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PhD Thesis

METHOD DEVELOPMENT AND VALIDATION FOR THE DETERMINATION OF 49 NEW PSYCHOACTIVE SUBSTANCES (NPS) IN SERUM, URINE AND HAIR BY ULTRA-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY - TANDEM MASS SPECTROMETRY (UHPLC-MS/MS): APPLICATION TO REAL SAMPLES

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Table of Contents:

List of tables and figures:

1. AUTHOR'S DECLARATION

"I declare that, except where explicit reference is made to the contribution of others, that this dissertation is the result of my own work and has not been submitted for any other degree at the Sapienza University of Rome or any other institution"

Signature……...

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2. ABSTRACT

Detection of new psychoactive substances (NPS), both in conventional and non-conventional biological samples, represents a hard challenge for forensic toxicologists. The number of newly NPS, increases each year. As soon as NPS are scheduled, new derivatives appear in the market. Despite the increasing number of NPS and the fact that fatal and acute intoxication cases have been already attributed to this novel class of compounds this phenomenon appears to be considerably underestimated, mainly due to the substantial lack of comprehensive screening methods for their detection in biological specimens. Development of analytical methods for the determination of NPS in biological specimens is of great importance to keep in pace with this phenomenon.

Thus, we sought to develop and validate a simple and rapid UHPLC–MS/MS screening method for the determination of 49 NPS belonging to different chemical classes (synthetic cannabinoids, cathinones, benzofurans, aminoindanes, phenethylamines, piperazines and piperidines) in serum, urine and hair extracts in a single run, following rapid and easy sample pre-injection treatment.

The method was very fast, easy to perform, cheap and minimum amount of sample (0.1 ml serum or urine and 50 mg hair) was required. Chromatography was carried out using an Acquity UPLC BEH reversed phase C18 column (2.1 x 75 mm, 1.7 μm) and a gradient elution with two solvents: 0.1% formic acid in water (solvent A) and acetonitrile (solvent B). The separated analytes were detected with a triple quadrupole mass spectrometer operated in multiple reaction monitoring (MRM) mode via positive electrospray ionization (ESI). The method was sensitive, linear from 1 to 100 ng/ml for serum and urine and from 1 to 100 pg/mg for hair, precise and accurate for most of the analytes. Matrix effects did not negatively affect the analytical sensitivity.

The validated method was successfully applied to authentic samples (serum, urine and hair) collected from an intoxication case after the consumption of NPS; and hair samples obtained by illicit drugs users who attended Drug Addiction Services or Care Emergency Departments (ED).

Keywords: new psychoactive substances, ultra-high performance liquid chromatography - tandem mass spectrometry, serum, urine, hair, toxicology

3. ABBREVIATIONS

2 C-B	2,5-dimethoxy-4-bromophenethylamine
4-APB	4-(2-aminopropyl)-benzofuran
4-FA	4-Fluoroamphetamine
5-APB	5-(2-aminopropyl)benzofuran
5-EAPB	5-(2-ethylaminopropyl)benzofuran
5F-ADB	Methyl (R)-2-[1-(5-fluoropentyl)-1H-indazole-3-carboxamido]-3,3-dimethylbutanoate
5-MAPB	5-(2-Methylaminopropyl)benzofuran
6-APB	6-(2-aminopropyl)-benzofuran
AM-2201	1-(5-fluoropentyl)-3-(1-naphthoyl)indole
AM-2233	1-[(N-methylpiperidin-2-yl)methyl]-3-(2-iodobenzoyl)indole
AM-694	1-(5-fluoropentyl)-3-(2-iodobenzoyl)indole
bk-MBDB	2-methylamino-1-(3,4-methylenedioxyphenyl)butan-1-one
BZP	1-benzylpiperazine
CB-13	Naphthalen-1-yl-(4-pentyloxynaphthalen-1-yl) methanone
DLLME	Dispersive Liquid/Liquid Microextraction
EI	Electron ionization
EMCDDA	European Monitoring Centre of Drug and Drug Addiction
ESI	Electrospray ionization
ETP	Ethylphenidate
GC-MS	Gas chromatography-mass spectrometry
IS	Internal standard

IV	Intravenous
JWH-007	1-pentyl-2-methyl-3-(1-naphthoyl)indole
JWH-016	(1-butyl-2-methyl-1H-indol-3-yl)-1-naphthalenyl-methanone
JWH-018	1-pentyl-3-(1-naphthoyl)indole
JWH-019	1-hexyl-3-(naphthalen-1-oyl)indole
JWH-073	Naphthalen-1-yl-(1-butylindol-3-yl) methanone
JWH-081	4-methoxynaphthalen- 1-yl- (1-pentylindol- 3-yl)methanone
JWH-098	4-methoxynaphthalen-1-yl-(1-pentyl-2-methylindol-3-yl)methanone
JWH-147	(1-hexyl-5-phenyl-1H-pyrrol-3-yl)-1-naphthalenyl-methanone
JWH-200	(1-(2-morpholin-4-ylethyl)indol-3-yl)-naphthalen-1-ylmethanone
JWH-203	1-pentyl-3-(2-chlorophenylacetyl)indole
JWH-210	4-ethylnaphthalen-1-yl-(1-pentylindol-3-yl)methanone
JWH-250	1-pentyl-3-(2-methoxyphenylacetyl)indole
JWH-251	1-pentyl-3-(2-methylphenylacetyl)indole
JWH-302	1-pentyl-3-(3-methoxyphenylacetyl)indole
JWH-307	(5-(2-fluorophenyl)-1-pentylpyrrol-3-yl)-naphthalen-1-ylmethanone
JWH-398	1-pentyl-3-(4-chloro-1-naphthoyl)indole
LC	Liquid chromatography
LC-HRMS	Liquid chromatography–high resolution mass spectrometry
LC-MS/MS	Liquid chromatography – tandem mass spectrometry
LC-QTOF MS	Liquid chromatography quadrupole time-of-flight mass spectrometry
LLE	Liquid-liquid extraction
LOD	Limit of detection

LOQ	Limit of quantification
mCPP	meta-Chlorophenylpiperazine
MDA	3,4-methylenedioxyamphetamine
MDAI	5,6-methylenedioxy-2-aminoindane
MDMA	3,4-methylenedioxymethamphetamine
MDPA	3,4-methylenedioxy-N-propyl-amphetamine
MDPV	Methylenedioxypyrovalerone
MeOH	Methyl alcohol (methanol)
MMB-2201	Methyl (1-(5-fluoropentyl)-1H-indole-3-carbonyl)-L-valinate
MP	Mobile phase
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NaOH	Sodium hydroxide
NPS	New psychoactive substances
QC	Quality control
RCS-4	1-pentyl-3-(4-methoxybenzoyl)indole
RCS-8	1-(2-cyclohexylethyl)-3-(2-methoxyphenylacetyl)indole
S/N	Signal to noise ratio
SoHT	Society of hair drug testing
SPE	Solid phase extraction
THC-d3	Tetrahydrocannabinol-d3
UHPLC-MS/MS	Ultra-high-performance liquid chromatography – tandem mass spectrometry
UNODC	United Nations Office on Drugs and Crime

WDR	World drug report
XLR-11	(1-(5-fluoropentyl)-1H-indol-3-yl) (2,2,3,3-tetramethylcyclopropyl)methanone
μ-SPE	Micro solid phase extraction

4. INTRODUCTION

4.1 New psychoactive substances (NPS)

In the recent years, an increasing global concern has been arisen over the rapid emergence of new substances in the market of illicit psychotropic drugs. New Psychoactive Substances (NPS) belong to several chemical classes, including but not limited to synthetic cannabinoids, cathinones, phenethylamines and piperazines and they are commonly sold via the internet as legal substitutes for classical drugs of abuse.

The number of newly NPS, as reported by the European Monitoring Centre of Drug and Drug Addiction (EMCDDA) and the United Nations Office on Drugs and Crime (UNODC), increases each year (1, 2). NPS have been synthesized to evade existing drug laws, usually by altering the chemical structures of illegal drugs or by finding compounds with different structures that produce effects similar to those of existing stimulant, hallucinogenic, psychedelic, sedative, dissociative or euphoric drugs. As soon as NPS are scheduled, new derivatives appear in the market. Therefore, according to the 2014 Flash Eurobarometer 3 per cent of young adults (ages 16-24) have reported using NPS (2).

Furthermore, the use of NPS as adulterants of stimulant recreational controlled drugs, has recently been documented as a new worrying phenomenon (3).

Despite the increasing number of NPS and the fact that fatal and acute intoxication cases have been already attributed to this novel class of compounds (4, 5) this phenomenon appears to be considerably underestimated, mainly due to the substantial lack of comprehensive screening methods for their detection in biological samples.

This rapid increase of NPS sets new challenges not only in drug prevention and legislation but also in clinical and forensic toxicology. The acute and chronic toxicity of many of these compounds is unknown and only little information is available on the pharmacology and toxicology, toxicokinetics, or detectability in body samples of such new compounds. There is a need for evidence-based treatment recommendations for acute intoxications and a demand for new strategies in analyzing these substances in clinical and forensic cases (6, 7).

Development of analytical methods for the determination of NPS both in traditional and alternative matrices is of great importance to investigate drug metabolism and to

associate intake to clinical outcomes and eventual intoxication symptoms, as well as to provide evidence in forensic cases (human performance and post-mortem cases).

4.1.1 Synthetic cannabinoids

Synthetic cannabinoids are a class of substances that differ from the cannabinoids occurred in cannabis plants but which also bind to cannabinoid receptors. They are usually marketed as designer drugs or sold in products claiming that they give the effects of cannabis. When these drugs are sprayed or otherwise soaked into a plant or other base material the blend is sometimes misleadingly marketed as synthetic marijuana. These synthetic marijuana products are sold for recreational drug use.

There are several psychoactive synthesized cannabinoid families such as AM-xxx, HU-xxx, JWH-xxx, CP-xx that are sprayed onto plant material that is then sold under brand names like K2 and Spice both of which are now often used as generic terms for any synthetic cannabinoid product.

They have been sold in head shops, online, and other stores. Studies have associated synthetic cannabinoid use with psychotic episodes days after use, some of which have resulted in death. These blends are often marketed as herbal incense or "herbal smoking blends", and the products are commonly consumed through smoking (8).

Synthetic cannabinoids frequently induce adverse effects which lead to hospitalization (9); they are potent drugs capable to cause intoxication and death (10). Many substances have been banned in many countries, although gaps remain and new compounds continue to emerge on a regular basis (8, 11).

As reported in the world drug report (WDR) 2016 the global market for synthetic NPS continues to be dominated by synthetic cannabinoids. In Europe, significant seizures of synthetic cannabinoids were recorded: 5.4 tons of synthetic cannabinoids were seized in 2014 (mainly in Cyprus and Turkey), compared with 1.2 tons in 2013. Moreover, between 2012 and 2014, most substances reported for the first time to the UNODC belonged to the group of synthetic cannabinoids (12).

4.1.2 Synthetic cathinones

Synthetic cathinones are an emerging class of designer drugs abused of due to their psychostimulant and hallucinogenic effects, similar to those of cocaine, 3,4-methylenedioxymethamphetamine (MDMA), amphetamines and methamphetamines (5). Indeed, they are marketed as cheap substitutes for the latter compounds. They are chemically related to cathinone, a stimulant found in the khat plant, which grows in East Africa and Southern Arabia, and people sometimes chew its leaves for their mild stimulant effects. Synthetic variants of cathinone can be much more potent than the natural product and, in some cases, very dangerous or even lethal (13). A number of synthetic cathinones related deaths has been published (5).

Synthetic cathinones, referred to as "bath salts" (14), can be found in the form of a brown or white crystal-like powder and are sold in small foil or plastic packages labelled as "not for human consumption", "jewellery cleaner", "plant food" or "phone screen cleaner". They are available online and in drug paraphernalia stores under a variety of brand names, such as Flakka, Cloud Nine, Lunar Wave, White Lightning, Bloom, Scarface and Vanilla Sky. In 2013, European countries reported more than 110 NPS products containing a combination of up to seven different NPS compounds sold as one product. Synthetic cannabinoids were found to be present in more than 55 % of these NPS products, and synthetic cathinones were present in more than 25 % (12).

In some countries, shortage of heroin and an increase in local availability of synthetic cathinones contributed to high-risk drug users switching to injecting NPS, primarily synthetic cathinones, as reported by WDR 2016. New synthetic cathinones are continuously emerging; although data collection for 2015 is still in progress, 75 new substances have been reported to UNODC for the first time, among which 20 belong to synthetic cathinones group compared to 21 newly emerged synthetic cannabinoids.

Moreover, global seizures of synthetic cathinones have been steadily increasing since they were first reported in 2010. Those seizures tripled between 2013 and 2014, reaching 1.3 tons. Most synthetic cathinones were seized in Europe and in East and South-East Asia (12).

4.1.3 Ethylphenidate (ETP)

Ethylphenidate (ethyl 2-phenyl-2-(piperidin-2-yl) acetate, ETP) is a psychostimulant NPS that inhibits reuptake of both dopamine and noradrenaline. It is an analogue of methylphenidate first reported to the EMCDDA in 2011 by the UK (15) and often sold under the street name 'Nopaine' or 'Gogaine'. ETP is also produced in vivo as a metabolite following the co-ingestion of methylphenidate and ethanol (16), first reported in two cases of methylphenidate overdose in 1999 (17). In 2014, ETP was first detected in postmortem blood following its abuse (18).

Recreational ETP use has been described on internet drug forums since 2010. ETP is thought to provide a stronger stimulant effect than cocaine and an empathogenic effect similar to ecstasy and mephedrone. Desirable effects for the user include euphoria, alertness, a general mood lift and increased social skills. However, this drug causes a range of unwanted effects, including chest pain, palpitations, agitation, nasal pain and irritation, bruxism, and abdominal and testicular pain. ETP has a far greater dopaminergic selectivity compared to methylphenidate, which may increase its dependence potential. Common routes of administration of ETP are insufflation and intravenous (IV) injection, with significant risks of infections associated with IV drug use (19, 20).

Some scientific reports on acute and chronic poisoning attributed to ETP and related fatalities have been published (18, 21-26). One of the major concerns about ETP**,** reported by users in more than one internet forums, is "a persistent impulse to redosing". The long-term abuse potential is hard to determine, even if its pharmacology suggests that there is a significant risk of abuse (25). Only a few ETP long-term effects have been reported and only one case report of dependence has been documented (22-24).

4.1.4 Benzofuran analogues

Benzofurans are psychoactive substances structurally very similar to the popular recreational drug MDMA and its active metabolite 3,4-methylenedioxyamphetamine (MDA). These compounds were originally synthesized for research purposes, specifically 5-(2-aminopropyl)-benzofuran (5-APB) and 6-(2-aminopropyl)-

benzofuran (6-APB) were synthesized to examine the role of the MDA dioxyle ring structure when interacting with serotonergic neurons.

These two benzofurans appeared in the drug market in 2010–2011. Since then, the presence of benzofurans in the illicit drug market has rapidly increased (27, 28). In 2012, 6-APB was amongst the most frequently offered NPS in online shops (29). Moreover, in 2013 benzofurans were one of the four most frequently detected NPS in the Netherlands (28). In Italy, 4-APB and 6-APB were also detected in seized materials analyzed in an Italian forensic toxicology laboratory in the period 2013-2015 (30).

Information regarding the desired effects of benzofurans is limited only to online user forums. These reports indicate increased empathy, euphoria, visual stimulation, appreciation for music and dancing and an increase in mood and self-acceptance, among positive effects (31-32). Nevertheless, users have reported multiple adverse effects of benzofurans, e.g., nausea, bruxism, dry mouth and eyes, diarrhea, sensitivity to light, palpitations, increased heart rate, blood pressure and temperature, hot flushes, headaches, drowsiness and clonus of the hands and feet. Also, psychological symptoms have been reported, such as hallucinations, depression, anxiety, panic attacks, insomnia, severe paranoia and psychosis. Furthermore, some users also reported an unpleasant 'comedown' that could last for several days (31-33). Main routes of administration of benzofurans include nasal insufflation of powder and ingestion.

To date, only a few scientific reports on acute benzofuran-related poisoning and fatal case reports are available in literature (32-38). There are no published reports on dependence to benzofurans (31). Neither have the long-term effects of regular use of these NPS been reported, nor has their chronic use been analytically confirmed by hair analysis.

4.2 Methods for the determination of NPS in biological samples

4.2.1 Blood and urine

Analytical methods for the simultaneous determination of NPS belonging to different chemical classes as well as case reports (acute intoxications/fatalities) where one or more NPS were detected in blood and/or urine have been reported in the scientific literature (4, 5, 39-41).

4.2.1.1 Blood

The blood concentration of different NPS varies from class to class and it highly depends on the route of administration. For instance, the concentration of the unchanged synthetic cannabinoids in blood is very low (ng/ml) even in fatal cases, whereas concentrations in the range of μg/ml were found in mephedrone related intoxications and fatalities (5, 42).

Adamowicz et al developed a fast and simple liquid chromatography – tandem mass spectrometry (LC-MS/MS) screening procedure for 143 NPS coming from different groups such as cathinones, phenethylamines, tryptamines, piperazines, piperidines, synthetic cannabinoids, arylalkylamines, arylcyclohexylamines, aminoindanes, and other drugs in blood. The sample pretreatment was easy and fast, employing only precipitation of 0.2 ml blood sample with 0.6 ml acetonitrile. The limit of detection (LOD) values were estimated for 104 drugs and ranged from 0.01 to 3.09 ng/ml. The extraction recoveries determined for 32 substances were between 1.8 and 133%. The procedure was successfully applied to the analysis of forensic blood samples in routine casework (43).

Vaiano et al developed and fully validated a LC–MS/MS screening method for the simultaneous detection of 69 substances, including 64 NPS belonging to different chemical classes and 5 ‘‘traditional’’ amphetamines in blood. The method was very fast, easy to perform and cheap as it only required the deproteinization of 0.2 ml blood sample with acetonitrile. The achieved limit of quantification (LOQ) values ranged from 0.1 to 0.5 ng/ml and the method was linear from 1 to 100 ng/ml. Precision and accuracy were acceptable at any quality control (QC) level and recovery efficiency ranged from 72 to 110%. Matrix effects did not negatively affect the analytical sensitivity. The validated method was successfully applied to three authentic samples allowing identification and quantification of: mephedrone and methamphetamine (post-mortem); ketamine, MDMA and MDA (post-mortem); and AB-FUBINACA (ante-mortem) (44).

Another research group developed and validated a method for the simultaneous determination of different classes of NPS (synthetic cannabinoids and their metabolites, cathinones and phenethylamines) directly on whole blood (0.1 ml) without anti-coagulants employing micro solid phase extraction (μ-SPE) and LC–MS/MS.

Recoveries ranged from 21% to 70%; matrix effect was lower than 15% for all the substances under investigation. LOQ values were 5 ng/ml for cathinones and phenethylamines, between 0.25 and 1 ng/ml for synthetic cannabinoids and up to 2.5 ng/ml for synthetic cannabinoid metabolites (45).

A gas chromatography – mass spectrometry (GC-MS) assay has been validated for qualitative and quantitative determination of a number of NPS belonging to synthetic cathinones and phenethylamines in 0.25 ml pericardial fluid and whole blood. The method included mixed-mode solid phase extraction, followed by microwave fast derivatization. Linearity ranged from 5 to 600 ng/ml. Intra- and inter-day precision ranged between 0.1 and 13.6%, while accuracy from 80 to 120% interval from the nominal concentration at all tested levels. The extraction efficiencies ranged from 76.6 to 112.8%. The method was applied to real samples (46).

Odoardi et al developed an ultra-high-performance liquid chromatography - tandem mass spectrometry (UHPLC-MS/MS) screening assay for the determination of 78 NPS of different classes in blood samples. The extraction of analytes was achieved by Dispersive Liquid/Liquid Microextraction (DLLME), a very rapid, cheap and efficient extraction technique that needs small amount of organic solvents. LODs ranged from 0.2 to 2 ng/ml. The method was then applied to 60 authentic specimens from forensic cases, demonstrating its suitability for the screening of a broad range of NPS (47).

4.2.1.2 Urine

Although detection of substances in blood suggests recent exposure and associate intoxication to the causative compound, urine is still the most preferred testing matrix in clinical and forensic settings (48).

Concheiro et al developed a liquid chromatography-high resolution mass spectrometry (LC-HRMS) method for the simultaneous determination of 40 novel psychoactive stimulants (8 piperazines, 4 designer amphetamines and 28 synthetic cathinones and 4 metabolites) in urine. The method was applied to real urine samples (n=62). One-hundred μl urine was subjected to solid phase cation exchange extraction. The chromatographic reverse-phase separation was achieved in 20 min (48).

A multi-component LC–MS/MS method was developed and validated for the detection and quantification of 11 designer benzodiazepines in urine. The method employed dilution of urine with internal standard (IS) and hydrolysis of any glucuronide conjugated forms. Separation of the analytes was achieved on a BEH Phenyl column, followed by tandem mass spectrometry (MS/MS) detection in positive electrospray ionization mode (ESI). The method was applied to study the occurrence of designer benzodiazepines in Sweden in 2014–2015, by analysis of 390 specimens retrieved from a routine drug testing laboratory. Forty percent of the latter specimens, selected based on a positive immunoassay benzodiazepine screening but a negative mass spectrometry (MS) confirmation for the common set of prescription benzodiazepines, were found positive to designer benzodiazepines. These findings stress the importance of employing and updating confirmation methods to include the increasing number of designer benzodiazepines appearing in the NPS market (49).

A research group developed and validated a LC-MS/MS method for simultaneous identification of traditional drugs of abuse, benzodiazepines and NPS in urine. The samples were undergone liquid-liquid extraction (LLE) and passed through a 0.22-mm polyvinylidene difluoride filter before injection into the chromatographic system. LOQ values ranged from 0.5 ng/mL to 31.3 ng/ml. The linearity ranged from 0.5 ng/ml to 200 ng/ml. The precision results were below 15.4% (intra-day) and 18.7% (inter-day). The assay was applied to 769 urine specimens. The most common drugs identified were ketamine, amphetamine, and opiates (50).

Bell et al developed a rapid multi-analyte screening method, using UHPLC-MS/MS for the analysis of 8 new designer drugs in urine following a 1:4 dilution of urine with mobile phase (MP). Although all target analytes were readily detected at 500 ng/ml, a cut-off of 1000 ng/ml was chosen to mirror the amphetamine screening cut-off commonly used for routine analysis of workplace drug testing samples. The authors concluded that direct analysis using LC-MS/MS offers an attractive way for the development of a rapid routine screen for NPS (51).

4.2.2 Hair

Hair testing for drugs of abuse was introduced over 60 years ago (52) and appears to be one of the most efficient tools to investigate drug-related history (53), due to the greater window of detection compared to that of blood and urine. Hair has been recently characterized as a peculiar tissue which ‘‘keeps memory’’ of the past history of drug intake of the subject (54).

Hair drug testing has gained increased interest and recognition over the past two decades and its application has been expanded in both forensic and clinical toxicology.

Hair is recognized as a complimentary testing matrix and is widely used with samples routinely collected during criminal investigations. Hair testing has been successfully applied for consumption history of classical drugs of abuse and it is used to monitor drug usage during drug rehabilitation programs (55); in post- mortem cases (56); in workplace drug testing (57); driving license regranting (58) and in child custody cases (59, 60). In the light of that, similarly to what happened with traditional drugs of abuse, assays for the determination of NPS and eventual metabolites in hair are in continuous development due to the high demand for the detection of the latter substances both in clinical and forensic cases.

The currently available methods for screening and quantitative analysis of NPS in hair were recently the subject of a comprehensive review with 54 references (61). The authors documented a wide variety of analytical methods for determination of NPS in hair and presented a concise table providing LOD and LOQ values as well as precision and accuracy for each method.

Methods for the determination of NPS in hair, are in continuous development together with some existing analytical methods for hair testing which are expanded to include as many NPS as possible due to an increasing demand to disclose their eventual presence in hair of users. Some studies have been carried out for the evaluation of the prevalence and diffusion of NPS. Re-analysis of previously tested hair samples for common drugs of abuse revealed some positive results for these new psychoactive compounds suggesting on the one hand their underestimated prevalence in users of recreational drugs and on the other a hand the need for NPS screenings to be routinely employed both in forensic and clinical toxicology.

Advances in MS technology enabled the improvement of sensitivity so that these novel substances, whose used doses are in the majority of cases unknown can be detected at low concentrations, i.e. pg per mg hair whereas traditional drugs of abuse are usually in the range of ng per mg keratin matrix.

Hyphenated mass spectrometric techniques are indispensable tools in clinical and forensic toxicology and doping control. Whereas GC-MS in the electron ionization (EI) mode plays a major role particularly in comprehensive screening procedures because a very large collection of reference spectra is available and the cost of the instrument is not excessive, LC coupled with different mass analyzer types is becoming more and more a standard technique for automated target screening procedures and particularly for high -throughput quantification. Indeed, LC-MS has shown to be an ideal supplement, especially for detection of more polar, unstable or low-dose drugs (62-67). Although very few GC-MS methods have been described for the determination of NPS in hair, the vast majority of studies conducted on this regard use LC-MS/MS or UHPLC–MS/MS. Moreover, liquid chromatography quadrupole time-of-flight mass spectrometry (LC-QTOF MS) and LC-HRMS have also been used with achieved LODs at decimal picograms.

However, limitations, common with the published procedures for traditional psychotropic drugs such as the difficulty in evaluating the real performance of the extraction procedure due to the lack of certified reference hair specimens with known drug content and lack of a protocol for washing procedures, should not be underestimated.

4.2.3 Alternative biological matrices

Determination of NPS and their metabolites in biological fluids or matrices other than blood or urine may be of interest in certain areas of drug concentration monitoring (68), since blood and urine drug testing may fail to document drug use when samples are collected at inconvenient times. Oral fluid is the only fluid which can be used successfully as a substitute for blood in therapeutic drug monitoring (69), while an individual's past history, can be obtained from hair or nails drug analysis. Drug concentrations in the bile and faeces can account for excretion of drugs and metabolites other than by the renal route. Furthermore, it is important that certain matrices (tears,

cerebrospinal fluid, bronchial secretions, peritoneal fluid and interstitial fluid) are analyzed, as these may reveal the presence of a drug at the site of action; others (fetal blood, amniotic fluid and breast milk) are useful for determining fetal and perinatal exposure to drugs.

For all these reasons, drug concentration measurement in nonconventional matrices and fluids, although sometimes expensive and difficult to carry out, should therefore be considered for inclusion in studies of the pharmacokinetics and pharmacodynamics of new drugs (68).

NPS have been determined in various alternative matrices including stomach content (70), vitreous humour (46, 71), meconium (72), brain, heart, lung, liver, kidney, spleen, and pancreas (42, 73, 74). However, the vast majority of the literature focuses on two alternative matrices: hair and oral fluid. In this concern, recently Øiestad et al. published a comprehensive review on the trends in analytical methods for the detection and/or quantification of NPS in oral fluid (75).

4.3 Ultra-high-performance liquid chromatography – tandem mass spectrometry (UHPLC-MS/MS)

(UHP)LC-MS/MS is a powerful hyphenated technique which combines the physical separation capabilities of liquid chromatography (LC) with the outstanding qualitative efficiency of MS. It has gained enormous growth both in forensic and clinical laboratories the last 20 years due to its superior analytical specificity over conventional high-performance liquid chromatography - mass spectrometry (HPLC - MS) and GC-MS (76). It is a powerful technique used for many applications which has very high sensitivity and selectivity. Generally, its application is oriented towards the general detection and potential identification of chemicals in the presence of other chemicals (in a complex mixture). The technique has both qualitative and quantitative uses such as identifying unknown compounds and quantifying the amount of a compound in a sample.

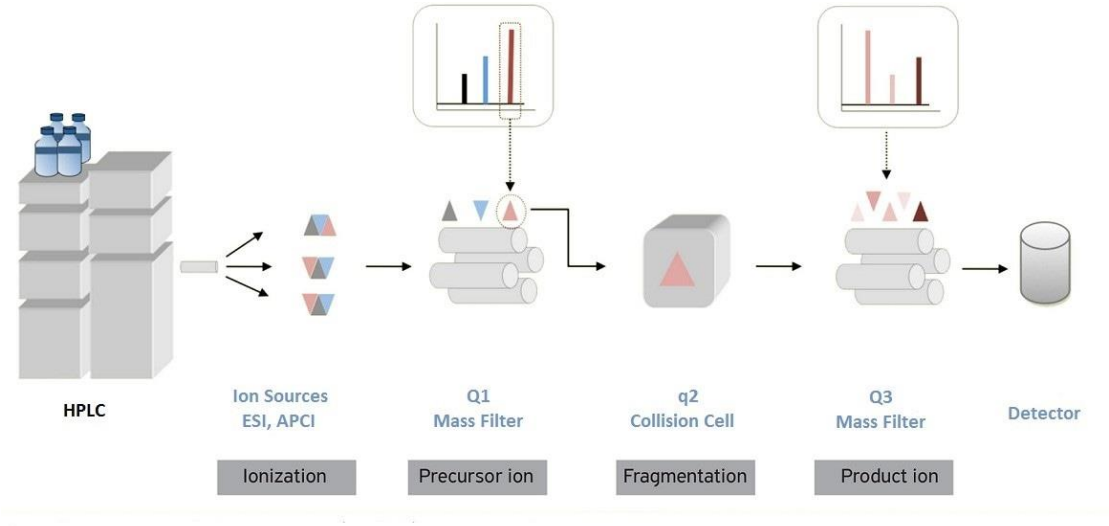


Figure 1: Triple quadrupole MS diagram (77)

Figure 2: From left to right: An ultra-high-performance liquid chromatography system (Waters Acquity UPLC) and a triple quadrupole mass spectrometer (Waters Xevo TQ) (78, 79)

5. AIMS

The aim of this project was the development and validation of a simple and rapid UHPLC–MS/MS screening method for the determination of 49 NPS belonging to different chemical classes (synthetic cannabinoids, synthetic cathinones, benzofurans, aminoindanes, phenethylamines, piperazines and piperidines) in serum, urine and hair extracts in a single run, following rapid and easy sample pre-injection treatment; and the application to authentic samples (serum, urine and hair) collected from an intoxication case after the consumption of NPS and hair samples obtained by illicit drugs consumers who attended Drug Addiction Services or Care Emergency Departments (ED).

6. EXPERIMENTAL

6.1 Chemicals and reagents

All drug standards as reported in Table 1 were supplied from LGC standards (Milan, Italy) apart from 5-(2-Methylaminopropyl)benzofuran hydrochloride (5-MAPB), 5-APB hydrochloride and 5-(2-ethylaminopropyl)benzofuran hydrochloride (5-EAPB) solutions which were purchased from Cayman Chemical (Cayman Chemical, MI, USA) and (±)-threo-Ethylphenidate (ETP) hydrochloride solution which was supplied from Sigma (Sigma-Aldrich, Barcelona, Spain).

Water, acetonitrile, formic acid and methyl alcohol were obtained from Sigma-Aldrich (Milano, Italy). Phree Phospholipid Removal tube, SPE columns were purchased from Phenomenex (Macclesfield, UK).

All other chemicals used for experiments were analytical reagent or HPLC grade from commercial resources.

Table 1. List of analytes with their chemical groups, retention times and quantification and confirmation MRM transitions together with optimized cone voltages and collision energies

			MRM transitions					
Analyte	**Chemical group**	**RT (min)**	**Quantification**			**Confirmation**		
			m/z	**CV (V)**	**CE (eV)**	**m/z**	**CV (V)**	**CE (eV)**
MDAI	Aminoindane	2.20	178.2 > 130.7	20	20	178.2 > 103.2	20	20
Methcathinone	Cathinone	2.20	164.2 > 131.0	20	20	164.2 > 146.2	20	20
Dimethylcathinone	Cathinone	2.25	178.3 > 105.1	26	20	178.3 > 72.2	26	20
Methylone	Cathinone	2.25	208.2 > 160.2	22	18	208.2 > 132.3	22	26
4-fluoromethcathinone	Cathinone	2.32	182.3 > 149.1	24	20	182.3 > 123.1	24	20
Ethylone	Cathinone	2.38	222.2 > 174.1	26	16	222.2 > 204.2	26	16
Methedrone	Cathinone	2.40	194.3 > 161.1	18	20	194.3 > 146.0	18	26
Buphedrone	Cathinone	2.45	178.3 > 91.1	22	20	178.3 > 160.0	22	12
4-FA	Phenethylamine	2.48	154.2 > 136.7	20	10	154.2 > 109.3	20	10
Butylone	Cathinone	2.48	222.2 > 174.2	22	18	222.2 > 131.3	22	34

Mephedrone	Cathinone	2.59	178.2 > 145.3	20	20	178.2 > 159.8	20	20
4 methylethcathinone	Cathinone	2.71	192.3 > 144.4	22	28	192.3 > 119.2	22	24
Pentedrone	Cathinone	2.75	192.3 > 132.1	26	18	192.3 > 174.2	22	14
Pentylone	Cathinone	2.76	236.2 > 188.3	24	18	236.2 > 131.2	24	36
5-APB	Benzofuran	2.80	176.4 > 131.0	15	15	176.4 > 159.4	15	12
m-CPP	Piperazine	2.85	197.2 > 154.0	22	22	197.2 > 119.2	22	25
3,4-dimethylmethcathinone	Cathinone	2.89	192.1 > 159.1	22	20	192.1 > 174.1	22	20
5-MAPB	Benzofuran	2.90	190.3 > 131.1	20	20	190.3 > 159.1	20	12
2 C-B	Phenethylamine	2.94	260.1 > 228.0	20	22	260.1 > 213.0	20	32
MDPV	Cathinone	2.96	276.3 > 175.1	20	22	276.3 > 126.3	20	22
5-EAPB	Benzofuran	3.03	204.4 > 131.1	20	20	204.4 > 159.0	15	15
ETP	Piperidine	3.26	248.3 > 84.2	26	15	248.3 > 248.3	26	5
Pravadoline	Cannabinoid	3.48	379.4 > 135.1	28	18	379.4 > 77.1	28	54
Naphyrone	Cathinone	3.54	282.2 > 141.4	34	20	282.2 > 155.2	34	28
AM-2233	Cannabinoid	3.57	459.2 > 231.0	26	34	459.2 > 98.1	26	28

JWH-200	Cannabinoid	3.74	385.3 > 155.1	30	20	385.3 > 114.2	30	26
MMB-2201	Cannabinoid	5.11	363.3 > 232.2	20	16	363.3 > 144.1	20	40
AM-694	Cannabinoid	5.50	436.2 > 231.0	38	26	436.2 > 203.1	38	44
5F-ADB	Cannabinoid	5.64	378.3 > 318.2	26	16	378.3 > 233.1	26	24
AM-2201	Cannabinoid	5.72	360.0 > 155.1	40	28	360.0 > 232.1	40	28
RCS-4	Cannabinoid	5.85	322.3 > 135.1	38	24	322.3 > 214.2	38	24
JWH-250	Cannabinoid	5.93	336.03 > 121.2	32	15	336.03 > 200.1	32	24
JWH-302	Cannabinoid	5.94	336.2 > 121.1	32	20	336.2 > 214.2	32	30
JWH-073	Cannabinoid	6.00	328.0 > 155.1	38	22	328.0 > 127.2	38	38
XLR-11	Cannabinoid	6.06	330.4 > 125.2	68	22	330.4 > 232.2	68	24
JWH-251	Cannabinoid	6.11	320.3 > 105.1	38	24	320.3 > 214.2	38	26
JWH-203	Cannabinoid	6.13	340.3 > 125.1	38	26	340.3 > 188.2	38	20
JWH-018	Cannabinoid	6.24	342.3 > 155.2	40	24	342.3 > 127.2	40	50
JWH-016	Cannabinoid	6.25	342.3 > 155.2	38	24	342.3 > 127.2	38	50
JWH-081	Cannabinoid	6.33	372.2 > 214.2	40	25	372.2 > 185.3	40	25

JWH-019	Cannabinoid	6.38	356.4 > 155.1	40	24	356.4 > 127.1	40	44
JWH-098	Cannabinoid	6.42	386.3 > 185.1	38	26	386.3 > 228.2	38	20
JWH-307	Cannabinoid	6.42	386.3 > 155.1	32	20	386.3 > 127.1	32	48
JWH-007	Cannabinoid	6.49	356.3 > 155.1	38	26	356.3 > 127.2	38	48
RCS-8	Cannabinoid	6.50	376.4 > 121.1	34	22	376.4 > 91.1	34	50
JWH-398	Cannabinoid	6.63	376.4 > 189.1	40	28	376.4 > 161.1	40	42
JWH-210	Cannabinoid	6.64	370.3 > 183.1	36	24	370.3 > 214.2	36	24
JWH-147	Cannabinoid	6.70	382.4 > 155.1	32	20	382.4 > 127.1	32	50
CB-13	Cannabinoid	7.13	369.3 > 155.1	36	24	369.3 > 171.1	36	28
MDPA	IS	2.80	222.2 >162.9	20	15			
THC-d3	IS	6.66	318.3 >196.3	20	25			

6.2 Biological material

Drug-free (blank) serum and urine samples, used for the development and validation of the method were obtained from a regional hospital from individuals with no drug-related history. Drug-free hair specimens were collected and donated by professional hairdressers.

6.3 Standard, working solutions, calibrators and QCs

All the standards were available at the concentration of 0.1 mg/ml in methyl alcohol and were kept at – 20 °C. Mix working solutions containing all the 49 analytes (see analyte list in Table 1) were prepared by dilution of the appropriate volume of stock standard solutions in methyl alcohol to obtain final concentrations of 1000, 100 and 10 ng/ml for hair testing and 1 and 0.1 μg/ml for serum and urine analysis. Mix IS working solution was prepared at the concentration of 0.5 μg/ml and 100 ng/ml for serum and urine analysis and hair testing, respectively. All working solutions were also stored at – 20 °C in amber glass vials.

Blank serum, urine and hair were evaluated to ensure absence of detectable analytes prior to fortification with working solutions to prepare calibrators and QCs. Addition of proper volume of mix standard working solutions in 1 ml blank serum, urine and 25 mg of hair created calibrators at 1, 5, 10, 50, 100 ng/ml, 1, 2.5, 5, 10, 50, 100 ng/ml and 1, 5, 10, 50, 100 pg/mg, respectively.

Three QC samples (low, medium, high) were prepared at the concentration of 1.2, 40 and 85 ng/ml in serum and 5, 40 and 85 ng/ml in urine. One ml of blank serum and urine were spiked with proper volumes of mix standard working solutions. The latter standards used for the fortification of blank serum and urine to yield QC samples were separately prepared from the ones used for the preparation of calibrators.

6.4 Hair preparation approaches evaluated during method development

Different extraction procedures were tested in order to choose the most suitable one for the majority of the analytes. The initial aim was to find an extraction method that could be used to simultaneously recover all the compounds. The following tests were carried

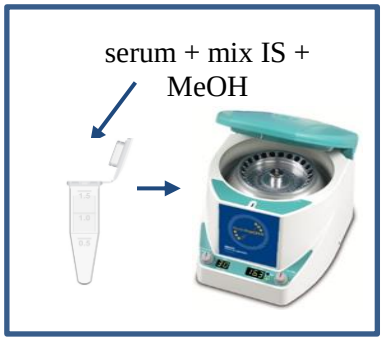
out: a) digestion of 25 mg hair using NaOH 1M (30 min at r.t) and extraction either with ethyl acetate or a mixture of hexane:ethyl acetate 80:20, evaporation, reconstitution in MP A:B, 80:20, b) the same as (a) but the digestion was performed by overnight incubation at 45°C, c) extraction of 25 mg hair in 1.5 ml methyl alcohol:CCl_3, 9:1, evaporation, reconstitution in MP A:B, 80:20, d) digestion of 25 mg hair using NaOH 1M (1 h at 90°C) and extraction after: 1) neutralization, 2) acidification, 3) without changing pH, either with ethyl acetate or a mixture of hexane:ethyl acetate 80:20, evaporation, reconstitution in MP A:B, 80:20 and e) treatment of 25 mg hair with 0.5 ml M3 reagent for 1 h at 100 °C, 1:4 dilution with water and direct injection.

Moreover, different reconstitution solvents, consisted of different percentages of MP A and B, were used to re-dissolve serum and hair dried extracts in order to choose the optimum one. Hundred percent MP A, 100% MP B, 20:80 MP A:B, 80:20 MP A:B, 50:50 MP A:B, 30:70 MP A:B and 70:30 MP A:B, were tested.

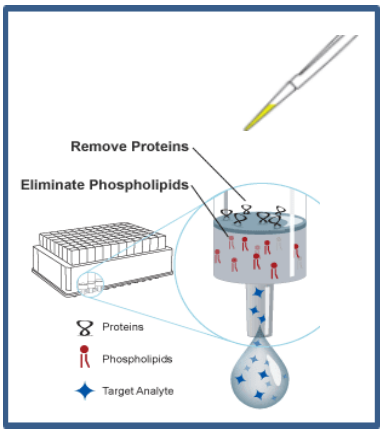
6.5 Sample preparation

6.5.1 Serum

Serum specimens (sample/QC/calibrator) (100 µl) were added with 10 µl mix IS solution at the concentration of 0.5 µg/ml (50 ng/ml) and 400 µl methyl alcohol. Vortex and ultracentrifugation at 13000 rpm for 10 minutes was followed. The supernatant was then collected and loaded directly into phospholipids removal cartridges. The cartridges (placed into clean glass vials) were vortex mixed and centrifuged at 3500 rpm at room temperature for 5 min. The filtrate was evaporated to dryness under gentle stream of nitrogen. The residue was reconstituted with 100 µl MP A:B, 80:20 and 10 µl were injected into the chromatographic system (See Figure 3). Whenever the real sample concentrations were found to exceed the highest calibration point, the extracts were appropriately diluted and re-injected into the chromatographic system.



Serum specimens (100 µl) were added with 10 µl mix IS solution and 400 µl MeOH. Vortex and ultracentrifugation at 13000 rpm for 10 minutes was followed.



The supernatant was then loaded directly into phospholipids removal cartridges. The cartridges (placed into clean glass vials) were vortex mixed and centrifuged at 3500 rpm at room temperature for 5 min.

The filtrate was evaporated to dryness under gentle stream of nitrogen. The residue was reconstituted with 100 µl MP A:B, 80:20 and 10 µl were injected into the UHPLC–MS/MS system.

Figure 3: Serum pre-injection treatment

6.5.2 Urine

Urine specimens (sample/QC/calibrator) were prepared for analysis by centrifuging at 13000 rpm for 10 minutes. Following the addition of 10 µl mix IS solution at the concentration of 0.5 µg/ml (50 ng/ml) to 100 µl sample, the samples were vortex mixed for 30 s and then diluted 1:4 (v/v) with MP A:B, 80:20. The samples (10 µl) were then directly injected into the UHPLC-MS/MS system. The procedure is schematically given in Figure 4. Whenever the real sample concentrations were found to exceed the highest calibration point, the extracts were opportunely diluted and re-injected into the chromatographic system.

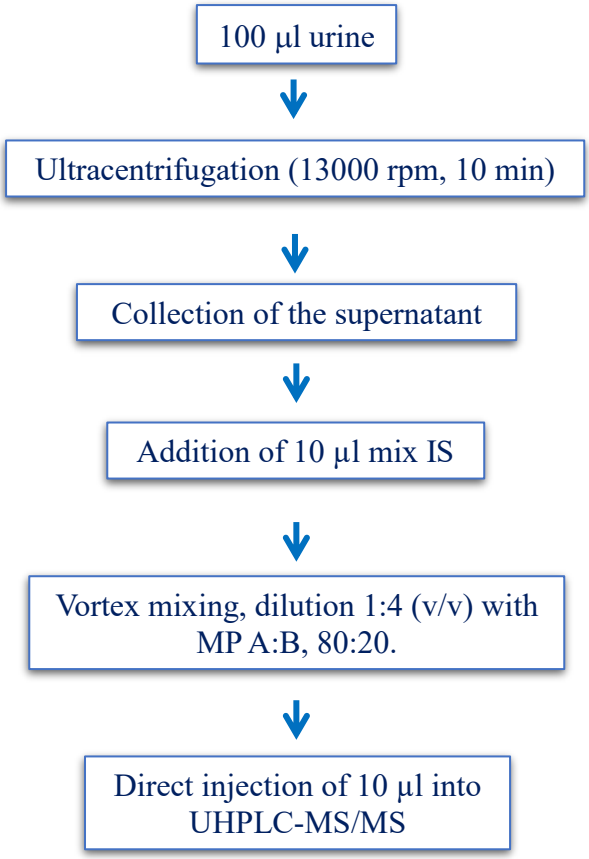


Figure 4: Urine pre-injection treatment

6.5.3 Hair

Hair samples were washed twice with deionized water and twice with dichloromethane and left to dry after the removal of solvent washes. Once dried, were cut into 2 segments of 2 cm each and put into 2 different labelled vials, indicating the proximal and distal segment. Subsequently, the specimens were cut into small pieces (<1mm) with clean scissors. Aliquots of 25 mg finely cut hair samples were weighed and added with 25 µl of mix IS solution (100 ng/ml) yielding a final concentration of 100 pg/mg.

A unique extraction procedure capable to recover all the analytes was not found due to the different physicochemical properties of the investigated substances; thus depending on the type of analytes to be extracted, two different procedures were followed:

(a) For cathinones, piperazines, aminoindanes and phenethylamines: Overnight incubation under sonication at 45°C with 300 µl HCOOH 0.1%, centrifugation at 3500 rpm for 5 min, injection of 10 µl of the extract into the chromatographic system;

(b) For cannabinoids: Addition of 1 ml NaOH 1 M, incubation at 90 °C for 1h, extraction with 1 ml ethyl acetate (twice). After vortex and centrifugation at 3500 rpm for 5 min, the organic phases were collected and combined. The extracts were evaporated to dryness under gentle stream of nitrogen and reconstituted in 100 µl MP A:B, 80:20. After vortex mix, ultracentrifugation at 13000 rpm for 10 minutes was followed and 10 µl were injected in UHPLC-MS/MS (See Figure 5). Whenever the real sample concentrations were found to exceed the highest calibration point, the extracts were opportunely diluted and re-injected into the chromatographic system.

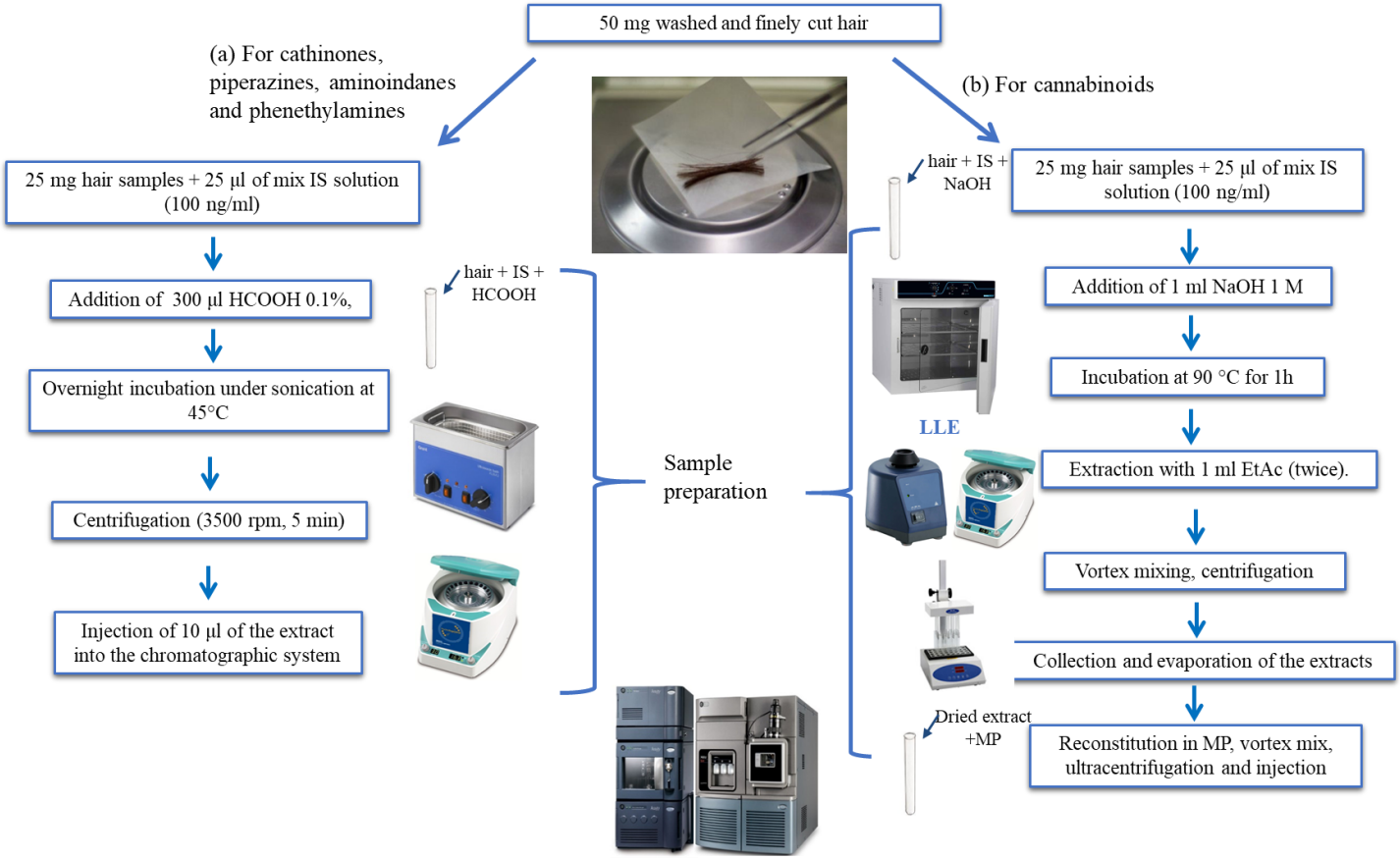


Figure 5: Hair pre-injection treatment

6.6 Ultra-high-performance liquid chromatography – tandem mass spectrometry (UHPLC-MS/MS)

Analytes in serum, urine and hair were detected using an ultra-high performance liquid chromatography system (Waters Acquity UPLC, Waters Corporation, Milan, Italy) coupled with a triple quadrupole mass spectrometer (Waters Xevo TQ, Waters Corporation). Chromatography was carried out using an Acquity UPLC BEH reversed phase C18 column (2.1 x 75 mm, 1.7 µm) and a gradient elution with two solvents: 0.1% formic acid in water (solvent A) and acetonitrile (solvent B). Solvent B was maintained 1% for the first 0.50 min. It was increased to 100% from 0.50 to 6.50 min, then decreased back to 1% from 6.51 to 7.50 min and held at 1% from 7.51 to 15.00 min for re-equilibration. The flow rate was kept constant at 0.30 ml/min during the analysis.

The separated analytes were detected with a triple quadrupole mass spectrometer operated in multiple reaction monitoring (MRM) mode via positive ESI. The applied ESI conditions were: capillary voltage 2.5 kV, desolvation temperature 600ºC, source temperature 150ºC, cone gas flow rate 30 l/h, desolvation gas flow rate 1000 l/h and collision gas flow rate 0.13 ml/min. Optimized cone energy (CV) voltages, MRM transitions, collision energy (CE) voltages and retention time for each analyte and IS are given in Table 1.

7. VALIDATION

Validation protocol applied in the present study included linearity, LOD and LOQ, precision, accuracy, matrix effect, recovery, and freeze-thaw stability for serum and urine, whereas only linearity, LOD and LOQ were evaluated for hair matrix (80).

Validation parameters were calculated using five different daily replicates of QC samples (low, medium, and high QC) along three subsequent working days. Linearity was determined by least-squares regression with $1/x^2$ weighting. Acceptable linearity was achieved when the coefficient of determination was at least 0.990 and the calibrators were quantified within ±20% at the LOQ and ±15% at higher concentrations. The LOD and LOQ were evaluated with decreasing analyte concentrations in the different biological matrices.

LOD was defined as the lowest analyte concentration that can be detected and identified with a given degree of certainty. Standard deviation (SD) of the mean noise level over the retention time window of each analyte was used to determine LOD. A minimum requirement for signal to noise ratio (S/N) of 3 is widely accepted. LOQ was the lowest concentration that met LOD criteria and a S/N of at least 10. Precision and accuracy were determined at the three QC samples concentrations by analyzing five replicates on three different days (n = 15) and expressed as the coefficient of variation (%) of the measured values and error (%) respectively, were expected to be less than 20%.

Over-the-curve samples (drug free samples fortified with all the analytes at a concentration five or 10 times higher than the highest calibration point) were tested for calibration curve fitting, precision and accuracy once they were appropriately diluted.

Matrix effects, recovery and process efficiency were determined using the experimental design proposed by Matuszewski et al. (81). Set 1 was five replicates of QC solutions prepared in the MP. Sets 2 and 3 were five replicates of blank samples fortified with QC solutions after and before extraction, respectively for serum and after and before dilution for urine. Matrix effects were determined by dividing mean peak areas of set 2 by set 1 multiplied by 100. Recovery was determined by comparing the mean peak areas of compounds under investigation obtained in set 3 to those in set 2 multiplied by 100. Process efficiency expressed as the ratio of the mean peak area of an analyte spiked before extraction (set 3) to the mean peak area of the same analyte standards (set 1) multiplied by 100. The effect of three freeze-thaw cycles (storage at –20°C) on the

compounds stability in serum and urine was evaluated by repeated analysis (n=3) of QC samples.

8. REAL SAMPLES

Real hair samples were collected from illicit drugs users who attended Drug Addiction Services or admitted to ED. Segmental head hair analysis was performed when hair length was equal or longer than 4 cm, whereas when shorter than 4 cm were analyzed in their full length. All the samples were preliminarily analyzed with routine methods employed in the collected laboratories for common drugs of abuse (opiates, amphetamines, cocaine, benzodiazepines, cannabis).

In our laboratory, an aliquot of these hair samples was received to be screened with the here described method for the presence of NPS since these new classes of substances are not routinely tested in roadside control, workplace drug testing and in the evaluation for traditional drug abstinence.

Moreover, serum, urine and hair samples were collected from a young individual, after acute intoxication related to consumption of drugs of abuse, and aliquots were sent to our laboratory. Briefly, a 24-year-old male with a diagnosis of schizoaffective disorder and drug abuse was brought to the ED of Hospital Universitari Son Espases, Palma de Mallorca, Spain by his partner and mother, who claimed that he had been presenting behavioral alterations during the previous week.

The psychiatric examination of the patient revealed a high psychomotor excitability with irritability and mydriasis. His speech was reiterative, expressed in a high tone and rate and focused on the repetition of world injustices.

His family explained that the patient had not been sleeping recently, and that they had found with him a powdery substance, apparently obtained on the Internet, which according to the website, was ETP.

A test and an electrocardiogram were performed, showing no alterations and a toxicological analysis was also carried out. In order to obtain analytical confirmation of acute use and chronic drug exposure, serum, urine and a 4-cm length hair sample were obtained at admission (t_0). The patient signed an informed consent form for the analysis of his biological samples. Approval for the study was obtained from the Hospital Ethics Committee. While waiting for results of toxicological analysis, the patient was admitted to the psychiatric unit for detoxification and clinical stability. A second urine sample was collected twelve hours after admission (t_1). After 36 hours,

the symptoms presented by the patient settled, and no decompensation of his schizoaffective disorder was observed. At this time, a second serum sample was obtained (t_2). Once the patient was stabilized he declared that he had been suffering from sub-depressive symptoms and had been taking in several occasions psychoactive substances and in particular ETP to improve his sexuality and to be more sociable. On day 8 of hospitalization a third set of urine and serum samples was collected (t_3). As the detoxification treatment was successful the patient was discharged with outpatient follow-up. Five days later, a new urine sample was obtained during a follow-up visit (t_4).

9. RESULTS - DISCUSSION

9.1 Method Validation

A UHPLC-MS/MS screening method was developed and validated for selective detection in serum, urine and hair of 49 NPS including synthetic cannabinoids, cathinones, phenethylamines, benzofurans, piperazines, aminoindanes, and piperidines. The list of compounds is presented in Table 1.

The gradient method allowed for separation of 49 substances in a 15-min run time. The retention times of compounds were from 2.2 to 7.13 min as given in Table 1.
No interfering peaks were observed in the drug-free serum and urine samples taken from 20 different sources. Similarly, none of the principal drugs of abuse (opiates, cocaine, cannabinoids, amphetamines type-stimulants) or common medications (antidepressants, benzodiazepines) interfered with the assay and with the accurate quantification of the low QC samples in serum and urine.

9.1.1 Serum

Linear calibration curves showed determination coefficients (R^2) equal to or higher than 0.99 (apart from Pravadoline, $R^2 = 0.9860$). LOD and LOQ values were adequate for the purpose of the present study (Table 2). The intra- and inter-assay precision and accuracy were in accordance with the internationally established acceptance criteria (Table 3).

Every analyte suffered from ion suppression, (results <100%) or enhancement (results >100%). Low recoveries were observed for some analytes (for example naphyrone). Recovery and matrix effect are given in Table 4. However, as mentioned by Peters et al (80), recovery is not among the validation parameters regarded as essential for method validation. Most authors agree, that the value for recovery is not important, as long as the data for LOQ, LOD, precision and accuracy (bias) are acceptable, as in the here described method. Nevertheless, some guidance documents request the determination of the recovery at high and low concentrations or even specify that the recovery should be greater than 50% (80). Most of the analytes met the latter criterion of an extraction recovery higher than 50%.

Regarding the following analytes: 5-APB, 5-MAPB, 5-EAPB and ETP, analytical recoveries obtained for the three different QC samples were always above 80%. The

intra- and inter-assay precision (% RSD) and accuracy (% error) values were always lower than 11%. The latter analytes showed no significant ion suppression/enhancement (< 15% analytical signal suppression due to matrix effect).

Selected MRM chromatograms of the serum extracts spiked with analytes at the concentration of 50 ng/ml are presented in Appendix 1.

Table 2. LOD, LOQ and linearity (coefficient of determination (R^2)) achieved for each analyte included in the screening method in serum

Compound	**LOD** (ng/ml)	**LOQ** (ng/ml)	**R^2** (1-100 ng/ml)
MDAI	0.1	0.3	0.9943
Methcathinone	0.3	1.0	0.9978
dimethylcathinone	0.1	0.3	0.9979
Methylone	0.3	1.0	0.9976
4-fluoromethcathinone	0.3	1.0	0.9988
Ethylone	0.1	0.3	0.9936
Methedrone	0.1	0.3	0.9958
Buphedrone	0.1	0.3	0.9975
4-FA	0.1	0.4	0.9905
Butylone	0.1	0.3	0.9921
Mephedrone	0.2	0.6	0.9972
4 methylethcathinone	0.1	0.4	0.9949
Pentedrone	0.1	0.3	0.9955
Pentylone	0.1	0.2	0.9953
5-APB	1.5	5	0.9965 (5-100 ng/ml)
m-CPP	0.3	1	0.9960
3,4-dimethylmethcathinone	0.1	0.3	0.9972
5-MAPB	1.5	5	0.9981 (5-100 ng/ml)
2 C-B	0.1	0.3	0.9961
MDPV	0.1	0.4	0.9938
5-EAPB	1.5	5	0.9936 (5-100 ng/ml)
ETP	1.5	5	0.9948 (5-100 ng/ml)
Pravadoline	0.1	0.3	0.9860
Naphyrone	0.3	0.9	0.9900

AM-2233	0.1	0.4	0.9917
JWH-200	0.1	0.2	0.9946
AM-694	0.1	0.2	0.9993
AM-2201	0.1	0.4	0.9942
RCS-4	0.1	0.3	0.9914
JWH-250	0.1	0.4	0.9981
JWH-302	0.1	0.2	0.9968
JWH-073	0.1	0.3	0.9904
XLR-11	0.3	1.0	0.9969
JWH-251	0.3	1.0	0.9979
JWH-203	0.3	1.0	0.9965
JWH-018	0.1	0.5	0.9964
JWH-016	0.1	0.3	0.9909
JWH-081	0.1	0.3	0.9959
JWH-019	0.3	1	0.9978
JWH-098	0.1	0.2	0.9968
JWH-307	0.1	0.2	0.9962
JWH-007	0.3	1	0.9984
RCS-8	0.2	0.6	0.9957
JWH-398	0.3	1	0.9967
JWH-210	0.1	0.3	0.9964
JWH-147	0.1	0.2	0.9946
CB-13	0.3	1	0.9961

Table 3. Intra- and inter day precision and accuracy in serum												
Analytes	**Intra-day precision % RSD**			**Intra-day accuracy % Error**			**Inter-day precision % RSD**			**Inter-day accuracy % Error**		
	Low QC	Medium QC	High QC	Low QC	Medium QC	High QC	Low QC	Medium QC	High QC	Low QC	Medium QC	High QC
MDAI	8.3	1.4	3.6	16.2	5.1	5.4	7.6	2.1	3.9	15.4	7.3	7.7
Methcathinone	13.5	6.0	4.4	14.7	4.3	13.6	14.2	8.6	5.9	19.8	8.4	12.4
Dimethylcathinone	9.9	2.3	7.7	19.1	7.0	9.9	8.0	4.3	7.9	17.6	10.5	7.6
Methylone	8.5	5.5	2.8	14.3	4.0	9.0	9.1	6.6	3.2	15.7	6.5	5.5
4-fluoromethcathinone	10.6	9.0	10.3	17.1	13.2	12.9	11.3	5.1	11.7	18.6	10.7	13.6
Ethylone	11.8	5.1	8.8	17.9	5.2	11.5	8.7	3.7	10.8	12.6	3.4	10.4
Methedrone	8.64	5.2	3.4	12.5	6.4	3.1	8.4	4.7	4.3	11.9	5.3	4.2
Buphedrone	5.7	3.2	3.7	18.9	4.0	5.0	3.8	4.2	4.6	16.7	6.6	3.8
4-FA	14.4	1.4	7.9	17.9	4.8	13.3	8.1	2.3	7.4	11.7	6.4	12.5
Butylone	4.3	5.6	0.5	14.2	5.8	1.1	4.4	5.6	0.5	15.8	4.8	2.4
Mephedrone	8.7	5.9	3.1	15.8	8.0	2.2	4.1	3.0	3.6	16.0	9.3	3.6
4-methylethcathinone	6.4	1.3	4.3	14.3	8.6	3.1	7.4	1.8	5.6	13.0	9.5	2.9
Pentedrone	3.7	2.0	1.2	16.9	1.4	1.8	6.9	1.9	5.3	15.5	3.8	4.2
Pentylone	8.6	7.3	3.0	12.0	5.2	2.8	9.9	6.5	3.8	10.5	6.0	3.3
m-CPP	12.3	11.4	9.1	19.5	7.6	6.2	18.6	14.7	12.0	17.2	8.3	6.7
3,4-dimethylmethcathinone	7.1	4.8	5.8	19.5	11.2	5.0	9.2	5.9	6.6	18.2	11.8	5.9
2 C-B	8.6	5.3	1.3	16.1	6.1	6.7	7.3	4.9	2.4	19.5	5.6	6.6
MDPV	10.3	4.1	9.6	19.8	14.5	8.5	15.8	5.2	6.3	13.3	13.4	12.5
Pravadoline	15.0	11.5	13.7	15.8	13.8	12.2	16.2	10.7	13.6	14.3	14.8	12.7
Naphyrone	17.7	14.6	13.7	19.1	14.3	13.8	18.4	11.3	13.2	16.0	12.6	12.3
AM-2233	8.3	5.5	7.2	18.2	14.9	9.4	9.0	8.7	6.0	14.0	13.6	11.3
JWH-200	12.5	5.3	14.0	14.1	6.7	10.3	13.6	7.1	13.2	16.7	8.5	12.6
AM-694	7.6	3.0	11.2	12.8	13.5	7.8	9.1	3.8	8.0	13.5	10.6	5.6
AM-2201	13.1	8.4	3.9	14.8	11.1	8.8	15.1	9.6	5.2	18.5	9.2	7.8

RCS-4	7.3	4.0	3.3	19.6	14.7	12.5	8.1	2.7	4.3	14.8	12.3	12.9
JWH-250	14.9	1.2	5.3	19.9	13.3	3.8	9.6	2.1	6.0	18.4	11.6	5.1
JWH-302	13.5	8.2	7.2	14.0	15.0	9.1	12.3	7.7	6.0	16.3	10.0	7.3
JWH-073	17.1	11.8	4.3	14.1	9.6	5.6	11.2	6.7	4.2	15.9	7.9	4.6
XLR-11	9.8	4.1	9.0	18.8	14.6	9.2	8.1	3.9	8.8	16.8	8.6	7.7
JWH-251	15.9	3.3	9.2	16.8	6.6	7.7	18.7	4.5	10.1	15.3	7.8	10.8
JWH-203	18.7	11.8	6.0	19.9	7.7	8.0	18.5	9.8	5.9	18.9	10.3	6.7
JWH-018	16.6	8.1	12.8	15.8	11.9	14.3	14.8	9.0	10.7	16.5	9.7	12.6
JWH-016	19.4	11.1	13.5	17.6	11.7	9.4	19.9	8.3	11.1	15.3	11.4	7.7
JWH-081	18.2	10.1	13.6	18.4	6.7	13.0	16.4	8.8	14.4	18.0	10.6	12.2
JWH-019	15.2	11.9	5.0	14.5	13.7	11.6	14.6	7.7	4.9	15.8	10.4	10.4
JWH-098	18.5	13.1	7.5	14.2	10.5	7.7	16.5	10.8	9.3	14.5	13.9	6.8
JWH-307	15.1	7.6	4.2	14.5	10.2	11.9	12.0	7.9	5.2	11.1	9.1	9.7
JWH-007	15.6	7.6	10.7	15.7	8.0	14.8	10.2	4.8	7.2	19.7	6.8	11.7
RCS-8	19.8	5.8	14.2	15.6	14.1	13.2	19.2	2.9	12.3	15.2	13.2	11.4
JWH-398	13.9	12.5	6.4	19.5	14.4	14.8	9.4	10.9	7.3	17.7	14.5	11.5
JWH-210	12.5	3.2	10.8	13.0	12.4	9.8	14.0	4.4	11.8	11.3	10.5	8.6
JWH-147	16.2	5.4	13.3	15.3	7.9	11.7	13.8	8.3	10.7	13.0	7.2	9.8
CB-13	17.7	13.5	12.7	16.4	13.6	12.1	18.9	10.6	13.6	15.2	14.7	11.5

Table 4. Recovery and matrix effect for each analyte in serum

Analytes	**Recovery (%)**			**Matrix effect (%)**		
	Low QC	Medium QC	High QC	Low QC	Medium QC	High QC
MDAI	74.8	80.1	48.7	115.2	104.1	120.6
Methcathinone	45.0	54.4	61.1	83.6	86.2	83.4
dimethylcathinone	85.0	64.4	51.6	77.8	75.2	77.0
Methylone	103.2	83.7	50.2	105.6	112.6	115.8
4-fluoromethcathinone	46.6	58.1	74.7	95.0	97.4	81.9
Ethylone	73.8	87.6	62.6	104.2	113.9	114.1
Methedrone	98.4	66.3	53.9	104.3	117.7	114.8
Buphedrone	64.1	67.9	54.9	118.2	103.6	120.4
4-FA	152.9	71.9	82.8	71.6	75.1	73.7
Butylone	73.8	79.2	63.1	108.3	113.0	117.7
Mephedrone	56.3	64.1	50.5	83.7	71.0	82.0
4-methylethcathinone	52.2	54.1	43.5	88.2	78.0	98.1
Pentedrone	81.7	60.4	42.3	97.9	71.8	93.9
Pentylone	204.1	159.5	133.9	103.3	114.5	120.6
m-CPP	78.9	64.6	52.7	123.5	119.9	108.1
3,4-dimethylmethcathinone	56.5	58.6	30.0	97.9	67.2	95.8
2 C-B	143.9	75.6	54.8	101.1	113.4	119.0
MDPV	117.4	58.2	75.5	108.6	105.6	112.0
Pravadoline	83.7	78.9	72.0	87.2	93.5	97.0
Naphyrone	33.8	25.6	27.0	89.4	87.6	90.3
AM-2233	65.5	94.9	74.2	75.2	68.4	73.1

JWH-200	97.3	92.5	91.2	86.0	67.1	70.8
AM-694	77.2	83.8	55.1	65.5	59.0	69.6
AM-2201	76.6	69.6	44.5	113.5	102.4	115.6
RCS-4	90.1	50.7	55.9	92.6	72.2	73.9
JWH-250	80.0	63.5	52.5	91.6	99.5	97.7
JWH-302	97.5	64.9	41.2	108.7	107.2	115.2
JWH-073	78.9	76.3	50.8	116.3	110.4	117.7
XLR-11	94.4	85.6	55.8	86.1	76.0	100.1
JWH-251	66.5	80.0	47.8	116.7	111.4	118.7
JWH-203	58.1	66.8	40.1	117.7	105.8	114.3
JWH-018	67.0	71.2	46.7	114.1	103.5	113.6
JWH-016	58.6	70.7	46.1	113.4	105.9	112.7
JWH-081	67.5	67.6	43.7	112.7	103.3	116.4
JWH-019	44.3	51.1	57.2	118.6	105.0	119.4
JWH-098	68.3	65.7	42.9	113.4	107.2	114.9
JWH-307	55.8	67.5	45.4	112.2	115.2	119.1
JWH-007	59.3	64.4	46.6	120.4	117.8	119.1
RCS-8	53.8	60.9	46.3	114.3	104.0	106.9
JWH-398	63.4	70.8	37.9	117.2	116.6	114.9
JWH-210	53.6	75.9	36.0	110.8	108.5	107.6
JWH-147	66.5	76.8	38.6	112.1	108.1	110.3
CB-13	55.0	67.1	27.8	123.1	119.3	116.7

9.1.2 Urine

Linear calibration curves showed determination coefficients (R^2) equal to or higher than 0.99 (apart from XLR-11, R^2= 0.9887 and RCS-8, R^2= 0.9840). LOD and LOQ values were adequate for the purpose of the present study, ranging from 0.1 to 9.3 and from 0.2 to 31.1 ng/ml, respectively (Table 5). The intra- and inter-assay precision and accuracy were in accordance with the internationally established acceptance criteria (Table 6).

Every analyte suffered from ion suppression, (results <100%) or enhancement (results >100%). Low recoveries were observed for some analytes. Recovery and matrix effect are given in Table 7.

Regarding the following analytes: 5-APB, 5-MAPB, 5-EAPB and ETP, analytical recoveries obtained for the three different QC samples were always above 80%. The intra- and inter-assay precision (% RSD) and accuracy (% error) values were always lower than 11%. The latter analytes showed no significant ion suppression/enhancement (< 15% analytical signal suppression due to matrix effect).

Selected MRM chromatograms of the serum extracts spiked with analytes at the concentration of 50 ng/ml are presented in Appendix 2.

Table 5. LOD, LOQ and linearity (coefficient of determination (R^2)) achieved for each substance included in the screening method in urine

Compound	**LOD** (ng/ml)	**LOQ** (ng/ml)	**R^2** (LOQ-100 ng/ml)
MDAI	0.2	0.7	0.9976
Methcathinone	1.3	4.4	0.9936
dimethylcathinone	0.8	2.7	0.9962
Methylone	1.8	5.9	0.9982
4-fluoromethcathinone	9.0	30.1	0.9999
Ethylone	0.4	1.2	0.9985
Methedrone	0.8	2.6	0.9999
Buphedrone	3.7	12.2	0.9991
4-FA	8.9	28.7	0.9973
Butylone	0.5	1.7	0.9975
Mephedrone	5.0	16.7	0.9956
4 methylethcathinone	0.5	1.5	0.9985
Pentedrone	0.3	1.0	0.9960
Pentylone	6.3	21.0	0.9912
5-APB	1.5	5	0.9936
3,4-dimethylmethcathinone	3.5	11.7	0.9972
5-MAPB	1.5	5	0.9993
2 C-B	9.3	31.1	0.9969
MDPV	0.6	1.9	0.9970
5-EAPB	0.5	2.5	0.9987
ETP	1.5	5	0.9954
Pravadoline	2.1	7.1	0.9912
Naphyrone	1.6	5.0	0.9968
AM-2233	0.2	0.6	1.000
JWH-200	0.1	0.4	0.9969
AM-694	0.1	0.3	0.9990
AM-2201	0.5	1.7	0.9952
RCS-4	0.2	0.5	0.9969
JWH-250	0.1	0.3	0.9985
JWH-302	0.6	2	0.9996
JWH-073	0.1	0.2	0.9988

XLR-11	0.1	0.2	0.9887
JWH-251	0.1	0.3	0.9996
JWH-203	0.1	0.3	0.9980
JWH-018	0.1	0.3	0.9997
JWH-016	0.2	0.6	0.9973
JWH-081	0.1	0.3	0.9978
JWH-019	1.3	4.4	0.9996
JWH-098	0.1	0.4	0.9956
JWH-307	0.1	0.2	0.9974
JWH-007	0.1	0.3	0.9978
RCS-8	0.1	0.3	0.9840
JWH-398	0.1	0.3	0.9978
JWH-210	0.1	0.3	0.9979
JWH-147	0.1	0.3	0.9990
CB-13	0.9	3.0	0.9971

Table 6. Intra- and inter day precision and accuracy in urine

Analytes	**Intra-day precision % RSD**			**Intra-day accuracy % Error**			**Inter-day precision % RSD**			**Inter-day accuracy % Error**		
	Low QC	Medium QC	High QC	Low QC	Medium QC	High QC	Low QC	Medium QC	High QC	Low QC	Medium QC	High QC
MDAI	15.1	7.3	1.5	19.4	13.3	10.6	10.8	3.8	1.1	18.8	11.3	8.4
Methcathinone	19.4	8.4	4.1	19.1	14.9	12.7	14.1	10.5	6.4	16.2	13.8	10.2
Dimethylcathinone	5.4	4.7	2.6	19.2	14.8	11.5	6.9	2.9	1.3	18.6	15.9	11.9
Methylone	NA	12.0	3.4	NA	14.4	10.6	NA	7.7	4.9	NA	10.7	8.3
4-fluoromethcathinone	NA	15.0	4.9	NA	11.5	8.5	NA	13.7	7.8	NA	12.6	7.4
Ethylone	9.6	8.5	4.4	17.7	14.6	5.4	8.4	6.4	3.8	15.6	11.8	3.4
Methedrone	3.7	2.4	1.5	16.1	8.2	4.9	2.9	2.7	1.5	19.8	17.0	4.9
Buphedrone	NA	12.6	5.7	NA	11.2	9.7	NA	10.2	7.4	NA	9.1	6.7
4-FA	NA	15.0	7.2	NA	14.6	5.1	NA	10.0	7.5	NA	13.9	5.0
Butylone	16.7	8.1	4.8	15.1	13.2	1.1	17.1	5.7	3.1	19.1	13.3	2.3
Mephedrone	NA	11.4	8.2	NA	10.7	7.2	NA	8.7	6.7	NA	13.0	7.9
4-methylethcathinone	13.0	7.9	2.0	19.2	9.6	5.4	12.6	7.3	3.2	17.6	8.8	4.3
Pentedrone	16.8	7.3	5.6	19.5	13.6	5.5	14.0	5.7	5.1	17.3	12.1	8.6
Pentylone	NA	11.8	3.8	NA	12.3	2.7	NA	9.5	3.8	NA	8.2	4
3,4-dimethylmethcathinone	NA	7.7	5.5	NA	11.3	8.3	NA	7.6	4.9	NA	13.8	6.4
2 C-B	NA	3.5	2.3	NA	14.0	10.5	NA	2.5	1.7	NA	13.1	6.2
MDPV	6.4	3.1	1.6	17.2	15.0	5.1	7.5	4.0	2.3	15.7	6.3	8.6
Pravadoline	NA	2.2	1.4	NA	8.2	1.1	NA	3.0	2.7	NA	7.3	2.6
Naphyrone	16.2	11.3	4.3	14.1	12.8	12.0	16.4	11.5	6.6	16.8	14.0	10.4
AM-2233	14.1	4.8	10.8	19.6	5.7	10.6	10.9	7.9	10.2	17.2	8.3	12.4.
JWH-200	8.7	7.6	4.4	17.1	10.7	9.8	5.4	4.4	2.5	14.5	12.3	9.5
AM-694	8.7	8.5	6.2	15.0	9.2	5.9	14.7	8.1	7.3	11.9	10.4	10.0

AM-2201	9.5	6.8	6.3	14.3	12.5	6.6	12.4	8.0	5.4	17.4	13.9	7.0
RCS-4	12.9	8.5	2.3	16.3	6.3	2.1	12.7	8.9	3.3	17.1	6.4	4.1
JWH-250	18.3	13.2	3.2	14.4	12.6	11.9	18.0	12.8	1.9	16.4	13.1	12.9
JWH-302	12.0	6.4	2.3	14.4	9.9	5.2	20.0	13.7	6.2	18.1	9.1	7.1
JWH-073	15.6	7.4	6.9	17.2	5.9	5.3	14.3	8.4	7.1	13.8	10.7	9.1
XLR-11	14.4	8.9	8.0	17.9	6.3	5.5	16.0	9.1	8.1	17.5	8.8	7.2
JWH-251	13.0	6.6	4.2	12.3	6.3	2.7	17.7	6.3	2.1	12.6	9.8	3.7
JWH-203	9.8	2.5	1.0	15.5	5.3	4.9	9.1	1.9	1.0	15.2	4.1	3.4
JWH-018	16.2	8.2	5.4	17.6	9.3	6.5	11.7	5.1	3.3	18.4	6.1	3.3
JWH-016	9.4	6.0	2.1	12.4	10.9	3.5	11.7	5.6	2.2	13.1	7.9	6.2
JWH-081	16.5	9.4	4.5	18.6	13.5	10.6	16.6	12.1	3.2	19.6	12.7	12.6
JWH-019	15.0	6.5	3.1	10.4	8.8	3.3	14.8	4.3	3.0	12.2	4.5	2.2
JWH-098	13.2	7.1	4.9	11.4	9.2	6.3	12.8	8.8	7.9	12.9	8.4	3.6
JWH-307	12.9	6.8	3.7	13.8	7.7	6.7	17.2	3.9	2.3	12.7	7.3	5.2
JWH-007	9.8	5.6	1.9	11.7	6.5	4.5	7.3	5.2	4.1	11.8	10.6	8.8
RCS-8	15.4	5.6	3.7	11.3	8.5	7.9	10.4	7.1	4.0	13.4	8.7	6.2
JWH-398	9.9	5.9	4.1	13.2	9.9	8.9	11.1	8.0	5.2	15.2	8.3	6.1
JWH-210	8.0	4.4	2.7	12.4	9.5	5.8	8.7	8.0	5.8	10.7	8.0	7.6
JWH-147	14.4	4.4	2.6	13.2	10.9	8.9	8.7	4.7	3.3	13.1	7.5	1.8
CB-13	10.1	6.8	4.4	12.9	11.9	11.1	12.5	9.2	2.7	14.4	10.4	10.2

Table 7. Recovery and matrix effect for each analyte in urine

Analytes	Recovery (%)			Matrix effect (%)		
	Low QC	Medium QC	High QC	Low QC	Medium QC	High QC
MDAI	85.1	92.6	84.5	78.2	73.4	75.6
Methcathinone	91.1	91.5	88.3	89.5	80.3	108.1
dimethylcathinone	105.4	104.9	79.1	78.8	78.0	93.3
Methylone	NA	104.9	79.1	NA	79.0	93.3
4-fluoromethcathinone	NA	107.1	94.7	NA	86.1	123.7
Ethylone	62.8	74.3	56.8	106.4	109.5	116.5
Methedrone	90.6	107.5	84.0	88.0	93.2	102.8
Buphedrone	NA	101.0	95.6	NA	75.7	78.4
4-FA	NA	98.4	86.7	NA	77.4	75.1
Butylone	88.3	103.7	94.6	116.6	111.6	119.5
Mephedrone	NA	100.5	90.5	NA	81.6	76.4
4-methylethcathinone	86.6	97.5	87.0	77.9	79.2	71.5
Pentedrone	67.2	77.2	62.3	90.3	90.2	93.1
Pentylone	NA	92.4	75.6	NA	129.0	117.1
3,4-dimethylmethcathinone	NA	111.4	91.8	NA	76.1	77.3
2 C-B	NA	54.3	59.4	NA	106.0	131.3
MDPV	63.3	85.8	78.1	89.5	76.4	94.6
Pravadoline	NA	115.6	90.6	NA	87.9	117.8
Naphyrone	98.5	83.2	91.0	75.1	79.4	75.5
AM-2233	84.3	98.9	89.2	79.1	78.9	76.3
JWH-200	112.5	102.0	95.5	81.3	74.1	88.9
AM-694	75.3	91.6	88.7	79.7	78.0	90.7
AM-2201	58.4	56.5	83.3	78.4	95.2	104.6
RCS-4	42.8	58.0	71.3	99.7	94.1	107.0
JWH-250	55.9	63.9	74.6	98.6	94.8	109.2
JWH-302	53.5	66.8	77.2	105.7	84.0	102.9

JWH-073	32.9	36.2	62.9	115.1	106.6	112.9
XLR-11	67.8	42.5	63.5	99.5	85.1	105.1
JWH-251	38.0	43.1	61.0	100.3	101.7	109.7
JWH-203	31.6	34.5	46.8	110.6	109.4	96.8
JWH-018	40.6	47.9	53.0	110.1	115.9	111.4
JWH-016	57.4	61.0	53.4	113.3	109.7	101.9
JWH-081	50.5	60.3	52.5	104.6	103.5	112.9
JWH-019	53.0	57.2	50.3	121.0	112.5	120.7
JWH-098	51.3	58.7	50.9	101.0	105.5	108.9
JWH-307	36.1	41.4	40.6	92.0	94.7	108.5
JWH-007	54.1	47.4	43.2	109.9	106.2	102.7
RCS-8	49.7	42.7	53.3	114.9	104.5	107.7
JWH-398	39.5	39.6	34.9	104.2	114.7	116.3
JWH-210	59.8	60.7	64.0	100.9	109.5	109.9
JWH-147	36.6	37.3	27.6	109.0	102.5	104.1
CB-13	53.2	55.3	55.0	108.2	105.2	105.0

9.1.3 Hair

Linear calibration curves showed determination coefficients (R^2) equal to or higher than 0.99. LOD and LOQ values, were acceptable for the purpose of the present study (Table 8). However, relatively high LOQ values were obtained for 4-FA and XLR-11, but were still adequate for the purpose of the method.

Selected MRM chromatograms of the serum extracts spiked with analytes at the concentration of 50 pg/mg are presented in Appendix 3.

Table 8. LOD, LOQ and linearity (coefficient of determination (R^2)) achieved for each substance included in the screening method in hair

Compound	**LOD** (pg/mg)	**LOQ** (pg/mg)	**R^2** (LOQ-100 pg/mg)
MDAI	0.3	0.9	0.9980
Methcathinone	2.0	6.5	0.9912
dimethylcathinone	0.7	2.3	0.9992
Methylone	0.7	2.4	0.9996
4-fluoromethcathinone	0.5	1.8	0.9995
Ethylone	2.0	6.7	0.9999
Methedrone	0.8	2.7	0.9966
Buphedrone	3.8	12.8	0.9915
4-FA	10.3	34.5	0.9996
Butylone	1.7	5.8	0.9998
Mephedrone	1.8	5.9	0.9986
4 methylethcathinone	0.2	0.7	0.9973
Pentedrone	0.5	1.7	0.9983
Pentylone	1.9	6.4	0.9997
5-APB	2	5	0.9995
m-CPP	2.3	7.8	0.9993
3,4-dimethylmethcathinone	1.2	4.0	0.9993
5-MAPB	2	5	0.9983
2 C-B	0.2	0.8	0.9999
MDPV	0.9	3	0.9918
5-EAPB	2	5	0.9998
Pravadoline	0.6	2.2	0.9969
Naphyrone	0.5	1.5	0.9998
AM-2233	2.8	9.2	0.9987
JWH-200	0.8	2.6	0.9937
AM-694	0.3	0.9	0.9965
AM-2201	0.2	0.6	0.9989
RCS-4	3.7	12.4	0.9960
JWH-250	1.3	4.5	0.9943
JWH-302	0.7	2.4	0.9997
JWH-073	0.3	0.9	0.9958

XLR-11	28.7	95.6	0.9968 (LOQ-500 pg/mg)
JWH-251	0.2	0.8	0.9985
JWH-203	0.5	1.6	0.9983
JWH-018	0.3	1.0	0.9936
JWH-016	0.4	1.2	0.9999
JWH-081	0.5	1.7	0.9952
JWH-019	0.3	1.0	0.9971
JWH-098	0.1	1.4	0.9996
JWH-307	0.4	1.2	0.9933
JWH-007	0.3	1.0	0.9999
RCS-8	2.6	8.6	0.9924
JWH-398	0.3	1.0	0.9964
JWH-210	0.4	1.1	0.9903
JWH-147	0.4	1.2	0.9996
CB-13	2.5	8.4	0.9975

9.2 Real samples

9.2.1 Intoxication related to 5-APB, 5-MAPB, 5-EAPB and ETP (serum, urine, hair)

In the case of the 24-year-old male, urine screening revealed 5-MAPB, 5-EAPB, 5-APB and ETP. Substances were then confirmed and quantified in urine and serum samples (Table 9 and Appendix 4). The pre-treatment performed for all the biological samples was slightly different from the here described one since it was adjusted to achieve a targeted measurement of these specific compounds and ritalinic acid. The exact procedure is described by Barceló et al (82).

To verify if also a repeated exposure to benzofurans and ETP occurred, segmental hair testing of two subsequent 2 cm segments was performed with both segments positive for 5-MAPB, 5-EAPB, 5-APB and ETP (Table 10).

Table 9: Serum and urine 5-APB, 5-MAPB, 5-EAPB, ETP and ritalinic acid concentrations found in the specimens collected at three different time intervals, following UHPLC-MS/MS analysis					
Sample	**5-APB (ng/mL)**	**5-MAPB (ng/mL)**	**5-EAPB (ng/mL)**	**ETP (ng/mL)**	**Ritalinic Acid (ng/mL)**
Serum $t_{0§}$	69.3	153.7	376.2	450.3	507.7
Serum $t_{2§}$	56.9	85.8	116.1	110.9	121.9
Serum $t_{3§}$	Neg	Neg	Neg	Neg	Neg
Urine $t_{0§}$	*	*	*	*	*
Urine $t_{1§}$	5172.3	12340.2	29880.8	3048.5	172041.5
Urine $t_{3§}$	77.5	8.5	3.7	Neg	100.0
Urine $t_{4§}$	Neg	Neg	Neg	Neg	9.8

§Times after the admission to the ED: $t_{0:}$ at 0 h; t_1: at 12 h; t_2: at 36 h, t_3: at 8 days and t_4: at 13 days.

* Insufficient volume of sample

Table 10: Hair 5-APB, 5-MAPB, 5-EAPB and ETP concentrations found after segmental analysis				
Sample	**5-APB (ng/mg)**	**5-MAPB (ng/mg)**	**5-EAPB (ng/mg)**	**ETP (ng/mg)**
Segment 1 (0-2 cm, proximal)	2.6	5.3	7.1	10.2
Segment 2 (2-4 cm, distal)	0.8	4.4	5.1	11.4

An acute intoxication accompanied by high psychomotor excitability, high irritability and mydriasis following the intake of benzofurans and ETP has been here described and analytically confirmed. Moreover, the use of segmental hair analysis has been also applied to demonstrate that the intoxication was not occasional, but likely due to chronic consumption of the same products in different occasions.

With respect to the detected NPS, it can be said that in the international literature there is only one other case of non-fatal acute overdose of ETP with analytical confirmation (21) and serum and urine values were much lower than those detected in the here described case. Moreover, only once ETP has been qualitatively identified in the hair

sample of a fatal case (18), while there is no analytical confirmation of repeated use in previously published case reports (22-24).

Conversely, to our knowledge up to date there is no analytical confirmation of 5-EAPB poisonings and very limited analytical data proving the consumption of 5-APB or 5-MAPB in some case reports (33-37). In addition, this is the first time that repeated use of these compounds has been objectively assessed by hair testing.

9.2.2 Hair samples

The developed method was used in our laboratory for analyses of forensic hair samples collected from drug abusers, drivers, and other individuals in cases where there was a need to prove or exclude the presence of NPS. Four out of the 10 samples analyzed were found positive to the following substances: 4-methylethcathinone; JWH-081; JWH-073 and JWH-250; and JWH-018. Interpretation of the findings is almost impossible mainly due to the lack of testing for metabolites and limited information on chemical and toxicological properties of these substances. Especially, when it comes to synthetic cannabinoids (main route of administration: smoking) external contamination could not be excluded.

The use of hair testing for forensic issues concerning NPS is still debatable. In particular, little information is currently available regarding the incorporation in the keratin matrix after use and the correlation between dose, use frequency, passive exposure, and resulting hair concentrations. Limitations and issues of hair NPS analysis still deserve substantial research and discussion within the scientific community, before a definitive interpretation of either a positive or negative result can be safely reported to the local authorities. The main issue when comes to the interpretation of the results are the discrimination between: (i) passive exposure vs. active consumption and (ii) sporadic vs. chronic use. Under these circumstances, any analytical finding from NPS hair testing should be cautiously interpreted by experienced forensic toxicologist (83).

Moreover, as for hair testing for common drugs, limitations and issues for hair NPS analysis exist. The Society of Hair Testing (SoHT) guidelines (84) state that external contamination must be taken into account when interpreting the findings and laboratories should evaluate the effectiveness of their wash procedures. In the methods for hair NPS testing reviewed by Kyriakou et al. (61) great heterogeneity has been noted

regarding decontamination step with some studies following extensive washing procedures and others none at all. Nonetheless, some authors used both aqueous and organic solvents, as proposed by SoHT for traditional drugs of abuse.

Another pitfall, common with the published procedures for traditional psychotropic drugs is the difficulty in evaluating the real performance of the extraction procedure due to the lack of certified reference hair specimens with known drug content.

10. CONCLUSIONS

NPS are the most recent challenge in clinical and forensic toxicology. They represent a great threat for EDs, which increasingly face intoxications due to undetectable substances with commonly available assays and whose health hazards are unknown. Hence, the development of analytical methods aiming at the detection of a broad-spectrum of compounds in conventional and non-conventional biological matrices is helpful.

We developed a sensitive UHPLC-MS/MS method for the simultaneous detection of 49 NPS in serum, urine and hair. The developed method allows a rapid screening analysis and requires only 0.1 ml serum and urine and 50 mg hair specimens. Such multi-analyte methods are necessary for both forensic and clinical laboratories due to the ever-growing spectrum of novel substances. The current method can be easily expanded to include a greater number of NPS and can be used in our laboratory in routine work for testing forensic biological samples collected from drug users, drivers and other individuals in cases where there is need to prove or exclude the presence of NPS. In general, our method can be utilized for clinical and forensic toxicology (human performance and post-mortem). Moreover, the method was already successfully applied to real cases.

11. FURTHER WORK

This methodology, as already mentioned, could be easily adapted by both clinical and toxicological laboratories and expanded to include more substances according to the needs of each laboratory.

Moreover, two ISs (JWH 018-d9 and mephedrone-d3) have been chromatographically characterized; optimized transitions, cone voltages and collision energies were also found thus permitting us to use them for the quantification of synthetic cannabinoids and cathinones, respectively.

The current method could also be used for the analysis of already tested samples that gave negative results for traditional drugs of abuse but there was a suspicion of drug consumption, to estimate the prevalence of NPS within tested population.

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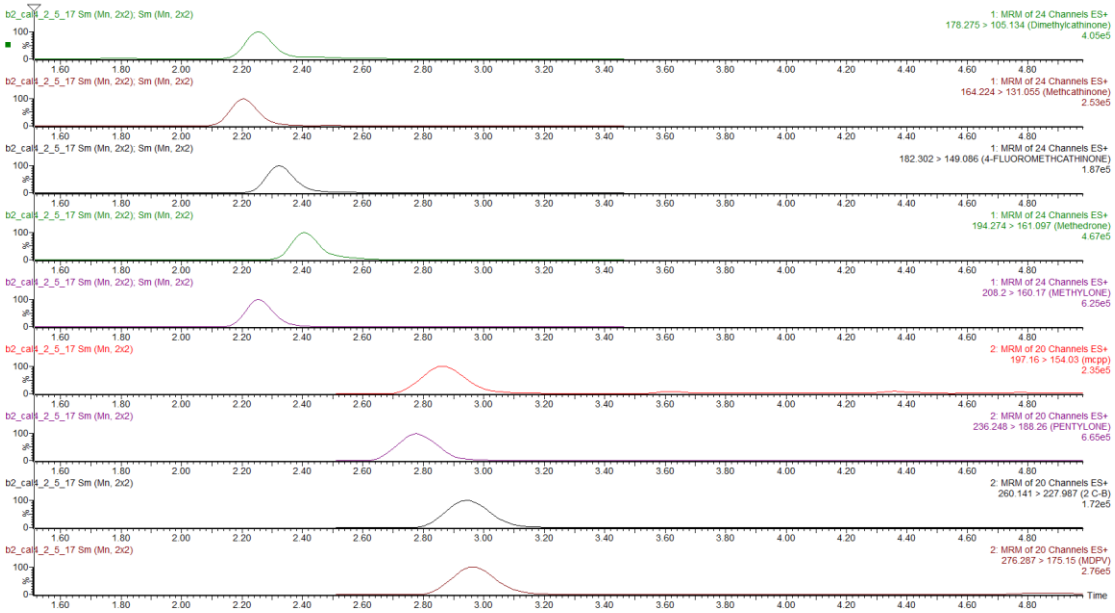
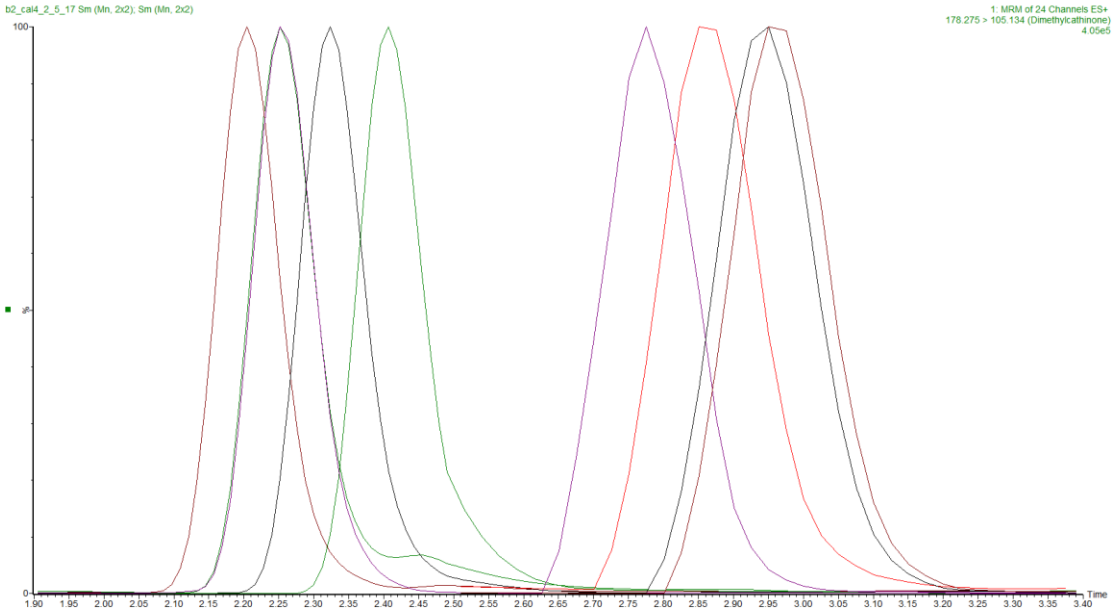
13. APPENDICES

1. Selected MRM chromatograms of the serum extracts spiked with analytes at the concentration of 50 ng/ml

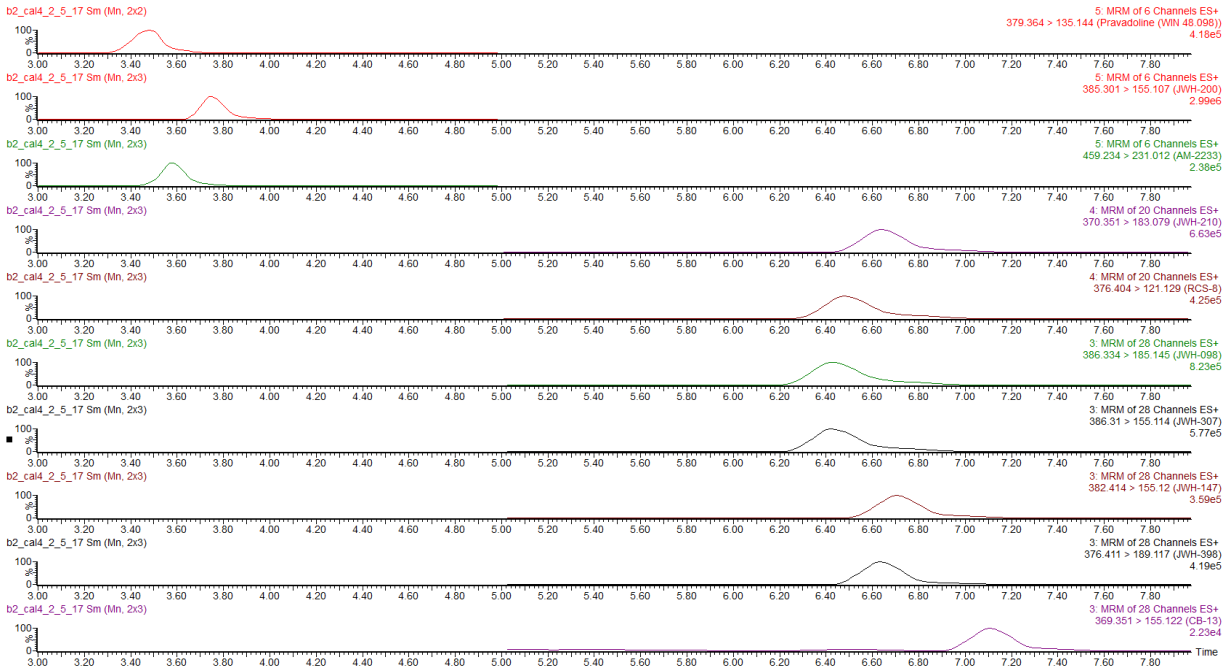
1.1 Smoothed chromatogram of the confirmation transitions (see Table 1) of selected cathinones: dimethylcathinone, methcathinone, 4-fluoromethcathinone, methedrone, methylone, m-cpp, pentylone, 2C-B, MDPV



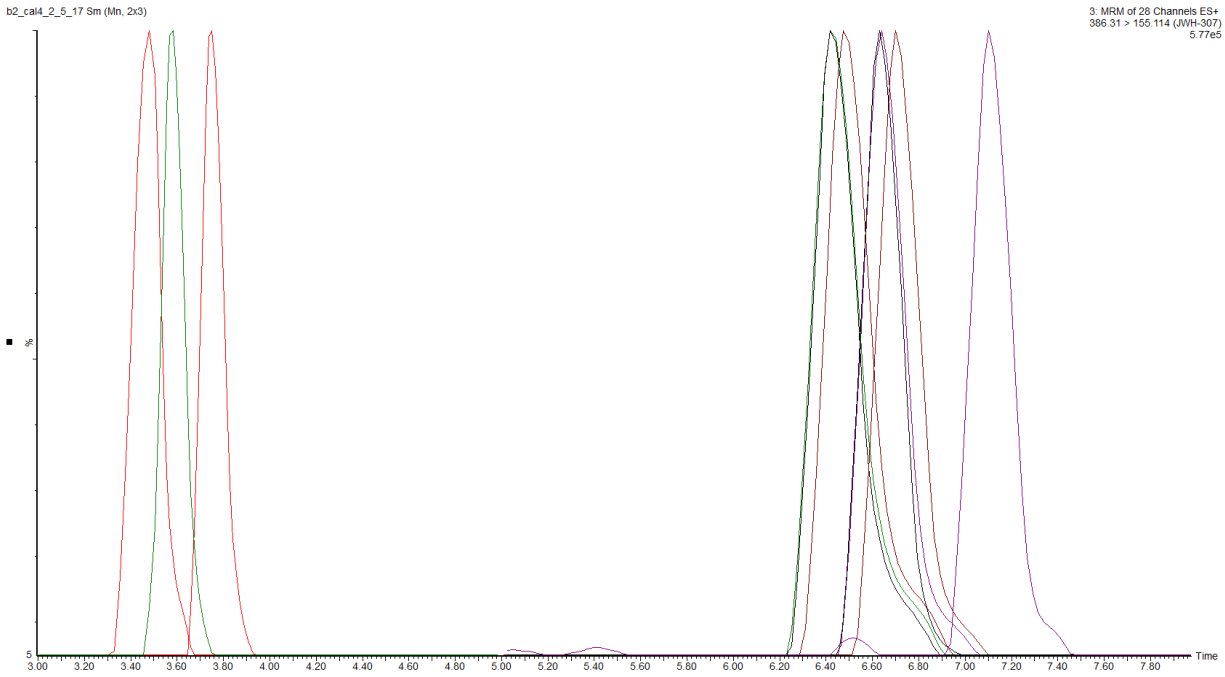
1.1.1 Overlay chromatogram of the analytes presented in the chromatogram 1.1



1.2 Smoothed chromatogram of the confirmation transitions (see Table 1) of selected cannabinoids: pravadoline, JWH-200, AM-2233, JWH-210, RCS-8, JWH-098, JWH-307, JWH-147, JWH-398, CB-13.

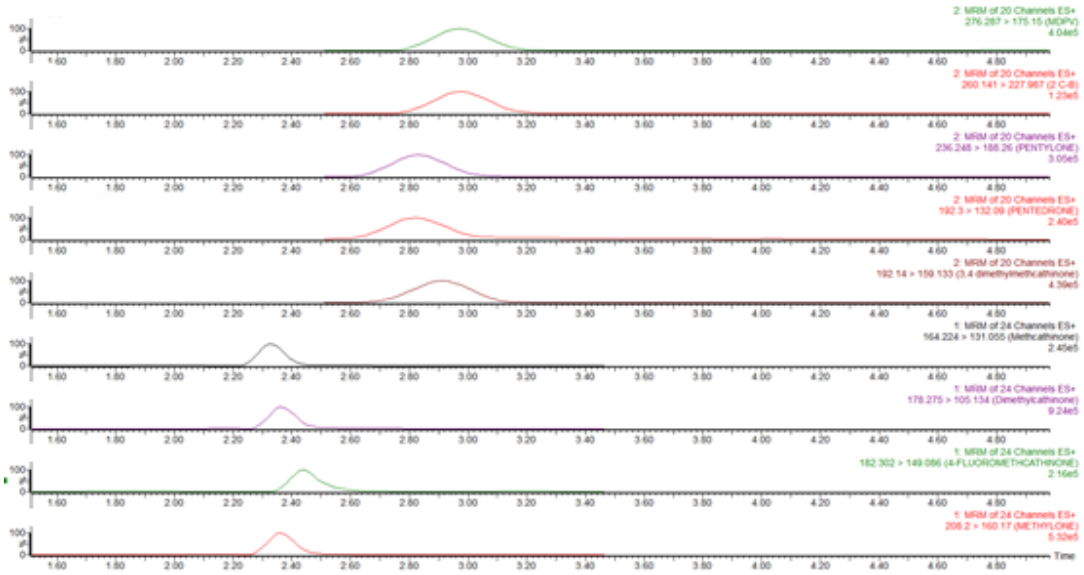


1.2.1 Overlay chromatogram of the analytes presented in the chromatogram 1.2

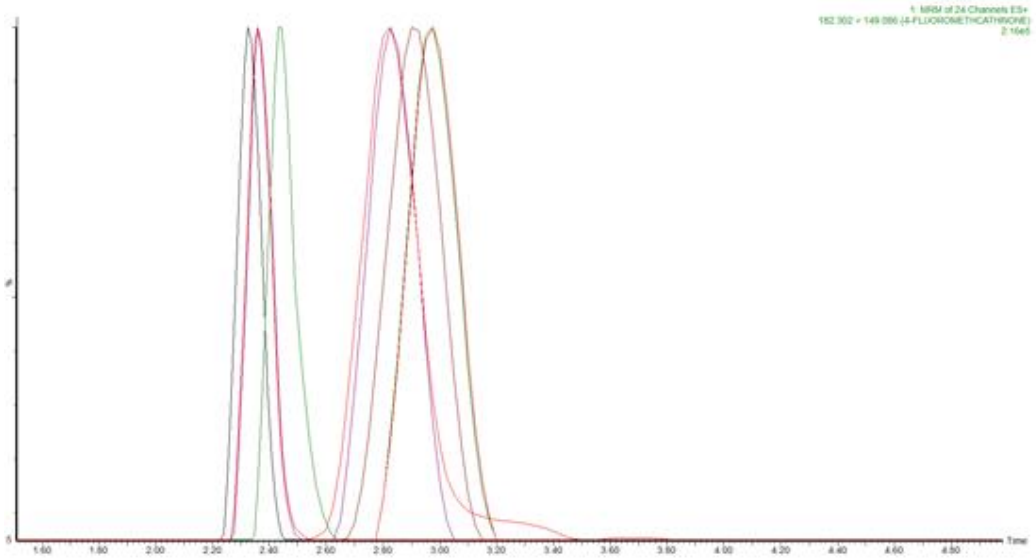


2. Selected MRM chromatograms of the urine extracts spiked with analytes at the concentration of 50 ng/ml

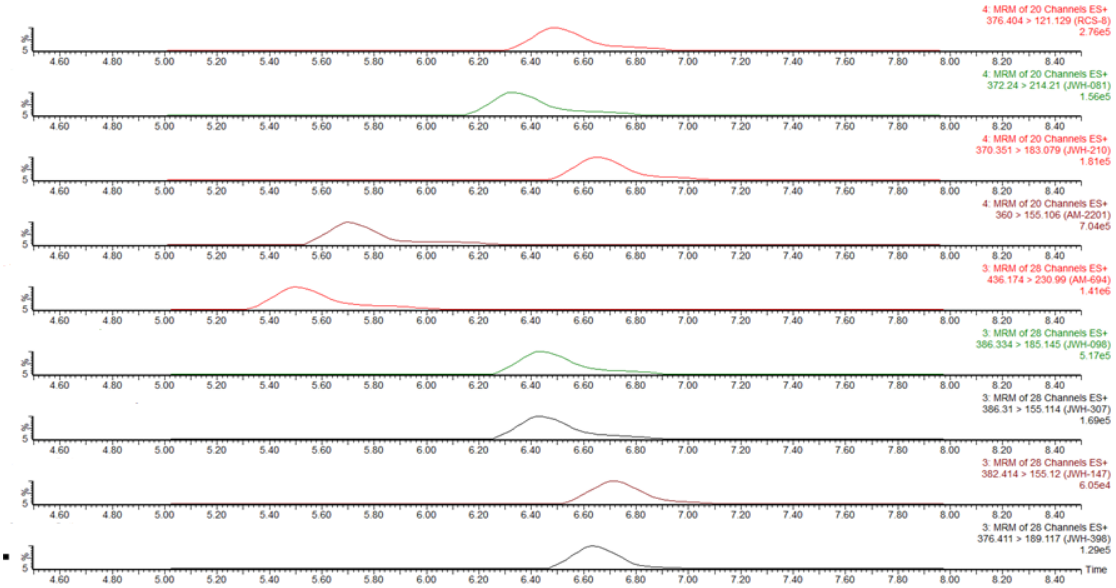
2.1 Smoothed chromatogram of the confirmation transitions (see Table 1) of selected cathinones: MDPV, 2C-B, pentylone, pentedrone, 3,4 dimethylmethcathinone, methcathinone, dimethylcathinone, 4-fluoromethcathinone, methylone.



2.1.1 Overlay chromatogram of the analytes presented in the chromatogram 2.1

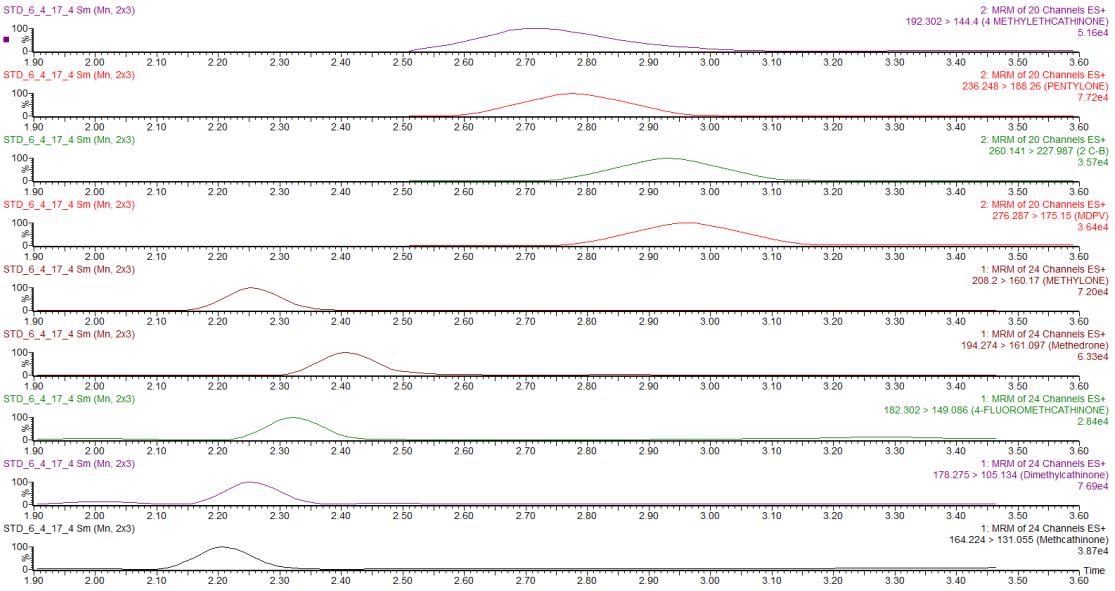


2.2 Smoothed chromatogram of the confirmation transitions (see Table 1) of selected cannabinoids: RCS-8, JWH-081, JWH-210, AM-2201, AM-694, JWH-098, JWH-307, JWH-147, JWH-398.

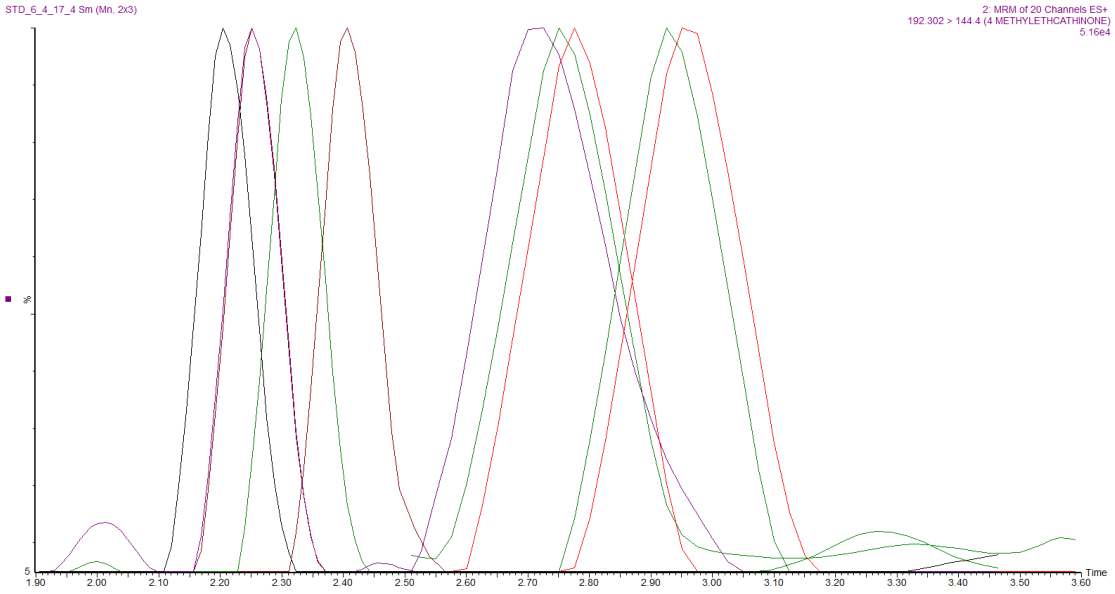


3. Selected MRM chromatograms of the hair extracts spiked with analytes at the concentration of 50 pg/mg

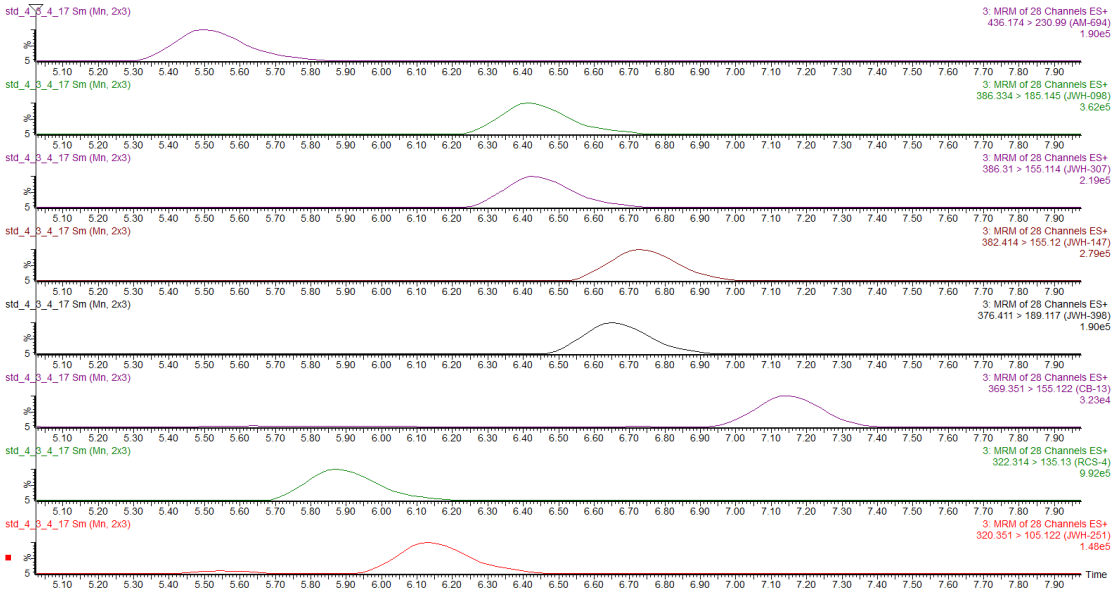
3.1 Smoothed chromatogram of the confirmation transitions (see Table 1) of selected cathinones: 4-methylethcathinone, pentylone, 2C-B, MDPV, methylone, methedrone, 4-fluoromethcathinone, dimethylcathinone, methcathinone.



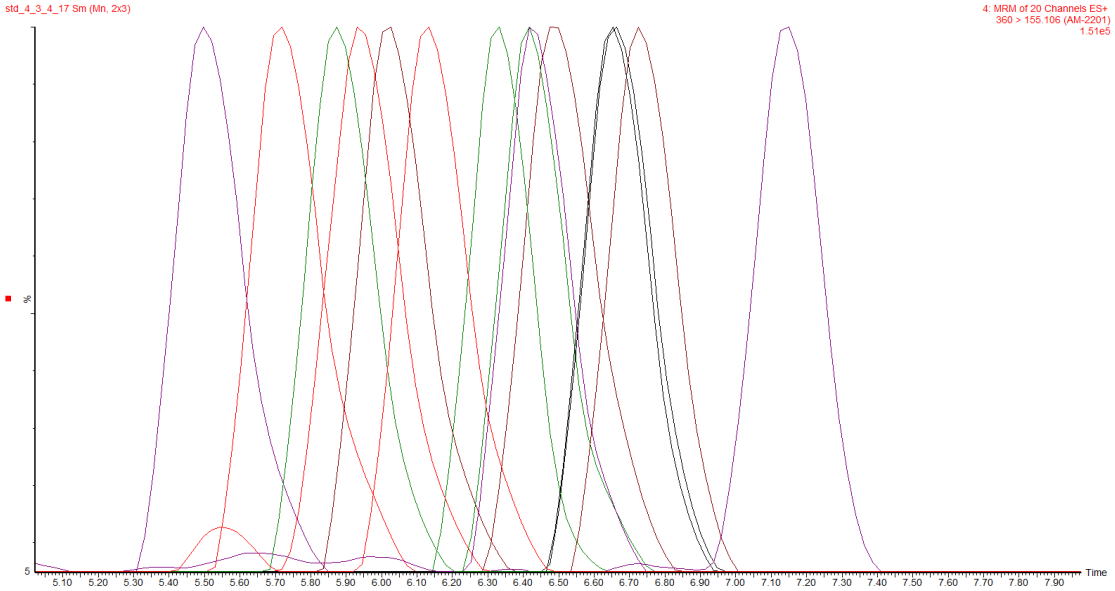
3.2 Overlay chromatogram of selected cathinones: pentedrone, 4-methylethcathinone, pentylone, 2C-B, MDPV, methylone, methedrone, 4-fluoromethcathinone, dimethylcathinone and methcathinone.



3.3 Smoothed chromatogram of the confirmation transitions (see Table 1) of selected cannabinoids: AM-694, JWH-098, JWH-307, JWH-147, JWH-398, CB-13, RCS-4 and JWH-251.

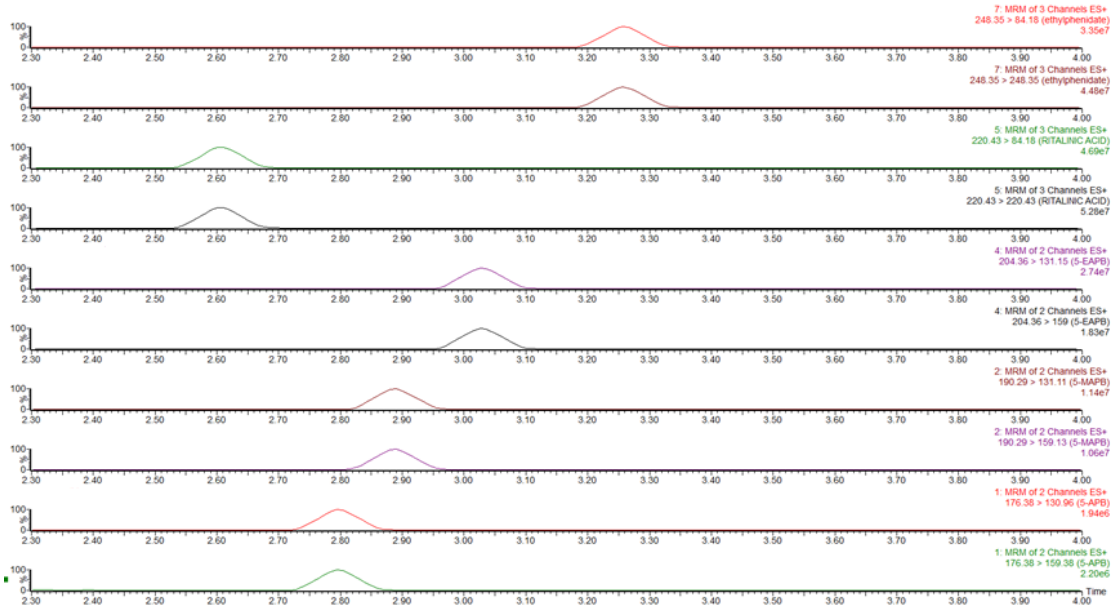


3.4 Overlay chromatogram of the confirmation transitions (see Table 1) of selected cannabinoids: RCS-8, AM 2201, AM-694, RCS-4, CB-13, JWH-251, JWH-398, JWH-147, JWH-307, JWH-098, JWH-073, JWH-250, JWH-081, JWH-210.

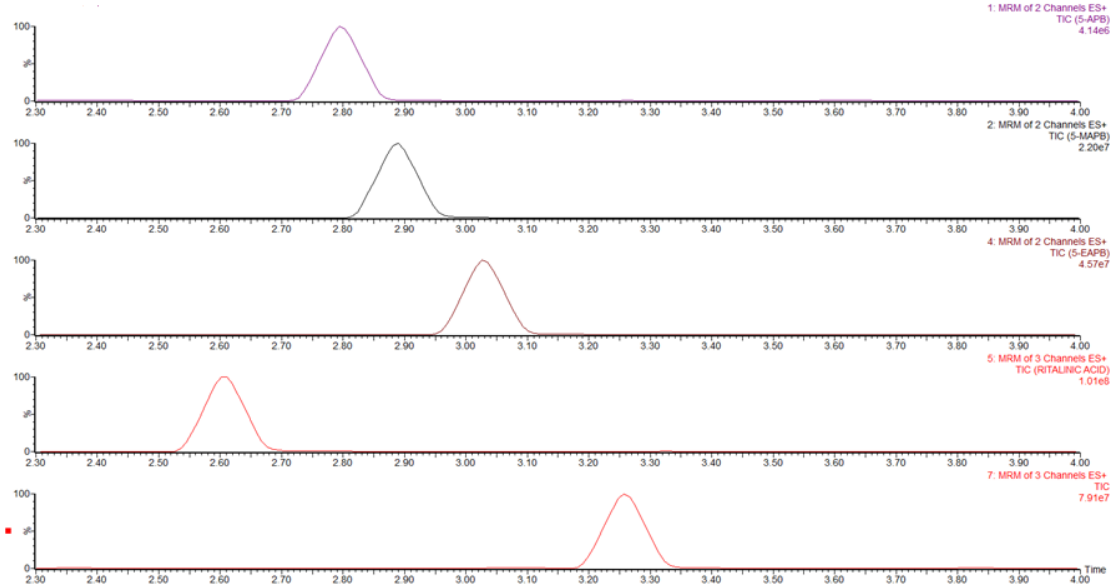


4. Selected MRM chromatograms of the serum, urine and hair extracts of a real intoxication case related to 5-APB, 5-MAPB, 5-EAPB and ETP

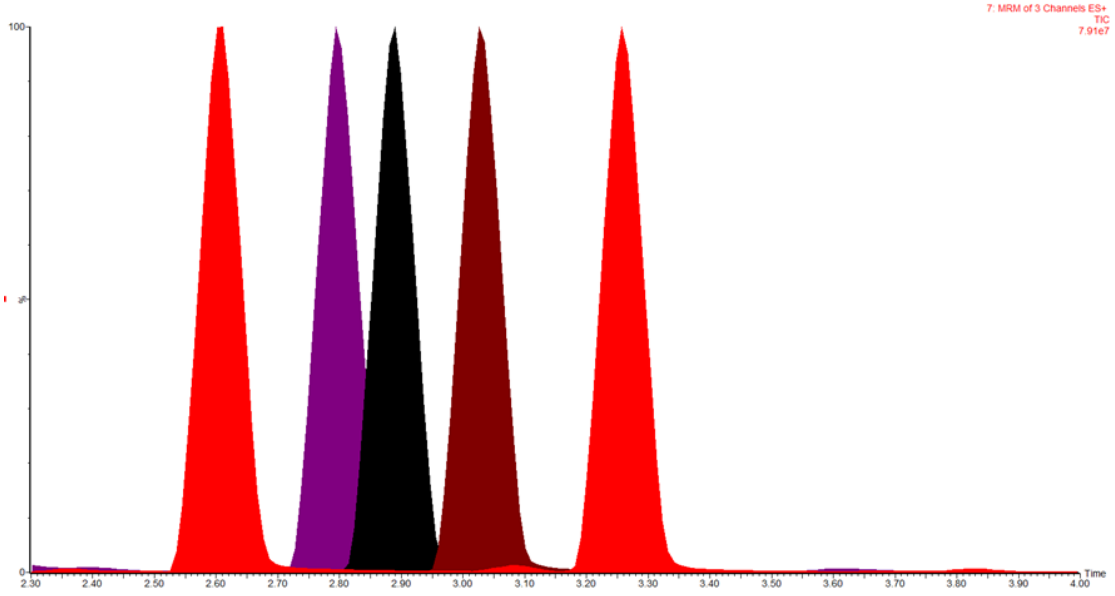
4.1 Serum t_0 quantification and confirmation transitions for detected compounds



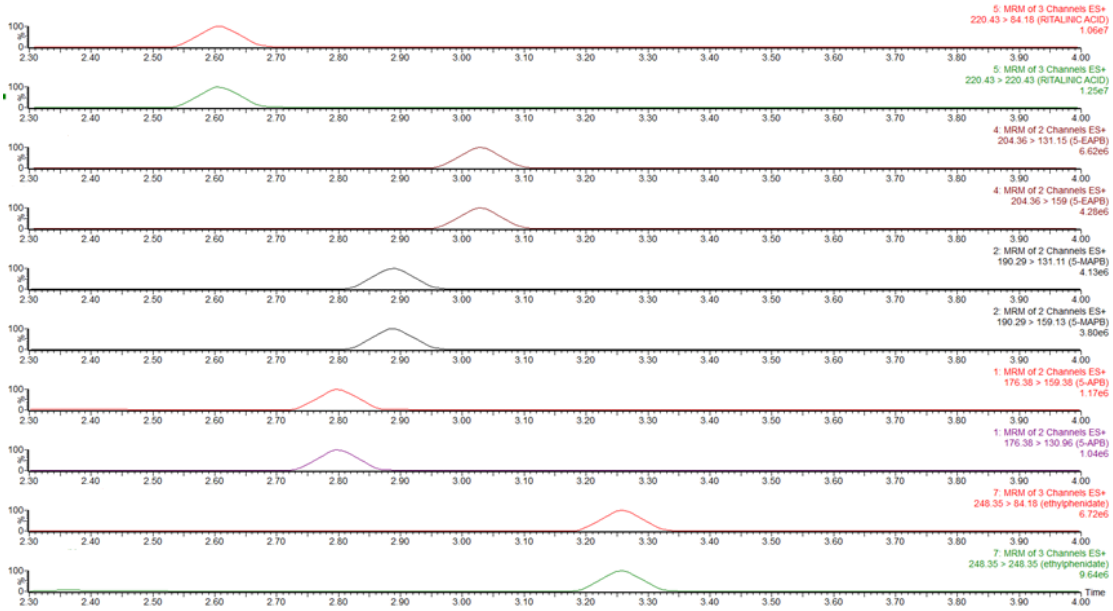
4.1.1 Serum t_0 TIC for detected compounds



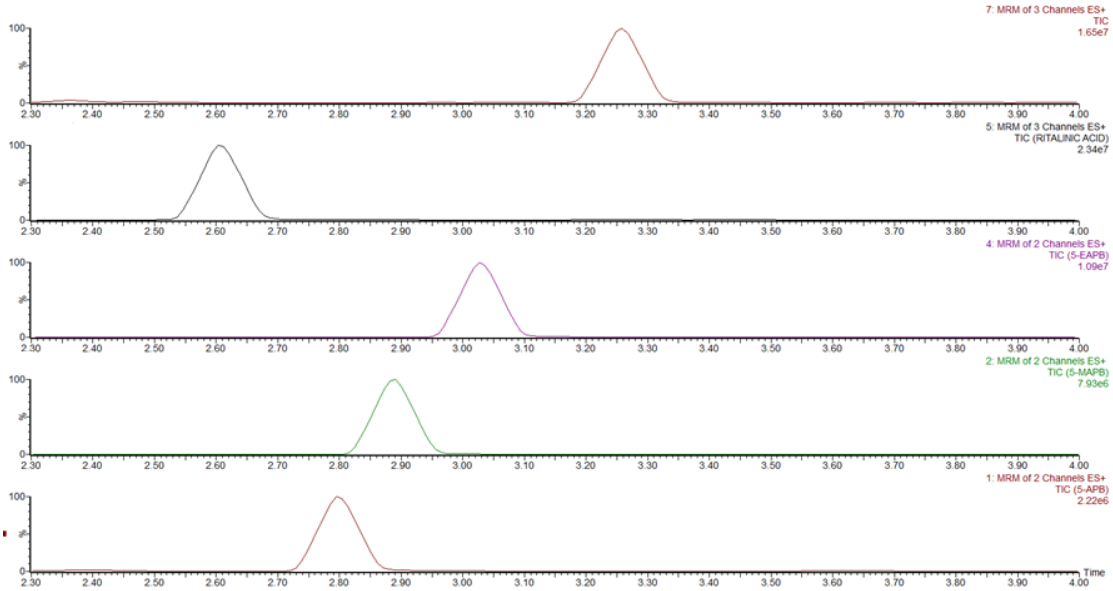
4.1.2 Overlay serum t_0



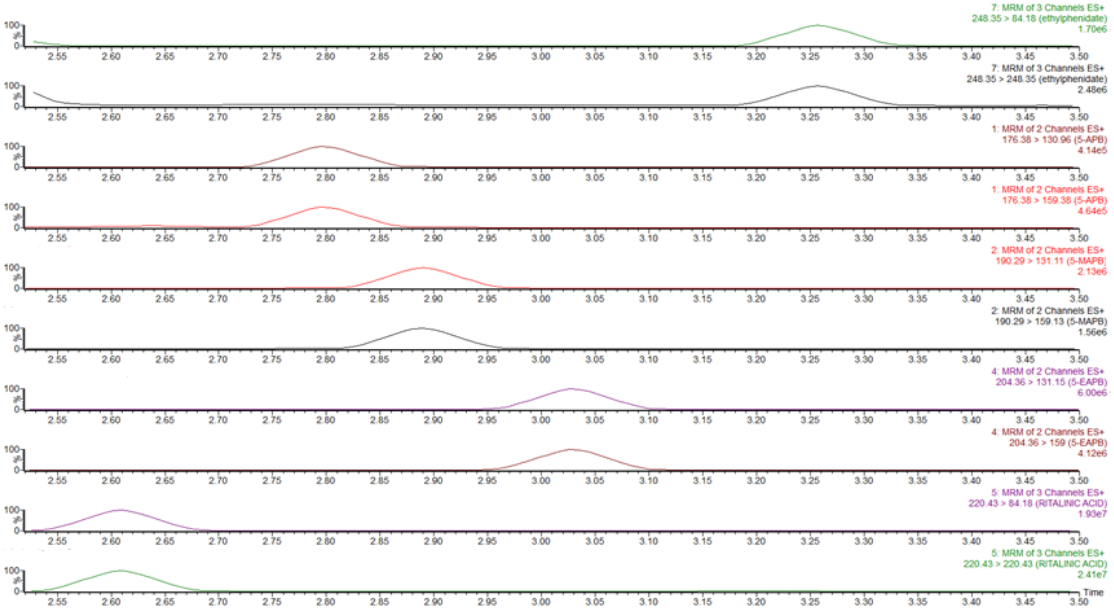
4.2 Serum t_1 quantification and confirmation transitions for detected compounds



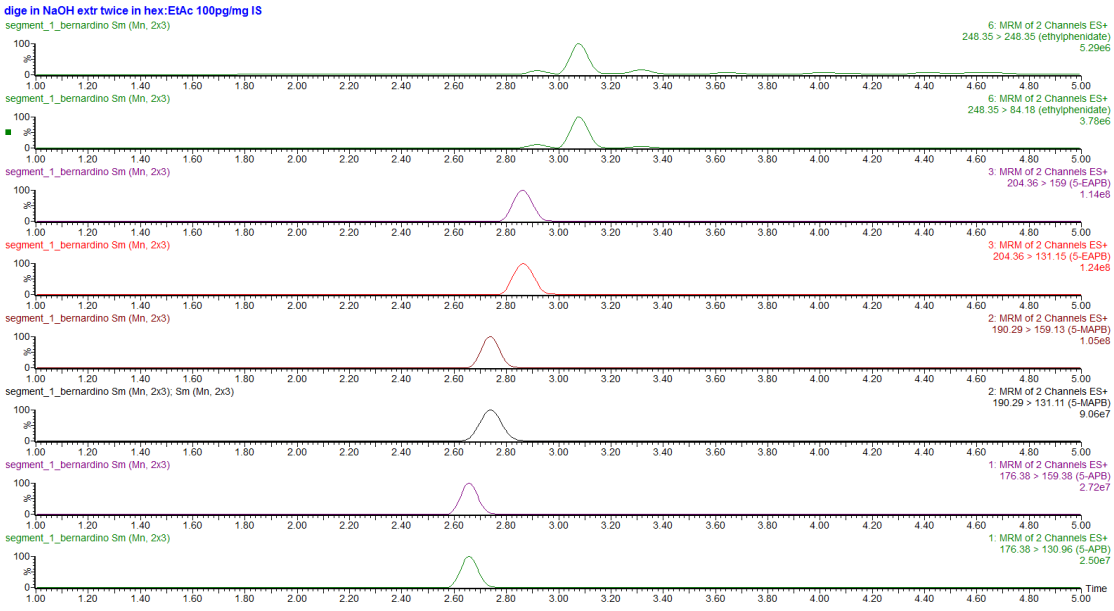
4.2.1 Serum t_1 TIC for detected compounds



4.3 Urine t_1 quantification and confirmation transitions for detected compounds



4.4 Hair segment 1 (0-2 cm, proximal) quantification and confirmation transitions for detected compounds



4.5 Hair segment 2 (2-4 cm, distal) quantification and confirmation transitions for detected compounds