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REVIEW ARTICLE

Recent Trends in Analytical Methods to Determine New Psychoactive Substances in Hair

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Abstract: New Psychoactive Substances (NPS) belong to several chemical classes, including phenethylamines, piperazines, synthetic cathinones and synthetic cannabinoids. Development and validation of analytical methods for the determination of NPS both in traditional and alternative matrices is of crucial importance to study drug metabolism and to associate consumption to clinical outcomes and eventual intoxication symptoms. Among different biological matrices**,** hair is the one with the widest time window to investigate drug-related history and demonstrate past intake.

The aim of this paper was to overview the trends of the rapidly evolving analytical methods for the determination of NPS in hair and the usefulness of these methods when applied to real cases.

A number of rapid and sensitive methods for the determination of NPS in hair matrix has been recently published, most of them using liquid chromatography coupled to mass spectrometry. Hair digestion and subsequent solid phase extraction or liquid-liquid extraction were described as well as extraction in organic solvents. For most of the methods limits of quantification at picogram per milligram hair were obtained. The measured concentrations for most of the NPS in real samples were in the range of picograms of drug per milligram of hair. Interpretation of the results and lack of cut-off values for the discrimination between chronic consumption and occasional use or external contamination are still challenging.

Methods for the determination of NPS in hair are continually emerging to include as many NPS as possible due to the great demand for their detection.

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1. INTRODUCTION

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 phenethylamines, piperazines, cathinones and synthetic cannabinoids. NPS have been synthesized to evade existing drug laws, usually by altering the chemical structures of illegal drugs or by finding drugs with different structures that produce effects similar to those of existing stimulant, hallucinogenic - psychedelic, sedative, dissociative or euphoric drugs. Furthermore, the use of new psychoactive substances (NPS) as adulterants of stimulant recreational controlled drugs, has recently been documented as a new worrying phenomenon [1].

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 [2, 3] this phenomenon appears to be considerably underestimated, mainly due to the substantial lack of comprehensive screening methods for the detection of NPS in biological samples.

The constant addition of new entries to the family of NPS, together with the wide physico-chemical variety of the single substances of this category makes their determination in biological specimens a demanding challenge in forensic and clinical toxicology [4]. Development of analytical methods for the determination of the latter substances both in conventional and non-conventional matrices is of great importance to investigate drug metabolism and to associate

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intake to clinical outcomes and eventual intoxication symptoms.

Determination of these drugs and their metabolites in biological fluids or matrices other than blood or urine may be of interest in certain areas of drug concentration monitoring [5], 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 [6], while an individual's past history, can be obtained from drug analysis of the hair or nails. 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, nails, cerebrospinal fluid, bronchial secretions, peritoneal fluid and interstitial fluid) are analysed, 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 [5].

Hair analysis appears to be one of the most efficient tools to investigate drug-related history [7], 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 [8].

Hair drug testing has gained increased interest and recognition over the past two decades and its application has been recently 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 programmes [9] in post- mortem cases [10] in workplace drug testing [11] driving license regranting [12] and in child custody cases [13, 14]. Moreover, hair testing has been proposed to complement and/or substitute traditional doping control strategies and corresponding test matrices [15, 16]. 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.

Although NPS have been determined in various alternative matrices including stomach content [17], vitreous humour [18], meconium [19], brain, heart, lung, liver, kidney, spleen, and pancreas [20-22], 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 [23].

In this paper we present the trends of the rapidly evolving analytical methods for the determination of NPS in hair. This review includes methods for hair testing of NPS using chromatographic assays up to March 2016; some describing simultaneous detection of a number of NPS from different chemical classes [24-29] in hair and others focusing on the determination of just one class [7, 19, 30-40]. Moreover, the usefulness of these methods when applied to real cases has also been discussed. An exhaustive Table (Table **1**) shows the overview of analytical methods for determination of NPS in hair, sorted by chemical class, reporting the most possible details concerning sample treatment, type of analytes separation, detection mode and validation parameters.

2. ANALYTICAL METHODS FOR THE DETERMINATION OF NPS IN HAIR

2.1. Analytical Methods for the Determination of Phenethylamines in Hair

Phenethylamines are a broad family of chemical variants of the core compounds, *i.e*., amphetamine, methamphetamine and MDMA [41]. 2 C-x is a general name of substituted phenethylamines which contain methoxy groups on the 2 and 5 positions of the benzene ring of phenethylamine and a variety of lipophilic substituents at position 4. The substances of this category show a prevalent hallucinogenic and psychedelic effect. The N-benzyl substituted phenethylamines (NBOMEs) are highly potent hallucinogens even in doses of micrograms, as the N-benzyl substitution of phenethylamines dramatically increases their affinity with the serotonin 2A (5-HT_{2A}) receptors [42].

2.1.1. 2 C-x Series

Two studies have been carried out for the determination of NPS belonging to the 2 C-x series in hair. Montenarh *et al.* developed a liquid chromatography tandem mass spectrometry (LC-MS/MS) screening method for the detection of the following substances: 2 C-P, 2 C-B, 2 C-D, 2 C-E, 2 C-I and 2 C-T, among other compounds of different chemical classes [29]. An amount of 20 mg washed and pulverized hair were extracted with diethyl ether-ethyl acetate mixture (1:1).

The analysis was carried out using LC-QTRAP operated in the positive electrospray ionization (ESI+) mode. Separation was achieved with gradient elution on a C18 column (2.1 × 150 mm, 3.5 μm) and the mobile phase consisted of 10 mM aqueous ammonium formate plus 0.1% formic acid pH 3.4 (eluent A) and acetonitrile plus 0.1% formic acid (eluent B).

Recovery and intraday precision was given only for the 2 C-P, with precision falling out of the acceptable criteria for the high control samples (63.7%) whereas LODs provided for all the above mentioned substances, ranged from 0.02 to 0.1 ng/mg. The new approach was tested for applicability by analyzing among others 13 hair samples**,** however no results on the eventual presence of 2 C-x compounds were provided.

Salomone *et al.* developed an ultra high performance liquid chromatography tandem mass spectrometry (UHPLC–MS/MS) assay for the determination of 31 stimulant and

Table 1. Overview of confirmation methods for determination of NPS in hair. Sorted by chemical class.

Substance	Method Assay	Extraction Method	Absolute Recovery (%)	Intraday Precision (RSD)	Interday Precision (RSD)	Intraday Accuracy (% Error)	Interday Accuracy (% Error)	LOD (pg/mg)	LOQ (pg/mg)	Refs.
Phenethylamines										
2 C-x series										
2 C-P	LC-MS/MS	LLE	72-130	12.4-63.7				50		[29]
	UHPLC-MS/MS	MeOH	96					1	2	[27]
2 C-B	LC-MS/MS	LLE						80		[29]
	UHPLC-MS/MS	MeOH	97					6.2	12	[27]
2 C-D	LC-MS/MS	LLE						50		[29]
2 C-E	LC-MS/MS	LLE						20		[29]
2 C-I	LC-MS/MS	LLE						80		[29]
2 C-T	LC-MS/MS	LLE						100		[29]
25H-NBOMe	UHPLC-MS/MS	MeOH	102					1	2	[27]
25C-NBOMe	UHPLC-MS/MS	MeOH	100					1.5	3	[27]
25B-NBOMe	UHPLC-MS/MS	MeOH	102					4.1	8.2	[27]
25I-NBOMe	UHPLC-MS/MS	MeOH	97					1.5	3	[27]
Other phenethylamines										
PMA	LC-MS/MS	SPE						10	50	[25]
	UHPLC-MS/MS	MeOH	98					8.8	18	[27]
PMMA	UHPLC-MS/MS	MeOH	97					1.3	2.6	[27]
4-MA	LC-MS/MS	Formic Acid		2-10	1-11			5	10	[24]
4-MTA	LC-MS/MS	SPE						20	50	[25]
	LC-MS/MS	Formic Acid		4-11	1-12			2	20	[24]
4-FA	LC-MS/MS	Formic Acid		3-15	1-15			2	5	[24]
	UHPLC-MS/MS	MeOH	94					1.6	3.2	[27]
	LC-MS/MS	MeOH and acidified MeOH						10		[28]
5-MAPB	UHPLC-MS/MS	MeOH	100					4.6	9.2	[27]
6-APB	UHPLC-MS/MS	MeOH	94					17	35	[27]
4-FMA	LC-MS/MS	MSPE	68.6 - 82	4.4-6.2	3-6.8	101.9-104.6[a]		2	10	[26]
Butylone	LC-MS/MS	Formic Acid		2-12	2-12			5	20	[24]
	UHPLC-MS/MS	MeOH	97					3.7	7.4	[27]
	LC-MS/MS	MeOH and acidified MeOH						10		[28]

(Table 1) Contd….

Substance	Method Assay	Extraction Method	Absolute Recovery (%)	Intraday Precision (RSD)	Interday Precision (RSD)	Intraday Accuracy (% Error)	Interday Accuracy (% Error)	LOD (pg/mg)	LOQ (pg/mg)	Refs.
Piperazines										
mCPP	LC-MS/MS	SPE						10	50	[25]
	LC-MS/MS	Formic Acid		0.2-12	3-12			5	20	[24]
	GC-MS	MSPE	92.7 - 100	7.70-16.17	2.01-9.64	-0.80-13.21	-5.03-6.36		50	[33]
	LC-MS/MS	MSPE	70.3 - 79.7	2.3-4	1.8-2.6	96.9-102.9[a]		5	10	[26]
	UHPLC-MS/MS	MeOH	91					3	6	[27]
	LC-MS/MS	MeOH and acidified MeOH						10		[28]
Benzylpiperazine	LC-MS/MS	Formic Acid		0.4-11	3-11			5	20	[24]
	LC-MS/MS	MeOH and acidified MeOH						10		[28]
TFMPP	GC-MS	MSPE	91.3 - 101.3	4.70-12.12	2.15-6.55	-8.45-10.07	-4.57-4.30		50	[33]
	LC-MS/MS	MSPE	61.5-68.9	1.8-3.3	1.5-3	99.8-103.7[a]		1	10	[26]
	LC-MS/MS	MeOH and acidified MeOH						10		[28]
MeOPP	GC-MS	MSPE	92-101.3	4.46-12.77	1.72-13.7	-8.45-18.53	-10.18-17.23		50	[33]
Cathinones										
α-PVP	UHPLC-MS/MS	MeOH	91					2	4	[27]
	LC-MS	Extrelut column		Within 9.4[b]	Within 9.4[b]			0.02 ng/10-mm	0.05 ng/10-mm	[35]
α-PBP	LC-MS	Extrelut column		Within 9.4[b]	Within 9.4[b]			0.02 ng/10-mm	0.05 ng/10-mm	[35]
Buphedrone	UHPLC-MS/MS	MeOH	79					4.2	8.4	[27]
Amfepramone	UHPLC-MS/MS	MeOH	81					4	8	[27]
3,4-DMMC	LC-MS/MS	Formic Acid		3-12	8-15			5	20	[24]
4-MEC	LC-MS/MS	Formic Acid		1-4	3-13			5	20	[24]
	UHPLC-MS/MS	MeOH	86					3	6	[27]
3-MMC	LC-HRMS	methanol:trifluoroacetic acid 9:1		Within 0.8	Within 6.9			20	100	[7]

(Table 1) Contd….

Substance	Method Assay	Extraction Method	Absolute Recovery (%)	Intraday Precision (RSD)	Interday Precision (RSD)	Intraday Accuracy (% Error)	Interday Accuracy (% Error)	LOD (pg/mg)	LOQ (pg/mg)	Refs.
Cathinones										
Ethcathinone	LC-MS/MS	Formic Acid		1-12	1-5			20	20	[24]
	UHPLC-MS/MS	MeOH	88					3.1	6.2	[27]
Ethylone	LC-MS/MS	Formic Acid		4-14	1-14			2	5	[24]
	LC-MS/MS	MeOH and acidified MeOH						10		[28]
Mephedrone	LC-MS/MS	Formic Acid		1-6	1-9			2	20	[24]
	LC-MS/MS	MSPE	51.1- 59.8	2.2 - 3	0.3 - 6.2	99-109.3[a]		1	2	[26]
	UHPLC-MS/MS	MeOH	81					2.4	4.8	[27]
	GC-MS	HCl (0.01 M): MeOH	>76	3.5 - 3.9	9.5 - 12.9	91.8 - 94.2	88 - 94.8	80	200	[45]
	LC-MS/MS	LLE	90.4-110.4	0.4 - 2.6	0.7 - 4.5	99.15-107.53[c]		2.5	5	[30]
	LC-MS/MS	MeOH and acidified MeOH						10		[28]
4-Methyle-phedrine	LC-MS/MS	LLE	98.4-106.2	0.6-4.92	0.7-4	98.52-114.59[c]		5	10	[30]
4-Methylnore-phedrine	LC-MS/MS	LLE	96.8-101.3	0.2-6.97	0.7-7.3	97.6-107.98[c]		5	10	[30]
Methedrone	LC-MS/MS	Formic Acid		1 - 13	1 - 12			2	20	[24]
	LC-MS/MS	MSPE	67.6- 72.4	3.3 - 5.6	2 - 7	103.9-106.2[a]		1	2	[26]
Methcathinone	LC-MS/MS	MeOH and acidified MeOH						10		[28]
Methylone	LC-MS/MS	Formic Acid		2 - 12	1 - 6			2	20	[24]
	UHPLC-MS/MS	MeOH	85					3.2	6.4	[27]
	LC-MS/MS	MSPE	70.8 - 74.4	2.2 - 2.8	2.8 - 4.7	102.2-109.3[a]		1	2	[26]
	LC-MS/MS	MeOH and acidified MeOH						10		[28]
Naphyrone	LC-MS/MS	Formic Acid		2 - 17	4 - 14			10	20	[24]
Pentedrone	LC-MS/MS	Formic Acid		3 - 16	1 - 15			2	20	[24]
	UHPLC-MS/MS	MeOH	87					3.9	7.8	[27]

(Table 1) Contd….

Substance	Method Assay	Extraction Method	Absolute Recovery (%)	Intraday Precision (RSD)	Interday Precision (RSD)	Intraday Accuracy (% Error)	Interday Accuracy (% Error)	LOD (pg/mg)	LOQ (pg/mg)	Refs.
Cathinones										
Pentylone	LC-MS/MS	Formic Acid		2 - 16	3 - 10			2	20	[24]
4-FMC	LC-MS/MS	MSPE	47.8 - 53.9	3 - 4.9	7.4 - 11.2	97.7-104.5[a]		5	10	[26]
	LC-MS/MS	Formic Acid		2-12	0.2-6			5	10	[24]
MDPV	LC-MS/MS	MSPE	75.8-85.0	2.6-5.4	0-3.9	102-104.6[a]		0.2	2	[26]
	UHPLC-MS/MS	MeOH	96					2	4	[27]
	LC-MS/MS	MeOH and acidified MeOH						10		[28]
	LC-MS/MS	Formic Acid		4-13	1-14			2	5	[24]
	LC-MS	Extrelut column		9.4[b]	9.4[b]			0.02 ng/ 10-mm	0.05 ng/ 10-mm	[35]
Cannabinoids										
Benzoylindoles										
AM2233	LC-MS/MS	MeOH		2 - 3	2 - 8			10	20	[24]
AM694	LC-MS/MS	MeOH		0.3 - 3	2 - 11			10	20	[24]
	LC-MS/MS	LLE						20		[29]
	UHPLC-MS/MS	LLE	93					0.8	2.6	[32]
	LC-MS/MS	Ethanol	84-96	3.1-7.8	4.1-7.8	-6.1-5.2[c]		0.5	0.5	[34]
	LC-QTOF MS	LLE							20	[8]
RCS-4	LC-MS/MS	LLE						100		[29]
	UHPLC-MS/MS	LLE	99					0.7	2.3	[32]
	LC-MS/MS	MeOH		1 - 10	2 - 12			10	20	[24]
	LC-MS/MS	Ethanol	81-97	3.1-6.3	4.5-8.7	-0.3-5.2[c]		0.5	0.5	[34]
RCS-4 ortho isomer	LC-MS/MS	Ethanol	83-98	2.6-7.1	2.6-6.8	-3.8-2.7[c]		0.5	0.5	[34]
WIN 48.098	UHPLC-MS/MS	LLE	91					0.7	2.3	[32]
	LC-MS/MS	MeOH		1 - 5	2 - 6			5	20	[24]
	LC-MS/MS	Ethanol	87-95	1.8-7.8	4.7-9.9	-6.4-1.9[c]		0.5	0.5	[34]
Naphthoylindoles										
AM-1220	UHPLC-MS/MS	LLE	106					0.4	1.3	[32]
AM2201	LC-MS/MS	MeOH						10	10	[24]
	LC-MS/MS	LLE						20		[29]
	UHPLC-MS/MS	LLE	88					0.7	2.3	[32]
	LC-MS/MS	Ethanol	85-97	2.4-4.8	4.6-10.3	-3.1 -4.8[c]		0.5	0.5	[34]
	LC-MS/MS	MeOH	74-93		0.3-4.8	-1.3-0.4[c]		0.05	0.1	[37]
AM-2201 N-4-OH M	LC-MS/MS	MeOH	95-107		2.5-3.2	-0.1-33[c]		0.05	0.1	[37]

(Table 1) Contd….

Substance	Method Assay	Extraction Method	Absolute Recovery (%)	Intraday Precision (RSD)	Interday Precision (RSD)	Intraday Accuracy (% Error)	Interday Accuracy (% Error)	LOD (pg/mg)	LOQ (pg/mg)	Refs.
Naphthoylindoles										
AM-2201 N-6-OHindole M	LC-MS/MS	MeOH	93-128		1-1.9	0.9-2.4[c]		0.05	0.1	[37]
JWH-007	LC-MS/MS	MeOH		1 - 6	1 - 8			5	10	[24]
	UHPLC-MS/MS	LLE	91					0.2	0.7	[32]
	LC-MS/MS	Ethanol	81-96	3-4.4	3.7-8.4	-6.9- -1.8[c]		0.5	0.5	[34]
JWH-015	LC-MS/MS	MeOH		1 - 3	1 - 9			2	10	[24]
	LC-MS/MS	LLE						500		[29]
	UHPLC-MS/MS	LLE	84					0.6	2	[32]
	LC-MS/MS	Ethanol	83-97	3.8-5.6	3.8-6.7	-0.1-1.3[c]		0.5	0.5	[34]
JWH-018	LC-MS/MS	MeOH		2-8	2 - 10			5	10	[24]
	LC-MS/MS	LLE						500		[29]
	UHPLC-MS/MS	LLE	102					0.9	3	[32]
	LC-MS/MS	Ethanol	83-97	1.8-6.7	4.1-8.2	-3.8-3.5[c]		0.5	0.5	[34]
	UHPLC-MS-MS	LLE		2.3-4.2	4.2-5.8	-3-0	-4.3- -1.1	0.18	0.59	[31]
	UHPLC-MS-MS	MeOH	78-94		1-5.6	-1-7.3[c]		0.05	0.1	[36]
	LC-QTOF MS	LLE							10	[8]
JWH-018 N-4-OH M	UHPLC-MS-MS	MeOH	93-95		5.7-6.3	1.2-3.4[c]		0.05	0.1	[36]
JWH-018 N-5-OH M	UHPLC-MS-MS	MeOH	98-100		6.2-8.5	1.8-2.6[c]		0.05	0.1	[36]
JWH-018 N-COOH M	UHPLC-MS-MS	MeOH	97-101		4.2-10	0.2-0.5[c]		0.05	0.1	[36]
JWH-019	LC-MS/MS	MeOH		0.3 - 10	3 - 7			5	20	[24]
	UHPLC-MS/MS	LLE	114					1	3.3	[32]
	LC-MS/MS	Ethanol	82-98	4.5-5.3	5.3-6.5	-2.6-4.2[c]		0.5	0.5	[34]
JWH-073	LC-MS/MS	MeOH		1 - 4	3 - 11			10	20	[24]
	LC-MS/MS	LLE						500		[29]
	UHPLC-MS/MS	LLE	100					0.5	1.6	[32]
	LC-MS/MS	Ethanol	83-98	5.9-8.2	7.8-11.6	-4.6- 1.9[c]		0.5	0.5	[34]
	UHPLC-MS/MS	MeOH	83-96		7.9-9.8	-1.5-3.6[c]		0.05	0.1	[36]
	UHPLC-MS-MS	LLE		1.9-3.4	4.4-8.2	-5.2-8.6	-6.1- -1.4	0.1	0.33	[31]
	LC-QTOF MS	LLE							10	[8]
JWH-073 N-3-OH M	UHPLC-MS/MS	MeOH	97-101		7-8.2	-3.5-2.6[c]		0.05	0.1	[36]
JWH-073 N-4-OH M	UHPLC-MS/MS	MeOH	94-98		6.6-10	-1.9- -1[c]		0.1	0.1	[36]
JWH-073 N-COOH M	UHPLC-MS/MS	MeOH	94-95		0.7-3.4	0.7-5.5[c]		0.05	0.1	[36]

(Table 1) Contd….

Substance	Method Assay	Extraction Method	Absolute Recovery (%)	Intraday Precision (RSD)	Interday Precision (RSD)	Intraday Accuracy (% Error)	Interday Accuracy (% Error)	LOD (pg/mg)	LOQ (pg/mg)	Refs.
Naphthoylindoles										
JWH-081	LC-MS/MS	MeOH		1 - 7	1 - 10			5	20	[24]
	UHPLC-MS/MS	LLE	95					0.6	2	[32]
	LC-MS/MS	Ethanol	85-99	3.2-6.9	5.9-9.5	-4.5-4[c]		0.5	0.5	[34]
	LC-QTOF MS	LLE							10	[8]
JWH-098	LC-MS/MS	MeOH		1 - 11	2 - 11			5	20	[24]
JWH-122	LC-MS/MS	MeOH		2 - 9	2 - 11			10	20	[24]
	LC-MS/MS	LLE						500		[29]
	UHPLC-MS/MS	LLE	106					0.9	3	[32]
	LC-MS/MS	Ethanol	82-97	2.8-7.1	5.3-7.1	-4.3-1.8[c]		0.5	0.5	[34]
	LC-MS/MS	MeOH	87-95		1.8-2.5	-0.6-3.3[c]		0.05	0.1	[37]
	LC-QTOF MS	LLE							20	[8]
JWH-122 N-5-OH	LC-MS/MS	MeOH	97-111		1.6-3.9	1.1-3.6[c]		0.05	0.1	[37]
JWH-200	LC-MS/MS	MeOH		1 - 7	6 - 13			5	10	[24]
	LC-MS/MS	LLE						20		[29]
	UHPLC-MS-MS	LLE		1.3-2.1	4.4-6.8	-4-6.1	-6.1-4.6	0.02	0.07	[31]
	UHPLC-MS/MS	LLE	106					0.4	1.3	[32]
	LC-MS/MS	Ethanol	86-95	1.6-5	4.7-8.1	-6.9- -0.5[c]		0.5	0.5	[34]
	LC-QTOF MS	LLE							10	[8]
JWH-210	LC-MS/MS	LLE	77 - 90	12.4 - 50.4				10		[29]
	UHPLC-MS/MS	LLE	114					0.7	2.3	[32]
	LC-MS/MS	Ethanol	95-97	3.7-8.9	8-9.5	-6.2- -0.1[c]		0.5	0.5	[34]
	LC-QTOF MS	LLE							10	[8]
WIN 55.212-2	UHPLC-MS/MS	LLE	110					0.8	2.6	[32]
	LC-MS/MS	Ethanol		6-8.2	6.3-9.9	-3.8-3.9[c]		0.5	0.5	[34]
MAM-2201	LC-MS/MS	MeOH	89-100		0.3-1.2	-0.2-1.9[c]		0.05	0.1	[37]
MAM-2201 N-COOH M	LC-MS/MS	MeOH	95-112		1.6-3.4	1.8-2.9[c]		0.05	0.1	[37]
MAM-2201 N-4-OH M	LC-MS/MS	MeOH	97-111		1-4.8	0.5-3.3[c]		0.05	0.1	[37]
JWH-398	LC-MS/MS	MeOH		1 - 5	1 - 10			5	10	[24]
	UHPLC-MS/MS	LLE	106					0.3	1	[32]
	LC-MS/MS	Ethanol	80-100	1.4-8.2	8.8-10.5	-4.5-1.1[c]		5	5	[34]
Phenylacetylindoles										
JWH-203	LC-MS/MS	LLE						50		[29]
	UHPLC-MS/MS	LLE	91					0.7	2.3	[32]
	LC-MS/MS	Ethanol	83-99	2.8-4.3	3.6-8.1	-6.1-0.3[c]		0.5	0.5	[34]

(Table 1) Contd….

Substance	Method Assay	Extraction Method	Absolute Recovery (%)	Intraday Precision (RSD)	Interday Precision (RSD)	Intraday Accuracy (% Error)	Interday Accuracy (% Error)	LOD (pg/mg)	LOQ (pg/mg)	Refs.
Phenylacetylindoles										
JWH-250	LC-MS/MS	MeOH		1 - 8	2 - 12			10	20	[24]
	LC-MS/MS	LLE						10		[29]
	UHPLC-MS/MS	LLE	98					0.5	1.6	[32]
	LC-MS/MS	Ethanol	84-96	4.2-4.5	4.2-9.2	-3.2-6.1[c]		0.5	0.5	[34]
	UHPLC-MS-MS	LLE		2.3-2.4	2.7-7.2	-4.5-2	-5 - -4.8	0.04	0.13	[31]
	LC-QTOF MS	LLE							10	[8]
JWH-251	LC-MS/MS	MeOH		3 - 10	2 - 12			10	20	[24]
	UHPLC-MS/MS	LLE	86					0.3	1	[32]
	LC-MS/MS	Ethanol	81-98	1.2-6.6	3-10.9	-4.3- -0.5[c]		0.5	0.5	[34]
JWH-302	LC-MS/MS	MeOH		1 - 3	1 - 5				0	[24]
RCS-8	LC-MS/MS	MeOH		2 - 7	2 - 15			5	10	[24]
	UHPLC-MS/MS	LLE	108					0.9	3	[32]
	LC-MS/MS	Ethanol	80-97	2.9-8.6	4.1-8.7	-4.4-3.1[c]		0.5	0.5	[34]
Naphthoylpyrroles										
JWH-030	LC-MS/MS	MeOH		1 - 4	1 - 10			10	10	[24]
JWH-147	LC-MS/MS	MeOH		1 - 2	1 - 13			10	20	[24]
JWH-307	LC-MS/MS	MeOH		1 - 9	1 - 9			10	20	[24]
	UHPLC-MS/MS	LLE	113					1.3	4.3	[32]
Other cannabinoids										
HU-210	UHPLC-MS/MS	LLE	112					24	80	[32]
	UHPLC-MS-MS	LLE		2.3-3.1	5.8-7.9	2.6-13.6	0.9-13.8	3	9.9	[31]
Methanandamide (AM-356)	LC-MS/MS	Ethanol	82-98	2.4-6.7	4.8-8.8	-6.3- -0.5[c]		5	5	[34]
XLR-11	LC-MS/MS	MeOH	84-103	3.1-7.9	8.3-11.7	-7.4-4.8	-6.6- -2.6	0.01	0.2	[38]
UR-144	LC-MS/MS	MeOH	94-105	6.1-6.2	8.6-9.8	-7- -4.9	-8.6- -5.2	0.01	0.2	[38]
UR-144 N-4-OH M	LC-MS/MS	MeOH	66-98	4.5-7.5	6.6-9.5	-6.2- -3.5	-8.5- -7.6	0.01	0.2	[38]
UR-144 N-5-OH M	LC-MS/MS	MeOH	58-64	10.1-11.1	7.1-9.8	-7.2- -5.9	-8- -5.6	0.01	0.2	[38]
UR-144 N-COOH M	LC-MS/MS	MeOH	94-105	6.5-10.2	10.1-10.4	-10.3- -7.8	-9.4- -9.2	0.01	0.2	[38]
XLR-11 N-4-OH M	LC-MS/MS	MeOH	87-103	5.5-7.6	6.4-9.3	-6.5- -1.9	-8.9- -3.4	0.2	0.2	[38]
CB-13	LC-MS/MS	MeOH		1 - 5	6 - 12			10	20	[24]
JWH-016	LC-MS/MS	MeOH		1 - 9	1 - 8			5	10	[24]

(Table 1) Contd….

Substance	Method Assay	Extraction Method	Absolute Recovery (%)	Intraday Precision (RSD)	Interday Precision (RSD)	Intraday Accuracy (% Error)	Interday Accuracy (% Error)	LOD (pg/mg)	LOQ (pg/mg)	Refs.
Other cannabinoids										
JWH-020	UHPLC-MS/MS	LLE	101					0.2	0.7	[32]
	LC-MS/MS	Ethanol	79-99	4.5-11.1	8-11.1	-6.3- -0.8[c]		0.5	0.5	[34]
JWH-201	LC-MS/MS	MeOH		1 - 4	2 - 11			2	10	[24]
AB-CHMINACA	LC-MS/MS	MeOH							2.5	[39]
5F-PB-22	LC-MS/MS	MeOH							1	[39]
Other NPS										
MXE	UHPLC-MS/MS	MeOH	98					1	2	[27]
4-MeO-PCP	UHPLC-MS/MS	MeOH	87					9	1.8	[27]
Diphenidine	UHPLC-MS/MS	MeOH	85					3.4	6.8	[27]

[a]: % target concentration
[b]: The method was linear from LOQ to 50 ng/10-mm hair segment, with maximum values for intraday and interday precisions in hair, tested at 0.4, 4, and 40 ng/10-mm hair segment being 9.4 %. LOD and LOQ values of 0.02 and 0.05 ng/10-mm, respectively were achieved.
[c]: not defined as intra- or inter-day accuracy.

psychedelic substituted phenethylamines and dissociative drugs in hair matrix [27]. Of the 2 C-x series 2 C-P, 2 C-B and 4 substances of the NBOMe group were tested. The method employed simple sample pre-treatment: extraction of 25 mg washed and cut hair sample in MeOH at 55 °C for 15 h.

All analyses were performed using an LC system interfaced to a QTRAP® 4500 mass spectrometer equipped with an electrospray Turbo Ion source operated in the positive ion mode. A C18 RRHD column (100 mm × 2.1 mm, 1.8 μm) protected by a C18 pre-column was used for the separation of the target analytes. Elution solvents were water/ formic acid 5mM (solvent A) and acetonitrile/methanol 80:20 plus 5 mM formic acid (solvent B). After an initial isocratic elution at 95% A for 0.5 min a linear gradient elution followed, before isocratic elution at 55% B and the total run time was 5.5 min.

Extraction recoveries together with limit of detection (LOD) and limit of quantification (LOQ) values suggested a specific and sensitive method.

The method was applied to 77 real cases which had been previously tested for traditional drugs of abuse; 23 authentic hair samples collected from MDMA and ketamine users (group A) and 54 authentic samples from driver's license regranting cases which had been found negative, when tested for common drugs of abuse (group B). Substances of 2 C-x series were not detected in any of the considered samples. The authors attributed the negative results either to possible delayed diffusion of these recent drugs among the Italian population or to the fact that these psychedelic phenethylamines are active at very low doses, reducing the detectable concentrations in hair, especially in the cases of sporadic intake. Thus, they stressed the need for further improvement of the sensitivity of the method to disclose possible presence of NBOMes at trace concentrations in hair, after active intake.

2.1.2. Other Phenethylamines

A total of five studies have been conducted for the determination of phenethylamines other than 2 C-x series in hair. A first group of authors developed a LC–ESI-MS/MS assay for the simultaneous analysis of NPS and traditional drugs in hair [25]. From the phenethylamine group PMA and 4-MTA, were determined. To 50 mg washed and finely cut hair samples internal standards (ISs) and phosphate buffer at pH 5.0 were added and incubated for 18 h at 45 °C. Vortex and centrifugation was followed by a solid phase extraction (SPE) with MCX®, Oasis®, Waters cartridges.

The separation and detection of the analytes was achieved by using LC coupled with an atmospheric pressure Ionization (API) 2000 MS/MS System equipped with a Turbo Ion Spray ionization source optimized in positive ionization mode. Chromatographic separation was carried out on an Atlantis® T3 column (150 x 2.1-mm I.D., 3 μm) and the MP consisted of (A) 2 mM ammonium formate, pH 3.0, and (B) acetonitrile/A (90:10, v/ v). Gradient elution was used and the total run time was 24 min.

LOD values of 10 and 20 pg/mg were achieved for PMA and 4-MTA, respectively and LOQ of 0.050 ng/mg for both substances.

This method was routinely applied to hair samples and two forensic cases were described. However no NPS were detected in examined samples, but traditional drugs of abuse.

A second group of authors, already mentioned for 2 C-x series, using the same UHPLC–MS/MS assay, determined PMA, PMMA, 4-FA, 5-MAPB, 6-APB and butylone after methanolic hair extraction achieving high recoveries ≥94% and LOD and LOQ values from 1.3-17 pg/mg and 2.6-35 pg/mg, respectively [27]. In one case, out of 77 investigated cases, hair was tested positive for 4-FA at the concentration of 55 pg/mg, together with other NPS and MDMA.

Strano-Rossi and co-workers described an UHPLC–MS/MS method, based on two different runs (*i.e*. different matrix extractions and chromatographic separations), for the determination of more than 50 NPS, belonging to different chemical classes in hair [24]. 4-MA, 4-MTA, 4-FA and butylone were among the investigated analytes. An aliquot of approximately 30 mg washed and cut hair was extracted by overnight incubation under sonication at 45°C using 0.1% formic acid in the presence of ISs. Analyses were performed using an UHPLC instrument coupled to triple quadrupole mass spectrometer. The column was a superficially porous Kinetex C18 column (2.6 μm, 100 × 2.1 mm) and the MPs used were: (A) 5 mM ammonium formate containing 0.1% formic acid and (B) methanol/acetonitrile 1:1 with 0.1% of formic acid. Two different MP gradients were employed; one for cannabinoids and one for cathinones and other stimulants because of too different chemical structures. Electrospray ionization (ESI) Jet-Stream was operated in positive mode. The method was proved to be reproducible and sensitive and applied to 50 samples coming from forensic cases (postmortem toxicological analyses, driving license regranting and workplace drug testing). None of the analytes under investigation were detected.

Hair samples from 325 routine cases, which originally tested positive for amphetamines or MDMA were reanalysed by Rust *et al*. in a study to evaluate the diffusion of NPS among typical MDMA and/or amphetamine users [28]. Hair fragments were decontaminated by a three-step washing procedure and extracted using a two-step extraction procedure (16 h, ultrasonication in methanol and 3 h, ultrasonication in acidified methanol). The analytes were separated using a liquid chromatography and detected by a linear ion trap quadrupole mass spectrometer operated in the ESI mode. Gradient elution was carried out on a Phenomenex Kinetex PFP column (2.6 mm, 50/2). The mobile phase consisted of 5mM ammonium formate buffer adjusted to pH 3.5 with formic acid (eluent A) and methanol containing 5mM ammonium formate (eluent B).

Among the compounds tested were 4-FA and butylone. 4-FA was identified in 4% of the cases under investigation.

Lendoiro *et al*. developed and fully validated a LC-MS/MS assay for the determination of 4-FMA among other psychoactive substances in hair [26]. Hair specimens (30 mg) were incubated in acidic methanol (0.1% HCl) and extracted by a mixed-mode SPE. Chromatographic separation was performed using an Atlantis T3 (3 μm; 2.1x50 mm) analytical column, and 2 mM ammonium formate pH 3 and acetonitrile as mobile phase. Precision and accuracy values within the acceptable range were obtained. Accuracy was expressed as the percentage of the nominal concentration, and was required to be within 85-115% of the target concentration. The validated method was applied to 16 hair samples, which were all found negative for 4-FMA.

2.2. Analytical Methods for the Determination of Piperazines in Hair

Piperazine-like compounds fall within the category of NPS and have already been found in the illegal drug market as substitute or constituent of MDMA in suspected MDMA seized pills. Piperazine-like compounds may act as stimulants and can produce euphoria, whereas some compounds of this class (*e.g*. TFMPP and mCPP) have small hallucinogenic potential [33].

Six studies described methods for the detection and quantification of piperazines in hair. In their previously mentioned multi-analyte LC–ESI-MS/MS assay after SPE, Imbert *et al*. included the determination of mCPP [25]. Using their UHPLC–MS/MS method after extraction in 0.1% formic acid Strano-Rossi *et al*. determined mCPP and benzylpiperazine [24]. Piperazines were not detected in none of the latter studies, when applied to real cases.

Another research group developed and validated a sensitive and simple gas chromatography-mass spectrometry (GC-MS) method for the identification and quantification of mCPP, TFMPP and MeOPP in hair [33]. A 20 mg decontaminated and cut hair sample was cleaned up by mixed mode solid-phase extraction after short hair incubation (40 min) with 1M NaOH at 50°C. Extracts were derivatized with N-methyl-N-(trimethylsilyl) trifluoroacetamide with 5% trimethylchlorosilane and p-tolylpiperazine (pTP) was used as IS. A gas chromatograph equipped with a mass selective detector and capillary column (30m×0.25mm I.D., 0.25μm film thickness) with 5% phenylmethylsiloxane was used for the analysis.

The method was applied to both post- and ante-mortem (subjects under antidepressant treatment with trazodone for the detection of mCPP and individuals undergoing psychiatric evaluation) real samples. Although results from the authentic hair samples were not presented, authors stated that the method could find application in forensic toxicology routine analysis.

Lendoiro *et al*. determined mCPP and TFMPP using the previously reported LC-MS/MS method which employed SPE for analytes extraction [26]. When the method was applied to 16 authentic hair specimens, ten positive results for mCPP were obtained in the range of 341.7-4000 pg/mg; however, in all cases trazodone identification (2085.3–4000 pg/mg) indicated a licit origin of mCPP as a metabolite of trazodone.

In their UHPLC–MS/MS multi-analyte assay Salomone *et al*. included hair testing of mCPP [27] and Rust and co-workers determined mCPP, benzylpiperazine and TFMPP using their method which employs 2 step extraction and LC-MS/MS analysis [28]. After the application of the latter method to 325 real cases, mCPP was found positive in 34

cases and TFMPP in one case. Five mCPP cases were also positive for trazodone.

2.3. Analytical Methods for the Determination of Synthetic Cathinones in Hair

Synthetic cathinones are chemical analogs of cathinone, a psychoactive monoamine alkaloid present in the shrub of Khat plant (Catha edulis). Cathinone derivatives are the β-keto (βk) analogues of the corresponding phenethylamine. Psychoactive stimulant substances belonging to the class of synthetic cathinones have been linked to varying degrees of agitation, confusional states, hyper arousal, and various types of psychotic behavior, up to and including a syndrome indistinguishable from excited delirium syndrome [43]. Moreover, synthetic cathinones can produce neurological and cardiovascular side-effects consistent with sympathomimetic toxicity [44].

Nine studies investigated the presence of synthetic cathinones in hair of possible consumers.

Namera *et al*. [35] were the first in detecting MDPV in hair sample coming from a deceased subject, whose cardiac blood contained 1200 ng/ml MDPV and 200 ng/ml α-PBP. Although the death was attributed to MDPV poisoning, hair test was performed to investigate chronic use. Hair samples were extensively washed with both aqueous and organic solutions and cut into 10 mm segments up to ten cm. Each segment was then treated with alkaline solution, in the presence of IS, and then extracted, evaporated and re-dissolved in methanol aqueous solution, before injected into the LC-MS system. Separation was achieved using a phenyl-hexyl column (150 mm x 2.1 mm i.d.,3 μm) with a mobile phase of methanol/ammonium formate solution (10 mM ammonia aqueous solution containing 0.1 % formic acid, pH 3.3) (35:65, v/v).

Segments analysed were positive for MDPV and α-PVP, with the highest MDPV concentration being 22 ng/10-mm hair, confirming previous intake of MDPV and α-PVP. Traces of α-PBP were also detected in the first segment (the one closet to the scalp). However, the concentrations provided per 10 mm and not per mg of hair did not allow any comparison with results from other studies. The authors reported that their findings suggest chronic MDPV and α-PVP use for at least 5 months, since both drugs were detected in the first 5 segments of 10 mm each.

In their multi-analyte method, Salomone *et al*. [27] determined α-PVP, buphedrone, amfepramone, 4-MEC, ethcathinone, mephedrone, methylone, pentedrone and MDPV after an extraction in methanol and determination in UHPLC–MS/MS. When they applied the method to 23 cases coming from MDMA and ketamine users, 3 were found positive to the latter drugs. In detail, in one case 4-MEC and mephedrone were detected at the concentration of 330 and 50 pg/mg, respectively; in another case, mephedrone was found at 59 pg/mg. In the last case of this group, α-PVP was detected together with methylone, MDPV and other NPS at the concentration of 1040 pg/mg, <LOQ and 120 pg/mg, respectively. In one out of 54 authentic samples from driver's license regranting which had been found negative, when tested for common drugs of abuse, methylone was quantified in a sample at 28 pg/mg.

In their above mentioned UHPLC–MS/MS method for NPS hair testing, Strano-Rossi *et al*. [24] included 3,4-DMMC, 4-MEC, ethcathinone, ethylone, mephedrone, methedrone, methylone, naphyrone, pentedrone, pentylone, 4-FMC and MDPV after hair treatment with formic acid 0.1%.

Three samples out of 50, coming from forensic cases, were positive for cathinones: MDPV at a concentration of 50 pg/mg in one case, 4-MEC at a concentration below LOQ in another and at 26 pg/mg in the last one.

In their LC-MS/MS method for the evaluation of the prevalence of NPS by hair testing, Rust *et al*. included the investigation of ethylone, mephedrone, methcathinone, methylone and MDPV in hair, after two step sample extraction with methanol and acidic methanol [28]. Methylone was detected in one out of 325 samples tested and mephedrone in 3% of the cases.

Pubic hair drug testing for the detection of 3-MMC and its metabolites, by means of liquid chromatography–high resolution/ high accuracy Orbitrap mass spectrometry (LC-HRMS), was used to document their potential intake by a drug dealer [7]. Pubic hair samples were decontaminated, pulverized using a ball mill, extracted in the presence of IS by overnight incubation with methanol:trifluoroacetic acid 9:1 and analysed.

The LC-HRMS system was equipped with a TFS Hypersil Gold PFP analytical column (2.1 x 100 mm, 1.9 μm particle size) and coupled to a TFS single-stage Exactive high energy collisional dissociation (HCD) MS system, interfaced with a TFS heated electrospray ionization HESI-II Ion Max source. MP A was water with 0.05% formic acid and 10 mM ammonium formate and MP B was acetonitrile with 0.05% formic acid.

Pubic hair specimen was positive for 3-methylmeth-cathinone (3-MMC) which was quantified at 25.8 ng/mg. 3-methylephedrine and 3-methylnorephedrine, metabolites of 3-MMC, were also detected in the same sample, suggesting 3-MMC intake by the drug dealer.

In their above reported multi-analyte LC-MS/MS assay, Lendoiro *et al*. [26] included hair testing of mephedrone, methedrone, methylone, 4-FMC and MDPV, after SPE sample treatment. However, none of those substances was detected in real cases tested.

After developing and validating a method for the determination of mephedrone in hair by GC-MS, Martin *et al*. reported the first series of mephedrone positive findings in hair samples [45]. Hair samples were decontaminated and incubated overnight in Soerensen buffer pH 7.0 in the presence of IS. Liquid-liquid extraction (LLE) was performed after alkalinisation of the incubation medium. The dry extract was derivatized, evaporated and reconstituted in ethyl acetate. Gas chromatographic separation was achieved with a 5-MS capillary column and detection by single ion monitoring in electron impact mode.

Thirteen out of 67 hair tested samples, were found positive for mephedrone with concentrations ranging from 0.2 to 313.2 ng/mg with a mean concentration of 26.8 ng/mg, showing enough method sensitivity to demonstrate both occasional and regular mephedrone intake.

Shah *et al*. were the first authors in developing and validating an UHPLC-MS/MS method for the quantification of not only mephedrone but also its two metabolites, 4-methylnorephedrine and 4-methylephedrine in hair [30]. Hair samples were enzymatically digested and extracted using LLE technique. The UHPLC-MS/MS system was operated in selective reaction monitoring (SRM) mode. The method was then applied to 154 real samples, 5 of which were confirmed as positive just for mephedrone (only one quantified at the concentration of 21.11 pg/mg).

Pichini *et al*. presented for the first time a case of a neonatal withdrawal syndrome in a baby born to a woman who was a chronic consumer of 4-methylethcathinone [19]. 4-MEC was identified and quantified by LC-MS/MS in neonatal meconium and in 4 subsequent 3 cm segments of maternal hair accounting for maternal consumption during the whole pregnancy (4.3, 4.0, 4.0 and 3.9 ng/mg hair starting from most proximal segment) demonstrating that maternal hair testing could provide useful information on chronic gestational use of 4-MEC in relation to neonatal outcome.

2.4. Analytical Methods for the Determination of Synthetic Cannabinoids in Hair

Synthetic cannabinoids are among the most commonly used NPS, which all act as agonists at the cannabinoid CB1 receptor. They are usually purchased as cannabis-like drugs, marketed as herbal blends and perceived as risk-free by inexperienced users. Synthetic cannabinoids may lead to severe health consequences, including tachycardia, anxiety, violent behavior, hallucinations and psychosis [46, 47].

2.4.1. Benzoylindoles

In their above reported method for hair NPS testing Strano-Rossi *et al*. [24] included a number of synthetic cannabinoids. The extraction process for these compounds differs from the previously mentioned one, due to the different chemical properties of cannabinoids. Briefly, thirty mg decontaminated and cut hair were incubated overnight under sonication at 45^{o}C with MeOH in the presence of JWH 210-d_9 as IS. Of benzoylindole group, AM2233, AM694, RCS-4 and WIN 48,098 were determined in hair. Out of 50 real cases, AM694 (30 pg/mg) was detected in one sample together with other synthetic cannabinoids of other chemical classes. Also Montenarh *et al*., included in their LC-MS/MS hair screening method cannabinoids of benzoyindole family: AM694 and RCS4, after extraction with diethyl ether-ethyl acetate mixture [29]. The method was applied to 13 hair samples but results were not given. Salomone *et al*. expanded a previously developed UHPLC-MS/MS method for the detection of 23 synthetic cannabinoids in hair specimens in order to include new emerging compounds [32]. Three out of the 23 cannabinnoids investigated come from the benzoyindole group *i.e*. AM694, RCS4 and WIN 48,098.

The newly developed and validated method was used for the evaluation of the consumption of the latter drugs over a large period of examination, and the diffusion of cannabimimetics among different populations of drug users. Prior to analysis hair specimens were undergone digestion with NaOH and extracted with a mixture of n-hexane/ ethylacetate. A reversed phase C18 column (100 mm × 2.1 mm, 1.7 μm), protected by a C18 VanGuard pre-column, was used for the separation of the analytes. Mobile phases were water/formic acid 20 mM plus ammonium formate 2 mM (solvent A) and acetonitrile/formic acid 20 mM (solvent B). Regarding instrumental analysis, ion source operated in the positive ion mode. The validated method was applied to 344 hair samples previously analysed for common drugs or alcohol; no cannabinoids of benzoylindole family detected.

Another sensitive, rapid and simple assay using LC-MS/MS was developed and fully validated by Hutter and co-workers for the determination of 22 synthetic cannabinoids in hair, 4 of them belonging to the benzoyindoles class: AM 694, RCS-4, RCS-4 ortho isomer and WIN 48,098 [34]. Extraction was achieved by 3-h ultrasonification of 50 mg hair in ethanol. Separation of the target analytes was carried out on a Luna Phenyl Hexyl column (50 mm × 2 mm, 5 μm) with an equivalent guard column (4 mm ×2 mm). Elution solvent A consisted of water with 0.2% formic acid and 2.0 mmol/L ammonium formate and solvent B was methanol. The method was proved to be precise, accurate, specific, linear and selective but no positive samples were found to these cannabinoids in examined cases.

A rapid screening method employing liquid chromato-graphy-quadrupole-time of flight mass spectrometry (LC-QTOF MS) was developed by Gottardo *et al*. for the detection of synthetic cannabinoids in hair [8]. From benzoylindoles, AM 694 was included in the assay. Hundred mg hair were cut and incubated overnight under strong basic conditions and were twice extracted by LLE. Organic layers from the two extractions were put together, evaporated, reconstituted in methanol and analysed. The separation was achieved on a Zorbax Eclipse XDB (2.1 x 150 mm, 5 μm) with gradient elution. 0.1% formic acid was the MP A and methanol was the MP B. The developed method was applied to 435 authentic hair samples from subjects to whom the driving license had been suspended/ withdrawn for ‘‘driving under the influence’’ of psychoactive drugs in order to evaluate the diffusion of these new compounds in the population, but none resulted positive for AM 694.

2.4.2. Naphthoylindoles

In their expanded UHPLC-MS/MS method for cannabinoids hair testing, Salomone *et al*. included the following cannabinoids of the naphthoylindole group: AM-1220, AM-2201, JWH-007, JWH-015, JWH-018, JWH-019, JWH-073, JWH-081, JWH-122, JWH-200, JWH-210, WIN 55,212-2 JWH-398 [32]. Fifteen real samples out of 344 tested were found positive for at least one synthetic cannabinoid of naphthoylindoles.

JWH-073 was detected in 11 samples out of 344 tested generally at low concentrations (mean 7.69 ± 14.4 pg/mg, median 1.9 pg/mg, range 1.6–50.5 pg/mg) followed by JWH-122 in 8 samples (mean concentration: 544 ± 968 pg/mg, median 28.4 pg/mg, range 7.4–2800 pg/mg). JWH-081 (range 8-194 pg/mg), JWH-018 (range 3.1-17.3 pg/mg),

JWH-210 (range 2.3-5.1 pg/mg), JWH-019 (range 3.8-4.1 pg/mg), and AM-1220 (1.3 pg/mg) were also detected.

AM-2201, JWH-007, JWH-015, JWH-018, JWH-019, JWH-073, JWH-081, JWH-098, JWH-122, JWH-200 and JWH-398 were present in the multi-analyte UHPLC–MS/MS method of Strano-Rossi *et al*. [24]. Three samples, out of 50 real cases, resulted positive for more than one synthetic cannabinoid. In the first case, JWH-073 (below LOQ), JWH-018 (20 pg/mg), JWH-081 (470 pg/mg) together with AM694 were determined. In another case, JWH-073 was detected at 2.1 ng/mg and JWH-018 at 90 pg/mg and finally in the third case JWH-073, JWH-018 and JWH-081 were all detected (<LOQ).

Likewise AM2201, JWH-015, JWH-018, JWH-073, JWH-122, JWH-200 and JWH-210 were included in the LC-MS/MS screening method of Montenarh *et al*., after extraction of the pulverized hair with diethyl ether-ethyl acetate mixture [29], but no positive result in real cases was obtained.

Hutter *et al*. determined 12 compounds of the naphthoylindole group: AM2201, JWH-007, JWH-015, JWH-018, JWH-019, JWH-073, JWH-081, JWH-122, JWH-200, JWH-210, WIN 55,212-2 and JWH-398, using the previously described LC-MS/MS cannabinoid-based method which employed ethanol extraction [34]. In 9 examined samples, the proximal hair segment tested positive for JWH-081 in 7 cases (range: 5.1-78 pg/mg), JWH-073 in 7 cases (range of 0.7-21 pg/mg), JWH-210 in 3 cases (range 0.5-5.2 pg/mg) and JWH-018 in two cases at 5.1 and 5.7 pg/mg, respectively. In the first segment, concentrations of up to 78 pg/mg JWH-081 were present. In segmental analysis, the levels of most substances increased from the first (closest to the scalp) to the third segment. The highest concentration was about 1100 pg/mg JWH-081. The findings of segmental hair tests in chronic users suggest side-stream smoke condensation as a major route of incorporation of the drugs in head.

Kim *et al*. developed and validated a LC/MS-MS method for the determination of 5 naphthoylindole-based synthetic cannabinoids and their metabolites in human and rat hair [37]. The following substances were invastigated: AM2201, AM-2201 N-4-OH M, AM-2201 N-6-OHindole M, JWH-122, JWH-122 N-5-OH, MAM-2201, MAM-2201 N-COOH M, MAM-2201 N-4-OH M. All analytes were extracted with methanol from washed and cut hair samples and the extracts were injected into LC–MS/MS with electrospray ion source in the positive ionization mode. AM-2201 and its monohydroxylated metabolites were detected in rat model and then the ratios of metabolite-to-parent drug were calculated to be used as criteria on external contamination. The effect of pigmentation on the distribution of AM-2201and its monohydroxylated metabolites in hair was also evaluated using animal model, showing no significant differences. Wide variation in concentrations of the 5 parent drugs and their metabolites was noticed when analyzing real hair samples coming from 9 cases of suspected of synthetic cannabinoids use; a range of 0.4–59.2 pg/mg for JWH-018, 0.1–0.8 pg/mg for JWH-073, 1.7–739.0 pg/mg for AM-2201, 0.1–402.0 pg/mg for JWH-122, 0.2–276.0 pg/mg for MAM-2201, 0.2–1.1 pg/mg for JWH-018N-COOH, 0.3–37.2 pg/mg for JWH-018 N-5-OH, 0.3 pg/mg for JWH-073 N-COOH, 0.4 pg/mg for AM-2201N-4-OH, 0.2–3.1 pg/mg for AM-2201 N-6-OHindole and 0.1–3.5 pg/mg for JWH-122 N-5-OH.

Three cannabinoids of the naphthoylindole category: JWH-018, JWH-073 and JWH-200 were investigated by Salomone *et al*. by hair digestion with NaOH and LLE extraction followed by analytes separation and detection with an UHPLC/MS-MS operating in the selected reaction monitoring mode [31]. A Zorbax XDB-C18 column 30 mm x 2.1 mm, 1.8 μm, protected by a C18 guard column, was used for the separation of the analytes. The elution solvents were water/formic acid 5mM (solvent A) and acetonitrile (solvent B). The method was successfully applied to the analysis of authentic samples collected from proven cannabis consumers. Fourteen out of 179 hair samples, two of which were pubic hair, collected by frequent users of THC-containing products, were found positive to at least one synthetic cannabinoid. JWH-018 was detected in 8 head hair samples and 1 pubic hair at the range of 0.6-70.5 pg/mg and 1.5 pg/mg, respectively. JWH-073 was determined in 7 head hair and 1 pubic hair specimen at the range of 0.5-413.3 pg/mg and 1.7 pg/mg, respectively. THC was always present in the samples tested positive for synthetic cannabinoids.

Also Kim *et al*. set up and validated an assay for the detection of JWH-018, JWH-073, and their metabolites in hair using UHPLC-MS-MS [36]. The analytes were extracted from hair with methanol; the extract was then filtered and analyzed by UHPLC–MS–MS with an electrospray ion source in positive-ionization mode. The validated method was then used for the investigation of the distribution of synthetic cannabinoids metabolites in hair; moreover the effect of hair pigmentation, by use of an animal model was also evaluated. The method was applied to authentic hair samples obtained by 18 individuals suspected of synthetic cannabinoids intake. The animal study showed that JWH-073 N-COOH M was the major metabolite of JWH-073 in rat hair, and hair pigmentation did not significantly affect the incorporation of JWH-073 and its metabolites into hair. JWH-018 (range: <LOQ-1700pg/mg), JWH-018 N-5-OH M (range: <LOQ-85 pg/mg), and JWH-073 (range: <LOQ-55 pg/mg) were detected in 18 real hair specimens.

In their LC-QTOF MS, Gottardo *et al*. method included the following substances: JWH-018, JWH-073, JWH-081, JWH-122, JWH-200 and JWH-210, after hair treatment under strong basic conditions and double LLE [8]. The latter substances were detected in 8 cases out of 435; two of which were found positive for more than one compound of naphthoylindoles.

JWH-018, JWH-073, JWH-081 and JWH-122 were detected in 3 (range 0.010 - 0.011 ng/mg), 2 (0.017 and 0.750 ng/mg), 4 (range 0.016 – 1.280 ng/mg) and 1 (0.125 ng/mg) cases, respectively.

2.4.3. Phenylacetylindoles

Montenarh *et al*. investigated also the following substances of the phenylacetylindole group using their LC-MS/MS screening method after extraction of the pulverized hair with diethyl ether-ethyl acetate mixture: JWH-203, and

JWH-250 [29]. The method was applied to 13 hair samples; however results were not provided. Likewise, Salomone *et al*. using their expanded method developed for the determination of cannabinoids in hair analysed for JWH-203, JWH-250, JWH-251 and RCS-8 [32]. JWH-250 found positive in 6 cases (range: 4.8-83.4 pg/mg) out of 15 cases tested positive for synthetic cannabinoids and a total of 334 samples.

Hutter *et al*. analysed for the same substances as Salomone *et al*. using the previously described LC-MS/MS cannabinoid-based method which employs ethanol extraction [31, 32, 34]. JWH-250 was detected in 8 real samples together with other synthetic cannabinoids at the range of 0.5-24 pg/mg.

Strano-Rossi *et al*. analysed the following substances of phenylacetylindole group using the previously mentioned UHPLC–MS/MS multi-analyte method after MeOH extraction: JWH-250, JWH-251, JWH-302 and RCS-8 [24], whereas In their first cannabinoid-based method in hair Salomone *et al*. and using their LC-QTOF MS method Gottardo *et al*. [8] analysed for JWH-250 [31]. JWH-250 detected in 8 authentic samples (range: 1.5-729.4 pg/mg) and in one case at the concentration of 12 pg/mg by Salomone *et al*. and Gottardo *et al*., respectively.

2.4.4. Naphthoylpyrroles

Two group of authors included naphthoylpyrroles in their assays: Strano-Rossi *et al*. determinates JWH-030, JWH-147 and JWH-307 [24], whereas Salomone *et al*. included only the JWH-307 in their previously described LC-MS/MS method [32]. None of these compounds detected when the latter methods were applied to real cases.

2.4.5. Other Cannabinoids

Using both their cannabinoid-based assays Salomone *et al*. [31, 32] investigated the presence of HU-210 in hair, whereas using their expanded LC-MS/MS method they checked for hair JWH-020 [32]. Applying the previously described LC-MS/MS cannabinoid-based method which employs ethanol hair extraction, Hutter *et al*. [34] determined methanandamide (AM-356) and JWH-020. None of these substances identified when the latter methods were applied to authentic specimens.

Park *et al*. [38] described a quantitative analytical method for the determination of XLR-11 and its metabolites in hair using LC-MS/MS, since the latter drug has been widely abused in South Korea recently. The authors stated that the detection of the metabolites in hair samples could exclude external contamination and prove actual consumption. The hair specimens were washed, cut and extracted in methanol. LC–MS/MS with electrospray ion source operating in positive-ionization mode was used for the analysis. The chromatographic separation was performed in a Zorbax Eclipse plus C18 (RRHD 2.1×100mm, 1.8μm). The validation results suggest that the method was accurate, selective, linear and precise.

It was then applied to 14 hair samples taken from individuals suspected of XLR-11 use. XLR-11 and 4 out of the 5 metabolites tested were detected (mainly UR-144 N-5-hydroxy metabolite and UR-144 N-pentanoic acid). Wide variation was noticed regarding the concentrations revealed from the analysis of the latter samples both for XLR-11 and its metabolites, with the concentration of the parent drug ranging from 0.8 to 5350.0 pg/mg. The concentration of XLR-11 as a parent drug was much higher compared to the metabolites. In one case only the parent drug was identified at low concentration leading the authors to suggest either external contamination of the hair sample or intake of impurity of other synthetic cannabinoids. UR-144 N-4-hydroxypentyl metabolite was not detected in authentic samples.

Strano-Rossi *et al*. investigated the following substances: CB-13, JWH-016 and JWH-201 using the previously reported UHPLC–MS/MS multi-analyte method after MeOH extraction [24]. None of these analytes were detected in the authentic samples.

Franz *et al*. [39] performed LC- MS/MS hair analysis of a real sample collected from a patient with a known history of abuse of synthetic cannabinoids. 5F-PB-22 and ABCHMINACA as well as their main metabolites 5F-PB-22 3-carboxyindole, PB-22 5-OH-pentyl, and AB-CHMINACA valine were identified in all segments. The authors evaluated the stability of the parent drugs in the hair matrix and under thermal stress, revealing that the three metabolites are also formed in externally contaminated hair after their storage at different conditions. LLOQs of 1 and 2.5 pg/mg were achieved for 5F-PB-22 and AB-CHMINACA, respectively.

Moreover, 5F-PB-22 3-carboxyindole and AB-CHMINACA valine were detected as degradation products in smoke condensate. Thus the authors concluded that caution must be taken when interpreting the 'metabolite' findings of compounds consisting of chemically labile amide/ ester bonds or 5-fluoro-pentyl side chains, taking into consideration the different mechanisms of formation and incorporation into hair.

A method for the detection of indole-derived cannabinoids (naphthoylindoles, phenylacetylindoles and benzoylindoles) in hair, oral-fluid, blood and urine has been developed using LC-MS/MS based on the use of precursor ion scan as the acquisition mode [40]. The authors proposed an alternative approach that focuses on the detection of specific molecular moieties, recurrent within the same pharmacological class of substances. The method was developed to recognize one or more common "structural markers", which correspond to mass spectral fragments coming from the specific moiety of the molecular structure that is common to the aminoalkylindole analogues and that is essential for their pharmacological categorization. Therefore, the assay is also suitable for identifying unknown compounds, given that they contain the targeted portion of the molecular structure.

2.5. Analytical Methods for the Determination of other NPS in Hair

Salomone *et al*. in their multi-analyte method included also MXE, 4-MeO-PCP and diphenidine after an extraction in methanol and determination by UHPLC–MS/MS [27]. Analyzing hair samples from MDMA or ketamine users, MXE was detected in three samples with a range of

concentration between 7.7 and 28 pg/mg and diphenidine in one sample at the concentration of 4400 pg/mg.

3. DISCUSSION AND CONCLUSION

The huge amount of methods detailed in the table and the results on real cases presented in the previous Sections suggest that methods for the determination of NPS in hair, are in continuous development together with some existing analytical methods for hair testing 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. Reanalysis 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. The presented results strongly support the use of hair analysis to monitor the diffusion of new psychoactive drugs in the users' community.

Advances in mass spectrometry technology enabled the improvement of sensitivity so that these novel substances, whose used doses are in the majority of cases unknown can be detected in 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 and will be indispensable tools in clinical and forensic toxicology and doping control. Whereas GC/MS in the 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/MS with different mass analyzer types is becoming more and more a standard technique for automated target screening procedures and particularly for high –throughput quantification. In fact LC/MS has shown to be an ideal supplement, especially for detection of more polar, unstable or low-dose drugs [48-53]. This latter evidence is supported by our results; 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, LC-QTOF MS and LC-HRMS have also been used with LODs at decimal picograms achieved.

With both types of analytes separation (GC or LC), the run time reported for the examined methods was quite short since the high throughput of analysis in pharmacotoxicological laboratories is now a principal crucial task in forensic toxicology and clinical pharmacotoxicology. Matrix decontamination is considered to be one of the main limitations in hair testing. The Society of Hair Testing (SoHT) guidelines 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 here presented methods, 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 Society of Hair Drug Testing for traditional drugs of abuse [54].

A pitfall, common with the published procedures for common 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; various extraction methods have been suggested to isolate NPS from hair such as methanol extraction or hydrolysis in alkaline solution followed by LLE or SPE depending on the chemical properties of the analytes.

Among the positive samples obtained from the analysis of authentic cases, the measured levels for most of the drugs were interestingly in the range of picograms of drug per milligrams of hair, either suggesting sporadic exposure to these substances or low rate of incorporation into the keratin matrix. However, only limited literature data concerning the detection of these new drugs in hair samples are currently available, making the interpretation of NPS concentrations in hair samples still ambiguous [27].

Moreover, the need for established cut-off values to discriminate between chronic consumption and occasional use (or external contamination) is underlined since epidemiological studies based on hair testing on NPS are still few and different ranges of concentration for different types of consumption patterns are far from being decided.

The results on hair samples compliment a series of investigation already presented in other papers applied in different biological matrices which can create a clinical set for the demonstration of consumption, which can be then associated with the clinical outcome with an intoxication or a fatality.

LIST OF ABBREVIATIONS

2 C-B	=	2,5-dimethoxy-4-bromophenethyl-amine
2 C-D	=	2,5-dimethoxy-4-methylphene-thylamine
2 C-E	=	2,5-dimethoxy-4-ethylphenethyl-amine
2 C-I	=	2,5-dimethoxy-4-iodophenethyl-amine
2 C-P	=	2,5-dimethoxy-4-(n)-propylphene-thylamine
2 C-T	=	2-(2,5-dimethoxy-4-(methylthio) phenyl)ethanamine or 4-methylthio-2,5-DMPEA
25B-NBOMe	=	2-(4-bromo-2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl)methyl] ethanamine
25C-NBOMe	=	2-(4-chloro-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl] ethanamine
25H-NBOMe	=	2-(2,5-dimethoxyphenyl)-N-(2-methoxybenzyl)ethanamine

25I-NBOMe	=	2-(4-iodo-2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl)methyl] ethanamine
3,4-DMMC	=	3,4-Dimethylmethcathinone
3-MMC	=	3-Methylmethcathinone
4-FA	=	4-Fluoroamphetamine
4-FMA	=	4-Fluoromethamphetamine
4-FMC	=	4-fluoromethcathinone or flephedrone
4-MA	=	4-Methylamphetamine
4-MEC	=	4-Methylethcathinone
4-MeO-PCP	=	4-Methoxyphencyclidine
4-MTA	=	4-Methylthioamphetamine
5F-PB-22	=	1-pentyfluoro-1H-indole-3-carboxylic acid 8-quinolinyl ester
5-MAPB	=	1-(Benzofuran-5-yl)-N-methylpropan-2-amine
6-APB	=	6-(2-aminopropyl)benzofuran
AB-CHMINACA	=	N-[(1S)-1-(Aminocarbonyl)-2-methylpropyl]-1-(cyclohexylmethyl)-1H-indazole-3-carboxamide
AM-1220	=	(R)-(1-((1-methylpiperidin-2-yl) methyl)-1H-indol-3-yl)(naphthalen-1-yl)methanone
AM2201	=	1-(5-fluoropentyl)-3-(1-naphthoyl) indole
AM2233	=	1-[(N-methylpiperidin-2-yl) methyl]-3-(2-iodobenzoyl)indole
AM694	=	1-(5-fluoropentyl)-3-(2-iodobenzoyl) indole
CB-13	=	naphthalen-1-yl-(4-pentyloxynaphthalen-1-yl)methanone
HU-210	=	(6aR,10aR)- 9-(Hydroxymethyl)-6,6-dimethyl- 3-(2-methyloctan-2-yl)- 6a,7,10,10a-tetrahydrobenzo [c] chromen- 1-ol
JWH-007	=	1-pentyl-2-methyl-3-(1-naphthoyl) indole
JWH-015	=	(2-Methyl-1-propyl-1H-indol-3-yl)-1-naphthalenylmethanone
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-020	=	(1-heptyl-1H-indol-3-yl)-1-naphthalenyl-methanone
JWH-030	=	naphthalen-1-yl-(1-pentylpyrrol-3-yl)methanone
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-122	=	(4-methyl-1-naphthyl)-(1-pentylindol-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-201	=	2-(4-methoxyphenyl)-1-(1-phentyl-1H-indol-3-yl)-ethanone
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
MAM-2201	=	(1-(5-fluoropentyl)-1H-indol-3-yl)(4-methyl-1-naphthalenyl)-methanone
mCPP	=	meta-Chlorophenylpiperazine
MDPV	=	Methylenedioxypyrovalerone
MeOPP	=	para-Methoxyphenylpiperazine
MXE	=	Methoxetamine
PMA	=	para-Methoxyamphetamine
PMMA	=	para-Methoxy-N-methylamphetamine
RCS-4	=	1-pentyl-3-(4-methoxybenzoyl) indole
RCS-8	=	1-(2-cyclohexylethyl)-3-(2-methoxyphenylacetyl)indole
TFMPP	=	3-Trifluoromethylphenylpiperazine
UR-144	=	(1-pentylindol-3-yl)-(2,2,3,3-tetramethylcyclopropyl)methanone
WIN 48,098	=	Pravadoline or (4-methoxyphenyl)-[2-methyl-1-(2-morpholin-4-ylethyl) indol-3-yl]methanone

WIN 55,212-2	=	(R)-(+)-[2,3-Dihydro-5-methyl- 3-(4-morpholinylmethyl)pyrrolo [1,2,3-de]-1,4-benzoxazin-6-yl]-1-napthalenylmethanone
XLR-11	=	(1-(5-fluoropentyl)-1H-indol-3-yl) (2,2,3,3-tetramethylcyclopropyl) methanone
α-PBP	=	α-Pyrrolidinobutiophenone
α-PVP	=	α-Pyrrolidinopentiophenone

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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