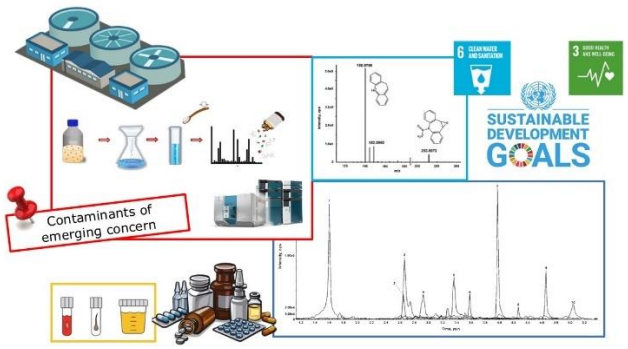
UNIVERSITY OF TURIN
DEPARTMENT OF CHEMISTRY

PhD Programme in Sustainable Development and Cooperation
Cycle: XXXVII

Analytical methods for monitoring pharmaceutical and illicit drugs in wastewater and biological matrices



by: Marta Massano

Tutor: Prof. Alberto Salomone
Co-tutor: Prof. Marco Minella
PhD Programme Coordinator: Prof. Francesca Barbero
Academic Years: 2021-2024
Academic Fields/Disciplines: Analytical chemistry

Torino, 2025

"La conoscenza è un paradosso, maggiore è l'apprendimento maggiore è la consapevolezza dell'enormità della propria ignoranza."

Arcane

Table of Contents

List of publications

Simultaneous determination of 137 drugs of abuse, new psychoactive substances, and novel synthetic opioids in meconium by UHPLC-QTOF

Ángela López-Rabuñal, Daniele Di Corcia, Eleonora Amante, **Marta Massano**, Angelines Cruz-Landeira, Ana de-Castro-Ríos, Alberto Salomone
Analytical and Bioanalytical Chemistry
https://doi.org/10.1007/s00216-021-03533-y

Development and validation of a UHPLC-HRMS-QTOF method for the detection of 132 New Psychoactive Substances and synthetic opioids, including fentanyl, in Dried Blood Spots

Marta Massano, Carola Incardona, Enrico Gerace, Pierre Negri, Eugenio Alladio, Alberto Salomone, Marco Vincenti
Talanta
https://doi.org/10.1016/j.talanta.2022.123265

Metabolic study of new psychoactive substance methoxpropamine in mice by UHPLC-QTOF-HRMS

Marta Massano, Enrico Gerace, Martina Borsari, Micaela Tirri, Christina Ververi, Eugenio Alladio, Marco Vincenti, Alberto Salomone
Drug Testing and Analysis
https://doi.org/10.1002/dta.3449

Wastewater surveillance of 105 pharmaceutical drugs and metabolites by means of ultra-high-performance liquid-chromatography-tandem high resolution mass spectrometry

Marta Massano, Alberto Salomone, Enrico Gerace, Eugenio Alladio, Marco Vincenti, Marco Minella
Journal of Chromatography A
https://doi.org/10.1016/j.chroma.2023.463896

Detection of fentanyl, synthetic opioids, and ketamine in hair specimens from purposive samples of American and Italian populations

Alberto Salomone, Martina Galletto, **Marta Massano**, Daniele Di Corcia, Joseph J. Palamar, Marco Vincenti
Journal of forensic science
https://doi.org/10.1111/1556-4029.15348

Trends in Reported and Biologically Confirmed Drug Use Among People Who Use Ecstasy in the Nightclub/Festival-Attending Population, 2016-2022

Joseph J. Palamar, Alberto Salomone, **Marta Massano**, Charles M. Cleland
Drug and Alcohol Dependence Reports

https://doi.org/10.1016/j.dadr.2023.100198

Five cases of unintentional exposure to BZO-4en-POXIZID among nightclub attendees in New York City

Joseph J. Palamar, **Marta Massano**, Alberto Salomone
Journal of Analytical Toxicology
https://doi.org/10.1093/jat/bkad086

Method development for the quantification of nine nitazene analogs and brorphine in Dried Blood Spots utilizing liquid chromatography - tandem mass spectrometry

Christina Ververi, Martina Galletto, **Marta Massano**, Eugenio Alladio, Marco Vincenti, Alberto Salomone
Journal of Pharmaceutical and Biomedical Analysis
https://doi.org/10.1016/j.jpba.2024.115975

Metabolic profile of N-ethylhexedrone, N-ethylpentedrone, and 4-chloromethcathinone in urine samples by UHPLC-QTOF-HRMS

Marta Massano, Melani Nuñez-Montero, Esther Papaseit, Olga Hladun, Clara Pérez-Maña, Mireia Ventura, Emilia Marchei, Eugenio Alladio, Enrico Gerace, Simona Pichini, Magi Farrè, Alberto Salomone
Journal of Pharmaceutical and Biomedical Analysis
https://doi.org/10.1016/j.jpba.2024.115994

Development and application of a sustainable approach for the determination of 95 pharmaceutical substances and metabolites in urban wastewater by means of ultra-high-performance liquid-chromatography-tandem mass spectrometry

Marta Massano, Dana Privitera, Eugenio Alladio, Enrico Gerace, Marco Minella, Marco Vincenti, and Alberto Salomone
INTERNATIONAL JOURNAL OF ENVIRONMENTAL ANALYTICAL CHEMISTRY
https://doi.org/10.1080/03067319.2024.2322098

Enhancing breast cancer screening with urinary biomarkers and Random Forest supervised classification: a comprehensive investigation

Eugenio Alladio, Fulvia Trapani, Lorenzo Castellino, **Marta Massano**, Daniele Di Corcia, Alberto Salomone, Enrico Berrino, Riccardo Ponzone, Caterina Marchio`, Anna Sapino, Marco Vincenti
Journal of Pharmaceutical and Biomedical Analysis
https://doi.org/10.1016/j.jpba.2024.116113

Chapter 1 | **Introduction**

Natural water chemistry has been a topic of interest for centuries and during this long period of investigation, many aspects of aqueous chemistry have been discovered, forgotten and rediscovered again, but the **chemical pollution** of water resources is one of the **major challenge** facing the humanity in this century [1]. Originally used in the 1990s to monitor the environmental impact of liquid household waste, the method has since also been used to **estimate substance intake and identify new consumption trends** [2]. Indeed, wastewater analysis has become a rapidly developing scientific discipline with the potential to monitor real-time data on geographical and temporal trends in substance consumption and the environmental contamination caused by them.

Concerning water, monitoring the quality of the water and its toxicological load is mandatory to guarantee citizens the consumption of pure water. It is necessary to extend access to clean water by planning actions able to treat contaminated water through environmentally sustainable technologies with the final aim of guaranteeing a low cost and safe water. Therefore, it is important to implement existing methodologies [3]. In fact, the physical, chemical and biological methods used in conventional wastewater treatment plants (WWTPs) are not always effective in removing the so-called contaminants of emerging concern (CECs)[4]. CECs are chemical substances from anthropogenic origin present in the environment at trace and ultra-trace levels [5,6]. CECs usually refer to a wide range of substances classified as pesticides, pharmaceuticals, personal care products, flame retardants, hormones, *etc.*, with pharmaceuticals and pesticides being most frequently detected due to their widespread use [7]. Among the contaminants of emerging concern (CECs), pharmaceutical drugs and personal care products (PPCPs) play an increasing role. The term PPCP comprises thousands of different chemical compounds, including prescription and **over-the-counter drugs**, **illicit drugs**, veterinary drugs, perfumes, creams, diagnostic and nutraceutical agents, and many others [3].

The use and abuse of pharmaceutical and illicit drugs is an increasingly widespread phenomenon both nationally and internationally; however, it is difficult to determine with certainty what substances are actually used by the population, the quantities consumed, or to follow the time trends and identify the emergence of new substances. The complexity of the phenomenon has further

increased in recent years, with the appearance on the illegal market of a wide range of new synthetic substances of abuse, generically called **New Psychoactive Substances (NPS)** [8]. My PhD project focused mainly on PPCPs, and particularly on the pharmaceutical and illicit drugs which are increasingly used in many Countries. The detection of these substances in waters has a dual goal: on one side, for their role as contaminants (e.g. for their effect on drug resistance phenomena or effects on aquatic flora), and secondly as their levels in waters can be used as indicators of consumption in certain population.

The present research aimed to develop new methods for the simultaneous determination of a wide panel of pharmaceutical, illicit drugs and their urinary metabolites in wastewater from municipal wastewater treatment plant. With these methods, it will therefore be possible to trace the type and quantity of substances collectively consumed by the populations served by the plants under study. Using **different biological matrices**, the main **metabolic pathways** of the newer **NPSs** and the extent to which they are modified in the body before being excreted and entering the sewage system were also investigated. Finally, a **collaboration** was initiated with partners in Italian and non-Italian cities to compare consumption trends and patterns of use of pharmaceutical and illicit drugs by different population.

The fundamental objective of this research is to propose useful methodologies to address some of the challenges posed by the United Nations (UN). The UN has established a 2030 Agenda that includes 17 Sustainable Development Goals (GOALS) covering a number of critical issues for the world. For the purposes of this study, special attention is given to **Goal 6** "Ensure the availability and management of all sustainable water and sanitation resources" and **Goal 3** "Ensure healthy lives and promote well-being for all at all ages" [9]. In order to achieve its objective, the thesis is presented as a collection of articles on the topic, published during the Ph.D. period.

References

[1] A. Shraim, A. Diab, A. Alsuhaimi, E. Niazy, M. Metwally, M. Amad, S. Sioud, A. Dawoud, Analysis of some pharmaceuticals in municipal wastewater of Almadinah Almunawarah, Arab. J. Chem. 10 (2017) S719–S729. https://doi.org/10.1016/j.arabjc.2012.11.014.

[2] Wastewater analysis and drugs — a European multi-city study | www.emcdda.europa.eu, https://www.emcdda.europa.eu/publications/html/pods/waste-water-analysis_en.

[3] Y. Deng, C. Guo, H. Zhang, X. Yin, L. Chen, D. Wu, J. Xu, Occurrence and removal of illicit drugs in different wastewater treatment plants with different treatment techniques, Environ. Sci. Eur. 32 (2020) 28. https://doi.org/10.1186/s12302-020-00304-x.

[4] J.C.G. Sousa, A.R. Ribeiro, M.O. Barbosa, C. Ribeiro, M.E. Tiritan, M.F.R. Pereira, A.M.T. Silva, Monitoring of the 17 EU Watch List contaminants of emerging concern in the Ave and the Sousa Rivers, Sci. Total Environ. 649 (2019) 1083–1095. https://doi.org/10.1016/j.scitotenv.2018.08.309.

[5] J.C.G. Sousa, A.R. Ribeiro, M.O. Barbosa, M.F.R. Pereira, A.M.T. Silva, A review on environmental monitoring of water organic pollutants identified by EU guidelines, J. Hazard. Mater. 344 (2018) 146–162. https://doi.org/10.1016/j.jhazmat.2017.09.058.

[6] S.D. Richardson, S.Y. Kimura, Water Analysis: Emerging Contaminants and Current Issues, Anal. Chem. 92 (2020) 473–505. https://doi.org/10.1021/acs.analchem.9b05269.

[7] A.B. Martínez-Piernas, P. Plaza-Bolaños, A. Gilabert, A. Agüera, Application of a fast and sensitive method for the determination of contaminants of emerging concern in wastewater using a quick, easy, cheap, effective, rugged and safe-based extraction and liquid chromatography coupled to mass spectrometry, J. Chromatogr. A 1653 (2021) 462396. https://doi.org/10.1016/j.chroma.2021.462396.

[8] N. Salgueiro-González, E. Zuccato, S. Castiglioni, Nationwide investigation on the use of new psychoactive substances in Italy through urban wastewater analysis, Sci. Total Environ. 843 (2022) 156982. https://doi.org/10.1016/j.scitotenv.2022.156982.

[9] THE 17 GOALS | Sustainable Development. https://sdgs.un.org/goals

Chapter 2 | **Wastewater-Based Epidemiology (WBE)**

Wastewater-based epidemiology (WBE) provides a comprehensive real-time framework of population attitude and health status [1]. WBE involves the chemical analysis of raw pooled wastewater for specific excreted biomarkers of interest to gain information on the consumption or exposure of a population to certain substances or microorganisms [2]. Therefore, WBE assesses the presence or quantity of a chemical or biological signal in a pooled sample of sewage, taken from the sewer network or wastewater treatment plant (WWTP), to gain information on various aspects of public health [3].

Conventional methods to estimate the rate of drugs use in a community already exist and are based on self-reported surveys, overdose/toxicological reports, and drug-related crime statistics [4–6]. However, traditional approaches such as self- reported surveys are commonly affected by high cost, delayed outcome, limited coverage, biases from nonresponse and unbalanced selection of sampled populations, with higher prevalence of drug abusers. For these reasons, sewage epidemiology established itself as a comprehensive, real-time, and cost-effective approach to reliably measure the average drug consumption within a community. The quantification of either the parent drugs or their human-specific metabolites in wastewater [7,8] is increasingly adopted to complement other conventional methods for the estimation of pharmaceuticals and drugs use/abuse in large communities.

2.1 Contaminants of emerging concern (CECs)

Chemical pollution of water resources is one of the major challenges humanity faces in this century as they are essential for human health, environmental sustainability and economic prosperity. However, this natural resource is under threat, making it essential to address the challenges of water-related sanitation and ecosystems [9]. One of the causes of water pollution are the *"emerging contaminants"*, that are chemical substances present in the environment at trace and ultra-trace levels [10,11]. However, once we focus on *"emerging contaminants",* we need to better define what is being targeted; as the definition of *"emerging"* is relative as what might have been an environmental contamination problem a decade or two ago, might not be the one today. In a

broader context, the attention could be extended from *emerging contaminants* (contaminants which have appeared only recently) to *Contaminants of Emerging Concerns* (contaminants which have been in the environment for a while but for which concerns have been raised more recently) (CECs)[12]. CECs include pharmaceuticals and personal care products (PPCPs), a group of emerging chemical pollutants characterised by significant bioactivity and medium solubility. It is a group of substances that "generally refers to any product used by individuals for personal health or cosmetic reasons or used by the agroindustry to improve the growth or health of livestock" (US-EPA).

The term PPCP contains thousands of different chemical compounds, such as prescription and over-the-counter drugs, veterinary drugs, perfumes, make-up, creams, diagnostic and nutraceutical agents, and many others. They are of great significance because, once taken by humans, they are excreted as such or partially metabolised, and, therefore, released into wastewater, thus still being bioactive in the environment and becoming a major risk to wildlife and aquatic ecosystems [13].

Interest in pharmaceuticals and their metabolites as environmental pollutants began in the 1970s, but it was not until recently that scientists began actively addressing the impact of these pollutants on the environment and living organisms. Some pharmaceuticals degrade upon consumption or are released into the environment, despite this, most remain unchanged, becoming persistent in the environment. It is well known that most of these chemicals remain bioactive even at extremely low concentrations after excretion from the body or after disposal in landfills and water, showing a tendency to accumulate in the food chain with a negative impact on the health of aquatic organisms and consumers [14]. Consequently, pharmaceuticals and their metabolites and by-products are a source of concern due to their potential ecological and environmental impacts. In addition, illicit drugs are also recognised as environmental contaminants and they are an emerging human health and ecological concern.

2.1.1 Xenobiotics in the environment: Factors of Interest/Consideration

The "xenobiotic" is defined as a substance of any kind, of natural or synthetic origin, that does not occur naturally in an organism. Xenobiotics are also substances that are not originally present in the environment but are introduced becoming a problem for the treatment efficiency of wastewater treatment systems being them present in large numbers with different characteristics thus increasing the removal difficulties. Wastewater, in environmental chemistry, is water that is

used in domestic, industrial and agricultural activities, and whose quality may be compromised by anthropogenic action, thus becoming unfit for the reuse. These waters, therefore, must necessarily be monitored and undergo purification before being released into the environment.

According to current literature [15], xenobiotics are continuously released into the environment in extremely large quantities, making humans the main source of contamination. (Figure 1). It has been calculated that more than 70-80% of pollution from pharmaceuticals or drugs has an anthropogenic origin, while all other sources of pollution (industrial, improper or illegal disposal, etc.) contribute the remaining 20-30%.

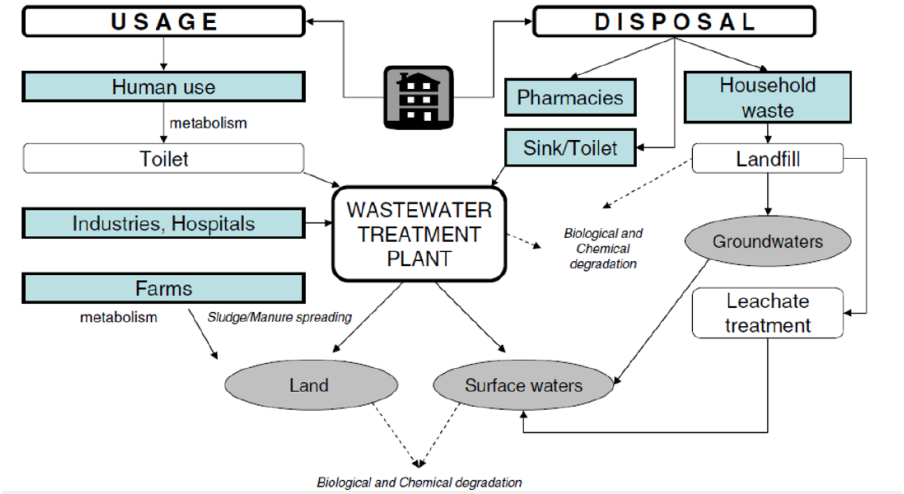


Figure 1 *The pathway of xenobiotics from use to release into the environment [16]*

Exposure of humans and animals to xenobiotic substances through the environment may be direct or indirect, and in the long term, pharmaceutical and drug pollutants could be responsible for chronic toxicity or other effects, including microbial resistance, endocrine disruption, growth inhibition, ecosystem destruction, cytotoxicity, mutagenicity, and teratogenicity [17]. Furthermore, although there is no systematic study proving a specific hazard or toxicity of drugs in the environment, the potential effects on wildlife have been widely demonstrated and they are known to be harmful to the aquatic environments. For example, the effects of bioaccumulation of these active ingredients in fish include endocrine effects, developmental alteration and behavioural changes [18,19].

The European Directive 91/271/EEC aims to protect human health and the environment in the EU from adverse effects (such as eutrophication) due to the state of urban wastewater. It sets Europe-wide standards for wastewater collection, treatment and discharge [20]. According to the directive of the European Parliament and the Council, strict and restrictive rules will apply to wastewater treatment by 2040. Specifically:

1) New limit values will be set for micropollutants requiring additional treatment, with application to large plants (10,000 population equivalent (p.e.)), in order to reduce the risk to the environment and public health.
2) A producer responsibility system targeted at pharmaceuticals and personal care products (the two main sources of harmful micropollutants) will be established to cover the additional costs of treating micropollutants and to incentivise the placing of less harmful products on the EU market.
3) Member States will have to better monitor and track non-domestic pollution in order to increase the possibilities of reuse of purified water and to reduce the risk of non-treatable substances being discharged into the environment and of treatment plants malfunctioning.
4) Member States will be required to organise cooperation between their health and wastewater authorities so that permanent surveillance of key public health parameters is in place.

2.1.2 Pharmaceutical drugs

In pharmacology, a pharmaceutical drug is an exogenous substance, organic or inorganic, natural or synthetic, capable of inducing functional changes in a living organism through physical, chemical or physical-chemical action. Pharmaceuticals and their residues in the environment are considered CECs, a definition used to distinguish them from "*conventional*" contaminants (i.e. metals such as mercury, lead, arsenic, etc.) or from those that have been defined as "priority" (DDT, PCB, PAH, etc.). Pharmaceuticals are present in the environment as an outcome of their production and formulation, from patient use, food production and improper disposal. Since they are designed to interact with a living system - such as the human body - and produce a pharmacological response at low dose, their presence raises environmental concern even at low concentration [21]. Consequently, the environmental risk to wildlife and aquatic

ecosystems is increased, with undesirable effects such as physiological, behavioural and reproduction alterations, and even death [22].

Although indication of pharmaceuticals and metabolites as potential environmental pollutants originated in the 1970 [23], only recently scientists have begun to actively address the impact of these pollutants on the environment and living organisms. Several active pharmaceutical ingredients and some of their metabolites proved to be bio-recalcitrant to the most common microorganisms used in civil wastewater treatment plants (WWTPs), allowing them to get through the conventional processes of wastewater treatment and thus enter the environment. Indeed, their hydrophobicity prevents their efficient partitioning into the solid phase used in water purification plants and/or their incorporation into spent sludge [11], increasing their spread into the aquatic environments [24]

The most common way for contamination of urban waters by the pharmaceuticals is via the unaltered excretion in urine and faeces, although different anthropogenic mechanisms should be assumed, namely:

1) Metabolism post-consumption; this occurs through the processes of converting substances into their more polar and more easily eliminated form (Figure 2).
2) Household disposal; unused (expired or unwanted) topical formulations or medicines are thrown in the sink/toilet or through the waste collection
3) Veterinary drugs, they are also excreted by animals in urine and faeces before being spread on the ground through the application of manure as fertiliser.

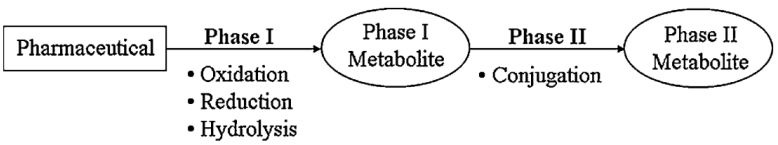


Figure 2 *Schematic representation of pharmaceutical biotransformation to increase their polarity (adapted from reference [25])*

Recent development of sensitive methods for identification and quantification of drugs enabled to define their distribution patterns in several environmental samples, thus highlighting the more relevant therapeutic classes in

terms of environmental contamination (Figure 3). These data could be useful to set out the most appropriate active substances to be used in ecotoxicity tests. According to data present in literature, scientific community has mainly concerned their attention on therapeutic classes such as, non-steroidal anti-inflammatory drugs, blood lipid lowering agents, antibiotics and sex hormones. For these reasons, the present study focused on pharmaceutical drugs belonging to some of these therapeutic classes.

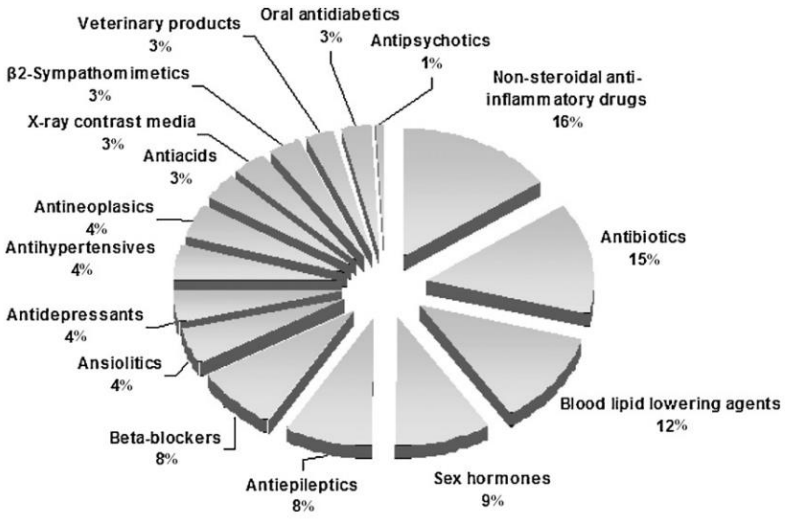


Figure 3 *Therapeutic classes detected in the environment, expressed in relative percentage. Data collected from 134 articles published between 1997 and 2009 [26]*

Among pharmaceutical prescriptions, non-steroidal anti-inflammatory drugs, cardiovascular drugs, antidepressants and antipsychotics are the most popular. In this study, therefore, drugs belonging to those classes that are among the most commonly consumed by the population as a whole were mainly taken into account:

1) **Analgesics:** also called painkillers, are pharmaceuticals that can relieve pain, such as headaches, or associated with inflammation, such as back pain; among these drugs, the most common is paracetamol. Also included within analgesics are opioids, derived from opium, which act by binding to opioid receptors present at both central nervous **s**ystem (CNS) and

peripheral nervous system (SNP) levels, modulating pain; they are widely used clinically for their efficacy in treating pain, both short- and long-term. Analytes belonging to this class and chosen for the study are: buprenorphine, dihydrocodeine, embutramide, hydromorphone, methadone, oxycodone, paracetamol, fenacetin, tapentadol, tramadol. Particularly interesting is the case of tramadol, which is the active ingredient in several common opioid painkiller prescriptions and recently classified in Italy as an illicit drug, suggesting that its use is not restricted to medical treatment, but also abused recreationally or misused for off-label treatments.

2) **Antidepressants:** a class of drugs widely used to treat mood disorders, such as depression and bipolar disorder. In fact, nowadays these drugs are also used to treat neuropathic pain, obsessive-compulsive disorders and smoking cessation therapy. The treated analytes that belong to this class are: amitriptyline, citalopram, clonidine, fluoxetine, fluvoxamine, mianserin, mirtazapine, paroxetine, trazodone.
3) **Antiepileptics:** these are administered in the presence of convulsions and repeated seizures. However, they may also be prescribed after a single epileptic seizure, if there is a high probability that seizures may recur, which does not necessarily imply the presence of the disease. Analytes treated are: carbamazepine, lamotrigine, oxcarbazepine. Of these, carbamazepine is one of the most frequently detected drugs in sewage treatment plants [27].
4) **Antipsychotics:** are used in the treatment of various psychopathological conditions, including psychotic disorders, severe mood disorders such as mania and delusional depression, states of psychomotor agitation and substance-induced psychosis, and behavioural disorders in dementia. Analytes treated are: chlorpromazine, clozapine, haloperidol, levamepromazine, olanzepine, periciazine, promazine, quetiapine, risperidone, tiapride, venlafaxine, ziprasidone.
5) **Benzodiazepines:** these are a class of psychoactive drugs with anxiolytic action that also produce anticonvulsant, anaesthetic and sedative effects. They are the most widely used drugs in the world (in cases of anxiety, panic attacks, severe and persistent difficulty in getting to sleep or in cases of sleep disturbed by prolonged nocturnal awakenings). Analytes treated in this case are: 7-aminonitrazepam (metabolite of nitrazepam), alprazolam, bromazepam, brotizolam, chlordiazepoxide and its metabolite

demoxepam, clobazam, clonazepam and its main urinary metabolite 7-aminoclonazepam, clotiazepam, delorazepam, desalchilflurazepam, diazepam and its metabolites nordiazepam, oxazepam and temazepam, diclazepam, flunitrazepam and its main urinary metabolite 7-aminoflunitrazepam, flurazepam, lorazepam, lormetazepam, midazolam, nordiazepam, triazolam, zolpidem.

6) **Cardiovascular drugs:** these are drugs that are directly or indirectly active on the cardiovascular system. Analytes treated in this class are: atenolol, bisoprolol, nebivolol, propafenone, ramipril, telmisartan, verapamil.
7) **Non-steroidal anti-inflammatory drugs (NSAIDs):** in addition to combating inflammation, they have pain-relieving and antipyretic capacities, which is why they can be prescribed for headaches, toothaches, menstrual cramps, sprains or strains, various forms of arthritis including rheumatoid arthritis and arthrosis, chronic neck pain or back pain. Analytes are: ketoprofen, ketorolac.
8) **Other drugs belonging to various classes:** atropine, biperiden, dextromethorphan, diphenidine, diphenhydramine, gliclazide, levamisole, lidocaine, loperamide, metformin, methylphenidate, metoclopramide, naloxone, oxybutynin, phendimetrazine, promethazine, scopolamine, ticlopidine, warfarin.

Some of the above-mentioned pharmaceutical drugs will be discussed in depth in the following chapters, observing their high concentrations in wastewater. In particular, this is the case of:

- **Paracetamol:** its high average concentration (above 10 µg/L), can be attributed to its widespread use in self-medication, benefiting from its availability without a prescription.
- **Metformin:** its high concentration (even higher than 15 µg/L) is due to its frequent prescription for the treatment of diabetes, reflecting the widespread use of this drug [28].
- **Lamotrigine and Carbamazepine**: used for neurological disorders, they have significant concentrations but also considerable variability. In particular, Carbamazepine is an analyte that is always present in wastewater as it is very persistent and the average removal efficiency by treatment plants is less than 10% [29].

2.1.3 Traditional drugs of abuse and New psychoactive substances

A drug is any natural or synthetic substance that, when ingested, produces a complex array of psychological and physical effects that alter bodily functions. Drugs may be legal like alcohol, tobacco etc., or can be illegal such as ecstasy, cocaine, cannabis, methamphetamine etc. [30]

Distinctive criteria for the classification of illicit drugs may be:

1) Historical, depending on their use in historical contexts.

2) Legislative, as in the Italian legal system there is the DPR 309/90 which reinstated the system of sanctions related to offences involving narcotic and psychotropic substances divided into four tables (I and III major penalties; II and IV minor penalties), plus a table V including medicines containing narcotic or psychotropic substances [31].

3) On the basis of their origin, natural (e.g. marijuana), synthetic (e.g. amphetamines) or semi-synthetic (e.g. heroin).

4) Chemical, according to which substances are grouped by reference to structural similarities.

5) Pharmacological or symptomatic, according to the effects the substance produces on the central nervous system.

In this classification, psychotropic substances are grouped within four further categories:

- **Analgesics and narcotics**, such as opiates, barbiturates, sedatives.
- **Stimulants**, such as cocaine, amphetamine and derivatives, GHB, antidepressants.
- **Hallucinogens or psychomimetics**, such as cannabinoids, LSD, mescaline, psilocin, scopolamine.
- **Anxiolytics**, such as benzodiazepines.

In detail, the screening method developed during this PhD focused on the following analytes: cocaine and its metabolite benzoylecgonine; cocaethylene; MDMA with its metabolite MDA; amphetamine; 6-monoacetylmorphine (6-MAM, metabolite of heroin); codeine with its metabolite morphine; methadone with its metabolite EDDP; buprenorphine with its metabolite norbuprenorphine; ketamine with its metabolite norketamine; THC-COOH (metabolite of THC).

As previously reported for pharmaceuticals, when drugs are consumed, a part is transformed within the body and excreted as metabolites, while the unreacted

part of the drug is excreted unchanged. Ultimately, drugs consumed together with their metabolites are excreted directly into the sewage system leading to wastewater treatment plants (WWTPs) [32]. However, over the years, some cases have been reported where aberrant loads of drugs of abuse were observed in sewage that could not be attributed solely to human consumption [33]. That is, cases in which higher percentages of the parent drug and very small (and sometimes absent) percentages of its metabolites were observed in the wastewater analysed. Such loads cannot be explained by consumption alone. The most likely origin of these high loads are direct discharges of synthetic wastes from drug production (clandestine laboratories) or cases where drug batches are dumped directly into watercourses to avoid detection and law enforcement targeting. Many studies show that chemical waste or by-products from illicit drug production gives rise to a specific chemical footprint that can be traced in wastewater and used for forensic purposes [34]. Furthermore, the discharge of synthetic chemical waste is a real threat to the normal operation of small wastewater treatment plants. Indeed, in addition to having an impact on the operation of the wastewater treatment plant, chemical waste poses a risk to the quality of the water. Therefore, illicit drugs, such as pharmaceuticals and their residues, present in the environment can also be considered CECs and therefore require appropriate monitoring procedures.

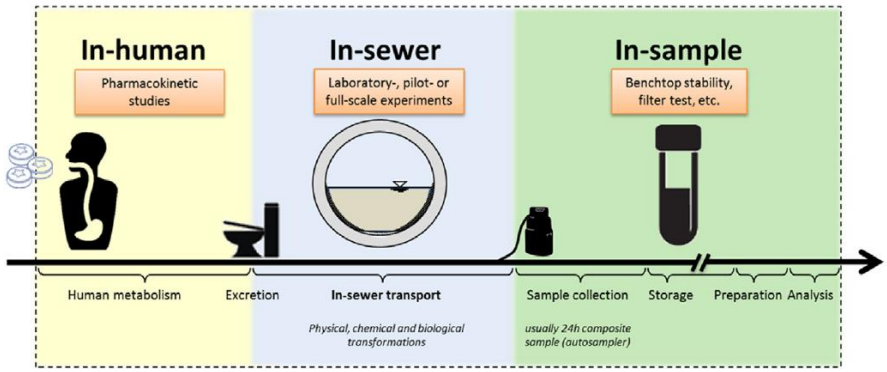


Figure 4 *Pathway from ingestion to wastewater analysis [35]*

Traditional illicit drugs are not the only psychotropic substances to be concerned about. In recent years, the diffusion of countless New Psychoactive Substances (NPS) in the illegal drug market introduced an unprecedented threat to the public health [36]. The adjective "new" does not necessarily indicate totally original compounds, but it may refer to substances initially synthesized and tested for their potential pharmaceutical properties and revalued years later as highly

potent drugs of abuse [37,38]. NPS are often categorized into synthetic cannabinoids, synthetic opioids, cathinones and hallucinogen, but this simplistic classification does not express adequately the variety and complexity of their potency, combined effects, and risk profiles that intersect categories and often differentiate compounds belonging to the same category [39]. Nowadays, the NPS abuse in U.S and Europe is proliferating at unprecedented rate and represents an increasing challenge to the established national and international drug policies. The most recent data [40] shows that drug producers continue to create new substances to avoid legal controls. Between 2016 and 2022, typically around 50 new psychoactive substances appeared on the market for the first time each year; this fell to 26 in 2023. In addition, around 400 previously reported new substances are detected on the market each year. Therefore, during my Ph.D. studies, I aimed to identify new metabolites of selected NPS, assuming that these same molecules are eliminated into the environment during human excretion and then potentially identified in wastewater samples. The ultimate goal is to incorporate the newly identified metabolites into wastewater protocols to be used in future studies. As it will be explained in more detail in later chapters, the investigated compounds were N-ethylhexedrone (NEH), N-ethylpentendrone (NEP), 4-chloromethcathinone (4-CMC) and methoxpropamine (MXPr)). After administration to human volunteers or rats, all samples (mainly urine) were analyzed by UHPLC-QTOF-HRMS to clarify the distribution of the parent drug and its metabolites in different biological matrices over time.

Finally, during my Ph.D., further studies have been undertaken to understand the actual prevalence of NPS in the population [41]. In particular, the opportunities and potential benefits of implementing dried blood spot (DBS) and keratin matrix analysis as complementary tools in forensic testing programmes are highlighted.

2.2 Wastewater surveillance and The Sustainable Development Agenda

The analysis of municipal wastewater is a developing field, involving scientists working in different research areas, including analytical chemistry, physiology, biochemistry, sewage engineering, spatial epidemiology and statistics, and conventional drug epidemiology [42]. This collaboration between multiple experts is just one example of the broader goals that European countries have committed to by 2030. In fact, water monitoring is just one of the goals of the 2030 Agenda. The 2030 Agenda for Sustainable Development is an action agenda for people, planet and prosperity, signed in September 2015 by the

governments of the 193 member countries of the United Nations. It incorporates 17 Sustainable Development Goals (SDGs) (Figure 5) into a major program of action with a total of 169 "targets," or goals, that European countries have pledged to achieve by 2030 [43].

As far as the present Ph.D. study is concerned, we would mainly focus on the Goals: Goal 3 ("Good health and well-being") and Goal 6 ("Clean water and sanitation").

Figure 5 *The 17 Sustainable Development Goals (SDGs)*

2.2.1 "Good health and well-being"- GOAL 3

The direct quote from the United Nations Regional Information Center (UNRIC) on Goal 3 tells: *"Ensuring healthy lives and promoting well-being for all at all ages is key to achieving sustainable development. Much progress has been made in increasing life expectancy and reducing some of the leading causes of death related to infant and maternal mortality. Significant progress has been made in access to safe water and sanitation. Nevertheless, much more effort is needed to completely eradicate a wide range of diseases and to address many different health problems, whether recent or long-standing"* [44].

Specifically, in the present work, we focused on the target reported in Goal 3:

1) **Target 3.5**: strengthen prevention and treatment of substance abuse, including drug abuse and harmful use of alcohol.

2) **Target 3.9**: by 2030, significantly reduce the number of deaths and diseases caused by hazardous chemicals and air, water and soil pollution and contamination.

During the Ph.D. project, this theme was conjugated in terms of monitoring drug and pharmaceuticals consumption trends by their levels in wastewater. The most common pharmaceutical drugs taken by people in different locations were studied to identify trends in consumption and medical prescriptions. Similarly, the study of psychoactive drug consumption trends in different realities was carried out, thus investigating in depth the prevalence of certain substances. Monitoring trends in pharmaceuticals and drugs use and accessing overall consumption levels is therefore a critical requirement for the development and targeting of effective interventions of every kind.

2.2.2 "Clean water and sanitation"- GOAL 6

The direct quote from the United Nations Regional Information Center (UNRIC) on Goal 6 tells: *"Accessible, clean water is an essential aspect of the world we want to live in. Our planet has enough clean water to achieve this goal. But due to poor infrastructure or economic mismanagement, millions of people, most of them children, die every year from diseases caused by inadequate water supply, sanitation and hygiene"*[45].

Specifically, in the present work, we focused on the target reported in Goal 6:

1) **Target 6.3**: improve water quality by 2030 by eliminating landfills, reducing pollution and the release of hazardous chemicals and wastes, halving the volume of untreated wastewater, and significantly increasing recycling and safe reuse worldwide.
2) **Target 6.6**: by 2030, expand international cooperation and support for water and sanitation activities and programs in developing countries, including water harvesting, desalination, water efficiency, wastewater treatment, and recycling and reuse technologies.

During the Ph.D. project, this issue was addressed by developing rapid and effective procedures for monitoring the concentration of drugs and pharmaceuticals in wastewater. Through collaboration with several wastewater treatment plants in northern Italy and Algeria, it was possible to test the effectiveness of the screening procedures developed. In addition, the ability to reduce the rates of these emerging contaminants was investigated by monitoring

the purification capacities between water leaving and entering the treatment plants. The detail of these studies is given in the following chapters.

2.2.3 EMCDDA (now EUDA) and the European surveillance

Despite the application of wastewater analysis for the detection of pharmaceuticals and drugs represents a rather recent monitoring approach, several European countries quickly identified it as an effective drug surveillance tool to assist public health and law enforcement officials [46]. From the outset, the former European Monitoring Agency for Drugs and Drug Addiction (EMCDDA), now renamed as European Union Drugs Agency (EUDA), has shown a strong interest in exploring the potential of wastewater analysis to complement and extend existing epidemiological tools. The Agency recognises in water analysis some clear advantages over other approaches. In particular, compared to surveys, it is not subject to response and nonresponse bias, also in light of the fact that users are often unaware of the substances actually present in the mix of adulterated drugs they are taking. The sewage method is a flexible tool because experiments can be designed to examine drug use in a particular area or to compare use between different areas during specific periods of the year or over successive years. As a result, the tool has the potential to provide timely information on geographic and temporal trends in short periods of time, including changing trends in specific locations or during special events or holidays (Figure 6).

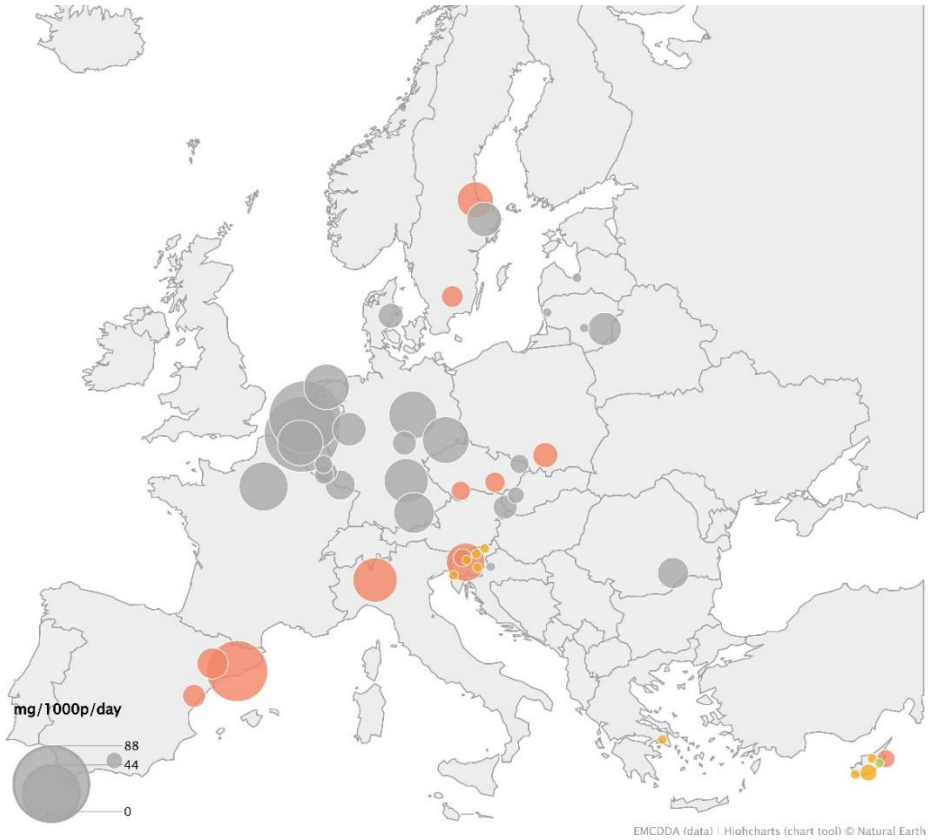


Figure 6 *Example of report from EMCDDA. Changes in the mean weekly ketamine metabolites from wastewater analyses in selected European cities between 2022 and 2023. Red = increase | Gray= decrease | Yellow = stable, with respect to previous year [46]*

The initial focus of investigation in this field was on the monitoring of environmental contamination caused by the excretion of prescription medicines. However, it was quickly realised that these approaches might lead to the possibility of assessing levels of illicit drug use by detecting their residues in the environment. It is important to remember that this type of approach has been made possible by modern analytical chemistry which offers a solution to some of the challenging limitations of the past. The use of advanced analytical techniques and the expertise of analysts are essential to decode the information that can be obtained from a complex matrix such as wastewater and to obtain accurate data on drug residues in wastewater at trace levels (ng/L or parts per trillion) [47].

To understand the importance of the phenomenon it is enough to know that in 2010 a Europe-wide network (Sewage analysis CORe group - Europe

(SCORE)) was established with the aim of standardizing the approaches used for wastewater analysis and coordinating international studies through the establishment of a common protocol of action [48]. The first activity of the SCORE group was a Europe-wide survey, conducted in 2011 in 19 European cities; based on the success of this initial study, comparable studies were undertaken in the following years, eventually covering 82 cities and 18 EU countries in 2020 [49].

Taking into account the growing attention of scientists to this topic and the increasing use and abuse of medicines and drugs in Italy, we believe that the present Ph.D. project can be of great relevance and offer a timely contribute to epidemiological studies on trends and patterns of drug use.

.

References

[1] E. Gagliano, D. Biondi, P. Roccaro, Wastewater-based epidemiology approach: The learning lessons from COVID-19 pandemic and the development of novel guidelines for future pandemics, Chemosphere 313 (2023) 137361. https://doi.org/10.1016/j.chemosphere.2022.137361.

[2] M.K. Wilson, K. Phung, A. Chappell, L.I. Pilkington, Wastewater-based Epidemiology to Investigate Spatio-Temporal Trends in Alcohol Consumption in Aotearoa, New Zealand, Chem. – Asian J. 19 (2024) e202301120. https://doi.org/10.1002/asia.202301120.

[3] J. O'Keeffe, Wastewater-based epidemiology: current uses and future opportunities as a public health surveillance tool, Environ. Health Rev. 64 (2021) 44–52. https://doi.org/10.5864/d2021-015.

[4] C.J. Banta-Green, J.A. Field, A.C. Chiaia, D.L. Sudakin, L. Power, L. de Montigny, The spatial epidemiology of cocaine, methamphetamine and 3,4-methylenedioxymethamphetamine (MDMA) use: a demonstration using a population measure of community drug load derived from municipal wastewater, Addict. Abingdon Engl. 104 (2009) 1874–1880. https://doi.org/10.1111/j.1360-0443.2009.02678.x.

[5] A.G. Asimakopoulos, K. Kannan, Neuropsychiatric pharmaceuticals and illicit drugs in wastewater treatment plants: a review, Environ. Chem. 13 (2016) 541–576. https://doi.org/10.1071/EN15202.

[6] B. Subedi, K. Kannan, Mass loading and removal of select illicit drugs in two wastewater treatment plants in New York State and estimation of illicit drug usage in communities through wastewater analysis, Environ. Sci. Technol. 48 (2014) 6661–6670. https://doi.org/10.1021/es501709a.

[7] B. Subedi, Estimation of Community Usage of Drugs Utilizing Sewage Epidemiology, Methods Mol. Biol. Clifton NJ 1810 (2018) 141–147. https://doi.org/10.1007/978-1-4939-8579-1_14.

[8] L.H. Restrepo-Vieira, F. Busetti, K.L. Linge, C.A. Joll, Development and validation of a direct injection liquid chromatography-tandem mass spectrometry method for the analysis of illicit drugs and psychopharmaceuticals in wastewater, J. Chromatogr. A 1685 (2022) 463562. https://doi.org/10.1016/j.chroma.2022.463562.

[9] Goal 6 | Department of Economic and Social Affairs, https://sdgs.un.org/goals/goal6.

[10] J.C.G. Sousa, A.R. Ribeiro, M.O. Barbosa, M.F.R. Pereira, A.M.T. Silva, A review on environmental monitoring of water organic pollutants identified

by EU guidelines, J. Hazard. Mater. 344 (2018) 146–162. https://doi.org/10.1016/j.jhazmat.2017.09.058.

[11] S.D. Richardson, S.Y. Kimura, Water Analysis: Emerging Contaminants and Current Issues, Anal. Chem. 92 (2020) 473–505. https://doi.org/10.1021/acs.analchem.9b05269.

[12] S. Sauvé, M. Desrosiers, A review of what is an emerging contaminant, Chem. Cent. J. 8 (2014) 15. https://doi.org/10.1186/1752-153X-8-15.

[13] R.A.M. Nassef, N.M.H. Rizk, I.E. Mousa, Recycle and treatment of wastewater produced from pharmaceutical plant, Res. J. Appl. Biotechnol. 5 (2019) 33–41. https://doi.org/10.21608/rjab.2019.76802.

[14] P. Verlicchi, M. Al Aukidy, A. Galletti, M. Petrovic, D. Barceló, Hospital effluent: investigation of the concentrations and distribution of pharmaceuticals and environmental risk assessment, Sci. Total Environ. 430 (2012) 109–118. https://doi.org/10.1016/j.scitotenv.2012.04.055.

[15] S.L. Bartelt-Hunt, D.D. Snow, T. Damon, J. Shockley, K. Hoagland, The occurrence of illicit and therapeutic pharmaceuticals in wastewater effluent and surface waters in Nebraska, Environ. Pollut. Barking Essex 1987 157 (2009) 786–791. https://doi.org/10.1016/j.envpol.2008.11.025.

[16] A. Nikolaou, S. Meric, D. Fatta, Occurrence patterns of pharmaceuticals in water and wastewater environments, Anal. Bioanal. Chem. 387 (2007) 1225–1234. https://doi.org/10.1007/s00216-006-1035-8.

[17] J. Wang, X. Hu, Ecopharmacovigilance: Current state, challenges, and opportunities in China, Indian J. Pharmacol. 46 (2014) 13–17. https://doi.org/10.4103/0253-7613.125158.

[18] T. Brodin, S. Piovano, J. Fick, J. Klaminder, M. Heynen, M. Jonsson, Ecological effects of pharmaceuticals in aquatic systems--impacts through behavioural alterations, Philos. Trans. R. Soc. Lond. B. Biol. Sci. 369 (2014) 20130580. https://doi.org/10.1098/rstb.2013.0580.

[19] J. Corcoran, M.J. Winter, C.R. Tyler, Pharmaceuticals in the aquatic environment: A critical review of the evidence for health effects in fish, Crit. Rev. Toxicol. 40 (2010) 287–304. https://doi.org/10.3109/10408440903373590.

[20] Trattamento delle acque reflue urbane | EUR-Lex, (n.d.). https://eur-lex.europa.eu/IT/legal-content/summary/urban-waste-water-treatment.html (accessed May 27, 2024).

[21] M. Paut Kusturica, M. Jevtic, J. Ristovski, Minimizing the environmental impact of unused pharmaceuticals: Review focused on prevention, Front.

Environ. Sci. 10 (2022) 1077974. https://doi.org/10.3389/fenvs.2022.1077974.

[22] K.E. Arnold, A.R. Brown, G.T. Ankley, J.P. Sumpter, Medicating the environment: assessing risks of pharmaceuticals to wildlife and ecosystems, Philos. Trans. R. Soc. B Biol. Sci. 369 (2014) 20130569. https://doi.org/10.1098/rstb.2013.0569.

[23] A. Shraim, A. Diab, A. Alsuhaimi, E. Niazy, M. Metwally, M. Amad, S. Sioud, A. Dawoud, Analysis of some pharmaceuticals in municipal wastewater of Almadinah Almunawarah, Arab. J. Chem. 10 (2017) S719–S729. https://doi.org/10.1016/j.arabjc.2012.11.014.

[24] A. Rodayan, M. Majewsky, V. Yargeau, Impact of approach used to determine removal levels of drugs of abuse during wastewater treatment, Sci. Total Environ. 487 (2014) 731–739. https://doi.org/10.1016/j.scitotenv.2014.03.080.

[25] C.G. Daughton, T.A. Ternes, Pharmaceuticals and personal care products in the environment: agents of subtle change?, Environ. Health Perspect. 107 Suppl 6 (1999) 907–938. https://doi.org/10.1289/ehp.99107s6907.

[26] L.H.M.L.M. Santos, A.N. Araújo, A. Fachini, A. Pena, C. Delerue-Matos, M.C.B.S.M. Montenegro, Ecotoxicological aspects related to the presence of pharmaceuticals in the aquatic environment, J. Hazard. Mater. 175 (2010) 45–95. https://doi.org/10.1016/j.jhazmat.2009.10.100.

[27] E. Donner, T. Kosjek, S. Qualmann, K.O. Kusk, E. Heath, D.M. Revitt, A. Ledin, H.R. Andersen, Ecotoxicity of carbamazepine and its UV photolysis transformation products, Sci. Total Environ. 443 (2013) 870–876. https://doi.org/10.1016/j.scitotenv.2012.11.059.

[28] E. Yavuz Guzel, A. Atasoy Aydin, İ.E. Gören, N. Unuvar, N. Daglioglu, Estimation of anti-diabetes drug metformin in Turkiye using wastewater-based epidemiology, Drug Test. Anal. (2024). https://doi.org/10.1002/dta.3646.

[29] Y. Zhang, S.-U. Geissen, C. Gal, Carbamazepine and diclofenac: removal in wastewater treatment plants and occurrence in water bodies, Chemosphere 73 (2008) 1151–1161. https://doi.org/10.1016/j.chemosphere.2008.07.086.

[30] N. Anzar, S. Suleman, S. Parvez, J. Narang, A review on Illicit drugs and biosensing advances for its rapid detection, Process Biochem. 113 (2022) 113–124. https://doi.org/10.1016/j.procbio.2021.12.021.

[31] DECRETO DEL PRESIDENTE DELLA REPUBBLICA 9 ottobre 1990, n. 309 - Normattiva, (n.d.). https://www.normattiva.it/uri-

res/N2Ls?urn:nir:presidente.repubblica:decreto:1990-10-09;309 (accessed June 20, 2024).

[32] L.H. Restrepo-Vieira, F. Busetti, K.L. Linge, C.A. Joll, Development and validation of a direct injection liquid chromatography-tandem mass spectrometry method for the analysis of illicit drugs and psychopharmaceuticals in wastewater, J. Chromatogr. A 1685 (2022) 463562. https://doi.org/10.1016/j.chroma.2022.463562.

[33] L. Bijlsma, E. Emke, F. Hernández, P. de Voogt, Investigation of drugs of abuse and relevant metabolites in Dutch sewage water by liquid chromatography coupled to high resolution mass spectrometry, Chemosphere 89 (2012) 1399–1406. https://doi.org/10.1016/j.chemosphere.2012.05.110.

[34] E. Emke, D. Vughs, A. Kolkman, P. de Voogt, Wastewater-based epidemiology generated forensic information: Amphetamine synthesis waste and its impact on a small sewage treatment plant, Forensic Sci. Int. 286 (2018) e1–e7. https://doi.org/10.1016/j.forsciint.2018.03.019.

[35] A.K. McCall, R. Bade, J. Kinyua, F.Y. Lai , P.K. Thai, A. Covaci, L. Bijlsma, A.L.N. van Nuijs, C. Ort, Critical review on the stability of illicit drugs in sewers and wastewater samples, Water Res. 88 (2016). https://doi.org/10.1016/j.watres.2015.10.040.

[36] New psychoactive substances: challenges for drug surveillance, control, and public health responses - The Lancet. https://www.thelancet.com/journals/lancet/article/PIIS0140-6736(19)32231-7/fulltext.

[37] D. Baumeister, L.M. Tojo, D.K. Tracy, Legal highs: staying on top of the flood of novel psychoactive substances, Ther. Adv. Psychopharmacol. 5 (2015) 97–132. https://doi.org/10.1177/2045125314559539.

[38] S.M. Aarde, M.A. Taffe, Predicting the Abuse Liability of Entactogen-Class, New and Emerging Psychoactive Substances via Preclinical Models of Drug Self-administration, Curr. Top. Behav. Neurosci. 32 (2017) 145–164. https://doi.org/10.1007/7854_2016_54.

[39] A. Shafi, A.J. Berry, H. Sumnall, D.M. Wood, D.K. Tracy, New psychoactive substances: a review and updates, Ther. Adv. Psychopharmacol. 10 (2020) 2045125320967197. https://doi.org/10.1177/2045125320967197.

[40] New psychoactive substances – the current situation in Europe (European Drug Report 2024) | www.emcdda.europa.eu. https://www.emcdda.europa.eu/publications/european-drug-report/2024/new-psychoactive-substances_en.

[41] M. Massano, C. Incardona, E. Gerace, P. Negri, E. Alladio, A. Salomone, M. Vincenti, Development and validation of a UHPLC-HRMS-QTOF method for the detection of 132 New Psychoactive Substances and synthetic opioids, including fentanyl, in Dried Blood Spots, Talanta 241 (2022) 123265. https://doi.org/10.1016/j.talanta.2022.123265.

[42] Wastewater analysis and drugs — a European multi-city study | www.emcdda.europa.eu,. https://www.emcdda.europa.eu/publications/html/pods/waste-water-analysis_en.

[43] Agenda 2030, ONU Ital. (2024). https://unric.org/it/agenda-2030/ (accessed June 20, 2024).

[44] UNO, Obiettivo 3: Assicurare la salute e il benessere per tutti e per tutte le età, ONU Ital. (2019). https://unric.org/it/obiettivo-3-assicurare-la-salute-e-il-benessere-per-tutti-e-per-tutte-le-eta/.

[45] UNO, Obiettivo 6: Garantire a tutti la disponibilità e la gestione sostenibile dell'acqua e delle strutture igienico-sanitarie, ONU Ital. (2019). https://unric.org/it/obiettivo-6-garantire-a-tutti-la-disponibilita-e-la-gestione-sostenibile-dellacqua-e-delle-strutture-igienico-sanitarie/.

[46] Wastewater analysis and drugs — a European multi-city study | www.emcdda.europa.eu. https://www.emcdda.europa.eu/publications/html/pods/waste-water-analysis_en.

[47] S. Castiglioni, E. Zuccato, E. Crisci, C. Chiabrando, R. Fanelli, R. Bagnati, Identification and measurement of illicit drugs and their metabolites in urban wastewater by liquid chromatography-tandem mass spectrometry, Anal. Chem. 78 (2006) 8421–8429. https://doi.org/10.1021/ac061095b.

[48] SCORE Network – Bringing together experts and knowledge on wastewater-based epidemiology. https://score-network.eu/.

[49] S. Castiglioni, K.V. Thomas, B. Kasprzyk-Hordern, L. Vandam, P. Griffiths, Testing wastewater to detect illicit drugs: state of the art, potential and research needs, Sci. Total Environ. 487 (2014) 613–620. https://doi.org/10.1016/j.scitotenv.2013.10.034.

Chapter 3 | **Wastewater surveillance of 105 pharmaceutical drugs and metabolites by means of ultra-high-performance liquid-chromatography-tandem high resolution mass spectrometry**

Marta Massano[1,2*], Alberto Salomone[1,2], Enrico Gerace[2], Eugenio Alladio[1,2], Marco Vincenti[1,2], Marco Minella[1]

1: Department of Chemistry, University of Turin, Italy

2: Centro Regionale Antidoping, Orbassano (TO), Italy

** Corresponding author: Marta Massano*

Email address: marta.massano@unito.it

The content of this Chapter is included in one published manuscript:

Wastewater surveillance of 105 pharmaceutical drugs and metabolites by means of ultra-high-performance liquid-chromatography-tandem high resolution mass spectrometry

***Marta Massano**, Alberto Salomone, Enrico Gerace, Eugenio Alladio, Marco Vincenti, Marco Minella*

Journal of Chromatography A

https://doi.org/10.1016/j.chroma.2023.463896

Keywords: environmental monitoring; pharmaceutical drugs; UHPLC-QTOF-HRMS; waste-based epidemiology; contaminants of emerging concern

Abstract

Water pollution from pharmaceutical drugs is becoming an environmental issue of increasing concern, making water quality monitoring a crucial priority to safeguard public health. In particular, the presence of antidepressants, benzodiazepines, antiepileptics, and antipsychotics require specific attention as they are known to be harmful to aquatic biota. In this study, a multi-class comprehensive method for the detection of 105 pharmaceutical residues in small (30 mL) water samples was developed according to fit-for-purpose criteria and then applied to provide wide screening of samples obtained from four Wastewater Treatment Plants (WWTPs) in northern Italy. The filtered samples (0.22 µm filters) were extracted by SPE, and then eluted. 5 µL of the concentrated samples were analyzed by a UHPLC-QTOF-HRMS method validated for screening purposes. Adequate sensitivity was recorded for all target analytes, with limits of detection below 5 ng/L for 76 out of 105 analytes. A total of 23 out of the 105 targeted pharmaceutical drugs was detected in all samples. Several further compounds were detected over wide concentration intervals, ranging from ng/L to µg/L. In addition, the retrospective analysis of full-scan QTOF-HRMS data was exploited to carry out an untargeted screening of some drugs' metabolites. As a proof of concept, it was investigated the presence of the carbamazepine metabolites, which is among the most frequently detected contaminants of emerging concern in wastewater. Thanks to this approach, 10,11-dihydro-10-hydroxycarbamazepine, 10,11-dihydro-10,11-dihydroxycarbamazepine and carbamazepine-10,11-epoxide were identified, the latter requiring particular attention, since it exhibits antiepileptic properties similar to carbamazepine and potential neurotoxic effects in living organism.

3.1 Introduction

Contaminants of emerging concern (CECs) are chemical substances from anthropogenic origin present in the environment at trace and ultra-trace levels [1,2]. CECs usually refer to a wide range of substances classified as pesticides, pharmaceuticals, personal care products, flame retardants, hormones, *etc*., with pharmaceuticals and pesticides being most frequently detected due to their widespread use [3].

Among the pharmaceutical prescriptions, non-steroidal anti-inflammatory, cardiovascular, anti-depressant, and antipsychotic drugs are the most represented. Several active pharmaceutical ingredients and some of their metabolites are known to substantially or partially survive the conventional processes of

wastewater treatment because they are bio-recalcitrant to the most common microorganisms used in the civil waste water treatment plants (WWTPs). Furthermore, the hydrophobicity of these drugs also prevents their efficient partition on the solid phase used for water purification in the plant, or their incorporation into the spent bacterial sludge [4], increasing their spreading into the aquatic environments. Monitoring the water quality is therefore crucial to safeguard the human health and protect the environmental biota.

In recent years, the persistence of xenobiotics in wastewater has also led to the development of wastewater-based epidemiology (WBE), which emerged as an essential complementary methodology for the evaluation of pharmaceutical and illicit drugs prevalence in selected populations. Conventional methods to estimate the rate of drugs use in a community already exist and are based on self-reported surveys, overdose/toxicological reports, and drug-related crime statistics [5–7]. Traditional approaches such as self-reported surveys are commonly affected by high cost, delayed outcome, limited coverage, and biases from nonresponse and unbalanced selection of sampled populations, with higher prevalence of drug abusers. For these reasons, sewage epidemiology established itself as a comprehensive, real-time, and cost-effective approach to reliably measure the average drug consumption within a community. The quantification of either the parent drugs or their human-specific metabolites in wastewater [8,9] is increasingly adopted to complement other conventional methods for the estimation of drugs use/abuse in large communities.

While the existing analytical procedures are generally addressed to the determination of specific classes of pharmaceutical products, the present study is aimed to create an unprecedented method for the simultaneous determination of a panel of hazardous pharmaceutical drugs in wastewater. It combines an effective solid phase extraction (SPE) of the analytes from the matrix followed by their detection by ultra-high-pressure liquid-chromatography (UHPLC) and time-of-flight high resolution mass spectrometry (TOF-HRMS). This analytical method achieved the semi-quantitative determination of 105 pharmaceutical drugs and provided a qualitative identification of their main metabolites. The targeted analytes included 11 antidepressants, 15 antipsychotics, 5 antiepileptics, 26 benzodiazepines, 3 barbiturates, 7 cardiovascular drugs, 3 non-steroidal anti-inflammatory drugs, 9 analgesics, and other 26 pharmaceutical drugs from different classes. The method was applied to real samples collected from wastewater influents in Northern Italy.

The selection of chemical compounds for this study was based on the literature information about the pharmaceutical residues most frequently found in wastewater [10–12] and those consistently identified in biological samples, as suggested by our own experience [17]. Our procedure was also designed to reduce the volume of sample analyzed, moving from the 100-250 mL typically reported in the literature [14–16] to only 30 mL of water.

3.2 Materials and methods

3.2.1 Introduction Reagents and standards

The 105 pure standards of the targeted drugs were purchased from either LGC Promochem SRL (Milan, Italy) or Sigma-Aldrich (Milan, Italy). Methanol, formic acid, and acetonitrile were provided by Sigma-Aldrich (Milan, Italy). Ultra-pure water was obtained using a Milli-Q® UF-Plus apparatus (Millipore, Bedford, MA, USA). Stock standard solutions were stored at −20 °C until used. Three compounds were used as the internal standards (IS), including two isotopically marked molecules (cocaine-D3, nitrazepam-D5, and coumachlor). A working solution mixture was prepared by dilution in methanol containing all 105 reference substances at the final concentration 1 μg/mL. Lastly, an internal standard mixture working solution containing the three selected IS was prepared in methanol at the final concentration of 1 μg/mL.

3.2.2 Real sample collection

The real samples were collected as 24 h composite samples of the inlet wastewater from four wastewater treatment plants located in the North West of Italy. The automatic sampler carries out a sampling cycle of 24-hourly aliquots, 350 mL of wastewater every 60 minutes, every day. The sampled water is collected in a refrigerated container and a 1 L aliquot of composite water is transferred into a glass container. Since the treatment plants involved in the present study asked to remain anonymous, they are identified as Sites 1, 2, 3, and 4. The aliquots, once taken from the sampler, are collected in refrigerated 1 L glass bottles and stored at −20°C until the moment of analysis.

3.2.3 Sample preparation

The spiked samples used in the method development and validation were prepared from ultra-pure water (Milli-Q® UF-Plus) fortified at five concentration levels (5, 10, 25, 100 and 1000 ng/L) with the working solution mixture.

Wastewater samples (100 mL) were centrifuged at 4000 rpm for 5 min and vacuum-filtered through 0.22 μm filter device (Steriflip-GP 50mL, 22um, Merck Life Science Srl). Then, a 30 mL aliquot of filtered wastewater or standard solution was spiked with the internal standards mixture (final concentration 25 ng/L) and extracted using an Oasis HLB solid phase extraction (SPE) cartridge (200 mg, 6 cm^3, Waters, Milford, MA). SPE cartridges were conditioned with 5 mL methanol and 5 mL ultrapure water, loaded with the entire samples volume, left to dry under vacuum for 30 min and eluted with 10 mL methanol. The eluate was dried for 4 hours at 40°C using a vacuum concentrator (Thermo Scientific™ Savant™ SpeedVac™). The dry residue was reconstituted with 50 μL methanol, centrifuged for 5 min at 13,000 g, and 5 μL of the supernatant was injected into the UHPLC system.

3.2.4 UHPLC-QTOF-HRMS analysis

UHPLC separation was performed with a Phenomenex Kinetex C18 column (100 × 2.1 mm, 1.7 μm) maintained at 45 °C on the SCIEX ExionLC™ AC system. The mobile phases consisted of water (A) and acetonitrile (B), both mixed with formic acid 5 mM. The LC flow rate was set at 0.5 mL/min and the mobile phase eluted under the following linear gradient conditions (A:B, v:v): isocratic elution at 95:5 for 0.5 min, from 95:5 to 5:95 in 7.5 min, isocratic elution at 5:95 for 0.5 min and final re-equilibration at the initial conditions for 2.5 min. The total run time was 11 min. All analyses were performed using a quadrupole/time-of-flight SCIEX X500R QTOF mass spectrometer (Sciex, Darmstadt, Germany) equipped with a Turbo VTM electrospray ion source operating in both positive- and negative-ion modes. Data acquisition involved the collection of a preliminary TOF high-resolution full scan mass spectrum, followed by a SWATH™ acquisition protocol which used a variable window setup (16 windows covering mass range from m/z 130.0 to 520.0 at 0.025 resolving power). The identification of the 105 target analytes was based on the coincidence of their retention times, precursor ion and characteristic fragment ion m/z values (mass error accepted ≤5 ppb) with those of the corresponding pure standards. Furthermore, the adoption of HRMS with SWATH™ acquisition mode enabled a retrospective investigation of the dataset files aimed to detect unexpected and untargeted compounds, for example the metabolites of contaminants identified in certain wastewater specimen. The full list of the target analytes is reported in Table 1. The internal standards were selected based on our previous experience [16].

3.2.5 Validation

Specificity, sensitivity, recovery, and calibration model for the analytical method described above were investigated. Specificity was ensured every time the compound signal was correctly extracted and identified by the instrument without interferences. In this HRMS study, specificity was verified when the chromatographic peak detected at the expected retention time was associated to a molecular ion affected by a m/z error lower than 5 ppb with respect to the theoretical exact mass. Sensitivity for each target analyte was expressed by its LOD value. LOD values were experimentally tested by spiking the aqueous matrix with the target analytes at increasing concentration levels (5, 10, 15 ng/L) and verifying the minimal concentration at which the observed instrumental signal-to-noise ratio (S/N) was higher than 3. The extraction recovery was determined by comparing the experimental results obtained from three water samples spiked at the concentration level of 20 ng/L, before and after the extraction step.

Table 1: *List of the 105 substances under study (target analytes).*

Compound	Formula	Charge	Precursor theoretical m/z	Fragment theoretical m/z	Retention time, min	Internal Standard
Antidepressants						
Amitriptyline	$C_{20}H_{23}N$	$[M+H]^+$	278.1903	91.0545	4.22	Cocaine-D3
Bupropion	$C_{13}H_{18}ClNO$	$[M+H]^+$	240.1150	131.0721	3.19	Coumachlor
Citalopram	$C_{20}H_{21}FN_2O$	$[M+H]^+$	325.1711	109.0453	3.84	Cocaine-D3
Clonidine	$C_9H_9Cl_2N_3$	$[M+H]^+$	230.0246	212.9972	1.88	Cocaine-D3
Fluoxetine	$C_{17}H_{18}F_3NO$	$[M+H]^+$	310.1413	265.1630	4.45	Cocaine-D3
Fluvoxamine	$C_{15}H_{21}F_3N_2O_2$	$[M+H]^+$	319.1628	71.0509	4.10	Cocaine-D3
Mianserin	$C_{18}H_{20}N_2$	$[M+H]^+$	265.1699	208.1124	3.79	Nitrazepam-D5
Mirtazapine	$C_{17}H_{19}N_3$	$[M+H]^+$	266.1652	195.0915	2.90	Nitrazepam-D5
Paroxetine	$C_{19}H_{20}FNO_3$	$[M+H]^+$	330.1500	192.1187	3.99	Coumachlor
Sertraline	$C_{17}H_{17}Cl_2N$	$[M+H]^+$	306.0811	158.9765	4.44	Nitrazepam-D5
Trazodone	$C_{19}H_{22}ClN_5O$	$[M+H]^+$	372.1586	176.0804	3.30	Nitrazepam-D5

Benzodiazepines and analogues						
7-Aminoclonazepam	$C_{15}H_{12}ClN_3O$	$[M+H]^+$	286.0742	121.0757	2.77	Nitrazepam-D5
7-Aminoflunitrazepam	$C_{16}H_{14}FN_3O$	$[M+H]^+$	284.1194	135.0916	3.07	Nitrazepam-D5
7-Aminonitrazepam	$C_{15}H_{13}N_3O$	$[M+H]^+$	252.1131	121.0760	1.97	Nitrazepam-D5
Alprazolam	$C_{17}H_{13}ClN_4$	$[M+H]^+$	309.0902	281.0698	4.30	Nitrazepam-D5
Bromazepam	$C_{14}H_{10}BrN_3O$	$[M+H]^+$	316.0080	182.0836	3.68	Nitrazepam-D5
Brotizolam	$C_{15}H_{10}BrClN_4S$	$[M+H]^+$	392.9571	314.0395	4.51	Cocaine-D3
Chlordiazepoxide	$C_{16}H_{14}ClN_3O$	$[M+H]^+$	300.0898	227.0499	3.35	Nitrazepam-D5
Clobazam	$C_{16}H_{13}ClN_2O_2$	$[M+H]^+$	301.0738	259.0630	4.64	Nitrazepam-D5
Clonazepam	$C_{15}H_{10}ClN_3O_3$	$[M+H]^+$	316.0484	270.0562	4.23	Nitrazepam-D5
Clotiazepam	$C_{16}H_{15}ClN_2O_S$	$[M+H]^+$	319.0666	278.0570	4.92	Nitrazepam-D5
Delorazepam	$C_{15}H_{10}Cl_2N_2O$	$[M+H]^+$	305.0243	140.0264	4.58	Nitrazepam-D5
Demoxepam	$C_{15}H_{11}ClN_2O_2$	$[M+H]^+$	287.0581	241.1100	4.19	Nitrazepam-D5
Desalchilflurazepam	$C_{17}H_{15}Cl_FN_3O$	$[M+H]^+$	332.0960	140.0257	4.94	Nitrazepam-D5
Diazepam	$C_{16}H_{13}ClN_2O$	$[M+H]^+$	285.0789	154.0413	4.84	Nitrazepam-D5

Diclazepam	$C_{16}H_{12}Cl_2N_2O$	$[M+H]^+$	319.0399	227.0502	5.10	Nitrazepam-D5
Diltiazem	$C_{22}H_{26}N_2O_4S$	$[M+H]^+$	415.1686	178.0305	3.82	Cocaine-D3
Flunitrazepam	$C_{16}H_{12}FN_3O_3$	$[M+H]^+$	314.0936	268.0991	4.44	Nitrazepam-D5
Flurazepam	$C_{21}H_{23}ClFN_3O$	$[M+H]^+$	388.1586	315.0672	3.71	Nitrazepam-D5
Lorazepam	$C_{15}H_{10}Cl_2N_2O_2$	$[M+H]^+$	321.0192	275.0144	4.20	Nitrazepam-D5
Lormetazepam	$C_{16}H_{12}Cl_2N_2O_2$	$[M+H]^+$	335.0349	289.0286	4.62	Nitrazepam-D5
Midazolam	$C_{18}H_{13}ClFN_3$	$[M+H]^+$	326.0855	291.1152	3.64	Nitrazepam-D5
Nordiazepam	$C_{15}H_{11}ClN_2O$	$[M+H]^+$	271.0633	140.0256	4.37	Nitrazepam-D5
Oxazepam	$C_{15}H_{11}ClN_2O_2$	$[M+H]^+$	287.0582	241.0528	4.09	Nitrazepam-D5
Temazepam	$C_{16}H_{13}ClN_2O_2$	$[M+H]^+$	301.0738	255.0679	4.47	Nitrazepam-D5
Triazolam	$C_{17}H_{12}Cl_2N_4$	$[M+H]^+$	343.0512	308.0822	4.37	Nitrazepam-D5
Zolpidem	$C_{19}H_{21}N_3O$	$[M+H]^+$	308.1757	236.1287	3.18	Nitrazepam-D5
Zopiclone	$C_{17}H_{17}ClN_6O_3$	$[M+H]^+$	389.1123	245.0225	2.78	Nitrazepam-D5
Barbiturates						
Amobarbital	$C_{11}H_{18}N_2O_3$	$[M-H]^-$	225.1245	41.9986	3.89	Nitrazepam-D5

Barbital	$C_8H_{12}N_2O_3$	$[M-H]^-$	183.0775	68.9012	2.25	Nitrazepam-D5
Secobarbital	$C_{12}H_{18}N_2O_3$	$[M-H]^-$	237.1245	41.9985	4.09	Nitrazepam-D5
Antipsychotic						
Amisulpride	$C_{17}H_{27}N_3O_4S$	$[M+H]^+$	370.1795	242.0477	2.55	Cocaine-D3
Aripiprazole	$C_{23}H_{27}Cl_2N_3O_2$	$[M+H]^+$	448.1553	285.0899	4.23	Coumachlor
Carbamazepine	$C_{15}H_{12}N_2O$	$[M+H]^+$	237.1022	194.0949	3.90	Nitrazepam-D5
Chlorpromazine	$C_{17}H_{19}ClN_2S$	$[M+H]^+$	319.1030	86.0962	4.42	Coumachlor
Clozapine	$C_{18}H_{19}ClN_4$	$[M+H]^+$	327.1371	270.0794	3.52	Nitrazepam-D5
Haloperidol	$C_{21}H_{23}ClFNO_2$	$[M+H]^+$	376.1474	165.0697	3.95	Coumachlor
Levomepromazine	$C_{19}H_{24}N_2OS$	$[M+H]^+$	329.1682	100.1121	4.23	Cocaine-D3
Olanzapine	$C_{17}H_{20}N_4S$	$[M+H]^+$	313.1481	256.0893	2.19	Nitrazepam-D5
Periciazine	$C_{21}H_{23}N_3OS$	$[M+H]^+$	366.1635	142.1223	3.89	Nitrazepam-D5
Promazine	$C_{17}H_{20}N_2S$	$[M+H]^+$	285.1420	86.0975	3.93	Cocaine-D3
Quetiapine	$C_{21}H_{25}N_3O_2S$	$[M+H]^+$	384.1740	253.0795	3.63	Nitrazepam-D5
Risperidone	$C_{23}H_{27}FN_4O_2$	$[M+H]^+$	411.2191	191.1174	3.32	Nitrazepam-D5

Tiapride	$C_{15}H_{24}N_2O_4S$	$[M+H]^+$	329.1530	256.0615	1.98	Cocaine-D3
Venlafaxine	$C_{17}H_{27}NO_2$	$[M+H]^+$	278.2115	58.0656	3.25	Cocaine-D3
Ziprasidone	$C_{21}H_{21}ClN_4OS$	$[M+H]^+$	413.1197	194.0373	3.65	Cocaine-D3
Zuclopenthixol	$C_{22}H_{25}ClN_2OS$	$[M+H]^+$	401.1449	271.0339	4.61	Nitrazepam-D5
Antiepileptic						
Lamotrigine	$C_9H_7Cl_2N_5$	$[M+H]^+$	256.0151	210.9820	2.73	Nitrazepam-D5
Oxcarbazepine	$C_{15}H_{12}N_2O_2$	$[M+H]^+$	253.0972	180.0810	3.58	Nitrazepam-D5
Pregabalin	$C_8H_{17}NO_2$	$[M+H]^+$	160.1332	55.0547	1.78	Nitrazepam-D5
Valproic acid	$C_8H_{16}O_2$	$[M-H]^-$	143.1078	98,7310	4.63	Nitrazepam-D5
Cardiovascular Drugs						
Atenolol	$C_{14}H_{22}N_2O_3$	$[M+H]^+$	267.1703	145.0638	1.60	Cocaine-D3
Bisoprolol	$C_{18}H_{31}NO_4$	$[M+H]^+$	326.2326	116.1068	3.38	Nitrazepam-D5
Nebivolol	$C_{22}H_{25}F_2NO_4$	$[M+H]^+$	406.1824	151.0561	4.24	Nitrazepam-D5
Propafenone	$C_{21}H_{27}NO_3$	$[M+H]^+$	342.2064	116.1067	4.12	Nitrazepam-D5
Ramipril	$C_{23}H_{32}N_2O_5$	$[M+H]^+$	417.2384	234.1497	3.97	Cocaine-D3

Telmisartan	$C_{33}H_{30}N_4O_2$	$[M+H]^+$	515.2442	497.2324	4.54	Nitrazepam-D5
Verapamil	$C_{27}H_{38}N_2O_4$	$[M+H]^+$	455.2904	165.0906	4.23	Nitrazepam-D5
Non-steroidal anti-inflammatory Drugs						
Ibuprofen	$C_{13}H_{18}O_2$	$[M-H]^-$	205.1234	161.1330	5.41	Coumachlor
Ketoprofen	$C_{16}H_{14}O_3$	$[M+H]^+$	255.1016	105.0328	4.58	Coumachlor
Ketorolac	$C_{15}H_{13}NO_3$	$[M+H]^+$	256.0968	105.0334	4.11	Coumachlor
Analgesics / opioids						
Buprenorphine	$C_{29}H_{41}NO_4$	$[M+H]^+$	468.3108	414.2637	3.85	Cocaine-D3
Dihydrocodeine	$C18H23NO_3$	$[M+H]^+$	302.1751	199.0756	1.84	Cocaine-D3
Embutramide	$C_{17}H_{27}NO_3$	$[M+H]^+$	294.2064	121.0644	4.30	Coumachlor
Hydromorphone	$C_{17}H_{19}NO_3$	$[M+H]^+$	286.1438	185.0588	1.48	Nitrazepam-D5
Methadone	$C_{21}H_{27}NO$	$[M+H]^+$	310.2165	105.0328	4.26	Cocaine-D3
Oxycodone	$C_{18}H_{21}NO_4$	$[M+H]^+$	316.1543	241.1062	2.07	Cocaine-D3
Paracetamol	$C_8H_9NO_2$	$[M+H]^+$	152.0706	110.0604	1.53	Coumachlor
Phenacetin	$C_{10}H_{13}NO_2$	$[M+H]^+$	180.1019	110.0606	3.29	Cocaine-D3

Tapentadol	$C_{14}H_{23}NO$	$[M+H]^+$	222.1852	107.0488	2.90	Cocaine-D3
Tramadol	$C_{16}H_{25}NO_2$	$[M+H]^+$	264.1958	58.0656	2.91	Cocaine-D3
Others						
Atropine	$C_{17}H_{23}NO_3$	$[M+H]^+$	290.1751	124.1124	2.52	Cocaine-D3
Biperiden	$C_{21}H_{29}NO$	$[M+H]^+$	312.2322	98.0965	4.33	Coumachlor
Dextromethorphan	$C_{18}H_{25}NO$	$[M+H]^+$	272.2009	215.1416	3.59	Cocaine-D3
Diphenhydramine	$C_{17}H_{21}NO$	$[M+H]^+$	256.1696	167.0840	3.68	Cocaine-D3
Diphenidine	$C_{19}H_{23}N$	$[M+H]^+$	266.1903	181.0996	3.71	Cocaine-D3
Disulfiram	$C_{10}H_{20}N_2S_4$	$[M+H]^+$	297.0582	116.0526	5.90	Cocaine-D3
Glibenclamide	$C_{23}H_{28}ClN_3O_5S$	$[M+H]^+$	494.1511	369.0270	5.45	Nitrazepam-D5
Gliclazide	$C_{15}H_{21}N_3O_3S$	$[M+H]^+$	324.1376	127.1225	4.86	Cocaine-D3
Levamisole	$C_{11}H_{12}N_2S$	$[M+H]^+$	205.0794	178.0687	2.00	Nitrazepam-D5
Lidocaine	$C_{14}H_{22}N_2O$	$[M+H]^+$	235.1805	86.0965	2.49	Cocaine-D3
Loperamide	$C_{29}H_{33}ClN_2O_2$	$[M+H]^+$	477.2303	266.1544	4.82	Coumachlor
Metformin	$C_4H_{11}N_5$	$[M+H]^+$	130.1087	71.0602	0.61	Cocaine-D3

Methylphenidate	$C_{14}H_{19}NO_2$	$[M+H]^+$	234.1489	84.0808	2.86	Cocaine-D3
Metoclopramide	$C_{14}H_{22}ClN_3O_2$	$[M+H]^+$	300.1473	227.0586	2.72	Cocaine-D3
Naloxone	$C_{19}H_{21}NO_4$	$[M+H]^+$	328.1543	310.1432	1.87	Cocaine-D3
Oxybutynin	$C_{22}H_{31}NO_3$	$[M+H]^+$	358.2377	142.1232	4.54	Nitrazepam-D5
Phendimetrazine	$C_{12}H_{17}NO$	$[M+H]^+$	192.1383	146.0960	2.09	Cocaine-D3
Promethazine	$C_{17}H_{20}N_2S$	$[M+H]^+$	285.1420	86.0960	3.93	Cocaine-D3
Scopolamine	$C_{17}H_{21}NO_4$	$[M+H]^+$	304.1543	138.0901	2.03	Cocaine-D3
Sildenafil	$C_{22}H_{30}N_6O_4S$	$[M+H]^+$	475.2122	58.0648	3.74	Nitrazepam-D5
Tadalafil	$C_{22}H_{19}N_3O_4$	$[M+H]^+$	390.1448	268.1082	4.36	Nitrazepam-D5
Ticlopidine	$C_{14}H_{14}ClNS$	$[M+H]^+$	264.0608	125.0144	3.17	Cocaine-D3
Vardenafil	$C_{23}H_{32}N_6O_4S$	$[M+H]^+$	489.2279	299.1100	3.55	Nitrazepam-D5
Warfarin	$C_{19}H_{16}O_4$	$[M+H]^+$	309.1121	250.1561	3.95	Cocaine-D3

The large number of target analytes and the limited number of isotopically-labeled IS make the present method suitable for screening and semi-quantitative purposes. For the initial and approximate quantification of the pharmaceutical compounds detected in the real wastewater samples, ultra-pure water was fortified with the working solution mixture at five concentration levels (5, 10, 25, 100 and 1000 ng/L), using cocaine-d3, nitrazepam-d5 and coumachlor as internal standards (IS). From the resulting solutions, a calibration model was built for each analyte, in which each calibration point was obtained in triplicate, at the five selected concentration levels.

3.2.6 Untargeted investigation of hazardous metabolites

The presence of drugs and their metabolic products in wastewater is mainly due to the urinary excretion of the consuming subjects. Acquisition of full scan high resolution mass spectra provides the chance of carrying out delayed retrospective analyses to verify the presence of drug metabolites, not directly targeted in the initial screening. Carbamazepine was selected as a typical model compound for testing the HRMS and the SWATH^{TM} acquisition method potential in real samples, since it is one of the drugs most frequently detected in wastewater treatment plants and in water bodies with clear and demonstrated environmental toxicity [17–19]. The metabolic pattern of carbamazepine is well known [20] and it is shown in Figure 1. Biotransformation includes oxidation, hydroxylation and hydrolysis transformation. The expected metabolites were identified in the real water samples based on the fragmentation patterns and the exact m/z of both their precursor and the fragment ions.

3.3 Results and discussion

3.3.1 Method validation

The developed method proved adequate for the individual detection of 105 target analytes and 3 internal standards in only 30 mL sample. Treating a low sample volume, i.e. 30 mL instead of 100-250 mL typically used, allowed us to reduce the preliminary steps, the volume of extraction solvent, and the energy consumption, making the whole procedure more sustainable for the environment. Also, loading a smaller volume of samples may extend the usability of the SPE cartridges. The chromatographic run was completed in only 11 min, including the final re-equilibration time (2.5 min). The fast data acquisition for a large number of target compounds within a single run is in agreement with the efficiency requirement needed for routine application. As shown in Table 1, all compounds

eluted in the first 5.10 min. The total elution and acquisition time was extended to 7.5 min to investigate the potential presence of unknown metabolites and/or contaminants in the retrospective data screening for untargeted analytes. The adoption of HRMS with SWATH^{TM} acquisition mode enables a retrospective investigation of the dataset files aimed to detect unexpected and untargeted compounds, for example the metabolites of contaminants positively identified in certain wastewater specimen. Any time a contaminant of emerging concern is repeatedly detected in wastewater, the retrospective investigation becomes an effective tool to reconsider the data without the need of repeating the analysis on stored samples, often no more available.

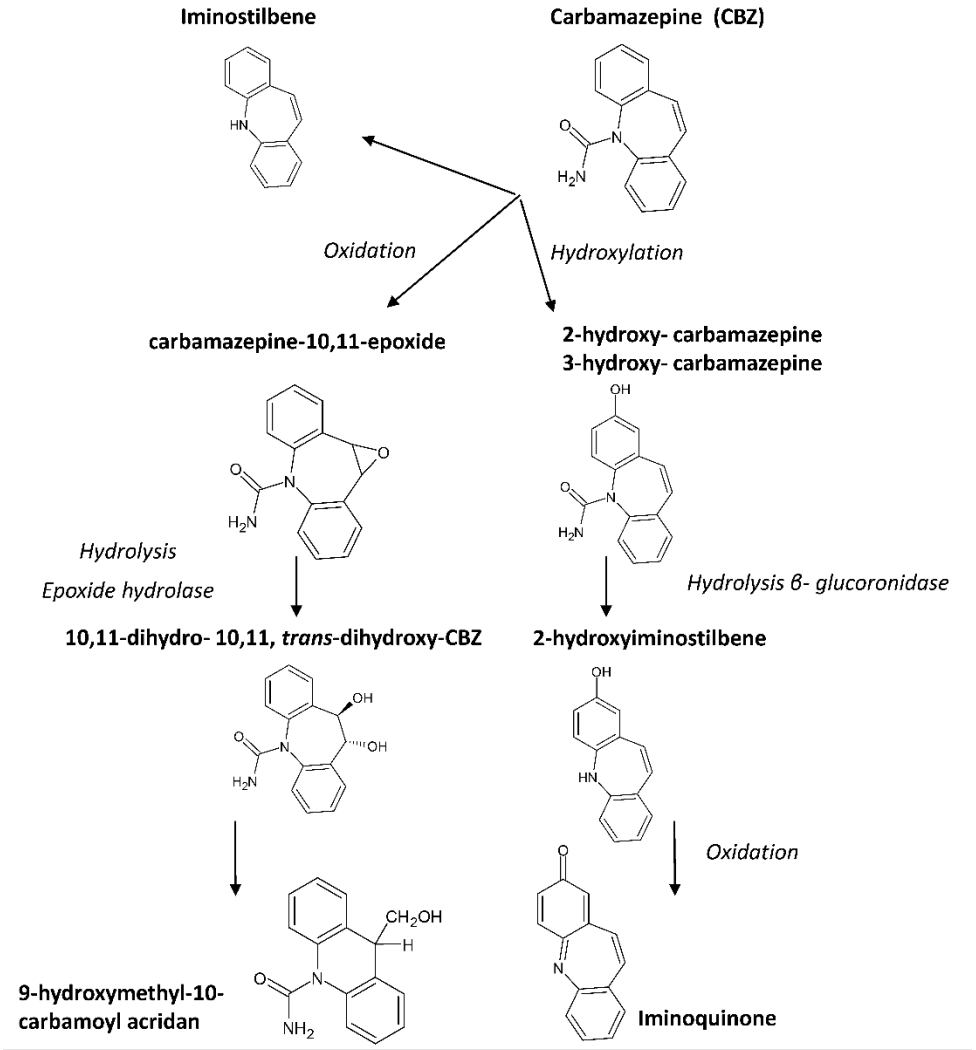


Figure 7 *Metabolic pathway of carbamazepine [23]*

This feature is of particular interest for the detection of metabolites. Furthermore, all coeluting substances could be quantified without interferences using the capability of high-resolution mass spectrometry that always provided significant differences in m/z values of precursor and characteristic fragment ion. In practice, all analytes were properly identified, with no interference in their signals and the specificity proved optimal, as each m/z peak showed a calculated mass error lower than 5 ppb.

The LOD, calibration, and recovery results obtained from the method validation experiments for the ultra-pure water samples fortified with 105 analytes and 3 internal standards are reported in the Tables S1 of the Supplementary Material. The LOD was verified by spiking pure water with decreasing concentrations (15, 10, 5 ng/L) until a response equivalent to three times the background noise was observed. This purely experimental process proved that for 76 analytes out of 105 (72%), a LOD lower than 5 ng/L was verified. For 13 analytes, the estimated LODs were between 5 and 10 ng/L, while the remaining 16 analytes (15%) showed LOD values between 10 and 15 ng/L. The estimated LODs are in agreement with the concentration ranges generally detected in wastewater and fully adequate for almost all the target analytes. In particular, the 5 ng/L limit represents the current lowest LOD value measured in several other studies [21–23].

The calibration curves were calculated from three replicates only, according to the semi-quantitative purpose of the present method and its application for screening a very large number of targeted and potentially untargeted analytes. For the same reason, application of the Mandel's test for non-linearity proved that the introduction of a quadratic term in the calibration curves, even when it improved the data-fitness, was not justified by the fit-for-purpose criteria followed in the present validation. Therefore, linear calibration was set up for all target analytes, whose ranges and equations are reported in Table S1.

The overall analytes' recovery was judged satisfactory, taking into account that several pre-concentration steps were involved in the procedure, including SPE and solvent evaporation of the extract, both leading to a potential loss of analytes. Recoveries higher than 70% was obtained for 62 out of 105 analytes and a recovery lower than 50% for 8 analytes only (Figure 2 & Table S1).

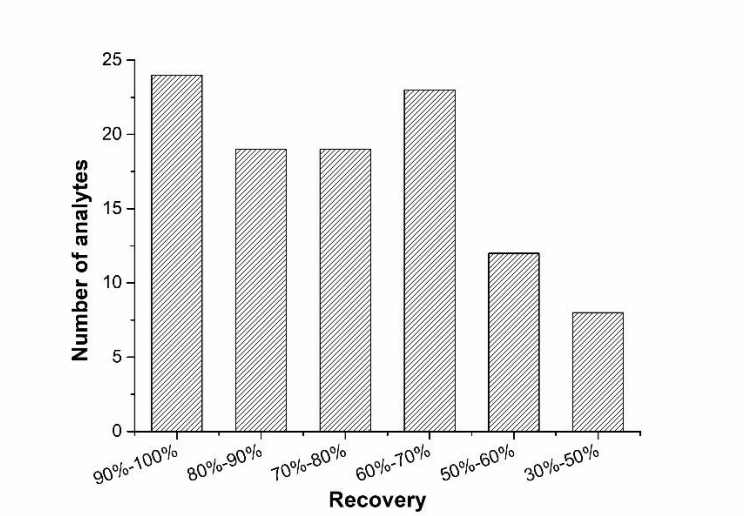


Figure 8 *Number of targeted analytes grouped by their percent recovery*.

3.3.2 Application to wastewater samples

The analytical method was applied to the analysis of inlet wastewater samples collected at four different urban wastewater treatment plants during year 2022. A total of 23 out of the 105 targeted pharmaceutical drugs was detected in almost all sites and limited differences were observed among several drugs arrays found at the different sampling sites. These homogeneous results suggest that similar drug prescriptions and consumption rates are uniformly distributed in northern Italy. Another possible reason for detecting some compounds rather than others may rely on their different physical and chemical properties (e.g., dissociation constants, partition coefficients, chemical stability) together with different metabolism and excretion kinetics.

Table 2 shows the average concentration of the pharmaceutical drugs found in the influent samples at the different WWTPs and Figure 3 shows an example of the extracted ion chromatogram (XIC) evidencing 10 of the pharmaceutical drugs found in a real sample of Site 4. The highest absolute concentration was detected for paracetamol (higher than 1 µg/L), which is currently consumed by a large percentage of general population. It is noteworthy that among the 20 best-selling active principle in Italy (according to the Federfarma 2021 report), several were identified, for example bisoprolol (concentration range 25-80 ng/L), nebivolol (found only in the site 1 at concentration higher than 60 ng/L) and ramipril (concentration range 10-25 ng/L), all belonging to the class of

cardiovascular drugs. Among these, also atenolol, propafenone, and telmisartan were consistently identified.

The classes of antidepressants, benzodiazepines, antiepileptics, and antipsychotic require particular attention as they are known to be harmful to the aquatic environments; for example, the effects of bioaccumulation of these active ingredients in fish include endocrine effects, developmental alteration and behavioral changes [24,25]. Among these, citalopram (concentration range 50-200 ng/L), lorazepam (concentration range 20-160 ng/L), trazodone (concentration range 5-20 ng/L) and carbamazepine (concentration range 100-600 ng/L) were detected in almost all samples. Particularly alarming is the case of tramadol (concentration range 40-215 ng/L) which is the active ingredient of several common opioid pain-relieving prescriptions. Recently in Italy, tramadol has also been classified among the illicit drugs, suggesting that its use is not restricted to medical therapies, but also abused for recreational purposes or misused for *off label* treatments.

The drug concentrations detected in 24-h representative samples are comparable or even higher than those observed in similar studies [26,27]. It is deduced that the total amounts of the screened pharmaceutical drugs released in the water acceptor bodies can be worryingly high, to the extent that constant monitoring may be required, particularly when scarce removal in the WWTP is expected.

Table 2 *Average concentration (ng/L) of the drugs found in the influent samples to the different WWTPs. The number of analyzed samples for each site is 2, 3, 4, and 6, for Site 1, 2, 3 and for 4, respectively.*

n.d. = not detected.

Compound	Site 1	Site 2	Site 3	Site 4
Antidepressants				
Bupropion	n.d	n.d	n.d	n.d
Citalopram	220	54	56	n.d
Mirtazapine	13	23	17	n.d
Trazodone	5	19	13	5
Benzodiazepine				

Lorazepam	29	76	160	24
Lormetazepam	9	75	160	9
Oxazepam	n.d	19	36	7
Temazepam	n.d	7	8	n.d
Antipsychotic				
Amisulpride	n.d	120	71	18
Carbamazepine	100	450	600	530
Quetiapine	n.d	39	22	11
Tiapride	n,d	n.d	5	n.d
Venlafaxine	n.d	> 1000	630	n.d
Antiepileptic				
Lamotrigine	n.d	350	860	n.d
Oxcarbazepine	n.d	380	200	n.d
Pregabalin	> 1000	n.d	n.d	n.d
Cardiovascular Drugs				
Atenolol	n.d	n.d	n.d	500
Bisoprolol	25	62	73	77
Nebivolol	68	n.d	n.d	n.d
Propafenone	220	95	44	30
Ramipril	17	26	n.d	n.d
Telmisartan	350	190	120	n.d
Non-steroidal anti-inflammatory Drugs				
Ketoprofen	320	48	420	900
Ketorolac	n.d	n.d	n.d	n.d
Analgesic/opioids				
Paracetamol	> 1000	n.d	≥ 1000	≥ 1000

Tapentadol	44	240	380	100
Tramadol	41	80	170	215
		Others		
Dextromethorphan	260	n.d	n.d	n.d
Gliclazide	32	18	180	n.d
Lidocaine	43	270	> 1000	82
Metoclopramide	n.d	18	19	n.d

For a plant with 100,000 inhabitants equivalent with a flow rate of about 24,000 m^3/day, it is possible to provide an estimate of the load (g/day) of a pharmaceutical drug arriving at a selected WWTP. Mass load of pharmaceutical drug residues can be determined using the following equation [28]:

$$Load\ (\frac{g}{day}) = concentration\ (\frac{ng}{L}) \times flow\ (\frac{\mathrm{L}}{\mathrm{day}}) \times \frac{100}{100 + stability(\%)} \times \frac{100}{100 - sorption\ (\%)} \times \frac{1}{10^9}$$

Taking tramadol and venlafaxine as model molecules and using the data reported in Table 3 (% of stability and sorption data were provided by the literature [29,30]), it is possible to calculate the mass of active ingredient present in the inlet wastewater entering the Site 3 treatment plant. The analytical results reported in Table 2 together with the water flow yield a mass load of about 10 g/day (≈ 4 Kg per year) for tramadol and about 20 g/day (≈ 7 Kg per year) for venlafaxine, respectively. These approximate calculations provide two important pieces of information: a) the total amounts of these drugs represent a significant threat to the survival and reproduction capabilities of living aquatic organisms [31,32] and b) highly efficient abatement procedures in the purification plants are needed to avoid significant release in the environment of pharmaceutical drugs.

Table 3 *Overview of parameters used in the sewage epidemiology calculations for each compound*

Compound	Concentration (ng/L)	Flow (L/day)	Stability (%) [a]	Sorption (%) [b]
Tramadol	380	2.40E+07	-11	1
Venlafaxine	630	2.40E+07	-20	0.4

[a] Stability change in raw wastewater at 19 °C after 12 h

[b] Average sorption to soil or sludge in collected wastewater samples

3.3.3 Untargeted screening for metabolites

A subsidiary scope of the present study was to verify if the analytical method based on full scan HRMS acquisition prove capable of identifying drug metabolites by means of untargeted screening strategies. Carbamazepine was selected as a model compound, due to its high environmental concern. In particular, previous studies have pointed out that certain metabolites raise as much concern for the aquatic environments as the corresponding parent drug [33–35]. The acquired data files were cross-examined in search of the expected metabolites [36]. The presence of 10,11-dihydro-10-hydroxycarbamazepine, 10,11-dihydro-10,11-dihydroxycarbamazepine, and carbamazepine-10,11-epoxide was instrumentally revealed and structurally characterized by the fragmentation pattern and exact mass of both their precursor and fragment ions (Table S2). In Figure 4, an example of the HRMS fragmentation pattern of one of the carbamazepine metabolites is reported.

Great attention was paid to the carbamazepine-10,11-epoxide as it is not only a metabolic oxidation product of carbamazepine, but also proved to possess antiepileptic properties similar to carbamazepine, possibly producing neurotoxic effects and having its own activity and environmental eco-toxicity [15]. The approximate concentration ratio between carbamazepine-10,11-epoxide and carbamazepine is higher than 3 in all analyzed samples (the signals intensity ratio between metabolite and precursor are reported in Table S3), suggesting a higher concentration of the metabolite with respect to the parent drug in wastewater. It is concluded that wastewater monitoring should include the most environmentally

relevant drug metabolites among the target analytes of acquisition and processing methods of analysis.

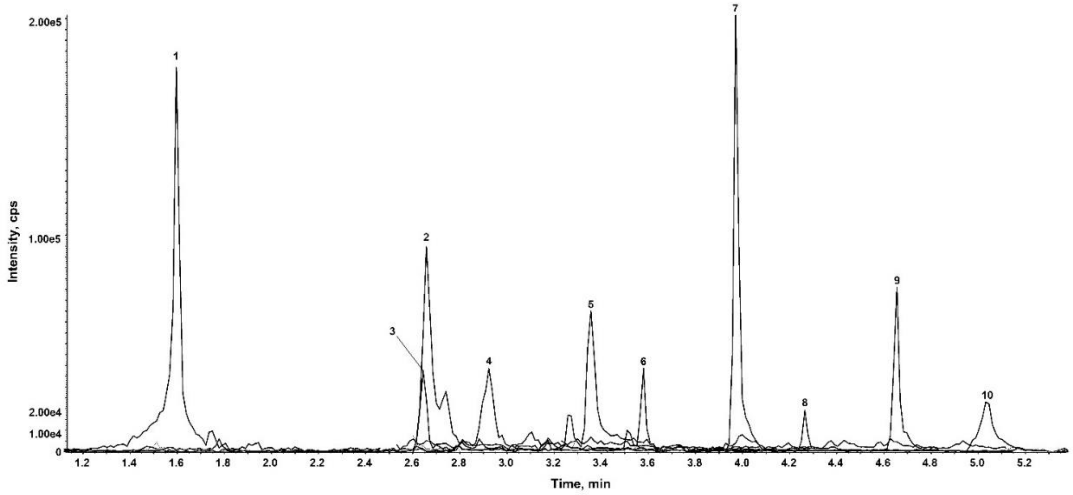


Figure 3 *Chromatographic profile of the 10 pharmaceutical drugs found in the Site 4 within a 1.2–5.2 min retention time interval. Extracted ion chromatograms (XICs) resulting from the optimized data acquisition method, built by the Scheduled Algorithm Pro in SCIEX OS Software. The numbered peaks correspond to: 1) Atenolol, 2) Tramadol, 3) Lidocaine, 4) Tapentadol, 5) Bisoprolol, 6) Amisulpride, 7) Carbamazepine, 8) Lorazepam, 9) Ketoprofen, and 10) Propafenone*

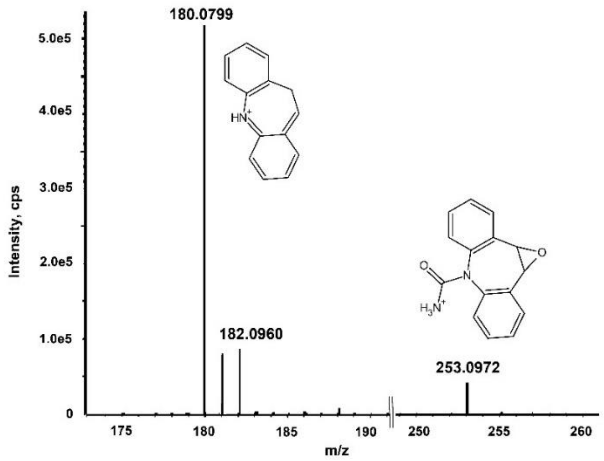


Figure 4 *HRMS fragmentation pattern of carbamazepine 10,11-epoxide*

3.4 Conclusions

The developed analytical method based on solid phase extraction of samples followed by UHPLC-QTOF-HRMS detection allowed the simultaneous quantification of 105 pharmaceuticals drugs and their metabolites in wastewater samples. The application of QTOF-HRMS technique allowed the combination of high-resolution full scan untargeted screening and targeted analysis, thus representing an effective method for fast and convenient environmental screening of drugs.

The collected data on the real samples are consistent with those available in the literature and confirm that many of the investigated pharmaceutical drugs are present in wastewater at a level that pose a health issue to the biota, considering also the increased risk associated with long-term simultaneous exposure to a mix of a large number of pharmaceutical products and their metabolites. In conclusion, the wastewater surveillance is essential not only to identify the pharmaceutical drugs used in the area, but also to monitor the purity of waters and the possible health risks for the inhabitants. In the future, analyses will be carried out i) to study the variation in the substances found over time and in the different territories at both intra- and inter-regional levels, ii) to evaluate the percentage abatement for the detected compounds in traditional WWTPs and, as a consequence, iii) to evaluate the real input of these CECs into the water acceptor bodies in term of total amount of released pollutants.

References

[1] J.C.G. Sousa, A.R. Ribeiro, M.O. Barbosa, M.F.R. Pereira, A.M.T. Silva, A review on environmental monitoring of water organic pollutants identified by EU guidelines, J. Hazard. Mater. 344 (2018) 146–162. https://doi.org/10.1016/j.jhazmat.2017.09.058.

[2] S.D. Richardson, T.A. Ternes, Water Analysis: Emerging Contaminants and Current Issues, Anal. Chem. 94 (2022) 382–416. https://doi.org/10.1021/acs.analchem.1c04640.

[3] A.B. Martínez-Piernas, P. Plaza-Bolaños, A. Gilabert, A. Agüera, Application of a fast and sensitive method for the determination of contaminants of emerging concern in wastewater using a quick, easy, cheap, effective, rugged and safe-based extraction and liquid chromatography coupled to mass spectrometry, J. Chromatogr. A 1653 (2021) 462396. https://doi.org/10.1016/j.chroma.2021.462396.

[4] S.D. Richardson, S.Y. Kimura, Water Analysis: Emerging Contaminants and Current Issues, Anal. Chem. 92 (2020) 473–505. https://doi.org/10.1021/acs.analchem.9b05269.

[5] C.J. Banta-Green, J.A. Field, A.C. Chiaia, D.L. Sudakin, L. Power, L. de Montigny, The spatial epidemiology of cocaine, methamphetamine and 3,4-methylenedioxymethamphetamine (MDMA) use: a demonstration using a population measure of community drug load derived from municipal wastewater, Addict. Abingdon Engl. 104 (2009) 1874–1880. https://doi.org/10.1111/j.1360-0443.2009.02678.x.

[6] A.G. Asimakopoulos, K. Kannan, Neuropsychiatric pharmaceuticals and illicit drugs in wastewater treatment plants: a review, Environ. Chem. 13 (2016) 541–576. https://doi.org/10.1071/EN15202.

[7] B. Subedi, K. Kannan, Mass loading and removal of select illicit drugs in two wastewater treatment plants in New York State and estimation of illicit drug usage in communities through wastewater analysis, Environ. Sci. Technol. 48 (2014) 6661–6670. https://doi.org/10.1021/es501709a.

[8] B. Subedi, Estimation of Community Usage of Drugs Utilizing Sewage Epidemiology, Methods Mol. Biol. Clifton NJ 1810 (2018) 141–147. https://doi.org/10.1007/978-1-4939-8579-1_14.

[9] L.H. Restrepo-Vieira, F. Busetti, K.L. Linge, C.A. Joll, Development and validation of a direct injection liquid chromatography-tandem mass spectrometry method for the analysis of illicit drugs and psychopharmaceuticals in wastewater, J. Chromatogr. A 1685 (2022) 463562. https://doi.org/10.1016/j.chroma.2022.463562.

[10] I. Pugajeva, J. Rusko, I. Perkons, E. Lundanes, V. Bartkevics, Determination of pharmaceutical residues in wastewater using high performance liquid chromatography coupled to quadrupole-Orbitrap mass spectrometry, J.

Pharm. Biomed. Anal. 133 (2017) 64–74. https://doi.org/10.1016/j.jpba.2016.11.008.
[11] D. Fatta-Kassinos, S. Meric, A. Nikolaou, Pharmaceutical residues in environmental waters and wastewater: current state of knowledge and future research, Anal. Bioanal. Chem. 399 (2011) 251–275. https://doi.org/10.1007/s00216-010-4300-9.
[12] J.O. Straub, Reduction in the environmental exposure of pharmaceuticals through diagnostics, Personalised Healthcare and other approaches. A mini review and discussion paper, Sustain. Chem. Pharm. 3 (2016) 1–7. https://doi.org/10.1016/j.scp.2015.12.001.
[13] K.S. Foppe, D.R. Hammond-Weinberger, B. Subedi, Estimation of the consumption of illicit drugs during special events in two communities in Western Kentucky, USA using sewage epidemiology, Sci. Total Environ. 633 (2018) 249–256. https://doi.org/10.1016/j.scitotenv.2018.03.175.
[14] S. Afsa, K. Hamden, P.A. Lara Martin, H.B. Mansour, Occurrence of 40 pharmaceutically active compounds in hospital and urban wastewaters and their contribution to Mahdia coastal seawater contamination, Environ. Sci. Pollut. Res. 27 (2020) 1941–1955. https://doi.org/10.1007/s11356-019-06866-5.
[15] A.M. Sulej-Suchomska, A. Klupczynska, P. Dereziński, J. Matysiak, P. Przybyłowski, Z.J. Kokot, Urban wastewater analysis as an effective tool for monitoring illegal drugs, including new psychoactive substances, in the Eastern European region, Sci. Rep. 10 (2020) 4885. https://doi.org/10.1038/s41598-020-61628-5.
[16] M. Vincenti, D. Cavanna, E. Gerace, V. Pirro, M. Petrarulo, D. Di Corcia, A. Salomone, Fast screening of 88 pharmaceutical drugs and metabolites in whole blood by ultrahigh-performance liquid chromatography-tandem mass spectrometry, Anal. Bioanal. Chem. 405 (2013) 863–879. https://doi.org/10.1007/s00216-012-6403-y.
[17] E. Donner, T. Kosjek, S. Qualmann, K.O. Kusk, E. Heath, D.M. Revitt, A. Ledin, H.R. Andersen, Ecotoxicity of carbamazepine and its UV photolysis transformation products, Sci. Total Environ. 443 (2013) 870–876. https://doi.org/10.1016/j.scitotenv.2012.11.059.
[18] M. Clara, B. Strenn, N. Kreuzinger, Carbamazepine as a possible anthropogenic marker in the aquatic environment: investigations on the behaviour of Carbamazepine in wastewater treatment and during groundwater infiltration, Water Res. 38 (2004) 947–954. https://doi.org/10.1016/j.watres.2003.10.058.
[19] E. De Laurentiis, S. Chiron, S. Kouras-Hadef, C. Richard, M. Minella, V. Maurino, C. Minero, D. Vione, Photochemical fate of carbamazepine in surface freshwaters: laboratory measures and modeling, Environ. Sci. Technol. 46 (2012) 8164–8173. https://doi.org/10.1021/es3015887.

[20] H. Breton, M. Cociglio, F. Bressolle, H. Peyriere, J.P. Blayac, D. Hillaire-Buys, Liquid chromatography-electrospray mass spectrometry determination of carbamazepine, oxcarbazepine and eight of their metabolites in human plasma, J. Chromatogr. B Analyt. Technol. Biomed. Life. Sci. 828 (2005) 80–90. https://doi.org/10.1016/j.jchromb.2005.09.019.
[21] S. Daouk, S. Fleury-Souverain, Y. Daali, Development of an LC-MS/MS Method for the Assessment of Selected Active Pharmaceuticals and Metabolites in Wastewaters of a Swiss University Hospital, CHIMIA 69 (2015) 684–684. https://doi.org/10.2533/chimia.2015.684.
[22] M. Gros, S. Rodríguez-Mozaz, D. Barceló, Rapid analysis of multiclass antibiotic residues and some of their metabolites in hospital, urban wastewater and river water by ultra-high-performance liquid chromatography coupled to quadrupole-linear ion trap tandem mass spectrometry, J. Chromatogr. A 1292 (2013) 173–188. https://doi.org/10.1016/j.chroma.2012.12.072.
[23] E. Gracia-Lor, M. Martínez, J.V. Sancho, G. Peñuela, F. Hernández, Multi-class determination of personal care products and pharmaceuticals in environmental and wastewater samples by ultra-high performance liquid-chromatography-tandem mass spectrometry, Talanta 99 (2012) 1011–1023. https://doi.org/10.1016/j.talanta.2012.07.091.
[24] T. Brodin, S. Piovano, J. Fick, J. Klaminder, M. Heynen, M. Jonsson, Ecological effects of pharmaceuticals in aquatic systems--impacts through behavioural alterations, Philos. Trans. R. Soc. Lond. B. Biol. Sci. 369 (2014) 20130580. https://doi.org/10.1098/rstb.2013.0580.
[25] J. Corcoran, M.J. Winter, C.R. Tyler, Pharmaceuticals in the aquatic environment: A critical review of the evidence for health effects in fish, Crit. Rev. Toxicol. 40 (2010) 287–304. https://doi.org/10.3109/10408440903373590.
[26] H. Ren, S. Yuan, J. Zheng, R. Luo, H. Qiang, W. Duan, Y. Zhao, P. Xiang, Direct injection ultra-performance liquid chromatography-tandem mass spectrometry for the high-throughput determination of 11 illicit drugs and metabolites in wastewater, J. Chromatogr. A 1685 (2022) 463587. https://doi.org/10.1016/j.chroma.2022.463587.
[27] A. Cardini, E. Pellegrino, L. Ercoli, Predicted and Measured Concentration of Pharmaceuticals in Surface Water of Areas with Increasing Anthropic Pressure: A Case Study in the Coastal Area of Central Italy, Water 13 (2021) 2807. https://doi.org/10.3390/w13202807.
[28] D.R. Baker, L. Barron, B. Kasprzyk-Hordern, Illicit and pharmaceutical drug consumption estimated via wastewater analysis. Part A: chemical analysis and drug use estimates, Sci. Total Environ. 487 (2014) 629–641. https://doi.org/10.1016/j.scitotenv.2013.11.107.
[29] D.R. Baker, B. Kasprzyk-Hordern, Critical evaluation of methodology commonly used in sample collection, storage and preparation for the

analysis of pharmaceuticals and illicit drugs in surface water and wastewater by solid phase extraction and liquid chromatography–mass spectrometry, J. Chromatogr. A 1218 (2011) 8036–8059. https://doi.org/10.1016/j.chroma.2011.09.012.

[30] D.R. Baker, B. Kasprzyk-Hordern, Multi-residue determination of the sorption of illicit drugs and pharmaceuticals to wastewater suspended particulate matter using pressurised liquid extraction, solid phase extraction and liquid chromatography coupled with tandem mass spectrometry, J. Chromatogr. A 1218 (2011) 7901–7913. https://doi.org/10.1016/j.chroma.2011.08.092.

[31] M. Buřič, K. Grabicová, J. Kubec, A. Kouba, I. Kuklina, P. Kozák, R. Grabic, T. Randák, Environmentally relevant concentrations of tramadol and citalopram alter behaviour of an aquatic invertebrate, Aquat. Toxicol. Amst. Neth. 200 (2018). https://doi.org/10.1016/j.aquatox.2018.05.008.

[32] M. Ziegler, H. Eckstein, H.-R. Köhler, S. Tisler, C. Zwiener, R. Triebskorn, Effects of the Antidepressants Citalopram and Venlafaxine on the Big Ramshorn Snail (Planorbarius corneus), Water 13 (2021) 1722. https://doi.org/10.3390/w13131722.

[33] J. Melin, A. Guillon, J. Enault, M. Esperanza, X. Dauchy, S. Bouchonnet, How to select relevant metabolites based on available data for parent molecules: Case of neonicotinoids, carbamates, phenylpyrazoles and organophosphorus compounds in French water resources, Environ. Pollut. Barking Essex 1987 265 (2020) 114992. https://doi.org/10.1016/j.envpol.2020.114992.

[34] A.S. Adeleye, J. Xue, Y. Zhao, A.A. Taylor, J.E. Zenobio, Y. Sun, Z. Han, O.A. Salawu, Y. Zhu, Abundance, fate, and effects of pharmaceuticals and personal care products in aquatic environments, J. Hazard. Mater. 424 (2022) 127284. https://doi.org/10.1016/j.jhazmat.2021.127284.

[35] D. Vione, L. Carena, The Possible Production of Harmful Intermediates Is the “Dark Side” Of the Environmental Photochemistry of Contaminants (Potentially Adverse Effects, And Many Knowledge Gaps), Environ. Sci. Technol. 54 (2020) 5328–5330. https://doi.org/10.1021/acs.est.0c01049.

[36] S. Shen, R. Elin, S. Soldin, Characterization of cross reactivity by carbamazepine 10,11-epoxide with carbamazepine assays, Clin. Biochem. 34 (2001) 157–8. https://doi.org/10.1016/S0009-9120(01)00186-2.

Supplementary materials

Table S2 *Results of LOD verified and Recovery (RE%)*

Compounds	LOD Verified (S/N>3) (ng/L)	Linear range tested (ng/L)	Equation	RE%
Antidepressants				
Amitriptyline	5	5-1000	$y = 2.27*10^{-2} x + 6.36*10^{-1}$	100
Bupropion	10	10-1000	$y = 6.12*10^{-3} x + 2,58*10^{-1}$	51
Citalopram	5	5-1000	$y = 5.22*10^{-4} x + 1.16*10^{-3}$	101
Clonidine	5	5-1000	$y = 5.04*10^{-3} x + 5.59*10^{-1}$	72
Fluoxetine	5	5-1000	$y = 7.79*10^{-3} x + 1.63*10^{-2}$	64
Fluvoxamine	15	25-1000	$y = 8.54*10^{-5} x + 8.10*10^{-3}$	58
Mianserin	15	25-1000	$y = 9.60*10^{-4} x + 1.00*10^{-2}$	86
Mirtazapine	5	5-1000	$y = 1.73*10^{-3} x + 3.18*10^{-3}$	63
Paroxetine	5	5-1000	$y = 5.79*10^{-4} x + 8.21*10^{-3}$	48
Sertraline	5	5-1000	$y = 2.00*10^{-3} x + 2.48*10^{-2}$	92
Trazodone	5	5-1000	$y = 1.94*10^{-3} x + 8.38*10^{-3}$	74
Benzodiazepines and analogues				
7-Aminoclonazepam	15	25-1000	$y = 4.24*10^{-4} x + 6.69*10^{-3}$	52
7-Aminoflunitrazepam	5	5-1000	$y = 8.39*10^{-4} x + 4.27*10^{-3}$	83
7-Aminonitrazepam	15	25-1000	$y = 7.55*10^{-4} x + 3.32*10^{-3}$	54
Alprazolam	5	5-1000	$y = 9.55*10^{-4} x + 1.55*10^{-4}$	94

Bromazepam	15	25-1000	$y = 1.16*10^{-3} x + 2.06*10^{-3}$	82
Brotizolam	5	5-1000	$y = 4.79*10^{-3} x + 1.82*10^{-2}$	74
Chlordiazepoxide	5	5-1000	$y = 1.93*10^{-3} x + 1.37*10^{-2}$	93
Clobazam	5	5-1000	$y = 1.35*10^{-3} x + 6.68*10^{-3}$	95
Clonazepam	5	5-1000	$y = 3.52*10^{-4} x - 3.36*10^{-3}$	94
Clotiazepam	10	10-1000	$y = 1.55*10^{-1} x - 2.15*10^{-3}$	78
Delorazepam	5	5-1000	$y = 4.65*10^{-4} x - 5.60*10^{-3}$	78
Demoxepam	5	5-1000	$y = 1.21*10^{-2} x + 6.04*10^{-3}$	70
Desalchilflurazepam	15	25-1000	$y = 1.55*10^{-4} x + 9.34*10^{-3}$	93
Diazepam	5	5-1000	$y = 5.38*10^{-4} x - 5.40*10^{-4}$	93
Diclazepam	5	5-1000	$y = 2.84*10^{-4} x - 2.63*10^{-4}$	77
Diltiazem	5	5-1000	$y = 5.68*10^{-4} x + 6.91*10^{-3}$	70
Flunitrazepam	5	5-1000	$y = 5.12*10^{-4} x - 6.81*10^{-3}$	100
Flurazepam	5	5-1000	$y = 5.03*10^{-3} x + 3.33*10^{-3}$	85
Lorazepam	5	5-1000	$y = 1.11*10^{-3} x + 2.49*10^{-4}$	91
Lormetazepam	5	5-1000	$y = 1.56*10^{-3} x + 1.21*10^{-2}$	82
Midazolam	5	5-1000	$y = 1.32*10^{-3} x - 2.19*10^{-3}$	94
Nordiazepam	5	5-1000	$y = 6.85*10^{-4} x - 8.25*10^{-3}$	83
Oxazepam	5	5-1000	$y = 2.18*10^{-3} x - 1.79*10^{-2}$	96
Temazepam	5	5-1000	$y = 2.81*10^{-3} x - 8.10*10^{-3}$	90
Triazolam	5	5-1000	$y = 1.01*10^{-3} x - 1.49*10^{-3}$	81
Zolpidem	5	5-1000	$y = 2.65*10^{-3} x - 3.38*10^{-3}$	90
Zopiclone	10	10-1000	$y = 1.63*10^{-4} x + 8.12*10^{-4}$	41
Barbiturates				

Amobarbital	15	25-1000	$y = 4.01*10^{-3} x -2.70*10^{-2}$	63
Barbital	15	25-1000	$y = 9.29*10^{-4} x -2.54*10^{-3}$	55
Secobarbital	10	10-1000	$y = 2.76*10^{-3} x +4.43*10^{-3}$	64
Antipsychotic				
Amisulpride	5	5-1000	$y = 5.50*10^{-4} x +9.46*10^{-3}$	67
Aripiprazole	15	25-1000	$y = 2.61*10^{-4} x -8.29*10^{-3}$	55
Carbamazepine	5	5-1000	$y = 3.61*10^{-3} x +2.74*10^{-2}$	82
Chlorpromazine	10	10-1000	$y = 1.38*10^{-1} x -7.01*10^{-2}$	54
Clozapine	5	5-1000	$y = 1.27*10^{-3} x -4.44*10^{-3}$	63
Haloperidol	5	5-1000	$y = 4.82*10^{-3} x +9.36*10^{-3}$	81
Levomepromazine	15	25-1000	$y = 1.48*10^{-4} x +3.51*10^{-4}$	86
Olanzapine	10	10-1000	$y = 7.48*10^{-4} x +2.16*10^{-4}$	33
Periciazine	5	5-1000	$y = 1.28*10^{-3} x +8.61*10^{-3}$	64
Promazine	10	10-1000	$y = 3.34*10^{-2} x -1.02*10^{-2}$	61
Quetiapine	5	5-1000	$y = 2.96*10^{-3} x +3.66*10^{-4}$	70
Risperidone	5	5-1000	$y = 2.65*10^{-3} x +2.65*10^{-4}$	62
Tiapride	5	5-1000	$y = 6.61*10^{-3} x +5.21*10^{-1}$	97
Venlafaxine	5	5-1000	$y = 8.91*10^{-3} x +4.64*10^{-2}$	67
Ziprasidone	5	5-1000	$y = 7.62*10^{-4} x -5.12*10^{-4}$	100
Zuclopenthixol	5	5-1000	$y = 3.69*10^{-2} x -1.41*10^{-2}$	50
Antiepileptics				
Lamotrigine	15	25-1000	$y = 3.62*10^{-5} x +1.71*10^{-3}$	69
Oxcarbazepine	5	5-1000	$y = 1.17*10^{-3} x -6.17*10^{-3}$	91
Tramadol	15	25-1000	$y = 2.22*10^{-2} x +1.40*10^{-2}$	33

Valproic acid	5	5-1000	$y = 2.86*10^{-4} x + 1.83*10^{-3}$	77
Cardiovascular Drugs				
Atenolol	5	5-1000	$y = 3.62*10^{-5} x + 3.31*10^{-3}$	46
Bisoprolol	5	5-1000	$y = 1.13*10^{-1} x - 2.11*10^{-2}$	57
Nebivolol	5	5-1000	$y = 4.92*10^{-2} x - 1.54*10^{-1}$	56
Propafenone	5	5-1000	$y = 1.47*10^{-3} x - 5.94*10^{-3}$	77
Ramipril	5	5-1000	$y = 1.70*10^{-2} x + 6.75*10^{-1}$	67
Telmisartan	5	5-1000	$y = 6.22*10^{-5} x - 5.69*10^{-4}$	86
Verapamil	5	5-1000	$y = 1.59*10^{-3} x - 5.89*10^{-3}$	88
Non-steroidal anti-inflammatory Drugs				
Ibuprofen	10	10-1000	$y = 1.18*10^{-4} x + 2.18*10^{-3}$	65
Ketoprofen	5	5-1000	$y = 7.93*10^{-4} x - 9.58*10^{-3}$	76
Ketorolac	5	5-1000	$y = 2.11*10^{-3} x + 2.19*10^{-3}$	87
Analgesics / opioids				
Buprenorphine	5	5-1000	$y = 8.48*10^{-4} x - 7.13*10^{-3}$	66
Dihydrocodeine	5	5-1000	$y = 2.50*10^{-4} x + 9.34*10^{-4}$	77
Embutramide	5	5-1000	$y = 3.19*10^{-3} x + 2.26*10^{-2}$	90
Hydromorphone	15	25-1000	$y = 8.14*10^{-4} x - 5,47*10^{-3}$	69
Methadone	5	5-1000	$y = 3.18*10^{-4} x + 5.74*10^{-3}$	101
Oxycodone	5	5-1000	$y = 4.84*10^{-3} x + 2.79*10^{-1}$	46
Paracetamol	10	10-1000	$y = 1.31*10^{-1} x + 5.91*10^{-1}$	80
Phenacetin	5	5-1000	$y = 2.67*10^{-4} x + 1.67*10^{-3}$	67
Tapentadol	5	5-1000	$y = 1.02*10^{-3} x + 8.77*10^{-3}$	90
Others				

Atropine	5	5-1000	$y = 3.84*10^{-4} x + 1.18*10^{-2}$	90
Biperiden	5	5-1000	$y = 2.64*10^{-1} x - 5.36*10^{-1}$	99
Dextromethorphan	5	5-1000	$y = 2.25*10^{-2} x + 7.42*10^{-1}$	83
Diphenhydramine	5	5-1000	$y = 1.01*10^{-3} x + 6.04*10^{-2}$	89
Diphenidine	5	5-1000	$y = 8.12*10^{-4} x - 6.76*10^{-3}$	74
Disulfiram	15	25-1000	$y = 1.04*10^{-4} x + 3.06*10^{-4}$	60
Glibenclamide	5	5-1000	$y = 1.67*10^{-2} x - 4.48*10^{-2}$	62
Gliclazide	5	5-1000	$y = 1.55*10^{-4} x + 1.78*10^{-2}$	83
Levamisole	10	10-1000	$y = 1.98*10^{-3} x - 1.77*10^{-3}$	70
Lidocaine	5	5-1000	$y = 2.38*10^{-2} x - 9.25*10^{-2}$	77
Loperamide	5	5-1000	$y = 4.76*10^{-3} x + 1.19*10^{-1}$	96
Metformin	15	25-1000	$y = 3.31*10^{-4} x + 9.36*10^{-2}$	56
Methylphenidate	5	5-1000	$y = 1.05*10^{-3} x - 9.88*10^{-3}$	86
Metoclopramide	5	5-1000	$y = 6.56*10^{-4} x + 8.94*10^{-2}$	75
Naloxone	5	5-1000	$y = 2.13*10^{-4} x + 1.50*10^{-2}$	36
Oxybutynin	5	5-1000	$y = 1.30*10^{-1} x - 1.26*10^{-1}$	85
Phendimetrazine	10	10-1000	$y = 1.10*10^{-4} x + 1.26*10^{-2}$	50
Promethazine	10	10-1000	$y = 3.29*10^{-2} x + 4.07*10^{-1}$	61
Scopolamine	5	5-1000	$y = 6.21*10^{-3} x + 2.76*10^{-1}$	39
Sildenafil	10	10-1000	$y = 7.63*10^{-2} x - 7.81*10^{-2}$	76
Tadalafil	5	5-1000	$y = 4.20*10^{-4} x + 4.60*10^{-4}$	63
Ticlopidine	15	25-1000	$y = 3.72*10^{-4} x - 5.31*10^{-3}$	61
Vardenafil	5	5-1000	$y = 5.93*10^{-3} x - 3.60*10^{-2}$	65
Warfarin	5	5-1000	$y = 1.05*10^{-2} x + 4.58*10^{-1}$	68

Table S2 *List of the metabolites of carbamazepine found in the samples and the details using for the qualitative identification*

Metabolite	Formula	Charge	Precursor theoretical m/z	Fragment theoretical m/z	Rt, min
10,11-Dihydro-10-hydroxycarbamazepine	$C_{15}H_{14}N_2O_2$	$[M+H]^+$	255.1128	194.0959	3.18
Carbamazepine 10,11-epoxide	$C_{15}H_{12}N2O_2$	$[M+H]^+$	253.0972	180.0806	2.93
10,11-Dihydro-10,11-dihydroxycarbamazepine	$C_{15}H_{14}N_2O_3$	$[M+H]^+$	271.1077	210.093	2.92

Table S3 *The ratio of the intensity of the signals detected between metabolite and precursor*

Sample origin	Ratio carbamazepine 10,11-epoxide / carbamazepine
Site 1	5
Site 2	4.4
Site 3	3.6
Site 4	3.4

Chapter 4 | **Development and application of a sustainable approach for the determination of 95 pharmaceutical substances and metabolites in urban wastewater by means of ultra-high-performance liquid-chromatography-tandem mass spectrometry**

Marta Massano[1*], Dana Privitera[1], Eugenio Alladio[1], Enrico Gerace[2], Marco Minella[1], Marco Vincenti[1,2], Alberto Salomone[1,2]

1: Department of Chemistry, University of Turin, Italy

2: Centro Regionale Antidoping, Orbassano (TO), Italy

** Corresponding author: Marta Massano*

Email address: marta.massano@unito.it

The content of this Chapter is included in one published manuscript:

Development and application of a sustainable approach for the determination of 95 pharmaceutical substances and metabolites in urban wastewater by means of ultra-high-performance liquid-chromatography-tandem mass spectrometry

Marta Massano, *Dana Privitera, Eugenio Alladio, Enrico Gerace, Marco Minella, Marco Vincenti, and Alberto Salomone*

INTERNATIONAL JOURNAL OF ENVIRONMENTAL ANALYTICAL CHEMISTRY
https://doi.org/10.1080/03067319.2024.2322098

Keywords: wastewater, pharmaceutical drugs, small-volume, UHPLC-MS/MS.

Abstract

In the last few years, the interest in pharmaceutical drugs and their metabolites as environmental pollutants is gaining growing importance. Regular monitoring and timely actions are decisive to implement appropriate water resource management strategies and to evaluate the efficiency of traditional or innovative wastewater treatment plants (WWTPs). This study describes the development and validation of an analytical procedure using 10 mL sample volume followed by direct-injection in ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) for the simultaneous determination of 95 pharmaceutical drugs and 10 of their main metabolites in wastewater. Adequate sensitivity was recorded for all target analytes, with limits of detection below 5 ng/L for 60 out of 95 analytes and recoveries exceeding 80% for all the analytes under study. A total of 42 target analytes were detected in almost all sites and limited differences were observed among several pharmaceutical drugs arrays found at the different sampling sites. In addition, an abatement yields higher than 50% was observed for only 12 of the 42 detected substances. The procedure, that combined direct injection and small sample volume collection, showed great potential and efficiency for the high-performance determination of pharmaceutical drugs in wastewater.

4.1 Introduction

Water resources are essential for human health, environmental sustainability and economic prosperity, making their pollution a major threat for humanity. Among the contaminants of emerging concern (CECs), pharmaceutical drugs and personal care products (PPCPs) play an increasing role [1]. The term PPCP comprises thousands of different chemical compounds, including prescription and over-the-counter drugs, veterinary drugs, perfumes, creams, diagnostic and nutraceutical agents, and many others [2]. Pharmaceuticals are present in the environment as an outcome of their production and formulation, and from patient use, food production, and improper disposal. Since they are designed to interact with a living system – namely, the human body - and produce a pharmacological response at low dose, their presence raises environmental concern even at low concentrations [3]. Among pharmaceutical prescriptions, non-steroidal anti-inflammatory, cardiovascular, anti-depressant and antipsychotic drugs are the most common. Several of these molecules are highly relevant because, once taken by humans, they are excreted unmodified or only partially metabolised, preserving a relevant pharmaceutical and biological activity in the environment.

Consequently, the environmental risk to wildlife and aquatic ecosystems is increased, with undesirable effects such as physiological, behavioural and reproduction alterations, and even death [4].

Although indication of pharmaceuticals and metabolites as potential environmental pollutants originated in the 1970s [5], only recently scientists have begun to actively address the impact of these pollutants on the environment and living organisms. In fact, it has been recently observed that the most common microorganisms used in civil wastewater treatment plants (WWTPs) are scarcely suitable for the biodegradation of various active pharmaceutical ingredients. The latter proved to be bio-recalcitrant for most microorganisms, allowing them to get through the conventional processes of wastewater treatment. The hydrophobicity of these drugs also prevents their efficient partition into the solid phase used in water purification plants, and/or their incorporation into the exhausted sludge [6], increasing their spread into the aquatic environments [7].

In recent years, the persistence of xenobiotics in wastewater also led to the development of wastewater-based epidemiology (WBE), which emerged as an essential complementary methodology for the evaluation of pharmaceutical and illicit drug prevalence in selected populations [8]. Regular monitoring and timely actions are decisive in developing appropriate water resource management strategies and pursuing the 17 UN Sustainable Development Goals (SDGs) (belonging to the 2030 Agenda for Sustainable Development) [9], in particular Goal 6 "*Ensuring the availability and management of all sustainable water and sanitation*" and Goal 3 "*Ensure healthy lives and promote well-being for all at all ages"*. Although no universal method is available to detect all pharmaceuticals reaching the ecosystem, several multi-analyte detection methods can be found in the literature [10]–[14]. However, the reported concentrations of the targeted compounds in wastewater samples proved to span from µg/L to ng/L [15],[16], establishing challenging limits of detection (LODs) and quantitation (LOQs) for the analytical methods devoted to their analysis. In order to achieve these low limits, most analytical methods use solid phase extraction (SPE) as a pre-concentration and purification technique, that removes matrix contaminants potentially capable of suppressing the analytes signal and decrease the sensitivity toward them [17]. However, SPE is time-consuming and involves the use of costly and environmentally-affecting consumables and additional solvents.

The present study aims to create a new method for the simultaneous determination of a wide panel of dangerous pharmaceutical drugs in wastewater using a sample preparation more in line with the principles of green chemistry

[18]. The procedure undertakes to eliminate, at least in the sample preparation steps, the use of any organic solvent and even the extraction of the analytes from the matrix, carrying out direct injection of the filtered sample into the chromatographic system, with consequent reduction of the collected sample volume. The proposed analytical method, based on ultra-high pressure liquid chromatography (UHPLC) coupled with triple quadrupole mass spectrometry (MS/MS), achieved the quantitative determination of 95 pharmaceutical drugs and 10 of their main metabolites. The list of analytes, already identified in a previous study [11] and selected from those most frequently detected in wastewater samples, included 10 antidepressants, 15 antipsychotics, 2 antiepileptics, 30 benzodiazepines, 7 cardiovascular drugs, 2 non-steroidal anti-inflammatory drugs, 10 analgesics, and other 19 pharmaceutical drugs from different classes. To our knowledge, no previous published studies reported the development of a direct-injection method for the analysis in urban wastewater of a large range of analytes in the ng/L range. A few published studies actually used the direct injection method, but for relatively small panels of pharmaceuticals [19],[20]. The method was then applied to real samples in order to prove the effective detection of the targeted analytes.

4.2 Materials and methods

4.2.1 Reagent and standards

The 95 pure standards of the targeted drugs were purchased from either LGC Promochem SRL (Milan, Italy) or Sigma-Aldrich (Milan, Italy) (purity ≥ 99%, concentration 1 mg/mL). Methanol, hydrochloric acid, formic acid, ammonium formate and acetonitrile were provided by Sigma-Aldrich (Milan, Italy) (purity ≥ 99%). Ultra-pure water was obtained using a Milli-Q® UF-Plus apparatus (Millipore, Bedford, MA, USA). The standard solutions were stored at −20 °C. Four compounds were used as the internal standards (IS), including cocaine-D3, nitrazepam-D5, coumachlor, and m-CPP-D8. A working solution mixture was diluted in methanol containing all 95 reference substances (the full list of the target analytes is presented in Table 1) at the final concentration of 1 μg/mL. Also, the internal standard solution, containing the four selected IS, was prepared in methanol at the final concentration of 1 μg/mL. The reagents, the rationale for the choice of the proper internal standards and the list of analytes used can be referred to those chosen in our previous study [11]. In particular, the internal standards chosen (although not coinciding with the analytes under study) showed characteristics suitable for the purpose

4.2.2 Real sample collection

Wastewater samples were collected at the inlet and outlet of three wastewater treatment plants located in north-western Italy. 350 mL of water were collected every 60 minutes (24-hour composite) in a refrigerated container. 1 L of each composite water sample was transferred into a glass container and stored at −20 °C until the analysis. As the study aimed to investigate the method performance on real samples and carry out an initial exploratory analysis of the pharmaceutical drugs present in the wastewater, only two samples (inlet and outlet) per plant were collected and the samples were analysed in duplicate. To preserve the anonymity of the treatment plants involved in the present study, they were identified as Sites 1, 2, and 3.

Table 3: *List of the 95 analytes, the monitored transitions, their instrumental parameters and the related internal standard. For each group, analytes are listed in alphabetical order. CE = Collision Energy*

Compound	Relative retention time, min	Precursor mass Q1 m/z	Fragments mass Q3 m/z	CE, V	Internal Standard
Antidepressants					
Amitriptyline	3.99	278.0	233.1	24	Cocaine-D3
			90.9	32	
Citalopram	3.47	325.0	262.0	26	Cocaine-D3
			109.1	34	
Clonidine	1.34	230.0	213.0	34	Cocaine-D3
			123.9	57	
Fluoxetine	4.12	310.1	148.2	12	Cocaine-D3
			44.1	44	
Fluvoxamine	3.89	319.1	200.1	20	Cocaine-D3
			71.0	35	
m-CPP	2.8	197.1	154.0	30	m-CPP-D8

			118.0	44	
Mianserin	3.38	265.0	208.1	27	Nitrazepam-D5
			58.1	45	
Mirtazapine	2.49	266.0	195.0	36	Nitrazepam-D5
			72.0	26	
Paroxetine	3.73	329.9	192.0	29	Coumachlor
			123.1	33	
Trazodone	3.36	372.1	176.0	50	Nitrazepam-D5
			148.0	48	
Benzodiazepines and analogues					
3-OH-Bromazepam	3.42	334.0	286.9	25	Nitrazepam-D5
			288.9	27	
7-Aminoclonazepam	2.81	286.1	121.1	39	Nitrazepam-D5
			222.2	35	
7-Aminoflunitrazepam	3.16	284.0	135.2	35	Nitrazepam-D5
			227.0	35	
7-Aminonitrazepam	1.94	252.0	120.9	35	Nitrazepam-D5
			94.0	48	
Alprazolam	4.6	309.0	280.9	36	Nitrazepam-D5
			205.1	56	
Bromazepam	3.98	318.0	182.2	42	Nitrazepam-D5
			182.0	43	
Brotizolam	5.09	395.0	314.0	32	Cocaine-D3
			314.0	32	

Chlordiazepoxide	3.47	300.1	227.1	31	Nitrazepam-D5
			283.2	21	
Clobazam	5.24	301.1	259.1	29	Nitrazepam-D5
			224.2	47	
Clonazepam	4.75	316.0	269.7	34	Nitrazepam-D5
			214.1	51	
Clotiazepam	5.46	319.0	291.0	30	Nitrazepam-D5
			154.0	38	
Delorazepam	5.26	304.9	139.9	39	Nitrazepam-D5
			242.1	37	
Demoxepam		287.0	180.0	33	Nitrazepam-D5
	3.99		242.3	47	
Desalchilflurazepam	5.02	289.0	139.9	38	Nitrazepam-D5
			226.0	38	
Diazepam	5.62	285.0	154.0	36	Nitrazepam-D5
			193.1	43	
Diclazepam	5.57	319.0	227.0	44	Nitrazepam-D5
			154.1	38	
Flunitrazepam		314.0	268.2	36	Nitrazepam-D5
	5		239.1	47	
Flurazepam	3.43	390.0	316.9	26	Nitrazepam-D5
			316.9	34	
Lorazepam	4.51	323.0	274.8	31	Nitrazepam-D5
			277.0	34	

Lormetazepam	5	337.0	288.9	30	Nitrazepam-D5
			291.0	28	
Midazolam	3.4	325.9	291.0	38	Nitrazepam-D5
			249.1	50	
Nordiazepam	4.74	271.0	140.0	37	Nitrazepam-D5
			208.0	38	
OH-Midazolam	3.65	342.1	323.9	30	Nitrazepam-D5
			203.1	37	
Oxazepam	4.38	287.0	231.0	30	Nitrazepam-D5
			269.0	19	
Temazepam	5.14	301.0	255.1	31	Nitrazepam-D5
			257.2	27	
Triazolam	4.93	343.0	308.0	37	Nitrazepam-D5
			314.9	39	
Zolpidem	3.11	308.1	92.0	63	Nitrazepam-D5
			220.1	60	
Zolpidem metabolite	2.15	338.1	265.2	52	Nitrazepam-D5
			293.1	38	
Zopiclone	2.37	389.0	245.0	25	Nitrazepam-D5
			246.9	25	
Antipsychotic					
Amisulpride	2.33	370.0	195.9	55	Cocaine-D3
			112.0	33	
Aripiprazole	3.97		284.9	36	Coumachlor

		449.9	287.1	34	
Carbamazepine	4.36	237.0	194.1	26	Nitrazepam-D5
			193.1	46	
Chlorpromazine	4.15	319.0	86.1	35	Coumachlor
			58.0	63	
Clozapine	3.2	326.9	270.0	31	Nitrazepam-D5
			192.1	57	
Haloperidol	3.95	376.0	123.0	53	Coumachlor
	3.59		164.9	33	
Levomepromazine	3.97	329.0	100.0	25	Cocaine-D3
			58.1.0	53	
Olanzapine	1.55	313.0	256.1	33	Nitrazepam-D5
			198.0	52	
Periciazine	3.63	366.0	142.1	30	Nitrazepam-D5
			114.0	37	
Promazine	3.65	285.1	86.1	24	Cocaine-D3
			58.1	57	
Quetiapine	3.36	384.1	253.1	31	Nitrazepam-D5
			279.1	34	
Risperidone	3.26	411.1	191.1	40	Nitrazepam-D5
			110.0	66	
Tiapride	1.46	329.2	256.1	45	Cocaine-D3
			213.1	60	
Venlafaxine	3.29	278.1	58.0	22	Cocaine-D3

			260.2	17	
Ziprasidone	3.65	413.1	166.0	41	Cocaine-D3
			177.0	37	
Antiepileptic					
Lamotrigine	2.26	255.9	144.9	47	Nitrazepam-D5
			211.0	36	
Oxcarbazepine	3.85	252.9	208.0	28	Nitrazepam-D5
			180.1	40	
Cardiovascular Drugs					
Atenolol	1.1	267.1	145.0	33	Cocaine-D3
			190.3	26	
Bisoprolol	3.34	326.2	116.1	35	Nitrazepam-D5
			74.1	38	
Nebivolol	3.98	406.2	151.1	35	Nitrazepam-D5
			123.0	38	
Propafenone	3.86	342.1	116.1	35	Nitrazepam-D5
			98.1	38	
Ramipril	3.9	417.2	234.1	29	Cocaine-D3
			91.1	86	
Telmisartan	4.76	515.2	497.0	47	Nitrazepam-D5
			276.0	62	
Verapamil	3.98	455.2	165.1	28	Nitrazepam-D5
			303.1	35	
Non-steroidal anti-inflammatory Drugs					

Ketoprofen	5	255.0	209.1	19	Coumachlor
			104.9	32	
Ketorolac	4.41	256.2	77.0	62	Coumachlor
			105.0	25	
Analgesics / opioids					
Buprenorphine	3.88	468.3	55.1	95	Cocaine-D3
			396.2	53	
Dihydrocodeine	1.35	302.2	199.1	42	Cocaine-D3
			128.1	75	
Embutramide	4.93	294.0	121.0	34	Coumachlor
			191.0	22	
Hydromorphone	0.85	286.1	185.1	39	Nitrazepam-D5
			157.1	53	
Methadone	4.01	310.0	265.1	20	Cocaine-D3
			105.0	33	
Oxycodone	1.5	316.0	241.0	38	Cocaine-D3
			256.1	35	
Paracetamol	1.5	152.0	110.0	22	Coumachlor
			65.0	39	
Phenacetin	3.54	180.0	110.1	29	Cocaine-D3
			138.0	21	
Tapentadol	2.84	222.1	107.0	40	Cocaine-D3
			121.0	26	
Tramadol	2.43	264.1	58.1	46	Cocaine-D3

			42.2	103	
Others					
Atropine	2.09	290.1	93.0	38	Cocaine-D3
			124.1	32	
Biperiden	3.9	312.0	98.1	31	Coumachlor
			70.3	68	
Dextromethorphan	3.26	272.2	171.1	50	Cocaine-D3
			215.1	34	
Diltiazem	3.59	415.0	178.0	32	Cocaine-D3
			150.0	58	
Diphenhydramine	3.37	256.1	167.0	20	Cocaine-D3
			165.1	54	
Diphenidine	3.33	266.1	181.1	24	Cocaine-D3
			102.9	48	
Gliclazide	5.37	324.1	127.1	22	Cocaine-D3
			110.1	41	
Levamisole	1.47	204.9	178.1	30	Nitrazepam-D5
			123.0	39	
Lidocaine	2.03	235.1	86.1	27	Cocaine-D3
			58.0	47	
Loperamide	4.53	477.0	266.1	34	Coumachlor
			210.1	64	
Metformin	0.48	130.1	71.2	26	Cocaine-D3
			60.0	19	

Methylphenidate	2.45	234.2	84.3	24	Cocaine-D3
			56.1	63	
Metoclopramide	2.25	299.9	227.0	26	Cocaine-D3
			184.0	41	
Naloxone	1.38	328.1	310.0	28	Cocaine-D3
			253.0	35	
Oxybutynin	4.22	358.0	124.0	27	Nitrazepam-D5
			72.1	52	
Phendimetrazine	1.65	192.0	148.1	28	Cocaine-D3
			115.0	44	
Promethazine	3.62	285.1	198.1	35	Cocaine-D3
			240.2	38	
Scopolamine	1.59	304.1	138.1	27	Cocaine-D3
			156.1	22	
Ticlopidine	2.82	264.1	154.0	24	Cocaine-D3
			88.9	77	
Warfarin	5.47	309.1	163.1	35	Cocaine-D3
			121.0	48	

4.2.3 Sample preparation

The spiked samples used in the method development and validation were prepared from ultra-pure water (Milli-Q® UF-Plus) fortified at six concentration levels (5-1000 ng/L) with the working solution mixture and the internal standards mixture (final concentration 25 ng/L). Wastewater samples (50 mL) were centrifuged at 4000 rpm for 5 min and vacuum-filtered through a 0.22 μm filter device (Steriflip-GP 50 mL, 22 μm, Merck Life Science Srl). Then, 10 mL of filtered wastewater was spiked with the internal standards mixture (final

concentration 25 ng/L) and added with a mixture of ammonium formate (2 mmol/L) and formic acid (0.02%). The samples were then vortexed, centrifuged for 10 min at 13,000 g, and 7 μL of the supernatant was injected into the UHPLC system.

4.2.4 Instrumentation

UHPLC separation was performed using a Phenomenex Kinetex C18 column (100 × 2.1 mm, 1.7 μm) maintained at 45 °C on the SCIEX ExionLCTM AC system. The mobile phases consisted of water (A) and acetonitrile (B), both mixed with formic acid 5 mM. The LC flow rate was set at 0.5 mL/min and the mobile phase eluted under the following linear gradient conditions (A:B, v:v): isocratic elution at 95:5 for 0.2 min, from 95:5 to 30:70 in 6.8 min, from 30:70 to 0:100 in 0.9 min, isocratic elution at 0:100 for 1 min and final re-equilibration at the initial conditions for 1.1 min. The total run time was 10 min. All analyses were performed using a mass spectrometer equipped with a quadrupole trap SCIEX triple Quad^{TM} 7500 mass spectrometer (Sciex, Darmstadt, Germany) system equipped with an OptiFlow Pro ion source with an analytical probe and E Lens. Electrospray ionization (ESI) was operated in the positive ion mode for all the analytes. For each transition, focusing voltages and collision energy (CE) were optimized under continuous analyte infusion. A single acquisition method consisting of 194 Multiple Reaction Monitoring (MRM) transitions (190 for the pharmaceutical drugs and 4 for the internal standards) was created using the Scheduled MRM programming in SCIEX OS software 2.0. Two MRM transitions were monitored for each target analyte. The full list of the target analytes, the transitions monitored and the corresponding instrumental parameters are reported in Table 1. The internal standards were selected on the basis of previous experience [21]. Since no extraction nor purification of the matrix (excluding filtration with 0.22 μm filter) was executed prior to injection, a control sample (mixture of the 95 analytes in water, concentration 100 ng/L) was injected at each analytical session to check the stability of the data acquired by the instrument.

4.2.5 Method validation

The validation strategy was based on the protocol recently published [22]. Each standard of ultra-pure water (Milli-Q® UF-Plus) fortified at six concentration levels (six concentration levels out of the following: 5, 10, 25, 50, 100, 500, 750, and 1000 ng/L, the lowest point of the curve was chosen according to the LOD of each analyte) was analyzed nine times in three working sessions (i.e., 3 replicates × 3 days × 6 calibrators) over ten days. This dataset of 54

analyses formed the groundwork on which the statistical evaluation of several validation parameters was founded, including calibration, intra- and inter-day trueness (expressed as bias%), repeatability and intermediate precision (expressed as percentage variation coefficient, CV%) (at 6 concentration levels), limit of detection (LOD), limit of quantification (LOQ). Recovery and stability parameters were evaluated with further independent experiments. An *ad hoc* Excel® sheet was built in-house to adapt the routine developed by Desharnais et al. [23]. All the equations employed to compute the validation parameters are reported elsewhere [24].

4.2.5.1. Validation parameters determination

Detailed explanation of the protocol used to estimate the different validation parameters can be found in a previously published article [25]. Briefly, the calibration curves were generated for each analyte from the peak-area ratio recorded between the analyte and the IS on the quantifier transition; the ratio was then plotted on the y-axis against the nominal analyte concentration to generate and estimate the curve that best fits the data distribution, with the support of statistical tests [22]–[26]. Heteroscedasticity was evaluated on the distribution of data points by comparing their variance at low, intermediate and high concentration levels. The order of each calibration model (linear vs. quadratic) was decided on the basis of Mandel's and lack-of-fit tests, supported by residual plot checking. The LOD was calculated from the linear portion of the calibration curve using the weighted Hubaux-Vos [27] method. LOD values were experimentally confirmed by spiking water with the target analytes at about the estimated LOD concentrations and verifying that the signal-to-noise ratio (S/N) was higher than 3.

The *recovery* was determined by comparing the experimental results obtained from three water samples fortified at two concentration levels 10 and 50 ng/L (selected to include analytes with both high and low LOD) with the working solution mixture, before and after the addition of the mixture of ammonium formate (2 mmol/L) and formic acid (0.02%). The *stability* was evaluated on separated water samples spiked with the analytes at 500 ng/L and stored for 1, 3, and 7 days at the temperatures of − 20 °C, 4 °C, and 25 °C, at pH 2 and 7, respectively. The choice of the concentration level (500 ng/L) was established with the aim of observing how and which of the factors under study (temperature, time and pH) affected the stability of the analytes during the sample storage period under conditions that allowed to detect all analytes in a single analysis. Acidic conditions (pH 2) were selected following literature reports stating that

acidification with hydrochloric acid increases the analytes stability in water [28] and simulate the reasonable conditions under which the samples can be transferred to the laboratory and stored before their analysis. Student t-test was performed on the collected data to evaluate the effect of the pH (pH =2 vs. pH =7), while ANOVA test was employed to evaluate the differences among the selected levels in terms of time (1 day, 3 days and 7 days) and temperatures (-20°C, 4°C and room temperature). pH 7 was chosen because it represents the average pH value measured in the samples received from the purification plants. Since a ±15% deviation from the nominal value is compatible with the experimental uncertainty, degradation effects were positively detected beyond this 15% limit [29].

4.3 Result and discussion

The method under study proved adequate for the individual detection of 95 target analytes and 4 internal standards at concentrations equal or lower than 10 ng/L, combined with an injection volume of only 7 µL. This achievement represented the main goal of the present study, which aimed to develop a new method for the simultaneous determination of an extended group of hazardous drugs in wastewater, under stringent conditions in terms of simplicity, sensitivity, and sustainability of sample pre-treatment. To this purpose, the low sample volume, i.e., 10 mL instead of the 100-250 mL typically treated, the elimination of SPE extraction step, the reduction of sample preparation time, and the consequent energy and material savings allowed us to reduce the treatment steps, costs, and analysis time, while improving the overall method's performance. In addition, the chromatographic run was completed in only 10 minutes, including the final re-equilibration time (1.1 minute). The rapid data acquisition for a large number of target compounds in a single run is in accordance with the efficiency constraints required for routine application. Finally, the AGREE metrics [30] was applied to verify the greenness of the proposed method, yielding an overall score of 0.61 which was considered more environmentally friendly and sustainable than both the method previously proposed and based on a SPE [11] - reporting a score of 0.44 (Figure 1) - and other methods based on alternative extraction procedures [11],[31].

The complete results of the validation experiments for wastewater samples fortified with 95 analytes at six concentrations levels (5-1000 ng/L) and 4 internal standards are reported in the Supplementary Tables S1 (calibration features) and S2 (LOD, accuracy, precision, recovery) and discussed hereafter. Table S1 reports

the outcome of the calibration process. Analysis of residues and variances of calibration data points at low, medium and high concentration levels showed that heteroscedastic distributions were present for all the target analytes, making the introduction of weighting factors in the calibration beneficial (either 1/x or $1/x^2$). Quadratic calibration models were chosen for 53 out of 95 analytes depending on the results of Mandel's and lack-of-fit tests.

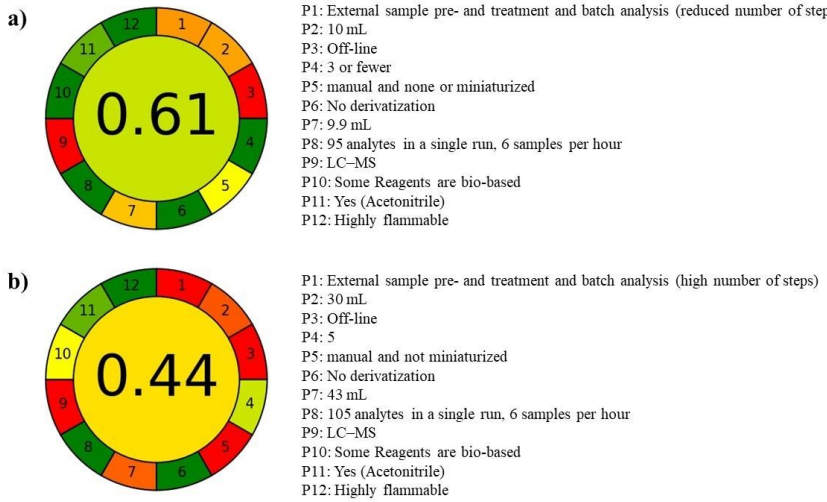


Figure 1: *AGREE metrics applied to a) the developed method (dilute and shoot) and comparison with b) SPE procedure [11]*

The LOD values, calculated using the corrected Hubaux-Vos algorithm, ranged from a minimum of 1 ng/L for tiapride up to a maximum of 44 ng/L for buprenorphine. The relatively large difference in the calculated LOD levels is due to the different chemical nature (alcohol moieties, amide-sulphur secondary structure, etc.) of the compounds in the panel. The purely experimental LOD verification proved that 60 analytes out of 95 (63%), yielded a LOD $\leq$ 5 ng/L. For 23 analytes, the estimated LODs were between 7.5 and 15 ng/L, while the remaining 12 analytes (12%) showed LOD values higher than 15 ng/L (Figure 2 and Table S2). The estimated LODs are in agreement with the concentrations generally detected in wastewater and adequate for almost all the target analytes.

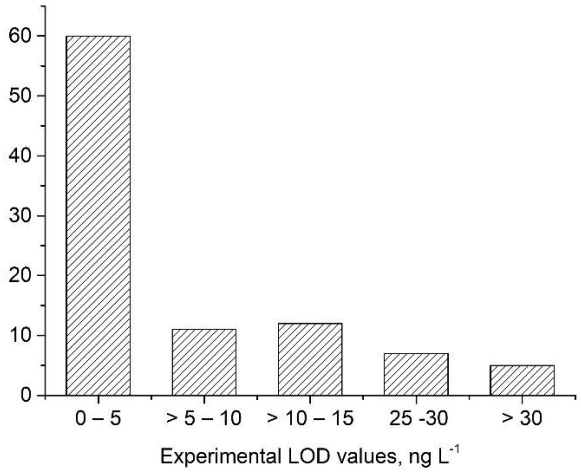


Figure 2. *Number of analytes yielding experimental LOD (ng/L) values within each concentration interval*

Considering in particular that only 7 μL of water sample was injected into the UHPLC system, the 5 ng/L detection limit achieved for most target analytes corresponds to about 35 fg injected which currently represents the lowest amount and one of the lowest LOD value measured with respect to several other studies [14],[32],[33]. Notably, no specific recommendations have yet been established for the detection of pharmaceutical drugs in wastewater, even if the issuance of a European Union directive concerning the monitoring of some CECs, with particular emphasis to some pharmaceutical drugs, in the wastewater leaving the purification plants is expected soon.

The overall recovery for all analytes proved satisfactory, with percentages higher than 80% obtained for all 95 analytes (Table S2). This result was expected, taking into account that no steps in the procedure is likely to produce a loss of analyte; this detail is important considering that few ng/L have frequently to be detected for several analytes.

Intermediate precision and repeatability (expressed as percentage variation coefficient, CV%) and trueness (expressed as bias %) for all 95 target analytes are reported in Table S2 as the mean of the bias% and CV% values obtained at all the six concentration levels. Notably, the validation procedure adopted in the present study allows precise and accurate calculation at all concentrations involved in the calibration process (6 calibration levels), not only at low,

intermediate and high concentrations, as most recommended validation protocols entail [34]. An example is reported in Figure S1, showing precision and trueness trends for amisulpride, an antipsychotic drug selected as representative of the substances brought to the attention of the future European Union directive. Precision data, described by CV% from repeated independent analyses, show rather homogeneous results within each class and between different classes of substances under study. Therefore, the observed data variability can realistically be attributed to the analytical method itself rather than to specific chemical properties of the investigated substances. Interestingly, the average intermediate precision result (CV% = 16%, N = 95) is only slightly higher than the intra-day variability (CV% = 12%, N = 95), as is expected for a substantially stable method and calibration.

The stability of the analytes solution mixtures was assessed by considering the effect of pH, temperature, and days of sample storage. Conditions were chosen to simulate possible storage scenarios, in order to assess (i) wastewater transfer from the sampling site to the laboratory (e.g. room temperature conditions at pH=7), (ii) short-term storage (e.g. at 4°C in the WWTP refrigerator) before shipment to the laboratory, and (iii) long-term storage in cold storage rooms at -20°C at different pH before analysis. The data were interpreted by means of trend lines that describe the variation of the analytes concentration at different storage temperatures and fixed pH during several storage intervals; and by identifying which factor, among temperature, pH, and time, produces a larger degradation. Student t-test and analysis of variance (ANOVA) tests [35] were applied on both the individual analytes and the entire classes (i.e., antidepressants, antipsychotics, antiepileptics, benzodiazepines, cardiovascular drugs, non-steroidal anti-inflammatory drugs, analgesics, and other pharmaceutical drugs from different classes). In this way, it was ascertained whether there was a significant incidence of a given parameter on the molecule stability. Overall, 45 out of 95 (47%) analytes showed a significant variation (based on the statistical tests performed) of stability for, at least, one of tested levels of the evaluated stability parameters, i.e., pH, temperature, and days of sample storage. Results are shown in Table S3a and S3b. Overall, the pH parameter was found to exhibit the greatest variability, and the classes most affected were: antiepileptics (3 out of 3 analytes with significant pH influence) and benzodiazepines (20 out of 26 analytes including 24 with significant pH influence and 2 with significant temperature effect); this probably showed that pH 2 might have an effect on these classes and makes pH=7 the best pH condition for the preservation of all analytes. An example is shown in

Figure S2, that shows boxplots illustrating the ratio between the analyte and the internal standard, at the two pH conditions, for clotiazepam and zolpidem. Figure S3, in the supplementary materials, shows the boxplots obtained for all the benzodiazepines under study. Additionally, Figure3 shows a volcano-like plot, revealing that 20 out of 26 benzodiazepines exhibited significant differences between pH 2 and pH 7. The x-axis of the plot represents the difference between the average values at pH 2 and pH 7 for all examined benzodiazepines, while the y-axis shows the -log10(p-value) obtained from the Student t-test. The red dashed line on the plot indicates the -log10(p-value) at a 95% significance level (p-value = 0.05).

In contrast, the time parameter (different days of storage) did not result in any significant decay in the stability of the compounds (refer to Figure S4 in the Supplementary Material).

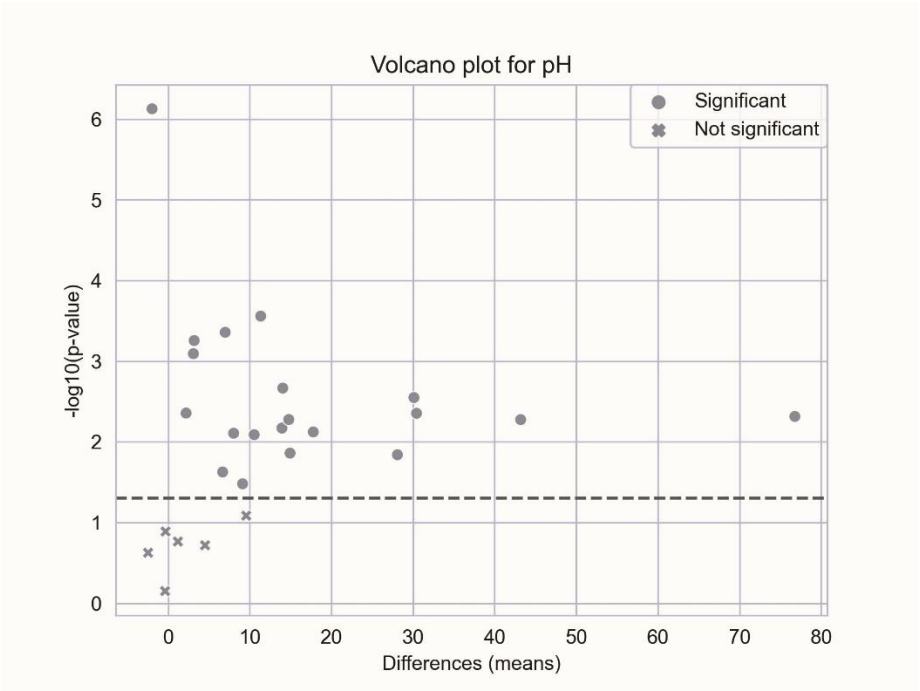


Figure 3. *Volcano-like plot, revealing that 20 out of 26 benzodiazepines exhibited significant differences between pH =2 and pH =7*

Analogous results were observed for the temperature, where significant differences among the tested temperature levels were only found for 6 out of 26 benzodiazepines (refer to Figure S5 in the Supplementary Material). We can

therefore state that it is possible to store wastewater samples correctly and without risk of major changes in stability (variations <15%) for up to 7 days at all temperatures reported in this study.

4.3.1 Application to the real samples

In order to test the effectiveness of the developed method and to study the spread of drugs in urban wastewater, samples of inlet and outlet wastewater collected at three different treatment plants were analysed during the year 2022. Out of the 95 monitored pharmaceutical drugs/metabolites, a total of 42 target analytes were detected in almost all sites. These homogeneous results suggest that similar drug prescriptions and consumption rates are uniformly distributed in northern Italy. Table 2 shows the average concentration (each sample was analysed in duplicate) of the pharmaceutical drugs quantified in the influent samples at the different WWTP. The highest absolute concentration (higher than 1 μg/L) was found for atenolol, telmisartan, ketoprofen, paracetamol, and metformin. While the high concentrations found for paracetamol and ketoprofen are not surprising, as both are among the most commonly used over-the-counter drugs, the high concentrations of metformin and telmisartan deserve more detailed explanation. Recent studies proved that metformin is the most consumed antidiabetic drug [36]. In fact, the common initial treatment for diabetes was based on metformin in both monotherapy and combination therapy, and patients initially treated with this pharmaceutical drug kept using the same therapy for long periods [37]. Also the high concentrations of telmisartan is not unaccountable: in fact, the *"National Report on medicines use in Italy"* OsMed 2021 [38] lists this pharmaceutical drug (together with ramipril, bisoprolol and metformin) among the thirty most consumed pharmaceutical ingredients in Italy.

Further drugs belonging to the classes of antidepressants, benzodiazepines, antiepileptics, and antipsychotics deserve particular attention for both their relatively high concentration detected in the real samples and their harmful effects on aquatic environments. Some consequences of bioaccumulation of these active ingredients in fish include endocrine effects, developmental alteration and behavioural changes [39]. Among these, venlafaxine (concentration range 550–800 ng/L), lorazepam (concentration range 90–150 ng/L), trazodone (concentration range 20–50 ng/L) and carbamazepine (concentration range 310–450 ng/L) were detected in almost all samples. In general, the drugs concentrations detected in the representative 24-hour samples of the present study are comparable or even higher than those observed in similar published studies

[40],[41]. For example, in the study by Cardini et al. [40] comparable concentrations above 200 ng/L were detected for carbamazepine and above 1000 ng/L for metformin. In contrast, atenolol concentrations detected in our samples were higher than in the previous study [40]. Further interest is raised by the case of zolpidem, a drug commonly used for insomnia with depressant activity on the central nervous system. Only its metabolite zolpidem (zolpidem carboxylic acid), not having any psychotropic activity, was detected in the tested wastewater samples (concentration range 110–290 ng/L).

Table 2: *Average concentration (ng/L) of the drugs found in the influent and effluent samples at the different WWTPs. The origin of the sample was anonymized by naming the different WWTPs as Site 1,2 and 3. The annotation n.d. means "not detected".*

	SITE 1		**SITE 2**		**SITE 3**	
	Inlet	**Outlet**	**Inlet**	**Outlet**	**Inlet**	**Outlet**
Antidepressants						
Amitryptline	8	9	11	8	24	10
Citalopram	33	76	60	90	68	79
m-CPP	8	7	7	5	6	6
Mirtazapine	25	20	14	15	18	20
Trazodone	45	27	32	21	47	47
Benzodiazepines and analogues						
3-OH-Bromazepam	41	39	37	25	40	40
Alprazolam	16	16	13	12	14	12
Lorazepam	154	148	133	98	111	101
Lormetazepam	86	63	79	56	63	61
Oxazepam	27	30	30	28	25	23
Zolpidem metabolite	295	288	244	114	175	170
Antipsychotics						
Amisulpride	144	150	135	140	188	139
Carbamazepine	446	396	353	315	353	337
Clozapine	14	15	20	25	18	30
Quetiapine	135	6	39	n.d.	52	n.d.
Tiapride	104	96	67	66	78	55
Venlafaxine	641	800	553	550	782	636
Antiepileptics						
Lamotrigine	478	>1000	435	>1000	362	>1000
Cardiovascular drugs						

Atenolol	>>1000	416	>>1000	107	>>1000	453
Bisoprolol	170	75	172	107	146	152
Propafenone	96	70	156	89	128	123
Ramipril	202	2	84	n.d	154	10
Telmisartan	>>1000	>1000	>>1000	>1000	>>1000	>1000
Verapamil	8	2	14	9	11	5
Non-steroidal anti-inflammatory drug						
Ketoprofen	>1000	272	>1000	163	>1000	313
Ketorolac	40	14	32	9	46	30
Analgesics/opioids						
Dihydrocodeine	71	36	61	15	46	38
Methadone	32	31	49	55	54	38
Paracetamol	>>1000	72	>>1000	n.d.	>>1000	n.d.
Tapentadol	659	641	405	369	497	390
Tramadol	126	130	120	125	189	188
Oxycodone	15	11	18	16	160	13
Others						
Dextrometorphan	20	18	12	12	16	14
Diltiazem	80	36	64	26	69	19
Diphenidramine	18	17	11	6	24	19
Gliclazide	42	19	17	17	93	43
Levamisole	12	12	14	14	18	18
Lidocaine	117	125	110	113	183	137
Metformin	>>1000	>1000	>>1000	254	>>1000	999
Metoclopramide	44	33	22	21	47	26
Naloxone	46	20	38	14	35	14
Ticlopidine	14	12	20	11	22	12

Several other compounds, as much as detected at lower concentration (5-10 ng/L), are of concern for their effects on the ecosystems (e.g. verapamil [42] detected at Site 2, Figure 4) and their contribution to the global environmental loading. As a matter of fact, a WWTP plant for 100,000 inhabitants' equivalent discharges on average approximately 24,000 m^3/day of water into the environment. As a consequence, the total amounts of pharmaceutical drugs released every day in the water acceptor bodies is approximately 2.4×10^7 higher than what is measured in 1 L sample, particularly in the event that scarce removal in the WWTP is expected.

Another interesting evidence of the present study is the scarce abatement rate of the targeted drugs achieved by the purification plant (Table 3). Table 3 reported the percentage abatement of each of the pharmaceutical drugs which was calculated based on the difference between the initial analyte concentration (C_0) (influent sample) and the final analyte concentration (C_f) (effluent sample), which was obtained from the calibration curve for each analyte in the sample solutions, using the following formula [43]:

$$\%\ abatement = \frac{C_0 - C_f}{C_0} x\ 100$$

By comparing the drug concentrations before and after the plant, an abatement rate ≥ 50% was observed for only 12 out of 42 analytes (29%), even when the analyte concentrations in the sample was low (i.e., without any overloading risk). For the remaining pharmaceutical drugs, no abatement was observed or - for few of them - even an increased concentration was recorded between the samples entering and leaving the plant (e.g. for citalopram, clozapine, and lamotrigine). An apparent "negative removal" was actually reported for some targeted pharmaceutical drugs in a previous study [44], which may be associated with an increased transformation of precursor compounds or parent compounds, or the hydraulic residence time, and/or the desorption from suspended solids in the wastewater treatment processes [45],[46]. The latter phenomenon draw attention on the need of careful treatment plant monitoring, before the treated water is discharged into the receiving water body, further highlighting that technological developments of WWTPs are mandatory to really remove the CECs monitored in this study. A few striking results of "negative removal" (lamotrigine and, to a lesser extent, citalopram) can be justified by chronological misalignment between the inlet and outlet sampling in combination with occasional peak concentrations for a specific drug.

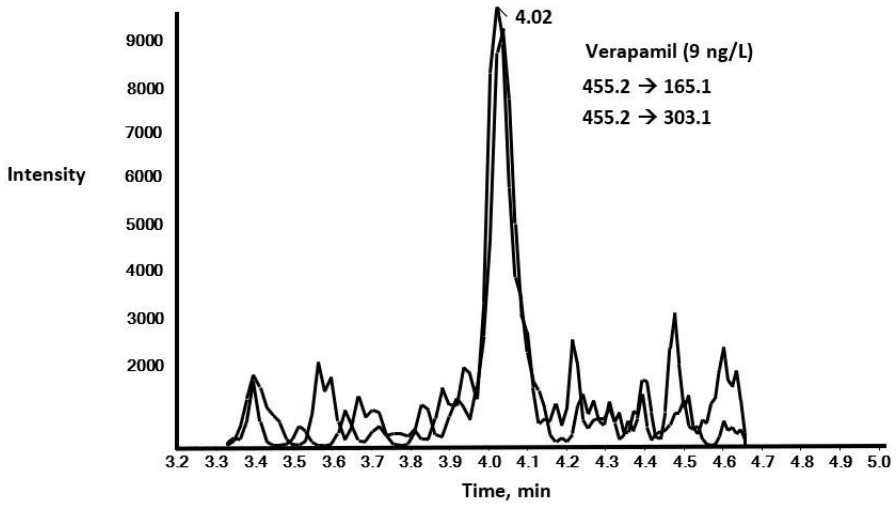


Figure 4. *Chromatographic profile of verapamil found in the Site 2. Extracted ion chromatogram (XIC) represent the outlet samples leaving the WWTP and detected at the concentration of 9 ng/L.*

Table 3: *Percentage (%) of abatement of different pharmaceutical drugs between influent and effluent samples of the various sites under study*

	Percentage (%) of abatement		
	Site 1	**Site 2**	**Site 3**
Antidepressants			
Amitryptline	-13	27	58
Citalopram	-130	-50	-16
m-CPP	13	29	0
Mirtazapine	20	-7	-11
Trazodone	40	34	0
Benzodiazepines and analogues			
3OH - Bromazepam	5	32	0
Alprazolam	0	8	14
Lorazepam	4	26	9
Lormetazepam	27	29	3
Oxazepam	-11	7	8
Zolpidem metabolite	2	53	3
Antipsychotics			
Amisulpride	-4	-4	26
Carbamazepina	11	11	5

Clozapine	-7	-25	-67
Quetiapine	96	100	100
Tiapride	8	1	29
Venlafaxine	-25	1	19
Antiepileptics			
Lamotrigine	-266	-196	-219
Cardiovascular drugs			
Atenolol	92	98	94
Bisoprolol	56	38	-4
Propafenone	27	43	4
Ramipril	99	100	94
Telmisartan	64	61	45
Verapamil	75	36	55
Non-steroidal anti-inflammatory drug			
Ketoprofen	91	94	89
Ketorolac	65	72	35
Analgesics/opioids			
Dihydrocodeine	49	75	17
Methadone	3	-12	30
Paracetamol	100	100	100
Tapentadol	3	9	22
Tramadol	-3	-4	1
Oxycodone	27	11	92
Others			
Dextrometorphan	10	0	13
Diltiazem	55	59	72
Diphenidramine	6	45	21
Gliclazide	55	0	54
Levamisole	0	0	0
Lidocaine	-7	-3	25
Metformin	88	98	96
Metoclopramide	25	5	45
Naloxone	57	63	60
Ticlopidine	14	45	45

In general, WWTPs have been historically designed to remove main nutrients such as carbon-, nitrogen-, and phosphorous-containing substrates, and to prevent microbial contaminations, and not to abate specific classes of contaminants. Therefore, the observed scarce drug removal rates are arguably related to unspecific treatment techniques [47]. The necessary technological evolution (i.e.,

the implementation of quaternary treatments) of water purification processes should be supported by appropriately targeted monitoring protocols such as the one proposed by the present study, also in the perspective of future European Union directives.

4.4 Conclusions

The method proposed in this study showed great potential as a practical and customary analytical tool for water analysis, since it is simple, fast, and highly efficient at the same time, while also allows to save important resources in terms of time and materials, making it more sustainable than the methods previously proposed by the literature. The greenness of the method was evaluated quantitatively using the recently developed AGREE metrics, resulting in an overall score of 0.61 and becoming a candidate green alternative for the analysis of pharmaceutical in water samples. Thanks to these features, the present procedure will be applied in future in high-throughput wastewater monitoring campaigns, thus enabling social and environmental protection actions.
The collected data on the real samples are consistent with those available in our previous study [11]and confirm that many of the investigated pharmaceutical drugs are present in wastewater at a level that pose a health issue to the biota.

References

[1] A.J. Ebele, M. Abou-Elwafa Abdallah, S. Harrad, Pharmaceuticals and personal care products (PPCPs) in the freshwater aquatic environment, Emerg. Contam. 3 (2017) 1–16. https://doi.org/10.1016/j.emcon.2016.12.004.

[2] J. Radjenovic, M. Petrovic, D. Barceló, Analysis of pharmaceuticals in wastewater and removal using a membrane bioreactor, Anal. Bioanal. Chem. 387 (2007) 1365–1377. https://doi.org/10.1007/s00216-006-0883-6.

[3] M. Paut Kusturica, M. Jevtic, J. Ristovski, Minimizing the environmental impact of unused pharmaceuticals: Review focused on prevention, Front. Environ. Sci. 10 (2022) 1077974. https://doi.org/10.3389/fenvs.2022.1077974.

[4] K.E. Arnold, A.R. Brown, G.T. Ankley, J.P. Sumpter, Medicating the environment: assessing risks of pharmaceuticals to wildlife and ecosystems, Philos. Trans. R. Soc. B Biol. Sci. 369 (2014) 20130569. https://doi.org/10.1098/rstb.2013.0569.

[5] A. Shraim, A. Diab, A. Alsuhaimi, E. Niazy, M. Metwally, M. Amad, S. Sioud, A. Dawoud, Analysis of some pharmaceuticals in municipal wastewater of Almadinah Almunawarah, Arab. J. Chem. 10 (2017) S719–S729. https://doi.org/10.1016/j.arabjc.2012.11.014.

[6] S.D. Richardson, S.Y. Kimura, Water Analysis: Emerging Contaminants and Current Issues, Anal. Chem. 92 (2020) 473–505. https://doi.org/10.1021/acs.analchem.9b05269.

[7] A. Rodayan, M. Majewsky, V. Yargeau, Impact of approach used to determine removal levels of drugs of abuse during wastewater treatment, Sci. Total Environ. 487 (2014) 731–739. https://doi.org/10.1016/j.scitotenv.2014.03.080.

[8] A.G. Asimakopoulos, K. Kannan, Neuropsychiatric pharmaceuticals and illicit drugs in wastewater treatment plants: a review, Environ. Chem. 13 (2016) 541–576. https://doi.org/10.1071/EN15202.

[9] THE 17 GOALS | Sustainable Development. https://sdgs.un.org/goals.

[10] A.M. Sulej-Suchomska, A. Klupczynska, P. Dereziński, J. Matysiak, P. Przybyłowski, Z.J. Kokot, Urban wastewater analysis as an effective tool for monitoring illegal drugs, including new psychoactive substances, in the Eastern European region, Sci. Rep. 10 (2020) 4885. https://doi.org/10.1038/s41598-020-61628-5.

[11] M. Massano, A. Salomone, E. Gerace, E. Alladio, M. Vincenti, M. Minella, Wastewater surveillance of 105 pharmaceutical drugs and metabolites by

means of ultra-high-performance liquid-chromatography-tandem high resolution mass spectrometry, J. Chromatogr. A 1693 (2023) 463896. https://doi.org/10.1016/j.chroma.2023.463896.

[12] S. Hussain, C. Ramnarayanan, T.S. Roopashree, Md.K. Anwer, N. Sreeharsha, A.B. Nair, Rapid, Precise and Affordable Estimation of Venlafaxine and Its Metabolites in Highly Polluted Effluent Waters: Proof-of-Concept for Methodology, Molecules 25 (2020) 4793. https://doi.org/10.3390/molecules25204793.

[13] S. Afsa, K. Hamden, P.A. Lara Martin, H.B. Mansour, Occurrence of 40 pharmaceutically active compounds in hospital and urban wastewaters and their contribution to Mahdia coastal seawater contamination, Environ. Sci. Pollut. Res. 27 (2020) 1941–1955. https://doi.org/10.1007/s11356-019-06866-5.

[14] E. Gracia-Lor, M. Martínez, J.V. Sancho, G. Peñuela, F. Hernández, Multi-class determination of personal care products and pharmaceuticals in environmental and wastewater samples by ultra-high-performance liquid-chromatography-tandem mass spectrometry, Talanta 99 (2012) 1011–1023. https://doi.org/10.1016/j.talanta.2012.07.091.

[15] J. Margot, L. Rossi, D.A. Barry, C. Holliger, A review of the fate of micropollutants in wastewater treatment plants, WIREs Water 2 (2015) 457–487. https://doi.org/10.1002/wat2.1090.

[16] B. Kasprzyk-Hordern, R.M. Dinsdale, A.J. Guwy, Illicit drugs and pharmaceuticals in the environment – Forensic applications of environmental data. Part 1: Estimation of the usage of drugs in local communities, Environ. Pollut. 157 (2009) 1773–1777. https://doi.org/10.1016/j.envpol.2009.03.017.

[17] F. Busetti, W.J. Backe, N. Bendixen, U. Maier, B. Place, W. Giger, J.A. Field, Trace analysis of environmental matrices by large-volume injection and liquid chromatography–mass spectrometry, Anal. Bioanal. Chem. 402 (2012) 175–186. https://doi.org/10.1007/s00216-011-5290-y.

[18] M. de la Guardia, S. Garrigues, The Concept of Green Analytical Chemistry, in: Handb. Green Anal. Chem., John Wiley & Sons, Ltd, 2012: pp. 1–16. https://doi.org/10.1002/9781119940722.ch1.

[19] C. Boix, M. Ibáñez, J.V. Sancho, J. Rambla, J.L. Aranda, S. Ballester, F. Hernández, Fast determination of 40 drugs in water using large volume direct injection liquid chromatography–tandem mass spectrometry, Talanta 131 (2015) 719–727. https://doi.org/10.1016/j.talanta.2014.08.005.

[20] C. Simarro-Gimeno, B. Garlito, E. Pitarch, F. Hernández, Evaluation of direct sample injection as a fast, no-sample handling, approach for the LC-MS/MS monitoring of pharmaceuticals in different water matrices, Microchem. J. 193 (2023) 108985. https://doi.org/10.1016/j.microc.2023.108985.

[21] M. Vincenti, D. Cavanna, E. Gerace, V. Pirro, M. Petrarulo, D. Di Corcia, A. Salomone, Fast screening of 88 pharmaceutical drugs and metabolites in whole blood by ultrahigh-performance liquid chromatography-tandem mass spectrometry, Anal. Bioanal. Chem. 405 (2013) 863–879. https://doi.org/10.1007/s00216-012-6403-y.

[22] E. Alladio, E. Amante, C. Bozzolino, F. Seganti, A. Salomone, M. Vincenti, B. Desharnais, Effective validation of chromatographic analytical methods: The illustrative case of androgenic steroids, Talanta 215 (2020) 120867. https://doi.org/10.1016/j.talanta.2020.120867.

[23] B. Desharnais, F. Camirand-Lemyre, P. Mireault, C.D. Skinner, Procedure for the Selection and Validation of a Calibration Model I—Description and Application, J. Anal. Toxicol. 41 (2017) 261–268. https://doi.org/10.1093/jat/bkx001.

[24] E. Alladio, E. Amante, C. Bozzolino, F. Seganti, A. Salomone, M. Vincenti, B. Desharnais, Experimental and statistical protocol for the effective validation of chromatographic analytical methods, Methods X 7 (2020). https://doi.org/10.1016/j.mex.2020.100919.

[25] M. Massano, C. Incardona, E. Gerace, P. Negri, E. Alladio, A. Salomone, M. Vincenti, Development and validation of a UHPLC-HRMS-QTOF method for the detection of 132 New Psychoactive Substances and synthetic opioids, including fentanyl, in Dried Blood Spots, Talanta 241 (2022) 123265. https://doi.org/10.1016/j.talanta.2022.123265.

[26] H. Gu, G. Liu, J. Wang, A.-F. Aubry, M. Arnold, Selecting the Correct Weighting Factors for Linear and Quadratic Calibration Curves with Least-Squares Regression Algorithm in Bioanalytical LC-MS/MS Assays and Impacts of Using Incorrect Weighting Factors on Curve Stability, Data Quality, and Assay Performance, Anal. Chem. 86 (2014). https://doi.org/10.1021/ac5018265.

[27] Andre. Hubaux, Gilbert. Vos, Decision and detection limits for calibration curves, Anal. Chem. 42 (1970) 849–855. https://doi.org/10.1021/ac60290a013.

[28] D.R. Baker, B. Kasprzyk-Hordern, Critical evaluation of methodology commonly used in sample collection, storage and preparation for the analysis of pharmaceuticals and illicit drugs in surface water and wastewater by solid phase extraction and liquid chromatography–mass spectrometry, J. Chromatogr. A 1218 (2011) 8036–8059. https://doi.org/10.1016/j.chroma.2011.09.012.

[29] A.M. Sulej-Suchomska, A. Klupczynska, P. Dereziński, J. Matysiak, P. Przybyłowski, Z.J. Kokot, Urban wastewater analysis as an effective tool for monitoring illegal drugs, including new psychoactive substances, in the Eastern European region, Sci. Rep. 10 (2020) 4885. https://doi.org/10.1038/s41598-020-61628-5.

[30] W. Wojnowski, M. Tobiszewski, F. Pena-Pereira, E. Psillakis, AGREEprep – Analytical greenness metric for sample preparation, TrAC Trends Anal. Chem. 149 (2022) 116553. https://doi.org/10.1016/j.trac.2022.116553.

[31] M.M. Nascimento, M.L. Nascimento, J. Pereira dos Anjos, R.L. Cunha, G.O. da Rocha, I. Ferreira dos Santos, P.A. de P. Pereira, J.B. de Andrade, A green method for the determination of illicit drugs in wastewater and surface waters-based on a semi-automated liquid-liquid microextraction device, J. Chromatogr. A 1710 (2023) 464230. https://doi.org/10.1016/j.chroma.2023.464230.

[32] M. Gros, S. Rodríguez-Mozaz, D. Barceló, Rapid analysis of multiclass antibiotic residues and some of their metabolites in hospital, urban wastewater and river water by ultra-high-performance liquid chromatography coupled to quadrupole-linear ion trap tandem mass spectrometry, J. Chromatogr. A 1292 (2013) 173–188. https://doi.org/10.1016/j.chroma.2012.12.072.

[33] S. Daouk, S. Fleury-Souverain, Y. Daali, Development of an LC-MS/MS Method for the Assessment of Selected Active Pharmaceuticals and Metabolites in Wastewaters of a Swiss University Hospital, CHIMIA 69 (2015) 684–684. https://doi.org/10.2533/chimia.2015.684.

[34] S.M.R. Wille, F.T. Peters, V. Di Fazio, N. Samyn, Practical aspects concerning validation and quality control for forensic and clinical bioanalytical quantitative methods, Accreditation Qual. Assur. 16 (2011) 279–292. https://doi.org/10.1007/s00769-011-0775-0.

[35] L. St»hle, S. Wold, Analysis of variance (ANOVA), Chemom. Intell. Lab. Syst. 6 (1989) 259–272. https://doi.org/10.1016/0169-7439(89)80095-4.

[36] Italy: consumption rate of antidiabetics by type 2021, Statista.

https://www.statista.com/statistics/1340887/consumption-of-antidiabetic-drugs-by-type-italy/

[37] A. Moreno Juste, E. Menditto, V. Orlando, V.M. Monetti, A. Gimeno Miguel, F. González Rubio, M.M. Aza–Pascual-Salcedo, C. Cahir, A. Prados Torres, G. Riccardi, Treatment Patterns of Diabetes in Italy: A Population-Based Study, Front. Pharmacol. 10 (2019) 870. https://doi.org/10.3389/fphar.2019.00870.

[38]Rapporto-OsMed-2021_EN.pdf, https://www.aifa.gov.it/documents/20142/1740782/Rapporto-OsMed-2021_EN.pdf

[39] J. Corcoran, M.J. Winter, C.R. Tyler, Pharmaceuticals in the aquatic environment: A critical review of the evidence for health effects in fish, Crit. Rev. Toxicol. 40 (2010) 287–304. https://doi.org/10.3109/10408440903373590.

[40] A. Cardini, E. Pellegrino, L. Ercoli, Predicted and Measured Concentration of Pharmaceuticals in Surface Water of Areas with Increasing Anthropic Pressure: A Case Study in the Coastal Area of Central Italy, Water 13 (2021) 2807. https://doi.org/10.3390/w13202807.

[41] H. Ren, S. Yuan, J. Zheng, R. Luo, H. Qiang, W. Duan, Y. Zhao, P. Xiang, Direct injection ultra-performance liquid chromatography-tandem mass spectrometry for the high-throughput determination of 11 illicit drugs and metabolites in wastewater, J. Chromatogr. A 1685 (2022) 463587. https://doi.org/10.1016/j.chroma.2022.463587.

[42] Z.-H. Li, P. Li, T. Randak, Ecotoxocological effects of short-term exposure to a human pharmaceutical Verapamil in juvenile rainbow trout (Oncorhynchus mykiss), Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 152 (2010) 385–391. https://doi.org/10.1016/j.cbpc.2010.06.007.

[43] T.G. Kebede, S. Dube, M.M. Nindi, Removal of Multi-Class Antibiotic Drugs from Wastewater Using Water-Soluble Protein of Moringa stenopetala Seeds, Water 11 (2019) 595. https://doi.org/10.3390/w11030595.

[44] C. Postigo, M.J. López de Alda, D. Barceló, Drugs of abuse and their metabolites in the Ebro River basin: occurrence in sewage and surface water, sewage treatment plants removal efficiency, and collective drug usage estimation, Environ. Int. 36 (2010) 75–84. https://doi.org/10.1016/j.envint.2009.10.004.

[45] I. González-Mariño, E. Gracia-Lor, N.I. Rousis, E. Castrignanò, K.V. Thomas, J.B. Quintana, B. Kasprzyk-Hordern, E. Zuccato, S. Castiglioni,

Wastewater-Based Epidemiology To Monitor Synthetic Cathinones Use in Different European Countries, Environ. Sci. Technol. 50 (2016) 10089–10096. https://doi.org/10.1021/acs.est.6b02644.

[46] M. Ostman, J. Fick, E. Näsström, R.H. Lindberg, A snapshot of illicit drug use in Sweden acquired through sewage water analysis, Sci. Total Environ. 472 (2014) 862–871. https://doi.org/10.1016/j.scitotenv.2013.11.081.

[47] Y. Deng, C. Guo, H. Zhang, X. Yin, L. Chen, D. Wu, J. Xu, Occurrence and removal of illicit drugs in different wastewater treatment plants with different treatment techniques, Environ. Sci. Eur. 32 (2020) 28. https://doi.org/10.1186/s12302-020-00304-x.

Supplementary materials

Table S1. *Calibration models for the targeted analytes: range, nature of the models, and equation.*

Compound	Calibration Range (ng/L)	Equation	Model	homoscedastic vs. heteroscedastic
		Antidepressants		
Amitriptyline	5-500	$y = -0.0106x^2 + 1.0696x – 0.0819$	Quadratic	heteroscedastic
Citalopram	5-500	$y = -0.0488x^2 + 3.5821x – 0.4396$	Quadratic	heteroscedastic
Clonidine	50-1000	$y = -0.0005x^2 + 0.0531x + 0.0102$	Quadratic	heteroscedastic
Fluoxetine	25-1000	$y = -0.0009x^2 + 0.0809x - 0.0371$	Quadratic	heteroscedastic
Fluvoxamine	25-1000	$y = -0.0015x^2 + 0.1293x – 0.0828$	Quadratic	heteroscedastic
m-CPP	10-750	$y = 0.0479x^2 + 5.4442x + 1.6057$	Quadratic	heteroscedastic
Mianserin	50-1000	$y = 0.0111x^2 + 3.4003x + 0.7747$	Linear	heteroscedastic
Mirtazapine	5-500	$y = -0.0007x^2 + 1.2672x + 0.1034$	Linear	heteroscedastic
Paroxetine	25-1000	$y = -0.0004x^2 + 3.0943x – 1.2671$	Linear	heteroscedastic

Trazodone	5-500	$y = -0.0007x^2 + 1.2672x + 0.1034$	Linear	heteroscedastic
Benzodiazepines and analogues				
3-OH-Bromazepam	50-1000	$y = 0.003x^2 + 0.6281x + 0.2431$	Quadratic	heteroscedastic
7-Aminoclonazepam	25-1000	$y = 0.0087x^2 + 0.8774x + 0.5256$	Quadratic	heteroscedastic
7-Aminoflunitrazepam	25-1000	$y = 0.0151x^2 + 1.9447x + 0.3606$	Quadratic	heteroscedastic
7-Aminonitrazepam	25-1000	$y = 0.0225x^2 + 4.7644x – 0.4215$	Quadratic	heteroscedastic
Alprazolam	5-500	$y = 0.0072x^2 + 1.2517x + 0.2861$	Linear	heteroscedastic
Bromazepam	25-1000	$y = 0.0056x^2 + 0.4192x + 0.4205$	Quadratic	heteroscedastic
Brotizolam	5-500	$y = -0.0033x^2 + 0.2373x – 0.019$	Quadratic	heteroscedastic
Chlordiazepoxide	5-500	$y = 0.0116x^2 + 3.7049x + 0.638$	Linear	heteroscedastic
Clobazam	5-500	$y = 0.0015x^2 + 2.9215x + 0.4157$	Linear	heteroscedastic
Clonazepam	25-1000	$y = 0.0017x^2 + 0.3532x + 0.1678$	Quadratic	heteroscedastic
Clotiazepam	5-500	$y = -0.0012x^2 + 8.4034x + 0.6697$	Linear	heteroscedastic
Delorazepam	5-500	$y = 0.0038x^2 + 0.9522x + 0.1791$	Linear	heteroscedastic
Demoxepam	25-1000	$y = 0.0023x^2 + 0.5946x + 0.3379$	Linear	heteroscedastic

Desalchilflurazepam	5-500	$y = 0.0047x^2 + 1.2888x + 0.2547$	Linear	heteroscedastic
Diazepam	10-750	$y = 0.0061x^2 + 1.586x + 0.3401$	Linear	heteroscedastic
Diclazepam	5-500	y = 0.0037x2 + 1.3278x + 0.2138	Linear	heteroscedastic
Flunitrazepam	10-750	$y = 0.0125x^2 + 2.9667x + 0.474$	Linear	heteroscedastic
Flurazepam	10-750	$y = 0.0504x^2 + 2.1044x + 0.3183$	Linear	heteroscedastic
Lorazepam	25-1000	$y = 0.0041x^2 + 1.6136x + 0.319$	Linear	heteroscedastic
Lormetazepam	25-1000	$y = 0.012x^2 + 2.4898x + 1.6192$	Quadratic	heteroscedastic
Midazolam	5-500	$y = 0.001x^2 + 5.0478x + 0.3355$	Linear	heteroscedastic
Nordiazepam	25-1000	$y = 0.0101x^2 + 1.4248x + 0.893$	Quadratic	heteroscedastic
OH-Midazolam	10-750	y = 0,0479x2 + 5,4442x + 1,6057 R^2 = 0,927	Quadratic	heteroscedastic
Oxazepam	25-1000	$y = 0.0024x^2 + 0.5363x + 0.3276$	Linear	heteroscedastic
Temazepam	25-1000	$y = 0.022x^2 + 5.5499x + 2,3275$	Linear	heteroscedastic
Triazolam	5-500	$y = 0,0017x^2 + 1,9179x + 0.1454$	Linear	heteroscedastic
Zolpidem	5-500	$y = -0.0016x^2 + 2.8734x + 0.1699$	Linear	heteroscedastic
Zolpidem metabolite	5-500	$y = 0.0326x^2 + 7.5396x + 0.7296$	Linear	heteroscedastic

Antipsychotic				
Amisulpride	5-500	$y = -0.0293x^2 + 2.0627x – 0.2391$	Quadratic	heteroscedastic
Aripiprazole	50-1000	$y = 0.0002x^2 + 0.1815x – 0.0862$	Linear	heteroscedastic
Carbamazepine	50-1000	$y = 0.0126x^2 + 11.769x + 1.9218$	Linear	heteroscedastic
Chlorpromazine	5-500	$y = 0.0249x^2 + 5.0307x – 0.4344$	Linear	heteroscedastic
Clozapine	5-500	$y = -0.001x^2 + 8.422x – 0.3972$	Linear	heteroscedastic
Haloperidol	5-500	$y = 0.0085x^2 + 10.476x – 0.7624$	Linear	heteroscedastic
Levomepromazine	10-750	$y = -0.0004x^2 + 1.0701x – 0.4578$	Linear	heteroscedastic
Olanzapine	50-1000	$y = 0.0339x^2 + 5.1726x – 12.277$	Linear	heteroscedastic
Periciazine	5-500	$y = 0.0286x^2 + 5.7066x + 0.3706$	Linear	heteroscedastic
Promazine	10-750	$y = -0.021x^2 + 2.7566x – 0.4112$	Quadratic	heteroscedastic
Quetiapine	5-500	$y = -0.00258x^2 + 7.8528x – 0.3586$	Linear	heteroscedastic
Risperidone	5-500	$y = 0.0961x^2 + 6.2635x – 0.1446$	Quadratic	heteroscedastic
Tiapride	5-500	$y = -0.0326x^2 + 2.2417x – 0.1828$	Quadratic	heteroscedastic
Venlafaxine	5-500	$y = -0.0082x^2 + 0.6179x – 0.0497$	Quadratic	heteroscedastic

Ziprasidone	50-1000	$y = -0.0002x^2 + 0.0311x + 0.0045$	Quadratic	heteroscedastic
Antiepileptic				
Lamotrigine	25-1000	$y = 0.00005x^2 + 0.1691x + 0.0677$	Linear	heteroscedastic
Oxcarbazepine	5-500	$y = 0.0367x^2 + 1.9818x + 0.4945$	Linear	heteroscedastic
Cardiovascular Drugs				
Atenolol	5-500	$y = -0.0129x^2 + 1.0063x – 0.0871$	Quadratic	heteroscedastic
Bisoprolol	5-500	$y = 0.0018x^2 + 2.1257x + 0.2557$	Linear	heteroscedastic
Nebivolol	10-750	$y = 0.0052x^2 + 0.7571x + 0.0775$	Quadratic	heteroscedastic
Propafenone	5-500	$y = 0.0164x^2 + 4.5359x + 0.2686$	Linear	heteroscedastic
Ramipril	50-1000	$y = -0.0097x^2 + 1.1404x + 0.2535$	Quadratic	heteroscedastic
Telmisartan	25-1000	$y = 0.0035x^2 + 0.1809x + 0.0709$	Quadratic	heteroscedastic
Verapamil	10-750	$y = 0.0126x^2 + 1.2564x + 0.0876$	Quadratic	heteroscedastic
Non-steroidal anti-inflammatory Drugs				
Ketoprofen	50-1000	$y = -0.0034x^2 + 6.2434x + 4.3736$	Linear	heteroscedastic
Ketorolac	25-1000	$y = 0.001x^2 + 1.5263x + 0.1863$	Linear	heteroscedastic

Analgesics / opioids				
Buprenorphine	25-1000	$y = -0.0004x^2 + 2.9617x + 1.1502$	Quadratic	heteroscedastic
Dihydrocodeine	10-750	$y = -0.0187x^2 + 1.2319x – 0.1038$	Quadratic	heteroscedastic
Embutramide	5-500	$y = -0.0061x^2 + 4.3327x + 0.1844$	Linear	heteroscedastic
Hydromorphone	5-500	$y = -0.0002x^2 + 1.1789x + 0.0536$	Linear	heteroscedastic
Methadone	5-500	$y = -0.0969x^2 + 7.8271x – 0.4545$	Quadratic	heteroscedastic
Oxycodone	5-500	$y = -0.0041x^2 + 0.4001x – 0.0275$	Quadratic	heteroscedastic
Paracetamol	50-1000	$y = -0,0004x^2 + 2,9617x + 1,1502$	Linear	heteroscedastic
Phenacetin	10-750	$y = -0.0049x^2 + 0.6625x + 0.591$	Quadratic	heteroscedastic
Tapentadol	5-500	$y = -0.0667x^2 + 4.7459x – 0.4745$	Quadratic	heteroscedastic
Tramadol	5-500	$y = -0.0446x^2 + 3.1937x – 0.267$	Quadratic	heteroscedastic
Others				
Atropine	5-500	$y = -0.0085x^2 + 0.9306x – 0.0579$	Quadratic	heteroscedastic
Biperiden	5-500	$y = -0.0547x^2 + 31.158x – 2.0282$	Linear	heteroscedastic
Dextromethorphan	5-500	$y = -0.0271x^2 + 2.2547x – 0.2633$	Quadratic	heteroscedastic

Diltiazem	5-500	$y = -0.033x^2 + 2.8559x – 0.1581$	Quadratic	heteroscedastic
Diphenhydramine	10-750	$y = -0.0142x^2 + 1.3092x – 0.0779$	Quadratic	heteroscedastic
Diphenidine	5-500	$y = -0.0787x^2 + 4.5275x – 0.6534$	Quadratic	heteroscedastic
EDDP	5-500	$y = -0,0034x^2 + 0,7223x - 0,0623$ $R^2 = 0,9678$	Linear	heteroscedastic
Gliclazide	50-1000	$y = -0.0035x^2 + 0.4128x – 0.0139$	Quadratic	heteroscedastic
Levamisole	5-500	$y = 0.0124x^2 + 17.873x + 1.3117$	Quadratic	heteroscedastic
Lidocaine	5-500	$y = -0.1525x^2 + 8.4437x + 0.1616$	Quadratic	heteroscedastic
Loperamide	5-500	$y = 0.1221x^2 + 8.7712x – 0.299$	Quadratic	heteroscedastic
Metformin	50-1000	$y = -0.0038x^2 + 0.6606x + 0.5512$	Quadratic	heteroscedastic
Methylphenidate	5-500	$y = -0.1847x^2 + 9.8691x – 1.1943$	Quadratic	heteroscedastic
Metoclopramide	5-500	$y = -0.1283x^2 + 9.0388x – 1.3012$	Quadratic	heteroscedastic
Naloxone	5-500	$y = -0.0105x^2 + 1.4502x – 0.1605$	Quadratic	heteroscedastic
Oxybutynin	10-750	$y = -0.0169x^2 + 1.8823x – 0.5777$	Quadratic	heteroscedastic
Phendimetrazine	5-500	$y = -0,0262x^2 + 1,7487x - 0,2506$ $R^2 = 0,9839$	Quadratic	heteroscedastic

Promethazine	10-750	$y = -0.0078x^2 + 1.2546x – 0.2186$	Quadratic	heteroscedastic
Scopolamine	5-500	$y = -0.0211x^2 + 1.6569x – 0.1287$	Quadratic	heteroscedastic
Ticlopidine	5-500	$y = -0.0431x^2 + 3.2484x – 0.3435$	Quadratic	heteroscedastic
Warfarin	25-1000	$y = -0.0035x^2 + 0.4128x – 0.0139$	Quadratic	heteroscedastic

Table S2. *Results of the analytical method validation: LOD, trueness, precision, and recovery. Trueness and precision data were obtained at 6 concentration levels (5- 1000 ng/L); the averaged values are reported.*

Compound	**LOD (ng/L) Hubaux-Vos**	**LOD Verified (S/N>3) (ng/L)**	**LOQ (ng/L) Hubaux-Vos**	**Intermediate Precision (mean CV%)**	**Repeatability (mean CV%)**	**Trueness (mean bias%) Intraday**	**Trueness (mean bias%) Interday**	**Recovery (RE%)**
Antidepressants								
Amitriptyline	3	3	7	15	11	-11	-5	106
Citalopram	2	2	4	13	8	-1	-1	96
Clonidine	23	15	45	2	11	-4	-16	99
Fluoxetine	29	15	59	1	23	-14	-16	99

Fluvoxamine	16	30	33	26	17	-6	1	104
m-CPP	3	2	5	16	7	-12	0	98
Mianserin	22	15	45	1	11	1	0	88
Mirtazapine	2	2	4	14	11	-13	-1	95
Paroxetine	10	7.5	21	12	11	-5	0	103
Trazodone	3	2	7	20	16	-11	1	83
Benzodiazepines and analogues								
3-OH-Bromazepam	14	15	28	1	8	0	-1	105
7-Aminoclonazepam	16	7.5	32	18	11	-6	-1	115
7-Aminoflunitrazepam	11	7.5	22	16	10	-3	0	114
7-Aminonitrazepam	12	10	25	18	10	-4	0	95
Alprazolam	4	5	9	19	10	-1	-21	114
Bromazepam	20	25	39	25	17	-4	0	86
Brotizolam	4	3	7	15	14	-10	-6	113
Chlordiazepoxide	4	5	9	33	11	-25	-6	108

Clobazam	4	5	9	17	10	-13	-7	112
Clonazepam	12	10	23	18	10	-5	0	90
Clotiazepam	3	2	6	20	10	-6	1	107
Delorazepam	5	5	10	17	11	-23	-16	98
Demoxepam	19	25	38	24	13	-4	1	101
Desalchilflurazepam	3	2	5	17	10	-16	0	98
Diazepam	4	7.5	9	14	8	-12	1	97
Diclazepam	4	5	9	17	9	-28	-16	98
Flunitrazepam	4	7.5	7	18	8	-9	-4	117
Flurazepam	5	5	10	16	14	-5	0	107
Lorazepam	15	15	30	18	12	-5	0	98
Lormetazepam	13	15	27	18	9	-1	0	101
Midazolam	3	3	7	18	13	-5	0	96
Nordiazepam	12	10	25	18	11	-3	0	100
OH-Midazolam	6	2	10	19	8	-11	2	104

Oxazepam	25	25	49	25	10	-7	2	95
Temazepam	19	25	38	20	12	-4	0	101
Triazolam	3	5	7	21	13	-16	1	108
Zolpidem	3	2	6	24	13	-6	0	85
Zolpidem metabolite	4	2	7	19	9	-6	0	87
Antipsychotic								
Amisulpride	2	2	4	10	9	-6	-1	118
Aripiprazole	32	50	63	1	13	0	3	100
Carbamazepine	4	2	7	21	1	-13	-10	104
Chlorpromazine	4	2	7	20	16	-8	1	99
Clozapine	3	2	6	18	15	-4	0	97
Haloperidol	3	2	6	16	10	-8	1	86
Levomepromazine	22	15	43	29	23	-1	-3	93
Olanzapine	26	15	51	1	21	-11	-11	83
Periciazine	2	2	5	18	15	-6	0	88

Promazine	6	5	12	13	12	-16	-14	94
Quetiapine	2	5	5	19	13	-7	-8	114
Risperidone	3	5	7	19	17	-5	0	95
Tiapride	1	2	3	9	8	-6	-1	97
Venlafaxine	2	2	4	11	10	-7	-4	101
Ziprasidone	37	50	73	2	19	-8	-11	88
Antiepileptic								
Lamotrigine	18	50	37	25	14	-13	3	95
Oxcarbazepine	5	3	10	21	1	-19	-18	87
Cardiovascular Drugs								
Atenolol	2	2	5	12	7	-7	-2	107
Bisoprolol	4	5	8	19	13	-2	-3	90
Nebivolol	7	5	12	19	16	-3	1	100
Propafenone	2	5	5	16	11	-6	0	97
Ramipril	21	15	42	1	10	-3	-6	102

Telmisartan	24	15	48	22	13	-5	0	100
Verapamil	7	5	13	20	13	-6	1	106
Non-steroidal anti-inflammatory Drugs								
Ketoprofen	15	15	30	1	10	1	0	116
Ketorolac	11	25	22	14	10	-5	0	96
Analgesics / opioids								
Buprenorphine	44	25	87	30	20	-100	-12	83
Dihydrocodeine	2	2	5	9	8	-37	-16	109
Embutramide	2	2	4	12	10	0	-3	84
Hydromorphone	4	3	8	28	11	-15	4	94
Methadone	4	2	9	16	14	-18	-14	197
Oxycodone	3	3	6	13	10	4	0	113
Paracetamol	30	50	59	1	13	-11	-3	110
Phenacetin	11	7.5	22	12	9	-82	-26	86
Tapentadol	2	7.5	4	10	8	5	0	111

Tramadol	2	2	4	12	7	4	-1	107
Others								
Atropine	2	2	5	12	13	-7	-1	129
Biperiden	3	2	6	16	13	-4	2	80
Dextromethorphan	2	2	4	10	10	-5	-1	109
Diltiazem	3	2	6	16	10	-4	0	106
Diphenhydramine	10	5	20	39	24	-28	3	119
Diphenidine	4	5	8	19	12	-11	-8	102
EDDP	2	2	3	11	7	-6	-1	90
Gliclazide	15	7.5	15	1	7	1	1	80
Levamisole	4	2	8	25	13	-10	3	93
Lidocaine	2	2	4	20	15	-21	-18	105
Loperamide	3	2	7	25	11	-5	2	89
Metformin	31	50	63	1	19	-17	-21	80
Methylphenidate	2	2	4	10	9	-8	-1	101

Metoclopramide	2	2	4	12	9	0	0	109
Naloxone	5	3	10	20	11	-12	0	117
Oxybutynin	5	2	10	20	12	-13	-8	106
Phendimetrazine	1.5	5	3	12	7	-6	-1	103
Promethazine	6	3	11	14	9	-4	0	97
Scopolamine	2	3	4	10	8	-7	-1	91
Ticlopidine	2	2	3	10	8	-5	-1	94
Warfarin	8	15	17	14	13	-11	-8	83

Table S3: a) *Parameter (pH, temperature, time) found to be the most significant in compound stability decay,* ***b)*** *Static test and p-value obtained on the relevant parameter in the stability study for benzodiazepines.*

a)

Compound	Significant parameter
Antidepressants	
Mianserin	pH
Mirtazapine	pH
Trazodone	pH
Benzodiazepines and analogues	
7-Aminoclonazepam	pH
7-Aminoflunitrazepam	pH
7-Aminonitrazepam	pH
Alprazolam	pH
Brotizolam	pH
Chlordiazepoxide	pH
Clobazam	pH
Clonazepam	Temperature
Clotiazepam	pH
Delorazepam	pH
Demoxepam	pH
Desalchilflurazepam	Temperature
Diazepam	pH
Diclazepam	pH
Flunitrazepam	pH

Flurazepam	pH
Lorazepam	pH
Lormetazepam	pH
Nordiazepam	pH
Oxazepam	pH
Zolpidem	pH
Zolpidem metabolite	pH
Antipsychotic	
Clozapine	pH
Levomepromazine	pH
Olanzapine	pH
Periciazine	pH
Quetiapine	pH
Risperidone	pH
Antiepileptic	
Lamotrigine	pH
Oxcarbazepine	pH
Cardiovascular Drugs	
Bisoprolol	pH
Nebivolol	pH
Telmisartan	pH
Verapamil	pH
Analgesics / opioids	
Embutramide	Temperature

Hydromorphone	pH
Others	
Gliclazide	pH
Levamisole	pH
Naloxone	Temperature
Oxybutynin	pH

b)

Benzodiazepines	Significant parameter	Statistical test	P-value
7-Aminoclonazepam	pH	t-test	4.4e-03
7-Aminoflunitrazepam	pH	t-test	1.4e-02
7-Aminonitrazepam	pH	t-test	4.4e-04
Alprazolam	pH	t-test	2.4e-02
Brotizolam	pH	t-test	7.4e-07
Chlordiazepoxide	pH	t-test	4.4e-03
Clobazam	pH	t-test	5.3e-03
Clonazepam	Temperature	ANOVA	9.8e-03
Clotiazepam	pH	t-test	4.8e-03
Delorazepam	pH	t-test	5.5e-04
Demoxepam	pH	t-test	7.8e-03
Desalchilflurazepam	Temperature	ANOVA	1.7e-03
Diazepam	pH	t-test	5.3e-03
Diclazepam	pH	t-test	8.1e-03

Flunitrazepam	pH	t-test	1.4e-02
Flurazepam	pH	t-test	7.5e-03
Lorazepam	pH	t-test	2.2e-03
Lormetazepam	pH	t-test	2.8e-03
Nordiazepam	pH	t-test	2.8e-04
Oxazepam	pH	t-test	8.0e-04
Temazepam	pH	t-test	3.3e-02
Zolpidem	pH	t-test	6.7e-03

Figure S1: *Intermediate precision and repeatability (CV%) and trueness (bias%) trends for the antipsychotic amisulpride at the different calibration levels*

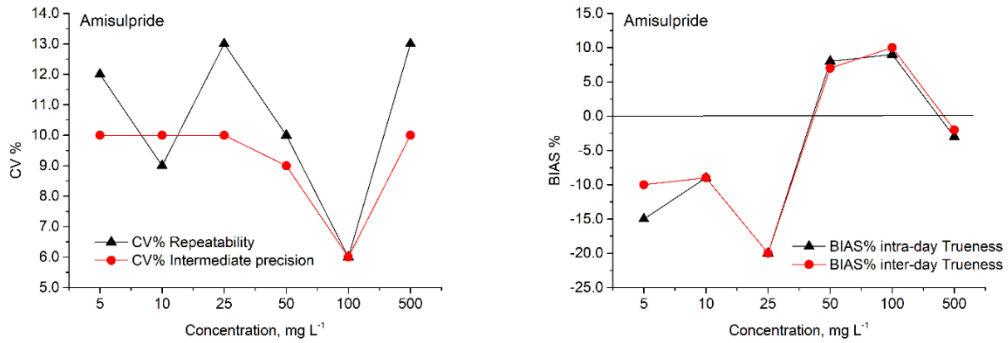


Figure S2: *Boxplot illustrating the ratio between the analyte and the internal standard of clotiazepam and zolpidem at the two different pH*

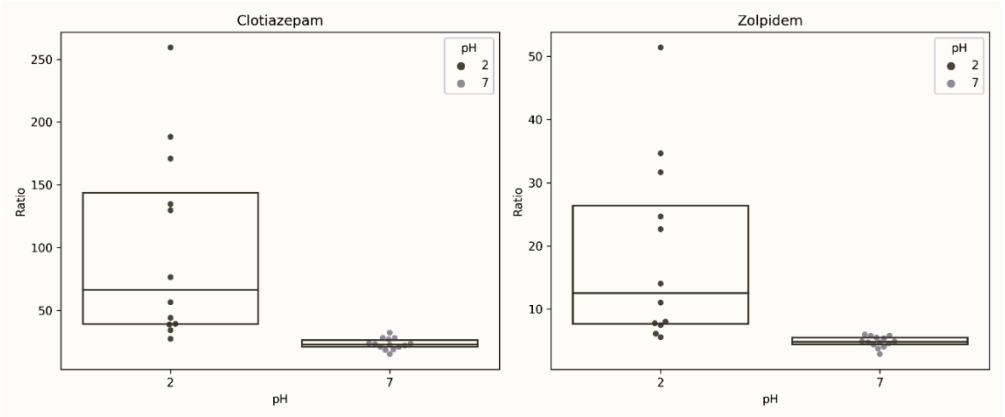


Figure S3: *Boxplot illustrating the ratio between the analyte and the internal standard of the benzodiazepines at the different pH*

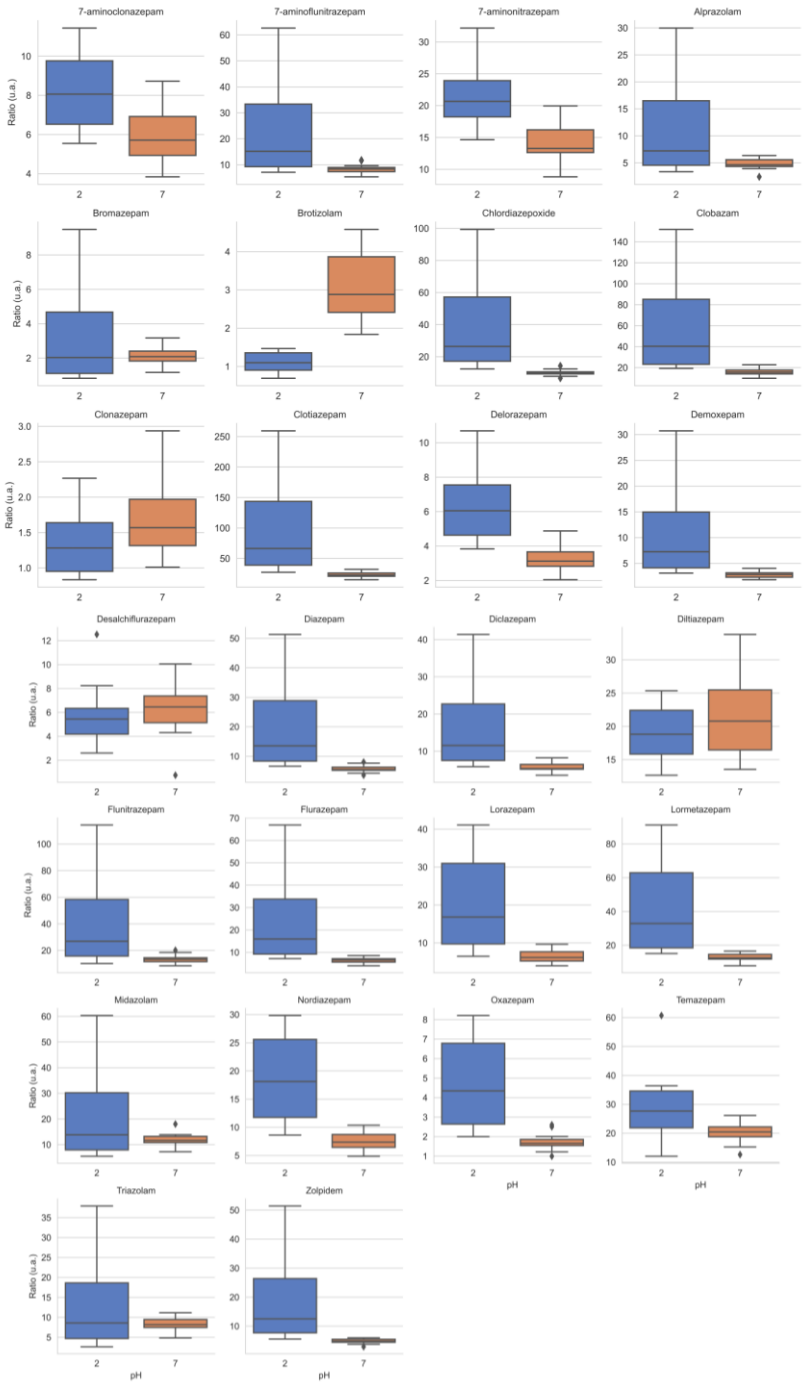


Figure S4: *Boxplot illustrating the ratio between the analyte and the internal standard of the benzodiazepines at the different time*

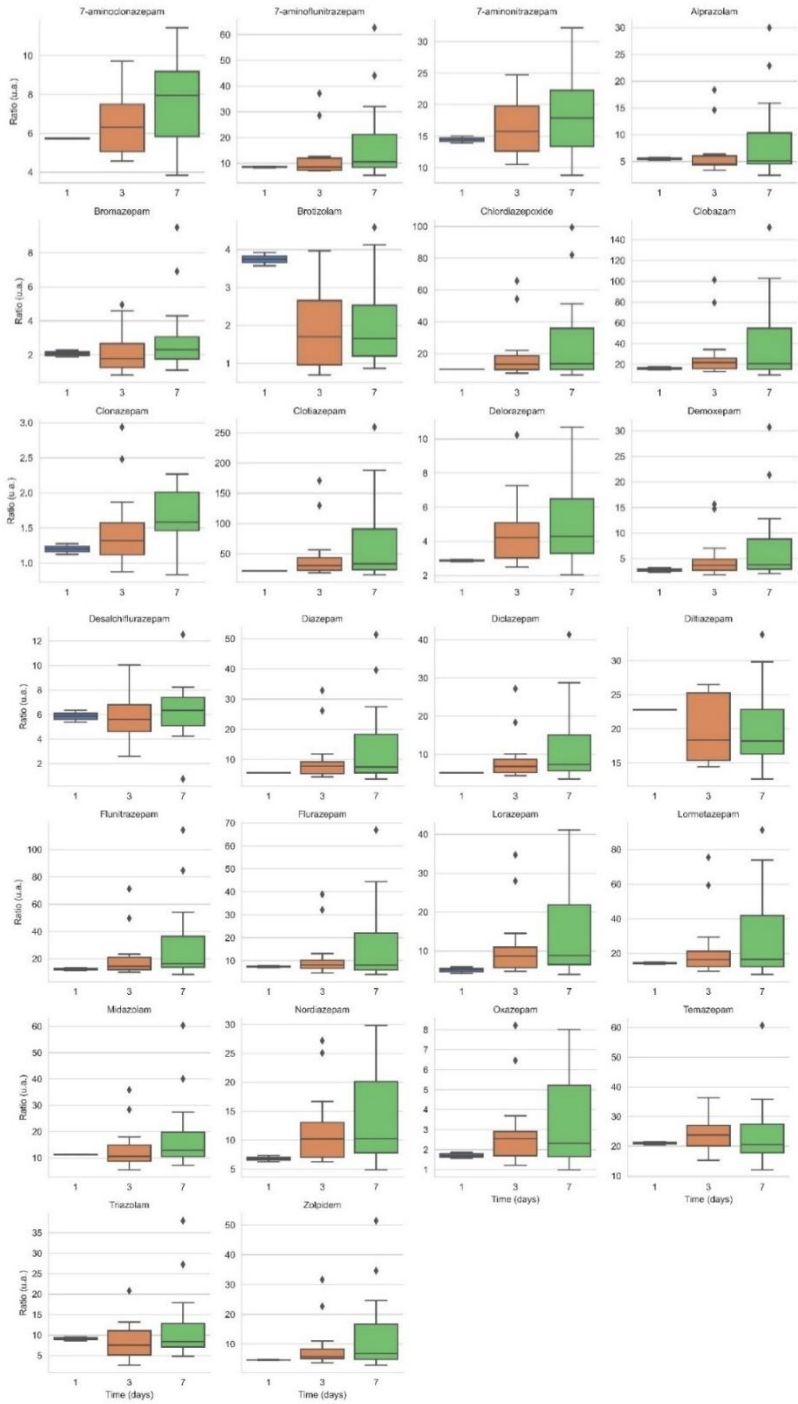
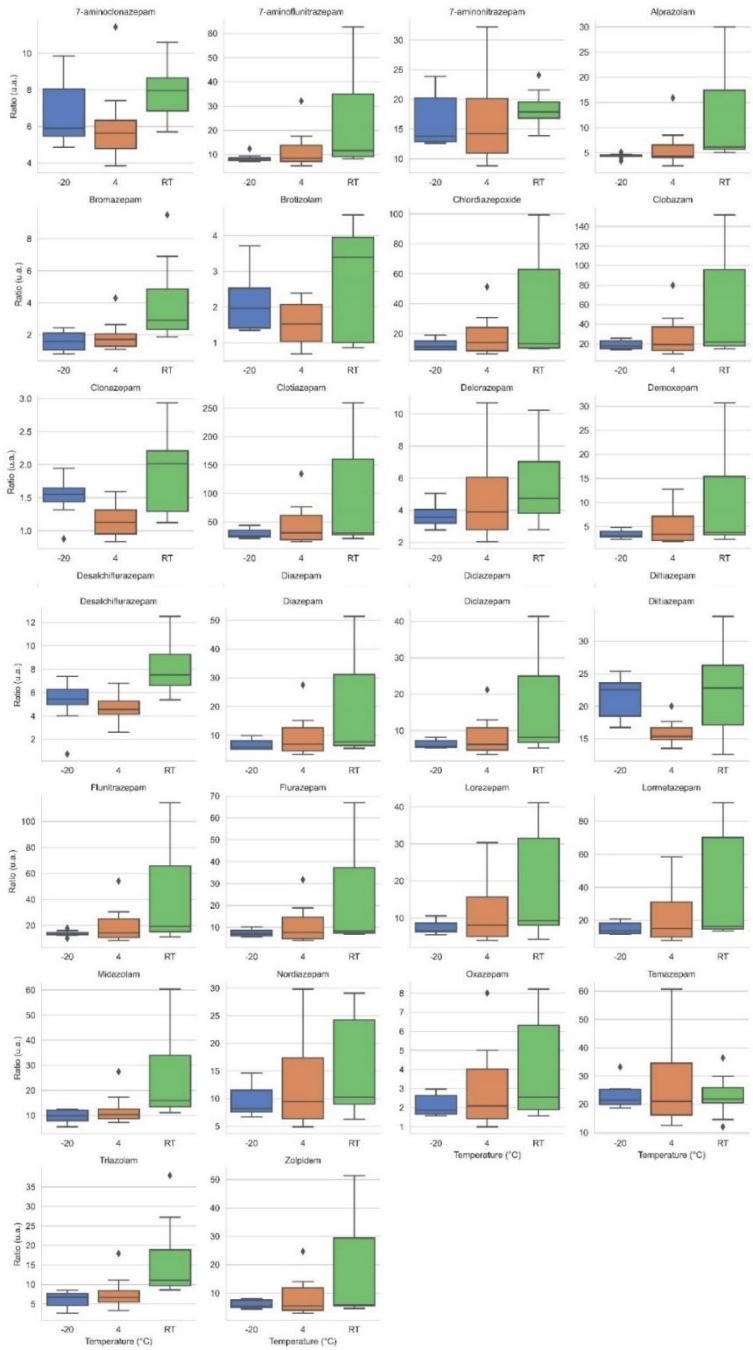


Figure S5: *Boxplot illustrating the ratio between the analyte and the internal standard of the benzodiazepines at the different temperature*



Chapter 5 | **Development a sustainable procedure for the determination of 16 illicit drugs and metabolites by means of ultra-high- performance liquid-chromatography-tandem mass spectrometry**

This Chapter is an extension of the method reported in Chapter 4 to allow the determination of 16 traditional drugs with the same procedure reported for pharmaceuticals. In this chapter we will describe the validation of the method that will be later used for the study reported in Chapter 6.

5.1 Introduction

The epidemiological analysis of drug use has gained considerable importance in the last two decades. This has not only involved the use of increasingly sensitive analytical techniques and the contribution and efforts of researchers around the world, but also the introduction of new matrices of choice, such as the wastewaters [1]. For the purposes of these analyses, wastewaters are usually sampled both before and after treatment by the local treatment plants. This double analysis makes possible to outline the real situation regarding the presence of pollutant components in the environment. The data obtained can be used for various purposes, such as, in the simplest case, for targeted investigations and controls on the drugs most consumed in a given area. Indeed, the interpretation of the obtained results can provide useful information on the level of consumption (close to real time) of illicit substances by the population present in the sampled geographical area [2]. Moreover, assuming that epidemiological analysis is characterized by the analysis of human consumption of drugs, it is necessary that the developed methods pay due attention not only to the presence of drugs, but also to the presence of their metabolites. In fact, only the presence of metabolites can confirm the consumption of illegal substances, as the products of biotransformation by the human body. In some cases, if the analysis does not reveal the presence of metabolites, attention should be shifted to the possibility that there is clandestine drug-producing activity in the geographic area covered by the search [3].

5.2 Materials and methods

5.2.1 Reagent and standards

The 16 pure standards of the targeted drugs were purchased from either LGC Promochem SRL (Milan, Italy) or Sigma-Aldrich (Milan, Italy). Methanol, formic acid, and acetonitrile were provided by Sigma-Aldrich (Milan, Italy). Ultra-pure water was obtained using a Milli-Q® UF-Plus apparatus (Millipore, Bedford, MA, USA). Stock standard solutions were stored at −20 °C until used. Three compounds were used as the internal standards (IS), including 13 isotopically marked molecules (Cocaine-D3, 6-MAM-D3, Morphine-D3, Methadone-D3, Amphetamine-D6, MDMA-D5, Buprenorphine-D4, Nor-buprenorphine-D3, Ketamine-D4, Nor-ketamine-D4, BZE-D3, EDDP-D3 and THC-COOH-D3). A working solution mixture was diluted in methanol containing all 16 reference substances (the full list of the target analytes is presented in Table 1) at the final concentration of 1 μg/mL. Also, the internal standard solution, containing the four selected IS, was prepared in methanol at the final concentration of 1 μg/mL.

Table 4: *List of the 20 analytes, the monitored transitions, their instrumental parameters and the related internal standard. For each group, analytes are listed in alphabetical order. CE = Collision Energy*

Compound	Relative retention time, min	Precursor mass Q1 m/z	Fragments mass Q3 m/z	CE, V	Internal Standard
Cocaine	2.5	304.1	183	35	Cocaine-D3
			82.1	37	
			104.9	49	
6-MAM	2.1	328.2	265.1	53	6-MAM-D3
			211.2	34	
			209	40	
Morphine	1.4	286.1	152.1	75	Morphine-D3
			201	35	
			165	56	

Methadone	3.3	310	265	20	Methadone-D3
			105	33	
			77	73	
Codeine	2.0	300	165	59	Cocaine-D3
			115	90	
		197.1	152	80	
Amphetamine	2.0	136	91	25	Amphetamine-D6
			119	11	
			65.1	46	
MDA	2.1	180	133.1	25	Amphetamine-D6
			135.1	25	
			163.1	15	
MDMA	2.1	194.1	163.2	18	MDMA-D5
			133	27	
			105.1	34	
Cocaethylene	2.7	317.9	196.1	27	Cocaine-D3
			82	39	
			108.1	47	
Buprenorphine	3.0	468.3	396.2	53	Buprenorphine-D4
			414.3	47	
			187	50	

Norbuprenorphine	2.6	414.1	340	41	Nor-Buprenorphine-D3
			152.1	101	
			165.1	83	
Ketamine	2.3	238	125	35	Ketamine-D4
			207.1	19	
			179	23	
Norketamine	2.2	224	207	17	Nor-ketamine-D4
			125	37	
			179	22	
Benzoylecgonine (BZE)	2.5	290	168.1	40	BZE-D3
			105.1	47	
			77.1	30	
EDDP	3.0	278.1	186.1	45	EDDP-D3
			249.1	38	
			234.1	50	
THC-COOH	5.2	345	299.1	27	THC-COOH-D3
			327.1	22	
			193.1	35	

5.2.2 Sample preparation

As we did for the procedure described in Chapter 4, the spiked samples used in the method development and validation were prepared from ultra-pure water (Milli-Q® UF-Plus) fortified at six concentration levels (5-1000 ng/L) with the working solution mixture and the internal standards mixture (final concentration 25 ng/L). The 10 mL of wastewater was added with a mixture of ammonium formate (2 mmol/L) and formic acid (0.02%). The samples were then vortexed, centrifuged for 10 min at 13,000 g, and 5 μL of the supernatant was injected into the UHPLC system.

5.2.3 Instrumentation

UHPLC separation was performed using a Phenomenex Kinetex C18 column (100 × 2.1 mm, 1.7 μm) maintained at 45 °C on the SCIEX ExionLCTM AC system. The mobile phases consisted of water (A) and acetonitrile (B), both mixed with formic acid 5 mM. The LC flow rate was set at 0.5 mL/min and the mobile phase eluted under the following linear gradient conditions (A:B, v:v): isocratic elution at 97:3 for 0.5 min, from 97:3 to 100% of B in 5.50 min, isocratic elution at 0:100 for 1.5 min and final re-equilibration at the initial conditions for 0.1 min and maintained for 1.5 min. The total run time was 8 min. All analyses were performed using a mass spectrometer equipped with a quadrupole trap SCIEX triple QuadTM 7500 mass spectrometer (Sciex, Darmstadt, Germany) system equipped with an OptiFlow Pro ion source with an analytical probe and E Lens. Electrospray ionization (ESI) was operated in the positive ion mode for all the analytes. For each transition, focusing voltages and collision energy (CE) were optimized under continuous analyte infusion. A single acquisition method consisting of 61 Multiple Reaction Monitoring (MRM) transitions (58 for the drugs and 13 for the internal standards) was created using the Scheduled MRM programming in SCIEX OS software 2.0. Three MRM transitions were monitored for each target analyte. The full list of the target analytes, the monitored transitions and the corresponding instrumental parameters are reported in Table 1. Since no extraction nor purification of the matrix was executed prior to injection, a control sample (mixture of the 16 analytes in water, concentration 100 ng/L) was injected at each analytical session to check the stability of the data acquired by the instrument.

5.2.4 Method validation

The protocol used for validation is the same as the one reported in Section 4.2.5 (Chapter 4) and is based on the validation protocol found in the literature

[4]. The accuracy, precision, sensitivity, recovery and calibration model of the above analytical method were analysed. The sensitivity for each target analyte was expressed by its LOD value. The LOD values were first calculated with Hubaux-Vos algorithm [5] and then verified by spiking the aqueous matrix with the target analytes at increasing concentration levels (the concentration selected around the calculated LOD), in order to verify the minimum concentration at which the observed instrumental signal-to-noise ratio (S/N) was greater than 3. The recovery was determined by comparing experimental results obtained from three water samples before and after acidification with ammonium formate and formic acid.

Recovery was evaluated at three different concentration levels: low, medium and high. Specifically:

- for cocaine, methadone, MDMA, cocaethylene, ketamine, benzoylecgonine, EDDP, the concentration levels of 10 ng/L, 100 ng/L and 1000 ng/L were chosen;
- for 6-MAM, morphine, codeine, norketamine, the concentrations 75 ng/L, 750 ng/L and 1000 ng/L were chosen;
- for buprenorphine and norbuprenorphine the concentration levels of 300 ng/L, 750 ng/L and 1000 ng/L were chosen;
- for amphetamine, concentration levels of 750 ng/L, 1000 ng/L and 1500 ng/L were chosen.

5.3 Result and discussion

The method under study proved adequate for the individual detection of 16 target analytes and 13 internal standards at concentrations equal or lower than 10 ng/L, combined with an injection volume of only 5 μL. The main objective of the present study, aimed to introduce a new panel of substances into the previously developed method (Chapter 4) for the simultaneous determination of an extended group of drugs in wastewater, was therefore achieved. As previously mentioned, the developed method aimed to meet the strict conditions in terms of simplicity, sensitivity and sustainability of sample pretreatment.

The complete results of the validation experiments for wastewater samples fortified with 20 analytes at six concentrations levels (5-1000 ng/L) are reported in the Table 2 (calibration features), Table 3 (LOD) and Table 4 (recovery) and discussed hereafter.

Table 2. *Calibration models for the targeted analytes*

Analyte	**Equation**	**Heteroschedastic / homoschedastic**	**Linear / quadratic**	**Weight**
6-MAM	$y = -0{,}0029x^2 + 0{,}9679x - 0{,}0295$	Heteroschedastic	Quadratic	$\frac{1}{x}$
Amphetamine	$y = -0{,}0089x^2 + 1{,}9897x - 33{,}609$	Homoschedastic	Quadratic	$\frac{1}{x}$
Buprenorphine	$y = 0{,}0312x^2 + 0{,}6939x + 6{,}7724$	Heteroschedastic	Quadratic	$\frac{1}{x}$
Benzoylecgonine	$y = -0{,}0019x^2 + 0{,}9425x + 0{,}2053$	Heteroschedastic	Quadratic	$\frac{1}{x}$
Cocaethylene	$y = -0{,}0125x^2 + 2{,}3071x - 0{,}1536$	Heteroschedastic	Quadratic	$\frac{1}{x}$
Cocaine	$y = 0{,}0021x^2 + 0{,}7785x + 0{,}833$	Heteroschedastic	Quadratic	$\frac{1}{x}$
Codeine	$y = -0{,}0128x^2 + 1{,}9865x - 0{,}3042$	Heteroschedastic	Quadratic	$\frac{1}{x}$
EDDP	$y = 0{,}0005x^2 + 0{,}4941x + 0{,}0033$	Heteroschedastic	Quadratic	$\frac{1}{x}$
Ketamine	$y = 0{,}0018x^2 + 0{,}9939x + 0{,}3225$	Heteroschedastic	Quadratic	$\frac{1}{x}$

MDMA	$y = -0{,}0878x^2 + 12{,}226x + 0{,}8028$	Heteroschedastic	Quadratic	$\frac{1}{x}$
Methadone	$y = -0{,}0329x^2 + 5{,}47x - 0{,}1377$	Heteroschedastic	Quadratic	$\frac{1}{x}$
Morphine	$y = -0{,}0017x^2 + 1{,}1118x + 0{,}4391$	Heteroschedastic	Quadratic	$\frac{1}{x}$
Norbuprenorphine	$y = 0{,}6148x + 0{,}4927$	Heteroschedastic	Linear	$\frac{1}{x}$
Norketamine	$y = 0{,}8742x - 0{,}1109$	Heteroschedastic	Linear	$\frac{1}{x}$
THC-COOH	$y = 0{,}001x^2 + 0{,}0635x + 0{,}3401$	Heteroschedastic	Quadratic	$\frac{1}{x}$

Table 3: *LOD using SCIEX Triple Quadrupole™ 7500 for traditional drugs of abuse*

	LOD Verified (ng/L)	**LOD (ng/L) Calculated Hubaux-Vos** [5]
Cocaine	1	1
Benzoylecgonine	1	1
Cocaethylene	1	1
Methadone	1	1
EDDP	1	1

MDMA	2	2
MDA	15	33
Amphetamine	50	44
Ketamine	1	1
Norketamine	3	3
6-MAM	3	3
Morphine	3	3
Codeine	3	4
Buprenorphine	15	15
Norbuprenorphine	40	67
THC-COOH	50	100

Table 4: *Recovery (RE%)*

	Low level (ng/L)	**Medium level (ng/L)**	**High level (ng/L)**
Cocaine	90	93	94
Methadone	99	98	100
MDMA	94	94	82
Cocaethylene	114	94	102
Ketamine	90	103	86
Benzoylecgonine	105	92	86
EDDP	84	88	85
	Low level (ng/L)	**Medium level (ng/L)**	**High level (ng/L)**
6-MAM	113	114	90

Morphine	119	108	89
Codeine	100	110	85
Norketamine	115	110	97
	Low level (ng/L)	**Medium level (ng/L)**	**High level (ng/L)**
Buprenorphine	89	92	100
MDA	96	91	112
Norbuprenorphine	114	94	86
	Low level (ng/L)	**Medium level (ng/L)**	**High level (ng/L)**
Amphetamine	91	103	106

Table 2 reports the outcome of the calibration process. Analysis of residues and variances of calibration data points at low, medium and high concentration levels showed that heteroscedastic distributions were present for all the target analytes (with the exception of amphetamine), making the introduction of weighting factors in the calibration beneficial (either $1/x$ or $1/x^2$).

The LOD values, calculated using the corrected Hubaux-Vos algorithm [5], ranged from a minimum of 1 ng/L for cocaine, benzoilecgonine, cocaethylene, methadone, EDDP and ketamine up to a maximum of 100 ng/L for THC-COOH. The relatively large difference in the calculated LOD levels is due to the different chemical composition (alcohol moieties, amide-sulphur secondary structure, etc.) of the compounds in the panel. The purely experimental LOD verification proved that 11 analytes out of 16 (69%), yielded a LOD $\leq$ 3 ng/L. While the remaining 5 analytes showed LOD values higher than 15 ng/L. The estimated LODs are in agreement with the concentrations generally detected in wastewater and adequate for almost all the target analytes. Notably, as in the case of pharmaceuticals, specific recommendations for the detection of illicit drugs in wastewater have not yet been established, although a European Union directive on the monitoring of certain ECCs.

The overall recovery for all analytes proved satisfactory, with percentages higher than 80% obtained for all 16 analytes at all three concertation levels considered. This result was expected, taking into account that no steps in the

procedure is likely to produce a loss of analyte; this detail is important considering that few ng/L have frequently to be detected for several analytes.

Intermediate precision and repeatability (expressed as coefficient of variation percent, CV%) and trueness (expressed as bias %) were calculated for all 16 target analytes. Notably, the validation procedure adopted in the present study allows precise and accurate calculation at all concentrations involved in the calibration process (6 calibration levels). On the assumption that, values less than 15% of the coefficient of variation in percent are considered optimal, while values between 15% and 20% are acceptable. In the entirety of the data obtained, the accuracy can be considered satisfactory for most analytes. 6-MAM, morphine and MDMA show only one higher CV% value greater than 20%, however, this being only slightly higher it can be considered acceptable, as it is occasional. Trueness was also found to be within acceptable limits, except for the fourth concentration level of norketamine and the second concentration level of amphetamine. However, in the overall evaluation of the data, the trueness obtained with the method used was optimal.

5.4 Conclusions and future perspectives

The presented method proved to be effective for the determination of 16 illicit drugs in aqueous matrix, and due to the excellent results obtained during the validation, the inclusion of the panel of substances (Chapter 4) under investigation in wastewater screening was carried out. With these assumptions it was thus proceeded with the agreement of the University of Algiers (Chapter 6). It was ensured the possibility to perform screening analysis on wastewater samples with the ability to identify and quantify 111 emerging contaminants (95 pharmaceutical drugs and 16 illicit drugs) exploiting a robust, efficient and sustainable method.

References

[1] M.M. Nascimento, M.L. Nascimento, J. Pereira dos Anjos, R.L. Cunha, G.O. da Rocha, I. Ferreira dos Santos, P.A. de P. Pereira, J.B. de Andrade, A green method for the determination of illicit drugs in wastewater and surface waters-based on a semi-automated liquid-liquid microextraction device, J. Chromatogr. A 1710 (2023) 464230. https://doi.org/10.1016/j.chroma.2023.464230.

[2] S. Castiglioni, E. Zuccato, E. Crisci, C. Chiabrando, R. Fanelli, R. Bagnati, Identification and measurement of illicit drugs and their metabolites in urban wastewater by liquid chromatography-tandem mass spectrometry, Anal. Chem. 78 (2006) 8421–8429. https://doi.org/10.1021/ac061095b.

[3] E. Emke, D. Vughs, A. Kolkman, P. de Voogt, Wastewater-based epidemiology generated forensic information: Amphetamine synthesis waste and its impact on a small sewage treatment plant, Forensic Sci. Int. 286 (2018) e1–e7. https://doi.org/10.1016/j.forsciint.2018.03.019.

[4] E. Alladio, E. Amante, C. Bozzolino, F. Seganti, A. Salomone, M. Vincenti, B. Desharnais, Effective validation of chromatographic analytical methods: The illustrative case of androgenic steroids, Talanta 215 (2020) 120867. https://doi.org/10.1016/j.talanta.2020.120867.

[5] Andre. Hubaux, Gilbert. Vos, Decision and detection limits for calibration curves, Anal. Chem. 42 (1970) 849–855. https://doi.org/10.1021/ac60290a013.

Chapter 6 | **Application of the sustainable analytical approach for the determination of pharmaceutical and drug substances in wastewater: a collaboration with the University of Algiers**

6.1 Introduction

As presented in previous chapters, wastewater analysis is emerging as a growing scientific discipline that offers an effective tool for monitoring pharmaceutical and illicit drug use in real time and across geographic areas. Because of the potential of this technique and the instrumentation available to the laboratory, I had the opportunity to apply the screening procedures described in chapters 4 and 5 to samples obtained through a collaboration with the University of Algiers.

Algeria is one of the southern Mediterranean countries, located in North Africa, with an area of 2,381,741 km2 and divided into 48 "wilayas". Nearly 80% of its territory is a desert area, where rainfall is almost non-existent and surface water resources are very scarce and limited mainly to the northern slope. For these reasons, Algeria is affected by water stress and is considered one of the poorest countries in terms of water potential, as the available quota per inhabitant and year is below the theoretical threshold of scarcity set by the World Bank (1000 m^3) [1,2]. The already high-risk water unavailability can be further exacerbated by wastewater pollution; including the class of newly emerging contaminants that counts pharmaceuticals and drugs. Moreover, in 2022, the drug use situation in Algeria was worrying, according to the Office for Combating Drugs and Drug Addiction. The police managed 304 cases of cocaine trafficking. In addition, 271 cases of cocaine possession and use were recorded, leading to the arrest of 113,012 people. The data covered also heroin (65 cases of trafficking and 133 cases of possession/use), cannabis (8,342 cases related to trafficking, marketing, possession, use and cultivation) and psychotropic substances (14,348 cases of trafficking, of which 97 related to international trafficking, and 23,841 cases of possession/use). In conclusion, more in-depth monitoring of actual pharmaceuticals and drug use appears necessary to establish a more effective control and prevention plan both in safeguarding the health of the population and in purifying wastewater from these emerging environmental contaminants.

6.2 Objective of the collaboration

This chapter will briefly discuss the application of the two previously validated robust, stable, precise and accurate analytical screening methods [3] on 134 real samples taken at three different sewage treatment plants located in Algeria: Berraki, Reghaia and Beni Messous (Figure 1). The collaboration project was to outline the actual situation regarding the presence of certain emerging contaminants in the environment and the capacity of the plants to purify wastewater from these compounds. In particular, the study is originated from an international agreement that the Chemistry Department of the University of Turin signed with the Faculty of Pharmacy of the University of Algiers to assess the epidemiology of drug and narcotic consumption in Algeria.

The project is designed to be divided into the following main parts distributed in the third year:

- **Creation of a collaboration with the Faculty of Pharmacy of the University of Algiers;**
- **Start a sampling campaign:** sampling was carried out at the inlet (n=67 samples A) and outlet (n=67 samples B) of the sewage treatment plants over a period of approximately 2 months, from 16/04/2023 to 31/05/2023;
- **Sample processing in the laboratory:** the analytical methods used in this study enabled the simultaneous quantitative analysis of 95 pharmaceuticals and 11 illicit drugs (these methods are presented in chapters 4 and 5);
- **Data processing:** in particular with the identification of the compounds present in the samples, the inter- and intra-week variability and the inter- and intra-plant variability.

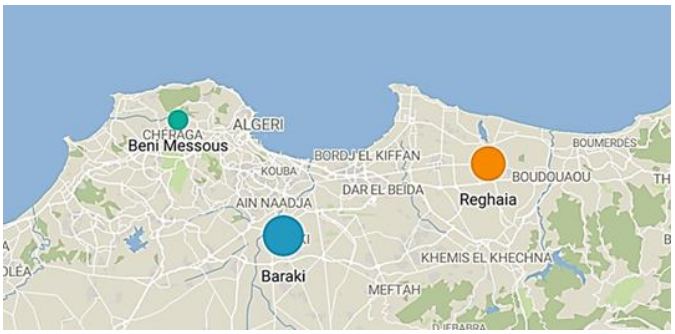


Figure 1 *Location of the three purification plants on Algerian territory: Berraki, Reghaia and Beni Messous*

6.3 Analytical procedure and results

The study was based on the use of two robust and effective procedures using a sample volume of 10 mL followed by direct injection in ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) (in Figure 2, sample pre-treatment) for the simultaneous determination of 95 pharmaceutical drugs and 11 illicit drugs in wastewater. The wastewater samples (50 mL) were centrifuged at 4000 rpm for 5 min and vacuum- filtered through a 0.22 μm filter device (Steriflip-GP 50 mL, 22 μm, Merck Life Science Srl). Then, 10 mL of filtered wastewater was spiked with the internal standards mixture (final concentration 25 ng/L) and added with a mixture of ammonium formate (2 mmol/L) and formic acid (0.02%). The samples were then vortexed, centrifuged for 10 min at 13,000 g and 7 μL of the supernatant was injected into the UHPLC system.

Sampling was carried out using an automatic sampler by taking a representative 24-hour sample at the inlet of the treatment plants and a representative sample at the outlet of the treatment plants, both obtained from a mixture of three samples taken at different distances (10 metres) and depths (50 cm) from each other, in order to gain a comprehensive understanding of the sampling situation. The samples did not undergo any pre-treatment in the purification plant; once taken, they were placed in 50 mL plastic falcons and, upon arrival at the laboratory, were centrifuged at 4500 rpm for 5 minutes, then acidified with HCl and, finally, stored in chillers with ice blocks at -20°C [4]. The storage conditions were chosen in order to ensure the stability of the molecules detected in the present study. The target molecules used to estimate pharmaceutical and drug use are, for the most part, fairly stable under sewage conditions (pH 6.5 and temperature 20°C); as stability after sampling is dependent on storage conditions, it was determined that the best storage method was to acidify the sample to pH 2 at -20°C to inhibit bacterial activity [5].

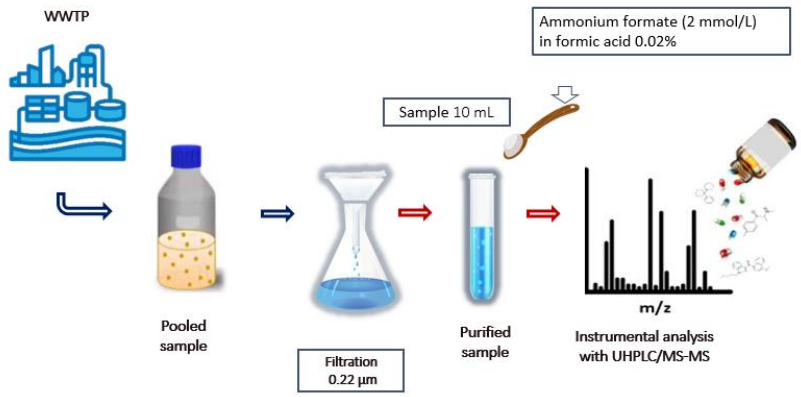


Figura 2 *Sample pre-treatment*

During the study, 67 samples were analysed at the inlet and as many at the outlet of three treatment plants in the Algiers wilaya to determine the prevalence of pharmaceutical and drug substances and their metabolites in the wastewater.

Figure 3 shows the significant trends observed in the three plants, expressed as a percentage of positivity over the three plants and for the totality of the samples. Detection of between 90% and 100% of substances such as Benzoylecgonine, Codeine, MDMA, Carbamazepine, Pregabalin, Tramadol, Lamotrigine, Amisulpride, Atenolol, Bisoprolol, Ketoprofen, Metformin and Lidocaine can be pointed out. These results highlight the need for specific approaches to manage these substances throughout the entire wastewater treatment process. Substances such as Quetiapine, Morphine and Paracetamol showed high detection rates (70%-90%), suggesting widespread use that would require special attention to mitigate their potential environmental impact. Compounds with moderate detection rates (20%-60%), such as EDDP, Amitriptyline, Diltiazem, Gliclazide, Telmisartan, Citalopram, Oxazepam, Dextromethorphan and Metoclopramide raise questions about their sources and the need for further investigation and increased surveillance, in the same way as those below 20%.

On the other hand, there is a total absence of THC-COOH. The lack of signals of the metabolite of THC is not due to the lack of consumption of the substance in the Algerian areas, but to the improper storage of the sample. In fact, if the water sample is aliquoted and acidified (for example, by bringing it to pH=2) before being properly filtered by completely removing the particulate matter, all

the THC-COOH is converted into its neutral and more lipophilic form and thus remains absorbed in the particulate matter present. For this reason, we unfortunately eliminated it during the filtration step on the 0.22 µm filter.

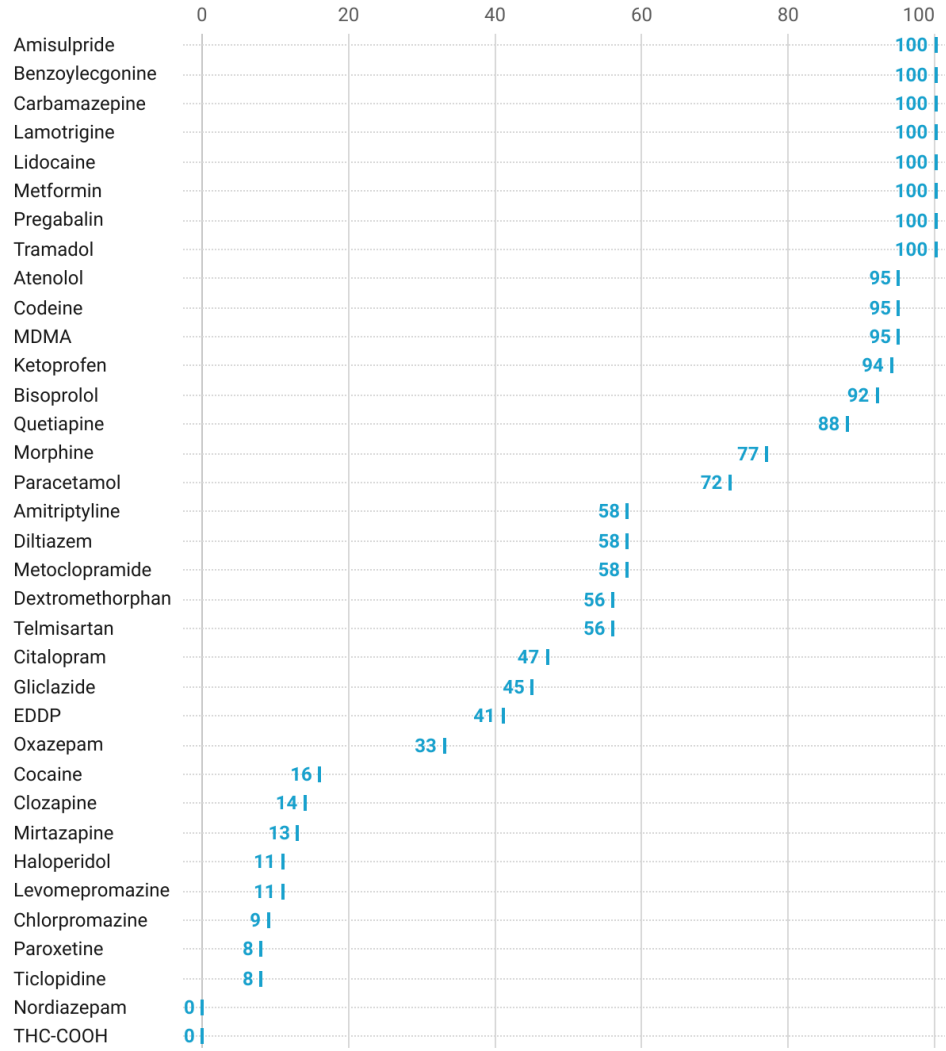


Figure 3 *Total percentages of presence of the substances in the samples analysed.*

Regarding the study of inter-weekly and intra-weekly variability, variability between WWTPs and variability between samples entering and leaving the plants, the Figure 4 shows the percentage frequency of significant variability for the three conditions. In particular, it is observed that the greatest variability

occurs between the different WWTPs studied, immediately followed by the observed difference between the amounts measured in the water entering and leaving the plants.

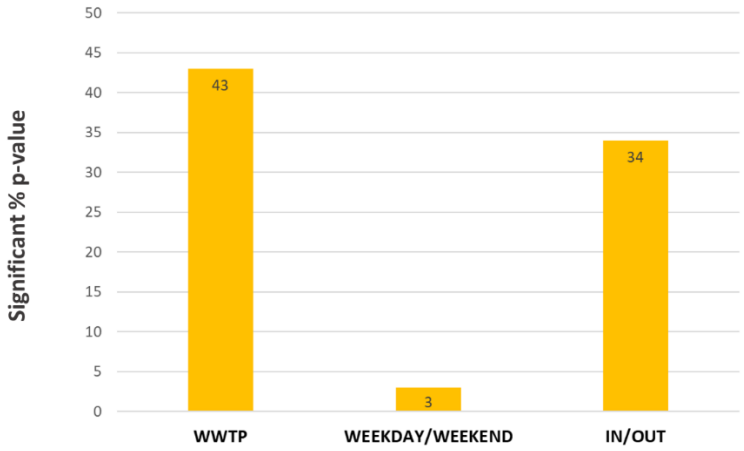


Figure 4 *Percentage of significant p-values measured for the three conditions: variability between different WWTPs, between weekdays and weekends, and between influent and effluent samples at plants.*

In detail, concerning the study of variability, significant results (p-value < 0.05) were observed only for some substances. For example (Tukey's Test Graph, Figure 5.1 and 5.2) for MDMA, different and statistically significant trends are observed between samples taken from different wastewater treatment plants and those referring to weekdays and weekends.

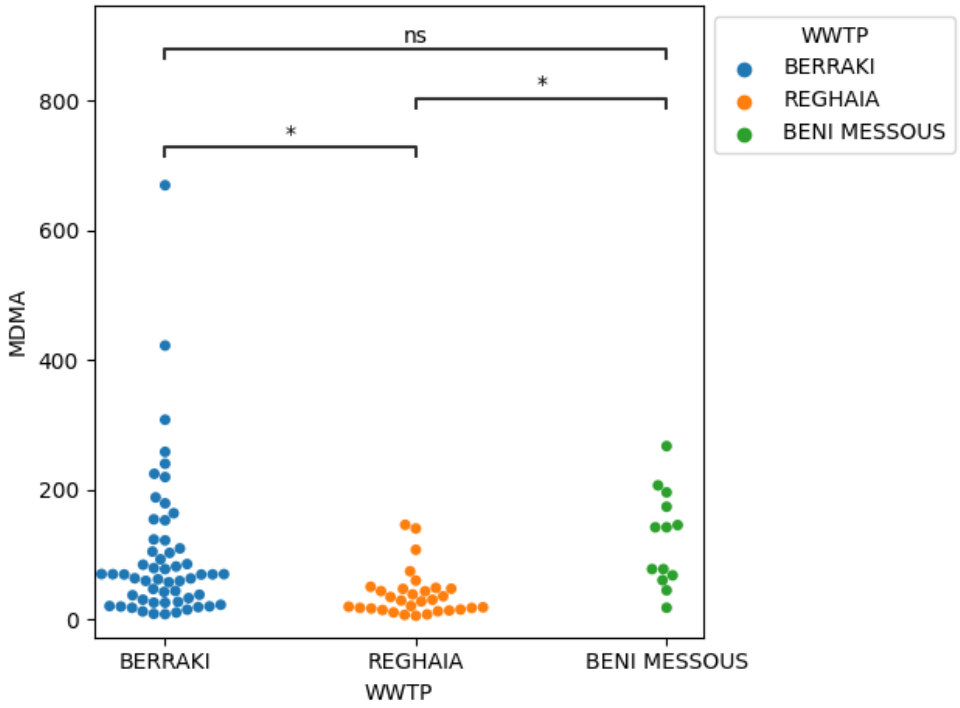


Figure 5.1 *Tukey test for MDMA in different purification plants. P-value Berraki vs. Reghaia = 0.01; p-value Reghaia vs. Beni Messous= 0.02; p-value Berraki vs. Beni Messous= 0.63.*

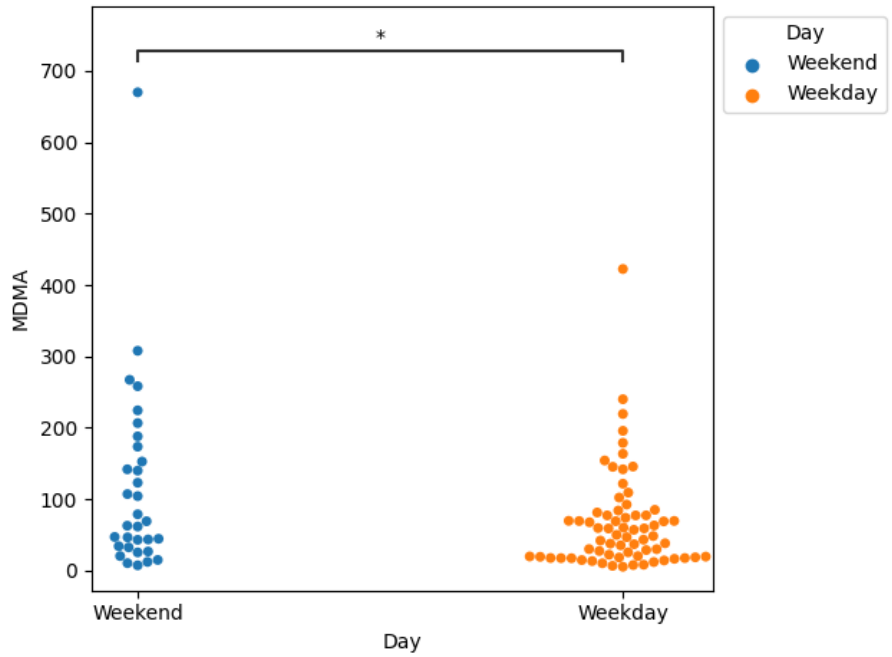


Figure 5.2 *Tukey's test for MDMA. P-value weekday/weekend= 0.02.*

Finally, interesting evidence is the poor abatement rate of some drugs from the sewage treatment plants. The abatement rate for each of the drugs (Table 1) is calculated based on the difference between the initial analyte concentration (C0) (influent sample) and the final analyte concentration (Cf) (effluent sample), obtained from the calibration curve for each analyte in the sample solutions, using the following formula [6]:

$$\%\ abatement = \frac{C0 - Cf}{C0}\ x\ 100$$

Comparing the concentrations of the drug before and after the purification process, an abatement rate ≥ 50% is observed for 15 out of 27 analytes (55%) in the Berraki plant. In the other two plants the number of analytes is lowered even further (11 in the Reghaia plant and 7 in the Beni Messous plant), even when the concentrations of the analytes in the sample were low (as in the case of Quetiapine, which has an average concentration of 20 ng/L), without any risk of deposition and retention of the compound in the plant. Particularly interesting is, also, the low removal (less than 50 percent) of some of the pharmaceutical compounds under investigation. In 37% of the cases, a higher concentration of the samples exiting the purification plants was recorded, as in the case of Citalopram, Oxazepam, Quetiapine, Carbamazepine, Bisoprol, Telmisartan, Dextromethorphan, Glicazide, Lidocaine and Metoclopramide, a phenomenon observed to different degrees in the three plants studied. Apparent "negative removal" has actually been proven for some pharmaceuticals in a previous study [7], and may be associated with increased transformation of precursor or parent compounds, hydraulic residence time, and/or desorption from suspended solids in wastewater treatment processes [8]. The latter phenomenon draws attention to the need for careful monitoring of treatment plants, prior to the discharge of treated water to the receiving water body, further highlighting the need for technological developments in sewage treatment plants.

Table 5 *Percentages (%) of abatement*

	BERRAKI (%)	**REGHAIA (%)**	**BENI MESSOUS (%)**
Antidepressant			
Amitriptyline	91	91	56
Citalopram	82	-286	-13
Mirtazapine	100	0	0
Paroxetine	100	100	0
Benzodiazepines and analogues			
Diltiazem	49	41	75
Oxazepam	22	-361	-363
Antipsychotics			
Amisulpride	42	25	27
Chlorpromazine	100	100	0
Clozapine	100	0	0
Haloperidol	100	0	0
Levomepromazine	100	100	0
Quetiapine	55	72	-317
Antiepileptic			
Carbamazepine	-4	17	-15
Lamotrigine	3	34	18
Pregabalin	91	85	88
Cardiovascular Drugs			
Atenolol	57	87	83
Bisoprolol	33	75	-27
Telmisartan	12	43	-13
Non-steroidal anti-inflammatory drugs			
Ketoprofen	44	67	28
Analgesics/opioids			
Paracetamol	87	78	39
Tramadol	4	16	80
Others			
Dextromethorphan	49	-130	-83
Gliclazide	48	-67	76
Lidocaine	8	-110	-128
Metformin	79	78	89
Metoclopramide	75	-18	-44
Ticlopidine	100	0	0

6.4 Conclusions

For the first time in Algeria, a study provides a profile of pharmaceutical and illicit drug use in the population, highlighting consumption trends. This demonstrates the importance of the wastewater epidemiological method to be considered as an effective tool to support harm reduction activities and prevention policies. These results could be the starting point for a large-scale study aimed at reducing risks from a management and health perspective. This research is distinguished by its comprehensive approach, including a variety of substances in an in-depth analysis of wastewater. By developing a detailed temporal model, the study offers a unique perspective on the daily dynamics of xenobiotic use. Evaluating the efficiency of sewage treatment plants contributes to the understanding of substance removal mechanisms, thus highlighting the crucial role of treatment infrastructure in environmental conservation while offering a holistic and objective perspective.

References

[1] T. Luo, R.S. Young, P. Reig, Aqueduct Projected Water Stress Country Rankings, (2015). https://www.wri.org/research/aqueduct-projected-water-stress-country-rankings.

[2] L. Bouchaala, N. Charchar, H. Sahraoui, A. Gherib, Assessment of wastewater biological treatment efficiency and mapping of WWTPs and LTPs in Algeria, J. Environ. Health Sci. Eng. 19 (2021) 1153–1169. https://doi.org/10.1007/s40201-021-00682-1.

[3] M. Massano, D. Privitera, E. Alladio, E. Gerace, M. Minella, M. Vincenti, A. Salomone, Development and Application of a Sustainable Approach for the Determination of 95 Pharmaceutical Substances and Metabolites in Wastewater by Means of Ultra-High-Performance Liquid-Chromatography-Tandem Mass Spectrometry, (2023). https://doi.org/10.2139/ssrn.4580564.

[4] L. Bijlsma, J.V. Sancho, E. Pitarch, M. Ibáñez, F. Hernández, Simultaneous ultra-high-pressure liquid chromatography-tandem mass spectrometry determination of amphetamine and amphetamine-like stimulants, cocaine and its metabolites, and a cannabis metabolite in surface water and urban wastewater, J. Chromatogr. A 1216 (2009) 3078–3089. https://doi.org/10.1016/j.chroma.2009.01.067.

[5] A.L.N. van Nuijs, J.-F. Mougel, I. Tarcomnicu, L. Bervoets, R. Blust, P.G. Jorens, H. Neels, A. Covaci, Sewage epidemiology--a real-time approach to estimate the consumption of illicit drugs in Brussels, Belgium, Environ. Int. 37 (2011) 612–621. https://doi.org/10.1016/j.envint.2010.12.006.

[6] T.G. Kebede, S. Dube, M.M. Nindi, Removal of Multi-Class Antibiotic Drugs from Wastewater Using Water-Soluble Protein of Moringa stenopetala Seeds, Water 11 (2019) 595. https://doi.org/10.3390/w11030595.

[7] C. Postigo, M.J. López de Alda, D. Barceló, Drugs of abuse and their metabolites in the Ebro River basin: occurrence in sewage and surface water, sewage treatment plants removal efficiency, and collective drug usage estimation, Environ. Int. 36 (2010) 75–84. https://doi.org/10.1016/j.envint.2009.10.004.

[8] I. González-Mariño, E. Gracia-Lor, N.I. Rousis, E. Castrignanò, K.V. Thomas, J.B. Quintana, B. Kasprzyk-Hordern, E. Zuccato, S. Castiglioni, Wastewater-Based Epidemiology To Monitor Synthetic Cathinones Use in Different European Countries, Environ. Sci. Technol. 50 (2016) 10089–10096. https://doi.org/10.1021/acs.est.6b02644.

Chapter 7 | **Metabolic profile of N-ethylhexedrone, N-ethylpentedrone, and 4-chloromethcathinone in urine samples by UHPLC-QTOF-HRMS**

Marta Massano[1,4*], Melani Nuñez-Montero[2], Esther Papaseit[2], Olga Hladun[2], Clara Pérez-Maña[2], Mireia Ventura[5], Emilia Marchei[3], Eugenio Alladio[1,4], Enrico Gerace[4], Simona Pichini[3], Magi Farrè[2], Alberto Salomone[1,4]

[1]Department of Chemistry, University of Turin, Italy

[2]Unit of Clinical Pharmacology Hospital Universitari Germans Trias i Pujol (HUGTiP-IGTP) and Universitat Autònoma de Barcelona, Barcelona, Spain

[3]National Centre on Addiction and Doping, Istituto Superiore di Sanità, 00161 Rome, Italy

[4]Centro Regionale Antidoping, Orbassano (TO), Italy

[5]Energy Control, Associació Benestar i Desenvolupament, 08012 Barcelona, Spain;

** Corresponding author: Marta Massano*

Email address: marta.massano@unito.it

The content of this Chapter is included in one published manuscript:

Metabolic profile of N-ethylhexedrone, N-ethylpentedrone, and 4-chloromethcathinone in urine samples by UHPLC-QTOF-HRMS

***Marta Massano**, Melani Nuñez-Montero, Esther Papaseit, Olga Hladun, Clara Pérez-Maña, Mireia Ventura, Emilia Marchei, Eugenio Alladio, Enrico Gerace, Simona Pichini, Magi Farrè, Alberto Salomone*

Journal of Pharmaceutical and Biomedical Analysis

https://doi.org/10.1016/j.jpba.2024.115994

Keywords: HRMS, metabolites, synthetic cathinone, Human urine, NPS

The following chapters deal with the study of the metabolic profiles of NPS that have recently appeared on the illicit market and the use of other biological matrices (dried blood spots and keratin matrix) to study the NPS use in the population.

Abstract

Forensic laboratories are constantly required to identify new drugs and their metabolites. N-ethylhexedrone (NEH, HEXEN), N-Ethylpentedrone (NEP), and 4-Chloromethcathinone (4-CMC, clephedrone) are synthetic substances structurally related to natural cathinone, alkaloid present in the leaves of the Catha edulis (Khat) plant. These synthetic cathinones (SC) are members of the heterogenous family of new psychoactive substances (NPS) that raised major concerns in scientific and forensic communities over the past years due to their widespread consumption. In this context, we investigated their metabolic profile using of UHPLC-QTOF-HRMS to elucidate the distribution of the parent drug and its metabolites in urine samples over time. Initially, both male and female volunteers were divided into three groups and eight subjects of each group were administered intranasally or orally with one SC (20-40 mg of NEH or NEP intranasal, 100-150 mg of 4-CMC oral). Urine samples were collected at 0-2 and 2-4 or 2-5 hours. Urine (50 µL) was diluted 1:2 with acetonitrile/methanol (95:5) and injected into the UHPLC-QTOF-HRMS. Phase-I and phase-II metabolites were identified on the basis of fragmentation patterns and exact masses. Several phase-I and glucuronide-phase-II metabolites were identified in urine samples. Keto group reduction, hydroxylation and dealkylation were the common metabolic pathways identified for all cathinones and the presence of NEH-glucuronide, NEP-glucuronide and 4-CMC-glucuronide was also relevant. Significant is the slower metabolite formation for 4-CMC, which was detected at

high concentrations in its original form even 5 hours after administration, due to its long half-life and low intrinsic clearance compared to the other SCs. UHPLC-QTOF-HRMS demonstrated a considerable capability to semi-quantify the three synthetic cathinones and identify the target metabolites with high reliability. The introduction of new target compounds improves the efficiency of toxicological screening analysis on real samples and extends the window of detection of the SCs in biological matrices.

7.1 Introduction

Nowadays, novel psychoactive substances (NPS) abuse in U.S [1] and Europe is proliferating at unprecedented rate and represents an increasing challenge to the established national and international drug policies. At the end of 2022, the EMCDDA was monitoring around 930 new psychoactive substances [2] and 41 were first reported in Europe in 2022. Generally, the NPS are categorized as synthetic cannabinoids, stimulants, depressant (benzodiazepines and opioids), and hallucinogens, but this classification does not express adequately the variety and complexity of their potency, combined effects, and risk profiles that intersect categories and often differentiate compounds belonging to the same category [3]. After synthetic cannabinoids, synthetic cathinones (SCs) were the second-largest category of NPS monitored by the EU Early Warning System and at the end of 2021 the EMCDDA monitored 162 cathinones [4]. SCs appeared in drug markets in the mid-2000s. Since their first appearance on the recreational drug market, these synthetic substances have changed their chemical structure mainly in an attempt to evade law enforcement. In recent years, both the US Drug Enforcement Agency and Council of the European Union have implemented control measures to prohibit their use and there has subsequently been a decline in their use [5].
Most of the cathinone seizures are powder, together with pills and similar products. These compounds have also been found as adulterants in “classical” illegal drugs such as cocaine or MDMA, suggesting that their prevalence of consumption could be underestimated [6], [7]. Their consumption represents an important public health problem, according to the most recent report from the United Nations Office on Drugs and Crime [1]. In addition to the data obtained by seizure analysis, the public health problem related to cathinones is also illustrated by numerous intoxication cases related to these substances, and even some fatalities [8–10]. When the first generation synthetic cathinones (methylone, mephedrone, MDPV) became illegal, a second and third generation emerged and a lack of information exists on their molecular structure and metabolic pathway,

making their identification in biological samples challenging. This is critical in the case of cathinones that undergo extensive/fast metabolic degradation, for which the identification of metabolites in urine constitutes the only possible way of attesting their consumption, in forensic and clinical contexts [11]. Therefore, understanding the mechanisms of action of these new drugs and the knowledge of their short- and long-term effects, their pharmacokinetic properties, and the correlation between the concentration in biological fluids and their activity has become crucial for public health reasons.

The main objective of the study presented here was indeed to develop a method for the investigation of the main metabolic pathways of the latest generation synthetic cathinones (N-ethylhexedrone, N-ethylpentedrone and 4-chloromethcathinone) in urine of both male and female human volunteers. Furthermore, the simultaneous semi-quantification of these synthetic cathinones was carried out in order to observe the variation of their concentration simultaneously with the formation of the metabolites. High performance of ultra-high-pressure liquid-chromatography (UHPLC) was combined with quadrupole time-of-flight high-resolution mass spectrometry (QTOF-HRMS) allowing the prediction of the metabolic profile on the basis of similar studies on analogous synthetic cathinones. Using this approach, it has been possible to hypothesize and confirm the structures of the main phase I and II metabolites, their exact masses, and their fragmentation patterns. Several studies on the metabolism of these three cathinones can be found in the literature; however, none of them studied the actual metabolic pattern in human urine but only attempt to reproduce the phenomenon in mice [12], human liver microsomes [13] or in vitro [14]. In the latter studies, the formation of metabolites was observed following reduction, glucuronidation, hydroxylation and combined N-deethylation and N-acetylation, N-deethylation and succinic conjugation and N-deethylation and adipic conjugation reactions. The presence of these metabolites, however, was observed after many hours (even 24 h) of exposure to the substances.

7.2 Materials and methods

7.2.1 Reagent and standards

All chemicals, including HPLC grade methanol, formic acid, and acetonitrile, were purchased from Sigma-Aldrich (Milan, Italy). Ultra-pure water was obtained using a Milli-Q® UF-Plus apparatus (Millipore, Bedford, MA, USA). For UHPLC-QTOF-HRMS semi-quantitative analysis, mephedrone-D_3 was purchased from LGC Promochem (Milan, Italy) (purity >99%, concentration 1 mg/mL). N-ethylhexedrone (NEH), N-Ethylpentedrone (NEP), and 4-

Chloromethcathinone (4-CMC) were kindly provided Cayman Chemical (Ann Arbor, Michigan, USA) (methanolic solution at a 0.1 mg/ mL concentration, purity provided by the supplier >99%). All working solutions were prepared in methanol at 1 μg/mL and stored at -20°C until used.

7.2.2 Study protocol

The urine samples used in this study were obtained from, 13 women (mean age: 32.2 years (range 24-54) and 11 men (mean age: 31.0 (range 23-40) enrolled at the Hospital Universitari Germans Trias i Pujol (HUGTiP-IGTP), in Badalona, Spain. The participants were recruited by word of mouth and snowball sampling through the harm reduction, non-governmental organization Energy Control (ABD). The protocol to investigate the potential for abuse and the human pharmacology of substances of abuse, including synthetic cathinones and cannabinoids, was approved by the local human research ethics committee (CEI-HUGTiP ref. PI-18-267). The study was conducted according to the Declaration of Helsinki recommendations (Fortaleza, 2013 and Spanish law on clinical investigation). All the participants were informed, both orally and in writing, and signed an informed consent prior to inclusion. The participants received monetary compensation for their participation. All participants had past recreational experience with cocaine, amphetamines, MDMA and synthetic cathinones, but they declared no recent use of the three cathinones under study. The 24 participants were divided into 3 groups of 8 subjects each: G1, self-administration of NEH intranasally, G2, self-administration of NEP intranasally and, G3, self-administration of 4-CMC orally. Each subject participated in only one session and so they only consumed one of the three cathinones under study. The doses and routes of administration of these substances were chosen on the basis of information obtained from internet forums, which is the main channel used to obtain this type of information for the time being and were in the range of those recommended in risk reduction organizations.

Table 1: *Characteristics six volunteers participating in the study and dosages administered*

Group	Compound	Volunteer	Sex	Dosage (mg)
G1	**NEH**	33	Female	30 mg
		34	Male	40 mg
		35	Male	30 mg
		36	Male	40 mg
		37	Female	30 mg
		38	Male	40 mg
		39	Male	30 mg
		40	Female	20 mg
G2	**NEP**	41	Male	40 mg
		42	Male	40 mg
		43	Female	20 mg
		44	Male	30 mg
		45	Male	40 mg
		46	Female	30 mg
		47	Female	30 mg
		48	Female	40 mg
G3	**4-CMC**	49	Female	150 mg
		50	Male	100 mg
		51	Female	100 mg
		52	Female	150 mg
		53	Male	150 mg

		54	Female	100 mg
		55	Female	100 mg
		56	Female	150 mg

The study design was observational, naturalistic, and prospective, with minimal intervention. The sessions took place on three different days, one for each substance, at a private club with ambient music. The ambient temperature in the private club was around 24°C. The sessions started at 3:00 p.m. and finished at 8:00–9:00 p.m. All the doses were self-administered and were also self-selected by each participant, based presumably on their previous experience. The drug samples were tested by Energy Control, a harm reduction organization that provides a drug checking service for users. Measures of the pharmacological effects were collected (data not presented in this manuscript). The study methodology was similar to that of other previously published studies [15], [16]. Tables 1 shows the characteristics of the study volunteers. Urine samples were collected at two ranges of time: G1 and G2 at 0-2 hours and 2-4 hours while for G3 at 0-2 and 2-5 hours after consumption. All samples were collected in 1.5-mL tubes and stored at -20°C before the analysis. The evolution of the three synthetic cathinones and their metabolites over time was monitored, noting the differences between male and female metabolic pathways and observing how they formed and evolved in the two-time ranges studied. Regarding the influence of the age factor on metabolism, the age groups of the volunteers were narrow (range 25-41 years, only one subject was 51 years old) and preliminary results did not indicate any age-related differences. Furthermore, there are no articles in the literature focusing on the effect of age in the metabolism of cathinone or similar compounds. There are several studies [17], [18] defining age as a key factor influencing the expression of cytochromes P450. Cytochromes involved in phase I metabolism. We will take advantage of this suggestion to investigate in the future.

7.2.2.1 Statistical data processing

The collected data were evaluated in the form of boxplots [19], comparing the time ranges of 0-2 hours vs. 2-4 hours or 2-5 hours. Different boxplots were created in order to observe possible differences in the presence of the cathinones and their metabolites in urine samples as a function of dosage and gender. Boxplots are valuable tools in data analysis, which allow easy comparison of

multiple groups and identification of data skewness and potential outliers within a dataset. In addition, Wilcoxon signed-rank test [20] was employed as non-parametric statistical test used to determine whether there was a significant difference between the paired observations in a dataset, in terms of sex and dosage, by comparing the two-time ranges examined. Wilcoxon test is an alternative to the paired t-test and is particularly useful when the data do not meet the assumptions required for parametric tests, such as when the data are not normally distributed or when the sample size is small, as in this case.

7.2.3 Urine sample collection and preparation

Briefly, 50 μL of the urine samples were initially added with the internal standard (mephedrone-D3 at final concentration of 100 ng/mL), and then they were diluted 1:2 with a frozen acetonitrile/methanol (95:5) mixture and vigorously stirred for 5 min. After centrifugation for 5 min at 13,000g, 5 μL of the supernatant was directly injected into the UHPLC system. No enzymatic hydrolysis of glucuronides was performed on phase II metabolites. In order to semi-quantify the possible presence of the three synthetic cathinones in the urine samples, and as the study did not include validation of the method used, a urine matrix previously tested as negative was used for the preparation of the calibration curve. The negative matrix was, therefore, fortified at five concentration levels (100, 500, 1000, 5000, 10,000 ng/mL) with each of the NEH, NEP and 4-CMC working solution and mephedrone-D3 was used as the internal standard (ISTD). The choice of calibration levels was based on the first publication concerning this experiment relating to the administration of cathinones to the healthy volunteers and the first investigation of their levels in real samples. [21]

7.2.4 Instrumental condition

UHPLC separation was performed on the SCIEX ExionLC™ AC system (Sciex, Darmstadt, Germany) using a Phenomenex Kinetex C18 column (100 x 2.1 mm, 1.7 μm) maintained at 45°C. The mobile phase was a mixture of water (A) and acetonitrile (B), both with 0.01% of formic acid. The LC flow rate was set at 0.5 mL/min and the mobile phase eluted under the following linear gradient conditions: (A:B, v:v) isocratic elution at 95:5 for 0.5 min, from 95:5 to 5:95 in 7.5 min, isocratic elution at 5:95 for 0.5 min and final re-equilibration for 2.5 min to the initial condition. The total run time was 10 min. All analyses were performed using a quadrupole/time-of-flight SCIEX X500R QTOF mass spectrometer (Sciex, Darmstadt, Germany) equipped with a Turbo VTM ion source operating in positive-ion electrospray ionization mode (full MS and

MS/MS parameters are available in Table S1). Data acquisition involved a preliminary TOF-MS high-resolution full scan followed by a SWATH™ acquisition protocol which used a variable window setup (18 windows covering mass range from m/z 100.0 to 500.0 at 0.025 resolving power), resulting in a final cycle time of 0.933 s. The variable windows technique allows the reduction of the size of the Q1 window in order to further improve the quality of the SWATH acquisition data, while maintaining a complete coverage of the mass range and optimal cycle times. In this case it was decided to use 22-Da windows as they allowed an optimal acquisition of the peaks, improving the specificity and reducing interference from possible co-eluting analytes. The qualitative identification of the target analyte (NEH, NEP and 4-CMC) was based on the coincidence of its retention times, precursor ion and characteristic fragment ion m/z values, while the tentative metabolites were identified by their fragmentation patterns and the exact masses of both their precursor and fragment ions (accepted mass error <5 ppm). To ensure the reliability of the data acquired by the instrument, an automatic calibration was set up every three samples using a solution of calibrators supplied by SCIEX. Data were acquired using the SCIEX OS 1.5 Software and raw data files were processed using the MarkerViewTM software from Sciex.

7.3 Results and discussion

7.3.1 Metabolism of NEH and NEP

In-house accurate mass library was built, considering the metabolic pathways already known for other structurally similar cathinones. NEH and NEP metabolites were investigated based on mephedrone (4-MMC) metabolism due to the similarity of its molecular structures [22–24]. Thus, similar metabolic reactions can be assumed, including reduction, hydroxylation, N-dealkylation and di-hydrogenation, similarly to 4-MMC. Figures 1 and 2 shows the expected phase I metabolites of NEP and NEH compounds. The conjugation reactions with glucuronic acid (phase II metabolism) were expected and these metabolites' structures were confirmed by the high-resolution mass spectra corresponding to new chromatographic peaks appearing in the urine samples collected after NEH and NEP administration. Candidate metabolites were singled out from the chromatographic profile of the full-scan analysis by checking the exact mass of the corresponding protonated molecular ion. Then, the elemental composition of the relative fragment ions and the rationality of its fragmentation pattern was checked in the MS/HRMS spectra to confirm the tentative metabolite's identification (Supplementary Figures S1 and S2). In accordance with the

literature [25], [26], a total of six metabolites (4 phase I and 2 phase II metabolites) for NEH and five for NEP (3 phase I and 2 phase II metabolites) were identified in urine samples, including phase I and glucuronated phase II metabolites. In detail, 1) at m/z 192.1382, nor-NEH, the metabolite formed by dealkylation of the ethyl chain, which subsequently affords hydroxy-nor-NEH upon reduction of α-keto group, observed 2) at m/z 194.1539. 3) at m/z 222.1852, hydroxy-NEH, stemming for reduction of the NEH keto group; 4) at m/z 238.1801, dihydroxy-NEH following hydroxylation of the aromatic ring. Finally, 5) NEH-Gluc and 6) NEH-nor-Gluc, respectively, by conjugation with glucuronic acid and subsequent reduction of the α-keto group. The same metabolites were identified for NEH and NEP, except for the di-hydroxylated form, which was only observed for NEH. This similarity in the metabolic pattern is due to the similarity of the two structures. In fact, NEH has only one more methyl in the alkyl chain than NEP. No sulfated metabolites were found (Table 2). This conjugation reaction represents a metabolic step that seems to be important in humans compared to glucuronidation and for this reason further experiments will be considered to confirm or exclude the presence of the sulfate conjugates in humans.

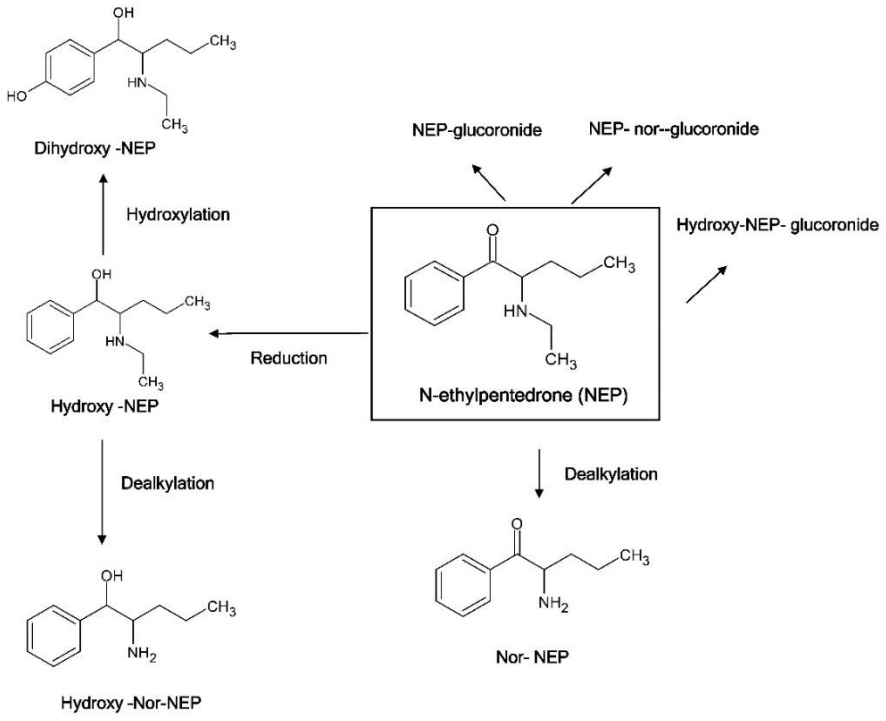


Figure 1: *Expected metabolic reactions of NEP*

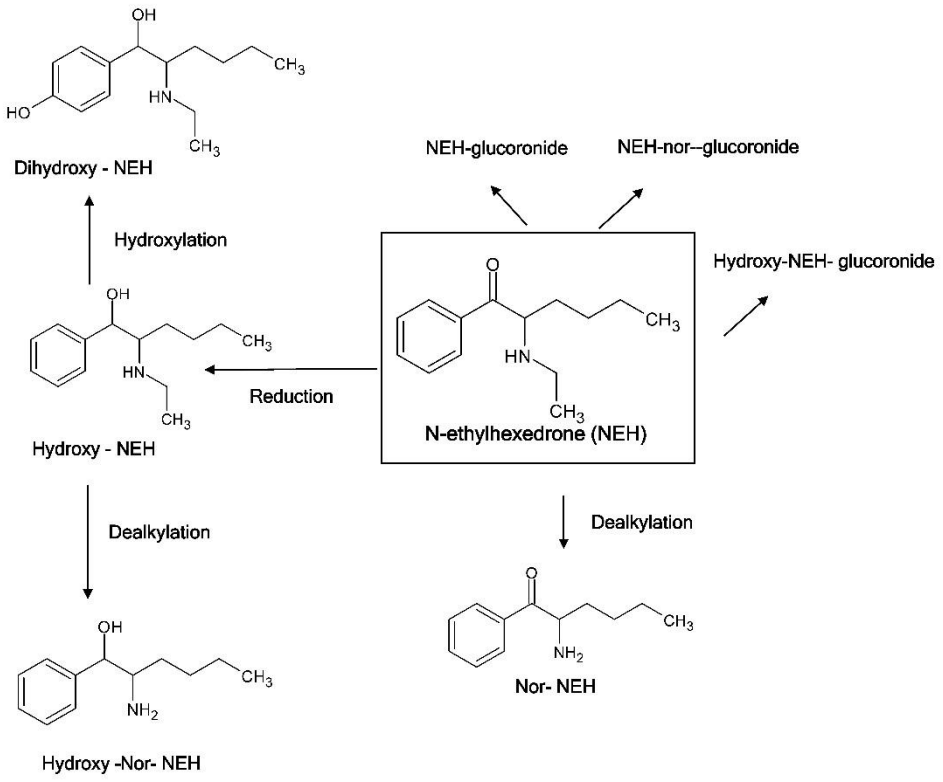


Figure 2: *Expected metabolic reactions of NEH*

Table 2. *Name, elemental composition, exact (theoretical) protonated mass, mass error found and retention time of the synthetic cathinones and their hypothesized metabolites in UHPLC-QTOF-HRMS.*

ID Compound	Elemental composition	$[M+H]^+$	t_r (min)	Found in urine 0-2 h (error mass <± 5 ppm)	Found in urine 2-4 or 2/5 h (error mass <± 5 ppm)
4-CMC	$C_{10}H_{12}ClNO$	198.0681	2.2	yes	yes
Nor-4-CMC	$C_9H_{10}ClNO$	184.0523	2.8	no	yes
Hydroxy-nor-4-CMC	$C_9H_{12}ClNO$	186.0680	0.4	no	yes
Hydroxy-4-CMC	$C_{10}H_{14}ClNO$	200.0836	2.2	yes	yes

Nor-4-CMC-Gluc	$C_{15}H_{20}ClNO_7$	362.1001		no	no
4-CMC-Gluc	$C_{16}H_{22}ClNO_7$	376.1157	7.8	yes	yes
N-Ethylhexedrone (NEH)	$C_{14}H_{21}NO$	220.1695	2.3	yes	yes
Nor-NEH	$C_{12}H_{17}NO$	192.1382	2.4	yes	yes
Hydroxy-NEH	$C_{14}H_{23}NO$	222.1852	2.6	yes	yes
Hydroxy-nor-NEH	$C_{12}H_{19}NO$	194.1539	2.3	yes	yes
Dihydroxy-NEH	$C_{14}H_{23}NO_2$	238.1801	2.5	yes	yes
NEH-Gluc	$C_{20}H_{31}NO_7$	398.2173	2.3	yes	yes
NEH-nor-Gluc	$C_{18}H_{27}NO_7$	370.1860	8.4	yes	yes
Hydroxy-NEH-Gluc	$C_{20}H_{31}NO_7$	398.2173		no	no
N-Ethylpentedrone (NEP)	$C_{13}H_{19}NO$	206.1539	2.3	yes	yes
Nor-NEP	$C_{11}H_{15}NO$	178.1226	2.2	yes	yes
Hydroxy-NEP	$C_{13}H_{21}NO$	208.1695	2.3	yes	yes
Hydroxy-nor-NEP	$C_{11}H_{17}NO$	180.1382	2.3	yes	yes
Dihydroxy-NEP	$C_{13}H_{21}NO_2$	224.1645		no	no
NEP-Gluc	$C_{19}H_{29}NO_7$	384.2016	3.3	yes	yes
NEP-nor-Gluc	$C_{17}H_{25}NO_7$	356.1703	2.4	yes	yes
Hydroxy-NEP-Gluc	$C_{19}H_{29}NO_7$	384.2016		no	no

Information on the concentration of NEH and NEP, their presence together with their metabolites in urine, the trends with which they occur in the two different time ranges (0-2 and 2-4h) and their possible different expression in male and female samples are shown in Table 3, Figure 3 and in supplementary Figures S3-S9. Concerning NEP, the trends of variation (evidenced by the trend line joining the same samples analysed in the two-time ranges) in the abundances of the compound and its metabolites can be observed in Figure 3 (30 mg dose) and Figure S3 (40 mg dose). Particularly interesting are the trends in Figure 3, which show that all metabolites increase in abundance after 2 hours after intake, while NEP decreases; this phenomenon is not observed in the case of the 40 mg. It can

therefore be assumed that an increase of 10 mg in the dose taken may cause a slowdown in metabolic processes or a saturation of the NEP metabolic pathway.

Concerning NEH, the trends of variation in the abundances of the compound and its metabolites can be observed in Figure S6 (30 mg dose) and Figure S7 (40 mg dose). For both the doses, only the increase in the signal intensity of the metabolite hydroxy-NEH, 2 hours after sample administration, can be appreciated; whereas for the other metabolites, no particular trend is observed between the two-time ranges. The inclusion of both male and female volunteers aims to consider the sexual dimorphism potentially related to pharmacodynamic and pharmacokinetic mechanisms that typically characterize NPS profiles [27]. In fact, such sex-related differences have already been described in the literature as variability factors influencing the pharmaco-toxicological benchmarks of various therapeutic drugs [28]. The study of this phenomenon for NEP and NEH compounds can be observed in Figures S4, S5 and S8, S9. However, only the hydroxy-NEP metabolite appears to be affected by a sex-related effect, showing a significant increase in women 2 hours after consuming the substance (Figure S4).

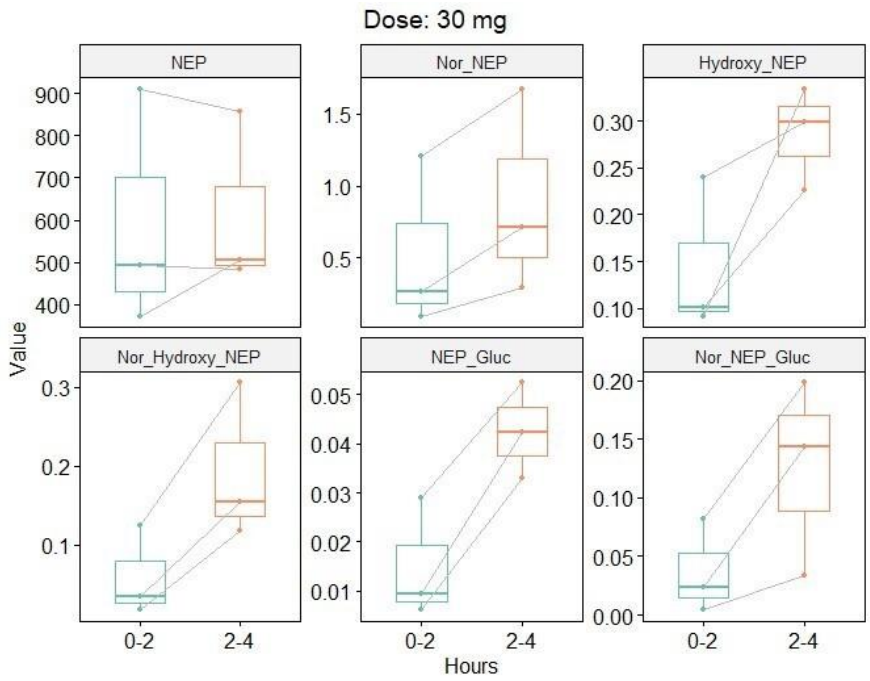


Figure 3: *Boxplot illustrating the ratio between the analyte and the internal standard of NEP and its metabolites at 0-2 and 2-4 hours after the administration of 30 mg dose*

7.3.2 Metabolism of 4-CMC

As expected, the metabolic profile of this cathinone (Figure 4) was very similar to the one observed for 4-chloroethoxyquinone (4-CEC) [29]. Three Phase I metabolites were identified (Table 2): 1) at m/z 200.0836, hydroxy-4-CMC, stemming from reduction of the 4-CMC keto group; 2) at m/z 184.0523, nor-4-CMC, the metabolite formed by N-demethylation, which subsequently affords hydroxy-nor-4-CMC upon reduction of α-keto group, observed 3) at m/z 186.0680. Similar to NEP and NEH, the phase II metabolite 4-CMC-glucoronate was identified and no sulphated metabolites were found. Candidate metabolites were identified from the chromatographic profile of the full-scan analysis by checking the exact mass of the corresponding protonated molecular ion, and the elemental composition of the relevant fragment ions was checked in the MS/HRMS spectra to confirm the identification of the provisional metabolite. An example of the MS/MS fragmentation spectrum of hydroxy-4-CMC, identified in group 3, sample 49 (range 2-5 hours after consumption of 4-CMC), is shown in Figure 5.

Compared to NEH and NEP, the formation of metabolites in the urine after intake of 4-CMC shows a different behaviour. In fact, based on $t_{1/2}$ values, 4-CMC is estimated to be a compound with low clearance ($t_{1/2}$ > 60 min) [14] and high half-life time ($t_{1/2}$ =105 min) [21], thus suggesting a lower propensity towards metabolic reaction and thus making 4-CMC abundant in the urine even 5 hours after administration (Table 3). Only the hydroxy-4-CMC metabolite was observed in both time ranges, while the others were detected only after 2 hours from administration. Furthermore, as can be seen from Figure 6, the hydroxy-4-CMC has a statistically significant increase in signal strength at dose of 150 mg (p-value = 0.02857), and for male and female volunteers together (p-value = 0.0006216). These results could identify this metabolic pathway as the preferred pathway for this synthetic cathinone.

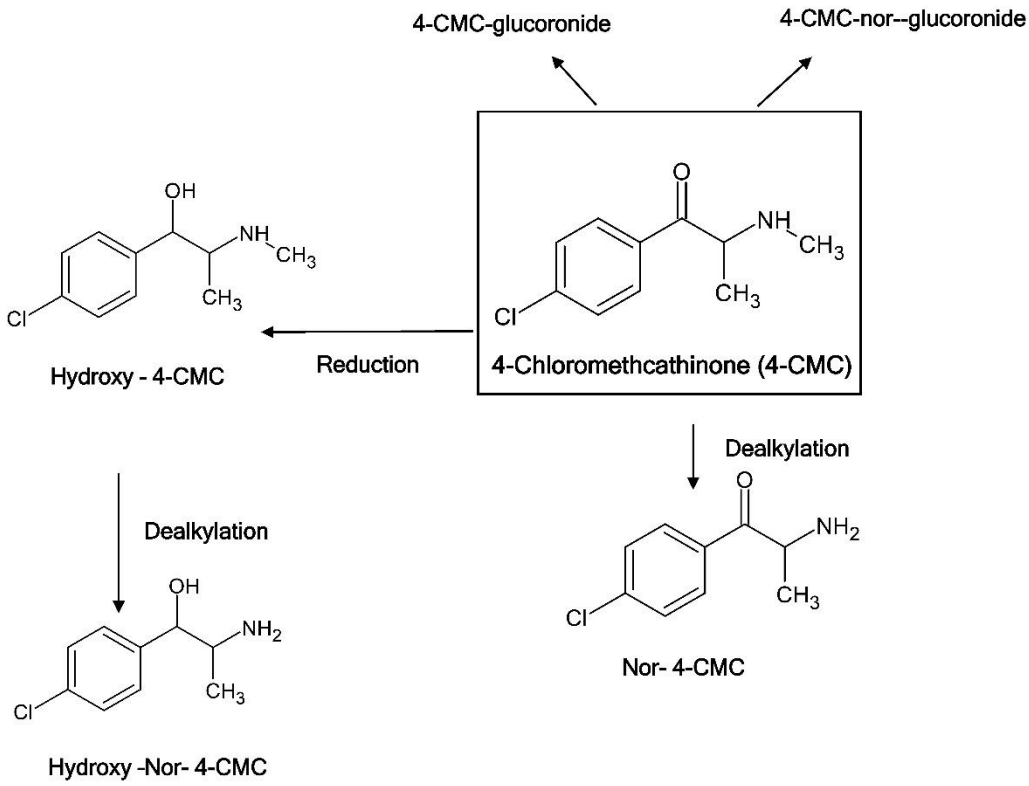


Figure 4: *Expected metabolic reactions of 4-CMC*

Table 3. *Concentration (ng/mL) of NEH, NEP and 4-CMC in urine sample*

NEH								
Volunteer	**33**	**34**	**35**	**36**	**37**	**38**	**39**	**40**
Time (h)	**Urine (ng/mL)**							
0-2	1630	403	290	484	309	no sample	15	887
2-4	2030	222	325	406	383	202	11	87
NEP								
Volunteer	**41**	**42**	**43**	**44**	**45**	**46**	**47**	**48**
Time (h)	**Urine (ng/mL)**							
0-2	8140	20	no sample	909	159	372	492	361
2-4	1770	347	1160	857	1230	504	483	119
4-CMC								

Volunteer	49	50	51	52	53	54	55	56
Time (h)	Urine (ng/mL)							
0-2	437	1270	429	156	601	4620	20300	126
2-5	8210	1100	7770	4460	5530	4280	5910	11500

7.4 Conclusions

The present study investigates the in vivo metabolism of NEP, NEH and 4-CMC on both male and female volunteers, in order to identify the main phase-I and phase-II metabolites and investigate their variation in the two observed time ranges. A significant feature of the resulting data is that a strong sexually dimorphic metabolism of hydroxy-NEP and hydroxy-NEH is observed. In particular, the hydroxy-metabolite showed a significant increase in women two hours after the consumption of the substances. The characterization of the main NEP, NEH and 4-CMC metabolites based on LC-HRMS and LC-MS/HRMS allowed the accurate mass determination of their protonated molecular ion and collisional activated fragment ions resulting in the reliable definition of their structure and the outlining of the major metabolic routes for the tested substance. The knowledge of the fragmentation pattern for both the parent drugs and their main metabolites will also allow to develop fit-for-purpose targeted analytical methods useful for the detection of them in biological specimens. In particular, hydroxy-NEP, hydroxy-NEH and hydroxy-4-CMC are suggested as target analytes in the toxicological analyses, so as to increase these synthetic cathinones detection time after intake and reduce the risk of false-negative results in the forensic cases. In fact, it is well known that characterizing of different species produced by the metabolic pathways of emerging psychoactive substances provide i) insight into mechanisms underlying their potential toxicity, and ii) useful data for their detection in biological samples. On the other hand, further studies may be required to speculate on metabolic pathway and related gender-based differences at other time points, so that the pharmacokinetics of these three synthetic cathinones can be further investigated.

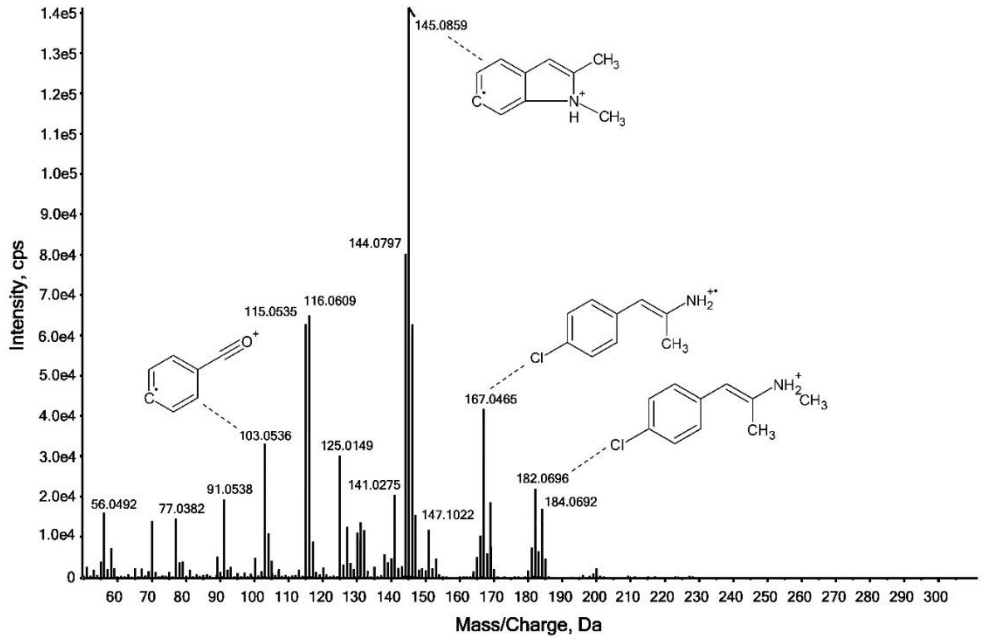


Figure 5: *HRMS fragmentation pattern of hydroxy-4-CMC. Sample 49, 2-5 hours after substance consumption*

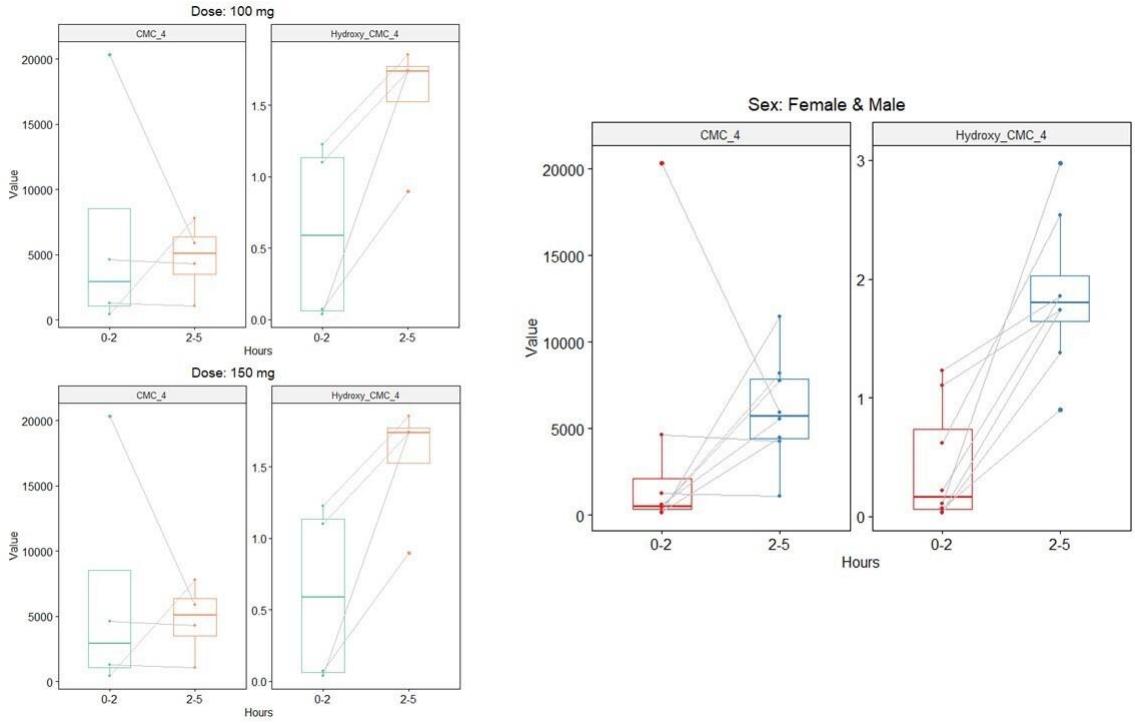


Figure 6: *Boxplots illustrating the ratio between the analyte and the internal standard of 4-CMC and its metabolites at 0-2 and 2-5 hours after the administration of 100 and 150 mg doses and for both male and female volunteers*

References

[1] «World Drug Report 2023», United Nations : Office on Drugs and Crime. www.unodc.org/unodc/en/data-and-analysis/world-drug-report-2023.html

[2] European Monitoring Centre for Drugs and Drug Addiction, *European drug report 2023*. in European drug report https://data.europa.eu/doi/10.2810/161905

[3] A. Shafi, A. J. Berry, H. Sumnall, D. M. Wood, e D. K. Tracy, New psychoactive substances: a review and updates, *Ther. Adv. Psychopharmacol.*, vol. 10, p. 2045125320967197, 2020, doi: 10.1177/2045125320967197.

[4] European Monitoring Centre for Drugs and Drug Addiction., *New psychoactive substances: 25 years of early warning and response in Europe: an update from the EU Early Warning System.* https://data.europa.eu/doi/10.2810/396103

[5] K. Layne, P. I. Dargan, e D. M. Wood, Chapter13 - Synthetic cathinones, in *Novel Psychoactive Substances (Second Edition)*, P. Dargan e D. Wood, A c. di, Boston: Academic Press, 2022, pp. 333–380. doi: 10.1016/B978-0-12-818788-3.00010-3.

[6] C.F. Oliver, J.J Palamar, A. Salomone, S.J. Simmons, H.L. Philogene-Khalid, N. Stokes-McCloskey, S.M. Rawls, Synthetic cathinone adulteration of illegal drugs, *Psychopharmacology (Berl.)*, vol. 236, fasc. 3, pp. 869–879, mar. 2019, doi: 10.1007/s00213-018-5066-6.

[7] C.V. Giné, I.F. Espinosa, M.V. Vilamala, New psychoactive substances as adulterants of controlled drugs. A worrying phenomenon?. Drug Testing and Analysis, 6(7-8), 819–824, 2014, doi:10.1002/dta.1610

[8] M. Majchrzak, R. Celiński, P. Kuś, T. Kowalska, e M. Sajewicz, The newest cathinone derivatives as designer drugs: an analytical and toxicological review, *Forensic Toxicol.*, vol. 36, fasc. 1, pp. 33–50, 2018, doi: 10.1007/s11419-017-0385-6.

[9] S. Zaami, R. Giorgetti, S. Pichini, F. Pantano, E. Marinelli, e F. P. Busardò, Synthetic cathinones related fatalities: an update, *Eur. Rev. Med. Pharmacol. Sci.*, vol. 22, fasc. 1, pp. 268–274, gen. 2018, doi: 10.26355/eurrev_201801_14129.

[10] M. Kraemer, A. Boehmer, B. Madea, e A. Maas, Death cases involving certain new psychoactive substances: A review of the literature, *Forensic Sci. Int.*, vol. 298, pp. 186–267, mag. 2019, doi: 10.1016/j.forsciint.2019.02.021.

[11] V. Uralets, S. Rana, S. Morgan, e W. Ross, Testing for Designer Stimulants: Metabolic Profiles of 16 Synthetic Cathinones Excreted Free in Human Urine, *J. Anal. Toxicol.*, vol. 38, fasc. 5, pp. 233–241, giu. 2014, doi: 10.1093/jat/bku021.

[12] J. Carrola, N. Duarte, P. Florindo, S. Henriques, G. da Silva, L. Bijlsma, R. Moreira, C. Correia, M. J. P. Á. Lopes, C.M. Sampayo, M.R. Bronze, Metabolism of N-ethylhexedrone and buphedrone: An in vivo study in mice using HPLC-MS/MS», *J. Chromatogr. B Analyt. Technol. Biomed. Life. Sci.*, vol. 1159, p. 122340, nov. 2020, doi: 10.1016/j.jchromb.2020.122340.

[13] R.P. Lopes, R.A. Ferro, M. Milhazes, M. Figueira, M.J. Caldeira, A.M.M. Antunes, H. Gaspar, Metabolic stability and metabolite profiling of emerging synthetic cathinones, *Front. Pharmacol.*, vol. 14, p. 1145140, 2023, doi: 10.3389/fphar.2023.1145140.

[14] C.A McNaney, D.M. Drexler, S.Y. Hnatyshyn, T.A. Zvyaga, J.O Knipe, J.V. Belcastro, M..Sanders,An Automated Liquid Chromatography-Mass Spectrometry Process to Determine Metabolic Stability Half-Life and Intrinsic Clearance of Drug Candidates by Substrate Depletion, *ASSAY Drug Dev. Technol.*, vol. 6, fasc. 1, pp. 121–129, feb. 2008, doi: 10.1089/adt.2007.103.

[15] E. Papaseit, E. Olesti, C. Pérez-Mañá, M. Torrens, F. Fonseca, M. Grifell, M. Ventura, R. de la Torre, M. Farré, Acute Pharmacological Effects of Oral and Intranasal Mephedrone: An Observational Study in Humans, *Pharmaceuticals*, vol. 14, fasc. 2, Art. fasc. 2, feb. 2021, doi: 10.3390/ph14020100.

[16] L. Martínez, N. La Maida, E. Papaseit, C. Pérez-Mañá, L. Poyatos, M. Pellegrini, S. Pichini, M. Ventura, L. Galindo, F.Paolo Busardò, M. Farré, Acute Pharmacological Effects and Oral Fluid Concentrations of the Synthetic Cannabinoids JWH-122 and JWH-210 in Humans After Self-Administration: An Observational Study», *Front. Pharmacol.*, vol. 12, p. 705643, ago. 2021, doi: 10.3389/fphar.2021.705643.

[17] Konstandi M, Johnson EO. Age-related modifications in CYP-dependent drug metabolism: role of stress. Front Endocrinol. 2023 May 24;14:1143835. doi: 10.3389/fendo.2023.1143835.

[18] M. T. Kinirons e M. S. O'Mahony, Drug metabolism and ageing, *Br. J. Clin. Pharmacol.*, vol. 57, fasc. 5, pp. 540–544, mag. 2004, doi: 10.1111/j.1365-2125.2004.02096.x.

[19] H. Wickham, *ggplot2*. in Use R! Cham: Springer International Publishing, 2016. doi: 10.1007/978-3-319-24277-4.

[20] D. Rey, M. Neuhäuser, Wilcoxon-Signed-Rank Test», in *International Encyclopedia of Statistical Science*, M. Lovric, A c. di, Berlin, Heidelberg: Springer, 2011, pp. 1658–1659. doi: 10.1007/978-3-642-04898-2_616.

[21] M. Nuñez-Montero, C. Lombroni, N. La Maida, M.C. Rotolo, S. Pichini, E. Papaseit, O. Hladun, M. Ventura, L. Poyatos, C. Pérez-Mañá, M. Farré, E. Marchei, GC-MS/MS Determination of Synthetic Cathinones: 4-chloromethcathinone, N-ethyl Pentedrone, and N-ethyl Hexedrone in Oral Fluid and Sweat of Consumers under Controlled Administration: Pilot Study, *Int. J. Mol. Sci.*, vol. 24, fasc. 11, p. 9387, mag. 2023, doi: 10.3390/ijms24119387.

[22] I. Linhart, M. Himl, M. Židková, M. Balíková, E. Lhotková, e T. Páleníček, Metabolic profile of mephedrone: Identification of nor-mephedrone conjugates with dicarboxylic acids as a new type of xenobiotic phase II metabolites, *Toxicol. Lett.*, vol. 240, fasc. 1, pp. 114–121, gen. 2016, doi: 10.1016/j.toxlet.2015.10.025.

[23] R.P. Lopes, R.A. Ferro, M. Milhazes, M. Figueira, M.J. Caldeira, A.M.M. Antunes, H. Gaspar, Metabolic stability and metabolite profiling of emerging synthetic cathinones, *Front. Pharmacol.*, vol. 14, p. 1145140, mar. 2023, doi: 10.3389/fphar.2023.1145140.

[24] J. Czerwinska, M. C. Parkin, C. George, A. T. Kicman, P. I. Dargan, e V. Abbate, Excretion of mephedrone and its phase I metabolites in urine after a controlled intranasal administration to healthy human volunteers, *Drug Test. Anal.*, vol. 14, fasc. 4, pp. 741–746, apr. 2022, doi: 10.1002/dta.3214.

[25] Z. Lin, Y. Chen, J. Li, Z. Xu, H. Wang, J. Lin, X. Ye, Z. Zhao, Y. Shen, Y. Zhang, S. Zheng, Y. Rao, Pharmacokinetics of N-ethylpentylone and its effect on increasing levels of dopamine and serotonin in the nucleus accumbens of conscious rats, *Addict. Biol.*, vol. 25, fasc. 3, p. e12755, mag. 2020, doi: 10.1111/adb.12755.

[26] J. Carrola, N. Duarte, P. Florindo, S. Henriques, G. da Silva, L. Bijlsma, R. Moreira, C. Correia, M. de Jesus Perry, Á. Lopes, C. de Mello-Sampayo, M. do Rosário Bronze, Metabolism of N-ethylhexedrone and buphedrone: An in vivo study in mice using HPLC-MS/MS, *J. Chromatogr. B*, vol. 1159, p. 122340, nov. 2020, doi: 10.1016/j.jchromb.2020.122340.

[27] L. Fattore, S. Altea, e W. Fratta, Sex differences in drug addiction: a review of animal and human studies, *Womens Health Lond. Engl.*, vol. 4, pp. 51–65, gen. 2008, doi: 10.2217/17455057.4.1.51.

[28] F. Franconi, V. Raparelli, V. Regitz-Zagrosek, Sex and gender landscape in pharmacology, *Pharmacol. Res.*, vol. 123, pp. 93–94, set. 2017, doi: 10.1016/j.phrs.2017.07.001.

[29] Y. Wang *et al.*, Pharmacokinetics and metabolomics of the new psychoactive substance 4-chloroethylcathinone, *Arab. J. Chem.*, vol. 16, fasc. 9, p. 105039, set. 2023, doi: 10.1016/j.arabjc.2023.105039.

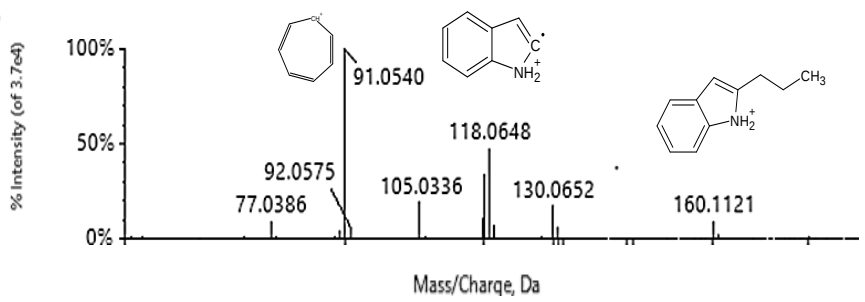
Supplementary materials

Table S1: *MS and MS/MS parameters used for the instrumental analysis*

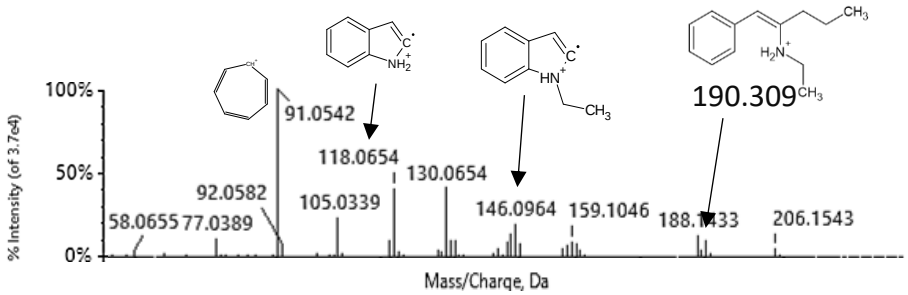
Source and Gas Parameters				
Ion gas 1 (psi)	Ion gas 2 (psi)	Curtain gas (psi)	CAD gas (psi)	Temperature (°C)
50	45	35	8	600
TOF MS				
TOF start mass (Da)	TOF stop mass (Da)	DP (V)	CE (V)	Accumulation time
100	500	65	10	0.1
TOF MS/MS				
TOF start mass (Da)	TOF stop mass (Da)	DP (V)	CE (V)	Accumulation time
50	500	65	35± 15	0.04

Figure S1: *HRMS fragmentation pattern of a) nor-NEP, b) hydroxy-NEP, c) hydroxy-nor-NEP, d) NEP-Gluc*

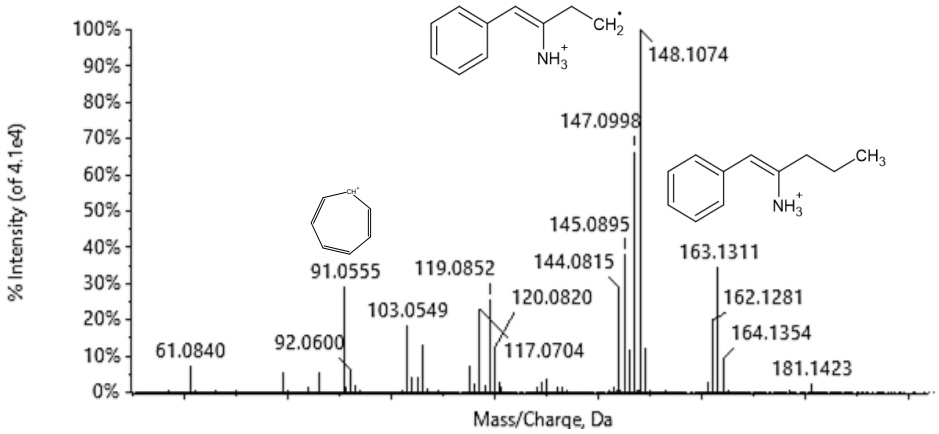
a)



b)


% Intensity (of 3.7e4)
100%
50%
0%
91.0542
118.0654
130.0654
92.0582
105.0339
58.0655
77.0389
146.0964
159.1046
188.1433
206.1543
190.309
Mass/Charge, Da

c)


% Intensity (of 4.1e4)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
148.1074
147.0998
145.0895
144.0815
163.1311
162.1281
164.1354
181.1423
91.0555
119.0852
120.0820
103.0549
92.0600
117.0704
61.0840
Mass/Charge, Da

d)

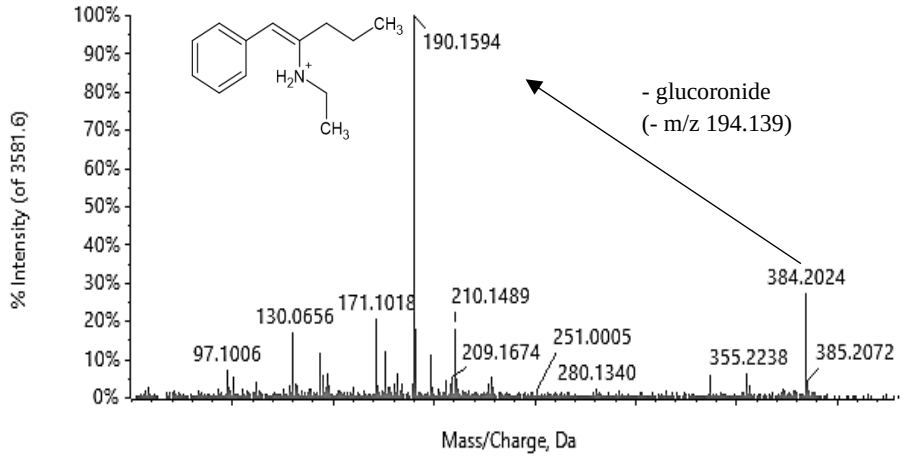
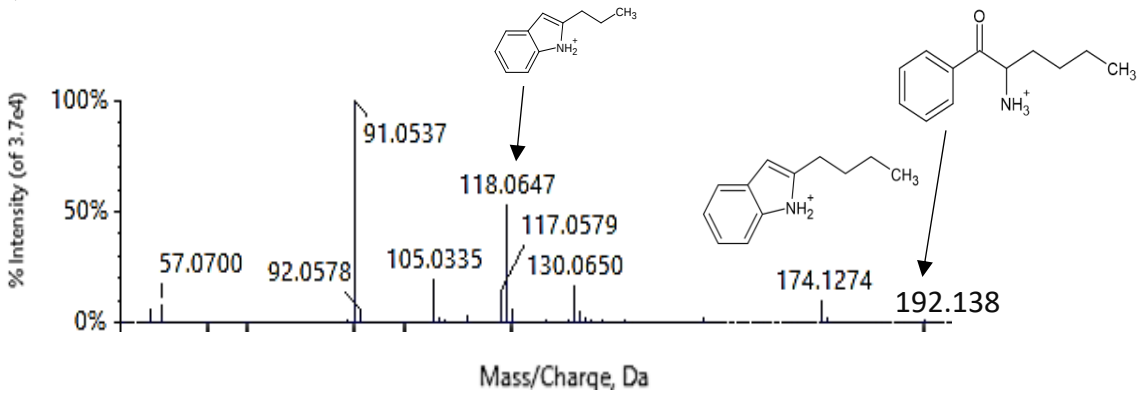
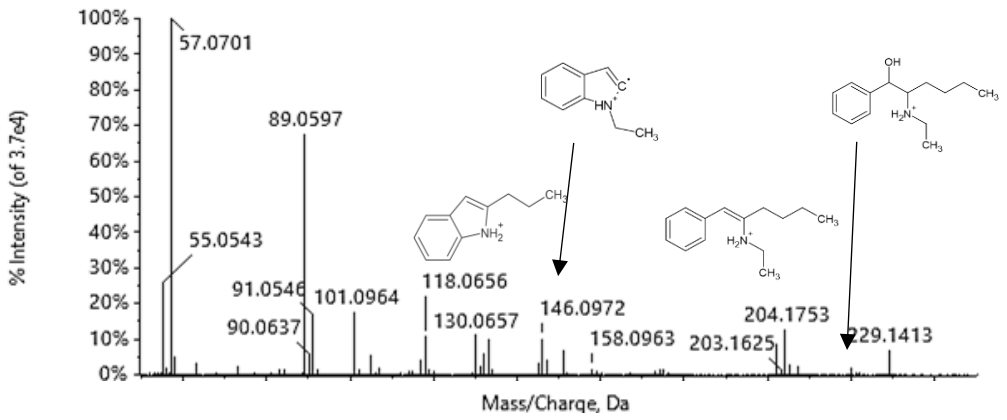

% Intensity (of 3581.6)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
190.1594
- glucoronide
(- m/z 194.139)
384.2024
210.1489
171.1018
130.0656
251.0005
97.1006
209.1674
280.1340
355.2238
385.2072
Mass/Charge, Da

Figure S2: *HRMS fragmentation pattern of a) nor-NEH, b) hydroxy-NEH, c) hydroxy-nor-NEH, d) dihydroxy-NEH, and e) NEH-Gluc*

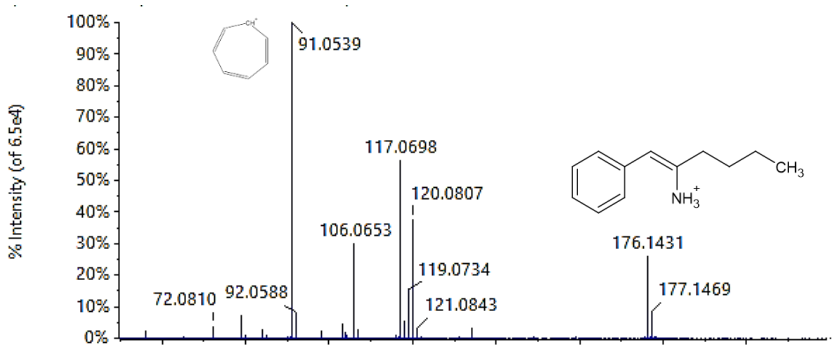
a)



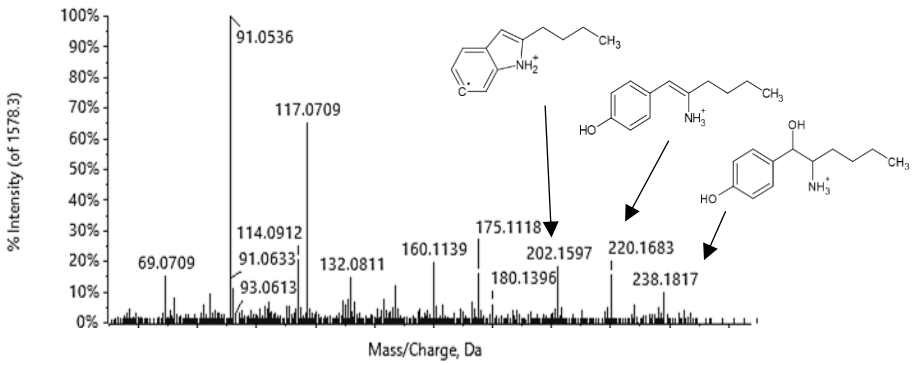
b)



c)



d)



e)

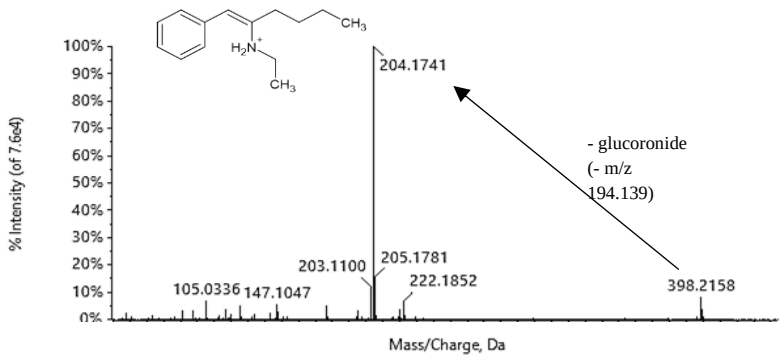


Figure S3: *Boxplots illustrating the ratio between the analyte and the internal standard of NEP and its metabolites at 0-2 and 2-4 hours after the administration of 40 mg dose*

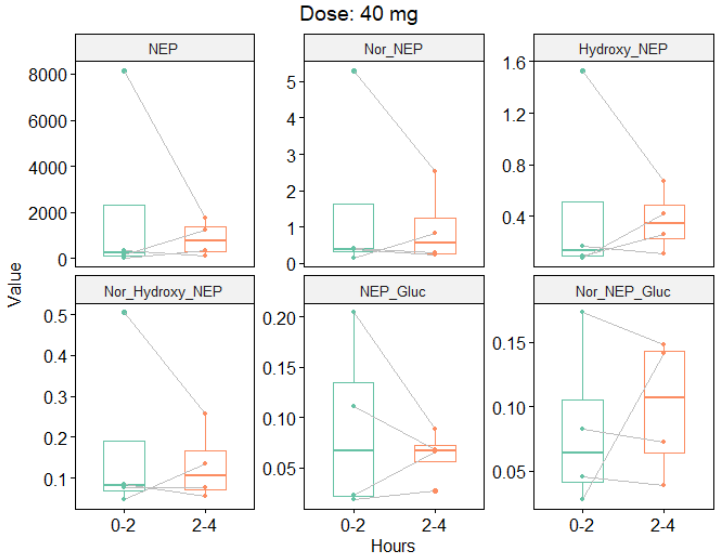


Figure S4: *Boxplots illustrating the ratio between the analyte and the internal standard of NEP and its metabolites at 0-2 and 2-4 hours after the administration for the female volunteers*

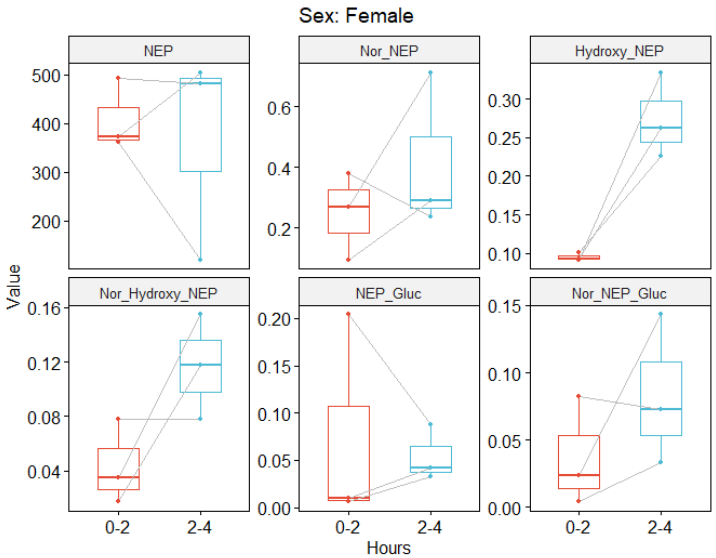


Figure S5: *Boxplots illustrating the ratio between the analyte and the internal standard of NEP and its metabolites at 0-2 and 2-4 hours after the administration for the male volunteers*

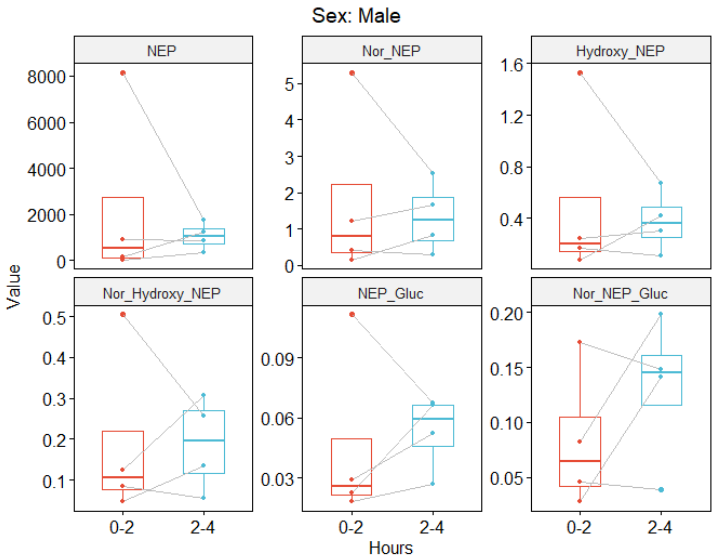


Figure S6: *Boxplots illustrating the ratio between the analyte and the internal standard of NEH and its metabolites at 0-2 and 2-4 hours after the administration of 30 mg dose*

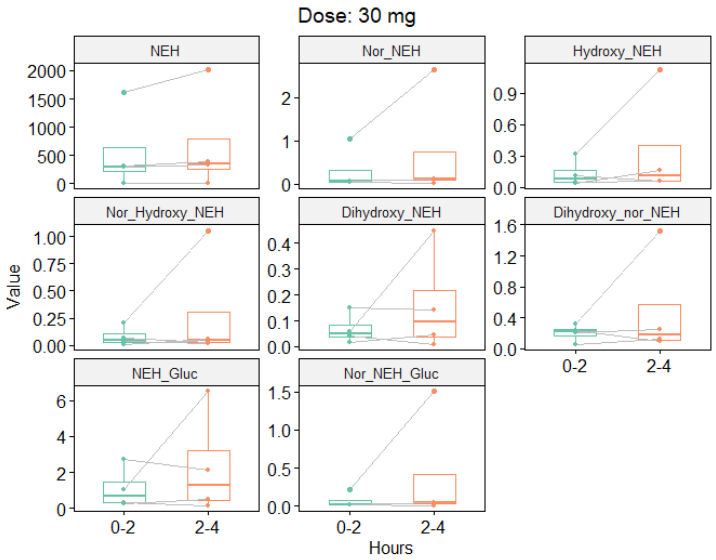


Figure S7: *Boxplots illustrating the ratio between the analyte and the internal standard of NEH and its metabolites at 0-2 and 2-4 hours after the administration of 40 mg dose*

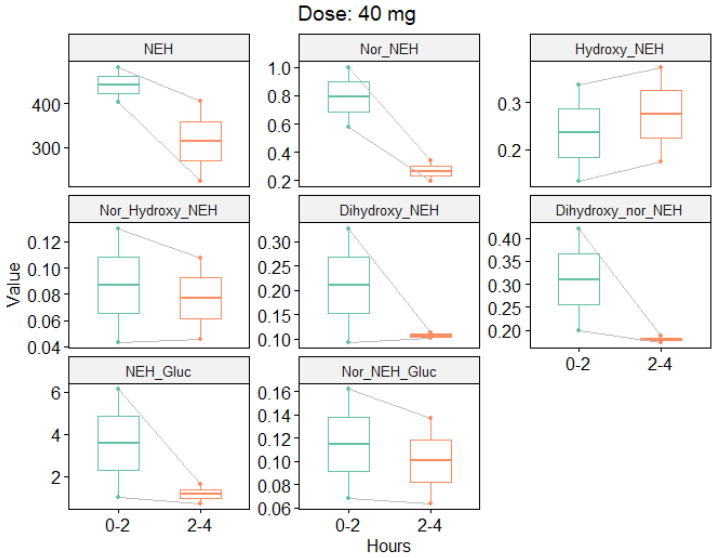


Figure S8: *Boxplots illustrating the ratio between the analyte and the internal standard of NEH and its metabolites at 0-2 and 2-4 hours after the administration for the female volunteers*

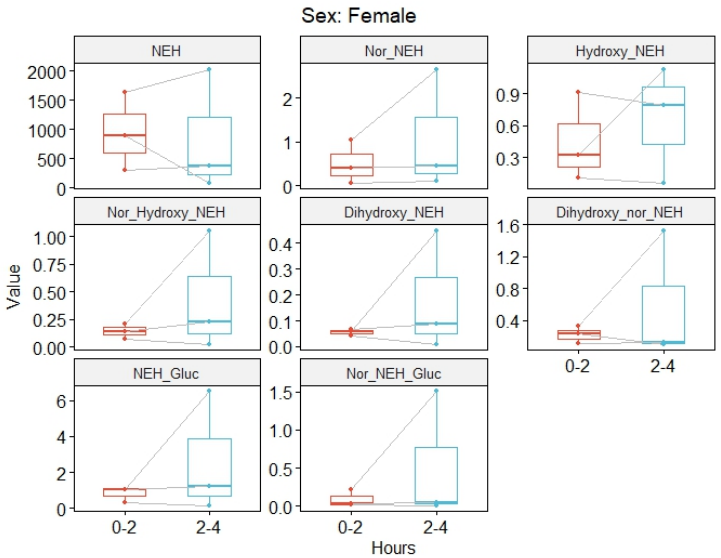
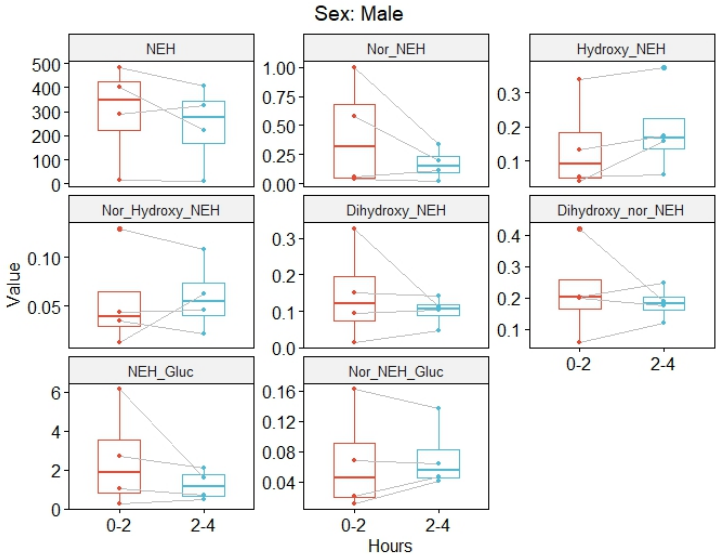


Figure S9: *Boxplots illustrating the ratio between the analyte and the internal standard of NEH and its metabolites at 0-2 and 2-4 hours after the administration for the male volunteers*



Chapter 8 | **Metabolic study of new psychoactive substance methoxpropamine in mice by UHPLC-QTOF-HRMS**

Marta Massano[1*], Enrico Gerace[2], Martina Borsari[1], Matteo Marti[3,4,5], Micaela Tirri[3], Christina Ververi[1], Eugenio Alladio[1], Marco Vincenti[1,2], Alberto Salomone[1,2]

[1]*Department of Chemistry, University of Turin, Italy*

[2] *Centro Regionale Antidoping, Orbassano (TO), Italy*

[3] *Department of Translational Medicine, Section of Legal Medicine, LTTA Center, University of Ferrara, Ferrara, Italy*

[4] *University Center for Studies on Gender Medicine, University of Ferrara, Ferrara, Italy*

[5]*Collaborative Center for the Italian National Early Warning System, Department of Anti-Drug Policies, Presidency of the Council of Ministers, Italy*

** Corresponding author: Marta Massano*

Email address: marta.massano@unito.it

The content of this Chapter is included in one published manuscript:

Metabolic study of new psychoactive substance methoxpropamine in mice by UHPLC-QTOF-HRMS

Marta Massano, *Enrico Gerace, Martina Borsari, Micaela Tirri, Christina Ververi, Eugenio Alladio, Marco Vincenti, Alberto Salomone*

Drug Testing and Analysis
https://doi.org/10.1002/dta.3449

Keywords: Methoxpropamine; NPS; metabolites; mice; HRMS

Abstract

Methoxpropamine (MXPr) is an arylcyclohexylamine dissociative drug structurally similar to 3-methoxyeticyclidine, ketamine and deschloroketamine, recently appeared in the European illegal market and was classified within the New Psychoactive Substances (NPS). Our study investigated the metabolism of MXPr to elucidate the distribution of the parent drug and its metabolites in body fluids and fur of 16 mice.

After the intraperitoneal administration of MXPr (1, 3, and 10 mg/kg), urine samples from 8 male and 8 female mice were collected every hour for 6 consecutive hours and then at 12-24 hours intervals. Additionally, plasma samples were collected 24 hours after MXPr (1 and 3 mg/kg) administration. Urine and plasma were diluted 1:3 with acetonitrile/methanol (95:5), and directly injected into the UHPLC-QTOF-HRMS system. The phase-I and II metabolites were preliminarily identified by means of the fragmentation patterns and the exact masses of both their precursor and fragment ions. Lastly, the mice fur was analyzed following an extraction procedure specific for the keratin matrix. Desmethyl-MXPr-glucoronide was identified in urine as the main metabolite, detected up to 24 hours after administration. The presence of NorMXPr in urine, plasma and fur was also relevant, following a N-dealkylation process of the parent drug. Other metabolites that were identified in fur and plasma included Desmethyl-MXPr and Dihydro-MXPr. Knowledge of the MXPr metabolites evolution is likely to support their introduction as target compounds in NPS toxicological screening analysis on real samples, both to confirm intake and extend the detection window of the dissociative drug MXPr in the biological matrices.

8.1 Introduction

The worldwide spread of new psychoactive substances (NPS) in the illicit drug market and their continuous increase in number and type, with the purpose of bypassing the controlled substance legislation, represents an unceasing defy for forensic scientists, clinicians and enforcement authorities [1,2]. Nowadays, NPS represent one of most important potential risk factor for public health [3].

Unfortunately, a lack of information exists on the metabolic pathway of NPS appearing in the drug scenario, making their identification in biological samples a controversial challenge, especially when the examined samples are limited and the drug is promptly and extensively metabolized.

NPS are often categorized as synthetic cannabinoids, stimulant, depressant (benzodiazepines and opioids), dissociative and hallucinogens, but this classification does not express adequately the variety and complexity of their potency, combined effects, and risk profiles that intersect categories and often differentiate compounds belonging to the same category [4].

A group of ketamine-like dissociative substances, based on the structure of arylcyclohexamines, represents a newly introduced subclass of NPS [5,6]. Among these, methoxpropamine (MXPr or 3-MeO-2′-oxo-PCPr) is an arylcyclohexylamine dissociative drug and a homologue of methoxetamine (MXE) that possesses structural similarities with 3-methoxyeticyclidine (3-MeO-PCE), ketamine and deschloroketamine [7]. Similarly to its analogs, MXPr acts as a potent antagonist of N-methyl-D-aspartate (NMDA) receptors [8]. Apparently, MXPr was first synthesized in Denmark in October 2019 and recently entered the chemical market for online sale [9]. It was then reported as an NPS by the European Monitoring Center for Drugs and Drug Addiction (EMCDDA) in 2020 and was identified for the first time in Italy in the same year [10].

As a recently introduced substance, little is known about MXPr pharmacology, toxicity and metabolism. Understanding the metabolic fate of the parent drug and identifying the phase I and II products is likely to represent a valid support to develop a targeted analytical method for biological samples, especially blood and urine. For this reason, the present work aimed to study the metabolic profile of MXPr, identify the main metabolites of phase I and II, and observe their pharmacokinetic evolution, in order to single out the most appropriate biomarkers for detecting MXPr abuse.

Quite often, *in vitro* tests and *in vitro* metabolic simulation, for example with human hepatocytes [11,12], human liver microsomes or with pooled human liver S9 fraction (pHLS9)[13] are performed to study NPS metabolism.

In our study, we preferred to develop *in vivo* models with male and female mice, possibly simulating the metabolic pathways in humans [14]. After administration of MXPr, urine, blood and fur were collected and then analyzed. Although an untargeted method was used for data acquisition, the approach used for data processing was typical of a targeted metabolomic analysis [15]. High performance of ultra-high-pressure liquid-chromatography (UHPLC) was combined with quadrupole time-of-flight high resolution mass spectrometry (QTOF-HRMS) allowing the prediction of MXPr metabolic profile on the basis of similar studies on the analogous MXE[16,17]. Using this approach, it has been

possible to hypothesize and confirm the structures of the main phase I and II metabolites, their exact masses and their fragmentation patterns.

8.2 Materials and methods

8.2.1 Reagent and standards

All chemicals, including methanol, formic acid and acetonitrile, were purchased from Sigma-Aldrich (Milan, Italy). Ultra-pure water was obtained using a Milli-Q® UF-Plus apparatus (Millipore, Bedford, MA, USA). For UHPLC-QTOF-HRMS quantitative analysis, ketamine-d4 was purchased from LGC Promochem (Milan, Italy) (purity >99%, concentration 1 mg/mL). Methoxpropamine was kindly provided by the Italian National Institute of Health (methanolic solution at a 0.02 mg/mL concentration, purity provided by the supplier >99%). All working solutions were prepared in methanol at 1 μg/mL and stored at – 20°C until used.

8.2.2 Mice study protocol

Overall, 16 ICR (CD-1®) mice (8 male and 8 female) weighing 30–35 g were grouped and exposed to a 12:12-h light-dark cycle (light period from 6:30 AM to 6:30 PM) at a temperature of 20–22°C and humidity of 45–55%. Each cage contained 5 mice with a floor area per animal of 80 cm^2 and minimum enclosure height of 12 cm. They were provided ad libitum access to food (Diet 4RF25 GLP; Mucedola, Settimo Milanese, Milan, Italy) and water. The experimental protocols performed in the present study were in accordance with the U.K. Animals (Scientific Procedures) Act of 1986 and associated guidelines and the new European Communities Council Directive of September 2010 (2010/63/EU). Experimental protocols were approved by the Italian Ministry of Health (license n. 335/2016-PR) and by the Animal Welfare Body of the University of Ferrara. According to the ARRIVE guidelines, all possible efforts were made to minimize the number of animals used, to minimize the animals' pain and discomfort.

MXPr was dissolved in Tween 80 (2%) and ethanol (5%), brought to the final volume with saline (0.9% NaCl). Mice were treated by intraperitoneal (i.p.) injection (volume of 4 μL/g) in subsequent days and divided into two groups (4 males and 4 females for each group). Group 1 (G1) were injected with all three doses of MXPr (1, 3, and 10 mg/kg; i.p.), while group 2 (G2) were injected only with dosage of 1 mg/kg. After the first injection of MXPr (1 mg/kg; i.p.) all mice (G1 and G2) were located in metabolic cages (Ugo Basile SRL, Gemonio (VA), Italy) for urine samples collection that was carried out individually for each

animal at a specific time point (protocol already validated in the laboratory [14]). Subsequently, urine samples were collected only from G1 mice group, after treatment on different days at different dosages (3 and 10 mg/kg; i.p.). After twenty days of wash out, all mice (G1 and G2) were re-treated twice with MXPr (1 and 3 mg/kg; i.p.) in consecutive days for blood and fur samples collection.

8.2.3 Urine samples collection and preparation

The urine samples were collected before MXPr administration (time 0; blank sample), then at 1, 2, 3, 4, 5, 6, 12 and 24 hours after administration. All samples were collected in 1.5 mL tubes and stored at –20 °C. Considering the extremely small volumes available, all urine samples were pooled by collection time and sex. The availability of the samples and the summary of the samples' collection is shown in Table S1. The evolution of MXPr and its metabolites over time was monitored, taking note of the differences between male and female metabolic pathways.

No enzymatic hydrolysis of glucuronides and sulphates was performed on phase II metabolites. Briefly, 50 μL of the urine samples were initially added with 2.5 μL of internal standard (ketamine-d4 at final concentration of 50 ng/mL), then they were diluted 1:3 with a frozen acetonitrile/methanol (95: 5) mixture and vigorously stirred for 5 min. After centrifugation for 5 min at 13,000 g, 5 μL of the supernatant was directly injected into the UHPLC system. To allow us to quantify the possible presence of MXPr in urine samples, a urinary matrix negative for the substance was fortified at five concentration levels (10, 50, 100, 500, 1000 ng / mL) with the MXPr working solution and ketamine-d4 was used as the internal standard (ISTD). Each calibration point was repeated in triplicate, for the urine matrix, at five concentration levels

8.2.4 Plasma samples collection and preparation

After 20 days of wash out, mice were re-administered with two MXPr doses (1 - 3 mg/kg; i.p.) in consecutive days, then the plasma samples were collected by submandibular withdrawal technique [18]. In this case, three samples were obtained: sample MG1 is the plasma pool relative to the male mice treated on day 1, while samples FG1 and FG2 are the plasma pools relative to the female mice treated at 1 mg/kg (day 1) and 3 mg/kg (day 2), respectively. For the plasma pretreatment, 50 μL of the samples were initially added with 2.5 μL of internal standard (ketamine-d4 at final concentration of 50 ng/mL), then they were diluted 1:3 with a frozen acetonitrile/methanol (95: 5) mixture, vigorously stirred for 5

min and placed in the cold room at –20°C for 15 min before centrifugation to facilitate the precipitation of plasma proteins. After centrifugation for 5 min at 13,000 g, 5 µL of the supernatant was directly injected into the UHPLC system and the concentration of MXPr was determined using a plasma sample of the mice, previously confirmed as negative to MXPr. The calibration curve was obtained in the range of concentration 10-1000 ng/mL.

8.2.5 Fur samples collection and preparation

Fur samples were collected one month in advance and at the end of the MXPr treatment. All fur samples were treated with a procedure developed on-purpose for the keratin matrix. Fur samples were pooled by sex in order to obtain a sufficient amount of fur for the analysis. About 50 mg of fur was decontaminated by an initial wash with 1 mL dichloromethane followed by a second wash with 1 mL methanol, each one performed under 3 min. stirring. The dried fur was pulverized using a six steel balls stirring in a Precellys® homogenizer. The pulverized samples were extracted by keeping them immersed in 0.5 mL methanol added with 2.5 µL of ISTD (ketamine-d4) at + 55 ± 5 ° C for 15 hours. At the end of the incubation, the tubes were centrifuged and 5 µL of the supernatant were injected into the UHPLC system. Finally, the fur of the mice, previously confirmed as negative to MXPr, was used to build the calibration curve ranged from 10 to 1000 pg /mg, allowing us to quantify the MXPr.

8.2.6 Instrumental condition

UHPLC separation was performed on the SCIEX ExionLC™ AC system (Sciex, Darmstadt, Germany) using a Phenomenex Kinetex C18 column (100 × 2.1 mm, 1.7 µm) maintained at 45°C. The mobile phase was a mixture of water (A) and acetonitrile (B), both with 0.01% of formic acid. The LC flow rate was set at 0.5 mL/min and the mobile phase eluted under the following linear gradient conditions: (A:B, v:v) isocratic elution at 95:5 for 0.5 min, from 95:5 to 5:95 in 7.5 min, isocratic elution at 5:95 for 0.5 min and final re-equilibration for 2.5 min to the initial condition. The total run time was 10 min. All analyses were performed using a quadrupole/time-of-flight SCIEX X500R QTOF mass spectrometer (Sciex, Darmstadt, Germany) equipped with a Turbo VTM ion source operating in positive-ion electrospray ionization mode (full MS and MS/MS parameters are available in the Supplementary Table S2). Data acquisition involved a preliminary TOF-MS high-resolution full scan followed by

a SWATH™ acquisition protocol which used a variable window setup (18 windows covering mass range from m/z 100.0 to 600.0 at 0.025 resolving power), resulting in a final cycle time of 0.933 s. The variable windows technique allows the reduction of the size of the Q1 window in order to further improve the quality of the SWATH acquisition data, while maintaining a complete coverage of the mass range and optimal cycle times. In this case it was decided to use 30 Da windows as they allowed an optimal acquisition of the peaks, improving the specificity and reducing interference from possible co-eluting analytes. The qualitative identification of the target analyte MXPr was based on the coincidence of its retention times, precursor ion and characteristic fragment ion m/z values, while the tentative metabolites were identified by their fragmentation patterns and the exact masses of both their precursor and fragment ions (accepted mass error < 5 ppm). To ensure the reliability of the data acquired by the instrument, an automatic calibration was set up every 3 samples using a solution of calibrators supplied by SCIEX. Data were acquired using the SCIEX OS 1.5 Software and raw data files were processed using the MarkerViewTM software from Sciex

8.3 Results and discussion

The alleged metabolites were investigated based on an extensive review of ketamine and MXE metabolism [17,18]. MXE was initially taken as reference because of the similarity of their molecular structures, where the N-ethyl group (MXE) has been replaced by a N-*n*-propyl group in MXPr (Figure 1a). Thus, similar metabolic reactions can be assumed, including reduction, hydroxylation, N-depropylation, dehydrogenation, O-desmethylation (Figure 1b), and di-hydrogenation of the cyclohexanone ring, similarly to ketamine. Figure 1c shows the expected phase I metabolites.

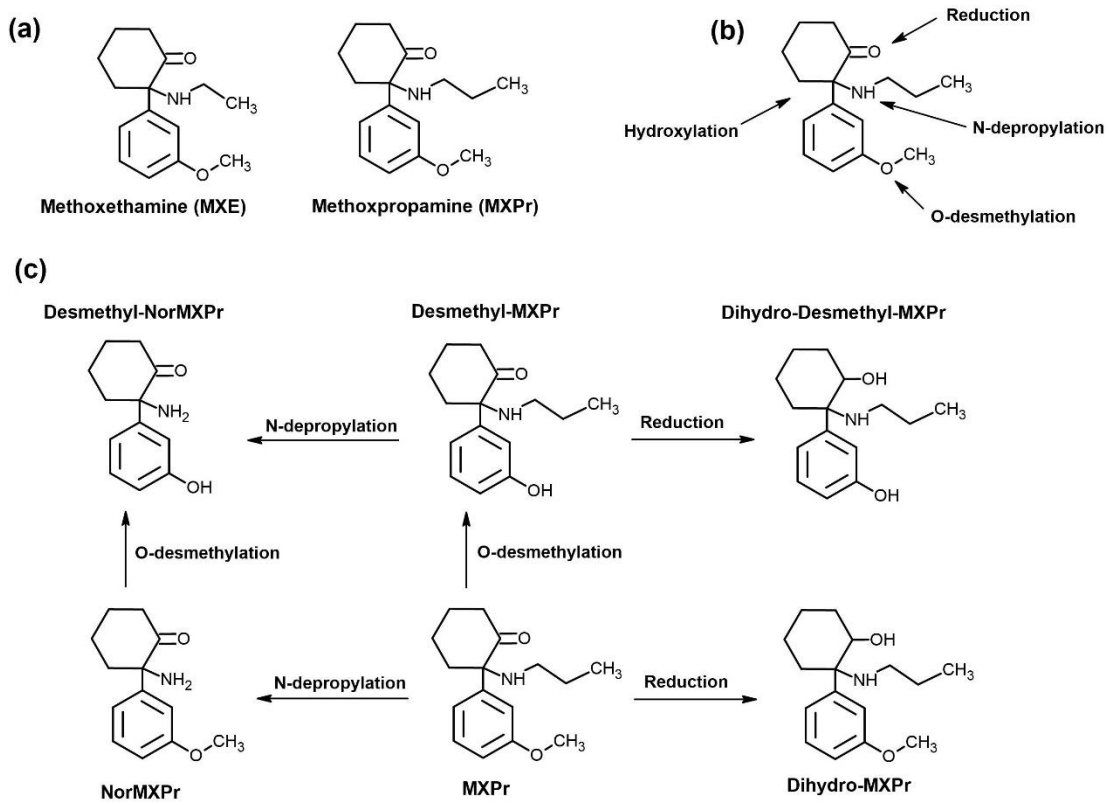


Figure 1: *(a) Structures of MXE and MXPr, (b) Expected metabolic reactions of MXPr and (c) Predicted metabolic pathway of MXPr*

Afterwards, the conjugation reactions with glucuronic acid (phase II metabolism) were expected and the confirmation of these metabolites' structures was deduced by the high-resolution mass spectra corresponding to new chromatographic peaks appearing in the urine samples collected after MXPr administration. We speculated that the same metabolic pathways, resulting from a single or a combination of the aforementioned biotransformation, would occur for MXPr. In our study, we developed in vivo models using male and female mice, possibly simulating human metabolic pathways. The inclusion of both male and female mice aims to consider the sexual dimorphism potentially related to pharmacodynamic and pharmacokinetic mechanisms that typically characterize NPS profiles [19]. In fact, such sex-related differences have already been described in the literature as variability factors influencing the pharmaco-toxicological benchmarks of various therapeutic drugs [20]. The urine pool from male mice treated with the highest concentration of MXPr (10 mg/kg) was

initially used for the detection of all metabolites, including the less abundant ones. A total of nine metabolites was identified in urine samples, including phase I and glucuronated phase II metabolites; no mono-hydroxylated nor sulfated metabolites were found (Table 1).

Table 1. *Name, elemental composition, exact (theoretical) protonated mass, mass error found and retention time of MXPr and its hypothesized metabolites in UHPLC-QTOF-HRMS.*

ID Compound	Elemental composition	$[M+H]^+$	Error (ppm)	t_r (min)	Found in mice urine
MXPr	$C_{16}H_{23}NO_2$	262.1802	-2.7	3.05	Yes
Desmethyl-MXPr	$C_{15}H_{21}NO_2$	248.1645	-3.8	2.46	Yes
Dihydro-Desmethyl-MXPr	$C_{15}H_{23}NO_2$	250.1802	-0.5	2.64	Yes
Desmethyl-NorMXPr	$C_{12}H_{15}NO_2$	206.1176	-0.5	2.00	Yes
NorMXPr	$C_{13}H_{17}NO_2$	220.1332	0.3	2.60	Yes
Dihydro-MXPr	$C_{16}H_{25}NO_2$	264.1958	-2.1	3.25	Yes
Desmethyl-MXPr-Gluc	$C_{21}H_{29}NO_8$	424.1966	-4.3	1.85	Yes
Desmethyl-NorMXPr-Gluc	$C_{18}H_{23}NO_8$	382.1496	-0.5	1.42	Yes
Dihydro-MXPr-Gluc	$C_{22}H_{33}NO_8$	440.2279	5.0	3.68	Yes
Dihydro-Desmethyl-MXPr-Gluc	$C_{21}H_{31}NO_8$	426.2122	0.7	2.08	Yes
Dihydro-NorMXPr	$C_{13}H_{18}NO_2$	221.1410	-	-	No
HydroxyMXPr	$C_{16}H_{23}NO_3$	278.1750	-	-	No
Hydroxy-NorMXPr	$C_{13}H_{17}NO_3$	236.1281	-	-	No
Desmethyl-Hydroxy-MxPr	$C_{15}H_{21}NO_3$	264.1594	>10	2.00	No
Hydroxy-Dihydro-MXPr	$C_{16}H_{25}NO_3$	280.1907	-	-	No
Desmethyl-Hydroxy-NorMxPr	$C_{12}H_{15}NO_3$	222.1124	>10	1.10	No
Dihydro-Hydroxy-NorMXPr	$C_{13}H_{18}NO_2$	221.1410	-	-	No
MXPr-Sulf	$C_{15}H_{20}NO_2OSO_3H$	342.1016	-	-	No

Dihydro-MxPr-Sulf	$C_{15}H_{22}NO_2OSO_3H$	344.1173	-	-	No
Desmethyl-MxPr-Sulf	$C_{14}H_{18}NO_2OSO_3H$	328.0860	-	-	No

Information on the presence of MXPr and its metabolites in urine at different time-points is available in Supplementary Materials Figure S2. Sulfation is a minor metabolic step in rodents while it seems to be more important in humans compared to glucuronidation. Therefore, further experiments will be considered to confirm or exclude the presence of the sulfate conjugates in humans. The candidate metabolites of MXPr were singled out from the chromatographic profile of the full-scan analysis by checking the exact mass of the corresponding protonated molecular ion. Then, the elemental composition of the relative fragment ions and the rationality of its fragmentation pattern was checked in the MS/HRMS spectra to confirm the tentative metabolite's identification (HRMS fragmentation patterns are available in Figure 2 and Supplementary Materials Figure S1). A chromatogram obtained from the analysis of a pooled urine sample collected 1 h after the 10 mg/kg MXPr administration is presented in Figure 3. Eight relevant chromatographic peaks were recorded, arising from the presence of MXPr plus seven metabolites. Among these, dihydro-MXPr and dihydro-MXPr-Gluc were not detected in the urine samples collected within the initial 3 h from the administration, whereas their presence was observed in the urine samples collected 4-5 h after the administration, suggesting a delayed formation and urinary excretion.

The presence of an intense peak (Figure 3, peak number 2; RT = 1.8 min) allegedly corresponding to desmethylmethoxpropamine-glucuronide (desmethyl-MXPr-Gluc) was observed in both high and low concentration treatments (1 and 10 mg/kg), suggesting that the metabolic pathway that produces it is the preferential one for MXPr excretion. The most abundant desmethyl-MXPr-Gluc peak during the excretion profile was observed 1h after the administration, with an average intensity about 16 times higher than the free phase desmetyl-MXPr, likely representing its phase I precursor.

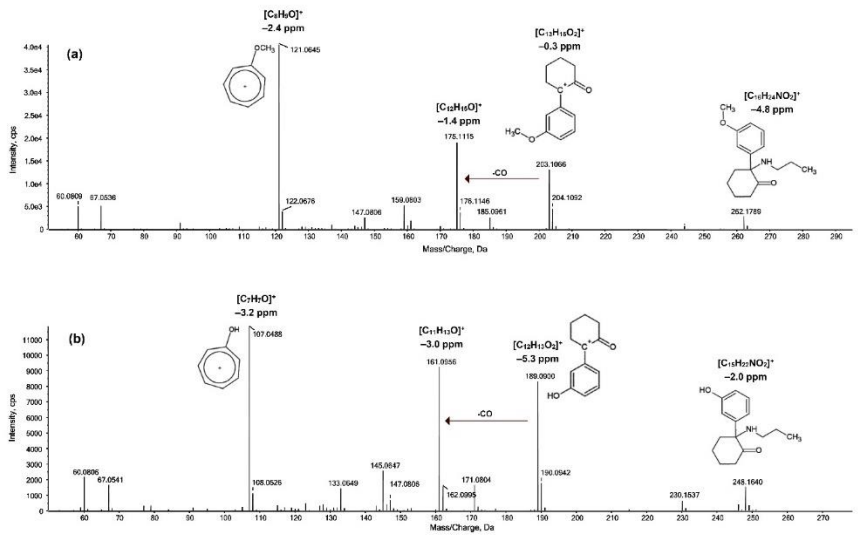


Figure 2 *HRMS fragmentation pattern of MXPr (a) and desmethyl-MXPr (b). The assumed molecular structures of the main fragments, their elemental composition, and the deviation from the exact mass (ppm) are reported.*

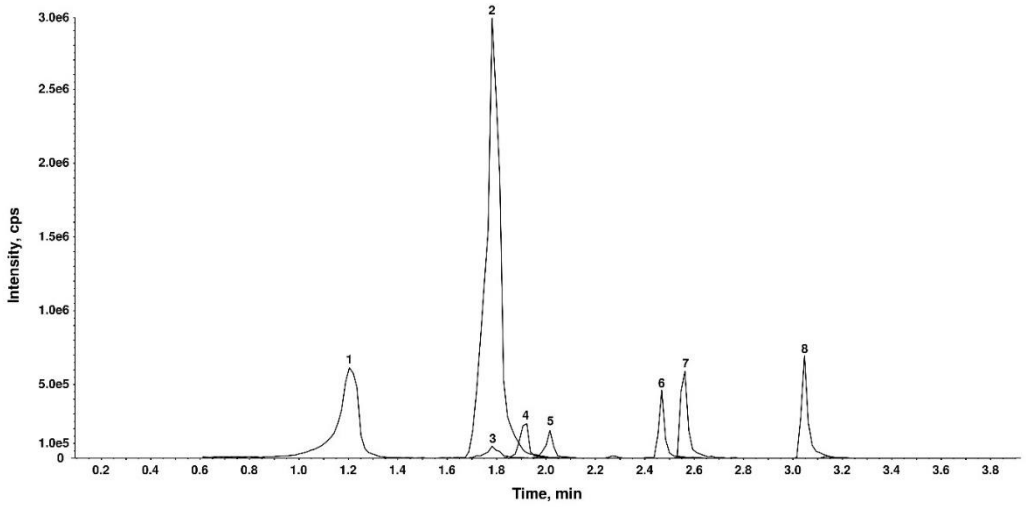


Figure 3: *Extracted ion chromatogram (XIC) obtained from the urine pool of male mice treated with the highest concentration of MXPr (10 mg / kg) and sampled 1 hour after treatment (1. Desmethyl-NorMxPr-Gluc, 2. Desmethyl-MxPr-Gluc, 3. Dihydro-Desmethyl-MxPr, 4. Desmethyl-NorMxPr, 5. Dihydro- Desmethyl-MxPr-Gluc, 6. Desmethyl-MxPr, 7. NorMxPr, 8. MxPr)*

MXPr was generally excreted as parent drug within the 24 h. Table 2 shows the approximate MXPr concentrations detected in the various urine samples. The identification of the main phase I metabolites is in agreement with the study by Goncalves R. et al. (2022) [21], which confirmed the presence of most of the phase II metabolites in urine collected from an MXPr consumer. The MXPr pharmacokinetcs profile shows an excretion peak after 1h, then a progressive decay along six to twelve hours, even if MXPr is still detectable 24 hours after administration of 3 and 10 mg/kg. The same trend is observed for other metabolites, such as NorMXPr and desmethyl-MXPr, that exhibit an excretion peak after one hour followed by a progressive decrease over time, leaving trace concentrations even at 24 h after administration (Supplementary Materials Figure S2).

Previous studies [22] identified CYP3A4 as the prevalent enzyme responsible for the N-dealkylation of ketamine, with secondary role played also by CYP2B6 and CYP2C9. In analogy, CYP3A4 is likely to be responsible for the MXPr metabolism to its metabolite NorMXPr. Extending the analogy, norketamine is known to be pharmacologically active, making it likely that NorMXPr also contributes to the overall MXPr toxicity. It should be noted that the relevant presence of NorMXPr in all urine samples makes it the main phase I metabolite, that persists in the urinary excretion even longer than the parent drug MXPr (Figure 4).

Some interesting differences were observed between the metabolites profiles of female and male mice, a phenomenon that could be attributed to sexually dimorphic metabolism [23], involving both phase I and phase II enzymes. Due to the unavailability of female mice urine samples at certain time intervals considered in this study, it is difficult to draw parallel pharmacokinetics curves and observe specific differences in the metabolites profile generated over time. However, it is possible to compare the variation in the levels of MXPr and its main metabolite NorMXPr, which definitely exhibit different ratios based on the mouse gender. In particular, the MXPr excretion peak was reached earlier in males than in females, whereas the peak MXPr concentration is significantly higher in female samples for all the administered doses (Figure 5a, b, c). Accordingly, NorMXPr reach higher concentrations in the male urine samples, both evidences suggesting that male mice have a faster metabolism of MXPr than female mice (Figures S4 in Supplementary Materials). Nevertheless, the analysis of only one pooled male and one pooled female sample extract is probably not enough to conclude that there are metabolism differences according to gender.

Further replicate measurements will be necessary to speculate about possible metabolism differences between males and females.

The analysis of fur and plasma samples did not allow the detection of all target analytes (the metabolites detected in plasma and fur are reported in Supplementary Materials - Table S3). MXPr and only the major phase I metabolites were observed in plasma (average concentration for the parent drug: 1.0 ng/mL) and fur (average concentration for the parent drug: 13 pg/mg). MXPr was eliminated as parent drug in blood before 24 h, while the presence of the nor-MXPr and dihydro-MXPr metabolites in the blood sample suggests these metabolites could be long-term drug use indicators. The plasma levels recorded after 10 mg/kg MXPr administration are consistent with those obtained in previous studies involving the administration of 10 mg/kg MXE [24]. Unlike ketamine, subjected to extremely rapid conversion to norketamine, the nor-derivatives of MXE and MXPr were detected in lower concentrations than the parent drugs [25].

Table 2. *Urine concentrations of MXPr (expressed in ng/mL) found at the different sampling time after the treatment.*

MXPr dosage	Sex	Collection time (h)							
		1	**2**	**3**	**4**	**5**	**6**	**12**	**24**
1 mg/kg	M	440	141	107	41	18	2	2	n.d.
	F	n/a	572	n/a	210	60	41	10	n.d
3 mg/kg	M	n/a	629	267	n/a	150	141	58	17
	F	n/a	n/a	n/a	n/a	1050	n/a	339	119
10	M	6730	3070	2870	1820	n/a	588	547	399
mg/kg	F	n/a	11300	8580	n/a	n/a	3348	1849	261

n.d.: not detected

n/a: sample not available

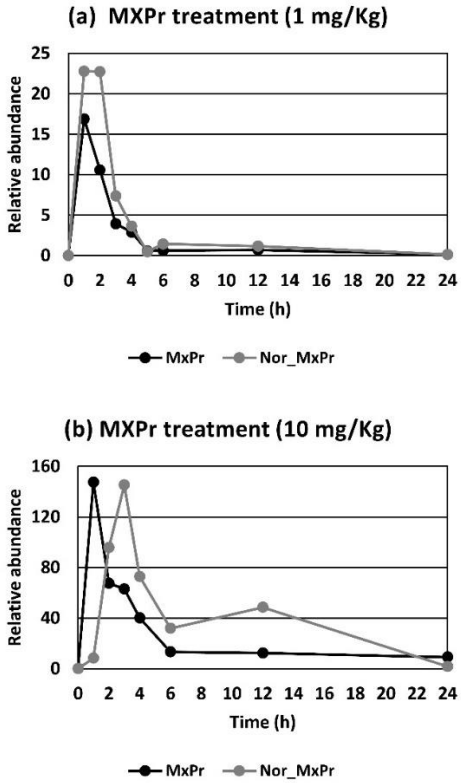


Figure 4: *MXPr and NorMXPr excretion trends at different h for samples: a) pooled urine of male mice (1 mg/Kg) and b) pooled urine of male mice (10 mg/Kg)*

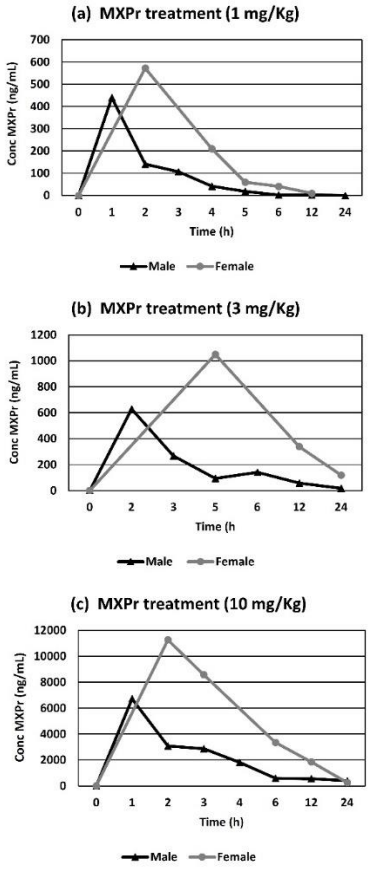


Figure 5: *Comparison of MXPr concentration in male and female mice urine for the three assays studied, respectively a) 1 mg / Kg, b) 3 mg / Kg, c) 10 mg / Kg*

Currently, very few data are available in the literature concerning the detection of MXPr and metabolites in biological samples. In one study, pooled human liver microsome (pHLM) assays were performed and analyzed using liquid chromatography quadrupole time-of-flight mass spectrometry (LC-QToF-MS) in order to detect MXPr metabolites. Three metabolites were identified including NorMXPr, desmethyl-MXPr, and dihydro-MXPr. Most of the phase II metabolites were confirmed to be present in urine and fur samples collected from mice treated with MXPr [10] .

A second study refers to the suicide of a drug abuser with past psychiatric disorders; postmortem blood and hair were collected and screened for the presence of xenobiotics by GC-MS and LC-HRMS [10]. The presence of high MXPr concentration was detected in hair (8 ng/mg) and blood (6400 ng/mL) samples, highlighting recent drug consumption before death. The blood sample turned out positive also for dihydro-MXPr, suggesting the inclusion of this metabolite in the screening procedure devoted to ascertain the detection time of MXPr intake.

8.4 Conclusions

The present study investigates for the first-time the in vivo metabolism of MXPr on both male and female mice, in order to identify the main MXPr phase-I and phase-II metabolites and delineate their pharmacokinetic profiles. A significant feature of the resulting data is that a strong sexually dimorphic metabolism of MXPr is observed. In particular, the parent drug undergoes a slower but more extensive metabolization in female mice than in male subjects.

The characterization of the main MXPr metabolites based on LC-HRMS and LC-MS/HRMS allowed the accurate mass determination of their protonated molecular ion and collisionally-activated fragment ions resulting in the reliable definition of their structure and the outlining of the major metabolic routes for the tested substance. The knowledge of the fragmentation pattern for both MXPr and its main metabolites will also allow to develop fit-for-purpose targeted analytical methods useful for the detection of MXPr and its metabolites in biological specimens. In particular, NorMXPr, DesmethylMXPr, and DihydroMXPr are suggested as target analytes in the toxicological analyses, so as to increase the MXPr detection time after intake and reduce the risk of false-negative results in the forensic cases. This may represent the major strength of this original investigation with particular mention to the translational value of these results. In fact, it is well known that characterizing in different species the metabolic

pathways of emerging psychoactive substances provide insight into mechanisms underlying their potential toxicity and data useful for their detection in biological samples. On the other hand, previous data document that some differences due to species-specific influences (i.e., different concentrations of metabolites or enzymatic polymorphism) can be observed relative to metabolism of many drugs [25]. Thus, despite the high similarity showed by the CYP3A4 enzyme mainly involved in the metabolism of MxPr and many other psychoactive drugs [25], further studies may be required to speculate on metabolic pathway and related gender-based differences.

References

[1] EU Drug Markets Report 2019, 260.

[2] EMCDDA–Europol 2017 Annual Report on the implementation of Council Decision 2005/387/JHA, Annu. Rep. (2017) 31.

[3] Current NPS Threats, U. N. Off. Drugs Crime. //www.unodc.org/unodc/en/scientists/current-nps-threats.html.

[4] A. Shafi, A.J. Berry, H. Sumnall, D.M. Wood, D.K. Tracy, New psychoactive substances: a review and updates, Ther. Adv. Psychopharmacol. 10 (2020) 2045125320967197. https://doi.org/10.1177/2045125320967197.

[5] M.R. Meyer, M. Bach, J. Welter, M. Bovens, A. Turcant, H.H. Maurer, Ketamine-derived designer drug methoxetamine: metabolism including isoenzyme kinetics and toxicological detectability using GC-MS and LC-(HR-)MSn, Anal. Bioanal. Chem. 405 (2013) 6307–6321. https://doi.org/10.1007/s00216-013-7051-6.

[6] H. Morris, J. Wallach, From PCP to MXE: a comprehensive review of the non-medical use of dissociative drugs, Drug Test. Anal. 6 (2014) 614–632. https://doi.org/10.1002/dta.1620.

[7] C.J. Botanas, J.B. de la Peña, I.J. Dela Peña, R. Tampus, R. Yoon, H.J. Kim, Y.S. Lee, C.G. Jang, J.H. Cheong, Methoxetamine, a ketamine derivative, produced conditioned place preference and was self-administered by rats: Evidence of its abuse potential, Pharmacol. Biochem. Behav. 133 (2015) 31–36. https://doi.org/10.1016/j.pbb.2015.03.007.

[8] T. Irie, D. Yamazaki, R. Kikura-Hanajiri, A potential of methoxpropamine to be a widespread recreational drug: it blocks NMDA receptors and inhibits NMDA receptor-mediated synaptic transmission in a brain preparation of mice, Forensic Toxicol. (2021). https://doi.org/10.1007/s11419-021-00571-0.

[9] M. Specka, T. Kuhlmann, J. Sawazki, U. Bonnet, R. Steinert, M. Cybulska-Rycicki, H. Eich, B. Zeiske, A. Niedersteberg, L. Schaaf, N. Scherbaum, Prevalence of Novel Psychoactive Substance (NPS) Use in Patients Admitted to Drug Detoxification Treatment, Front. Psychiatry 11 (2020) 569. https://doi.org/10.3389/fpsyt.2020.00569.

[10] S. Mestria, S. Odoardi, G. Biosa, V. Valentini, G. Di Masi, F. Cittadini, S. Strano-Rossi, Method development for the identification of methoxpropamine, 2-fluoro-deschloroketamine and deschloroketamine and their main metabolites in blood and hair and forensic application, Forensic Sci. Int. 323 (2021) 110817. https://doi.org/10.1016/j.forsciint.2021.110817.

[11] L.H.J. Richter, V. Flockerzi, H.H. Maurer, M.R. Meyer, Pooled human liver preparations, HepaRG, or HepG2 cell lines for metabolism studies of new psychoactive substances? A study using MDMA, MDBD, butylone, MDPPP, MDPV, MDPB, 5-MAPB, and 5-API as examples, J. Pharm. Biomed. Anal. 143 (2017) 32–42. https://doi.org/10.1016/j.jpba.2017.05.028.

[12] S.K. Manier, C. Felske, N. Eckstein, M.R. Meyer, The metabolic fate of two new psychoactive substances - 2-aminoindane and N-methyl-2-aminoindane - studied in vitro and in vivo to support drug testing, Drug Test. Anal. 12 (2020) 145–151. https://doi.org/10.1002/dta.2699.

[13] L. Wagmann, F. Frankenfeld, Y.M. Park, J. Herrmann, S. Fischmann, F. Westphal, R. Müller, V. Flockerzi, M.R. Meyer, How to Study the Metabolism of New Psychoactive Substances for the Purpose of Toxicological Screenings—A Follow-Up Study Comparing Pooled Human Liver S9, HepaRG Cells, and Zebrafish Larvae, Front. Chem. 8 (2020). https://www.frontiersin.org/articles/10.3389/fchem.2020.00539 (accessed December 22, 2022).

[14] S. Mestria, S. Odoardi, S. Federici, S. Bilel, M. Tirri, M. Marti, S. Strano Rossi, Metabolism Study of N-Methyl 2-Aminoindane (NM2AI) and Determination of Metabolites in Biological Samples by LC-HRMS, J. Anal. Toxicol. 45 (2021) 475–483. https://doi.org/10.1093/jat/bkaa111.

[15] C.H. Johnson, J. Ivanisevic, G. Siuzdak, Metabolomics: beyond biomarkers and towards mechanisms, Nat. Rev. Mol. Cell Biol. 17 (2016) 451–459. https://doi.org/10.1038/nrm.2016.25.

[16] E.L. Menzies, S.C. Hudson, P.I. Dargan, M.C. Parkin, D.M. Wood, A.T. Kicman, Characterizing metabolites and potential metabolic pathways for the novel psychoactive substance methoxetamine, Drug Test. Anal. 6 (2014) 506–515. https://doi.org/10.1002/dta.1541.

[17] E.D. Kharasch, R. Labroo, Metabolism of ketamine stereoisomers by human liver microsomes, Anesthesiology 77 (1992) 1201–1207. https://doi.org/10.1097/00000542-199212000-00022.

[18] M. Tirri, R. Arfè, S. Bilel, G. Corli, B. Marchetti, A. Fantinati, F. Vincenzi, F. De-Giorgio, C. Camuto, M. Mazzarino, M. Barbieri, R.M. Gaudio, K. Varani, P.A. Borea, F. Botrè, M. Marti, In Vivo Bio-Activation of JWH-175 to JWH-018: Pharmacodynamic and Pharmacokinetic Studies in Mice, Int. J. Mol. Sci. 23 (2022) 8030. https://doi.org/10.3390/ijms23148030.

[19] L. Fattore, S. Altea, W. Fratta, Sex Differences in Drug Addiction: A Review of Animal and Human Studies, Womens Health 4 (2008) 51–65. https://doi.org/10.2217/17455057.4.1.51.

[20] F. Franconi, V. Raparelli, V. Regitz-Zagrosek, Sex and gender landscape in pharmacology, Pharmacol. Res. 123 (2017) 93–94. https://doi.org/10.1016/j.phrs.2017.07.001.

[21] R. Goncalves, N. Castaing, C. Richeval, D. Ducint, K. Titier, E. Morvan, A. Grélard, A. Loquet, M. Molimard, Methoxpropamine (MXPr) in powder, urine and hair samples: Analytical characterization and metabolite identification of a new threat, Forensic Sci. Int. 333 (2022) 111215. https://doi.org/10.1016/j.forsciint.2022.111215.

[22] R. Goncalves, N. Castaing, C. Richeval, D. Ducint, K. Titier, E. Morvan, A. Grélard, A. Loquet, M. Molimard, Methoxpropamine (MXPr) in powder, urine and hair samples: Analytical characterization and metabolite identification of a new threat, Forensic Sci. Int. 333 (2022) 111215. https://doi.org/10.1016/j.forsciint.2022.111215.

[23] A. Leskanicova, O. Chovancova, M. Babincak, L. Verboova, Z. Benetinova, D. Macekova, J. Kostolny, B. Smajda, T. Kiskova, Sexual Dimorphism in Energy Metabolism of Wistar Rats Using Data Analysis, Molecules 25 (2020) 2353. https://doi.org/10.3390/molecules25102353.

[24] R.R. Horsley, E. Lhotkova, K. Hajkova, B. Jurasek, M. Kuchar, T. Palenicek, Detailed pharmacological evaluation of methoxetamine (MXE), a novel psychoactive ketamine analogue-Behavioural, pharmacokinetic and metabolic studies in the Wistar rat, Brain Res. Bull. 126 (2016) 102–110. https://doi.org/10.1016/j.brainresbull.2016.05.002.

[25] T. Páleníček, M. Fujáková, M. Brunovský, M. Balíková, J. Horáček, I. Gorman, F. Tylš, B. Tišlerová, P. Soš, V. Bubeníková-Valešová, C. Höschl, V. Krajča, Electroencephalographic spectral and coherence analysis of ketamine in rats: correlation with behavioral effects and pharmacokinetics, Neuropsychobiology 63 (2011) 202–218. https://doi.org/10.1159/000321803

Supplementary materials

Table S1: *Availability of the samples and the summary of the samples' collection*

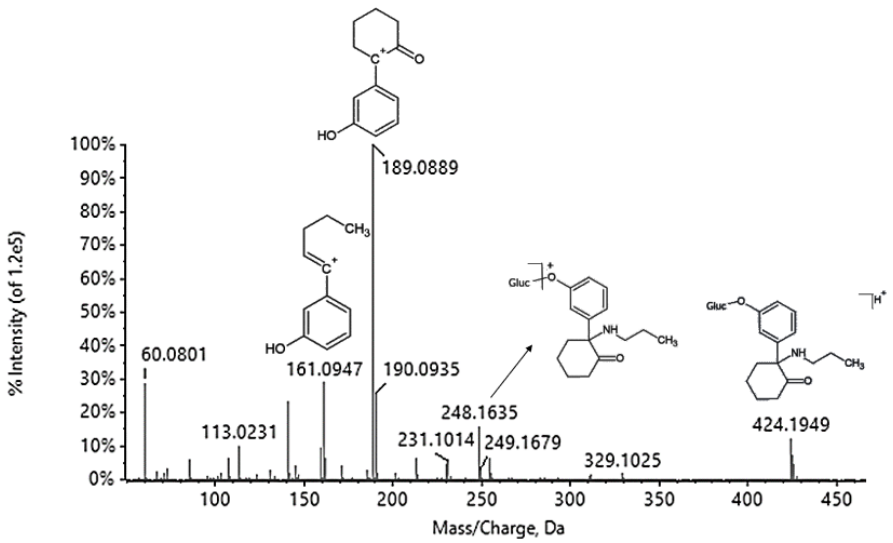
Inj. dose	Sex	Inj. day	Sampling time (h) and availability								
			0	**1**	**2**	**3**	**4**	**5**	**6**	**12**	**24**
1 mg/kg	M	03/05/21	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
(G1)	F	03/05/21	Yes	No	Yes	No	Yes	Yes	Yes	Yes	Yes
1 mg/kg	M	04/05/21	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
(G2)	F	04/05/21	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
10 mg/kg	M	05/05/21	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes
(G1)	F	05/05/21	No	No	Yes	Yes	No	No	No	Yes	Yes
3 mg/kg	M	06/05/21	No	No	Yes	Yes	No	Yes	Yes	Yes	Yes
(G1)	F	06/05/21	No	No	No	No	No	Yes	No	Yes	Yes

Table S2: *MS and MS/MS parameters used for the instrumental analysis*

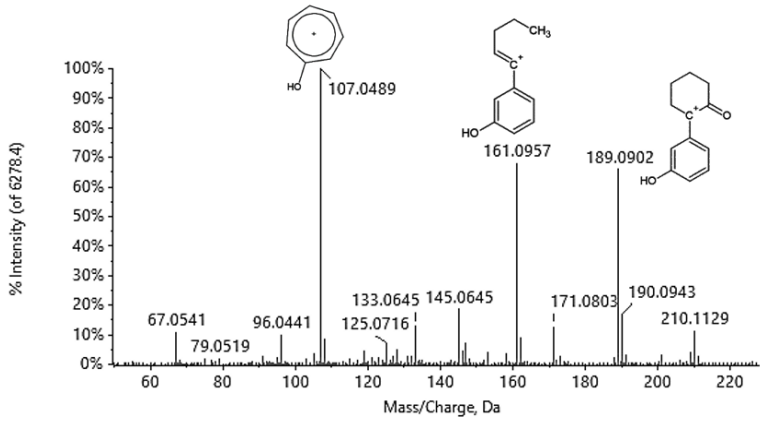
Source and Gas Parameters				
Ion gas 1 (psi)	Ion gas 2 (psi)	Curtain gas (psi)	CAD gas (psi)	**Temperature (°C)**
50	**45**	**35**	**8**	**600**
TOF MS				
TOF start mass (Da)	TOF stop mass (Da)	DP (V)	CE (V)	**Accumulation time**
100	**600**	**65**	**10**	**0.1**
TOF MS/MS				
TOF start mass (Da)	TOF stop mass (Da)	DP (V)	CE (V)	**Accumulation time**
50	**800**	**65**	**35± 15**	**0.04**

Figure S1: *HRMS fragmentation pattern of a) Desmethyl- MXPr-gluc, b) Desmetyl-NorMXPr, c) Desmetyl-NorMXPr-gluc, d) Dihydro-Desmetyl-MXPr, e) Dihydro-Desmetyl-MXPr-gluc, f) Nor-MXPr*

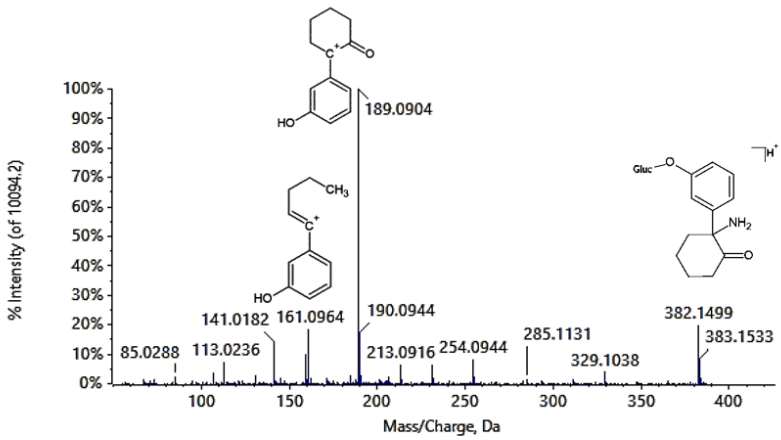
a)



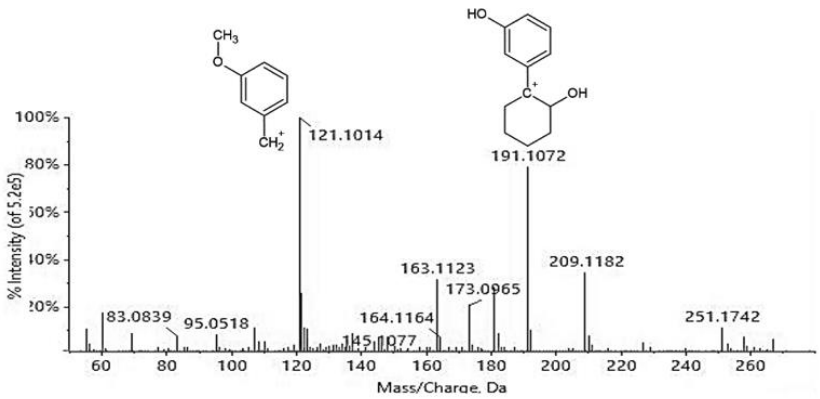
b)


% Intensity (of 6278.4)
Mass/Charge, Da
107.0489
161.0957
189.0902
67.0541
79.0519
96.0441
125.0716
133.0645
145.0645
171.0803
190.0943
210.1129

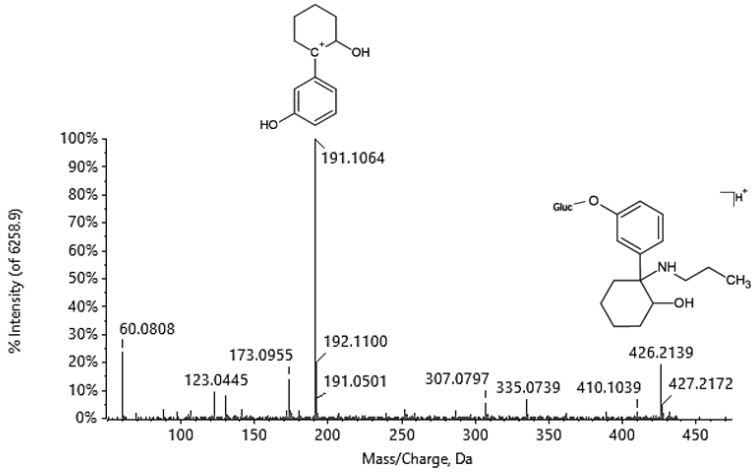
c)


% Intensity (of 10094.2)
Mass/Charge, Da
189.0904
141.0182
161.0964
190.0944
85.0288
113.0236
213.0916
254.0944
285.1131
329.1038
382.1499
383.1533

d)


% Intensity (of 5.265)
Mass/Charge, Da
121.1014
191.1072
83.0839
95.0518
163.1123
173.0965
164.1164
209.1182
251.1742

e)


% Intensity (of 6258.9)
Mass/Charge, Da
191.1064
60.0808
173.0955
192.1100
123.0445
191.0501
307.0797
335.0739
410.1039
426.2139
427.2172
Gluc
NH
CH3
OH
HO
H+

f)

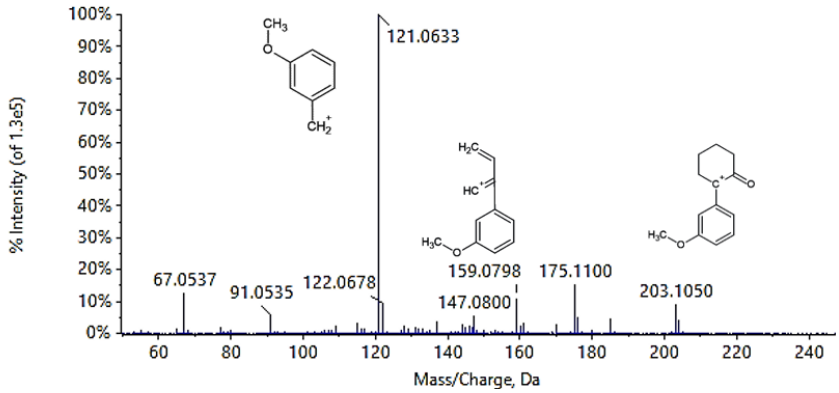

% Intensity (of 1.3e5)
Mass/Charge, Da
121.0633
67.0537
91.0535
122.0678
147.0800
159.0798
175.1100
203.1050
CH3
CH2+
H2C
HC+
H3C
O

Figure S2: *MXPr and metabolites excretion trends with and without Desmethyl-MxPr-GLuc at different h for samples: a) pooled urine of male mice (10 mg/Kg) with Desmethyl-MxPr-GLuc, b) pooled urine of male mice (1 mg/Kg) with Desmethyl-MxPr-GLuc, c) pooled urine of male mice (10 mg/Kg) without Desmethyl-MxPr-GLuc, b) pooled urine of male mice (1 mg/Kg) without Desmethyl-MxPr-GLuc*

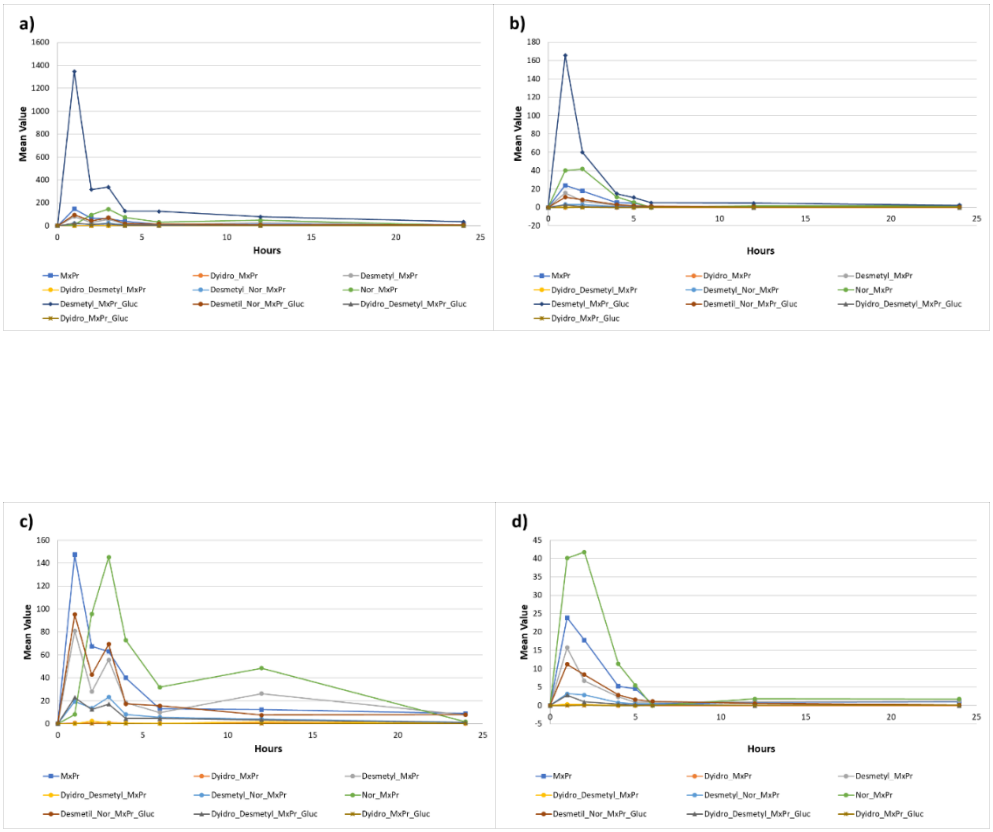
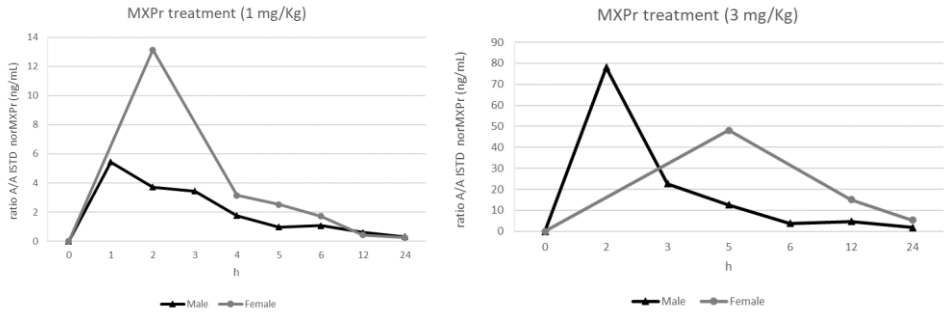


Figure S3: *Comparison of NorMXPr signal intensity in male and female mice for the three assays studied (1, 3, 10 mg /Kg, respectively)*



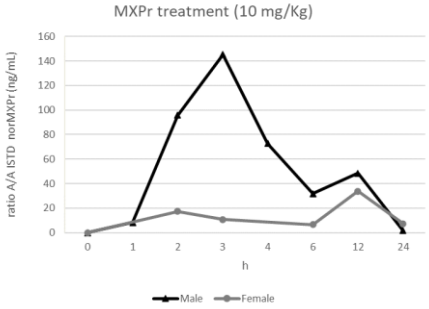


Table S3: *Metabolites detected in plasma and hair*

Compound	**Plasma**		**Fur**	
	Male	**Female**	**Male**	**Female**
MXPr	yes	yes	yes	yes
Desmethyl-MXPr	no	no	yes	yes
Dihydro-Desmethyl-MXPr	no	no	no	no
Desmethyl-NorMXPr	no	no	no	no
NorMXPr	yes	yes	yes	yes
Dihydro-MXPr	no	yes	no	no
Desmethyl-MXPr- Gluc	no	no	yes	no
Desmethyl-NorMXPr-Gluc	no	no	no	no
Dihydro-MXPr-Gluc	no	no	no	no
Dihydro-Desmethyl-MXPr-Gluc	no	no	no	no

Chapter 9 | **Development and validation of a UHPLC-HRMS-QTOF method for the detection of 132 New Psychoactive Substances and synthetic opioids, including fentanyl, in Dried Blood Spots**

Marta Massano[1*], Carola Incardona[1], Enrico Gerace[2], Pierre Negri[3], Eugenio Alladio[1], Alberto Salomone[1,2], Marco Vincenti[1,2]

1: Department of Chemistry, University of Torino,

2: Centro Regionale Antidoping e di Tossicologia "A. Bertinaria", Regione Gonzole 10/1, 10043, Orbassano, Torino, Italy

3: SCIEX, Redwood City, CA, 01701, USA

** Corresponding author: Marta Massano*

Email address: marta.massano@unito.it

The content of this Chapter is included in one published manuscript:

Development and validation of a UHPLC-HRMS-QTOF method for the detection of 132 New Psychoactive Substances and synthetic opioids, including fentanyl, in Dried Blood Spots

***Marta Massano**, Carola Incardona, Enrico Gerace, Pierre Negri, Eugenio Alladio, Alberto Salomone, Marco Vincenti*

Talanta

https://doi.org/10.1016/j.talanta.2022.123265

Keywords: NPS, fentanyl, dried blood spots, validation, QTOF, HRMS, opioids

Abstract

Dried Blood Spots (DBS) represents a promising micro-sampling technique in the field of forensic toxicology to carry out minimally invasive blood sample collection. In DBS, cheap, fast and easy sampling is combined with effortless store and transport. These properties aimed us to develop and validate a quick and easy procedure for the detection of a large and diverse range of emerging and alarming New Psychoactive Substances (NPS). A drop of whole blood sample was collected on a DBS card and dried for 3 hours, from which a total of 132 analytes (including NPS, synthetic opioids NSO and metabolites) plus 13 deuterated internal standards could be extracted using 500 µL of a methanol/acetonitrile mixture (3:1, v/v) and subsequently separated and identified by means of ultra-high-performance liquid-chromatography (UHPLC) coupled to high resolution mass spectrometry (HRMS). The extraction efficiency proved to be reproducible with yields ranging from 30% to 100% depending on the different classes of drugs. Trueness, repeatability, and intermediate precision fulfilled acceptance criteria for almost all synthetic opioids, cathinones and hallucinogens (bias and CV% below ±20%); in particular, the aggregate inter-day trueness data showed extremely limited deviation from the expected concentrations (−10% < bias% < +10%) for 114 target analytes out of 132. The calculated limits of detection ranged from 1.3 to 6.3 ng/mL, consistently exceeding the values experimentally tested. Moderate ion suppression was observed for most analytes, partly caused by blood fortification itself. Good stability of the target analytes at −20 °C, 4 °C, and 35 °C on DBS cards after drying was observed, even for long periods of time. Optimal storage condition appeared to be at 4 °C resulting in virtually no drugs degradation for up to 40 days. The novel analytical method based on DBS sampling, verified on venous whole blood real samples previously tested positive with our routine procedure, conveys remarkable potential in analytical toxicology, clinical analysis, and doping control.

9.1 Introduction

Drug abuse has certainly represented a dramatic social and health issue for a long time, but in recent years the diffusion of countless New Psychoactive Substances (NPS) in the illegal drug market introduced an unprecedented threat to the public health [1]. The adjective "new" does not necessarily indicate totally original compounds, but it may refer to substances initially synthesized and tested for their potential pharmaceutical properties and revalued years later as highly potent drugs of abuse [2,3]. NPS are often categorized into synthetic

cannabinoids, synthetic opioids, cathinones and hallucinogen, but this simplistic classification does not express adequately the variety and complexity of their potency, combined effects, and risk profiles that intersect categories and often differentiate compounds belonging to the same category [4].

Nowadays, the NPS abuse in U.S and Europe is proliferating at unprecedented rate [5] and represents an increasing challenge to the established national and international drug policies. Difficulties associated with NPS monitoring include their high number, the speed with which they enter and exit the illegal drug market, the restricted and partial information about them, the incomplete knowledge about their composition and mixing, the variable and often unknown potency, and the difficulty for toxicological laboratories to find the analytical standards to provide confirmatory testing [6]. New analytical approaches and in-depth investigations of the different biological matrices, either conventional or alternative, are thus needed for effective monitoring, surveillance, drug control, and public health campaigns aimed to reduce this drug-related harm [7].

Dried blood spot (DBS) is a sampling technique based on the collection of a whole blood drop on a filter paper. It has been widely used in the diagnostic screening of neonatal metabolic disorders [8] and is now available for clinical diagnostics [9] as well as other applications, including forensic toxicology [10,11] and antidoping analyses [12]. With the development of modern mass spectrometers, several studies have been published on the use of DBS to monitor the blood concentration of a single or multiple target substances for neonatal diagnosis [13], drugs of abuse [14] and therapeutic drugs [15]. The collection of whole blood dried on a piece of filter paper provides several advantages including simplicity, speed, resistance to manipulations, and enhanced stability of the target analytes at room temperature. Compared to conventional venous blood sampling, the typical 30-50 µL collection of capillary blood, obtained by finger, ear, or heel prick, is minimally invasive. The minimum space occupied by the sampling paper as well as the reduced effects of environmental degradation result in a facilitated storage and shipment of DBS specimens [16]. The continuous sensitivity improvement enabled by mass spectrometric techniques encourages further a minimal blood volume handling, such as the one involved in DBS [17].

The present study aims to highlight the opportunities and potential benefits arising from the implementation of DBS as a complementary tool in forensic control programs to monitor the NPS diffusion. To this purpose, the present approach combines DBS sampling with ultra-high-pressure liquid-chromatography

(UHPLC) coupled to time-of-flight high resolution mass spectrometry (TOF-HRMS). The analytical method developed in this study achieved the simultaneous qualitative and quantitative analysis of 132 NPS/NSO analytes and metabolites (their full list is available in Table 1), with the goal of reaching adequately low LOQ levels using a simple and rapid extraction procedure.

Table 6. *List of the 132 NPS/NSO substances under study (target analytes).*

Compounds	Formula	Precursor theoretical Mass	Fragment theoretical Mass	Rt	ISTD
5-CHLORO-AB-PINACA	$C_{18}H_{25}ClN_4O_2$	365.17388	249.0776	5.07	JWH-081 D9
5-CHLORO-THJ018	$C_{23}H_{21}ClN_2O$	377.14152	249.0771	7.12	JWH-081 D9
5-F-AB-PINACA	$C_{18}H_{25}FN_4O_2$	349.20343	233.1066	4.61	JWH-081 D9
5-F-APINACA	$C_{23}H_{30}FN_3O$	384.24457	135.1168	7.18	JWH-081 D9
5-F-APP-PICA	$C_{23}H_{26}FN_3O_2$	396.20818	232.1111	4.93	JWH-081 D9
5-F-APP-PINACA	$C_{22}H_{25}FN_4O_2$	397.20343	233.1065	5.06	JWH-081 D9
5-F-CUMYL-PINACA	$C_{22}H_{26}FN_3O$	368.21327	233.1064	6.33	JWH-081 D9
5-F-NNEI MAPHTHYL isomer	$C_{24}H_{23}FN_2O$	375.18672	232.1112	6.42	JWH-081 D9
5-F-PB22	$C_{23}H_{21}FN_2O_2$	377.16598	232.1113	6.09	JWH-081 D9
AB-CHMINACA	$C_{20}H_{28}N_4O_2$	357.2285	241.1333	5.63	JWH-081 D9

AB-FUBINACA	$C_{20}H_{21}FN_4O_2$	369.17213	253.075	4.90	JWH-081 D9
AB-PINACA	$C_{18}H_{26}N_4O_2$	331.21285	215.1181	5.18	JWH-081 D9
ADBICA	$C_{20}H_{29}N_3O_2$	344.23325	214.121	5.41	JWH-081 D9
ADB-PINACA	$C_{19}H_{28}N_4O_2$	345.2285	215.1163	5.55	JWH-081 D9
AM-1220	$C_{26}H_{26}N_2O$	383.21179	98.0958	4.14	JWH-081 D9
AM-2201	$C_{24}H_{22}FNO$	360.17582	155.0488	6.49	JWH-081 D9
AM-2233	$C_{22}H_{23}IN_2O$	459.09279	98.0963	3.77	JWH-081 D9
AM-694	$C_{20}H_{19}FINO$	436.05682	230.9297	6.12	JWH-081 D9
APP-FUBINACA	$C_{24}H_{21}FN_4O_2$	417.17213	253.0755	5.23	JWH-081 D9
CB-13	$C_{26}H_{24}O_2$	369.18491	155.049	7.37	JWH-081 D9
CUMYL PEGACLONE	$C_{25}H_{28}N_2O$	373.22744	255.1475	6.66	JWH-081 D9
JWH-007	$C_{25}H_{25}NO$	356.20089	155.0483	7.30	JWH-081 D9
JWH-015	$C_{23}H_{21}NO$	328.16959	155.0493	6.48	JWH-081 D9
JWH-018	$C_{24}H_{23}NO$	342.18524	155.0493	6.84	JWH-081 D9
JWH-019	$C_{25}H_{25}NO$	356.20089	169.0651	7.46	JWH-081 D9

JWH-020	$C_{26}H_{27}NO$	370.21654	155.0483	7.75	JWH-081 D9
JWH-073	$C_{23}H_{21}NO$	328.16959	155.0487	6.73	JWH-081 D9
JWH-081	$C_{25}H_{25}NO_2$	372.19581	185.0593	7.15	JWH-081 D9
JWH-098	$C_{26}H_{27}NO_2$	386.21146	155.0481	7.32	JWH-081 D9
JWH-122	$C_{25}H_{25}NO$	356.20089	169.0644	7.46	JWH-081 D9
JWH-147	$C_{27}H_{27}NO$	382.21654	155.0481	7.79	JWH-081 D9
JWH-203	$C_{21}H_{22}ClNO$	340.14627	125.015	7.01	JWH-081 D9
JWH-210	$C_{26}H_{27}NO$	370.21654	183.0802	7.57	JWH-081 D9
JWH-250	$C_{22}H_{25}NO_2$	336.19581	121.0643	6.50	JWH-081 D9
JWH-251	$C_{22}H_{25}NO$	320.20089	214.1219	6.85	JWH-081 D9
JWH-302	$C_{22}H_{25}NO_2$	336.19581	214.1209	6.50	JWH-081 D9
JWH-307	$C_{26}H_{24}FNO$	386.19147	155.0486	7.29	JWH-081 D9
JWH-398	$C_{24}H_{22}ClNO$	376.14627	189.0102	7.68	JWH-081 D9
MAB-CHMINACA	$C_{21}H_{30}N_4O_2$	371.24415	241.1313	5.90	JWH-081 D9
MAM-2201	$C_{25}H_{24}FNO$	374.19147	169.0646	6.64	JWH-081 D9

MDMB-CHMINACA	$C_{22}H_{31}N_3O_3$	386.24382	241.1324	7.22	JWH-081 D9
MDMB-CHMICA	$C_{23}H_{32}N_2O_3$	385.24857	240.1382	6.75	JWH-081 D9
MMB-2201	$C_{20}H_{27}FN_2O_3$	363.20785	232.1116	5.61	JWH-081 D9
PB-22	$C_{23}H_{22}N_2O_2$	359.1754	214.1203	6.72	JWH-081 D9
RCS-4	$C_{21}H_{23}NO_2$	322.18016	135.0439	6.50	JWH-081 D9
RCS-8	$C_{25}H_{29}NO_2$	376.22711	121.0644	7.46	JWH-081 D9
STS-135	$C_{24}H_{31}FN_2O$	383.24932	135.1157	7.17	JWH-081 D9
UR-144	$C_{21}H_{29}NO$	312.23219	125.0954	7.43	JWH-081 D9
UR-144-5-OH	$C_{21}H_{29}NO_2$	328.22711	125.0957	5.88	JWH-081 D9
WIN-48	$C_{23}H_{26}N_2O_3$	379.20162	135.0429	3.75	JWH-081 D9
WIN-55	$C_{27}H_{26}N_2O_3$	427.20162	155.0482	5.56	JWH-081 D9
XLR-11	$C_{21}H_{28}FNO$	330.22277	125.0944	6.79	JWH-081 D9
25B-NBOMe	$C_{18}H_{22}BrNO_3$	380.08558	121.0651	3.94	MDPV D8
25C-NBOMe	$C_{18}H_{22}ClNO_3$	336.1361	121.0657	4.02	25I-NBOMe D3
25H-NBOMe	$C_{18}H_{23}NO_3$	302.17507	121.0654	3.59	25I-NBOMe D3

25I-NBOMe	$C_{18}H_{22}INO_3$	428.07172	121.0639	4.10	25I-NBOMe D3
2C-B	$C_{10}H_{14}BrNO_2$	260.02807	243.0025	2.80	MDPV D8
2C-P	$C_{13}H_{21}NO_2$	224.16451	207.1355	3.45	mCPP D8
3-4 DMMC	$C_{12}H_{17}NO$	192.13829	159.103	2.73	MDPV D8
4-F-METCAT	$C_{10}H_{12}FNO$	182.09757	149.0631	1.66	Mephedrone D3
4-MEC	$C_{12}H_{17}NO$	192.13829	174.1258	2.47	MDPV D8
5-EAPB	$C_{13}H_{17}NO$	204.13829	131.0479	2.74	MDPV D8
6-APB	$C_{11}H_{13}NO$	176.10699	131.0492	2.38	MDPV D8
ALFA-PVP	$C_{15}H_{21}NO$	232.16959	91.0535	2.91	MDPV D8
Buphedrone	$C_{11}H_{15}NO$	178.12264	131.0700	1.83	MDPV D8
Butylone	$C_{12}H_{15}NO_3$	222.11247	174.0917	2.25	MDPV D8
Ethylone	$C_{12}H_{15}NO_3$	222.11247	174.0916	2.13	PCP D5
Ketamine	$C_{13}H_{16}ClNO$	238.09932	125.0149	2.42	MDPV D8
mCPP	$C_{10}H_{13}ClN_2$	197.084	154.0414	2.70	mCPP D8
MDPV	$C_{16}H_{21}NO_3$	276.15942	126.1278	2.90	MDPV D8
Mephedrone	$C_{11}H_{15}NO$	178.12264	144.0810	2.27	Mephedrone D3
Methedrone	$C_{11}H_{15}NO_2$	194.11756	146.0602	2.09	MDPV D8
Methylone	$C_{11}H_{13}NO_3$	208.09682	160.0763	1.78	MDPV D8
MXE	$C_{15}H_{21}NO_2$	248.16451	203.1058	2.64	MDPV D8
N-Ethylcathinone	$C_{11}H_{15}NO$	178.12264	132.0808	1.83	MDPV D8
N-ethylpentylone	$C_{14}H_{19}NO_3$	250.14377	202.1211	2.79	MDPV D8
PCP	$C_{17}H_{25}N$	244.20598	91.0534	3.27	PCP D5

PCP-4MEO	$C_{18}H_{27}NO$	274.21654	121.0638	3.41	PCP D5
Pentedrone	$C_{12}H_{17}NO$	192.13829	145.0883	2.50	Mephedrone D3
Pentylone	$C_{13}H_{17}NO_3$	236.12812	188.1065	2.70	MDPV D8
PMA	$C_{10}H_{15}NO$	166.12264	121.0641	2.10	MDPV D8
PMMA	$C_{11}H_{17}NO$	180.13829	121.0651	2.18	MDPV D8
Ritalinic acid	$C_{13}H_{17}NO_2$	220.13321	84.0806	2.30	MDPV D8
Trazodone	$C_{19}H_{22}ClN_5O$	372.15856	176.0804	3.30	MDPV D8
4-ANPP	$C_{19}H_{24}N_2$	281.20123	105.0689	3.44	Fentanyl D5
4-Fluorobutyrfentanyl	$C_{23}H_{29}FN_2O$	369.23367	188.1434	3.98	Fentanyl D5
4-methylfentanyl	$C_{23}H_{30}N_2O$	351.24309	202.1577	3.71	Fentanyl D5
3-methylnorfentanyl	$C_{15}H_{22}N_2O$	246.1732	69.6693	2.81	Fentanyl D5
Acetyl Fentanyl	$C_{21}H_{26}N_2O$	323.21179	188.1434	3.10	Fentanyl D5
Acrylfentanyl	$C_{22}H_{26}N_2O$	335.21179	188.1417	3.35	Fentanyl D5
AH-7921	$C_{16}H_{22}Cl_2N_2O$	329.1182	172.9545	3.50	Fentanyl D5
Alfentanil	$C_{21}H_{32}N_6O_3$	417.26087	268.1776	3.49	Fentanyl D5
Butyrfentanyl	$C_{23}H_{30}N_2O$	351.24309	188.1417	3.69	Fentanyl D5
Butyryl Fentanyl (carboxy metabolite)	$C_{23}H_{28}N_2O_3$	381.21727	188.1417	3.04	Fentanyl D5
Carfentanyl	$C_{24}H_{30}N_2O_3$	395.23292	113.059	3.65	Fentanyl D5
Cyclopropylfentanyl	$C_{23}H_{28}N_2O$	349.22744	188.1419	3.54	Fentanyl D5
Despropionyl p-Fluorofentanyl	$C_{19}H_{23}FN_2$	299.1918	105.0691	3.60	Fentanyl D5
Fentanyl	$C_{22}H_{28}N_2O$	337.22744	188.1434	3.55	Fentanyl D5
FuranilFentanil	$C_{24}H_{26}N_2O_2$	375.2067	188.1434	3.67	Fentanyl D5

Hydrocodone	$C_{18}H_{21}NO_3$	300.15942	199.0761	2.16	Fentanyl D5
MT-45	$C_{24}H_{32}N_2$	349.26383	181.0999	4.12	Fentanyl D5
OCFENTANYL	$C_{22}H_{27}FN_2O_2$	371.21293	188.1418	3.25	Fentanyl D5
Remifentanil	$C_{20}H_{28}N_2O_5$	377.2071	113.0603	2.92	Fentanyl D5
Sufentanil	$C_{22}H_{30}N_2O_2S$	387.21008	238.1268	3.82	Fentanyl D5
Tramadol	$C_{16}H_{25}NO_2$	264.19581	58.0648	2.71	Fentanyl D5
U-47700	$C_{16}H_{24}C_{l2}N_2O$	331.13385	284.061	3.42	Fentanyl D5
Valerylfentanyl (carboxy metabolite)	$C_{24}H_{30}N_2O_3$	395.23292	113.0589	3.65	Fentanyl D5
4-ACETOXY DIPT	$C_{18}H_{26}N_2O_2$	303.2067	160.0747	3.10	MDPV D8
4-ACETOXY DMT	$C_{14}H_{18}N_2O_2$	247.1441	174.0899	2.58	MDPV D8
5-MeO-DALT	$C_{17}H_{22}N_2O$	271.18049	174.0912	3.10	MDPV D8
5-MeO-DIPT	$C_{17}H_{26}N_2O$	275.21179	174.091	3.31	MDPV D8
5-METOXY AMT	$C_{12}H_{16}N_2O$	205.13354	147.0672	2.25	PCP D5
DMT	$C_{12}H_{16}N_2$	189.13862		2.06	Mescaline D9
HARMINE	$C_{13}H_{12}N_2O$	213.10224	170.0826	2.88	MDPV D8
LSD	$C_{20}H_{25}N_3O$	324.20704	223.1227	3.13	MDPV D8
Mescaline	$C_{11}H_{17}NO_3$	212.12812	195.1019	1.98	PCP D5
Mitragynine	$C_{23}H_{30}N_2O_4$	399.22783	174.0914	3.69	MDPV D8
Psilocin	$C_{12}H_{16}N_2O$	205.13354	132.0808	1.78	MDPV D8
Norfentanyl	$C_{14}H_{20}N_2O$	233.16484	144.0808	2.46	Norfentayl D5
5-F-ADB	$C_{20}H_{28}FN_3O_3$	378.21875	233.1061	6.17	JWH-081 D9

ADB-FUBINACA	$C_{21}H_{23}FN_4O_2$	383.18778	253.0747	5.19	JWH-081 D9
β-Phenylfentanyl	$C_{28}H_{32}N_2O$	413.25874	188.1398	4.27	Fentanyl D5
Butyrilnorfentanyl	$C_{15}H_{22}N_2O$	247.18049	84.0800	3.00	Fentanyl D5
Ethylphenidate	$C_{15}H_{21}NO_2$	248.16451	84.0797	3.05	MDPV D8
Ethyltryptamine	$C_{12}H_{16}N_2$	189.13862	130.0643	2.65	MDPV D8
Furanylnorfentanyl	$C_{16}H_{18}N_2O_2$	271.1441	84.0798	2.55	Fentanyl D5
Hydroxy-fentanyl	$C_{22}H_{28}N_2O_2$	353.22235	204.135	3.11	Fentanyl D5
Hydroxy-thiofentanyl	$C_{20}H_{26}N_2O_2S$	359.17878	192.0829	2.94	Fentanyl D5
Fentanyl	$C_{26}H_{28}N_2O$	385.22744	188.1434	3.91	Fentanyl D5
5-MeO-DMT	$C_{13}H_{18}N_2O$	219.14919	58.0691	2.22	MDPV D8
Phenylacetylfentanyl	$C_{27}H_{30}N_2O$	399.24309	188.1408	3.05	Fentanyl D5
Eutylone	$C_{13}H_{17}NO_3$	236.12812	188.1060	2.64	MDPV D8
PCP-D5	$C_{17}H_{20}[2H]_5N$	249.23736	96.0854	3.24	
AH-7921 D6	$C_{16}H_1[2H]_6C_{l2}N_2O$	335.15586	172.9546	3.64	
Norfentayl D5	$C_{14}H_{15}[2H]_5N_2O$	238.19622	84.0800	2.40	
Oxycodone D6	$C_{18}H_{15}[2H]_6NO_4$	322.192	304.1792	1.93	
Fentanyl D5	$C_{22}H_{23}[2H]_5N_2O$	342.25882	188.1416	3.50	
JWH-250 D5	$C_{22}H_{20}[2H]_5NO_2$	341.22719	121.0637	6.73	
JWH-081 D9	$C_{25}H_{16}[2H]_9NO_2$	381.2523	185.0577	7.24	
JWH-018 D9	$C_{24}H_{14}[2H]_9NO$	351.24173	155.0485	7.03	
Mephedrone D3	$C_{11}H_{12}[2H]_3NO$	181.14147	148.1064	2.35	
mCPP D8	$C_{10}H_5[2H]_8ClN_2$	205.13422	158.0661	2.65	
25I-nBOMe D3	$C_{18}H_{19}[2H]_3INO_3$	431.09055	124.0835	4.09	

Mescaline D9	$C_{11}H_8[2H]_9NO_3$	221.18461	171.0921	1.90
MDPV D8	$C_{16}H_{13}[2H]_8NO_3$	284.20963	134.1778	2.97

9.2 Reagents and Standards

All chemicals, including methanol, formic acid and acetonitrile, were purchased from Sigma-Aldrich (Milan, Italy). Ultra-pure water was obtained using a Milli-Q® UF-Plus apparatus (Millipore, Bedford, MA, USA). All stock standard solutions were prepared in methanol at 1 mg/mL and stored at –20 °C until used. Working solution of 132 analytes (identified among the most common synthetic cannabinoids, synthetic opioids, synthetic cathinones and hallucinogens monitored nationally and internationally) and internal standard solution containing 13 deuterated NPSs, were prepared at the final concentration of 1 µg/mL by dilution with methanol. The analytical standards of the target analytes and deuterated internal standards were purchased from LGC Promochem (Milan, Italy) and Sigma-Aldrich (Milan, Italy) (purity > 99%, concentration between 0.1 mg/mL and 1 mg/mL), or kindly provided by the National Early Warning System (provided at a concentration of 0.02 mg/mL). DBS cards (FTA™ DMPK C) were purchased from Whatman™ GE Healthcare (UK).

9.3 Sample Preparation

For the preparation of spiked specimens used in the analytical method development and validation, a standard blood matrix was obtained by mixing different aliquots of blank whole blood obtained from volunteers (laboratory personnel) after having signed an informed consent. The absence of all the investigated analytes was verified by means of standard procedures routinely used in our lab for blood testing. The blank samples were then fortified at six concentration levels (5, 7.5, 10, 25, 50, 100 ng/ mL) with an NPS working solution.

The blood samples were vigorously stirred prior to spotting a 30 µL aliquot on a DBS collection card using a calibrated pipette. The spots were allowed to dry for at least three hours at room temperature. The entire spots on the DBS card were punched out and transferred into an Eppendorf tube together with 500 µL methanol/acetonitrile (3:1, v/v) mixture. A 12.5 µL solution of the deuterated internal standards (ISTD) was added to the tubes to reach a final concentration of 25 ng/mL. After ultrasonication (215/860 W, 50/60 Hz) for 30 min at room temperature, the tubes were centrifuged for 5 min at 13000 g. The

supernatant was transferred into a fresh tube prior to evaporating the solvent under a gentle nitrogen flow at room temperature. The dry residue was reconstituted with 50 µL methanol, centrifuged for 5 min at 13000 g, and 5 µL of the supernatant was injected into the UHPLC system.

9.4 Instrumentation

UHPLC separation was performed with a Phenomenex Kinetex C18 column (100 × 2.1 mm, 1.7 µm) maintained at 45 ºC on the SCIEX ExionLC™ AC system. The mobile phases consisted of water (A) and acetonitrile (B), both with formic acid 5 mM. The LC flow rate was set at 0.5 mL/min and the mobile phase eluted under the following linear gradient conditions: (A:B, v:v) isocratic elution at 95:5 for 0.5 min, from 95:5 to 5:95 in 7.5 min, isocratic elution at 5:95 for 0.5 min and final re-equilibration for 2.5 min to the initial condition. The total run time was 10 min.

All analyses were performed using a quadrupole/time-of-flight SCIEX X500R QTOF mass spectrometer (Sciex, Darmstadt, Germany) equipped with a Turbo VTM ion source operating in electrospray positive-ion mode (full MS and MS/MS parameters available in the Supplementary Table S1). Data acquisition involved a preliminary TOF-MS high-resolution full scan followed by a SWATH™ acquisition protocol which used a variable window setup (18 windows covering mass range from *m/z* 99.5 to 575.0 at 0.025 resolving power), resulting in a final cycle time of 0.933 sec. The qualitative identification of the 132 target analytes was based on the coincidence of their retention times, precursor ion and characteristic fragment ion *m/z* values (mass error accepted < 5 ng/mL) (Table S1) as identification parameters. Data were acquired using the SCIEX OS 1.5 Software.

9.5 Method validation

The validation strategy was based on a protocol recently published [18]. Each standard of blank whole blood fortified at six concentration levels (5, 7.5, 10, 25, 50, 100 ng/mL) was analysed nine times in three working sessions (i.e., 3×3) along ten days. This dataset of 54 analysis formed the groundwork on which the statistical evaluation of several validation parameters was founded, including calibration, intra- and inter-day trueness, repeatability and intermediate precision (at 6 concentration levels), limit of detection (LOD), limit of quantification (LOQ). Recovery, matrix effect and stability parameters were evaluated with further independent experiments. Other independent analyses on purposely

spiked samples were performed later to verify the actual detection of the target analytes at the calculated LODs (see the following paragraphs). An *ad hoc* Excel® sheet was built in-house to adapt the routine developed by Desharnais et al. [19]. All the equations employed to compute the validation parameters have been omitted from this text and can be found elsewhere [20].

9.5.1 Calibration

Calibration curves were generated from the peak-area ratio between each analyte and the ISTD quantifier transitions; the ratio was then plotted on the y-axis against the nominal analyte concentration to generate and estimate the curve that best fits and predicts the data distribution, with the support of statistical tests [21]. In the first step of the calibration process, homoscedastic vs. heteroscedastic distribution of the data points was evaluated by analyzing the residues distribution (9 data-points at 6 concentration levels) and comparing the variances at low, medium and high concentration levels. If the variance increased with concentration, the system proved heteroscedastic ($p < 0.05$) and a weighted model was adopted, using either a $1/x$ weighting factor (when the variance increased proportionally to the concentration) or a $1/x^2$ weighting (when a quadratic increase of the variance was observed). Then, the order of the calibration model (linear vs. quadratic) was selected on the basis of Mandel and lack-of-fit tests, using a significance level of 95%.

9.5.2 LOD and LOQ

The Hubaux-Vos method [22] was used for the calculation of the LOD values. The original method is based on the hypothesis that the data distribution is homoscedastic. Since this condition is usually not met, the weighting factors were included in the Hubaux-Vos calculation of the LOD, as described in the Currie's method [23]. LOQs were attributed to the lowest concentration within the respective calibration range yielding trueness values within the accepted limit (typically, bias% < ±20%).

The calculated LOD values were then experimentally tested by spiking the blank matrix with the target analytes at the approximate LOD concentrations and verifying that the signal-to-noise ratio (S/N) was higher than 3.

9.5.3 Trueness and Precision

The procedure used to calculate the intra-day trueness (expressed as bias %) considers separately the three calibration curves of the same day: two of these are cyclically used for the construction of a calibration model, which is used to

perform a back-calculation of the points of the third curve. The concentration values calculated by repeating the procedure for the three days are used to determine the bias %. The calculation of the inter-day trueness is similar, but the calibration constructed with the six data-points at each level collected in two days is cyclically used to back-calculate the concentration relative to the data-points of the third day. The results are then averaged.

Repeatability was independently assessed for the three days of analysis. For each day of validation, the calibration model was used to calculate the concentrations of the three experimental replicates; then, the coefficient of variation (CV%) was determined for each concentration level by averaging the precision obtained for the three days. The intermediate precision followed a similar procedure, that make use of the nine replications collected during all three days. In practice, the protocol used for calculating trueness and precision is based on the data collected for the 9 calibration curves, obtained in the three days [24].

4.5.4. Matrix effect and extraction recovery

The matrix effect was estimated at the concentration level of 10 ng/mL by comparing the experimental results obtained from three blank whole blood samples and MeOH solutions, equally spiked after the extraction step [25]. The ionization suppression/enhancement for each target analyte was expressed as the mean percentage ratio between the two measured signals. Owing to coelution, several spiked analytes are potentially present within a single ESI droplet, resulting in the possibility that the matrix effect may be partially due to their compresence, which is unlikely in a real blood sample. For this reason, the matrix effect for the compounds whose ion suppression exceeded 50% was tested again by spiking the blank blood with the single substance.

The extraction recovery was determined by comparing the experimental results obtained from three whole blood samples spiked at the concentration level of 100 ng/mL, before and after the extraction step. It was expressed as the mean percentage ratio between the two signals with its uncertainty expressed as extraction repeatability (CV%) from the three replications. Also in this case, the recovery was measured again for the analytes whose value was lower than 50% in order to evaluate a potential correlation between recovery and the compresence of analytes commonly present/absent in the same real sample.

9.5.5 Stability

Stability was evaluated at low (10 ng/mL) and high (100 ng/mL) concentrations levels. Following the deposition of the drop of blood and after

three hours of drying, the samples were placed at the temperature conditions of –20 °C, 4 °C and 35 °C and stored before analysis for, respectively, 1, 3, 7 and 40 days. The conditions were chosen to simulate the possible storage scenarios, from the mildest to the most critical conditions, so as to evaluate i) the transfer of the cards from the sampling site (*e.g.*, workplace) to the testing laboratory, ii) a short-term storage of the cards before analysis, and iii) a long-term storage of the cards, in the event that a counter-analysis is requested after a long time interval, for example when a positive result is challenged by the sample donor. The stability conditions were observed through trend lines that describe the variation of the concentration calculated at the different storage temperatures and after different storage intervals. Since a 15% deviation from the nominal value is compatible with the experimental uncertainty, the presence of degradation effects was positively detected above this 15% limit [26].

9.6 Application on real samples

The applicability of the developed procedure on real samples was verified on venous whole blood samples previously analyzed and tested positive by means of a previously published UHPLC-MS/MS method, which is periodically reviewed and updated according to new compounds entering the market [27]. At the time of analysis, the blood real specimens were obtained by venous sampling and then stored until analysis under controlled conditions, at the temperature of 4 °C. A drop of blood was spotted on the card and then treated as previously described. Whenever the effective drug concentration exceeded the calibration range, the samples were diluted to fit the quantitation range considered in the curve. The results obtained from the present method based on DBS and our standard procedure on venous blood were then compared to evaluate the performance of the new procedure in identifying and quantifying some of the analytes presented in Table 1.

9.7 Results and discussion

The present method proved adequate for the individual detection of all 132 target analytes and 13 internal standards at concentrations equal or lower than 10 ng/mL. In this work, a C18 column was used to obtain the best separation of the investigated compounds. The chromatographic run was completed in 10 min, including the final re-equilibration time, in agreement with the efficiency requirement needed for routine application. As shown in Table 1, 90 out of 132 compounds elute in the first 4.5 min, while 38 of the remaining analytes (mostly synthetic cannabinoids) elute between 6.5 and 7.75 min. Even when co-elution of

chromatographic peaks was observed, high resolution mass spectrometry guaranteed separate quantifications of the coeluting substances by means of their differences in precursor ion and characteristic fragment ion *m/z* values (mass error accepted < 5 ng/mL). A typical example of extracted ion chromatogram is presented in Figure 1, in which the group of 24 synthetic opioids - spiked in the whole blood at 10 ng/mL concentration - is represented.

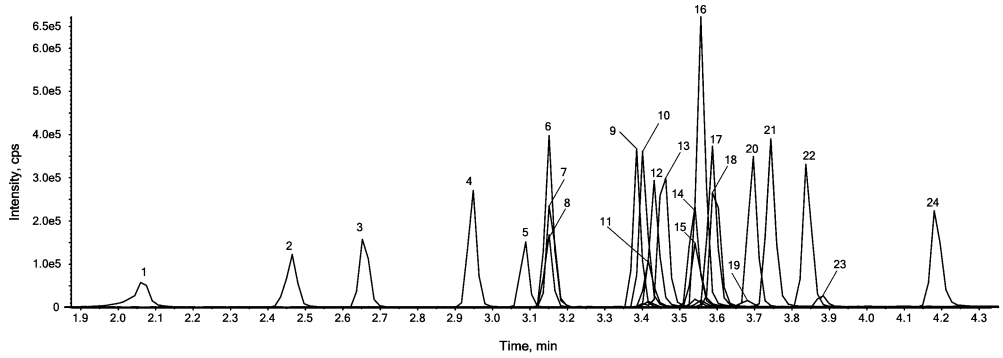


Figure 9. *Chromatographic profile of 24 synthetic opioids included in the panel within a 1.9 - 4.3 min retention time interval. Extracted ion chromatograms (XICs) resulting from the optimized data acquisition method, obtained from the 10 ng/mL neat standard mixture. Method was built using the Scheduled Algorithm Pro in SCIEX OS Software. In the figure: 1) Hydrocodone, 2) Norfentanyl, 3) Butyryl norfentanyl, 4) Remifentanil, 5) Butyryl fentanyl carboxy metabolite, 6) Acetyl Fentanyl, 7) Ocfentanyl, 8) Carfentanyl, 9) Alfentanil, 10) Acrylfentanyl, 11) U-47700, 12) 4-ANPP, 13) Fentanyl, 14) Despropionyl p-fluorofentanyl, 15) AH-7921, 16) FuranilFentanil, 17) Butyrylfentanyl, 18) Cyclopropylfentanyl, 19) Valeryl fentanyl carboxy metabolite 20) 4-F-Butyrylfentanyl, 21) 4-Methyl fentanyl, 22) Tramadol, 23) Sufentanil, 24) MT-45.*

The complete results of the validation experiments for DBS samples fortified with 132 analytes at six concentrations levels (5, 7.5, 10, 25, 50 and 100 ng/mL) and 13 internal standards are reported in the Supplementary Tables S2-S7. Table S2 reports the outcome of the calibration process. Analysis of residues and variances of calibration data points at low, medium and high concentration levels showed that heteroscedastic distributions were present for all the target analytes, making the introduction of weighting factors in the calibration (either $1/x$ of $1/x^2$) beneficial. Moreover, the quadratic term of the calibration model proved statistically significant for all but one (JWH-015) tested substances. Consequently, a quadratic calibration model was chosen for all analytes.

The LOD values, calculated using the corrected Hubaux-Vos algorithm, ranged from 1.3 ng/mL for ethylphenidate and 1.8 ng/mL for 4-F-butyrylfentanyl up to 6.3 ng/mL for UR-144. The LOD was then verified by spiking blank blood samples with decreasing concentrations (7.5, 5, 2 ng/mL) until a response equivalent to three times the background noise was observed. This verification process proved that for 74 analytes out of 132 (56%), a 2 ng/mL LOD was experimentally verified, namely a lower LOD value than the calculated ones (Table S3). This evidence suggests that the Hubaux-Vos algorithm corrected with Currie's method provide reliable yet conservative LOD values.

The experimental results obtained for LOD values confirmed that the overall method sensitivity is adequate for the detection of NPS extracted from DBS in routine applications. In fact, the 2 ng/mL limit consistently assessed in the present study represents the actual lower LOD measured in several other studies [28–30] and an acceptable requirement for NPS detection in blood, based on information available from case reports [31–33]. On the other hand, specific recommendations for DBS detection in blood have not been established yet, as long as little clinical information is available at the moment.

The extraction recovery was found to depend on the different classes of drugs considered (Figure S4), being higher for the class of synthetic cannabinoids (range 50%-100%) particularly for the JWH-series, and lower for synthetic cathinones and hallucinogens (range 30%-60%) and fentanyl analogues and synthetic opioids (range 30-50%). Relatively low extraction recoveries are expected from DBS cards [34], also taking into account that a large number of analytes have to be extracted simultaneously from the same dried droplet. However, the extraction repeatability measured from three replications proved to be satisfactory for the large majority of substances (CV% lower than 15% for 113 out of 132) [26], with the peculiar exception of the JWH-series that combines high extraction yield with relatively lower repeatability (10 out of 17 showed CV% in the range 15%-28%).

Further tests were performed in order to evaluate a possible correlation between the recovery yield of fentanyl, norfentanyl and ketamine and their compresence, as they are frequently present in the same real sample. The results showed (Table S5) that the extraction recovery did not vary significantly, no matter if only these three substances were spiked in the blank blood or all 132 analytes were simultaneously present in the sample.

Intermediate precision and repeatability (expressed as percent variation coefficient, CV%) and trueness (expressed as bias %) for all 132 target analytes are reported in Table S3. Notably, the validation procedure adopted in the present study allows precise and accurate calculation at all concentrations involved in the calibration process (6 calibration levels), not only at low, intermediate and high concentrations, as most recommended validation protocols entail. An example is reported in Figure 2, showing precision and trueness trends for AH-7921. The repeatability and intermediate precision and trueness trend for all the classes of compounds studied is reported in the Supplementary Figure S6.

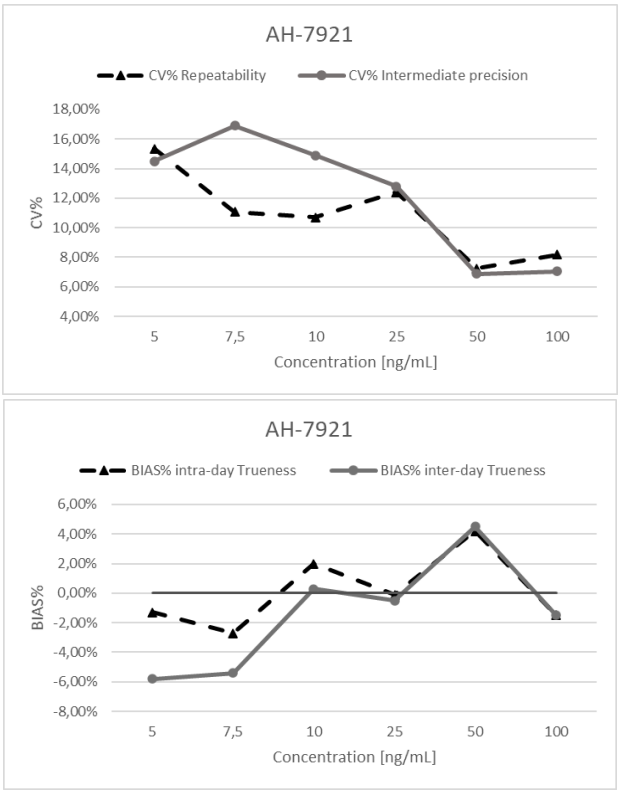


Figure 2. *Intermediate precision and repeatability (CV%) and trueness (bias%) trends for the synthetic opioid AH-7921 at the different calibration levels.*

In particular, the aggregate inter-day trueness data are reported in Figure 3, showing extremely limited deviation from the expected concentrations (-10% < bias% < +10%) for 114 target analytes out of 132. The inter-day trueness is an especially important performance parameter because it provides a reasonable

estimation of the quantitative measurement reliability under routine conditions and – indirectly – the quality of calibration. This positive outcome partly relies on its averaging the single results of repeated determinations, forasmuch as the random sources of variability that influence the precision results (extraction yield, matrix effect, *etc.*) may find equalization. Therefore, it is highly recommended to collect blood droplets on three separate DBS cards and average their results whenever accurate quantitative determinations are needed.

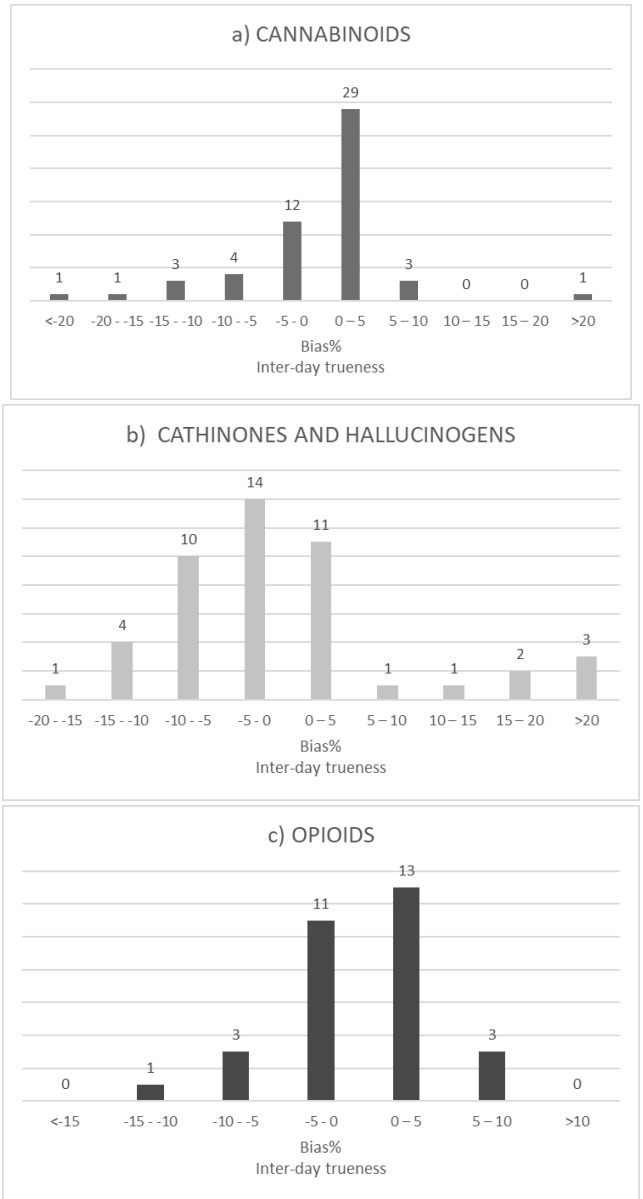


Figure 3. *Inter-day trueness (bias%) trends for the class of a) synthetic cannabinoids, b) cathinones and hallucinogens and c) synthetic opioids.*

Precision data, described by CV% from repeated independent analyses, show rather homogeneous results within each class and between different classes of substances under study (Supplementary Table S3 and Figure S6). Therefore, the observed data variability can realistically be attributed to the analytical method itself rather than to specific chemical properties of the investigated substances or their interaction with the DBS card. Quite interestingly, the average intermediate precision result (CV% = 15.5%, N = 132) is only slightly higher than the intra-day variability (CV% = 11.4%, N = 132), as is expected for a substantially stable method and calibration.

Electrospray ionization produced moderate ion suppression, resulting in matrix effect values ranging between negligible to –50% for synthetic cannabinoids (average –29%), between negligible to –40% for synthetic cathinones and hallucinogens (average –24%) and fentanyl analogues and synthetic opioids (average –27%) (Table S7). Due to the presence of many spiked analytes inside the single blood drop and the crowding of peaks around certain retention times (for example, in the interval 3.4-4.0 min), we considered the possibility that part of the matrix effect could be attributed to their coelution and interaction. To test this hypothesis, we measured again the matrix effect using blood droplets spiked with only the five substances whose ion suppression value exceeded –50% in the first set of experiments. Indeed, ion suppression decreased for all target analytes, on average from –62% to –29%. In particular, the effect reduction for 5-chloro-TH-J018 was from –69% to –9%, for AM-2201 from –57% to –43%, for JWH-007 from –66% to –7%, for MMB-2201 from –58% to –38%, and for WIN-48 from –59% to –49%. These results suggest that the recorded ion suppression initially attributed to the blood matrix is likely to be influenced by the coelution of several spiked analytes. In real samples, the simultaneous presence of more than five drugs in a single blood sample is quite implausible to occur, even in the worst cases. In conclusion, the ion suppression data reported in Table S6 should be considered as upper limits observed under stressed conditions rather than expected values in real toxicological contexts. Again, whenever accurate quantitative determination of a single substance is needed, specific experiments should be planned to complete the validation for that specific substance and an on-purpose calibration model may be prudentially built, so as to avoid overestimation of the matrix effect.

The largest part of the target analytes showed good stability on DBS cards after drying, even for long periods of time. At both concentration levels tested,

deviations from the nominal value fell within 15% for most of them. Quite obviously, the NPS stability proved to be slightly more affected when a storage temperature of 35 °C was maintained, in combination with a high storage period and low analyte concentration. Figure 4 reports an example of this trend. In particular, synthetic cannabinoids – and especially the JWH group – showed lower stability than the other classes of NPS. An example of particularly poor stability is evident in Figure 5 for JWH-015: on one hand, acceptable conservation (> 90%) is guaranteed during the initial 7 days at any storage temperature, on the other hand it is evident that significant degradation occurred after 40 storage days even if the DBS cards were maintained at –20 °C. In summary, the stability data suggest that storage of the DBS cards at –20 °C is not essential, the most convenient storage condition apparently being at 4 °C. As a matter of fact, extremely limited degradation is observed at 4 °C for periods fully compatible with routine analytical processing, while an acceptable level of preservation is maintained up to 40 days for most NPS. Specific caution should be exercised in counter-analyses involving the confirmation of JWH-series positive testing. Further studies will be needed to evaluate the compounds stability in real samples, similarly to what has been done on post-mortem samples for psychoactive substances [11]. Indeed, spiked samples may not always display the same stability profile as real samples. The proven stability of NPS on DBS cards, combined with ease of sampling and minimal storage volume, represents a key asset of the DBS technique confirming its use as an alternative and innovative sampling method in troublesome conditions, for example when the sampling site is far from the laboratory and/or few days are required after sampling until the analysis is made possible.

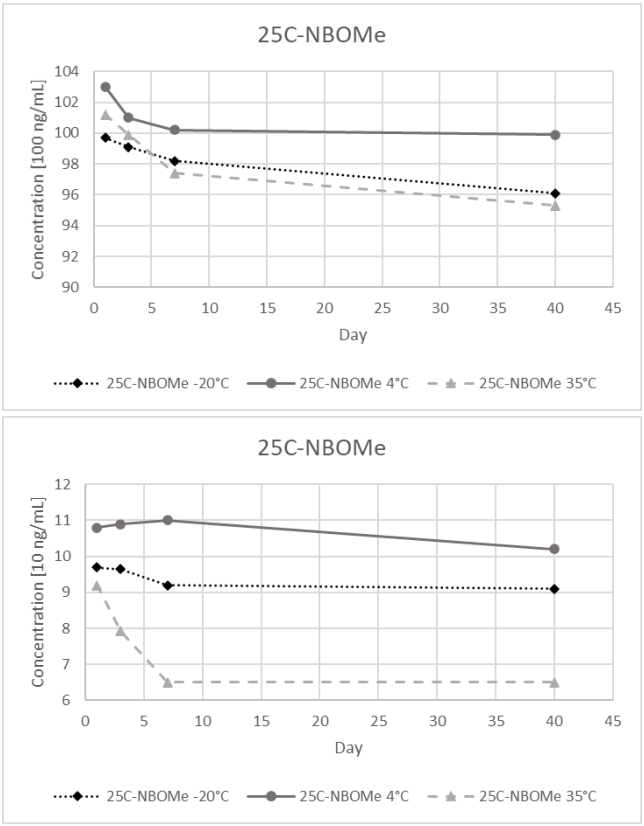


Figure 4. *Stability of 25C-NBoMe at different temperature conditions (–20, 4, 35 °C) during 1, 3, 7, 40 days.*

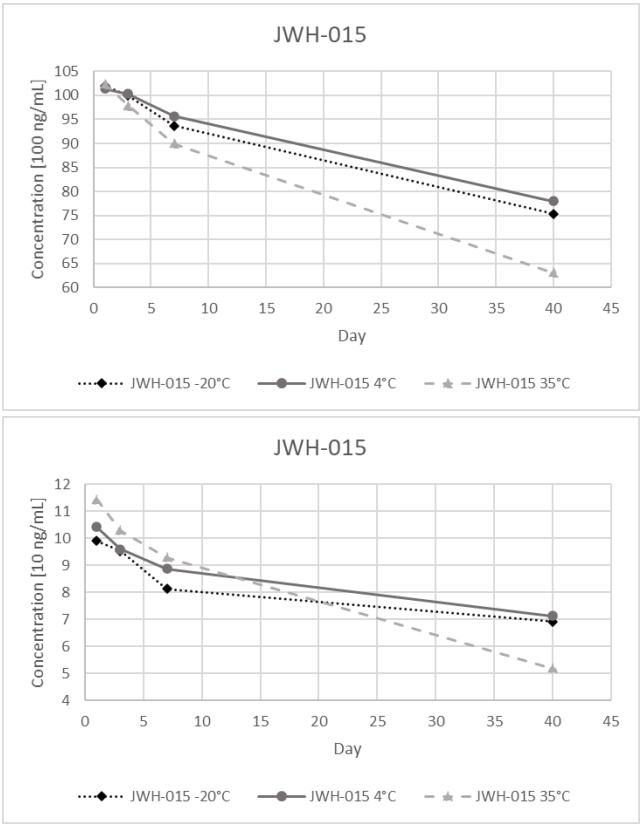


Figure 5. *Stability of JWH-015 at different temperature conditions (–20, 4, 35 °C) during 1, 3, 7, 40 days.*

9.8 Application to real samples

After completing the validation, the present method was applied to seven venous whole blood samples which previously tested positive to ketamine and fentanyl in our laboratory. The results summarized in Table 2 show that the new procedure based on DBS sample collection allowed confirmation of the ketamine and fentanyl positive testing, whenever their concentrations was higher than the corresponding LOQ of the method. Also, the quantitative results proved consistent with those measured with the routine procedure based on large blood volume sampling. These results demonstrate the ability of the new technique to detect and correctly quantify the substances present in the sample.

Table 2. *Ketamine and fentanyl concentrations determined on venous whole blood (ng/mL) from real toxicology casework and measured with both the routine laboratory*

procedure and the present DBS method. Values between parentheses are rough estimations below LOQ; n.d. = not detected.

ID Sample	Routine method (UHPLC–ESI-MS/MS)		DBS method (UHPLC-QTOF-HRMS)	
	Ketamine (ng/mL)	Fentanyl (ng/mL)	Ketamine (ng/mL)	Fentanyl (ng/mL)
S1	n.d.	0.3	n.d.	< LOQ
S2	37.5	n.d.	42.0	n.d.
S3	493	n.d.	597	n.d.
S4	447	0.2	600	n.d.
S5	74.0	2.0	88.0	< LOQ (~2.3)
S6	405	n.d.	700	n.d.
S7	275	2.1	140	< LOQ (~2.5)

9.9 Conclusions

The need to test biological samples for drugs of abuse keeps increasing in several social contexts, especially in workplace and road controls, so as to guarantee safer conditions for workers, drivers, and third parties, but the progressive introduction of NPS in the illegal market significantly complicated these tests and expanded the number of targeted analytes. It is nonetheless essential to discriminate the subject who are under the effect of drugs from those who may have consumed drugs days earlier, i.e. out of the control context. The matrix of choice for this discrimination is blood, but blood sampling is prevented in almost all circumstances by ethical and practical reasons.

The present method overcomes both problems by combining the use of UHPLC-QTOF-HRMS instrumentation with a simple and minimally invasive DBS sample collection for detecting as many as 132 traditional drugs and NPS selected from EMCDDA reports [35] and articles [36,37], together with their metabolites, on only 30 μL whole blood. The analytical method's validation confirmed its reliability for the extraction and accurate analysis of this wide array of structurally different NPS within an adequate concentration interval (typically, 5-100 ng/mL).

The initial comparison on real toxicology samples between our traditional routine procedure based on high volume blood sampling and the new DBS procedure provides a preliminary confirmation of the potential applicability of the latter technique on a vast scale in several fields, including workplace drug testing, road controls, and drug monitoring in clinical and forensic contexts.

Prospectively, the new method will be applied on real capillary whole blood samples in comparison with venous blood. Further investigation will be devoted to the hematocrit effect (HT) and its influence on the volumetric blood sampling method, either a single drop deposited on the card (requiring a corrective factor for the HT effect) or other micro-sampling systems (VAMS).

References

[1] A. Peacock, R. Bruno, N. Gisev, L. Degenhardt, W. Hall, R. Sedefov, J. White, K.V. Thomas, M. Farrell, P. Griffiths, New psychoactive substances: challenges for drug surveillance, control, and public health responses, Lancet 394 (2019) 1668–1684. https://doi.org/10.1016/S0140-6736(19)32231-7.

[2] D. Baumeister, L.M. Tojo, D.K. Tracy, Legal highs: staying on top of the flood of novel psychoactive substances, Ther. Adv. Psychopharmacol. 5 (2015) 97–132. https://doi.org/10.1177/2045125314559539.

[3] S.M. Aarde, M.A. Taffe, Predicting the abuse liability of entactogen-class, new and emerging psychoactive substances via preclinical models of drug self-administration, Curr. Top. Behav. Neurosci. 32 (2017) 145–164. https://doi.org/10.1007/7854_2016_54.

[4] A. Shafi, A.J. Berry, H. Sumnall, D.M. Wood, D.K. Tracy, New psychoactive substances: a review and updates, Ther. Adv. Psychopharmacol. 10 (2020) 2045125320967197. https://doi.org/10.1177/2045125320967197.

[5] Observatoire européen des drogues et des toxicomanies, ed., European drug report: trends and developments, Publications office of the European Union, Luxembourg, 2021.

[6] A. Salomone, G. Gazzilli, D. Di Corcia, E. Gerace, M. Vincenti, Determination of cathinones and other stimulant, psychedelic, and dissociative designer drugs in real hair samples, Anal. Bioanal. Chem. 408 (2016) 2035–2042. https://doi.org/10.1007/s00216-015-9247-4.

[7] H.R. Sumnall, M. Evans-Brown, J. McVeigh, Social, policy, and public health perspectives on new psychoactive substances, Drug Test. Anal. 3 (2011) 515–523. https://doi.org/10.1002/dta.310.

[8] C.C. Scriver, A simple phenylalanine method for detecting phenylketonuria in large populations of newborn infants, by Robert Guthrie and Ada Susi, Pediatrics, 1963;32:318-343, Pediatrics 102 (1998) 236–237.

[9] K. Malsagova, A. Kopylov, A. Stepanov, T. Butkova, A. Izotov, A. Kaysheva, Dried blood spot in laboratory: directions and prospects, Diagn. Basel Switz. 10 (2020) E248. https://doi.org/10.3390/diagnostics10040248.

[10] C.P. Stove, A.-S.M.E. Ingels, P.M.M. De Kesel, W.E. Lambert, Dried blood spots in toxicology: from the cradle to the grave? Crit. Rev. Toxicol. 42 (2012) 230–243. https://doi.org/10.3109/10408444.2011.650790.

[11] M. Moretti, A. Manfredi, F. Freni, C. Previderé, A.M.M. Osculati, P. Grignani, L. Tronconi, C. Carelli, C. Vignali, L. Morini, A comparison between two different dried blood substrates in determination of psychoactive

substances in postmortem samples, Forensic Toxicol. 39 (2021) 385–393. https://doi.org/10.1007/s11419-020-00567-2.

[12] M. Thevis, T. Kuuranne, A. Thomas, H. Geyer, Do dried blood spots have the potential to support result management processes in routine sports drug testing? - Part 2: Proactive sampling for follow-up investigations concerning atypical or adverse analytical findings, Drug Test. Anal. 13 (2021) 505–509. https://doi.org/10.1002/dta.3011.

[13] A. Deep, P. Kumar, A. Kumar, A. Thakkar, Dry blood spot technique: a review, Int. J. Pharm. Sci. Rev. Res. 15 (2012) 90–94.

[14] N. Sadones, S. Capiau, P.M.M. De Kesel, W.E. Lambert, C.P. Stove, Spot them in the spot: analysis of abused substances using dried blood spots, Bioanalysis 6 (2014) 2211–2227. https://doi.org/10.4155/bio.14.156.

[15] H.Y. Tey, H.H. See, A review of recent advances in microsampling techniques of biological fluids for therapeutic drug monitoring, J. Chromatogr. A. 1635 (2021) 461731. https://doi.org/10.1016/j.chroma.2020.461731.

[16] R.V. Oliveira, J. Henion, E. Wickremsinhe, Fully-automated approach for online dried blood spot extraction and bioanalysis by two-dimensional-liquid chromatography coupled with high-resolution quadrupole time-of-flight mass spectrometry, Anal. Chem. 86 (2014) 1246–1253. https://doi.org/10.1021/ac403672u.

[17] W. Harry Hannon, B.L. Therrell, Overview of the history and applications of dried blood samples, in: Dried Blood Spots, Wiley-Blackwell, 2014: pp. 1–15. https://doi.org/10.1002/9781118890837.ch1.

[18] E. Alladio, E. Amante, C. Bozzolino, F. Seganti, A. Salomone, M. Vincenti, B. Desharnais, Effective validation of chromatographic analytical methods: the illustrative case of androgenic steroids, Talanta 215 (2020) 120867. https://doi.org/10.1016/j.talanta.2020.120867.

[19] B. Desharnais, F. Camirand-Lemyre, P. Mireault, C.D. Skinner, Procedure for the selection and validation of a calibration model i-description and application, J. Anal. Toxicol. 41 (2017) 261–268. https://doi.org/10.1093/jat/bkx001.

[20] E. Alladio, E. Amante, C. Bozzolino, F. Seganti, A. Salomone, M. Vincenti, B. Desharnais, Experimental and statistical protocol for the effective validation of chromatographic analytical methods, MethodsX 7 (2020) 100919. https://doi.org/10.1016/j.mex.2020.100919.

[21] H. Gu, G. Liu, J. Wang, A.-F. Aubry, M.E. Arnold, Selecting the correct weighting factors for linear and quadratic calibration curves with least-squares regression algorithm in bioanalytical LC-MS/MS assays and impacts of using incorrect weighting factors on curve stability, data quality, and assay performance, Anal. Chem. 86 (2014) 8959–8966. https://doi.org/10.1021/ac5018265.

[22] Andre. Hubaux, Gilbert. Vos, Decision and detection limits for calibration curves, Anal. Chem. 42 (1970) 849–855. https://doi.org/10.1021/ac60290a013.

[23] L.A. Currie, Detection and quantification limits: origins and historical overview, Analytica Chimica Acta 391 (1999) 127-134. https://doi.org/10.1016/S0003-2670(99)00105-1.

[24] B. Desharnais, F. Camirand-Lemyre, P. Mireault, C.D. Skinner, Procedure for the selection and validation of a calibration model II-Theoretical basis, J. Anal. Toxicol. 41 (2017) 269–276. https://doi.org/10.1093/jat/bkx002.

[25] Scientific Working Group for Forensic Toxicology, Scientific Working Group for Forensic Toxicology (SWGTOX) standard practices for method validation in forensic toxicology, J. Anal. Toxicol. 37 (2013) 452–474. https://doi.org/10.1093/jat/bkt054.

[26] S. Capiau, H. Veenhof, R.A. Koster, Y. Bergqvist, M. Boettcher, O. Halmingh, B.G. Keevil, B.C.P. Koch, R. Linden, C. Pistos, L.M. Stolk, D.J. Touw, C.P. Stove, J.-W.C. Alffenaar, Official international association for therapeutic drug monitoring and clinical toxicology guideline: development and validation of dried blood spot–based methods for therapeutic drug monitoring, Ther. Drug Monit. 41 (2019) 409–430. https://doi.org/10.1097/FTD.0000000000000643.

[27] M. Vincenti, D. Cavanna, E. Gerace, V. Pirro, M. Petrarulo, D. Di Corcia, A. Salomone, Fast screening of 88 pharmaceutical drugs and metabolites in whole blood by ultrahigh-performance liquid chromatography–tandem mass spectrometry, Anal. Bioanal. Chem. 405 (2013) 863–879. https://doi.org/10.1007/s00216-012-6403-y.

[28] Y. Wang, Y. Shi, Y. Yu, L. Chen, J. Jiang, J. Long, P. Xiang, G. Duan, Screening of synthetic cathinones and metabolites in dried blood spots by UPLC–MS-MS, J. Anal. Toxicol. 45 (2020). https://doi.org/10.1093/jat/bkaa106.

[29] S. Sadler Simões, A. Castañera Ajenjo, M.J. Dias, Dried blood spots combined to an UPLC-MS/MS method for the simultaneous determination of

drugs of abuse in forensic toxicology, J. Pharm. Biomed. Anal. 147 (2018) 634–644. https://doi.org/10.1016/j.jpba.2017.02.046.

[30] M. Concheiro, S. Anizan, K. Ellefsen, M.A. Huestis, Simultaneous quantification of 28 synthetic cathinones and metabolites in urine by liquid chromatography-high resolution mass spectrometry, Anal. Bioanal. Chem. 405 (2013) 9437–9448. https://doi.org/10.1007/s00216-013-7386-z.

[31] J. Welter-Luedeke, H.H. Maurer, Relevance of published blood concentrations of new psychoactive substance for rational case interpretation, WIREs Forensic Sci. 1 (2019) e1174. https://doi.org/10.1002/wfs2.1174.

[32] K. Shintani-Ishida, K. Saka, M. Nakamura, K.-I. Yoshida, H. Ikegaya, Experimental study on the postmortem redistribution of the substituted phenethylamine, 25B-NBOMe, J. Forensic Sci. 63 (2018) 588–591. https://doi.org/10.1111/1556-4029.13583.

[33] M. Diestelmann, A. Zangl, I. Herrle, E. Koch, M. Graw, L.D. Paul, MDPV in forensic routine cases: psychotic and aggressive behavior in relation to plasma concentrations, Forensic Sci. Int. 283 (2018) 72–84. https://doi.org/10.1016/j.forsciint.2017.12.003.

[34] R.A. Koster, D.J. Touw, J.-W.C. Alffenaar, Dried blood spot analysis; facing new challenges, J. Appl. Bioanal. 1 (2015) 38–41. https://doi.org/10.17145/jab.15.007.

[35] The EU Early Warning System on new psychoactive substances (NPS) | www.emcdda.europa.eu. https://www.emcdda.europa.eu/publications/topic-overviews/eu-early-warning-system

[36] F. Vincenti, A. Gregori, M. Flammini, F. Di Rosa, A. Salomone, Seizures of new psychoactive substances on the Italian territory during the COVID-19 pandemic, Forensic Sci. Int. 326 (2021) 110904. https://doi.org/10.1016/j.forsciint.2021.110904.

[37] J. Lyden, I.A. Binswanger, The United States opioid epidemic, Semin. Perinatol. 43 (2019) 123–131. https://doi.org/10.1053/j.semperi.2019.01.001.

Supplementary materials

Table S1. *MS and MS/MS parameters used for the instrumental analysis*

Source and Gas Parameters				
Ion gas 1 (psi)	Ion gas 2 (psi)	Curtain gas (psi)	CAD gas (psi)	Temperature (°C)
50	**45**	**35**	**8**	**600**
TOF MS				
TOF start mass (Da)	TOF stop mass (Da)	DP (V)	CE (V)	Accumulation time
100	**575**	**65**	**10**	**0.1**
TOF MS/MS				
TOF start mass (Da)	TOF stop mass (Da)	DP (V)	CE (V)	Accumulation time
50	**575**	**65**	**35± 15**	**0.04**

Table S2. *Calibration models for the targeted analytes: range, weighting correction, order, and equation.*

Compound	**Calibration Range (ng/mL)**	**Weight**	**Model**	**Equation**
5-Chloro-AB-PINACA	5 - 100	1/x	Quadratic	$-6.8\times10^{-4}\,x^2 + 1.2\times10^{-2}\,x - 7.9\times10^{-4}$
5-Chloro-TH-J018	5 - 100	$1/x^2$	Quadratic	$1.4\times10^{-6}\,x^2 + 1.1\times10^{-3}\,x - 6.5\times10^{-6}$
5-F-AB-PINACA	5 - 100	1/x	Quadratic	$-2.3\times10^{-3}\,x^2 + 2.2\times10^{-2}\,x - 1.7\times10^{-3}$

5-F-ADB	7.5 - 100	1/x	Quadratic	$-2.0\times10^{-4}\,x^2 + 2.2\times10^{-3}\,x - 2.2\times10^{-4}$
5-F-APINACA	5 - 100	1/x	Quadratic	$1.2\times10^{-3}\,x^2 + 6.1\times10^{-2}\,x - 3.8\times10^{-2}$
5-F-APP PICA	5 - 100	1/x	Quadratic	$-3.0\times10^{-4}\,x^2 + 1.6\times10^{-2}\,x + 6.2\times10^{-5}$
5-F-APP PINACA	5 - 100	1/x	Quadratic	$-1.6\times10^{-3}\,x^2 + 1.6\times10^{-2}\,x - 1.2\times10^{-2}$
5-F-CUMYL PINACA	5 - 100	$1/x^2$	Quadratic	$-8.0\times10^{-3}\,x^2 + 1.6\times10^{-2}\,x + 3.6\times10^{-5}$
5-F NNEI 2'-naphthyl isomer	5 - 100	1/x	Quadratic	$2.9\times10^{-4}\,x^2 + 9.4\times10^{-3}\,x + 7.0\times10^{-4}$
5-F-PB22	5 - 100	$1/x^2$	Quadratic	$-7.5\times10^{-4}\,x^2 + 2.4\times10^{-2} - 1.8\times10^{-3}$
AB-CHMINACA	5 - 100	1/x	Quadratic	$-9.0\times10^{-4}\,x^2 + 1.6\times10^{-2}\,x - 1.1\times10^{-3}$
AB-FUBINACA	5 - 100	1/x	Quadratic	$-6.7\times10^{-4}\,x^2 + 8.1\times10^{-3}\,x - 4.6\times10^{-4}$
AB-PINACA	5 - 100	1/x	Quadratic	$-3.0\times10^{-3}\,x^2 + 2.7\times10^{-2}\,x - 2.4\times10^{-3}$
ADB-FUBINACA	7.5 - 100	1/x	Quadratic	$-1.8\times10^{-5}\,x^2 + 3.0\times10^{-4}\,x - 4.4\times10^{-6}$
ADBICA	5 - 100	1/x	Quadratic	$-2.9\times10^{-3}\,x^2 + 3.0\times10^{-3}\,x - 1.8\times10^{-3}$
ADB-PINACA	5 - 100	1/x	Quadratic	$2.9\times10^{-4}\,x^2 + 1.6\times10^{-2}\,x - 8.3\times10^{-4}$
CB-13	5 - 100	1/x	Quadratic	$1.5\times10^{-4}\,x^2 + 1.2\times10^{-3}\,x - 1.1\times10^{-4}$
AM-1220	5 - 100	1/x	Quadratic	$5.5\times10^{-4}\,x^2 + 6.0\times10^{-3}\,x - 5.4\times10^{-4}$

AM-2201	5 - 100	$1/x^2$	Quadratic	$-1.7\times10^{-5}\,x^2 + 2.2\times10^{-3}\,x - 2.1\times10^{-5}$
AM-2233	5 - 100	1/x	Quadratic	$-4.2\times10^{-4}\,x^2 + 6.3\times10^{-3}\,x - 3.1\times10^{-4}$
AM-694	5 - 100	1/x	Quadratic	$-3.0\times10^{-4}\,x^2 + 8.0\times10^{-3}\,x - 6.7\times10^{-4}$
APP-FUBINACA	5 - 100	1/x	Quadratic	$-8.1\times10^{-4}\,x^2 + 1.0\times10^{-2}\,x - 6.9\times10^{-4}$
CUMYL-PeGACLONE	5 - 100	$1/x^2$	Quadratic	$-2.0\times10^{-4}\,x^2 + 1.9\times10^{-2}\,x - 3.1\times10^{-5}$
JWH-007	5 - 100	1/x	Quadratic	$2.9\times10^{-5}\,x^2 + 2.1\times10^{-3}\,x - 7.6\times10^{-5}$
JWH-015	5 - 100	$1/x^2$	Linear	$-1.3\times10^{-2}\,x^2 + 1.0\times10^{-2}\,x - 4.0\times10^{-4}$
JWH-018	5 - 100	1/x	Quadratic	$3.9\times10^{-4}\,x^2 + 1.5\times10^{-2}\,x - 5.3\times10^{-4}$
JWH-019	5 - 100	$1/x^2$	Quadratic	$-9.3\times10^{-5}\,x^2 + 1.4\times10^{-3}\,x - 2.5\times10^{-5}$
JWH-020	5 - 100	1/x	Quadratic	$-4.5\times10^{-5}\,x^2 + 7.0\times10^{-4}\,x - 4.5\times10^{-5}$
JWH-073	5 - 100	1/x	Quadratic	$-5.3\times10^{-4}\,x^2 + 6.9\times10^{-3}\,x - 4.5\times10^{-4}$
JWH-081	5 - 100	1/x	Quadratic	$-1.5\times10^{-3}\,x^2 + 2.2\times10^{-2}\,x - 1.8\times10^{-3}$
JWH-098	5 - 100	1/x	Quadratic	$1.0\times10^{-4}\,x^2 + 4.2\times10^{-3}\,x - 6.3\times10^{-5}$
JWH-122	5 - 100	$1/x^2$	Quadratic	$-1.0\times10^{-4}\,x^2 + 1.4\times10^{-3}\,x - 2.7\times10^{-5}$
JWH-147	5 - 100	1/x	Quadratic	$-2.7\times10^{-4}\,x^2 + 2.7\times10^{-3}\,x - 3.6\times10^{-4}$

JWH-203	5 - 100	1/x	Quadratic	$-2.1\times10^{-4}\,x^2+1.8\times10^{-3}\,x-2.3\times10^{-4}$
JWH-210	5 - 100	$1/x^2$	Quadratic	$3.3\times10^{-5}\,x^2+1.0\times10^{-3}\,x+1.9\times10^{-5}$
JWH-250	5 - 100	1/x	Quadratic	$-7.1\times10^{-3}\,x^2+8.7\times10^{-2}\,x-6.9\times10^{-3}$
JWH-251	5 - 100	$1/x^2$	Quadratic	$5.6\times10^{-3}\,x^2+1.5\times10^{-1}\,x-1.3\times10^{-3}$
JWH-302	5 - 100	1/x	Quadratic	$-5.9\times10^{-4}\,x^2+5.3\times10^{-3}\,x-6.5\times10^{-4}$
JWH-307	5 - 100	1/x	Quadratic	$-2.0\times10^{-4}\,x^2+4.1\times10^{-3}\,x+7.3\times10^{-5}$
JWH-398	5 – 100	1/x	Quadratic	$-8.2\times10^{-5}\,x^2+6.0\times10^{-4}\,x-5.6\times10^{-5}$
MAB-CHMINACA	5 - 100	1/x	Quadratic	$-1.2\times10^{-3}\,x^2+1.1\times10^{-2}\,x-1.0\times10^{-3}$
MAM-2201	5 - 100	1/x	Quadratic	$-2.5\times10^{-5}\,x^2+3.1\times10^{-3}\,x+1.8\times10^{-5}$
MDMB-CHMICA	5 - 100	$1/x^2$	Quadratic	$2.0\times10^{-3}\,x^2+1.2\times10^{-1}\,x-6.0\times10^{-4}$
MDMB-CHMINACA	5 - 100	$1/x^2$	Quadratic	$-4.0\times10^{-3}\,x^2+4.8\times10^{-2}\,x-1.0\times10^{-3}$
MMB-2201	5 - 100	1/x	Quadratic	$-5.9\times10^{-3}\,x^2+9.4\times10^{-2}\,x+1.5\times10^{-3}$
PB-22	5 - 100	1/x	Quadratic	$-4.0\times10^{-3}\,x^2+3.9\times10^{-2}\,x-2.8\times10^{-3}$
RCS-4	5 - 100	1/x	Quadratic	$-1.0\times10^{-2}\,x^2+1.1\times10^{-1}\,x-7.5\times10^{-3}$
RCS-8	5 - 100	1/x	Quadratic	$-4.4\times10^{-4}\,x^2+2.8\times10^{-3}\,x-4.3\times10^{-4}$

STS-135	5 - 100	1/x	Quadratic	$-1.0\times10^{-2}\,x^2+6.7\times10^{-2}\,x-9.5\times10^{-3}$
UR-144	5 - 100	1/x	Quadratic	$-6.6\times10^{-4}\,x^2+3.7\times10^{-3}\,x-5.9\times10^{-4}$
UR-144-5-OH	5 - 100	1/x	Quadratic	$-2.4\times10^{-3}\,x^2+2.0\times10^{-2}\,x-1.6\times10^{-3}$
WIN-48	5 - 100	1/x	Quadratic	$-2.0\times10^{-3}\,x^2+2.0\times10^{-2}\,x-1.3\times10^{-3}$
WIN-55	5 - 100	1/x	Quadratic	$-2.2\times10^{-3}\,x^2+1.7\times10^{-2}\,x-1.7\times10^{-3}$
XLR-11	5 - 100	1/x	Quadratic	$-3.1\times10^{-4}\,x^2+4.2\times10^{-3}\,x-3.0\times10^{-4}$
25B-NBOMe	5 - 100	1/x	Quadratic	$-2.4\times10^{-4}\,x^2+8.3\times10^{-3}\,x-5.5\times10^{-4}$
25C-NBOMe	5 - 100	$1/x^2$	Quadratic	$8.6\times10^{-4}\,x^2+7.9\times10^{-3}\,x+1.5\times10^{-4}$
25H-NBOMe	5 - 100	$1/x^2$	Quadratic	$4.2\times10^{-4}\,x^2+9.2\times10^{-3}\,x+1.1\times10^{-4}$
25I-NBOMe	5 - 100	$1/x^2$	Quadratic	$4.0\times10^{-4}\,x^2+9.1\times10^{-3}\,x+4.7\times10^{-5}$
2C-B	5 - 100	1/x	Quadratic	$-1.6\times10^{-4}\,x^2+1.1\times10^{-3}\,x-1.5\times10^{-4}$
2C-P	5 - 100	1/x	Quadratic	$-1.3\times10^{-3}\,x^2+1.0\times10^{-2}\,x-1.2\times10^{-3}$
3-4-DMMC	5 - 100	1/x	Quadratic	$-5.5\times10^{-4}\,x^2+3.5\times10^{-3}\,x-5.1\times10^{-4}$
4-Acetoxy-DiPT	5 - 100	1/x	Quadratic	$-2.1\times10^{-4}\,x^2+3.8\times10^{-3}\,x-1.6\times10^{-4}$
4-Acetoxy-DMT	5 - 100	1/x	Quadratic	$-1.7\times10^{-4}\,x^2+3.0\times10^{-3}\,x-2.0\times10^{-4}$

4-F-Methcathinone	5 - 100	1/x	Quadratic	$3.0\times10^{-3}\ x^2 + 7.5\times10^{-2}\ x - 2.3\times10^{-3}$
4-MEC	5 - 100	1/x	Quadratic	$-3.0\times10^{-4}\ x^2 + 2.1\times10^{-3}\ x - 2.9\times10^{-4}$
5-EAPB	5 - 100	$1/x^2$	Quadratic	$-7.5\times10^{-6}\ x^2 + 4.0\times10^{-3}\ x + 2.4\times10^{-5}$
5-Methoxy AMT	7.5 - 100	1/x	Quadratic	$-2.8\times10^{-4}\ x^2 + 1.9\times10^{-3}\ x - 2.6\times10^{-4}$
5- Methoxy DALT	5 - 100	1/x	Quadratic	$-4.7\times10^{-4}\ x^2 + 3.5\times10^{-3}\ x - 4.4\times10^{-4}$
5- Methoxy DMT	5 - 100	$1/x^2$	Quadratic	$3.4\times10^{-5}\ x^2 + 1.5\times10^{-3}\ x - 1.5\times10^{-5}$
5- Methoxy DiPT	5 - 100	1/x	Quadratic	$-6.2\times10^{-4}\ x^2 + 4.1\times10^{-3}\ x - 5.8\times10^{-4}$
Eutylone	5 - 100	1/x	Quadratic	$-3.2\times10^{-3}\ x^2 + 4.3\times10^{-2}\ x - 3.2\times10^{-3}$
6-APB	7.5 - 100	1/x	Quadratic	$-8.2\times10^{-4}\ x^2 + 5.5\times10^{-3}\ x - 7.5\times10^{-4}$
Buphedrone	5 - 100	$1/x^2$	Quadratic	$-3.9\times10^{-5}\ x^2 + 1.0\times10^{-2}\ x + 3.8\times10^{-6}$
Butylone	5 - 100	1/x	Quadratic	$-1.0\times10^{-4}\ x^2 + 3.4\times10^{-3}\ x - 2.4\times10^{-5}$
DMT	5 - 100	1/x	Quadratic	$-1.5\times10^{-2}\ x^2 + 1.2\times10^{-1}\ x - 1.4\times10^{-2}$
Ethylone	7.5 - 100	1/x	Quadratic	$-7.7\times10^{-4}\ x^2 + 5.8\times10^{-3}\ x - 8.4\times10^{-4}$
Ethylphenidate	5 - 100	$1/x^2$	Quadratic	$-6.0\times10^{-5}\ x^2 + 4.1\times10^{-3}\ x - 1.1\times10^{-5}$
Ethyltryptamine	5 - 100	$1/x^2$	Quadratic	$-2.7\times10^{-5}\ x^2 + 4.1\times10^{-3}\ x + 1.0\times10^{-6}$

Harmine	5 - 100	1/x	Quadratic	$-4.7\times10^{-4}\,x^2+2.9\times10^{-3}\,x-4.4\times10^{-4}$
Ketamine	5 - 100	1/x	Quadratic	$-4.3\times10^{-4}\,x^2+3.0\times10^{-3}\,x-4.2\times10^{-4}$
LSD	5 - 100	1/x	Quadratic	$-9.0\times10^{-4}\,x^2+3.5\times10^{-3}\,x-1.0\times10^{-3}$
mCPP	5 - 100	$1/x^2$	Quadratic	$1.0\times10^{-4}\,x^2+2.5\times10^{-3}\,x-1.8\times10^{-5}$
MDPV	5 - 100	1/x	Quadratic	$-1.6\times10^{-4}\,x^2+1.2\times10^{-3}\,x-1.3\times10^{-4}$
Mephedrone	5 - 100	1/x	Quadratic	$-4.2\times10^{-3}\,x^2+3.5\times10^{-2}\,x-4.3\times10^{-3}$
Mescaline	5 - 100	1/x	Quadratic	$-8.6\times10^{-4}\,x^2+8.9\times10^{-3}\,x-7.8\times10^{-4}$
Methedrone	5 - 100	$1/x^2$	Quadratic	$7.0\times10^{-4}\,x^2+6.5\times10^{-3}\,x+5.6\times10^{-5}$
Methylone	5 - 100	1/x	Quadratic	$-5.0\times10^{-4}\,x^2+3.8\times10^{-3}\,x-4.4\times10^{-4}$
MXE	5 - 100	1/x	Quadratic	$-1.0\times10^{-4}\,x^2+3.0\times10^{-3}\,x-2.2\times10^{-4}$
Mitragynine	5 - 100	1/x	Quadratic	$-1.7\times10^{-4}\,x^2+1.3\times10^{-3}\,x-1.4\times10^{-4}$
N-Ethylcathinone	5 - 100	$1/x^2$	Quadratic	$-3.0\times10^{-4}\,x^2+1.0\times10^{-2}\,x+3.1\times10^{-6}$
N-Ethylpentylone	5 - 100	$1/x^2$	Quadratic	$-5.8\times10^{-5}\,x^2+2.3\times10^{-3}\,x-1.4\times10^{-5}$
PCP	5 - 100	$1/x^2$	Quadratic	$-5.1\times10^{-3}\,x^2+4.5\times10^{-2}\,x-5.0\times10^{-4}$
4-MeO-PCP	5 - 100	$1/x^2$	Quadratic	$9.3\times10^{-5}\,x^2+5.6\times10^{-3}\,x-1.2\times10^{-5}$

Pentedrone	5 - 100	1/x	Quadratic	$-5.3\times10^{-4}\,x^2 + 4.4\times10^{-3}\,x - 5.6\times10^{-4}$
Pentylone	5 - 100	1/x	Quadratic	$-4.5\times10^{-4}\,x^2 + 3.0\times10^{-3}\,x - 4.0\times10^{-4}$
PMA	5 - 100	$1/x^2$	Quadratic	$-9.7\times10^{-5}\,x^2 + 3.2\times10^{-3}\,x - 2.7\times10^{-5}$
PMMA	5 - 100	$1/x^2$	Quadratic	$-5.9\times10^{-4}\,x^2 + 1.6\times10^{-2}\,x - 1.7\times10^{-4}$
Psilocin	5 - 100	$1/x^2$	Quadratic	$3.3\times10^{-5}\,x^2 + 1.5\times10^{-3}\,x + 8.4\times10^{-6}$
Ritanilic acid	5 - 100	$1/x^2$	Quadratic	$1.3\times10^{-5}\,x^2 + 7.0\times10^{-4}\,x - 1.8\times10^{-6}$
Trazodone	5 - 100	1/x	Quadratic	$-1.0\times10^{-4}\,x^2 + 3.3\times10^{-3}\,x + 1.5\times10^{-5}$
α-PVP	5 - 100	1/x2	Quadratic	$-5.5\times10^{-3}\,x^2 + 4.9\times10^{-2}\,x - 5.0\times10^{-4}$
3-Methylnorfentanyl	5 - 100	1/x2	Quadratic	$-1.4\times10^{-3}\,x^2 + 6.1\times10^{-2}\,x - 6.0\times10^{-4}$
4-ANPP	5 - 100	1/x	Quadratic	$-1.5\times10^{-4}\,x^2 + 2.0\times10^{-3}\,x - 1.4\times10^{-4}$
4-F-Butyrylfentanyl	5 - 100	1/x2	Quadratic	$1.3\times10^{-6}\,x^2 + 3.6\times10^{-3}\,x - 1.2\times10^{-5}$
4-Methyl fentanyl	5 - 100	1/x	Quadratic	$-2.1\times10^{-4}\,x^2 + 2.7\times10^{-3}\,x - 2.4\times10^{-4}$
Acetyl fentanyl	5 - 100	1/x	Quadratic	$-4.9\times10^{-4}\,x^2 + 6.3\times10^{-3}\,x - 7.6\times10^{-4}$
Acrylfentanyl	5 - 100	1/x2	Quadratic	$-5.6\times10^{-3}\,x^2 + 8.0\times10^{-2}\,x - 6.0\times10^{-4}$
AH-7921	5 - 100	1/x2	Quadratic	$2.1\times10^{-3}\,x^2 + 3.4\times10^{-2}\,x + 9.4\times10^{-5}$

Alfentanyl	5 - 100	1/x2	Quadratic	$7.6\times10^{-5}\ x^2 + 4.0\times10^{-3}\ x + 3.1\times10^{-5}$
Butyrylfentanyl	5 - 100	1/x	Quadratic	$-\ 8.0\times10^{-3}\ x^2 + 8.2\times10^{-2}\ x - 6.9\times10^{-3}$
Butyryl fentanyl carboxy metabolite	5 - 100	1/x2	Quadratic	$-\ 2.1\times10^{-3}\ x^2 + 3.9\times10^{-2}\ x - 3.0\times10^{-4}$
Butyryl norfentanyl	5 - 100	1/x	Quadratic	$-\ 3.1\times10^{-4}\ x^2 + 6.3\times10^{-3}\ x - 3.4\times10^{-4}$
Carfentanyl	5 - 100	1/x	Quadratic	$-\ 2.0\times10^{-4}\ x^2 + 1.8\times10^{-3}\ x - 2.2\times10^{-4}$
Cyclopropylfentanyl	5 - 100	1/x2	Quadratic	$-\ 3.4\times10^{-3}\ x^2 + 6.0\times10^{-2}\ x - 5.0\times10^{-4}$
Despropionyl p-fluorofentanyl	5 - 100	1/x	Quadratic	$-\ 1.4\times10^{-5}\ x^2 + 2.2\times10^{-3}\ x - 2.7\times10^{-5}$
Fentanyl	5 - 100	1/x	Quadratic	$-\ 4.5\times10^{-4}\ x^2 + 1.2\times10^{-2}\ x - 6.4\times10^{-4}$
Furanylfentanyl	5 - 100	1/x2	Quadratic	$-\ 5.8\times10^{-4}\ x^2 + 1.2\times10^{-2}\ x - 1.6\times10^{-4}$
Furanylnorfentanyl	5 - 100	1/x2	Quadratic	$2.2\times10^{-6}\ x^2 + 3.0\times10^{-3}\ x - 1.4\times10^{-5}$
Hydrocodone	5 - 100	1/x	Quadratic	$-\ 6.8\times10^{-4}\ x^2 + 8.8\times10^{-3}\ x - 7.6\times10^{-4}$
MT-45	5 - 100	1/x2	Quadratic	$1.0\times10^{-4}\ x^2 + 4.5\times10^{-3}\ x - 6.5\times10^{-5}$
Norfentanyl	5 - 100	1/x	Quadratic	$-\ 2.1\times10^{-3}\ x^2 + 4.4\times10^{-2}\ x - 2.3\times10^{-3}$
Ocfentanyl	5 - 100	1/x	Quadratic	$-\ 4.3\times10^{-3}\ x^2 + 4.2\times10^{-2}\ x - 3.4\times10^{-3}$
OH-Fentanyl	5 - 100	1/x	Quadratic	$-\ 1.5\times10^{-4}\ x^2 + 1.3\times10^{-3}\ x - 1.3\times10^{-4}$

OH-Thiofentanyl	5 - 100	1/x	Quadratic	$-2.3\times10^{-4}\,x^2 + 1.4\times10^{-3}\,x - 2.1\times10^{-4}$
Phenylacetyl fentanyl	5 - 100	1/x	Quadratic	$-1.3\times10^{-4}\,x^2 + 1.7\times10^{-3}\,x - 1.6\times10^{-4}$
4-Phenylfentanyl	5 - 100	1/x	Quadratic	$-7.6\times10^{-4}\,x^2 + 6.0\times10^{-3}\,x - 7.8\times10^{-4}$
Remifentanyl	5 - 100	1/x	Quadratic	$-2.0\times10^{-3}\,x^2 + 1.4\times10^{-3}\,x - 1.0\times10^{-4}$
Sufentanyl	5 - 100	1/x	Quadratic	$-7.2\times10^{-5}\,x^2 + 7.0\times10^{-4}\,x - 5.2\times10^{-5}$
Tramadol	5 - 100	1/x	Quadratic	$-6.2\times10^{-4}\,x^2 + 5.0\times10^{-3}\,x - 7.0\times10^{-4}$
U-47700	5 - 100	1/x2	Quadratic	$5.6\times10^{-5}x^2 + 2.9\times10^{-3}\,x - 2.6\times10^{-6}$
Valeryl fentanyl carboxy metabolite	5 - 100	1/x	Quadratic	$-6.8\times10^{-5}\,x^2 + 1.7\times10^{-3}\,x - 7.5\times10^{-5}$
β-Phenylfentanyl	5 - 100	1/x2	Quadratic	$-5.4\times10^{-3}\,x^2 + 6.8\times10^{-2}\,x - 6.0\times10^{-4}$

Table S3. *Results of the analytical method validation: LOD, LOQ, trueness, and precision. Trueness and precision data were obtained at 6 concentration levels (5, 7.5, 10, 25, 50, 100 ng/mL); the averaged values are reported.*

Compound	LOD (ng/mL) Hubaux-Vos	LOD Verified (S/N>3) (ng/mL)	Repeatability (mean CV%)	Intermediate Precision (mean CV%)	Trueness (mean bias%) Intraday	Trueness (mean bias%) Interday
Synthetic Cannabinoids						
5-Chloro-AB-PINACA	3.5	2	11.7	15.6	4.2	1.7
5-Chloro-TH-J018	5.3	5	18.8	29.3	0.7	-3.6
5-F-AB-PINACA	5.0	2	13.4	23.5	0.04	1.1
5-F-PB22	3.9	2	9.5	13.5	-2.2	0.6
5-F-APINACA	4.4	2	7.5	21.2	-5.1	3.4
5-F-APP PICA	3.1	2	14.3	19.3	0.2	-3.0
5-F-APP PINACA	4.9	2	10.5	23.5	-3.7	4.3
5-F-CUMYL PINACA	2.3	2	11.7	14.6	0.9	-0.8

5-F NNEI 2'-naphthyl isomer	4.4	5	11.2	24.2	-4.7	-1.3
AB-CHMINACA	4.6	2	11.6	22.5	4.6	6.0
AB-FUBINACA	4.4	5	13.6	21.4	-1.7	4.1
AB-PINACA	4.8	2	11.2	20.8	-2.13	-1.9
ADBICA	4.5	2	14.1	2.2	-0.06	0.9
ADB-PINACA	4.8	2	10.8	20.5	-3.0	2.1
CB-13	3.9	5	11.7	11.4	-17.1	-5.5
AM-1220	5.2	5	14.8	23.4	-2.4	2.1
AM-2201	2.6	2	6.8	14.2	0.4	1.0
AM-2233	3.5	5	13.3	20.0	4.9	5.1
AM-694	4.3	5	11.9	17.4	6.6	4.5
APP-FUBINACA	5.0	5	13.0	24.3	-7.1	1.9
CUMYL-PeGACLONE	2.6	2	16.1	16.3	-5.9	0.5
JWH-007	2.2	2	10.4	10.0	-8.3	2.9

JWH-015	3.2	5	14.8	23.7	18.2	2.4
JWH-018	3.0	5	12.3	13.0	-0.8	2.1
JWH-019	3.2	5	15.8	19.9	2.7	1.2
JWH-020	3.6	5	11.4	15.5	-8.5	1.2
JWH-073	3.9	5	14.8	20.4	2.4	-2.4
JWH-081	3.9	2	12.1	15.0	3.4	3.6
JWH-098	2.2	2	11.3	11.8	2.3	1.6
JWH-122	3.3	2	18.0	20.1	-0.4	-16.4
JWH-147	5.7	5	16.9	15.6	12.2	1.7
JWH-203	5.0	5	13.1	13.9	0.04	-3.9
JWH-210	3.7	5	13.5	16.8	-0.5	-1.9
JWH-250	4.1	2	11.9	16.1	-8.3	-2.8
JWH-251	2.8	2	13.1	15.4	0.4	1.2
JWH-302	5.7	5	14.1	21.0	0.6	-0.3
JWH-307	2.6	2	15.5	15.9	-2.3	-2.1

JWH-398	4.0	5	11.6	15.7	1.4	-7.4
MAB-CHMINACA	5.2	2	10.7	24.8	-5.2	2.3
MAM-2201	3.5	5	15.9	18.4	-2.3	2.5
MDMB-CHMICA	2.4	2	12.4	14.7	0.2	-10.7
MDMB-CHMINACA	4.0	2	14.9	25.4	1.3	1.1
MMB-2201	3.5	2	14.9	22.2	0.3	-3.7
PB-22	4.6	2	14.6	21.1	-5.2	1.4
RCS-4	4.5	2	14.7	22.6	-1.4	4.9
RCS-8	6.2	5	25.1	17.5	-10.4	-13.4
STS-135	5.6	2	15.1	13.6	2.3	-10.6
UR-144	6.3	5	13.0	18.5	-24.1	-22.5
UR-144-5-OH	4.6	5	11.8	20.8	-20.4	-6.9
WIN-48	4.3	2	12.2	20.5	0.4	3.0
WIN-55	5.2	2	11.7	22.4	4.3	-5.1
XLR-11	4.6	5	13.2	24.3	1.2	5.1

5-F-ADB	6.0	7.5	15.6	25.0	-7.1	2.4
ADB-FUBINACA	3.4	7.5	12.9	17.4	-2.2	0.6
Synthetic Cathinones and Hallucinogens						
25B-NBOMe	4.5	2	7.4	13.1	2.6	1.6
25C-NBOMe	3.8	2	8.9	12.5	13.9	31.3
25H-NBOMe	3.6	2	10.4	18.2	-1.3	-1.5
25I-NBOMe	4.2	2	9.5	15.4	2.8	-1.4
2C-B	5.5	5	12.2	15.5	-10.9	-10.9
2C-P	4.9	2	9.2	16.6	-0.05	-16.1
3-4-DMMC	6.0	5	10.7	17.3	-9.3	9.1
4-Acetoxy-DiPT	3.1	5	11.1	15.6	2.4	37
4-Acetoxy-DMT	4.4	5	10.9	15.6	11.9	3.3
4-F-METCAT	3.7	5	10.2	13.4	1.6	2.9
4-MEC	5.4	2	11.7	13.4	-10.9	-7.5
5-EAPB	2.2	2	8.3	10.9	0.3	-0.7

5-Methoxy AMT	5.7	7.5	11.0	14.3	-9.6	-7.2
5- Methoxy DALT	5.1	2	9.5	14.1	-0.3	-0.3
5- Methoxy DMT	4.8	5	9.2	12.3	3.3	-0.01
5- Methoxy DiPT	5.6	2	8.9	12.9	-8.5	-8.9
ALFA-PVP	2.6	2	11.6	18.6	0.8	-10.9
6-APB	5.5	7.5	11.8	15.1	-9.9	-9.7
Buphedrone	2.5	2	8.6	9.5	-0.6	0.3
Butylone	2.3	2	9.7	13.2	0.4	-0.2
DMT	4.6	5	10.7	13.2	-9.1	-4.1
Ethylone	6.0	7.5	7.0	13.5	-2.7	-7.2
Ethylphenidate	1.3	2	7.5	8.0	0.3	0.4
Ethyltryptamine	1.8	2	10.9	11.6	0.4	-0.01
Harmine	6.3	5	11.3	17.2	-6.3	-11.3
Ketamine	5.6	5	10.8	15.2	-4.5	-8.1
LSD	5.1	2	11.8	15.4	-2.3	-6.6

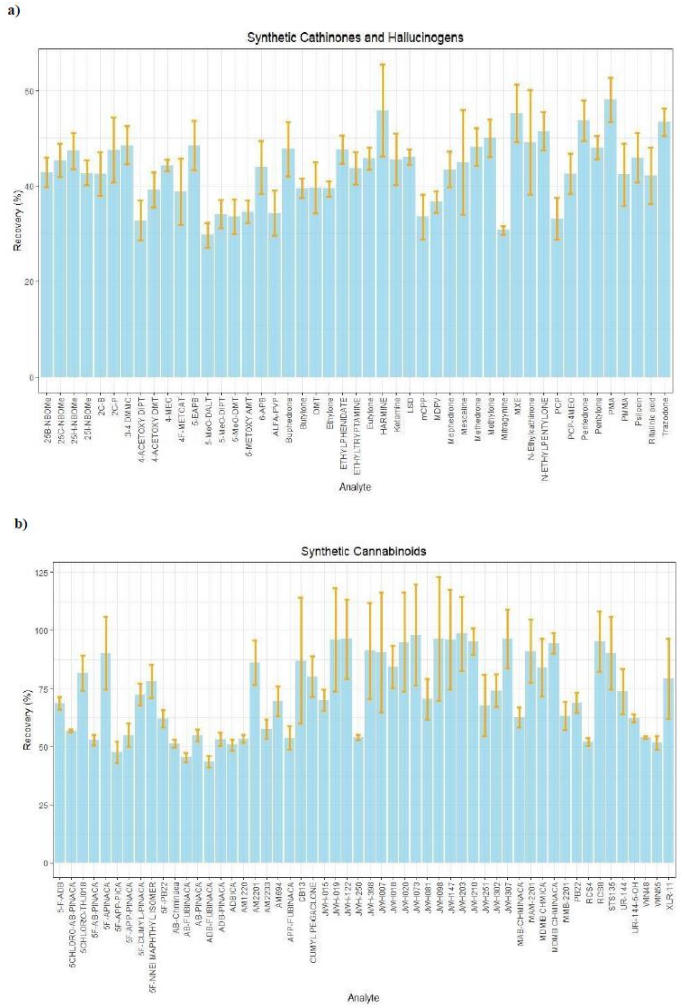
mCPP	4.8	5	9.7	17.1	13.5	18.1
MDPV	4.3	5	8.8	13.1	-6.7	-5.7
Mephedrone	5.2	5	12.3	15.7	-6.5	-1.3
Mescaline	4.1	5	11.1	16.4	1.7	2.4
Methedrone	2.5	2	9.1	11.9	-4.4	-0.3
Methylone	4.7	5	11.5	13.3	-3.9	-5.8
MXE	3.6	2	9.1	10.7	0.4	3.5
Mitragynine	5.0	5	12.4	17.6	-5.4	-4.6
N-Ethylcathinone	2.2	2	10.7	14.5	1.7	0.8
N-Ethylpentylone	2.7	2	10.1	9.9	13.1	17.3
PCP	2.1	2	11.0	14.4	-10.4	-11.7
4-MeO-PCP	3.6	5	13.7	15.1	15.3	21.4
Pentedrone	5.4	2	10.8	14.4	-0.7	-1.4
Pentylone	5.8	2	7.8	12.9	-16.6	-7.8
PMA	4.0	5	14.6	18.9	18.3	20.2

	3.6	5	12.2	15.0	8.9	12.2
PMMA						
Psilocin	3.4	5	7.0	7.5	0.2	-0.1
Ritanilic acid	2.4	2	12.4	14.7	-0.8	-0.6
Trazodone	3.1	5	11.1	15.6	-1.0	2.7
Eutylone	3.8	2	10.2	13.7	2.8	5.0
Fentanyl analogous and synthetic opioids						
3-Methylnorfentanyl	2.2	2	9.3	11.7	1.7	-0.5
4-ANPP	3.2	2	7.5	8.9	2.1	5.4
4-F-Butyrylfentanyl	1.8	2	10.1	11.6	1.5	-0.04
4-Methyl fentanyl	3.7	2	8.8	10.8	2.3	6.2
Acetyl fentanyl	5.3	2	9.9	11.8	-0.8	4.1
Acrylfentanyl	4.3	5	9.7	10.9	0.4	-5.0
AH-7921	2.4	2	10.8	13.6	0.1	-1.4

Alfentanyl	2.0	2	7.6	9.1	-0.9	-0.4
Butyrfentanyl	3.6	2	10.4	14.6	2.3	1.4
Butyryl fentanyl carboxy metabolite	4.5	2	7.0	9.9	0.2	1.1
Butyryl norfentanyl	3.1	5	9.9	14.3	-1.7	2.5
Carfentanyl	4.8	5	9.7	11.8	-4.4	-2.6
Cyclopropylfentanyl	2.3	2	10.5	14.7	0.8	0.2
Despropionyl p-fluorofentanyl	2.6	2	9.1	14.1	1.4	1.3
Fentanyl	3.2	2	7.9	11.1	-0.5	1.3
Furanylfentanyl	2.3	2	9.7	14.6	-2.4	0.2
Furanylnorfentanyl	2.2	2	12.1	12.6	0.1	-0.3
Hydrocodone	3.4	5	9.9	11.4	0.3	4.6
MT-45	2.8	2	10.5	13.3	0.5	-0.7

Norfentanyl	3.0	5	13.0	13.5	1.9	0.9
Ocfentanyl	3.1	2	8.1	8.8	-1.3	3.3
OH-Fentanyl	4.2	5	10.6	13.3	-2.3	-1.5
OH-Thiofentanyl	6.0	5	8.8	12.9	-5.4	-11.7
Phenylacetyl fentanyl	4.2	2	11.5	13.7	1.0	6.1
Phenylfentanyl	5.3	2	10.3	13.3	-2.5	-4.7
Remifentanyl	4.3	5	9.5	10.7	1.1	-5.1
Sufentanyl	3.5	5	11.5	11.0	-4.1	-0.4
Tramadol	5.8	2	7.9	9.5	-11.0	-5.9
U-47700	2.3	2	10.7	12.5	-1.9	-0.4
Valeryl fentanyl carboxy metabolite	3.3	5	9.8	14.9	3.8	3.9
β-Phenylfentanyl	2.5	2	10.3	12.7	-0.5	1.3

Figure S4. *Recovery trend (RE%) and their CV% for the three classes of NPS.* ***a)*** *Cathinones and hallucinogens;* ***b)*** *synthetic cannabinoids;* ***c)*** *synthetic opioids.*



c)

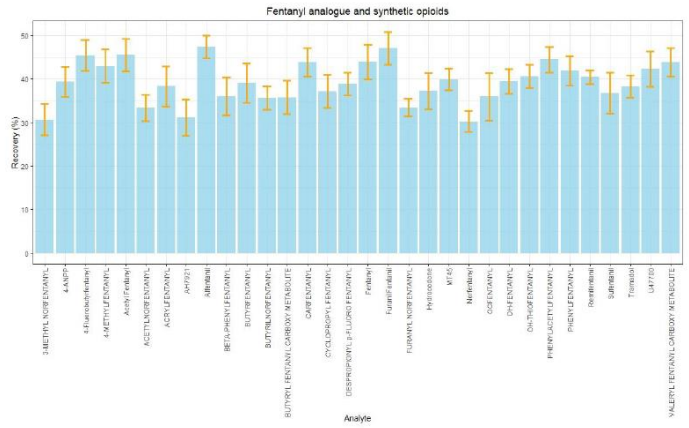
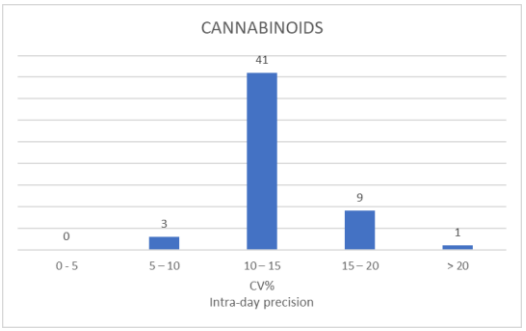
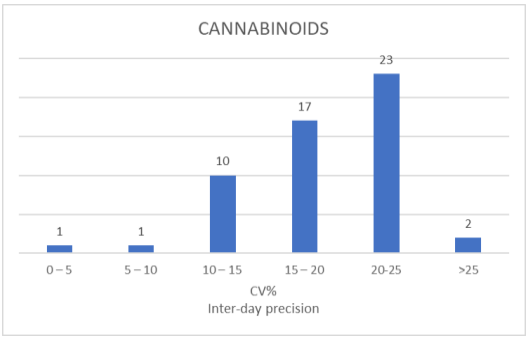


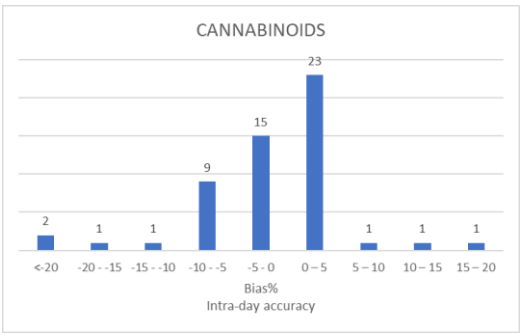
Table S5. *Comparison of the recovery data from DBS samples spiked with respectively* ***a)*** *all 132 target analytes in the blood drop;* ***b)*** *only the 3 listed analytes.*

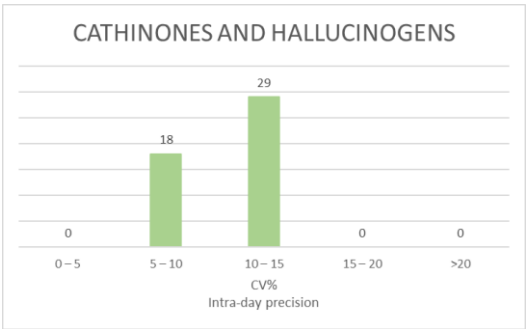
Compound	**Recovery % (CV%)** (100 ng/mL)	
	Working solution (132 compounds)	Three targeted analytes
Synthetic Cathinones and Hallucinogens		
Ketamine	46 (12%)	44 (4%)
Fentanyl analogous and synthetic opioids		
Fentanyl	44 (9%)	40 (5%)
Norfentanyl	30 (8%)	39 (4%)

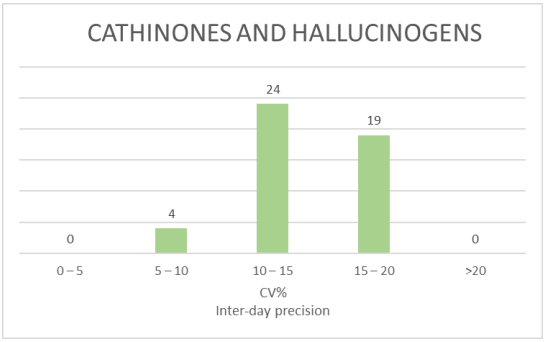
Figure S6. *Repeatability (intra-day), intermediate (inter-day) precision, and intra-day trueness expressed collectively (number of substances) for the three classes: synthetic cannabinoids, synthetic cathinones and hallucinogens, synthetic opioids.*

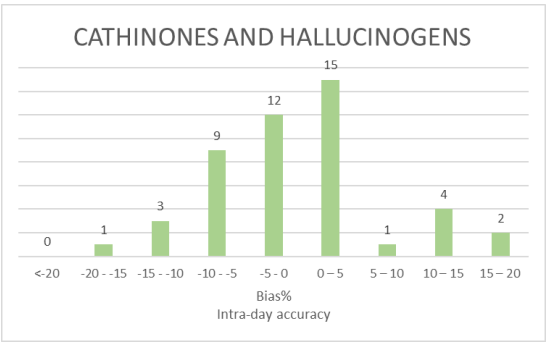


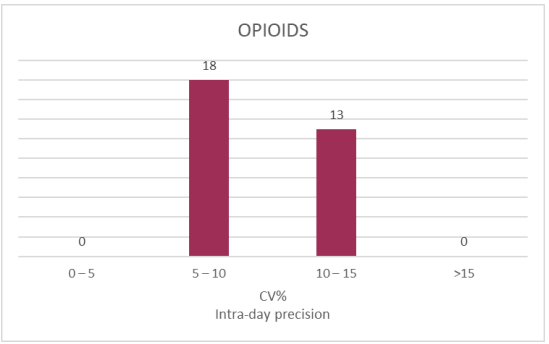


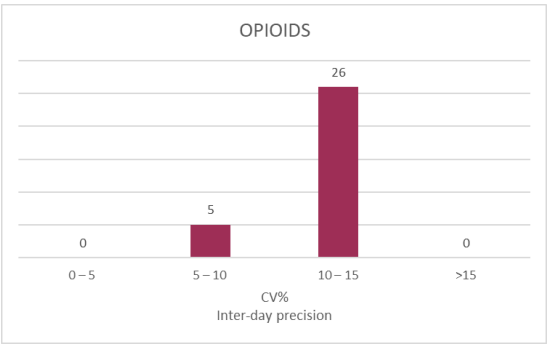





CATHINONES AND HALLUCINOGENS
0
4
24
19
0
0 – 5
5 – 10
10 – 15
15 – 20
>20
CV%
Inter-day precision


CATHINONES AND HALLUCINOGENS
0
1
3
9
12
15
1
4
2
<-20
-20 - -15
-15 - -10
-10 - -5
-5 - 0
0 – 5
5 – 10
10 – 15
15 – 20
Bias%
Intra-day accuracy


OPIOIDS
0
18
13
0
0 – 5
5 – 10
10 – 15
>15
CV%
Intra-day precision


OPIOIDS
0
5
26
0
0 – 5
5 – 10
10 – 15
>15
CV%
Inter-day precision

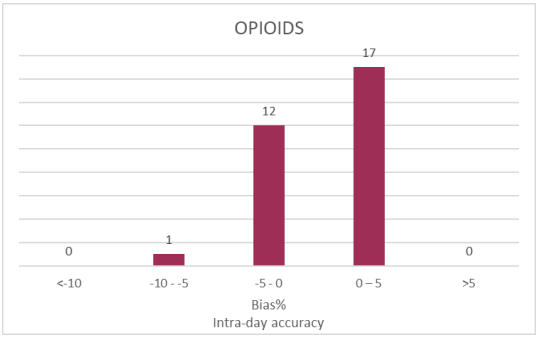


Table S7. *Matrix effect (%) and Recovery (%) for the 132 targeted analytes collectively spiked in two different DBS cards respectively at concentrations of 10 ng/mL and 100 ng/mL.*

Compound	**Matrix effect (±%)**	**Recovery % (CV%)**
	Low = 10 ng/mL	High = 100 ng/mL
Synthetic Cannabinoids		
5-Chloro-AB-PINACA	-19	57 (1%)
5-Chloro-TH-J018	-69	83 (8%)
5-F-AB-PINACA	-27	53 (4%)
5-F-APINACA	-43	90 (17%)
5-F-PB22	-7	62 (9%)
5-F-APP PICA	-32	48 (9%)
5-F-APP PINACA	-34	55 (9%)
5-F-CUMYL PINACA	-10	72 (7%)
5-F-NNEI 2'-naphthyl isomer	-35	78 (9%)
AB-CHMINACA	2	51 (3%)
AB-FUBINACA	10	45 (4%)

AB-PINACA	-30	55 (5%)
ADBICA	-19	51 (5%)
ADB-PINACA	1	53 (5%)
CB-13	-47	89 (31%)
AM-1220	-37	54 (3%)
AM-2201	-57	86 (11%)
AM-2233	-40	58 (7%)
AM-694	-10	70 (9%)
APP-FUBINACA	-42	54 (9%)
CUMYL-PeGACLONE	-19	80 (11%)
JWH-007	-66	92 (27%)
JWH-015	-30	70 (7%)
JWH-018	-43	86 (10%)
JWH-019	-46	97 (23%)
JWH-020	-42	97 (22%)
JWH-073	-22	98 (19%)
JWH-081	-44	72 (13%)
JWH-098	-43	95 (28%)
JWH-122	-46	98 (17%)
JWH-147	-42	96 (22%)
JWH-203	-38	98 (16%)
JWH-210	-43	95 (3%)
JWH-250	6	54 (2%)
JWH-251	-23	68 (19%)

JWH-302	-21	74 (9%)
JWH-307	-43	95 (12%)
JWH-398	-41	93 (26%)
MAB-CHMINACA	4	63 (7%)
MAM-2201	-37	90 (16%)
MDMB-CHMICA	-33	84 (5%)
MDMB-CHMINACA	-6	95 (15%)
MMB-2201	-58	63 (10%)
PB-22	-15	69 (6%)
RCS-4	-41	52 (3%)
RCS-8	-42	94 (14%)
STS-135	-43	90 (17%)
UR-144	-44	74 (13%)
UR-144-5-OH	-36	62 (3%)
WIN-48	-59	54 (1%)
WIN-55	-49	52 (6%)
XLR-11	-24	79 (22%)
5-F-ADB	-17	69 (4%)
ADB-FUBINACA	-43	44 (6%)
Synthetic Cathinones and Hallucinogens		
25B-NBOMe	-38	43 (7%)
25C-NBOMe	-16	45 (8%)
25H-NBOMe	-10	47 (8%)
25I-NBOMe	-33	42 (6%)

2C-B	-17	42 (11%)
2C-P	-35	48 (14%)
3-4-DMMC	-38	48 (8%)
4-Acetoxy-DiPT	-5	33 (13%)
4-Acetoxy-DMT	-29	39 (9%)
4-F-METCAT	-9	39 (18%)
4-MEC	-34	44 (3%)
5-EAPB	-21	48 (11%)
5-Methoxy AMT	-21	35 (7%)
5- Methoxy DALT	-30	30 (9%)
5- Methoxy DMT	-12	34 (11%)
5- Methoxy DiPT	-22	34 (13%)
ALFA-PVP	-29	34 (14%)
6-APB	-21	44 (13%)
Buphedrone	-2	48 (12%)
Butylone	-20	40 (5%)
DMT	-15	34 (13%)
Ethylone	-38	39 (4%)
Ethylphenidate	-19	48 (6%)
Ethyltryptamine	-22	44 (8%)
Harmine	-30	56 (17%)
Ketamine	-29	46 (12%)
LSD	-35	46 (4%)
mCPP	-35	34 (14%)

MDPV	-34	37 (6%)
Mephedrone	-22	43 (9%)
Mescaline	-5	45 (24%)
Methedrone	-30	48 (8%)
Methylone	-28	50 (8%)
MXE	-27	55 (11%)
Mitragynine	-35	31 (3%)
N-Ethylcathinone	-2	49 (22%)
N-Ethylpentylone	-25	51 (8%)
PCP	-20	33 (13%)
4-MeO-PCP	-36	43 (10%)
Pentedrone	-34	54 (8%)
Pentylone	-30	48 (5%)
PMA	-14	58 (8%)
PMMA	-2	42 (15%)
Psilocin	-34	46 (11%)
Ritanilic acid	-28	42 (14%)
Trazodone	-30	53 (5%)
Eutylone	-5	46 (5%)
Fentanyl analogous and synthetic opioids		
3-Methylnorfentanyl	-11	31 (12%)
4-ANPP	-38	39 (9%)
4-F-Butyrylfentanyl	-34	45 (8%)
4-Methyl fentanyl	-35	43 (9%)

Acetyl fentanyl	-35	46 (8%)
Acrylfentanyl	-16	38 (12%)
AH-7921	-17	31 (14%)
Alfentanyl	-36	47 (6%)
Butyrfentanyl	-15	39 (12%)
Butyryl fentanyl carboxy metabolite	-9	36 (11%)
Butyryl norfentanyl	-40	36 (8%)
Carfentanyl	-39	44 (8%)
Cyclopropylfentanyl	-16	37 (10%)
Despropionyl p-fluorofentanyl	-42	39 (7%)
Fentanyl	-38	44 (9%)
Furanylfentanyl	-33	47 (8%)
Furanylnorfentanyl	-19	33 (6%)
Hydrocodone	-10	37 (11%)
MT-45	-42	40 (6%)
Norfentanyl	-10	30 (8%)
Ocfentanyl	-15	36 (15%)
OH-Fentanyl	-20	40 (7%)
OH-Thiofentanyl	-31	41 (7%)
Phenylacetyl fentanyl	-32	44 (7%)
Phenylfentanyl	-34	42 (8%)
Remifentanyl	-23	40 (4%)
Sufentanyl	-28	37 (13%)

Tramadol	-34	38 (7%)
U-47700	-34	42 (10%)
Valeryl fentanyl carboxy metabolite	-39	44 (8%)
β-Phenylfentanyl	-19	36 (12%)

Chapter 10 | **Detection of fentanyl, synthetic opioids, and ketamine in hair specimens from purposive samples of American and Italian populations**

Alberto Salomone[1,2]*, Martina Galletto[1], Marta Massano[1], Daniele Di Corcia[2], Joseph J. Palamar[3], Marco Vincenti[1,2]

[1]*Department of Chemistry, University of Turin, Italy*

[2]*Centro Regionale Antidoping, Orbassano (TO), Italy*

[3]*Department of Population Health, New York University Grossman School of Medicine, New York, New York, USA*

The content of this Chapter is included in one published manuscript:

Detection of fentanyl, synthetic opioids, and ketamine in hair specimens from purposive samples of American and Italian populations

Alberto Salomone, Martina Galletto, Marta Massano, Daniele Di Corcia, Joseph J. Palamar, Marco Vincenti
Journal of Forensic Science
https://doi.org/10.1111/1556-4029.15348

Keywords: Fentanyl, Hair analysis, Ketamine, MDMA, New psychoactive substances, Synthetic opioids

Abstract

With the current crisis related to the diffusion of fentanyl and other novel opioids in several countries and populations, new and effective approaches are needed to better elucidate the phenomenon. In this context, hair testing offers a unique perspective in the investigation of drug consumption, producing useful information in terms of exposure to psychoactive substances. In this research, we applied targeted ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) analytical methods to detect novel synthetic and prescription opioids and other common controlled psychoactive drugs in the keratin matrix. A total of 120 hair samples were analyzed from the United States (US) and Italy, segmented when longer than 6 cm, and then analyzed. In the 60 samples (83 segments in total) analyzed from a purposive sample of data collected in the US, fentanyl was detected in 14 cases (16.9%), with no detection of nitazens or brorphine. We also detected fentanyl metabolites, despropionyl-p-fluorofentanyl, and prescription opioids. In the 60 samples collected in Italy (91 segments in total), ketamine was the most prevalent compound detected (in 41 cases; 45.1%), with ketamine demonstrating a strong correlation with detection of amphetamines and MDMA, likely due to co-use of these substances in recreational contexts. Several common drugs were also detected but no exposure to fentanyl or its analogues were detected. Results of this retrospective exploration of drug use add to increasing evidence that hair testing can serve as a useful adjunct to epidemiology studies that seek to determine biologically confirmed use and exposure in high-risk populations

10.1 Introduction

In the past decade, illicit opioid use has progressed from nonmedical use of legal analgesic drugs such as hydrocodone, oxycodone, and tramadol, to the diffusion of illicitly manufactured fentanyl and its analogues, often referred to as "fentalogs" [1, 2]. In addition, a new class of synthetic opioids referred to as nitazenes has been recently reported in several illegal drug markets [3]. Many of these novel synthetic opioids (NSOs) are considered particularly risky due to their high potency and because they are often introduced into the market as cutting/adulterant agents of drugs such as heroin or simply as cheaper substitutes for other drugs [4, 5].

While heroin was the first drug to become frequently replaced or cut with NSO, these compounds began to appear to counterfeit pills representing common prescription drugs (e.g., oxycodone, alprazolam) and other illegal powder drugs

such as cocaine [5-10], raising a major health concern for unaware users. In parallel, however, preference for fentanyl has increased among some populations, leaving doubt regarding whether fentanyl is more demand-led or supply-led [11]. However, it is important to note that currently, the synthetic opioid crisis is centered in North America. In 2022, in the United States (US), there were 71,238 deaths linked to use of synthetic opioids such as fentanyl [12], and in 2021, there were at least 10,000 fentanyl seizures in the US which weighed over 10,000 kg in total [13]. In Europe, however, among 12 countries providing seizure data to the European Union Early Warning System, in 2021, there were only 187 recorded fentanyl seizures (weighting 5.5 kg in total) [14]. Heroin has largely been replaced by fentanyls in the US but heroin is still the most common illicit opioid in Europe [14]. In Italy, an average of 0.74 tons of heroin have been seized annually between 2011 and 2021 [14].

Despite differences in the opioid and other drug landscapes between the US and Europe, new and effective approaches are needed to monitor shifting drug-related phenomena. For example, in the US, in 2021, there was an increase to 24,538 deaths linked to cocaine use and 32,856 deaths linked to other psychostimulant (mainly methamphetamine) use [12], but the vast majority of such cases involve co-use of opioids [15]. A greater understanding of course (or co-exposure) of illicit fentanyl, opioids, and other psychoactive drugs can help adapt and improve existing interventions aimed to reduce overdose mortality, together with broad integrated public health strategies based on overdose education and prevention and to support the drugs debate [16-18].

In this context, hair analysis has proved to be an easy and effective tool to investigate the prevalence of use of psychoactive substances, since the keratin matrix allows for the detection of past drug exposure and for the investigation of the chronological profile of the exposure to one or multiple compounds. Furthermore, hair analysis is now based on multiclass methods for both well-known and emerging compounds, allowing for the investigation of different consumption patterns, including co-use of common drugs (including prescription opioids), as well as occasional vs. frequent NSO use or exposure [19-21]. While several papers have described multianalyte screening methods capable of detecting NSOs [22], few have presented results from real samples [23-25]. Polydrug use has generally been shown to be common based on the aforementioned studies, involving several psychoactive substances and not only heroin [26, 27]. In general, fentanyl has been the most frequently detected

compound among the class of fentalogs [8, 19], suggesting that it is the most prevalent molecule while the less common analogues tend to be co-used with other drugs and are thus not consumed in isolation. Other typical matters of current discussion (in order to provide a definitive interpretation of positive versus negative results) are: (i) the meaning of quantitative results, in terms of occasional and frequent use or exposure), and (ii) the identification of proper metabolites to discriminate direct exposure from potential external contamination.

In this paper, we present our ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) analysis of a subset of hair samples collected in the US and in Italy based on purposive sampling methods. All samples were screened for fentalogs, prescription opioids, nitazens, brorphine, and other common controlled psychoactive drugs.

10.2 Materials and methods

10.2.1 Reagent and standards

All chemicals, including methanol, formic acid, and acetonitrile, were purchased from Sigma-Aldrich while ultra-pure water was obtained using a Milli-Q@ UF-Plus apparatus (Millipore). The analytical standards of the target analytes and deuterated internal standards (norfentanyl-D5, fentanyl-D5 and oxycodone-D6) were purchased from LGC Promochem and Sigma-Aldrich (purity >99%, concentration between 0.1 mg/mL and 1mg/mL), or kindly provided by the Italian National Early Warning System (provided at a concentration of 0.02 mg/mL). The list of target analytes is presented in Table 1. All stock standard solutions were prepared in methanol at 1 mg/ mL and stored at -20°C until used. Working solution of 42 analytes (identified among the most common synthetic opioids and those recently observed by the warning systems) and internal standard solution were prepared at the final concentration of 1 μg/mL by dilution with methanol.

Table 7: *List (ordered by RT) of the monitored transitions, their instrumental parameters and the related internal standard for the screened compounds.*

Compound	Retention time, min	Precursor mass Q1 m/z	Fragments mass Q3 m/z	CE (V)	EP (V)	Internal Standard
Acetyl norfentanyl	2.10	219.1	84.1	23	8	Fentanyl-D5
			55.1	48		

			56.1	42		
Methoxyacetyl	2.10	249.1	84.1	22		Fentanyl-D5
norfentanyl			55.1	55	8	
			56	40		
Oxycodone	2.10	316.0	298.1	25		Oxycodone-
			241.1	38	8	D6
			256.1	34		
Hydrocodone	2.20	300.0	199.1	42		Fentanyl-D5
			171.1	51	8	
			128.0	74		
Norfentanyl	2.60	233.0	84.1	24		Norfentanyl-
			150.1	22	8	D5
			55.0	50		
Metodesnitazene	2.60	338.1	100.0	23		Fentanyl-D5
			72.0	53	8	
			121.0	50		
3-methyl Norfentanyl	2.70	247.1	98.1	23	8	Fentanyl-D5
			150.1	26		
			69.0	42		
Furanyl Norfentanyl	2.70	271.2	84	20	8	Fentanyl-D5
			56.1	41		
			55	54		
Tramadol	2.80	264.1	58.1	46		Fentanyl-D5
			246.1	15	8	
Butiryl Norfentanyl	3.00	247.2	84.0	24		Fentanyl-D5
			177.2	21	8	
			55.0	55		
Etodesnitazene	3.00	352.1	100.1	26		Fentanyl-D5
			71.9	57	8	
			107.1	60		
Remifentanyl	3.10	377.1	317.2	22		Fentanyl-D5
			228.0	27	8	
			116.1	37		
	3.10	381.1	188.2	34		Fentanyl-D5
Butyrylfentanyl			105.1	56	8	
carboxy metabolite			260.1	34		
OH-thioentanyl	3.10	359.1	192.1	32		Fentanyl-D5
			146.1	32	8	

			111.0	50		
Valeryl p-fluoro fentanyl	3.20	395.1	188.2	32		Fentanyl-D5
			105.0	57	8	
			274.1	33		
Acetylfentanyl	3.20	323.0	188.2	38		Fentanyl-D5
			105.0	38	8	
			103.0	86		
Ocfentanyl	3.20	371.1	105.1	50	8	Fentanyl-D5
			188.2	31		
			134.0	38		
beta-OH-fentanyl	3.30	353.2	186.1	32		Fentanyl-D5
			204.2	28	8	
			335.2	26		
4-ANPP	3.40	281.0	188.2	24	8	Fentanyl-D5
			105.0	41		
			103.0	63		
Alfentanyl	3.50	417.0	268.3	24		Fentanyl-D5
			197.2	35	8	
			165.0	47		
Acrylfentanyl	3.50	335.1	188.2	30		Fentanyl-D5
			105.0	50	8	
			132.1	42		
Despropionyl-p-fluorofentanyl	3.50	299.2	188.1	24		Fentanyl-D5
			105.0	39	8	
			134.0	32		
Flunitazene	3.50	371.1	100.1	33		Fentanyl-D5
			109.1	65	8	
			72.1	58		
Fentanyl	3.50	337.1	188.2	32	8	Fentanyl-D5
			105.0	49		
			132.1	42		
Metonitazene	3.50	383.0	100.0	26		Fentanyl-D5
			72.1	58	8	
			121.0	38		
U-47700	3.50	328.9	204.1	36		Fentanyl-D5
			286.1	24	8	
			206.1	34		
4-methylfentanyl	3.60	351.1	91	51		Fentanyl-D5

			202.1	30	8	
			119.1	35		
AH-7921	3.60		173.0	40		Fentanyl-D5
		329.0	284.1	23	8	
			286.1	24		
Furanilfentanyl	3.60	375.0	188.2	28		Fentanyl-D5
			105.0	52	8	
			103.0	82		
	3.70	402.0	218.2	29		Fentanyl-D5
Brorphine			104.1	63	8	
			218.2	35		
Carfentanyl	3.70	395.0	335.2	25	8	Fentanyl-D5
			246.1	34		
			113.0	34		
Cyclopropylfentanyl	3.70	349.1	188.1	32		Fentanyl-D5
			105.0	51	8	
			132.0	40		
N-pyrrolidino	3.70	395.0	98.0	27	8	Fentanyl-D5
etonitazene			107.0	70		
			56.0	82		
Isotonitazene	3.70	411.2	100.0	20	8	Fentanyl-D5
			106.9	52		
			72.0	42		
Butyrylfentanyl	3.80	351.2	188.2	31		Fentanyl-D5
			105.1	49	8	
			230.2	31		
Phenyl fentanyl	3.80	385.2	188.2	29	8	Fentanyl-D5
			105	51		
			134.2	36		
Sufentanyl	3.90	387.0	238.1	26		Fentanyl-D5
			355.1	26	8	
			111.0	46		
4-F-butylfentanyl	3.90	369.1	188.1	33		Fentanyl-D5
			105.0	55	8	
			248.1	33		
Phenylacetyl fentanyl	4.10	399.2	105	55		Fentanyl-D5
			188.2	32	8	
			134.1	39		
MT-45	4.20	349.1	181.1	36	8	Fenatnyl-D5
			166.2	46		

			169.2	25		
beta-phenyl Fentanyl	4.30	413.2	188.2	35	8	Fentanyl-D5
			105.0	55		
			292.1	37		
Butonitazene	4.4	425.2	100.1	31		Fentanyl-D5
			72.0	67	8	
			107.0	75		

10.2.2 Sample collection and preparation

In this study, we focus on two purposive samples of adults from the US and from Italy. Hair samples were collected in 2022 in the US (60 samples, from an ongoing rapid street reporting surveillance study being conducted throughout various US cities by the National Drug Early Warning System) [28] and in Italy (60 samples, from harm reduction services in Northern Italy), according to international guidelines [29]. With regard to the 60 US samples, we focused on samples provided by participants who reported past-12month use of heroin and/or fentanyl (n=18), 21 participants reporting past-12-month use of at least one novel psychoactive substance (NPS; who did not report heroin or fentanyl use; n=21), and a random sample of 21 participants who did not report heroin, fentanyl, or NPS use (n=21). In order to nullify any further risk related to data sharing and to safeguard the privacy of sample donors, in the US, all samples were collected anonymously. Italian samples were made anonymous by alphanumeric codes and used only in our laboratory. The risk of re-identification was also nullified. Furthermore, subjects provided informed consent to be tested for drug exposure. The study protocol for hair sample collection and testing for US samples was reviewed and approved by the Institutional Review Board (IRB) of the University of Florida. The study protocol for hair sample collection in Italy was approved by the Bioethical Committee of the University of Turin.

All samples were analyzed up to a maximum of the proximal 12 cm, since the study aimed to explore the intake of drugs in the 12 months prior to collection (assuming a normal hair growth rate of 1 cm per month). When hair was ≤ 6 cm, it was analyzed in its entire length. When hair was longer than 6 cm (54 samples), two segments were prepared for analysis (with one representing roughly the past six months and the other representing roughly the previous 6-12 months). Therefore, a total of 174 separate segments was analyzed. The targeted screening for common drugs was performed using previously published and fully validated methods [30, 31]. Existing procedures for novel opioids [32, 33] were adjusted to

expand the panel of screened molecules. A partial validation was performed, aimed to verify the method sensitivity and the quality of the calculated concentrations. The limits of detection are presented in Table S1, while data for trueness and precision at three different concentration levels are presented in Table S2.

All samples were treated with a procedure developed on-purpose for the keratin matrix. About 50 mg of hair was decontaminated by an initial wash with 1-mL dichloromethane followed by a second wash with 1-mL methanol, each one performed under 3 min stirring. The dried hair was pulverized using six steel balls stirring in a Precellys® homogenizer. The pulverized samples were extracted by keeping them immersed in 0.5 mL methanol added with 2.5 μL of an internal standards mixture (final concentration of 0.01 ng/mg) at +55 ± 5 °C for 15 h. Lastly, the organic phase was collected and an aliquot of 3 μL was directly injected into the UHPLC–MS-MS system. A calibration curve in the range 10-250 pg/mg was also prepared by spiking the proper quantities of analytical standards into a blank hair sample.

10.2.3 Instrumentation

UHPLC separation was performed with a Phenomenex Kinetex C18 column (100 × 2.1 mm, 1.7 μm) maintained at 45 °C on the SCIEX ExionLC™ AC system. The mobile phases consisted of water (A) and acetonitrile (B), both with formic acid 5 mM. The LC flow rate was set at 0.5 mL/min and the mobile phase eluted under the following linear gradient conditions: (A:B, v:v) isocratic elution at 95:5 for 0.5 min, from 95:5 to 5:95 in 7.5 min, isocratic elution at 5:95 for 0.5 min and final re-equilibration for 1.5 min to the initial condition. The total run time was 10 min. All analyses were performed using a mass spectrometer equipped with a quadrupole trap SCIEX triple Quad™ 7500 mass spectrometer (Sciex, Darmstadt, Germany) system equipped with an OptiFlow Pro ion source with an analytical probe and E Lens. The ionization source was operated with electrospray ionization (ESI) in the positive mode. For each transition, compound-specific parameters such as collision energy (CE) were also optimized after infusion of the standard solution. A single acquisition method was created using the Scheduled MRM algorithm in SCIEX OS software 2.0. Three MRM transitions were monitored for each targeted analyte. The full list of the target analytes, the monitored transitions, and their instrumental parameter are reported in Table 1.

10.2.4 Statistical analysis

We used descriptive statistics to describe the number of segments testing positive for various drugs in each country, and among positive cases we also described the range of levels of molecules detected. Within the Italian sample, we also computed Spearman correlations to determine the extent to which level of detection of each drug was correlated. Python version 3.11.3 has been used to compute the correlation matrices, involving numpy, pandas and seaborn libraries.

10.3 Results and discussion

10.3.1 Testing for NSO

In the 60 samples collected in the US (comprising of 83 segments), at least one opioid was detected in 16 segments (19.3%). Fentanyl was detected above the LOD (estimated at 5 pg/mg) in 14 segments (16.9%). The range of measured concentrations of fentanyl was extremely wide, ranging from 13 pg/mg through 7300 pg/mg, with a mean value of 1377 pg/mg and a median of 382 pg/mg. Only four segments measured below 100 pg/mg (0.1 ng/mg). In eight segments, external contamination was excluded because the metabolite norfentanyl was also detected in the range 32 pg/mg-2300 pg/mg, with a mean value of 809 pg/mg and a median of 209 pg/mg. Another promising marker of active fentanyl use, beta-hydroxyfentanyl [34], was detected in six cases, in the range 17 pg/mg-1400 pg/mg. However, when beta-hydroxyfentanyl was present, norfentanyl was as well. Overall, the main metabolites were detected in the majority of hair samples testing positive for fentanyl supporting the possibility to ascertain active use. Acetylfentanyl, which is suggestive of clandestine production, was detected in three cases (range: 129 pg/ mg - 265 pg/mg) and 4-ANPP, which is a precursor of fentanyl, in seven cases (range: 23 pg/mg-2200pg/mg), confirming that these two molecules are often present in hair samples from people exposed to fentanyl, as a by-product of either metabolism or synthesis of fentanyl. One further fentalog, despropionyl-p-fluorofentanyl, was detected in one sample at the concentration of 25 pg/mg, together with fentanyl at 2900 pg/mg. The sporadic occurrence of the other fentalogs has different possible explanations: (i) low prevalence within the populations assessed at the time of the sample collection, (ii) poor incorporation or low stability in the keratin matrix, and/or (iii) insufficient sensitivity of the analytical method in relation to the low effective dosage. A summary of results is presented in Table 2.

Three prescription opioids were detected in seven segments (8.4%), usually together with fentanyl. Only two segments (collected from the same subject) followed a different trend, with fentanyl below the LOD and hydrocodone

measured at 37 pg/mg and 46 pg/mg, respectively. The trace level of fentanyl detected might indicate unintended exposure as an adulterant or contaminant if the drugs were obtained illegally. The nitazene compounds and brorphine, which appeared to have a significant presence in the NPS opioid market in 2019 and 2020 [3], were not detected. Although the number of samples analyzed in this study was relatively small, the non-detection of emerging opioids is coherent with the modern drug scenario, in which the typical life cycle of a new substance is generally short. Most new drugs appear to remain in circulation less than six months and up to one year but then rapidly decline, disappear, and then are replaced by other newly emerging synthetic substances [3].
Novel and prescription synthetic opioid identification was much less common in the 60 samples collected in Italy. In particular, fentalogs were never detected, while only five segments (5.5%) were positive for at least one compound such as hydrocodone, oxycodone, or tramadol. Two subjects were positive for all three prescription opioids. In one case, two segments were obtained from the same sample, showing the same trend of consumption (hydrocodone at 19 and 22 pg/mg, respectively).

10.3.2 Testing for common drugs

Samples were considered positive in accordance with international cut-offs for parent drugs and metabolites [35]. In the group of samples from the US, cocaine was the most prevalent substance found in the samples, with 19 segments (22.9%) resulting above the cut-off for either cocaine or its metabolite benzoylecgonine (BZE).

Cocaethylene was detected above 0.05 ng/mg only in five cases. The 6-acetylmorphine (6-MAM) as marker of heroin use was identified in five segments (6.0%), and all samples positive for 6-MAM also tested positive for BZE. While it is not possible to discriminate whether cocaine and heroin were taken simultaneously or in rapid sequence, the fact that the two substances were used in the same six months is remarkable. Use of cannabis-derived products was verified by the presence of A^9 -tetrahydrocannabinol (THC) in only eight segments (9.6%). It is noteworthy that the frequent use of amphetamine/methamphetamine/MDMA as a whole was observed in 26 segments (31.3%), of which 14 tested positive also to cocaine.

Table 2 *Summary of results obtained from 60 real hair samples collected in the US. All concentrations are in pg/mg. Two segments from the same sample are referred to as a and b*

Sample	**Fentanyl**	**Norfentanyl**	**4-ANPP**	**β-OH-fentanyl**	**Other fentalogs**	**Prescription opioids**
1	90	-	-	-	-	-
2	384	43	29	71	-	-
3	501	69	-	-	-	-
4a	2900	1700	747	1400	Acetyl fentanyl 157 Despropionyl p-fluorofentanyl 25	Tramadol 1400
4b	2900	2000	737	1300	Acetyl fentanyl 129	Tramadol 1200
5a	-	-	-	-	-	Hydrocodone 37
5b	-	-	-	-	-	Hydrocodone 46
6	15	-	-	-	-	Hydrocodone 26 Tramadol 13 Oxycodone 128
7	13	-	-	-	-	-
8	103	-	-	-	-	-
9	331	32	23	-	-	-
10	1800	150	73	60	-	-
11	143	-	-	-	-	-
12a	2800	268	223	17	-	Hydrocodone 78 Tramadol 25
12b	7300	2300	2200	980	Acetyl fentanyl 265	Tramadol 1000
13	61	-	-	-	-	-

Ketamine was detected in only one segment, in contradiction with the increasing trend recently reported, especially in the New York City area [36, 37].

An exhaustive comparison of patterns of drug use between the US and Italy based on the group of results hereby presented is not possible, nor is this the goal of our research. However, a striking difference emerges from the results obtained from the samples collected in Italy. Among 91 segments, a total of 41 (45.1%) tested positive for exposure ketamine in the range 245-8500 pg/mg (mean value 2324 pg/mg, median 1496 pg/mg). The large majority of samples positive for ketamine also tested positive for MDMA and/or cocaine, showing a trend of potential co-use of stimulating and dissociative substances. Overall, THC was still the most prevalent parent drug, with 73 positive segments (80.2%). The use of heroin, proven by the presence of 6-MAM, was identified in eight segments (8.8%).

Correlation matrices for the measured levels of common drugs in the Italian population of hair samples are presented in Figure 1. High correlation coefficients suggest that subjects who were more exposed to ketamine were more exposed to amphetamine, possibly due to a co-use of the substances in certain recreational contexts.

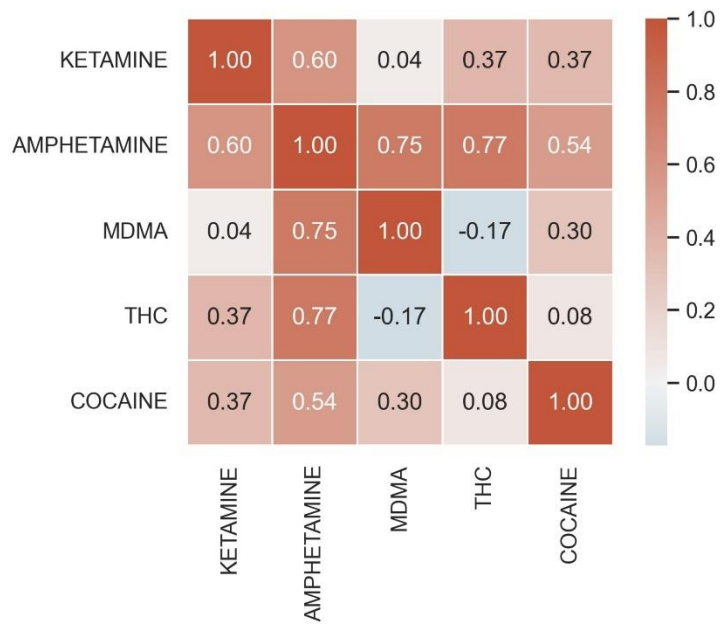


Figure 1: *Correlation matrix for the measured levels of common drugs of abuse in the Italian population.*

In this study, we investigated samples from two different populations within these two countries, and results should not be directly used to indicate prevalence of drug use, as we used purposive sampling. As such, results are not generalizable to US or Italian populations, but rather present a snapshot of drug use within select populations in each country. Indeed, all cases of synthetic opioid detection were in the US, but we focused on a sample in which many participants reported recent synthetic opioid use. Most use of common party drugs such as ketamine and MDMA were detected in the Italian sample, but we must keep in mind that these were individuals receiving harm reduction services associated with nightlife. People who attend nightclubs in particular tend to report higher prevalence of use of such drugs than the general population [38-40]. Prevalence of past-year ketamine use among young adults is estimated to be <1% in both the US and in Europe [14, 41], although seizures of the drug appear to be increasing at a similar rate [14, 42]. While prevalence of past-year heroin use is estimated to be <1% in both the US and Europe [14, 43], in the US, synthetic opioids such as fentanyl analogs indeed are more available and have been involved in hundreds of thousands of deaths in recent years [13, 44]. As such, it is important to note both where biological specimens are collected but also the populations from which they are obtained. This is because results will vary in particular across high-risk populations (e.g., nightclub attendees, people who utilize drug checking services) and the general population.

10.4 Conclusions

Hair analysis can help to retrospectively explore trends in drug use, and incorporating hair testing into epidemiology studies or surveillance studies can provide opportunity for relatively rapid dissemination of results (including public alerts) to both the scientific community and populations at risk. In this analysis focusing on hair samples collected in the US and in Italy, we tested for use or exposure to fentalogs, prescription opioids, and more common controlled drugs including ketamine. Results suggest that currently fentalogs continue to be a US (or North American) phenomenon, with no detected cases in Italy despite high prevalence of detection of other drugs within this country.

Thanks to the longer detection window of hair (in comparison to much shorter detection windows provided by urine, saliva, and blood), drug exposures occurring 1-2 weeks up through a year before hair collection can provide retrospective results to inform scientists and public health practitioners about the diffusion of drugs in their countries. Hair analysis results based on real hair

samples can provide information regarding both intentional and unintentional exposure to NPS/NSO, both with and without use of common controlled drugs. As such, hair testing can serve as an addition to epidemiology studies that seek to incorporate biological testing with survey research. The combination of surveys and hair testing can thus be used to monitor drug exposure in a more effective manner than using surveys or biological testing alone.

References

[1] D. Ciccarone, The triple wave epidemic: Supply and demand drivers of the US opioid overdose crisis, Int J Drug Policy. 2019;71:183-8. https://doi.org/10.1016/j.drugpo.2019.01.010

[2] M. Zoorob, Fentanyl shock: The changing geography of overdose in the United States. Int J Drug Policy. 2019; 70:40-6. https://doi.org/10.1016/j.drugpo.2019.04.010

[3] MM. Vandeputte, AJ. Krotulski, DM. Papsun, BK. Logan, CP. Stove, The rise and fall of isotonitazene and brorphine: Two recent stars in the synthetic opioid firmament. J Anal Toxicol. 2022;46:115-21. https://doi.org/10.1093/jat/bkab082

[4] MK. Laing, Ti L, A. Marmel, S. Tobias, AM. Shapiro, R. Laing, An outbreak of novel psychoactive substance benzodiazepines in the unregulated drug supply: Preliminary results from a community drug checking program using point-of-care and confirmatory methods. Int J Drug Policy. 2021;93:103169. https://doi.org/10.1016/j.drugpo.2021.103169

[5] W. Tupper, K. McCrae, .I. Garber, M. Lysyshyn, E.Wood, Initial results of a drug checking pilot program to detect fentanyl adulteration in a Canadian setting. Drug Alcohol Depend. 2018;190:242- https://doi.org/10.1016/j.drugalcdep.2018.06.020

[6] S. Tobias, AM. Shapiro, CJ. Grant, P. Patel, M. Lysyshyn, Drug checking identifies counterfeit alprazolam tablets. Drug Alcohol Depend. 2021;218:108300. https://doi.org/10.1016/j.drugalcdep.2020.108300

[7] C. May, C. Downey, JD. Power, PV. Kavanagh, An unusual detection of tert-butyl-4-anilinopiperidine-1-carboxylate in seizures of falsified 'Xanax' tablets and in items in a suspected heroin seizure submitted by Irish law enforcement. Drug Test Anal. 2020;12(9):1387-92. https://doi.org/10.1002/dta.2884

[8] JJ. Palamar, A. Salomone, R. Bigiarini, M. Vincenti, P. Acosta, B. Tofighi, Testing hair for fentanyl exposure: A method to inform harm reduction behavior among individuals who use heroin. Am J Drug Alcohol Abuse. 2019;45(1):90-6. https://doi.org/10.1080/00952990.2018.1550652

[9] P. DiSalvo, G. Cooper, J. Tsao, M. Romeo, LK. Laskowski, G. Chesney, Fentanyl-contaminated cocaine outbreak with laboratory confirmation in New York City in 2019. Am J Emerg Med. 2020;40:103-5. https://doi.org/10.1016/j.ajem.2020.12.002

[10] JN. Park, E. Rashidi, K. Foti, M. Zoorob, S. Sherman, GC. Alexander, Fentanyl and fentanyl analogs in the illicit stimulant supply: Results from U.S. drug seizure data, 2011-2016. Drug Alcohol Depend. 2021;218:108416. https://doi.org/10.1016/j.drugalcdep.2020.108416

[11] SG. Mars, D. Rosenblum, D. Ciccarone, Illicit fentanyls in the opioid street market: Desired or imposed? Addiction. 2019;114(5):774-80. https://doi.org/10.1111/add.14474

[12] Centers for Disease Control and Prevention. U.S. overdose deaths in 2021 increased half as much as in 2020 – but are still up 15%. 2022. Available from: https://www.cdc.gov/nchs/pressroom/nchs_press_releases/2022/202205.htm.

[13] JJ. Palamar, D. Ciccarone, C. Rutherford, KM. Keyes, TH. Carr, LB. Cottler, Trends in seizures of powders and pills containing illicit fentanyl in the United States, 2018 through 2021. Drug Alcohol Depend. 2022:109398. https://doi.org/10.1016/j.drugalcdep.2022.109398

[14] European Monitoring Centre for Drugs and Drug Addiction. European Drug Report 2023: Trends and Developments; 2023. Available from: https://www.emcdda.europa.eu/publications/european-drug-report/2023_en.

[15] M. Kariisa, L. Scholl, N. Wilson, P. Seth, B. Hoots, Drug overdose deaths involving cocaine and psychostimulants with abuse potential - United States, 2003-2017. MMWR Morb Mortal Wkly Rep. 2019;68(17):388-95. https://doi.org/10.15585/mmwr.mm6817a3

[16] S. Martinez, JD. Jones, L. Brandt, ANC. Campbell, R. Abbott, SD.Comer, The increasing prevalence of fentanyl: A urinalysis-based study among individuals with opioid use disorder in New York City. Am J Addict. 2021;30(1):65-71. https://doi.org/10.1111/ajad.13092

[17] JJ. Palamar, A. Salomone, MJ. Barratt, Drug checking to detect fentanyl and new psychoactive substances. Curr Opin Psychiatry. 2020;33(4):301-5. https://doi.org/10.1097/yco.0000000000000607

[18] H. Bach, V. Jenkins, A. Aledhaim, S. Moayedi, SM. Schenkel, HK. Kim, Prevalence of fentanyl exposure and knowledge regarding the risk of its use among emergency department patients with active opioid use history at an urban medical center in Baltimore, Maryland. Clin Toxicol. 2020;58(6):460-5. https://doi.org/10.1080/15563650.2019.1657583

[19] A. Salomone, R. Bigiarini, JJ. Palamar, C. McKnight, L. Vinsick, E. Amante, Toward the interpretation of positive testing for fentanyl and its analogs in real hair samples: Preliminary considerations. J Anal Toxicol. 2020;44(4):362-9. https://doi.org/10.1093/jat/bkz102

[20] IA. Larabi, M. Martin, I. Etting, G. Pfau, Y. Edel, JC. Alvarez. Development and validation of liquid chromatography-tandem mass spectrometry targeted screening of 16 fentanyl analogs and U-47700 in hair: Application to 137 authentic samples. Drug Test Anal. 2020;12(9):1298-308. https://doi.org/10.1002/dta.2868

[21] J. Chas, R. Bauer, IA. Larabi, G. Peytavin, P. Roux, E. Cua, Evaluation of drug abuse by hair analysis and self-reported use among MSM under PrEP: Results from a French substudy of the ANRS-IPERGAY Trial. J Acquir Immune Defic Syndr. 2021;86(5):552-61. https://doi.org/10.1097/qai.0000000000002610

[22] KB. Palmquist, MT. Truver, EN. Shoff, AJ. Krotulski, MJ. Swortwood, Review of analytical methods for screening and quantification of fentanyl analogs and novel synthetic opioids in biological specimens. J Forensic Sci. 2023; online ahead of print. https://doi.org/10.1111/1556-4029.15282

[23] NA. Platosz, TM. Binz, MR. Baumgartner, E. Lendoiro, A. de Castro, M. Concheiro, Quantification of Classic, Prescription and synthetic opioids in hair by LC-MS-MS. J Anal Toxicol. 2021;45(9):943-9. https://doi.org/10.1093/jat/bkaa152

[24] F. Freni, M. Moretti, D. Radaelli, C. Carelli, AMM. Osculati, L. Tronconi, Determination of fentanyl and 19 derivatives in hair: Application to an Italian population. J Pharm Biomed Anal. 2020;189:113476. https://doi.org/10.1016/j.jpba.2020.113476

[25] MDM. Ramírez Fernández, SMR. Wille, D. Jankowski, V. Hill, N. Samyn, Development of an UPLC-MS/MS method for the analysis of 16 synthetic opioids in segmented hair, and evaluation of the polydrug history in fentanyl analogue users. Forensic Sci Int. 2020;307:110137. https://doi.org/10.1016/j.forsciint.2019.110137

[26] I. Rammohan, S. Jain, Sun S, Marks C, Milloy MJ, Hayashi K, et al. Latent polydrug use patterns and the provision of injection initiation assistance among people who inject drugs in three North American settings. Drug Alcohol Rev. 2023. https://doi.org/10.1111/dar.13687

[27] J. van Amsterdam, M. Pierce, W.van den Brink, Predictors and motives of polydrug use in opioid users. A narrative review. Curr Opin Psychiatry. 2023;36(4):301-7. https://doi.org/10.1097/yco.0000000000000875

[28] LB. Cottler, BA. Goldberger, SJ. Nixon, CW. Striley, E. Barenholtz, ND. Fitzgerald, Introducing NIDA's new National Drug Early Warning System.

Drug Alcohol Depend. 2020;217:108286. https://doi.org/10.1016/j.drugalcdep.2020.108286

[29] A. Salomone, L. Tsanaclis, R. Agius, P. Kintz, MR. Baumgartner, European guidelines for workplace drug and alcohol testing in hair. Drug Test Anal. 2016;8(10):996-1004. https://doi.org/10.1002/dta.1999

[30] D. Di Corcia, A. Salomone, E. Gerace, Analysis of drugs of abuse in hair samples by ultrahigh-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). Methods Mol Biol. 2018;1810:107-14. https://doi.org/10.1007/978-1-4939-8579-1_10

[31] D. Di Corcia, F. D'Urso, E. Gerace, A. Salomone, M. Vincenti, Simultaneous determination in hair of multiclass drugs of abuse (including THC) by ultra-high performance liquid chromatography-tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 2012;899:154-9. https://doi.org/10.1016/j.jchromb.2012.05.003

[32] A. Salomone, JJ. Palamar, E. Gerace, D. Di Corcia, M.Vincenti, Hair testing for drugs of abuse and new psychoactive substances in a high-risk population. J Anal Toxicol. 2017;41(5):376-81. https://doi.org/10.1093/jat/bkx020

[33] A. Salomone, JJ. Palamar, Bigiarini R, Gerace E, Di Corcia D, Vincenti M. Detection of fentanyl analogs and synthetic opioids in real hair samples. J Anal Toxicol. 2019;43(4):259-65. https://doi.org/10.1093/jat/bky093

[34] A. Salomone, D. Di Corcia, P. Negri, M. Kolia, E. Amante, E. Gerace, Targeted and untargeted detection of fentanyl analogues and their metabolites in hair by means of UHPLC-QTOF-HRMS. Anal Bioanal Chem. 2021;413(1):225-33. https://doi.org/10.1007/s00216-020-02994-x

[35] D. Favretto, G. Cooper, M. Andraus, F. Sporkert, R. Agius, B. Appenzeller, The Society of Hair Testing consensus on general recommendations for hair testing and drugs of abuse testing in hair. Drug Test Anal. 2023; online ahead of print. https://doi.org/10.1002/dta.3526

[36] E. Arango, A. Toriello, Z. Rosario, G.Cooper, Increasing prevalence of ketamine in drivers in New York City including the identification of 2-fluoro-deschloroketamine. J Anal Toxicol. 202145(8):799-806. https://doi.org/10.1093/jat/bkab057

[37] JJ. Palamar, KM. Keyes, Trends in drug use among electronic dance music party attendees in New York City, 2016-2019. Drug Alcohol Depend. 2020;209:107889. https://doi.org/10.1016/j.drugalcdep.2020.107889

[38] JJ. Palamar, A. Le, C. Rutherfold, KM. Keyes, Exploring potential bellwethers for drug-related mortality in the general population: A case for sentinel surveillance of trends in drug use among nightclub/festival attendees. Subst Use Misuse. 2022;58(2):188-97. https://doi.org/10.1080/10826084.2022.2151315

[39] JJ. Palamar, C. Rutherford, CM. Cleland, KM. Keyes, Concerts, bars, parties, and raves: Differential risk for drug use among high school seniors according to venue attendance. Subst Abus. 2022;43(1):785-91. https://doi.org/10.1080/08897077.2021.2010253

[40] JJ. Palamar, M. Griffin-Tomas, DC. Ompad, Illicit drug use among rave attendees in a nationally representative sample of US high school seniors. Drug Alcohol Depend. 2015;152:24-31. https://doi.org/10.1016/j.drugalcdep.2015.05.002

[41] JJ. Palamar, C. Rutherford, KM. Keyes, Trends in ketamine use, exposures, and seizures in the United States. Am J Public Health. 2021;111(11):2046-9. https://doi.org/10.2105/ajph.2021.306486

[42] JJ. Palamar, ST. Wilkinson, TH. Carr, C. Rutherford, LB. Cottler. Trends in illicit ketamine seizures in the US from 2017-2022. JAMA Psychiatry. 2023;80(7):750-751. https://doi.org/10.1001/jamapsychiatry.2023.1423

[43] Center for Behavioral Health Statistics and Quality. Results from the 2021 National Survey on Drug Use and Health: Detailed tables. Rockville, MD: Substance Abuse and Mental Health Services Administration; 2022. Available from https://www.samhsa.gov/data/report/2021-nsduh-detailed-tables.

[44] Hedegaard H, Miniño AM, Warner M. Drug overdose deaths in the United States, 1999-2019. NCHS Data Brief. 2020;394:1-8. Available from https://www.cdc.gov/nchs/data/databriefs/db394-H.

Supplementary materials

Table S1 *Limits of Detection (LOD) for the screened compounds. LOD were calculated from five injections of the lowest point of calibration curve.*

Compound	LOD (pg/mg)
4-ANPP	1.7
Acetylfentanyl	2.8
Carfentanyl	2.2
Fentanyl	3.7
Furanilfentanyl	1.6
Hydrocodone	6.2
Norfentanyl	3.3
Remifentanyl	0.6
Tramadol	4..3

Alfentanyl	0.8
Oxycodone	1.7
Sufentanyl	3.5
U-47700	3.6
4-F-butylfentanyl	2.2
4-methylfentanyl	1.0
Acrylfentanyl	5.1
AH-7921	3.5
Butyrylfentanyl	1.8
Ciclopropylfentanyl	2.8
MT-45	1.5
Ocfentanyl	1.6

3-methyl Norfentanyl	1.2
Acetyl norfentanyl	1.7
Butyrylfentanyl carboxy metabolite	1.9
Despropionyl-p-fluorofentanyl	3.1
Valeryl p-fluoro fentanyl	3.5
Beta-OH-fentanyl	0.8
Beta-phenyl Fentanyl	1.2
Butiryl Norfentanyl	1.7
Furanyl Norfentanyl	1.2
Methoxyacetyl norfentanyl	5.5
OH-ThioFentanyl	1.0
Phenylacetyl fentanyl	5.0

Phenyl fentanyl	1.8
Brorphine	0.7
Butonitazene	3.0
Etodesnitazene	5.4
Flunitazene	1.6
Metodesnitazene	8.0
Metonitazene	3.1
N-pyrrolidino etonitazene	4.1
Isotonitazene	5.6

Table S2 *Precision and trueness values at three different concentrations, calculated with five replicates. A partial validation was performed for the compounds which were detected in real samples.*

Compound	**Concentration (pg/mg)**	**Precision (CV%)**	**Trueness (BIAS%)**

Fentanyl	10	14%	11%
	50	15%	-4%
	200	18%	-1%
Norfentanyl	10	5%	-20%
	50	6%	8%
	200	7%	0.1%
4-ANPP	10	18%	14%
	50	17%	2%
	200	12%	5%
β-OH-fentanyl	10	19%	15%
	50	20%	-8%
	200	20%	-1%
Acetyl fentanyl	10	5%	-14%
	50	8%	14%
	200	12%	-4%
Despropionyl p-fluorofentanyl	10	17%	18%
	50	19%	-17%
	200	17%	-0.2%
Tramadol	10	12%	-18%
	50	15%	9%
	200	10%	-1%
Hydrocodone	10	20%	-20%
	50	18%	16%
	200	19%	-2%
Oxycodone	10	14%	-12%
	50	7%	-2%
	200	15%	-1%

Chapter 11 | **Five cases of unintentional exposure to BZO-4en-POXIZID among nightclub attendees in New York City**

Joseph J. Palamar[1*], Marta Massano[2], Alberto Salomone[2,3]

[1]*Department of Population Health, New York University Grossman School of Medicine, New York, New York, USA*

[2]*Department of Chemistry, University of Turin, Italy*

[3]*Centro Regionale Antidoping, Orbassano (TO), Italy*

The content of this Chapter is included in one published manuscript:

Five cases of unintentional exposure to BZO-4en-POXIZID among nightclub attendees in New York City
Joseph J. Palamar, Marta Massano, Alberto Salomone
Journal of Analytical Toxicology
https://doi.org/10.1093/jat/bkad086

Abstract

A new class of synthetic cannabinoids called OXIZIDs has emerged in recent years. This class consists of compounds with oxindole cores and hydrazide/hydrazone linker moieties and has often been described as being designed to circumvent a Chinese class-wide ban that was effective as of 1 July 2021. However, through hair testing of nightclub attendees in New York City—a high-risk population for recreational drug use— we have evidence suggesting exposures to an OXIZID called BZO-4en-POXIZID (4en-pentyl MDA-19) prior to the effective ban. Through analysis of 6 cm segmented hair samples from attendees collected in 2021, we detected five cases of exposure. Specifically, we detected a cluster of three cases based on hair samples collected on 20 June 2021, and then two additional cases from samples collected on 16 July 2021. Four of these hair samples were long enough to analyze two 6 cm hair segments (representing approximately two 6-month timeframes) and three of four of these cases tested positive for repeated exposure (for an estimated exposure over 6 months prior to hair collection). All cases included young adult females reporting past-year cannabis use but all tested negative for tetrahydrocannabinol exposure. Three cases also reported past-year use of cocaine, ecstasy, and/or ketamine, and four cases tested positive for exposure to cocaine, 3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxyamphetamine (MDA), methamphetamine and/or eutylone. These subjects were exposed to BZO-4en-POXIZID—likely as an adulterant in other drugs, and these cases are among the first documented cases which occurred approximately half a year before the Chinese legislative ban.

11.1 Introduction

A new class of synthetic cannabinoids called OXIZIDs (Figure 1) has emerged in recent years. This class consists of compounds with oxindole cores, and the hydrazide/hydrazone linker moieties which have often been described as being designed to circumvent a Chinese class-wide synthetic cannabinoid (SC) ban [1–5]. The ban was announced on 11 May 2021, and it has been effective as of 1 July 2021 [6]. Since the ban targets all compounds containing one of seven general core structures, manufacturers appear to have moved towards the synthesis of SCs with alternative core components. These include SCs with oxindole cores and hydrazide/hydrazone linker moieties not banned by the recent Chinese legislation. These SCs are based on the substance MDA-19 (2) and include 4en-pentyl MDA-19 (BZO-4en-POXIZID).

A decade ago, SC use was highly prevalent in the USA. In 2011, after cannabis, this class of drugs was the most prevalent drug class used among 12th graders, although the prevalence of past-year use substantially decreased from 11.4% in 2011 to 3.2% in 2022 [7]. The number of SC related poisonings in the USA also substantially decreased from 6,968 reported poisonings in 2011 to 524 in 2022 [8]. However, there have been notable outbreaks of SC intoxications in recent years, including New York City (NYC) [9]. In addition, SCs included as adulterants in other drugs including cannabis products has occurred throughout parts of the world [10–12].

There have also been clusters of overdoses in the USA in which purported ecstasy (3,4methylenedioxymethamphetamine [MDMA]) was adulterated with SCs [13]. As such, there has been increasing concern among international public health organizations about illicitly purchased drugs (including cannabis) containing SCs [14].

Since the Chinese class-wide legislation on SCs in 2021, few studies have investigated the distribution of OXIZIDs, or more specifically BZO-4en-POXIZID (4en-pentyl MDA19) [1–5]. This compound was first synthesized over a decade ago and reportedly has a high affinity for type 1 (CB_1) and type 2 (CB_2) cannabinoid receptors [15]. It is not currently controlled in the USA, although a recent law was passed in North Dakota banning the substance [16]. Furthermore, only a few documented submissions in the USA have contained BZO-4en-POXIZID. On 14 November 2021, US Customs and Border Protection inspected the first known batch of BZO-4en-POXIZID [17]. Two parcels were shipped from China and inspected in Maryland, each containing ∼1kg of powder, with one package testing positive for BZO-4enPOXIZID. The second submission received by NPS Discovery in Philadelphia, PA from Indianapolis, ID tested positive for BZO-4en-POXIZID [18]. This compound was also identified by the US Drug Enforcement Administration in the first quarter of 2022 in the southern region of the country [19].

In this report, we focus on five cases of detected BZO-4en-POXIZID exposure within participants surveyed and hair tested in NYC as part of an epidemiological study monitoring drug use and new psychoactive substance (NPS) exposure among nightclub attendees.

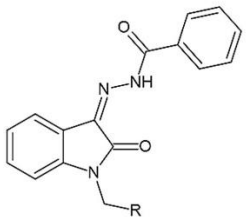


***Figure** 1. Chemical structure of the OXIZID class.*

11.2 Materials and methods

11.2.1 Study details

In this study, adults (age ≥18 years) were surveyed before entering nightclubs and dance festivals in NYC. Participants were given an anonymous survey inquiring about past-year use of approximately 100 drugs, and participants were asked if they were willing to provide a hair sample for subsequent analysis. A total of 350 adults were surveyed in 2021 (from July through December); 100 participants provided a hair sample, and of these, 69 samples were adequate in size to be tested. Hair analysis was conducted in March 2023. All methods were approved by the first author's institutional review board.

11.2.2 Collection of specimens

Using clean scissors, staff cut a small amount of hair from the vertex region, as close to the scalp as possible. All but one participant provided head hair, with one providing armpit hair. One sample was collected from each individual. Short hair (e.g., <2cm) did not preclude hair submission eligibility. Hair samples were folded into a piece of tin foil and sealed in envelopes labelled with the participant's study ID number. Hair samples were stored in a locked drawer at room temperature before being shipped to the toxicology lab for analysis.

11.2.3 Reagents and standards

All chemicals, including methanol, formic acid, and acetonitrile, were purchased from Sigma-Aldrich (Milan, Italy) (purity ≥ 99% for all) while ultra-pure water was obtained using a Milli-Q® UF-Plus apparatus (Millipore, Bedford, MA, USA).

11.2.4 Sample preparation

All hair samples were treated with a procedure developed for the keratin matrix. Approximately 50mg of hair was decontaminated by an initial wash with 1mL dichloromethane followed by a second wash with 1mL methanol, each performed under 3min of stirring. After complete removal of the solvent wash, the hair was dried at room temperature overnight. The samples were then pulverized using six steel balls stirring in a Precellys® homogenizer (Bertin Technologies, France). Half a milliliter of methanol and 2.5µL of an internal standard mixture (0.01 ng/mg) were added to the pulverized samples, and the specimen tubes were sealed and incubated at 55°C±5°C for 15h. The organic phase was collected, and an aliquot of 5µL was directly injected into the ultra-high-performance liquid chromatography (UHPLC) system.

11.2.5 Instrumentation

Targeted screening for traditional drugs of abuse was performed using previously published methods [20, 21]. A list of target analytes (n=155) is presented in Supplementary Table S1. The untargeted approach was based on an existing method [22]. All methods have been validated and have been routinely used in our laboratory. In particular, the selectivity was evaluated, as recently proposed [23].

UHPLC separation was performed on the SCIEX ExionLC™ AC system (Sciex, Darmstadt, Germany) using a Phenomenex Kinetex C18 column (100 × 2.1mm, 1.7µm) maintained at 45°C. The mobile phase was a mixture of water (A) and acetonitrile (B), both with 0.01% of formic acid. The LC flow rate was set at 0.5mL/min. Mobile phase starting conditions were eluted under the following linear gradient conditions: starting conditions were 5% B hold for 0.5min and increased to 95% over 7.5min. Using a linear gradient, 95% B was maintained for 0.5min, and a final re-equilibration was programmed for 1.5min to the initial gradient conditions. The total run time was 10min. All analyses were performed using a SCIEX X500R quadrupole time of-flight mass spectrometer (Sciex, Darmstadt, Germany) equipped with a Turbo VTM source operated in positive ion electrospray ionization mode. Data acquisition involved a preliminary TOF-MS high-resolution full scan followed by a Sequential Window Acquisition of All Theoretical Mass Spectra (SWATH)™ acquisition protocol which used a variable window setup (18 windows covering mass range from 100.0 to 600.0m/z at 0.025 resolving power), resulting in a final cycle time of 0.933 s. The variable windows technique allows the reduction of the size of the Q1 window in order to

further improve the quality of the SWATH acquisition data, while maintaining a complete coverage of the mass range and optimal cycle times. In this case, it was decided to use 30Da windows as they allowed an optimal acquisition of the peaks, improving the specificity and reducing interference from possible co-eluting analytes. The qualitative identification of the target analyte BZO-4enPOXIZID was based on the identification of precursor ion (m/z 334.1550) and characteristic fragment ion m/z values with mass error <5 ppm as identification parameters. Data were acquired using SCIEX OS 1.5 Software and raw data files were processed using the MarkerView^{TM} software from Sciex.

11.3 Case details

The five cases were surveyed (with hair samples collected) on 20 June 2021 (*n*=3) and on 16 July 2021 (*n*=2), with 2–3 participants surveyed in June completing the survey at about the same time, suggesting they might have arrived at the venue together. As shown in Table 1, all cases were female, and four provided a hair sample ≥12cm in length. This permitted segmental analysis—with four cases each having two analyzable (i.e., sufficient quantity) hair segments, corresponding roughly to 0–6 months and 6–12 months before collection, respectively. All cases reported past-year cannabis use, and none reported past-year SC use. Cases 1, 2 and 4 reported having vaped in the past year, but none reported vaping of cannabis or tetrahydrocannabinol (THC) products. Case 1 also reported use of cocaine, ecstasy, amphetamine (nonmedical use), ketamine and 2C-I; Case 2 also reported use of ecstasy; and Case 5 also reported use of cocaine and ecstasy.

All five cases tested positive for BZO-4en-POXIZID exposure. Three of the four cases which provided enough hair for segmental analysis tested positive in two segments, with these segments corresponding roughly to the past 6 months and the previous six months. In addition, three cases also tested positive for exposure to cocaine, MDMA, amphetamine, ketamine and/or eutylone, and these three cases all reported past-year use of various party drugs. Cases 3 and 4, however, only reported cannabis use, with one testing positive for exposure to methamphetamine and the other not testing positive for exposure to any other drugs.

Case	Date of collection	Sex	Age	Past-year reported drug use	Toxicology results (ng/mg)
1	20 June 2021	F	27	Cannabis Ecstasy Cocaine Amphetamine Ketamine 2C-I	**Segment 1:** BZO-4en-POXIZID Cocaine 0.64 BZE 0.18 Amphetamine 1.0 Ketamine 0.21 **Segment 2:** BZO-4en-POXIZID Cocaine 0.87 BZE 0.36 Ketamine 0.19
2	20 June 2021	F	23	Cannabis Ecstasy	**Segment 1:** BZO-4en-POXIZID Cocaine 0.16 Amphetamine 0.87
3	20 June 2021	F	36	Cannabis	**Segment 1:** BZO-4en-POXIZID **Segment 2:** BZO-4en-POXIZID
4	16 July 2021	F	25	Cannabis	**Segment 1:** BZO-4en-POXIZID Methamphetamine 0.17 **Segment 2:** BZO-4en-POXIZID
5	16 July 2021	F	23	Cannabis Ecstasy Cocaine	**Segment 1:** BZO-4en-POXIZID Cocaine 0.06 BZE 0.23 MDMA 2.60 MDA 1.0 Eutylone 0.28 **Segment 2:** Cocaine 0.08 BZE 0.62 MDMA 0.93 ketamine 0.14 MDA 0.17 Eutylone 0.03

Table 1 *Case Details*

11.4 Results and discussion

11.4.1 BZO-4en-POXIZID identification

Because of the continuous fluctuation of the NPS landscape, targeted analysis based on a well-defined panel of analytes may miss the identification of molecules which are present in analyzed samples. Taking advantage of untargeted high resolution-mass spectrometry (HRMS)-based screening, a qualitative identification of NPS (https://www.cfsre.org/npsdiscovery/scope-recommendations) was performed. The candidate BZO-4en-POXIZID was singled out from the chromatographic profile of the full-scan analysis by checking the exact mass of the corresponding protonated molecular ion. Then, the elemental composition of the relative fragment ions and the rationality of its fragmentation pattern were checked in the MS/HRMS spectra to confirm the presumptive molecule identification (HRMS fragmentation patterns are presented in Figure 2). The fragmentation spectrum was then compared to results from a recently published study [2].

Among the 69 analyzable samples, we detected five cases of exposure to BZO-4en-POXIZID which appear to have occurred prior to the Chinese ban on SCs. In fact, in three of four cases providing hair long enough for segmental analysis, repeated exposure was detected suggesting additional exposure six or more months prior to hair collection. This provides evidence that BZO-4en-POXIZID was available on the recreational drug market (possibly in the NYC area) months before such compounds were thought to originally have emerged. To our knowledge, the first cases of BZO-4en-POXIZID discovered prior to our study were in November 2021 and January 2022, respectively [17, 18]. None of the five cases testing positive for exposure reported past-year SC use, suggesting unintentional exposure. While inaccurate self-report (e.g., underreporting of drug use) is possible, the items on the survey that assess past-year drug use have been shown to have strong or almost perfect test–retest reliability (κ=0.88–1.00) [24]. People in this high-risk population also tend to use common party drugs such as ecstasy, but SC use is relatively rare [25]. Further, at the end of the survey, all five cases answered that they responded to all questions honestly. This is important because it has been found that those who report answering less than all questions honestly on this survey are more likely to provide discordant responses regarding drug use [24]. As such, what is more likely was unintentional exposure to BZO-4en-POXIZID as an adulterant in other drugs used.

Previous studies have noted that BZO-4en-POXIZID (and pentyl MDA-19) has tended to be detected in the form of yellow powder [1, 3, 26] and one lab detected such compounds in e-liquid for vaping [1]. If in powder form, it is possible that the drug was mixed into drugs purported to be ecstasy,

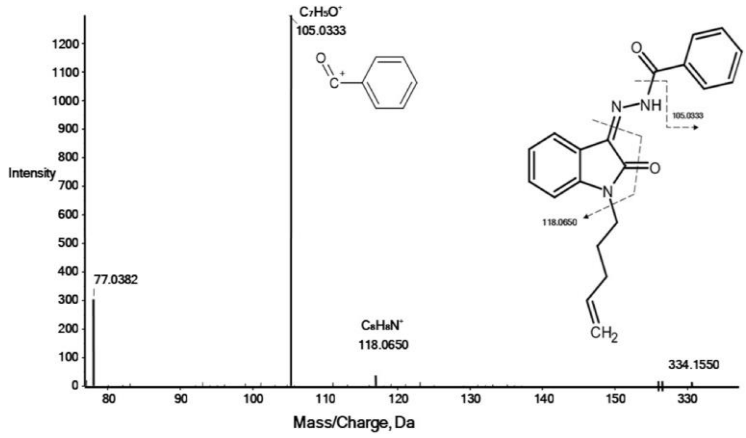


***Figure** 2. HRMS fragmentation pattern of BZO-4en-POXIZID detected in the samples.*

cocaine, or ketamine, although if BZO-4en-POXIZID powder was yellow in nature, it is possible that the mixture could have been visible to consumers ingesting the substances. Although three cases reported past-year vaping (likely of nicotine), none reported vaping cannabis or THC products. As such, it is unknown whether exposure through vape cartridges was likely. Most cases reported using and/or tested positive for a variety of party drugs, but despite all cases reporting cannabis use, none tested positive for THC exposure. However, THC exposure can be difficult to detect in hair particularly when use is infrequent [27, 28]. Historically, of the party drugs commonly used in this population, ecstasy in particular has been known to contain a wide variety of adulterants [29, 30], and extensive unintentional exposure to synthetic cathinones and methamphetamine in particular have been detected in hair samples submitted by people in this NYC population who use ecstasy [31]. While to our knowledge, studies have not detected SCs as adulterants in purported ecstasy, in 2015, a cluster of 11 individuals at a university in Connecticut overdosed on a drug purported to be "Molly" (powder ecstasy) which was later found to contain the SC AB FUBINACA [13]. Two students were in critical condition after the exposure, and one was revived after heart failure.

It is important to keep in mind that standard drug tests (e.g., dip sticks) cannot detect the presence of SCs so the prevalence of related poisonings from BZO-4en-POXIZID or SCs is unknown. Relatedly, many studies focusing on SC use are limited to poisoning cases, which limits ability to estimate the prevalence of *use*—regardless of whether a poisoning occurs. We are not aware whether these cases experienced adverse effects due to exposure, but we believe the strength of this epidemiology study is that we were able to detect potential clusters of repeated unknown exposure.

11.5 Conclusions

We detected five cases of unreported exposure to BZO-4enPOXIZID—likely as an adulterant in other drugs. We believe these findings are unique—first, because these were detected in a largescale epidemiology survey study rather than in poisonings; and second, we believe these are among the first detected cases of exposure—which appear to have occurred many months prior to the Chinese SC ban. We recommend that more studies monitor potential exposure to novel compounds without relying on poisonings.

References

[1] Liu, C.M., Hua, Z.D., Jia, W., Li, T. Identification of AD18, 5F-MDA-19, and pentyl MDA-19 in seized materials after the class-wide ban of synthetic cannabinoids in China. *Drug Testing and Analysis*, (2022) **14**, 307–316.

[2] S. Watanabe, S. Baginski, T. Iwai, R. Matsushita, M. Takatsu, T. Nakanishi, Systematic in vitro metabolic profiling of the OXIZID synthetic cannabinoids BZO-4en-POXIZID, BZO-POXIZID, 5F-BZO-POXIZID, BZO-HEXOXIZID and BZO-CHMOXIZID. *Journal of Analytical Toxicology*, (2023) **47**, 455–463.

[3] M.H. Deventer, K. Van Uytfanghe, I.M.J. Vinckier, F. Reniero, C. Guillou, C.P. Stove, Cannabinoid receptor activation potential of the next generation, generic ban evading OXIZID synthetic cannabinoid receptor agonists. *Drug Testing and Analysis*, (2022) **14**, 1565–1575.

[4] K.Z.H. Lee, Z. Wang, C.Y. Fong, E.M.L. Goh, H.Y. Moy, E.C.Y. Chan, Identification of optimal urinary biomarkers of synthetic cannabinoids BZO-HEXOXIZID, BZO-POXIZID, 5F-BZO-POXIZID, and BZO-CHMOXIZID for illicit abuse monitoring. *Clinical Chemistry*, (2022) **68**, 1436–1448.

[5] M.H. Deventer, K. Van Uytfanghe, I.M.J. Vinckier, F. Reniero, C. Guillou, C.P. Stove. New generic ban evading synthetic cannabinoids shaking up the market? *Toxicologie Analytique Et Clinique*, (2022) **34**, S2.

[6] United Nations Office on Drugs and Crime. (2021) *May 2021– China: Announcement to Place Synthetic Cannabinoids Under Generic Control*.

[7] R.A. Miech, L.D. Johnston, M.E. Patrick, P.M. O'Malley, J.G. Bachman, J.E. Schulenberg, *Monitoring the Future National Survey Results on Drug Use*, (2023) *1975–2022: Secondary School Students*. Ann Arbor: Institute for Social Research, The University of Michigan. https://monitoringthefuture.org/wp-content/ uploads/2022/12/mtf2022.pdf.

[8] American Association of Poison Control Centers. (2022) *Synthetic Cannabinoids Data*. https://www.aapcc.org/track/syntheticcannabinoids

[9] A.J. Adams, S.D. Banister, L. Irizarry, J. Trecki, M. Schwartz, R. Gerona, Zombie" outbreak caused by the synthetic cannabinoid AMB-FUBINACA in New York. *New England Journal of Medicine*, (2017) **376**, 235–242.

[10] E. Gerace, F. Seganti, D. Di Corcia, M. Vincenti, A. Salomone, GC-MS identification and quantification of the synthetic cannabinoid MDMB-4en-

PINACA in cannabis-derived material seized in the Turin metropolitan area (Italy). *Current Pharmaceutical Design*, (2022) **28**, 2618–2621.

[11] M.C. Monti, J. Zeugin, K. Koch, N. Milenkovic, E. Scheurer, K. Mercer-Chalmers-Bender, Adulteration of low-delta9-tetrahydrocannabinol products with synthetic cannabinoids: Results from drug checking services. *Drug Testing and Analysis*, (2022) **14**, 1026–1039.

[12] P.E. Oomen, D. Schori, K. Tögel-Lins, D. Acreman, S. Chenorhokian, A. Luf, Cannabis adulterated with the synthetic cannabinoid receptor agonist MDMB-4en-PINACA and the role of European drug checking services. *International Journal of Drug Policy*, (2022) **100**, 103493.

[13] Department of Justice U.S. Attorney's Office District of Connecticut. (2015) *Two Men Charged with Distributing Synthetic Drugs That Caused* Overdoses at Wesleyan University. https:// www.justice.gov/usao-ct/pr/former-wesleyan-student-who-distr ibuted-synthetic-drugs-caused-overdoses-sentenced-prison#:~ :text=LONERGAN%20and%20Kramer%20were%20arrested, charge%20on%20November%2012%2C%202015

[14] European Monitoring Centre for Drugs and Drug Addiction. (2022) *European Drug Report 2022: Trends and Developments*. Publications Office of the European Union, Luxembourg. https://www.emcdda.europa.eu/publications/edr/trendsdevelopments/2022_en

[15] P. Diaz, J. Xu, F. Astruc-Diaz, H.M. Pan, D.L. Brown, M. Naguib, Design and synthesis of a novel series of N-alkyl isatin acylhydrazone derivatives that act as selective cannabinoid receptor 2 agonists for the treatment of neuropathic pain. *Journal of Medicinal Chemistry*, (2008) **51**, 4932–4947.

[16] Sixty-eighth Legislative Assembly of North Dakota. (2023) *Regular Session Commencing Tuesday, January 3, 2023. Senate Bill no. 2093*. https://www.ndlegis.gov/assembly/68-2023/regular/ documents/23-8099-04000.pdf

[17] US Customs and Border Protection. (2022) *CBP Officers and Scientists Identify Two New Synthetic Cannabinoid Analogues*. https://www.cbp.gov/newsroom/local-media-release/cbp-officersand-scientists-identify-two-new-synthetic-cannabinoid

[18] The Center for Forensic Science Research & Education. (2022) *BZO-4en-POXIZID*. https://www.cfsre.org/nps-discovery/ monographs/bzo-4en-poxizid

[19] US Drug Enforcement Administration. (2022) *NFLIS Drug Snapshot: March 2022*. https://www.nflis.deadiversion.usdoj.gov/nflisdata/docs/NFLIS_Snapshot_March_2022.pdf

[20] D. Di Corcia, A. Salomone, E. Gerace, Analysis of drugs of abuse in hair samples by ultra high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). *Methods in Molecular Biology*, (2018) **1810**, 107–114.

[21] D. Di Corcia, F. D'Urso, E. Gerace, A. Salomone, M. Vincenti, Simultaneous determination in hair of multiclass drugs of abuse (including THC) by ultra-high performance liquid chromatography-tandem mass spectrometry. *Journal of Chromatography B*, (2012) **899**, 154–159.

[22] A. Salomone, D. Di Corcia, P. Negri, M. Kolia, E. Amante, E. Gerace, Targeted and untargeted detection of fentanyl analogues and their metabolites in hair by means of UHPLC-QTOF-HRMS. *Analytical and Bioanalytical Chemistry*, (2021) **413**, 225–233.

[23] S.M.R. Wille, B. Desharnais, S. Pichini, A.D. Trana, F.P. Busardò, D.K. Wissenbach, Liquid chromatography highresolution mass spectrometry in forensic toxicology: what are the specifics of method development, validation and quality assurance for comprehensive screening approaches? *Current Pharmaceutical Design*, (2022) **28**, 1230–1244.

[24] J.J. Palamar, A. Le, P. Acosta, C.M. Cleland, Consistency of self-reported drug use among electronic dance music party attendees. *Drug and Alcohol Review*, (2019) **38**, 798–806.

[25] J.J. Palamar, A. Le, C.M. Cleland, K.M. Keyes, Trends in drug use among nightclub and festival attendees in New York City, 2017-2022. *International Journal of Drug Policy*, (2023) **115**, 104001.

[26] Nacionalni Forenzicni Laboratorij. (2022)ˇ *Analytical Report: BZO-4en-POXIZID (C20H19N3O2)*.https://www.policija.si/apps/nfl_response_web/0_Analytical_Reports_final/BZO-4enPOXIZID-ID-3051-21_report.pdf

[27] M. Taylor, R. Lees, G. Henderson, A. Lingford-Hughes, J. Macleod, J. Sullivan, Comparison of cannabinoids in hair with self-reported cannabis consumption in heavy, light and non-cannabis users. *Drug and Alcohol Review*, (2017) **36**, 220–226.

[28] J.J. Palamar, A. Le, H. Guarino, P. Mateu-Gelabert, A comparison of the utility of urine- and hair testing in detecting self-reported drug use among young adult opioid users. *Drug and Alcohol Dependence*, **200**, 161–167.

[29] T.M. Brunt, C. Nagy, A. Bucheli, D. Martins, M. Ugarte, C. Beduwe, Drug testing in Europe: monitoring results of the Trans European Drug Information (TEDI) project. *Drug Testing and Analysis*, (2017) **9**, 188–198.

[30] C. Vidal Gine, M. Ventura Vilamala, I. Fornis Espinosa, C. Gil Lladanosa, N. Calzada Alvarez, A. Fito Fruitos, Crystals and tablets in the Spanish ecstasy market 2000-2014: are they the same or different in terms of purity and adulteration? *Forensic Science International*, (2016) **263**, 164–168.

[31] J.J. Palamar, A. Salomone, E. Gerace, D. Di Corcia, M. Vincenti, C.M. Cleland, Hair testing to assess both known and unknown use of drugs amongst ecstasy users in the electronic dance music scene. *International Journal of Drug Policy*, (2017) **48**, 91–98.

Supplementary materials

Table S1*Target Analytes*

Common Drugs	**Synthetic Opioids**	**Synthetic Cathinones**	**(SCs continued)**
Cocaine	Fentanyl	α-PVP	JWH-307
Benzoylecgonine	Norfentanyl	3,4-MDPV	JWH-398
MDMA	Acetyl Fentanyl	Butylone	RCS-4
MDA	Furanyl Fentanyl	Methylone	WIN-48
Ketamine	Acryl Fentanyl	Ethylone	JWH-015
Methamphetamine	Cyclopropylfentanyl	Pentylone	RCS-8
Amphetamine	4-Methyl Fentanyl	N-Ethylpentylone	WIN-55
THC	Butyryl Fentanil	Ethcathinone	AM-1220
PCP	4F-Butyryl Fentanyl	4F-Methcathinone	JWH-210
Common Psychedelics	4-ANNP	3,4-DMMC	JWH-016
Mescaline	3-Methyl Norfentanyl	Mephedrone	JWH-098
Psilocybin	Acetyl Norfentanyl	Buphedrone	JWH-147
LSD	Ocfentanil	Pentedrone	JWH-302
Psilocin	U-47700	Methedrone	AM-2233
Tryptamines	AH-7921	Mexedrone	MAM-2201
DMT	MT-45	Naphyrone	AB-PINACA
4-Acetoxy-DiPT	**Benzodiazepines**	**Other Novel Stimulants**	5F-AB-PINACA
4-Acetoxy-DMT	Zolpidem	4-FA	ADB-PINACA
4-HO-DET	Zopiclone	PMA	5F-APINACA
5-Methoxy AMT	Triazolam	PMMA	5-CHLORO-AB-PINACA
5-Methoxy DALT	Lorazepam	2C-B	AB-FUBINACA
5-Methoxy DPT	Lormetazepam	2C-P	APP-FUBINACA

5-Methoxy MiPT	Diazepam	2C-B	ADBICA
NBOMe	Nordiazepam	4-MEC	AKB-48 (APINACA)
25I-NBOMe	Oxazepam	5-MAPB	CB-13
25H-NBOMe	Temazepam	5-EAPB	MAB-CHMINACA
25B-NBOMe	Prazepam	6-APB	AB-CHMINACA
25C-NBOMe	Flunitrazepam	MDEA	PB-22
Novel Dissociatives	Clonazepam	**Synthetic Cannabinoids (SCs)**	5F-PB22
MXE	Bromazepam	JWH-018	STS-135
4-MeO-PCP	Alprazolam	JWH-073	XLR-11
Diphenidine	Nitrazepam	JWH-200	UR-144
Prescription Opioids	Demoxepam	JWH-250	UR-144-5-OH
Tramadol	Desalkylflurazepam	AM-694	MMB-CH
Oxycodone	Midazolam	AM-2201	MDMB-CHMICA
Oxymorphone	Delorazepam	JWH-007	5-CHLORO-THJ-018
Codeine	Pyrazolam	JWH-019	5F-APP-PICA
Morphine	Flubromazepam	JWH-020	5F-APP-PINACA
Hydrocodone	Nifoxipam	JWH-081	5F-CUMYL-PINACA
Methadone		JWH-122	5-F-NNEI NAPHTHYL ISOMER
		JWH-203	CUMYL-PEGACLONE
		JWH-251	MMB-2201

Chapter 12 | **Conclusions**

The main goal of this Ph.D. thesis was to develop robust, reliable and sustainable methods for the simultaneous determination of a large panel of illicit drugs and pharmaceuticals in wastewater. This objective was fully achieved, first by developing an SPE-based method that allowed the identification and quantification of pharmaceuticals, and then by improving this method according to the principles of sustainable chemistry. Indeed, the method proposed in this study showed great potential as a practical and routine analytical tool for water analysis as it is simple, fast and at the same time highly efficient. Moreover, it saves important resources in terms of time and materials, making it more in line with the principles of sustainable chemistry than previously proposed methods in the literature. Thanks to all these features, the present method can be applied in future high-performance wastewater monitoring campaigns, thus enabling social and environmental protection measures.

In addition, as my Ph.D. research focused more broadly on substances of toxicological interest, it was decided to extend the study to the broad family of NPS. In particular, the metabolic pathways of some of the newer NPS identified in the illicit market have been studied. This study is deemed particularly important in the field of forensic toxicology as it would provide (i) information on the mechanisms underlying the potential toxicity of these substances and (ii) useful data for their detection in biological samples and their excretion in wastewater, once the proper markers (i.e. the metabolites) are identified. Finally, to confirm drug use and exposure in high-risk populations, a combination of biological and nonbiological sample-based approaches should be more effective. In this context, the results of this retrospective investigation on aqueous matrix were joined by growing evidence derived from hair analysis and the -recently introduced- minimally invasive technique of DBS sampling.

In conclusion, the development of these methods has allowed the creation of many collaborations, both in Italian and in non-Italian countries, with the aim of highlighting the actuality and the real emergency related to the presence of these CECs and to follow the objectives reported in the Agenda 2030.

Acknowledgements

First of all, I would like to thank Prof. Alberto Salomone for being a good supervisor and for stimulating me to work with perseverance, a critical eye and always better.

Thanks to Prof. Marco Vincenti and my co-supervisor Prof. Marco Minella for the excellent advice and suggestions they gave me during my Ph.D. studies.

Thanks to Prof. Frederic Been and Prof. Fabio Gosetti for the time spent revising my thesis and for the important suggestions.

Thanks to Prof. Eugenio Alladio for his support and productive collaboration.

Thanks to all the CAD people for their help and cooperation. Above all, thanks to Enrico for sharing with me his experience and enthusiasm for forensic chemistry, and to Daniele for teaching me the secrets of LC-MS.

Thanks to Dr. Christina Ververi for accompanying me on this unforgettable PhD journey. Nothing would have been the same without her.

Thanks to Federica and Valentina for sharing with me every teamwork and every difficult moment of this PhD.

Thanks to Monica e Marta for being the best friends anyone could ever have and for their support and patience since my first year at university.

Thanks to my family, always on my side. Special thanks to my mum for being the biggest fan of my life.

Finally, Giuseppe: thank you for being the best part of my day, every day.