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Analytical detection of New Psychoactive Substances in biological samples: a Systematic Review

M. G. Santos*, R. J. A. Nascimento, V. Vescovi

[a] *Programa de Pós-graduação em Ciências Forenses/ Universidade Federal do Sul e Sudeste do Pará, 68507590, Marabá-Pará, Brasil*

**Endereço de e-mail para correspondência:michelegou103@gmail.com*

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Resumo

Novas substâncias psicoativas (NSP) representam um desafio analítico significativo devido à sua diversidade estrutural e ao surgimento acelerado no mercado de drogas. Diante desse cenário, este trabalho teve como objetivo revisar sistematicamente os principais métodos analíticos validados recentemente para a identificação de NSP em matrizes biológicas, além das técnicas de preparo de amostras associadas. A revisão foi conduzida seguindo as diretrizes PRIMA-S, com seleção de estudos baseados nos critérios de inclusão e exclusão predefinidos, resultando na análise de 73 artigos. Entre as matrizes biológicas analisadas, o sangue e a urina foram as mais recorrentes, sendo a extração em fase sólida o método de preparo de amostra mais utilizado. Observou-se ainda uma tendência crescente na busca por procedimentos de preparo mais ágeis, simples e com menor toxicidade (redução no consumo de solventes tóxicos). Quanto às técnicas analíticas, a cromatografia líquida acoplada à espectrometria de massas de alta resolução, empregando especialmente com colunas C18, destacou-se pela sua seletividade e especificidade. Quanto às classes de NSP, os estimulantes foram os mais frequentes. Portanto, diante dos resultados apresentados nesta revisão, é fundamental ressaltar que não existe um método único ideal para a identificação de NSP. Dada a constante modificação estrutural dessas substâncias, uma abordagem combinada, utilizando diferentes ferramentas analíticas, torna-se frequentemente necessária. Além disso, a atualização contínua das técnicas pelas autoridades é essencial para acompanhar a evolução desses compostos.

Palavras-Chave: método analítico; validação; NSP; matrizes biológicas; toxicologia forense.

Abstract

New psychoactive substances (NPS) represent a significant analytical challenge due to their structural diversity and rapid emergence in the drug market. Given this scenario, this study aimed to systematically review the main validated analytical methods recently developed for the identification of NPS in biological matrices, as well as the associated sample preparation techniques. The review was conducted following PRISMA guidelines, with study selection based on predefined inclusion and exclusion criteria, resulting in the analysis of 73 articles. Among the biological matrices analyzed, blood and urine were the most frequent, and solid-phase extraction was the most commonly used sample preparation method. Additionally, there was a growing trend toward the development of faster, simpler, and less toxic (reduction in the consumption of toxic solvents) sample preparation procedures. Regarding analytical techniques, liquid chromatography coupled with high-resolution mass spectrometry, particularly using C18 columns, stood out for its selectivity and specificity. Among NPS classes, stimulants were the most frequently detected. Therefore, based on the results presented in this review, it is essential to emphasize that there is no single ideal method for identifying NPS. Given the constant structural modifications of these substances, a combined approach using different analytical tools is often necessary. Furthermore, continuous updates to techniques by authorities are crucial to keep up with the evolution of these compounds.

Keywords: analytical method; validation; NPS; biological matrices; forensic toxicology.

1. INTRODUCTION

In recent years, a large number of new psychoactive substances (NPS) have entered the market and changed the landscape of recreational drugs, becoming a cause for concern around the world. The development and use of NPS represent a significant public health challenge, given their capacity to induce more effects on the human body than conventional drugs [1].

These substances can be analogs of existing controlled drugs, newly synthesized chemicals or even derivatives of failed drugs or even controlled drugs, such as benzodiazepines [2], which are used to mimic the psychoactive effects of controlled drugs [3]. The term "new" does not necessarily mean new compounds, but substances that have recently appeared on the drug market and are not under the control of the authorities [4].

The NPS detection represents the main analytical challenge for in-field instruments and laboratories in charge to detect and quantify these compounds [5,6]. From 2012 to 2023, a total of 1,245 different NPS were reported to the United Nations Office on Drugs and Crime (UNODC) by 142 countries and territories [7]. Among these, stimulants and synthetic cannabinoid receptor agonists were the two largest groups, accounting for 61% of all reported NPS [7].

From an analytical point of view, the main difficulties in detecting and identifying NPS have been associated with the great structural diversity of NPS that enter the market, even within the same chemical class, and the speed with which these new molecules appear and also disappear from the market, thus requiring the constant development of new analytical protocols [8]. A well-known example is JWH-018, one of the first synthetic cannabinoids detected in products such as "Spice." After its prohibition, structural analogues like JWH-073 quickly emerged, with the main difference being the side chain attached to the indole group of the molecule [9]. These modifications aim to circumvent legal restrictions and demonstrate the agility of manufacturers in altering chemical structures to evade regulations, further complicating detection using traditional analytical methods [3].

Correctly identifying illicit substances in the body is extremely important, both in clinical and forensic toxicology. Although drug testing is routine in Forensic Laboratories, new analytical challenges have arisen with the emergence of NPS. Several techniques, such as immunoassay [10], and different chromatography tests, each with its advantages and disadvantages, are being increasingly improved for better and more accurate identification of NPS. In this context, considering the broad structural diversity of the various NPS classes developed to circumvent legislation and the challenges faced by authorities in detecting these compounds, the objective of this study was to conduct a systematic review of the main analytical methods recently validated for the identification of NPS in biological matrices, as well as the primary sample preparation techniques employed in the detection process. This review focused on studies published between 2018 and June 2024, aiming to elucidate the most frequently used techniques for identifying NPS by substance class. Furthermore, the strengths and limitations of each method are discussed, with the goal of providing relevant insights to support the implementation or modernization of public policies in the field of forensic science.

2. METHODOLOGY

This study is characterized as exploratory research that seeks to present and analyze the current NPS detection methods used by forensic laboratories and researchers. The methodology employed in this work was based on the PRISMA-S checklist (Preferred Reporting Items for Systematic reviews and Meta-Analyses Statement) [11], which is a guide for conducting systematic reviews.

The searches were carried out in the PubMed database (National Center for Biotechnology Information, US National Library of Medicine, Bethesda, MD, USA), which is the largest source of references for scientific articles in the biomedical environment [12] and is the

database that presents the most relevant studies on the subject with greater reliability and specificity in this topic. To capture a broad spectrum of research, our search strategy employed the combined English descriptors: *new psychoactive substances* AND *analytical methods*, without specifying individual techniques. The search encompassed studies published between January 2018 and June, 2024.

The studies were selected through an eligibility criterion, based on inclusion and exclusion criteria. The inclusion criteria considered were: (1) original research articles (peer reviewed) that deals with validated identification methods, (2) research reports that deals with the identification of NPS of any class and (3) studies based on human biological samples. The exclusion criteria considered were: (1) review articles, (2) studies focused exclusively on non-NPS drugs, (3) studies with non-human biological samples or other types of samples, such as wastewater.

The data extracted from these articles, as presented in Table 1, includes the following information: (1) First Author (year); (2) Type of NPS; (3) Biological sample; (4) Sample preparation method; (5) Analytical technique; (6) Column/Detector; (7) LOD (min-max); (8) LOQ (min-max). This information was obtained from the text, tables, figures, and graphs within the articles.

3. RESULTS AND DISCUSSION

3.1 General characteristics of the studies

The searches conducted on the PubMed online research platform yielded a total of 275 articles, of which 189 were discarded based on the eligibility criteria, and 86 were read in full. After reviewing the full texts, 12 articles were discarded because they did not meet the eligibility criteria, resulting in a total of 73 articles collected (Figure 1). The article's analysis followed the strategy described hereafter: the article's first selection was carried out based on the evaluation of the title, abstract, and keywords of the studies found; then, the works considered relevant were evaluated according to the inclusion and exclusion criteria defined for this study.

The articles included in the review covered different classes of NPS, such as synthetic hallucinogens, synthetic stimulants, synthetic opioids and synthetic cannabinoids, which have been extracted from biological samples, such as urine, whole blood, post-mortem blood, oral fluid, hair, nails, plasma, meconium and others. The main information extracted from the articles included in this review is synthesized in Table 1 and will be discussed in the following topics.

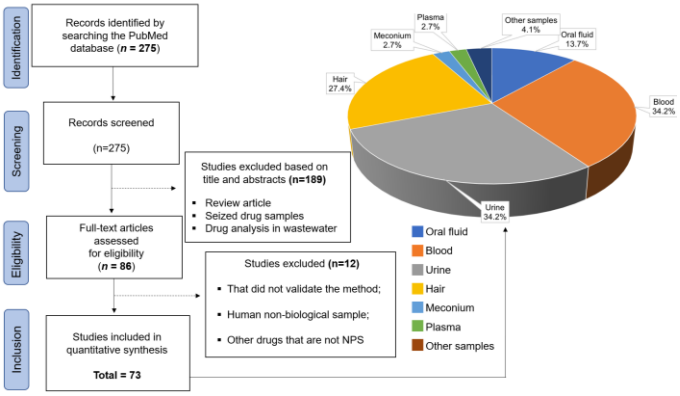

Figure 1: Flowchart of study selection and distribution of samples used in the analyses

3.1.1 Analysis of biological samples

The 73 studies included in this review used different biological matrices to identify NPS: nail, oral fluid, plasma, hair, meconium and mainly blood and urine (Figure 1). The selection of biological matrices for drug analysis depends on the specific purpose of the test [13]. The compounds tested may vary in their detection window, procedure for obtaining the sample (invasive or non-invasive) and cost [14].

Blood and urine samples were the most commonly used matrices in the studies (34.2%). The blood sample was used to detect synthetic opioids [15], synthetic cathinones [16], designer benzodiazepines [17], phenethylamines [18], synthetic cannabinoids and amphetamines, such as 3,4-methylenedioxy-N-ethylamphetamine (MDEA) e methylenedioxypyrovalerone (MDPV) [19]. Only two studies (2.7%) used plasma, which is the liquid matrix of blood, to identify mephedrone [20] and designer benzodiazepines [21]. The predominance of blood samples in this research is not surprising, given their

established role in forensic toxicology [14]. Blood tests offer high analytical accuracy and are well-suited for scenarios in which precise quantification is critical, such as specific drug screenings [22]. They enable the detection of both the presence and concentration of substances within a narrow time window, making them particularly effective for assessing recent drug use and potential impairment. However, the utilization of blood samples for drug analysis presents certain limitations. The invasive nature of blood collection, necessitating venipuncture by qualified personnel, significantly elevates costs and renders on-site collection impractical in many circumstances [13]. Furthermore, blood samples are susceptible to false negative results due to factors such as rapid metabolism and elimination of certain NPS, low concentrations of analytes at the time of collection, or delays between drug intake and sample collection, which can hinder detection [22].

Urine samples were used for detection of different classes of NPS, primarily for synthetic stimulants like phenethylamines [18,23–29], mephedrone [20,30], amphetamines, including 3,4-methylenedioxyamphetamine (MDA) and 3,4-methylenedioxymethamphetamine (MDMA) [19,23,31]. Other drug classes detected in urine included synthetic sedatives [21,31–33], synthetic cannabinoids [25,34–36], hallucinogens [33,37], and synthetic opioids [15,37–39].

Compared to a blood sample, urine is readily accessible by non-invasive procedures (except when supervised collection is necessary), and virtually all drug metabolites are excreted in the urine [40]. Furthermore, urine offers the possibility to document drug consumption with a longer detection window, from hours to a few days after ingestion [41]. However, the biggest challenge for urine NPS testing is that new substances are constantly appearing on the market, and their metabolic profiles are still unknown. Therefore, specific determination in urine becomes a difficult task, since there is a need for characterization and synthesis of adequate metabolites. In addition, several compounds can result in the same metabolites, which makes it difficult to identify the substance actually ingested [40].

Hair sample was used in 27.4% of studies to identify amphetamine derivatives, synthetic cannabinoids, opioids, cathinones, fentanyl, dissociative anesthetics, and phenethylamines [23,31,32,37,42–49]. The analysis of hair samples is the method that has a greater detection window, extending to several months or even years, according to the length of the hair shaft [50]. Due to its long detection window, it can provide information to recreate the history and state of drug abuse of the individual [51]. An additional advantage lies in the ease and non-invasiveness of sample collection [52].

The oral fluid was used in 13.7% of studies, mainly for synthetic cathinones [13,36,53–55], and synthetic cannabinoids [34–36]. Nevertheless, the main disadvantage of using this sample is the variability in acidity and saliva volume. This is a significant issue when analyzing NPS because many of these drugs, including amphetamines and cannabis, are known to reduce saliva production [14]. Although the modern literature on analytical methodologies applied to this alternative biological matrix is still limited, there have been several technological advances in forensic toxicology, such as screening tests and more selective techniques for alternative matrices [56,57].

Another biological sample employed in this research was meconium, the newborn's first stool. Meconium has been utilized to detect illicit drug use during pregnancy [58]. It offers valuable insights into maternal and neonatal risks, aiding in the development of preventive measures and essential medical interventions [59]. Two studies specifically analyzed meconium samples to identify synthetic cannabinoids and their metabolites, synthetic cathinones, stimulants, hallucinogens and their metabolites, and synthetic opioids and their metabolites [37,60]. Meconium analysis offers a significant advantage with its wide detection window, spanning the last 20 weeks of pregnancy. Its ease of collection and non-invasive nature for the newborn make it a convenient tool [58]. Despite that, the presence of substantial interfering substances can complicate analysis and potentially reduce sensitivity compared to alternative methods [61]. Another important limitation is the timing

of sample collection: since meconium is typically passed within the first few days after birth, delayed collection or early passage in utero may result in missed or incomplete samples. Furthermore, meconium formation begins around the 12th week of gestation, meaning that drug exposure occurring earlier in pregnancy may not be detected [62].

Post mortem samples of the vitreous humor, bile, and gastric contents also were used for drug testing. In the study by Gicquel [46] these samples were used to identify arylcyclohexylamines, designer benzodiazepines, stimulants, and synthetic cannabinoids. Another interesting biological matrix alternative is the nail. Liu et al. [63] were able to identify and quantify around 106 substances in 294 nail samples from drug users. Nevertheless, due to the low concentration of the submitted analyte, it is necessary to use a high-sensitivity analytical method.

Some studies have analyzed more than one biological fluid in the same forensic case. For example, in the study by Alexandridou et al. (2020) [38], the researchers developed and validated a GC-MS method for the detection of NPS without prior derivatization in whole blood and urine. The comparison between the two matrices revealed important differences for toxicological practice. Blood was used for the quantification of five NPS and was considered the most appropriate matrix for evaluating pharmacological effects and intoxication. For this reason, it is often the primary choice in clinical and forensic contexts. The urine sample was used for qualitative detection, allowing the identification of six NPS, including methylone, which could not be quantified in blood. This matrix is useful for screening and confirming recent use, although it does not directly reflect active levels in the body. Both matrices demonstrated effective recovery (80–120%) and low detection limits (LOD: 0.002–0.08 µg/mL), enabling fast and sensitive analysis. Despite that, while no interferences were detected in urine samples, multiple chromatographic peaks co-eluted with methylone (5.13 min) in blood samples, preventing accurate quantification of this NPS in blood using the method.

In the study by Mestria et al. (2021) [48], which investigated three ketamine analogs, methoxpropamine (MXPr), 2-fluoro-deschloroketamine (FDCK), and deschloroketamine (DCK)—in blood and hair from a suicide case, the authors detected and characterized these compounds using LC-HRMS and LC-MS/MS techniques. In blood, it was possible to directly detect and quantify the compounds MXPr (6400 ng/mL), FDCK (1300 ng/mL), and DCK (40 ng/mL), as well as some metabolites (dihydro-MXPr, nor-FDCK, dihydro-FDCK, dihydro-nor-FDCK, and dihydro-DCK) in very low concentrations. This suggested recent intake, with little time for metabolism before death, allowing for a direct correlation with the fatal event. In the hair sample, chronic use of FDCK and MXPr was evident due to high concentrations (16 ng/mg and 8 ng/mg, respectively). DCK was not detected, as in the blood, and only FDCK metabolites were found (nor-FDCK, dihydro-FDCK, dihydro-nor-FDCK). Therefore, comparing results from different biological matrices in the same case provides important toxicological information, as the combined analysis of two or more matrices enhances the robustness of toxicological interpretation, such as assessing acute intoxications, determining cause of death, analyzing metabolites, and identifying chronic or repeated substance use, among others.

3.1.2 Analysis of sample preparation methods

All the biological samples mentioned above are complex, containing phospholipids, inorganic salts, proteins, and organic compounds. To minimize the interference of these substances during drug identification, it is common to perform sample preparation, where the target analytes are concentrated or isolated to levels suitable for detection, producing better results [64]. The choice of sample preparation technique depends on the matrix, the physical and chemical properties of the NPS under investigation, and the level of sensitivity and specificity required for a specific analysis [65].

Regarding the methods used for sample preparation, only a few articles performed simple dilution,

totaling five studies (6.8%) primarily involving urine samples [19,66]. Lin et al. [67] developed and reported a new colorimetric detection method for fentanyl preparing the samples by simple dilution with deionized water. This method was also observed in the works of Razavipanah et al. [20] that designed a new electrochemical sensor to detect mephedrone, while Fan et al. [66] described the use of simple urine centrifugation and filtration as a sample preparation step in the detection of 13 different synthetic cathinones.

In this review, solid-phase extraction (SPE) was the predominant extraction technique, employed in 52.1% (38) of the reviewed studies, being mainly used to detect synthetic cathinones in hair samples [30,32,37,44,68–70]. Other substances, such as synthetic cannabinoids [24,32,37], synthetic opioids, hallucinogens, stimulants [37], and sedatives and dissociatives [32,69], have also been identified using various forms of SPE.

The SPE procedure typically consists of four steps, conditioning, loading, washing and elution, and the method's selectivity depends on the nature of the molecule of interest, the type of solid sorbent, and the solutions used in each step [13]. This method is considered fast and easy to handle, as it involves the removal of proteins through the addition of a reagent, which can be an organic solvent, acid, or even salt, and can be applied to a wide range of analytes [71]. There are minor variations of the SPE method, such as microextraction approaches, found in studies analyzed for different categories of NPS.

The Micro-Extraction by Packed Sorbent (MEPS) technique was used in three studies on oral fluid samples [55] and plasma [21]. Solid-Phase Microextraction (SPME) was applied in a study on oral fluid samples [34], and Micro-Solid-Phase Extraction (μ-SPE or MI-SPE) in urine samples [72] and oral fluid [54,55], respectively. The MIP SPE method uses Molecularly imprinted polymers (MIP) as the solid phase. These polymers are designed to selectively recognize specific molecules, making MIP SPE highly selective for target compounds, such as specific drugs. This method is ideal when high specificity in extraction is required. The SPME method performs the extraction process on a microscale, using a fiber coated with a solid phase.

Other interesting techniques are the Headspace Solid-Phase Microextraction (HS-SPME) method, used by Anzillotti et. al. [34] in the preparation of samples containing synthetic cannabinoids present in oral fluid, and the Sorbent-Assisted Liquid-Liquid Extraction (SALLE) method, which combines elements of liquid-liquid extraction (LLE) with SPE, used by Staeheli [25] in the preparation of samples containing synthetic cannabinoids present in urine.

The second most widely used method for the preparation of biological samples was LLE (42.5%), which involves the separation of analytes between two immiscible liquid phases, where the analyte is transferred from one liquid phase (usually the sample) to another liquid phase (typically an organic solvent). However, due to their limitations, such as unsatisfied sensitivity, time-sonsuming procedures, and high operational cost, novel samples preparation techniques, have been developed to improve the sensitivity, streamline process and address other emerging challenges [73].

Variations of LLE found in the studies included Pressurized Liquid Extraction (PLE), which involves extraction with solvents under pressure; Dispersive Liquid-Liquid Microextraction (DLLME), which disperses solvent in a liquid sample; and Parallel artificial liquid membrane extraction (PALME), which uses a liquid membrane for extraction. Liquid-Phase Microextraction (LPME) is a liquid-phase extraction method that utilizes very small volumes of solvent to extract analytes from a sample. It is a sensitive and efficient technique, generally applied to extract compounds present in low concentrations. Finally, the SHS-HLLME technique, or " Switchable Hydrophilicity Solvent based Homogenous Liquid–Liquid Microextraction," represents a modern approach to liquid-phase microextraction, designed to be fast and efficient for the extraction of analytes from liquid samples.

In recent years, other extraction procedures known as green extraction methods have been applied, as they require reduced amounts of samples, solvents, and

reagents [74]. One such method is the QuEChERS technique (Quick, Easy, Cheap, Effective, Rugged, and Safe), which was used in a single study (1.4%) [19], for the identification of 84 different classes of NPS. Other techniques identified in the studies include protein precipitation (PP), primarily for blood samples [39,75] and urine samples [18,39]. All quantitative data of sample preparation methods are summarized in Figure 2.

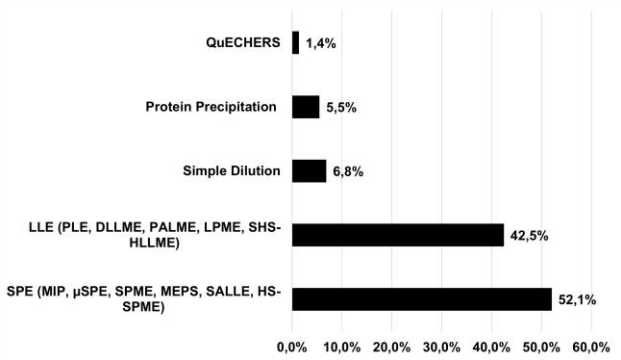

Figure 2: Percentage distribution of sample preparation methods used for the detection of New Psychoactive Substances (NPS) in biological matrices. *Abbreviations:* **µ-SPE:** (micro)solid extraction; **DLLME:** Liquid/Dispersive Liquid Microextraction; **HS-SPME:** Headspace-solid phase microextraction; **LLE:** Liquid-Liquid Extraction; **MEPS:** packaged sorbent microextraction; **MIP:** Molecularly imprinted polymers; **PALME**: Parallel artificial liquid membrane extraction; **PLE:** Pressurized liquid extraction; **QuEChERS:** Quick, Easy, Cheap, Effective, Rugged and Safe; **SALLE:** Salting-out liquid-liquid extraction; **SPE:** Solid Phase Extraction; **SHS-HLLME:** Switchable Hydrophilicity Solvent based Homogenous Liquid–Liquid Microextraction; **SPME:** Solid-phase microextraction.

3.1.3 Analysis of analytical techniques

The most commonly used analytical techniques in the studies were Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), or liquid chromatography coupled to high-resolution mass spectrometry (HRMS) employed in 35 studies (47.9%), for identification primarily of synthetic cathinones, also designer benzodiazepines, opioids, and synthetic cannabinoids (Figure 3).

LC is a generic term that encompasses several variations of the technique, including HPLC (high-performance liquid chromatography) and UHPLC (ultra-high-performance liquid chromatography). The main differences between HPLC and UHPLC lie in column technology and operating pressure, while HPLC uses columns packed with particles ranging from 3 to 5 micrometers and operates at pressures up to 6,000 psi, UHPLC employs smaller particles, typically less than 2 micrometers, and operates at much higher pressures, reaching 15,000 psi or more. As a result, UHPLC offers greater efficiency, improved resolution, and shorter analysis times, although it requires more specialized instrumentation [76]. The UHPLC was the second most frequently used technique UHPLC, used in 22 studies (30.1%), for identification of all the different classes of NPS. This method is widely used in biological fluids, enabling increased detection and reduced spectral interference from endogenous compounds in the biological matrix [77].

The third most common technique was gas chromatography-mass spectrometry (GC-MS), used in 13 studies (17.8%), mainly used for the identification of synthetic cathinones [78] and synthetic opioids [79], and only two studies used this technique for the identification of synthetic cannabinoids [34,80].

As shown in Figure 3, some analytical techniques in the reviewed studies were used infrequently, each representing only 1.4% of the total methods employed. These include approaches such as colorimetric detection for fentanyl identification [67], DART-QqQMS (Desorption Atmospheric Pressure Photoionization coupled with triple quadrupole mass spectrometry) to identify different NPS [81], CE-HRMS (capillary electrophoresis coupled with high-resolution mass spectrometry) to identify synthetic cathinones and tryptamines [27], MS-MS (general tandem mass spectrometry technique) for different NPS [82], MIP to identify mephedrone [20], and SFC (supercritical fluid chromatography) to identify synthetic cathinones and phenethylamines [18]. Although rarely used, these techniques may offer specific advantages such as speed, selectivity, or suitability for certain sample types and target compounds, especially in exploratory contexts, rapid screening, or when more conventional methods are limited.

The remaining techniques for the identification and quantification of NPS, applied in the studies evaluated in this review, are presented in Figure 3.

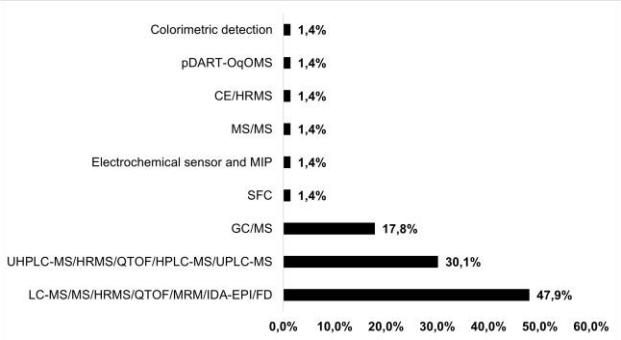

Figure 3: Percentage distribution of analytical techniques used for the detection of New Psychoactive Substances (NPS) in biological matrices. *Abbreviations:* **CE/HRMS:** Capillary Electrophoresis / High-Resolution Mass Spectrometry**; GC-MS:** Gas Chromatography – Mass Spectrometry; **LC-MS/MS/HRMS/QTOF/MRM/IDA-EPI/FD:** Liquid Chromatography – Tandem Mass Spectrometry / High-Resolution Mass Spectrometry / Quadrupole Time-of-Flight / Multiple Reaction Monitoring / Information-Dependent Acquisition – Enhanced Product Ion / Fluorescence Detection; **MIP:** Molecularly Imprinted Polymer; **MS/MS:** Tandem Mass Spectrometry; **pDART-QqQMS:** Portable Direct Analysis in Real Time – Triple Quadrupole Mass Spectrometry; **SFC:** Supercritical Fluid Chromatography; **UHPLC-MS/HRMS/QTOF/HPLC-MS/UHPLC-MS:** Ultra-High Performance Liquid Chromatography – Mass Spectrometry / High-Resolution Mass Spectrometry / Quadrupole Time-of-Flight / High Performance Liquid Chromatography – Mass Spectrometry / Ultra-High Performance Liquid Chromatography – Mass Spectrometry.

3.1.4 Analysis of NPS classes

Most studies analyzed more than one class of NPS, including stimulants, depressants, or CNS disruptors. However, the stimulant group was the most prevalent, accounting for 67% (Figure 4). This class includes synthetic cathinones, aminoindanes, piperazines, amphetamine-type stimulants, and their derivatives. The second most frequently identified class was CNS disruptors, which appeared in 58% of the studies and includes synthetic cannabinoids and synthetic hallucinogens. The third most identified class was CNS depressants, present in 47% of the studies; this group includes synthetic opioids and designer benzodiazepines.

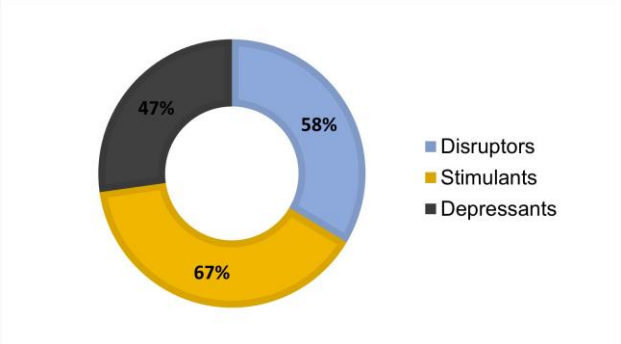

Figure 4: Distribution of the main classes of New Psychoactive Substances (NPS) identified in the studies.

The predominance of multiple classes of emerging NPS in the analyzed studies, especially stimulants, followed by CNS disruptors and depressants, highlights the chemical and functional diversity of these compounds, whose constant structural modifications pose a regulatory challenge. Given this complexity and the speed at which new variants are introduced to the market, traditional control mechanisms based solely on the individual listing of substances become insufficient.

In Brazil, aiming to more effectively combat the emergence and spread of NPS, a generic control system was also implemented [83]. This model establishes a basic chemical structure and its possible molecular variations, extending control to all substances that fit these descriptions. In this way, entire groups of compounds are banned simultaneously, preemptively regulating potential new drugs. The method is particularly effective against NPS, which often arise from minor modifications to already controlled molecules in an attempt to circumvent existing regulations [83]. Between 1999 and April 2025, through 94 Collegiate Board Resolutions (RDCs), Agência Nacional de Vigilância Sanitária (ANVISA) included a significant number of new substances in its control list [84].

3.2 Bioanalysis of Synthetic Stimulants

The NPS classified as synthetic stimulants, according to UNODC, are phenethylamines, amphetamines, or cathinones, and they are the most abundant representatives in the NPS market [85]. Synthetic cathinones are normally consumed in high doses, as most of them have less stimulating power due to the difficulty of crossing the blood-brain barrier and reaching the brain [86]. As a result, the concentrations of these substances in biological matrices can vary considerably. For example, the average concentration of mephedrone in the blood of fatal cases was 2,663 ng/mL, with a range of 51 to 22,000 ng/mL [87], while the concentration of mephedrone in the plasma of healthy individuals after oral administration was 122.6 ng/mL, with a range of 52 to 218 ng/mL [88]. These variations present significant analytical challenges, as detection methods must be sensitive enough to identify trace

amounts of some substances while being robust enough to quantify others at much higher concentrations.

In this research, the most applied technique for identifying this class of NPS was UHPLC, followed by LC-MS and GC-MS. The GC-MS is a technique that has been applied for the determination of NPS in urine samples, that applies to volatile psychoactive compounds and possesses a relatively short run time. Therefore, a large number of compounds can be screened, for this reason it is a very suitable technique for analysis of synthetic cathinones, and these drugs are commonly consumed in the form of mixtures [89]. In the studies utilizing GC for the analysis of synthetic cathinones, Agilent J&W HP-5ms columns with 5% phenylmethylsiloxane were commonly employed. Specifically, two column configurations were referenced: one measuring 30 m x 0.25 mm I.D. x 0.25 µm film thickness [38,68], and another with 30 m x 0.32 mm I.D. x 0.25 µm film thickness [16]. These columns are recognized for their low polarity, minimal bleed, high precision, and versatility in high performance analysis, along with their ability to withstand high temperatures. However, this technique requires the derivatization of the analytes [90].

An alternative to this technique is the LC which allows the determination of compounds with a wide range of polarity, low volatility, and thermolability with the application of more generic sample treatment strategies [91]. For this reason, a large number of synthetic cathinones were analyzed by this technique, and different columns were applied, such as the pentafluorophenylpropyl (PFPP) column, which is an excellent choice for the retention and selectivity of compounds containing amine and charged bases, such as synthetic cathinones [23], and mephedrone and metabolites [30], being very suitable for LC-MS instrumentation due to its reliability and efficiency with acidic mobile phases [92]. Other columns such as Phenomenex Kinetex Biphenyl, the ACQUITY HSS C18 column, and Atlantis T3, were also used in different biological materials, such as blood [19], urine [33] meconium [37,60], and hair [93].

UHPLC was applied in twelve studies of synthetic stimulants, mainly synthetic cathinones and amphetamines, and different types of biological samples. The main differences of this technique are the chromatographic columns used, in the studies included in this review, most used the HSS C18 ACQUITY column, which has significantly reduced dimensions, with excellent performance, greater retention, and longer useful life [46]. López Rabuñal et al. [37] employed the UHPLC coupled to quadrupole time-of-flight mass spectrometry (QTOF-MS) in meconium and hair samples to identify synthetic cathinones. This newly developed technique provides quick and efficient access to detailed information regarding the nature of compounds and their complex mixtures. It has been widely used in various fields to analyze various materials, including the analysis of NPS metabolites [21,32,37].

Other techniques were also employed, such as the supercritical fluid chromatography (SFC) technique in the study of Borovcová et al. [18] to analyze synthetic cathinones and phenethylamines in urine samples. This separation method resembles gas and liquid chromatography, but uses a supercritical fluid as the mobile phase, which allows a shorter chromatographic separation time [94]. Another technique used was capillary electrophoresis-mass spectrometry (CE-MS) to identify synthetic cathinones and phenethylamines in urine samples [27]. This is a separation technique based on the movement of ions under electrophoretic and/or electro-osmotic forces produced by applying an electric field with a mass spectrometer [95]. The technique is especially suitable for polar and ionic compounds in complex polar matrices, and it is also possible to identify metabolites of substances [96].

Only one electrochemical technique was identified in our research, which was the development of an ultrasensitive and selective electrochemical sensor by Razavipanah et al. [20], to detect mephedrone in urine and plasma samples. The sensor, developed by the researchers, showed high efficiency in detection limits, as well as good stability, reproducibility, and repeatability.

3.3 Bioanalysis of synthetic cannabinoids

Synthetic cannabinoids (CS) are substances that interact with the CB1 and CB2 endocannabinoid receptors and cause cannabimimetic effects similar to Δ9-tetrahydrocannabinol (THC), the main psychoactive constituent of cannabis [1]. According to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) [97], synthetic cannabinoids can be classified into up to seven main groups, namely the naphthoylindoles, which are JWH-018, JWH-073, JWH-200 and JWH-210, naphthylmethylindole, naphthoylpyrroles, naphthylmethylindenes, phenylacetylindoles, the classic cyclohexylphenols and cannabinoids such as HU-210 or nabilone). In addition, new molecules with different structures continue to be synthesized, which makes it difficult to create legislation to control them [5].

Only two studies have utilized GC-MS for detection and identification of synthetic cannabinoids. Alexandridou [38] used GC-MS to identify JWH-018 and AM-2201, while Anzillotti [34] used it to identify JWH-(019, 081, 122, 200, and 250), HU-211, AM-2201, and others.

In this research, the detection and identification of synthetic cannabinoids have been primarily conducted using LC-MS techniques, owing to their high accuracy, precision and detection sensitivity. as demonstrated by Staeheli et al. [25]. They developed a sensitive LC-MS/MS method to detect 75 synthetic cannabinoids in urine by comparing MS/MS spectra to an internal library. The high structural similarity of synthetic cannabinoids suggests that this method can potentially identify other compounds not included in the study.

Although HPLC has been widely used, UHPLC has gained significant attention in recent years [36,37]. As mentioned previously, this technique, is basically an advanced form of liquid chromatography that uses high pressure to achieve superior peak resolution and sensitivity [98]. UHPLC-HRMS offers faster analysis times and can be applied even when pure standards are unavailable, enabling the identification of various synthetic cannabinoid classes and their metabolites [99].

3.4 Bioanalysis of Synthetic Hallucinogens

Hallucinogens are a pharmacologically diverse group of compounds capable of producing unique alterations of consciousness; these psychoactive substances produce a profile of changes in thoughts, perceptions, and emotions, often including profound alterations in the perception of reality [100]. Among the synthetic hallucinogens, there are tryptamines, NBOMES and 2C drugs, and anesthetic dissociatives such as, ketamine, norketamine, methoxetamine, phencyclidine and methoxpropamin, among others.

In this study, chromatographic techniques LC-MS and GC-MS were widely employed, demonstrating high analytical performance in both screening and elucidating the composition and structural characteristics of unknown compounds. These methods proved particularly valuable in forensic cases where no prior information on drug intake was available, as exemplified by Matey et al.[69] who analyzed synthetic hallucinogens in real-world scenarios. The most common columns used were hydrophobic, with phenyl and C18 groups being the predominant choices.

In another study, the same group worked on the identification of ketamine and its derivatives, in hair samples [44]. Ketamine is a dissociative anesthetic abused by an increasing number of young people as a “club drug” and is often distributed in “club drugs”, “raves” and parties [101]. In this case, the researchers employed GC, a technique known for its short analysis time and ability to trace numerous compounds. A capillary column (30 m, 0.25 mm i.d., 0.25mm thick film of 5% phenylmethylsiloxane), coupled with an MS/EI detector in selected-ion monitoring mode was used. The technique was applied to 1,189 forensic hair samples, detecting over 60 positive cases of ketamine and its derivatives. Due to its enhanced detection capability, accuracy, and precision, the validated method met the analytical requirements of the Spanish National Institute of Toxicology and Forensic Sciences (INTCF).

Therefore, both LC-MS and GC-MS have still been the most applied methods for identifying synthetic

hallucinogens, although some other works have reported different methods, such as Capillary electrophoresis mass spectrometry, to identify tryptamines in urine samples [27], and UHPLC in meconium [37], oral fluid samples for identification of 2C drugs [54] and tryptamines [36].

Despite being recent techniques, still little studied, they are effective and perform well for detecting sample mixtures. Capillary electrophoresis, for example, is a modern tool for analyzing a wide range of compounds in complex samples, such as urine, as it allows the separation and identification of various analytes, from small ions to high molecular weight protein complexes [102], being many times more efficient than traditional methods, since it reduces the complexity of the analyte mixture that enters the mass spectrometer, resulting in reduced ion suppression and a more direct interpretation of mass spectrometry data.

Table 1. Extraction of data from included studies

Nº	First author (year)	NPS Class	Sample	Sample preparation method	Analytical Technique	Column / Detector	LOD (min - máx)	LOQ (min -máx)
1	**Adamowicz (2020)** [15]	Fentanyl analogues (synthetic opioid)	Blood	LLE	LC-MS/MS*	Kinetex C18, column (Phenomenex)	0.01 - 0.02 ng/ml	0.1 - 100 ng/ml to Acetylfentanyl and 0.2 - 100 ng/ml to Sufentanil
2	**Aldubayyan (2022)** [28]	Synthetic cathinones	Urine	LLE	LC-MS/MS	HSS T3 (C18)	0.09-0.49 ng/mL	1 ng/mL
3	**Aldubayyan (2023)** [103]	Synthetic cathinones	Blood	LLE	LC-MS/MS	HSS T3 (C18)	0.1-1.45 ng/ml	1-5 ng/ml
4	**Alexandridou (2020)** [38]	Synthetic cathinones, synthetic opioids and synthetic cannabinoids.	Blood Urine	LLE	GC-MS	Agilent J&W HP-5 ms capillary column	0.08 µg/ml to mephedrone and 0.02 to JWH-018	0.25 µg/ml to mephedrone and 0.05 to JWH-018
5	**Antunes (2021)** [16]	4-CEC, α-PVP, 4-Cl-PVP and MDPV (Synthetic cathinones)	Blood	SPE	GC-MS	Phenylmethylpolysiloxane capillary column (HP-5)	1 – 10 ng/ml	25 - 800 ng/mL
6	**Anzillotti (2019)** [34]	Synthetic cannabinoids	Oral fluid	HS-SPME and DI-SPME	GC-MS	DB-5 capillary column (5% phenylmethylpolysiloxane)	1 - 10 ng/ml	1 - 10 ng/ml
7	**Ares-Fuentes (2021)** [21]	Clonazolam, deschloroe-tizolam, nifoxipam, flubromazolam and meclonazepam (designers benzodiazepines) and zolpidem, zaleplon and zopiclone (three z-hypnotic drugs)	Plasma	MEPS	UHPLC–MS/MS*	ACQUITY UPLCTMBEH Shield RP18 column	0.5 - 5 ng/ml	1-10 ng/ml
8	**Banaszkiewicz (2020)** [17]	Designer benzodiazepines	Blood	LLE	LC–MS/MS	C18 column	0.01 - 0.33 ng/ml	1 ng/ml
9	**Barone (2024)** [104]	Multiple NPS	Hair	LLE	UHPLC–MS/MS	C18 column	4-40 pg/mg	-
10	**Barone (2023)** [105]	Multiple NPS	Blood	LLE	LC-MS/MS	C18 column	0.95-65 ng/mL	0.32-130 ng/mL
11	**Borovcová (2018)** [18]	Synthetic cathinones and phenethylamines	Urine	PP	SFC and UHPLC	BEH Phenyl RP18 C8	0.01 - 5 ng/ml	0.02- 4.22 ng/mL
12	**Caixia Guo (2023)** [106]	Synthetic benzodiazepines	Blood	SPE	LC-MS/MS	Fluorophenyl Propil	0.1-10 ng/ml	-

13	**Calò (2020)** [35]	Synthetic cannabinoids	Oral fluid	PP	HPLC-MS/MS	Agilent® Pursuit XRs C18 column	0.9 ng/ml (JWH081) - 831 ng/ml (CP47497-C7)	2.9 ng/ml (JWH081) - 2769 ng/ml (CP47497-C7)
14	**Chen (2023)** [107]	Multiple NPS	Urine	LLE	LC-MS/MS	Biphenyl column	0.05-5 ng/mL	0.1-5 ng/mL
15	**Cláudia (2019)** [68]	Synthetic cathinones and phenethylamines	Blood	SPE	GC-MS-EI	capillary column with 5% phenyl-methylpolysiloxane (HP-5MS)	5-40 ng/ml	5-40 ng/ml
16	**Czerwinska (2019)** [30]	Mephedrone and metabolites (Synthetic cathinones)	Blood	SPE	LC-MS/MS* (Quadrupole mass spectrometer coupled) and HESI operated in positive ion mode.	Selectra® pentafluorophenylpropyl column	50–500 pg/mL	200–2000 pg/mL
17	**Fabresse (2019)** [23]	Multiple NPS	Hair	LLE	HESI and LC-HRMS	Two reversed-phase columns were evaluated: Accucore™ phenylhexyl and Accucore™ pentafluoro-phenyl.	0.01 - 5 ng/ml	0.2 - 15,61 ng/ml
18	**Fabris (2023)** [108]	Synthetic cannabinoids	Blood	SHS-HLLME	LC-MS/MS	C18 column	0.01-0,08 ng/mL	0.1 ng/mL
19	**Fabris (2023)** [109]	Synthetic cathinones	Urine and Blood	DLLME	UHPLC-MS	C18 column	0.2-1.0 ng/mL	1.0-10 ng/mL
20	**Fabris (2024)** [110]	Multiple NPS	Blood	PALME	LC-MS/MS	C18 column	0.1-0.75 ng/mL	1 ng/mL
21	**Fan (2020)** [66]	Synthetic cathinones	Urine	Simple dilution	LC-MS/MS coupled to triple quadrupole linear mass spectrometer equipped with an ESI	Phenomenex Kinetex1 Biphenyl column	0.1 - 0.5 ng/ml	0.5–1.0 ng/mL
22	**Fernández (2019)** [53]	Synthetic cathinones	Oral fluid	DLLME	UPLC-MS/MS.	The Acquity UPLC™ BEH Shield RP 18 analytical column	0.25 - 5 ng/ml	500 ng/ml
23	**Di Francesco (2024)** [111]	Multiple NPS	Oral fluid	LLE	LC-MS/MS	C18 column	0.01-10 ng/mL	0.03-15 ng/mL
24	**García-Atienza (2023)** [112]	Synthetic cannabinoid	Oral fluid	SPE	LC-FD	C18 column	0.6-0.8 µg/L	2.0-2.6 µg/L
25	**Garneau (2021)** [39]	Synthetic opioids and designer benzodiazepines	Post-mortem blood Urine	PP	LC–MS/MS operated in ESI and MRM	Agilent Zorbax Eclipse Plus C18 column	Blood and Urine 0.05 - 20 ng/ml	-
26	**Gicquel (2021)** [46]	Arylcyclohexylamines, designer benzodiazepines, synthetic stimulants and synthetic cannabinoids	Peripheral and cardiac blood Urine Vitreous humor Bile Gastric contents Hair	LLE	LC-HRMS detection together with NMR spectroscopy	ACQUITY HSS C18 column	5-100 µg/L	10-2000 pg/mg
27	**Gottardo (2020)** [27]	Synthetic cathinones, phenethylamines and tryptamines	Urine	LLE	CE-HRMS		10 - 15 ng/ml	25 - 50 ng/ml

28	**Gundersen (2019)** [24]	Synthetic cannabinoids	Urine	SPE	LC-QTOF-MS	Zorbax Eclipse Plus C18 Rapid Resolution HD speaker	0.1 – 17.5 ng/mL	0.01–5 ng/mL
29	**Hsu (2024)** [81]	Multiple NPS	Urine	SPE	pDART-QqQ-MS	-	2-113.33 ng/mL	20-75 ng/mL
30	**Huang (2023)** [113]	Synthetic cannabinoids	Hair	SPE	UHPLC-MS	C18 column	0.0025-0.05 ng/mg	-
31	**Ji (2023)** [82]	Multiple NPS	Blood and Urine	LLE	MS-MS	-	1-100 ng/mL for urine and 0.3-50 ng/mL for blood	5-200 ng/mL for urine and 1-200 ng/mL for blood
32	**Kim (2018)** [80]	Synthetic stimulants (AP Derivatives (4FA, 4FMA, 4CA, PMA, 4CMA, 6APB, PMMA and 6MAPB and 1 aminoindan analogue (MDAI).	Urine	LLE	GC-MS	capillary column (DB-5MS)	0.5 - 2.5 ng/ml	2 - 25 ng/ml
33	**Kleis (2022)** [114]	Synthetic cannabinoids, stimulants, hallucinogens and benzodiazepines	Serum	SPE	LC–QTOF–MS	EC-C18 Poroshell column	1-10 ng/mL	-
34	**Kutzler (2024)** [115]	Synthetic cannabinoids	Hair	SPE	LC-MS	C18 column	1.8–34 pg/mg	-
35	**Lesne (2023)** [116]	Synthetic hallucinogens	Oral fluid	MEPS	LC-MS	Hydrophobic column (Varian PLRP-S 300A°)	0.09-1.22 µg/L	0.29-4.06 µg/L
36	**Lin (2021)** [67]	Fentanyl	Urine	Simple dilution	Visual colorimetric detection using Rose Bengal (RB)	-	0.7 mg/L	-
37	**Ling Goh (2023)** [70]	Synthetic cannabinoids and synthetic cathinones	Urine	SPE	LC- QTOF–MS	Biphenyl column	1.5-7.5 ng/mL (synthetic cannabinoids and metabolites) 15 ng/mL (synthetic cathinones and other NPS).	-
38	**Liu (2022)** [63]	Synthetic cathinones, opioids and synthetic cannabinoids	Nail	SPE	UPLC-MS/MS	ACQUITY UPLC®HSS C18 column	2.5-25 pg/mg	5-50 pg/mg
39	**López-Rabuñal (2019)** [60]	Synthetic cathinones	Meconium	SPE	LC-MS/MS	Atlantis T3 - reversed-phase (C18)	0.1 – 1 ng/g	1 - 2 ng/g
40	**López-Rabuñal (2021)** [37]	Multiple NPS	Meconium Maternal Hair	SPE	UHPLC-QTOF Mass Spectrometry; LC-MS/MS for maternal hair	Phenomenex Kinetex C18 column	0.04 - 2.4 ng/g	-
41	**Machado (2023)** [117]	Alpha-pyrrolidinohexanophenone (α-PHP) synthetic cathinones,	Blood	SPE	GC-MS	Phenylmethylpolysiloxane capillary column (HP-5)	5 ng/mL	10 ng/mL
42	**Maida (2022)** [49]	Synthetic cathinones	Hair	SPE	UHPLC-HRMS	Phenyl-Hexyl	2 pg/mg	5 pg/mg

43	**Massano (2022)** [118]	Synthetic cannabinoids, synthetic cathinones, synthetic hallucinogens and synthetic opioids	Dried Blood Spots (DBS)	SPE	UHPLC-HRMS	Phenomenex Kinetex C18	1.3-6.3 ng/ml	2 - 7.5 ng/ml
44	**Matey (2019)** [44]	Ketamine and norketamine	Hair	LLE and SPE	GC-MS	Capillary column	Ketamine 0.2 ng/mg and Norketamine 0.05 ng/mg	Ketamine 0.5 ng/mg Norketamine 0.05 ng/mg
45	**Matey (2022)** [69]	Methoxetamine and arylcyclohexylamine class	Hair	LLE and SPE	LC-HR-MS/MS and GC-MS	GC-MS: Column 5% phenylmethylpolysiloxane LC-HR-MS: Column: phenylhexyl	50-250 pg/mg	2-5 pg/mg
46	**Mestria (2021)** [48]	Methoxpropamine (MXPr), 2-fluoro-deschloroketamine (FDCK), deschloroketamine (DCK)	Blood Hair	DLLME	LC-HRMS or LC-MS/MS	J&W scientific 5% phenyl-methylsiloxone capillary column	Blood: 0.5 ng/ml for LC-MS/MS and to 10 ng/ml for LC-HRMS. Hair: 0.01 ng/mg for LC-MS/MS and 0.05 ng/mg for LC-HRMS	Blood: 2 ng/ml Hair: 0.05 ng/mg
47	**Musial (2022)** [42]	Synthetic cannabinoids, synthetic cathinones and designer benzodiazepines	Hair	SPE	LC-MS/MS	Phenomenex Kinetex C18	-	0.025-1.25 ng/mg
48	**Musile (2023)** [45]	Synthetic cathinones	Hair	SPE	LC-MS	Phenomenex Kinetex C18	0.065-0.125 ng/mg	10 ng/mg
49	**Musile (2020)** [31]	Amphetamine, synthetic opioid, synthetic depressants/sedatives	Hair	LLE	UHPLC-Ion Trap MS equipped with a ESI source	Acclaim® RSLC 120 C18 column	0.01- 0.25 ng-mg	-
50	**Olesti (2020)** [33]	Synthetic hallucinogens (2C drugs), synthetic stimulant, designer benzodiazepines	Urine	Simple dilution	LC-MS/MS	Acquity UPLC BEH C18	0.3 - 2.5 ng/ml	1 - 5 ng/ml
51	**Orfanidis (2021)** [19]	Synthetic cathinones, synthetic cannabinoids, amphetamines, fentanyl and designer benzodiazepines	Post-mortem Blood	QuEChERS	UHPLC-MS-MS*	Acquity column BEH C18	0.01 - 9.07 ng/ml	0.03 - 27.2 ng/mL
52	**Pascali (2022)** [119]	Synthetic cannabinoids	Oral fluid	LLE	LC-MS/MS	Pursuit XRs Ultra (Unspecified alkyl group)	0.001-2.275 ng/ml	0.004-7.583 ng/ml
53	**Pascual-Caro (2023)** [29]	Synthetic cathinones, opioids and others	Urine	SPE	LC-MS/MS	Phenomenex C18	0.003-0.5 ng/ml	0.05-1.5 ng/ml
54	**Razavipanah (2018)** [20]	Mephedrone	Urine Plasma	Simple dilution	An electrochemical MIP sensor based on sol-gel MIP technology	-	0.8 nM	-
55	**Rubicondo (2023)** [43]	Multiple NPS	Hair	SPE	LC-MS/MS	Zorbax Eclipse Plus C18	0.03-9 pg/mg	0.07-10 pg/mg

56	**Salomone (2021)** [47]	Fentanyl analogues	Hair	Simple dilution	UHPLC-QTOF-HRMS	Phenomenex Kinetex C18	0.2 - 1.2 pn/mg	0.4 - 2.4 pg/mg
57	**Salomone (2023)** [120]	Fentanyl, synthetic opioids, and ketamine	Hair	SPE	UHPLC–MS/MS	C18 column	17-7300 pg/mg	-
58	**Sánchez-González (2019)** [72]	Synthetic cathinones	Urine	µ-SPE and MIP	HPLC-MS/MS	Kinetex reversed phase column 2.6ÿ C18 100 Å	0.14 - 1.51 µg L^{-1}	0.48 - 5.03 µg L^{-1}
59	**Sara Júlio (2023)** [78]	Synthetic cathinones	Blood	SPE	GC-MS	Agilent HP5-Ms Capillary column	800 ng/ml	5 ng/ml
60	**Schüller (2023)** [121]	Nitazene (synthetic opioids)	Blood	LPME	UHPLC–MS/MS	Biphenyl column	0.01-0.1 nM	0.1-0.5 nM
61	**Simão (2023)** [122]	Ketamine and norketamine	Hair	SPME	GC-MS	Agilent J&W HP-5 ms capillary column	0.01 ng/mg for Ketamine and 0.05 ng/mg for norketamine	0.05 ng/mg
62	**Sorribes-Soriano (2019)** [54]	Amphetamines, synthetic cathinones and 4 2C drugs	Oral fluid	MIP-SPE	UHPLC	BEH C18 column	0.03 - 1.3 µg L^{-1}	-
63	**Sorribes-Soriano (2019)** [55]	Dichloropane	Oral fluid	MEPS	GC-MS	HP-5 ms capillary column	70 µg/L	200 µg/L
64	**Staeheli (2019)** [25]	Synthetic cannabinoids	Urine	SALLE	LC–MS/MS MRM−IDA−EPI	Phenomenex Synergi Polar RP column	0.05 - 2.5 ng/ml	-
65	**Tomczak (2018)** [75]	"(1-(4-chlorophenyl)-2-(methylamino)-1-propanone) or 4-CMC"	Blood	PP	GC-MS in electronic ionization mode	Phenomenex ZB-5 MS capillary column	0.3 ng/ml	1 ng/ml
66	**Trana (2020)** [36]	Synthetic cannabinoids, fentanyl analogues, synthetic cathinones, tryptamines and phenethylamine	Blood Urine Oral fluid	LLE	HPLC-MS-MS*	Waters Oasis reversed phase column	Blood: 0.03 - 0.35 ng/mL Oral Fluid: 0.03- 0.25 ng/mL Urine: 0.02 - 0.25 ng/mL	Blood: 0.08 - 1 ng/mL Oral fluid 0.07- 0.8 ng/mL Urine: 0.06 - 0.5 ng/mL
67	**Vincenti (2019)** [32]	Synthetic sedatives-dissociatives, synthetic cannabinoids, synthetic cathinones, amphetamines and 2C drugs	Hair	PLE and DLLME, also had LLE and SPE	UHPLC–HRMS/MS	Kinetex XB C18 and Kinetex PFP by Phenomenex	0.1 - 5 pg/mg	5 - 50 pg/mg
68	**Walton (2022)** [123]	Synthetic opioid	Blood Urine	LLE	LC-MS	Agilent InfinityLab Poroshell C-18	0.1 ng/ml	0.5 ng/ml
69	**Yang (2022)** [124]	Amphetamines and methamphetamines	Postmortem blood Urine	LLE	LC-MS/MS	Zorbax SB-Aq	0.5 ng/ml	5 ng/ml
70	**Yang (2024)** [125]	Synthetic cannabinoids	Hair	SPE	LC-MS/MS	C18 column	10-15 pg/mg	25-40 pg/mg
71	**Yen (2024)** [126]	Synthetic cathinones,	Urine	LLE	GC-MS	capillary column with 5% pheny (DB-5ms)	0.79-1.01 ng/mL	-
72	**Zhai (2023)** [127]	Phenethylamines and their derivatives	Hair	SPE	UHPLC-MS/MS	Biphenyl column	0.5-10 pg/mg	1-20 pg/mg

73	**Zhao (2024)** [128]	Amphetamine	Urine	SPME	UHPLC-MS	C18 column	0.01-0.02 ng/mL	0.02-0.05 ng/mL

Abbreviations: * Method is in tandem; **µ-SPE**: (micro)solid extraction; **CE – HRMS**: Capillary electrophoresis mass spectrometry; **DBS**: Dried Blood Spots; **DI-SPME:** Direct immersion-solid phase microextraction; **DLLME**: Liquid/Dispersive Liquid Microextraction; **ESI:** electrospray ionization; **GC-MS:** gas chromatography coupled to a mass spectrometer; **HESI:** Hot Electrospray Ionization; **HS-SPME**: Headspace-solid phase microextraction; **HRMS:** High Resolution Mass Spectrometry; **LC-MS**: liquid chromatography coupled to a mass spectrometer; **LC-FD:** liquid chromatography with fluorescence detection; **LLE:** Liquid-Liquid Extraction; **LOD:** Detection limit; **LOQ:** Limit of quantification; **LPME:** liquid-phase microextraction; **MEPS:** packaged sorbent microextraction; **MIP:** Molecularly imprinted polymers; **MRM:** Multiple reaction monitoring; **PALME:** Parallel artificial liquid membrane extraction; **pDART-QqQ-MS:** paper-loaded direct analysis in real time triple quadrupole mass spectrometry; **PLE:** pressurized liquid extraction; **PP:** protein precipitation ; **QTOF:** quadrupole time-of-flight instrumentation; **QuEChERS:** Quick, Easy, Cheap, Effective, Rugged and Safe; **SALLE:** salting-out liquid-liquid extraction; **SFC:** Supercritical fluid chromatography; **SHS-HLLME:** Switchable Hydrophilicity Solvent based Homogenous Liquid–Liquid Microextraction,; **SPE:** Solid Phase Extraction; **SPME:** solid-phase microextraction; **UHPLC:** Ultra-High Performance Liquid Chromatography.

3.5 Bioanalysis of Synthetic Depressants/Sedatives

NPS depressants or sedatives refer to a group of substances that are capable of decreasing brain activity, causing the user to become "sedated", or even disinterested in the situations around them; this is because such drugs reduce the amount of heartbeat, reducing blood circulation in the body and brain, which can lead to death [129]. Within this class, designed benzodiazepines and synthetic opioids stand out.

In the review, different methodologies were also applied, with the UHPCL technique being the most used to identify these compounds. Although the works present different columns used, the C18 column is present in most of them. It is also possible to observe that different sample preparation methods were applied depending on the sample type.

In the work of Lin et al. [67], a recognition strategy for the colorimetric detection of fentanyl was developed; this was the only work included in the review that applied a colorimetric method in a fast, economical, selective, and sensitive way. They used a colorimetric indicator called Rose Bengal (RB), a hydrophilic dye widely used in microbiology techniques [130] and in the quantification of the hydrophobicity of enzyme immobilization support [131]. Fentanyl, and its analogs, is an opioid that has been increasingly observed as an additive in several illicit drugs, such as heroin and cocaine [132]. Lin et. al [67] identified Fentanyl present in urine samples, without any previous sample preparation, and through the molecular interactions between fentanyl and the dye used, the colorimetric assay was effective in identifying the compound in amounts as low as 10 $mg.L^{-1}$, making it possible to visualize the color change with the naked eye, it is important to note that other opioids can also be applied in this colorimetric method developed.

Other studies have only worked with designer benzodiazepines and Z-hypnotic drugs [21], which are substances increasingly used for self-medication or recreational purposes without real knowledge of their numerous adverse effects [133]. These substances were analyzed through plasma and blood samples. For plasma samples, the preparation was based on the MEPS technique followed by analysis in UHPLC-MS/MS, this combination was validated and effectively confirmed the presence of these compounds in human plasma, which may go unnoticed in other analytical tests.

In the work of Banaszkiewicz et. al [17], they used whole blood to determine four benzodiazepine designers and 3 Z-hypnotic drugs, through the method based on mass spectrometry in tandem liquid chromatography and the sample preparation method was LLE. The validated method was applied to 145 samples of toxicological cases, allowing information on the prevalence of the use of these substances to be obtained. The most frequently determined compounds were nor-dazepam in 87 cases (60%), diazepam in 81 cases (55.9%), temazepam in 72 cases (49.7%), oxazepam in 56 cases (38.7%) and midazolam in 36 cases (24.8%).

4. CONCLUSION

This study reviewed the main analytical methods recently validated for identifying NPS in biological matrices from 2018 to June 2024. The most applied analytical technique in the studies included in this review was LC-MS, with some of them applying the high-resolution technique (HRMS) due to its high specificity

when dealing with NPS. The group of stimulants was the most prevalent in the majority of studies, as this class of NPS is the one that generates the newest substances, according to UNODC data. Chromatographic separations were performed using hydrophobic interaction columns, employing phenyl and alkyl groups with varying hydrophobicity (C8 and predominantly C18). It's important to note that in this review, only Borovcová et al. [18] studied the effect of different polar groups (phenyl and alkyl groups present in the stationary phase) on NPS identification, meaning that the majority of authors did not extensively address the potential impact of different stationary phases on analyte separation efficiency in the column.

Blood and urine samples were the most commonly used for identifying substances in the stimulants and cannabinoids group. In contrast, hair samples were primarily used to identify substances in the hallucinogens and sedative depressants group. Despite traditional matrices (such as blood, hair, and urine) remaining the primary choice for sample collection, the literature reflects a growing use of alternative matrices, such as oral fluid, which has gained prominence as a non-invasive alternative to blood.

Regarding analyte extraction, SPE methods and their variations were the most commonly applied, followed by the LLE method and its variations for different types of samples. However, miniaturized techniques have gained relevance in these analyses due to their consideration as 'greener' and generally more cost-effective alternatives to conventional procedures.

It is important to emphasize that the most of the studies reviewed in this work followed the guidelines of the Scientific Working Group on Forensic Toxicology (SWGTOX) in their methodological validation. The SWGTOX guidelines, recently updated to the ANSI/ASB 036 standard, establish minimum requirements for method validation in forensic toxicology. These guidelines include the evaluation of parameters such as bias and precision, linearity, carryover, matrix effects, interferences, ionization suppression/enhancement, stability, LOD and LOQ, ensuring greater methodological reliability [134].

Despite that, some limitations were observed. In a small portion of the studies, external validation with authentic samples, a crucial step to demonstrate the method's practical applicability, was not reported. The absence of this test may compromise the practical relevance of the developed solutions and reduce their potential regulatory impact, as evidenced in the study by Sara Júlio (2023) [78], where a newly validated methodology for detecting cathinones failed in all six authentic cases tested. Additionally, another point that caught our attention was the way external validation was conducted, which varied significantly among the studies. While some researchers used authentic samples with previously positive results, others analyzed samples from volunteers (considered suspicious and not suspicious) without prior analysis of the analyte. Another divergent point was the sample size, ranging from isolated cases to studies with thousands of cases. This lack of uniformity may hinder the comparison between methods and the generalization of results, highlighting the need for greater standardization in external validation test.

It's crucial to emphasize the responsibility of toxicologists in understanding the strengths and limitations of analytical techniques and biological matrices to critically evaluate drug test results, as obtaining analytical standards that can serve as references for NPS identification is a significant challenge. It's fundamental to highlight that there is no ideal method for NPS identification, as the combination of different analytical tools may be necessary for assessing these substances. However, HRMS excels in its ability to collect many mass spectra per second and can help elucidate the structure of unknown compounds, as is the case with NPS. Low-resolution mass spectrometry (LRMS) is the standard technique widely used in clinical and forensic toxicology laboratories. This is because of its user-friendliness, the availability of reference libraries, and lower costs. Despite that, HRMS is gaining increasing importance, particularly in comprehensive analyses with no specific target in mind.

Furthermore, more studies on pharmacokinetic parameters are needed to understand the ultimate fate of these substances in the body and the metabolites generated, allowing for determining the best biological sample to be used in detection tests. This will lead to a more precise interpretation of distribution studies in the body and facilitate their detection in various biological samples, aiding in the selection of the most suitable analytical method for NPS identification.

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