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MASTER THESIS

Retrospective target and suspect screening of new psychoactive substances in raw wastewater by Liquid Chromatography – Quadrupole-Time-of-flight Mass Spectrometry (LC-QTOF-MS)

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CHEMIST

ATHENS

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ΕΡΕΥΝΗΤΙΚΗ ΕΡΓΑΣΙΑ ΔΙΠΛΩΜΑΤΟΣ ΕΙΔΙΚΕΥΣΗΣ

Αναδρομική σάρωση στοχευμένων και ύποπτων νέων ψυχοδραστικών ουσιών σε ανεπεξέργαστα λύματα με LC-QTOF-MS

ΚΩΝΣΤΑΝΤΙΝΑ ΔΙΑΜΑΝΤΗ

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ΕΠΙΒΛΕΠΩΝ ΚΑΘΗΓΗΤΗΣ:

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ΗΜΕΡΟΜΗΝΙΑ ΕΞΕΤΑΣΗΣ 20/10/2017

ABSTRACT

Every substance that is consumed ends up either unchanged or as a metabolite in sewer systems, so the analysis of raw wastewater samples can provide valuable information on the estimation of their consumption. This approach, which is known as wastewater-based epidemiology (WBE), can be applied in monitoring of the usage of New Psychoactive Substances (NPS). These compounds are legal replacements of established narcotic and psychotropic drugs with slightly modified chemical structures and similar or new effects.

This thesis reports the retrospective target and suspect screening of NPS in raw wastewater samples collected on March 2015, 2016, 2017 from the main wastewater treatment plant (WWTP) of Athens. Solid-phase extraction (SPE) with four different sorbent materials that covered a broad range of analytes was used for sample treatment. Extracts were analyzed with reversed-phase liquid-chromatography coupled to quadrupole-time-of-flight mass spectrometry (RPLC-QTOF-MS) and the data were acquired through broad-band Collision Induced Dissociation (bbCID) mode, which provided information on parent and fragment ions without pre-selection of analytes in one run. Validation was performed based on representative compounds of the wide-scope screening method. A database of approximately 200 NPS was used for target screening and the detection was based on mass accuracy, retention time, isotopic pattern and fragmentation products. For the suspect screening, a list of approximately 500 NPS was built and information, such as mass accuracy, predicted retention times and MS/MS library data, led to the tentative identification of the NPS candidates.

Following the aforementioned procedures, few of the investigated compounds were detected in raw wastewater. 15 NPS, some of them for the first time, were detected through target screening in wastewater and the results indicated an occasional use during the week and over the years. 6 NPS were tentatively identified through suspect screening, while the difficulties in the identification of more NPS to a better confidence level were discussed.

SUBJECT AREA: Environmental Analytical Chemistry

KEYWORDS: New Psychoactive Substances, Wastewater-based epidemiology, LC-QTOF-MS, Target screening, Suspect screening

ΠΕΡΙΛΗΨΗ

Κάθε ουσία που καταναλώνεται καταλήγει είτε αμετάβλητη είτε ως μεταβολίτης στα συστήματα αποχέτευσης, οπότε η ανάλυση δειγμάτων ανεπεξέργαστων λυμάτων μπορεί να παρέχει πολύτιμες πληροφορίες για την εκτίμηση της κατανάλωσής τους. Αυτή η προσέγγιση, γνωστή ως επιδημιολογία βασισμένη στα απόβλητα, μπορεί να εφαρμοστεί στην παρακολούθηση της χρήσης νέων ψυχοδραστικών ουσιών (ΝΨΟ). Οι ενώσεις αυτές αποτελούν νόμιμες αντικαταστάσεις των καθιερωμένων ναρκωτικών και ψυχοτρόπων ουσιών με ελαφρώς τροποποιημένες χημικές δομές και παρόμοιες ή νέες επιδράσεις.

Η διατριβή αυτή αναφέρεται στην αναδρομική σάρωση στοχευμένων και ύποπτων ΝΨΟ σε δείγματα ανεπεξέργαστων λυμάτων που συλλέχθηκαν τον Μάρτιο του 2015, 2016, 2017 από το κύριο κέντρο επεξεργασίας λυμάτων (ΚΕΛ) της Αθήνας. Για την προκατεργασία των δειγμάτων χρησιμοποιήθηκε η εκχύλιση στερεάς φάσης με τέσσερα διαφορετικά προσροφητικά υλικά που κάλυπταν μεγάλο φάσμα αναλυτών. Τα εκχυλίσματα αναλύθηκαν με υγροχρωματογραφία αντίστροφης φάσης συζευγμένη με φασματομετρία μαζών με υβριδικό τετράπολο-αναλυτή χρόνου πτήσης (RPLC-QTOF-MS) χρησιμοποιώντας τη λειτουργία bbCID, η οποία παρείχε πληροφορίες για τα πρόδρομα ιόντα και τα θραύσματα, χωρίς προεπιλογή των αναλυτών και με μία ανάλυση. Επικύρωση πραγματοποιήθηκε χρησιμοποιώντας αντιπροσωπευτικές ενώσεις της εφαρμοζόμενης ευρείας μεθόδου σάρωσης. Μία βάση δεδομένων περίπου 200 ΝΨΟ χρησιμοποιήθηκε για τη στοχευμένη σάρωση και η ανίχνευση βασίστηκε στην ακρίβεια μάζας, στο χρόνο ανάκτησης, στο ισοτοπικό προφίλ και στα προϊόντα θραυσματοποίησης. Για τη σάρωση ύποπτων ενώσεων δημιουργήθηκε μια λίστα 500 περίπου ΝΨΟ και πληροφορίες, όπως η ακρίβεια μάζας, οι προβλεπόμενοι χρόνοι ανάσχεσης και MS/MS δεδομένα από βιβλιοθήκες, οδήγησαν στον πιθανό προσδιορισμό υποψήφιων ΝΨΟ.

Σύμφωνα με τις προαναφερθείσες διαδικασίες, λίγες από τις εξεταζόμενες ενώσεις ανιχνεύθηκαν στα ανεπεξέργαστα λύματα. 15 ΝΨΟ, μερικές εκ των οποίων για πρώτη φορά, ανιχνεύθηκαν μέσω στοχευμένης σάρωσης στα λύματα και τα αποτελέσματα υποδεικνύουν περιστασιακή χρήση τους κατά τη διάρκεια της εβδομάδας και μέσα στα έτη. 6 ΝΨΟ προσδιορίστηκαν πιθανώς μέσω της σάρωσης ύποπτων ενώσεων, ενώ

συζητήθηκαν οι δυσκολίες στην ταυτοποίηση περισσότερων ΝΨΟ σε ένα καλύτερο επίπεδο εμπιστοσύνης.

ΘΕΜΑΤΙΚΗ ΠΕΡΙΟΧΗ: Περιβαλλοντική Αναλυτική Χημεία

ΛΕΞΕΙΣ ΚΛΕΙΔΙΑ: Νέες ψυχοδραστικές ουσίες, Επιδημιολογία βασισμένη στα απόβλητα, LC-QTOF-MS, Στοχευμένη σάρωση, Σάρωση ύποπτων ενώσεων

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PREFACE

This master thesis was performed at the laboratory of Analytical Chemistry, Department of Chemistry, National and Kapodistrian University of Athens under the supervision of Associate Professor Nikolaos S. Thomaidis.

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CHAPTER 1

Introduction

1.1 New Psychoactive Substances

As defined from the Council Decision 2005/387/JHA, New Psychoactive Substance means a new narcotic drug or a new psychotropic drug in pure form or in a preparation that has not been scheduled under the 1961 United Nations Single Convention on Narcotic Drugs or the 1971 United Nations Single Convention on Psychotropic Substances respectively and may pose a threat to public health comparable to the substances listed in the Conventions [1].

These New Psychoactive Substances (NPS), also known as ‘designer drugs’, ‘legal highs’, ‘research chemicals’, may not be newly developed, but are newly available as products on the drugs market [2]. They are marketed as ‘legal’ replacements to illicit drugs and they are produced by slightly changing the structure of controlled substances in order to evade legislation and give similar or new psychotropic experiences [3]. NPS are included in different chemical families and have various pharmacological effects. By the end of 2016, more than 620 NPS were being monitored by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), while 66 new substances were reported to the European Union (EU) Early Warning System (EWS) for the first time in Europe in 2016 [4].

New Psychoactive Substances are produced on a commercial scale in clandestine synthetic drug production facilities by organized crime groups. Their synthesis can emerge deliberately or accidentally by making a new substance of an uncontrolled precursor chemical. Because of the fact that they are legal replacements to controlled drugs, they are sold openly in specialized ‘head shops’ in cities and online. Alongside the ‘designer drugs’, ‘legal highs’, ‘research chemicals’, which are aimed to recreational use, NPS are often sold as ‘food or dietary supplements’. These products are used by people wanting to enhance their body and mind and are openly sold in fitness

shops and online. In the group of NPS are also included prescribed medicines that are misused or imported illegally outside the EU [2].

Although NPS were reported to the EU EWS at a rate of one per week in 2016, the overall number of new detections was lower than in previous years. This can be explained by the fact that some European countries have introduced bans and other measures to target producers and retailers of NPS and high street shops, as well as imports of NPS outside the EU (mainly from China) [4].

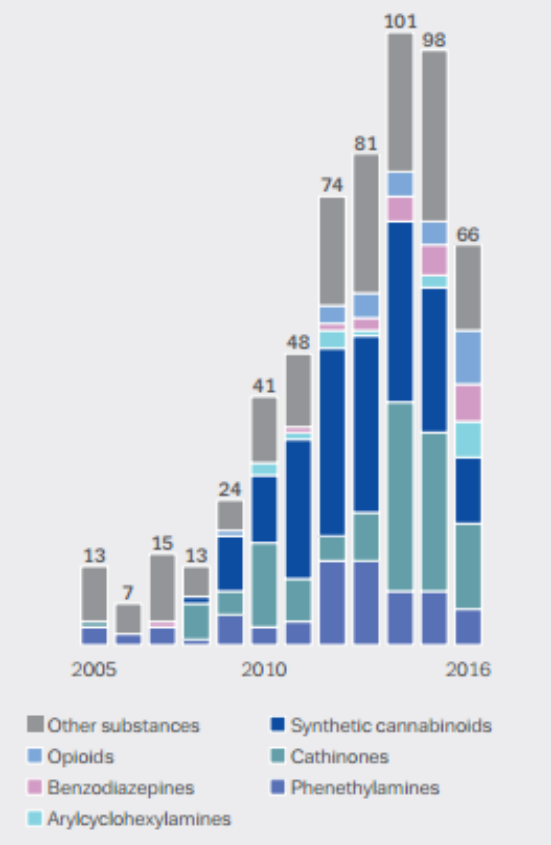

Figure 1: Number and categories of NPS notified to the EU EWS for the first time, 2005-2016 [4]

Currently, there is limited or no information available regarding the chemical stability, the pharmacodynamic profile, the metabolism, the effects and the potential acute toxicity, especially when the substances first appear on the drugs market. However, the number of cases of intoxications and deaths is constantly increasing, so this phenomenon is a great matter of concern for Public Health [5, 6].

1.2 Classes of New Psychoactive Substances

1.2.1 Synthetic cannabinoids

Synthetic cannabinoids were first detected in Europe towards the end of 2008 and today are the largest group of new substances monitored by the EMCDDA [2]. Despite the fact that they share no structural relationship, synthetic cannabinoids mimic the effects of delta-9-tetrahydrocannabinol (THC), which is largely responsible for the major psychoactive effects of cannabis [4, 7]. The synthetic cannabinoid classes are the JWH-series of aminoalkylindoles and the CP-series of cyclohexylphenols, as well as the ultrapotent indazole class and their ester analogues (e.g. AMB-FUBINACA, 5F–AMB, MDMB-CHMICA), capable of producing severe agitation and psychosis at very small doses. Limited pharmacologic and pharmacokinetic data are available on newer agents given their rapid production and introduction to the market [7].

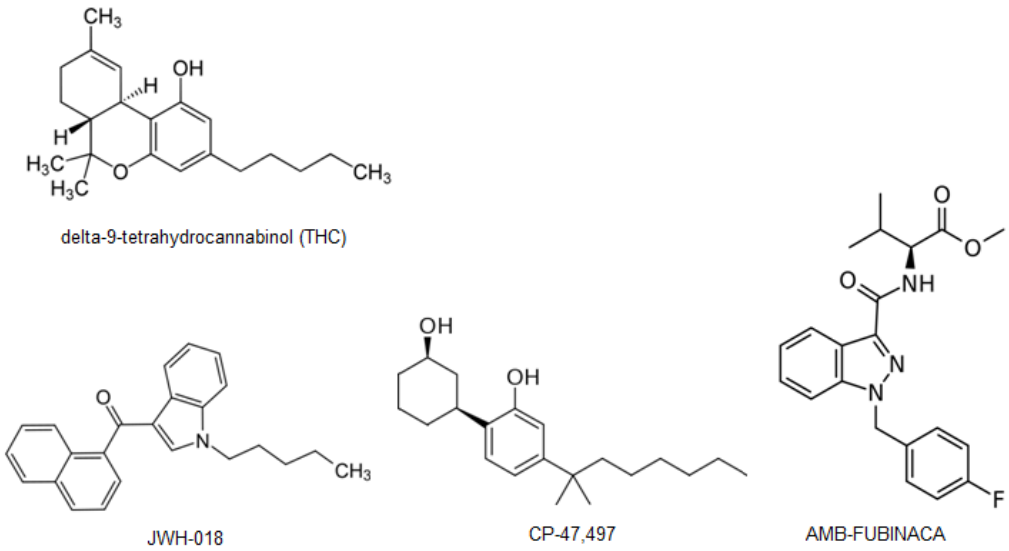

Figure 2: Structures of THC and selected synthetic cannabinoids

1.2.2 Synthetic cathinones

Synthetic cathinones is the second largest group of new substances monitored by the EMCDDA [2]. They represent a large family of β-keto phenethylamine derivatives chemically related to the parent compound cathinone, one of the psychoactive alkaloids present in the Khat plant (Catha

edulis) [5]. Given their structural similarities with dopamine, methamphetamine, and 3,4-methylenedioxymethamphetamine (MDMA), synthetic cathinones possess both amphetamine and serotonergic properties [7]. They have stimulant and hallucinogenic effects [7] and are used as legal replacements for MDMA, amphetamine and cocaine [2]. The most commonly used synthetic cathinones are 4-methylmethcathinone (4-MMC, mephedrone), methylone, 4-methylethcathinone (4-MEC), 3,4-methylenedioxypyrovalerone (MDPV), pentedrone, α-pyrrolidinovalerophenone (α-PVP) [5]. Their pharmacokinetic and pharmacodynamic data remain limited [7].

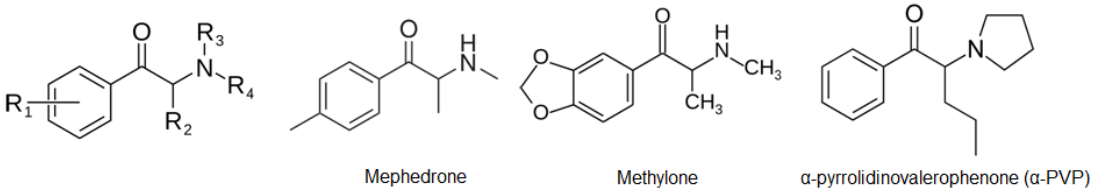

Figure 3: General structure of substituted cathinones and structures of selected synthetic cathinones

1.2.3 Phenethylamines

The most common group of phenylethylamine derivatives reported as NPS is the 2C drugs. These compounds are 2C-X series analogs of mescaline. The name '2C' refers to the two carbons between the benzene ring and amino group in the chemical structure. Of particular clinical relevance is the N-methoxybenzyl-substituted class of 2C phenylethylamines (NBOMe), created by attaching a modified phenyl ring to the 2C–X structure. 2C drugs have hallucinogenic properties and are marketed as having affects similar to lysergic acid diethylamide (LSD) and 3,4-methylenedioxymethamphetamine (MDMA). Serious health complications and fatal overdoses have brought the abuse in this new group of substances to the public's attention [7, 8].

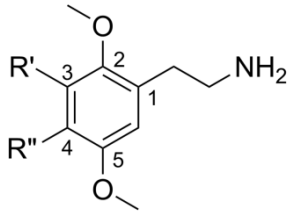

Figure 4: General structure of 2C-X series

1.2.4 New benzodiazepines

Benzodiazepines are a large group of substances widely prescribed for the treatment of anxiety, insomnia, muscle spasms, alcohol withdrawal and epilepsy, but there is also a widespread illegal use of these substances for recreational purposes. Designer benzodiazepines are often developed by pharmaceutical companies and have recently appeared in online shops as ‘research chemicals’, despite never been marketed. Today more than 50 different substances of benzodiazepine-type, such as diclazepam, flubromazepam, pyrazolam, clonazolam, deschloroetizolam, flubromazolam, nifoxipam, cinazepam, are available on the illegal market, including both prescribed substances and benzodiazepines produced for recreational use. Depressant effects and varying potencies of these designer benzodiazepines represent a concern, as well as reports on intoxications and deaths due to them [9].

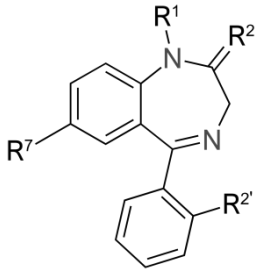

Figure 5: General structure of substituted benzodiazepines

1.2.5 New synthetic opioids

New opioids are of special concern for public health. This is because they are often highly potent and are sold as heroin to unsuspecting users, and thus pose a high risk of overdose and death. For example, the fentanyls are a family of drugs that have caused hundreds of deaths in Europe and the United States [2]. Other new synthetic opioids that have recently appeared on the recreational drug market are AH-7921 and MT-45, which have analgesic properties comparable to those exerted by morphine [10].

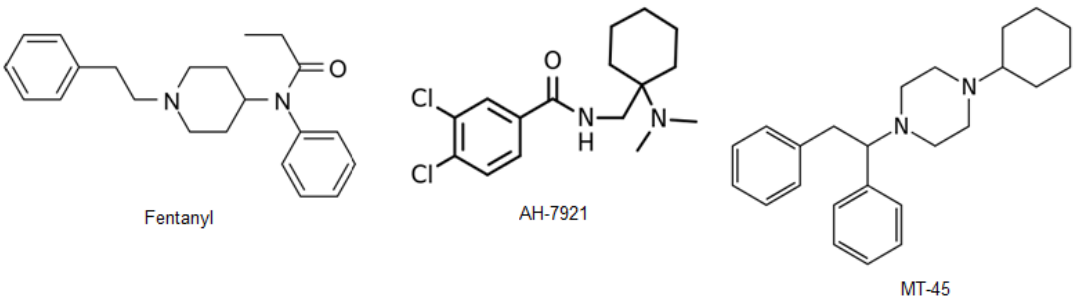

Figure 6: Structures of selected synthetic opioids

1.2.6 Arylcyclohexylamines

The arylcyclohexylamine class includes phencyclidine (PCP) and ketamine analogues. Methoxetamine is a legal alternative to ketamine, while 3-methoxy-phencyclidine, 4-methoxy-phencyclidine and 3-methoxy-eticyclidine are legally available alternatives to phencyclidine. It is likely that many chemical analogues of this family of drugs will be found to possess the characteristic dissociative anaesthetic properties of ketamine and phencyclidine [11].

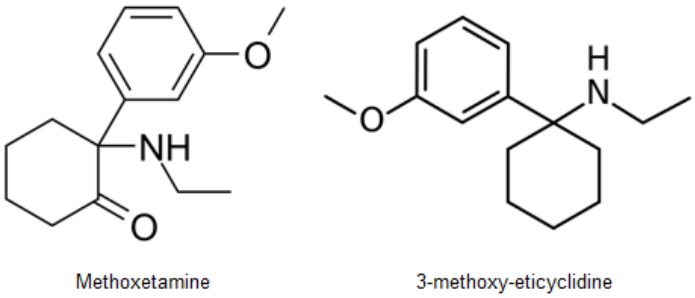

Figure 7: Structures of selected arylcyclohexylamines

1.2.7 New synthetic tryptamines

New synthetically produced tryptamine hallucinogens, such as alpha-methyltryptamine (AMT), 5-methoxy-N,N-dimethyltryptamine (5-MeO-DMT) and 5-methoxy-N,N-diisopropyltryptamine (5-MeO-DIPT), are claimed as the next-generation designer drugs that legally replace LSD. The principal structural feature that gives the hallucinogenic properties to tryptamine analogues is the indole nucleus. Different structural modifications result to

diverse molecules with dissimilar chemical properties, which consequently have the ability to induce different states of mind and behaviors. Available information on these new tryptamine derivatives is very scarce, while their use has been related with intoxications and deaths over the last years [12].

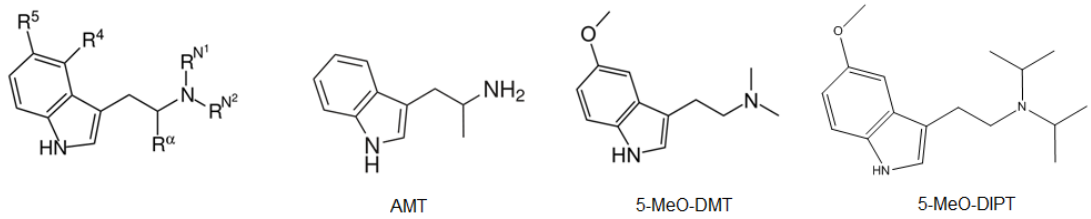

Figure 8: General structure of substituted tryptamines and structures of selected new synthetic tryptamines

1.2.8 Arylalkylamines

The most common substances of this class are the aminopropylbenzofurans (APB), such as 1-(Benzofuran-5-yl)-*N*-methylpropan-2-amine (5-MAPB) and the isomers 5-APB and 6-APB, and the substance methylthienylpropamine (MPA). 5- and 6-APB, which have become popular on the recreational drug market, are structurally related to 3,4-methylenedioxymethamphetamine (MDMA) and 3,4-methylenedioxyamphetamine (MDA) and have both psychostimulant and hallucinogenic properties [10, 13].

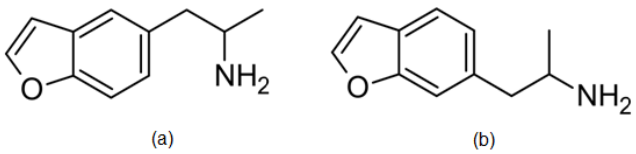

Figure 9: The isomers 5-APB (a) and 6-APB (b)

1.2.9 Aminoindanes

The basic structure of aminoindanes makes them conformationally ring analogues of amphetamine. Internet websites offering synthetic compounds as 'research chemicals' have recently been advertising 5,6-methylenedioxy-2-aminoindane (MDAI), 5, 6-methylenedioxy-N-methyl-2-aminoindane (MDMAI), 5-iodo-2-aminoindane (5-IAI), and 5-methoxy-6-methyl-2-aminoindane (MMAI) [14]. Recent research shows that aminoindanes share similar pharmacological

properties with 3,4-methylenedioxymethamphetamine (MDMA). At present, there is very limited information about their acute and chronic human toxicity. The extent of current availability and trends in recreational use of this class of drugs is, also, uncertain [3].

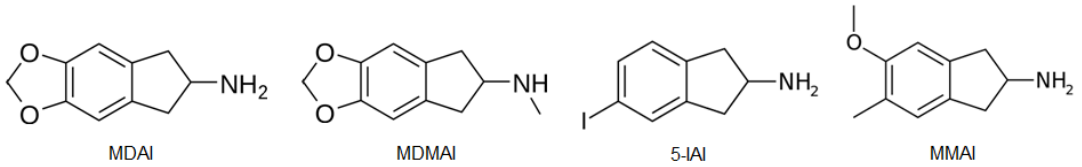

Figure 10: Structures of selected aminoindanes

1.2.10 Piperazine derivatives

The piperazine derivatives include 1-benzylpiperazine (BZP), 1,3-trifluoromethylphenylpiperazine (TFMPP), 1-(3-chlorophenyl) piperazine (mCPP) and 1-(4-methoxyphenyl) piperazine (MeOPP). They are increasingly being substituted for MDMA, either as single constituents or more commonly as mixtures of piperazine derivatives. Their toxicity is mostly due to excessive sympathomimetic effects. Piperazine derivatives appear to have mild to moderate potential for abuse and dependency [3].

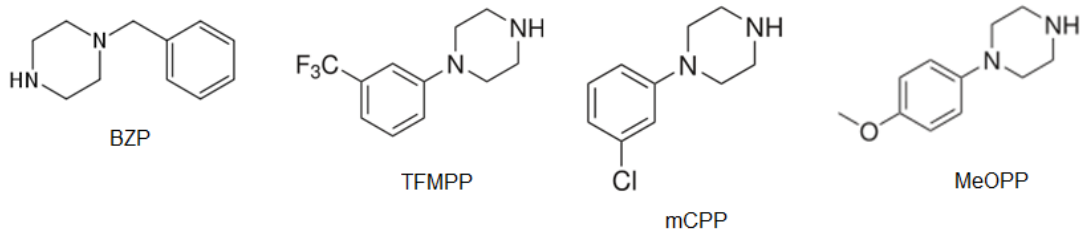

Figure 11: Structures of selected piperazines

1.2.11 Piperidines & Pyrrolidines

Several new dissociative piperidine derivatives have appeared on the recreational drug market, such as diphenidine and 2-methoxydiphenidine (2-MXP), as well as pyrrolidine derivatives, such as 2-(diphenylmethyl)pyrrolidine (desoxy-D2PM). They are suspected to be associated with a number of adverse health effects [10, 15].

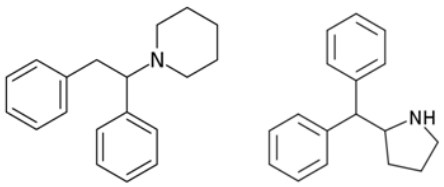

Figure 12: Structures of selected piperidines and pyrrolidines

1.2.12 Plants & Extracts

Although most New Psychoactive Substances are synthetic chemicals, some of them have natural origin. Chewing the plant Khat (Catha edulis) produces sympathomimetic and CNS-stimulating effects due to its pharmacologically active components, cathinone and, to a lesser extent, cathine (norpseudoephedrine) [16, 17]. Kratom (Mitragyna speciosa) has as main psychoactive components in its leaves the alkaloids mitragynine, 7-hydroxymitragynine, speciociliatine, speciogynine, paynantheine. Small doses of Kratom produce cocaine-like stimulation, while larger doses cause morphine-like sedative-narcotic effects [16, 18]. The plant Salvia divinorum is used as a legal herbal hallucinogen. The effective dose of salvinorin A, the active non-alkaloidal ingredient of the plant, is comparable to that of the synthetic hallucinogens lysergic acid diethylamide (LSD) or dimethoxybromoamphetamine (DOB) [16, 19].

1.2.13 Others

In this category, other NPS that have been reported to EU EWS to lesser extent and cannot be regarded to any of the above categories are included, as well as pharmaceuticals that have been reported to be mixed with illicit drugs and NPS in order to increase their desirable effects.

1.3 Occurrence of New Psychoactive Substances in Greece

During 2011-2016, 62 new psychoactive substances were identified for the first time in Greece, mostly synthetic cathinones and cannabinoids, as shown in **Table 1**. From all the NPS of the table, only 2-MMC is controlled in our country, while the new phenethylamine 1-phenethyl-4-hydroxypiperidine was identified for the first time in EU in Greece in 2016 [20, 21].

Table 1: New Psychoactive Substances detected in Greece during 2011-2016, according to Greek Documentation and Monitoring Centre for Drugs [20]

CHEMICAL CLASSES	SUBSTANCES	N
Synthetic cannabinnoids	AB-FUBINACA, 5-Fluoro-AB-Pinaca, MDMB-CHMICA, AB-CHMINACA, CUMYL-5FPINACA, 5F-AKB48, NM-2201, EAM-2201, AM-2201, MAM-2201, JWH-018, JWH-210, JWH-122, JWH-073, JWH-250, RCS-4, UR-144, Mepiramim, JWH-203, ADBICA, AB-001, JWH-208, 5F-ADB, XLR11/5FUR-144, AKB48/APINACA	25
Cathinones	MDPBP, Pentedrone, Clephedrone, N-ethylnorpentedrone, 3-FMC, 4-MEC, 3-MCC, Butylone, MDPV, Methylone, Mephedrone, alpha-PVP, 2-MMC, 4-CI-a-α-PVP, 2-MEC, Ethylone, Ephylone	17
Phenethylamines	25B-NBOMe, 25N-NBOMe, 2C-E, 4-FA, 25I-NBOMe, 2-PEA, MDPA, 1-phenethyl-4-hydroxypiperidine	8
Other substances	Modafinil, epirocaine, GBL, Iso-ethcathinone, phenibut	5
Indolalkylamine (tryptamine)	5-Meo-MiPT, 5-MeO-MALT	2
Arylcyclohexylamines	Methoxetamine	1
Arylalcylamines	6-MAPB	1
Piperidines & pirrolidines	Ethylphenidate, 2-DPMP	2
Opioids	Tramadol	1
	OVERAL	**62**

During 2012-2016, National Organization for Medicines recalled products that were sold in haberdashery shops and tobacco shops in southern Greece, which contained synthetic cannabinoids. Intoxications and deaths related to NPS use were reported during 2011-2016, which were associated with synthetic cathinones (MDPV, alpha-PVP, Pentedrone, Mephedrone, CUMYL-5FPINACA), synthetic cannabinoids (JWH-122, JWH-210, JWH-250), GHB / GBL, the plant Salvia divinorum, pregabalin and quetiapine, together with opioids, benzodiazepines, other pharmaceuticals and alcohol [20, 21].

1.4 Wastewater-based epidemiology

Wastewater-based epidemiology (WBE) is an innovative approach in monitoring public health and lifestyle habits by analyzing raw wastewater. It relies on the fact that almost everything that population consumes or is exposed to is excreted as parent compound or metabolite in urine and faeces and ends up in the sewer network. Thus, researchers can identify and quantify selected substances in raw wastewater samples, calculate the daily sewer loads of them and back-calculate the usage by taking into account the human excretion of target residues (pharmacokinetic data) and their stability in wastewater (environmental transformation rate data). Then, normalization is applied using information on the population served by the treatment plant in order to facilitate comparison among the cities [22, 23].

This idea was firstly proposed in 2001 by Daughton [22] who tried to bridge environmental and social sciences. He proposed the analysis of influent wastewater from sewage treatment facilities for trace elements in order to collect data that reflect community usage of illicit and abused drugs. Zuccato et al. [24] applied this proposal in 2005 and the following years WBE had extensively been applied in monitoring illicit and licit drug use in different locations all over the world [25-30]. Recently, WBE has been expanded to monitoring NPS use [31-43].

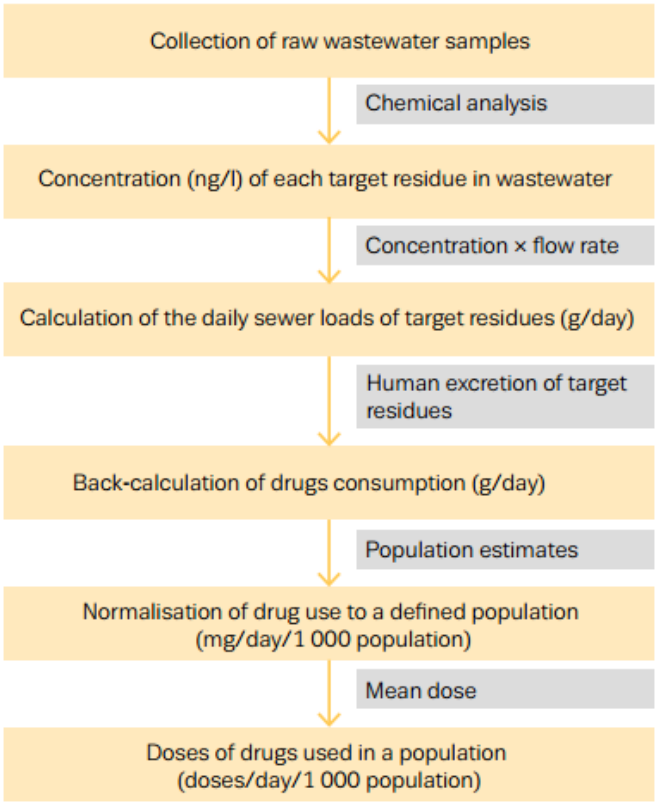

Figure 13: The main steps of the procedure of wastewater-based epidemiology [23]

WBE is a non-invasive and near-real-time technique of monitoring community usage of substances [23]. It is a complementary epidemiological tool to other established monitoring tools, such as population surveys, consumer interviews, medical records and crime statistics [24]. These conventional techniques are time-consuming and complex and moreover, self-reporting data of drug usage may be unreliable, due to the fact that it is a hidden and highly stigmatised behaviour and users are often unaware of the actual mix of substances they take [23]. The analysis of municipal wastewater provides us with the opportunity to obtain more reliable and timely information in short timeframes on geographical and temporal trends [23, 28], as well as correlate these data with socio-economic phenomena [30]. However, it should not be overestimated, because it is subject to different kind of uncertainties regarding sampling, analysis, stability of substances, metabolism, back-calculation of usage, estimation of population size [23].

Although WBE has successfully been applied to established illicit drugs, monitoring of NPS is a challenging task. The main challenges are the big number of new synthetic compounds that enter the market, which may become bigger by slightly changing the structure of them in order to produce new ones, the small size of the new psychoactive substance market with respect to the illicit drugs market and the lack of pharmacokinetic studies, metabolic profiles and excretion rates of these new drugs [44]. Thus, it is difficult to choose the suitable biomarker, which means the specific chemical substance selected as target drug residue in wastewater and used for back-calculating drug consumption values.

CHAPTER 2

Detection of New Psychoactive Substances in raw wastewater – Literature review

2.1 Introduction

The detection of new psychoactive substances in wastewater is a promising approach to understand the extent of their use by population, but meanwhile it is a challenging task. The concentrations of NPS in influent wastewater are much lower than in human biological fluids. Their low concentrations and the complex and unknown composition of raw wastewater matrix which includes a large number of other substances with different physicochemical properties make it difficult to identify them reliably. Advanced analytical techniques are required, so liquid chromatography coupled to high resolution mass spectrometry is the technique of choice for such compounds because of its excellent selectivity and sensitivity [23].

2.2 Sample treatment

The common technique that is used for extraction, clean-up and pre-concentration of contaminants in water is Solid Phase Extraction (SPE). The usual steps of SPE include the conditioning of the sorbent in the cartridge, the loading of the sample, where analytes interact with the sorbent and impurities pass through, the wash-up, the drying and finally the elution of the analytes.

SPE is the most appropriate technique for isolation of the target compounds from the aqueous matrix, as matrix components interfere with the analytical measurement and cause signal suppression or enhancement due to co-eluting matrix constituents of samples during ionization in LC-MS, and mainly when using ESI as source [45, 46]. Also, SPE leads to the enrichment of the final extract, so as to achieve the low limits of detection (LODs) required for determining environmental concentrations. As far as the wastewater, extraction provides the lowest recoveries compared to other types of aqueous

matrix, because the analytes are more affected by matrix components in sewage water. However, these low recoveries and potential losses from SPE are usually corrected well by adding appropriate deuterated compounds as internal standards [45].

Different extraction sorbents can be used for compounds with different physicochemical properties. On the other hand, it is often wanted to compare different kinds of study in a single analysis or use the data retrospectively, as described in session 2.3.2. For this reason, generic sample treatments are used for one single extraction of all analytes, as far as possible, in wastewater matrix. Kern et al. [47] used four different SPE cartridge materials simultaneously in order to have broad enrichment efficiency.

2.3 Analytical techniques – Liquid Chromatography coupled to Mass Spectrometry (LC-MS)

LC–MS is a sophisticated hyphenation of analytical techniques which enables the determination of organic pollutants in complex environmental matrices. A range of different LC-MS technologies have been put forward in recent years for the analysis of mixtures of many known and unknown compounds at low concentrations in complex matrices [48, 49].

2.3.1 Reversed Phase Ultra High Performance Liquid Chromatography (RP-UHPLC)

UHPLC uses small-diameter particles in the stationary phase and short columns and provides fast and high resolution separation that increases LC-MS sensitivity and minimizes matrix interference arising from minimal sample preparation [50, 51]. UHPLC is commonly performed in reversed-phase (RP) mode using C18 columns. The mobile phase consists of an aqueous and an organic solvent. Methanol and acetonitrile are commonly used as organic solvents. In some methods, the mobile phase is acidified with small percentages by volume of acetic or formic acid in order to improve ionization

of the compounds in the positive ionization mode [45]. Gradient elution programs are preferred for better and faster separations.

2.3.2 High-resolution mass spectrometry (HRMS)

In most WBE studies, the analytical methods that are developed include liquid chromatography coupled to tandem mass spectrometry using low resolution mass analyzers, usually triple quadrupole (QqQ), because this technique is reliable for qualitative and quantitative determination of selected/known biomarkers [46].

On the other hand, the use of liquid chromatography coupled to high-resolution mass spectrometry allows the wide-scope screening of parent compounds, metabolites and transformation products that may be known, suspect or unknown, so it can be used for the investigation of the growing and diverse group of NPS, as well as their metabolites and TPs, and for the estimation of the trends in their use by population [46, 52].

Among the possible ionization techniques in LC-MS, electrospray ionization (ESI) is the most widely used, compared with atmospheric pressure chemical ionization (APCI) or the more recent atmospheric pressure photoionization (APPI) [48].

LC-HRMS has an excellent performance on qualitative applications thanks to the high mass accuracy and the selectivity in full-scan acquisition mode that ensure reliable detection and identification, while more and more studies use LC-HRMS for complete analysis, both identification and quantification [46]. With full-spectrum accurate-mass data, a theoretically unlimited number of analytes that are present in a sample can be identified, because the acquisitions have been made as ‘all ions all the time’ [49]. The simultaneous determination of a broad number of compounds in one injection, with a corresponding reduction of time and costs, and even when reference standards are not available, make LC-HRMS one of the current trends in environmental analytical chemistry [52]. Moreover, investigation can be performed in a retrospective way in order to detect compounds that initially were not considered, even after years, without additional analysis of the

samples. This ability is advantageous, because in some occasions, samples might already have been discarded or the analytes have been degraded [46].

Time-of-flight (TOF) is one of the most used HRMS analyzers and it is easily coupled to ultra high performance liquid chromatography (UHPLC). Mass resolution typically ranges from 20,000 up to 80,000 FWHM and mass accuracy is lower than 2 ppm. Hybrid configurations, such as Quadrupole-Time-of-flight (QTOF), increase the potential of the analyzer for screening purposes and provide relevant structural information by obtaining accurate-mass product-ion spectra after MS/MS experiments [46]. Product-ion spectra can be obtained with either data dependent acquisition or data independent acquisition, where the instrument automatically switches after a full-scan-mode acquisition to a product-ion scan mode as the second scan event in the scan cycles [49].

2.3.2.1 Data Dependent Acquisition (DDA)

In this acquisition, there is firstly a full scan which is defined as the survey scan and data are processed “on-the-fly” to determine the candidates of interest based on predefined selection criteria, such as intensity threshold or suspect inclusion list. If the selection criteria are met, MS/MS analysis is then triggered and MS/MS scans (data-dependent) are performed [46, 48]. With this acquisition, ‘clean’ spectra with structural information are obtained in one injection. However, if the number of candidates of interest is big, the number of scans is decreased, so there are less data points that affect the detectability of the chromatographic peak [46].

2.3.2.2 Data Independent Acquisition (DIA)

With this acquisition, there is no need to pre-select the precursor ion. Full-scan spectra at different collision energies are obtained in one injection. This acquisition provides simultaneously accurate mass data of parent compounds and fragment ions in a single run using two scans, one at low and one at high collision energy. By applying low energy (LE) in the collision cell, no

fragmentation is performed. A full-scan spectrum is obtained that provides information for the parent ion (the (de)-protonated molecule) and, in some cases, the adduct ions and the in-source fragments. By applying high energy (HE) in the collision cell, fragmentation is performed and a spectrum similar to MS/MS experiments is obtained. This approach is called all-ions MS/MS, MS^E or bbCID, according to the QTOF manufacturer [46].

2.4 Data treatment – Approaches in HRMS screening

After the sample preparation and the LC-HRMS analysis, raw data can be treated with three different approaches, target, suspect and non-target screening. A systematic workflow for all three approaches is shown in **Figure 14**.

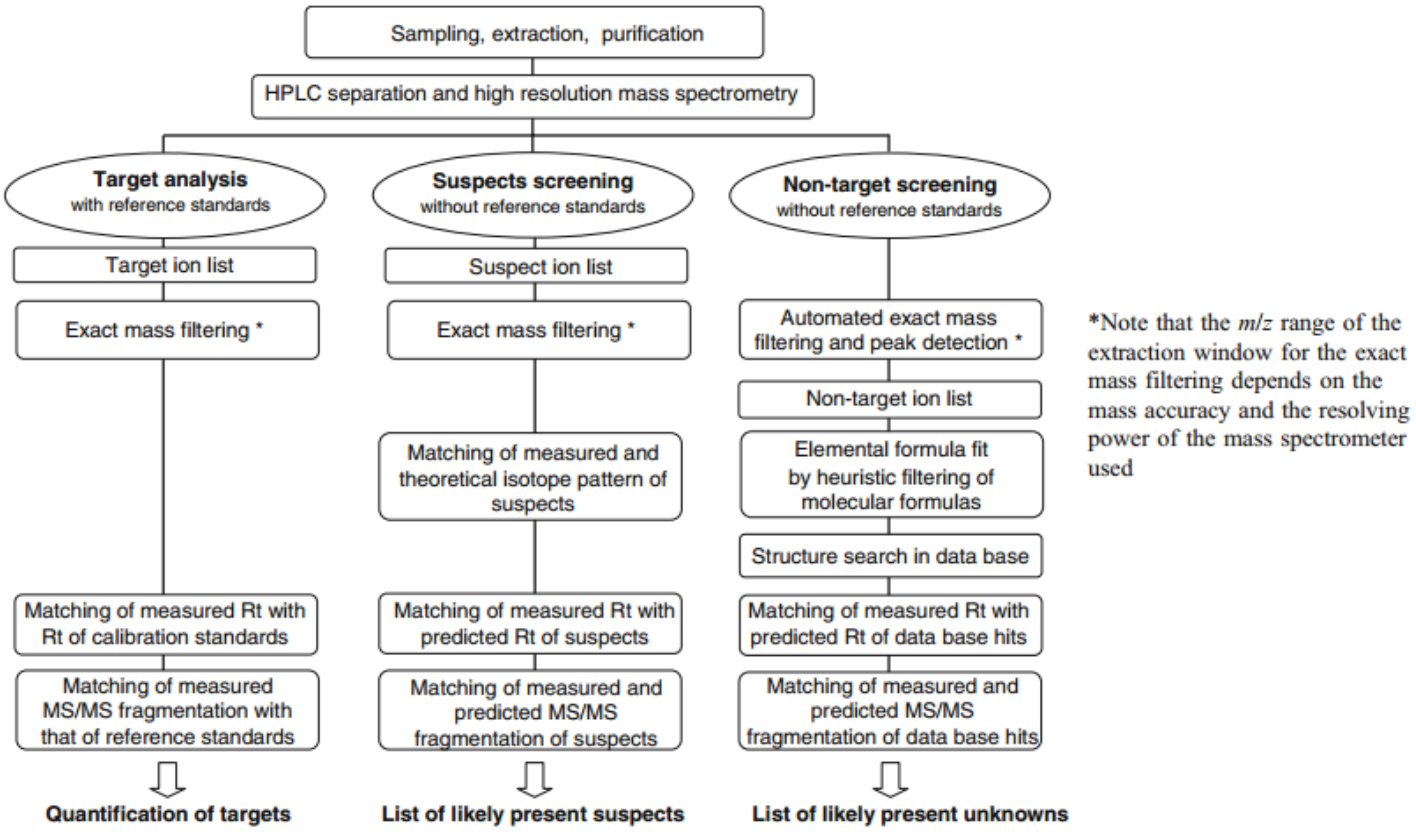

Figure 14: Systematic workflow for target, suspect and non-target screening by LC-HRMS/MS [48]

2.4.1 Target screening

In this approach, an in-house developed database is used for the screening of a large number of compounds. The information included in the database is based on the analysis of the available reference standards [46]. The reference standard is necessary for comparison of the retention time, the MS spectrum profile (precursor ion, adducts, in-source fragments), as well as the MS/MS spectrum (fragment ions and ion ratios) [53]. Quantitation can be performed in full-scan mode, but requires greater effort than in LC-LRMS methods where Single Reaction Monitoring (SRM) mode is used [46, 53].

2.4.2 Suspect screening

In this approach, a list of suspect compounds that are possible to be found in specific samples is built. The screening is based only on the exact m/z of the expected ions, which, in case of the ESI source, are usually the pseudomolecular ions $[M+H]^+$ and $[M-H]^-$, except for some compounds which exclusively show adduct formation. Molecular formula and structure are known, so this information can be efficiently used in the identification and confirmation process [48]. Absence from blank samples, mass accuracy, isotopic pattern, retention time prediction, ionization efficiency and information on fragment ions reported in the literature are parameters that can facilitate tentative identification of suspect candidates [48, 54].

2.4.3 Non-target screening

In non-target methodologies, samples are searched for compounds without any previous information on them. These unknown compounds are actually new, unexpected or not searched ones in specific samples. Identification is a challenge in this approach, as more than one elemental formula and several plausible structures are obtained for a given unknown compound detected in a sample [46]. Except for the elucidation of unknowns, non-target screening is used for the identification of metabolites and transformation products, arising from in vivo and in vitro experiments, in-silico modeling and degradation

laboratory studies [46, 49]. In this case, the number of chemically meaningful structures, which can be assigned to an unknown peak, is limited to structures that show a close relationship with the parent compound and also, an adequate control sample or time series is available [48].

2.4.4 Confidence in the identification procedure

2.4.4.1 Confidence in target screening

The confirmation of positive findings in target screening can be performed by attributing identification points (IPs). According to the 2002/657/EC guideline, 4 IPs are required for unequivocal confirmation, and for HRMS instruments with resolution higher than 10,000, the precursor ion earns 2 IPs and the product ions earn 2.5 IPs [55]. This means that one single HRMS/MS transition can confirm the detection of a substance, which is risky when there are several co-eluting isomers [39]. Another fact is that resolving power may largely vary between HRMS instruments, which makes the definition of general criteria difficult [49]. More precise criteria for the use of mass accuracy and mass resolution have to be implemented to define clearly the requirements for a reliable confirmation in LC-HRMS [48]. Bletsou et al. [53] proposed an identification points system for HRMS analysis in order to take full advantage of the capabilities of HRMS instruments.

2.4.4.2 Confidence in suspect and non-target screening

An identification strategy through five levels of confidence has been proposed for HRMS screening by Schymanski et al. [56], as described in **Figure 15**. Level 1 corresponds to the confirmed structure by the use of a reference standard, level 2 to a probable structure using literature or diagnostic data, level 3 to tentative candidate(s) with possible, not exact, structures, level 4 to an unequivocal molecular formula and level 5 to the exact mass. Non-target screening starts from level 5 and suspect screening from level 3 and, as identification confidence increases, they reach ‘better’ levels up to level 1. Target screening starts by definition from level 1. If the evidence of the sample

and the evidence of the reference standard (target) or the tentative candidate (suspect) do not match, then the component associated with the target or suspect should become a ‘non-target of interest’ and ‘downgrade’ to level 5 [57].

Generally, in both suspect and non-target screening, reference standards are required for ultimate and unambiguous confirmation, but should be purchased in a final stage, when solid well-found evidence exists on the presence of the compound in the sample [46].

Moreover, complementary techniques can be used for evaluation of possible candidates, such as NMR, a powerful structure elucidation technique, although this requires sufficiently high concentrations and often an isolation of the unknown compound [48].

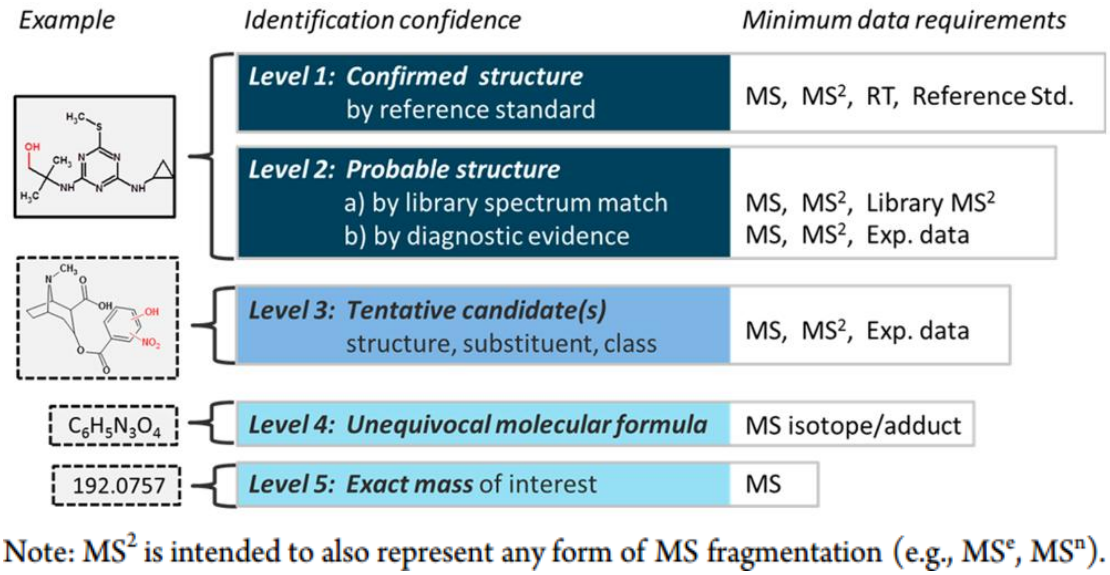

Figure 15: Identification confidence levels in HRMS [56]

2.5 Qualitative validation

It is difficult to ensure that a wide-scope screening method can detect and identify all compounds included in the target list. Reference standards are obviously required for a final confirmation of the identity, but also are needed to perform method validation. Qualitative validation is normally performed with

selected compounds from the target list that are taken as a model, due to the extreme difficulties to validate the method for the huge number of compounds that might be included in the target list [46]. The validation dataset should be chosen according to some rules that would guarantee its representativeness, such as selection of analytes from all classes of compounds in the database, with different physicochemical properties, assessed in terms of retention time, and also, analytes that are ionized in positive and negative mode [53].

2.6 Occurrence of New Psychoactive Substances in the aqueous environment – Analytical methods performed

So far, there are a lot of different studies for the determination of selected NPS in wastewater by LRMS [31-38, 40-42, 58-60], while, few studies deal with the detection of a broad range of NPS, together with controlled drugs of abuse, in wastewater matrix by HRMS [39, 43, 52, 61, 62].

In the following text, an overview of the analytical procedures and methods that have been applied in NPS detection in wastewater is performed. The first paragraphs refer to LC-MS analysis, while there is a separate reference to different developed methods.

For the extraction of NPS from the wastewater matrix, the most used sorbent is Oasis MCX (Waters, Milford Massachusetts, USA), a mixed-mode strong cation-exchange reversed-phase sorbent [34-36, 38-41, 59]. Also, Oasis HLB (Waters, Milford Massachusetts, USA), a strongly hydrophilic, reversed-phase sorbent with unique hydrophilic-lipophilic balance, has been used in different NPS studies as an extraction sorbent in cartridges [33, 43, 61] or disks (in combination with off-line automated SPE) [52], as well as PolyClean 2H (Cheshire Sciences, Chester, UK), a mixed hydrophilic/hydrophobic sorbent [37], and Strata-X (Phenomenex, Torrance, CA, USA), a reversed-phase sorbent with three mechanisms of retention, pi-pi bonding, hydrogen bonding, hydrophobic interactions [62].

For the separation of NPS in wastewater, C8 [34] and C18 columns [31, 33, 36, 39, 40, 41, 52, 59, 60- 62] have mainly been used. In some cases, an ether-linked phenyl reversed-phase column [38] and a pentafluorophenyl

reversed-phase column [32, 37] were used for better separation according to the developed method. In a LC-HRMS wide-scope suspect screening method for the detection of (il)licit drugs, pharmaceuticals of abuse and NPS, a biphenyl reversed-phase column was used [43]. On the other hand, Kinyua et al. [35] used hydrophilic interaction liquid chromatography (HILIC) for the detection of 7 NPS (arylalkylamines, arylcyclohexylamines, phenethylamines, synthetic cathinones), because methiopropamine was not well retained in C18-based chromatography.

Regarding LC-LRMS, most of the developed methods for the determination of selected NPS use triple quadrupole (QqQ) [33-38, 40, 41, 59] or quadrupole-ion trap (QIT) [31, 32, 60] as mass analyzers.

Regarding LC-HRMS, for the target screening of emerging psychoactive substances, together with traditional illicit drugs, pharmaceuticals and main urinary metabolites, in wastewater samples, quadrupole-time-of-flight (QTOF) mass spectrometer has widely been used either in DDA mode [61, 62] or in DIA mode [52]. Causanilles et al. [43] developed a suspect screening workflow by LC-HRMS. A qualitative screening of 2,000 (il)licit drugs, pharmaceuticals of potential abuse, NPS, metabolites and TPs was applied in wastewater samples collected prior and during a city festival, where there was a higher possibility of users of recreational substances and consequently higher residual concentrations of used NPS were expected. That was performed using an all-ions MS/MS acquisition (DIA) by LC-QTOF-MS, and then a DDA by LC-LTQ-Orbitrap-MS using an inclusion list with the masses previously tentatively identified. González-Mariño et al. [39] discussed the difficulties of acquiring MS/MS fragmentation in case of NPS, likely because of the low intensity of the $[M+H]^+$ ions in wastewater. Thus, he used linear ion trap-Orbitrap (LTQ-Orbitrap) mass spectrometer and performed DDA with inclusion lists for both target and suspect screening, but not in one analysis.

NPS are better ionized in the positive ionization mode, so most of the NPS studies use ESI (+) [31-38, 40, 41, 43, 52, 59, 60-62]. In one study, ESI (-) is used for the determination of the synthetic cannabinoid CP 47, 497 [37].

Apart from the LC-MS methodologies, some studies propose different procedures for the determination of NPS, together with established illicit drugs, in influent wastewater. González-Mariño et al. [42] developed an alternative method to common SPE-LC-MS procedures. He used ultra-high performance supercritical fluid chromatography coupled to tandem mass spectrometry (UHPSFC-MS/MS), combined with liquid-liquid extraction (LLE), for the determination of THC, 3 cannabinnoid metabolites and 4 synthetic cannabinnoid metabolites of the JWH-series. Mwenesongole et al. [58] presented a method based on solid phase extraction and gas chromatography-mass spectrometry (GC-MS) for the determination of 25 traditional and newly emerged drugs of abuse.

As mentioned in chapter 1, there are a lot of different NPS that are consumed in a lesser extent than classical illicit drugs and also, there is a huge dilution in wastewater. Thus, it is expected that very low concentrations of NPS residues will be determined in wastewater. Indeed, recent WBE studies for known NPS in wastewater have determined very low values (ng/L) of NPS and NPS below the LOQ [31-38, 40, 41, 52, 58, 59, 60-62] or none at all [42].

A literature review on the occurrence of NPS in the aqueous environment worldwide is presented in **Table 2**.

Table 2: Detected New Psychoactive Substances in the aqueous environment

New Psychoactive Substance	Sample Type	Sampling Point	Sampling Date	Reference
1-(2-methoxyphenyl)piperazine (oMeOPP)	Pooled wastewater	Pissoirs, central Oslo, Norway	-	[44]
2,5-dimethoxy-4-bromophenethylamine (2C-B)	Influent wastewater	main WWTP in Amsterdam, the Netherlands	Summer 2012 just prior to and during a festival that attracted ~300,000 visitors to the city	[43]
3,4-methylenedioxypyrovalerone (MDPV)	Influent wastewater	3 WWTPs in Adelaide, Australia	2009-2011	[32]
	Influent wastewater	2 WWTPs (Helsinki, Lappeenranta), Finland	May 2012	[59]
	Influent wastewater	Milan, Italy	March 2015	[41]
	Influent wastewater	Oslo, Norway	March 2015	[41]
	Influent wastewater	Zurich, Switzerland	March 2015	[41]
	Influent wastewater	13 STPs in cities of China	August, September 2014, May-September 2015	[40]
	Effluent wastewater	6 STPs in cities of China	August, September 2014, May-September 2015	[40]
3-methoxy-4-methylamphetamine (MMA)	Influent wastewater	main WWTP in Amsterdam, the Netherlands	Summer 2012 and 2014 just prior to and during a festival that attracted ~300,000 visitors to the city	[43]
3-trifluoromethylphenylpiperazine	Influent wastewater	7 WWTPs, UK	June 2010	[63]

(TFMPP)	Effluent wastewater	7 WWTPs, UK	June 2010	[63]
	River water	6 river locations, UK	June 2010	[63]
	Influent wastewater	3 WWTPs in Adelaide, Australia	2009-2011	[32]
4-fluoroamphetamine (4-FA)	Influent wastewater	main WWTP in Amsterdam, the Netherlands	Summer 2012 just prior to and during a festival that attracted ~300,000 visitors to the city	[43]
4-fluorophenylpiperazine (4-FPP)	Influent wastewater	WWTP, Cambridge, UK	-	[58]
4-methoxymethamphetamine (PMMA)	Influent wastewater	STP in Zurich, Switzerland	August 2013	[35]
4'-methyl-alpha-pyrrolidinohexanophenone (MPHP)	Influent wastewater	3 WWTPs, Spain	-	[62]
	Effluent wastewater	3 WWTPs, Spain	-	[62]
4'-methyl-alpha-pyrrolidinopropiophenone (MePPP)	Influent wastewater	WWTP, Cambridge, UK	-	[58]
4-methylethcathinone (4-MEC)	Effluent wastewater	Plaszow WWTP, Krakow, Poland	May 2012	[61]
alpha-Pyrrolidinovalerophenone (a-PVP)	Influent wastewater	3 WWTPs (Kamari, Fira, Karterados), Santorini island, Greece	July 2013	[37]
	Influent wastewater	3 WWTPs, Spain	-	[62]
	Effluent wastewater	3 WWTPs, Spain	-	[62]
Benzylpiperazine (BPZ)	Influent wastewater	7 WWTPs, UK	June 2010	[63]

	Effluent wastewater	7 WWTPs, UK	June 2010	[63]
	River water	6 river locations, UK	June 2010	[63]
	Inlet sewage	onsite WWTP in a festival, Australia	2010, 2011	[31]
	Influent wastewater	3 WWTPs in Adelaide, Australia	2009-2011	[32]
	Influent wastewater	36 STPs in cities of China	August, September 2014, May-September 2015	[40]
Bufotenine	River water	Surface waters, Turia River, Spain	-	[62]
Butylone	Influent wastewater	STP in Boechout, Belgium	December 2013	[35]
	Influent wastewater	STP in Zurich, Switzerland	August 2013	[35]
	Influent wastewater	WWTP, Cambridge, UK	-	[58]
CP47,497	Influent wastewater	5 WWTPs (Kamari, Fira, Karterados, Emporio, Ia), Santorini island, Greece	July 2013	[37]
	Effluent wastewater	5 WWTPs (Kamari, Fira, Karterados, Emporio, Ia), Santorini island, Greece	July 2013	[37]
Ethylamphetamine	Influent wastewater	3 WWTPs, Spain	-	[62]
	Effluent wastewater	3 WWTPs, Spain	-	[62]

Ethylone	Influent wastewater	STP in Antwerp-Zuid, Belgium	December 2013	[35]
	Influent wastewater	STP in Zurich, Switzerland	August 2013	[35]
Flephedrone	Influent wastewater	central WWTP, Zagreb, Croatia	May, June 2015	[38]
JWH-122	Inlet sewage	Hamar, Norway	July 2012	[34]
	Influent wastewater	3 WWTPs (Kamari, Karterados, Emporio), Santorini island, Greece	July 2013	[37]
	Effluent wastewater	4 WWTPs (Kamari, Fira, Karterados, Emporio), Santorini island, Greece	July 2013	[37]
JWH-018 N-5-hydroxypentyl	Inlet sewage	3 cities (Oslo, Bergen Hamar) in Norway	July 2012	[34]
JWH-210	Influent wastewater	2 WWTPs (Kamari, Emporio), Santorini island, Greece	July 2013	[37]
	Effluent wastewater	5 WWTPs (Kamari, Fira, Karterados, Emporio, Ia), Santorini island, Greece	July 2013	[37]
Ketamine	Influent wastewater	3 WWTPs (Antwerpen-Zuid, Brussel-Noord, Deurne), Belgium	April, May 2012	[33]
	Influent wastewater	STPs (Milan, Rome, Naples, Turin, Bologna, Verona, Florence, Bari, Pescara, Cagliari, Perugia, Merano, Gorizia, Nuoro, Potenza,	2010-2013	[36]

		Terni), Italy		
	Influent wastewater	WWTP in Oslo, Norway	February 2014	[52]
	Influent wastewater	central WWTP, Zagreb, Croatia	December 2014, June 2015	[38]
	Influent wastewater	WWTP, Cambridge, UK	-	[58]
Mephedrone	Inlet sewage	onsite WWTP in a festival, Australia	2010, 2011	[31]
	Influent wastewater	3 WWTPs in Adelaide, Australia	2009-2011	[32]
	Effluent wastewater	Plaszow WWTP, Krakow, Poland	May 2012	[61]
	Influent wastewater	STPs (Bologna, Florence), Italy	2010-2013	[36]
	Influent wastewater	Bristol, United Kingdom	March 2015	[41]
	Influent wastewater	Brussels, Belgium	March 2015	[41]
	Influent wastewater	Copenhagen, Denmark	March 2015	[41]
	Influent wastewater	Oslo, Norway	March 2015	[41]
	Influent wastewater	Utrecht, The Netherlands	March 2015	[41]
	Influent wastewater	Zurich, Switzerland	March 2015	[41]
	Influent wastewater	central WWTP, Zagreb, Croatia	May, June 2015	[38]
	Influent wastewater	WWTP, Cambridge, UK	-	[58]
meta-Chlorophenylpiperazine	Influent wastewater	main WWTP in Amsterdam, the	Summer 2012 just prior to and	[43]

(mCPP)		Netherlands	during a festival that attracted ~300,000 visitors to the city	
	Influent wastewater	WWTP, Cambridge, UK	-	[58]
Methedrone	Secondary effluent wastewater	WWTP, Velika Gorica, Croatia	March 2015	[38]
Methoxetamine (MXE)	Influent wastewater	5 STPs (Antwerp-Noord, Antwerp-Zuid, Antwerp-Deurne, Boechout, Ninove), Belgium	December 2013, March-April 2014	[35]
	Influent wastewater	STP in Zurich, Switzerland	August 2013	[35]
	Influent wastewater	WWTP in Oslo, Norway	February 2014	[52]
Methylbenzylpiperazine (MBPZ)	Influent wastewater	WWTP, Cambridge, UK	-	[58]
Methylhexanamine (DMAA)	Influent wastewater	main WWTP in Amsterdam, the Netherlands	Summer 2012 just prior to and during a festival that attracted ~300,000 visitors to the city	[43]
Methylone	Inlet sewage	onsite WWTP in a festival, Australia	2010, 2011	[31]
	Influent wastewater	3 WWTPs in Adelaide, Australia	2009-2011	[32]
	Inlet sewage	WWTP, South East Queensland, Australia	February 2011 - August 2013	[60]
	Influent wastewater	STP in Zurich, Switzerland	August 2013	[35]
	Influent wastewater	WWTP in Oslo, Norway	February 2014	[52]

	Influent wastewater	Bristol, United Kingdom	March 2015	[41]
	Influent wastewater	Copenhagen, Denmark	March 2015	[41]
	Influent wastewater	Utrecht, The Netherlands	March 2015	[41]
	Influent wastewater	central WWTP, Zagreb, Croatia	May, June 2015	[38]
	River water	Sava river, downstream of the main wastewater outfalls of Zagreb, Croatia	June 2015	[38]
Norketamine	Influent wastewater	3 WWTPs (Antwerpen-Zuid, Brussel-Noord, Deurne), Belgium	April, May 2012	[33]
	Influent wastewater	central WWTP, Zagreb, Croatia	June 2015	[38]

CHAPTER 3

Scope

The widespread appearance of New Psychoactive Substances on illicit drugs market, the limited data on their effects and toxicity for humans and the reports of intoxications and deaths make the monitoring of public usage of such substances an important demand. Almost every NPS that is consumed ends up as parent compound or metabolite in sewer systems, so the analysis of raw wastewater can provide valuable information regarding the NPS use of the population served by the WWTP.

LC-HRMS allows the wide-scope screening of NPS, their metabolites and transformation products with an acquisition of accurate-mass full spectrum data. These data can be used for target, suspect and non-target screening, as well as retrospective screening, years after the treatment of samples without additional analysis of them.

Recent studies focus on the determination of selected NPS in wastewater by LC-LRMS and available reference standards. However, efforts for screening of a wide range of NPS in wastewater by LC-HRMS are very limited.

The scope of this study is the retrospective target and suspect screening of NPS in raw wastewater samples from the main wastewater treatment plant of Athens that were collected during 2015-2017. For this reason, there was an application of a generic sample preparation for the enrichment of the extracts with a broad range of analytes with different physicochemical properties, as well as a data independent acquisition by LC-HRMS, where with one injection and no pre-selection of analytes, information were obtained for both parent compounds and fragment ions. Consequently, a qualitative approach can be performed that may indicate the use of specific NPS constantly or occasionally during the week and over the years. Moreover, specific challenges that emerge through the application of retrospective target and suspect screening of such substances with low concentrations in a complex matrix are discussed.

CHAPTER 4

Materials and Methods

4.1 Chemicals and Materials

For the sample preparation, the glass fiber filters (GFF, pore size 0.7 μm) that were used for wastewater filtration were obtained from Millipore (Cork, Ireland). The empty solid phase extraction polypropylene tubes (6 mL) and the cartridge sorbent materials Sepra ZT (Strata-X), Sepra ZT-WCX (Strata-X-CW) and ZT-WAX (Strata-X-AW) were purchased from Phenomenex (Torrance, USA), while the Isolute ENV+ sorbent material and the frits (20 μm, 6 mL) were purchased from Biotage (Ystrad Mynach, UK). Regenerated cellulose (RC) syringe filters (diameter 15 mm, pore size 0.2 μm) were obtained from Phenomenex (Torrance, CA, USA). Regarding the chemicals of the sample preparation, methanol was HPLC grade and was purchased from Fischer Scientific (Loughborough, UK) and ethylacetate ≥ 99.5% (GC), ammonia solution 25% for analysis and formic acid 98-100% for analysis were purchased from Sigma Aldrich (Steinheim, Germany).

All the solvents for the LC-QTOF-MS analysis were UHPLC-MS grade. Methanol was purchased from Merck (Darmstadt, Germany) and the eluent additives ammonium formate, ammonium acetate and formic acid 99% were purchased from Fluka (Buchs, Switzerland). Ultrapure water was provided by a Milli-Q purification apparatus (Millipore Direct-Q UV, Bedford, MA, USA).

The reference standards that were used for method validation were obtained from the companies presented below. 2-Phenethylamine, Acetyl-Fentanyl, Alprazolam, Amphetamine, Bromazepam, Clonazepam, Diazepam, Ephedrine, Fentanyl, Flunitrazepam, Flurazepam, Gabapentin, Ketamine, Lorazepam, MDA, MDEA, MDMA, Medazepam, Methamphetamine, Midazolam, Nitrazepam, Nor-Diazepam, Nor-Ephedrine, Nor-Fentanyl, Nor-Ketamine, Oxazepam, Prazepam, Pregabalin, Remifentanyl, Temazepam, Tetrazepam, Venlafaxine, all high-purity individual standards (> 98%), solutions or solids, were purchased from LGC Promochem (Molsheim,

France). Benzylpiperazine, JWH-018, JWH-073, Mephedrone were purchased from Cerilliant Corp. (Round Rock, TX, USA), as certified solutions. alpha-PVP, JWH-210, MePPP were purchased from Cayman Chemical Company (East Ellsworth, MI, USA), as certified solutions and crystallized solids. JWH-122 was purchased from LGC (Mercatorstrass, Germany) and JWH-250 from Toronto Research Chemicals (Toronto, Canada, USA). Tramadol was of high-purity grade (more than 90%), and were purchased from Sigma-Aldrich (Steinheim, Germany). 1[(4-chlorophenyl) phenyl methyl]piperazine, 1-3-Trifluoromethylphenyl-Piperazine, Atomoxetine, Bupropion, Diphenhydramine, Memantine, o-Chlorophenyl-Piperazine were kindly offered from Eawag (aquatic research institute, Zurich, Switzerland).

Regarding the internal standards that were used for all the analysis from 2015 to 2017 and the method validation, Atrazine-d5, Cocaine-d3, Codeine-d6, Diazepam-d5, Ketamine-d4, Morphine-d3 were purchased from LGC Promochem (Molsheim, France). BZP-d7 and Mephedrone-d3 were purchased from Cerilliant Corp. (Round Rock, TX, USA), while Sulfadiazine-d4, Sulfadimethoxin-d4, Sulfadimidine-d4 were purchased from Toronto Research Chemicals (Toronto, Canada). Flunixin-d3 and Meloxicam-d3 were donated by the Veterinary Drug Residues Laboratory of the State General Laboratory of Cyprus, while Amisulpride-D5, Amphetamine-D6, Atenolol-D7, Atorvastatin-D5, Benzotriazole-5-Methyl-D6, Carbamazepine-D8, Cetirizine-D8, Citalopram-D6, Lamotrigine-13C3 d3, Metformin-D6, Metronidazole-D4, Ranitidine-D6, Ritonavir-D6, Saccharin-13C6, Tramadol-D6, Valsartan-13C5 15N, Venlafaxine-D6 were kindly offered from Eawag (aquatic research institute, Zurich, Switzerland).

4.2 Sampling and Storage

24-hour flow-proportional composite influent wastewater samples were collected from the main wastewater treatment plant in the greater Athens area in Greece, which is located in Psyttalia Island.

The Psyttalia Wastewater Treatment Plant (WWTP) is one of the biggest in Europe and worldwide and its capacity is 5,200,000 population equivalents.

The residential population connected to the WWTP based on official census in 2011 is 3,700,000 and the average wastewater flow is approximately 730,000 m^3/day. The Psyttalia WWTP facilities include wastewater pretreatment on the Attica mainland and then primary treatment and advanced secondary biological treatment using activated sludge processes on Psyttalia Island. The wastewater effluents are being received by the Saronic Gulf [64].

Wastewater sampling was performed by the method of flow-proportional composite sampling, which consists of a combination of numerous discrete samples (aliquots) that are taken over known flow intervals [65]. The analysis of the sampled material, collected over a period of time, will represent the average performance of a WWTP during the collection period.

Raw wastewater samples were collected from 2015 to 2017. Every year, March was the chosen month for the sampling in order to have temporal homogeneity. So, the sampling was performed for 7 or 8 consecutive days of March of 3 consecutive years in order to estimate the trends during the week and over the years. The exact sampling dates and the average flow rates are shown in **Table 3**.

Table 3: Sampling dates and average flow rate of influent wastewater in Psyttalia WWTP

Sampling dates	Average flow (m^3/day)
04-11/03/2015	820,870
16-23/03/2016	701,850
08-14/03/2017	919,729

After sampling, raw wastewater samples were kept in pre-cleaned high-density polyethylene (HDPE) bottles. Immediately after arrival at the laboratory, they were vacuum filtered through glass fiber filters (GFF) with a pore size of 0.7 μm in order to remove suspended solids that may clog the adsorbent bed during SPE. Finally, they were stored in the dark at 4 ^oC until analysis.

4.3 Sample preparation

Sample treatment and extraction were carried out based on the protocol of Kern et al. [47] with few variations. 100 mL of wastewater sample aliquots were adjusted to pH 6.5 (±0.2) with few drops of formic acid 0.1 M and an IS mix solution (which may differed through the years of analysis) was spiked in each sample. To achieve sufficient enrichment for a broad range of compounds, SPE with mixed bed multilayer cartridges was used for sample clean-up and pre-concentration. These in-house SPE cartridges consisted of 200 mg of Strata-X (polymeric reversed phase sorbent for extraction of neutral and aromatic compounds) and a mixture of 100 mg of Strata-X-AW (weak anion exchanger for extraction of acidic compounds with pK_a<5), 100 mg of Strata-X-CW (weak cation exchanger for extraction of basic compounds with pK_a>8) and 150 mg of IsoluteENV+ (polymeric reversed phase sorbent for extraction of polar compounds). The conditioning of the cartridges was performed with 3 mL methanol and 3 mL water. The samples were loaded to the SPE cartridges and then they were dried under vaccum at a flow rate of 10 mL/min for 0.5 to 1 h. The elution of the analytes from the adsorbent material was performed by a basic solution (4 mL of ethylacetate/methanol (50/50 v/v) containing 2% ammonia hydroxide (v/v)), followed by an acidic solution (2 mL of ethylacetate/methanol (50/50 v/v) containing 1.7% formic acid (v/v)). The extracts were evaporated under a gentle nitrogen stream to a final volume of 50 μL and finally reconstituted to a final volume of 500 μL methanol/water 50/50. Every extract was filtered directly into a 2 mL vial using a syringe fitted with a 0.2 μm RC membrane filter in order to remove the solid particles that were still present and may cause blockage of the column filter, and then they were ready for LC-HRMS/MS analysis.

4.4 Instrumentation

An Ultra-High Performance Liquid Chromatography (UHPLC) system (UltiMate 3000 RSLC, Thermo Fisher Scientific, Germany) coupled to a Quadrupole-Time of Flight Mass Spectrometer (QTOF-MS) (Maxis Impact, Bruker Daltonics, Bremen, Germany) was used for the analysis of the

samples. The UHPLC apparatus consists of a solvent rack degasser, a binary pump with solvent selection valve (HPG-3400), an auto-sampler and a column. The QTOF-MS apparatus consists of an Electrospray Ionization (ESI) source operating in positive and negative mode.

Figure 16: UHPLC-QTOF-MS, Maxis Impact, Bruker Daltonics

In our analysis, two separate reversed-phase chromatographic runs were performed for positive and negative ESI mode. An Acclaim RSLC 120 C18 column (2.1 × 100 mm, 2.2 μm) (Dionex Bonded Silica Products, Thermo Scientific, Dreieich, Germany), preceded by an ACQUITY UPLC BEH C18 1.7 μm guard column of the same packaging material (VanGuard Pre-Column, Waters, Dublin, Ireland), and thermostated at 30 ^{o}C, was used. In the positive ESI mode, the aqueous mobile phase consisted of 90% H_2O, 10% CH_3OH, 5 mM $HCOONH_4$, 0.01% HCOOH and the organic mobile phase consisted of CH_3OH, 5 mM $HCOONH_4$, 0.01% HCOOH. In the negative ESI mode, the aqueous mobile phase consisted of 90% H_2O, 10% CH_3OH, 5 mM CH_3COONH_4 and the organic mobile phase consisted of CH_3OH, 5 mM CH_3COONH_4. The gradient elution program was the same for both ionization modes and applied changes in mobile phase and in flow rate. It started with 1.0% of organic phase (flow rate 0.200 mL/min) for 1 min, increasing to 39.0% by 3 min (flow rate 0.200 mL/min), and then to 99.9% (flow rate 0.400 mL/min) in the following 11 min. These almost pure organic conditions were kept constant for 2 min (flow rate 0.480 mL/min) and then initial conditions were

restored within 0.1 min, kept for 3 min and then the flow rate decreased to 0.200 mL/min for the last minute. The injection volume was set to 5 μL.

The operating parameters of the ESI interface were the following: capillary voltage 2500 V for positive and 3000 V for negative mode, end plate offset 500 V, nebulizer pressure (N_2) 2.0 bar, drying gas (N_2) 8.0 L/min, drying temperature 200 ^{o}C.

Data were acquired through a Data Independent Acquisition (DIA) scan mode, called broad-band Collision Induced Dissociation (bbCID), which provided both MS and MS/MS spectra simultaneously using two different collision energies with a scan rate of 2 Hz and a mass range of 50-1000 Da. Low collision energy (4 eV) provided a full scan spectrum (MS) and high collision energy (25 eV) provided a spectrum where all ions were fragmented (bbCID MS/MS).

An external calibration of the QTOF mass spectrometer was performed with a sodium formate solution before analysis. Also, a calibrant injection was performed automatically at the beginning of each run and the segment of 0.1-0.25 min was used for internal calibration. The calibrant solution of sodium formate consisted of 10 mM sodium formate clusters in a mixture of water : isopropanol 1:1. The theoretical exact masses of calibration ions with formulas $Na(NaCOOH)_{1-14}$ in the range of 50−1000 Da were used for calibration. The instrument provided a typical resolving power of 36,000-40,000 during calibration.

Bruker's software that was used for raw data analysis was DataAnalysis 4.3, TASQ Client 1.0, TargetAnalysis 1.3.

4.5 Method validation

A representative validation dataset of 49 NPS and illicit drugs with similar structures with NPS was used in order to evaluate linearity, accuracy, precision, matrix effects and detectability of the screening method. The compounds of the validation dataset and some of their fragments in positive ESI mode are shown in **Table 4**. These selected compounds represented

almost all the classes of NPS in the database and had several physicochemical properties, so they eluted all over the chromatogram.

Linearity was studied for each compound by analyzing standard solutions at 8 different concentrations ranging from 25-200 μg/L. Using these calibration curves, the instrumental limits of detection (ILOD) were calculated by multiplying the standard error by 3 and dividing it by the slope, and the instrumental limits of quantification (ILOQ) by multiplying the ILOD by 3.3.

Accuracy was assessed with recovery experiments. Method recovery was calculated by dividing the peak area of the spiked samples by the peak area of the matrix-matched samples at 500 ng/L. The initial samples were analyzed for determination of the analytes of the validation dataset and if the sample already contained the analyte, its peak area was subtracted from the peak area of the spiked sample and the peak area of the matrix-matched sample. Precision was expressed as method repeatability in terms of relative standard deviation (%RSD) in 4 spiked samples at 500 ng/L. After the calculation of the matrix factor by dividing the peak area of matrix-matched samples by the peak area of the standard solutions, matrix effect was assessed by the equation: %Matrix Effect = (Matrix Factor - 1) × 100. The method limits of detection (MLOD) and quantification (MLOQ) were calculated by dividing the ILOD and ILOQ respectively by the matrix factor and then by dividing the results with 200, which is the pre-concentration factor.

Table 4: Validation dataset

Class	Compound name	CAS Number	Molecular formula	Calculated m/z of $[M+H]^+$	Retention time (min)	Fragm 1	Fragm 2	Fragm 3	Fragm 4
Cannabinoids	JWH-018	(209414-07-3)	C24H23N1O1	342.1852	12.41	155.0491	214.1226	145.0648	
	JWH-073	(208987-48-8)	C23H21N1O1	328.1696	11.88	155.0491	200.107	145.0648	127.0542
	JWH-122	(619294-47-2)	C25H25N1O1	356.2009	12.89	169.0648	214.1226		
	JWH-210	(824959-81-1)	C26H27N1O1	370.2165	13.24	183.0804	214.1226	155.0855	144.0444
	JWH-250	(864445-43-2)	C22H25N1O2	336.1958	11.83	121.0648	200.1434	214.1226	303.1618
Cathinones	alpha-PVP	(14530-33-7)	C15H21N1O1	232.1696	5.08	91.0542	105.0335	126.1277	84.0808
	Bupropion	(34911-55-2)	C13H18N1O1Cl1	240.115	5.76	131.073	57.0699	166.0418	139.0309
	Mephedrone	(1189805-46-6)	C11H15N1O1	178.1226	4.49	145.0886	144.0808	91.0542	119.0855
	MePPP	(1313393-58-6, 28117-80-8)	C14H19NO	218.1539	4.76	98.0964	119.0855	147.0804	
	Nor-Ephedrine	(14838-15-4)	C9H13NO	152.107	3.54	91.0542	115.0542	134.0964	117.0699
Arylalkylamines/ Arylcyclohexylamines/ Phenethylamines	Atomoxetine	(83015-26-3)	C17H21NO	256.1696	7.63	44.0495			
	Ketamine	(6740-88-1)	C13H16ClNO	238.0993	4.61	125.0153	67.0542	179.0622	220.0888
	Nor-Ketamine	(35211-10-0)	C12H14ClNO	224.0837	4.69	125.0153	67.0542	163.0309	
	2-Phenethylamine	(64-04-0)	C8H11N	122.0964	3.57	105.0699	79.0542	95.0491	77.0386

	Amphetamine	(300-62-9)	C9H13N	136.1121	4.16	91.0542	65.0386		
	MDA	(4764-17-4)	C10H13N1O2	180.1019	4.19	105.0699	79.0542	135.0441	133.0648
	MDEA	(82801-81-8)	C12H17N1O2	208.1332	4.39	105.0699	135.0441	133.0648	163.0754
	MDMA	(42542-10-9)	C11H15N1O2	194.1176	4.18	105.0699	135.0441	79.0542	133.0648
	Methamphetamine	(537-46-2)	C10H15N1	150.1277	4.21	91.0542	65.0386	119.0855	
Benzodiazepines	Alprazolam	(28981-97-7)	C17H13N4Cl1	309.0902	8.36	281.0714	274.1213		
	Bromazepam	(1812-30-2)	C14H10N3O1Br1	316.008	7.28	182.0839	209.0947	288.0131	80.0495
	Clonazepam	(1622-61-3)	C15H10N3O3Cl1	316.0483	7.61	270.0554	302.0453	241.0527	207.0917
	Diazepam	(439-14-5)	C16H13N2O1Cl1	285.0789	9.53	193.0886	154.0418	222.1152	257.084
	Flunitrazepam	(1622-62-4)	C16H12N3O3F1	314.0935	7.83	268.1006	300.0905	239.0979	286.0986
	Flurazepam	(17617-23-1)	C21H23N3O1Cl1F1	388.1586	6.61	315.0695	100.1121	288.0586	
	Lorazepam	(846-49-1)	C15H10N2O2Cl2	321.0192	8.36	275.0137	229.0527	303.0086	
	Medazepam	(2898-12-6)	C16H15N2Cl1	271.0997	10.22	207.1043	91.0542	242.0731	
	Midazolam	(59467-70-8)	C18H13N3Cl1F1	326.0855	8.63	291.1166	244.0324		
	Nitrazepam	(146-22-5)	C15H11N3O3	282.0873	7.79	236.0944	268.0842	207.0917	
	Nor-Diazepam	(1088-11-5)	C15H11N2O1Cl1	271.0633	9.23	140.0262	208.0995	165.0209	91.0542
	Oxazepam	(604-75-1)	C15H11N2O2Cl1	287.0582	8.43	241.0527	269.0476	231.0684	

	Prazepam	(2955-38-6)	C19H17N2O1Cl1	325.1102	10.58	271.0633	140.0262		
	Temazepam	(846-50-4)	C16H13N2O2Cl1	301.0738	7.94	255.0684	193.0886	228.0575	
	Tetrazepam	(10379-14-3)	C16H17N2O1Cl1	289.1102	10.54	253.1335	81.0699	225.1022	
Opioids	Acetyl-Fentanyl	(3258-84-2)	C21H26N2O						
	Fentanyl	(437-38-7)	C22H28N2O1	337.2274	6.04	188.1434	105.0699	132.0808	134.0964
	Nor-Fentanyl	(1609-66-1)	C14H20N2O1	233.1648	4.68	84.0808	55.0542	56.0495	57.0335
	Remifentanyl	(132875-61-7)	C20H28N2O5	377.2071	5.08	228.123	113.0597	261.1598	317.186
	Tramadol	(27203-92-5)	C16H25N1O2	264.1958	4.88	58.0651			
Piperazine derivates	Benzylpiperazine	(2759-28-6)	C11H16N2	177.1386	4.23	91.0542			
	1-3-Trifluoromethylphenyl-Piperazine	(15532-75-9)	C11H13F3N2	231.1104	5.93				
	1[(4-chlorophenyl) phenyl methyl]piperazine	(303-26-4)	C17H19ClN2	287.131	8.53				
	o-Chlorophenyl-Piperazine		C10H13ClN2	197.084	5.08				
Others	Diphenhydramine	(58-73-1)	C17H21N1O1	256.1696	6.63	167.0855	165.0699	152.0621	
	Ephedrine	(299-42-3)	C10H15N1O1	166.1226	3.76	91.0542	115.0542	117.0699	133.0886

	Gabapentin	(60142-96-3)	C9H17N1O2	172.1332	3.78	55.0178	95.0855	67.0542	91.0542
	Memantine	(19982-08-2)	C12H21N	180.1747	6.91	107.0855	163.1481	121.1012	
	Pregabalin	(148553-50-8)	C8H17N1O2	160.1332	3.88	55.0542	83.0855	142.1226	
	Venlafaxine	(93413-69-5)	C17H27N1O2	278.2115	6.14	58.0651	121.0648	147.0804	215.143

4.6 Retrospective target and suspect screening for the determination of New Psychoactive Substances

4.6.1 Target screening

A database of approximately 200 new psychoactive substances was used for the target screening of the raw wastewater samples in the positive ESI mode. The database contained precursor ions, retention time, adducts, in-source fragments and bbCID MS/MS fragments. This information was acquired from the analysis of the standard solutions of NPS, which were available in the laboratory, with the bbCID method, or was part of the manufacturer's database, Bruker's ToxScreener 2.1, which was built with the same bbCID method. Information of 10 NPS in the negative ESI mode was available, so these analytes were also screened in this mode.

The raw data were processed with Bruker's TASQ Client 1.0 and DataAnalysis 4.3. The TASQ method in TASQ Client 1.0 created in all samples the Extracted Ion Chromatogram (EIC) of the precursor ion of the compounds included in the database with a mass error window of ±0.005 Da.

Every peak that was detected for a target compound was evaluated according to some parameters that were set to the method and after manual inspection. The first one was the mass accuracy, which refers to the difference between the accurate mass (measured) and the exact mass (theoretical) and is expressed in mDa or ppm. The second one was the retention time shift, which refers to the difference between the measured retention time and the one that is recorded to the database. The last parameter was the isotopic fitting, which refers to the correlation between the theoretical and the experimental isotopic pattern. Its calculation is based on the standard deviation of the masses and the intensities for all isotopic peaks and is expressed by the mSigma value. Lower mSigma value indicates better isotopic fitting.

The screening parameters that were set to the method in both positive and negative ESI mode were an area threshold of 1000 counts and an intensity threshold of 500 counts. Regarding the mass accuracy, peaks having this value higher than 2.5 mDa and 5 ppm were rejected. Regarding the retention time, peaks having this value higher than 0.2 min were also rejected. The

mSigma threshold was set to 200. However, this value was only considered as a positive confirmation and not for rejecting peaks, because strong matrix effects combined with low concentration levels of analytes may affect the isotopic pattern results and give a bad mSigma value, although the compound may be present.

In order to confirm the screening results, bbCID MS/MS fragments were examined, as well as adducts and in-source fragments in full scan MS.

Apart from the EIC of the precursor ion of a compound, the TASQ method created with the same mass error window the EICs of its adducts, in-source and bbCID MS/MS fragments, so the fitting of their chromatographic profiles were inspected and evaluated. Except for TASQ Client 1.0, DataAnalysis 4.3 was used for the inspection and evaluation of the bbCID mass spectra.

For the identification and confirmation of the analytes, the Identification Points (IPs) system that has been proposed for HRMS analysis by Bletsou et al. [53] was used. Precursor ion (mass accuracy) and retention time earn together 2 IPs, while isotopic fitting earns 0.5 IP. Furthermore, each of the in-source and bbCID MS/MS fragments (mass accuracy) earns 2.5 IPs.

4.6.2 Suspect screening

A suspect list of approximately 500 new psychoactive substances was built according to information from the HighResNPS database [66] and EWS reports. Only the exact mass of the compounds in this database was used as prior information.

The raw data were analyzed with Bruker's TargetAnalysis 1.3 and DataAnalysis 4.3. It was assumed that all suspect compounds produced $[M+H]^+$ when they ionized by the positive ESI source, so the TargetAnalysis method created in every sample the EICs of the pseudomolecular ions with a mass error window of ±0.005 Da.

Some parameters were automatically applied in the detection of the peaks that corresponded to the suspect compounds, such as area and intensity higher than 1000 and 500 counts respectively and mass accuracy below 2.5

mDa and 5 ppm. The parameters in area and intensity were set at very low values regarding the low concentrations of NPS that have been reported in wastewater. However, with these thresholds, random noise peaks were detected, so manual inspection were needed for their rejection. Moreover, peaks that also occurred in the procedural blank were rejected.

For all remaining peaks that referred to certain parent compounds, the bbCID EICs of these compounds were created. These bbCID peaks should have had lower intensity comparing with the initial peak in full-scan MS or not exist at all, because the precursor ions were being fragmented in bbCID. So, if the intensity of the bbCID peak was higher, it meant that this peak did not correspond to the suspect compound, but to an in-source fragment of another compound that eluted in this retention time. Thus, these peaks were rejected, too.

Then, the experimental retention time of the peaks was compared with the predicted retention time from an in-house QSRR (Quantitative Structure-Retention Relationship) retention time prediction model [67]. Its prediction relies on the chemical structure similarity of the suspect compound with the compounds of the training set that was used to model retention time. As far as the applicability domain of the model, there are four regions (boxes) in the bubble plots that refer to four different levels of acceptance for the predicted retention time. Box 1 means that the chemical structure of the compound is very similar to the training set used to build the model and the error is less than 1 min. Box 2 means that the structure is diverse or the observed error is relatively accepted comparing the chemical structural effect and the error is less than 2 min. Box 3 means that the residuals are high and the predicted retention time is questioned. Finally, box 4 means either that the model is not applicable for the suspect compound, if the bubble size is huge, or that the suspect compound is false positive and it does not be corresponded to the given retention time, if the bubble size is tiny. In conclusion, measured retention time is accepted in boxes 1 and 2 and rejected in box 4 when the bubble size is very small. In all other cases, the retention time prediction results are not reliable and other methods of confirmation should be applied, such as bbCID MS/MS fragments.

After the comparison of the experimental and predicted retention time, full-scan MS was manually inspected. Peaks that referred to isotopes with ^{13}C, ^{2}H, etc. were rejected, the isotopic pattern for suspect compounds with Cl, Br, S (distinctive isotopic signature) was carefully examined, isotopic fitting < 200 mSigma was considered as a positive confirmation and adducts were screened.

Finally, for the identification of the suspect compounds at a higher confidence level, the presence of fragment ions was also evaluated. The bbCID spectrum does not provide 'clean' structural information, as no pre-selection of analytes is occurred; however, the bbCID MS/MS fragments can provide valuable information and are used for identification of analytes in wide-scope screening methods and in retrospective analysis. EICs of bbCID MS/MS fragments were carefully checked to be parallel to the EICs of the precursor ions in full-scan MS. Fragments in bbCID were checked to be in agreement with literature or library spectrum data. The libraries that were used were HighResNPS [66], MassBank [68] and mzCloud [69]. If no literature information or library data were available, the experimental information (diagnostic fragments) was used in order to explain the possible structure.

In order to arrange all the above steps of the workflow and communicate confidence in the identification procedure, the Identification Confidence Levels of Schymanski et al. [56] were used. In suspect screening, the identification starts from level 3 that refers to tentative candidate(s) and reaches level 1 that refers to a confirmed structure, if a reference standard is available. Level 2 refers to probable structure and is divided into level 2a (library), in which data from MS/MS spectrum match literature or library spectrum data, and level 2b (diagnostic), in which no standard or literature information are available, but no other structure fits the experimental information **(Figure 15)**.

CHAPTER 5

Results and Discussion

5.1 Validation results

As mentioned in chapter 4.5, for the evaluation of linearity, accuracy, precision, matrix effects and detectability of the screening method, a representative validation dataset of 49 NPS and illicit drugs with similar structures with NPS was used.

Regarding linearity, the slope, the intercept and the correlation coefficient (R^2) of the standard solution calibration curve for each compound are presented in **Table 5**.

The instrumental limits of detection (ILODs) and the instrumental limits of quantification (ILOQs) that were calculated from the data of the calibration curves are presented in **Table 6**. ILODs for most analytes were 15-25 μg/L **(Figure 17)**.

Table 5: Validation results - Linearity: Slope, intercept and correlation coefficient (R^2) of the standard solution calibration curve of 8 different concentrations ranging from 25-200 μg/L for each compound

Class	Analyte	Slope (b)	Standard error (S_b)	Intercept (a)	Standard error (S_a)	Correlation coefficient (R^2)
Cannabinoids	JWH-018	$58.0 \cdot 10^2$	$3.3 \cdot 10^2$	$3.7 \cdot 10^4$	$4.2 \cdot 10^4$	0.98
	JWH-073	$78.2 \cdot 10^2$	$4.2 \cdot 10^2$	$9.3 \cdot 10^4$	$5.4 \cdot 10^4$	0.98
	JWH-122	$48.4 \cdot 10^2$	$2.6 \cdot 10^2$	$2.1 \cdot 10^4$	$3.3 \cdot 10^4$	0.98
	JWH-210	$54.0 \cdot 10^2$	$3.0 \cdot 10^2$	$1.8 \cdot 10^4$	$3.8 \cdot 10^4$	0.98
	JWH-250	$50.2 \cdot 10^2$	$2.9 \cdot 10^2$	$5.5 \cdot 10^4$	$3.6 \cdot 10^4$	0.98
Cathinones	alpha-PVP	$92.6 \cdot 10^2$	$3.3 \cdot 10^2$	$12.9 \cdot 10^4$	$4.2 \cdot 10^4$	0.993
	Bupropion	$128.7 \cdot 10^2$	$3.5 \cdot 10^2$	$22.5 \cdot 10^4$	$4.4 \cdot 10^4$	0.995
	Mephedrone	$127.1 \cdot 10^2$	$5.5 \cdot 10^2$	$7.5 \cdot 10^4$	$6.9 \cdot 10^4$	0.99

	MePPP	$162.9 \cdot 10^2$	$5.7 \cdot 10^2$	$28.2 \cdot 10^4$	$7.2 \cdot 10^4$	0.993
	Nor-Ephedrine	$121.0 \cdot 10^2$	$3.9 \cdot 10^2$	$31.2 \cdot 10^4$	$5.0 \cdot 10^4$	0.994
Phenethylamines/ Arylcyclohexylamines/ Arylalkylamines	Atomoxetine	$143.3 \cdot 10^2$	$4.4 \cdot 10^2$	$26.1 \cdot 10^4$	$5.5 \cdot 10^4$	0.994
	Ketamine	$72.4 \cdot 10^2$	$2.0 \cdot 10^2$	$10.2 \cdot 10^4$	$2.6 \cdot 10^4$	0.995
	Nor-Ketamine	$37.4 \cdot 10^2$	$1.0 \cdot 10^2$	$7.6 \cdot 10^4$	$1.3 \cdot 10^4$	0.995
	2-Phenethylamine	1821	77	$19.2 \cdot 10^3$	$9.8 \cdot 10^3$	0.99
	Amphetamine	$72.3 \cdot 10^2$	$1.1 \cdot 10^2$	$7.1 \cdot 10^4$	$1.4 \cdot 10^4$	0.999
	MDA	4190	90	$9.3 \cdot 10^4$	$1.1 \cdot 10^4$	0.997
	MDEA	$40.1 \cdot 10^3$	$1.0 \cdot 10^3$	$5.9 \cdot 10^5$	$1.3 \cdot 10^5$	0.996
	MDMA	$65.6 \cdot 10^2$	$1.5 \cdot 10^2$	$7.1 \cdot 10^4$	$1.9 \cdot 10^4$	0.997
	Methamphetamine	$65.4 \cdot 10^2$	$2.0 \cdot 10^2$	$2.7 \cdot 10^4$	$2.6 \cdot 10^4$	0.994
Benzodiazepines	Alprazolam	$78.8 \cdot 10^2$	$3.7 \cdot 10^2$	$8.8 \cdot 10^4$	$4.7 \cdot 10^4$	0.99
	Bromazepam	999	29	$8.2 \cdot 10^3$	$3.7 \cdot 10^3$	0.995
	Clonazepam	1201	44	$16.4 \cdot 10^3$	$5.6 \cdot 10^3$	0.992
	Diazepam	$125.0 \cdot 10^2$	$6.3 \cdot 10^2$	$18.6 \cdot 10^4$	$7.9 \cdot 10^4$	0.99
	Flunitrazepam	1954	47	$18.5 \cdot 10^3$	$6.0 \cdot 10^3$	0.997
	Flurazepam	$64.7 \cdot 10^2$	$2.7 \cdot 10^2$	$5.6 \cdot 10^4$	$3.3 \cdot 10^4$	0.990
	Lorazepam	232	11	$3.6 \cdot 10^3$	$1.4 \cdot 10^3$	0.99
	Medazepam	$93.2 \cdot 10^2$	$3.9 \cdot 10^2$	$12.0 \cdot 10^4$	$5.0 \cdot 10^4$	0.99
	Midazolam	$156.6 \cdot 10^2$	$6.4 \cdot 10^2$	$22.0 \cdot 10^4$	$8.1 \cdot 10^4$	0.990
	Nitrazepam	1327	62	$17.1 \cdot 10^3$	$7.8 \cdot 10^3$	0.99
	Nor-Diazepam	1365	82	$1.0 \cdot 10^4$	$1.0 \cdot 10^4$	0.98
	Oxazepam	108.9	7	$26.6 \cdot 10^2$	$9.8 \cdot 10^2$	0.98
	Prazepam	$57.1 \cdot 10^2$	$3.1 \cdot 10^2$	$7.0 \cdot 10^4$	$3.9 \cdot 10^4$	0.98
	Temazepam	1347	58	$24.5 \cdot 10^3$	$7.3 \cdot 10^3$	0.99
	Tetrazepam	$48.8 \cdot 10^2$	$1.9 \cdot 10^2$	$9.2 \cdot 10^4$	$2.4 \cdot 10^4$	0.991
Opioids	Acetyl-Fentanyl	$130.4 \cdot 10^2$	$3.8 \cdot 10^2$	$22.9 \cdot 10^4$	$4.8 \cdot 10^4$	0.995

	Fentanyl	$129.5 \cdot 10^2$	$5.4 \cdot 10^2$	$19.4 \cdot 10^4$	$6.9 \cdot 10^4$	0.99
	Nor-Fentanyl	$89.9 \cdot 10^2$	$2.4 \cdot 10^2$	$17.5 \cdot 10^4$	$3.1 \cdot 10^4$	0.996
	Remifentanyl	$91.0 \cdot 10^2$	$2.6 \cdot 10^2$	$15.7 \cdot 10^4$	$3.3 \cdot 10^4$	0.995
	Tramadol	$194.9 \cdot 10^2$	$6.9 \cdot 10^2$	$27.6 \cdot 10^4$	$8.7 \cdot 10^4$	0.993
Piperazine derivates	Benzylpiperazine	$98.1 \cdot 10^2$	$4.9 \cdot 10^2$	$12.3 \cdot 10^4$	$6.2 \cdot 10^4$	0.99
	1-3-Trifluoromethylphenyl-Piperazine	$165.1 \cdot 10^2$	$2.9 \cdot 10^2$	$35.0 \cdot 10^4$	$3.7 \cdot 10^4$	0.998
	1[(4-chlorophenyl) phenyl methyl]piperazine	$71.0 \cdot 10^2$	$3.2 \cdot 10^2$	$10.3 \cdot 10^4$	$4.0 \cdot 10^4$	0.99
	o-Chlorophenyl-Piperazine	$107.9 \cdot 10^2$	$4.7 \cdot 10^2$	$20.9 \cdot 10^4$	$5.9 \cdot 10^4$	0.99
Others	Diphenhydramine	$167.3 \cdot 10^2$	$4.8 \cdot 10^2$	$35.0 \cdot 10^4$	$6.1 \cdot 10^4$	0.995
	Ephedrine	$128.4 \cdot 10^2$	$4.5 \cdot 10^2$	$24.5 \cdot 10^4$	$5.7 \cdot 10^4$	0.993
	Gabapentin	1448	60	$21.1 \cdot 10^3$	$7.6 \cdot 10^3$	0.99
	Memantine	$102.6 \cdot 10^2$	$5.2 \cdot 10^2$	$12.4 \cdot 10^4$	$6.6 \cdot 10^4$	0.98
	Pregabalin	489	21	$6.9 \cdot 10^3$	$2.6 \cdot 10^3$	0.99
	Venlafaxine	$179.1 \cdot 10^2$	$4.8 \cdot 10^2$	$26.1 \cdot 10^4$	$6.0 \cdot 10^4$	0.996

Table 6: Validation results - ILODs & ILOQs

Class	Analyte	ILOD (µg/L)	ILOQ (µg/L)
Cannabinoids	JWH-018	28	91
	JWH-073	26	87
	JWH-122	26	87
	JWH-210	27	90
	JWH-250	28	91
Cathinones	alpha-PVP	17	57
	Bupropion	13	44

	Mephedrone	21	69
	MePPP	17	56
	Nor-Ephedrine	16	52
Phenethylamines/ Arylcyclohexylamines/ Arylalkylamines	Atomoxetine	15	49
	Ketamine	14	45
	Nor-Ketamine	14	45
	2-Phenethylamine	21	68
	Amphetamine	7.2	24
	MDA	10	34
	MDEA	12	41
	MDMA	11	37
	Methamphetamine	15	50
Benzodiazepines	Alprazolam	23	76
	Bromazepam	14	47
	Clonazepam	18	59
	Diazepam	18	59
	Flunitrazepam	12	39
	Flurazepam	20	66
	Lorazepam	23	76
	Medazepam	21	68
	Midazolam	20	66
	Nitrazepam	23	74
	Nor-Diazepam	29	96
	Oxazepam	27	88
	Prazepam	27	88
	Temazepam	21	69
	Tetrazepam	19	61

Opioids	Acetyl-Fentanyl	14	47
	Fentanyl	20	67
	Nor-Fentanyl	13	43
	Remifentanyl	14	46
	Tramadol	17	57
Piperazine derivates	Benzylpiperazine	24	80
	1-3-Trifluoromethylphenyl-Piperazine	8.6	28
	1[(4-chlorophenyl) phenyl methyl]piperazine	22	71
	o-Chlorophenyl-Piperazine	21	70
Others	Diphenhydramine	14	46
	Ephedrine	17	56
	Gabapentin	20	66
	Memantine	25	81
	Pregabalin	21	68
	Venlafaxine	13	43

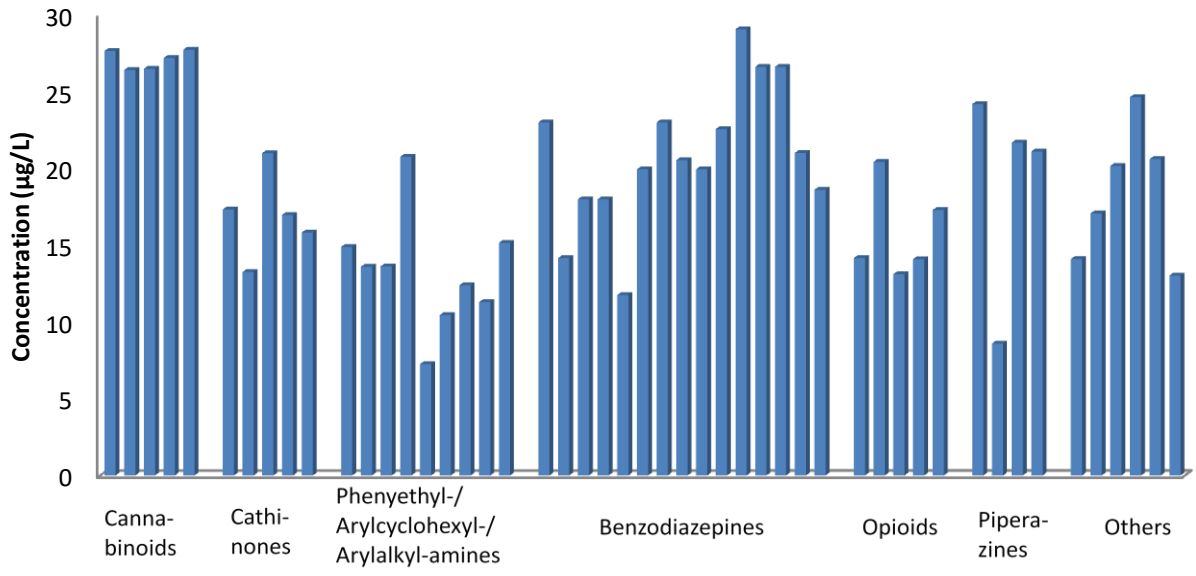

Figure 17: ILODs (µg/L) for representative NPS and illicit drugs of every class

Recovery experiments were performed at 500 ng/L. The majority of the analytes had satisfactory recoveries between 70-100%, as shown in **Figure 18**. Method repeatability in terms of %RSD in 4 spiked samples at 500 ng/L was below 25% for all analytes **(Figure 19)**. **Figure 20** presents the method limits of detection (MLODs), which were 250-750 ng/L for most analytes, except cannabinoids, which had worst sensitivity and their MLODs were 1.5-6.5 μg/L.

The results for recoveries, repeatability, matrix effects, MLODs and MLOQs are presented in total in **Table 7**.

Table 7: Validation results - Recoveries, repeatability, matrix effects, MLODs and MLOQs

Class	Analyte	%Recovery	%RSD (n=4)	%Matrix Effect	MLOD (μg/L)	MLOQ (μg/L)
Cannabinoids	JWH-018	97	24	-98	6.4	21
	JWH-073	84	9.6	-94	2.2	7.1
	JWH-122	99	19	-97	4.6	15
	JWH-210	104	10	-98	5.5	18
	JWH-250	101	5.6	-91	1.6	5.1
Cathinones	alpha-PVP	83	2.5	-83	0.50	1.6
	Bupropion	52	4.1	-85	0.46	1.5
	Mephedrone	26	19	-78	0.48	1.6
	MePPP	78	6.0	-84	0.53	1.8
	Nor-Ephedrine	82	7.2	-84	0.51	1.7
Phenethylamines/ Arylcyclohexylamines/ Arylalkylamines	Atomoxetine	106	3.3	-88	0.64	2.1
	Ketamine	94	8.2	-88	0.57	1.9
	Nor-Ketamine	56	21	-85	0.46	1.5
	2-Phenethylamine	57	13	238	0.031	0.10
	Amphetamine	80	5.8	-52	0.075	0.25

	MDA	70	8.8	-74	0.20	0.67
	MDEA	101	5.3	-89	0.56	1.9
	MDMA	93	6.5	-89	0.50	1.7
	Methamphetamine	100	4.9	-72	0.27	0.90
Benzodiazepines	Alprazolam	89	4.6	-83	0.66	2.2
	Bromazepam	106	17	-84	0.44	1.4
	Clonazepam	94	4.8	-81	0.47	1.6
	Diazepam	93	3.3	-81	0.46	1.5
	Flunitrazepam	92	5.6	-82	0.32	1.1
	Flurazepam	116	9.2	-90	0.98	3.2
	Lorazepam	80	21	-53	0.24	0.80
	Medazepam	91	3.3	-81	0.54	1.8
	Midazolam	90	6.9	-83	0.57	1.9
	Nitrazepam	94	2.1	-78	0.51	1.7
	Nor-Diazepam	81	7.4	-69	0.47	1.6
	Oxazepam	92	8.9	-63	0.36	1.2
	Prazepam	96	6.6	-75	0.54	1.8
	Temazepam	93	2.6	-63	0.28	0.93
	Tetrazepam	96	5.0	-81	0.50	1.6
Opioids	Acetyl-Fentanyl	105	8.5	-88	0.59	1.9
	Fentanyl	118	7.6	-89	0.90	3.0
	Nor-Fentanyl	87	10	-90	0.66	2.2
	Remifentanyl	80	4.5	-89	0.61	2.0
	Tramadol	84	8.3	-76	0.37	1.2
Piperazine derivates	Benzylpiperazine	89	5.2	-85	0.80	2.6
	1-3-Trifluoromethylphenyl-Piperazine	87	5.0	-91	0.49	1.6

	1[(4-chlorophenyl) phenyl methyl]piperazine	100	4.7	-92	1.3	4.3
	o-Chlorophenyl-Piperazine	88	6.0	-89	0.97	3.2
Others	Diphenhydramine	96	5.5	-87	0.56	1.8
	Ephedrine	75	4.7	-87	0.64	2.1
	Gabapentin	91	7.6	-77	0.44	1.4
	Memantine	99	4.4	-79	0.59	1.9
	Pregabalin	72	5.3	-51	0.21	0.70
	Venlafaxine	81	5.9	-86	0.45	1.5

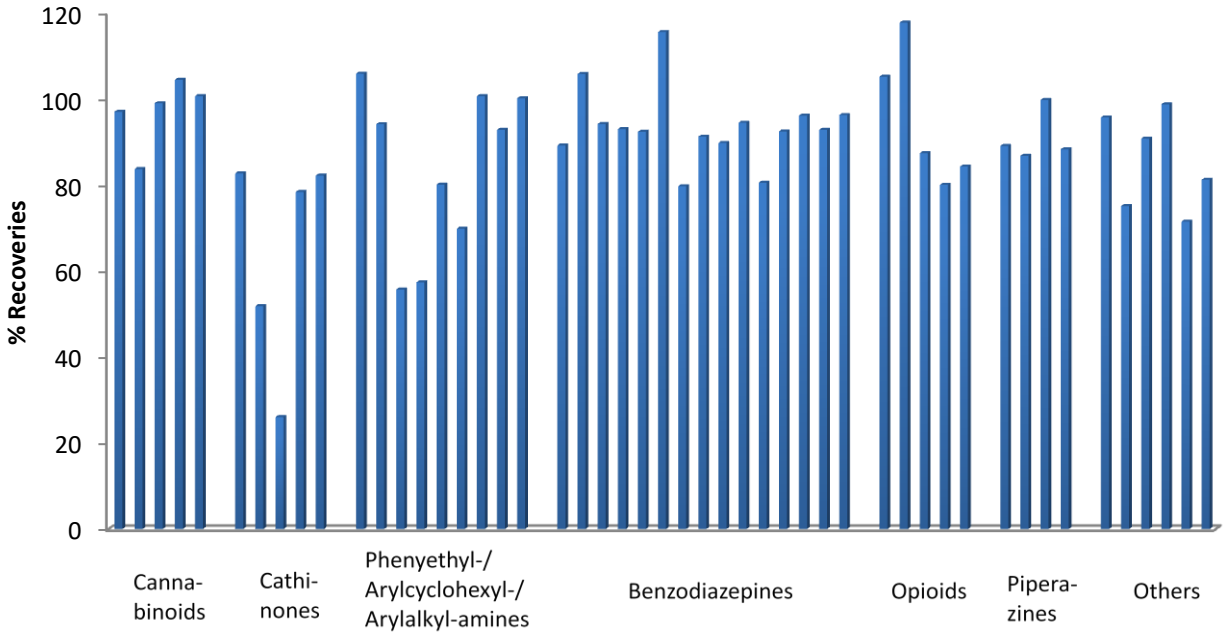

Figure 18: %Recoveries for representative NPS and illicit drugs of every class

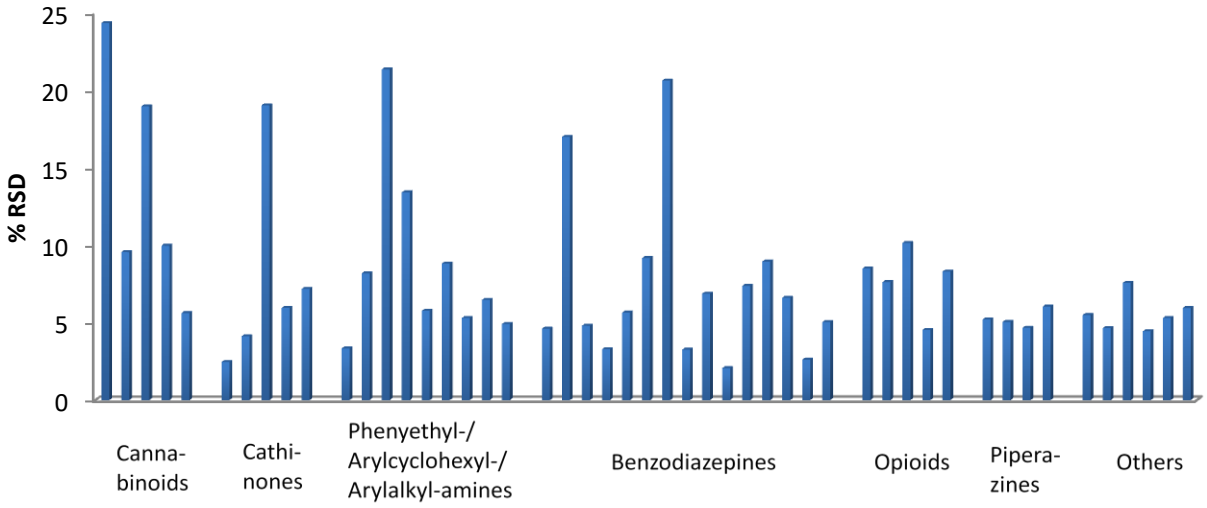

Figure 19: Repeatability (%RSD) for representative NPS and illicit drugs of every class

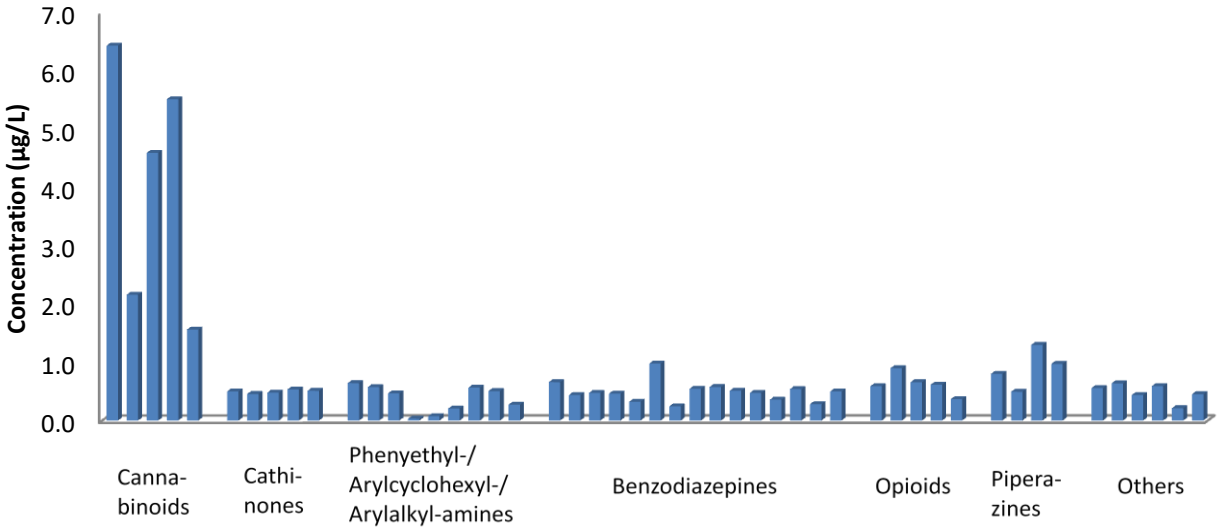

Figure 20: MLODs (µg/L) for representative NPS and illicit drugs of every class

5.2 Target screening results

5.2.1 Sampling of March 2015

8 influent wastewater (IWW) samples were collected consecutively from 04.03.2015 to 11.03.2015. 6 NPS were detected at least in five out of eight samples from target screening approach, as well as 9 pharmaceuticals that are related to NPS use.

The phenethylamines 2-Phenethylamine, PMA (para-methoxyamphetamine) and PMMA (para-Methoxy-N-methylamphetamine) were detected in all samples. 2-Phenethylamine and PMMA fulfilled all the screening parameters, mass accuracy < 2.5 mDa and < 5 ppm, retention time shift < 0.2 min, isotopic fitting < 200 mSigma, while PMA fulfilled the parameters of mass accuracy and retention time shift. PMA was confirmed by the presence of fragment ion 121, so it earned 4.5 IPs, and 2-Phenethylamine was confirmed by the presence of 3 fragment ions, so it earned more than 5 IPs. Two other phenethylamines, Methoxetamine and N-Ethyl-Amphetamine, were detected in 5 and 6 samples respectively and earned 2 IPs, as two screening parameters were achieved; mass accuracy and retention time shift. The same two screening parameters were also achieved by MDAI (5.6-methylenedioxy-2-aminoindane) of the class of aminoindanes, which was detected in 6 samples and earned 2 IPs.

O-Desmethyl-Tramadol, a metabolite of the opioid Tramadol, and Diphenhydramine, Gabapentin, GHB (Gamma-Hydroxybutyric acid), Memantine, Orphenadrine, Pregabalin, Quetiapine and Venlafaxine, which are pharmaceuticals that are also related to NPS use, were detected in all samples. Most of them fulfilled all the screening parameters and were confirmed by the presence of fragment ions, as their concentrations in wastewater are higher than those of NPS, so their precursor ions have higher intensities (signals) and more information regarding fragment ions can be retrieved.

Table 8: New Psychoactive Substances detected in raw wastewater of Athens on March 2015

New Psychoactive Substance	Class	Number of samples (out of 8 in total)	Identification Points
MDAI (5.6-methylenedioxy-2-aminoindane)	Aminoindanes	6	2
2-Phenethylamine	Phenethylamines	8	>5
Methoxetamine		5	2
N-Ethyl-Amphetamine		6	2
PMA (para-methoxyamphetamine)		8	4.5
PMMA (para-Methoxy-N-methylamphetamine)		8	2.5
O-Desmethyl-Tramadol	Opioids	8	5
Diphenhydramine	Others	8	4.5
Gabapentin		8	2.5
GHB (Gamma-Hydroxybutyric acid)		8	2.5
Memantine		8	5
Orphenadrine		8	>5
Pregabalin		8	>5
Quetiapine		8	2.5
Venlafaxine		8	>5

5.2.2 Sampling of March 2016

On March 2016 from 16.03 to 23.03, 8 consecutive IWW samples were collected and 8 NPS and 8 pharmaceuticals related to NPS use were detected.

The phenethylamines 2-Phenethylamine, PMA and PMMA were detected in all samples and fulfilled the screening parameters of mass accuracy < 2.5 mDa and < 5 ppm, retention time shift < 0.2 min, isotopic fitting < 200 mSigma. PMA earned 4.5 IPs due to the presence of 1 fragment ion and 2-Phenethylamine earned more than 5 IPs due the presence of 3 fragment ions. MePPP (4'-Methyl-α-pyrrolidinopropiophenone) and MBZP (Methylbenzylpiperazine) were detected in 1 and 7 samples respectively and earned 2.5 IPs, as they fulfilled all the screening parameters. MDAI and Methoxetamine were detected in 7 and 5 samples respectively and earned 2 IPs by fulfilling the criteria of mass accuracy and retention time shift.

All the pharmaceuticals detected in raw wastewater of 2015, were also detected in 2016, except GHB.

Table 9: New Psychoactive Substances detected in raw wastewater of Athens on March 2016

New Psychoactive Substance	Class	Number of samples (out of 8 in total)	Identification Points
MDAI (5.6-methylenedioxy-2-aminoindane)	Aminoindanes	7	2
2-Phenethylamine	Phenethylamines	8	>5
MePPP (4'-Methyl-α-pyrrolidinopropiophenone)		1	2.5
Methoxetamine		5	2
PMA (para-methoxyamphetamine)		8	4.5
PMMA (para-Methoxy-N-methylamphetamine)		8	2.5
MBZP (Methylbenzylpiperazine)	Piperazine derivatives	7	2.5
Ethylphenidate	Piperidines & pyrrolidines	5	2
O-Desmethyl-Tramadol	Opioids	8	2.5

Diphenhydramine	Others	8	4.5
Gabapentin		8	2.5
Memantine		8	5
Orphenadrine		8	>4.5
Pregabalin		8	5
Quetiapine		8	>5
Venlafaxine		8	>5

5.2.3 Sampling of March 2017

Target screening was successfully applied in 7 IWW samples that were collected every day from 08.03.2017 to 14.03.2017. 10 NPS were detected, each of them at least in one of seven samples, as well as the aforementioned 9 pharmaceuticals that are related to NPS use.

AB-CHMINACA, DMT (Dimethyltryptamine), MBZP (Methylbenzylpiperazine) and Methedrone were detected in one sample. These compounds earned 2 IPs, as two screening parameters were achieved; mass accuracy < 2.5 mDa and < 5 ppm and retention time shift < 0.2 min. Bufotenin (5-OH-DMT) was detected in 6 out of 7 samples, fulfilled the same two screening parameters, but also was confirmed by the presence of the fragment ion 132, so it earned 4.5 IPs.

DMAA (Methylhexanamine) was detected in five samples, while Methoxyphenamine, PMA (para-methoxyamphetamine) and PMMA (para-Methoxy-N-methylamphetamine) were detected in all samples. All these phenethylamines earned 2.5 IPs, as they fulfilled all the screening parameters; mass accuracy < 2.5 mDa and < 5 ppm, retention time shift < 0.2 min, isotopic fitting < 200 mSigma. Another compound in this group, 2-Phenethylamine, was detected in all samples, fulfilled all the screening criteria, and also confirmed by the presence of 2 fragment ions, so it earned more than 5 IPs.

Table 10: New Psychoactive Substances detected in raw wastewater of Athens on March 2017

New Psychoactive Substance	Class	Number of samples (out of 7 in total)	Identification Points
Bufotenin (5-OH-DMT)	Indolalkylamines	6	4.5
DMT (Dimethyltryptamine)		1	2
MBZP (Methylbenzylpiperazine)	Piperazine derivatives	1	2
AB-CHMINACA	Synthetic cannabinoids	1	2
Methedrone	Synthetic cathinones	1	2
2-Phenethylamine	Phenethylamines	7	>5
DMAA (Methylhexanamine)		5	2.5
Methoxyphenamine		7	2.5
PMA (para-methoxyamphetamine)		7	2.5
PMMA (para-Methoxy-N-methylamphetamine)		7	2.5
O-Desmethyl-Tramadol	Opioids	7	2.5
Diphenhydramine	Others	5	4.5
GHB (Gamma-Hydroxybutyric acid)		2	2
Gabapentin		7	5
Memantine		7	2.5
Orphenadrine		7	>4.5
Pregabalin		7	2.5
Quetiapine		7	>5
Venlafaxine		7	5

5.2.4 Discussion

15 New Psychoactive Substances were detected in raw wastewater samples from Athens during 2015-2017. From them, 2-Phenethylamine, DMAA, MePPP, N-Ethyl-Amphetamine, PMMA, as well as the pharmaceuticals Diphenhydramine, Gabapentin, Memantine, O-Desmethyl-Tramadol, Orphenadrine, Pregabalin, Quetiapine and Venlafaxine, have been reported in raw wastewater from Athens in 2014 in a previous study by Bletsou et al. [53]. Bufotenin, DMAA, MePPP, Methedrone, Methoxetamine, N-Ethyl-Amphetamine, MBZP, PMMA have been reported in IWW, EWW or river water in European countries, such as Belgium, Croatia, Norway, Spain, Switzerland, UK and the Netherlands, during 2012-2015 **(Table 2)**. To the author's knowledge, AB-CHMINACA, DMT, Ethylphenidate, MDAI, Methoxyphenamine and PMA are reported for the first time in raw wastewater. From the total of the detected NPS, 2-Phenethylamine, AB-CHMINACA, Ethylphenidate and Methoxetamine are in agreement with the NPS that have been reported by the Greek Documentation and Monitoring Centre for Drugs **(Table 1)**.

5.3 Suspect screening results

Suspect screening was successfully applied retrospectively in the influent wastewater (IWW) samples that were collected at Saturdays of 07.03.2015, 19.03.2016, 11.03.2017. This day was preferred, as it is considered that recreational drugs use is increased during weekends [30]. All the tentative candidates of NPS that fulfilled the screening parameters and were not excluded as outliers by the QSRR prediction model are presented and evaluated in the following subchapters. After their tentative identification, they were screened in all samples that were collected from 2015 to 2017 (8 IWW from 2015, 8 IWW from 2016, 7 IWW from 2017) in order to check their presence all over the week.

5.3.1 Candidates of level 2a

Six NPS were tentatively identified at level 2a by matching their bbCID MS/MS fragments with library MS/MS data. The isotopic fitting and the presence of characteristic adducts in full-scan mode strongly confirmed some of the candidates. The chemical structures of all six candidates were within the applicability domain of the QSRR prediction model and according to the prediction, the differences between the measured and the predicted retention time were less than 2 min, which was acceptable for the confirmation of the structure.

The precursor ion of N-methyl-2-AI fulfilled the screening parameters of mass accuracy < 2.5 mDa and < 5 ppm and isotopic fitting < 200 mSigma. The adduct ion with NH_4 was also detected. According to the QSRR prediction model, the measured RT was accepted with an error less than 1 min. The bbCID MS/MS fragments were compared the MS/MS spectrum of mzCloud library and 2 fragments matched **(Figure 21a, b)**. The EICs of bbCID MS/MS fragments had the same chromatographic profile to the EICs of the precursor ions in full-scan MS **(Figure 21c)**. Thus, N-methyl-2-AI reached level 2a. This compound is the N-methylated derivative of 2-Aminoindane and is analogous to amphetamine. It has stimulant properties and is easily accessible through webshops. It has been reported in blood samples and hair samples from autopsies or other forensic cases in EU [70, 71], but it is the first time reported in wastewater.

The values for mass accuracy and isotopic fitting of $[M+H]^+$ of DL-4662 were below the screening criteria and the measured RT was accepted with an error less than 2 min, according to the QSRR prediction model. The bbCID MS/MS fragment 107 matched with the MS/MS spectrum of mzCloud library. DL-4662 is one of the latest synthetic cathinone derivatives and is the first time that is reported to be present in wastewater.

The mass accuracy of the precursor ion of N-hydroxy MDA was lower than 2.5 mDa and 5 ppm, and the adduct ions with Na and K were detected. The measured RT was accepted with an error less than 2 min, according to the QSRR prediction model. The bbCID MS/MS fragments were compared with

the MS/MS spectrum of mzCloud library and 2 fragments matched. N-hydroxy MDA, which is reported in wastewater for the first time, is the N-hydroxy homologue of MDA and is popular as drug of abuse that causes empathy and euphoria at low doses and agitation, delirium and hallucination at high doses [72].

Mass accuracy for PMEA (para-Methoxy-N-ethylamphetamine) was below the screening limits, measured RT was accepted according to the QSRR prediction and 3 bbCID MS/MS fragments matched the mzCloud spectrum data **(Figure 22)**. PMEA is the N-ethylated analogue of PMA and is sold as a designer drug that has stimulant properties. It is the first time that is reported in wastewater.

The precursor ions for Benzydamine and NMP (N-Methylpyrrolidone) fulfilled the screening parameters of mass accuracy and isotopic fitting. For NMP, the adduct ions with Na and K were detected. According to the QSRR prediction model, the measured RT for both compounds was accepted with an error less than 1 min, and also, their bbCID MS/MS fragments matched the MS/MS spectra of MassBank and mzCloud libraries. Benzydamine, available as the hydrochloride salt, is an indolic, nonsteroidal anti-inflamatory drug (NSAID). NMP is used as a cosmetic ingredient and as an intermediate in the pharmaceutical industry. Both are mentioned in EWS lists of NPS, as they have been reported in intoxication cases and have been considered as potentially new substances of abuse.

Phenibut, which is sold on the Internet without a prescription as a supplement and is used as a nootropic and recreational drug, was initially considered as candidate of level 2a, as the $[M+H]^+$ fulfilled the screening parameters of mass accuracy < 2.5 mDa and < 5 ppm and isotopic fitting < 200 mSigma, the measured RT was accepted with an error less than 1 min and 2 bbCID MS/MS fragments matched the MS/MS library spectrum data. However, the illicit drug MDA (3.4-Methylenedioxyamphetamine) has the same molecular formula and elutes in the same RT. bbCID MS/MS fragments of MDA with the method applied are known, but none of them was detected. Although MDA was spiked in the IWW samples and indeed it existed in them, only the

purchase of the standard solution of Phenibut can inform if the two compounds co-elute or the peak that was detected only corresponds to MDA.

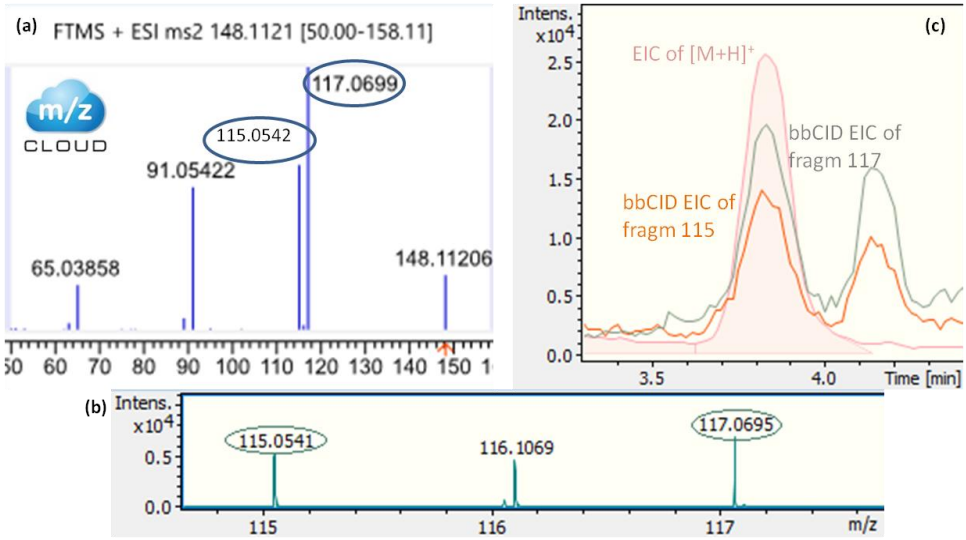

Figure 21: (a) mzCloud MS/MS data of N-methyl-2AI acquired with ESI-LTQ-Orbitrap-MS, (b) bbCID MS fragments of N-methyl-2AI that matched mzCloud MS/MS data, (c) EIC of the precursor ion of N-methyl-2AI in full-scan MS and EICs of bbCID MS fragments

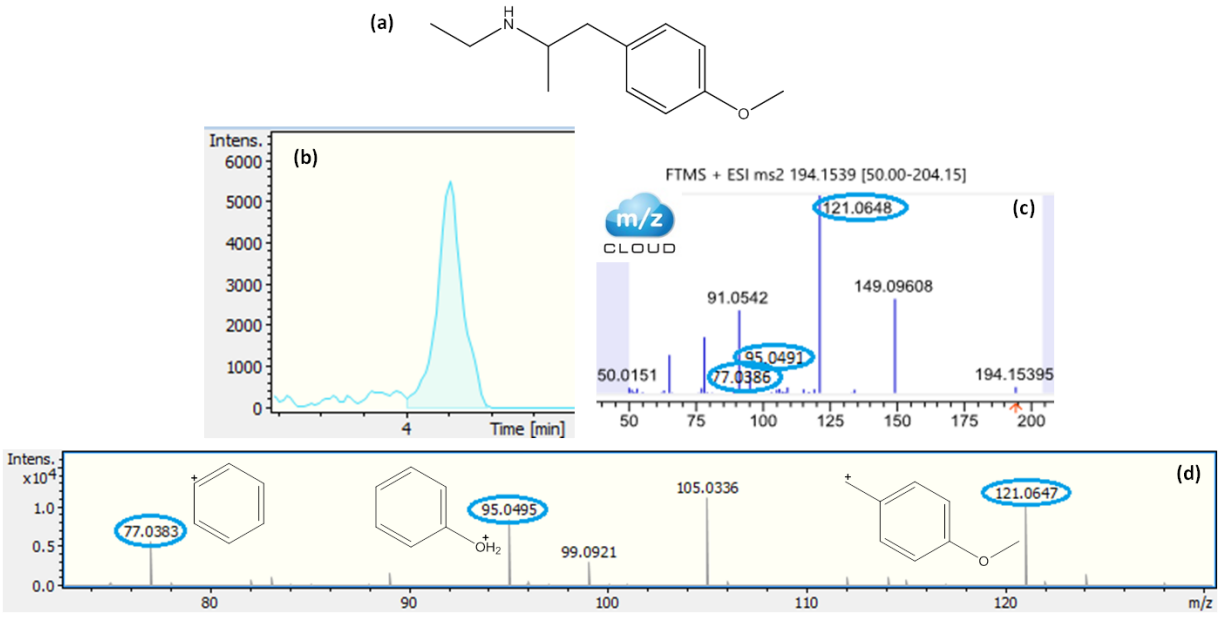

Figure 22: (a) Chemical structure of PMEA, (b) EIC of $[M+H]^+$ ion of PMEA (mass accuracy < 2.5 mDa and < 5 ppm), $t_{R\,(exp)}$=4.2 min, (c) mzCloud MS/MS data of PMEA acquired with ESI-LTQ-Orbitrap-MS, (d) bbCID MS fragments of PMEA that matched mzCloud MS/MS data

Table 11 summarizes the suspect screening results at level 2a with information of the identification parameters. All the tentative candidates at level 2a were detected in all samples collected during 2015-2017 and are reported for the first time in raw wastewater.

5.3.2 Candidates of level 2b

For the NPS candidates that are presented in **Table 12**, no literature or library spectrum data were available for confirmation of the structure, according to the author's knowledge. Thus, diagnostic bbCID MS/MS fragments were used in order to explain the possible structure. These fragments were carefully checked to have the same chromatographic profile with the parent compound, because bbCID MS/MS fragments may correspond to other compounds that elute in close retention times.

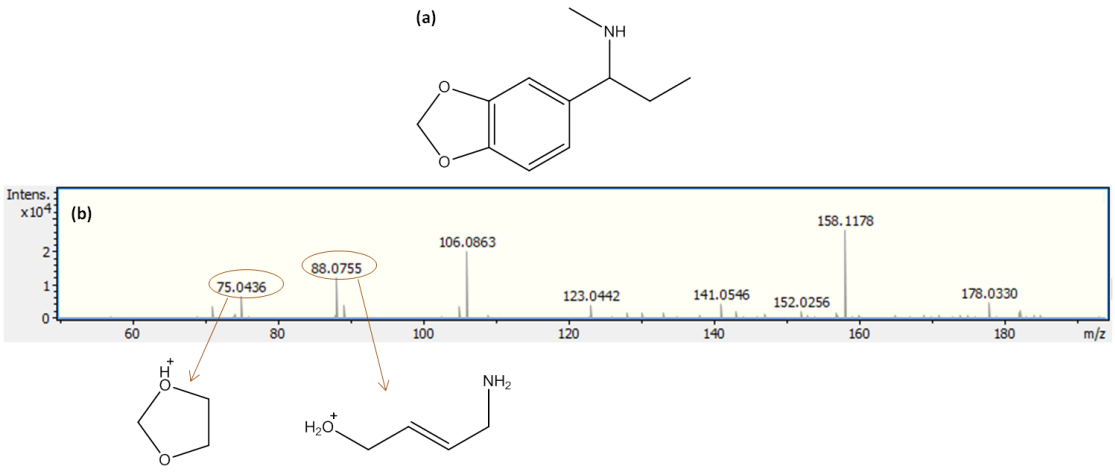

Figure 23: (a) Chemical structure of M-ALPHA, (b) bbCID MS and possible fragment structures that could explain the structure of M-ALPHA

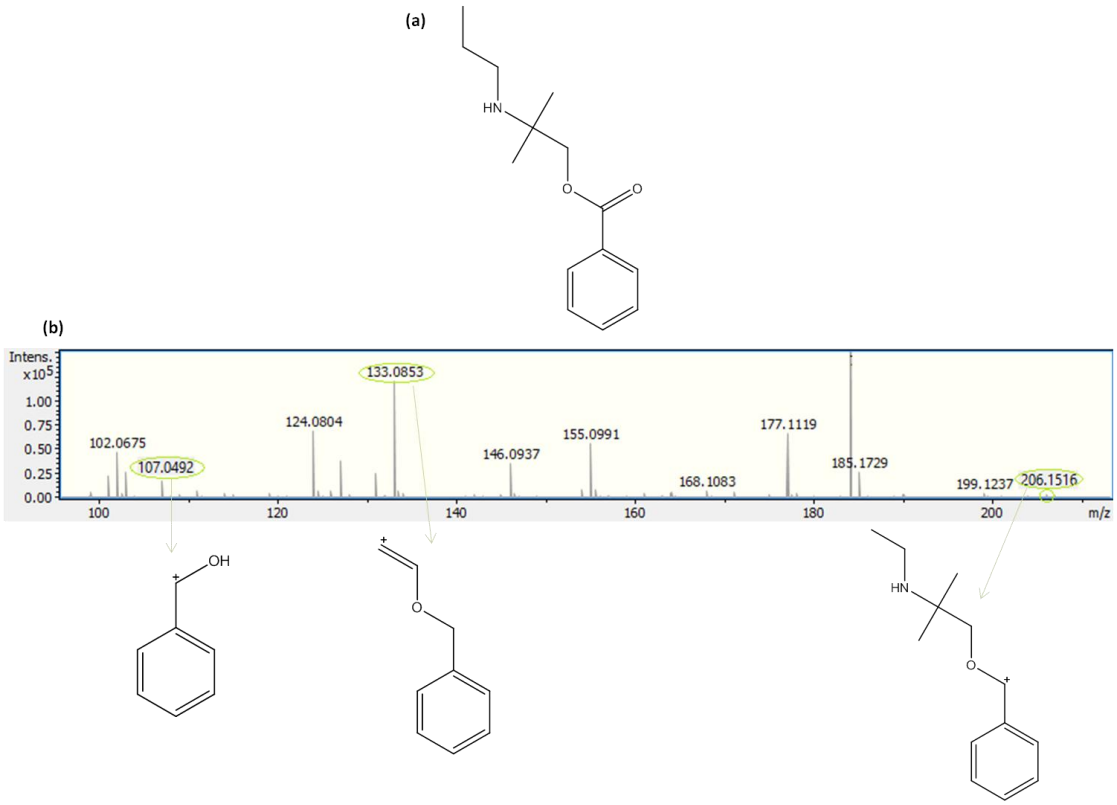

Figure 24: (a) Chemical structure of Epirocaine, (b) bbCID MS and possible fragment structures that could explain the structure of Epirocaine

Table 11: NPS candidates in level 2a

Class	NPS candidate	Molecular formula	Calculated m/z of $[M+H]^+$	Measured RT (min)	Predicted RT (min) by SVM	Isotopic fitting < 200 mSigma	Adducts	bbCID MS/MS fragments matched with MS/MS library data	Year of detection	Number of samples
Aminoindanes	N-methyl-2AI	C10H13N1	148.1121	3.81	3.49	✓	$[M+NH_4]^+$	115.0542, 117.0699	2015, 2016, 2017	23
Synthetic Cathinones	DL-4662	C15H23N1O3	266.1751	3.48	5.47	✓	-	107.0491	2015, 2016, 2017	23
Phenethylamines	N-hydroxy MDA	C10H13N1O3	196.0968	5.68	4.49	×	$[M+Na]^+$, $[M+K]^+$	77.0386, 103.0542	2015, 2016, 2017	23
	PMEA	C12H19N1O1	194.1539	4.21	4.58	×	-	77.0386, 95.0491, 121.0648	2015, 2016, 2017	23
Others	Benzydamine	C19H23N3O1	310.1914	7.76	7.94	✓	-	86.0964	2015, 2016, 2017	23
	NMP	C5H9N1O1	100.0757	3.11	3.02	✓	$[M+Na]^+$, $[M+K]^+$	58.0287, 82.0651	2015, 2016, 2017	23

Table 12: NPS candidates in level 2b

NPS candidate	Molecular formula	Calculated m/z of $[M+H]^+$	Measured RT (min)	Predicted RT (min) by SVM	Isotopic fitting < 200 mSigma	Adducts*	Year of detection*
N-methyl aminorex derivative	C10H12N2O1	177.1022	3.68	4.31	✓	$[M+Na]^+$	2015, 2016, 2017
M-ALPHA	C11H15N1O2	194.1176	5.38	4.17	✓	-	2015, 2016, 2017
Methallylescaline	C14H21N1O3	252.1594	6.18	5.56	×	-	2015, 2016, 2017
LTI-701	C20H21F1N2O1	325.1711	6.78	-**	✓	-	2015, 2016, 2017
Epirocaine	C14H21N1O2	236.1645	7.18	5.83	×	-	2015, 2016, 2017

**at least in one sample of each year*

***the retention time prediction results are questionable and other methods of identification, such as MS/MS fragments, should be applied*

5.3.3 Structural isomers in NPS suspect list

The suspect list that was built included a lot of structural isomers. As mentioned in chapter 1, NPS are produced by slightly modifying the structures of established illicit drugs, so it is expected that isomeric compounds will also be produced. These isomers often produce common fragment ions and it is difficult to identify them properly using MS/MS data. In the following paragraphs, such cases that emerged during suspect screening of NPS are mentioned and evaluated.

5-Methoxy-Methylone and N-Me-bk-MMDA-2 are positional structural isomers and produce common fragment ions. The peak for m/z = 238.1074, which refers to the precursor ion of these isomers, had mass accuracy lower than 2.5 mDa and 5 ppm, and also, adduct ions with Na and K were detected. bbCID MS/MS fragment 105 matched the MS/MS data from mzCloud for 5-Methoxy-Methylone. No MS/MS data were available for N-Me-bk-MMDA-2, but this fragment could explain its structure, too. The predicted RTs for both compounds were almost the same, according to the QSRR model. So, MS/MS information and RT prediction could not distinguish and identify these isomers. The same happened with 3.4-DMAR and 4.4'-DMAR.

For m/z = 150.1277, which refers to the precursor ion of the structural isomers 3-amino-1-phenyl-butane, 4-methylamphetamine and N.N-dimethylphenethylamine, two peaks at 3.69 min and 6.53 min were detected with mass accuracy lower than 2.5 mDa and 5 ppm. According to the QSRR prediction model, the predicted retention times were 4.40 min for 3-amino-1-phenyl-butane, 4.69 min for 4-methylamphetamine and 4.03 min for N.N-dimethylphenethylamine. MS/MS fragments 105.0699, 133.1012, 103.0542 were reported in HighResNPS database for 4-methylamphetamine. The fragment 105 was present in the bbCID mass spectrum that corresponded to the chromatographic peak at 3.69 min, but this fragment could also explain the structures of the other two isomers. For the bbCID mass spectrum that corresponded to the chromatographic peak at 6.53 min, there was no match with the aforementioned MS/MS fragments. However, the diagnostic bbCID fragment 117 could explain the structure for more than one of these isomers.

Consequently, common fragmentation of these isomers makes their identification difficult. Similarly, two peaks were detected for m/z = 222.1489, which refers to the isomers 3.4-MDPA and N-ethylphenmetrazol, with mass accuracy < 2.5 mDa and < 5 ppm and isotopic fitting < 200 mSigma. Predicted RTs were accepted for both isomers for both peaks. bbCID MS/MS fragments 91 and 103 that were detected for both peaks matched the MS/MS library data from mzCloud for 3.4-MDPA, while no library data were available for N-ethylphenmetrazol. However, bbCID fragments 91 and 103 could explain the structure of N-ethylphenmetrazol, too.

For m/z = 240.1594, which refers to the precursor ion of the structural isomers 3C-E and Proscaline, a peak at 7.28 min was detected in all years. This peak fulfilled the screening parameters of mass accuracy and isotopic fitting. According to the QSRR prediction model, the predicted retention times for these compounds were very close and the measured RT was accepted for both compounds with an error less than 2 min. No library or literature MS/MS data were available, so the diagnostic bbCID fragments were examined. The EICs of the bbCID fragments 58 and 163 had the same chromatographic profile to the EICs of the precursor ion in full-scan MS, but they could explain the structure for both compounds **(Figure 25)**.

Consequently, isomeric compounds such as those described above cannot be identified by MS/MS information and remain at level 3.

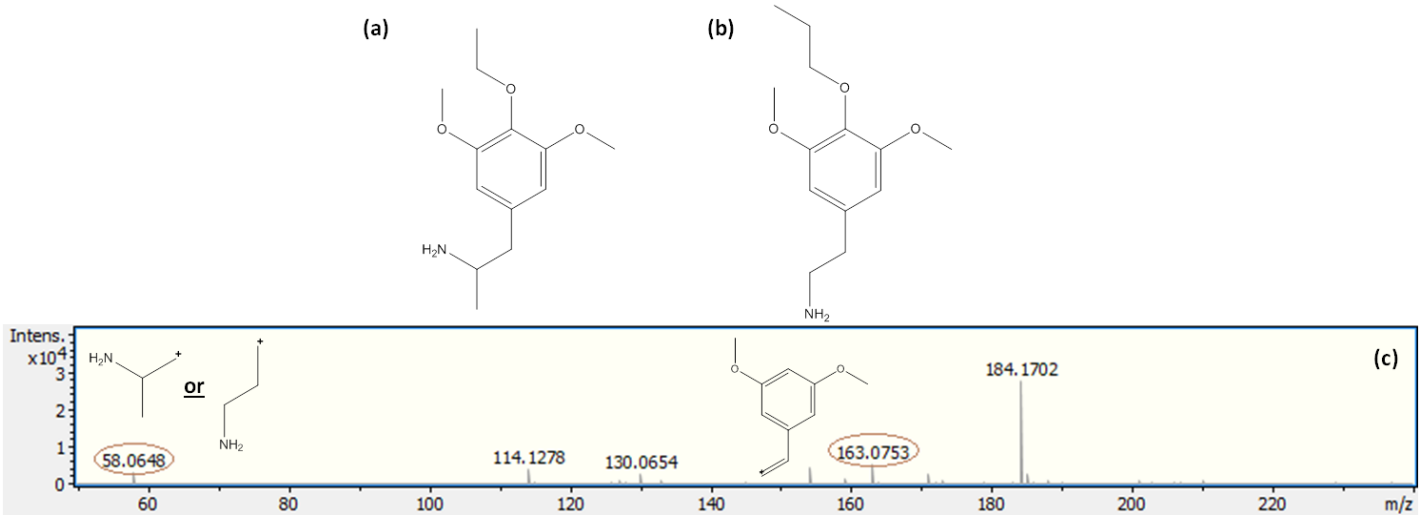

Figure 25: (a) Chemical structure of 3C-E, (b) Chemical structure of Proscaline, (c) bbCID MS and predicted fragments for 3C-E and Proscaline

On the other hand, the two peaks at 3.14 min and 6.78 min that were detected with mass accuracy below 2.5 mDa and 5 ppm for m/z = 224.1281 were matched by the QSRR prediction model with MDHOET and EFLEA respectively, which are two isomeric phenethylamines from the suspect list. No library MS/MS data were available, so diagnostic fragments were used in order to explain the possible structures. The structure of MDHOET was tentatively identified by experimental evidence **(Figure 26)**, so it reached level 2b. The peak at 6.78 min had low intensity and the few bbCID MS/MS fragments that were produced could not explain the structure of EFLEA, so it remained at level 3.

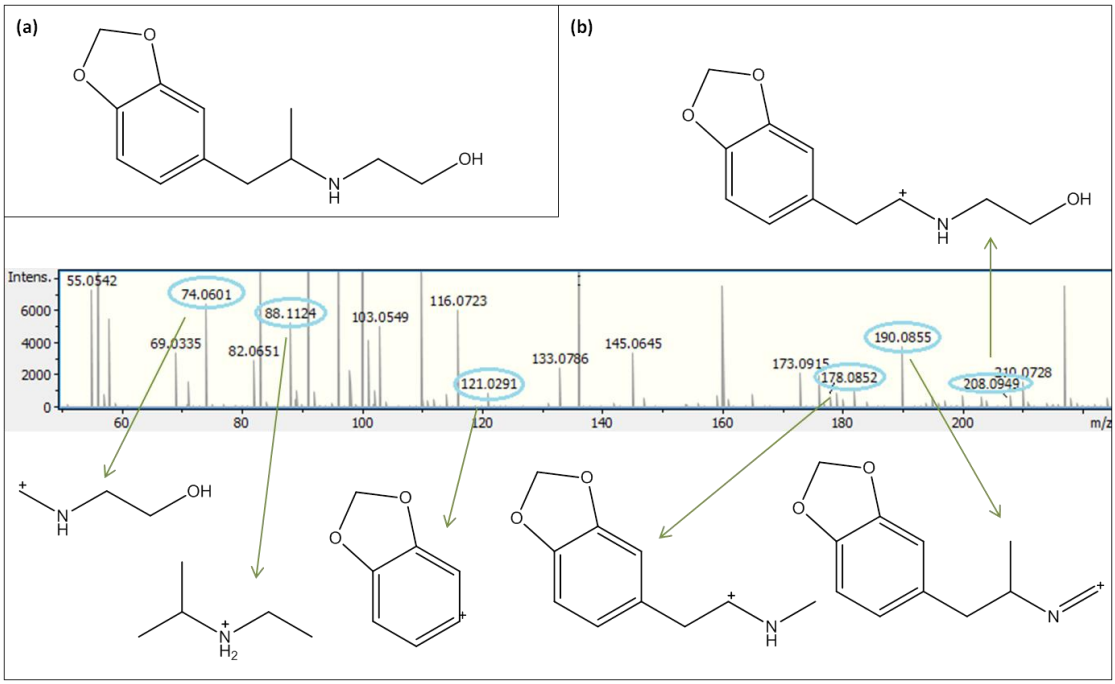

Figure 26: (a) Chemical structure of MDHOET, (b) bbCID MS and possible fragment structures that could explain the structure of MDHOET

5.3.4 Candidates of level 3 and false positive results

There were some precursor ions of specific NPS from the suspect list that, although they fulfilled the screening parameters and some other confirmatory criteria (for example retention time prediction, isotopic fitting, presence of adducts), their bbCID MS/MS fragments did not match with library spectrum data.

As mentioned before, mass spectra acquired from a data independent acquisition (DIA) are not ‘clean’ spectra, because all ions are fragmented, so the bbCID spectrum in a very small time interval and after background subtraction still includes fragments from both the suspect precursor ion and all the co-eluting ions. For NPS that occur in very low concentrations in wastewater and the intensities of their $[M+H]^+$ ions are very low, even after the pre-concentration of samples, there is a possibility of no fragmentation. Therefore, the fragments of bbCID mass spectrum may not correspond to the suspect compound and thus, they do not match the MS/MS spectrum of libraries or literature.

Another aspect is that MS/MS data from spectral libraries and literature may be obtained with different instrumental configurations (QTOF, LTQ-Orbitrap) and different fragmentation modes, which may result to different fragment ions and mainly different abundance of them. This is another reason that should be considered when there is no match in fragment ions.

In **Table 13**, all NPS candidates that started from level 3, but their bbCID MS/MS fragments did not match with library MS/MS data, are presented. Further investigation is needed in order to explain if they are NPS residues or other isobaric compounds that occur in wastewater, which means that these masses are false positive results and should ‘downgrade’ to level 5. If wastewater extracts are still available and are stored in suitable conditions in order to avoid degradation of analytes, further analysis could be performed. Data dependent acquisition (DDA) with inclusion lists of these candidates and more than one injection could be applied.

Table 14 presents some NPS candidates with mass accuracy lower than 2.5 mDa and 5 ppm, for which MS/MS library data were not available, but also their fragmentation was very low and few bbCID MS/MS fragments that were produced could not explain the possible structure. These are NPS candidates of level 3 that should be also carefully further investigated, whether they are false positive results and should ‘downgrade’ to level 5 or not.

Table 13: NPS candidates in level 3 that their bbCID MS/MS fragments did not match with library MS/MS data

NPS candidate	Molecular formula	Calculated m/z of $[M+H]^+$	Measured RT (min)	Predicted RT (min) by SVM	Isotopic fitting < 200 mSigma	Adducts*	Year of detection*
1-Aminoindan	C9H11N1	134.0964	3.18	2.66	×	-	2016
2-Aminoindan				3.13			
Nor-mephedrone	C10H13N1O1	164.107	5.86	3.88	✓	-	2015, 2016, 2017
Mexedrone	C12H17N1O2	208.1332	2.84	3.91	✓	-	2015
2-MeO-Ketamine	C14H19N1O2	234.1489	6.71	4.66	×	-	2015, 2016, 2017
4'-Methoxy-alpha-PPP (MOPPP)				-**			
Allylescaline (peak 1)	C13H19N1O3	238.1438	2.66	4.87	×	-	2015, 2017
Viloxazine (peak 1)				4.78			
Allylescaline (peak 2)			3.14	4.87	×	-	2015
Viloxazine (peak 2)				4.78			
Allylescaline (peak 3)			3.43	4.87	×	$[M+K]^+$	2016
Viloxazine (peak 3)				4.78			
4-AcO-DMT	C14H18N2O2	247.1441	3.74	3.92	✓	$[M+Na]^+$	2015, 2016, 2017
5-MeO-DPT	C17H26N2O1	275.2118	5.54	5.98	✓	-	2017

3-MeO-PCMo	C17H25N1O2	276.1958	7.86	5.89	✓	$[M+Na]^+$	2017
5-MeO-DiBF				-**			
4F-alpha-PEP / 4F-PV8	C17H24F1N1O1	278.1915	8.61	7.05	×	-	2015, 2016, 2017
HDMP-28 (methylnaphthidate)	C18H21N1O2	284.1645	8.98	-**	✓	$[M+NH_4]^+$	2015, 2016
25H-NBOH (peak 1)	C17H21N1O3	288.1594	8.38	-**	✓	-	2015, 2016, 2017
25H-NBOH (peak 2)			8.74		✓	$[M+Na]^+$, $[M+K]^+$	2015, 2016, 2017
alpha-pyrrolidinononaphenone (alpha-PNP)	C19H29N1O1	288.2322	8.26	8.31	✓	-	2015, 2016, 2017
3.4-DMeO-alpha-PVP	C17H25N1O3	292.1907	8.26	6.09	✓	-	2015, 2016, 2017
4-MeO-alpha-PV9	C19H29N1O2	304.2271	9.99	7.73	×	-	2017
Nitracaine	C16H24N2O4	309.1809	5.94	-**	×	-	2015, 2016, 2017
5-fluoropentyl-3-pyridinoylindole	C19H19F1N2O1	311.1554	6.81	-**	✓	-	2015, 2016, 2017
Noopept (peak 1)	C17H22N2O4	319.1652	4.51	6.02	×	-	2015, 2016, 2017
Noopept (peak 2)			6.41		✓	$[M+Na]^+$	2015, 2016, 2017
Noopept (peak 3)			6.69		✓	$[M+Na]^+$, $[M+K]^+$	2015, 2016, 2017

*at least in one sample of each year

**the retention time prediction results are questionable and other methods of identification, such as MS/MS fragments, should be applied

Table 14: NPS candidates in level 3 with few bbCID MS fragments that could not explain the possible structures

NPS candidate	Molecular formula	Calculated m/z of $[M+H]^+$	Measured RT (min)	Predicted RT (min) by SVM	Isotopic fitting < 200 mSigma	Adducts	Year of detection*
1-Ethynyl-cyclohexanol (ECX)	C8H12O1	125.0961	5.54	6.31	×	-	2015, 2016
LY2183240	C17H17N5O1	308.1506	10.78	-**	✓	-	2017
PRE-084	C19H27N1O3	318.2064	6.78	7.55	×	-	2016, 2017

*at least in one sample of each year

**the retention time prediction results are questionable and other methods of identification, such as MS/MS fragments, should be applied

CHAPTER 6

Conclusions

The analysis of raw wastewater for the detection of the target compounds is the first step in WBE and was applied in our study in order to detect NPS in wastewater of Athens. The application of a generic sample treatment using SPE with four different extraction sorbents and a data independent acquisition mode by LC-HRMS allowed the retrospective analysis of wastewater samples collected the last few years and gave the opportunity to estimate if NPS are consumed occasionally or more frequently during the week and over the years.

Target screening was applied based on some performance criteria, such as mass accuracy, retention time, isotopic pattern, MS/MS information, and IPs were attributed in order to facilitate confidence. 49 NPS and illicit drugs with similar structures with NPS were used as a representative model to validate the wide-scope screening with a database of approximately 200 NPS. The target screening results indicated the occasional detection of 15 NPS during 2015-2017, alongside with pharmaceuticals associated to the potential of abuse, but that should not be directly linked to NPS usage. Up to our knowledge, AB-CHMINACA, DMT, Ethylphenidate, MDAI, Methoxyphenamine and PMA were detected for the first time in the aqueous environment.

The application of suspect screening with a list of approximately 500 NPS, using only exact mass as prior information, tentatively identified 6 NPS by matching their bbCID MS/MS fragments with library MS/MS data and by the use of advanced chemometrics in order to check the plausibility of the retention time. So, Benzydamine, DL-4662, N-hydroxy MDA, N-methyl-2-AI, NMP and PMEA were tentatively reported for the first time in wastewater and were present all days of the week of every year. For some other NPS that library MS/MS data were not available for comparison, diagnostic bbCID MS/MS fragments were used for the explanation of the NPS candidates. Moreover, the challenges on the identification of NPS due to their low signals and consequently their low fragmentation were discussed and evaluated, as

well as the difficulties of tentatively identifying NPS that are structural isomers and produce common fragment ions.

The results of our research assured that there is NPS consumption in the population of Greece, but in lower frequency and in lower concentrations compared with established illicit drugs, such as reports from the Greek Documentation and Monitoring Centre for Drugs have mentioned.

To conclude, such a study can indicate the presence of NPS in the wastewater matrix. However, back-calculation of NPS use is subject to many limitations and yet, it cannot be achieved in the same level as established illicit drugs. The results are mainly qualitative and give information about the frequency of NPS detection in different places and over time.

ABBREVIATIONS – ACRONYMS

2C-B	2,5-dimethoxy-4-bromophenethylamine
2-MMC	2-methylmethcathinone
2-MXP	2-methoxydiphenidine
4-FA	4-fluoroamphetamine
4-FPP	4-fluorophenylpiperazine
4-MEC	4-methylethcathinone
4-MMC	4-methylmethcathinone
5-IAI	5-iodo-2-aminoindane
5-MAPB	1-(Benzofuran-5-yl)-*N*-methylpropan-2-amine
5-MeO-DIPT	5-methoxy-N,N-diisopropyltryptamine
5-MeO-DMT	5-methoxy-N,N-dimethyltryptamine
AMT	alpha-methyltryptamine
APB	Aminopropylbenzofurans
APCI	Atmospheric pressure chemical ionization
APPI	Atmospheric pressure photoionization
bbCID	broad-band Collision Induced Dissociation
BPZ	Benzylpiperazine
CNS	Central Nervous System
DDA	Data Dependent Acquisition
desoxy-D2PM	2-(diphenylmethyl)pyrrolidine
DIA	Data Independent Acquisition
DMAA	Methylhexanamine
DMT	Dimethyltryptamine
DOB	Dimethoxybromoamphetamine
EIC	Extracted Ion Chromatogram
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
ESI	Electrospray Ionization
EU	European Union
EWS	Early Warning System
FWHM	Full Width at Half Maximum
GC-MS	Gas Chromatography – Mass Spectrometry
GFF	Glass fiber filters

GHB	Gamma-Hydroxybutyric acid
HDPE	High-density polyethylene
HE	High energy
HRMS	High-resolution mass spectrometry
ILOD	Instrumental limit of detection
ILOQ	Instrumental limit of quantification
IP	Identification point
IS	Internal standard
IWW	Influent wastewater
LC-HRMS	Liquid chromatography – High-resolution mass spectrometry
LC-LRMS	Liquid chromatography – Low-resolution mass spectrometry
LC-MS	Liquid Chromatography – Mass Spectrometry
LE	Low energy
LLE	Liquid-liquid extraction
LOD	Limit of detection
LSD	Lysergic acid diethylamide
LTQ-Orbitrap	Linear ion trap-Orbitrap
MBPZ	Methylbenzylpiperazine
mCPP	meta-Chlorophenylpiperazine
MDA	3,4-methylenedioxyamphetamine
MDAI	5,6-methylenedioxy-2-aminoindane
MDMA	3,4-methylenedioxymethamphetamine
MDMAI	5,6-methylenedioxy-N-methyl-2-aminoindane
MDPV	3,4-methylenedioxypyrovalerone
MeOPP	1-(4-methoxyphenyl) piperazine
MePPP	4’-Methyl-α-pyrrolidinopropiophenone
MLOD	Method limit of detection
MLOQ	Method limit of quantification
MMA	3-methoxy-4-methylamphetamine
MMAI	5-methoxy-6-methyl-2-aminoindane
MPA	Methylthienylpropamine
MPHP	4'-methyl-alpha-pyrrolidinohexanophenone
MS/MS	Tandem mass spectrometry
MXE	Methoxetamine

NMP	N-Methylpyrrolidone
NPS	New Psychoactive Substance
oMeOPP	1-(2-methoxyphenyl)piperazine
PCP	Phencyclidine
PMA	para-Methoxyamphetamine
PMEA	para-Methoxy-N-ethylamphetamine
PMMA	4-Methoxymethamphetamine
QIT	Quadrupole-ion trap
QqQ	Triple quadrupole
QSRR	Quantitative Structure-Retention Relationship
QTOF	Quadrupole-Time-of-flight
RC	Regenerated cellulose
RP	Reversed-phase
RSD	Relative standard deviation
RT	Retention time
SPE	Solid phase extraction
SRM	Single Reaction Monitoring
TFMPP	1,3-trifluoromethylphenylpiperazine
THC	delta-9-tetrahydrocannabinol
TOF	Time-of-flight
TP	Transformation product
UHPLC	Ultra high performance liquid chromatography
UHPSFC-MS/MS	Ultra-high performance supercritical fluid chromatography - tandem mass spectrometry
WBE	Wastewater-based epidemiology
WWTP	Wastewater Treatment Plant
α-PVP	α-pyrrolidinovalerophenone

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