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MONITORING OF NEW PSYCHOACTIVE SUBSTANCES IN THE HAIR OF PATIENTS WITH A HISTORY OF ADDICTION. EVALUATION OF THE PUBLIC HEALTH IMPACT.

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Abstract

Introduction. The term New Psychoactive Substances (NPS) encompasses a broad category of drugs, including molecules synthesized more than 80 years ago which have become available on the market in recent years, and molecules which have been used for some time in the medical field, whose illicit use for recreational purposes has recently exploded [8]. The analysis of NPS usually requires mass spectrometry (MS) based techniques. The aim of our study was to define the prevalence of NPS consumption in patients with a history of drug addiction followed by Public Services for Pathological Addictions (Ser.DP), with the purpose of highlighting the effective presence of NPS within the metropolitan area of Bologna and evaluating the association of consumption of NPS and drugs of abuse (DOA).

Materials and methods. Sustained by literature, a multi-analyte UHPLC-MS/MS method for the identification of 127 NPS (phenethylamines, arylcyclohexylamines, synthetic opioids, tryptamines, synthetic cannabinoids, synthetic cathinones, designer benzodiazepines) and 15 classic drugs of abuse (DOA) in hair samples was developed and validated according to International Guidelines [112]. Samples pretreatment consisted of washing steps and overnight incubation at 45°C in an acid mixture of methanol and water. After cooling, supernatant were injected into the chromatographic system coupled with a tandem mass spectrometry detector.

Results. Successful validation was achieved for almost all of the compounds. The method met all the required technical parameters. LOQ was set from 4 to 80 pg/mg The developed method was applied to 107 cases (85 males and 22 females) of clinical interest. Out of 85 hair samples resulting positive to classical drugs of abuse, NPS were found in twelve (8 male and 4 female).

Conclusion. The present methodology represents an easy, low cost, wide-panel method for the detection of 127 NPS and 15 DOA in hair samples. Such multi-analyte methods facilitates the study of the prevalence of drugs abused that will enable the competent control authorities to obtain evidence-based reports regarding the critical spread of the threat represented by NPS.

Monitoring of New Psychoactive Substances in the hair of patients with a history of addiction. Evaluation of the public health impact.

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Chapter 3. Experimental study

Semi-systematic and IUPAC names

(±)-cis-3-methyl Norfentanyl: N-[(3R,4S)-3-methylpiperidin-4-yl]-N-phenylpropanamide

(±)-trans-3-methyl Norfentanyl: N-[(3R,4R)-3-methylpiperidin-4-yl]-N-phenylpropanamide

αET (α-Ethyltryptamine): 1-(1H-indol-3-yl)butan-2-amine

α-PHP (alpha-pyrrolidinohexiophenone): 1-phenyl-2-pyrrolidin-1-ylhexan-1-one

β-Hydroxy fentanyl: N-[1-(2-hydroxy-2-phenylethyl)piperidin-4-yl]-N-phenylpropanamide

β-Hydroxythiofentanyl: N-[1-(2-hydroxy-2-thiophen-2-ylethyl)piperidin-4-yl]-Nphenyl propanamide

β-Phenyl fentanyl: N-(1-phenethylpiperidin-4-yl)-N,3-diphenylpropanamide

2-F-DCK (2-fluoro-deschloroketamine): 2-(2-Fluorophenyl)2—methylamino-cyclohexanone

2-Methyl AP-237: 2-methyl-1-butyryl-4-cinnamylpiperazine

EDDP (2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine)

3,4-DMMC (3,4-dimethylmethcathinone): 1-(3,4-dimethylphenyl)-2-(methylamino)propan-1-one

3-methoxy PCE (3-methoxy eticyclidine): N-ethyl-1-(3-methoxyphenyl)-cyclohexanamine

3-MMC (3-methylmethcathinone): 2-(Methylamino)-1-(3-methylphenyl)-1-propanone

3,4 MD-alfa-PHP (3,4-Methylenedioxy-α-pyrrolidinohexanophenone): 1-(1,3-benzodioxol-5-yl)-2-(1-pyrrolidinyl)-1hexanone

4-AcO-DiPT (4-acetoxy-N,N-Diisopropyltryptamine): 3-[2-[bis(1-ethylethyl)amino]ethyl]-1H-indol-4-ol

4-ANPP: N-phenyl-1-(2-phenylethyl)piperidin-4-amine

4-OH-DET (4-hydroxy Diethyltryptamine): 3-[2-(diethylamino)ethyl]-1H-indol-4-ol

4-FMC (4-Fluoromethcathinone): 1-(4-fluorophenyl)-2-(methylamino)propan-1-one

4F-MDMB-BUTICA: methyl (S)-2-(1-(4-fluorobutyl)-1H-indole-3-carboxamido)-3,3-dimethylbutanoate

4-MEC (4-Methylethcathinone): 2-(ethylamino)-1-(4-methylphenyl)propan-1-one

5Cl-AB-PINACA (5-cloro-AB-PINACA): *N*-[(2*S*)-1-amino-3-methyl-1-oxobutan-2-yl]-1-(5-chloropentyl)indazole-3-carboxamide

5Cl-THJ-018 (5-chloropentyl JWH 018 indazole analog): 1-(5-Chloropentyl)-1H-indazol-3-yl](1-naphthyl)methanone

5-EAPB (5-(2-Ethylaminopropyl)Benzofuran): 1-(1-benzofuran-5-yl)-N-ethylpropan-2-amine

5F-ADB: methyl (2R)-2-[[1-(5-fluoropentyl)indazole-3-carbonyl]amino]-3,3-dimethylbutanoate

5F-AKB48 (APINACA N-(5-fluoropentyl) analog): N-((3s,5s,7s)-adamantan-1-yl)-1-(5-fluoropentyl)-1H-indazole-3-carboxamide

5F-APP-PICA (PX-1): N-(1-amino-1-oxo-3-phenylpropan-2-yl)-1-(5-fluoropentyl)indole-3- carboxamide

5F-APP-PINACA (PX-2): N-(1-amino-1-oxo-3-phenylpropan-2-yl)-1-(5-fluoropentyl)-1Hindazole-3-carboxamide

5F-CUMYL-P7AICA (SGT-263): 1-(5-fluoropentyl)-N-(2-phenylpropan-2-yl)-1H-pyrrolo[2,3-b]pyridine-3-carboxamide

5F-Cumyl-PEGACLONE: 5-(5-Fluoropentyl)-2-(2-phenylpropan-2-yl)-2,5-dihydro-1Hpyrido[4,3-b]indol-1-one

5F-Cumyl-PICA: 1-(5-fluoropentyl)-*N*-(2-phenylpropan-2-yl)indole-3-carboxamide

5F-Cumyl-PINACA: 1-(5-fluoropentyl)-N-(2-phenylpropan-2-yl)indazole-3-carboxamide

5F_EDMB-PICA: ethyl(S)-2-(1-(5-fluoropentyl)-1H-indole-3-carboxamido)-3,3-dimethylbutanoate

5F-EMB-PICA: N-[[1-(5-fluoropentyl)-1H-indol-3-yl]carbonyl]-L-valine

5F-EMB-PINACA: Ethyl 2-[[1-(5-fluoropentyl)indazole-3-carbonyl]amino]-3-methyl-butanoate 5

5F-MDMB-P7AICA: methyl (S)-2-(1-(5-fluoropentyl)-1H-pyrrolo[2,3-b]pyridine-3- carboxamido)-3,3-dimethylbutanoate

5F-MDMB-PICA (5F-MDMB-2201): methyl (2S)-2-{[1-(5-fluoropentyl)-1H-indole-3- carbonyl]amino}-3,3-dimethylbutanoate

5F-NNEI 2'-Naphthyl Isomer: 1-(5-Fluoropentyl)-N-(naphthalen-2-yl)-1H-indole-3-carboxamide

5-HTP (5-hydroxytryptophan): 2-amino-3-(5-hydroxy-1H-indol-3-yl)propanoic acid

5-MeO-AMT (5-methoxy-α-methyltryptamine): 1-(5-methoxy-1H-indol-3-yl)propan-2-amine

5-MeO-DALT (N,N-di allyl-5-methoxy tryptamine): N-[2-(5-methoxy-1H-indol-3-yl)ethyl]-N-prop-2-enylprop-2-en-1-amine

5-MeO-DMT (5-methoxy-N,N-dimethiltryptamine): 2-(5-methoxy-1H-indol-3-yl)-N,N-dimethylethanamine

5-MeO-DPT (5-methoxy-N,N-Dipropyltryptamine): N-[2-(5-methoxy-1H-indol-3-yl)ethyl]-N-propylpropan-1-amine

5-MeO-MiPT (5-methoxy-N-methyl-N-isopropyltryptamine): N-[2-(5-methoxy-1H-indol-3-yl)ethyl]-N-methylpropan-2-amine

6-mono-acetyl-morphine (6-MAM): (9-hydroxy-3-methyl-2,4,4a,7,7a,13-hexahydro-1H-4,12-methanobenzofuro[3,2-e]isoquinolin-7-yl)

acetyl fentanyl: N-Phenyl-N-[1-(2-phenylethyl)-4-piperidinyl]-acetamide

acetyl norfentanyl: N-phenyl-N-piperidin-4-ylacetamide

ADB-FUBINACA: N-[(2S)-1-amino-3,3-dimethyl-1-oxobutan-2-yl]-1-[(4-fluorophenyl)methyl]-1H-indazole-3- carboxamide

alfentanyl: N-[1-[2-(4-ethyl-5-oxotetrazol-1-yl)ethyl]-4-(methoxymethyl)piperidin-4-yl]-Nphenylpropanamide

AM-2201: [1-(5-fluoropentyl)indol-3-yl]-naphthalen-1-ylmethanone

AM-2233: (2-iodophenyl)-[1-[(1-methylpiperidin-2-yl)methyl]indol-3-yl]methanone

AM-694: [1-(5-fluoropentyl)indol-3-yl]-(2-iodophenyl)methanone

Amphetamine (AMP): 1-phenylpropan-2-amine

AP-237 (1-Butyryl-4-cinnamylpiperazine): 1-[4-(3-phenyl-2-propen-1-yl)-1-piperazinyl]-1-butanone

APB (alpha-Methyl-5-benzofuranethanamine): 1-(1-benzofuran-yl)propan-2-amine

APP-FUBINACA: N-[(2S)-1-amino-1-oxo-3-phenylpropan-2-yl]-1-[(4- fluorophenyl)methyl]indazole-3-carboxamide

bentazepam: 5-phenyl-1,3,6,7,8,9-hexahydro-[1]benzothiolo[2,3-e][1,4]diazepin-2-one

BEC (benzoilecgonine): (1R,2R,3S,5S)-3-benzoyloxy-8-methyl-8-azabicyclo[3.2.1]octane-2-carboxylic acid

brorphine: 3-[1-[1-(4-bromophenyl)ethyl]piperidin-4-yl]-1H-benzimidazol-2-one

buphedrone: 2-(methylamino)-1-phenylbutan-1-one

butyryl fentanyl: N-phenyl-N-[1-(2-phenylethyl)piperidin-4-yl]butanamide

butyryl fentanyl carboxy metabolite: 4-oxo-4-(N-[1-(2-phenylethyl)piperidin-4- yl]anilino)butanoic acid

butyryl norfentanyl: (5Z,8Z,11Z,14Z,16R)-16-hydroxyicosa-5,8,11,14-tetraenoic acid

buthylone: 1-(1,3-benzodioxol-5-yl)-2-(methylamino)butan-1-one

butonitazene: 2-[2-[(4-butoxyphenyl)methyl]-5-nitrobenzimidazol-1-yl]-N,N-diethylethanamine

cannabidiol (CBD): 2-[(1R,6R)-3-methyl-6-prop-1-en-2-ylcyclohex-2-en-1-yl]-5-pentylbenzene-1,3-diol

carfentanil: methyl 1-(2-phenylethyl)-4-(N-propanoylanilino)piperidine-4-carboxylate

clonazolam: 6-(2-chlorophenyl)-1-methyl-8-nitro-4H-[1,2,4]triazolo[4,3-a][1,4]benzodiazepine

cocaine (COC): methyl (1R,2R,3S,5S)-3-benzoyloxy-8-methyl-8-azabicyclo[3.2.1]octane-2-carboxylate

cocaethylene (COCAET): ethyl (1R,2R,3S,5S)-3-benzoyloxy-8-methyl-8-azabicyclo[3.2.1]octane-2-carboxylate

codeine (COD): (4R,4aR,7S,7aR,12bS)-9-methoxy-3-methyl-2,4,4a,7,7a,13-hexahydro-1H-4,12-methanobenzofuro[3,2-e]isoquinolin-7-ol

cumyl-PEGACLONE (SGT-151): 5-pentyl-2-(2-phenylpropan-2-yl)-2,5-dihydro-1H-pyrido[4,3-b]indol-1-one

cyclopropylfentanyl: N-phenyl-N-[1-(2-phenylethyl)piperidin-4-yl] cyclopropanecarboxamide
delta-9-tetrahydrocannabinol (delta-9-THC):
despropionyl para-fluorofentanyl: N-(4-fluorophenyl)-1-phenethylpiperidin-4-amine
diclazepam: 7-chloro-5-(2-chlorophenyl)-1-methyl-3H-1,4-benzodiazepin-2-one
dimethylcathinone: 2-(Dimethylamino)-1-phenylpropan-1-on
ecgonine methyl estere (EME):
ethcathinone: 2-(ethylamino)-1-phenylpropan-1-one 6
ethylone: 1-(1,3-benzodioxol-5-yl)-2-(ethylamino)propan-1-one
ethylphenidate: ethyl 2-phenyl-2-piperidin-2-ylacetate
etizolam: 7-(2-chlorophenyl)-4-ethyl-13-methyl-3-thia-1,8,11,12-tetrazatricyclo[8.3.0.02,6]trideca-2(6),4,7,10,12-pentaene
etodesnitazene: 2-[2-[(4-ethoxyphenyl)methyl]benzimidazol-1-yl]-N,N-diethylethanamine
euthylone: 1-(1,3-benzodioxol-5-yl)-2-(ethylamino)butan-1-one
fentanyl: N-phenyl-N-[1-(2-phenylethyl)piperidin-4-yl]propenamide
flualprazolam: 8-chloro-6-(2-fluorophenyl)-1-methyl-4H-[1,2,4]triazolo[4,3-a][1,4]benzodiazepine
flunitazene: N,N-diethyl-2-[2-[(4-fluorophenyl)methyl]-5-nitrobenzimidazol-1-yl]ethanamine
furanyl fentanyl: N-phenyl-N-[1-(2-phenylethyl)piperidin-4-yl]furan-2-carboxamide
furanyl norfentanyl: N-phenyl-N-piperidin-4-ylfuran-2-carboxamide
isobutyryl fentanyl: 2-methyl-N-phenyl-N-[1-(2-phenylethyl)piperidin-4-yl]propanamide
isotonitazene: N,N-diethyl-2-[5-nitro-2-[(4-propan-2-yloxyphenyl)methyl]benzimidazol-1-yl]ethanamine
JWH-007: (2-methyl-1-pentylindol-3-yl)-naphthalen-1-ylmethanone
JWH-016: (1-butyl-2-methylindol-3-yl)-naphthalen-1-ylmethanone
JWH-018: (1-pentyl-1H-indol-3-yl)-1-naphthalenyl-methanone
JWH-019: (1-hexyl-1H-indol-3-yl)(naphthalen-1-yl)methanone
JWH-081: (4-Methoxynaphthalen-1-yl)(1-pentyl-1H-indol-3-yl)methanone
JWH-098: (4-methoxynaphthalen-1-yl)-(2-methyl-1-pentylindol-3-yl)methanone
JWH-122: (4-Methylnaphthalen-1-yl)(1-pentyl-1H-indol-3-yl)methanone
JWH-200: [1-(2-morpholin-4-ylethyl)indol-3-yl]-naphthalen-1-ylmethanone
JWH-203: 2-(2-chlorophenyl)-1-(2-methyl-1-pentyl-1H-indol-3-yl)ethanone
JWH-210: (4-ethyl-1-naphthalenyl)(1-pentyl-1H-indol-3-yl)-methanone
JWH-250: 2-(2-methoxyphenyl)-1-(1-pentyl-1H-indol-3-yl)ethanone
JWH-251: 2-(3-methylphenyl)-1-(1-pentyl-1H-indol-3-yl)ethanone
JWH-302: 2-(3-methoxyphenyl)-1-(1-pentylindol-3-yl)ethenone

JWH-398: (4-chloronaphthalen-1-yl)(1-pentyl-1H-indole-3-yl)methanone
ketamine (KETA): 2-(2-chlorophenyl)-2-(methylamino)cyclohexan-1-one
MAPB (5-(N-Methyl-2-aminopropyl)benzofuran): 1-(benzofuran-yl)-N-methylpropan-2-amine
MDMB-4en-PICA: methyl (2S)-3,3-dimethyl-2-[(1-pent-4-enylindole-3-carbonyl)amino]butanoate
MDMB-4en-PINACA: methyl (S)-3,3-dimethyl-2-(1-(pent-4-en-1-yl)-1H-indazole-3- carboxamido)butanoate
MDMB-CHMICA: methyl (2S)-2-{[1-(cyclohexylmethyl)-1H-indole-3-carbonyl]amino}3,3- dimethylbutanoate
Methylenedioxypirovalerone (MDPV): 1-(1,3-benzodioxol-5-yl)-2-(pyrrolidin-1-yl)pentan-1-one
mephedrone (4-Methyl MCAT, 4-MMC): 2-(Methylamino)-1-(4-methylphenyl)propan-1-one
methadone (MTD): 6-(dimethylamino)-4,4-diphenylheptan-3-one
methamphetamine (METH): (2S)-N-methyl-1-phenylpropan-2-amine
methylenedioxyamphetamine (MDA): 1-(1,3-benzodioxol-4-yl)propan-2-amine
methylenedioxymethamphetamine (MDMA): 1-(1,3-benzodioxol-5-yl)-N-methylpropan-2-amine
methcathinone(MCAT): 2-(methylamino)-1-phenylpropan-1-one
methedrone (4-Methoxy MCAT): 1-(4-methoxyphenyl)-2-(methylamino)propan-1-one
methoxpropamine: 2-(3-methoxyphenyl)-2-(propylamino)cyclohexan-1-one
methoxy acetyl fentanyl: 2-methoxy-N-phenyl-N-[1-(2-phenylethyl)-4-piperidinyl]-acetamid
methoxy acetyl norfentanyl: 2-methoxy-N-phenyl-N-piperidin-1-ium-4-ylacetamide
methylone: 1-(1,3-benzodioxol-5-yl)-2-(methylamino)propan-1-one
metodesnitazene: N,N-diethyl-2-[2-[(4-methoxyphenyl)methyl]benzimidazol-1-yl]ethanamine
metonitazene: N,N-diethyl-2-[2-[(4-methoxyphenyl)methyl]-5-nitrobenzimidazol-1-yl]ethanamine
MMB2201 (5F-AMB-PICA): methyl (2S)-2-{[1-(5-fluoropentyl)-1H-indole-3- carbonyl]amino}-3-methylbutanoate
morphine (MOP): (4R,4aR,7S,7aR,12bS)-3-methyl-2,4,4a,7,7a,13-hexahydro-1H-4,12-methanobenzofuro[3,2-e]isoquinoline-7,9-diol
N-ethyl-penthylone: 1-(1,3-benzodieoxol-5-yl)-2-(ethylamino)pentan-1-on
N-pyrrolidino etonitazene: 2-[(4-ethoxyphenyl)methyl]-5-nitro-1-[2-(1-pyrrolidinyl)ethyl]-1H-benzimidazole
N,N-Dimethyltryptamine (DMT): 2-(1H-indol-3-yl)-N,N-dimethylethanamine
norfentanyl: N-phenyl-N-piperidin-4-ylpropanamide
norketamine: 2-amino-2-(2-chlorophenyl)cyclohexan-1-one
O-PCE (deschloro-N-ethyl-ketamine): 2-(ethylamino)-2-phenyl-cyclohexanone
ocfentanyl: N-(2-fluorophenyl)-2-methoxy-N-[1-(2-phenylethyl)piperidin-4-yl]acetamide

p-fluoro-furanyl fentanyl: N-(4-fluorophenyl)-N-[1-(2-phenylethyl)piperidin-4-yl]furan-2-carboxamide

pentylone: 1-(1,3-benzodioxol-5-yl)-2-(methylamino)pentan-1-on

phenylfentanyl: N-phenyl-N-[1-(2-phenylethyl)-4-piperidyl]benzamide

phenylacetyl fentanyl: N,2-diphenyl-N-[1-(2-phenylethyl)piperidin-4-yl]acetamide

pravadoline (WIN 48,098): (4-methoxyphenyl)-[2-methyl-1-(2-morpholin-4-ylethyl)indol-3-yl] methanone

psylocibin: [3-[2-(dimethylamino)ethyl]-1H-indol-4-yl] dihydrogen phosphate

RCS-4: (4-methoxyphenyl)(1-pentyl-1H-indol-3-yl)methanone

RCS-8: 1-[1-(2-cyclohexylethyl)-1H-indol-3-yl]-2-(2-methoxyphenyl)ethenone

ritalinic acid: 2-phenyl-2-piperidin-2-ylacetic acid

UR-144: (1-pentyl-1H-indol-3-yl)(2,2,3,3-tetramethylcyclopropyl)-methanone

valeryl fentanyl carboxy metabolite: 5-oxo-5-(N-[1-(2-phenylethyl)piperidin-4- yl]anilino)pentanoic acid

Introduction

Over the past 20 years, the world drug market has seen the introduction of a new group of substances characterized by a great variety of chemical compounds, known as “New Psychoactive Substances” (NPS). The acronym NPS encompasses a wide range of compounds including molecules which were synthesized more than 80 years ago and have never been clinically approved due to their strong side effects, drugs which have been used in the medical field for some time but whose recreational use has only recently been discovered, and finally, molecules which have recently been synthesized from classic drugs of abuse. The structure of the majority of these newly synthesized and newly marketed substances derives directly from the "old” ones; that is to say, they are characterized by a structural skeleton which is identical or at least similar to the basic chemical structure of the classic drugs but presents minimal conformational or compositional modifications causing alterations in pharmacological effects thus allowing them to pass as legal. These substances are receiving increasingly positive feedback from users because, in spite of their extreme toxicity (much higher than that of common drugs), they are made more appealing as they are easily available (even on the web), have a low cost and legal status as they are not yet controlled by narcotics conventions. According to the United Nations Office on Drugs and Crime (UNODC), NPS are defined as “substances of abuse, either in a pure form or a preparation, that are not controlled by the 1961 Single Convention on Narcotic Drugs or the 1971 Convention on Psychotropic Substances, but which may pose a public health threat”. [1] Globalization combined with web technologies have contributed to the development of NPS and since 2015 approximately 400 NPS are detected every year. This phenomenon has also caught on in Italy and in order to keep up to date, it is essential to highlight both the prevalence of NPS abuse and possible combinations with classic substances. For this reason, extensive scientific research has been carried out in the available literature to study and characterize the evolving phenomenon in depth. The traits of the new drugs market, the chemical aspects and pharmacological effects and the legal efforts by competent authorities such as the UNODC (in the global context) and the EMCDDA (European Monitoring Centre for Drugs and Drugs Addiction), working to combat NPS around the world, have been analyzed and highlighted. In recent years, the analytical and interpretative point of view has been complicated and further exacerbated by the dynamism of the market for these "designer" or "new psychoactive" drugs. For this reason, forensic toxicology has mainly shifted its focus and its efforts from classic drugs of abuse to NPS. This is also evident in the increase in the number of scientific publications on new psychoactive substances over the last twenty years (from 29 articles per year in 2010 to 335 in 2022). Increment that mirrors the rapid spread of NPS.

Indeed, this increase seems to be in line with the rapid development of NPS over the years and perfectly reflects the efforts to increase common knowledge of the phenomenon in order to activate the competent authorities to carry out awareness-raising campaigns regarding this obvious threat to public health.

The main health risk posed by these new drugs lies in their similarity to traditional drugs. A similarity which leads users to believe that their same effect and dose are the same. This misunderstanding often has serious, if not fatal, consequences. In addition, the dissimilarities allow users to pass drug tests because these new drugs are not recognizable, so minor modifications in the chemical structure mean that they generally fail to be detected by the screening procedures commonly used in forensic laboratories. In recent years, however, modern forensic toxicology can rely on a wide variety of innovative procedures, certified standards and innovative analytical tools to successfully respond to these analytical challenges. As observed in literature, the techniques commonly used for the unambiguous determination of chemicals include gas chromatography/mass spectrometry (GC/MS), high performance liquid chromatography (HPLC), liquid chromatography/mass spectrometry (LC/MS) and tandem mass spectrometry (MS/MS).

Given the above global scenario, in order to characterize the quality and rate of NPS consumption in the metropolitan area of Bologna, we analyzed three hundred hair samples from specific safety-sensitive social groups, such as drivers and workers, followed by the Public Services for Pathological Addictions (Ser.DP). The experimental study proceeded with the initial bibliographic research of the methods already published for the analysis of NPS in the hair. Next, an innovative method for the simultaneous identification of a considerable number of NPS was developed and validated. Finally, to analyze the presence of NPS in our city and the ways in which they are intertwined with the world of classical drugs, the analytical method was executed on the keratin samples of the patients involved. Individual NPS detection must be reported to the national early warning system, which communicates with national and international authorities. The aim of the report is to make the authorities aware of the real presence of new substances, so that they can implement mechanisms to combat their impact on public health.

Chapter 1. New Psychoactive Substances (NPS)

1. NPS: a global phenomenon

The drug world is constantly evolving. Over recent decades, an increasing number of NPS have appeared on the drug market. The “cannibal drug” or “flakka” (synthetic cathinones), the “zombie” drug or “krokodil” (synthetic opiods), the “ayahuasca” (tryptamines), the so-called “N-Bomb”, “B-Fly,” e “Dr. Death” (phenethylamines), are just some of the novelties that are making their entry into the world of drugs, under the emerging macro-category of NPS [2],[3].

NPS act on the same receptors as "traditional" drugs (cannabis derivatives, cocaine, amphetamines/methamphetamines, opioids) but with psychotropic effects which also occur at very low doses with greater receptor affinity and potency. In most cases, a single functional or chemical group constitutes the only difference between the NPS and the parent recreational drug. This change not only alters the molecule’s pharmacological properties, but also makes the use of that molecule legal, simply because the derivative is not registered on the list of narcotic products—making it a “legal high”. Due to their very low cost (5 to 20 euros per gram) and ready availability (facilitated by deliveries via the Internet), NPS are an emerging public health problem. [4]

NPS are a diverse and rapidly evolving group of substances available on the global illicit drug market (e.g. smart shop, internet, "dark net") as substitutes for controlled substances. They have been described as a "growing global epidemic.” [5], [6] UNODC defines NPS as “substances of abuse, either in a pure form or a preparation, that are not controlled by the 1961 Single Convention on Narcotic Drugs or the 1971 Convention on Psychotropic Substances, but which may pose a public health threat”.[1]

However, definitions of NPS may vary from country to country, reflecting differences in national legislation rather than pharmacological or structural classification. [6] UNODC further specifies that "the term 'new' does not necessarily refer to new invented substances, but to substances that have recently become commercially available." Indeed, some NPS were first synthesized more than 80 years ago but only recently have their chemistry or synthesis process been slightly modified to escape national and international legislation.[7] Known on the market as 'designer drugs,' "legal highs," "bath salts," and "research chemicals”, NPS traditionally encompassed synthetic substances, but under terms such as “designer drugs” they have recently been expanded to include other psychoactive substances which mimic the effects of illicit drugs and are produced by introducing slight modifications to the chemical structure of controlled substances to circumvent drug controls.

These substances are often associated with a label saying, “not intended for human consumption”. Chemically and pharmacologically speaking, Ketamine must be considered one of the oldest NPS, similarly, other NPS such as phenethylamines and piperazines. To date, there is no universally accepted method to categorize NPS. Initially, they were functionally classified into only three broad categories (stimulants, hallucinogens and depressants) based on the effects they produced on the entire organism. [6] More recently, also following their evolution which has seen the development of more and more new compounds, a more pragmatic classification system has been defined, going on to divide NPS into the following four groups: synthetic stimulants, synthetic cannabinoids, synthetic hallucinogens, and synthetic depressants (the latter include synthetic opioids and designer benzodiazepines). [6]

Considering the chemical structure and the effects, on the other hand, the group of NPS may be divided into seven categories: synthetic cannabinoids, synthetic cathinones, phenethylamines, arylcyclohexylamines (phenciclydine-type substances), synthetic opioids, designer benzodiazepines and tryptamines [5]

The aforementioned categories correlate with the identification of the major groups of substances characterizing the market at present, namely synthetic cannabinoids (e.g. APINACA, JWH-018), synthetic cathinones (e.g. 4-methylethcathinone (4-MEC) and α-pyrrolidinopentiophenone (α – PVP)), phencyclidine-type substances (e.g. methoxetamine (MXE)), phenethylamines (e.g. 2C-E and 25H-NBOMe), piperazines (e.g. benzylpiperazine (BZP) and 1-(3-chlorophenyl) piperazine (mCPP)), tryptamines (e.g. α-methyltryptamine (AMT)), synthetic opioids (e.g. fentanyl analogues) and designer benzodiazepines. [8]

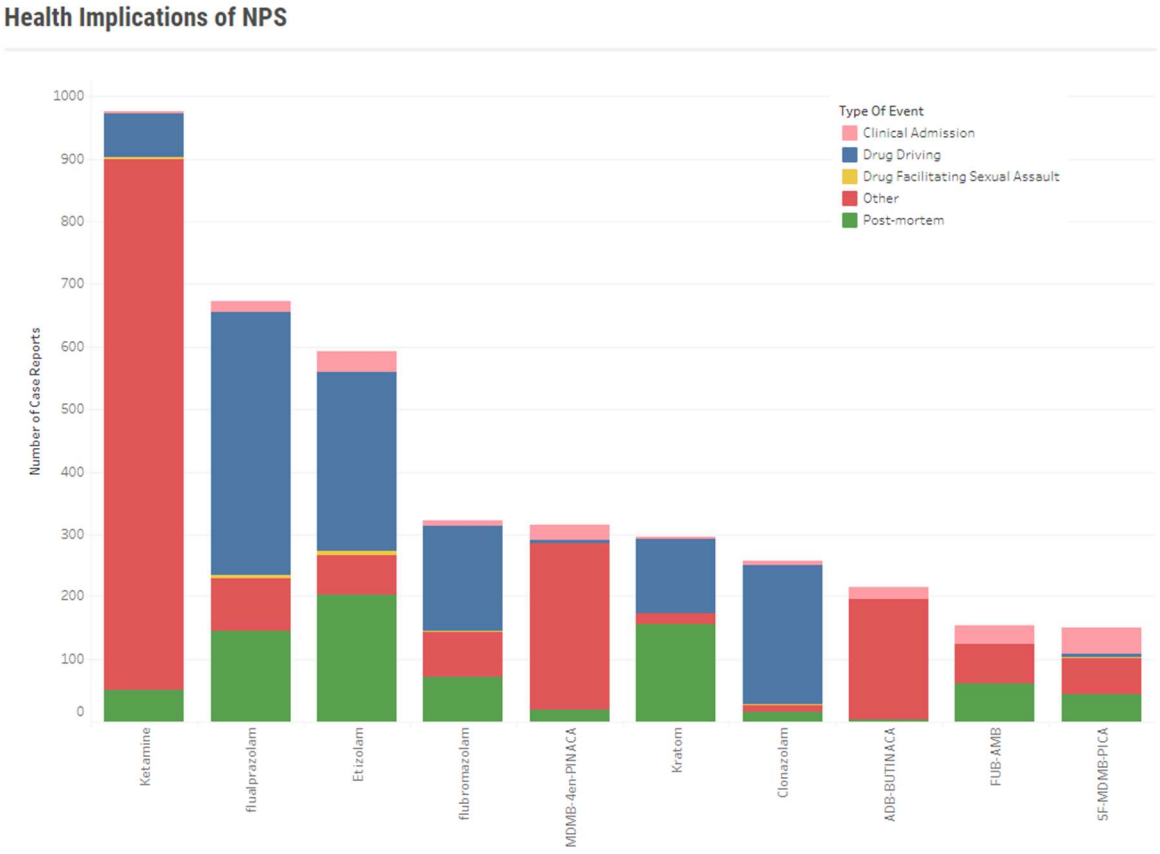
1.2. **The risks of NPS: a public health threat**

The rapid explosion in the number of ever renewed psychoactive substances on the global market represents a significant public health risk and consequently a challenge to drug policy. Unfortunately, knowledge of the adverse health effects and social damage caused by NPS is still sadly inadequate, especially given the paucity of analytical and legislative tools available.

In turn, this precariousness is a result of the lack of knowledge as to the present situation of NPS on the market, rendering the challenge for the prevention and treatment of intoxication caused by new psychoactive substances is considerable. In short, it is all one great vicious circle it is crucial we interrupt. In addition to the sanitary problems already encountered with traditional illicit drug use, NPS are an aggravation of the public health threat.

Understanding the health risks associated with their use is an important element of the analysis that UNODC is undertaking to support the prioritization of NPS for international review by the World Health Organization. The chart below (Figure 1) shows the 10 most common NPS in toxicological cases reported, classified according to effect group, region and type of event that led to the submission of the sample for analysis. In many cases, more than one substance was identified.

Figure 1: Ten NPS most frequently mentioned in toxicology cases reported to UNODC by type of event that led to the submission of the sample for analysis.



Source : data from the UNODC EWA on NPS (https://www.unodc.org/unodc/en/scientists/ewa/data.html)

Public health, at present, is mainly affected by the huge lack of knowledge of NPS toxicity, the borderline between a "safe" dose and a fatal dose, and the unknown adverse health effects they produce [9-13].

Moreover, drug users who consume these new substances often do not even know the true identity of the substance they are assuming because new products containing NPS do not provide or provide very little information as to their composition [10, 14, 15]. There are, unfortunately, countless cases in which the addict finds himself consuming an NPS without knowing it, because it is used by drug dealers as an adulterant. [16]

In general, the side effects of NPS range from convulsions to agitation, aggression, and acute psychosis as well as the potential development of addiction. There are many cases of hospitalization for acute intoxication cause NPS. As previously mentioned, information on the long-term adverse effects or risks of NPS are not known or are, at any rate, very limited.

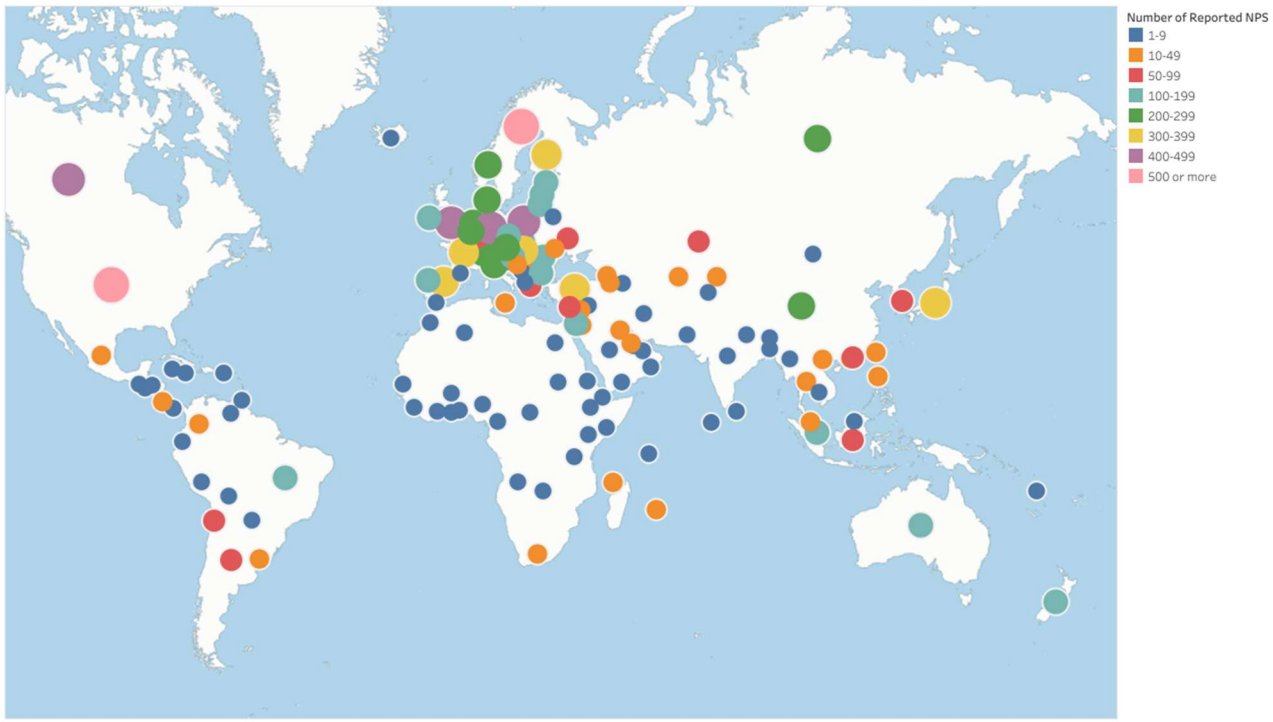
Another important aspect is that purity and composition of products containing NPS are often unknown, which places users at high risk often including cases of poly-substance use. [1]

Considering the significant increase in the number of hospitalizations or deaths from NPS overdoses reported by various poison centers, many NPS appear to have addictive properties as well as peripheral toxicological effects. Being aware of the behavioral, neurochemical and electrophysiological effects caused can be extremely helpful in identifying them. [5]

1.3. **The diffusion of NPS on the drug market**

As of December 2021, governments and laboratories have reported over 1000 new psychoactive substances in more than 130 countries (Figure 2) to the UNODC Early Warning Advisory (EWA). [1]

Figure 2 : The map shows the number of NPS reported by countries/territories to the UNODC EWA.



Source : data from the UNODC EWA on NPS (https://www.unodc.org/unodc/en/scientists/ewa/data.html)

The prevalence of NPS in the drug market is unprecedented; indeed it was estimated that in 2015 (considered the peak of prevalence) the rate of new substances coming onto the market was one compound per week. In recent years, the number of new psychoactive substance detections has declined. Moreover, the nature of the market has changed, with a relative decrease in the number of new stimulants and synthetic cannabinoids detected, and an increase in the number of new opioids and benzodiazepines available. [6]

The EU Early Warning System (EWS) for NPS, operated by the EMCDDA, is the first regional early-warning mechanism set up to monitor and respond to uncontrolled new drugs. One of the latest EU EWS reports states that the European market for new psychoactive substances has been affected by the restrictions on their production and export recently imposed by China, one of the main countries of origin. Interestingly, no new fentanyl derivatives were detected in Europe in 2020-2021, but 15 new, uncontrolled synthetic opiates, including 9 potent benzimidazole opiates. In addition, in 2021, also in Europe, four new synthetic cannabinoids «OXIZID» were identified. The rapidly changing profile of the NPS market raises concerns about the uncertainty and ambiguity regarding their chemical, metabolic, and toxicity profiles, and the consequent physical, social and mental health damage. [6]

In Italy

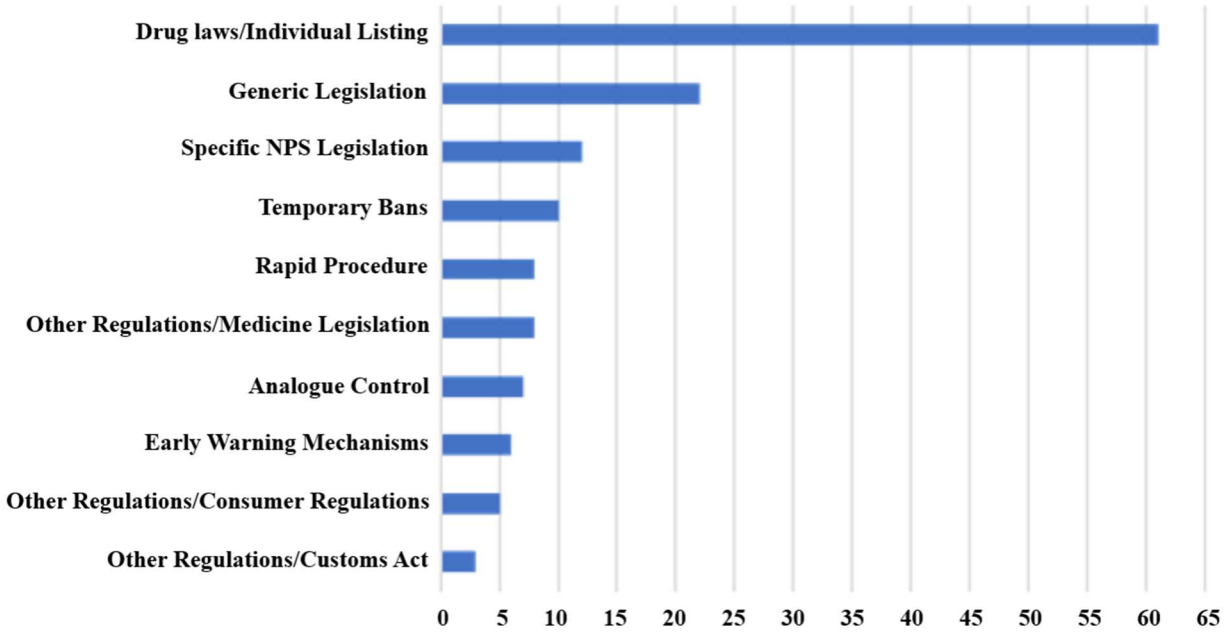
In Italy, the national early warning system is lead by the "SNAP" (Sistema di Allerta Precoce) project, headed by the National Health Institute. The SNAP represents a knowledge hub for policymakers, laboratories and law enforcement on NPS trends, adverse effects (toxicological and pharmacological), national legislative responses and substance analysis. From its account it appears that seizures of NPS have also increased in Italy. The lockdown measures which affected our country (and not only), probably influenced the retail trade of classic narcotics (cocaine, marijuana, ecstasy, heroin, etc.) and may even have favored the development of more digitized ways of distributing substances. During 2020 alone, forty-four NPS were identified, most of them belonging to the category of synthetic cathinones. The detection of these new substances on the national market, with reports sent to SNAP for 'seizure of NPS', led to the issuing of new decrees by the Ministry of Health to update the tables, adding 74 new substances to those previously controlled. This is the mechanism that we must constantly support with the development of ever new analytical methodologies which allow us to be at the forefront in this fight. [17]

1.4. **Legal situation of NPS**

As mentioned in previous paragraphs, legal status is not regulated by international drug control conventions for all of the new substances, and for this reason it may differ significantly from country to country. The most detected NPS on the market have been subject to international control under the UN Conventions (e.g. mephedrone in 2015; the synthetic cannabinoid ADB-FUBINACA in 2019). On a national level, some countries have designed legislative ploys to include the highest possible number of NPS in their national prohibited list. For example, in the United Kingdom (UK) in 2016 a law on psychoactive substances was introduced which effectively created a 'general ban' of all current and future NPSs (with some exemptions). However, the legislation has been criticized for the imprecise definition of "psychoactivity".

As of 2021, more than 60 countries have implemented and renewed legal instruments, modifying existing legislation. With the aim of protecting public health from the dynamic NPS market, some countries have sought to explore a wide range of legislative responses (Figure 3) to the specific challenges posed by the wide range of NPS.

Figure 3: Type of legislative responses explored by some countries with the aim of contrasting NPS phenomenon



Source : data from the UNODC EWA on NPS

In countries where there has been a dramatic increase in the development of new psychoactive substances, corresponding legislation has been introduced which invokes the principle of "chemical similarity" to an already controlled substance, so that substances not explicitly mentioned in the legislation can be monitored. [1]

Over the last decade, the number of NPS has doubled, thus representing a critical challenge for governments, the scientific community and civil society [18]. Currently, not all NPS are under international control and not all countries have established appropriate control measures. In some countries, such as Poland and the UK, existing laws, such as consumer safety legislation, are used to ostracize the distribution of NPS. In others (Hungary, Finland, Italy, France, Denmark, etc.), existing drug laws or processes have been extended or adapted; in Ireland, Austria, Portugal, Romania and Sweden, new legislation has been developed [19].

Despite attempts to control the use of psychoactive substances around the world, a growing diversity (in the type and number) of NPS continues to emerge among recreational drugs, especially as manufacturers attempt to circumvent drug legislation [9-11, 20-29]. In Europe, at least 50 new substances are detected every year, for every substance that is controlled and therefore declared illegal, one or more structurally modified counterparts are introduced onto the legal market, in a seemingly endless spiral. [1,9,10,20-22,29-32]

For example, an NPS called naphyrone appeared in the UK as a legal substitute for mephedrone, which had been classified as an illicit substance a few months earlier [33,34]. In 2017, the European institutions approved new legislation to speed up the procedure for responding to NPS, including them in the official definition of "drugs" [35]. This legislation focuses on early warning, risk assessment and control measures, while promoting the rationalization, acceleration and increase of NPS knowledge, including toxicological studies, the development of analytical chemical methodologies for NPS detection, etc. [7]

1.5. **NPS: attempted classification**

There is no universally accepted method of categorizing NPS. According to Zapata et al [7]. Several are the criteria that can be considered for the classification of NPS. The most popular criterion is represented by the pharmacological effect (Hallucinogens, Stimulants, Depressants). However, classifications according to the origin (Natural, Synthetic and Semisynthetic) or legal status (1961 Single Convention on Narcotic Drugs and its subsequent updated editions) of the substances are also common. But previous classifications, whilst undoubtedly important in the fields of medicine and law, have one major flaw, they do not take chemistry into account at all.

For simpler comprehension, here we have decided to follow the classification suggested by [5], which examines both chemical structure and effects, and according to which the group of NPS may be divided into seven categories: synthetic cannabinoids, synthetic cathinones, phenethylamines, arylcyclohexylamines (phenciclydine-type substances), synthetic opioids, designer benzodiazepines and tryptamines.

1.5.1. *Synthetic cannabinoids*

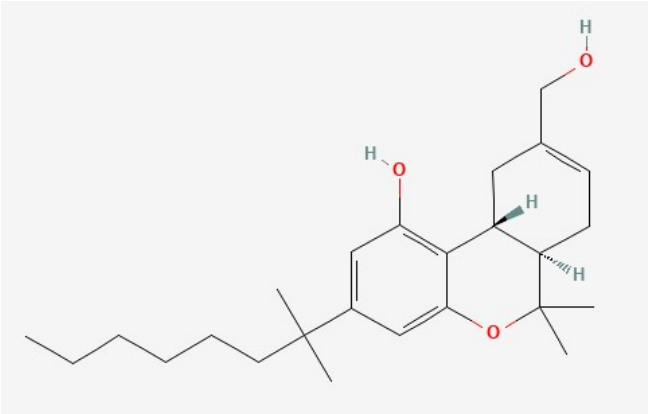
Synthetic cannabinoids (SCs) are molecules with a psychotropic behavior like Δ9-tetrahydrocannabinol (THC), the primary active principle in cannabis. In their pure state these substances come in liquid (oil) or solid form but are often laced onto herbal products (the vegetable mixture is usually sprayed with a solution composed of cannabinoids). [36] Usually smoked or taken orally, they are sold as 'Spice Gold', 'Spice Silver', 'Spice Diamond', 'K2', 'Bliss', 'Black Mamba', 'Bombay Blue', 'Blaze', 'Genie', 'Zohai', 'JWH -018, -073, -250', 'Kronic', 'Yucatan Fire', 'Skunk', 'Moon Rocks', 'Mr. Smiley'.

SCs bear structural features that allow binding to one of the known cannabinoid receptors in the brain (CB1) and in other organs. More correctly indicated as Synthetic Cannabinoid Receptor Agonists (SCRA). In vitro studies have shown that some synthetic compounds bind more strongly to this receptor than THC, producing the same psychotropic effect but with higher intensity at lower doses, causing much more serious side effects. Initially they were used for therapeutic purposes in the treatment of chronic pain. However, it proved difficult to separate medical properties from unwanted psychoactive effects. A clear demonstration are JWH compounds which were developed as new pharmaceutical therapeutical products in research for drug-receptor interactions from the synthesis of analogues of THC and its metabolites by Professor John William Huffman [37]

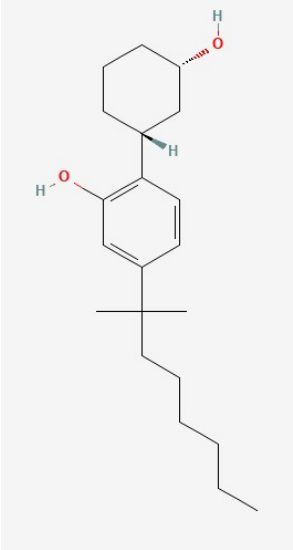
According to their chemical structure, it is possible to divide SCs into 3 separate subcategories: Classics, Non-classics and Hybrids (see one example for each subcategory in Figure 4). The cannabinoids belonging to the first group are characterized by a THC-like chemical structure (e.g. HU-210). Among the "non-classical" cannabinoids are "CP compounds" (cyclohexylphenols or 3-arylcyclohexanols) so called for the cyclopentadienil complex characterizing its structure. Among the hybrids, we find an emerging variety represented by aminoalkylindoles such as naphthoylindoles (e.g. JWH-018), phenylacetylindoles (e.g. JWH-250), and benzoylindoles (e.g. AM-694) [40].

Figure 4: Chemical structures of the three types of SCs (one for each subcategory)

Classical cannabinoids (THC-like compounds): HU-210

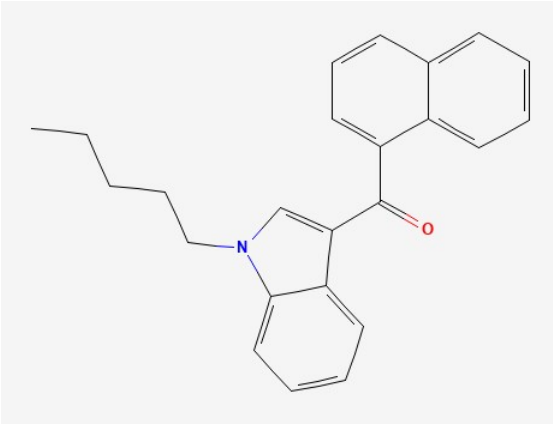


Non-classical cannabinoids (CP compounds): CP-47,497

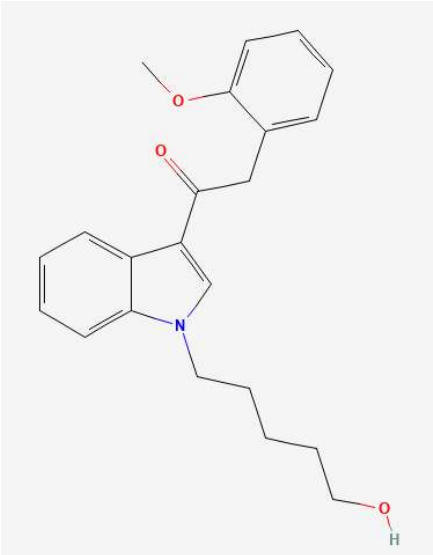


Hybrid cannabinoids (Aminoalkylindoles)

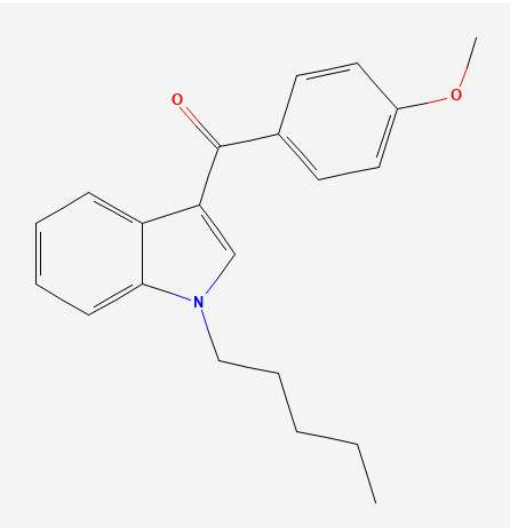
Naphthoylindoles: JWH-018



Phenylacetylindoles: JWH-250



Benzoylindoles: RCS-4



The first SC were identified in 2008 in preparations called “herbal mixtures” or “herbal blends” (i.e., Spice) and sold as air fresheners or incense. According to the “2012 UNODC” survey, the most widespread SC was JWH-018, followed by JWH-073, JWH250 and JWH-081. Over the last decade, however, the most common cannabinoid detected was recognized as MDMB-4en-PINACA but at the start of 2021 this was replaced by ADB-BUTINACA. [5]

The increasing structural diversity of the new SCs, demonstrated by the introduction on the market of compounds such as APINACA (AKB-48), an adamantyl indazole carboxamide, and AB-PINACA, an aminocarbonyl indazole carboxamide, would appear to be driven by the manufacturers desire to circumvent some of the national legislative responses put in place to counter the 'previous generations' of SCs. [37]

1.5.2. *Synthetic cathinones*

Synthetic cathinones (SCAs) are newly synthesized compounds which derive structurally from cathinone, the principal active ingredient in the leaves of the khat plant (catha edulis). We could consider cathinone as a prototype from which all other SCAs were created. Indeed, SCAs are commonly marked by a β-keto group on the phenethylamine side chain. They are often sold as 'research chemicals', 'plant food', 'bath salts' or 'glass detergent', as a powder (white or brown), pill or capsule (often sold as ecstasy). Most synthetic derivatives are ingested but can be injected. Mephedrone is commonly nasally insufflated, injected, ingested by swallowing a powder wrapped in paper (‘bombing’) or mixed into a beverage.

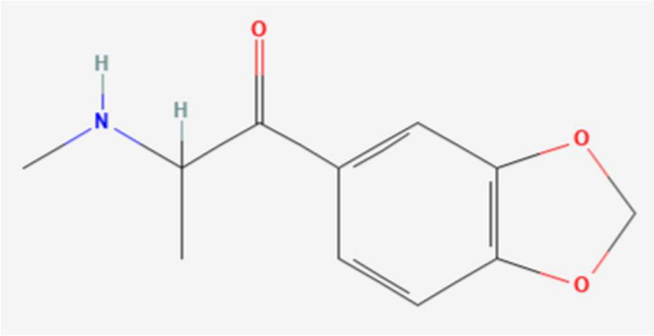
SCAs mediate the actions of dopamine, norepinephrine and/or serotonin, mimicking the effects of traditional drugs such as cocaine, amphetamine, methamphetamine, and ecstasy, acting as central nervous system stimulants (CNS). But, unlike the parent drugs, SCAs show a reduction in the potency of the stimulating effect due to the β-keto group, as it makes the molecule less suitable for crossing the blood-brain barrier. [39]. Cardiac, psychiatric, and neurological signs are some of the adverse effects reported. [40].

SCAs appeared in drug markets in the mid-2000s. In 2005, methylone, an analogue of MDMA, was the first SCA reported to the European Monitoring Centre on Drugs and Drug Addiction (EMCDDA). On the market we identify (Figure 5) amphetamine-type analogue, i.e. cathinone, mephedrone, and methylone which are structurally related to amphetamine, methamphetamine and MDMA respectively; pyrovalerone-type analogue (3,4-methylenedioxypyrovalerone and naphyrone) and from 2010 onwards other SCAs used such as butylone, 4-methylethcathinone, 4-fluoromethcathinone, naphyrone, 3-fluoromethcathinone, methedrone, and, to a lesser extent,

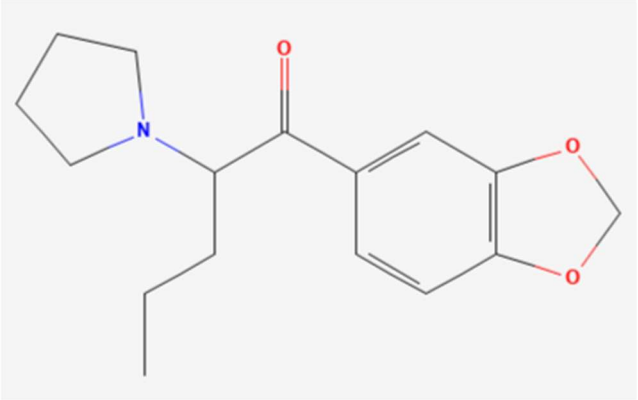
3,4-dimethyl- methcathinone, α-pyrrolidinopentiophenone (α-PVP), buphedrone, pentedrone and α-pyrrolidinopropiophenone (α-PPP).

Figure 5: Some of the structures typical of SCAs.

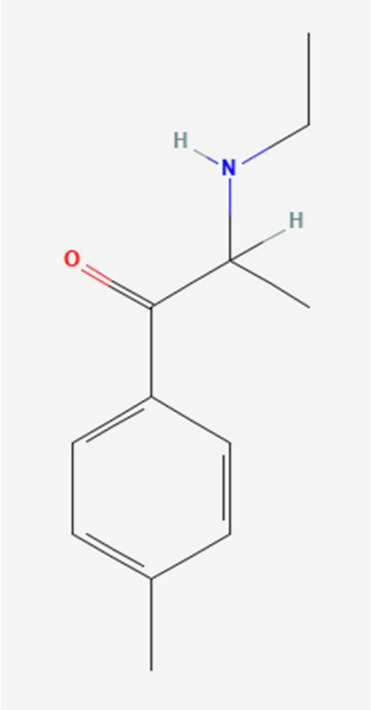
Amphetamine-type: methylone



Pyrovalerone-type: 3,4-Methylenedioxipilovalerone (3,4-MDPV)



And others: e.g. 4-methylethcathinone (4-MEC)



Some SCAs had been patented as antidepressant and antiparkinsonian agents [41], but very few have been exploited clinically predominantly on account of their abuse and dependence potential. For instance, whereas diethylcathinone (amfepramone) is used as an appetite suppressant, pyrovalerone, initially marketed for use as an appetite suppressant and in the treatment of chronic fatigue, was later withdrawn due to abuse and dependency in users [42].

1.5.3. ***Phenethylamines***

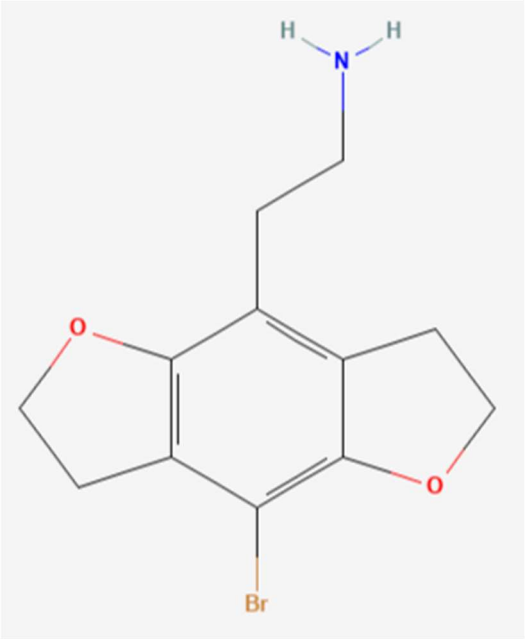
Phenethylamines (PEAs) are a class of substances, either natural or synthetic, with observed stimulant and psychoactive effects. This group, besides including parent drugs such as amphetamines, methamphetamines and MDMA, all controlled under the 1971 Convention [43], also includes newly synthetized molecules which can be included in the definition of NPS and be divided into numerous subgroups based on the different substitution on the aromatic ring, the alkyl chain and the nitrogen atom. In particular (Figure 6), we can distinguish the "2C" series, characterized by methoxy groups in positions 2 and 5 and any other substituent on the aromatic ring (2C-B and 2C-I), the "D" series (DOI, DOC), similar to the 2Cs but with a methyl on the side chain, the "NBOMe" series, also composed of derivatives of the 2C series but with a group N-benzyl-methoxy (25B-NBOMe and 25C-NBOMe), the 4-fluoroamphetamine (4-FA), "FLY" and "Dragonfly", respectively tetrahydrobenzodifuranic (2C-B-Fly) and benzodifuranic (Bromo-Dragonfly), and many others (e.g., p-methoxymethamphetamine or PMMA). [44].

Commercial names include ‘Europa’ for 2C-E; ‘4-FMP’, ‘para-fluoroamphetamine’, ‘RDJ’ for 4-FA; and ‘4-MMA’, ‘Methyl-MA’ for PMMA. PEAs are usually sold as pills, except for FLY compounds that are commonly sold in powder form, while oral doses (on a slip of blotter paper) are usually available for “D substances”. Ingestion is the most common route of administration. Similar to parent drugs, NPS’s phenethylamines also act as central nervous system stimulants. However, this group also includes classical hallucinogens (psychedelics) which mediate specific serotonin receptor activities and produce hallucinations. The substances in these groups mimic the effects of traditional drugs such as 2C-B, LSD and DMT but may also possess residual stimulant activity.

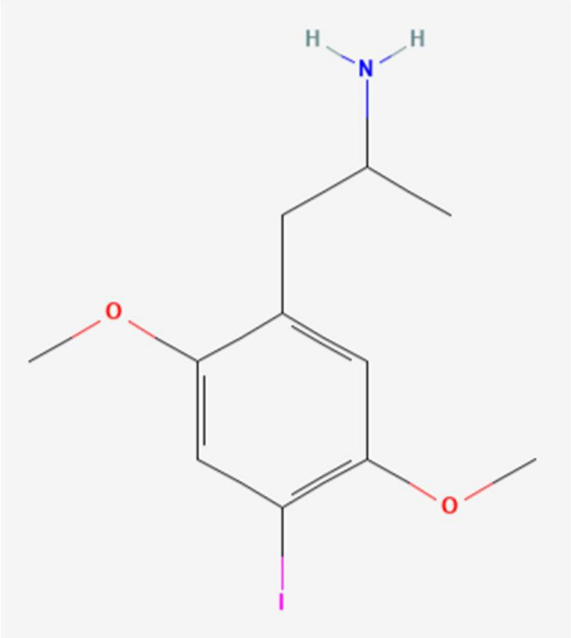
Since 2009 substances such as 2C-E, 2C-I, 4-FA and PMMA have been commonly reported by different countries and regions, and since 2011 UNODC reports including 4-FMA, 5-APB, 6-APB and 2C-C-NBOMe have increased exponentially. From simple variations in the mescaline molecule, a hallucinogenic alkaloid mainly contained in peyote, other powerful psychedelic substances have been obtained, such as, for example, 4-bromo-2,5-dimethoxy phenethylamine (2C-B) synthesized by Shulgin in 1974. [45].

Figure 6: Some of the structural characteristics of the PEA group

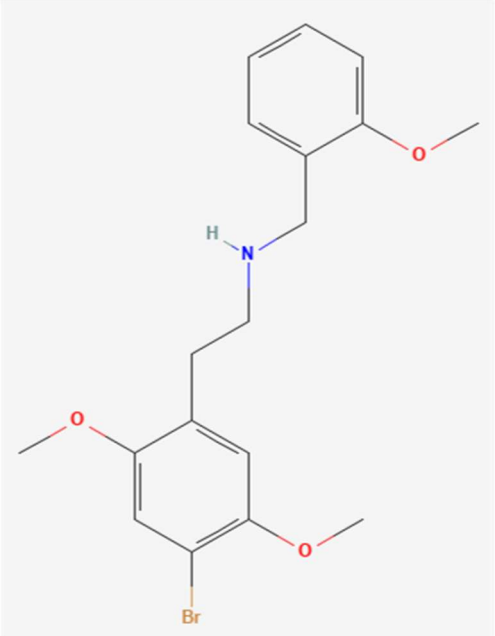
"C" series: 2C-B Fly



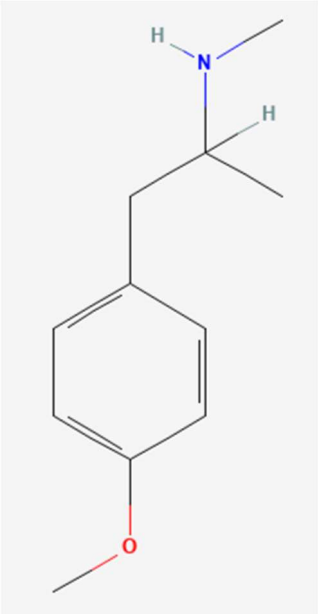
"D" series: DOI



"NBOMe" series: 25B-NBOMe



Others: e.g. p-methoxymethamphetamine (PMMA)



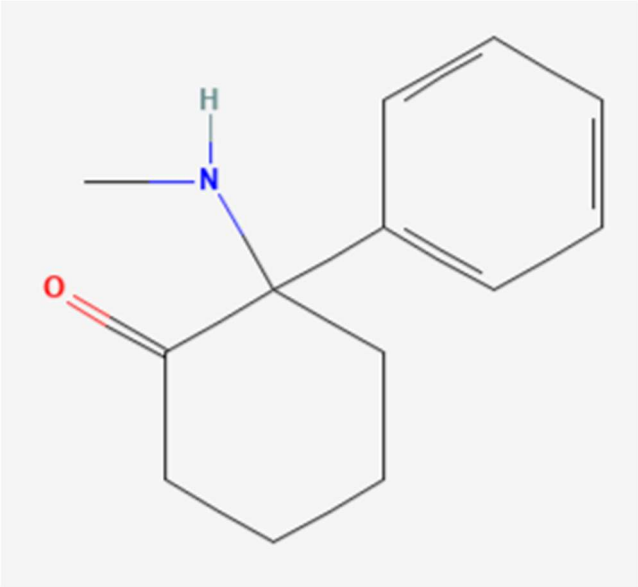
The series of PEAs differ from each other simply due to slight changes in chemical structure. The effects of these substances are strongly dose dependent. Indeed, it is known that their psychoactive effects range from the mere stimulating effect at lower doses, to the hallucinogenic and entactogenic, i.e., psychoactive substances that increase feelings of love and union with others, at higher doses. [46]. Many of these substances are sold on the drug market as a substitute for "ecstasy", this could be very dangerous because of their different adverse effects and weight and purity of doses.
As an example, PMMA in combination with PMA (a substance listed in Schedule I of the 1971 United Nations Convention on Psychotropic Substances), has been frequently found in tablets which carry a similar logo to 'ecstasy'.[50] PMA, PMMA and 4-methylthioamfetamine have been more frequently associated with incidental deaths than other phenethylamines.

1.5.4. *Arylcyclohexylamines*

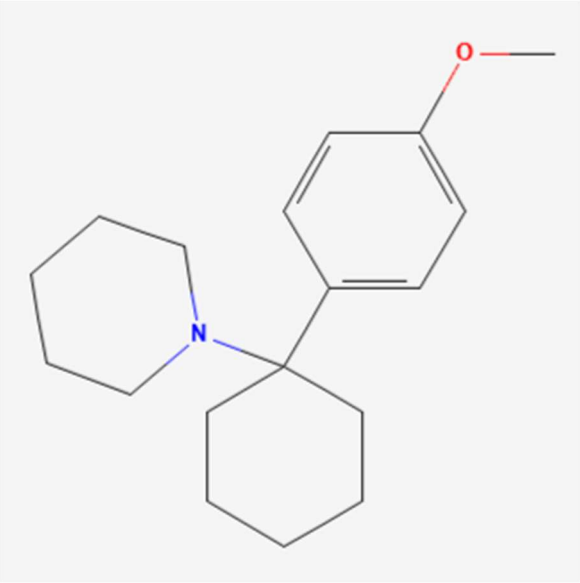
New substances, showing a structural similarity to phencyclidine and ketamine, are classified as arylcyclohexylamines (ACH). Currently, on the market, the best-known member of the group is ketamine but the historical substance of the group is phenyclidine (PCP). [4] PCP was first synthesized in the 50s, sold as an injectable anaesthetic under the trade names Sernyl and Sernylan until it was withdrawn from the market due to intensely negative psychological effects, such as dysphoria, confusion, delirium and psychosis. [50,51] The psychedelic properties of PCP led to a new chapter in its history as a street drug ("angel dust") and made it the first of many synthetic drugs to appear on the market as an illicit recreational substance. [50]. Later in history, PCP was used as starting point for ketamine synthesis with the purpose of developing new ACH anaesthetics with analgesic properties [5]
Similarly to SCAs and PEAs, ACH have a predominantly SNC stimulating action which resembles that of classic stimulant drugs, but we must not forget its strong dissociative characteristics [4] Indeed, first-generation PCP derivatives retain a cyclohexane ring, in order to maintain antagonistic activity against the N-methyl-D-aspartate receptor (NMDA) and therefore result in dissociative effects. Derived through modification of the aryl ring, that is, through the addition of an alkyl chain or substitutions of the amino group, the ACH family can be distinguished in three main subfamilies (Figure 7): ketamine-like molecules, PCP-like molecules and ethylcyclidine-like molecules (PCE-like). [47]

Figure 7: Some chemical structures of ACH

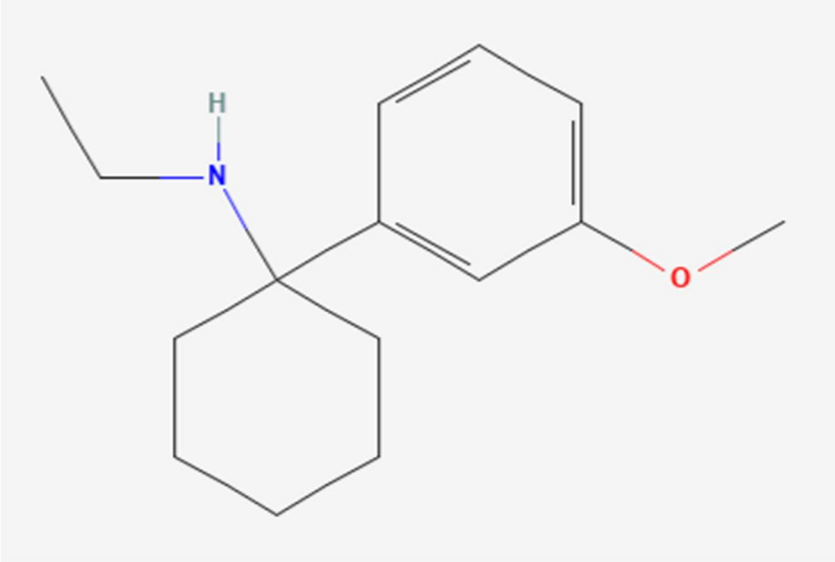
Ketamine-like: Deschloroketamine (DCK)



PCP-like: 4-methoxy-PCP



PCE-like: 3-methoxy-PCE



Focusing on ketamine and its derivatives, “DCKs” and the fluorinated derivative 2F-deschloroketamine (2F-DCK) are the least well-described ACH derivatives on the drug market. In Europe, the most widespread ACH appears to be methoxetamine. Its effects are reportedly similar to those of ketamine, but last longer and are more intense, the doses are also very different, meaning that the switch from ketamine is particularly dangerous.

Among the structural analogues of PCP and PCE that are more widely available on the market we find newer substances such as 3-methoxyethylcyclidine (3-meo-PCE), 4-methoxyphenyclidine (4-meo-PCP), which are often sold as "research reagents" in powder form. PCP and structural analogues have dissociative properties, reputed hallucinogenic and sedative effects.

Nasal and oral administration routes are the most common, although cases of intravenous, anal and sublingual administration have been described [51-53]. Well-known is although the frequently co-consumed of ACH substances with other drugs of abuse, including alcohol, THC, amphetamines or cocaine.

These cocktails are likely to modify the pharmacokinetics, pharmacodynamics and toxicity profile of these drugs. [4]

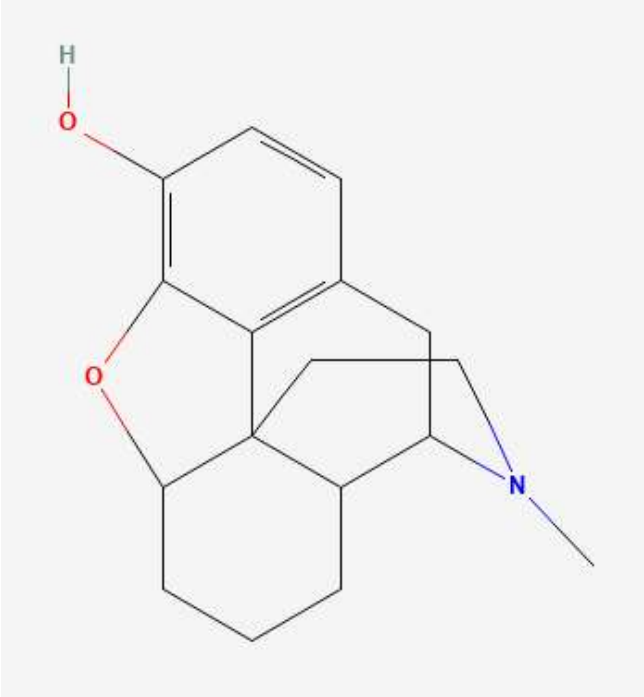
1.5.5. *Synthetic opioids*

Synthetic opioids (SOs) are laboratory substances which act on nerve cells and consequently on mental processes. They were developed with a therapeutic purpose for use as analgesic drugs. Their mechanism of action consists in partial agonistic interaction with opioid receptors coupled with G proteins in the brain and spinal cord, with selectivity for the μ-opioid receptor. [54, 55] The μ receptors are those mainly responsible for the toxic effects represented by nausea, euphoria, muscle stiffness, respiratory depression, and sedation; furthermore, they are the main players governing the phenomena of tolerance and dependence caused by prolonged use. [56, 57] The effects of opioids derive directly from their pharmacological composition. Their chemical structure involves 3 types of structure (Figure 8) based on the 4,5-epoxy morphine ring (e.g. morphine-related), phenylpiperidines (e.g. fentanyl-related) and non-fentanyl-related structure (e.g. nitazene-related).

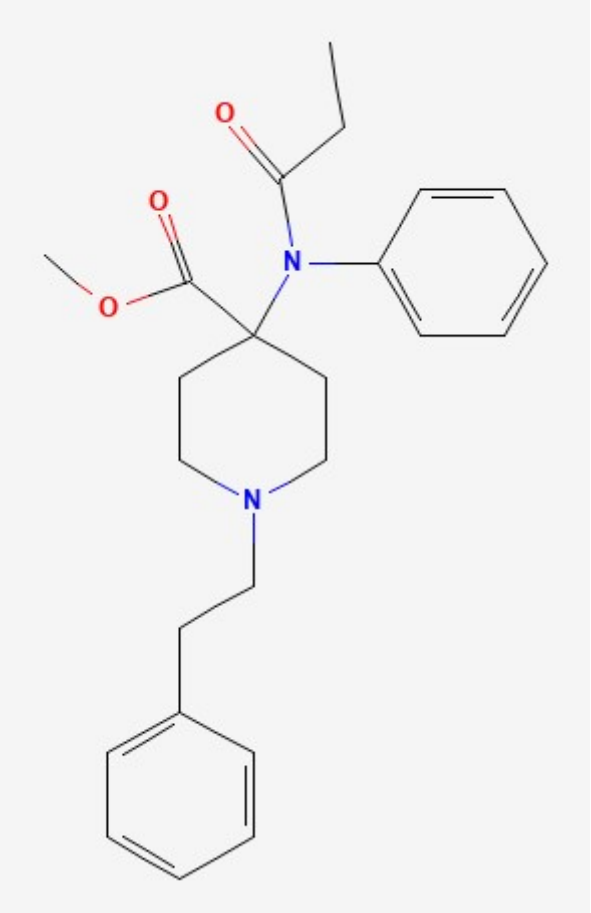
SOs are synthetized from 4-aniline-N-phenethylpiperidine (ANPP) and N-phenethyl-4-piperidone (NPP). SOs are marketed in powder, tablet or liquid forms. Routes of intake include orally, sniffing, smoking or injection. Acetylfentanyl has already been found in the form of nasal spray and MT-45 powder in herbal smoke mixtures that associate SCs. Rectal or sublingual routes of administration have also been reported, such as AH-7921. [58] With the advent of electronic cigarettes, formulation for their vaporization has also entered the market. [59]

Figure 8: Some of the structural characteristics of the SOs group

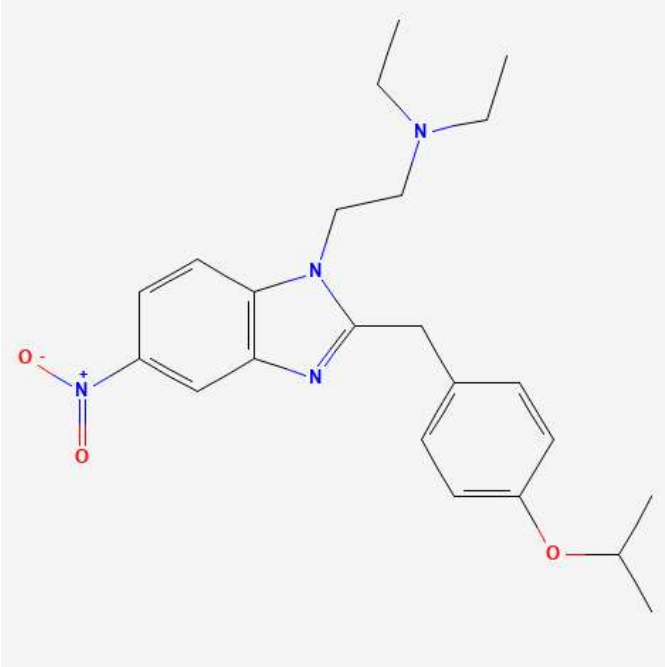
Morphine-related: Desmorphine



Fentanyl-related: carfentanyl



Nitazene-related: isotonitazene



In 2019, the opioid market was mostly dominated by fentanyl analogues but after several bans countered their spread, the illicit market has shifted its attention to synthetic non-fentanyl opioids, called "nitazenes". These drugs were developed 60 years ago as potential painkillers but were never approved by the authorities for clinical use.

Since 2019, when they began to appear, in just two years, reports of new SOs concern analogues of nitazenes 7 times out of 9. The danger of these new compounds is increased by the identification of more types of nitazene analogue in single sample reports. [60] The alternative modes of consumption implemented by users, other than pharmaceutical, do not allow us to predict toxicity. A striking example is represented by the habit of injecting the liquid contained in fentanyl patches, intended for transcutaneous use. [61]

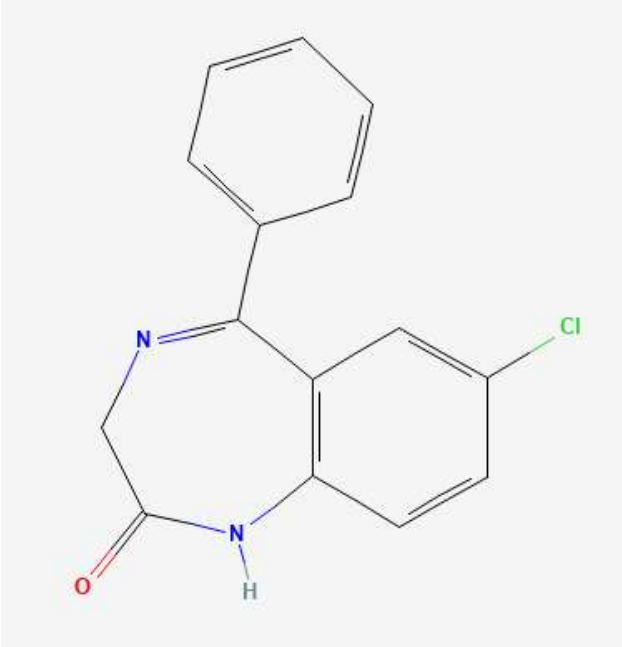
1.5.6. ***Designer benzodiazepines***

Benzodiazepines (BDZs) are synthetic substances normally marketed as tablets, capsules and occasionally as injectable substances. BDZs have been designed as the main pharmacotherapies for anxiety, panic attacks, sleep disorders and epilepsy, and were used as myorelaxants during surgical and orthopedic procedures [62]. BZDs, are substances with a depressive action of the Central Nervous System (CNS), acting by facilitating the binding of the inhibitory neurotransmitter GABA (Gamma-Aminobutyric Acid) to various GABA receptors throughout CNS. [63] and inducing side effects such as drowsiness, dizziness, fatigue, dysarthria, loss of coordination, headache and amnesia. Moreover, BDZs have the potential to create addiction [64], as confirmed by clinical trials in which subjects in long-term treatment experienced dependence and tolerance. [62] As the relevant risk of abuse is well documented, it was not surprising that the United Nations Commission on Narcotic Drugs' decided to place under Schedule IV, more than 30 BDZs circulating on the market [65-69]. This has led to a decrease in the non-medical consumption of BDZs and opioids, a reduction even more exacerbated by the rapid spread of new synthetic molecules in all respects definable Designer Benzodiazepines (DBDZ). [70,71]

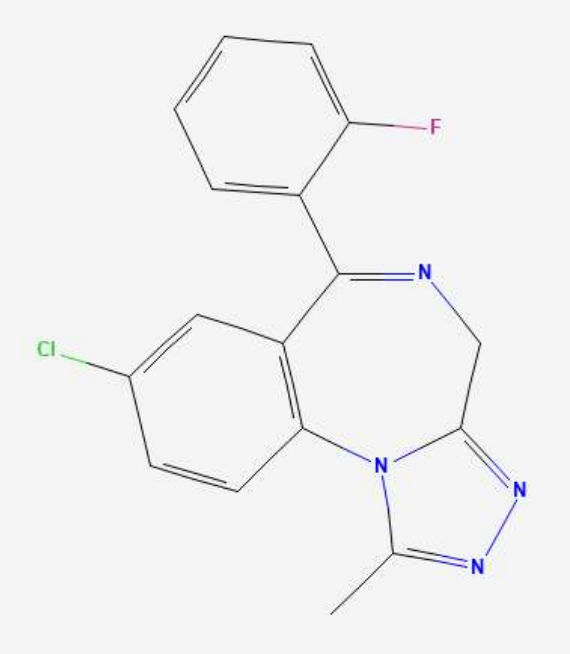
These NPS have the same chemical structure as the legal BZD. The generation of a large number of new synthetic compounds is possible by slightly altering the nucleus of the chemical structure in different positions. Mainly creating 1,4-benzodiazepines, triazolobenzodiazepines and thienotriazolodiazepines (Figure 9). The more recent benzodiazepine has a triazole ring fused to the nucleus of diazepine 1.4 and electron-withdrawing groups (bromine, chlorine, nitro etc.) in the R8 position which increase affinity for the GABA receptor [62].

Figure 9: Chemical structures of three designer benzodiazepine as an example of the chemical structure which characterizes the group.

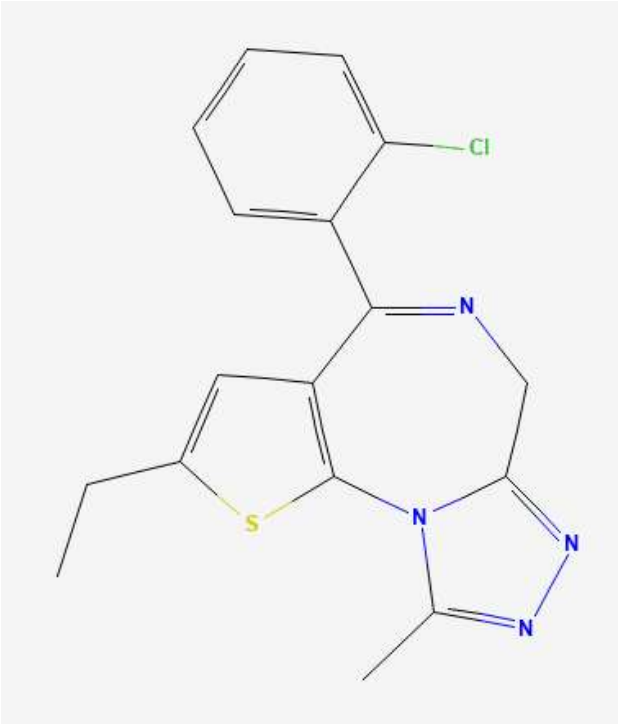
Classic benzodiazepines: nordazepam



Triazolobenzodiazepines: flualprazolam



Thienotriazolodiazepines: etizolam



Phenazepam and nimetazepam were the first DBZD identified in Europe on the internet in 2007, followed by etizolam in 2011. Unfortunately, even in the case of newly synthetized benzodiazepines, evidence has emerged of concurrent BDZ misuse with other drugs, a well-known phenomenon increasing health and safety concerns. [72] The number of DBZD confiscations and undercover purchases rose in the US from 2391 in 2018 to 6194 in 2019 according to the US National Forensic Laboratory Information System. [73-76]. Produced in clandestine laboratories, DBZD do not meet the same strict approval requirements as legal pharmaceuticals and may contain variable amounts of active ingredients or contaminants, i.e. NSO and other NPS [77]. Users are generally unaware of the presence of contaminants in a product, resulting in an increasing number of adverse health events for DBZD, including emergency admissions and death investigations [78-80]. There is also increasing DBDZ prevalence in driving impairment and road traffic incidents [81, 82]. According to the UNODC, between 2019 and April 2020, DBZD were identified in 48% and 83% of post-mortem and Driving Under the Influence of Drug (DUID) cases, respectively, with flualprazolam, flubromazolam and etizolam as the most frequently detected substances. [77, 83] Due to the high abuse potential and life-threatening consequences of DBZD use, between 2020 and 2021 clonazolam, diclazepam, etizolam, flualprazolam and flubromazolam were listed in Schedule IV of the Convention of Psychotropic Substances of 1971. [84].

Primary motivations for DBDZ misuse are the possibility to manage the acute effects of stimulants or to compensate and attenuate the symptoms of abstinence with DBDZs' hypnotic and anxiolytic effects. Unfortunately, they can also cause 'high' effects in certain subjects. One of the most dangerous features of DBDZs is the mechanism which gives the psychotropic action a very slow beginning and a very long half-life, so clearly the risk is that users take more doses than necessary, because they do not feel the effect of the first.

1.5.7. ***Tryptamines***

Tryptamines (T) are a group of monoamine alkaloids in which the main chemical skeleton is a tryptamine, a natural alkaloid. Marketed as vegetable formulations (mushrooms or Ayahuasca, usually sold as dried preparations) or synthetic (sold as capsules, tablets, powders or in liquid form), they are chosen by their users for their hallucinogenic and alienating properties. The group includes both some natural neurotransmitters such as serotine and compounds with hallucinogenic activity such as dimethyltryptamine (DMT) the hallucinogenic active ingredient of decoctions of Ayahuasca, used by some local populations, and like psylocibin, present in some hallucinogenic mushrooms.

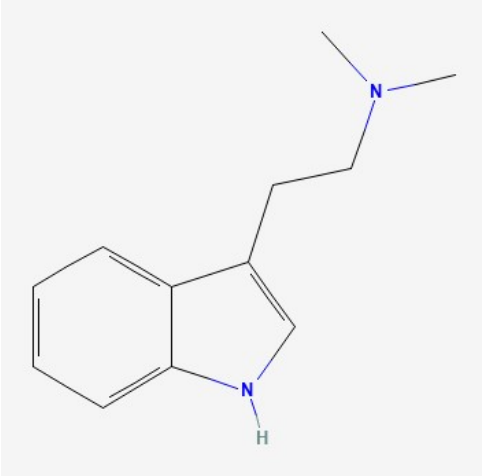
As for synthetic formulations, originating from research studies, they are now in circulation as new psychoactive substances.

Examples include 5-MeO-DMT, 5-MeO-DPT, AMT, 4-AcO-DMT, 4-AcODiPT, 5-HTP, psilocin, psilocybin, DET, DMT, etriptamina, 5-MeO-DALT, 5-MeOMiPT, 4-AcO-DMT. [85]

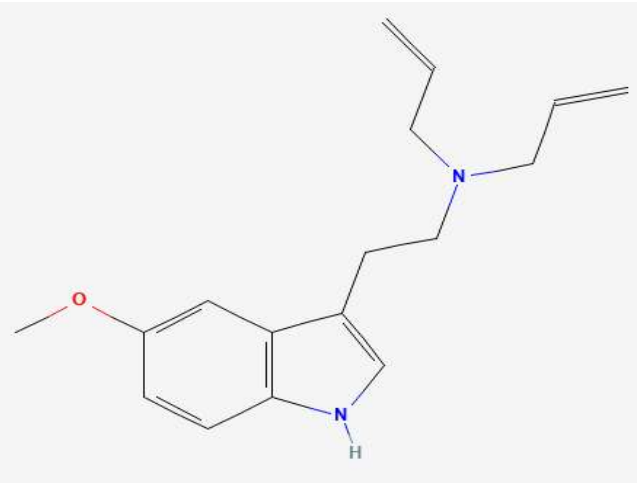
As described by Baumeister D. et al. [86], T fit within the hallucinogenic subcategory (together with phenethylamines). Like many hallucinogens, T modulates serotonin activity by acting on the 5-HT2A receptor. They are the result of decarboxylation of tryptophan (an amino acid) and are chemically characterized by an indole, a bicyclic combination of a benzene ring and a pyrrole ring, with an amino group attached to a 2-carbon side chain (Figure 10). T group includes compounds such as alpha-methyltryptamine (AMT), N,N-dimethyltryptamine (DMT), N,N-diallyl-5-methoxytryptamine (5-MeO-DALT) and 5-methoxy-N,N-disopropyltyptamine (5-MeO-DIPT) 'foxy methoxy'. They possess an indole ring structure, a bicyclical combination of a benzene ring and a pyrrole ring, with an amino group attached to a 2-carbon side chain. [87]

Figure 10: Some of the structural characteristics of tryptamines

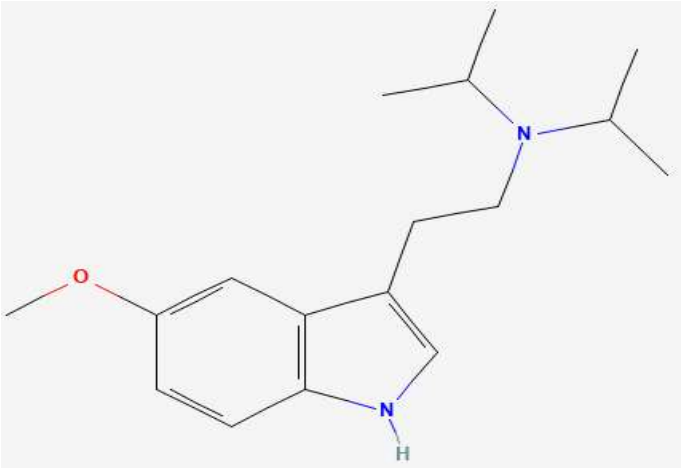
N,N-dimethyltryptamine (N,N-DMT)



N,N-diallyl-5-methoxytryptamine (5-MeO-DALT)



5-methoxy-N,N.disopropyltryptamine (5-MeO-DiPT)



Chapter 2. Toxicological aspects of NPS hair testing

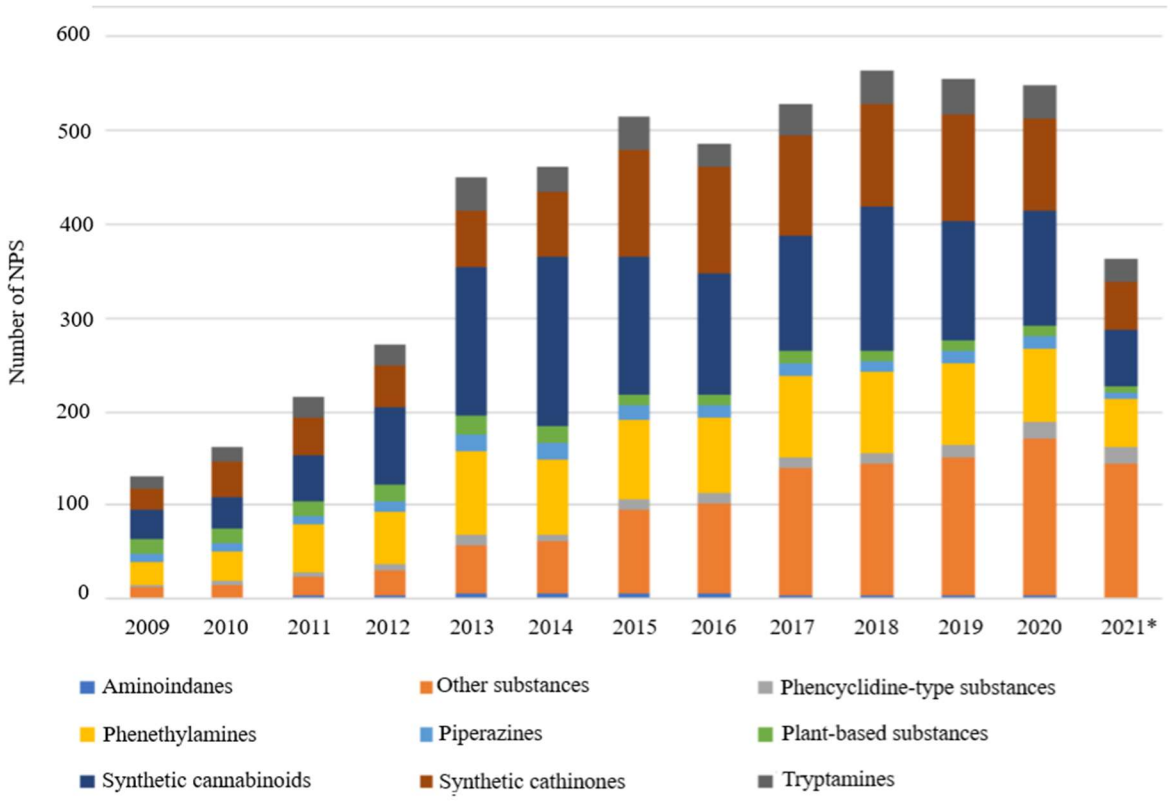
2.1. NPS and analytical challenges

Epidemiological studies carried out in the United States and Europe show that the consumption of NPS spreads indifferently among all subclasses of the population (student, user-type or "psychonaut", drug addict, prisoner) although there was a clear prevalence of abuse in the low age groups (especially among adolescents). This very real prevalence could have its roots in the fact that most are still legal and can easily be obtained on the "dark web". [5]

However, an accurate study of NPS use is hampered by numerous objective difficulties:

- 1,124 substances were reported to the UNODC Early Warning Advisory between 2009 and December 2021 (Figure 11), resulting in problems of analysis and recognition of the molecule;
- continuous turnover of substances, for example alpha-PVP, the cathinone most used in 2010 is gradually disappearing to make room for new types of cathinones.

Figure 11: NPS reported to UNODC each year, by UNODC classification substance group, 2009-2021



Source: UNODC Early Warning Advisory on NPS, 2022.

Currently most of the information comes from the analysis of confiscation by law enforcement officers, while data on biological material (from living or deceased subjects) are limited. These aspects result in inevitable recognition problems, of both a clinical and analytical nature. From an analytical point of view, the high turnover and especially the number of circulating NPS, cause considerable difficulties in the research and identification of compounds in biological samples. Valid identification and determination must comply with the minimum analytical standards defined by the scientific community. To achieve the minimum performance criteria defined by the scientific community for valid recognition, each laboratory requires, at the least, certified standard solutions (non-existent or not easily available) and high performance laboratory equipment. [88]

To date, few laboratories are equipped with validated methods and analytical techniques that allow research into the most widespread NPS.

2.2. The analysis of keratin matrix in forensic toxicology

The Forensic Toxicologist usually manages conventional biological matrices such as whole blood and urinary matrix but also unconventional matrices such as gastric content, oral fluids, sweat, keratin matrix (head hair, pubic hair and nails) and other tissues, for the purpose of determining the timing of events, the degree of intoxication, and the damage resulting from different patterns of drug and alcohol abuse by using highly specific and sensitive techniques. Each matrix has a different time window of detection; usually blood and oral fluids can provide indications only over narrow time windows (of hours or a day, respectively); information from a wider window of time may be provided by urine, sweat (some weeks) and by the keratin matrices (one to six months).

Nowadays, the practice of forensic toxicology embraces three major subdivisions: postmortem forensic toxicology, forensic drug testing, and human performance toxicology. Among these, human performance toxicology deals with the relationship between the presence of a drug in an individual and changes in attitude or performance on assigned tasks. Human performance toxicology is widely applied in the following areas: occupational safety (D.lgs 81/2008); sports competitions (anti-doping), rehabilitation programs, driving license renewal. [89]

In some of the above-mentioned areas, health checks for the absence of drug addiction and the use of narcotic or psychotropic substances by workers performing tasks involving particular safety risks, or the safety and health of third parties, are regulated by the measures set down on 18th September 2008, published in the Official Gazette No. 236 of 8/10/2008. [90]

These sectors should be characterized by a constant monitoring process which requires regular or causal drug tests to confirm fitness for work, athletic performance or simply to confirm the sobriety status of former drug addicts in treatment at public pathological addiction services.

Short time window information given by blood or saliva are not always appropriate for this type of monitoring. The only conventional matrix characterized by a longer detection window, namely urine, is also the matrix most easily adulterated by monitored subjects. For this reason, to obtain certain information on consumption over a longer time period, the matrix of choice is that of keratin. The advantages of this matrix lie in the non-invasiveness of the collection, in a drug detection window extending considerably from one to six months (as already mentioned). The value of keratin matrix analysis in identifying drug users should be increasingly appreciated. It would have wide applications in pre-employment screening, forensic science, clinical application and doping control.

Hair testing

The hair bulb has its own life cycle divided into three phases: anagenic, catagenic and telogenic. The first one is the only phase in which drugs would be incorporated. Therefore, in the proximal part of the hair, close to the skin, it is possible to detect a temporal exposure close to the intake, while in the distal part, towards the tip, a more distant exposure is detected over time. Moreover, since the rate of hair growth is approximately 1 cm/month, segmental analysis per cm of hair can provide information on the history and nature of consumption of a substance in each of the months corresponding to the segment analyzed. It is important to note that beyond 6 cm of hair, determining the analytes in the hair becomes quite difficult as the matrix is consumed.

With regard to the collection of the sample, it is preferable to take it from the back of the head (vertex), as close as possible to the scalp; it is believed that this region of the head is associated with a minimum variability in the inter-individual rate of hair growth.

The hair matrix of choice for the analysis of narcotic drugs is represented by head hair, but if its extraction is not possible (e.g. baldness, or shaving), alternative extraction sites may be used, such as the chest, pubic hair, armpits or face (beard hair). However, the growth rate and dormancy (telogenic phase) of hairs taken from these areas are different from the growth rate and dormancy of the head hair itself. Therefore, it is not possible to trace back a time window of substance use, but only to confirm or exclude previous use. [90]

Another feature which makes the hair matrix preferable to others is the storage mode. Indeed, unlike other biological matrices which require special storage conditions (4°C, -20°C or -80°C), the hair matrix can simply be stored at room temperature and away from light.

Hair analysis can be considered the “gold standard" in the biomonitoring of toxic substances and is the method of choice for the retrospective assessment of past, chronic, sub-chronic exposure to xenobiotics. [6, 91]

2.3. NPS hair testing: overview of the literature

In order to deal with the NPS traffic market, characterized by the continuous evolution of new synthesized substances, it is necessary to use versatile and cutting-edge analytical methodologies, able to detect an ever increasing number of NPS, simultaneously. For this reason, academic, research, and forensic institutions continually update their analytical methodologies in order to identify emerging NPS. Indeed, there are more and more publications reported in literature focusing simultaneously on their analytical determination together with that of classic traditional substances. (Table 1).

Herein we made a selection and a brief elaboration of the characteristics of the analytical method published on Pubmed between 2013 and 2023 (January) regarding "NPS hair testing". Since the aim of our study is to develop a method of simultaneous detection of many substance (NPS and classical drugs), only multi-analyte methods were included. As the table shows, only half of the methods comprise all NPS classes as subjects of investigation and determination; the volume of keratin matrix for the analysis is between 20 and 50 mg and the pre-analytical technique of extraction and purification are slightly different. The prerogative of almost all methods is the implementation of analyte separation through liquid chromatography (except for the method of Anzillotti et al. 2020). [102] The innovative nature of this chromatographic technique allows the separation of large and small complex molecules without the need for special sample purification techniques and provides excellent coupling to mass spectrometry technologies such as high resolution and tandem mass spectrometry.

However, it is not easy to develop a method that includes enough NPS, as these drugs are continuously introduced onto the black market and the reference standards, necessary for the development of analytical methods for their revelation, are not immediately available. [9,10,20-22,103]

To reduce the supply on the NPS market and to implement effective health intervention strategies, the ability to determine and identify NPS is fundamental. Subsequently, the collection of accurate data is imperative for effective policymaking. Unfortunately, competent institutions in different countries face important challenges in the recognition of NPS in both confiscated materials and biological specimens of drug users. Analytical detection of NPS by second-tier analysis requires standard solutions, methodologies and analytical equipment not yet accessible to all laboratories. Therefore, their identification in biological samples, as well as in samples confiscated or collected, represents one of the greatest difficulties. These aspects are extremely important especially when considering the legal, health and social consequences of NPS [104-107].

Table 1: Selection and elaboration of 11 analytical papers involving multi-analyte determination

year of publication	first author	compounds	sample	treatment	instrumentation	LOQ	references
2012	Rust K.Y.	14 synthetic cathinones and piperazines	20–30 mg	two steps, methanol (5 ml, 16 h, ultrasonication) and methanol acidified with HCl (3 ml, 3 h, ultrasonication).	LC-MS/MS: Applied Biosystems 5500 Q Trap	10 to 50pg/mg	[92]
2014	Strano-Rossi S.	50 substances (SCs, SCAs, ketamine, piperazines and amphetamine-type substances—ATS)	30 mg	under sonication at 45°C overnight with (a) water 0,1% HCCOOH (b) MeOH;	LC-MS/MS: Agilent 6460 triple quadrupole	2-20 pg/mg	[93]
2015	Montenarh D.	130 substances (illicit drugs, prescription drugs and NPS)	20 mg	LLE with diethyl ether-ethyl acetate mixture (1:1) after hydrolysis (90 min at 40°C)	LC–MS/MS: AB SCIEX 3200 Q-TRAP	5pg-15ng	[94]
2016	Salomone A.	31 stimulant and phenethylamines, and dissociative drugs	25 mg	incubation at 55 °C for 15 h in methanol	LC–MS/MS: AB Sciex 4500 Q-Trap	1.8-35 pg/mg	[95]

year of publication	first author	compounds	sample	treatment	instrumentation	LOQ	references
2017	Boumba V.A.	132 NPS (SCAs, SCs, stimulants, piperazines and others)	20 mg	0.1 M HCl in methanol	LC–MS/MS: Sciex 4500 Q-Trap	1-100 pg/mg	[96]
2017	Montesano C.	15 substances (SCAs, SCs, 2C-T-4, 4-fluorophenylpiperazine, methoxetamine)	20 mg	pressurized liquid extraction (PLE) followed by solid-phase extraction (SPE) clean-up	LC-HRMS : Thermo Scientific Q-Exactive	8 to 50pg/mg	[97]
2017	Lendoiro E.	classic ATS, SCAs, synthetic piperazines, piperazine and medicines	30 mg	incubation with acid methanol (0.1% HCl) + a mixed-mode solid-phase extraction	LC-MS/MS: Quattro Micro TM API ESCI triple quadrupole	2-20 pg/mg	[98]
2019	Fabresse N.	284 substances (72 NPS)	20-50 mg	Acid hydrolisis and 2 pH LLE	LC-HRMS : Thermo Scientific Q-Exactive	1-500 pg/mg	[99]

year of publication	first author	compounds	sample	treatment	instrumentation	LOQ	references
2020	Niebel A.	35 synthetic cathinones and piperazines	50 mg	2x [methanol/acetonitrile/H2O with ammonium formate (25:25:50) solution shaken at 40 °C for 18 h	LC-MS/MS: Agilent 6460 triple quadrupole	6-9.5 pg/mg	[100]
2020	Mannocchi G.	87 NPS and 32 other drugs	25 mg	incubation for 60 min at 100°C in M3® reagent	LC-MS/MS: Waters XEVO TQ-S Micro	1 to 30pg/mg	[101]
2020	Anzillotti L.	methadone, cannabinoids and synthetic cannabinoids	30 mg	incubation with basic solution + LLE	GC-MS: Agilent 5977B single quadrupole	50pg/mg -1 ng/mg	[102]

UNODC, through its program of laboratories and forensic services, continues to help Member States develop and strengthen their capacity to reveal and determine NPS by developing and disseminating recommended laboratory testing methods for newly controlled substances and to provide chemical reference standards to help the laboratory analyze NPSs in seized materials and biological samples. The UNODC Scientific and Forensic Services program provides the technical means to support synthetic drug law enforcement activities in different regions.

Although some progress has been made at a global level in quali-quantitative determination of NPS, in many countries this remains a major challenge in terms of monitoring and reporting, informed therapeutic intervention and in the implementation of planning decisions. [108]

As stated by Couto et al. [109] "Due to the lack of standard analytical methods for NPS, their identification has certainly become an analytical toxicological challenge, because whenever an analytical method is applied to a new drug, a different derivative quickly emerges, often able to circumvent existing methods in a stealthy manner". The above quotation implies an increase in the probability of false negative analysis reports. The secret lies in an in-depth understanding the of chemical structure and chemical similarities/ differences between NPS. Knowledge that could be gained through the unification and sharing of the fundamental chemical data of NPS resulting in improved regulation and detection of new substances. In 2019 the Global Commission on Drug Policy reported that the classification of psychoactive substances must be urgently improved to better consistency[28,2].

Chapter 3. Experimental study

3.1. Aims of the study

Up to November 2021, through the EWA system over 1100 individual NPS were identified. The sheer volume of these newly synthetized substances and their chemical variability create relevant analytical challenges in order to detect them in biological samples and confiscated batches. To date, there are few analytical methods successfully validated for the simultaneous identification of all NPS classes in hair samples. The aim of our study was to:

- develop and validate a reliable and high-throughput UHPLC/MSMS method for the determination of 127 New Psychoactive Substances (parent and metabolite) and 15 classic drugs of abuse (parent and metabolite).
- apply the newly validated method on samples of keratin matrix taken from specific safety-sensitive social groups, such as drivers and workers, followed by public services for pathological addictions (Ser.DP) in the metropolitan area of Bologna, with the aim of evaluating quality and rate of consumption of NPS, in conjunction with classic abuse drugs too. The collection of keratin samples was carried out by professional figures of Ser.D.P. with subsequent toxicological analysis in the Laboratory of Forensic Toxicology of the University of Bologna.

3.2. Material and methods

3.2.1. *Development and validation of an LC-MS/MS method for NPS in keratin matrices*

The present analytical method was developed at the Laboratory of Forensic Toxicology in the Department of Medical and Surgical Sciences of the University of Bologna and was validated for keratin matrix analysis. The method included almost 150 substances from synthetic cannabinoids, synthetic cathinones, synthetic opioids, other synthetic hallucinogens/stimulants, designer benzodiazepines to classic drugs of abuse. The analysis was based on an acid hydrolysis with methanolic:water acid solution and direct injection in the instrumental analysis by liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS). Through a single extraction it is possible to simultaneously search for the presence of both classic drugs and new psychoactive drugs. All the standards of New psychoactive substances were provided by the National Health Institute and Comedical s.r.l. (Trento, Italy) within the national project "SNAP", allowing the laboratories involved in the project, to detect potentially health-threatening phenomena related to the emergence of new drugs and new modes of use as early as possible. [110]

Chemicals and reagents

The National Health Institute and Comedical s.r.l. (Italy, Trento) within the national project "SNAP" provide standards of 3,4-methylmethcathinone, 4-fluoromethcathinone, 4-methylethcathinone, AM-2201, AM-2233, AM-694, buphedrone, butylone, ethcathinone, ethylone, JWH-007, JWH-016, JWH-019, JWH-081, JWH-098, JWH-122, JWH-203, JWH-210, JWH-251, JWH-302, JWH-398, 3,4-methylenedioxypyrovalerone, methcatinone, methedrone, methylone, pentylone, RCS-4, RCS-8 and pravadoline at a concentration of 100 µg/ml; (±)-cis-3-methyl norfentanyl, (±)-trans-3-methyl norfentanyl, α-ethyl-triptamine, β-hydroxyfentanyl, β-hydroxythiofentanyl, β-phenyl fentanyl, 4-AcO-DiPT,4-ANPP, 5/6-APB, 5-Cl-THJ 018, 5-EAPB, 5F-ADB, 5F-APP-PICA (PX-1), 5F-APP-PINACA(PX-2), 5F-CumylPINACA, 5F-NNEI 2'-Naphthyl Isomer, 5/6-MAPB, 5-MeO-AMT, 5-MeO-DALT, 5-MeO-DMT, 5-MeO-DPT, 5-MeO-MiPT, AB-CHMINACA, AB-FUBINACA, acetyl fentanyl, acetyl norfentanyl, ADB-FUBINACA, alfentanyl, APP-FUBINACA, butyryl fentanyl, butyryl fentanyl carboxy metabolite, butyryl norfentanyl, carfentanyl, Cumyl-PEGACLONE (SGT-151), cyclopropylfentanyl, despropionyl para-fluorofentanyl, ethylphenidate, fentanyl,furanyl norfentanyl, JWH-018, JWH-200, JWH-250, MDMB-CHMICA, mephedrone, methoxyacetyl norfentanyl, MMB-2201, N,N-dimethylcathinone, N,N-dimethyltryptamine, norfentanyl, phenylfentanyl, phenylacetyl fentanyl, ritalinic acid and valeryl fentanyl carboxy metabolite at a concentration of 50 µg/ml; 2-fluoro deschloroketamine, 3-methoxy PCE, deschloro-N-ethyl-ketamine, bentazepam, clonazolam, diclazepam, etizolam, 5-fluoro CUMYL-P7AICA, 5-fluoro CUMYL-PeGACLONE, 5-fluoro MDMB-7-PAICA, 5-fluoro MDMB-PICA, UR-144, 3',4'-Methylenedioxy-α-pyrrolidinohexiophenone, ethylone, euthylone, N-ethyl pentylone, α-pyrrolidinohexanophenone, furanyl fentanyl, isobutyryl fentanyl, isotonitazene, methoxyacetyl fentanyl, ocfentanyl, para-fluoro-furanyl fentanyl, 2-methyl AP-237, AP-237 at a concentration of 20 µg/ml; 5-Methylmethiopropamine, methoxpropamine, brorphine, butonitazene, etodesnitazene, flunitazene, metodesnitazene, metonitazene, N-pyrrolidino etonitazene, 4-fluoro MDMB-BUTICA, 5-fluoro CUMYL-PICA, 5-fluoro EDMB-PICA, 5-fluoro EMB-PICA, ADB-4en-PINACA, MDMB-4en-PICA, MDMB-4en-PINACA, MDMB-4en-PINACA butanoic acid metabolite, 3-methylmethcathinone at a concentration of 10 µg/ml; standard of amphetamine, metamphetamine, methylenedioxyamphetamine, methylenedioxymethamphetamine, cocaine, cocaetilene, ecgonine methyl estere, ketamine, norketamine, methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, morphine, codeine, 6-mono-acetyl-morphine, delta-9-tetrahydrocannabinol, cannabidiolo at a concentration of 1 mg/ml and benzoilecgonine, internal standards (fentanyl-d5, ketamina-d4, jwh-122-d9, nordiazepam-d5) at a concentration of 100 µg/ml were obtained from Sigma Aldrich (Steinheim, Germany) and LGC Standards Ltd (Milano, Italy).

Standard compounds were stored according to supplier recommendations until their use.

2-isopropanol, acetone, acetonitrile, dichloromethane, formic acid and methanol were purchased from Sigma-Aldrich (Germany). All reagents and solvents were of LC/MS grade. Ultra-pure water was obtained from PURELAB® chorus 1, Elga Veolia. Drug-free keratin matrices was supplied by the Laboratory of Forensic Toxicology (Bologna, Italy).

Calibration standards and quality control

Individual stock solutions of the listed standards were used to prepare one working mixture at 100 ng/mL in methanol and were stored at −20 °C until use.

Internal standards mixture containing fentanyl-d5, ketamina-d4, jwh-122-d9, nordiazepam-d5 was prepared at a concentration of 1 µg/mL. Drug-free hair samples were obtained from laboratory staff and used for the preparation of calibration curves and for matrix effect studies. Calibration points and Quality controls (QCs) were prepared spiking aliquots of the 100 ng/mL standards working mixture on the matrix (excluding the higher calibration point, the addition were diluted to a final volume of 100 µL with methanol [93]). Then, each point was vortexed and left to dry overnight. Quality Control (QC) were spiked with an indipendent working solution.

Sample preparation

Each sample of keratin matrix was washed with dicloromethane, methanol and acetone. After drying, each sample was finely cut and an aliquot of 25 mg was weighted in a centrifuge glass tube. Then the sample was added with 300 microliters of methanolic-water (70:30, v/v) 0,1% formic acid solution and put overnight in a thermoblock at 45°C.

UHPLC–MS/MS conditions

The instrumental analysis was performed using a Waters Acquity (Ultra High Performance Liquid Chromatography) UHPLC® (Milford, MA), coupled to a quadrupole mass detector Waters Xevo TQD with an electrospray ion source (ESI) operating in positive mode. Chromatographic separation was achieved on an Acquity UPLC® HSS C18 column (1.8 µm, 2.1 × 150 mm from Waters, Italy, Milan) set at 40 °C and injection volume was 5 µL. The mobile phases used were A – water 0.1% formic acid and B – acetonitrile 0.1% formic acid. Gradient elution was as follows: Mobile phase B starting concentration was 10%, linearly increased to 40% at 8.0 min, further increased to 95% at 13.0 min, kept constant for 1.5 min, decreased to the starting conditions in 0.5 min, and kept at 10% for 2 min for equilibration. Total run time was 17 min. Flow rate was set at 0.4 ml/min. The autosampler was cooled down to 10°C.

The MS was operated with positive ionization in Multiple Reaction Monitoring (MRM) mode. Specific MRM transitions and collision energies were recovered both from the rapid LC-MS/MS method for the detection of 182 novel psychoactive substances in whole blood already published [111] and from literature, on substances tuned with the same MS‐device. Parameter optimization was achieved through a series of experiments carried out by consecutive injection of individual standard solution at a concentration of 1 µg/mL. At least two characteristic transitions were chosen for each analyte. Due to the high number of analytes, two different MS methods were developed, one for substances included in Panels 1 and one for substances included in Panel 2 (including a selection of classic drugs of abuse). A total of two injections were carried out, one per MS/MS method. Optimized MS parameters were as follows: capillary voltage 3.50 kV, desolvation gas temperature 400°C, source gas flow (nitrogen) desolvation rate 800 L/h, cone 20 L/h, gas in collision argon.
The optimal transitions, respective cone and collision energies, retention time and internal standard (IS) used for validation of all compounds are summarized in **Table 2**.

Table 2 Retention times, multiple reaction monitoring transitions (MRM) and collision energies of analytes of interest and their internal standards (ISs)

Compound	RT	Precursor ion	Quantifier ion (CE (V))	Qualifier ion(s) (CE (V))	Cone (V)
New Psychoactive Substances					
(±)-cis-3-methyl Norfentanyl	4,32	247	98.1 (18)	69.1 (29)	25
(±)-trans-3-methyl Norfentanyl	4,18	247	98.1 (18)	69.1 (29)	25
(R)-5-fluoro ADB	12,4	378	233 (20)	318 (14)	45
2-fluoro deschloroketamine	3,02	222,1	109.0 (50)	204.1 (20)	38
2-Methyl AP-237	5,61	287,2	91.1 (50)	117.1 (16)	28
3-methoxy PCE	6,14	234,2	121.1 (28)	189.0 (15)	12
3-methylmethcathinone	3,45	178,1	160.0 (13)	144.9 (22)	30
3,4 MD-alfa-PHP	6,32	290,1	135.0 (33)	140.1(35)	50
3,4-dimethylmethcathinone	4,54	192	174 (13)	159 (22)	30

Compound	RT	Precursor ion	Quantifier ion (CE (V))	Qualifier ion(s) (CE (V))	Cone (V)
4-acetoxy DiPT	5,31	303	114 (18)	160 (28)	15
4-ANPP	6,7	281	105 (22)	188 (14)	22
4-fluoro MDMB-BUTICA	12,0	363,2	144.0 (40)	218.1 (34)	60
4-Fluoromethcathinone	2,25	182	149 (15)	164 (10)	20
4-hydroxy DET	2,69	233,1	86.1 (18)	160 (14)	16
4-methylethcathinone	3,8	192	145.3 (18)	174 (14)	35
5-chloro AB-PINACA	10,74	366	145 (44)	249 (20)	36
5-chloro THJ 018	13,61	377,2	248.9 (16)	212.9 (24)	25
5-EAPB	4,6	204,1	131 (20)	91 (30)	24
5-fluoro AKB48 {5F-API-NACA)	13,96	384	135 (24)	106.9 (45)	35
5-fluoro APP-PICA	10,8	396,3	232 (26)	144 (44)	25
5-fluoro APP-PINACA	10,68	397,3	233 (22)	145 (46)	20
5-fluoro CUMYL-P7AICA	11,95	368,2	119.1 (52)	174.1 (36)	62
5-fluoro CUMYL-Pe-GACLONE	11,7	391,2	119 (36)	273.2 (18)	50
5-fluoro CUMYL-PICA	12,7	367,2	249.1 (25)	206.1 (35)	50
5-fluoro CUMYL-PINACA	12,9	368,3	233 (18)	250 (10)	32
5-fluoro EDMB-PICA	12,8	391,2	232.1 (30)	144.0 (55)	40
5-fluoro EMB-PICA	12,5	377,2	144.0 (40)	232.1 (24)	36
5-fluoro NNEI 2'-naphtyl isomer	12,88	375,3	232 (20)	144 (42)	22
5-hydroxytryptophan	1,13	221,1	134 (17)	204 (11)	28
5-methoxy AMT	3,26	205,1	173 (22)	147 (20)	22
5-methoxy DALT	5,38	271,2	110 (14)	174 (18)	24
5-methoxy DMT	3,14	219,1	58 (12)	130.1 (46)	20
5-methoxy DPT	6,11	275,2	114 (16)	174 (14)	14
5-methoxy MiPT	4,08	247,1	86 (14)	174 (16)	10

Compound	RT	Precursor ion	Quantifier ion (CE (V))	Qualifier ion(s) (CE (V))	Cone (V)
5/6-APB	3,71	176,2	91 (26)	77 (40)	22
5/6-MAPB	4,1	190,2	159.1 (10)	131 (18)	22
5F-MDMB-P7AICA	11,8	378,1	145.1 (40)	233.2 (24)	45
5F-MDMB-PICA	12,43	377	145.1 (40)	233.2 (20)	36
AB-CHMINACA	12,04	357,4	145 (46)	241.2 (28)	38
AB-FUBINACA	10,78	369,3	253 (24)	109 (40)	20
acetyl fentanyl	5,72	323	105 (36)	188 (20)	25
acetyl norfentanyl	2,44	219,2	84.1 (18)	55.2 (36)	25
ADB-FUBINACA	11,33	383	253 (25)	338 (10)	25
alfa-PHP	6,18	246,2	91.1 (22)	105.1 (28)	60
alfentanil	6,65	417,1	197.1 (26)	268.1 (16)	24
alpha-ethyltryptamine	4,18	189,2	130 (16)	58.1 (16)	26
AM 2201	12,94	360	127 (40)	155 (28)	45
AM 2233	8,22	459	98 (34)	112 (22)	45
AM-694	12,61	436	231 (28)	202.7V(40)	45
AP-237	5,16	273,2	117.1 (14)	91.1(48)	24
APP-FUBINACA	10,92	417,3	109.0 (40)	253.0 (24)	20
bentazepam	6,55	297,2	139.0 (38)	166.1 (28)	34
beta-hydroxy fentanyl	6	353	335 (16)	204 (38)	35
beta-hydroxythiofentanyl	5,62	359	192 (22)	111 (38)	25
beta-phenyl fentanyl	9,83	413,6	105 (44)	188 (26)	35
brorphine	7,23	400,1	218.1 (22)	104 (50)	50
buphedrone	3,38	178	160 (10)	130.3 (32)	30
butonitazene	9,92	425,2	100 (20)	106.9 (52)	46
buthylone	2,71	222	204 (11)	174 (20)	25
butyryl fentanyl	7,7	351,2	105 (40)	188.1 (22)	30

Compound	RT	Precursor ion	Quantifier ion (CE (V))	Qualifier ion(s) (CE (V))	Cone (V)
butyryl fentanyl carboxy metabolite	5,49	381	105 (42)	188 (45)	25
butyryl norfentanyl	4,87	247,0	84 (28)	55 (36)	25
carfentanil	7,87	395,2	113 (32)	105 (52)	22
clonazolam	8,82	354,1	308 (26)	326 (24)	10
CUMYL-PeGACLONE	13,07	373,3	255.1 (10)	185.1 (24)	30
cyclopropyl fentanyl	7,45	349,2	105 (30)	188.1 (20)	25
deschloro-N-ethyl-ketamine	3,54	218,1	173.1(8)	145.1 (16)	38
despropionyl para-fluorofentanyl	7,1	299	105 (38)	188 (16)	15
diclazepam	11,39	321,1	89.3 (60)	165.1 (54)	20
dimethylcathinone	2,29	178,1	72 (22)	105.3 (20)	30
ethcathinone	2,65	177,7	72 (16)	105.2 (22)	30
ethylone	2,76	222	146 (26)	204 (38)	30
ethylphenidate	5,57	248,1	84,1	56	50
etizolam	10,18	343	138.1 (42)	314.1 (26)	36
etodesnitazene	3,91	352,2	100 (18)	106.9 (40)	52
euthylone	3,58	236,1	174 (36)	188.1 (16)	35
fentanyl	6,58	337,2	105,2	188,2	35
flunitazene	6,63	371,2	100 (26)	72 (34)	60
furanyl fentanyl	7,3	375,1	105 (38)	188 (18)	16
furanyl norfentanyl	3,95	271	84 (18)	55 (38)	16
isobutyryl fentanyl	7,9	351,3	105.1 (42)	188.1 (22)	30
isotonitazene	8,4	411,2	106.9 (46)	100 (22)	50
JWH-007	13,55	356	127 (48)	155 (26)	45
JWH-016	13,26	342	127 (44)	155 (34)	45
JWH-018	13,63	342	127 (25)	155 (34)	45

Compound	RT	Precursor ion	Quantifier ion (CE (V))	Qualifier ion(s) (CE (V))	Cone (V)
JWH-019	13,97	356	127,0001	228 (26)	45
JWH-081	13,91	372	185 (26)	157.1 (40)	45
JWH-098	14,04	386	185 (26)	127 (34)	45
JWH-122	13,76	356	169 (26)	141 (40)	45
JWH-200	8,6	385	114 (46)	155 (42)	20
JWH-203	13,64	340	125 (28)	214 (22)	45
JWH-210	14,3	370	183 (26)	214 (24)	45
JWH-250	12,98	336	91 (50)	121 (32)	45
JWH-251	13,24	320	105 (22)	214 (15)	45
JWH-302	12,9	336	213.9 (30)	143.9 (44)	45
JWH-398	14,04	376	189 (26)	161 (48)	45
ketamine	3,62	238,2	125.1 (25)	220.2 (15)	20
MDMB-4en-PICA	12,8	357,21	212.1 (38)	130.1 (20)	36
MDMB-4en-PINACA	13,33	358,5	213.1 (31)	298.19 (20)	45
MDMB-CHMICA	13,2	385	240 (24)	144 (46)	20
MDPV	4,95	276	126.2 (25)	135 (23)	29
mephedrone	3,4	178,3	160.2 (12)	145.1 (18)	20
methcathinone	2,24	163,9	131 (20)	104.8 (22)	30
methedrone	2,83	194	176 (8)	161 (13)	20
methoxpropamine	5,12	262,2	203.1 (10)	121.1 (30)	40
methoxyacetyl fentanyl	5,57	353,3	188 (20)	84.1 (18)	30
methoxyacetyl norfentanyl	2,31	249	84 (14)	55 (38)	15
methylone	2,34	208	160.21 (15)	132.1 (27)	30
metodesnitazene	2,88	338,2	100.1 (22)	72.1 (34)	48
metonitazene	6,37	383,2	100 (22)	121 (34)	48
MMB2201	11,87	363,3	231.9 (12)	143.9 (38)	34

Compound	RT	Precursor ion	Quantifier ion (CE (V))	Qualifier ion(s) (CE (V))	Cone (V)
N-ethyl pentylone	4,69	250,2	202.2 (18)	232.2 (13)	24
N-pyrrolidino etonitazene	7,29	395,21	98.1 (22)	107 (22)	52
N,N-dimethyltriptamine	3,01	189,2	58 (12)	117 (34)	20
norfentanyl	3,66	233,1	84.3 (20)	55.3 (34)	25
norketamina	3,4	224,1	125 (20)	207.1 (10)	20
ocfentanyl	5,85	371,2	105.2 (40)	188.2 (24)	30
p-fluoro-furanyl fentanyl	7,68	393,2	105.1 (40)	188.1 (22)	25
pentylone	3,56	236	188 (18)	218 (10)	35
phenyl fentanyl	8,38	385	105 (46)	188 (24)	40
phenylacetyl fentanyl	9,28	399	188 (24)	105 (46)	46
pravadoline	7,47	379,2	135 (24)	114 (32)	45
RCS-4	13,25	322	135 (24)	106.8 (40)	20
RCS-8	14,1	376	121 (26)	91 (40)	45
ritalinic acid	3,47	220,1	84.1 (20)	56 (46)	20
UR144	14,27	312,4	125.1 (22)	144.1 (38)	42
valeryl fentanyl carboxy metabolite	5,7	395	188 (26)	105 (44)	40
Classic Drugs of Abuse (DOA)					
6-monoacetylmorphine	2,61	328,1	165.1 (40)	181.2 (40)	30
amphetamine	2,65	136,1	119.1 (8)	91.1 (15)	20
benzoylecgonine	3,65	290,1	168.1 (20)	105.1 (33)	30
cannabidiol	12,01	315,15	123.1 (32)	193.15 (20)	35
cocaetilene	6,16	318,1	196.1 (20)	82.1 (30)	30
cocaine	5,11	304,2	182.26 (20)	82.3 (28)	30
codeine	2,16	300,1	215.1 (25)	199.2 (27)	30
delta-9-tetrahydrocannabi-nol	13,5	315,2	193.1 (22)	123 (34)	35

Compound	RT	Precursor ion	Quantifier ion (CE (V))	Qualifier ion(s) (CE (V))	Cone (V)
ecgonine methyl ester	0,91	200,2	82.1 (23)	182.1 (17)	35
2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine	8,18	278,2	234.2 (26)	186.2 (35)	50
methylenedioxyampheta-mine	2,75	180,1	163.1 (10)	133.1 (18)	20
methylenedioxymetham-phetamine	2,86	194,1	163 (14)	133.1 (20)	20
methadone	8,61	310,3	105.1 (32)	265.2 (14)	30
methamphetamine	2,57	150,1	119.1 (10)	91.1 (12)	20
morphine	1,32	286	165.1 (40)	153 (40)	35
Internal Standards (ISs)					
fentanyl-d5	6,56	342,2	105.2 (38)	188.2 (30)	40
JWH-122 d9	14,05	365,2	114.9 (35)	169 (35)	50
ketamine-d4	3,28	242,2	129.1 (30)	242 (10)	35
nordiazepam-d5	8,89	276,1	165.1 (28)	213.0 (28)	50

Method validation

The method was validated according to the European Medicine Academy (EMA) guidelines [112], evaluating the following analytical parameters for all analytes: selectivity, linearity, accuracy, precision, limit of quantification (LOQ), matrix effect, recovery.

Selectivity

Drug-free hair samples from six different sources were analyzed to assess selectivity, to determine the interference by endogenous hair constituents at the retention times of our analytes of interest. Absence of interfering compounds was accepted if the response was less than 20% of the lower limit of quantification (LLOQ) for the analytes and 5% for the IS.

Carry-over.

Immediately after the Upper Limit of Quantification (ULOQ) of every calibration curve, we analyzed replicates of blank sample to determine the carry-over. Results for blank sample following ULOQ should not be greater than 20% of the LLOQ and 5% for the IS.

Linearity and Lower Limit of Quantification (LLOQ)

Seven-point calibration curves were prepared by spiking appropriate amounts of working solution in blank hair samples to obtain final concentrations ranging from LLOQ to 640pg/mg. All the curves included a blank sample spiked with IS only (zero sample). Quantification was achieved by plotting the peak area ratios of the single analyte and the coupled IS. Masslynx Software (Waters, USA) was used for this scope. Back-calculated concentrations should be within ±15% of the nominal concentrations and at least 75% of the calibration points must fulfill this criterion. The LLOQ was selected as the lowest concentration point with an accuracy and precision of ± 20 %, and a S/N > 10.

Precision and accuracy

Intra and inter day precision (coefficient of variation CV%) and accuracy (bias%) were determined at four concentration levels: LOQ, QC Low (LQC), QC Medium (MQC) and QC High (HQC). Intra-day assay was established processing 6 replicates of each QC and LOQ on the same day. Inter day assay was established processing 6 replicates of each QC and LOQ on three different days. Accuracy and precision were obtained by bias calculation and relative errors, through Masslynx software (Waters, USA). Accuracy and precision of ±15% for QC levels and of ±20% for LOQ, were required.

Matrix Effect and Extraction Recovery

Percent Matrix Effect (ME) and Extraction Recovery (ER) were calculated at three concentration levels (Low, Medium and High) by means of the following equations:

- ME (%) =B/A x 100.
- ER (%) =C/B x 100.

Where:

A= analyte/IS mean peak area ratio obtained by injecting *extraction solvent (N=3) spiked at three concentration levels.*

B= analyte/IS mean peak area ratio obtained by injecting *drug-free matrix extracts (N=3) spiked at three concentration levels* ***before*** *extraction.*

C= analyte/IS mean peak area ratio obtained by injecting *drug-free matrix extracts (N=3) spiked at three concentration levels* ***after*** *extraction.*

ME and RE were tested by analyzing blank hair matrices from six different sources. The CV of the ME calculated should not exceed 15 %.

3.2.2. *Application of LC-MS/MS method in patients with a history of addiction*

NPS are an increasingly critical phenomenon which has also emerged in Italy. Indeed, there are several reported cases of acute intoxication caused by NPS. Over the past decade, institutions have been fighting against their quantity and chemical diversification, which require constant updating of the analytical methods of detection, with the addition of new molecules to ensure their identification as intoxicants. In order to continually update, it is therefore imperative to highlight both the prevalence of consumption of these new substances and their possible combinations with classic substances of abuse. The developed method was applied to some patients followed by the drug addiction service of the Bologna public services. Every year, the drug addiction service sends around 300 samples to the forensic toxicology laboratory in Bologna in order to search for common narcotic substances (cannabinoids, cocaine and metabolites, amphetamine-like drugs, opiates). The drug addiction service treats patients with current or previous diagnosis of substance use disorder (DSM-5), for clinical, therapeutic, and preventive purposes. For the present study, a residual aliquot of the analyses, rendered completely anonymous, was stored, and analyzed for the NPS panel search. The type and number of positive findings were then reported in order to explore the use of NPS in this specific category of patients, which to date is not known in Italy.

3.3. **Results**

3.3.1. *Development and validation of an LC-MS/MS method for NPS in keratin matrices*

Method validation

Successful validation was achieved for almost all of the analytes. Validation parameters, especially linearity, accuracy, precision, and limit of quantification (LOQ) are shown in Table 3.

Selectivity

Six drug-free hair samples coming from different sources were scrutinized in order to check the eventual presence of interfering peaks in MRM chromatograms where our analytes were expected to elute. No interfering peaks due to endogenous substances were detected.

Carry-over.

MRM chromatograms of drug-free hair samples running immediately after the ULOQ (640 pg/mg) showed no peak of our analytes of interest or IS, thus confirming that carry-over was negligible.

Linearity and limit of quantification (LOQ)

The method exhibits linear calibration functions for all the analytes of interest in the tested range, with R2 always better than 0.99 except for the compounds highlighted in Table 3 (4-Fluoromethcathinone, 5-chloro AB-PINACA, 5-fluoro CUMYL-PINACA). A weighting factor 1/x was adopted. LOQs were 4 pg/mg for all the substances, except for AB-CHMINACA, MDMB-CHMICA, mephedrone, methcathinone, 6-MAM, morphine, which LOQ was at 10 pg/mg; 5F-MDMB-BUTICA, 5-fluoro CUMYL-PeGACLONE, 5-Hydroxxythriptophan, cannabidiol, delta-9-THC which LOQ was at 40 pg/mg and, finally, 4-Fluoromethcathinone and 5-chloro AB-PINACA which LOQ was at 80 pg/mg. Overlay of some MRM chromatograms obtained at LOQ of one compound for NPS classes are shown in Figure 12.

Table 3: Coefficients of determination (R^2) and LOQ for substances. Compounds with different LOQ and/or linearity <0.99 are highlighted.

Compounds	R^2	LOQ (pg/mg)
New Psychoactive Substances		
(±)-cis-3-methyl Norfentanyl	0,991	4
(±)-trans-3-methyl Norfentanyl	0,994	4
(R)-5-fluoro ADB	0,991	4
2-fluoro Deschloroketamine	0,994	4
2-Methyl AP-237	0,991	4
3-methoxy PCE	0,995	4
3-Methylmethcathinone	0,990	4
3,4 MD-alfa-PHP	0,991	4
3,4-Dimethylmethcathinone (3,4.DMMC)	0,993	4
4-acetoxy DiPT	0,991	4
4-ANPP	0,999	4
4-fluoro MDMB-BUTICA	0,991	40
4-Fluoromethcathinone (flefedrone)	0,975	80
4-hidroxy DET	0,997	4

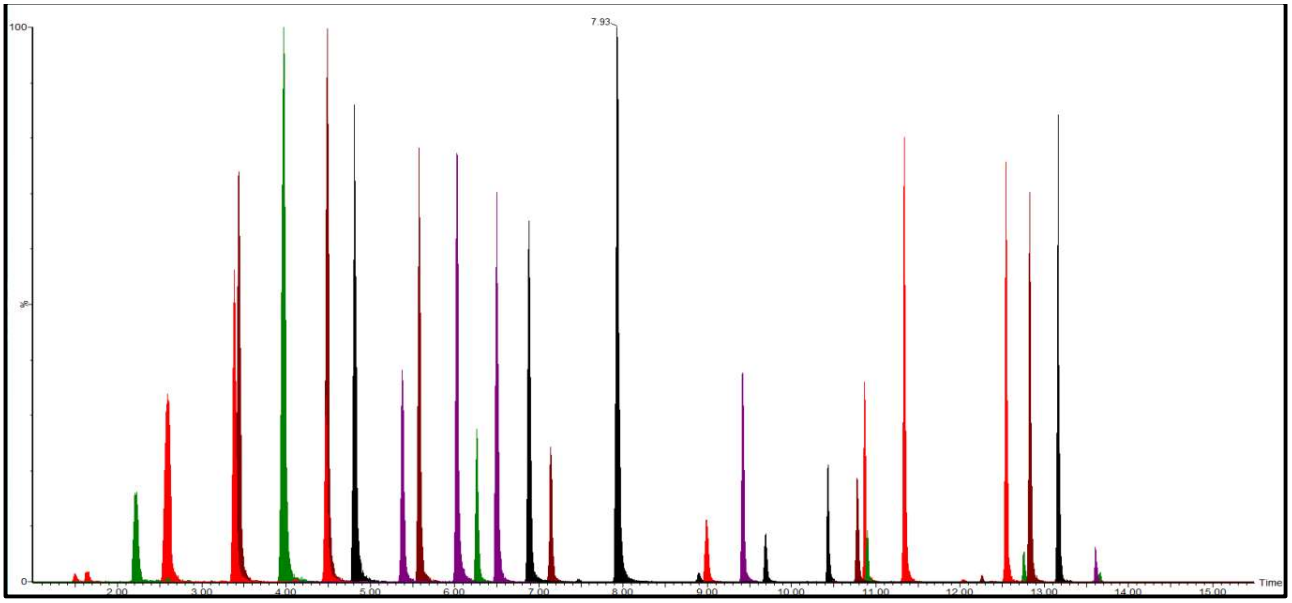
Compounds	R^2	LOQ (pg/mg)
4-Methylethcathinone	0,991	4
5-chloro AB-PINACA	0,975	80
5-chloro THJ 018	0,994	4
5-EAPB	0,991	4
5-fluoro AKB48 {5F-APINACA)	0,998	4
5-fluoro APP-PICA	0,991	4
5-fluoro APP-PINACA	0,991	4
5-fluoro CUMYL-P7AICA	0,991	4
5-fluoro CUMYL-PeGACLONE	0,990	40
5-fluoro CUMYL-PICA	0,991	4
5-fluoro CUMYL-PINACA	0,987	4
5-fluoro EDMB-PICA	0,990	4
5-fluoro EMB-PICA	0,990	4
5-fluoro NNEI 2'-naphtyl isomer	0,998	4
5-Hydroxytryptophan	0,993	40
5-methoxy AMT	0,991	4
5-methoxy DALT	0,992	4
5-methoxy DMT	0,992	4
5-methoxy DPT	0,993	4
5-methoxy MiPT	0,993	4
5/6-APB	0,997	4
5/6-MAPB	0,992	4
5F-MDMB-P7AICA	0,994	4
5F-MDMB-PICA	0,994	4
AB-CHMINACA	0,999	10
AB-FUBINACA	0,990	4
acetyl fentanyl	0,992	4
acetyl norfentanyl	0,995	4
ADB-FUBINACA	0,991	4
alfa-PHP	0,992	4
alfentanil	0,993	4
alpha-Ethyltryptamine	0,991	4
AM 2201	0,991	4
AM 2233	0,994	4

Compounds	R^2	LOQ (pg/mg)
AM-694	0,992	4
AP-237	0,992	4
APP-FUBINACA	0,992	4
Bentazepam	0,990	4
beta-hydroxy Fentanyl	0,991	4
beta-hydroxythiofentanyl	0,991	4
beta-Phenyl fentanyl	0,993	4
Brorphine	0,991	4
Buphedrone	0,991	4
Butonitazene	0,990	4
Buthylone	0,992	4
Butyryl fentanyl	0,990	4
Butyryl fentanyl carboxy metabolite	0,994	4
Butyryl norfentanyl	0,991	4
Carfentanil	0,993	4
Clonazolam	0,995	4
CUMYL-PeGACLONE	0,990	4
Cyclopropyl fentanyl (hydrochloride)	0,994	4
deschloro-N-ethyl-Ketamine	0,992	4
Despropionyl para-Fluorofentanyl	0,992	4
Diclazepam	0,991	4
Dimethylcathinone	0,992	4
Ethcathinone	0,991	4
Ethylone	0,991	4
Ethylphenidate	0,995	4
Etizolam	0,993	4
Etodesnitazene (etazene)	0,991	4
Euthylone	0,992	4
Fentanyl	0,994	4
Flunitazene	0,990	4
Furanyl fentanyl	0,993	4
Furanyl norfentanyl	0,990	4
Isobutyryl fentanyl	0,992	4
Isotonitazene	0,993	4

Compounds	R^2	LOQ (pg/mg)
JWH-007	0,996	4
JWH-016	0,991	4
JWH-018	0,994	4
JWH-019	0,994	4
JWH-081	0,991	4
JWH-098	0,991	4
JWH-122	0,990	4
JWH-200	0,993	4
JWH-203	0,990	4
JWH-210	0,997	4
JWH-250	0,990	4
JWH-251	0,991	4
JWH-302	0,990	4
JWH-398	0,998	4
Ketamina	0,992	4
MDMB-4en-PICA	0,999	4
MDMB-4en-PINACA	0,995	4
MDMB-CHMICA	0,995	10
3,4-MDPV	0,993	4
Mephedrone	0,992	10
Methcathinone	0,996	10
Methedrone	0,995	4
Methoxpropamine (mxpr)	0,993	4
Methoxyacetyl fentanyl	0,991	4
Methoxyacetyl norfentanyl	0,991	4
Methylone	0,991	4
Metodesnitazene	0,994	4
Metonitazene	0,991	4
MMB2201	0,991	4
N-ethyl Pentylone	0,992	4
N-Pyrrolidino Etonitazene	0,991	4
N,N-DMT	0,993	4
Norfentanyl	0,990	4
Norketamina	0,993	4
Ocfentanyl	0,994	4

Compounds	R^2	LOQ (pg/mg)
p-fluoro-Furanyl fentanyl	0,992	4
Pentylone	0,990	4
Phenyl fentanyl	0,990	4
Phenylacetyl fentanyl	0,996	4
Pravadoline (win48,098)	0,994	4
RCS-4	0,991	4
RCS-8	0,990	4
Ritalinic acid	0,992	4
UR-144	0,993	4
Valeryl fentanyl carboxy metabolite	0,992	4
Classic Drugs of Abuse (DOA)		
6-Mam	0,990	10
Amphetamine	0,993	4
Benzoylecgonine	0,991	4
Cannabidiol	0,990	40
Cocaetilene	0,994	4
Cocaine	0,991	4
Codeine	0,991	4
Delta-9-THC	0,997	40
Ecgonine methyl ester	0,995	4
EDDP	0,991	4
MDA	0,992	4
MDMA	0,991	4
Methadone	0,990	4
Methamphetamine	0,990	4
Morphine	0,991	10

Figure 12: Chromatogram overlay of some compounds are reported: methylone, 4-OH-DET, deschloro-N-ethyl-ketamine, IS 1, 5-methoxy-MiPT, 5-EAPB, N-ethyl pentylone, MDPV, 2-methyl AP-237, acetyl fentanyl, 3,4 MD-alfa-PHP, bentazepam, alfentanil, brorphine, pravadoline (win 48,098), AM2233, IS 2, butonitazene, etizolam, 5F-APP-PICA, diclazepam, APP-FUBINACA, ADB-FUBINACA, 5F-CUMYL-P7AICA, AM2201, RCS4, MDMB-4en-PINACA, JWH251, 5F-AKB48, IS 3.



Precision and Accuracy

Intra and inter day precision (coefficient of variation CV%) and accuracy (bias%) of all analytes accordingly fit with the requirements of EMA guidelines, with the single exception of an occasional outlier highlighted with "*". All the data are shown in Table 4.

Table 4: Average percentage values of intra and inter day precision (coefficient of variation CV%) and accuracy (bias%). (*value that exceeded the limit required by the guidelines)

Compound	**Intra-day precision (Average CV%; n=6)**				**Inter-day precision (Average CV%; n=18)**				**Accuracy (Average bias%; n=18)**			
	LOQ	**LQC**	**MQC**	**HQC**	**LOQ**	**LQC**	**MQC**	**HQC**	**LOQ**	**LQC**	**MQC**	**HQC**
New Psychoactive Substances												
(±)-cis-3-methyl Norfentanyl	14.1	4,0	2,0	1,0	7.3	2.3	4.2	0.7	11.9	5.1	4.6	4.9
(±)-trans-3-methyl Norfentanyl	13.5	3.9	12.2	0.7	0.2	3.5	3,0	0.2	12.5	5,0	5.4	5.6
(R)-5-fluoro ADB	14.1	11.5	13.8	1.7	8.9	5.8	4,0	1.3	18	3.4	3.6	4.6

Compound	Intra-day precision (Average CV%; n=6)				Inter-day precision (Average CV%; n=18)				Accuracy (Average bias%; n=18)			
	LOQ	LQC	MQC	HQC	LOQ	LQC	MQC	HQC	LOQ	LQC	MQC	HQC
2-fluoro Deschloroketa-mine	11.1	3.8	11.1	0.8	20.3	1.6	8.3	1.1	6.4	7.2	4.3	0.6
2-Methyl AP-237	5	3,0	2.7	9.4	15.8	7.1	5.5	1,0	8.5	1.5	6.9	3.5
3-methoxy PCE	8.4	13.7	8.9	7.3	17.3	11.5	4.8	0.9	2.3	4.6	5.3	5,0
3-Methylmethca-thinone	13.5	3.6	8.4	2.3	20.4	2,0	3,0	0.6	7.6	1.5	10.2	0.3
3.4 MD-alfa-PHP	8.3	4.7	0.5	2.7	15.4	2,0	13	2,0	13.6	6.9	3.8	8.2
3.4-Dimethylme-thcathinone (3.4.DMMC)	6.9	10.5	3.8	2.6	17.8	4	2.8	5.3	12.7	5.4	7.3	2.1
4-acetoxy DiPT	1.6	1.8	1.6	12.5	6.5	6.7	7.2	8.8	14.2	4.0	5.0	1.7
4-ANPP	3.3	5	0.2	2.8	11.1	2.0	4.5	0.2	12.3	6.4	7.5	2.3
4-fluoro MDMB-BUTICA	9.2	5.3	4.8	2.2	12.8	13.6	1.5	1.2	12.8	7.4	7.9	4.5
4-Fluoromethca-thinone (flefe-drone)	19.2	4.0	6.8	1.7	11.1	10.7	6.9	1.8	18.1	5.0	3.6	5.6
4-hydroxy DET	18.6	2.4	14.5	3,0	16.9	8.3	1.3	10.4	15.9	4.2	12.6	7.3
4-Methylethcathi-none	12.5	3.0	0.7	2	6.7	12.4	8.4	10.5	1.8	6.4	11.9	0.3
5-chloro AB-PI-NACA	15.6	14	9	10.9	11.4	14.6	7.2	1.3	4.6	2.5	3.8	4.0
5-chloro THJ 018	16.6	11.2	2.5	9.7	5,0	1.6	2.0	15.4	3.9	4.0	1.4	0.1
5-EAPB	14.8	4.8	12.4	5.6	7.5	2.9	2.7	1.7	15.6	9.7	2.9	6.6
5-fluoro AKB48 {5F-APINACA)	21.2	3,0	8.6	9	15.1	2.0	6.4	2,0	8.7	13.4	4.6	9.9
5-fluoro APP-PICA	8.1	3.4	10.5	7.3	4.8	3.6	9.6	1.1	9.6	9.9	3.2	5.5
5-fluoro APP-PI-NACA	11.3	10.8	10.7	1.3	14.8	2.5	14.9	0.1	7.4	5.6	3.3	13.7
5-fluoro CUMYL-P7AICA	17.1	3	8.7	5.1	1.1	4.2	4.5	0.9	17.5	3.0	2.7	9.3
5-fluoro CUMYL-PeGACLONE	18.4	7.1	4.0	0.8	11.9	6.5	4.3	1.1	11.1	0.4	13.7	3.0
5-fluoro CUMYL-PICA	16.5	2.1	3.0	10.1	7.1	11.5	4.9	2.4	5.3	8.5	9.3	5.2
5-fluoro CUMYL-PINACA	11.8	6.7	6.1	7.2	16.1	8.6	1.4	3.5	24.6	3.4	8.5	1.1

Compound	Intra-day precision (Average CV%; n=6)				Inter-day precision (Average CV%; n=18)				Accuracy (Average bias%; n=18)			
	LOQ	LQC	MQC	HQC	LOQ	LQC	MQC	HQC	LOQ	LQC	MQC	HQC
5-fluoro EDMB-PICA	4.9	6.4	6.9	7.8	10.0	12.5	2.7	3.0	7.4	3.3	14.4	5.0
5-fluoro EMB-PICA	16.3	13.1	11.3	5.9	7.5	7.2	1.1	1.6	2.1	7.4	16.8	3.0
5-fluoro NNEI 2'-naphtyl isomer	11.7	10.6	2.0	1.1	9.6	3.0	12.3	2.0	8.1	0.6	4.8	1.4
5-Hydroxytrypto-phan	9.2	6.1	4.0	4.8	9.6	3.5	12.1	2.9	7.9	2.9	0.7	4.0
5-methoxy AMT	15.1	11.4	3.0	2.6	9.1	3.0	4.4	1.7	9.8	5.0	0.2	0.8
5-methoxy DALT	16.8	8.4	14	3.6	7.1	10.4	4.7	1.1	15.7	14.9	7.4	1.8
5-methoxy DMT	4.0	4.4	6.4	9.4	7.0	1.4	2.8	1.4	19.2	14	9.1	0.3
5-methoxy DPT	20.6	13.0	22.8	8.9	4.2	7.5	12.6	7.2	18.9	11.8	12.3	6.4
5-methoxy MiPT	8.2	0.8	10.9	1.4	15.7	4.5	3.9	2.1	5.2	9.3	12.2	2.8
5/6-APB	14.0	6.9	7.1	4.5	5.3	2.9	7.2	6.9	17.3	6.3	9.1	0.1
5/6-MAPB	16.4	8.3	6.9	5.2	13.1	6.9	5.2	1.5	20.9	5.6	8.4	2.2
5F-MDMB-P7AICA	9.5	5.9	14.9	13.1	13.1	10.6	25.5	7.6	19.7	9.7	0.7	0.3
5F-MDMB-PICA	12.3	14	9.6	2.9	10.4	7.0	2.9	0.6	22.5*	1.5	3.5	0.8
AB-CHMINACA	24.2*	15.8	6.0	1.3	11.1	6.6	17.7	11.4	12.0	2.4	2.0	2.9
AB-FUBINACA	9.3	16.4	8.6	4.2	3.2	3.4	0.3	5.6	14.3	0.8	3.1	3.5
acetyl fentanyl	15.9	17.5	6.7	1.9	21.9*	8.2	6.3	4.9	19	12.5	2.2	5.1
acetyl norfentanyl	1.2	3.0	5.2	1.2	10.1	1.9	9.9	1.1	15.1	0.6	1.7	0.9
ADB-FUBINACA	18.2	12.3	4.7	9.2	22.7*	8.2	2.5	2.2	5.1	5.0	1.6	8.1
alfa-PHP	15.0	2.1	2.2	8.8	2.2	8.9	15.2	2.4	13.5	7.3	2.4	8.5
alfentanil	10.9	6.4	7.7	3.1	23.6*	1.3	9.4	3.2	19.5	6.3	1.3	7.4
alpha-ethyltrypta-mine	4.6	5.4	8.2	5.5	24.1*	17.4	5.5	1.6	15.0	4.0	6.3	0.2
AM 2201	17.9	2.0	4.1	4.1	8.9	18.6	4.8	1.2	14.3	3.0	1.3	2.9
AM 2233	8.9	2.9	4.8	1.2	1.2	12.1	14.1	5.9	7.9	7.2	9.4	0.2
AM-694	13.7	6.1	5.0	1.8	16.6	8.8	11.2	4.8	17.2	0.2	1.9	1.5

Compound	Intra-day precision (Average CV%; n=6)				Inter-day precision (Average CV%; n=18)				Accuracy (Average bias%; n=18)			
	LOQ	LQC	MQC	HQC	LOQ	LQC	MQC	HQC	LOQ	LQC	MQC	HQC
AP-237	3.6	3.8	12.1	3.1	10.3	9.7	6.0	4.9	16.5	4.3	0.7	4.0
APP-FUBINACA	10.2	3.7	12.1	0.7	2.3	12.3	1.0	1.3	20.7	0.5	4.4	2.3
bentazepam	1.5	4.0	9.1	5.4	9.8	2.2	8.1	2.9	10.4	4.7	0.5	1.7
beta-hydroxy Fentanyl	1.9	5.6	2.8	5.2	9.7	8.0	14.8	0.3	5.6	8.7	6.8	1.7
beta-hydroxythiofentanyl	15.6	16.3	6.0	12.7	7.4	13.2	9.2	6.9	8.5	8.8	2.9	0.2
beta-Phenyl fentanyl	7.2	9.2	7.4	1.3	9.3	11.5	13.3	7.2	15.8	3.2	5.3	2.6
brorphine	4.4	0.6	4.2	0.6	7.8	8.9	2.0	2.4	7.0	6.2	3.2	0.5
buphedrone	5.3	1.9	7.0	8.6	16.8	3.6	1.9	1.6	4.6	3.2	3.2	6.0
butonitazene	14.9	14.5	7.8	0.8	3.0	8.2	14.5	8.0	2.1	1.6	5.3	2.7
buthylone	13.9	7.3	4.8	0.4	16.2	1.5	7.3	1.6	1.6	7.8	6.2	0.3
butyryl fentanyl	17.6	11.4	6.9	0.6	7.7	2.3	11.4	8.0	22.3*	6.8	2.5	7.7
butyryl fentanyl carboxy metabolite	11.4	1.2	4.9	6.5	0.4	1.7	1.2	0.7	4.2	12.2	1.4	5.2
butyryl norfentanyl	10,6	9.3	9.7	1.9	8.5	13.3	2.5	2.9	6.0	9.8	2.7	11.3
carfentanil	7.5	8.5	3.1	1.3	14.8	3.3	6.1	1.1	1.5	2.8	5.5	5.7
clonazolam	15.8	13.3	2.6	2.6	15.0	9.0	5.2	6.7	16.0	1.5	0.6	2.2
CUMYL-PeGACLONE	22.4*	6.4	3.6	1.1	17.3	2.1	2.7	1.3	15.1	5.3	5.4	0.6
cyclopropyl fentanyl (hydrochloride)	14.8	11.5	2.2	0.7	3.6	2.6	6.1	3.0	6.9	3.7	6.6	3.2
deschloro-N-ethyl-Ketamine	16.5	8.5	6.5	6.1	11.4	12.5	10.9	4.2	11.3	21.8	10.6	2.4
despropionyl para-Fluorofentanyl	12.9	8.4	8.6	3.0	16.6	1.0	9.2	1.5	12.0	1.3	4.2	5.3
diclazepam	15.1	6.0	16.3	6.9	7.0	11.3	9.4	5.0	13.0	13.5	4.0	4.0
dimethylcathinone	17.8	8.6	25.4	6.5	6.2	14.4	7.9	14.4	13.5	14.6	8.5	2.1
ethcathinone	4.6	8.3	5.6	12.9	11.0	10.8	10.2	0.9	3.2	5.4	4.3	0.2
ethylone	18.6	10.6	21.7	6.2	17.0	2.5	5.1	1.6	14.0	13.3	3.8	3.9
ethylphenidate	17.1	13.7	9.2	8.1	11.6	4.9	6.6	11.5	20.7*	7.3	2.5	2.8

Compound	Intra-day precision (Average CV%; n=6)				Inter-day precision (Average CV%; n=18)				Accuracy (Average bias%; n=18)			
	LOQ	**LQC**	**MQC**	**HQC**	**LOQ**	**LQC**	**MQC**	**HQC**	**LOQ**	**LQC**	**MQC**	**HQC**
etizolam	7.0	4.9	12.8	3.1	9.9	4.0	4.8	5.1	12.4	12.1	4.0	0.8
etodesnitazene (etazene)	20.2	3.3	12.8	8.9	14.0	4.4	3.6	2.8	7.2	12.4	14.6	0.3
euthylone	7.1	8.6	13.2	2.8	5.4	8.1	5.0	13.3	12.0	4.1	5.9	2.9
fentanyl	17.5	2.3	4.3	0.9	14.4	4.4	3.6	2.8	5.6	6.1	1.8	3.8
flunitazene	16.4	6.3	11.5	2.8	16.2	1.7	3.7	5.5	8.9	8.4	7.8	1.6
furanyl fentanyl	2.7	16.5	4.2	13.5	3.3	10.1	12.6	3.8	14.9	14.7	1.6	1.7
furanyl norfenta-nyl	15.3	14.5	19.5	7.4	11.6	14.3	10.6	6.7	11.9	12.8	2.0	0.1
isobutyryl fentanyl	16.4	7.3	7.2	8.2	4.2	3.5	2.4	11.3	12.1	8.6	3.9	1.7
isotonitazene	3.0	13.5	11.1	10.3	4.0	9.3	8.7	6.0	5.8	1.5	3.8	1.4
JWH-007	10.3	7.1	7.9	5.3	8.7	6.8	4.9	2.6	21.7*	6.1	2.1	3.3
JWH-016	19.2	3.2	9.7	2.7	15.6	14.4	3.6	9.4	0.6	3.5	6.8	0.4
JWH-018	21.1*	10.9	8.8	3.3	10.2	10.9	9.1	3.7	19.6	6.4	8.6	4.8
JWH-019	2.2	5.0	8.6	10.8	7.3	7.3	5.7	1.9	8.1	6.2	2.3	1.4
JWH-081	14.3	14.9	9.2	1.8	0.5	5.3	4.7	8.1	8.9	4.9	4.9	2.3
JWH-098	17.9	1.7	13.7	11.8	11.9	6.8	6.2	5.2	3.7	5.6	2.7	2.3
JWH-122	15.8	15.7	8.9	10.6	15.7	10.8	8.6	11.3	15.8	1.2	0.1	3.1
JWH-200	6.8	5.9	3.0	9.0	4.7	14.6	7.8	14.4	13.3	13.8	6.2	11.3
JWH-203	18.4	12.5	1.2	10	0.5	4.2	7.0	9.4	14.7	11.1	9.6	4.8
JWH-210	22.4*	15.6	9.9	7.8	11.6	6.2	2.1	22.7	11.8	1.6	6.6	0.9
JWH-250	16.5	2.9	5.6	6	2.3	2.7	0.6	17.2	13.8	6	6	2.5
JWH-251	13.0	1.1	13.4	5.9	2.4	4.7	1.4	16.5	7.8	4.3	2.6	3.4
JWH-302	15.9	2.2	4.5	5	2.9	0.4	1.2	20.7	4.9	18.9	0.3	0.3
JWH-398	27.1*	11.7	8.1	1.6	8.2	9	14.7	10.4	8.2	2.7	4.6	1.2
ketamina	7.1	1.4	13	3.0	5.2	7.7	17.1	5.6	3.4	14.8	6.9	1.0
MDMB-4en-PICA	8.0	4.1	13.1	4.1	6.4	13.8	9.3	0.9	7.3	9.3	7.4	2.4
MDMB-4en-PI-NACA	16.1	11.2	5.1	0.7	13.7	3.1	0.8	6.5	7.9	3.7	1.1	9.7

Compound	Intra-day precision (Average CV%; n=6)				Inter-day precision (Average CV%; n=18)				Accuracy (Average bias%; n=18)			
	LOQ	LQC	MQC	HQC	LOQ	LQC	MQC	HQC	LOQ	LQC	MQC	HQC
MDMB-CHMICA	5.3	15.5	10.5	0.5	12.5	16.7	5.9	10.7	15.8	1.3	3.1	6.4
MDPV	9.3	2.6	20.3	5	12.4	0.3	8.1	11.1	3.1	5.3	7.9	4.3
mephedrone	2.8	10.1	12	4.4	1.1	15.7	13.7	22.3	6.4	3.9	9.8	2.2
methcathinone	12.2	5.1	2.1	6.8	11.9	4.1	13.5	11.0	0.5	2.4	10.9	0.8
methedrone	1.0	4.3	9.3	8.1	6.3	8.6	24	6.9	10	5.9	5.7	0.3
methoxpropa-mine(mxpr)	13.6	3.8	3.1	1.6	6.8	13.3	13.6	7.0	10.4	13.7	6.1	0.9
methoxyacetyl fentanyl	4.5	6.8	6.4	11	11.4	11.3	14.7	12.1	4.8	3.6	3.7	1.2
methoxyacetyl norfentanyl	18.6	11.6	0.2	15	8.5	6.1	7.6	8.5	3.3	5	1.5	6.1
methylone	1.0	3.3	5.9	1.3	14.5	6.1	0.8	8	16.5	12.2	4.1	1.8
metodesnitazene	0.4	2.4	12.9	2.4	1.0	11.9	6.6	4.8	14.2	5.5	2.1	6.5
metonitazene	2.9	24.6	5.3	3.9	2.4	5.9	13.7	3.6	0.5	14.3	7.2	2.4
MMB2201	8.4	10.8	11	0.6	4.2	10.8	6.2	14.3	12.5	11.4	6	1.9
N-ethyl pentylone	3.0	18.5	5.4	1.3	15.3	13.2	4.3	7.2	13.7	5.3	0.9	1.2
N-pyrrolidino eto-nitazene	17.5	1.3	5.7	4.5	15.5	8	5.3	1.9	10.5	4.8	1.5	0.2
N.N-DMT	22.8*	16.4	6.6	1.6	5.1	7.3	0.4	3.6	1.8	11.1	5.1	2.8
norfentanyl	10.4	17.1	0.7	4.1	5.3	7.6	2	2.4	5.7	6.1	3.7	7.2
norketamina	13.8	12.5	0.7	3.7	20.0	2.3	6.6	14.2	15.5	15.4	0.6	1.3
ocfentanyl	13.3	1.9	3.4	1.6	10.2	13.3	3.9	6.5	7.9	9.1	13	0.2
p-fluoro-furanyl fentanyl	3.8	10.5	13.6	2.3	16.2	8	2.7	14	8.4	3.9	1.6	4.2
pentylone	6.9	14.3	11.3	9.6	0.2	0.8	5.2	11.1	5.0	1.5	1.2	0.7
phenyl fentanyl	1.9	14.2	17.2	6.6	14.4	4.9	6.3	10.6	7.5	9	3.2	4.7
phenylacetyl fenta-nyl	9.4	2.3	2.8	3.2	5.7	12.2	8.1	17.9	2.2	11.6	4.4	0.4
pravadoline (win48.098)	13.1	14.5	2.3	0.1	6.4	11.6	8.1	11.3	2.6	5.2	7.6	4.1
RCS-4	14.1	5.5	0.5	0.4	6	14,0	13	4.1	14.1	12.4	7.4	3.6
RCS-8	20.7	5.8	0.6	0.1	6.3	7.3	8.8	1.5	7.6	15.2	7.2	1.5
ritalinic acid	17.2	11.9	4.5	2.7	13.8	4.1	6.9	4.6	9.9	5.7	2.6	1.9

Compound	Intra-day precision (Average CV%; n=6)				Inter-day precision (Average CV%; n=18)				Accuracy (Average bias%; n=18)			
	LOQ	**LQC**	**MQC**	**HQC**	**LOQ**	**LQC**	**MQC**	**HQC**	**LOQ**	**LQC**	**MQC**	**HQC**
UR144	6.3	11.2	11.9	11.1	7	5.2	6.3	4	9.5	8.1	13.5	5.1
valeryl fentanyl carboxy metabolite	11.4	11.1	11.1	10.1	6	0.7	13.8	13	3.6	1.5	1.9	5.1
Classic Drugs of Abuse (DOA)												
6-Mam	15.4	11.4	11.2	7.3	13.9	1.3	5.3	6.1	2.1	4.3	2.4	4.3
amphetamine	17.2	5.9	12.7	14.3	1.2	6.3	8.1	0.3	12	7.1	4	10.6
benzoylecgonine	11.7	14.9	4	11.3	16.7	10.6	9.6	5.1	2.4	8	6	4.3
cannabidiol	15.7	13.2	10.6	9.6	8.3	7.5	2.2	5.5	8	11.1	6.4	1
cocaetilene	17.9	3.7	8.5	4.9	13.5	9	11.6	0.8	8.5	10.1	10.8	6
cocaine	4	9.8	12.3	2.6	8.5	3.1	5.3	8.9	2.9	11.9	4	2.8
codeine	20.8	13.4	15.8	6.2	4.3	4.3	2.4	9.8	9.6	3.1	11.4	6.2
celta-9-THC	15	6,7	6,2	4,9	13,4	3,7	2,7	14	15,2	6,4	3.9	4,4
ccgonine methyl ester	9.6	2	0,3	3,6	8,3	1,7	5,9	8,5	3,8	11.4	2,1	4
EDDP	18.7	7.9	6,6	14,7	17,4	12,2	4,4	0,2	15,2	4.7	6	2,3
MDA	3.3	11.8	11,1	6,1	12,7	3.1	1,3	4,5	8.3	0.4	13,3	7,4
MDMA	14	1,7	4,2	6,9	1,2	14,4	6,2	2,2	4.7	4,4	5,3	2,3
methadone	1.6	13.1	8,3	12,1	10.4	6,6	5	3,8	2,3	9,3	9,3	17
methamphetamine	18,8	2,7	0.1	0,5	13,9	1.5	15,9	16.3	8,6	2,1	5,3	7,3
morphine	13,5	0,8	8,8	12,6	4,6	14	6	3,7	4,9	2,6	3,1	1.8

Matrix Effect and Extraction of Recovery

%ME and %ER yield were calculated at three concentration levels (Low, Medium, High). With the chosen extraction procedure, matrix effect and extraction recovery of analytes under investigation were always acceptable. The matrix effect ranged from between 99-113%*for LQC; 87-103% for MQC, for 88-91% HQC (range of the average value calculated for all the analytes). The extraction recovery was always above 80%, ranging 82-89%*for LQC; 87-94% for MQC, for 88-101% HQC.

3.3.2. *Application of the LC-MS/MS method in patients with a history of addiction*

The newly developed method was applied on 110 residual aliquots of 300 keratin samples from patients under drug monitoring by public services for pathological addictions of the National Health System. More than half of the samples could not be analyzed due to insufficient matrix availability. The patients showing positivity to both NPS and Drugs of abuse (DOA) are listed in Table 5. Out of a total of 110 analyzable samples, 85 matrices were found positive to classic drugs of abuse, of those 12 were also positive to NPS (8 male and 4 female, the 25-50 age group was that most frequently resulting positive). New substances without the association of at least one class of traditional drugs were not detected. Negative data are not shown.

Table 5: Positivity detection of NPS in association with Drugs of abuse (DOA). Gender, age, sample type and substances or classes of substances defining the positivity are listed (M=male; F=female).

N° case (M/F)	age (yrs)	sample type	NPS detected (concentration in pg/mg)	Classes of DOA detected
5 (F)	18	hair	fentanyl (10.6)	opiates
28 (M)	21	hair	ketamine (10.3); norketamine (<loq)	cannabinoids, methadone and metabolite
35a (M)	25	hair	ketamine (11.9), norketamine (<loq), fentanyl (4.3), 4-f-methcathinone (<loq), buphedrone (<loq), RCS-4 (6.9)	cocaine, opiates and amphetamine-like substances
43 (M)	43	hair	ethylone (8.4)	cannabinoids, cocaine, opiates and metabolites
52 (F)	57	hair	ketamine (62.3), norketamine (14.9)	cannabinoids, cocaine, methadone and metabolites
53 (M)	50	hair	ketamine (20.6), norketamine (4.8), fentanyl(<loq), acetyl fentanyl (7.9)	cocaine, opiates and amphetamine-like substances
67 (F)	26	hair	ketamine (50.2), norketamine 9.8)	cocaine, amphetamine-like substances methadone and metabolites

N° case (M/F)	age (yrs)	sample type	NPS detected (concentration in pg/mg)	Classes of DOA detected
75 (M)	33	hair	ketamine (8.3), norketamine (<loq), mephedrone10.9), fentanyl(25), furanyl fentanyl(<loq)	cocaine, amphetamine-like substances and metabolites
77 (M)	58	hair	fentanyl (6.9)	cocaine, opiates, methadone and metabolite
78 (M)	27	hair	ketamine (19.8), norketamine (4.5)	cannabinoid, cocaine and metabolites
89 (F)	27	hair	3-methylmethcathinone (4.1), flualprazolam(<loq), 5F-EMB PICA (5.9)	cocaine, opiates methadone and metabolites
91 (M)	26	hair	ritalinic acid (<loq)	cocaine, opiates methadone and metabolites

3.4. Discussion

The drug market and the technological developments within its distribution have undergone significant periods of growth and change, increasing the number, type and availability of new drugs. Over the past 25 years, consumers have been exposed to an escalating risk from the continuous introduction of substances belonging to a growing number of chemical classes characterized by increased potency and toxicity. It is therefore essential to further strengthen the global early warning system for NPS in order to identify transboundary health threats, communicate risks and improve awareness and response. [113].

Multiagency and multidisciplinary networks created to rapidly detect, assess and respond to health and social threats caused by NPS were established as drug early warning systems and were represented, at a global level, by the UNODC Early Warning Advisory (EWA) while the European Union Early Warning System (EU EWS), operated by the EMCDDA works at European level. Nationally, several countries, 29 to date, have set up individual projects of early warning and actively participate in the EWS program. National early warning systems, such as the “SNAP” project in Italy and EU EWS, mutually exchange information on the chemical identification of NPSs from forensic and toxicology laboratories. This approach allows the collection and rapid reporting of event-based information on the manifestation of harm caused by NPS at a national level. [114]

However, illicit drugs must not be forgotten. Indeed, the emergence of these new substances has not completely replaced the old drugs, and we have also seen interaction between illicit drugs and the NPS markets heightened. This interaction poses a high level of risk for the users, who often find themselves unknowingly taking highly potent and toxic substances they are unfamiliar with. Indeed, we have also witnessed the adulteration and illegal sale of controlled drugs and NPS products. It is in this context that the role of forensic toxicology and all those laboratories working with the EWA system are important. The collaboration of researchers, technical analysts and other professionals involved, over the past 25 years, has provided valuable data on the rapid growth and revolution in the distribution and danger posed by these new substances. As an integral part of this mechanism the aim of this study was to explore the use of NPS in the metropolitan area of Bologna, by carrying out multi-analyte characterization on the hair samples of a subpopulation of drivers and workers followed by public services for pathological addictions (Ser.DP). For this purpose, after deep scientific research of the literature an effective and reliable method was developed and tested for the simultaneous identification and quantification of a very wide number of “new” drugs (newly synthesized or simply substances newly used as recreational) along with classic “old” and prescription ones.

The choice of the psychotropic compounds to be included in the analysis was based on epidemiological data related to the consumption of prescription and classic drugs of abuse in the metropolitan area of Bologna (for the “old drugs”) [115] and by the availability of NPS certified standards provided by the National Health Institute and Comedical s.r.l. (Italy, Trento) within the national “SNAP" project.

The type of information required guided the selection of the matrix to be submitted for analysis. Indeed, hair matrix is the “gold standard" in the context of long-term biomonitoring and may be considered the method of choice for retrospective assessment of toxicological abuse or environmental exposure. In the absence or insufficiency of head hair, keratin matrices such as axillary and inguinal hair have been used. The latter are always valid matrices of accumulation to obtain information relative to past chronic or sub-chronic consumption, but it is not possible to achieve temporally precise information. Hair sampling is not very invasive, can be performed by non-specialized health care personnel, is difficult to alter and hardly exceeds half a gram in weight. For these reasons, and in line with the literature (Table 1), only 25 milligrams of matrix were used for the evaluation of NPS consumption.

The chemical heterogeneity of the substances belonging to the large and diverse group of NPS is a major difficulty in the analysis and interpretation of forensic toxicological data. The issue mainly lies in the minor structural modifications that complicate detection by instruments, both in confiscated drugs and in biological fluids which are subjected to chemical-toxicological analysis.

The rapid and constant appearance of new substances on the market aggravates this challenge whose keystone is embodied by the dynamism of new generation analytical equipment. Indeed, analytical methods using instruments such as liquid chromatography coupled to tandem mass spectrometry or high-resolution mass spectrometry can easily be applied to new compounds of interest once certified standards become available for development. However, the diversity of the substances of interest often requires the use of different and specific extraction techniques, the use of specific mobile phases to optimize the ionization of the target compounds, and ad hoc chromatographic and spectrometric conditions which facilitate the affinity and mass separation of the exact NPS subgroup. All this with the aim of achieving sound identification and accurate quantification of the substances in question. Therefore, forensic toxicology laboratories often find themselves having to decide between the development of a single method, multi-analyte but generally with poor sensitivity and with considerable difficulties in terms of quantification, or the development of several methods, one specific to each class of compounds, with the need to then analyze the same sample multiple times.

This option entails a waste not only of matrix, which, especially as far as keratin is concerned, may not be available in large quantities, but also the consumption of reagents and laboratory material, increasing costs and analytical time.

For the evaluation of NPS consumption in the sample of patients in the experimental study, the developed method built-in several compounds belonging to different classes of NPS. In particular, it contained synthetic cannabinoids, synthetic opioids, synthetic cathinones, designer benzodiazepines and other substances. Taking into consideration publications such as Boumba et al. [96] and Strano-Rossi et al. [93], a single extractive procedure was performed, resulting in overnight hydrolysis at 45 degrees in 0.1% FA methanolic (70): water (30) mixture solution. The selection of the extraction phase was made considering the peak shape of the MRM signal and the quantity of NPS extracted. The increased volume of methanol allows us to extract the majority of synthetic cannabinoids, whose detection is inhibited by the percentage of water. However, the aqueous fraction must be maintained as it improves the shape of the peak. Future tests could be carried out to further reduce the percentage of water with the aim of improving the efficiency of extraction of synthetic cannabinoids.

Scientific research in literature for the parameters of MS/MS detection was rigourously carried out. The injection of individual certified standard was executed for all the analytes to optimize the parameters of spectrometric detection. As already mentioned, the choice of a multi-method caused the sensitivity of the detector to decrease, as a result the lowest level of the selected calibration range (4 pg/mg) was not reached by all the compounds, e.g. mephedrone, 6-MAM, morphine, AB-CHMINACA, methcathinone, MDMB-CHMICA, 4-fluoro MDMB-BUTICA, 5-fluoro CUMYL-PeGACLONE, 5-hydroxytryptophan, cannabidiol, delta-9-THC, 4-fluoromethcathinone, 5-chloro

AB-PINACA. However, this limit may be overrun by adding a concentration or/and sonication step of the extract in the post-hydrolysis sample preparation.

Calibration range was selected in accordance with NPS concentrations typically found in literature. [116] [117.] Thus, the calibration interval chosen, with a range of 4 to 640 pg/mg, is consistent with that suggested by the main literature studies and is suitable for forensic analysis. It should be noted that in this study an attempt has been made to achieve low concentration levels in order to detect even the smallest amounts of compounds present in the hair, which may indicate sporadic use of the new substance alone or as an adulterant of traditional drugs of abuse. In other words, for a proper study of consumption quality we preferred to limit false negatives as much as possible.

The multi-analyte UPLC-MS/MS method developed was successfully validated according to the EMA guidelines. [112] Drug-free samples of keratin matrix from different sources and different origins were obtained to assess the presence of endogenous components and, in particular, whether this presence could co-elute and indeed interfere with the signal of the analytes considered. Taking into account the phenotype variability of the Italian population, hair of different color (blonde, black, brown, white), structure (straight or curly) and finally of different origin (head, inguinal and axillary) were subjected to the study. The multi-analyte method exhibits good linear calibration functions for all the analytes of interest and good precision and accuracy at all concentrations evaluated, including the lowest level. The new validation criteria were met for all substances tested. The greatest difficulties were encountered with sensitivity, because, as already mentioned, the simultaneous characterization of dozens of molecules penalized the detection at low concentrations of some substances. In the future, it will be possible to increase sensitivity by improving or adding sample treatment steps prior to instrumental analysis.

Among the limitations of this study and possible future developments, it should be emphasized that, a shortcoming of our multi-method is the number of internal standards. Four internal standards were selected, representing the most important and globally marketed group of NPS. Previously, another multi-method was successfully validated and published and applied to whole blood for the characterization of 182 NPS, also using few internal standards compared to the number of compounds considered. In the future, it would be more appropriate to include within the analysis a greater number of internal standards in order to obtain at least one reference molecule for each class included. So far, nordiazepam-d5, ketamine-d4, JWH-122-d9 and fentanyl-D5 have been found to be satisfactory for the assessment of precision and accuracy, but better results can be expected using specific internal standards with a chromatographic and mass behaviour more similar to the analytes of interest. Since there was already a multi-method for NPS, it is important to highlight the necessity of implementing a multi-method on hair due to the diversity that can be obtained from these two matrices.

Whole blood is normally investigated to obtain information on very recent consumption, e.g. in cases of acute or fatal intoxication and in the evaluation of driving under the influence (DUI). The hair matrix, instead, provides information on drug accumulation over time, therefore it can be used for our purpose of monitoring habits, as a therapeutic approach in a health care context.

As required by international protocols, precursor and two product ions were monitored for the identification of each compound, based on retention time, and good separation was obtained for all substances, in particular for all those isomeric compounds from synthetic cannabinoids, synthetic cathinones and fentanyls; it was not possible to obtain chromatographic separation of a couple of compounds belonging to the benzofuran group (5-6APB and 5-6MAPB).

The developed method can be profitably applied not only in long-term biomonitoring assessment in a clinical-forensic context but also in retrospective estimation of recent past exposure to xenobiotics in a post-mortem context.

The experimental study was proposed in order to attain a more in-depth understanding of the prevalence of consumption of these new substances in our city and the way in which they are intertwined with the world of classic drugs. For this purpose, the multi-analyte method was executed on the anonymized keratin samples of some patients followed by the drug addiction service of Bologna. Out of three hundred people subjected to hair sampling and to routine chemical-toxicological analysis with an already validated method, a total of 107 samples, including 85 males and 22 females, had a residual aliquot available for further analysis and which was submitted to the newly validated hair analysis. The sampling was carried out according to the guidelines promoted by the National Health Institute [90]. As to the nature of the sample, all samples were black or brown colored; 76 hairs from the scalp, 9 hairs from the other regions (inguinal, axillary, chest) were analyzed. In 107 samples analyzed, 22 (21%) samples resulted negative to all drugs tested; with regard to the positive samples, 69 (81%) belong to male and 16 (19%) to female. With regard to gender, we found that the number of women who were positive for at least one NPS was 33% (n=4). Regarding age, positive findings were detected above all in the 26-50 age group (n=49) respect to the 18-25 age group (n=12) and over 51 years (n=21). This positivity prevalence regarding the age is also reflected in the group of subjects that resulted in an NPS positivity.

A total of 13 different NPS, belonging to the classes of synthetic cathinones, synthetic opioids, synthetic cannabinoids and designer benzodiazepines, were detected. In four cases a combination of more than one NPS class consumption was revealed. In all the NPS positivity cases the presence of classical drugs was also proven, demonstrating with clear evidence the strong interconnection between the consumption of new substances with traditional ones.

Specifically, synthetic cathinones were always found in association with cocaine, while the presence of methadone, opiates, amphetamines and cannabinoids was alternated. Furthermore, fentanyl and other fentanyl-related compounds were always associated with opiates, although in most cases cocaine was also present. According to the results, ketamine is the most commonly present NPS, indeed out of twelve samples, seven were found positive to ketamine and its principal metabolites (norketamine). A possible therapeutic origin of ketamines and fentanyls' positivities has been excluded. As a matter of fact, each hair sample analysed was associated with an anamnestic questionnaire, in which long-term therapies were outlined. With regard to a single exposure positivity, we analysed hair segments with an average length of 3 cm, thus diluting the possible single administration. In fact, in the literature there are some cases of single dose positivity, but the hair was analysed in 1cm-long segments. [118]

These analytical results are consistent with the presence of NPS on the market today, as established in the literature review. [116] Indeed, the most widespread and marketed classes are synthetic cathinones and synthetic cannabinoids while the most widespread new substance is ketamine.

The role of forensic toxicology in collecting and interpreting population data (such as sex, age and circumstances of consumption) in relation to the prevalence of usage of these substances, highlights useful aspects for the future development of targeted prevention and control strategies. The assumption is that if a large population were subjected to NPS hair analysis, then the relevant results obtained would provide epidemiological data on NPS trends and the extent of their use in the community. With this purpose, another future goal is the application of the multi-analyte method on the hair samples of drug addicts who come into contact with the work of road units trying to shield the severe health emergency given by the drug addiction and the dangers related to it, including the phenomenon of NPSs. As a result of the experimental study, analytically based NPS feedback in biological samples should be reported to the control authorities to trigger the prioritization of NPSs for international review by the World Health Organization and their qualification as illegal, in order to contrast their impact on public health.

3.5. Conclusion

The NPS phenomenon is constantly and rapidly evolving within the drug panorama, a rate of one new drug launches per week (in 2015) representing the wake-up-call that is essential to evaluate the real prevalence of abuse of these substances in the population most in contact with this world: drug addicts. Every year, the drug addiction service treats patients with current or previous diagnosis of substance use disorder (DSM-5), for clinical, therapeutic, and preventative purposes. In order to explore the consumption habits of NPS and classic drugs of abuse in this specific category of the population, an approach based on LC-MS/MS systems for the characterization in hair samples of several analytes including synthetic cannabinoids, synthetic opioids, synthetic cathinones, stimulants, benzodiazepines and others, and classic drugs of abuse was developed and discussed. Despite the use of a rather non-specific extraction procedure, such as that of formic acid in a water and methanol mixture, almost all analytes showed a good signal and a clear definition from the noise given by the matrix to the lowest calibration point. Good linearity and satisfactory accuracy and precision were achieved for most of the selected compounds. Future research is orientated towards analytical developments and improvements, such as the use of additional internal standards, the identification of LOD and LOQ at lower concentrations by adding an extract concentration step in the pre-analytical phase, and the application of the NPS method developed on a large scale of samples. Finally, given the analytical difficulties and limitations discussed here, the development of these multi-analyte methods facilitates the study of the prevalence of drug abuse. Moreover, the possibility of being constantly updated with the availability of certified standards for newly introduced drugs on the market should enable evidence-based reports to the competent control authorities regarding the urgent spread of the threat represented by NPS.

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