present study, to analyze one case with some herbal blends and powders in a total of 17 products seized by the authorities in smartshops, a simple and fast organic extraction procedure followed by an UPLC–MS/MS method was applied. The seized materials were mostly in the form of dried plant materials ($n = 14$) and three were in the form of powders. Twenty-seven compounds were selected among the most prevalent according with the literature and the identification was verified by comparing the mass spectra with data from authentic reference material and published data [12]. The results obtained are presented in Table 6. From the 17 products analyzed five did not match with any of the compounds tested and the remaining twelve revealed the presence of at least one synthetic cannabinoid. A total of ten different synthetic cannabinoids were detected. The main substances confirmed according to the comparison with authentic reference material and published data were: AM-2201, JWH-018, JWH-073, JWH-203, JWH-210 and JWH-250. In one product (named Texas) it was found HU-210. In the product named CM21 it was detected a peak that most probably corresponds to the JWH-022 and in the DNB product a peak that corresponds to the RCS-4. All the synthetic cannabinoids were found as adulterants in dried plant materials, namely "herbal incenses" or "potpourris" labeled with "not for human consumption". None of the powders

showed any synthetic cannabinoid, which was already expected due to the fact that powders or pills formulations are normally associated to stimulant compounds, while dried plant materials to synthetic cannabinoids. On two products from the fourteen dried plant materials analyzed none of the substances included in the method were confirmed or suspected to be present. On the remaining twelve products, in only one was identified a single substance, the JWH-122, the others revealed a synthetic cannabinoids mixture with two or more different compounds. Despite the fact that no quantification was performed for the plant materials, it is possible to establish that for the products with a mixture of different substances there is one or two substances, present in an higher amount. The other(s) substances present in lower or trace amount, are probably result of the manufacturing process of the synthetic cannabinoid or from contamination during the commercial preparation of the dried plant materials. It is also important to notice that despite the fact that new substances are available nearly every week the most popular and likely very effective synthetic cannabinoids of the past, such as the JWH-018 and JWH-073 are still available and being used, as confirmed by the results obtained for the seized products, with the presence of JWH-018 in eight products and of JWH-073 in three products, and by the results obtained for the urine samples. Therefore, the constant monitoring of these substances, in the clinical and forensic context, remains necessary. On the other hand, the analysis of the seized products allowed us to identify some of the new substances available in the Portuguese market and therefore a priority concerning their inclusion in the method in a near future.

4. Conclusions

The increasing popularity and availability of synthetic cannabinoids in all kinds of formulations recently became in Portugal a considerable public concern. Therefore, the requirement for the implementation of regulatory control actions, and the demand for the development of analytical methodologies that allows identifying and quantifying the presence of synthetic cannabinoids in different "legal high" products and/or their metabolites in biological matrices, has increased. For that reason, an UPLC–MS/MS method was developed and fully validated for the confirmation and quantification of synthetic cannabinoids in urine samples, and was subsequently applied to authentic samples allowing the notification of the first cases in Portugal. The method developed presents all the advantages of the UPLC–MS/MS technology with a reduced run time and improved sensitivity and selectivity when compared with other methodologies such as the high performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) and GC–MS. In addition, the method developed allows the simultaneous analysis of THC and metabolites, an important feature since from our data the consumption of synthetic cannabinoids together or sequentially to cannabis is a reality and therefore their confirmation could be fundamental to a complete and correct interpretation of the toxicological results. In addition, the qualitative analysis of cannabinoid compounds in 17 "legal high" products using a simple and fast organic extraction procedure followed by an UPLC–MS/MS method allowed the detection and identification of ten different synthetic cannabinoids. The identification of the substances that are available in Portugal allows us to orientate our future work, in terms of the substances that should be a priority to search for, in the clinical and forensic context.

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